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Oxidative Phosphorylation, A History of Unsuccessful Attempts: Is It Only An Experimental Problem?

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It is due to the predominance of the logic based on the inductive reasoning the commonly held view that the purpose of a scientist is that of making observations, of verifying theories through observations and of proposing theories through the generalization of observations. There is an opposite view, however, which starts from the assumption that empirical sciences are systems of theories.[1] In this type of logic, theories are conjectures proposed by the scientist to explain and predict natural processes, and cannot be inferred from observations, although they must be compatible with them. Empirical sciences are thought as including two types of statements: universal and specific. The formers are hypotheses with the character of natural laws, the latters assert the occurrence of single events.[1] The validity of both statements is decided empirically. However there is an asymmetric relationship between the two. The universal statements can never be verified but only refuted by specific statements (experimental observations). On the other hand a specific statement can be verified through another observation. A theory can be disproven if there exists at least one class of events forbidden for the theory. A theory which has a larger number (in comparison to another) of potential falsifiers, and can therefore more easily be tested and refuted, has also a larger empirical content. In the following, I shall try to show the impact of this logic of scientific knowledge on current hypotheses and future theories of energy conservation.

Comments on the Chemical or Conformational Hypotheses

"Can you describe any possible observations which, if they are actually made, would refute your theory? If you cannot, then your theory has clearly not the character of an empirical theory; for if all conceivable observations agree with your theory then you are not

entitled to claim of any particular observation that it gives empirical support to your theory".[2]

The "chemical" hypothesis has had a considerable influence for almost twenty years in the field of oxidative phosphorylation, and it has been the first attempt of symbolic analysis of the process.

A symbolic formulation of electron transport coupled phosphorylation is:

$$AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP \quad (1)$$

The "chemical" hypothesis[3,4] has been recently formulated as[5]:

$$AH_2 + B + C\ (I) \rightleftharpoons A \sim C\ (I) + BH_2 \quad (2)$$

$$A \sim C\ (I) + ADP + P_i \rightleftharpoons A + C + ATP \quad (3)$$

Sum

$$AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP \quad (4)$$

$A \sim C\ (I)$ indicates a high energy intermediate where the redox energy is conserved in a covalent bond between one of the products of the redox reaction and a ligand C. The "conformational" hypothesis[6–8] has been written[5] as:

$$AH_2 + B \rightleftharpoons A^* + BH_2 \quad (5)$$

$$A^* + ADP + P_i \rightleftharpoons A + ATP \quad (6)$$

Sum

$$AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP \quad (7)$$

A* indicates the conservation of the redox energy into a rearrangement of the protein structure of a redox carrier.[5] The chemical and conformational hypotheses have been considered as "variants of a single hypothesis", otherwise denoted as the C hypothesis.[5]

All scientific theories exclude a specific class of events.[1] A theory speaks about empirical reality only in so far as it sets limits to it. Every theory can thus be put into the form "such and such does not happen".[2] The observation of the event forbidden by the theory constitutes a refutation of the theory. By using this type of analysis, the empirical content of equations (2–7) can be formulated as such: "There is no conversion of redox into chemical energy (say ATP) which does not involve: (a) (chemical)-a covalent bond with a redox carrier or; (b) (conformational)-a conformational rearrangement of a redox carrier". A disproof of these hypotheses requires the observation of a process of energy transduction without either the formation of a covalent bond or a conformational change at the level of the respiratory chain. I maintain that the class of the potential falsifiers of these

hypotheses, as they stand in equations (2–7) is practically empty. Until they are expressed in such general terms it is impossible to make specific predictions leading to falsifying experiments. Hence, their empirical content is negligible and they are of little help to the experimentalist. This criticism is not directed to a chemical or conformational hypothesis in general, but to the view of considering equations (2–7) *as* a hypothesis.

Attempts have been made to propose specific mechanisms. Although some of them are of considerable interest, an analytical discussion is out of place in the present context. On the other hand I shall briefly consider the view that shifts of the α band of cytochrome b and of the midpoint potentials of cytochromes b and a upon energization[9, 10] are in support of the formulation given in equations (2–7). Two problems arise here, one is experimental, the other theoretical. The experimental question concerns the amount of information on membrane conformation which can be obtained from the red shift as compared, for example, to other extrinsic probes such as those used in many other laboratories. That the importance of the red shift derives from its being "primary" because of its faster kinetics, in respect to the other dyes, is irrelevant because this may be due to an additional diffusion step present in the latter but not in the former case. On the other hand it might be easily predicted that the red shift will be a difficult source of structural information because (a) it is difficult to decide whether the source of the shift is an intramolecular rearrangement[9, 10] or it concerns the environment of the chromophore,[11] (b) there are no known models to which compare the "energy state" of cytochrome b. Point (b) is a criticism also to the molecular interpretation of the shift of the midpoint potentials. From a theoretical standpoint, the observations are "in accord" with the chemical or conformational hypotheses simply because equations (2–7) are compatible with "any observation". However they can be equally accounted for by the chemiosmotic hypothesis as far as this hypothesis is equally elusive. The cytochrome's shifts cannot be used to refute, or therefore to support, a particular mechanism of oxidative phosphorylation.

Comments on the Chemiosmotic Hypothesis

This hypothesis[12, 13] is a mixture of both precise and of more general statements. In the former case it has a high empirical content and it has been put under stringent experimental test whereas in the latter it suffers the same objections as the chemical-conformational hypotheses. Some of its basic formulations can be stated as follows.

"The conversion of redox into chemical energy does not require high energy intermediates of the A ~ C or C ~ I type". The disproof

of this statement requires the isolation of the high energy intermediates. Since these intermediates are however not defined in chemical terms, the class of the potential falsifiers of this statement is empty.

"The process of electron transport coupled phosphorylation does not occur without (a) a precise arrangement of the electron and hydrogen carriers, (b) a certain stoicheometric transfer of protons, (c) a membrane potential of a certain magnitude and (d) an impermeability of the mitochondrial membrane to diffusion of charged species."

Hypotheses (a) and (d) have been most fruitful. Although I do not think that the arrangement of the respiratory loops proposed by Mitchell, is feasible, it must be conceded that this proposal has given a great impetus to studies of the topography of the membrane. The requirement for a restriction to charge translocation has led not only to a new approach for studying the mechanism of action of uncouplers[14–16] but also to the discovery of the anion carriers[17] (the proposal for which was also included in the hypothesis).

On the other hand strong experimental evidence has been provided that hypotheses (b) and (c) are not correct. The stoicheometry of the proton translocation per energy rich bond during the conversion of chemical into osmotic energy or vice versa is 4 and not 2 as predicted by the chemiosmotic hypothesis.[18–23] Indeed Cockrell *et al.*[18] have reported a $K^+/\sim$ ratio of 3 and 7, for the respiration and ATP driven uptake, respectively. We have obtained a ratio of about 4 in both cases.[19–23] The stoichiometry of the H^+/K^+ exchange during active transport is variable and dependent on the internal and external concentrations of H^+ and K^+ whereas in the chemiosmotic hypothesis it should be constant and independent of the H^+ and K^+ concentrations.[21, 24, 25] Finally the osmotically driven ATP synthesis takes place in the presence of an osmotic potential (as calculated from the H^+ and K^+ concentration gradients) which is half that predicted by the chemiosmotic hypothesis.[22, 24–26]

Also, experiments on the saturation kinetics[27] and the dependence of the $K/\sim$ ratio on the H^+ and K^+ concentrations[21] strongly oppose an electrophoretic ion diffusion down an electrical gradient.[25] However the question of the transmural potential will presumably remain open for a certain time due to the difficulty of performing conclusive refuting experiments. On the other hand the Occam's razor here applies: what is needed for a membrane potential in active ion uptake once its role in ATP synthesis is questioned? My opinion is that the chemiosmotic hypothesis has had more success in predicting some general properties of the mitochondria than in devising a specific mechanism for oxidative phosphorylation.

The Basic Properties of the Mitochondrial Membrane

I propose that these be individuated as follows:

1. *Coupling of transport to metabolism and transformation of scalar into vectorial reactions.* The property is shared by the Na/K^+ ATPase reaction of the plasma membrane. According to the Curie's theorem "the effects cannot be more asymmetric than the causes". If one assumes that the forces of chemical affinity are scalar, a dilemma arises as to the conversion of a scalar into a vectorial reaction. It has been pointed out however that the enzyme catalyzed group transfer reaction occurs in an anisotropic microscopic non-aqueous phase as to show in reality a vectorial character.[28] The orientation of all the enzyme molecules within a membrane is required in order to avoid a random distribution in a homogeneous phase which renders isotropic the chemical reaction. A fundamental difference between our and Mitchell's concept for active transport in mitochondria, is that the former requires a specific geometric arrangement of membrane groups which are uniquely involved in ion translocation, whereas in the latter the vectorial character concerns only the operation of the respiratory chain and of the ATPase. Our concept would be refuted by the observation that active transport entails an electrophoretic diffusion of cations down an electrical gradient.

2. *Propagation of high energy state in membrane structure.* This property is unique to the mitochondrial membrane although it may be compared with the conformational change proposed to accompany the action potential in the nerve membrane. The redox energy is primarily conserved in a particular conformation of the membrane structure rather than in a covalent bond or a conformational change involving an electron carrier. This concept, may be formalized as follows:

$$AH_2 + B + M \rightleftharpoons A + BH_2 + M^* \tag{8}$$

$$M^* + P_i + ADP \rightleftharpoons M + ATP \tag{9}$$

Sum

$$AH_2 + B + P_i + ADP \rightleftharpoons A + BH_2 + ATP \tag{10}$$

Where M and M* indicate a low and high energy state of the membrane. Whatever the energy conserving site, the high energy state can be propagated along the membrane without formation of covalent bonds or diffusion of low molecular weight high energy intermediates. The concept expressed in equations (8–9) will be refuted by one of the following observations.

(a) The occurrence of site specificity for reaction of ADP, uncouplers, and inhibitors of oxidative phosphorylation. These effects are predicted in equations (2–7) since the compounds $A \sim C$ (I) or A*

vary according to the site in the respiratory chain. On the other hand they are excluded in equations (8–9) where M* is common for the three sites.

(b) Presence of energy conservation and respiratory control in soluble electron transport systems, devoid of membrane organization. In equations (2–7) the respiratory control is due to bringing a respiratory carrier into an inhibited form A ~ C (I) or A*. In equations (8–9) the respiratory control is due to the fact that the transfer of reducing equivalents from A to B, at the site where energy is conserved, must occur across the membrane M, and is inhibited when the membrane is in the energized state M*. Equations (8–9) therefore imply that the high energy state is a property of the environment and becomes manifest only when electron transport takes place in a lipid-rich multimolecular assembly with specific geometric and structural requirements. However equations (8–9) do not require an alternation of hydrogen and electron carrier as in the chemiosmotic hypothesis.

It may be argued that by writing down equations (8–9) I have exposed myself to the same criticism of elusiveness used for equations (2–7). My answer is: (a) the equations (8–9) are not a hypothesis for oxidative phosphorylation; they are simply a symbolic means for expressing the alternative concept that energy is not conserved in respiratory chain components and does not involve the existence of low molecular weight high energy intermediates; (b) although equations (8–9) are vague, I have proposed some observations through which my concept may be refuted; I expect the proponents of equations (2–7) to suggest similar falsifying experiments for their concepts.

Membrane Structure, Carrier "Gating" and Nucleophilic Sites

The growing of a theory for oxidative phosphorylation necessarily involves a multistep process. This means to start by proposing *very narrow range hypotheses* which have a precise empirical content and lead to definite predictions and experimental tests. After suitable controls broader hypotheses can be proposed which should be tested also by utilizing the concepts derived from the simpler hypotheses. And so on until the general principles underlying the mechanism of energy transduction may be included in a theory of oxidative phosphorylation.

The present proposals of our laboratory for active transport and oxidative phosphorylation are based on three general concepts.

1. *The mitochondrial membrane is formed by a core of polypeptide chains buried in hydrocarbon regions.*[25, 27, 29] The polar sites of the membrane proteins are partly exposed or "bare" (in respect to the aqueous phase) and partly unexposed; i.e., not accessible to hydrophilic ions.

Transport across the membrane and coupling of ion fluxes involves interaction with these sites. Specific transport mechanisms are required (for example the presence of extrinsic ionophores) when the interaction involves the unexposed polar groups (carriers). This proposal implies a membrane structure of the type envisaged by Lenard and Singer[30] and Wallach and Zahler,[31] rather than the classical lipid bilayer. Furthermore this proposal is alternative to those implying electrophoretic, electrogenic ion diffusion across the membrane and coupling of fluxes through membrane potential. Instead we assume that the electrophoretic step is in series with another involving an electrostatic interaction with a fixed negative charge system. The term electroneutral exchange[21,27] indicates that the coupling of the various forms of energy, including the ion fluxes which constitute the osmotic energy, does not occur through a transmembrane electrical potential, but rather involve common chemical reactions within the membrane. Electroneutrality is also a stringent thermodynamic requirement for an electrostatic interaction in a hydrophobic environment.

2. *The accessibility of membrane hydrophobic and polar groups for the aqueous phase is modified when the membrane undergoes an energy-linked rearrangement.* The geometric properties of the groups which undergo an energy-linked protonation and therefore operate as proton carriers, are dependent on the metabolic state of the membrane ("gating" of the carrier).[22,25] This proposal is alternative to those implying either a free diffusion of protons across the membrane or a translocation through respiratory loops and ATPase and explains the high and low rates of proton translocation in the energized and deenergized membrane, respectively.

3. *The energy-linked conformational change involves the formation of highly nucleophilic sites.*[22,23,25,26,29,32,33,35,36] The proton donating and acceptor properties of membrane groups are markedly modified during transition from the deenergized to energized state. Such changes of apparent pK_a may be due to: (a) enhanced hydrophobicity[34] with restriction to ionization of ion pairs and/or increased activity of protons or (b) increased strength of specific groups due to intramolecular rearrangement of the electron configuration. The formation of superacid or superbasic regions, where substrates are more easily protonated or deprotonated, can be utilized for acid-base catalysis (ATP synthesis) and operation of the proton pump. Our proposal gives an alternative explanation to a number of recent observations. The shifts of the absorption band and of the midpoint potentials are not due to the formation of high energy forms of the cytochromes but rather to pK_a shifts in the cytochrome environments. The increased uptake of organic anions to energized submitochondrial

particles is not due to diffusion down an electrical gradient (positive inside) or attraction to positive charges, but rather to an entropy driven process accompanying a decrease of electrostatic repulsion in certain membrane regions.[37] Finally uncouplers act by transferring protons in the lipid phase. However the uncoupling effect is not due to the H^+ movement across the membrane to collapse an electrical potential but to the interaction, within the membrane, of the protons with the energy-linked nucleophiles or to an increase of H^+ ions activity in certain membrane regions.[26, 29] The sink for the protons is due to the increase of electron density in some groups of the membrane proteins, rather than to the low electrochemical potential of protons in the inner aqueous mitochondrial phase. We are at present actively engaged in deriving from these very general ideas simple experimental questions to put under stringent test. A number of observations have been made which appear to be in accord with the general formulation of the problem.

It may be argued that these proposals are still far from the "great" problems of oxidative phosphorylation. On the other hand they have the advantage to be possibly expressed in chemical terms and lead to definite questions. The answer to these questions may permit to move the preliminary steps toward a structural analysis of the membrane during energy transduction.

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