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Review

Chemiosmotic coupling in oxidative and photosynthetic phosphorylation ☆,☆☆

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ABSTRACT

50 years ago Peter Mitchell proposed the chemiosmotic hypothesis for which he was awarded the Nobel Prize for Chemistry in 1978. His comprehensive review on chemiosmotic coupling known as the first "Grey Book", has been reprinted here with permission, to offer an electronic record and easy access to this important contribution to the biochemical literature. This remarkable account of Peter Mitchell's ideas originally published in 1966 is a landmark and must-read publication for any scientist in the field of bioenergetics. As far as was possible, the wording and format of the original publication have been retained. Some changes were required for consistency with BBA formats though these do not affect scientific meaning. A scanned version of the original publication is also provided as a downloadable file in Supplementary Information. See also Editorial in this issue by Peter R. Rich. Original title: CHEMIOSMOTIC COUPLING IN OXIDATIVE AND PHOTOSYNTHETIC PHOSPHORYLATION, by Peter Mitchell, Glynn Research Laboratories, Bodmin, Cornwall, England.

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1. Introduction

1.1. The basic question of the coupling mechanism

The process of electron transport phosphorylation consists of the flows of two sets of particles: the oxido-reduction (o/r) particles (e.g. 2H, H^-, or $2e^-$), and the hydro-dehydration (h/d) particles, popularly identified as "energy-rich" squiggle (~) bonds terminating at adenosinetriphosphate (ATP). The enzymes and catalytic carriers that channel these flows in mitochondria, chloroplasts, and microorganisms are so organised that there is a variable degree of coupling between them. The end result of the coupling between the flows through the o/r and h/d pathways in oxidative phosphorylation in mitochondria is that, for the equivalent of each pair of electrons traversing the respiratory chain, up to 3 anhydrobond equivalents may normally traverse the h/d pathway from adenosine diphosphate plus inorganic phosphate ($ADP+P_i$) to water. In photosynthetic phosphorylation the stoichiometry is less certain, and it is thought that either one or two anhydrobond equivalents may traverse the h/d pathway per electron pair equivalent traversing the o/r pathway (see [1–3]).

It has long been thought that coupling between oxido-reduction and phosphorylation depends upon the existence of certain "energy-rich" chemical intermediates that are common to the o/r and h/d pathways [4–9]; and that if only the identity of these intermediates and the details of their reaction mechanisms could be elucidated, an understanding of the coupling mechanism would be obtained (see reviews by: [10–12,264]). Thus, in spite of some awareness that a more liberal approach might fruitfully be adopted [13–15], the study of the question of the coupling mechanism has continued to be ruled by the well-trodden and familiar tenets of the chemical coupling conception, no matter how fantastic the resulting tissue of hypothesis. The object of the present review is to overlook this customary restriction of perspective and to pose the central question of electron transport phosphorylation in the elementary form: How are the flows in the o/r and h/d pathways coupled to each other? In answer to this question I shall develop the view that coupling may occur through a chemiosmotic type of mechanism [16–18] that does not require chemical intermediates common to oxido-reduction and phosphorylation. It will be desirable to compare the relative merits of the orthodox chemical coupling hypothesis with the chemiosmotic coupling hypothesis; and to facilitate this comparison we shall begin by considering the former in a suitable idiom.

1.2. Outline of the chemical hypothesis

In the context of electron transport phosphorylation, the so-called energy-rich or squiggle bond means no more than a potential site for occupation by water or for hydrolysis; and squiggle bonds flowing one way are formally equivalent to the elements of water flowing the other way. It is therefore more appropriate to describe the currency of the h/d pathway as anhydro particles than as "energy-rich" bonds. The flows in the o/r chain and in the h/d pathway differ in an important thermodynamic respect. The range of potential energy change of the o/r particles as they traverse the respiratory chain between substrate and oxygen in oxidative phosphorylation depends on the substrate. It is

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[1] Peter Mitchell (1921–1992).

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equivalent to about 0.8 electron volts (eV) for the passage of one electron or its equivalent from the fumarate/succinate couple to oxygen, and about 1.1 eV from the nicotinamide-adenine dinucleotide o/r couple (or $NAD^+/(NADH+H^+)$ couple) to oxygen at neutral pH and at atmospheric pressure. The potential across the h/d pathway, on the other hand is not directly dependent upon the substrate, but goes from water in the physiological media to water in equilibrium with the ATP/(ADP + P_i) couple poised at physiological levels. It would go from about 55 M water (giving almost complete ATP hydrolysis) to about 5 μM water when the ATP/ADP ratio would be poised about centrally in equilibrium with 10 mM P_i at neutral pH. This is easily verified by writing the hydrolysis equilibrium for the reaction $ATP + H_2O \rightleftharpoons ADP + P_i$ unconventionally to include the elements of water, thus,

$$\frac{\{ADP\} \times \{Pi\}}{\{ATP\}} = K'\{H_2O\} \tag{0}$$

where the variable $K'\{H_2O\}$ is equal to the so-called hydrolysis constant, and the curly brackets denote activities. It is evident that the work done when water is pulled from an activity of 5 μM to an activity of 55 M is equal to the free energy of hydrolysis of ATP, which is about 10,000 calories per mole or 0.42 eV per molecule of water at a P_i concentration of 10 mM and at neutral pH [19,20].

The basic chemical conception of coupling in oxidative phosphorylation is represented by the diagram of Fig. i, following the conventions of [10]. In this diagram it is not possible to represent the cyclic oxidoreduction of the carrier, CH, as it is not known whether CH stands for the oxidised or the reduced carrier. We must be content with the hypothesis that it represents either one or the other and that consequently CH + IOH can react to form C~I + H_2O only once per 2 electron equivalent o/r cycle of CH. It is customary to assume that the hypothetical anhydro compounds C~I, X~I, and X~P would all be about half hydrolysed in equilibrium with the same water potential as the centrally poised ATP/ADP couple or with about 5 μM water. Thus the transition of the dehydration-coupling electron transport carrier between the states of C~I + H_2O and CH + IOH corresponds approximately to the work done in dehydrating $(ADP+P_i)^- + H^+$ to give ATP + H_2O, or about 0.42 eV per molecule of ATP. The physical process of dehydration, requiring work of some 0.42 eV, is supposed to be done by the carrier, C, as it passes cyclically through the transition states associated with the passage of 2 electrons or their equivalent through the o/r chain. The effective o/r potential drop across the coupling carrier, C, will thus be diminished by about 210 mV when the poise of the ATP/ADP couple is about central, and this "back E.M.F." will rise with the ratio of concentration of ATP to (ADP + P_i), causing the phenomena

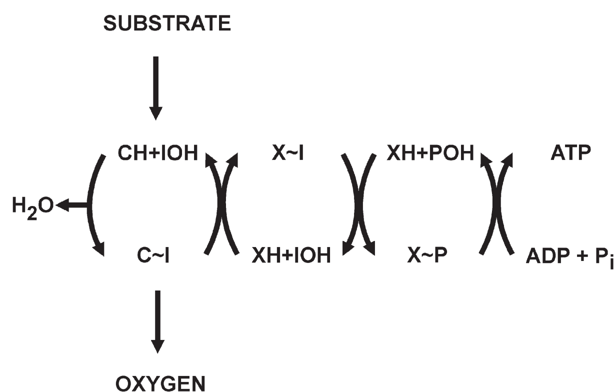


Fig. i. Outline of the chemical coupling hypothesis for one phosphorylation site in oxidative phosphorylation. The substance CH is a member of the respiratory chain which is assumed to be able to form the anhydride C~I as a result of oxido-reduction. It is not agreed as to whether C in C~I is the oxidised or reduced form of the respiratory carrier. The hypothetical pathway connecting the hypothetical C~I with ATP through X~I is self explanatory.

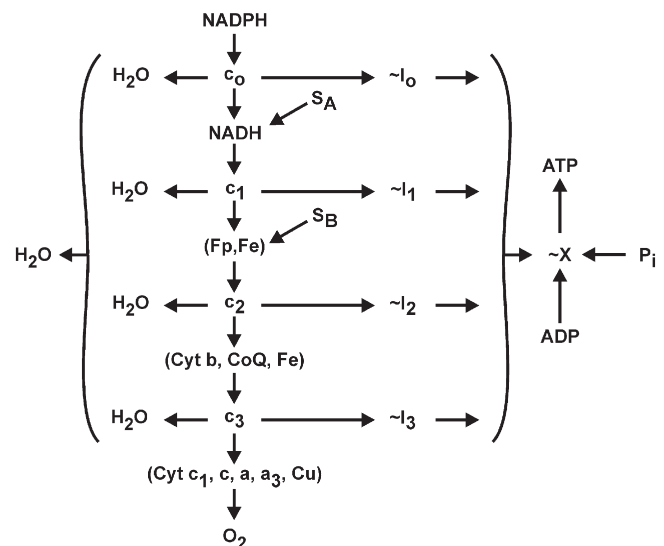


Fig. ii. Outline of chemical coupling hypothesis of oxidative phosphorylation showing four phosphorylation sites including the transhydrogenase site. The substrates S_A and S_B represent NAD-linked and FAD-linked substrates. Other conventions, as used in the text, follow [10].

of "crossover" and "reversed electron transport". Continuing in this idiom, Fig. ii summarises the main features of the present chemical theory of the reactions involved in oxidative phosphorylation in terms of the flows of divalent o/r particles(e.g. $2e^-$, 2H, or H^-) of the respiratory chain represented vertically on the left, and of the h/d particles represented horizontally between the brackets. Under appropriate conditions all the flows except that between oxygen and cytochrome oxidase (a, a_3, Cu) are supposed to be reversible, but for the sake of clarity the arrows point in the forward direction of the flow of reducing equivalents in the o/r chain, and in the direction of flow of

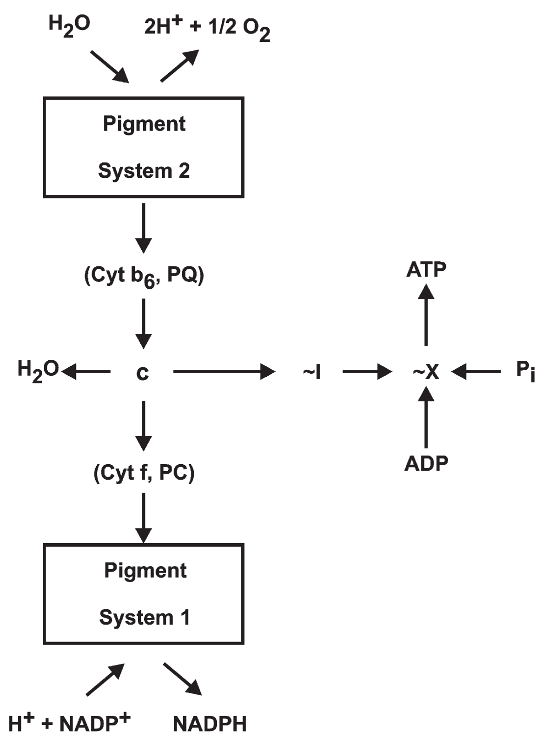


Fig. iii. Outline of chemical hypothesis of coupling in non-cyclic photophosphorylation, following [2]. Conventions as in Fig. ii except PQ and PC which stand respectively for plastoquinone and plastocyanin.

the various components of the h/d chain, indicated in the diagram. The carriers C_O, C_I, C_2, and C_3 are supposed to represent the four, as yet, unidentified members of the respiratory chain at the four coupling sites, corresponding to the hypothetical CH/C~I of Fig. i. Substrates reacting at the level of NADH and succinate are indicated by S_A and S_B respectively. For non-cyclic photophosphorylation, an analogous scheme is shown in Fig. iii, following [2].

1.3. The question of the existence of C~I intermediates

It is relevant to remark at this point (see [15]) that during the twenty odd years since the problem of the coupling mechanism in electron transport phosphorylation was first studied, no generally accepted coupling intermediate common to the o/r and h/d pathways has been isolated or characterised, and no unequivocal evidence for the existence of coupling intermediates of this kind has been obtained. Dr. E.C. Slater has pointed out to the author that the high-energy carrier intermediates of the type that he first represented on paper in 1953 might be particularly difficult to isolate in the high-energy state because of their lability and because of their relatively low concentration in terms of the energy-rich bond characteristic—an argument that deserves to be taken very seriously. On the other hand, the belief has been widely accepted that the energy-rich coupling intermediates (C_1~I_1, C_2~I_2, C_3~I_3 etc.) must exist because there is no feasible alternative means of coupling electron transport to phosphorylation.

1.4. Objects of the chemiosmotic hypothesis

My main object in proposing a working chemiosmotic hypothesis of oxidative and photosynthetic phosphorylation five years ago [16] was threefold: (i) To provide a simple rationale for the organisation of the components of the o/r and h/d pathways in the lipid membrane systems of mitochondria and chloroplasts; (ii) To formulate a type of coupling that would require no intermediates, like C~I, directly linking oxido-reduction to hydro-dehydration, so that future work need no longer be so dependent upon or so circumscribed by the belief in the C~I intermediates; and (iii) To acknowledge the elusive character of the C~I intermediates by admitting that they may not exist.

The development of the chemiosmotic hypothesis depended upon the formulation of a group of postulates defining the minimum requirements of a workable system. In view of the novelty of this subject it will be of interest to describe how these basic postulates were derived.

2. Derivation of the chemiosmotic postulates

2.1. The anisotropic o/r system

The pioneer work of Lund [21], and Stiehler and Flexner [22] led to the suggestion by Lundegardh [23] that if oxido-reduction through the cytochrome system were anisotropically organised across a membrane, as in a fuel cell [24], H^+ ions would be produced on one side and consumed on the other (Fig. 1). Lundegardh's suggestion is an obvious starting point for the development of osmotic coupling concepts because it could, in theory, achieve the first essential step—the conversion of the free energy of electron transport to the osmotic potential of the proton concentration difference across a proton-impermeable membrane (see [25]). The second step, required to complete the coupling process, would be the coupling of ATP synthesis to the effective return flow of protons across the membrane. Davies and Ogston [26] and Davies and Krebs [27] suggested that ATP might be synthesised via a second electron-transport system orientated in the membrane like the first, but driven in reverse by the proton concentration difference produced by the first electron-transport system. However, this suggestion as to the possible mechanism of coupling in oxidative phosphorylation did not meet with success because, apart from other difficulties, it fails to eliminate the necessity for the chemical coupling step between electron transport and phosphorylation; for the synthesis of ATP would presumably have to be chemically coupled to the supposed second electron transport system.

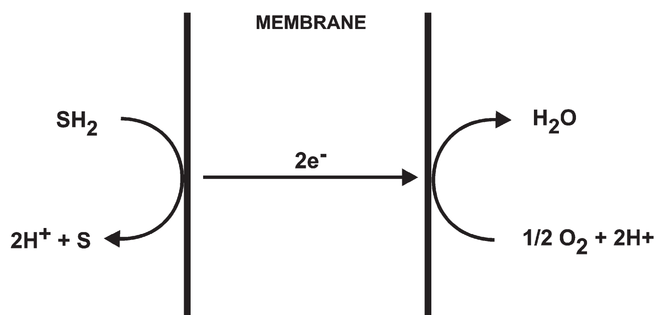


Fig. 1. Electron translocating oxido-reduction system, after [23].

2.2. The anisotropic h/d system

The progress of ideas appears to have been inhibited at this point by the circumstance that metals are very specific conductors of electrons. This circumstance has apparently fostered the prejudice that oxido-reduction reactions are unique in having electromotive properties, and that only oxido-reduction reactions can be directly coupled to ion translocation or to the separation of H^+ and OH^- ions in electrochemical systems. However, the development of ideas related to the concept of group translocation [17,28,29] has led to the suggestion that, given an enzyme or catalytic carrier that acts as a specific conductor of OH^- or O^{2-} groups, hydro-dehydration reactions can be organised to provide the electromotive power of electrochemical cells in much the same way as oxido-reduction reactions (see [30,31]). The reversible anisotropic ATPase system of the chemiosmotic hypothesis was based upon this concept [16]. It was shown, as illustrated in Fig. 5A, that if the active centre region of a membrane-located ATPase were specifically accessible to OH^- ions from one side only, to H^+ ions from the other side only, and to water as H_2O from neither side, ATP hydrolysis would be reversibly coupled to the translocation of OH^- groups or ions across the system with a stoichiometry of one OH^- translocated (equivalent to one proton translocated in the opposite direction) per ATP hydrolysed. It was therefore logical to postulate that the function of the known membrane-located ATPase systems of mitochondria and chloroplasts is effectively to couple ATP synthesis to the flow of protons back across the membrane.

2.3. An electric component of the protonmotive force

According to the more primitive form of the chemiosmotic coupling hypothesis, ATP hydrolysis and substrate oxidation would each generate a difference of concentration of protons in the same direction across a proton-impermeable membrane, so that, if the proton concentration difference could become big enough, electron transport would reverse ATP hydrolysis, and ATP hydrolysis would exert a back pressure on electron transport. Unfortunately, the pH difference corresponding to the proton concentration difference required to reverse ATP hydrolysis through the ATPase system appears to be too large for this primitive protonmotive mechanism to work simply on the basis of the osmotic pressure of the protons. The mechanism was accordingly sophisticated by postulating that the coupling membrane has a low permeability to ions generally and not only to protons, so that the electron transport and ATPase systems could be coupled through the sum of the electrical pressure difference and the osmotic pressure difference (i.e. the electrochemical potential difference) of protons that would thus be conserved across the membrane. Under such

conditions it would be possible for the major part of the electrochemical potential difference to be due to the membrane potential, and there need be only a relatively small pH difference [16].

2.4. Exchange-diffusion systems

While the introduction of the foregoing sophistication solved one problem, it created another; for, the membrane potential that would now be required to reverse the ATPase reaction would cause the ions of opposite sign of charge to the internal aqueous phase to leak in through the coupling membrane. To prevent swelling and lysis, the ion leakage would have to be balanced by extrusion of ions against the electrical gradient. It was therefore necessary to postulate that the coupling membrane contains exchange diffusion systems—analogous to the system first described by Ussing [32]—that strictly couple the exchange of anions against OH^- ions and of cations against H^+ ions [16].

2.5. Sophistication of the anisotropic o/r system: The o/r loop

It appears, superficially, that the flow of electrons across the membrane resulting from the oxido-reductions in the electron transport chain, and the effective flow of protons across the membrane through the ATPase system during ATP synthesis, should represent a closed circuit if we assume complete ion-tightness of the coupling membrane. A closed circuit cannot, however, consist partly of hydroxyl ions or protons and partly of electrons. The apparent difficulty arises because we have not specified the directions of access of oxygen and substrate to the primitive o/r system illustrated in Fig. 1. When we resolve this ambiguity by showing both oxidant and reductant originating on the same side of the membrane, the o/r chain is effectively bent back on itself into an o/r loop as shown in Fig. 2.

It will be seen that this sophistication of Lundegardh's linear system translocates hydrogen groups one way and electrons the other way, and that it thus gives a net translocation of protons. The arms of the o/r loop in Fig. 2 are shown as circuits of hydrogen and electron carriers, to illustrate the mechanism by which the flows of hydrogen atoms and electrons may occur. For the sake of simplicity, the flows of the chemical particles are shown only by single lines in the other illustrations in this review. The diagrams should not be taken to signify partiality towards either the current flow or bimolecular interaction models of electron and hydrogen transfer as discussed by Chance et al. [33].

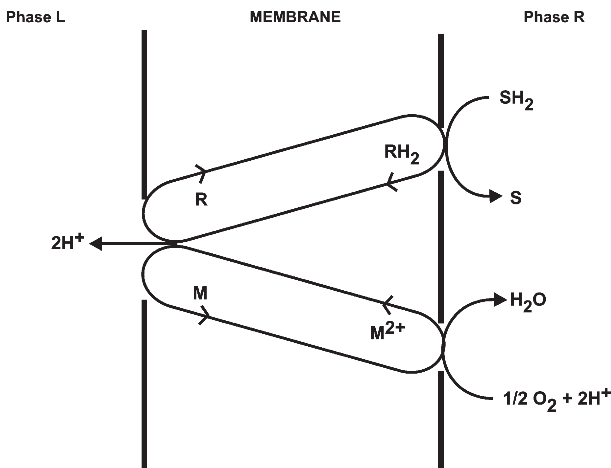


Fig. 2. Proton translocating oxido-reduction loop composed of a hydrogen carrier (R/RH_2) and an electron carrier (M/M^{2+}).

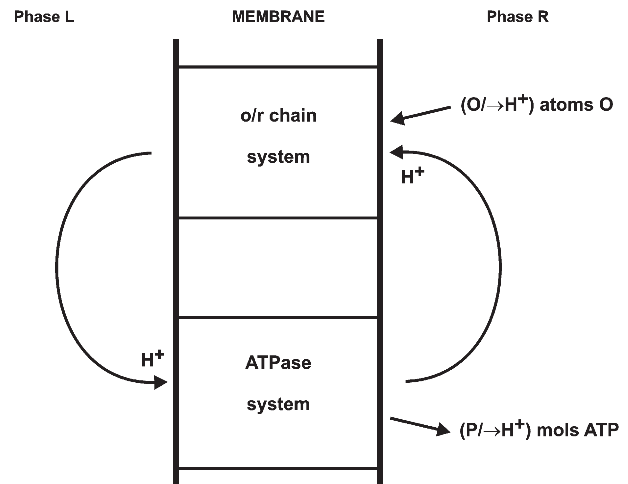


Fig. 3. Stoichiometry of chemiosmotic coupling. The circulation of one proton is caused by the utilisation of a certain number ($O/\rightarrow H^+$) of oxygen atoms, and causes the synthesis of a certain number ($P/\rightarrow H^+$) of ATP molecules. The P/O quotient is the product $(P/\rightarrow H^+)\times(\rightarrow H^+/O)$.

2.6. The coupling proton circuit and P/2e values

Fig. 3 illustrates the coupling between the respiratory chain o/r loop system and the reversible anisotropic ATPase system by means of the proton current flowing cyclically between them across the coupling membrane. It will be appreciated that the P/O or P/2e quotient of the chemiosmotic system depends upon the ratio of the number of protons translocated (written $\rightarrow H^+$) per ATP synthesised in the reversible ATPase system to the number of protons translocated per electron pair equivalent traversing the respiratory chain, or $P/2e = (P/\rightarrow H^+)x(\rightarrow H^+/O)$. As P/2e quotients depend on the substrate undergoing oxidation, it was necessary to postulate that the respiratory chain is looped across the membrane more than once, and that the stoichiometry of proton translocation depends upon the point at which the substrate feeds reducing equivalents into the chain. My original suggestion as to the arrangement of the respiratory chain is reproduced in Fig. 4, which shows the effective translocation of 3 protons per O for oxidation of an NAD-linked substrate, and 2 protons per O for oxidation of a substrate (e.g. succinate) utilising the part of the chain from flavoprotein (Fp) only. It should be noted that one of

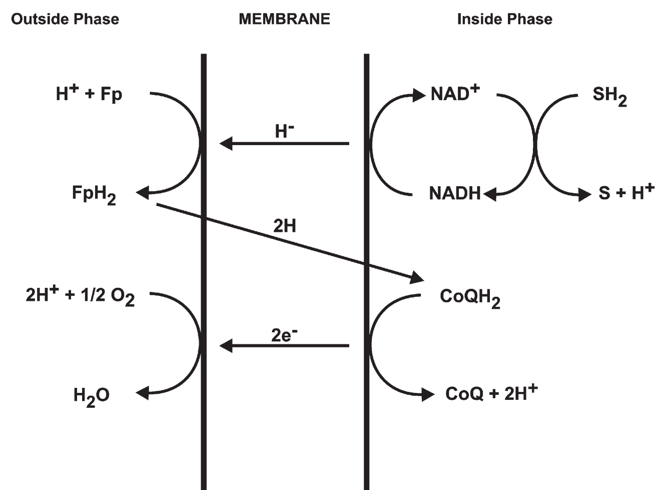


Fig. 4. Folding of respiratory chain as suggested by Mitchell [16]. Substrate (SH_2) is oxidised via nicotinamide-adenine dinucleotide (NAD). Flavoprotein (Fp), and coenzyme Q (CoQ), Succinate oxidation would utilise the part of the chain from Fp only.

the air loops of Fig. 4 is incomplete because the path of entry of the substrate, SH_2, into the system from the right is omitted from the diagram. The looped arrangement of the o/r chain in Fig. 4 makes use of a similar principle to that used by Davies [34,35] to explain the multiple secretion of H^+ ions in gastric mucosa; and we shall define and extend this principle in more detail in Section 4.

2.7. Polarity of the protonmotive force relative to the coupling membrane system

The proton translocating o/r and h/d systems of the chemiosmotic hypothesis might presumably be orientated in the coupling membrane so as to translocate protons either inwards or outwards relative to the closed side of the vesicular membrane system; but, provided that the o/r and h/d systems were orientated correctly relative to each other, the chemiosmotic coupling principle should not be dependent upon the direction of proton translocation. For the sake of argument, I originally depicted the o/r and h/d systems as translocating protons inwards during substrate oxidation and ATP hydrolysis in both mitochondria and chloroplasts [16]. Jagendorf and Hind [36], Neumann and Jagendorf [37], and Jagendorf and Neumann [38] confirmed that protons do, in fact, appear to pass in through the grana membrane of fragmented spinach chloroplasts during electron transport; and there is support for the view that ATP synthesis is coupled to the outward flow of protons through the reversible ATPase of the grana membrane [39]. In intact mitochondria from rat liver, however, it has been shown that protons are translocated outwards during substrate oxidation and during ATP hydrolysis ([17, 40]); and this also appears to be the direction of proton translocation in certain bacteria [41,42]. It would therefore seem that the anisotropic ATPase and respiratory chain systems may be orientated so as to translocate protons inwards through the chloroplast grana or lamellae, but outwards through the plasma membrane of certain bacteria and through the cristae membrane of intact mitochondria.

2.8. Summary of the basic postulates

It will now be useful to summarise the basis of the chemiosmotic coupling hypothesis in the form of four essential postulates; for, these postulates can be used, on the one hand, for the further development of the theory of chemiosmotic coupling, and on the other hand, as the target for critical experiments designed to show that the chemiosmotic hypothesis may be untenable.

1. The membrane-located ATPase systems of mitochondria and chloroplasts are hydro-dehydration systems with terminal specificities for water and ATP; and their normal function is to couple reversibly the translocation of protons across the membrane to the flow of anhydro-bond equivalents between water and the couple ATP/(ADP + P_i).
2. The membrane-located oxido-reduction chain systems of mitochondria and chloroplasts catalyse the flow of reducing equivalents, such as hydrogen groups and electron pairs, between substrates of different oxido-reduction potential; and their normal function is to couple reversibly the translocation of protons across the membrane to the flow of reducing equivalents during oxido-reduction.
3. There are present in the membrane of mitochondria and chloroplasts substrate-specific exchange-diffusion carrier systems that permit the effective reversible trans-membrane exchange of anions against OH^- ions and of cations against H^+ ions; and the normal function of these systems is to regulate the pH and osmotic differential across the membrane, and to permit entry and exit of essential metabolites (e.g. substrates and phosphate acceptor) without collapse of the membrane potential.
4. The systems of postulates 1, 2, and 3 are located in a specialised coupling membrane which has a low permeability to protons and to anions and cations generally.

3. The proton translocating ATPase system

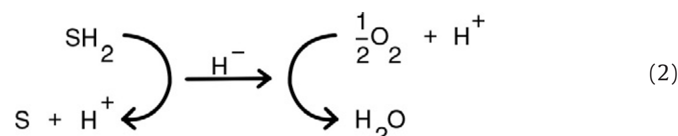
3.1. Proton translocating hydro-dehydration reactions

Direct measurements of proton translocation driven by ATP hydrolysis in intact rat liver mitochondria have recently shown that very nearly 2 protons are translocated outwards per ATP hydrolysed [40]. The simple anisotropic ATPase system outlined in the original version of my hypothesis was intended to account for the translocation of only one proton per ATP hydrolysed [16]. We shall therefore proceed to develop the concept of the anisotropic ATPase system in more general terms, and also in a more detailed chemical idiom than before.

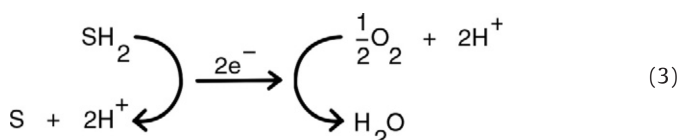
As intimated in the previous section, the general mechanism of coupling between hydrodehydration and proton translocation can be neatly described by writing h/d reactions as pairs of half reactions connected by the flow of OH^- or O^{2-} groups in much the same way as one writes o/r reactions as half reactions connected by the flow of hydride ions or electrons. The o/r reaction,

$$SH_2 + \frac{1}{2}O_2 \rightarrow S + H_2O \quad (1)$$

can be written vectorially as a pair of monoelectrogenic half reactions between left hand and right hand phases, connected by the flow of hydride ions,



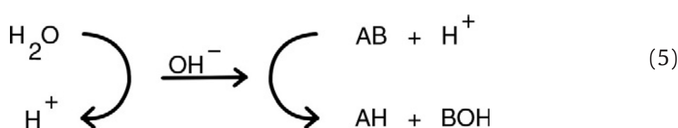
or as a pair of di-electrogenic half reactions connected by the flow of pairs of electrons,



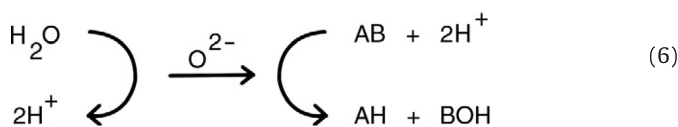
Similarly, the h/d reaction,

$$AB + H_2O \rightarrow AH + BOH \quad (4)$$

can be written vectorially as a pair of monoelectrogenic half reactions, connected by the flow of hydroxyl ions,



or as a pair of di-electrogenic half reactions connected by the flow of O^{2-} groups,



It will be appreciated that, as we are considering aqueous phases on the left and right hand sides, the flow of OH^- or O^{2-} one way is equivalent to the flow of H_2O the same way and one or two protons respectively the other way. For the present purposes, attention will

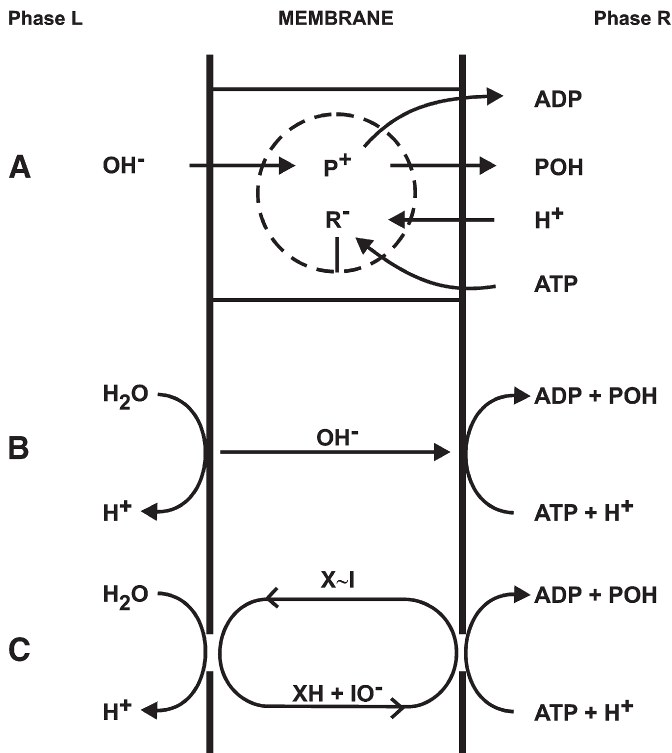


Fig. 5. Proton translocating reversible ATPase system of type I: A, original version [16]; B, as half reactions; C, mechanism via the anhydride, X–I.

be focused upon the equivalent proton flows, and we shall ignore the compensating flows of H_2O until a later stage of the argument.

Comparison of A and B, Fig. 5, shows that the mono-electrogenic ATPase reaction described as half reactions connected by OH^- translocation (type I ATPase or ATPase I) is formally equivalent to my original description of the anisotropic ATPase. Fig. 6A shows the closely analogous di-electrogenic ATPase reaction as half reactions connected by O^{2-} translocation (ATPase II).

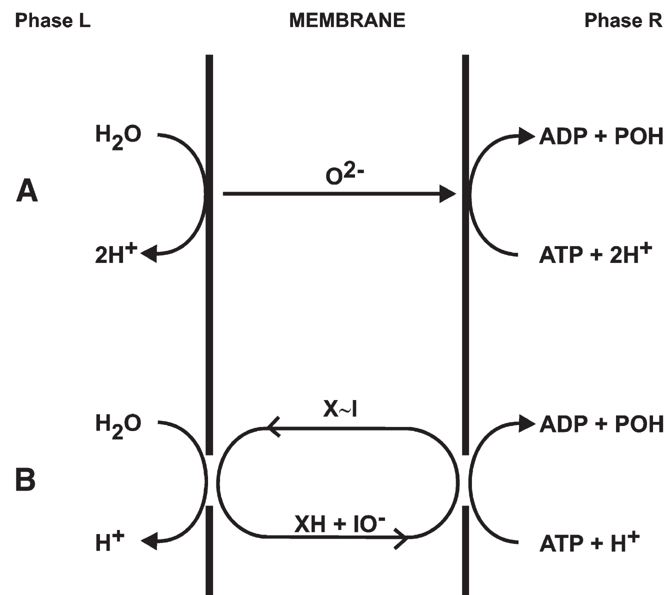


Fig. 6. Proton translocating reversible ATPase system of type II: A, as half reactions; B, mechanism via the anhydride, X–I.

3.2. ATPase I and ATPase II: Mechanism and poise of equilibrium

We are now in a position to consider how the translocation of OH^- or O^{2-} through the ATPase system might be catalysed specifically by an appropriate chemical mechanism. Figs. 5C and 6B indicate what is probably the simplest type of translocation mechanism consistent with present knowledge. These diagrams suggest essentially that OH^- or O^{2-} could be translocated to the right by the flows of the groups $XH + IO^-$ or $X^- + IO^-$ respectively to the right, and the return flow of the corresponding anhydride X~I to the left. The suggested mechanisms for the ATPases I and II are thus essentially similar, and the more detailed treatment of the ATPase II system which follows can be applied to ATPase I with appropriate quantitative adjustments.

The overall ATPase II reaction can be represented as

$$ATP + H_2O + 2H_R^+ \rightleftharpoons ADP + POH + 2H_L^+ \tag{7}$$

The suffixes L and R stand for the aqueous phases on the left and right of the coupling membrane respectively. Denoting electrochemical activities by curly brackets and writing inorganic phosphate as POH, the normal hydrolysis equilibrium for ATP can be written

$$\frac{\{ADP\} \times \{POH\}}{\{ATP\}} = K'\{H_2O\}_{aq}. \tag{8}$$

The electrochemical activity of water in the aqueous phases L and R is represented as $\{H_2O\}_{aq}$, and the product $K'\{H_2O\}_{aq}$ is equal to the hydrolytic constant as normally defined. When the hydrolytic reaction is strictly coupled to the translocation of 2 protons from phase R to phase L per ATP hydrolysed as described by Eq. (7),

$$\frac{\{ADP\} \times \{POH\}}{\{ATP\}} = K'\{H_2O\}_{aq} \frac{\{H^+\}_R^2}{\{H^+\}_L^2} \tag{9}$$

provided that ADP, POH, and ATP all participate in the equilibrium from the same phase. When there is a membrane potential of ΔE millivolts (mV) between phases L and R, positive in phase L, by definition of pH and electrochemical potential,

$$\log_{10} \frac{\{H^+\}_L}{\{H^+\}_R} = pH_R - pH_L + \frac{\Delta E}{Z} \tag{10}$$

where Z = 2303RT/F, F is the Faraday, and R is the gas constant. From Eqs. (9) and (10),

$$\log_{10} \frac{\{ATP\}_L}{\{ATP\}_L\{POH\}_L} = 2\left(pH_R - pH_L + \frac{\Delta E}{Z}\right) - \log_{10}\left(K'\{H_2O\}_{aq}\right). \tag{11}$$

At 300°K, Z is close to 60 mV, and the hydrolysis constant for ATP is close to 5 [19,20]; and hence, substituting ΔpH for $pH_L - pH_R$, Eq. (11) can be written approximately as

$$\log_{10} \frac{\{ATP\}_L}{\{ADP\}_L} \simeq \log_{10}\{POH\}_L - 2\Delta pH + \frac{\Delta E}{30} - 5. \tag{12}$$

As the electrochemical activities of ATP, ADP, and POH all refer to the same phase, they can be approximately equated with concentrations (written [ ]), and assuming [POH] to be 0.01 M,

$$\log_{10} \frac{\{ATP\}}{\{ADP\}} \simeq \frac{\Delta E}{30} - 2\Delta pH - 7 \tag{13}$$

The electrochemical potential difference of protons necessary to poise the ATP/ADP couple central under these conditions is thus given, in mV, by

$$\Delta E - 60\Delta pH \doteq 210 \quad (14)$$

In other words, a pH differential alone of 3.5 units (acid in phase L), or a membrane potential alone of 210 mV, or a combination, such as a pH differential of one unit and a membrane potential of 150 mV, would poise the ATP/ADP couple central under these conditions. We may conveniently refer to the electrochemical potential difference of protons across the coupling membrane as the proton motive force (by analogy with electromotive force or E.M.F.). A convenient unit for the P.M.F. is the millivolt; but we must bear in mind that the P.M.F. includes the osmotic component of the electrochemical potential difference of protons across the membrane, and that it is therefore different from the membrane potential by an amount corresponding to the pH differential or, writing Δp for the P.M.F.,

$$\Delta p \doteq \Delta E - 60\Delta pH \quad (15)$$

In the case of the ATPase I system of Fig. 5, the P.M.F. required to poise the ATP/ADP centrally is twice as great (e.g. 420 mV or −7 pH units) as in the case of the ATPase II system of Fig. 6 (see [16,18]).

3.3. Further discussion of ATPase II

The mechanism suggested for the reversible anisotropic ATPase II reaction in Fig. 6 is illustrated in more detail in Fig. 7, which shows the h/d system catalysing ATP synthesis. This scheme represents the translocational and chemical transitions of the acidic groups XH and IOH supposed to be involved in the active centre regions of the ATPase enzyme complex. Both the chemical transitions and the translocational transitions are assumed to occur spontaneously as reversible diffusional movements within the catalytic system. It must be understood that Fig. 7 shows the diffusional movements of the groups X and I relative to the ATPase enzyme complex in a highly diagrammatic form. For example, the groups X and I may move over only very small distances compared with the total thickness of the coupling membrane during the catalytic activity of the system (see [29]). The transitions are represented cyclically, and the arrows point in the forward direction of ATP synthesis for the sake of formal clarity only. The actual direction of the reversible transitions would depend upon the poise of the electrochemical potentials of the components feeding into and out of the system. The ATPase is represented in three functional parts, A, B, and C, corresponding respectively to X–I hydrolase, X–I translocase, and X–I synthetase, but the functional "parts" need not correspond to physically separable enzyme particles. We shall discuss later in this section the possible relationship between the functional parts of the ATPase of Fig. 7 and the physically separable parts of the ATPase systems of mitochondria and chloroplasts.

There are two main criteria by which one can judge whether hypothetical mechanisms, such as that of Fig. 7, might work in practice. (i) The system must represent the correct stoichiometry, so that the overall reaction would naturally poise at the known thermodynamic equilibrium; (ii) The intermediates or transition states through which the components of the system would pass as equilibrium was approached should all occur in sufficient concentration (or be sufficiently probable) to permit the reactions to proceed at the known overall reaction velocity. Using these criteria, we can appreciate that the proposed intermediate X–I of the ATPase must have a sufficiently low hydrolysis constant at the high electrochemical potential of H^+ in phase L to come into reversible equilibrium with water according to the reaction,

$$X{-}I + H_2O \rightleftharpoons XH + IOH \quad (16)$$

like an ester having a standard free energy of hydrolysis of some −3,000 cal or a hydrolysis constant of about 10^2 M. On the other hand, the intermediate X~I must come into equilibrium with the ATP/(ADP + POH) couple in phase R through the reaction,

$$X{\sim}I + POH + ADP \rightleftharpoons X^- + IO^- + 2H^+ + ATP \quad (17)$$

so that, at the low electrochemical potential of H^+ in phase R, X~I must have a standard free energy of hydrolysis equivalent to some −10,000 cal, or a hydrolysis constant not far from 10^5 M. This implies that, as the system vibrates between states in which the X–I

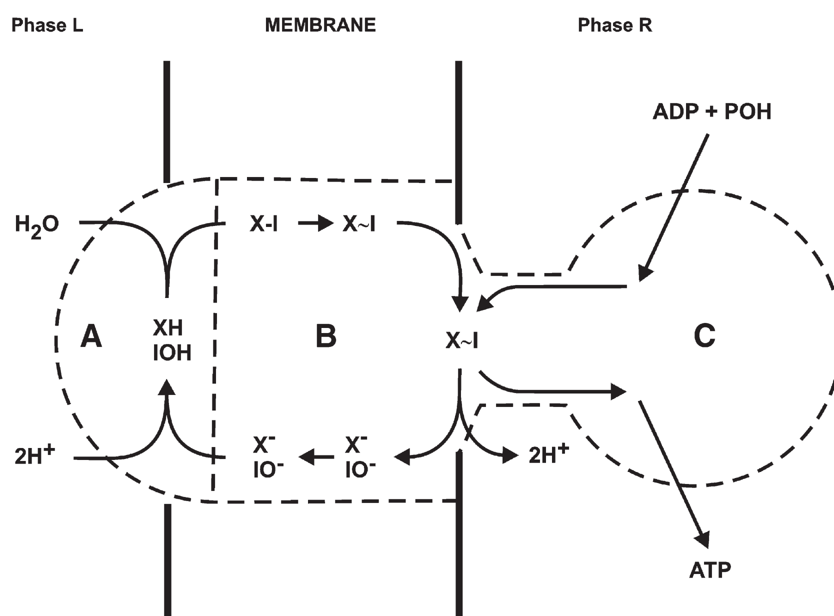


Fig. 7. Diagram of ATPase II showing suggested functional regions: A, X–I hydrolase: B, X–I translocase: C, X–I synthetase. Regions A plus B may be identified approximately with F_O, and region C with F_1 or with F_1 plus inhibitor. In intact mitochondria, phase R would be the matrix or internal cristae phase, whereas in chloroplasts, phase L would be the internal grana phase. The arrows point in the directions corresponding to ATP synthesis.

anhydride is alternately accessible to phases L and R, the anhydride must change reversibly from being X–I when in contact with phase L to X~I when in contact with phase R, and the so-called hydrolysis constant must vary accordingly. At first sight it may seem to the reader that it would be impossible for the X–I to X~I transition to occur spontaneously in this way. We can appreciate, however, that the X–I to X~I transition could indeed occur as depicted, by analogy with the dependence of the free energy (ΔG) of hydrolysis of an anhydride X–I on $\{H^+\}$ in an isotropic system. In the case of an anhydride that hydrolyses to give two acidic groups, such as XH and IOH, provided that the pH slope of the entropy were not very large (see [43]) the pH slope of the −ΔG of hydrolysis, above the effective pK values of the two acidic groups, would be approximately 2.303×2RT cal (or about 2800 cal) per pH unit. We can therefore think of the transition of X–I to X~I in the ATPase of Fig. 7 as being due, not effectively to the pumping of energy into X–I to convert it to X~I, but rather to the lowering of the ground state energy for X–I hydrolysis by some 10,000 cal on going from phase L to phase R. In the absence of overwhelming entropy effects, it would be reasonable to suppose that the operation of the simple form of ATPase mechanism shown in Fig. 7 would require the effective pK values of the acidic groups XH and IOH to be a little below the normal pH of phase L, or in the region of pH 6. The driving force on the X–I synthetase reaction described by Eq. (17) would be due, in part, to the low concentration of H^+, and in part to the low concentrations of X^- and IO^- on the right side of the membrane. The low local concentration of H^+ in phase R would be due both to the positive electric potential relative to more distant parts of this phase and to the relatively low "bulk" level of H^+ ion concentration in this phase maintained by the respiratory chain system. But the low concentration of X^- and IO^- in phase R would be due only to the tendency of the negative potential in this phase to force these negatively charged groups to the left across the membrane.

3.4. Practical reversal of the ATPase reaction

3.4.1. Spatially isotropic conditions

It is evident from the foregoing discussion that the reversible kinetic function of the hypothetical anisotropic ATPase system under the influence of the appropriate P.M.F. would not have a close counterpart in an isotropic system, containing, for example, the isolated ATPase, free of osmotically functional membrane. On the other hand, thermodynamic considerations suggest that the ATP-synthesising function of the ATPase might possibly be reproducible under spatially isotropic conditions, if the equivalent of the spatial anisotropy were imposed on the time dimension. For example, by alternating the spatially isotropic system between appropriate extremes of proton activity, one might expect, for each cycle of alternation, a maximum ATP synthesis corresponding to the X–I anhydride content of the enzyme system at acid pH. The author has discussed the feasibility of such experiments with Dr. E. Racker, and exploratory work is now under way in the latter's laboratory.

3.4.2. Spatially anisotropic conditions

According to the chemiosmotic hypothesis, the h/d or ATPase system of mitochondria and chloroplasts is coupled to the o/r or respiratory chain system by means of the proton current, and there is no chemical link between the o/r and h/d systems. Equilibrium between ATP and water should be catalysed by the ATPase at a fairly central poise of the ATP/(ADP + P_i) couple when balanced by the appropriate P.M.F., independently of the source of the P.M.F. It follows that, in the case of ATPase II, if a P.M.F. corresponding to 200 to 250 mV or −3 to −4 pH unit were artificially imposed for a short time—for example, by equilibrating a suspension of mitochondria at a high pH and suddenly lowering the pH in presence of ADP + P_i, or vice versa in the case of a chloroplast suspension—there should be a brief synthesis of ATP; and the maximum possible amount of synthesis should be equal to half the number of protons titrating across the membrane during the pH differential decay. In the case of ATPase I, twice as much ATP could theoretically be synthesised, but twice the P.M.F. (i.e. about 450 mV or −7 pH units) would be required to poise the ATP/(ADP + P_i) couple in favour of ATP synthesis.

During the investigation of two stage photosynthetic phosphorylation in spinach chloroplast preparations, which happened to require an acid medium for the first energy-accepting (light) stage and an alkaline medium containing ADP and P_i for the second (dark) stage, Hind and Jagendorf [44] noticed that, when the pH values of the two stages were respectively 4.6 and 8.0, controls which had not been illuminated in the first stage synthesised a considerable amount of ATP in the second stage. This important accidental observation is relevant to the predictions of the chemiosmotic hypothesis, and has led to a more detailed study of acid/base dark phosphorylation in spinach chloroplasts [39], from which the authors conclude that the chemiosmotic explanation of the phosphorylation mechanism compares favourably with other possible explanations. Jagendorf and Uribe [39] observed that the amount of ATP synthesised by the chloroplasts was more dependent upon the range over which the pH was changed from the acid medium of the first stage to the alkaline medium containing ADP + P_i in the second stage, than upon the absolute initial and final pH values—implying that ATP synthesis was due to the pH differential established briefly across the membrane rather than to the pH change of the whole system. The amount of ATP synthesised was much increased by the presence of a wide range of anionic buffers including, for example, succinate and phthalate, which may act mainly by increasing the total proton flux across the grana membrane. Under appropriate conditions, pH shifts of 4.0 and 3.0 units respectively produced about 200 μmol and 40 μmol ATP per mg chlorophyll, when the concentration of P_i was about 1.0 mM and the ratio of [ATP]/[ADP] was respectively about 0.3 and 0.05 at the termination of ATP synthesis. These [ATP]/[ADP] ratios compare with theoretical maximum ratios for ATPase II of about 1.0 and 0.01 respectively calculated from Eq. (11), assuming the site of ATP synthesis to be at about pH 7 during the proton flow across the ATPase system. This, and other similar comparisons between the observations and the predicted behaviour based on ATPase II, show that there is moderately good agreement. Further, the observations could not possibly be accounted for by the activity of an ATPase of type I. Jagendorf and Uribe [39] have pointed out that the amount of ATP that can be synthesised is equivalent to some 100 ATP per cytochrome f, thus making it unlikely that synthesis could occur via an energy-rich carrier intermediate unless the intermediate were cyclically involved in oxido-reduction or hydro-dehydration, driven somehow by the pH gradient.

It is of interest to try to estimate whether the proton flux through the membrane could account for the observed ATP synthesis. For example, about 40 μmol ATP were found to be synthesised per g chloroplast protein when the pH shift was from pH 4 to pH 8 and 10 mM succinate was present in the acid stage. The titration of succinate from pH 4 to pH 8 involves the neutralisation of about 1.5 proton equivalents per mole succinate. Assuming that the succinate would have equilibrated across the grana membrane during the initial acid stage (50% of it would be unionised), and that it would diffuse out slowly in comparison to the rate of ATP synthesis during the second (alkaline outside) stage, the proton flux due to the neutralisation of the succinate would be 15 U μmol/g chloroplast protein, where U is the volume (in ml/g protein) of the internal water space of the grana in the acid stage. If the grana were rather swollen so that U was, say 10 or 20 ml/g protein, the proton flux of some 150 to 300 μequiv/g protein might be sufficient to give a flux of 80 μequiv protons through ATPase II to yield 40 μequiv ATP. If the grana had an internal volume of, say 5 ml/g protein, the flux of only some 75 μequiv protons/g protein would not account for the observed ATP synthesis in terms of the buffering power of the succinate. The

internal buffering power of the grana discs themselves is not known. The relevant experimental information concerning the internal volume of the grana and their internal buffering power would help considerably in the interpretation of the mechanism of acid/base phosphorylation.

Jagendorf and Uribe [39] have pointed out that the potent inhibitory effect of ethylenediamine tetraacetate (EDTA) on acid/base phosphorylation is in keeping with the fact that EDTA is known to remove the chloroplast coupling factor [45] which appears to be a component of the membrane-located chloroplast ATPase system [46,47]. McCarty and Racker [48] have confirmed that spinach chloroplasts catalyse an acid/base dark phosphorylation of ADP, and have shown that it is specifically inhibited by an antiserum to the chloroplast coupling factor and by the antibiotic Dio-9 which affects chloroplasts in much the same way as oligomycin affects mitochondria [49]. The evidence invites the simple interpretation that the acid/base phosphorylation of ADP by chloroplasts is catalysed by a membrane located ATPase system which has similar properties to the hypothetical ATPase II.

At the time of writing, experiments designed to detect ATP synthesis induced by a pH gradient across the coupling membrane of mitochondria and submitochondrial particles are in progress in several laboratories, and I have privately received some preliminary reports of negative results. In such experiments, it is important to appreciate that if the coupling membrane has a low permeability to anions and cations, as postulated, the flow of protons through the ATPase system, resulting from the establishment of an artificial pH differential, would build up an opposing membrane potential unless there were an electrically compensating flow of one or more other ionic species. In this respect the experiments of Jagendorf's group are not completely understood, and it may be that their success depends upon the grana membrane of fragmented chloroplast preparations being artificially permeable to certain ions (see [50]). At all events, it is essential to recognise the following criteria in designing experiments intended to test the chemiosmotic synthesis of ATP via the membrane-located ATPase system: (i) The driving force on ATP synthesis is the P.M.F. and not the pH differential (see Eq. (12)); (ii) A P.M.F. of some 210 mV (equivalent to −3.5 pH units with no membrane potential) should be required to drive ATP synthesis at a central poise of the ATP/ADP couple via ATPase II in the presence of 10 mM P_i (iii) The amount of ATP synthesised via ATPase II should be given by the total number of protons passing across the coupling membrane at a P.M.F. sufficient to drive ATP synthesis at the existing ATP/ADP poise, multiplied by the proportion of the total proton flux which passes specifically through the ATPase system; (iv) The synthesis of ATP, driven by a pH differential, may be stimulated by specific reagents, such as gramicidin or valinomycin, that can collapse the membrane potential without collapsing the pH differential (see Section 7.2.1).

It follows from the third criterion above that ATP synthesis, driven by an artificial pH differential, should be susceptible to uncoupling by the classical proton conducting reagents such as nitrophenols or carbonyl cyanide phenylhydrazones (see Sections 7.2.3 and 7.2.4), and Jagendorf and Uribe [39] have shown that this is, in fact, the case for the fragmented spinach chloroplast system.

3.5. Coupling factors and the ATPase system

It was discovered by two different research groups at about the same time [51,52] that the disintegration of beef heart mitochondria by sonic oscillation in the presence of EDTA [53], or by shaking with glass beads [54,55], releases soluble coupling factor protein that can restore phosphorylating activity to the respiratory chain of depleted mitochondrial fragments under appropriate conditions. The best characterised of the coupling factor material so far obtained from such experiments is a protein of molecular weight 280,000, called F_1 which exhibits a Mg^{2+}-dependent ATPase activity [11,56,57], A considerable proportion of the F_1 extracted from mitochondria [54] may exhibit little or no ATPase activity because of the presence of several different inhibitory substances [11,56,58] which prevent expression of ATPase, but stabilise and do not inhibit coupling activity, One of these inhibitors was shown by Pullman and Monroy [59] to be a polypeptide of molecular weight about 15,000, which becomes firmly attached to F_1, Another protein, F_2, which contributes to the restoration of phosphorylation in depleted particles [11] may also be closely associated with F_1, Pullman and Monroy [59] have summarised the view of their research group as to the function of F_1 in the following terms. "The course of this work was guided by the hypothesis that hydrolysis of ATP by F_1 represented a reversal of the terminal transphosphorylation reaction of respiratory chain phosphorylation, followed by hydrolysis of the intermediate. This hypothesis included the consideration that in intact mitochondria the hydrolytic potentiality of the enzyme is suppressed and ADP is the obligatory acceptor of the phosphate group. Accordingly, the ATPase activity of F_1 is not essential to its coupling activity and in effect represents an abnormal activity occurring as a result of its displacement from the structural organization of the mitochondria."

It seems to be reasonable to suggest that the function of the F_1 or F_1-inhibitor fragment (possibly in association with F_2) corresponds to the X–I synthetase of my hypothesis (see Fig. 7), catalysing the reaction,

$$XH + IOH + ATP \rightleftharpoons XI + POH + ADP \quad (18)$$

and that when the ATPase activity of F_1 is expressed, the elements of H_2O artificially take the place of XH and IOH in reaction 18. In recent electron micrograph studies [11,60,61] the factor F_1 (possibly in association with F_2) has been tentatively identified with the characteristic spherical units that appear to be attached by stalks to the inner side of the cristae membrane of whole mitochondria, and to the outer side of the cristae membrane of sonically disintegrated mitochondria [62–65].

After isolation, the ATPase activity of F_1 has been shown to be oligomycin-insensitive, but sensitivity to oligomycin can be restored by adsorption of F_1 on sonically prepared beef heart mitochondrial particles, or by combination with a "comminuted membrane particle", called F_O, that can be isolated by sonic disintegration of mitochondrial particles after trypsin and urea treatment [66,67]. Treatment of F_O alone with oligomycin will inhibit the ATPase activity when the F_O–F_1 complex is subsequently formed, and F_1, exhibiting normal ATPase activity, can be recovered from the oligomycin-inhibited F_O–F_1 complex [66]. It seems probable, therefore, that oligomycin combines with a component of F_O in the F_O–F_1 complex and that F_O must somehow be an essential functional participant in the ATPase activity of the complex. The generally accepted view of the coupling mechanism in oxidative phosphorylation requires, of course, that the reversible ATPase activity of the coupling factor complex should be chemically coupled to oxido-reduction in the respiratory chain. The original preparations of F_O [67] contained "the entire chain of respiratory catalysts", phospholipid, and factors such as the structural protein or F_4 [68,69]. The fact that a reconstituted system of oxidative phosphorylation could be obtained with a combination of F_O, F_1 and F_2 [67] suggested that the o/r components of F_O were chemically coupled to the reversal of the ATPase activity of F_1 [11], and that "although the F_O preparation is still very crude, it represents the most highly degraded form of an actively phosphorylating respiratory chain that has been obtained thus far" [67]. The observation that F_O confers oligomycin sensitivity on F_1 could thus be interpreted as a confirmation of the idea that oligomycin inhibits the "phosphorylating respiratory chain" or the "energy-linked respiratory chain" (involving the non-phosphorylated high-energy compounds, $C_1{\sim}I_1$ etc.) but not the "non energy-linked respiratory chain" (see [11]).

According to the chemiosmotic view of the coupling mechanism the so-called "energy-linked respiratory chain" and the "non energy-linked respiratory chain" are chemically identical, and there is no chemical connection between the reversible ATPase system and the respiratory chain system. Accordingly, the inhibitory action of oligomycin on the reversible ATPase need not be dependent upon the chemically separate o/r processes of the respiratory chain, and one need not wish to implicate respiratory chain carriers in the relationship between F_1 and F_O. Dr. E. Racker has recently informed me that he and Dr. Y. Kagawa have succeeded in reducing the phospholipid and respiratory enzyme content of F_O preparations to low levels by treatment with cholate. The new preparations of cholate-treated F_O confer oligomycin- sensitivity on the ATPase activity of F_1 in presence of phospholipid, and it seems unlikely that any components of the respiratory chain could be involved in the functional relationship between F_O and F_1.

The facts at present available seem to suggest that part of the F_O complex of the cristae membrane may correspond to the X–I translocase of my hypothesis and part to the X–I hydrolase (Fig. 7), and that the site of oligomycin inhibition is located in the X–I translocase or X–I hydrolase functional regions of the ATPase II system. Thus, we would consider the oligomycin insensitive ATPase reaction catalysed by isolated F_1 to compare with Eq. (18),

$$H_2O + ATP \rightarrow POH + ADP \quad (18A)$$

while the oligomycin-sensitive ATPase of the complete ATPase II system would be represented by

$$XH_R + IOH_R + ATP \rightarrow XI_R + POH + ADP \quad (18)$$

$$XI_R \rightarrow XI_L \quad (19)$$

$$H_2O + XI_L \rightarrow X_L^- + IO_L^- + 2H_L^+ \quad (20)$$

$$2H_R^+ + X_L^- + IO_L^- \rightarrow XH_R + IOH_R \quad (21)$$

$$\text{Sum}: 2H_R^+ + H_2O + ATP \rightarrow 2H_L^+ + POH + ADP \quad (22)$$

It has been shown that the $^{32}P_i$–ATP exchange reaction of intact mitochondria and phosphorylating mitochondrial particles is not catalysed by F_1 or by depleted particles of cristae membrane, but that it reappears when F_1 is readsorbed by the depleted particles under conditions corresponding to those required for coupled phosphorylation, but without oxido-reducible substrates [54]. This would be consistent with the view that F_1 corresponds to the X–I synthetase and determines the terminal nucleotide specificity of the ATPase system of my hypothesis; but that the $^{32}P_i$–ATP exchange can occur only when the complete ATPase of Fig. 7 is present in a membrane system across which a P.M.F. in equilibrium with a significant concentration of X–I can be conserved. It is interesting to note that the X–I synthetase reaction of my hypothesis need not involve an X–P intermediate of significant half life, and that a significant ^{14}C–ATP–ADP exchange reaction is not, therefore, obligatory [70]. The fact that ADP must be present to enable arsenate to stimulate respiration in intact mitochondria [71], and that the ATP-hydrolysing activity of non-respiring mitochondria is not appreciably stimulated by arsenate (see [72]) would also be in conformity with my formulation of the ATPase reaction.

There is relatively little information about the membrane-located ATPase systems of chloroplasts, but the facts available encourage the suggestion that these systems may turn out to be fundamentally similar to the mitochondrial ATPase [46,47,73]. It is of interest that the chromatophores of *Rhodospirillum rubrum* contain an oligomycin-sensitive ATPase [74], and that the ATP–P_i and ADP–ATP exchange reactions catalysed by *R. rubrum* chromatophores resemble those of mitochondria, suggesting that phosphorylation is catalysed by the ATPase operating in reverse [75]. Virtually nothing is known of the possible relationship between ATPases and oxidative phosphorylation in bacteria (see [76]).

4. The proton translocating oxido-reduction chain

4.1. The o/r loop: Further development

The translocation of charge by oxido-reduction depends fundamentally upon the o/r reaction being heterolytic so that it can occur as an electron transport between two half reactions. The concept of the proton translocating o/r loop, which has evolved from the linear electron translocating system, as described in Section 2.5, depends not only upon the heterolytic property of the o/r reactions, but also upon a transition between different currencies of oxido-reduction at the junction between the two arms of the o/r loop system. Some of the main divalent o/r currencies in the respiratory chain can be represented as $2e^-$, H^-, and 2H (corresponding to the zero, one or two hydrogen transfer characteristics described in the original version of my hypothesis); and Fig. 8 shows three possible o/r loops that can be constructed using pairs of these currencies. It will be noted that the type I loops translocate one proton in the 2 electron equivalent o/r process, while the type II loop translocates two protons in the 2 electron equivalent o/r process.

It should be appreciated that the coupling between o/r reactions and the translocation processes must be specified on the one hand by the uniqueness of the oxido-reduction changes, channelled by the chemical catalytic function of the enzymes and catalytic carriers, and on the other hand by the uniqueness of the translocation reactions, channelled by the physical catalytic function of the enzymes and catalytic carriers in the coupling membrane. Owing to this dual specification, the overall translocation reaction catalysed across a given o/r loop would not be directly determined by the chemical changes corresponding to the oxido-reduction reactions, but would be determined indirectly by these changes through the mediation of the

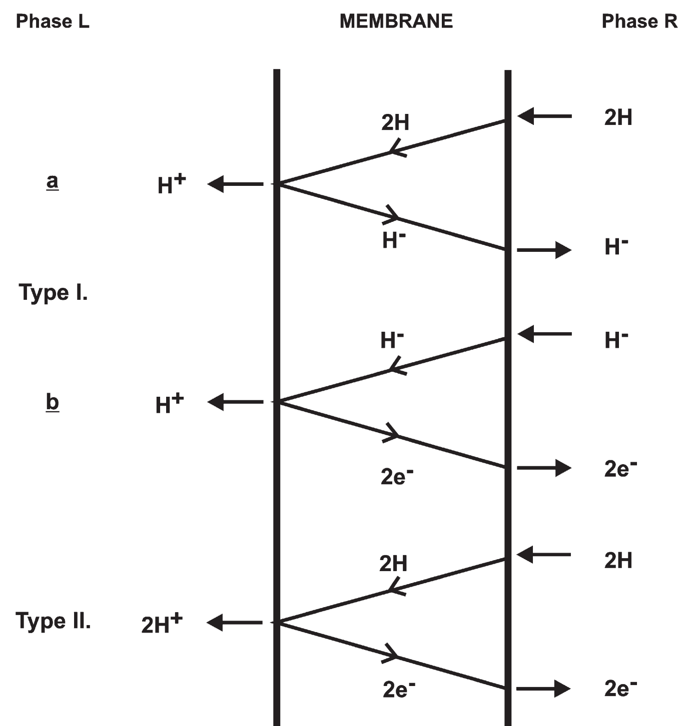


Fig. 8. Possible proton translocating oxido-reduction loops of type I and type II, translocating one and two protons respectively per two electron transfer equivalent.

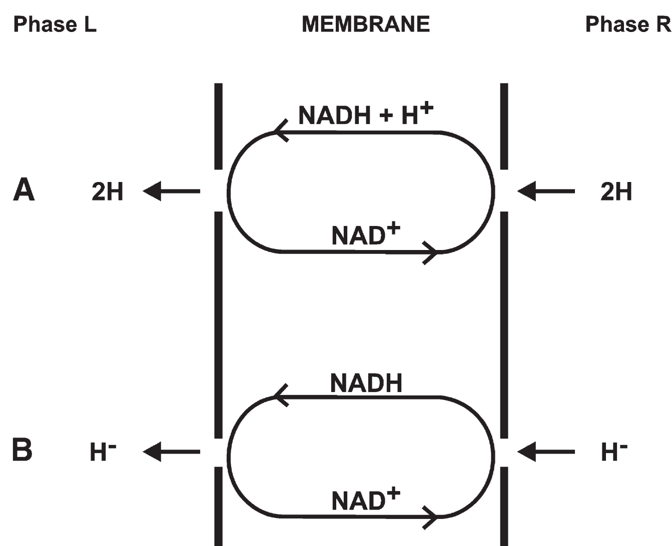


Fig. 9. Effect of translocational specificity upon the currency of oxido-reduction: A, specificity for 2H groups: B, specificity for H^- ions.

translocational specificities of the catalytic system. For example, the o/r couple $NAD^+/(NADH + H^+)$ could act as a carrier of hydrogen groups (2H) or of hydride ions (H^-), depending upon whether the essentially cyclic translocation process catalysed was that of ($NADH + H^+$ minus NAD^+) translocation, as shown in Fig. 9A, or (NADH minus NAD^+) translocation, as shown in Fig. 9B. The general principle of the proton translocating o/r loop depends upon the differential flows of hydrogen atoms (or their equivalent) and electrons in the two arms of the loop. In the least sophisticated case this differential would be determined only by the electrovalency–covalency changes of the two o/r couples, the neutralising ions arising from electrovalency changes, such as OH^- in the reaction, $2Fe^{2+} + 1/2O_2 + H_2O \rightarrow 2Fe^{3+} + 2OH^-$, or H^+ in the reaction, $SH_2 + M^{2+} \rightarrow S + M + 2H^+$, being excluded from participation in the translocation reactions by the catalytic carrier specificities. However, the cases in which, for example, reduction of a substrate results in the effective formation of a strong acid that can be internally neutralised, such as NAD^+ to ($NADH + H^+$) as considered above, or of a weak acid that may or may not ionize, such as R_1–S–S–R_2 (R_1–SH, R_2–SH), permit a more sophisticated approach to the decisive function of the translocational specificities. The translocation of R_1–S–S–R_2 minus (R_1–S^-,R_2–S^-) could, for example, account for electron translocation in the lower arm of the type II loop of Fig. 8. It is not proposed to develop these considerations further at the present stage of presentation of my hypothesis, but it is clear that this, more sophisticated, aspect of the chemiosmotic concept would be important should a further development become warranted. The work done in translocating n equivalents of protons from right to left across the coupling membrane is given by

$$\Delta G = nRT \cdot \ln \frac{\{H^+\}_L}{\{H^+\}_R} \tag{23}$$

where { } means electrochemical activity, and L and R refer to the left and right hand phases respectively. Writing the electrochemical potential difference of protons as the P.M.F. in mV,

$$\Delta G = nZ\Delta p \tag{24}$$

where Z = 2303RT/F, and Δp is the P.M.F., positive on the left. As the terminal oxidant and reductant of the o/r loop systems are in the same aqueous phase, the work done in transferring n protons at equilibrium can be equated to the work done by the transport of an electron pair equivalent across the loop having an o/r potential span of ΔE′,

$$\Delta G = nZ\Delta p = 2Z\Delta E' \tag{25}$$

or

$$\Delta p = \frac{2}{n}\Delta E' \tag{26}$$

where the sign of ΔE′ is written positive for a loop in which the more hydrogen-rich of the two currencies of oxido-reduction is nearer the substrate end of the chain, and the junction between the currencies is on the left. It follows from Eq. (26) that, at a P.M.F. of 210 mV (which would poise the ATP/ADP couple central through the ATPase II system at a [Pi] of 10 mM), the type II loop would require an o/r potential span of 210 mV, while the type I loop would require a span of 105 mV.

If the driving E.M.F. of the oxido-reduction across the o/r loop were not in equilibrium with the P.M.F. across the membrane, the net driving force (f) on the oxido-reduction reaction would be given by

$$f = \frac{2}{n}\Delta E' - \Delta p \tag{27}$$

As the rate of the forward electron and hydrogen transfer reaction would be partly governed by the magnitude of f, factors that increased the P.M.F. (Δp) relative to the o/r potential span (ΔE′) across the loop would cause control or reversal of electron and hydrogen transfer.

4.2. Mitochondrial o/r loops

The phenomena of crossover and the association between phosphorylation and electron and hydrogen transfer through different groups of carriers in the respiratory chain of mitochondria [4,6] have defined three main regions of the respiratory chain system from NADH to oxygen within which coupling between the flows of the o/r and h/d particles appears to occur. These coupling regions are represented by the spans: NAD^+ to flavoprotein; flavoprotein to cytochrome b; cytochrome b to cytochrome a. There has been a tendency to permit the presumed coupling sites to slip towards the oxygen end of the o/r scale (see e.g. [7]) which seems to have been prompted less by experimental fact than by the feeling that the o/r span between oxygen and cytochrome c should be fully utilised [9]. In fact, only one phosphorylation is coupled to respiration between cytochrome c and oxygen [77]. The overall standard o/r potential-span between NAD and cytochrome a, of some 600 mV, is fairly equally divided between the three spans, which respectively have standard potentials of about 250 mV, 150 mV, and 200 mV.

According to the chemiosmotic hypothesis, the regions of coupling in the respiratory chain represent the regions of folding into proton translocating o/r loops, and we shall refer to Loops 1, 2 and 3, corresponding to the respective regions from NAD to cytochrome a. It has been shown [40], that the oxidation of succinate and β-hydroxybutyrate by intact rat liver mitochondria is accompanied by the translocation of close to 4 protons and 6 protons respectively outward through the coupling membrane. It would seem, therefore, that Loop 1 translocates 2 protons per electron pair equivalent and that Loops 2 and 3 translocate a total of 4 protons. We have also observed that the antimycin-sensitive oxidation of succinate and β-hydroxybutyrate by low concentrations of ferricyanide in intact rat liver mitochondria is accompanied by the translocation of approximately 2 less protons than the corresponding oxidations by oxygen when allowance is made for the protons produced during the reduction of ferricyanide on the outer side of the coupling membrane by an organic electron donor. As ferricyanide has been shown to react with the respiratory chain primarily at the level of cytochrome c or c_1 [78–80], so that electron transport should thus bypass

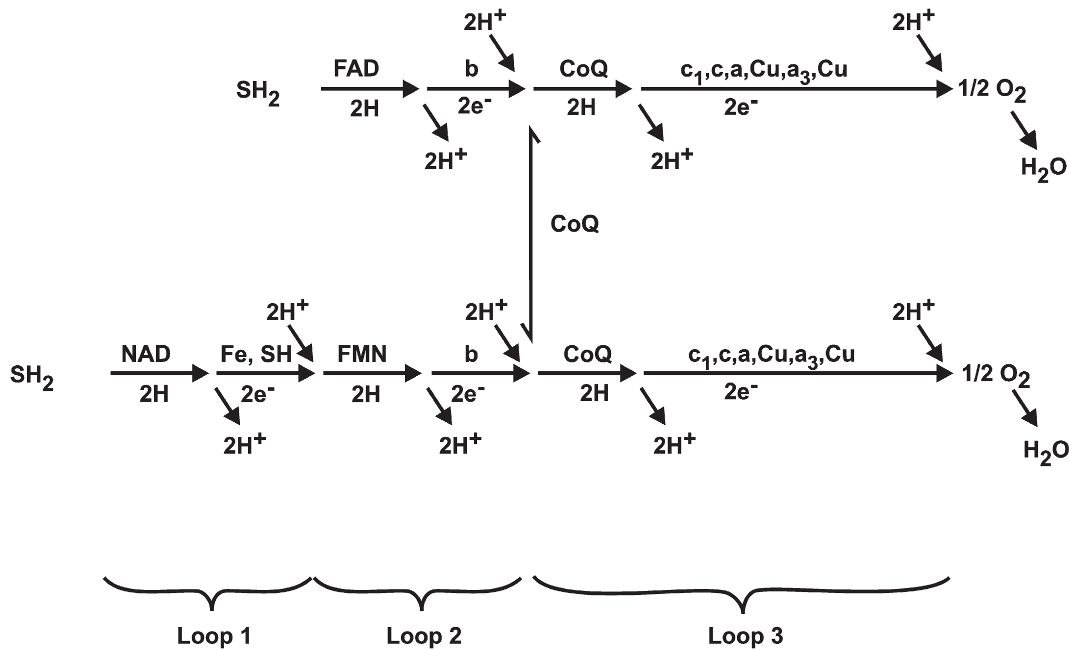


Fig. 10. Suggested respiratory chains for oxidation of substrates (SH_2) linked through: A, FAD; and B, NAD. The o/r loops are indicated by the brackets and by the points of entry and exit of H^+ at the junctions between (2H) and electron ($2e^-$) currencies of oxido-reduction. Abbreviations are as in the text except for cytochromes, described by lower case letters only.

Loop 3, our observations suggest that Loops 1, 2 and 3 each translocate 2 protons per electron pair equivalent, and that they may be of type II, Fig. 8. This conclusion is supported by our observation that the antimycin-insensitive dehydrogenation of tetramethyl-p-phenylenediamine (TMPD) by oxygen in intact rat liver mitochondria gives a limiting H^+/O quotient of 2 (Mitchell and Moyle, unpublished).

The loop systems suggested for the succinate oxidase and NADH oxidase of the intact respiratory chain are shown in Fig. 10, A and B. We shall consider the possible existence of Loop O, corresponding to the energy-linked pyridine nucleotide transhydrogenase [81] in Section 4.5.

4.3. "Energy coupling" with o/r loops

As pointed out in my original exposition [16], the interaction between the loops would have the effect of making the o/r potential spans across the loops "drift together", and we shall now consider more precisely what is meant by this. Eq. (27) gives the relationship between the force (f) on the oxido-reduction process across a given o/r loop (conveniently expressed in mV), the o/r potential span ($\Delta E'$) across the loop and the P.M.F. (Δp) across the membrane. The o/r potential span across the loop would be given by a standard potential and the poise of the relevant oxidants and reductants. Let us write for the poise of the reactants in the hydrogen-carrying arm of the loop, in equilibrium with phase R,

$$(E')_H = (E'_m)_H + Z\log_{10}\sqrt[j]{\frac{\{OX_H\}}{\{RED_H\}}}, \tag{28}$$

and for the poise of the electron-carrying arm, in equilibrium with phase R,

$$(E')_e = (E'_m)_e + Z\log_{10}\sqrt[k]{\frac{\{OX_e\}}{\{RED_e\}}}, \tag{29}$$

where E′ means the midpoint potential [31], j and k are the respective numbers of electron equivalents donated per mole of the hydrogen carrier and accepted per mole of the electron carrier, and Z = 2303RT/F. It follows that

$$\Delta E' = (E'_m)_e - (E'_m)_H + Z\log_{10}\sqrt[k]{\frac{\{OX_e\}}{\{RED_e\}}}\cdot\sqrt[j]{\frac{\{RED_H\}}{\{OX_H\}}} \tag{30}$$

Substituting in Eq. (27) and writing $\Delta E'_m$ for $(E'_m)_e - (E'_m)_H$,

$$f = \frac{2}{n}\left[\Delta E'_m + Z\log_{10}\sqrt[k]{\frac{\{OX_e\}}{\{RED_e\}}}\cdot\sqrt[j]{\frac{\{RED_H\}}{\{OX_H\}}}\right] - \Delta p, \tag{31}$$

where the oxidant/reductant activities are those in equilibrium with phase R. For a type II loop, in which, for example, we may represent the hydrogen carrier as S/SH_2 and the electron carrier as Fe^{3+}/Fe^{2+},

$$f = \Delta E'_m + \frac{Z}{2}\log_{10}\left[\frac{\{Fe^{3+}\}^2}{\{Fe^{2+}\}^2}\cdot\frac{\{SH_2\}}{\{S\}}\right] - \Delta p, \tag{32}$$

where the oxidant/reductant activities are those in equilibrium with phase R. When the system was near chemiosmotic equilibrium, and substituting 60 mV for Z, the poise of the carriers would be given by

$$\log_{10}\left[\frac{\{Fe^{2+}\}^2}{\{Fe^{3+}\}^2}\cdot\frac{\{S\}}{\{SH_2\}}\right] = \frac{\Delta E'_m - \Delta p}{30} \tag{33}$$

In other words, the poise of the pair of couples involved in an o/r loop would settle at a point that differs from the normal poise, given by the midpoint span $\Delta E'_m$, as though the P.M.F. were simply subtracting its value from the midpoint span. It follows that as the o/r system of Loop 1, Loop 2 and Loop 3 came into equilibrium with the P.M.F. across the coupling membrane, the carrier poise across

each loop would adjust itself according to Eq. (33). It should be noted that the overall carrier poise in a given loop, e.g.

$$\frac{\{Fe^{2+}\}^2}{\{Fe^{3+}\}^2} \cdot \frac{\{S\}}{\{SH_2\}},$$

would be unity if $\Delta E'_m - \Delta p$ were zero, and the extent to which the poise would differ from unity would depend upon the value of $\Delta E'_m - \Delta p$. A rise in Δp would cause the hydrogen carrier to become reduced relative to the electron carrier. The absolute change of poise of either carrier in a given o/r loop, caused by a change in Δp, would depend on the relative degrees of anchorage of the potentia1s of either carrier to the potential of the neighbouring o/r component in the respiratory chain.

Eq. (33) shows that the P.M.F. developed by respiratory activity across one loop would have the effect of "reversing electron transport" in the other loops because the o/r couples in each loop are poised against a common value of Δp. Similarly, it will be noted that a P.M.F. generated by any other means, such as by the hydrolysis of ATP through the proton-translocating ATPase, would contribute to the reversal of electron transport. Conversely, forward electron transport through the chain of loops, and the corresponding change of o/r poise of the carriers, would be promoted if the P.M.F. across the membrane were lowered, for example, by the flow of protons back through the ATPase II system in presence of phosphate acceptor (see Section 7.1), or by proton leakage across the coupling membrane, catalysed by proton-conducting uncoupling agents (see Section 7.2.3).

The foregoing elementary treatment of the interaction between oxido-reduction across an o/r loop and the P.M.F. across the coupling membrane shows how a rise in "energy coupling" with the o/r chain (i.e. a rise in Δp) should cause the hydrogen carriers to become more reduced and the electron carriers to become more oxidised in the region of the membrane in contact with phase R. It should be understood, however, that a more detailed exposition, including a treatment of the effects of the P.M.F. on the spatial distribution of the components of the o/r couples across the thickness of the membrane, is required to describe the relationship between the state of the system and the overall content of oxidised and reduced forms of the carriers. It must suffice for the present to point out that the overall poise of a given member of the respiratory chain, measured, for example, by means of a spectrophotometer, would not necessarily correspond to the actual o/r state of the carrier at any point in the type of anisotropic system described by my hypothesis, since the same o/r couple may be expected to be poised over a range of different potentials across the thickness of the coupling membrane.

4.4. Possible composition of Loops 1, 2, and 3 in mitochondria

As in the case of the hypothetical coupling sites of the chemical hypothesis, owing to the incomplete knowledge of the properties and identities of the component carriers of the respiratory chain system, any attempt to name the hydrogen and electron carriers involved in the o/r loops at the present time is bound to be somewhat speculative. It is profitable, however, to attempt to bring some order to this difficult question.

To account for three o/r loops of type II, three electron carriers and three hydrogen carriers would be required, and these would have to succeed each other alternately down the respiratory chain, starting at the substrate end with a hydrogen carrier and finishing with an electron carrier at the oxygen end. Contrary to the view expressed by Dixon and Webb [82] with which I concurred in a recent article [18], the reducing equivalents carried by cytochrome c and cytochrome oxidase (consisting of the a,Cu; a_3,Cu complex) have been shown by o/r titrations [83–87,265] to be accounted for by the valency change of the metal ions and thus to be in the currency of electrons. The titration of cytochrome b of beef heart particles with succinate strongly suggests that cytochrome b is an electron carrier [88]; and the similarities between cytochromes c and c_1 [89–91] may justify the assumption that cytochrome c_1 is an electron carrier also. Taking account of the required locations of the three o/r loops in the respiratory chain, cytochrome b might be the electron carrier of Loop 2 and cytochrome c or cytochrome a might be the electron carrier of Loop 3. The hydrogen carriers of Loops 2 and 3 would have to be on the substrate side of the suggested electron carriers, and the more obvious possibilities would be coenzyme Q (CoQ), and flavin mononucleotide (FMN) or flavin-adenine dinucleotide (FAD) respectively. The part of the respiratory chain from the FMN region to NAD is less well characterised even than the rest, and suggestions concerning Loop 1 are therefore correspondingly more speculative. The most obvious possible hydrogen carrier for Loop 1 would be NAD, and the recent evidence for the participation of a labile sulphide-nonhaem iron component in oxido-reduction through the FMN region of the NADH dehydrogenase system [92–94] invites the suggestion that the nonhaem iron may be the electron carrier of Loop 1. However, as shown in Section 4.1, metallic carriers are not the only vehicles for transporting electrons. It would be possible, for example, for the electron carrier of Loop 1 to be a pair of SH groups which could move cyclically, one way oxidised as R_1–S–S–R_2, and the other way reduced and deprotonated as (R_l–S^-,R_2–S^-). As it has been observed [95] that there are at least four functionally different types of SH group in addition to the labile sulphide in the NADH dehydrogenase, we shall, for the time being, represent the electron carrier of Loop 1 tentatively as Fe, SH.

My suggestions as to the composition of the loops involved in oxidation of FAD-linked (e.g. succinate) and NAD-linked substrates are illustrated in A and B of Fig. 10 respectively. Two separate chains are shown for the sake of clarity, and the fact that the pathway from CoQ to oxygen is common to NAD-linked and FAD-linked oxidations is indicated by a CoQ connection between the two chains, consistent with the fact that CoQ, like NAD, is present in excess of other carriers. The chains represented linearly in Fig. 10 are shown in looped configuration in Fig. 11 so as to illustrate the sidedness of the suggested systems.

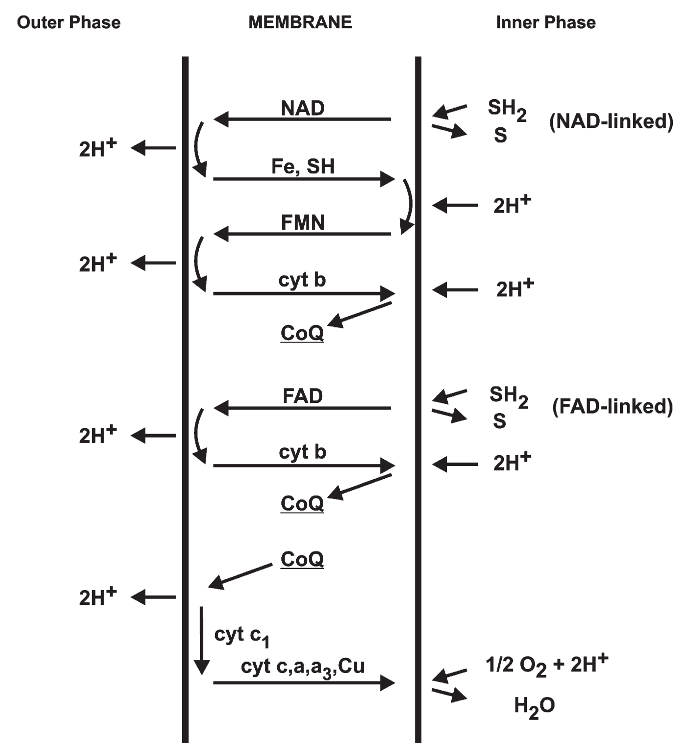


Fig. 11. Suggested folding of the proton translocating respiratory chain for oxidation of NAD-linked and FAD-linked substrates in mitochondria. The chain is shown branching at CoQ.

4.5. Survey of mitochondrial electron and hydrogen transfer systems

4.5.1. Loop 2 and Loop 3 regions

From the point of view of the chemical pathway of electron and hydrogen transfer, Figs. 10 and 11 follow the generally accepted pattern, except for the placing of CoQ, which is usually supposed to be on the substrate side of cytochrome b (see [10,12,96]), and except for the FAD-FMN region which is only now undergoing intensive biochemical study. The position of CoQ shown in Figs. 10 and 11 corresponds to that originally suggested, perhaps fortuitously, by Hatefi [97], and on the grounds of o/r potential by Moret [98].

It should be understood that the representation of the loops of the respiratory chain in Fig. 11 is highly diagrammatic, and is intended primarily to illustrate the accessibility of the o/r currency junctions to the aqueous phases on either side of the coupling membrane in relation to the sequence of the carrier components connecting substrate and oxygen. The abstract idea of the “folding” of the “linear” sequence of respiratory carriers into air “loops” would be expected to be represented in reality by subtleties of conformation and packing of the catalytic carrier molecules that would facilitate the appropriate electronic and nuclear movements according to the normal mechanisms of enzyme catalysis. One of the more obvious consequences expected of the effectively looped arrangement of the sequence of carriers in the mitochondrial membrane would be a tendency to short-circuit. Further, it would be expected that the functional activity of the system would be especially sensitive to physical displacement of the spatially related catalytic carrier and structural components of the lipoprotein membrane fabric.

In the Keilin–Hartree type of “non-phosphorylating preparations” of heart muscle, kinetic measurements have been taken to indicate that the greater part of the cytochrome b lies off the main respiratory chain [99,100, 101]; and Redfearn and Pumphrey [78] and Chance and Redfearn [102] have shown that in the same type of “non-phosphorylating preparations” the bulk of the CoQ present, like the cytochrome b, is not oxidised or reduced fast enough to be situated on the main respiratory chain between succinate and oxygen. On the other hand, in intact mitochondria and phosphorylating mitochondrial particles, the kinetics of the oxidoreduction of both cytochrome b and CoQ during forward electron and hydrogen transfer ([4,103–107,108]) and during reversed electron and hydrogen transfer [77,109] are in accord with the requirements for the functional participation of these carriers in the main electron and hydrogen transfer chain. The kinetics of oxido-reduction.of cytochrome b in particles prepared by sonic disintegration of mitochondria suggest that “the modification of mitochondrial structure during disruption may alter the accessibility or form of cytochrome b as it becomes displaced from the phosphorylating respiratory chain” [77]. It would seem that one of the main differences between intact mitochondria and certain “non-phosphorylating preparations” is that the respiratory chain of the latter has been damaged so that both cytochrome b and CoQ have become partially dislocated [101,102]. In keeping with this interpretation, we should consider, as an extreme example of the short-circuiting artefact, the respiratory particles that catalyse oxidoreduction between substrate and oxygen after extraction of virtually all the CoQ [110]. The short-circuiting of the o/r loops would, of course, impair the proton translocating function of the respiratory chain system, and would correspondingly impair the coupling between oxido-reduction and phosphorylation.

Part of the change of behaviour of cytochrome b observed when the mitochondrial respiratory chain system is subjected to various disintegration procedures may be related to the fact that the standard o/r potential of cytochrome b is influenced by the structural matrix in which it is situated in the mitochondrial membrane. Holton and Colpa-Boonstra [88] have estimated that·the midpoint potential (E'_m) of cytochrome b in nonphosphorylating heart muscle particles is +77 mV at pH 7.4, whereas Goldberger et al. [111] have shown that the E'_m of isolated cytochrome b is −340 mV at pH 7.0, and that cytochrome b can be reduced by $CoQH_2$ analogues (E'_m about +100 mV at pH 7.0) only when it is made more oxidising by combination with structural protein. Deeb and Hager [112] have similarly observed that crystalline cytochrome b_1 of *Escherichia coli* has an E'_m of −340 mV at pH 7.0 and that this is 350 mV more negative than the value found in crude preparations. Deeb and Hager [112] have also noted, for the *E. coli* respiratory chain system, that “in contrast to cytochrome b_1 in vivo which is nonautoxidisable, crystalline cytochrome b_1 as well as b_1 which is attached to cell membrane fragments is rapidly autoxidisable. Therefore, it appears that mechanical breakage of the cell membrane brings about a subtle change in the environment of cytochrome b_1 which affects its reaction with oxygen.”

Antimycin A specifically blocks electron and hydrogen transfer in the respiratory chain at a point close up to cytochrome c_1 on the substrate side, and its main effect is seen, both in nonphosphorylating preparations [78,80,79] and in phosphorylating preparations [103,107,108] as an oxidation of cytochrome c and c_1 and a reduction of cytochrome b and CoQ. The amount of antimycin A required to give the full inhibitory effect in rat liver mitochondria is not more than one molecule per pair of cytochrome b haem groups, and there is a delay in the development of inhibition suggesting a complex process involving a dislocation of some component [79,80].

Brown et al. [113] have shown that there is a considerable leak across the antimycin A-sensitive site which can be blocked by certain alkyl hydroxynaphthoquinones. Indeed, the alkyl hydroxynaphthoquinones which stop the leak across the antimycin A-sensitive site completely block oxido-reduction across this site in the absence of antimycin, and there is evidence that the antimycin A-resistant reaction may be due to a bypass or short-circuit through endogenous quinones that can be extracted with organic solvents [113]. The isolated succinate-CoQ reductase [114] and succinate-dichlorophenol indophenol reductase [115] fragments of the respiratory chain are inhibited by alkyl hydroxynaphthoquinones but not by antimycin A, indicating that the former inhibit the reaction of the hydrogen acceptor with cytochrome b at the oxygen end of Loop 2. The antimycin A-inhibited region can be artificially bypassed by TMPD in intact mitochondria with loss of only the phosphorylation that is coupled to electron and hydrogen transfer in the cytochrome b or Loop 2 region [116,117]. The TMPD bypass causes oxidation of cytochrome b and reduction of cytochrome c, and the artificial hydrogen carrier is therefore believed to couple the reduction of cytochrome c_1 or c to the oxidation of cytochrome b; but it is not at all clear, according to the usual view of the components of the respiratory chain, why the phosphorylation in this region should be lost [117]. According to Fig. 11, the TMPD would short-circuit the CoQ–cytochrome b span, so that Loop 2 and Loop 3 would coalesce to form a single hybrid loop.

The substitution of ferricyanide at low concentration (0.2 mM) for molecular oxygen in the oxidation of succinate or NAD-linked substrates by intact mitochondria results in the loss of only the phosphorylation in the Loop 3 or cytochrome c region ([118], and see [119]), so that P/2e ratios approaching 2 for NAD-linked substrates and approaching 1 for succinate are obtained. At low concentrations of ferricyanide, the reduction of ferricyanide by intact mitochondria is antimycin A sensitive and evidently occurs at the level of cytochrome c or c_1 [118,120], but at higher concentrations, the ferricyanide reacts with the respiratory chain on the substrate side of the antimycin A-sensitive site, probably at the same point as the TMPD bypass. In mitochondrial fragments, as opposed to intact mitochondria, oxidation of succinate or NAD by ferricyanide is relatively antimycin A insensitive[79,121] and the ferricyanide presumably reacts at the FAD and Fe, SH-FMN levels respectively in the succinate and NADH oxidising chains. These regions of reaction of ferricyanide are evidently inaccessible in intact mitochondria [79,118]. The phosphorylation coupled to electron and hydrogen transfer in the cytochrome

c or Loop 3 region of the respiratory chain must be functional in antimycin A-sensitive mitochondria since P/2e quotients approaching unity are obtained with ferrocyanide or TMPD as substrates [122,123].

4.5.2. Relevant disintegration and reintegration studies

Some comment is required concerning the evidence about the respiratory chain derived from disintegration and reintegration studies. It is generally agreed that these studies cannot be used as the foundation on which to build knowledge of the intact respiratory chain because experience has shown that the parts of the system do not naturally fall back into a unique functional pattern after being disturbed from their native positions (see [96,124]). Moreover, the physical particles into which the system tends to fragment during degradation may depend upon the type of force to which the system is subjected, and in any case the physical entities obtained may not correspond to the functional entities existing in the intact system. It is evident that the short-circuiting and substitution effects that would be expected to cause problems of interpretation in frank disintegration and reintegration studies are essentially similar to the effects that have often unexpectedly been responsible for problems of interpretation in studies of less drastically treated preparations.

It may be permissible to compare the appropriate Loop 2–Loop 3 regions of Fig. 11 with the succinate–cytochrome c reductase particle described by Takemori and King [115]. Accordingly, the cytochrome b–c_1 fragment of the succinate–cytochrome c reductase [115] or the similar CoQH_2–cytochrome c reductase isolated by the Madison group [125,126] might be compared with the cytochrome b–c_1 region of Figs. 10 and 11. However, we should have to assume that the endogenous CoQ present in the b–c_1 or CoQH_2 cytochrome c reductase preparations [115,127] couples the oxidation of cytochrome b to the reduction of cytochrome c_1 within the particles. Further the activity of the b–c_1 preparations as CoQH_2–cytochrome c reductases for lower CoQ homologues [127], and the stimulation of the coupling between b–c_1 preparations and soluble succinate dehydrogenase or succinate–CoQ reductase preparations by lower CoQ homologues [128] would have to be classed as artefacts. Hatefi [126] has pointed out that “the presence and the function of a cytochrome b type hemoprotein in the preparations of succinic–Q reductase have not been explained” (see [129]). The NADH–CoQ reductase preparations [130,131] also tend to contain cytochrome b. A possible explanation might be, according to Figs. 10 and 11, that the functional succinate–CoQ reductase or Fe–CoQ reductase would correspond to Loop 2 and would, like the L-lactate dehydrogenase of yeast [266] or the formate dehydrogenase–cytochrome b_1 complex of *E. coli* [132], consist essentially of a f1avo-haemoprotein. It is relevant that a recent kinetic study of the reconstituted succinate oxidase system [133] “demonstrated an intimate relationship between the succinate dehydrogenase and cytochrome b molecules”, and suggested that electron and hydrogen transfer from cytochrome b to oxygen was mediated along alternative parallel carrier routes. The observation that deoxycholate extracts of beef heart mitochondria particles behave kinetically towards natural substrates and other reducing agents as though three functionally distinct cytochrome b fractions were present in the respiratory chain [134], would also be consistent with my suggestion that the respiratory chain stems from oxygen through a shared set of carriers to CoQ, and branches from CoQ via separate cytochrome b-linked chains (Fig. 11).

The succinate–CoQ reductase is distinguished from other fragments of the respiratory chain by being very sensitive to the lipid-soluble iron-chelator thenoyltrifluoroacetone ([114,135]: [126]), and this property is consistent with the fact that thenoyltrifluoroacetone is a potent inhibitor of succinate oxidation, but not of NADH oxidation by the intact respiratory chain system [136]. The fact that thenoyltrifluoroacetone inhibits oxidation of 2,6-dichlorophenol indophenol by succinate, catalysed by succinate–cytochrome c reductase, while antimycin A and substituted naphthoquinones inhibit cytochrome c reduction but not 2,6-dichlorophenol indophenol reduction by succinate [115] confirms that the site of action of thenoyltrifluoroacetone is closer to the substrate end of the respiratory chain than the antimycin A-sensitive site and that the former site is on the succinate-oxidising branch of the chain. As thenoyltrifluoroacetone does not inhibit the soluble succinate dehydrogenase-catalysed reduction of phenazine methosulphate by succinate [135] it has been suggested that nonhaem iron, or the thenoyltrifluoroacetonesensitive component, couples the succinate dehydrogenase flavoprotein to the next component in the chain, customarily assumed to be CoQ [126], but represented by cytochrome b in Fig. 11. On the basis of an EPR signal at $g = 1.90$ and the presence of nonhaem iron in the b–c_1 preparations, Rieske et al. [137] have suggested that nonhaem iron may also be involved in the cytochrome b–c_1 segment of the respiratory chain on the oxygen side of cytochrome b.

The cyanide-sensitive cytochrome oxidase, containing cytochromes a and a_3 and functional Cu [85,138,139] is shown as the electron-carrying arm of Loop 3 in Figs. 10 and 11.

4.5.3. Loop 1 region

The coupling of electron and hydrogen transfer to phosphorylation in the NAD-FMN region of the respiratory chain is specific for the oxidation of NAD-linked substrates in the mitochondria of many animal and plant species; but the fact that endogenous NADH oxidation in yeast “mitochondria” is not accompanied by a “site 1” phosphorylation [140] indicates that this fragile coupling region may not occur universally in mitochondria and in microbial respiratory chain systems. The Loop 1 region of the respiratory chain appears to be represented by the so-called NADH–CoQ reductase [131] of which NADH-dehydrogenase [141] is the terminal part. At the present stage of the work on NADH dehydrogenase, the definition of the corresponding physical fragment of the respiratory chain is somewhat indistinct because the retention of the normal functional properties of this region depends upon the integrity of a particle that is rather more labile than, for example, the succinate dehydrogenase flavoprotein fragment of the succinate-oxidising respiratory chain (see [136]). As Massey and Veeger [96] have aptly remarked, the clear cut identification of the respiratory chain NADH dehydrogenase “must await the demonstration that it can react with the next member of the electron transport chain. This, of course, must await the clear cut identification of the next member of the electron transport chain!” The difference between the NADH dehydrogenase and the NADH–CoQ reductase is likewise ill-defined at present, since the definition of NADH–CoQ·reductase may vary from preparation to preparation. Indeed, there is some doubt as to whether the NADH–CoQ reductase of Hatefi et al. [131] can properly be taken to represent the native system coupling NADH oxidation to reduction of endogenous CoQ since it reacts fast with the unnatural quinones called CoQ_1 and CoQ_2, but not significantly with the higher natural homologues (see also [142,143]).

The electron and hydrogen transfer function in the Loop 1 or NAD-FMN coupling region of the intact respiratory chain is characterised by remarkable lability towards treatments or reagents that tend to loosen or dislocate the structural integrity of the native system (see [136,146]). In this respect the Loop 1 region resembles the Loop 2 or cytochrome b region, except that in the case of Loop 1 the lability may be more pronounced. Amongst the many non- or semi-specific reagents that inhibit electron and hydrogen transfer in Loop 1, including even sub-lytic concentrations of Triton X-100 [144], Amytal has received the most intensive study, and it has been shown that the fish poison rotenone and other rotenoids react very specifically and irreversibly, blocking the flow of reducing equivalents at the same point as Amytal, on the substrate side of cytochrome b and CoQ but on the oxygen side of FMN [267,268,105,145,146]. In conformity with this important fact it has been established that when the

NADH dehydrogenase is assayed with ferricyanide as electron acceptor, either in situ in the respiratory chain, or after careful isolation, neither Amytal [121,147] nor rotenone [146] cause significant inhibition. It has also been observed that rotenone does not inhibit the rapid exchange of hydrogen between [4B-^{3}H]NADH and water, presumed to be catalysed by the NADH dehydrogenase of sonically prepared beef heart mitochondrial particles [148]. Inhibition by rotenone is complete in rat liver mitochondria when only 24 to 28 mμmol has been taken up per g protein [145], and a characteristic delay between rotenone uptake and the onset of inhibition of the NADH oxidase of beef heart mitochondria, and other features of the inhibitory process [146], suggest that the site of uptake is different from the site of inhibition and that inhibition may result from a subtle conformational change similar to that induced by Amytal and other diverse inhibitory agents.

Colpa-Boonstra and Slater [149] observed that Amytal-inhibited mitochondria catalysed an antimycin-sensitive oxidation of vitamin K_3. Following up this observation it has been shown that vitamin K_3 will bypass the Amytal- [150] and rotenone- [145] sensitive site, permitting intact mitochondria to oxidise NAD-linked substrates with a P/O quotient approaching 2. This oxidation is antimycin A sensitive and dicoumarol sensitive, and vitamin K_3 evidently permits oxidation of NADH via the dicoumarol-sensitive DT diaphorase and cytochrome b. Added support is thus given to the view that cytochrome b represents the substrate end of Loop 2. Presumably the substrate or outer side of cytochrome b is accessible to vitamin K_3 in intact mitochondria (see Fig. 11). The Amytal and rotenone-sensitive site of the NADH-oxidising branch of the respiratory chain would seem to represent the counterpart of the thenoyltrifluoroacetone-sensitive site of the succinate-oxidising branch.

The NADH dehydrogenase of the respiratory chain is distinguished not only by its lability, but also by a high nonhaem iron and labile sulphide content [131]. Lusty et al. [93] have observed that the NADH dehydrogenase of beef heart mitochondria contains 16 to 18 Fe atoms and 27 labile sulphide groups per flavin. These groups, both in the intact respiratory chain and in the isolated dehydrogenase, appear to be associated with an EPR signal at $g = 1.94$ [92], the kinetics of appearance and disappearance of which imply that a labile sulphide-nonhaem iron complex may be a functional oxido-reduction link in the dehydrogenase. Treatments, such as warming, that disrupt the labile structure of the NADH dehydrogenase have been shown to give a simultaneous loss of the reactivity with ferricyanide, and disappearance of the $g = 1.94$ signal. These treatments also cause a loss of labile sulphide and iron, and a delayed emergence of artificial cytochrome c reductase activity. A considerable proportion of the labile sulphide is mineralised during the change of the enzyme from NADH dehydrogenase to artificial cytochrome c reductase, but although the iron content of the high molecular weight dehydrogenase falls, mineral iron is not recoverable by chelators [92,93].

The so-called NADH–CoQ reductase, containing NADH dehydrogenase, flavoprotein, nonhaem iron, CoQ, lipid, and small quantities of cytochromes b and c_1 ([131,135]; and see [130]) differs from the NADH dehydrogenase in being sensitive to Amytal and rotenone: and, as in the intact respiratory chain, the site of inhibition of electron and hydrogen transfer is at some point on the oxygen side of the flavoprotein. This site does not correspond to the site of the component giving the EPR signal at $g = 1.94$, presumed to be the Fe component, because the oxidation and reduction of this component by ferricyanide and NADH are Amytal and rotenone insensitive. It would therefore seem that the site of the lesion resulting from warming and other treatments that cause loss of ferricyanide reactivity of the dehydrogenase is nearer the NADH end of the chain than the lesion produced by Amytal and rotenone. The apparent complexities of the NADH-terminal part of the respiratory chain have been taken to imply that additional unknown o/r components may be present [136,142,151].

4.5.4. Phosphorylating and non-phosphorylating respiratory chains

Within the broad limits of the present knowledge of the respiratory carrier systems, there is no evidence for the participation of the respiratory chain in chemical reactions other than those of oxido-reduction during the conduction of reducing equivalents from substrates to oxygen. In other words, in spite of an intensive search, there is no chemical evidence to support the widely canvassed view that the respiratory chain system can exist in alternative "phosphorylating" and "non-phosphorylating" forms, supposed to be distinguished by the participation of chemically-linked "energy transfer" reactions in the former, and their absence or non-participation in the latter. Under the circumstances, there is a case for admitting that the chemical function of the respiratory chain may be no more than it has long seemed to be, namely, the conduction of electrons and hydrogen atoms from substrates to oxygen. The physical organisation of the respiratory chain in mitochondrial and bacterial membranes is undoubtedly complex, and the changes or loss of function resulting from treatment with various kinds of inhibitor or "denaturing" agent are, in many cases, explicable in terms of the dislocation of one component or another, or in terms of the short-circuiting or substitution of one region or another of the respiratory chain system. The view of the respiratory chain, according to the chemical coupling hypothesis, would require us to believe that the chemical complexity of the respiratory chain is considerably greater than it seems, and that the known physical complexity has some functional significance that is, as yet, a matter of conjecture. On the other hand, the view of the respiratory chain, according to the chemiosmotic coupling hypothesis, does not require us to bias, one way or the other, the chemical and physical facts as far as they are known at present.

4.6. Loop 0: The energy-linked pyridine nucleotide transhydrogenase

The reduction of NADP by NADH resembles reversed electron and hydrogen transfer nearer the oxygen end of the respiratory chain in that it is coupled to ATP hydrolysis or to oxido-reduction elsewhere in the respiratory chain system (see [10]). Present estimates suggest that the reduction of one mole of NADP is equivalent to the hydrolysis of one mole of ATP or the translocation of one proton pair equivalent [152,153], but the data might be compatible with twice this estimate of NADP reduction per ATP or per proton pair. As Lee et al. [148] have shown that the 4A-hydrogen atom of NADH is transferred to NADP without coming into equilibrium with water, the type II loop of Fig. 12A could not account for the coupling of the transhydrogenase; but the coupling might possibly be accounted for by the type I loop of Fig. 12B. An alternative possibility is that the transhydrogenase could be coupled to proton translocation via the intermediate translocation of a metallic cation (M) as illustrated in Fig. 12C. Proton translocation of rather low stoichiometry has been shown to accompany the transhydrogenase reaction catalysed by beef heart mitochondrial particles [154], but further experimental data are required before details of the coupling mechanism can be usefully discussed.

4.7. Photon-energised electron and hydrogen transfer in photophosphorylation

Space will not permit a full discussion of this topic, and at all events, present knowledge will hardly justify more than the following brief and comparatively speculative treatment.

In 1961, Duysens, Amesz and Kamp showed that two photochemical systems, called System 1 and System 2, are involved in photosynthesis in certain algae [263]. The previous year, Hill and Bendall [155] had pointed out that if two light-driven reactions of chloroplasts caused respectively the reduction of cytochrome b_6 and the oxidation of cytochrome f, the thermochemical flow of electrons back from cytochrome b_6 to cytochrome f could be coupled to phosphorylation in the chloroplast, exactly as electron transfer from cytochrome b to

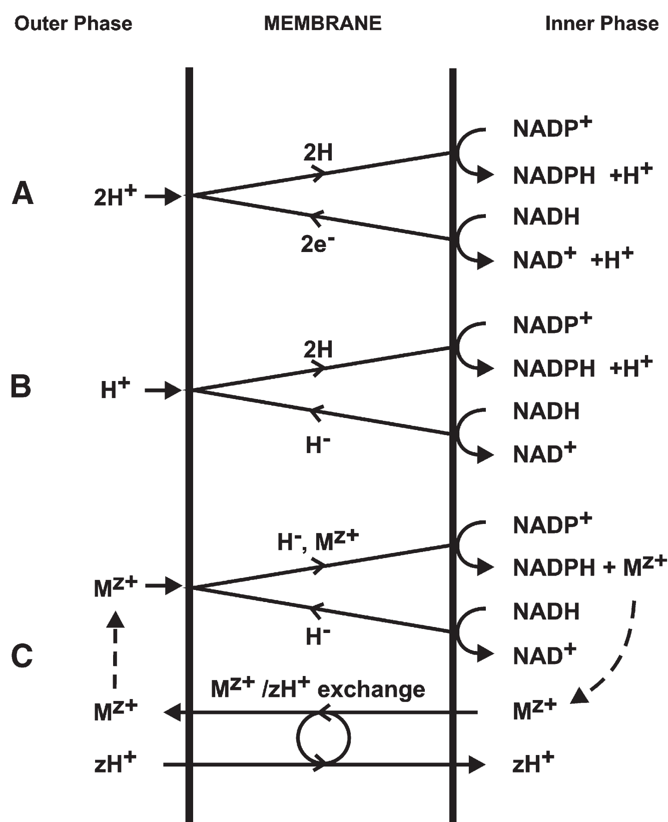


Fig. 12. Possible types of proton translocating oxido-reduction loop for the transhydrogenase or Loop 0 region of the respiratory chain of mitochondria: A, type II loop; B, type I loop; C, specialised loop operating via the cation, M^{z+} of valency z, coupled to proton translocation by a M^{Z+}/zH^{+} exchange diffusion carrier (see Section 6.2).

cytochrome c is coupled to phosphorylation in the mitochondrion. Rumberg [156] has recently verified that cytochrome b is involved in the o/r chain of photosynthesis, and it is evident that plastoquinone (PQ) is a functional hydrogen carrier, adjacent to cytochrome f (see [157]), or adjacent to cytochrome b [156]. Allowing myself some prejudice in the placing of PQ, I have attempted an elementary synthesis of the facts, centred on the foregoing observations, in Fig. 13A. Assuming that the effective o/r loops were of type II. ($n=2$), and taking it that the chloroplast ATPase is of type II (see Section 3) a P/2e quotient of 2 would be obtained. An alternative possibility is that the loop including pigment System 2 might translocate hydride ions ($n=1$) or hydrogen groups ($n=0$) rather than electron pairs, and the P/2e quotient could accordingly be 1.5 or 1.0. As can be seen from Fig. 13A, the latter possibility would be equivalent to the non-participation of System 2 in the translocation reactions per se. These tentative suggestions, it should be emphasised, are intended only to serve as examples of the application of the chemiosmotic coupling principles to photophosphorylation.

Shen and Shen [158] and Hind and Jagendorf [159] observed that photophosphorylation of ADP could be catalysed by preparations of spinach chloroplasts in separate light and dark stages. In the light stage, the chloroplasts were illuminated without phosphate acceptor, and acquired a short-lived energy-state or substance, described as X_E. In the dark stage, the incubation of X_E-containing chloroplasts with ADP and P_i resulted in the synthesis of ATP. At pH 5 or 6, the acquisition of X_E during illumination of the chloroplasts is accompanied by the development of a pH differential, as predicted by my hypothesis, and there is evidently a close correlation between the pH differential and X_E [36,38]. The pH differential (or X_E) appears to possess most of the kinetic requirements for involvement as an intermediary in photophosphorylation [37,44,160]. The fact that in the fragmented chloroplast preparations of Jagendorf's group, the optimum pH for X_E and pH differential formation was well on the acid side of pH 7, whereas the pH optimum of the dark stage, and for normal one-stage photophosphorylation, was on the alkaline side of pH 7, suggested that X_E might be an "energy reservoir" and might not be an obligate intermediary. However, we have found that the pH-dependence of the development of the pH differential is affected by the method of preparing the chloroplasts, and that with relatively intact chloroplasts the pH differential development is almost pH-independent between pH 6 and 8 (Mitchell and Moyle, unpublished). This observation may account for the fact that [159] observed efficient X_E formation at neutral pH, while [158] did not.

According to the chemiosmotic coupling hypothesis, it is not the pH differential, but the P.M.F. which poises oxido-reduction against ATP hydrolysis. The pH differential is only one component of the P.M.F., and if the membrane potential varied independently of the P.M.F., the pH differential would also vary independently of the P.M.F. It follows that the pH change that develops when chloroplasts are illuminated may be the outward sign of the development of a P.M.F. across the coupling membrane, and the present experimental evidence argues in favour of this view.

Baltscheffsky and von Stedingk [161] have recently observed that the chromatophores of *R. rubrum* change the pH of their suspension medium on illumination, as do chloroplasts. The evidence available at present [1,162] prompts the speculative suggestion of Fig. 13B, for cyclic photophosphorylation catalysed by chromatophores. Two o/r loops are shown, one involving the photosynthetic pigment system (PPS), cytochrome c_2 (cyt c_2), and CoQ, and the other involving cytochrome b (cyt b), and flavoprotein (Fp). Under physiological conditions, Fp is supposed to be reduced on the right hand side of PPS, while cyt c_2 is oxidised on the left, thus completing the cycle. If the electron and hydrogen transfer were blocked near cytochrome b with antimycin A, and phenazine methosulphate were used to restore photophosphorylation [1], only the loop involving PPS, cyt c_2, and CoQ would be operative.

4.8. Effects of alternation between hydrogen and electron carriers

Several consequences should follow from the alternation of hydrogen and electron carriers down the respiratory chain as described by Fig. 10. One of the most important of these would be the exchange of protons between hydrogenated carriers and water. Drysdale and Cohn [163] observed that rat liver mitochondria catalyse a rapid exchange of deuterium between NADH and deuterated water, and that the artificial NADH-cytochrome c reductase behaves similarly. More recently, Lee et al. [133,148] have observed that a rapid transfer, and an equally rapid exchange of tritium between the 4B H-atom of NADH and water are catalysed by beef heart submitochondrial particles during NADH oxidation, and when there is no net NADH oxidation, respectively. The exchange reaction was not inhibited by cyanide, antimycin A or rotenone. As the transhydrogenase was shown to transfer hydrogen between the 4A position of NAD and the 4B position of NADP without water exchange, and the DT diaphorase was shown to catalyse hydrogen transfer from the 4A position of NADH or NADPH without water exchange, it was inferred that the NADH-water exchange of the 4B proton was catalysed by the NADH dehydrogenase of the particles. It is significant that whereas the NADH dehydrogenase of the respiratory chain thus appears to catalyse a rapid NADH-water proton exchange, as required by the formulation of Figs. 10 and 11, the other NADH oxidising systems, DT diaphorase, microsomal NADH-cytochrome b_5 reductase, and "external NADH-cytochrome c reductase" of liver mitochondria, catalyse hydrogen transfer from NADH without NADH-water proton exchange (see [133,148,164]). The observation of Gawron et al. [165] that Keilin–Hartree type heart mitochondrial preparations catalyse a transfer of tritium from succinate to NAD during reversed electron

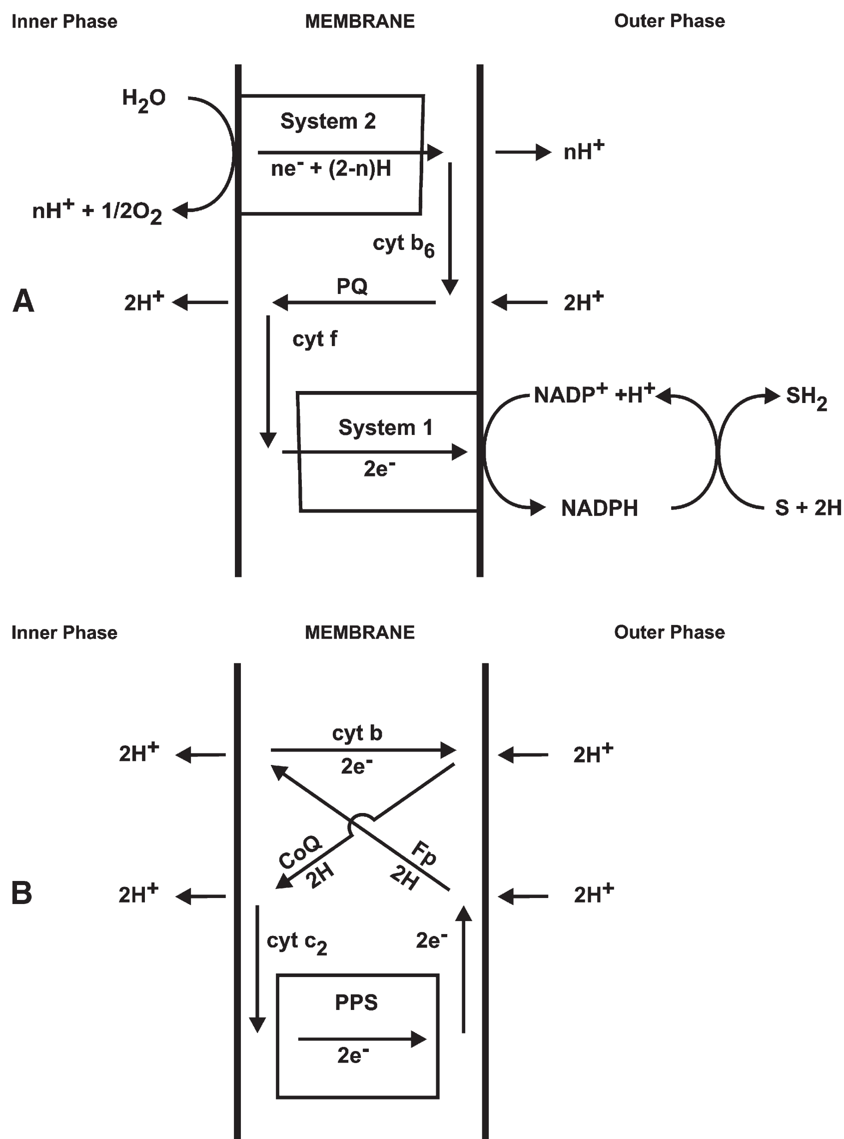


Fig. 13. A. Possible proton translocating oxido-reduction system for noncyclic photophosphorylation in chloroplasts, giving $\rightarrow H^+/2e^- = 4$, 3, or 2, corresponding to $P/2e^- = 2$, 1.5, or 1 with ATPase II, according to whether $n = 2$, 1, or 0 respectively. B, Possible proton translocating oxido- reduction system for cyclic photophosphorylation in chromatophores, giving $\rightarrow H^+/2e^- = 4$, corresponding to $P/2e^- = 2$ with ATPase II.

and hydrogen transfer should probably be attributed to exchange via malate dehydrogenase and not to transfer through the respiratory chain [166].

Another consequence of the alternation of hydrogen and electron carriers along the respiratory chain should be the occurrence of a characteristically large hydrogen isotope effect that should be rather well distributed over the length of the chain. An effect that appears to answer to this description has been observed by Tyler and Estabrook [167].

5. The coupling membrane

The fourth main postulate of the chemiosmotic hypothesis is that there exists a so-called coupling membrane, probably to be identified with the cristae and grana membrane of mitochondria and chloroplasts respectively, and with the plasma membrane and chromatophore membrane of bacteria. The proton-translocating respiratory chain and ATPase systems are assumed to be orientated in this membrane so that a displacement of protons through either system would enable the processes of oxido-reduction and phosphorylation to be coupled by the proton circuit operating at an effective pressure, or P.M.F., of some 250 mV.

In mitochondria, giving a respiratory control ratio of 5, the coupling through the proton circuit would have to occur without loss of more than 20% of the proton current by leakage through the coupling membrane. Evidently: (a) the main hydrophobic laminar fabric of the coupling membrane, which we shall call the osmotic barrier, would have to be very impermeable to ions generally; and (b) the principle of electrical neutrality would not apply individually to the inner and outer aqueous phases separated by the membrane, but it would apply to the three phase system of inner, outer, and membrane phase. As it has been customary amongst those working on ion transport in mitochondria and chloroplasts to regard the electrical neutrality of the internal and external aqueous phases as though demanded a priori by physical principle, I shall begin by giving some explanation and qualification of the latter conclusion.

5.1. Quantitative significance of charge displacement through the coupling membrane

The osmotically resistant membrane of sphered rat liver mitochondria has been shown to have an electrical capacity of about 1 $\mu F/cm^2$ ([168]; and see [18]). As the capacity (C) is the ratio of

charge displacement (ΔQ) to electric potential difference (ΔE), we can calculate as follows:

$$C = \Delta Q / \Delta E \tag{34}$$

1 F = 1 C/V
1 C = 6.28×10^{18} electronic charges (e)
1 mol = 6.02×10^{23} molecules
ΔQ = 62.8 e/mV $\mu^2 = 1.05 \times 10^{-14}$ equivalents/mV cm^2

In other words, in the charging process, about 63 electronic charges per μ^2, or about 10^{-14} equivalents per cm^2 would be displaced through the coupling membrane per mV potential difference across it. For a membrane potential of 200 mV the surface density of charge displacement would therefore be about 12,500 electronic charges per μ^2 or about 2 mμequiv/cm^2. To assess the quantity of charge displacement per unit weight of mitochondria or per unit volume of internal aqueous phase, it is necessary to estimate the area of the coupling membrane. I have shown elsewhere that the cristae membrane of rat liver mitochondria has an area of about 40 m^2/g mitochondrial protein or per ml internal phase [18]. Assuming that the coupling membrane can be identified with the cristae membrane, we can calculate that the electric displacement per mV potential difference corresponds to about 4 mμequiv charge/g protein or 4 μnormal charge in the internal phase. For the potential of some 200 mV required by the chemiosmotic hypothesis during oxidative phosphorylation, the charge displacement would correspond to about 0.8 μequiv/g protein, or an internal concentration of about 0.8 mM monovalent ion. We can compare this, in rat liver mitochondria, with cytochromes each present at about 0.2 μmol, ubiquinone at about 1.5 μmol, and NAD at about 5 μmol/g protein. Thus, owing to the large interfacial area, the electric displacement between the two aqueous phases does, in fact, correspond to a significant displacement of chemical substance from one phase to the other.

5.2. Energy storage capacity of coupling membrane system

The electric and osmotic components of the P.M.F. are each associated with a capacity of the chemiosmotic system; and the appropriate intensity-capacity products would represent the "energy storage capacity" of the system. We can readily estimate the energy storage capacity due (i) to the membrane potential, and (ii) to the pH differential if we know the relevant capacity factors. We shall assume that ATP synthesis can proceed via the ATPase II system between an [ATP]/([ADP]x[P_i]) poise of 10^4 and unity (corresponding to a membrane potential from 270 mV to 150 mV, or a pH differential from 4.5 to 2.5 units). The data of Section 5.1 show that, in rat liver mitochondria, the discharge of the membrane potential by 120 mV would be accompanied by the translocation of 480 mμequiv charge/g protein, and if this occurred as the translocation of protons via ATPase II, it would result in the synthesis of 240 mμmol ATP/g protein. The internal acid–base buffering capacity of rat liver mitochondria in the region of pH 7 is equivalent to about 20 μequiv protons/pH unit g protein [40]. Assuming the buffering power of the outer medium to be relatively large, the equalisation of a differential of 2 pH units by the passage of protons through ATPase II would synthesise 20 μmol ATP. Hence, for the same potential differential the energy storage capacity associated with the pH differential would be nearly a hundred times that associated with the membrane potential.

In order to facilitate the following calculation, Dr. A. Jagendorf kindly estimated that the membrane area of the grana discs of spinach chloroplasts would be about 160 m^2/g chloroplast protein—about four times as great as for the cristae of rat liver mitochondria. We do not yet have an estimate of the internal acid–base buffering power in this case, but assuming that it is about the same as for rat liver mitochondria, the energy storage capacity associated with the pH differential, calculated as in the foregoing, would be equivalent to about 20 μmol ATP/g protein, and the energy storage capacity associated with the membrane potential would be equivalent to only about 1 μmol ATP/g protein. The amounts of the energy-rich intermediate X_E obtained on illuminating spinach chloroplasts in the absence of phosphate acceptor [37,158] ranged up to a maximum yield corresponding to 20 μmol ATP/g protein. It is evident that the energy storage capacity associated with the pH differential might account for the quantity of X_E found, but the energy storage capacity associated with the membrane potential could not. These considerations are relevant to the discussion as to whether the pH differential corresponding to X_E is an obligatory intermediate between oxido-reduction and phosphorylation in chloroplasts, or whether it is only an energy reservoir on a side branch of the direct line of coupling [36]. It seems likely that the greater part of the P.M.F. may be a membrane potential under normal conditions, and that only after damaging the chloroplasts does the pH differential—with its relatively large energy storage capacity—represent the main component of the P.M.F. (see Section 6).

5.3. Membrane permeability and the conservation of the electric displacement

The early measurements of the dependence of mitochondrial "packed volume" or light scattering upon the osmotic pressure and solute composition of the suspension medium (see [13]) indicated that the osmotically functional membrane system of mitochondria is duplex, in keeping with knowledge of mitochondrial morphology. The inner, more osmotically inaccessible, aqueous compartment could be tentatively identified with the cristae matrix, and its relatively impermeable limiting membrane could be identified with the cristae membrane. The outer, more accessible, aqueous compartment could be identified with the region between the cristae membrane and the relatively pervious outer mitochondrial wall. Observations reviewed by Lehninger [169], and more recent work discussed at a symposium (see [170]) strongly support this view of mitochondrial structure. Lehninger [13] drew attention to the similarities between the membrane systems of mitochondria and certain bacteria [171], and there can now be little doubt that it is legitimate to compare the outer membrane of mitochondria with the bacterial cell wall, and the cristae membrane with the cytochrome-containing plasma membrane of bacteria (see [172]). In rat liver mitochondria, the volume of aqueous medium in the internal and external compartments each amount to very approximately 1 ml/g mitochondrial protein [173–175].

The low permeability of the mitochondrial cristae membrane to non-electrolytes containing 5 OH groups or more is fairly well established [13]; and recent observations by Chappell and Crofts [176] confirm and substantially extend earlier suggestions that the osmotic barrier component of the cristae membrane has a low permeability to monovalent cations [177,174], to monovalent anions [178], and to anions of higher valency [175]. Rather crude titration experiments on rat liver mitochondria [42,179] suggested that the cristae membrane has a low permeability even to protons (presumably as H_3O^+), and this has been confirmed by more refined techniques [40,180]. We have shown that after a pH differential has been established across the mitochondrial membrane system, either by adding acid or alkali to the suspension medium, or as a result of respiration or ATP hydrolysis, the rate of neutralisation of the pH differential across the membrane system is low, even when specific membrane potential-collapsing reagents such as calcium salts or valinomycin (see Sections 7.2.1, 7.2.2) are present. Therefore we can conclude that the osmotic barrier component of the cristae membrane greatly impedes the free diffusion of protons, and hydroxyl ions. We have also observed that submitochondrial particles, prepared either by sonic disintegration [154] or by digitonin treatment (Mitchell,

Moyle and Lee, unpublished) of beef heart mitochondria, possess an osmotically functional membrane that impedes acid–base equilibrium between the interior of the particles and the suspension medium.

Unfortunately, measurements of the diffusion of anions and cations through the mitochondrial membrane system have not yet been done quantitatively enough to permit the calculation of permeability coefficients. Robertson et al. [178] give a value for chloride permeation in carrot and beet mitochondria corresponding to a diffusion coefficient of about 5×10^{-14} cm^2/s for a membrane 10 mμ thick. This is equivalent to a permeability coefficient of 5×10^{-8} cm/s, which is fairly typical of lipid membranes [181]. However, since we now know that mitochondria are not freely permeable to cations, the foregoing value for chloride permeability cannot be taken at its face value.

The phosphate acceptor controlled respiration rate of rat liver mitochondria corresponds to the consumption of about 30 μg atom oxygen per min per g mitochondrial protein with succinate as substrate (see e.g. [144]). Since 4 protons are translocated through the coupling membrane per oxygen atom reduced, in the controlled steady state the cyclic flux of protons would be about 2 μμequiv/s g protein, or about 5 μμequiv/cm^2 s. This corresponds to a comparatively low rate of net charge permeation if the controlling electric potential across the membrane is some 200 to 250 mV, as required by my hypothesis. As pointed out elsewhere [18], the flux of sodium ions through the plasma membrane of the frog's sartorius muscle fibre, under an electrical potential difference of 100 mV, is about 4 μμequiv/cm^2 s [182]. The latter flux rate is of the same order as that calculated for the mitochondrion, but the electric potential assumed to exist across the mitochondrial coupling membrane is at least twice as great as that of the muscle fibre membrane. Formerly, it was thought that a potential approaching 500 mV might be required across the coupling membrane [18]. The present assumption of 200 to 250 mV is more in keeping with the accepted tenets of cell physiology, but it is still comparatively high. Obviously, measurements of the mitochondrial membrane potential, and observations on the quantitative relationship between the membrane potential and the ion fluxes, are required to settle this question satisfactorily.

Comparatively little is known of the osmotic properties of the limiting membrane of chloroplast lamellae or grana (see [50,183]), or of the chromatophores of photosynthetic bacteria (see [161]).

6. The proton circuit network

6.1. Exchange diffusion systems

The third main postulate of the chemiosmotic hypothesis is that the diffusion of ions other than protons (or OH^- ions) down the electrical gradient across the coupling membrane, and their accumulation in osmotically disruptive concentrations in the internal phase, must be counterbalanced by specific extrusion in exchange for protons or OH^- ions [16]; and further, that the entry of certain substrates up the electrical gradient must be facilitated by specific exchange against H^+ or OH^- ions, either directly or indirectly [17]. There are two aspects of specific osmotic linkage phenomena that can show experimentally. The more obvious one is the specificity with which certain solutes are accepted through the membrane. The other aspect is the stoichiometric relationship between the entry or exit of the specific solute and the movement of other osmotically and electrically potent particles, such as protons or hydroxyl ions across the coupling membrane.

An atractyloside-sensitive ATP/ADP translocation system, exhibiting a higher nucleotide specificity than the reversible mitochondrial ATPase has recently been demonstrated in mitochondria of rat liver and beef heart [184–189]. It is not known at present for which of the ionic forms of ATP and ADP the ATP/ADP translocation system is specific, or whether the translocation of ATP or ADP occurs with or against that of specific cations or anions. Recent work from Chappell's laboratory (see [176]) suggests that there are specific translocation systems in rat liver mitochondria for the entry of inorganic phosphate [186], and for citrate, malate [144], and other Krebs cycle acids (see also [190]). The specificity of these systems appears to be fairly high, and it is of special interest that Chappell's group is obtaining evidence that the translocation systems for the anionic substrates are selective with respect to the ionic species and effectively catalyse exchange diffusion of the anions against hydroxyl ions ([176]; Chappell, personal communication). In my laboratory, we have observed that protons exchange with sodium ions across the coupling membrane of rat liver mitochondria, and the high temperature coefficient, pH dependence, and other characteristics of the reaction suggest that it is mediated by a proton/ cation exchange diffusion carrier system (Mitchell and Moyle, unpublished).

It would seem that experimental evidence for the postulated anion and cation exchange diffusion systems is beginning to come to light, and it may, perhaps, be opportune to draw attention to the importance of measuring, not only the substrate specificity of these systems, but also the stoichiometry of the ionic exchange reactions catalysed across the coupling membrane.

6.2. Coupling between proton, anion, and cation circuits

We shall now proceed to a closer integration of the conception of chemical and osmotic events in proton transport phosphorylation by including the exchange diffusion systems in the proton flow diagram. To obtain some formal simplification we shall use the expression “effective proton translocation” to mean the sum of the translocation of H^+ and OH^-, the sign of the vector for $\rightarrow OH^-$ being minus that for $\rightarrow H^+$. In the coupled translocation of H^+ or OH^- ions with cations or anions (e.g. Ca^{2+} and OH^- travelling together as $CaOH^-$, or exchange diffusion of H^+ against K^+) we shall treat OH^- translocation one way as H^+ translocation the other, so that all coupled translocations will appear formally as if involving protons.

During steady state oxidative or photosynthetic phosphorylation the net rate of effective proton translocation would be zero, or

$$\left(\rightarrow H^+\right)_{o/r} + \left(\rightarrow H^+\right)_{h/d} + \left(\rightarrow H^+\right)_D + \sum\left(\rightarrow H^+\right)_{nC^z} = 0 \quad (35)$$

All the terms in Eq. (35) refer to the effective rates of proton translocation. The first three terms refer to net effective proton translocation through the o/r system, through the h/d system, and by diffusion (D) respectively. The fourth term refers to the effective proton translocation coupled to the translocation of cations or anions (C) of valency $+z$ or $-z$ respectively, the number n being used to denote the number of anions or cations translocated in the same direction relative to each proton. Negative values of n denote that the direction of translocation of C is opposite to that of the protons with which the translocation is coupled. The summation sign ($\sum$) refers to the fact that there may be more than one term of that type. The flow diagram of respiration-driven ATP synthesis in Fig. 14 illustrates how Eq. (35) describes the postulated proton circuits across the coupling membrane of mitochondria. The proton current flowing outward through the o/r system, $(\rightarrow H^+)_{o/r}$, is supposed to return through the membrane by four main parallel routes: by the reversible ATPase system synthesising ATP, $(\rightarrow H^+)_{h/d}$ by non-coupled diffusion through the membrane, $(\rightarrow H^+)_D$, and by coupled diffusion e.g. against cations, and with anions, $S(\rightarrow H^+)_{nC^z}$.

Owing to the reciprocity of the relationships in which proton translocation is supposed to be coupled to the translocation of other ions, we can write equations of the type

$$n\left(\rightarrow H^+\right)_{nC^z} = \left(\rightarrow C^Z\right)_{\frac{1}{n}H^+} \quad (36)$$

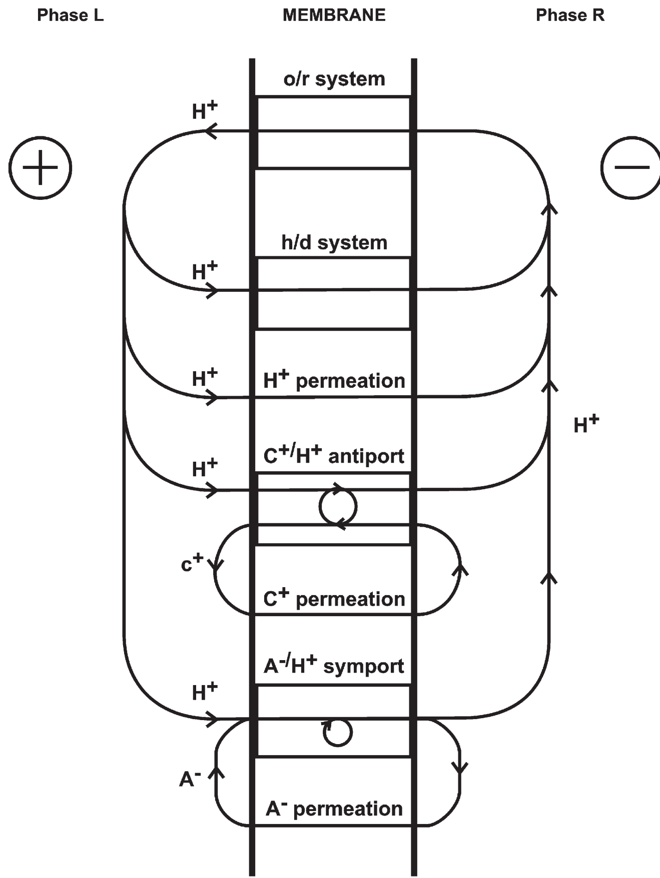


Fig. 14. Coupling through proton circuits. Translocation of protons through oxido-reduction (o/r system) is shown driving the proton current through the reversible ATPase (h/d system), synthesising ATP. Dissipation of proton current occurs through the permeation of H^+, through exchange diffusion of H^+ against cations(C^+/H^+ antiport) and with anions (A^-/H^+ symport). The latter two dissipation rates are controlled in the steady state by cation (C^+) and anion (A^-) permeation respectively. The symbols C^+ and A^- do not denote the valency of the cations and anions, or their stoichiometry of translocation. The plus and minus signs show the electrical polarity.

connecting the effective proton, anion and cation translocations. In the case of anions or cations that do not undergo chemical transformation, the net transport of the ion must be zero in the steady state, or

$$\left(\rightarrow C^Z\right)_{\frac{1}{n}H^+} + \left(\rightarrow C^Z\right)_D = 0 \tag{37}$$

From Eqs. (36) and (37), in the case of nontransformable ions,

$$n\left(\rightarrow H^+\right)_{nC^z} + \left(\rightarrow C^Z\right)_D = 0 \tag{38}$$

so that Eq. (35) can be written as

$$\left(\rightarrow H^+\right)_{o/r} + \left(\rightarrow H^+\right)_{h/d} + \left(\rightarrow H^+\right)_D - \sum \frac{1}{n}\left(\rightarrow C^Z\right)_D = 0 \tag{39}$$

Fig. 14 illustrates how the rates of non-coupled diffusional flow of anions and cations may determine the dissipation of proton current via the coupled translocation systems in the steady state.

In the non-steady state, we can describe the rate of change of the pH differential by

$$-B\frac{d(\Delta pH)}{dt} = \left(\rightarrow H^+\right)_{o/r} + \left(\rightarrow H^+\right)_{h/d} + \left(\rightarrow H^+\right)_D + \sum\left(\rightarrow H^+\right)_{nC^z} \tag{40}$$

where B represents the quantity of protons passing through the membrane, $d(\rightarrow H^+)$, per unit change of pH differential, or

$$B = \frac{d(\rightarrow H^+)}{d(\Delta pH)} \tag{41}$$

The rate of change of membrane potential can similarly be described by

$$M\frac{d(\Delta E)}{dt} = \left(\rightarrow H^+\right)_{o/r} + \left(\rightarrow H^+\right)_{h/d} + \left(\rightarrow H^+\right)_D + \sum(nz+1)\left(\rightarrow H^+\right)_{nC^z} + \sum z\left(\rightarrow C^Z\right)_D \tag{42}$$

where

$$M = \frac{d(\rightarrow H^+)}{d(\Delta E)} \tag{43}$$

In rat liver mitochondria, assuming the buffering power of the outer medium to be relatively high, the coefficients $-B$ and M are about 20 μequiv/pH unit g protein, and 4.2 mμequiv/mV g protein respectively as shown in Sections 5.1 and 5.2. Eqs. (40) and (42) show that if the membrane potential and pH differential built up from zero without leakage of ions other than protons through the membrane, and without participation of the exchange diffusion systems, $M\Delta E = -B\Delta pH$; and substituting the numerical values for M and B, and expressing ΔE and ΔpH in mV, $\Delta E/-Z\Delta pH = 80/1$.

Hence, some 99% of the P.M.F. would be in the form of the membrane potential. The large potential across the coupling membrane would, in practice, cause the permeation of ions down the electric gradient, and this would depress the membrane potential without changing the pH differential unless a compensating process were operating. We visualise, as the compensating process, the specific exchange diffusion, for example of K^+ against H^+($n = -1$, $z = 1$, in term 4 of Eqs. (40) and (42)), which would result in a depression of $-\Delta pH$ but no change in ΔE. Different values of n and z could enable the processes catalysed through the exchange diffusion systems to transform $-\Delta pH$ to ΔE to a lesser or greater extent. If, for the sake of argument, Ca^{2+} travelled against H^+ ($n = -1$, $z = 2$) the pH differential would rise as the membrane potential fell, other things being equal. On the other hand, if Ca^{2+} travelled against Na^+ or K^+ (either indirectly via $\rightarrow H^+$ or directly through a shared carrier), the membrane potential would fall, but the pH differential would remain unchanged, other things being equal. It can readily be shown that during steady state activity of the chemiosmotic system the competition between the translocation of ions through the specific exchange diffusion systems, and the permeation of the ions back through the membrane would give rise to a balance between the ΔE and $-Z\Delta pH$ components of the P.M.F. In general, the higher the velocity constants of the exchange diffusion reactions compared with the permeation constants, the greater would be the proportion of the P.M.F. represented by ΔE; and making reasonable assumptions as to the characteristics of the exchange diffusion reactions and the permeability of the coupling membrane, the steady state values of $\Delta E/-Z\Delta pH$ would make the pH differential represent no more than a few percent of the P.M.F. This conclusion is in keeping with the fact that large pH differences across the coupling membranes of mitochondria, bacteria and chloroplasts have not been recorded experimentally.

7. The integral process of proton transport phosphorylation

7.1. Respiratory control

It has been observed that the onset of oxidoreduction through the electron and hydrogen transfer chain in mitochondria [40] and in bacteria [42] is accompanied by a net output of protons, and that in

chloroplasts [38] and chromatophores [161] it is accompanied by a net intake of protons. However, as oxido-reduction continues, a steady state is soon reached in which there is little or no net acid production or consumption. As indicated in the previous section, this steady state condition is thought to involve a cyclic flow of protons across the membrane.

In the case of rat liver mitochondria, oxidising succinate in the controlled state with no phosphate acceptor, the net outward proton flux through the o/r system would be about 2 μequiv/s g protein (see Section 5.3). The net inward flow through the h/d system would be blocked because ATPase II would be in the X~I form, there being no phosphate acceptor. However, the sum of the net inward flow of protons through the diffusion pathway and through the exchange diffusion carrier pathway would be equal to 2 μequiv/s g protein. Owing to the restriction of the return flow of protons, the P.M.F. would be high and the back pressure on oxido-reduction would be sufficient to slow respiration to the "controlled" rate by the mechanism considered in Section 4.3. The poise of the ATP/ADP couple in State 4 is not precisely known, but assuming that the ATP/ADP ratio may reach 100 at a P_i concentration of 10 mM [4,191,192], the controlling P.M.F. would be some 270 mV (Eq. (15)), of which we might expect about 250 mV to be represented by the membrane potential (see Section 6.2). The total effective o/r spans from cytochrome oxidase to succinate and from cytochrome oxidase to NAD-linked substrates in equilibrium with a P.M.F. of 270 mV would be 540 mV and 810 mV respectively. Assuming the succinate/fumarate couple to be poised at about −100 mV (corresponding to about 1000 succinate molecules per fumarate molecule), the equilibrium poise of the NAD and cytochrome a would respectively be about −370 mV and +440 mV. Hence, if the poise of State 4 corresponded to equilibrium, the NAD would be about 98% reduced, and the cytochrome a would be more than 99% oxidised.

On adding phosphate acceptor to the mitochondria in State 4, the phosphorylation coupled flow of protons through ATPase II would start up, and this would result in a fall in the P.M.F. to a new steady-state value, characteristic of State 3. The observed rise in the rate of respiration to about 160 μg atom O/g protein min, corresponding to a control ratio of 5 (e.g. [144]), would bring the net rate of outward proton translocation through the o/r system to about 10 μequiv/s g protein. The net inward rate of proton movement, also corresponding to about 10 μequiv/s g protein in the steady state, would now be made up of about 2 μequiv/s g protein (or less, owing to the fall in the P.M.F.) through the diffusion and exchange diffusion carrier pathways and about 8 μequiv/s g protein, synthesising about 4 μequiv ATP/s g protein, through ATPase II. Owing to the closed characteristic of the proton circuits, we should not expect large changes of external pH during the State 4/State 3 transitions. On the other hand we would expect a significant change in the P.M.F., the greater part of which would consist of the membrane potential. If the NAD^+ were 50% reduced, and the cytochrome a were 10% reduced in State 3 [4,193], giving an o/r span of 660 mV, the equilibrium P.M.F. would correspond to about 220 mV, and the equilibrium succinate/fumarate poise would remain at −100 mV. It has not yet been possible to measure the membrane potential directly, but we shall consider some evidence for its existence in the following section.

7.1.1. Formation of the coupling membrane potential

We have shown that during a brief burst of respiratory or ATPase activity, rat liver mitochondria translocate protons outwards, and that the observed pH change of the suspension medium can be interpreted as showing a stoichiometric translocation of protons through the o/r and h/d systems [154]. The observations of [38] and of [161] on the inward translocation of protons in briefly illuminated chloroplasts and chromatophores are interpreted qualitatively in a similar way, although in these systems $\rightarrow H^+/2e$ quotients have not yet been measured. The success of this type of experiment is dependent upon there being some "backlash" between the o/r and h/d systems, so that the protons translocated by either system during a brief burst of act from rest would not immediately pass back across the membrane in the closed circuit that operates in the steady state [40]. The main natural "backlash" would presumably correspond to the quantity of charge (in the form of protons translocated through the o/r and h/d system) required to build up the membrane potential towards the steady state in which the protons would be sucked back through the membrane as fast as they appear. As shown in Section 6.1, the translocation of only about 1 μequiv protons/g protein in rat liver mitochondria should bring the membrane potential to its presumed respiratory control value of some 250 mV. In practice the observed $\rightarrow H^+/O$ and $\rightarrow H^+/P$ quotients in rat liver mitochondria isolated by an orthodox procedure were not appreciably lowered until the amounts of oxygen reduced or ATP hydrolysed were equivalent to the translocation of some 10 μequiv protons/g protein [40]. At that point, however, there was a sharp cut-off in the further appearance of protons during oxido-reduction or ATP hydrolysis, and the rate of respiration (or its proton translocation equivalent in ATP hydrolysis) fell from that characteristic of State 3 to that characteristic of State 4 [180]. Our interpretation is that movable ionic constituents of the mitochondrial system are present to the extent of some 10 μequiv charge, and that the migration or orientation of these charged particles or structures, as the electric field builds up across the membrane, is mainly responsible for the "backlash". We presume that the transition of the system from State 3, or its equivalent, to State 4, or its equivalent, occurs because the membrane potential builds up to the point at which it successfully balances the forward o/r or h/d chemical pressure. If this interpretation were correct, it would be expected that a type of reagent that would specifically collapse the membrane potential component of the P.M.F. should release respiratory control and effectively increase the observed "backlash" to an indefinitely high value.

Pressman's important discovery that the antibiotic polypeptides, valinomycin and gramicidin, release respiratory control and simultaneously cause the uptake of K^+ and output of H^+ [194–196] suggested to us that the appropriate type of reagent had, in fact, been found. Chappell and Crofts [186] confirmed Pressman's observations and showed that gramicidin and valinomycin confer upon natural lipid membranes a specific permeability to certain cations. Chappell and Crofts [176] further found that gramicidin and valinomycin had similar effects upon artificial and natural lipid membranes, thus showing that the specific ion-conducting property was resident in the polypeptide molecules themselves. Ogata and Rasmussen [197] have observed that valinomycin permits K^+ to compete with Ca^{2+} in rat liver mitochondria, and conclude that valinomycin acts by making the membrane permeable to K^+ ions. Azzi and Azzone [198] arrived at a similar conclusion from swelling and shrinkage studies. It seems, therefore, that valinomycin and gramicidin should be ideal membrane potential-collapsing reagents.

The effects of valinomycin and gramicidin on stoichiometric proton translocation measurements, using rat liver mitochondria in a KCl medium, were found to be as predicted [180]. Within the region of the normal "backlash", the polypeptides had no effect or slightly increased the observed pH shift or proton translocation stoichiometry. However, no cut-off in proton translocation was observed at the "backlash" limit corresponding to about 10 μequiv protons/g protein, and the usual transition from the State 3 to the State 4 rate of o/r or h/d activity did not occur. At the suggestion of Dr. J.B. Chappell, we found the effect of low concentrations of calcium chloride (100 μM) to be similar to that of gramicidin or valinomycin.

The foregoing observations are in accord with the hypothesis that the greater part of the P.M.F. normally consists of a membrane potential, and supports the view that a stoichiometric proton displacement through the o/r and h/d systems can be observed only under special circumstances [40]. I shall discuss further evidence for the electrogenic

character of proton translocation through the o/r and h/d systems in connection with the mechanism of action of uncouplers (Section 7.2).

We should note that the present discussion is mainly concentrated upon oxidative phosphorylation in mitochondria because there is a relative abundance of experimental information to draw upon. It is possible that in photophosphorylation, catalysed by chloroplasts, the proportion of the P.M.F. represented by $-Z\Delta pH$ may be higher than in mitochondria, because the different polarity of the photophosphorylation system might permit a low pH within the grana discs or lamellae without destructive effects. However, the fact that the relative movements of protons and potassium ions are the same in chloroplasts as in mitochondria [50] suggests that the mitochondrial and chloroplast systems may be closely analogous.

7.1.2. The mechanism of action of oligomycin

It is now generally recognised that oligomycin acts exclusively on the h/d system, and that the inhibition of respiration caused by oligomycin in well coupled mitochondria is due to a respiratory control effect, consequent upon the inactivation of the reversible ATPase and the virtual exclusion of phosphate acceptor (see [10]). The mechanism of release of oligomycin-induced respiratory inhibition by uncoupling agents is the same as the mechanism by which the uncouplers cause the State 4 to State 3 transition in normal mitochondria (see Section 7.2).

Lee and Ernster [153,199] have recently discovered that, in submitochondrial particles, the efficiency of the interaction between electron and hydrogen transfer in different parts of the respiratory chain can be increased by excess oligomycin, and that the efficiency of oxidative phosphorylation can be increased in depleted (e.g. EDTA-treated) submitochondrial particles by titrating with oligomycin to an appropriate end-point. It is possible that the displacement of the factor F_1 in the preparation of “normal” or “depleted” submitochondrial particles permits the access of water to the right hand side of the F_O or X–I translocase/X–I hydrolase system as depicted in Fig. 7, Section 3.5. If this were the case, a leakage of proton current through this system would accompany the cyclic dehydration of XH and IOH and hydrolysis of X~I. Inhibition of the X–I translocase/X–I hydrolase by excess oligomycin would prevent such leakage. Moreover, if the F_O system were slightly more accessible to or had a slightly higher affinity for oligomycin when F_1 were removed than when F_1 were present, appropriate titration of depleted submitochondrial particles with oligomycin could result in the selective inhibition of the naked F_O sites through which proton leakage was occurring, and increase the phosphorylation efficiency through the remaining intact ATPase II complexes.

7.1.3. Relationship between kinetics of oxido- reduction and proton translocation

According to the concept of the proton translocating o/r loop, the effective passage of protons through the coupling membrane is due to the hydrogen- and electron-conducting respiratory carriers, which are supposed to be so organised as to carry hydrogen atoms across the membrane one way and electrons the other way. The kinetic characteristics of oxido-reduction and proton translocation in a given segment of the o/r chain must accordingly be very closely related, since they refer to one and the same process.

Chance and his associates [200–203] have shown that the half times for oxidation of the intact chain of cytochromes a_3, a, c and c_1 by molecular oxygen lie between some 2 ms and 20 ms at 25°. Referring to Figs. 10 and 11, it will be noted that the passage of electron. from cytochrome c_1 through the span to oxygen would not be associated with the appearance of protons on the outer surface of the membrane of intact mitochondria, although it would be associated with the absorption of protons on the inside through the half reaction $1/2O_2 + 2e^- + 2H^+ \rightarrow H_2O$. The initial production of protons in the outer medium would accompany the oxidation of CoQ by cytochrome c_1 in Loop 3 and, subsequently, further protons would be produced during the oxidation of Fp by cytochrome b in Loop 2 and during the oxidation of NADH by Fe, SH in Loop 1. Chance has frequently pointed out that, compared with the other cytochromes, the kinetic behaviour of cytochrome b tends to be anomalous both in “non-phosphorylating” and in “phosphorylating” respiratory chain systems (see e.g. [203]). This may be due to the presence of CoQ between cytochrome c_1 and cytochrome b and to the existence of several independent cytochrome b components (see Section 4.5). However, the time for half oxidation of the Fp components of the initially fully reduced respiratory chain of intact rat liver mitochondria in the presence of β-hydroxybutyrate has been given as some 45 ms at 25 °C [200]. Taking the amount of Fp to be some 0.5 μequivalents per g mitochondrial protein, half oxidation through Loop 2 and about twice this o/r flow through Loop 3 would produce 1.5 μg ion H^+ per g mitochondrial protein. The oxidation of NADH would ensue, so that within the first 50 ms after adding oxygen we might anticipate that as much as 2 μg ion H^+ should have been liberated on the surface of an initially anaerobic suspension of rat liver mitochondria in the presence of β-hydroxybutyrate. The steady rate of β-hydroxybutyrate oxidation in State 3 corresponds to about 0.5 μg atom O/g protein s, or to the translocation of about 3.0 μg ion H^+/g protein s. In our stoichiometric proton translocation measurements [40] we routinely injected saline containing some 50 mμg atom O into anaerobic rat liver mitochondrial suspensions equivalent to some 35 mg protein, or some 1.5 μg atom O/g mitochondrial protein. At the steady-state rate, about 3.0 sec should have been required for complete reduction of the oxygen injected. Taking account of the initial rapid kinetics the total time should be shortened, but it is not clear by how much (see [203]). Assuming that the State 3 rate would supervene after the initial liberation of 2 μg ion H^+ in the outer medium, we would expect some 2 μg ion H^+ to have appeared in the first 50 ms, followed by 3.0 μg ion H^+ per sec during a further 2.3 s. In the corresponding case of succinate oxidation, the steady-state oxidation rate is about three times faster than for β-hydroxybutyrate, and the proton translocation process should be complete in less than 1 s.

The usual time constants for glass electrode pH-measuring systems are in excess of 1 s and it is somewhat difficult technically to obtain short time constants in such systems. Dr. Moyle and I have recently initiated some studies which show that proton translocation during β-hydroxybutyrate oxidation under the conditions described above is half complete in 1.6 s, or less, and that the rate of proton translocation at this time is not less than 2.94 μg ion H^+/s g protein, corresponding to the consumption of 0.49 μg atom O/s g protein. The rate of proton translocation during succinate oxidation has been shown to be at least twice as fast as for β-hydroxybutyrate oxidation, but we have not yet been able to reduce the time constant of our recording system sufficiently to measure this rate.

Chance has suggested that the kinetics of proton translocation can be studied by using a coloured pH indicator and rapid spectrophotometric recording techniques (personal communication, and see proceedings of F.E.B.S. meeting, Warsaw, 1966). Chance's preliminary observations have led him to question whether the observed rates of proton translocation are fast enough to be in accord with the predictions of my hypothesis. There are special technical difficulties associated with the use of pH indicators to measure the pH of one phase of an essentially three phase system such as a mitochondrial suspension. It is evident from Dr. Chance's studies (reported verbally at the American Cell Biology meeting in Philadelphia, 1965) that bromthymol blue may indicate the pH of the inner aqueous mitochondrial phase, or the outer aqueous phase, or possibly the membrane phase, or all three. There are obvious problems of interpretation that may further arise from the migration of the indicator as a result of differences of electrical potential in the system. When such difficulties have been overcome, the pH indicator technique promises to be especially valuable for fast kinetic studies.

7.2. The mechanism of uncoupling

7.2.1. Cation conductors

As discussed in the previous section, gramicidin and valinomycin enhance the permeability of natural and artificial membranes to specific cations. No other direct biochemical activity of these compounds is known, and it is therefore appropriate to seek to explain the potent uncoupling action of gramicidin and valinomycin in terms of the known effect on membrane permeability. Referring to Fig. 14, it will be seen that the steady state cyclic flow of cations is depicted as being driven by the proton current through the relevant exchange diffusion systems. We assume that a mitochondrial suspension, respiring in a KCl medium, would be extruding K^+ ions in exchange for H^+ via the exchange diffusion carrier system, at a rate sufficient to compensate for the inward leakage of K^+ ions. Thus the dissipation of the proton current through the exchange diffusion system would depend upon the potassium ion permeability of the coupling membrane. If the potassium ion permeability were specifically raised by the presence of gramicidin or valinomycin in the coupling membrane, the enhanced flow of potassium ions across the membrane would, other things being equal, increase the dissipation of the proton current through the exchange diffusion system in the steady state, and thus decrease respiratory control. Further, if anions such as phosphate, for which there are exchange diffusion carriers, were present in the medium, the enhanced entry of K^+ on the one hand, and the resulting increased pH differential on the other hand (see Section 6.2) would be expected to lead to the uptake of anions with the potassium ions and to mitochondrial swelling or lysis [176]. The interesting observation of Pressman (personal communication) that the addition of valinomycin to a respiring mitochondrial suspension may cause a rapid uptake of K^+ (down the electric potential gradient) although there may have been a net output of K^+ (exchange of K^+ against H^+ faster than net permeation of K^+) just prior to the addition of the valinomycin would be explained in terms of the model described here and would not require any other "active" potassium pumping device.

The chemiosmotic hypothesis can evidently account rather simply for the main characteristics of the uncoupling and osmotic actions of gramicidin and valinomycin [194,196,204,205]. Chappell and Crofts [176,186] have studied the osmotic effects of gramicidin and valinomycin in considerable detail, and have interpreted their observations along lines that are largely in accord with the hypothesis discussed in this review. There are, however, certain discrepancies that arise from the fact that they, like Moore and Pressman [194], have assumed that the membrane system is electrically neutral, whereas I assume that the electric potential across the membrane is directly responsible, for example, for K^+ uptake in presence of gramicidin or valinomycin, or for Ca^{2+} uptake without any added catalyst (see discussion between Chappell and Mitchell in [170]).

7.2.2. Divalent cations

The divalent metal ions Ca^{2+}, Sr^{2+}, and Mn^{2+} have recently been shown to exhibit a special type of stoichiometric uncoupling effect. The addition of a small amount of the divalent metal salt to a mitochondrial suspension respiring in State 4 gives rise to a discreet burst of respiration, during which the divalent cation is taken up by the mitochondria [193,206–211]. Chance [193] has summarised the characteristic action of small amounts of Ca^{2+} in the following terms: "four distinctive phenomena occur simultaneously upon addition of low concentrations of calcium or ADP and P_i to mitochondrial suspensions. Coincident with the addition of either of these substances (in phosphate-supplemented mitochondria): (a) the steady states of the respiratory carriers jump to new and characteristic oxidation-reduction levels (State 4 to 3 transition); (b) electron transport is stimulated: (c) light scattering changes are initiated; (d) in the case of ADP phosphorylation, hydrogen ion accumulation occurs, and in the case of calcium accumulation, extrusion occurs." The similar action of Ca^{2+} and ADP in bringing about the State 4 to State 3 transition originally led Chance to suggest that "calcium was expended in the reaction with mitochondria in the same sense that ADP was expended" (see [193]). This view has given to the idea that "calcium reacts with all three energy conservation sites of mitochondria, and at a point in the energy transfer pathway which is at the level of either the nonphosphorylated or phosphorylated intermediate". In the same paper, however, Chance [193] points out that the reaction of Ca^{2+} with mitochondria shows a half-time of only 70 ms at 26°, so that "any intervening process between the arrival of calcium at the outer membrane of mitochondria and its subsequent reaction with respiratory enzymes of the crista are either non-existent or non-rate-limiting."

According to the thesis developed here, the reaction of the divalent anions with respiratory enzymes or their hypothetical intermediates would indeed be non-existent. The speed and extent of respiratory stimulation would be attributed to the rapidity and completeness of the collapse of the membrane potential, as the divalent anion was sucked in down the electrical gradient (possibly in exchange for an alkali metal ion or a proton). The temporary State 4 to State 3 transition, with its characteristic changes of carrier o/r levels, would occur because the membrane potential is the major part of the P.M.F., the o/r state of the respiratory carriers being controlled by the P.M.F. as described in Section 4.3. The collapse of the membrane potential, accompanied by respiratory stimulation, would cause an appearance of protons in the suspension medium phase because the rate at which they were drawn into the internal mitochondrial phase would have decreased, while their rate of translocation to the surface would have increased. However, the activity of the electrogenic proton translocating respiratory chain would, in due course, compensate stoichiometrically for the charge displacement corresponding to the uptake of the divalent cation, the membrane potential would rise to the normal control value, and the system would return to State 4. The similarity between the effects of $ADP + P_i$ and Ca^{2+}, which Chance has stressed, would be expected because Ca^{2+} would cause the P.M.F. to fall and would set in motion the State 4 to State 3 transition by collapsing the membrane potential: similarly $ADP + P_i$ would cause the P.M.F. to fall and would set in motion the State 4 to State 3 transition by reacting with X~I in ATPase II and permitting a rapid flow of protons through this system down the P.M.F. gradient. However, whereas the appearance of protons in the medium would accompany Ca^{2+} uptake as shown above, the disappearance of protons from the medium would accompany $ADP + P_i$ uptake because the proton circulation stimulated by the acceptance of water from $ADP + P_i$ in ATPase II would not cause any appreciable change of balance between the contributions of $-Z\Delta pH$ and ΔE to the P.M.F., but, as is well known, the dehydration of $ADP + P_i$ is accompanied by proton absorption near pH 7.

The appearance of protons in the suspension medium during divalent metal uptake has an important corollary. When no penetrating anion is present (e.g. phosphate) to act as effective *Gegenion* during divalent cation uptake by mitochondria, the cation uptake has been shown to be limited by the occurrence of a new inhibited state, which Chance [193,212] has described as State 6. In this state, induced by calcium, the calcium ions are supposed to form an inhibited complex with certain carriers etc. It is interesting to note, however, that after a mitochondrial suspension has accumulated divalent cation with resultant acidification of the suspension medium, the breakage of the membrane with Triton X-100 causes pH neutralisation [262,213]. It would seem, therefore, that State 6 may well be due simply to the circumstance that when no other anion is available to replace hydroxyl ion during divalent metal uptake, the interior of the mitochondria becomes alkaline.

Rossi and Azzone [210] have shown that although the stoichiometry of absorption of divalent cations, based on oxygen uptake or on

proton output, varies according to the detailed experimental conditions, the ratio of proton output to oxygen uptake is relatively constant. They observe an output of approximately $4H^+$ per O reduced for calcium uptake by rat liver mitochondria oxidising succinate, and about $6H^+$ per O when glutamate is the substrate. These interesting observations appear to support the mechanism of cation uptake discussed here.

7.2.3. Dinitrophenol and other proton conductors

It has long been thought that the classical uncoupling agents, exemplified by 2,4-dinitrophenol (DNP), react with one or more of the hypothetical energy-rich intermediates of oxidative phosphorylation, and thereby cause the entry of water into the $ADP+P_i$ dehydrating system. Many suggestions have been made as to which hypothetical energy-rich intermediate or intermediates is or are attacked (see [10,11]) and there does not at present appear to be a consensus of opinion on this matter. It has been observed that DNP and other uncouplers hasten acid–base equilibrium across the membrane of certain bacteria and of rat liver mitochondria [40,42,179], and this appears to have been confirmed for rat liver mitochondria by Chappell and Crofts [176], and for chloroplasts by Jagendorf and Neumann [37]. A closer examination of the acid–base equilibrium across the coupling membrane has revealed some subtleties that shed a new light on the mechanism of uncoupling by proton-conducting reagents.

Dr. Moyle and I have observed that the effect of DNP or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CFCCP) on the pH differential across the membrane system of rat liver mitochondria depends upon whether the pH differential was created, (a) by respiratory or ATPase activity, or (b) by adding acid or alkali to the suspension medium. In case (a), 50 μM DNP or 0.5 μM CFCCP cause a rapid and complete collapse of the pH differential. In case (b), however, when HCl is used to create the pH differential in an anaerobic KCl suspension medium, the addition of the same concentrations of the above uncouplers causes only a limited rapid phase of pH equilibration. As the classical uncouplers represent a special type of weak lipid-soluble acid, there are two main kinds of proton conduction reaction that they might catalyse across the membrane. One kind of reaction would permit exchange of H^+ against another cation such as K^+, and the other would permit only the net conduction of H^+. There are chemical reasons for thinking that the latter might be the more likely [179]. If the classical uncouplers were, in fact, specific proton conductors, they would not be expected to equilibrate a pH differential created by the addition of HCl on one side of a membrane, impermeable to both K^+ and Cl^-. The failure of the Cl^- ion to accompany the passage of H^+ across the membrane would create a membrane potential that would arrest equilibration. However, if the membrane were made specifically permeable to a cation, say K^+, and the concentration of KCl were fairly high on both sides of the membrane, the migration of K^+ would collapse the membrane potential, and the equilibration of the pH differential could go effectively to completion. The effect of the specific cation conductor valinomycin, on pH equilibration in case (b) was accordingly investigated. We found that, in the absence of DNP or CFCCP, the presence of 10 μg valinomycin/g mitochondrial protein did not significantly hasten pH equilibration when HCl was used to create the pH differential. But when 50 μM DNP or 0.5 μM CFCCP were added, the pH differential collapsed as rapidly and as completely as in the case (a) type of experiment. The addition of valinomycin in the case (a) type of experiment had virtually no effect.

The simple conclusions to be drawn from these observations are as follows: (1) DNP and CFCCP are specific proton conductors; (2) The state of the mitochondrial suspension after the outward translocation of protons through the o/r or h/d system differs in a subtle way from the state of the suspension after the external pH has been brought to·the same value by the addition of HCl. In the former case the suspension consists effectively of "mitochondria acid" (the mitochondria being the anion),·because the acidification of the suspension medium and the creation of the membrane potential occur in a single integral protogenic process, as though the mitochondria had simply increased their acidic strength. In the latter case the suspension consists of mitochondria + HCl, and the internal and external aqueous phases are relatively electrically neutral. These conclusions are, of course, integral with the view that the proton translocation through the o/r and h/d systems of mitochondria is electrogenic. Chance et al. [214] have drawn attention to the fact that the dinitrophenol type of uncoupling agent alters the steady state levels of the respiratory carriers more rapidly than does ADP plus phosphate. This relatively rapid effect would be expected to result from the collapse of the P.M.F. by the proton-conducting type of uncoupler.

A considerable number of reagents are now known to resemble DNP and CFCCP in their uncoupling activity. As I pointed out some years ago [179], the DNP-type of uncoupling reagent appears to be distinguished by the possession of two or more alternative weakly acidic groups between which the proton-bonding electron can pass by way of π-orbitals. The following are some examples of this protonconducting class of uncoupler: Nitrophenols, halogenophenols [215–218], dicoumarol, tricyanoaminopropene, carbonylcyanidephenylhydrazones [219], nitro-and chlorobenztriazoles, 3,5-dihalogeno-4-hydroxybenzonitrile(see [220]), β-nitrostyrenes, benzalmalononitriles [221], tetrachloro-2-trifluoromethylbenzimidazoles [222].

7.2.4. Uncoupling of photophosphorylation

Until comparatively recently ammonium salts were the only recognised uncoup1ers of photophosphorylation (see [223]), and it was thought that DNP did not exert an uncoupling action in photosynthetic systems of higher plants. However, Neumann and Jagendorf [224] demonstrated that DNP can be seen to uncouple photophosphorylation in spinach chloroplasts under appropriate conditions; and CFCCP, which is one of the more potent uncouplers of oxidative phosphorylation, is now known to be one of the best uncouplers of photophosphorylation also (see [2]). Gramicidin uncouples both oxidative and photophosphorylation [223]. Although certain substances are about equally effective in uncoupling oxidative and photophosphorylation, there appears to be a systematic difference in potency of the proton conducting type of reagent that may be related to the different polarity of the two systems. The mitochondrial system, which extrudes protons during activity, and is presumed to have a negative electric potential in the internal phase is preferentially uncoupled by weak acids (as listed above), and is not uncoupled by ammonium salts [225]; whereas the chloroplast system, which takes up protons during activity and is presumed to have a positive electric potential in the internal phase is preferentially uncoupled by ammonium salts and by other weak lipid-soluble bases [226]. It is relevant that oxidative phosphorylation in mitochondria is uncoupled by certain polyvalent cations, whereas photophosphorylation in chloroplasts is uncoupled by certain polyvalent anions [227]. It is tempting to conclude that the rate-limiting step in the cyclic proton-ferrying action of the proton-conducting uncouplers is generally the one involving the passage of charge across the membrane. In the acidic uncoupler the charged form is the anion, of which the electron would have to pass outwards across the mitochondrial membrane, assisted by the electric potential gradient. In the basic uncoupler the charged form is the cation, which would have to pass outwards across the grana membrane, assisted by the electric potential gradient. We have argued that swelling and uncoupling of mitochondria by divalent cations is driven by the internal negative potential. Similarly we would argue that swelling and uncoupling in chloroplasts is driven by the internal positive potential.

7.2.5. Other uncouplers

Uncoupling by detergents and other membrane-lytic agents needs no explanation in terms of the chemiosmotic hypothesis; and the

arguments concerning uncoupling by o/r reagents that can bypass sections of the o/r chain are essentially similar for my hypothesis as for the chemical coupling hypothesis. Uncoupling by arsenate, potentiated by ADP [71], could be explained in an orthodox way in terms of the ATPase II system discussed in Section 3.5.

7.2.6. Respiratory inhibition by uncouplers

It has been observed that the proton-conducting type of uncoupler, such as DNP and its derivatives, may inhibit respiration at high concentrations [228], and an attempt has been made to use this fact as evidence for the reaction of DNP analogues with the hypothetical high-energy intermediates of the electron and hydrogen transfer chain [215]. The regions of the respiratory chain inhibited by the dinitrophenols have been shown to correspond approximately to the Amytal-sensitive and antimycin A-sensitive sites. No inhibition was found in the region of "the third phosphorylation site between cytochrome c and O_2", although this site "is as readily uncoupled as the others" [228]. These observations have been discussed by Hemker [215,228] in terms of the scheme described by Hulsmann [229], incorporating the reactions

$$C{\sim}I + \varnothing \rightarrow C + \varnothing{-}I \quad (a)$$

$$\varnothing{-}I \rightleftharpoons \varnothing + I \quad (b)$$

in which C ~ I is an energy-rich carrier compound, and ∅ is an inhibitor or uncoupler. The difference between an uncoupler and a respiratory inhibitor is supposed by Hulsmann to be a quantitative one, depending upon the poise of the equilibrium in Eq. (b). The stimulation or inhibition of respiration is described by the reactions between neighbouring respiratory carriers B and C as follows (compare Fig. ii):

$$CH_2 + I + B \rightleftharpoons C{\sim}I + BH_2 \quad (c)$$

$$C{\sim}I + \varnothing \rightarrow C + \varnothing{-}I \quad (a)$$

$$\varnothing{-}I \rightleftharpoons \varnothing + I \quad (b)$$

$$\text{Sum}: CH_2 + B \xrightarrow{\varnothing} C + BH_2 \quad (d)$$

And the stimulation or inhibition of ATPase activity supposed to originate from each coupling site is described by the reactions

$$ATP + I \rightleftharpoons ADP + I{\sim}P \quad (e)$$

$$I{\sim}P + C \rightleftharpoons Pi + C{\sim}I \quad (f)$$

$$C{\sim}I + \varnothing \rightarrow C + \varnothing{-}I \quad (a)$$

$$\varnothing{-}I \rightleftharpoons \varnothing + I \quad (b)$$

$$\text{Sum}: ATP \xrightarrow{\varnothing} ADP + Pi \quad (g)$$

Stimulation of the o/r and ATPase reactions is explained by the hydrolysis of C ~ I by ∅, via reactions a and b; and inhibition is explained by the withdrawal of I from the reactions by binding in ∅–I. As Hemker [228] found that respiratory inhibition by DNP at high concentrations was mainly at Site 1, less at Site 2, and that there was no inhibition at Site 3, the component I in the foregoing scheme would have to be different and specific for each site (compare Fig. ii). The optimum DNP concentration for ATPase stimulation was found to be more than ten times greater than the optimum for respiratory stimulation, but this was thought not to be prejudicial to the scheme because the rate-limiting effect of sequestration of the I compounds need not be quantitatively the same in the ATPases as in the o/r sequences supposed to occur at each phosphorylation site. As the rate-limiting processes are depicted in the respiratory chain, it would be expected that the pattern of stimulation and inhibition by DNP and DNP analogues would be the same for all NAD-linked substrates. It was found, however, that this was not at all the case [215]. The discrepancy was interpreted as follows "On the basis of the difference in the relation between $p(C_{aq})_{opt}$ and pH for different NAD-linked substrates it is concluded that mitochondrial NAD^+ associated with the different substrates is present in different compartments, and that the whole phosphorylation system connected with a particular substrate must be localised in the same mitochondrial compartment. The compartments differ in their lipophilic character." The building of second order hypothesis upon first order hypothesis, exemplified here, and not uncommon in the literature of oxidative and photosynthetic phosphorylation, is known to be a rather hazardous practice.

7.2.7. ATPase activity and the o/r state of the carriers

The explanation of uncoupling and inhibition in the respiratory chain, based on the scheme of Hulsmann [229], quoted above, is integral with the requirement that the ATPase activities at the three hypothetical coupling sites shall be strongly dependent upon the o/r state of the respiratory carriers; for, according to Eq. (f), if the carrier C became completely reduced to CH_2, the ATPase activity should be zero. The observations of Wadkins and Lehninger [269] and Chefurka [230] have been quoted as giving experimental evidence for such a dependence of ATPase activity upon the o/r state of the carriers, but a close scrutiny of the experimental facts shows that a different interpretation would be more in keeping with other facts and observations.

Wadkins and Lehninger [269] found that the rate of the ATP–P_i exchange reaction catalysed by digitonin particles from rat liver mitochondria was much lower when the particles were incubated under reducing conditions than when they were incubated under oxidising conditions, with the important proviso that the concentration of P_i was kept very low. At physiologically normal P_i concentrations, there was little or no effect. There was similarly an inhibitory effect on the ATPase activity of the digitonin particles under reducing conditions in the absence of added magnesium, but, in the presence of 3 mM $MgCl_2$, incubation under reducing conditions had little effect on the ATPase activity compared with oxidising conditions. The o/r state of the carriers in whole rat liver mitochondria was found to have no effect on the ATPase activity. There was, however, an inhibitory effect of reduction on the ATP–P_i exchange reaction in whole rat liver mitochondria, but again this was only seen at very low P_i concentrations and in experiments of short incubation time. In no case was it shown that after incubation under oxidising conditions, the ATP–P_i exchange or ATPase activity could be inhibited by imposing reducing conditions. The simplest conclusion would therefore seem to be that under the special circumstances found by Wadkins and Lehninger [269] to result in higher exchange or ATPase activity under oxidising than under reducing conditions of incubation, the rate of the reaction was limited by the integrity of the particles or mitochondria, and that oxidising conditions caused more swelling or loosening than reducing conditions.

Chefurka [230] was able to observe that the ATPase activity of whole rat liver mitochondria was depressed by reducing conditions, obtained by adding 1 to 10 mM KCN, or by the addition of substrates when air was excluded. However, these effects could be obtained only when the mitochondrial concentration corresponded to some 0.2 mg protein/ml. It was pointed out that no satisfactory explanation for the failure of reducing conditions to inhibit ATPase activity at mitochondrial concentrations corresponding to some 2 mg protein/ml could be given. The inhibition of the expression of the ATPase by incubation under reducing conditions was shown to be reversible in the sense that a change to oxidising conditions after incubation in reducing conditions would permit expression of the ATPase; but no experiment was described to show that the ATPase activity could be

inhibited by reducing conditions after the ATPase had been activated under oxidising conditions. There seems to be little doubt that the results of Chefurka [230], like those of Wadkins and Lehninger [269], can best be explained by a trivial effect of the incubation conditions upon the integrity of the mitochondria.

Boyer et al.[231–233] found that the o/r state of the respiratory chain had little or no effect on the ATP–P_i exchange in intact mitochondria or sonic particles. Low [234] found that the Mg^{2+}-requiring ATPase of 0.1% deoxycholate-treated rat liver mitochondria was enhanced by reduction with succinate or dithionite. Myers and Slater [235] concluded from their careful and well documented study of the ATPase of rat liver mitochondria: "The addition to liver mitochondria of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (diphosphopyridine nucleotide, cytochrome c, the 2:3 dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the adenosine triphosphatase activity. It appears very unlikely then that a member of the respiratory chain is directly involved in the hydrolysis of adenosine triphosphate."

In view of the facts, there would appear to be a very high risk indeed attached to the defence of the scheme of Hulsmann [229] discussed above. Nevertheless a third order hypothesis has actually been proposed to explain the lack of effect of the o/r state of the respiratory chain on the ATPase activity. Lee and Ernster [152] have suggested that "when the carriers are in the redox state unfavourable for the formation of C~, the concentration of available I increases, and this condition triggers an uncoupler-induced dissociation if I–X by the reactions" described as follows,

$$I{\sim}X + \varnothing \rightleftharpoons X + \varnothing{-}I \qquad \text{(h)}$$

$$\varnothing{-}I \rightleftharpoons \varnothing + I \qquad \text{(i)}$$

Thus, it would be assumed that the uncouplers interact both at the hypothetical coupling sites $C_1{\sim}I_1$, $C_2{\sim}I_2$, $C_3{\sim}I_3$ and in the h/d chain at ~X of Fig.(ii).This is a similar assumption to that made by Chance [193] with respect to the sites of action of Ca^{2+} ions.

7.2.8. Enzyme inhibition by lipid-soluble reagents generally

Returning to consider the inhibitory effect of high concentrations of uncoupling reagents, such as alkyl dinitrophenols, on the respiratory enzymes and the ATPase of mitochondria [215,228], it should be borne in mind that electron and hydrogen transfer through the Amytal-sensitive site is very susceptible to inhibition by a great variety of lipid-soluble reagents, and that the antimycin sensitive site is also rather easily affected, as discussed in Section 4.5. It is also relevant that DNP is known to inhibit enzymes that do not participate in the respiratory chain, for example, hexokinase [236]. Weinbach and Garbus [237] have observed that DNP is taken up avidly by mitochondria and that much of the DNP-binding is due to interaction with mitochondrial proteins. Hemker [228] found that the addition of ethanol to a suspension of mitochondria treated with DNP or with the 4-isooctyl derivative had the effect of diluting the uncoupler at the sites of activity in the mitochondria. Howland [238] has observed a similar type of releasing effect of hydrophobic uncoupling agents on inhibition of succinate oxidation by hydrolapachol, and has drawn the following conclusion: "The fact that uncoupling concentrations of different uncouplers release inhibition to different extents indicates that the release is not only a property of their uncoupling action per se and suggests that some form of competition may be operative. In addition, high concentrations of inhibitor override the effects of uncouplers, also pointing to a competitive situation." It would seem that inhibitory and competitive effects of hydrophobic substances on the o/r and h/d reactions of oxidative phosphorylation may often require no more than trivial explanations.

7.3. Site specific reagents

7.3.1. Oxidative phosphorylation

If it were correct that the coupling of oxido-reduction to phosphorylation is mediated through chemical intermediates ($C_1{\sim}I_1$, etc.) common to the o/r and h/d chains, one might expect that, over the course of time, reagents would be discovered with specific reactivities for the coupling intermediates. It has been suggested that guanidine and its derivatives represent just such a class of compounds [195,239–245]. The main circumstance that has led to the view that guanidine and its derivatives react with the coupling intermediates is that respiratory inhibition is promoted by the conditions of vigorous respiration and tight coupling that would cause the accumulation of the hypothetical C~I compounds, and is released by uncouplers that are supposed to dissociate or hydrolyse C~I. The circumstance that has led to the idea of the site specificity of the supposed interaction between the guanidines and the C~I intermediates is that respiratory inhibition may be localised in the Amytal-sensitive region, or in the antimycin A-sensitive region, depending upon the guanidine derivative.

As I have pointed out elsewhere, [18] a simple explanation of the action of guanidine and its derivatives can be given in terms of the chemiosmotic hypothesis, as follows. Pressman and Park [245] have shown that guanidine competes with Mg^{2+} for entry into mitochondria, and enhances the uptake of P_i. Guanidine and its lipid-soluble derivatives, being cations, are presumably drawn into mitochondria down the electric gradient, and the degree of accumulation (and the inhibitory effect) is dependent upon vigorous, controlled, respiration because these are the circumstances maximising the P.M.F. and the membrane potential. The presence of the guanidine, concentrated, within the mitochondrial cristae and in the coupling membrane would be expected to result in the inhibition of electron and hydrogen transfer through the respiratory chain at susceptible points. The main point, at which most of the guanidine derivatives act is in the delicate Site 1 region, which is blocked even by sub-lytic concentrations of Triton X-100. The less susceptible point, at which, for example, phenethyl-biguanide (DBI) reacts is in the rather easily disrupted cytochrome b region, which is sensitive to antimycin A and hydroxyquinoline-N-oxide (see Section 4.5). The reversal of the guanidine induced respiratory inhibition by uncouplers would be explained in terms of the chemiosmotic hypothesis, since the uncouplers would collapse the membrane potential, and release the accumulated guanidine. The observation of Pressman [245], that respiratory inhibition by DBI in the antimycin A-sensitive region is slowly reversible by uncouplers generally, whereas the inhibition of the sensitive region by other guanidine derivatives is moderately reversible by hydrophilic uncouplers such as DNP, but not so reversible by dicoumarol and other lipophilic uncouplers, may reflect the special susceptibility of the Amytal-sensitive region of the respiratory chain to dislocation by diverse lipophilic reagents as discussed in Section 4.5.3. Schäfer [246] observed that very lipophilic biguanides are uncouplers, and has shown that n-heptylbiguanide blocks the respiratory chain at the same point as Amytal, while it uncouples succinate oxidation. He draws the conclusion that "n-heptylbiguanide behaves as an uncoupler of the second phosphorylating site, whereas it is an inhibitor of the first." My interpretation would be that n-heptylbiguanide increases the ionic permeability of the coupling membrane because it is a detergent, and that it also acts like Amytal or Triton X-100 in blocking the respiratory chain. Guillory and Slater [247] have recently argued for some specificity towards Site 3 in the case of decamethylenediguanidine (synthalin) on the grounds that it inhibits the acceptor-stimulated oxidation of tetramethyl-p-phenylenediamine by mitochondria from rat liver. However, the experimental results presented by Guillory and Slater [247] imply that synthalin partially uncouples the TMPD oxidation and also inhibits the reaction of the reversible ATPase with phosphate acceptor, rather than that there is

evidence for a specific reaction between synthalin and the hypothetical Site 3.

7.3.2. Photophosphorylation

Baltscheffsky and Arwiddson [248] observed that valinomycin at low concentrations would eliminate approximately half the phosphorylative activity of chromatophores from *R. rubrum* under normal (physiological) conditions of cyclic photophosphorylation. When, however, the electron and hydrogen transfer chain was blocked by 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and the blockage was bypassed by the addition of phenazine methosulphate, the phosphorylation associated with this so-called PMS-system was resistant to valinomycin. Further, it was found that in the PMS-system nearly twice as many light quanta were consumed as in the physiological system for each molecule of ATP produced [249]. More recently Baltscheffsky and von Stedingk [161] have observed that the so-called physiological system gives rise to a pH shift during illumination (see Section 4.7), whereas the PMS-system does not. Horio and Yamashita [250] have also shown that part of the phosphorylative activity of *R. rubrum* chromatophores is more susceptible to inactivation by Triton X-100 than the rest. These observations have led to the suggestion that the valinomycin-sensitive phosphorylation is similar to that occurring in oxidative phosphorylation in mitochondria, and is associated with electron and hydrogen transfer in the region of the o/r chain blocked by HQNO, whereas the phosphorylation associated with the PMS-system involves a different energy transfer mechanism. According to Baltscheffsky [1] “the latter energy transfer pathway appears by different criteria to be uniquely lacking the hypothetical intermediate X–I”. It is remarkable however, that phosphorylation through both sites postulated by Baltscheffsky and co-workers is blocked by low concentrations of oligomycin [161].

Avron and Shavit [223] have shown that in Swiss-chard chloroplasts certain uncoupling agents, notably gramicidin S, acetyl guanidine and CFCCP, uncouple phosphorylation associated with ferricyanide reduction more effectively than that dependent upon PMS as co-factor. They suggest that a possible explanation may be the existence of two phosphorylation sites, one linked to the ferricyanide photoreduction pathway, related to System II, and the other linked to the PMS-mediated pathway, related to System I. Baltscheffsky [1] has recently discussed similar evidence of differential inhibitory effects of desaspidin in chloroplasts observed by his group. Unlike CFCCP, desaspidin inhibits phosphorylation in chloroplasts with PMS as co-factor at much lower concentrations than non-cyclic photophosphorylation using OH-as electron donor (see also [270]).

In the case of photophosphorylation in chloroplasts it does not seem that the present evidence warrants the interpretation that the phosphorylation reaction accompanying non-cyclic oxido-reduction differs fundamentally from that accompanying the cyclic type of oxido-reduction. According to the chemiosmotic hypothesis, phosphorylation would occur through the reversible ATPase II system in either case, and the different susceptibilities of the phosphorylation associated with non-cyclic and cyclic types of oxido-reduction to various inhibitors and uncouplers would be explained in terms of the susceptibilities of the different o/r and photosynthetic pigment systems involved. On the other hand, in the case of the photophosphorylation in chromatophores, there appears to be a case for considering the possibility that one phosphorylation may be coupled to oxidoreduction by a chemical type of mechanism, and that another phosphorylation may be coupled to oxidoreduction by a chemiosmotic type of mechanism.

7.4. Reversal of electron and proton transport

According to the chemiosmotic hypothesis, the synthesis or hydrolysis of ATP via the h/d or ATPase system is linked reversibly to the translocation of protons across the coupling membrane, and if the protons pass through the ATPase in one direction, ATP synthesis will occur, but if they pass in the reverse direction, ATP hydrolysis will occur. The oxido-reduction reactions through one or more of the o/r loops would similarly reverse if the P.M.F. exceeded the mid-point potential span across the o/r loop system, or, using Eq. (31), if the quantity f, given by

$$f = \frac{2}{n}\left[\Delta E'_m + Z\log_{10}\sqrt[k]{\frac{\{OX_e\}}{\{RED_e\}}}\cdot\sqrt[j]{\frac{\{RED_H\}}{\{OX_H\}}}\right] - \Delta p$$

were negative, n being the number of protons translocated by the loop system. In the case of the transhydrogenase system of Loop 0 (Section 4.6), $\Delta E'_m$ is only 4 mV [152], and is thus negligible compared with $-\Delta p$ under normal conditions. In Loop 0, therefore, the normal direction of proton translocation, and electron and hydrogen transfer would be the reverse of that occurring in Loop 1, Loop 2 and Loop 3, and would be driven by the P.M.F. When Δp had its normal magnitude of some +250 mV, the reversal of electron and hydrogen transfer would occur through any of Loops 1, 2, or 3 if the o/r state of the carriers across the loop were appropriately poised to make f negative. In these cases, however, the direction of the P.M.F., denoted by the sign of Δp is always the same, and corresponds to the net outward translocation of protons in intact mitochondria.

There should be an especially interesting consequence if the sign of Δp were artificially reversed by reversing the direction of the chemical force on oxido-reduction. For example, if the transhydrogenase of Loop 0 were operated under conditions of initially zero P.M.F., and the reaction $NADP^+ + NADH \rightarrow NADPH + NAD^+$ were catalysed spontaneously, there should be a net translocation of protons in the direction opposite to that observed normally. This experiment has been done with sonic particles from beef heart mitochondria [154], and although the observed stoichiometry was low, we were able to confirm that the direction of proton translocation was indeed reversed when the direction of oxido-reduction was reversed. We have also found it possible to reverse the normal direction of oxido-reduction through the part of the respiratory chain between succinate dehydrogenase and a point on the oxygen side of the antimycin A-sensitive site, probably corresponding to cytochrome c. This has been done in intact beef heart mitochondria by reducing internal fumarate with an externally added artificial electron donor. The reaction was sensitive to antimycin A, and caused the translocation of protons inwards instead of outwards across the mitochondrial membrane system [154]. These observations would be difficult to explain in terms of the chemical coupling hypothesis.

7.5. Osmotic pressure and water flow

As indicated in Section 3.1, the ATPase II system would not actually transport protons inwards during ATP synthesis in mitochondria, but would transport O^{2-} outwards. The effect of the outward transport of O^{2-} would be equivalent to the translocation of $2H^+$ inwards and H_2O outwards. Hence,in State 3, for example, water would be chemically transported outwards through the membrane. This water flux is not, however, likely to contribute significantly to the osmotic steady state of the mitochondria, because the rate of equilibration of water by diffusion across the coupling membrane would be expected to be high, compared with that of the main solutes present in the internal and external aqueous phase. Similar considerations would apply to water movement in chloroplasts and bacteria. Accordingly, the reversible o/r-linked and h/d-linked swelling and shrinkage changes of mitochondria, chloroplasts, and bacteria can probably be ascribed mainly to the flow of water, as such, under its own activity gradient, following the metabolically linked translocation of osmotically active solutes.

8. The sidedness of the chemiosmotic system

One of the most stringent requirements of the chemiosmotic hypothesis is that the membrane systems of mitochondria, chloroplasts, and bacteria across which the o/r and h/d systems are presumed to be asymmetrically organised should exhibit a sidedness. Owing to this fundamental requirement, it has been possible to use a powerful argument against acceptance of the chemiosmotic view of the coupling mechanism.

It has been known for a number of years that sonically disintegrated mitochondria will catalyse oxidative phosphorylation [7], energy-linked transhydrogenation [153], and other reversed electron transfer reactions [251]. The mitochondrial fragments used in such studies differ from intact mitochondria in that the soluble enzymes and intermediates such as NAD and ATP are no longer retained behind a membrane as in intact mitochondria; and moreover, whereas, in intact mitochondria, only the endogenous NAD, NADP, etc. can participate directly in energy-linked electron and hydrogen transfer reactions, in the sonically fragmented system, externally added NAD, NADP, etc. are able to react directly with the enzymes catalysing the energy-linked reactions. One would naturally interpret these facts as indicating that the closed membrane system of mitochondria, separating inner and outer aqueous phases, is not necessary for the coupling of oxidation to phosphorylation, and it would follow that the chemiosmotic hypothesis should be abandoned. However, further thought shows that there is another possible interpretation of the behaviour of the particles obtained by sonic disintegration of mitochondria. Electron microscopy has shown that these sonic particles are vesicular and are not open membrane fragments. Further, it has been pointed out by Lee and Ernster [153] that the stalked spherical bodies that are seen in negatively stained electron micrographs on the inside of the cristae membrane of intact mitochondria [252,253], appear to be present on the outside of the vesicular sonic particles [63,65]. It is conceivable, therefore, that the limiting membrane of the sonic particles may be the osmotically functional coupling membrane, and that the membrane of the particles may be inside out so that the side normally accessible only to internal NAD, ATP, substrates etc. would be directly accessible to the NAD, ATP, substrates etc. in the suspension medium. If this were the case, we should be able to detect the presence of the coupling membrane by titrating suspensions of the sonic particles with acid or alkali and observing the time course of acid–base equilibration with a sensitive pH meter as we have done in intact mitochondria. We should also observe that respiratory or ATPase activity would be accompanied, not by the outward, but by the inward translocation of protons. In a preliminary report of such experiments [154] we have been able to confirm that the sonic particles from beef heart mitochondria appear to possess an osmotically functional membrane, and that, judging from the direction of proton translocation during oxidation of succinate or NADH, and during hydrolysis of ATP, the membrane is inside out. Similar measurements on particles isolated from beef heart mitochondria by digitonin treatment, following the method of [254], have shown that, in this case, the polarity of the membrane is the same as that of intact mitochondria and opposite to that of the sonically prepared particles (Mitchell, Moyle and Lee, unpublished). Fig. 15 illustrates the relationship between the intact mitochondrion and the sonic and digitonin particles.

Our observations on the sidedness of the mitochondrial membrane system bring into a focus a number of observations on the differences between the behaviour of mitochondria or digitonin particles on the one hand and of sonic particles on the other. During studies on ATP-driven reversed electron and hydrogen transfer in particles derived from pigeon heart mitochondria, Chance and Fugmann [255] and Lee [256] found that digitonin particles oxidise exogenous cytochrome c, but reduce only endogenous NAD, whereas sonic particles reduce exogenous NAD, but oxidise only endogenous cytochrome c. The digitonin particles contain considerable quantities of endogenous

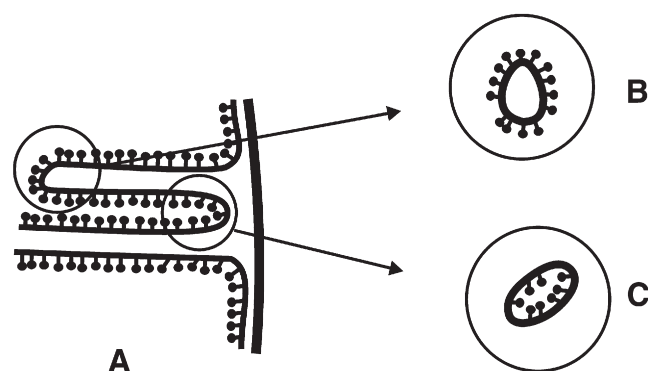


Fig. 15. Diagram showing relative sidedness of mitochondrial cristae membrane: A, in intact mitochondria; B, in particles obtained by sonic disintegration; C, in particles obtained by digitonin treatment. The stalked spherical particles are shown on the side of the membrane away from which protons are translocated during respiration and ATP hydrolysis.

NAD, while sonic particles contain practically none (see also [257]). Chance [123] and Jacobs and Sanadi [200] showed that much of the endogenous cytochrome c is readily liberated from intact mitochondria by swelling them in 15 mM KCl and subsequently washing them in 150 mM KCl. On the other hand, the endogenous cytochrome c of sonic particles is apparently trapped behind the membrane, for it is not liberated by the above procedure (Lee, personal communication).

Summarising these observations, it would seem that in intact mitochondria the cytochrome c is present on the surface of the cristae membrane, and the components with which it reacts (cytochrome a and cytochrome c_1) must also be accessible to cytochrome c from outside. On the other hand, as originally suggested by Lehninger [258], NAD^+ and NADH can react with the respiratory chain system only from the inside of the cristae membrane. External NADP can react directly with the transhydrogenase system in sonic particles, but not in intact mitochondria [153], and so the reactive site for $NADP^+$ and NADPH must likewise be present on the inner side of the cristae membrane of intact mitochondria. It is relevant that the respiratory chain system is now known to be present in the laminar part of the cristae membrane, and that the stalked spheres on the inner surface of the membrane contain the part of the reversible ATPase system that reacts with ATP, ADP, and P_i (see [61]). It has been shown that the endogenous ADP of mitochondria is phosphorylated within the cristae, and that the movement of ADP and ATP between the external medium and the site of phosphorylation is mediated by an atractylate-sensitive translocation system (see [188]). The atractylate-sensitive reaction is absent in sonic particles [251], but is partially intact in digitonin particles [259]. It now seems clear that the atractylate-sensitive system is present in the cristae membrane, and that it is required in intact mitochondria and in digitonin particles to permit external ADP and ATP to gain access to the terminal member of the reversible ATPase system. In sonic particles, however, the membrane is inside out, and although the atractylate-sensitive system is probably present in the membrane it would not be functional as an essential intermediary between external ADP and ATP and the reversible ATPase system. The same considerations apply to the other specific translocation systems for cations and anions, including P_i and substrates, discussed in Section 6.

Vasington [260] found that digitonin particles exhibit an energy-linked uptake of calcium ions as do intact mitochondria, but the system for calcium ion uptake appeared to be absent from sonic particles. This observation may have the simple explanation that in the sonic particles calcium ions are expelled rather than being drawn in by the membrane potential, because the polarity of the membrane is reversed. A similar explanation may apply to the observation of [247] that the powerful inhibitory effects of hexyl guanidine on

respiration in intact mitochondria do not occur in sonic mitochondrial particles (see also Section 7.3.1).

In chloroplasts, the direction of proton translocation, driven by light-activated oxido-reduction is inwards through the grana membrane, and we should therefore expect the polarity of the membrane with respect to the reversible ATPase system to be similar to that of sonic mitochondrial particles. Electron micrographs do, in fact, show the stalked spheres on the external surface of the grana [261], and the latent ATPase which is thought to correspond to the terminal component of the reversible ATPase is evidently sufficiently exposed at the surface of the particles to react with protein and become inactivated by a specific antibody [48].

9. Summary

The object of the chemiosmotic hypothesis is to explain the coupling between oxido-reduction and phosphorylation without assuming the existence of chemical intermediates common to the oxido-reduction and phosphorylation pathways. The chemiosmotic hypothesis is based upon four postulates that can be used, on the one hand for the further development of the theory of chemiosmotic coupling, and on the other hand for the design of experiments intended to test the validity of the hypothesis.

The chemiosmotic system consists of four basic parts, corresponding to the four postulates:

1. The proton translocating reversible ATPase system.
2. The proton translocating oxido-reduction chain.
3. The exchange diffusion systems, coupling proton translocation to that of anions and cations.
4. The ion-impermeable coupling membrane, in which systems 1, 2, and 3 reside.

There are two possible reversible ATPase systems, called ATPase I and ATPase II, which may translocate one and two protons respectively per ATP hydrolysed. The ATPase systems of both chloroplasts and mitochondria may be of type II. The best characterised of the so-called coupling factors appear to correspond to parts of the ATPase II system of mitochondria and chloroplasts. The mechanism of proton translocation by ATPase II can be explained in terms of the reactions of an anhydride X–I, formed between two acidic groups XH and IOH in the ATPase system.

The translocation of protons by the membrane-located o/r chain is explained in terms of o/r loops, each consisting of one hydrogen and one electron carrier. The chain is thought to consist of several o/r loops in series, the protons being taken in and given out on opposite sides of the membrane at the points of alternation between the hydrogen and electron carriers.

The operation of the proton translocating ATPase and o/r chain systems in an ion-tight membrane would create both a pH differential and a membrane potential, conveniently described together as a protonmotive force (P.M.F.) by analogy with electromotive force. The presence of the exchange-diffusion systems would regulate the internal pH and would enhance the membrane potential component of the P.M.F. at the expense of the pH differential.

Coupling between oxido-reduction and phosphorylation can be described by a circulating proton current connecting the ATPase and o/r systems at a P.M.F. of some 250 mV. The interaction between oxido-reduction and phosphorylation in this kind of "fuel cell" arrangement would permit the two systems to come into equilibrium and would allow some reversibility of both oxido-reduction and hydration-dehydration reactions. The hypothetical system provides a model of respiratory control and reversed electron transport. The chemiosmotic model also accounts very simply for the phenomena of uncoupling and ion accumulation in mitochondria and chloroplasts.

Measurements of proton translocation in mitochondria, bacteria, and chloroplasts have shown that in the latter case, the protons pass inwards through the grana membrane, but in the former two cases the protons pass outwards. In digitonin fragments of mitochondria, the direction of proton translocation is as in intact mitochondria, but in sonic fragments of mitochondria the direction of proton translocation is reversed, and it is evident that the membrane of the sonic fragments is effectively inside out. The sidedness of the chemiosmotic system is one of its most interesting and characteristic features.

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