Biochimica et Biophysica Acta, 768 (1984) 257–292
Elsevier

BBA 86115

A MINIMAL HYPOTHESIS FOR MEMBRANE-LINKED FREE-ENERGY TRANSDUCTION

THE ROLE OF INDEPENDENT, SMALL COUPLING UNITS

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(Received February 29th, 1984)

Contents

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Abbreviations: DCCD, *N*,*N'*-dicyclohexylcarbodiimide; PS I, II, Photosystem I, II.

I. Summary

Experimental data are reviewed that are not in keeping with the scheme of 'delocalized' protonic coupling in membrane-linked free-energy transduction. It turns out that there are three main types of anomalies: (i) rates of electron transfer and of ATP synthesis do not solely depend on their own driving force and on $\Delta\tilde{\mu}_H$, (ii) the ('static head') ratio of ΔG_P to $\Delta\tilde{\mu}_H$ varies with $\Delta\tilde{\mu}_H$ and (iii) inhibition of either some of the electron-transfer chains or some of the $\vec{H}^+$-ATPases, does not cause an overcapacity in the other, non-inhibited proton pumps. None of the earlier free-energy coupling schemes, alternative to delocalized protonic coupling, can account for these three anomalies.

We propose to add a fifth postulate, namely that of the coupling unit, to the four existing postulates of 'delocalized protonic coupling' and show that, with this postulate, protonic coupling can again account for most experimental observations.

We also discuss: (i) how experimental data that might seem to be at odds with the 'coupling unit' hypothesis can be accounted for and (ii) the problem of the spatial arrangement of the electrical field in the different free-energy coupling schemes.

II. Introduction

The concept that the H^+ electrochemical potential difference, $\Delta\tilde{\mu}_H$, acts as the coupling intermediate between redox or light-driven H^+ pumps on one side and ATPase H^+ pumps on the other [1,2] has been of great predictive and practical value in the analysis of events associated with membrane-linked biological free-energy transduction [3]. The following postulates [1,4] have been shown to be correct: (i) the primary (respiration- or photoreaction-linked electron-transfer chain, or bacteriorhodopsin) as well as the secondary free-energy transducing enzyme complexes ($\vec{H}^+$-ATPase) can couple transmembrane proton movement to scalar chemical reactions, (ii) the free-energy transducing membranes have a limited permeability for protons and (iii) they contain electrophoretic or proton-linked substrate transport systems. Also, transmembrane electric potentials and pH differences exist under energized conditions [5].

The general features of the $\Delta\tilde{\mu}_H$-linked coupling scheme having been accepted, a major question now addresses the precise mechanism by which the primary and secondary H^+ pumps are coupled together. This implies the identification of the phases at the two sides of the membrane between

which the protons are transferred. Mitchell [6] tentatively identified these with the bulk aqueous phases inside and outside the free-energy coupling organelles. More recently [7], he specified that, on their way to the $\vec{H}^+$-ATPase, the protons would not necessarily flow all the way through the bulk aqueous phase, but might follow pathways closer to the membrane. Yet [2,7], the proton conductivity of the bulk aqueous phases would be so high that differences in electrochemical potential for protons could never amount to more than small humps of value less than the thermal energy kT (see also below). Consequently, energy coupling would be completely characterized by one electrochemical potential difference for protons between two homogeneous aqueous phases separated by the energy-coupling membrane.

However, this elaboration of the protonic coupling scheme appears to be increasingly in conflict with experimental results. While recent reviews of such conflicting results have tended to be primarily analytical in nature, our purpose will be here merely to order the anomalies. Furthermore, we shall propose that protonmotive free-energy coupling should be interpreted as the resultant of an array of, rather independent, small free-energy coupling units operating as proton microcircuits within the energy-coupling membrane [8].

III. Experimental anomalies confronting the three-phase chemiosmotic coupling scheme

IIIA. Anomaly 1: no unique correlation between $\Delta\tilde{\mu}_H$ and fluxes

In the three-phase interpretation of protonic coupling ('at the physiological level') which we shall call 'delocalized coupling' [9], electron transport in the respiratory or photo-redox chain is coupled to proton transport across the membrane. The latter generates a difference ($\Delta\tilde{\mu}_H$) between the purportedly homogeneous electrochemical proton potentials in the two bulk aqueous phases bordering the membrane. When this $\Delta\tilde{\mu}_H$ reaches a sufficient magnitude, it may drive the reaction catalyzed by the proton translocating ATPase backwards, so that ADP is phosphorylated. It is implied that the passage from redox free energy to ATP free energy of hydrolysis (phosphate potential) will always occur through Gibbs free energy of the form of $\Delta\tilde{\mu}_H$. Respiratory control (i.e., increase in electron flow upon addition of ADP and phosphate) is explained via a decrease in $\Delta\tilde{\mu}_H$ caused by the initiation of proton flux through the $\vec{H}^+$-ATPase, i.e., respiration senses phosphorylation only through $\Delta\tilde{\mu}_H$. Since ion transport also affects respiration through $\Delta\tilde{\mu}_H$, the relation between respiratory rate and $\Delta\tilde{\mu}_H$ should not depend on how $\Delta\tilde{\mu}_H$ is depressed. It should also be independent on the properties of the passive H^+ leakage. Likewise phosphorylation rate should be uniquely dependent on the magnitude of $\Delta\tilde{\mu}_H$. Both predictions, however, are not verified by the results of the corresponding experiments.

Padan and Rottenberg [10] found that the effect of ADP or of valinomycin plus K^+ on respiration and $\Delta\tilde{\mu}_H$ were not equivalent in that, at equal $\Delta\tilde{\mu}_H$ values, respiration was stimulated less by the ionophore than by ADP: the electron-transfer chain seemed to sense the effect of ADP not only through the effect of the latter on $\Delta\tilde{\mu}_H$. More generally, the variation of oxidation rate with $\Delta\tilde{\mu}_H$ in mitochondria was different for different methods employed to vary $\Delta\tilde{\mu}_H$ [11–13]. Since addition of ADP may result in a decrease of the passive leak rate via F_0-F_1 ATPase, it has been contended that the 'anomalous' relationship between J_O and $\Delta\tilde{\mu}_H$ be due to this latter effect. However, such an effect of ADP should not cause a shift in the relationship between electron-transfer rate and $\Delta\tilde{\mu}_H$. Yet, ADP was found to stimulate respiration *more* than uncouplers, at the same $\Delta\tilde{\mu}_H$.

In heart submitochondrial particles, respiratory control in the absence of a closed membrane has been claimed [14,15]. These observations, not supported by adequate $\Delta\tilde{\mu}_H$ assays, may be considered not strictly compatible with those reported previously which still indicate correlations between J_O and $\Delta\tilde{\mu}_H$ although more complex than predicted by delocalized protonic coupling.

In the same manner as the respiratory rate J_O is not uniquely dependent on $\Delta\tilde{\mu}_H$, also the phosphorylation rate, J_P is not uniquely dependent on $\Delta\tilde{\mu}_H$. In chromatophores the variation of the rate of ATP synthesis with $\Delta\tilde{\mu}_H$ was only little when the latter was depressed by addition of protonophorous uncouplers, but strong when the depression was caused by reducing the intensity of

illumination or adding an inhibitor of electron transfer [16–18]. In mitochondria [19] addition of respiratory inhibitors resulted in a marked depression of J_{ATP} with little or no change of $\Delta\tilde{\mu}_H$ [20]. In contrast, depression of $\Delta\tilde{\mu}_H$ by 50 mV through ion transport had no effect on J_{ATP} [19,21]. This suggests that the dependence of J_{ATP} on $\Delta\tilde{\mu}_H$ is not independent of the way the latter is varied [13,16–23].

There are several separate reports for large variations of J_{ATP} in the presence of hardly detectable changes in $\Delta\tilde{\mu}_H$ Refs. 24–28, cf. Refs. 276 and 277. Sorgato et al. [24], for instance, showed that in submitochondrial particles 50% inhibition of succinate oxidation by malonate resulted in almost 50% inhibition of the rate of ATP synthesis without appreciably changing $\Delta\tilde{\mu}_H$. In chromatophores, photophosphorylation correlated with cytochrome *c*-420 content rather than with transmembrane proton movement [29,30].

Other reports exist of low levels of $\Delta\tilde{\mu}_H$, with only little effect on ATP synthesis. Mitochondria with a membrane potential of some 20 mV positive inside (as measured by electrode impalement) have been claimed to be capable of ATP production [31]. Unfortunately, parallel determinations of $\Delta\tilde{\mu}_H$ with the isotope distribution technique, have not been reported. Uncoupler resistant mutants of *Bacillus megaterium* synthesized ATP after $\Delta\tilde{\mu}_H$ had been annihilated by protonophorous uncouplers [32]. Membrane vesicles of the same cells synthesized ATP at the low $\Delta\tilde{\mu}_H$ found in the absence of ionophores [33].

In principle, one of these reports [24] might be explained within the bounds of the delocalized protonic coupling hypothesis by postulating that the dependence of the phosphorylation and the oxidation rates on $\Delta\tilde{\mu}_H$ is so steep to be within the experimental error of the $\Delta\tilde{\mu}_H$ measurements [34]. Indeed [35,36], already in the case of simple, enzyme kinetics, reactions rates depend on free-energy differences in a sigmoidal, rather than proportional, fashion, with a steep dependence of the rate on the free-energy difference in one region [5] and hardly any dependence in other regions. The former region might be that where the rates of ATP synthesis varied strongly with little variatoin in $\Delta\tilde{\mu}_H$ [19,20,24]. This suggestion, however, would not account for the most crucial anomaly, namely that the dependence of the pumping rate on $\Delta\tilde{\mu}_H$ is different for different modes of variation of the latter [10–13,16–22]. Indeed it is this nonunequivocal dependence that constitutes anomaly one.

This type of observation is not limited to cases of free-energy transduction between electron transport chains and $\bar{H}^+$-ATPases. For alanine uptake into *Rhodopseudomonas sphaeroides* cells Elferink et al. [37] showed that the dependence of the uptake rate on the membrane potential (the pH gradient was kept constant) was different when the membrane potential was varied by varying the light intensity, as compared to when, at high light intensity, different amounts of protonophorous uncoupler were present.

Although findings like these have been interpreted as being in conflict with Mitchell's views and have led to alternative coupling schemes [10,32,38,39], they could in principle fit into the delocalized protonic coupling scheme by postulating that in addition to the free-energy transducing link between the electron transport chain and the $\bar{H}^+$-ATPase, there exist 'allosteric' links between primary and secondary proton pumps [40–43] though not by postulating that there exists a $\Delta\tilde{\mu}_H$-controlled gated H^+ flux through the $\bar{H}^+$-ATPase.

An allosteric effect of a redox component that would equilibrate with the redox state of one of the components of the respiratory chain, on the $\bar{H}^+$-ATPase (i.e., activating or deactivating the enzyme), is certainly among the possibilities that deserve further study in view of the reported effects of natural and artificial redox mediators on $\bar{H}^+$-ATPase and other free-energy transducing enzymes [43–51]. The ATPase inhibitor protein or, in photosynthetic bacteria and chloroplasts, a subunit of F_1, could be candidates for this role [52,53]. Markwell et al. [54] and Horton [55], citing work of Baker and co-workers [56] that would indicate inhibition of thylakoid protein kinase by low ATP/ADP ratios (see also Ref. 57), proposed that this may be a physiological way in which distribution of photon energy between Photosystem I and Photosystem II would be regulated. It would be only one step further to propose that through protein phosphorylation the ATP/ADP ratio would directly regulate the rate of electron transfer. A factor of significance may be the observed [58] movement of light-harvesting chlorophyll *a/b*

complexes from the Photosystem-II-rich 'apressed' membranes to the Photosystem-I- and $\vec{H}^+$-ATPase-rich [59] unstacked regions, upon phosphorylation of thylakoid membranes (Rev. 61).

According to Skulachev [73,134] the allosteric link between redox and ATPase H^+ pump would be represented by the capacity of the redox pump to raise the proton electrochemical potential difference of the neighbouring ATPase above the kinetic threshold for ATP synthesis.

IIIB. Anomaly 2: imbalance of ΔG_P and $\Delta\tilde{\mu}_H$ at static head

The experiments of this second group cannot be explained by allosteric links. Typically, respiring mitochondria are allowed to phosphorylate some added ADP until they reach the steady-state condition characterized by the absence of any further net ATP synthesis; state 4. It is apparent from the non equilibrium thermodynamic analysis that the term 'static head' is a proper definition for state 4 [63]. We shall here describe the $\vec{H}^+$-ATPase as a completely coupled pump and assume the absence of flux through the $\vec{H}^+$-ATPase in state 4. In the delocalized protonic-coupling interpretation the reaction catalyzed by the $\vec{H}^+$-ATPase:

$$n_H^P \cdot H^{+\,out} + ADP + P \rightleftharpoons ATP + n_H^P \cdot H^{+\,in} \qquad (1)$$

has then come to equilibrium: its Gibbs free-energy change, consisting of the Gibbs free energy of hydrolysis of ATP (i.e., the phosphate potential ΔG_P) plus n_H^P-times the electrochemical potential difference for protons ($\Delta\tilde{\mu}_H$), must equal zero:

$$\Delta G_P + n_H^P \cdot \Delta\tilde{\mu}_H = 0 \qquad (2)$$

Consequently, under 'state 4' conditions the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ is predicted to be constant and equal to the proton pumping stoichiometry of the $\vec{H}^+$-ATPase:

$$\Delta G_P/(-\Delta\tilde{\mu}_H) = n_H^P \qquad (3)$$

(in mitochondria, $\Delta\tilde{\mu}_H$ would be negative, according to our definition). The equality sign is valid only, when the state-4 condition represents a true equilibrium for the proton translocating ATPase and not a kinetically restricted state [64,65].

In the early experiments [66] the ratio $\Delta G_P/|\Delta\tilde{\mu}_H|$ was found already slightly to exceed the theoretical value of 2 proposed by Mitchell [4]. Subsequently, observed ratios markedly exceeded 3 (and even 6) and, what is really inconsistent with Eqn. 3 (irrespective of the true value of n_H^P), varied with varying $\Delta\tilde{\mu}_H$ [12,38,39,65–68]. Wilson and Forman [68] observed a similar rise of $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio. However, in their experiments a possible role of substrate level phosphorylation cannot be excluded.

The reported electrogenicity of the adenine-nucleotide translocator [70] would have the effect of augmenting n_H^P by 1, but could not explain such a variation with $\Delta\tilde{\mu}_H$. Furthermore variation of $\Delta G_P/|\Delta\tilde{\mu}_H|$ with $\Delta\tilde{\mu}_H$ has been observed in systems in which the role of the adenine-nucleotide translocator has been eliminated (i.e., in submitochondrial particles [8,71] and in chloroplasts [72,73]) and in systems where adenine nucleotide translocation is irrelevant [74–75,130]. Similar results have been obtained in studies where transport is the energetic output process, e.g., the steady-state lactose gradient across the membrane of *E. coli* cells [78] decreased less than the proton gradient as the latter was decreased with a protonophorous uncoupler.

Postulated 'allosteric' effects (e.g., by the protonophores on the $\vec{H}^+$-ATPase) cannot alone explain these observations, as the equilibrium condition of Eqn. 2 is not affected by allosteric interference with the catalyst and the reaction catalyzed by the $\vec{H}^+$-ATPase is (essentially) in equilibrium. In particular, it has been shown that the $\vec{H}^+$-ATPase does not shut down, since the dependence of $\Delta G_P/|\Delta\tilde{\mu}_H|$ on $\Delta\tilde{\mu}_H$ is the same whether the added adenine nucleotide is AMP or ATP [65]. Baccarini-Melandri et al. [16] stressed that the ATPase proton pump may be intrinsically uncoupled ('slipping' [79–81]). Then, the ratio of ΔG_P to $|\Delta\tilde{\mu}_H|$ under 'state-4' conditions would not equal the intrinsic stoichiometry, but the phenomenological stoichiometry multiplied by the degree of coupling, two parameters defined in Non-Equilibrium Thermodynamics [63]. However, such slip in the $\vec{H}^+$-ATPase would cause the state-4 ΔG_P-to-$|\Delta\tilde{\mu}_H|$ ratio to become *lower* than the intrinsic $\vec{H}^+$/ATP stoichiometry [65,82,83] so that with the observed ratios exceeding 6 [39,65,71,84] the in-

trinsic stoichiometry of the H^+-ATPase would have to exceed 6. Under conditions of ATP hydrolysis the ratio of ΔG_P to $|\Delta\tilde{\mu}_H|$ should then exceed this intrinsic stoichiometry of 6 or more, but the values found for these ratios are consistently lower than 5 [66,67,85–89]. Consequently, it can be concluded that intrinsic uncoupling within the $\vec{H}^+$-ATPase cannot alone account for the observed increase of the $\Delta G_P/\Delta\tilde{\mu}_H$ ratio at low $\Delta\tilde{\mu}_H$.

A somewhat different question is whether the variations of the $\Delta G_P/\Delta\tilde{\mu}_H$ ratio with $\Delta\tilde{\mu}_H$ could be accounted for by genuine $\Delta\tilde{\mu}_H$ dependence of the stoichiometry of the ATPase H^+ pump. Such a $\Delta\tilde{\mu}_H$ dependence would demand a quite special catalytic mechanism for the proton pump (with feed-back controls adjusting the pumping stoichiometry to $\Delta\tilde{\mu}_H$). Moreover, if the intrinsic proton pumping stoichiometry of the $\vec{H}^+$-ATPase at low $\Delta\tilde{\mu}_H$ would indeed be so much higher than at physiological magnitudes of $\Delta\tilde{\mu}_H$, one would expect the dependence of the rate of ATP-synthesis on $\Delta\tilde{\mu}_H$ to be rather smooth, because ATP synthesis should be thermodynamically feasible at low magnitudes of $\Delta\tilde{\mu}_H$. Experiments by various groups (see under anomaly 1) have shown that at least in some cases, rather the opposite is the case: the rate of ATP synthesis is almost a 'step-function' of $\Delta\tilde{\mu}_H$. Concordantly, $\Delta\tilde{\mu}_H$-dependence of the pumping stoichiometry cannot, by itself, account for anomaly 1. We conclude that variable stoichiometry in itself does not explain anomalies 1 and 2.

The presumed presence of a protonophore binding protein at or near the mitochondrial $\vec{H}^+$-ATPase [90,91] might suggest that the increasing concentrations of protonophores used to decrease $\Delta\tilde{\mu}_H$ in some of the above experiments, cause an increasing modification of the $\vec{H}^+$-ATPase, the modified enzyme molecules having increased proton-pump stoichiometries. However, the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio also increases when $\Delta\tilde{\mu}_H$ is lowered in the absence of protonophores [65]. Moreover, the static-head imbalance between $\Delta\tilde{\mu}_H$ as input force and an output force has been also observed when the output force is the redox-potential difference across 'site 1' in submitochondrial particles. In this system the $\vec{H}^+$-ATPase is the primary pump and complex I is the secondary pump and the $\Delta\tilde{\mu}_H$ generated by the $\vec{H}^+$-ATPase drives the reversal of the redox H^+ pump at site I [75,84,267]. Depression of $\Delta\tilde{\mu}_H$ by either inhibition of the $\vec{H}^+$-ATPase, or by uncouplers or by a combination of various permeant ions was always accompanied by a marked increase of the $\Delta G_O/\Delta\tilde{\mu}_H$ ratio.

A high ΔG_P in the presence of low $\Delta\tilde{\mu}_H$ may also be observed without addition of protonophoric agents or respiratory inhibitors; alkalophilic bacteria maintain a ΔG_P of 46–51 kJ/mol under conditions of high alkalinity of the environment [77]. At this high pH, the pH is acidic inside and the overall $\Delta\tilde{\mu}_H$ is decreased to values around 50 mV or lower. The alkalophilic bacteria may therefore be considered as a naturally occurring model demonstrating alternatives to the delocalized protonic coupling scheme.

The conclusions based on the above group of experiments depend on the validity of the ΔpH and $\Delta\psi$ assays, especially at low values of the sum of the two. However, the argument of the improper assessment of $\Delta\tilde{\mu}_H$ can be made less crucial by the following consideration. The possible and systematic errors implicit in the ion redistribution method used for $\Delta\tilde{\mu}_H$ assays (such as the assumption that the probe binding is negligible, or that the activity coefficients for the probe are close to 1, or that equilibrium conditions have been attained) generally lead to *overestimation* of the $\Delta\tilde{\mu}_H$ value, and especially so under conditions of very low $\Delta\tilde{\mu}_H$ [90–92]. The correction for such possible error would result in even higher $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratios, which would fortify, rather than eliminate, the essence of the anomaly.

IIIC. Anomaly 3: direct cross-talk between energy transducers as revealed by dual inhibitor titrations

The idea [95–106,8,75] behind the dual inhibitor titrations is that a delocalized protonic coupling device essentially consists of two proton pumps in series, with a 'delocalized' intermediate in between. With 'delocalized' we mean that every energized proton produced by a 'primary' proton pump is available to all 'secondary' proton pumps in the same vesicular unit. When considering steady-state flow through the pathway catalyzed by these two enzymes (such as would take place in 'state 3' of oxidative phosphorylation), the question may be asked as to which of the two proton

pumps limits (controls) the flux. Experimentally, this problem can be investigated by measuring the effect of an added inhibitor of one of the two proton pumps on the flow [98–101,107–110]. The 'rule of thumb' (for the rigorous treatment, see Refs. 8 and 83) is that the extent to which one of the enzymes controls the flux should be decreased when the other enzyme is inhibited. In other words: inhibition of the primary H^+ pump should decrease the effect on the flux of low concentrations of the inhibitor of the secondary H^+ pump (and vice versa).

This approach has been applied to the ATP energized 'reversal' of 'site 1' oxidative phosphorylation in submitochondrial particles: partial inhibition of this 'reversal' with oligomycin made rotenone (an inhibitor of the NADH: ubiquinone oxidoreductase) more rather than less effective as an inhibitor of NAD reduction by succinate [8,95,97,103]. Symmetrically, rotenone made oligomycin more effective. In oxidative phosphorylation in submitochondrial particles (Refs. 75, 95 and 97; cf. Refs. 11 and 276), bacteria [27,102] and in photophosphorylation [41,98,99,112] with many combinations of primary and secondary proton pump inhibitors or activators, analogous results were obtained.

Photosynthetic systems have the advantage that modulation of illumination rather than the addition of a xenobiotic agent can be used to modulate the activity of the primary proton pumps. The inhibitory effect of DCCD on photophosphorylation by chromatophores was not decreased by increasing the dark time between flashes by two orders of magnitude [17,100,112].

Some objections have been raised against the dual inhibitor titrations. The most fundamental of them arises from the suspicion that the predictions of the titration, in the absence of more specific kinetic models for energy coupling, might not permit a clear distinction between the delocalized or the localized coupling hypothesis [278,279]. Another objection concerns the fact that one or both inhibitors may cause changes in the $\Delta\tilde{\mu}_H$ values that modify the kinetic properties of the pumps. Note that in many of these titrations the changes of $\Delta\tilde{\mu}_H$ were not directly measured. In fact, this is part of the strength of the approach; provided that the reaction rates vary in a neat fashion with $\Delta\tilde{\mu}_H$ [10,11,19,84,115,116], the dual inhibitor titrations' assay of the pool character of the high free-energy intermediate does not depend on the validity of methods for the measurement of $\Delta\tilde{\mu}_H$.

An approach [99] somewhat related to the dual inhibitor titrations, considered the effectiveness at which protonophorous uncouplers inhibit electron-transfer driven phosphorylation [99,101,104–106] or ATP-hydrolysis-driven reverse electron transfer [8,75,97,103]. In the delocalized coupling scheme, it would be expected that inhibition of the $\Delta\tilde{\mu}_H$-consuming proton pump would lead to an increase in $\Delta\tilde{\mu}_H$, thus rendering the protonophore less effective in depressing the output flux. However, the opposite holds true [8,75,99,103]. On the other hand, contrasting conclusions, based on methodological objections, have been reached [113].

Other observations also pertinent to the cross-talk effects, but comprising $\Delta\tilde{\mu}_H$ assays, will be discussed later in subsection VIC. One of these observations will be simulated with the electric analog network and shown to require the coupling unit concept.

The direct cross talk indicated by anomaly 3 could again conceivably be due, as anomaly 1, to some sort of allosteric control of the primary over the secondary proton pump. Anomaly 2, however, could not be explained this way. Hence, together, the three anomalies constitute a strong case against delocalized protonic coupling: given that all possible ways-out of the above three anomalies (e.g., allosteric control, complete failure of all $\Delta\tilde{\mu}_H$ measurements, highly unusual $\Delta\tilde{\mu}_H$ dependence of the proton pumps) are not realistic, the delocalized interpretation [2,7] of protonic coupling proposed by Mitchell [1] is in contrast with the experimental evidence.

IV. Alternative coupling schemes

Fig. 1A–C gives three schemes of energy coupling, alternative to delocalized protonic coupling. In all these three, $\Delta\tilde{\mu}_H$ is not the only high free-energy intermediate. Scheme A [32,118] is the only one that retains $\Delta\tilde{\mu}_H$ as a central high free-energy intermediate. It suggests, however, that free-energy transduction between respiratory chain and $\vec{H}^+$-ATPase can also occur without the involvement of

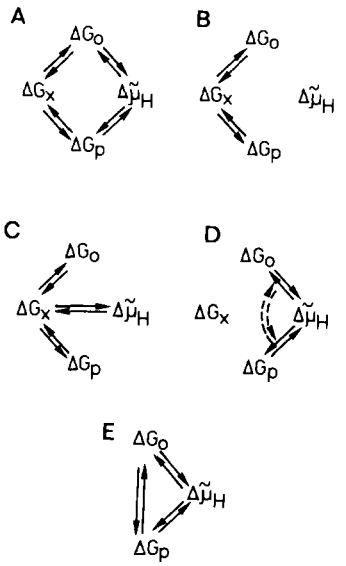


Fig. 1. Schemes summarizing the various coupling hypotheses alternative to the 'delocalized' chemiosmotic coupling hypothesis. (A) A coupling hypothesis where free energy can be transduced either through $\Delta\tilde{\mu}_H$ or through some other high-energy intermediate. (B) The hypothesis in which $\Delta\tilde{\mu}_H$ is irrelevant to free-energy transduction [119,122] intramembrane protons being the high free-energy intermediates [119]. (C) The hypothesis in which free energy passes through an additional high free-energy intermediate symbolized as ΔG_X and identified as: a molecular bond with high free energy of hydrolysis [123], a strained conformation of (part of) a protein [124] or of the entire energy-coupling membrane [134], protons close to the membrane [26,66,67,117,140,177] or protons inside 'proto-neural networks' [131]. (D) The allosteric interaction hypothesis. This scheme is identical to the delocalized chemiosmotic scheme, except that allosteric interactions (i.e., without free-energy transduction) (dashed lines) are postulated between the electron-transport chain and the $\vec{H}^+$-ATPase [22,138–144]. (E) The 'parallel coupling' hypothesis [10] where there is both direct (through $\Delta\tilde{\mu}_H$) and indirect free-energy transduction.

$\Delta\tilde{\mu}_H$. In the latter case, an as yet undefined high free-energy state of some sort would serve as the high free-energy intermediate. Scheme B states that $\Delta\tilde{\mu}_H$ has nothing to do with free-energy transduction [119–121].

Scheme C accepts that $\Delta\tilde{\mu}_H$ would occasionally correlate with the one, central high free-energy intermediate. The different hypotheses fitting into this scheme differ in the identity proposed for the central high free-energy intermediate, ΔG_X [32,123–125]. Williams [119] proposed that the respiratory chain would pump protons into the energy-coupling membrane, rather than across it. He [121,126,127] stressed that local acidity [119], anhydricity [120] and many more energetic terms would accompany the high free-energy state of the proton within the membrane (see also Ref. 128). Alternatively, the high-free-energy protons have been proposed to be close to the membrane but on the aqueous side of the interface [66,117,129,130]. It has been stressed that the high-free-energy protons should be on the average closer to supposed respiratory chain-$\vec{H}^+$-ATPase supercomplexes than to sites of transmembrane proton back leakage [46]. It has also been proposed that the protons may reside in special proton channels on the membrane surface or inside the membrane (Refs. 106, 131 and 132; cf. also Refs. 133, 265 and 231).

All hypotheses summarized in Fig. 1 can explain the type-1 anomaly discussed in the preceding section: both scheme A and scheme C allow correlations between $\Delta\tilde{\mu}_H$ and oxidation or phosphorylation rate to differ depending on whether $\Delta\tilde{\mu}_H$ is varied by affecting the respiratory chain, the membrane permeability for protons or the $\vec{H}^+$-ATPase. According to scheme B the relationships between the $\Delta\tilde{\mu}_H$ values and the flux rates are coincidental [122].

Because of the non-zero proton back leakage, none of the three schemes in Fig. 1A–C predicts the interconversion of $\Delta\tilde{\mu}_H$ and ΔG_P to be in complete equilibrium. Thus, in terms of these three schemes it is to be expected that Eqn. 3 will not describe the 'state 4' ratios of $\Delta\tilde{\mu}_H$ to ΔG_P in their dependence on $\Delta\tilde{\mu}_H$. For scheme C the latter conclusion is true only for the experiments in which $\Delta\tilde{\mu}_H$ is varied by varying the proton permeability of the membrane and not for the titrations with respiratory inhibitors [65]. Under conditions of progressive inhibition of respiration, the ratio of ΔG_P to $\Delta\tilde{\mu}_H$ would be expected to be largely independent of $\Delta\tilde{\mu}_H$ [65]. Likewise, it is not evident in the scheme A that the variation of $\Delta G_P/|\Delta\tilde{\mu}_H|$ with $\Delta\tilde{\mu}_H$ would be independent of whether $\Delta\tilde{\mu}_H$ were varied by inhibiting the respiratory chain or by adding a protonophore. Yet, this has been observed [65,75].

Anomaly 3, suggesting direct cross-talk between electron-transfer chain and $\vec{H}^+$-ATPase rather than

communication through a generalized high free-energy state, has not yet been discussed as a test of any of the schemes in Fig. 1. In schemes A, B and C, direct (i.e., not through the 'pool' ΔG_X) cross talk between electron-transfer chains and $\vec{H}^+$-ATPases is not expected. As a consequence these schemes cannot account for anomaly 3.

Thus the scheme of electron-transfer-linked phosphorylation cannot be such that all redox enzymes contribute to the filling of one common free-energy reservoir out of which all individual $\vec{H}^+$-ATPases can then use free energy (e.g., in the form of an 'energized' proton) to drive the phosphorylation of ADP. The absence of a common free-energy pool is central to the coupling unit concept developed in this article. Fig. 2 makes this elaboration of Fig. 1 explicit. Green et al. [134] also discussed repeating units as the basis for the catalysis of free-energy transduction but concluded that the units would form a pool.

The parallel coupling model (Fig. 1E, Refs. 10 and 117) is somewhat similar to the model of scheme A. However, it does not explicitly postulate the existence of an additional high free-energy intermediate characterized by a ΔG_X but only a 'direct free-energy' transfer from ΔG_O to ΔG_P. Recently [239,240], the nature of this direct interaction was suggested to consist of surface diffusion of protons. The effectiveness of the transfer of protons would then depend on the distance between the primary and the secondary proton pump. At certain temperatures the degree of coupling would be increased [239] due to a phase transition in the membrane which could bring the pumps closer together. Since at other temperatures there still is coupling, it is not clear whether the proposal implies that the protons pumped by one electron-transfer chain could only be used by one (or a few) ATPases, or whether the proton, if it would not encounter an active ATPase, would diffuse on until it would found such an active $\vec{H}^+$-ATPase. In the latter case a thermodynamic potential could be ascribed to the surface protons, making the parallel coupling hypothesis an elaboration of scheme A in Fig. 1. In the former interpretation of parallel coupling, electron transfer chains and $\vec{H}^+$-ATPases would function as coupling units in much the same fashion as suggested in this paper.

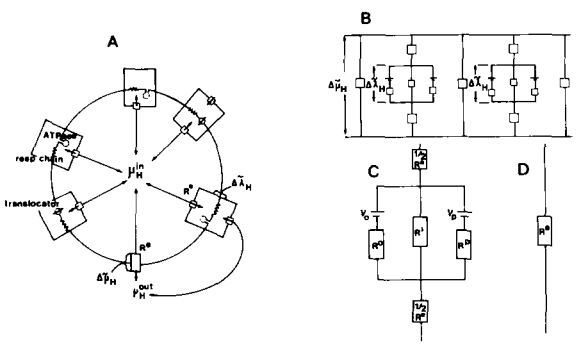


Fig. 2. (A) the presently proposed minimum hypothesis for coupling-unit-dependent energy coupling ('mosaic protonic coupling'). (B) The electric (non-equilibrium thermodynamic) representation of (A). The scheme consists of the juxtaposition of two elements: n coupling units (C) and m bulk-phase-to-bulk-phase proton leaks (D). Symbols are R for resistances and V for electromotive forces. Capacitances [cf. 130] have been left out for simplicity. A coupling unit consists of two batteries (one 'O', for the electron-transfer chain and one, 'P', for the $\vec{H}^+$-ATPase) with electromotive forces V_O and V_P and internal resistances R^O and R^P, respectively), a resistance with respect to proton back leakage (R^i) but with common connections to the two bulk phases through finite resistances (R^*). It should be noted that the 'internal resistances' R^O and R^P do not refer to novel proton leakage pathways but rather to the enzymatic activity of the respective proton pump [cf. 83].

V. Devising a minimal hypothesis

The experimental results that have led to the rather general acceptance [3] of protonic coupling indicate the presence of a closed biological membrane with primary (electron-transport chain) and secondary ($\vec{H}^+$-ATPase) proton pump inserted in non-random orientation. Each primary pump will locally pump protons across the membrane and deposit them in a region ('domain') close to its active site. These protons may then either leak back across the membrane, enter into the bulk aqueous phase, or pass along side the membrane. If the site at which the primary proton pump dissociates the proton, the site where the secondary proton pump may associate the proton, and the entrance to the proton leaks, would all be in open aqueous connection, then normal exchange of protons between all these sites would be so rapid (compared to the turnover rate of the enzymes) [135] that the distinction between these proton sites and pathways would have no physical meaning [7]. Without extra 'barriers' we would

arrive at 'delocalized' protonic coupling: the protons in the bulk phase would be free-energetically equivalent to the protons near enzymes and leaks. However, we include the postulate that primary and secondary proton pump are not randomly laterally distributed but ordered, either structurally, or functionally, with respect to one another, so that the space into which the former pumps and the space from which the latter abstracts a proton constitute, at least during a time corresponding to that required to synthesize ATP (i.e., for up to at least 100 ms), one functional compartment that are not identical to the bulk aqueous phases bordering the membrane [38]. The nature of these proton spaces (or 'domains' [136]) is perhaps most easily visualized when the primary and the secondary proton pump are next to each other in the membrane [66,137], but the proximity can also be assumed to be functional [131,132,136,138,265, 231]. Indeed the pumps would not have to remain permanently together if lateral diffusion [266] would cause sufficiently frequent encounters of primary and secondary H^+ pumps in energy-coupling membranes. Van Kooten [75] and Slater (Slater, E.C., personal communication) have recently proposed such models. In our view the combination of a few primary and secondary proton pumps, utilizing the same proton space constitutes a 'coupling unit'. A consequence is that the different individual proton domains are partially isolated one from another (depending on the barriers, see below) so that the different individual coupling units may act independently. As will be discussed later, the independent behaviour of each coupling unit leads to a fluctuation of the proton thermodynamic potentials among the various units. Hence a proper name for such free-energy coupling mechanism is 'mosaic protonic coupling'. However, we shall use below the term 'coupling unit' concept as a short notation for a free-energy transduction mechanism involving such fluctuating potentials.

VI. Explaining the anomalies with the coupling-unit postulate

VIA. An electric-network analogue

In the present section we shall discuss the ability of the coupling unit concept to explain anomalies 1, 2 and 3. To render the discussion more quantitative an electric network analogue has been designed (Fig. 2B, C, D). For simplicity, capacitances have been left out. The network is an electrical representation of the functional organization of a free-energy-transducing organelle through a number of coupling units as depicted in Fig. 2A. The functional organization of Fig. 2A is not meant to imply strictly the presence of only one primary and one secondary proton pump in the same coupling unit but it allows also the case of a few primary and secondary proton pumps belonging to the same coupling unit. The 'free energy' of the energized proton in the ith region (the proton space of the ith coupling unit) is denoted as $\Delta\tilde{\lambda}_{H_i}$. The presence of a finite resistance (R^*) between the bulk aqueous phase and the coupling unit implies that, even under steady-state conditions, the magnitudes of the $\Delta\tilde{\lambda}_{H_i}$'s generally differ from the bulk-phase-to-bulk-phase proton electrochemical potential difference, $\Delta\tilde{\mu}_H$.

Haraux and de Kouchkovsky [139] have included an extra electrical resistance between the part of the proton space (or domain) into which the protons are pumped by the primary redox-driven proton pump and the part from which the protons are extracted by the secondary ATP-synthesizing proton pump. Their conclusion was based on the observation that the dependences of the rates of e^- transfer [140] and of ATP synthesis [141] on $\Delta\tilde{\mu}_H$ were affected by the substitution of ^1H_2O with ^2H_2O. However, isotope substitution effects on the reaction rates of the proton pumps themselves should be more rigidly excluded.

As discussed in the section on anomaly 1 the dependence of the respiratory rate on $\Delta\tilde{\mu}_H$ varies according to the way $\Delta\tilde{\mu}_H$ is varied, i.e., at equivalent $\Delta\tilde{\mu}_H$ values the respiratory rate is higher in the presence of ADP than in the presence of uncouplers [10,11]. Hence the plot of J_O vs. $\Delta\tilde{\mu}_H$ is steeper in the presence of ADP than in the presence of uncouplers [11]. In terms of the mosaic protonic coupling hypothesis this is due to the fact that ADP drains energy directly from the proton space of the coupling unit while the uncoupler also increases the proton permeability at the level of the bulk phase. Hence ADP would affect $\Delta\tilde{\lambda}_H$ more than $\Delta\tilde{\mu}_H$ and vice versa for the uncoupler.

Fig. 3 shows the simulation of the differential

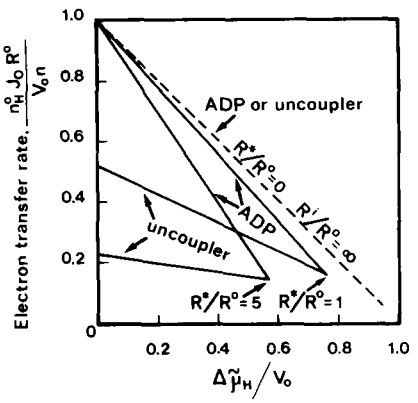


Fig. 3. Predicted relationship between electron-transfer rates J_O and $\Delta\tilde{\mu}_H$ during stimulation of the electron-transfer rate by either ADP or uncouplers. The symbols have the meaning specified in Fig. 2 and in the Appendix (see Scheme I). The equations for the stimulation by ADP (Eqn. A-6) and for the stimulation by uncouplers (Eqn. A-9) are derived in subsection A-II of the Appendix. The dashed line, which corresponds to the limit case of delocalized protonic coupling, is plotted according to Eqn. A-10. The assumptions are: $nR^e/mR^O = 10$ and $R^i/R^O = 10$.

effects of ADP and uncouplers on the relationship between J_O and $\Delta\tilde{\mu}_H$. In essence, three conditions have been considered, namely a ratio of the proton-domain-to-bulk-phase resistance (R^*) to the redox proton pump inner resistance (R^O) equal to 0, 1 and 5. The first condition is that corresponding to a completely delocalized free-energy transducing system. The dotted line indicates that in the case of a free equilibration of protons between coupling unit and bulk phase, the relationship between J_O and $\Delta\tilde{\mu}_H$ is independent of whether respiration is activated by ADP or by uncouplers. The other two conditions correspond to the existence of a 'barrier' to the free diffusion of protons between each coupling unit and the bulk phase. The continuous lines indicate that the difference between the slopes of the plots J_O vs. $\Delta\tilde{\mu}_H$ for the two conditions (respiration in stimulated by ADP versus uncoupler), becomes more significant with increasing domain resistance.

Let's consider now the observation that the dependence of the rate of ATP synthesis on $\Delta\tilde{\mu}_H$ differs depending on the way $\Delta\tilde{\mu}_H$ is varied [14–25]. The finding is that the rate of ATP synthesis is markedly sensitive to electron-transfer inhibitors [14,16,19,20,27] with negligible $\Delta\tilde{\mu}_H$ depression. On the other hand J_P is much less sensitive to ion transport or to uncoupler: J_P may be partially or even little affected in the presence of extensive depression of $\Delta\tilde{\mu}_H$ [19,21,142, cf. 37]. In terms of the coupling unit concept, inhibition of some of the primary pumps by electron-transport inhibitors depresses the level of the corresponding $\Delta\tilde{\lambda}_{H_i}$, what leads to a proportional elimination of the coupling units in terms of their making ATP. On the other hand elimination of only some of the primary pumps could yield little effect on the level of $\Delta\tilde{\mu}_H$, kept to its almost normal level by the other active coupling units [114]. Increased ion transport can lead to depression of the proton electrochemical gradient at the level of the bulk phase ($\Delta\tilde{\mu}_H$) but much less so at the coupling unit ($\Delta\tilde{\lambda}_{H_i}$).

Fig. 4 shows a simulation of these differential effects of respiratory inhibitors and uncouplers on the relationship between J_P and $\Delta\tilde{\mu}_H$. The three conditions considered are those corresponding as in Fig. 3 to a ratio of the proton domain resistance

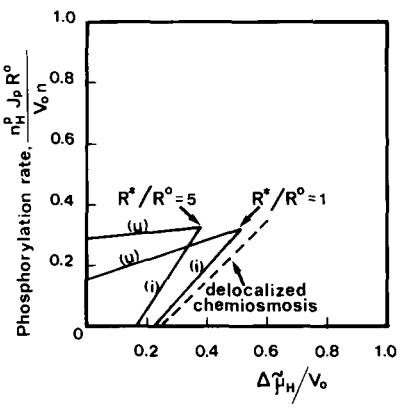


Fig. 4. Predicted relationship between rates of ATP synthesis J_P and $\Delta\tilde{\mu}_H$ during titrations with either uncouplers or electron-transfer inhibitors. The symbols have the meaning specified in Fig. 2 and in the Appendix. Lines (u) (titrations with uncouplers) are plotted according to Eqn. A-14; lines (i) (titrations with electron-transfer inhibitors) are obtained from Eqn. A-25. The assumptions are $R^i/R^O = 10$; $nR^e/mR^O = 10$; $R^P/R^O = 1$, $V_P/V_O = 0.25$. Other assumptions as to the R^* resistance are indicated in the figure. The dashed line (delocalized coupling) is plotted according to Eqn. A-26. Eqns. A-14, A-25 and A-26 are derived in subsection A-III of the Appendix.

to the redox proton pump resistance equal to 0, 1 and 5. The dotted line indicates that for a complete delocalization of proton in the delocalized model, the relationship between J_P and $\Delta\tilde{\mu}_H$ is independent of whether respiratory inhibitors or protonophorous uncouplers are used to vary $\Delta\tilde{\mu}_H$. The other continuous lines on the other hand indicate that the higher the resistance of the proton domain to the free diffusion of protons, the larger is the difference in slopes in the plot J_P vs. $\Delta\tilde{\mu}_H$. It may be noted at this point that simulations such as those of Figs. 3 and 4 can be obtained even in the absence of an internal leak in the coupling unit (i.e., whilst taking $R^O/R^i = 0$).

The considerations elaborated above in the case of Figs. 3 and 4 may be used to explain other phenomena which appear at variance with the delocalized protonic coupling scheme. For example the phosphorylation of ADP at low $\Delta\tilde{\mu}_H$ [19,77,144] is probably due to the fact that under those conditions the high resistance of the proton domain, R^*, is capable of maintaining a high $\Delta\tilde{\lambda}_H$ in the coupling unit in spite of the low $\Delta\tilde{\mu}_H$ between the bulk phases. A similar explanation could also, in principle, be applied for the case of ATP synthesis in giant mitochondria under conditions where the $\Delta\psi$ recorded by electrode impalement is claimed to be very low [31]. This might also be the case for the respiration driven ATP synthesis in uncoupler resistant mutants of *Bacillus megaterium* [32,76]. Note that in this system ATP synthesis is still sensitive to uncouplers when driven by a K^+ diffusion potential, which does involve bulk phase $\Delta\tilde{\mu}_H$.

Consider now the observations referred to in anomaly 2, namely that the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio increases with the decrease of $|\Delta\tilde{\mu}_H|$ [38,39,66–69]. According to the coupling unit concept, in state 4 ΔG_P is near equilibrium with the $\Delta\tilde{\lambda}_H$ of the individual coupling units, but not with the bulk phase $\Delta\tilde{\mu}_H$. Furthermore, addition of protonophorous agents does not result in the same extent of depression of the proton electrochemical potential in the bulk phase and in the coupling units, in spite of the fact that all membranes may be rendered equally permeable by a given uncoupler concentration. The reason for this is that protons are pumped directly into the proton space of the coupling unit, and may only subsequently reach the bulk phase. Hence the backflow of protons catalyzed by the uncoupler is more easily compensated in the coupling unit than in the bulk phase. Clearly the more marked the difference between $\Delta\tilde{\lambda}_H$ and $\Delta\tilde{\mu}_H$, the more apparent the increase of the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio in static head mitochondria.

Fig. 5 shows a simulation of the relationships between the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio and $\Delta\tilde{\mu}_H$ as obtained by varying the magnitude of the passive proton permeability through the membrane (R^e/m). Three conditions have been selected, namely ratios of proton domain resistance to redox proton pump resistance of 0, 1 and 5. The dashed line indicates that for a negligible proton domain resistance (R^*), the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio remains constant with the variation of $\Delta\tilde{\mu}_H$. On the other hand, the higher proton domain resistance, the more marked the rise of the $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio with decreasing $|\Delta\tilde{\mu}_H|$.

The simulations shown in Figs. 3–5 refer either to flow-force relationships or to force ratios during the coupled operation of primary and secondary

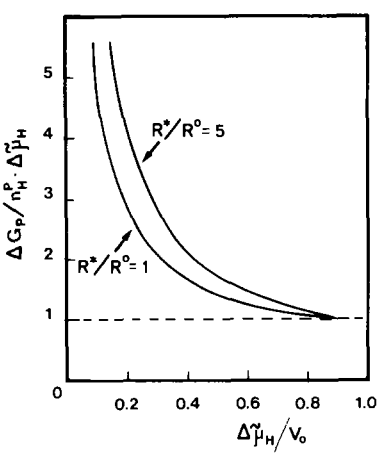


Fig. 5. Predicted relationship between $\Delta G_P/|\Delta\tilde{\mu}_H|$ and $\Delta\tilde{\mu}_H$ as varied in the presence of increasing uncoupler concentrations. The symbols have the meaning specified in the Appendix. Continuous lines are plotted according to Eqn. A-29. The assumptions are $R^i/R^O = 10$ and R^*/R^O as indicated in the figure. Eqn. A-29 is balanced also for $\Delta G_P/|\Delta\tilde{\mu}_H|$ when $\Delta\tilde{\mu}_H$ is changed by electron-transfer inhibitors if inhibition of the redox pump in a unit is assumed to block completely the unit (see text and subsection A-IV of the Appendix). The dashed line corresponds to the limit case $R^*/R^O \to 0$ (delocalized coupling).

pumps. However, the coupling unit concept can explain other phenomena arising during the operation of the primary pumps alone and which also seem to be in conflict with the delocalized protonic coupling model. One of the most striking of these phenomena is the observation that addition of small oxygen pulses to anaerobic mitochondria and bacteria does not result in proton extrusion of the correct stoichiometry [106,145–148]. This means inter alia that the minimal amount of protons, calculated to be necessary to charge the membrane, cannot be detected without a parallel counter-flow of permeant cations (or co-flow of permeant anions). Note that under the same conditions the consumption of oxygen is accompanied by an apparent rise of $\Delta\psi$ [147] as indicated by the $\Delta\psi$ probe, merocyanine [148]. Presumably, activation of the primary proton pump leads to proton pumping into the proton space of the coupling unit but not into the bulk phase. The coupling unit protons apparently cannot be detected by assay systems suited for protons in outer aqueous phase such as pH electrodes or water-soluble pH indicators (see also Ref. 101).

Another long-standing debate concerns the stoichiometry of the primary or secondary proton pumps taken individually (for reviews, see Refs. 149 and 150). As already extensively discussed elsewhere [80] part of these discrepancies depend on the fact that what is actually measured is the ratio of the input and output flows of the pump ('the flow ratio') and not its stoichiometry. The flow ratios depend on the force ratio as indicated by the nonequilibrium thermodynamic treatment [63,80]. Satisfactory as this analysis may be, it would still not explain why the observed flow ratio at negligible force ratio may vary with the number and rate of turnovers of the pump (Refs. 75, 130 and 151, in contrast to Ref. 99). This latter variation suggests that the proton-pumping activity may be affected to a different extent depending on whether the output force determining the operation of the pump is the local or the bulk phase proton electrochemical potential ($\Delta\tilde{\lambda}_H$ and $\Delta\tilde{\mu}_H$, respectively). Indeed, the number of turnovers required to raise the proton potential is higher in the latter than in the former case due to the much higher capacity of the bulk phase. Furthermore, the magnitude of the two forces may vary with variations of the proton domain resistance R^* or with the presence of ions affecting this resistance.

VIB. The direct cross-talk as a consequence of the special nature of the energized state

Above we have used an electric-network analogue of our proposal of coupling unit to show that it can account for anomalies of the types 1 and 2. However, strictly speaking, applications of electric network theory to coupling unit schemes is not correct. The use of Kirchoff's laws in connection with proton currents flowing into or out of a local proton domain presupposes the local proton potential to be in steady state (i.e., essentially invariant in time). The fact that we propose the local proton domain to extend no further than to one (or a few) primary and one (or a few) secondary proton pumps, makes such a steady-state assumption unrealistic, even when a macroscopic steady-state output flux is observable.

It has been calculated [153] that in energy coupling particles with a diameter of about 30 nm, a membrane potential sufficient to sustain ATP synthesis, may already be generated by the uncompensated transfer across the membrane of some 25 elementary charges. The number of $\vec{H}^+$-ATPases per particle can be estimated at about ten [99,154]. Therefore, the analogous calculation carried out for a coupling unit (i.e., a combination of an $\vec{H}^+$-ATPase and an electron-transfer-driven proton pump), leads to the conclusion that only of the order of two translocated charges are needed to create the local electric potential sufficient to drive ATP formation. The very fact that this number is so small would give rise to strong fluctuations in $\Delta\tilde{\lambda}_H$ [268].

There are some experimental indications of the number of protons that would be necessary to fill the domain up to a level sufficient for ATP synthesis [22,29,41,75,136,142,144]. For instance [144], in chromatophores, when ADP is present throughout, at low phosphate potential, ATP synthesis already begins after one turnover of the primary proton pump: in these light-driven systems the content of the proton domain in the energized state is only one set of protons more than in the de-energized state (a set of protons being defined as the number of protons necessary

to synthesize one molecule of ATP when the system is in steady state). Interestingly, at higher phosphate potentials, more than one turnover is necessary in order for net ATP synthesis to start [144]: the number of protons in the proton domain necessary to initiate ATP synthesis increases with increasing ATP/ADP ratios.

Given that the number of 'local' protons is so small, steady-state assumptions with respect to local proton potentials will be inapplicable, so that the predictions by the electric network analogue may not be fully correct.

Similarly, since the number of extra protons in the proton domain would be rather small, the definition of the thermodynamic potential of those protons becomes problematic [155,156,258]. Locally, fluctuations are no longer negligible. Also, the symbol $\Delta\tilde{\lambda}_H$, used by us for the local proton gradient, should not be taken to indicate that the 'high free energy' stored would reside solely in a proton's electrochemical potential difference. The proton may still reside on a protein. In that case, it is not appropriate to separate the free energy of the proton from the total free energy of the proton-protein complex [157; cf. 126]. Hence $\Delta\tilde{\lambda}_H$ is not a thermodynamic potential, but just a symbol for the fluctuating proton activity and 'membrane energization' in the proton domain i. Moreover, the proton once pumped into the proton domain, has a finite time of retention there, defined by the probabilities of going back the same way it came (in the case of a kinetically reversible primary proton pump), of leaking (or slipping) backwards, of moving into the bulk phase, or of moving back through the $\vec{H}^+$-ATPase. Even if one were to define the ensemble average or the time average for $\Delta\tilde{\lambda}_H$, the interpretation of the meaning of that parameter would be problematic [268], though it could under certain conditions correlate with the rate, or extent, of ATP formation, or with the 'energized state'. Inhibition of the primary pump will affect the number (n) of energized domain rather than the values of $\Delta\tilde{\lambda}_{H_i}$ in the single domains. The concept that the electron transfer chain and the $\vec{H}^+$-ATPase function as a unit [8,38,130] now becomes clear: a single turnover of the former will generate sufficient local electrical potential to induce ATP synthesis in the $\vec{H}^+$-ATPase that belongs to the same coupling unit. This accounts for the phenomenon that ATP synthesis depends on the number of active respiratory chains rather than on the magnitude of $\Delta\tilde{\mu}_H$; (see also Refs. 101, 153 and 158).

Likewise inhibition of the secondary pump will effectively block the entire coupling unit provided that the retention time of the $\vec{H}^+$-ATPase inhibitor on the enzyme is comparable with the life time of the high-energy protons in the same domain. Thus, the observation that electron transport chain and $\vec{H}^+$-ATPase together act as functional unit when attacked by all-or-none type of inhibitors (anomaly 3) can be understood in terms of the minimal hypothesis presented in the above section. This analysis holds, however, provided that the protonic currents through the bulk phases to the non-inhibited coupling units are low.

VIC. Use of the coupling-unit postulate to explain the cross-talk characteristics

In the previous sections the flow-force relationships and the force ratios in static head have been simulated with the electric network analogue. In these simulations the only assumption used was that of a resistance R^* which limits the proton diffusion from the coupling units proton domain into the adjacent bulk aqueous phase. This assumption is sufficient to account for the anomalies of type 1 (i.e., different dependence of the rates of electron transfer and ATP synthesis on $\Delta\tilde{\mu}_H$ depending on how the latter is varied). Also it accounts for part of anomaly 2 (i.e., variation of the state-4 $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio with $\Delta\tilde{\mu}_H$ for the case where the latter is varied by titration with a protonophorous uncoupler).

In further simulations based on the electrical network, the second part of anomaly-2, i.e., variation of the state-4 $\Delta G_P/|\Delta\tilde{\mu}_H|$ ratio in titrations with respiratory inhibitor, could not be reproduced. Neither could we make the electric network analogue show that the cross talk effects of the dual inhibitor titrations (anomaly 3) would follow directly from the coupling unit concept. The reason why such a direct cross talk between electron-transfer chain and $\vec{H}^+$-ATPase is not found in the electrical network simulation, is that in the case of inhibition of the respiratory chain, the $\vec{H}^+$-ATPase of that same coupling unit could still make ATP

by using protons coming from the bulk phase and reaching the $\vec{H}^+$-ATPase through the resistance R^*. We shall first analyze additional experimental results which are suggestive of direct cross-talk between respiratory chain and $\vec{H}^+$-ATPase within the same coupling unit.

The first is the experiment in which the effect of limited amounts of respiratory inhibitors on $\Delta\tilde{\mu}_H$ in state-3 mitochondria was compared to the effect of the same amounts in state-4 mitochondria. The extents of depression of $\Delta\tilde{\mu}_H$ were almost identical under the two conditions [19]. Thiscould be readily understood if indeed respiratory inhibitors would just eliminate coupling units: the bulk-phase-to-bulk-phase $\Delta\tilde{\mu}_H$ is a function of $\Delta\tilde{\lambda}_H$ with a relationship determined by the ratio of the total resistance between the local proton domains and the bulk phase (i.e., R^*/n) to the total resistance for passive proton leakage across the membrane (outside the coupling units, i.e., R^e/m). The transition from state-4 to state-3 mitochondria would result in a drop in $\Delta\tilde{\lambda}_H$ and hence in $\Delta\tilde{\mu}_H$, but the effects of respiratory inhibitors would be unaffected by such a transition. More simply, the respiratory inhibitors depress the H^+ fluxes through the unit parallel to the abolition of the primary pumps. This renders the titrations of the state 4 and the state 3 mitochondria undistinguishable if $\Delta\tilde{\mu}_H$ is normalized by its magnitude in the absence of inhibitor.

We have simulated the above experiment both in terms of the delocalized protonic coupling scheme (Fig. 6A) and in terms of the coupling unit scheme (Fig. 6B). In the latter simulation we have used the electric network analogue of Fig. 2B supplemented with the assumption that inhibition of a fraction α of the electron-transfer chains would essentially eliminate a fraction α of the coupling units (i.e., reduce n to $n(1-\alpha)$. The consequences of this assumption can be inferred from Fig. 6B. In the case of the relationship between state-4 $\Delta\tilde{\mu}_H$ and fraction of inhibited electron-transfer chain, the pattern is similar for delocalized and localized protonic coupling, Fig. 6A and B. On the other hand, for the relationship between state-3 $\Delta\tilde{\mu}_H$ and fraction of inhibited electron transfer chain, there is a more marked depression of $\Delta\tilde{\mu}_H$ in delocalized with respect to localized protonic coupling. This is in contrast

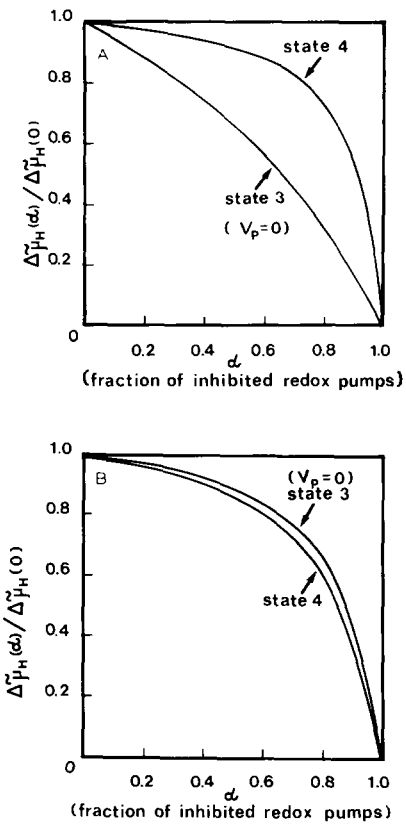


Fig. 6. Predicted relationships between the levels of $\Delta\tilde{\mu}_H$ in state 3 and state 4 during titrations with electron-transfer inhibitors according to a delocalized coupling scheme (Fig. 6A) and a protonic coupling unit scheme (Fig. 6B). The details of these network simulations are discussed in Subsection A-V of the Appendix. The curves in Fig. 6A for the titrations in state 3 and 4 are obtained from Eqns. A-30 and A-31, respectively. The correspondent curves for a protonic coupling unit scheme (Fig. 6B) are obtained from Eqns. A-32 and A-33. The assumptions are $nR^e/mR^O = 10$, $R^P/R^O = 1$, $R^*/R^O = 1$ and $R^i/R^O = 10$. $A_P \equiv n_H^P V_P$.

with what observed experimentally [19]. Experiments of this kind may prove useful to ascertain quantitatively the extent of 'direct cross talk' between primary and secondary proton pumps.

The second type of experiments compares state-3 mitochondria to state-4 mitochondria to which so much protonophorous uncoupler has been added that they respire at the state-3 rate. In terms of any protonic coupling hypothesis, whether delocalized or localized, this implies that the rate of H^+ circulation is the same, either through the

uncoupler, or through the ATPase H^+ pump pathway. If one now starts to eliminate the H^+ pumps, the pattern of the relationship between respiratory rate and $\Delta\tilde{\mu}_H$ should be similar or different according to whether the H^+ backflow is unaffected or modified by the respiratory inhibitor. At equal depression of the respiratory rate the depression of $\Delta\tilde{\mu}_H$ should be more marked when the H^+ pathway is not affected by the respiratory inhibitor as compared to when the H^+ pathway is affected by the inhibitor. Indeed, the experimental result is that inhibition of respiration decreases strongly $\Delta\tilde{\mu}_H$ if a protonophorous uncoupler is present, but very slightly if ADP plus phosphate are present ('state 3') [152].

Fig. 7 shows the results of a simulation of the variation of the rate of ATP synthesis with $\Delta\tilde{\mu}_H$ for the cases where the latter is varied either through titration with a protonophorous uncoupler (assumed to increase the proton back leakage outside the coupling units: the results turn out to be straight lines), or through titration with a respiratory inhibitor (assumed to eliminate coupling units: the results turn out to be curved lines). Comparison of these results to the results of the simulation based on the 'classical' electric network theory (Fig. 4) shows little difference except for the prediction that in the titration with respiratory inhibitor the rate of phosphorylation should not vary linearly with $\Delta\tilde{\mu}_H$ but rather almost quadratically. The reason for this phenomenon may be phrased as follows: with decreasing $|\Delta\tilde{\mu}_H|$ the rate of phosphorylation decreases not only because $\Delta\tilde{\mu}_H$ is decreased (which by itself would give a linear effect) but also because H^+-ATPases are effectively eliminated: the two effects together will act quasi-quadratically. It may be noted that this nonlinear dependence of ATP synthesis on $\Delta\tilde{\mu}_H$ at low magnitudes of $|\Delta\tilde{\mu}_H|$ is in keeping with experimental results (e.g., Ref. 18).

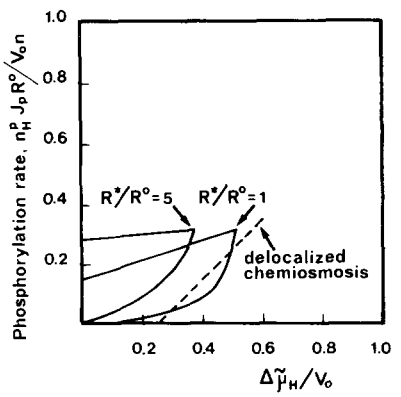


Fig. 7. Predicted relationship between rates of ATP synthesis J_P and $\Delta\tilde{\mu}_H$ during titrations with electron-transfer inhibitors and with protonophores. The curved lines (titrations with electron-transfer inhibitors) are drawn according to Eqn. A-27. This equation can be obtained from a coupling unit network assuming that the inhibitoin of the redox pump in a coupling unit causes the complete block of that unit (see text and subsection A-III of the Appendix). The stright line (titrations with uncouplers) are plotted utilizing Eqn. A-14. The following assumptions are made: $V_P/V_O = 0.25$; $R^i/R^O = 10$, $nR^e/mR^O = 10$, $R^P/R^O = 1$ and R^*/R^O as indicated in the figure. The dashed line represents the relationship obtained for a delocalized coupling scheme (Eqn. A-26).

We have also analyzed how at static head ('state 4') $\Delta G_P/|\Delta\tilde{\mu}_H|$ should vary with $\Delta\tilde{\mu}_H$ using either protonophore or respiratory inhibitor titration to vary the latter. The relevant equation is:

$$\frac{\Delta G_P}{n_H^P \Delta\tilde{\mu}_H} = 1 + \frac{mR^*}{nR^e} \tag{4}$$

Addition of a protonophore will increase the number (m) of transmembrane proton leaks, while addition of a respiratory inhibitor would decrease the number of coupling units (n). Clearly, in either case the ratio should increase. It turns out that m and n can be simultaneously eliminated from the above equation:

$$\left|\frac{\Delta G_P}{n_H^P \Delta\tilde{\mu}_H}\right| = 1 + \frac{\dfrac{V_O}{R^O|\Delta\tilde{\mu}_H|} - \dfrac{1}{R^O} - \dfrac{1}{R^i}}{\dfrac{1}{R^i} + \dfrac{1}{R^O} + \dfrac{1}{R^*}} \tag{5}$$

Consequently, the increase of $\Delta G_P/|\Delta\tilde{\mu}_H|$ with decreasing $|\Delta\tilde{\mu}_H|$ should be the same for the addition of a protonophorous uncoupler and of a respiratory inhibitor (Fig. 5). It should be stressed that for the respiratory inhibitor titration Eqn. 5 can be obtained only by assuming that inhibition of the primary pump brings about the parallel abolition of the secondary pump in the coupling unit. In fact, the experimental observation is that $\Delta G_P/|\Delta\tilde{\mu}_H|$ does indeed increase with decreasing $|\Delta\tilde{\mu}_H|$ independent of whether $|\Delta\tilde{\mu}_H|$ is reduced by

adding a protonophorous uncoupler or a respiratory inhibitor [8,65,75]. Previously [65], this has been interpreted as evidence against an electric network interpretation of localized protonic coupling. We now suggest that it is the 'mosaic' statistical nature of free-energy transduction that may account for this observation.

In the simulations with the adjusted version of the electric network analogue, we have assumed ohmic flow-force relationships for proton movement as well as complete coupling (i.e., absence of molecular slip) of the proton pumps. Yet, calculation showed (see Fig. 8) that, under conditions where there is no net ATP synthesis, inhibition of redox proton pumps does not lead to a proportional decrease of $|\Delta\tilde{\mu}_H|$: there is an apparent excess of proton pumps. The more pronounced the curvature of the dependence of $\Delta\tilde{\mu}_H$ on redox rate, the smaller R^i, as compared to R^* and R^O. 'Ohmic behaviour' (Fig. 8, dashed line) would be obtained for vanishing back leakage within the coupling units ($R^i = \infty$).

That the $\Delta\tilde{\mu}_H$ generated by a proton pump does not drop linearly with pumping rate, is a widely reported observation [20,68,79,81,114,159] and has

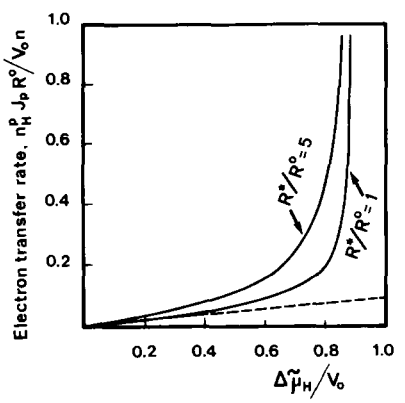


Fig. 8. Predicted relationship between $\Delta\tilde{\mu}_H$ and electron-transfer rate during titrations with electron-transfer inhibitors. The corresponding equation (A-36) is derived in subsection A-VI of the Appendix, assuming that the inhibition of the redox pump in a coupling element is equivalent to the elimination of that coupling element in the network representation. The assumptions are: $nR^c/mR^O = 10$, $R^i/R^O = 10$ and R^*/R^O as indicated in the figure. The dashed line, plotted according to Eqn. A-37, corresponds to the limit case $R^*/R^O \to 0$, $R^i/R^O \to \infty$ (delocalized coupling).

been attributed to nonohmic conductance of the membrane [87,114,159] or to slip in the proton pump [79–81,160,161]. Here, this nonlinear behaviour is predicted by an electric network consisting of ohmic components only. In fact, for the hybrid electric analogue it is immaterial whether R^i is a real proton back leak within the coupling unit or a molecular slip such as proposed by Pietrobon et al. [79–81]. The possibility that different 'sites' of oxidative phosphorylation may charge different proton domains [cf. 132] with different resistances (R^i) with respect to local protonic back leakage, would account for the observation that the extent of nonlinearity of the relationship between respiration and $\Delta\tilde{\mu}_H$ is different for each 'site' [79, cf. 81].

From the discussion presented in this section it appears that the value of the resistance R^* plays a crucial role in determining the extent of localization of the process of free-energy transduction. Indeed for a value of R^* tending to zero the system behaves as completely delocalized and bulk phase $\Delta\tilde{\mu}_H$ behaves as a competent thermodynamic and kinetic intermediate in free-energy transduction. On the contrary, for R^* tending to infinity, the system behaves as completely localized and the value of bulk $\Delta\tilde{\mu}_H$ may have little relation with the actual driving force at the site of ATP synthesis.

VII. The nature of the local resistance

It has been suggested [130,139,162] that conduction of protons along the surface of the free-energy transducing membrane might be so rapid that protons pumped by the electron transfer chain would be near the $\bar{H}^+$-ATPase before they would have had time to move into the bulk phase. The turnover time of the $\bar{H}^+$-ATPase, however, is typically some 10–100 ms. This can be calculated from the phosphorylation rates and the number of F_1 binding sites in mitochondrial membranes [163,265]. The results of this calculation would be decreased to 1–10 ms, if less than 10% of F_1 would be active owing to intrinsic regulatory properties [164]. Consequently, the proton, once it has arrived at the ATPase, would still have about that time to equilibrate with the bulk phase. Below we will

show that, in the absence of extra diffusion barriers, that time would be more than sufficient. In view of the reversibility of the $\bar{H}^+$-ATPase under ('state 4-uncoupled) conditions in which experimental indications for localized energy coupling are still found [65], it seems unlikely that the proton is trapped irreversibly by the $\bar{H}^+$-ATPase [141,75].

In the absence of a barrier between the site at which the pumped proton is released by the primary proton pump and the bordering bulk aqueous phase, the proton would delocalize according to free (Brownian) diffusion. The equation for Brownian motion in three dimensions is [165,166]:

$$\Delta^2 = 6D\tau$$

where Δ^2 is the average squared displacement of the substance considered during a time interval τ and D is the diffusion coefficient. The diffusion coefficient for a proton in water is about 10^{-4} cm^2/s [167]. The viscosity of the matrix can be higher than that of ordinary water due to the high protein concentration. However, since protein tend to increase rather than to decrease proton diffusion [169], the true diffusion coefficient is not expected to be far below this value. Taking dimensions of the mitochondrial matrix space as 1 μm by 1 μm by 1 μm (which may be compared with the mass of 0.1 pg [168]) the proton (or proton 'hole') would be expected to have seen all corners of the mitochondrial matrix space in about 50 μs: without a special device keeping the proton from moving into the bulk aqueous phase, no significant electrochemical gradient could survive even a period of $1/1000 \times$ the turnover-time of the $\bar{H}^+$-ATPase.

Using the first diffusion law of Fick:

$$J_H = D\frac{\Delta C_H}{\Delta x} \simeq DC_H\frac{\frac{\Delta\tilde{\mu}_H}{\Delta x}}{RT}$$

one can estimate how small the steady state inhomogeneity in electrochemical proton gradient in the absence of extra diffusion barriers could be. A typical rate of proton pumping would be 0.1 μmol H^+/min per mg protein. The surface area of the inner mitochondrial membrane has been estimated at about 400 cm^2/mg protein [6]. Taking a distance of 0.5 μm (approx. 25 nm for a submitochondrial particle or a chromatophore) for the distance between the surface and the center of the 'bulk aqueous phase' a concentration gradient of some 4 nM (0.2 nM for a submitochondrial particle) is calculated through the use of the above formula assuming that all proton current flows through the bulk phase. At pH 7 this amounts to a pH difference between the surface of the membrane and the center of the bulk aqueous phase of less than 0.02 units (and less than 0.001 units for submitochondrial particles). Thus proton diffusion through aqueous phases is too rapid to allow for the steady-state (cf. below) proton electrochemical potential to be significantly heterogeneous in such phases (Refs. 2 and 7, in contrast to Ref. 170).

We conclude that rather than trying to formulate mechanisms for rapid surface conduction [171] one should try to envisage ways in which equilibration of protons on the membrane's surface with the bulk aqueous phase can be appreciably retarded.

Using neutral red as a pH probe for the thylakoid interior, Junge et al. [172] indeed measured a relaxation-time constant of some 100 μs, in line with the above calculations. They concluded that the proton would diffuse into the inner bulk phase before it would be consumed by the $\bar{H}^+$-ATPase. (The same group had obtained evidence for the existence of a barrier for proton measurement, but only at the outside of the thylakoid [173]). More recently, however, the same group [133,174] established that such orthodox chemiosmotic behaviour is not observed in freshly isolated chloroplasts. It may be noted that groups reporting anomalies with respect to delocalized protonic coupling in thylakoids have indeed used freshly isolated materials (Refs. 175–177, cf. also Ref. 75). Also, Aflalo and Shavit [178] observed that hypotonic treatment of thylakoids removed the kinetic features of the supposed limitation of light-driven ATP synthesis by a diffusional limitation towards ADP.

Hong and Junge [174] proposed an explanation for the retardation of the proton movement they observed: since in free-energy coupling organelles the internal space generally has a buffer capacity of approx. 1–10 mM/pH unit [179, cf. 180], a proton at pH 7 is only for 10^{-5} part of the time a

free proton, and for $1-10^{-5}$ part a bound proton. Assuming that the buffer does not rapidly diffuse, the effective diffusion coefficient of that proton would be reduced by a factor of 10^5. Consequently, relaxation times of a proton gradient would be 5 s rather than 50 μs. Thus Hong and Junge [174] tentatively attributed the retarded diffusion of protons they had observed, to this presumed retardation effect caused by the binding and 'debinding' to the buffer.

In Ref. 265 one of us has analyzed the implications of this contention in more detail. This analysis showed that the presence of buffering groups cannot explain that there would be an increased difference between the local proton electrochemical potential and the bulk phase electrochemical potential in the steady-state. In other words, the effect of the buffer capacity would not be that of increasing the resistance (e.g., R^*) in Fig. 2 but only that of increasing a local capacitance. Increased local buffer capacity would not therefore reduce the time-average of the difference in pH between the bulk and the local space.

Anomaly 2, however, suggests that there would also be a significant stationary difference between the local and the bulk-phase proton potential (i.e., between $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\lambda}_{H,}$ in Fig. 2). This anomaly cannot then be accounted for by the local presence of proton buffers. Likewise, although indeed differences in dependence of phosphorylation rate on ΔpH between conditions with inhibited electron transfer and conditions with protonophore are expected as the result of local buffering these differences are expected to be below experimental resolution (i.e., on the order of 0.2 pH units only).

From the above discussion we conclude that the postulate of proton domains at a proton electrochemical potential distinct from that of both aqueous bulk phases, does imply the presence of devices (lipidic or proteinaceous) that prevent the proton from escaping to the bulk phase (see Refs. 131, 265 and 231). It will be one of the most interesting aspects of future research to examine the nature of these devices. We may also mention the observation that the proton bound to the Schiff's base of retinal in bacteriorhodopsin exchanges only 'slowly' (i.e., at the time-scale relevant for mosaic protonic coupling) with bulk phase protons [182].

It may be visualized that the magnitude of the resistance between the proton domain and the adjacent bulk phase lies somewhere between zero (delocalized coupling) and infinity (fully localized coupling). On the basis of the simulations (Figs. 3–8) the extra resistance may be calculated as 5-fold smaller than the resistance for the leakage of protons between the bulk aqueous phases (R^e), taken per coupling unit. Thus one would estimate an order of magnitude of 0.1 kΩ per mg mitochondrial protein, or a conductance of approx. 1 $/\Omega m^2$ (some 1000-fold larger than the conductance through typical phospholipid bilayers [183]. In view of the presumably rather strong dependence of the rate of ATP synthesis on $\Delta\tilde{\lambda}_H$, such a resistor would account for an all-or-none effect of inhibition of an electron-transfer chain on the ATP synthesis by the adjacent $\vec{H}^+$-ATPase.

With respect to the identity of the devices, the analysis of the proteins coded by the (alkalophilic *Bacillus*) DNA responsible for the transformation of *B. subtilis* to an alkalophilic phenotype [184] might be a promising approach. These and related topics are discussed in detail elsewhere [265,231]. Recent work of Gutman et al. [135,186–188] has led to some insight into possible properties of the proton domains (page 188). In this respect the experiments of Ryrie and Jagendorf [189] showing that the rate of H^+ exchange between chloroplast F_1 and the surrounding medium depended on the free-energy state of the thylakoid membrane may be pertinent.

Experiments by Theg et al. (Ref. 142 and cf. Ref. 75) revealed a special proton space in the thylakoid membranes, where protons derived from water oxidation by Photosystem II are invisible for neutral red. In the absence of proton-translocating ionophores, that space would be saturated with protons, but in their presence protons within the space would be able to equilibrate with the bulk aqueous phase during the dark periods between the applied light flashes [143]. A problem with the interpretation of these experiments is, however, the uncertain position of the pH-probe neutral red. The group of Dilley has concluded that the modification of the chloroplast photosystem by acetic anhydride is controlled by a local pH which differs both from the internal and the external pH. In contrast with earlier reports [132,136,138,195],

it is not clear if the domains of the two photosystems are separate from each other [196].

Also, the kinetic heterogeneity of the carotenoid band shift and other electric-field indicators has been interpreted as differentiating between bulk-phase and more local protons (Refs. 176, 177, 190 and 191; see also Ref. 75 in contrast to Refs. 192–194). The phase that would reflect the local protons is observed when ATP or Photosystem I is used for energization [176,191], so that local protons would only seem to be relevant in the free-energy transduction around Photosystem I. On the other hand, the proton domain inferred by the group of Junge (finding a proton domain invisible for neutral red that could be specifically populated with Photosystem II protons, [139,174]) seems to be confined to Photosystem II. Possibly, free-energy transduction at the two photosystems involves two different proton domains, which are differently observed by the different ways of probing them [132]. Experimental systems of ATP-synthesis induction by a macroscopic electric field [197–199] may yield more information.

Van Dam [200,201] suggested that the close apposition of the mitochondrial cristae membranes [202] may be the structural basis for areas with restricted proton diffusion in mitochondria. It is known that such close apposition varies during variations of the metabolic state [203,204] as well as during osmotic swelling [205,206]. Nesbitt and Berg [207] showed that the inner aqueous space of spinach thylakoid is highly viscous especially when they are energized. Albertsson [208] presented evidence for strong interactions between the sides of the thylakoids that face the lumen. It will be matter of future investigation to assess whether these factors play a role as diffusion barriers for protons.

Recently, Boyer [273] has proposed an extreme mode of direct (electrostatic) coupling between redox and ATPase proton pump such that the $\vec{H}^+$-ATPase would already be induced to make ATP as the electron transfer chain would be on its way to translocate a charge across the membrane.

VIII. Objections against the coupling-unit hypothesis

VIIIA. Inaccessibility of the proton pumps towards their scalar substrates

Unless they were highly proton specific, the existence of diffusion barriers around the coupling units could pose a problem to substrate availability for respiration or phosphorylation. Indeed, indications for hindered access of ADP to the chloroplast coupling factor have been reported by Aflalo and Shavit [178]. Van Dam [200,201] solves the problem by suggesting that the proton barrier would be on the 'cytosolic' side of the inner mitochondrial membrane (due to the apposition of the cristae), whereas the substrates are utilized on the inner side of the membrane. Other possibilities would be that pumps involved in free-energy transduction sequester protons and the other substrates and products in distinct domains, with different diffusional barriers towards the bulk phase, or that the translocators would reside within the coupling unit (cf. Refs. 209 and 210; see, however, Ref. 211; cf. the glutamate microcompartmentation [212] and the direct functional interaction of creatine kinase and the adenine-nucleotide translocator [213,214]).

VIIIB. The capacitance of the proton domains: too low to account for the observed free-energy stores?

The fact that R^* is not infinite implies that bulk phase protons may be implied in energy coupling. Hence, one would expect that upon sudden inhibition of respiration, ATP synthesis would continue for some time. Because bulk phase protons would in such a case, through movement back into the local domain and then through the $\vec{H}^+$-ATPase, drive the synthesis of ATP, the amount of ATP made would correspond to the number of protons in the proton domains (which we estimate to be on the order of the number of coupling units, i.e., approx. 0.2 nmol/mg protein) plus the capacity in the form of $\Delta\tilde{\mu}_H$ (some 5 nmol/mg protein). Consequently, the observation [215] of an ATP-synthesis capacity under such conditions of some 6 nmol/mg protein would not necessarily be in complete conflict with our coupling unit hypothe-

sis. It may further be added that in these experiments there were no adequate controls for: (i) the myokinase activity, (ii) the time required for the inhibitor to act and (iii) the operation of substrate level phosphorylation [215].

VIIIC. Acid-base phosphorylation

Following the observations of Jagendorf and Uribe [216], a number of workers [217–221] have found that an artificially imposed $\Delta\tilde{\mu}_H$ can lead to the net synthesis of ATP in a variety of systems. Typically, the relationship between the applied $\Delta\tilde{\mu}_H$ and the quasi-steady-state rate of phosphorylation was measured. Invariably, a threshold of typically some 150 mV exists, below which phosphorylation is negligible, whilst above this threshold there is a steep dependence of the rate of phosphorylation on the magnitude of the (clamped) $\Delta\tilde{\mu}_H$. Although, at very high values of the applied $\Delta\tilde{\mu}_H$, phosphorylation rates as high as those driven by electron transport may be observed [219,222] it is a jump in logic to state that this alone proves that $\Delta\tilde{\mu}_H$ is the only intermediate in electron-transport phosphorylation. To show this, it is necessary, at the very least, to show that rates of phosphorylatoin driven either by electron transport or by an artificially imposed $\Delta\tilde{\mu}_H$ depend unequivocally, and to an equal extent, on the magnitude of the $\Delta\tilde{\mu}_H$ under the different conditions. This has not been done to date. Again, the possibility of some proton movement through R^* (i.e., that R^* is not assumed to be infinite) may account for the observation of the ATP synthesis driven by artificially imposed $\Delta\tilde{\mu}_H$. Correspondingly, in alkalophilic bacteria and in uncoupler resistant mutants, the protonic isolation between the respiratory-chain $\vec{H}^+$ ATPase supercomplexes and the aqueous bulk phase would be nearly complete.

VIIID. Uncouplers

The view of the localization of the high free-energy state in units smaller than an entire vesicular structure such as a chloroplast thylakoid [223] or a chromatophore [154] may seem in conflict with the observation that for the most active uncoupler the number of molecules needed for uncoupling is smaller than the number of electron-transfer chains or the number of $\vec{H}^+$-ATPases. However, in these systems the uncoupler titre depends strictly on the electron-transfer rate. When similar titrations were carried out with intact mitochondria where the number of coupling units would be some 20 000 per vesicular structure, the number of ionophores needed for complete uncoupling was much closer to (i.e., 0.2-times [224] or 1-time [39]) the number of proposed coupling units than to (i.e., they were 4000–20 000-times) the number of mitochondria. Importantly, the uncoupler may rapidly move from one proposed coupling unit to another [99,104, 224,225,105]. Indeed, it was found that reducing the rate of phosphorylation either by slowing the rate of electron transport [105,226] or by decreasing the number of active $\vec{H}^+$-ATPases (Refs. 99, 105 and 75; see Ref. 8 for the analogous effect with ATP-driven reversed electron transport), reduced the amount of protonophore required to give full uncoupling. This was understood by assuming some direct interaction between $\vec{H}^+$-ATPase and electron-transfer chain (cf. also Refs. 227–230).

VIIIE. Distant location of primary and secondary proton pumps

Another piece of potential evidence against localization of the high-free-energy state is the electron microscopic evidence for distant locations of the chloroplast $\vec{H}^+$-ATPase and most of Photosystem II (Refs. 232 and 59, in contrast to Ref. 233) as well as the *Halobacterium halobium* $\vec{H}^+$-ATPase and of the bacteriorhodopsin patches. Also, in the latter system evidence for localized protonic coupling has been obtained [74,269,75]. An uncertainty here is whether the significant fraction of the Photosystem II (the PS II fraction, cf. Ref. 234) and the bacteriorhodopsin that might still be close to the $\vec{H}^+$-ATPase, cannot account for the light-driven ATP synthesis. Also, it has been doubted [235] whether the bacteriorhodopsin, when it is functional in light-driven ATP synthesis in *H. halobium,* is indeed present in the crystalline form in the purple membrane patches. The spatial separation between PS II and the chloroplast $\vec{H}^+$-ATPase is accompanied by a similar distance between PS II and Photosystem I [234]. To account

for the cooperation between PS I and PS II necessary for non-cyclic photophosphorylation, Anderson [59] proposed that plastoquinone would act as a membraneous lateral carrier of redox equivalents between the grana and the stroma lamellas. Anderson estimated lateral diffusion of plastoquinone to exceed 40 nm/ms, whereas the observed reduction of plastocyanine by plastoquinone takes some 20 ms. Millner et al. [236] verified the prediction of this hypothesis that electron transfer would be reduced when the plastoquinone concentration in the membrane would be decreased by fusion with liposomes if and only if no exogenous plastoquinone was added. Proton-linked-free-energy transduction occurs on the same time-scale [237]. Therefore the existence of a similar device for free-energy transfer would solve the problem of the distant locations of PS II and the $\vec{H}^+$-ATPase in chloroplast (cf. Refs. 75 and 238). Different degrees of localization of free-energy transduction, depending on membrane fluidity and hence on the possibility of the proton pumps to interact directly, has been implied by the work of Rottenberg (Refs. 162, 239 and 240, cf. Ref. 241). Furthermore, it has been pointed out that the functional contact between primary proton pumps and $\vec{H}^+$-ATPase does not necessarily imply a physical contact [101,131,231].

VIIIF. Concentrations of proton pumps: do they match?

The coupling unit hypothesis predicts that the total number of secondary proton pump would roughly match (although on the basis of their stoichiometries and turnover rates) with the total number of primary proton pumps in a free-energy coupling membrane. In bovine-heart, rat-heart and rat-liver submitochondrial particles there is 1 F_1 per bc_1 complex [242, 163, 243] and about 1 F_1 per 2.5 classical coupling sites [244]. In *Rs. sphaeroides* there is roughly a 1-to-1 stoichiometry of $\vec{H}^+$-ATPase and reaction centers [245]. In rat-liver mitochondria there are about as many adenine-nucleotide translocators as F_1s [70,246]. However, such stoichiometries may not always match. The question may be raised whether, in the case of the presence of more than one primary proton pump per coupling unit, elimination of one of them would still eliminate the entire coupling unit (cf. anomaly 3). We here note that a steep dependence of the rate of ATP synthesis on $\Delta\tilde{\lambda}_H$ together with the presence of a threshold (e.g., due to the ambient ΔG_P) would allow the depression in $\Delta\tilde{\lambda}_H$ due to elimination of only one out of, for instance, five primary proton pumps, to have such an effect.

IX. Electric-field profiles

The extrusion of the protons would (immediately, without secondary ion movement) create a transmembrane electrical potential difference, because of the positive charge of the protons [153,247]: it is as if an electric capacitor is charged. With the known specific capacitance of biological membranes, such membrane potentials could be estimated to be already of the order of 0.1 V after the translocation of 0.5 nmol protons per mg protein [6]. Indeed in most systems the electric potential difference is thought to exceed the pH difference [248].

This analogy with a spherical electric capacitor imposes important limitations to other proposed coupling mechanisms, if they are to explain the absence of a bulk phase to bulk phase electric potential difference: even without any movement of ions from bulk phase to the membrane or vice versa, the translocation of free protons from the inside to the outside of the topologically closed coupling membrane would necessarily lead to such an electric potential difference between the bulk phases (67, 249, 65; for some experimental follow-up, see Ref. 250). This will be illustrated with the help of Fig. 9. The transition from Fig. 9A to Fig. 9B corresponds to the translocation of protons from the inner side of the membrane to the outer side (as a result of the action of the electron-transfer chains, or the $\vec{H}^+$-ATPase). For simplicity we assume that sufficient buffer capacity is present everywhere in the system, so that we can limit the discussion to electric potentials. In the 'delocalized protonic coupling' scheme (Fig. 9C1) the protons (or hydroxyl ions) can move freely into the bulk aqueous phase that is at the same side of the membrane. The bulk phases are then equipotential. In Fig. 9D1 the field lines are depicted: in this situation, therefore, there is no difference in electric potential between positions in the bulk phase

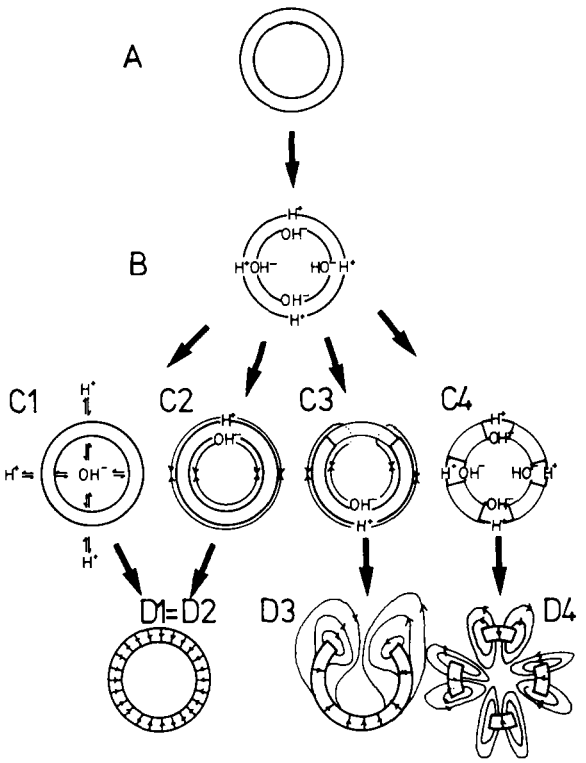


Fig. 9. The electric or protonic potential field in the different protonic coupling hypotheses. In (A) the energy-coupling organelle is shown prior to charge separation. In (B) the primary charge separation has taken place. In (C) the fates of the protons and hydroxyls are shown in the various coupling models: in (C1), the delocalized chemiosmotic coupling hypothesis, the protons (hydroxyl ions) on either side of the membrane equilibrate with the respective bulk aqueous phase; in (C2), the protons do not equilibrate with the bulk aqueous phase, but rapidly move across the membrane surface. The resulting electric fields for these two coupling theories are the same: (D1–2). In (C3), the 'localized chemiosmotic' coupling hypothesis, the protons move on the surface of the membrane, but the membrane consists essentially of two regions, one that contains a large number of energy-coupling units, whereas the other is leaky towards protons. The resulting electric field has been sketched in (D3). (C4) represents the 'mosaic protonic' coupling hypothesis presented in this article. Here the protons do not rapidly move across the membrane, but remain localized within one coupling unit. In between the coupling units, the membrane is leaky towards protons. The resulting electric-field profile is sketched in (D4). Note that every coupling unit has its own localized field. The latter phenomenon and the exact picture of the field lines will depend on the location and magnitudes of the electric resistances; this figure can only be used for illustrative purposes.

and positions on the membrane surface.

The coupling schemes proposed by Rottenberg [162,239] and Kell [130] contended that under stationary-state conditions a significant fraction of the protons would not equilibrate with the bulk phases, but would be rapidly moving on the membrane surface (cf. Refs. 251–253 and 139; perhaps through anionic phospholipid headgroups [171]) (Fig. 9C2). The situation is again analogous to a spherical capacitor: the electric-field profile becomes identical to that for the 'delocalized protonic coupling' scheme (Fig. 9D1). An important consequence is that even if the protons do not equilibrate with the bulk phase, the primary charge separation (Fig. 9A and B) will still lead to a difference in electric potential between the bulk phases. Consequently, the proposals of Rottenberg [162,239], Kell [130] and Haraux and de Kouchkovsky [139] do not successfully account for the energetic incompetence of the measured electric-potential difference with respect to the observed phosphorylation potentials.

Van Dam et al. [67] stressed that to allow the bulk-phase-to-bulk-phase electric-potential difference to be smaller than the electric potential across the free-energy coupling units themselves, the charge separation would have to be discontinuous. They assumed the proton leaks of free-energy transducing membranes [180] to be present at a location distinct from the free-energy coupling units (65 and Fig. 9C3). From the estimated electric, or 'protic' field profile (Fig. 9D3), it can be deduced that, in this proposal, the electric-potential difference between the bulk phases is lower than that across the free-energy coupling units, provided that the electric resistances between the proton domains and the bulk phases are of the same order of magnitude as the electric resistances for proton leakage between the two bulk phases.

In Fig. 2 (characteristic for the minimal hypothesis presented here [8,254]), the free-energy coupling membrane is not divided into one free-energy transducing and one proton leaking part, but it is stressed that the membrane may consist of an array of different elements, some of which contain both electron-transport chain and $\tilde{H}^+$-ATPase and others of which contain proton leaks (yet others may contain other combinations of proton pumps and leaks). Indeed sufficient

ultrastructural evidence shows that free-energy transducing organelles do not resemble the spherically symmetric bilayer structures assumed in Fig. 9A. Both in mitochondria [206] and in chloroplast [255] the free-energy transducing membranes are highly folded. If indeed the assumption of (i) complete conductivity of presumed aqueous spaces and (ii) large electrical resistances of membraneous spaces, were not completely correct, this structural detail would have important implications for the field lines in Fig. 9. The corresponding electric field profile (Fig. 9D4) would become like a conglomerate of electric dipoles. The exact profile of the electric field would depend on such factors as the spacing between the active coupling units, the electric conductivity of the barriers between the proton domains and the bulk aqueous phases and the leakiness of the leak elements. Zymanyi and Garab [256] have calculated electric potential profiles for theoretical conditions partly analogous to the ones we have attributed here to the minimum hypothesis, and found profiles that are partly comparable to those proposed in Fig. 9D4. This figure also shows that bulk-phase-to-bulk-phase electric potential differences will arise, but will be lower than those across the free-energy coupling units themselves, the difference again depending on numerous physical parameters in the system. Also, the electric potential across the membrane will vary with the precise positions between which it is measured, i.e., depending on whether one samples at a leak or a coupling unit [257, 258, 140, 139, 259, 141].

Williams [260] takes this point to the extreme when stating that there is no relationship between bulk-phase-to-bulk-phase electric-potential difference and the local charge separations. In fact, in his proposal, there are only two explicit phases present (in contrast to the very large number of phases in the hypothesis presented here): the membrane phase, containing the high free-energy protons and the aqueous phase. The orientation of the dipoles in Fig. 9B with respect to one another were not explicitly specified by Williams. The electric potential part of the free energy of the energized state was not considered the most important part, but part of the binding free-energy of the proton to the local site. This is one of the points where the minimal hypothesis presented here differs from the one proposed by Williams [120,121,261,127]. Our minimal hypothesis assumes that the majority of the free-energy coupling units have the same orientation with respect to the free-energy coupling membrane. Moreover, we do think that the electric term of the proton free energy (though not its concentration term) can still be described by a formulation in terms of Coulombs law (integrated over space). In theory it should be possible to calculate the electric field profiles [cf. 271,256].

X. Conclusion

A minimal hypothesis has been proposed that accounts, in particular, for three lines of experimental evidence apparently in conflict with the delocalized interpretation of the protonic coupling hypothesis: (i) non-unequivocal determination of fluxes by $\Delta\tilde{\mu}_H$, (ii) increasing thermodynamic incompetence between output and input force of the secondary pump at decreasing $\Delta\tilde{\mu}_H$; (iii) effective concomitant elimination of electron transport chains and $\vec{H}^+$-ATPases in titrations with inhibitors of either. To the four original postulates of the chemiosmotic hypothesis our hypothesis adds one additional postulate namely that our redox and ATPase proton pumps operate in functional coupling units, consisting of one (or a few) of either, electrically isolated from the aqueous bulk phases through a finite resistance.

Since in any free-energy transducing organelle there may be more than one type of primary (e.g., electron-transfer chain site I, site II, site III, bacteriorhodopsin, $\vec{H}^+$-ATPase in anaerobic organisms) and secondary (e.g., $\vec{H}^+$-ATPase, proton-linked translocators) proton pumps operative at the same time, the combinations of primary and secondary proton pumps constitute an array of coupling units. This array would be fixed when considered at the time-scale of some 10 ms, though it might well rearrange at time-scales of 100 ms and longer. Another property of the proposed coupling scheme is that, when the energy-coupling organelle (e.g., the mitochondrion) is in steady state, a close look at its coupling membrane would reveal a fluctuating mosaic of energized and unenergized domains: the fraction of proton domains that would be energized would not change with time, but that fraction would be made up of

different individual coupling units at different times. In contrast to this, in the 'delocalized' protonic coupling scheme the proton potential is essentially independent of space and time all over any of the two phases bordering the energy-coupling membrane.

Appendix

A-I. General comments on the network representation

In an attempt to analyze the steady-state thermodynamic implications of the mosaic protonic coupling scheme we utilize a network representation (Fig. 2), which consists of an array of coupling elements (Fig. 2C) and transmembrane leaks (Fig. 2D). Meaningful relationships can be obtained from this electric analogue when the electric currents and the electronmotive forces of the network are identified with proton fluxes and protonmotive forces, respectively. In order to account for the coupling between proton fluxes and redox (and ATPase) fluxes the following assumptions are made:

$$\begin{aligned} j_O &= i_O/n_H^O \\ j_P &= i_P/n_H^P \\ A_O &= -\Delta G_O = n_H^O V_O \\ A_P &= -\Delta G_P = n_H^P V_P \end{aligned} \qquad \text{(A-1)}$$

where n_H^O and n_H^P are the stoichiometries of the redox and ATPase proton pumps, $A_O = -\Delta G_O$ and $A_P = -\Delta G_P$ the affinities of the electron-transport and ATPase reactions, j_O and j_P the contributions of each coupling element to the redox and ATPase reaction rates. i_O and i_P are the electric (protonic) currents through the R^O's and R^P's, respectively; V_O and V_P the electron (proton)motive forces of the generators of each coupling element in the network (see Fig. 2). The simple assumption A-1 is equivalent to postulating a strictly stoichiometric coupling within each proton pump; within each single coupling unit, a partial uncoupling is assured by the R^i resistance. The condition of steady state for proton fluxes is implicit in the application of Kirchoff's laws to the electric circuit of Fig. 2.

To achieve a more general and synthetic presentation of the results obtained in the network analysis, all the relevant quantities have been normalized to the input parameters (i.e., protonmotive forces and resistances have been divided by V_O and R^O, respectively, and proton fluxes by the V_O/R^O ratio).

A-II. Relationship between the rate of electron transfer, J_O, and $\Delta\tilde{\mu}_H$ during stimulation of electron flow by either ADP or uncouplers under state-4 conditions

An electric circuit equivalent to the arrangement depicted in Fig. 2A, including an array of n coupling elements and m transmembrane leaks of resistance R^e, is given in Scheme I. By applying Kirchoff's laws to this scheme we obtain the following relations for the currents i_c, i_O, i_i, i_P, through the resistances R^*, R^0, R^i and R^P, respectively, of each coupling element:

$$i_c = \frac{m}{n}\frac{\Delta\tilde{\mu}_H}{R^e} \qquad \text{(A-2)}$$

$$i_O = \frac{V_O - \left(\Delta\tilde{\mu}_H + \frac{m}{n}\frac{R^*}{R^e}\Delta\tilde{\mu}_H\right)}{R^O} \qquad \text{(A-3)}$$

$$i_i = -\Delta\tilde{\mu}_H \frac{\left(1 + \frac{m}{n}\frac{R^*}{R^e}\right)}{R^i} \qquad \text{(A-4)}$$

$$i_P = \frac{V_P - \left(\Delta\tilde{\mu}_H + \frac{m}{n}\frac{R^*}{R^e}\Delta\tilde{\mu}_H\right)}{R^P} \qquad \text{(A-5)}$$

Introducing the rate of total electron transfer for n coupling units $J_O = nj_O$, according to Eqn. A-1, Eqn. A-3 is rewritten as:

$$\frac{n_H^O J_O}{n} = \frac{V_O - \Delta\tilde{\mu}_H\left(1 + \frac{m}{n}\frac{R^*}{R^e}\right)}{R^O}$$

and in a normalized form:

$$\frac{n_H^O J_O R^O}{V_O n} = 1 - \frac{\Delta\tilde{\mu}_H}{V_O}\left(1 + \frac{mR^*/R^O}{nR^e/R^O}\right) \qquad \text{(A-6)}$$

If the ratios m/n, R^*/R^O and R^e/R^O are kept

contant, *Eqn. A-6 describes the dependence of the rate of electron flow upon* $\Delta\tilde{\mu}_H$, *when this parameter is changed by varying the affinity of the ATPase reaction,* e.g., by changing the concentration of ADP.

State 4 (i.e., static head for phosphorylation) is attained when the i_P current of each coupling element is zero. This condition implies that the equation at each node O takes the form:

$$i_O + i_i = i_c \tag{A-7}$$

Combining Eqn. A-7 with A-2, A-3 and A-4, we obtain:

$$\frac{m\Delta\tilde{\mu}_H}{nR^e} = \frac{V_O R^i - \Delta\tilde{\mu}_H(R^i + R^O)}{R^O R^i + R^*(R^i + R^O)} \tag{A-8}$$

The effect of different degrees of uncoupling at the level of transmembrane proton leaks is simulated by varying the number m of the resistances R^e. Since we are interested in the relationship between J_O and $\Delta\tilde{\mu}_H$ when $\Delta\tilde{\mu}_H$ is changed by uncoupling agents under state-4 conditions, $(m/n)\,\Delta\tilde{\mu}_H/R^e$ in Eqn. A-3 is substituted by Eqn. A-8, yielding after some rearrangements:

$$i_O = \frac{V_O(R^i + R^*) - R^i\Delta\tilde{\mu}_H}{R^O R^i + R^* R^O + R^* R^i}$$

which gives for J_O the following normalized relation:

$$\frac{n_H^O J_O R^O}{V_O n} = \frac{\dfrac{R^i}{R^O} + \dfrac{R^*}{R^O} - \dfrac{R^i}{R^O}\dfrac{\Delta\tilde{\mu}_H}{V_O}}{\dfrac{R^i}{R^O} + \dfrac{R^*}{R^O} + \dfrac{R^* R^i}{(R^O)^2}} \tag{A-9}$$

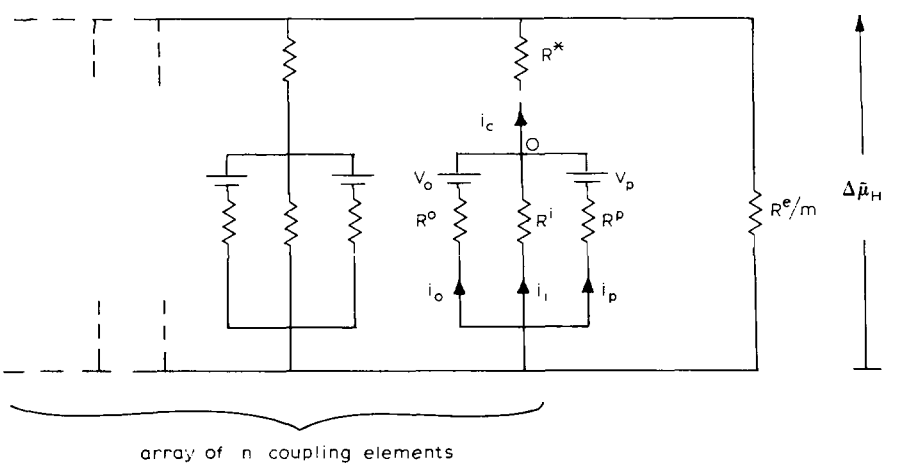
Eqn. A-9 describes the state-4 dependence of J_O *upon* $\Delta\tilde{\mu}_H$ *as varied by uncouplers.* In general, Eqns. A-6 and A-9 give rise to distinct dependences $J_O - \Delta\tilde{\mu}_H$ (see Fig. 3).

The behaviour of a delocalized chemiosmotic coupling scheme formed by perfectly intrinsically coupled coupling units can be obtained from our mosaic network as a limit case, when $R^* \to 0$ and $R^i \to \infty$. It is immediate to verify that in this limit both Eqns. A-6 and A-9 reduce to:

$$\frac{n_H^O J_O R^O}{V_O n} = 1 - \frac{\Delta\tilde{\mu}_H}{V_O} \tag{A-10}$$

A-III. Relationship between rates of ATP synthesis, J_P, *and* $\Delta\tilde{\mu}_H$ *during titrations with either uncouplers or electron-transfer inhibitors*

A procedure analogous to that employed in the preceding section can be followed to obtain a relationship between the rate of ATP synthesis J_P and $\Delta\tilde{\mu}_H$ when $\Delta\tilde{\mu}_H$ is decreased by uncouplers. Under state 3 conditions the equation at node O



Scheme I

(see Scheme 1) is:

$$i_O + i_i + i_P = i_c \tag{A-11}$$

Combining Eqn. A-11 with Eqns. A-2–A-5 we obtain:

$$\frac{m\Delta\tilde{\mu}_H}{nR^e} = \frac{\frac{V_O}{R^O} + \frac{V_P}{R^P} - \Delta\tilde{\mu}_H\left(\frac{1}{R^O} + \frac{1}{R^i} + \frac{1}{R^P}\right)}{1 + R^*\left(\frac{1}{R^O} + \frac{1}{R^i} + \frac{1}{R^P}\right)} \tag{A-12}$$

Substitution of $(m/n)\,\Delta\tilde{\mu}_H/R^e$ in Eqn. A-5 by Eqn. A-12 yields:

$$i_P = \frac{-\frac{\Delta\tilde{\mu}_H}{R^*R^P} + \frac{V_P}{R^P}\left(\frac{1}{R^*} + \frac{1}{R^O} + \frac{1}{R^i}\right) - \frac{V_O}{R^PR^O}}{\frac{1}{R^*} + \frac{1}{R^O} + \frac{1}{R^i} + \frac{1}{R^P}} \tag{A-13}$$

Introducing the rate of phosphorylation $J_P = -ni_P = -nj_P/n_H^P$ (the minus sign in the phosphorylation flux is a consequence of the definition of positive i_P for ATP hydrolysis), and normalizing gives:

$$\frac{n_H^P J_P R^O}{nV_O} = \frac{\frac{\Delta\tilde{\mu}_H (R^O)^2}{V_O R^* R^P} - \frac{V_P R^O}{V_O R^P}\left(\frac{R^O}{R^*} + 1 + \frac{R^O}{R^i}\right) + \frac{R^O}{R^P}}{\frac{R^O}{R^*} + \frac{R^O}{R^i} + \frac{R^O}{R^P} + 1} \tag{A-14}$$

Eqn. A-14 describes the dependence of J_P on $\Delta\tilde{\mu}_H$ in state 3, when changing the ratio of the number of perfectly functional coupling units vs. that of leaks, e.g., through the addition of uncouplers.

The action of an inhibitor of electron flow can be mimicked assuming that, under conditions of partial inhibition, the network representation of the transducing organelle includes an array of n_i coupling elements in which R^O is infinite and an array of $(n - n_i)$ uninhibited coupling elements. Such an arrangement is given in Scheme II. The following equations, based on Kirchoff's laws, hold for each active coupling element:

$$-R^*i_c + V_P - R^Pi_P = \Delta\tilde{\mu}_H \tag{A-15}$$

$$-R^*i_c - R^ii_i = \Delta\tilde{\mu}_H \tag{A-16}$$

$$-R^*i_c + V_O - R^Oi^O = \Delta\tilde{\mu}_H \tag{A-17}$$

$$i_O + i_i + i_P = i_c \tag{A-18}$$

The solution of the system A-15–A-18 yields:

$$i_c = \frac{R^iR^PV_O + R^OR^iV_P - \Delta\tilde{\mu}_H(R^iR^P + R^OR^P + R^OR^i)}{R^OR^iR^P + R^*(R^iR^P + R^OR^P + R^OR^i)} \tag{A-19}$$

$$i_P = \frac{V_P}{R^P} - \frac{R^*\left[R^iR^PV_O + R^OR^iV_P - \Delta\tilde{\mu}_H(R^iR^P + R^OR^P + R^OR^i)\right]}{R^P\left[R^OR^iR^P + R^*(R^iR^P + R^OR^P + R^OR^i)\right]} - \frac{\Delta\tilde{\mu}_H}{R^P} \tag{A-20}$$

The correspondent equations for the currents i'_c, i'_P through the inhibited coupling element are easily obtained from Eqns. A-19 and A-20 when $R^O \to \infty$:

$$i'_c = \frac{R^iV_P - \Delta\tilde{\mu}_H(R^P + R^i)}{R^iR^P + R^*(R^P + R^i)} \tag{A-21}$$

$$i'_P = \frac{V_P}{R^P} - \frac{R^*\left[R^iV_P - \Delta\tilde{\mu}_H(R^P + R^i)\right]}{R^P\left[R^iR^P + R^*(R^P + R^i)\right]} - \frac{\Delta\tilde{\mu}_H}{R^P} \tag{A-22}$$

Both i_P and i'_P contribute to the rate of phosphorylation J_P; thus, according to the assumptions (A-1):

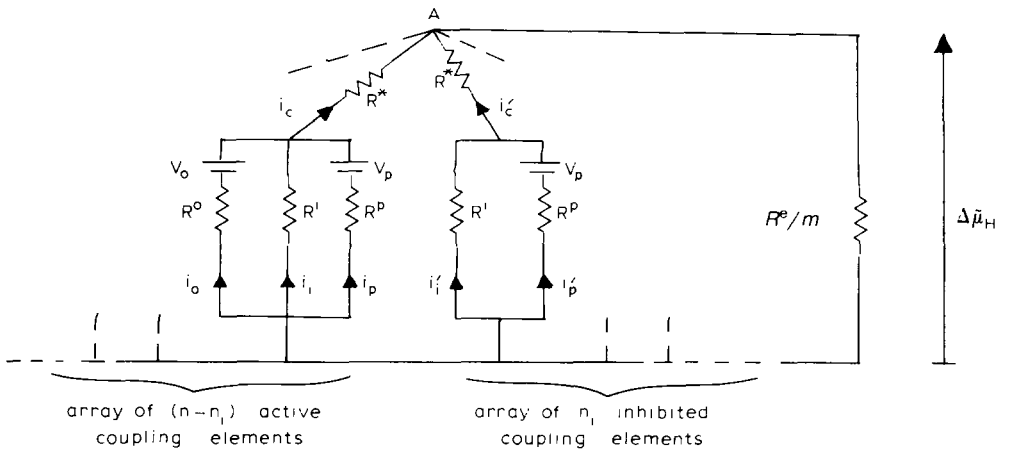
$$n_H^P J_P = -i_P(n - n_i) - n_i i'_P \tag{A-23}$$

In order to obtain a relationship between J_P and $\Delta\tilde{\mu}_H$ when the number of inhibited coupling elements n_i is varied, the dependence upon n_i in Eqn. A-23 is eliminated making use of the equation at node A of Scheme II:

$$(n - n_i)i_c + n_i i'_c = m\Delta\tilde{\mu}_H/R^e \tag{A-24}$$

Combining Eqn. A-23 with Eqns. A-19–A-22 and A-24, rearranging and normalizing we finally get the result:

$$\frac{n_H^P J_P R^O}{nV_O} = \frac{\Delta\tilde{\mu}_H R^O}{V_O R^P}\left(1 + \frac{mR^*/R^O}{nR^e/R^O}\right) - \frac{V_P R^O}{V_O R^P} \tag{A-25}$$



Scheme II

Eqn. A-25 expresses the dependence of J_P upon $\Delta\tilde{\mu}_H$ when the ratio of active and inhibited coupling units is changed.

In the limit $R^* \to 0$, $R^i \to \infty$ (delocalized chemiosmosis) Eqns. A-14 and A-25 reduce to the same equation:

$$\frac{n_H^P J_P R^O}{nV_O} = \frac{R^O}{R^P}\left(\frac{\Delta\tilde{\mu}_H}{V_O} - \frac{V_P}{V_O}\right) \tag{A-26}$$

As pointed out in the text we are also interested in simulating the effect of redox inhibitors, assuming that inhibition of the redox pump in a coupling unit causes the complete block of that unit. This assumption, in a simulation of electron-transfer inhibition in terms of the mosaic network implies merely the variation of the number of coupling units in the circuit of Scheme I (keeping the number of leaks constant). This is because the n_i inhibited coupling elements of Scheme II are in this case completely inactivated, and therefore do not contribute to proton currents. A procedure analogous to the one outlined above yields the normalized relation:

$$\frac{n_H^P J_P R^O}{nV_O} = \frac{\frac{m}{n}\frac{R^O}{R^e}\frac{R^*}{R^O}\frac{R^O}{R^P}\left[1+\frac{R^O}{R^*}\frac{\Delta\tilde{\mu}_H}{V_O} - \frac{V_P}{V_O}\left(1+\frac{R^O}{R^i}+\frac{R^O}{R^*}\right)\right]}{\left(\frac{V_P}{V_O}\frac{R^O}{R^P}+1\right)\frac{V_O}{\Delta\tilde{\mu}_H} - \frac{R^O}{R^P} - \frac{R^O}{R^i} - 1} \tag{A-27}$$

where n indicates the total number of coupling units, active in the absence of electron transfer inhibitors.

A-IV. Relationship between $\Delta G_P/\Delta\tilde{\mu}_H$ ratio and $\Delta\tilde{\mu}_H$ as varied in the presence of increasing uncoupler concentrations under state-4 conditions

Under state-4 conditions ($i_P = 0$) Eqn. A-5 gives:

$$V_P = \Delta\tilde{\mu}_H + \frac{m}{n}\frac{R^*\Delta\tilde{\mu}_H}{R^e} \tag{A-28}$$

Combining this equation with Eqn. A-8 and assumption A-1 we obtain *the equation*:

$$\frac{A_P}{n_H^P\Delta\tilde{\mu}_H} = \frac{V_P}{\Delta\tilde{\mu}_H} = 1 + \frac{\frac{V_O}{\Delta\tilde{\mu}_H} - \frac{R^O}{R^i} - 1}{\frac{R^O}{R^*} + \frac{R^O}{R^i} + 1} \tag{A-29}$$

which describes the dependence of the force ratio upon $\Delta\tilde{\mu}_H$ as varied by uncouplers.

The same equation is also obtained when $\Delta\tilde{\mu}_H$ is changed by electron-transfer inhibitors if we assume (see subsection A-III) that inhibition of the redox pump in a unit is equivalent to a complete block of the unit. Under this assumption, in fact, $V_P/\Delta\tilde{\mu}_H$ is simply determined by the ratio m/n, and it is of course irrelevant whether this ratio is changed by varying m or n.

A-V. Levels of $\Delta\tilde{\mu}_H$ in state 3 and state 4 during titrations with electron-transfer inhibitors

The response of $\Delta\tilde{\mu}_H$ to inhibition of electron flow under state-3 and state-4 conditions can be easily calculated for a delocalized protonic coupling scheme. When $R^* \to 0$ and $R^i \to \infty$ the network of Scheme I reduces to an array of a number n of V_O and a number n of V_P generators in parallel with the transmembrane resistance R^e/m. If α indicates the fraction of inhibited redox pumps (V_O), under conditions of partial inhibition, the network will include a number n of V_P generators and a number $n(1-\alpha)$ of V_O generators (active redox pumps). By applying Kirchoff's laws to such a circuit the following equation is obtained:

$$\frac{\Delta\tilde{\mu}_H}{V_O} = \frac{R^O V_P / R^P V_O + 1 - \alpha}{\frac{mR^O}{nR^e} + 1 - \alpha + \frac{R^O}{R^P}} \tag{A-30}$$

When the system attains static head for phosphorylation (state 4), $\Delta\tilde{\mu}_H = V_P$, (the currents i_P through the R^P resistances are 0) and Eqn. A-30 yields:

$$\frac{\Delta\tilde{\mu}_H}{V_O} = \frac{1-\alpha}{\frac{mR^O}{nR^e} + 1 - \alpha} \tag{A-31}$$

Eqns. A-30 and A-31 yield the dependence of $\Delta\tilde{\mu}_H$ on the fraction α of inhibited redox pumps in state 3 and 4, respectively, for a delocalized protonic coupling scheme.

Analogous relationships between $\Delta\tilde{\mu}_H$ and the fraction α of inhibited redox pumps will be derived now from the mosaic network (i.e., for a mosaic protonic coupling scheme), assuming that inhibition of the redox pump in a coupling unit completely deactivates the unit (see subsection A-III). Thus, under this assumption, $\Delta\tilde{\mu}_H$ is obtained from the analysis of a network (Scheme I) which includes an array of $n(1-\alpha)$ coupling elements.

From the equation at each node O of Scheme 1:

$$i_O + i_i + i_P = i_c$$

and making use of Eqns. A-2–A-5 where n has been replaced by $n(1-\alpha)$ the following equation can be obtained:

$$\frac{\frac{\Delta\tilde{\mu}_H}{V_O}}{} = \frac{(1-\alpha)\left(\frac{R^i}{R^O}\frac{R^P}{R^O} + \frac{V_P}{V_O}\frac{R^i}{R^O}\right)}{\frac{m}{n}\frac{R^O}{R^e}\frac{R^iR^P}{R^{O2}} + \left(\frac{R^iR^P}{R^{O2}} + \frac{R^P}{R^O} + \frac{R^i}{R^O}\right)\left(1 - \alpha + \frac{mR^O}{nR^e}\frac{R^*}{R^O}\right)} \tag{A-32}$$

Under state-4 conditions ($i_P = 0$) the same procedure gives:

$$\frac{\Delta\tilde{\mu}_H}{V_O} = \frac{\frac{R^i}{R^O}(1-\alpha)}{\frac{m}{n}\frac{R^O}{R^e}\frac{R^i}{R^O} + \left(\frac{R^i}{R^O} + 1\right)\left(1 - \alpha + \frac{m}{n}\frac{R^O}{R^e}\frac{R^*}{R^O}\right)} \tag{A-33}$$

These equations are equivalent to Eqns. A-30 and A-31 but for a protonic-coupling unit scheme.

A-VI. Relationship between $\Delta\tilde{\mu}_H$ and J_O in state 4 during titration with electron-transfer inhibitors

As pointed out in subsection A-III, if we assume that the inhibition of the proton pump in a coupling element is synonymous of a complete block of the unit, the simulation of electron-transfer inhibition can be obtained simply by varying the number of coupling units at constant R^e/m, in the circuit of Scheme I. According to this assumption the total proton current associated with redox pumps is given by (see Eqn. A-3 and A-1):

$$n_H^O J_O = \frac{n_a V_O - \left(n_a \Delta\tilde{\mu}_H + \frac{mR^*\Delta\tilde{\mu}_H}{R^e}\right)}{R^O} \tag{A-34}$$

where n_a indicates the actual number of active coupling units in a free-energy transducing organelle. Under state-4 conditions (see eqn. A-8):

$$n_a = \frac{m\Delta\tilde{\mu}_H\left(R^OR^i + R^*(R^O + R^i)\right)}{R^e\left(V_OR^i - \Delta\tilde{\mu}_H(R^i + R^O)\right)} \tag{A-35}$$

Substitution of n_a in Eqn. A-34 by Eqn. A-35 and

normalization yield:

$$\frac{n_H^O J_O R^O}{nV_O} = \frac{\Delta\tilde{\mu}_H R^O m}{V_O R^e n} \frac{\left(1 + \frac{R^* R^O}{R^O R^i} - \frac{\Delta\tilde{\mu}_H}{V_O}\right)}{1 - \frac{\Delta\tilde{\mu}_H}{V_O}\left(1 + \frac{R^O}{R^i}\right)} \qquad \text{(A-36)}$$

where n indicates the total number of coupling units per vesicle, active in the absence of electron-transfer inhibitors. *Eqn. A-36 describes how* $\Delta\tilde{\mu}_H$ *depends on the rate of the redox pumps when a number of coupling units is blocked by an electron transfer inhibitor.*

For a delocalized coupling scheme (i.e., for a network representation including an array of V_O and V_P generators in parallel with a transmembrane leak of resistance R^e/m) the following equation is readily obtained:

$$\frac{n_H^O J_O R^O}{nV_O} = \frac{\Delta\tilde{\mu}_H m R^O}{nR^e V_O} \qquad \text{(A-37)}$$

Notes added in proof (Received November 22nd, 1984)

(i) (Subsection IIIB)

A reinvestigation of ΔG_P and $\Delta\tilde{\mu}_H$ in mitochondria in state 4 yielded $\Delta G_P/\Delta\tilde{\mu}_H$ ratios that were constant; they did not increase upon addition of protonophores or respiratory inhibitor [272]. Further investigations under representative experimental conditions will have to decide whether this observation invalidates the experimental bases [12,38,39,65–68] for anomaly 2 in mitochondria. Alternatively, it might reflect that the barrier that prevents proton escape to the bulk aqueous phases is fragile.

(ii) (Section IV)

The model of Konings and colleagues [47,49], which accounted for allosteric interactions between the electron-transfer chains and secondary proton pumps such as H^+-ATPases and translocators through redox centers on the latter, has now been extended to include free-energy transduction through such a parallel redox pathway [270]. Thus the model has shifted from D to A in Fig. 1.

(iii) (Subsection VIB)

The expectation that free-energy coupling, when organized in a large number of small coupling units where the latter contain only some two protons, would not follow usual kinetic, or current-voltage rate, equations, has been confirmed by theoretical analyses of such systems [268,274,275]. The same studies demonstrated that ATP synthesis might well occur in cases where the average local proton potential would be thermodynamically incompetent if compared to ΔG_P.

(iv) (Section III)

It has been suggested that many of the empirical anomalies described in Section III can be explained in terms of the delocalized chemiosmotic coupling hypothesis, if the preparations studied would have been sufficiently heterogeneous [280].

(v) (Subsection IIIB)

It has been suggested [281] that the thermodynamic insufficiency of $\Delta\tilde{\mu}_H$ in a number of systems is due to the fact that in those systems Na^+ has taken the role of H^+.

Acknowledgements

H.V.W. wishes to thank Catia Sorgato, Marieke Elferink, Hagai Rottenberg, Jan Berden, Karel van Dam, Klaas Hellingwerf, Marc Symons, Olaf van Kooten, Piet de Jonge, Rob van de Bend, Ruud Kraayenhof, Sam Helgerson, Simon de Vries, Steven Theg, Wolfgang Junge and other colleagues for discussions. He also wishes to thank and acknowledge Drs. Mitchell and Williams for clarifying to him many important aspects of protonic coupling schemes. D.B.K. wishes to thank the Science & Engineering Research Council, U.K., for financial support, and many colleagues for discussions. G.F.A. and H.V.W. wish to thank Daniela Pietrobon, Mario Zoratti, Dieter Walz and Jörg Stucki for many stimulating discussions. B.A.M. and G.V. acknowledge the financial support of C.N.R. of Italy (Grant no. 81.00179.04). G.V. acknowledges stimulating discussions with M. Meuti and L. Sportelli.

H.V.W., D.B.K. and G.F.A. would like to mention that some of the important elements of the new proposal have been foreshadowed by the fundamental work of McClare [158].

We thank Mrs. Franca Schiavon Mazzari for typing the manuscript.

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