The hypothesis of localized chemiosmosis is unsatisfactory

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The hypotheses of 'localized' chemiosmosis have been put to an experimental test in the system of oxidative phosphorylation by rat liver mitochondria. We find that the variation of the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ with $\Delta\tilde{\mu}_H$ does not depend on how $\Delta\tilde{\mu}_H$ is varied. This is in conflict with hypotheses of localized chemiosmosis. Of all coupling hypotheses, only the parallel-coupling hypothesis can explain the observations, unless variation of the H^+ /ATP stoichiometry of the ATPase proton pump is accepted. In the latter case, 'macroscopic' chemiosmosis can explain the observations equally well as hypotheses of localized chemiosmosis. It is concluded that either variation of H^+ /ATP stoichiometries must be accepted, or that the parallel-coupling hypothesis should be reformulated so that it becomes open to experimental tests.

The importance of transmembrane proton movement for energy conservation in oxidative and photophosphorylation is now almost generally accepted (Boyer *et al.*, 1977). This concept originated in Mitchell's (1961) chemiosmotic hypothesis. Some phenomena, however, seem to be difficult to explain by the chemiosmotic-coupling hypothesis. One of these is the observation (Padan & Rottenberg, 1973) that the relation between oxidation rate and $\Delta\tilde{\mu}_H$ depended on the mechanism by which the latter parameter was varied (e.g. by addition of ADP versus addition of protonophore). [See also Azzone *et al.* (1978), Sorgato & Ferguson (1979) and Holian & Wilson (1980); and for photophosphorylation, see Ort (1976), Ort *et al.* (1976a,b), Baccarini-Melandri *et al.* (1977), Casadio *et al.* (1978), Melandri & Baccarini-Melandri (1979) and Melandri *et al.* (1981).] However, this observation could also be explained through a supposed allosteric effect of added adenine nucleotides on the activity of the respiratory chain. The extra link did not have to be the energy-transducing link postulated by the 'parallel-coupling' hypothesis (Padan & Rottenberg, 1973) to be able to explain these results (cf. Melandri & Baccarini-Melandri, 1979).

At least one falsification of the chemiosmotic coupling hypothesis has, however, been reported [Wiechmann *et al.* (1975); Azzone *et al.* (1977a,b); Baccarini-Melandri *et al.* (1977); Michel & Oosterhelt (1980)] that cannot be explained by an allosteric effect; it results from a measurement at equilibrium for the proton-translocating ATPase. This conflict between the chemiosmotic-coupling

hypothesis and experimental results arises in comparisons of the $\Delta\tilde{\mu}_H$, the transmembrane bulk-phase-to-bulk-phase electrochemical potential difference for protons, and the phosphate potential, ΔG_p , when the maximum amount of added ADP has been phosphorylated ('State 4'; Chance & Williams, 1955). These are the conditions under which the phosphate potential has increased to such a high value that it has reached equilibrium with the protonmotive force. The total Gibbs-free-energy change of the proton-translocating ATPase reaction then equals zero, so that the ratio of phosphate potential to the protonmotive force should be equal to the number of protons pumped by the ATPase per molecule of ATP hydrolysed. Nicholls (1974) was the first to measure both the phosphate potential and $\Delta\tilde{\mu}_H$ in one incubation. The comparison of the two yielded the conclusion that their ratio, 2.3 (Nicholls, 1974), was slightly higher than was compatible with the H^+ /ATP stoichiometry postulated by Mitchell (1967). Uncertainties as to the exact values of the activity coefficients of rubidium and acetate in the mitochondrial matrix, or the size of the relevant matrix water space (Garlid, 1979) may reduce the significance of this discrepancy. When other authors (Azzone *et al.*, 1977a,b, 1978; Wiechmann *et al.*, 1975; Van Dam *et al.*, 1978a) considered much lower values of $\Delta\tilde{\mu}_H$, a more prominent anomaly arose: the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio increased at low values of $\Delta\tilde{\mu}_H$, whereas the chemiosmotic-coupling hypothesis predicts that this ratio remains constant.

Azzone *et al.* (1977b) interpreted their results by a model that is only slightly more explicit

than the parallel-coupling hypothesis. Oxidative phosphorylation would resemble a 'molecular-energy machine' (McClare, 1971) rather than a chemiosmotic 'fuel cell'. The direct energy transmission in the former would obviate the potential danger of energy dissipation through thermal equilibrium. This model was similar to an earlier model [Williams (1961), evaluated by Mitchell (1977)] that contended that the high-energy state might be identical with the electrochemical potential difference of protons between the aqueous phases and the membrane phase. Unfortunately, these three models share a property with the other existing models such as the conformational hypothesis (Boyer, 1965), namely that they are stated not exactly enough to be falsifiable (Popper, 1963).

Van Dam *et al.* (1978a) tried to remove this problem of non-testability when they formulated their version of 'localized chemiosmosis'. They postulated that, on average, the respiratory-chain complexes and the proton-translocating ATPases are closer to each other than to the sites in the mitochondrial inner membrane at which proton leakage might occur. Moreover, protons close to these respiratory-chain-ATPase supercomplexes

would meet a resistance if they attempted to equilibrate with the external or internal phase. In Fig. 1, this resistance is denoted as R_H^* . If there is a significant proton leakage current through the proton leaks in the membrane, then the proton-motive force measured from external bulk phase to inner bulk phase (the $\Delta\tilde{\mu}_H$) would be lower than the protonmotive force across the mitochondrial inner membrane in the vicinity of the respiratory-chain-ATPase supercomplexes [the relevant protonmotive force, denoted as $\Delta\tilde{\lambda}_H$, formerly (Van Dam *et al.*, 1978a) denoted as Δp]. By using the analogy of a simple electric network as a first-order quantitative approach, Van Dam *et al.* (1978a) expressed the relation between the local protonmotive force experienced by the respiratory chains and the H^+ -translocating ATPases, $\Delta\tilde{\lambda}_H$, and the electrochemical potential difference for protons between bulk external and bulk matrix space, $\Delta\tilde{\mu}_H$, as:

$$\Delta\tilde{\mu}_H/\Delta\tilde{\lambda}_H = 1/(1 + R_H^*/R_H^1) \quad (1)$$

Consequently the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ would be:

$$\Delta G_p/\Delta\tilde{\mu}_H = n_H^p(1 + R_H^*/R_H^1) \quad (2)$$

Fig. 1. A testable scheme of localized chemiosmosis

For simplicity's sake the mitochondrial inner membrane is represented as the skin of a sphere. On the left-hand side the respiratory chain and the ATPase are located close together. The proton leakage is postulated to be on average farther away from the respiratory chains than the H^+ -transporting ATPases. This is stressed by depicting the proton leakage on the far right-hand side. The postulated resistance, R_H^* , between the region close to the respiratory chain-ATPase area and the bulk inner and outer phase are depicted as well as the resistance, R_H^1 , of the proton leaks. $\Delta\tilde{\lambda}_H$ (formerly called Δp ; Van Dam *et al.*, 1978a) is the electrochemical potential difference for protons between the internal 'local' space and the external 'local' space, $\Delta\tilde{\mu}_H$ is the electrochemical potential difference for protons between the internal 'bulk' space and the external 'bulk' space.

Here n_H^p is the number of protons pumped per molecule of ATP hydrolysed, including the proton involved in adenine nucleotides and phosphate transport. R_H^1 is the resistance of the mitochondrial inner membrane towards proton movement.

The explanation for the high phosphate potential/ $\Delta\tilde{\mu}_H$ ratios found at low $\Delta\tilde{\mu}_H$ values was that all such determinations had been performed under conditions of (supposedly) increased proton permeability of the mitochondrial inner membrane (either induced by the addition of uncouplers or by swelling). The low values for $\Delta\tilde{\mu}_H$ had thus always been accompanied by low values of R_H^1 . According to the abovementioned formula, this would explain the observed high ratio of phosphate potential to $\Delta\tilde{\mu}_H$.

Van Dam *et al.* (1978a) gave no structural basis for the extra compartment, but Kell (1979) found a physical basis for localized chemiosmosis in a comparison of mitochondrial proton pumping with electrode reactions. His arguments gave a good impression of how anomalous electric potential and pH profiles may occur, but would by themselves not explain a steady-state difference in proton electrochemical potential between two points in a single (internal or external) phase if no processes were operating that kept the protons from equilibrating within any single phase. Van Dam *et al.* (1978a) suggest a candidate for such a process: the flux of protons from the region close to the respiratory-chain-ATPase supercomplexes through the bulk phases and the proton leaks in the mitochondrial inner membrane to the matrix bulk phase and the local phase on the inner side of the membrane. Kell (1979) also indicated that the discrepancies between the results of different laboratories (for a review, see Brand, 1977) in experiments devised to determine H^+/O stoichiometries may be accounted for by localized chemiosmosis. A similar interpretation can be given to studies of oxidative phosphorylation in leaky skeletal-muscle submitochondrial particles (Komai *et al.*, 1976; Scott *et al.*, 1978) and to the absence of a membrane potential (Tedeschi, 1980) in single mitochondria that phosphorylated ADP.

The explicitness of the Van Dam formulation of localized chemiosmosis makes the hypothesis testable. We have now performed this test, and show that the results cannot be explained by localized chemiosmosis.

Materials and methods

Rat liver mitochondria (5 mg of protein/ml), isolated as described by Hogeboom (1955) but as modified by Myers & Slater (1957), were incubated in a 1.55 ml oxygraph vessel (thermostatically controlled at 298 K) containing an oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH,

U.S.A.) and a potassium-selective combination electrode (type 10 405 3754; Ingold, Urdorf, Switzerland) in 40 mM-sucrose/170 mM-mannitol/0.8 mM-EDTA (sodium salt)/1.25 mM-Tris phosphate/7 mM-MgCl₂, pH 7.3), saturated in oxygen. Rotenone (7 µg), valinomycin (39 µg), as well as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or sodium malonate (pH 7), were added before Tris succinate (25 mM, pH 7.3). When the oxygen electrode indicated 45% of oxygen saturation, 0.5 µmol of ADP (sodium salt) was added. At 10% of oxygen saturation a 500 µl sample was layered on to silicone oil [0.5 ml, AR 200/AR 20, 3:4 (v/v) (Wacker G.m.b.H., München, Germany)], floating on 200 µl of 15% HClO₄ in a 1.5 ml Eppendorf centrifuge tube (Werkheiser & Bartley, 1957). Simultaneously with starting the centrifugation an 800 µl sample was injected into 200 µl of 15% HClO₄, rapidly mixed and put on ice/water. The supernatant of the latter sampling was neutralized to pH values between 6.0 and 7.0 with 0.15 or 0.5 M-Mops (4-morpholinepropanesulphonic acid)/1.5 M-KOH and again stored on ice to be frozen (below 160 K) within 5 h. After thawing, this sample was used to determine adenine nucleotide concentrations by means of standard enzymic procedures (Bergmeyer, 1970). In the fractions below and above the silicone-oil layer of the former sample, potassium and phosphate were determined by flame photometry and the Chen (Chen *et al.*, 1956) procedure. Under the conditions of Chen *et al.* (1956), 10% of ATP is determined as phosphate. We did not correct for this. The phosphate potential was calculated by using a standard free-energy change (ΔG) of 30.75 kJ/mol (Rosing & Slater, 1972). $\Delta\tilde{\mu}_H$ was calculated by assuming equilibration of K⁺ (Rottenberg, 1975) and phosphoric acid (Chappell & Crofts, 1966) across the mitochondrial inner membrane. Matrix volume and sucrose-inaccessible space were taken as 1.5 and 3 µl/mg of protein respectively. In parallel experiments with ³H₂O, [¹⁴C]sucrose and ⁸⁶Rb⁺, matrix volume and sucrose-inaccessible space varied from 1.4 and 2.4 µl/mg respectively at the lowest, to 1.9 and 3.4 µl/mg respectively at the highest, membrane-potential value.

The experiments with ascorbate as the substrate were essentially similar, except that they were performed simultaneously rather than sequentially. To 1.5 ml of medium [0.20 M-mannitol/2.5 mM-Tris, phosphate/10 mM-(Tris) Mops/1 mM-EGTA/7 mM-MgCl₂ (pH 7.5)/⁸⁶RbCl (10 µl, 0.08 µCi)/[¹⁴C]5,5-dimethylxazolidine-2,4-dione (0.10 µCi, 10 µl)/³H₂O (5 µl)] incubated in scintillation vials in a rotatory shaking water bath at 298 K, 5 mg of mitochondria, protein, 6 µg of rotenone, 24 µg of valinomycin, and 2.5 mM-Tris ascorbate, pH 7.5, plus various amounts of *NNN'*-tetramethyl-*p*-phenylene-

diamine, or 40 nmol of *NNN'*-tetramethyl-*p*-phenylenediamine plus variable amounts of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, were added. Then 150 nmol of Tris AMP was injected. Four representative experiments were run in the oxygraph vessel. Sampling was after sufficient time for the incubation mixture with the highest *NNN'*-tetramethyl-*p*-phenylenediamine and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone concentrations still to have a pO_2 of at least 10%. No significant differences between oxygraph and water-bath runs were observed. ΔpH and the membrane potential were calculated by assuming equilibration of the acid form of 5,5-dimethyloxazolidine-2,4-dione and of Rb^+ . ^3H_2O (5 Ci/litre), $[U-^{14}C]$ sucrose (50 mCi/litre, 12 μM) and $^{86}RbCl$ (0.10 Ci/litre) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[2-^{14}C]$ 5,5-dimethyloxazolidine-2,4-dione [0.10 Ci/litre (in water) 2 mM], was obtained from New England Nuclear Corp., Boston, MA, U.S.A.

Results

Experiments analogous to that represented by the open circles in Fig. 2 led the groups of Azzone (Azzone *et al.*, 1977b, 1978) and Van Dam (cf. Wiechmann *et al.*, 1975; Van Dam *et al.*, 1978a) to the development of their version of the hypothesis of localized chemiosmosis. Mitochondria oxidizing

succinate in the presence of added ADP were allowed to reach the steady state in which the oxidation rate and the membrane potential (as judged from the potassium electrode) reached their minimum and maximum value respectively (State 4). Then the extramitochondrial phosphate potential and $\Delta\tilde{\mu}_H$ were determined. All arguments in the present paper pertain to the intramitochondrial as well as to the extramitochondrial phosphate potential. Since measurement of the former is prone to systematic experimental errors, we measured the latter, as both the proton-translocating ATPase and the translocator of adenine nucleotides are supposed to be at equilibrium under State-4 conditions. This experiment was repeated in the presence of various concentrations of added protonophores and the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ was plotted against $\Delta\tilde{\mu}_H$ (O in Fig. 2). Contrary to the predictions of the chemiosmotic hypothesis, but consistent with the localized-chemiosmosis hypothesis, this value increases at low values of $\Delta\tilde{\mu}_H$.

The exact formulation of the hypothesis of localized chemiosmosis (cf. Van Dam *et al.*, 1978a, and the introduction above) explains this observation on the basis of the fact that the variation in $\Delta\tilde{\mu}_H$ is brought about by varying the proton leakiness of the mitochondrial inner membrane. Only then the extent to which the measured $\Delta\tilde{\mu}_H$ is an underestimation of the relevant $\Delta\tilde{\lambda}_H$ increases with decreasing $\Delta\tilde{\mu}_H$. This suggests a test for the hypothesis of localized chemiosmosis: if $\Delta\tilde{\mu}_H$ is

Fig. 2. Dependence of $\Delta G_p / \Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$ with succinate as substrate

$\Delta\tilde{\mu}_H$ is varied either with 0–33 mM-malonate (●, ■) or with protonophore (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 0–0.2 nmol/mg of protein) (○). ● and ○ are from one experiment. ■ is from an essentially identical experiment using ^{86}Rb and $[^{14}C]$ 5,5-dimethyloxazolidine-2,4-dione distribution to measure $\Delta\tilde{\mu}_H$.

varied without varying the proton permeability of the mitochondrial inner membrane, then the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ should be independent of $\Delta\tilde{\mu}_H$. This situation was obtained by performing the above-mentioned experiment in the presence of various amounts of malonate, an inhibitor of succinate dehydrogenase. Thus $\Delta\tilde{\mu}_H$ is varied by varying the activity of its generator, the oxidative proton pump, rather than its dissipator, the proton leakage. The results of this experiment are represented by ● in Fig. 2.

In contrast with the prediction by the hypothesis of localized chemiosmosis, the ratio of phosphate potential to $\Delta\tilde{\mu}_H$ was still dependent on $\Delta\tilde{\mu}_H$. The dependence was actually quite similar to the dependence in the protonophore experiment.

This finding was not specifically the result of the method of determination of $\Delta\tilde{\mu}_H$. The ■ symbols in Fig. 2 indicate the variation of the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio with $\Delta\tilde{\mu}_H$ in a malonate titration experiment, in which we used [^{14}C]5,5-dimethyloxazolidine-2,4-dione and $^{86}\text{Rb}^+$ uptake to monitor ΔpH and the membrane potential. To measure matrix and adherent space we performed parallel runs with ^{86}Rb , [^{14}C]sucrose and $^3\text{H}_2\text{O}$.

To make sure that we were not misled by an (unexpected) uncoupling effect of malonate, we performed an analogous experiment with a catalyst rather than an inhibitor. We used ascorbate as the substrate and varied the oxidation rate by adding

various amounts of *NNN'*-tetramethyl-*p*-phenylenediamine. Fig. 3 shows similar results to Fig. 2 in that the phosphate-potential/ $\Delta\tilde{\mu}_H$ ratio also increases with decreasing $\Delta\tilde{\mu}_H$ (●). Variation of protonophore concentration at high *NNN'*-tetramethyl-*p*-phenylenediamine concentration (○) gives closely similar dependences of $\Delta G_p/\Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$. Again the protonophore points fall on the same curve as the low *NNN'*-tetramethyl-*p*-phenylenediamine points. Thus the above-mentioned falsification of the hypothesis of localized chemiosmosis is confirmed.

Discussion

The experiments reported in the present paper showed the following. The effect of variation of the $\Delta\tilde{\mu}_H$ in rat liver mitochondria on the State-4 $\Delta G_p/\Delta\tilde{\mu}_H$ ratio is independent of whether $\Delta\tilde{\mu}_H$ is varied through protonophore titration or through variation of the activity of the respiratory chain. The latter variation is expected to have no, or at least a very different, effect on the proton permeability of the mitochondrial inner membrane. Since localized chemiosmosis predicts a variation in the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio only when the proton permeability of the mitochondrial inner membrane is changed, we conclude that the variation of this ratio cannot be explained by the hypothesis of localized chemiosmosis as formulated by Van Dam *et al.* (1978a).

To the experiment with malonate the objection may be made that, at higher concentrations (e.g. at the 33 mM used), malonate might act as a protonophore and thus cause a decrease in R_H^1 (the ohmic resistance of the mitochondrial inner membrane towards proton permeation). However, in view of the strong inhibitory effect of malonate on the rate of succinate oxidation (results not shown), we conclude that it must at least have diminished $\Delta\tilde{\mu}_H$ in part through diminishing the rate of proton pumping. It seems unlikely, therefore, that the relation of $\Delta G_p/\Delta\tilde{\mu}_H$ to $\Delta\tilde{\mu}_H$ would be so similar to the relation observed with protonophore. Moreover, the ascorbate-variable *NNN'*-tetramethyl-*p*-phenylenediamine experiment is insensitive to this type of argument. Here $\Delta\tilde{\mu}_H$ increases parallel to the increasing concentration of *NNN'*-tetramethyl-*p*-phenylenediamine. The known uncoupling activity of *NNN'*-tetramethyl-*p*-phenylenediamine (which was indeed observed at higher concentrations and was the reason why the *NNN'*-tetramethyl-*p*-phenylenediamine concentration was not increased further) would tend to level off the dependence of $\Delta G_p/\Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$. Yet the increase of the ratio $\Delta G_p/\Delta\tilde{\mu}_H$ at lower $\Delta\tilde{\mu}_H$ values is still observed.

Now the question must be raised as to whether this falsification of the hypothesis of localized chemiosmosis, as formulated by Van Dam *et al.* (1978a) and in the introduction above, can be taken

Fig. 3. Dependence of $\Delta G_p/\Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$ with ascorbate as the substrate

$\Delta\tilde{\mu}_H$ is varied with either 0–50 μM -*NNN'*-tetramethyl-*p*-phenylenediamine (●) carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (0–0.1 nmol/mg of protein) (○).

as a falsification of other hypotheses of localized chemiosmosis. Fig. 4 gives the most general formulations of the three main groups of coupling hypotheses. It is seen that group b contains all hypotheses of localized chemiosmosis as well as the chemical, conformational and Williams (1961) hypotheses. For the present discussion the essential differences between the groups are that, in the chemiosmotic-coupling hypothesis, energy transduction from oxidation to phosphorylation occurs only through $\Delta\tilde{\mu}_H$, whereas in the group-b hypotheses it occurs through an as-yet-unmeasurable energetic intermediate that is in energy-transducing contact with $\Delta\tilde{\mu}_H$. The parallel-coupling hypothesis is again different, because it allows two ways of energy transduction to occur in parallel. A summary of expectations of the different coupling hypotheses under the two experimental conditions of the present study (protonophore versus malonate) is given in Table 1. The chemiosmotic hypothesis is inconsistent with both the results of the protono-

phore and the results of the malonate experiment, whereas the parallel-coupling hypothesis (Padan & Rottenberg, 1973) is consistent with both. We have already concluded that the hypothesis of localized chemiosmosis as formulated by Van Dam *et al.* (1978a) is consistent with the results of the protonophore experiment, but not with the results of the malonate experiment. If one would elaborate the other b-type coupling hypotheses in such a manner that the high-energy intermediate can be treated as having a free energy ΔG_p , which can be converted into $\Delta\tilde{\mu}_H$ by a physico-chemical reaction, then near-equilibrium irreversible thermodynamics (Katchalsky & Curran, 1967; Westerhoff & Van Dam, 1979) allows the formulation of equations analogous to eqns. (1) and (2) ($\Delta\tilde{\lambda}_H$ being replaced by ΔG_p). Consequently all b-type hypotheses would be refuted by the malonate and *NNN'*-tetramethyl-*p*-phenylenediamine effects on the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio.

One may, however, contend that the difference in

Fig. 4. Current coupling hypotheses

The hypotheses of localized chemiosmosis of Azzone *et al.* (1977) and Van Dam *et al.* (1978a) are placed in the same group as the hypotheses of Williams (1961) and Kell (1979), i.e. b2. Likewise the chemical-coupling hypothesis (Slater, 1953) as modified by Slater (1967) and the conformational hypothesis (Boyer, 1965) are placed together in group b1. The chemiosmotic-coupling hypothesis (Mitchell, 1961) and the parallel-coupling hypothesis (Padan & Rottenberg, 1973) fall into single categories (a and c respectively). The energy-transducing links (double arrows) as well as the dissipative reactions (curved arrows) are shown.

Table 1. Predictions of the different coupling hypotheses (cf. Fig. 4) for the outcome of the protonophore and the malonate (*NNN'*-tetramethyl-*p*-phenylenediamine) experiments with the conclusions that can be drawn from comparison with the experimental results (see also the text)

	Observation	Theory			
		Chemiosmotic (a)	Chemical/ conformational (b1)	Localized chemiosmosis (b2)	Parallel coupling (c)
Variation of $\Delta G_p/\Delta\tilde{\mu}_H$ with protonophore?	Yes	No	Yes	Yes	Probably
As above with malonate?	Yes	No	No*	No*	Possibly
Hypothesis falsified?	—	Yes	Yes†	Yes†	No
As above if variable H^+ /ATP accepted?	—	No	No	No	No

* Certainly if near-equilibrium thermodynamics can be applied, probably on the basis of the qualitative argument given in the text.

† Cf.*

free energy between the high-energy intermediate ΔG_p and $\Delta\tilde{\mu}_H$ may become so high that the assumed ohmic flux/force relationships no longer apply (Prigogine *et al.*, 1948), so that also eqns. (1) and (2) would fail. In that case, however, it still seems unlikely that shutting off the tap at the $\Delta\tilde{\mu}_H$ -generating reaction by inhibiting respiration would have the same effect on the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio as draining at the other end, by dissipating the $\Delta\tilde{\mu}_H$. Fig. 2 suggests that the effects of malonate and protonophore are identical at equal $\Delta\tilde{\mu}_H$. Fig. 3 suggests the same for *NNN'*-tetramethyl-*p*-phenylenediamine and protonophore.

The latter argument can be made stronger by the following. If indeed the ATP-forming reaction with well-defined stoichiometry n_H^+ is in equilibrium with $\Delta G_{\sim}$ ($\Delta\tilde{\lambda}_H$), then the variation of the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio reflects a variation of the $\Delta G_{\sim}/\Delta\tilde{\mu}_H$ ratio. If one then considers a point on the curve of Fig. 2 (or Fig. 3), one effectively considers two experimental conditions (i.e. one in the presence of malonate and the other in the presence of protonophore) with identical $\Delta\tilde{\mu}_H$, $\Delta G_p/\Delta\tilde{\mu}_H$, and therefore ΔG_p , and $\Delta G_{\sim}$. However, the oxidation rates are very different. Reconsidering the schemes b in Fig. 4 one must then conclude (cf. Nicholls, 1974) that under the two conditions there would be very different rates of formation of $\Delta\tilde{\mu}_H$ from $\Delta G_{\sim}$ at identical values of $\Delta\tilde{\mu}_H$, $\Delta G_{\sim}$ and even all the other parameters. Table 2 sharpens this point even further by actually showing two sets of points and a control point. Within each set, $\Delta\tilde{\mu}_H$, ΔG_p , and essentially all other parameters have similar values, whereas the oxidation rate in the protonophore case is much higher than in the malonate case. [The slight inhibition of uncoupled succinate oxidation by high concentrations of protonophores has been attributed to rate limitation at the site of succinate translocation (Kraaijenhof & Van Dam, 1969) and is irrelevant to the present

argument.] Thus it appears that the $\Delta G_{\sim}$ -to- $\Delta\tilde{\mu}_H$ reaction could have two very different rates at nearly identical concentrations of its substrates and products (remember that ΔG_p is in equilibrium with $\Delta G_{\sim}$).

There are only a few ways out of this paradox. The first is that all b-type hypotheses are incorrect. A second one would be that the reaction rate of the $\Delta G_{\sim}$ -to- $\Delta\tilde{\mu}_H$ reaction would indeed be highly dependent on the concentrations of its substrates and products, so that the minor differences in Table 2 would still be relevant. This possibility can be refuted, because if for instance the higher rate of oxidation in the protonophore case would be due to an imperceptibly higher ATP concentration, then one can find a malonate point with an even higher ATP concentration but a still much lower oxidation rate.

An important way out might seem the contention that a dissipative cycle is missing in Fig. 4. This could be the continuous dissipation of ΔG_p , not via $\Delta\tilde{\mu}_H$ dissipation but through catalysis of scalar ATPase by fragments of broken mitochondria in the preparation. Then the assumed energetic equivalence of ΔG_p and $\Delta G_{\sim}$ (see above) would no longer be valid. This contention is certainly correct, but probably quantitatively unimportant. In our mitochondrial preparations State-4 oxidation is routinely only about 15% inhibited by atractyloside (see also Lemasters & Sowers, 1979). The flow from $\Delta G_{\sim}$ to ΔG_p would be one-fifth of the flow from $\Delta G_{\sim}$ to $\Delta\tilde{\mu}_H$. Moreover, at identical low values of $\Delta\tilde{\mu}_H$, the flow from $\Delta G_{\sim}$ to $\Delta\tilde{\mu}_H$ and therefore $\Delta G_{\sim}$ itself would be higher in the protonophore than in the malonate experiment. Since the flow between $\Delta G_{\sim}$ and ΔG_p is far from its maximum (State 3) magnitude, this higher $\Delta G_{\sim}$ should have been reflected by an increase in ΔG_p as well. This has not been observed (cf. Table 2).

Table 2. *Parameter values at corresponding protonophore and malonate points*

The points are five of the points shown in Fig. 2. Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; J_o , oxidation rate.

[Malonate] (mM)	15	—	33	—	—
[FCCP] (nmol/mg of protein)	—	0.27	—	0.67	—
[ATP] _{ex.} (μM)	66	56	12	59	119
[ADP] _{ex.} (μM)	87	78	30	12	90
[P] _{ex.} (mM)	1.7	1.8	1.6	1.5	2.0
[K ⁺] _{ex.} (mM)	0.91	1.0	1.7	1.4	1.3
[P] _{in.} (mmol/kg of protein)	56	60	39	43	187
[K ⁺] _{in.} (mmol/kg of protein)	119	197	44	33	192
ΔpH	0.84	0.86	0.67	0.77	1.37
Δψ (mV)	91	101	42	39	93
Δμ _H (kJ/mol)	13.6	14.6	7.8	8.2	16.8
ΔG _p (kJ/mol)	45.8	45.5	44.3	45.0	46.8
ΔG _p /Δμ _H	3.4	3.1	5.6	5.5	2.9
J_o (mmol/min per kg)	25	351	12	277	138

Other possible dissipative cycles would be a 'squigglase' [i.e. a reaction straightforwardly dissipating $\Delta G_{\sim} (\Delta \tilde{\lambda}_H)$]. Such a reaction would not solve the abovementioned paradox, as ΔG_p would still be a measure of $\Delta G_{\sim}$. The same is true for an inherently slipping oxidative proton pump (Pietrobon & Azzone, 1980). A slipping ATPase would have implications similar to those discussed in the preceding paragraph.

Missing dissipative cycles may, however, complicate the picture if it is assumed that protonophores affect them rather than the $\Delta \tilde{\mu}_H$ dissipative cycle (Hanstein, 1976; Katre & Wilson, 1978, 1980; Pietrobon & Azzone, 1980). If such a protonophore action would outweigh its protonophorous activity, then the abovementioned paradox would disappear. Especially if the protonophore would inherently uncouple the oxidative proton pump, no difference between the malonate and the protonophore results would be expected. In view of what is known about the mechanism of action of protonophores (e.g. McLaughlin & Dilger, 1980; Terada & Van Dam, 1975), we consider it unlikely that the effect of protonophores occurs largely through inherent uncoupling of the redox proton pumps.

Finally, there is another possibility. This affects the assumption that ΔG_p reflects $\Delta G_{\sim} (\Delta \tilde{\lambda}_H)$. It cannot yet be excluded that the stoichiometry of the number of protons pumped per number of ATP molecules hydrolysed depends on $\Delta \tilde{\mu}_H$. It is noteworthy that, if a variable H^+ /ATP pump stoichiometry is accepted, the chemiosmotic-coupling hypothesis itself could also explain the observations; there is no need to assume localized phenomena on the basis of experiments of the type reported here. Although the idea of variable stoichiometries is not at all popular in bioenergetics (probably in view of the fact that they could explain almost anything), reports of variable stoichiometries are becoming numerous [Schuldiner & Fishkes, 1978; Ramos & Kaback, 1977 (but see Zilberstein *et al.*, 1979; Booth *et al.*, 1979; Lanyi & Silverman, 1979; Ten Brink & Konings, 1980; Paterson *et al.*, 1980; Leblanc *et al.*, 1980]. For all these reported findings, other explanations remain possible (cf. West, 1980).

Other explanations exist that accommodate both the protonophore and the malonate results within the framework of the chemiosmotic-coupling hypothesis. The first is relatively trivial, i.e. that our methods of measuring $\Delta \tilde{\mu}_H$ (in this case the silicone-oil centrifugation method) could be erroneous. The protonophore experiment has, however, also been performed by using potassium-electrode data to calculate the membrane potential. These experiments gave essentially the same results (Van Dam *et al.*, 1978b). Moreover, protonophore and malonate would not be expected to cause the same errors.

Another explanation of this type would be that the intramitochondrial water space we measure with our sucrose/ ^3H_2O method does not dissolve our tracer ions (Luck, 1976; Garlid, 1979). Then, especially at lower water contents, we might highly underestimate the $\Delta \tilde{\mu}_H$ (cf. Tedeschi, 1980). The lowest water space we measured was $1.4 \mu\text{l}/\text{mg}$ of protein, which is still more than twice the value usually found for osmotically inactive matrix water (Bentzel & Solomon, 1967; Harris & Van Dam, 1968; Garlid, 1979). Moreover, if the water space we measure was totally irrelevant as a solvent for the intramatrix potassium, and therefore the membrane potential would remain constant on titration with protonophore or respiratory inhibitor, then it would be unclear why potassium leaves the matrix space when such a titration is performed.

A further, possibly important, objection against our interpretation of our experiments might be directed against our assumption of equilibrium of the proton-translocating ATPase under State-4 conditions. It might be proposed that the decrease of oxidation at State 4 is due to a kinetic limitation of the ATP-synthesizing machinery, the concentration of ADP becoming kinetically limiting. Our view that in State 4 the H^+ -translocating ATPase is in dynamic equilibrium, is based on the observation of Lee & Eiler (1953) (see also Ernster *et al.*, 1954 and Boyer *et al.*, 1956) that in State 4 there is still a high forward rate of [^{32}P]ATP synthesis from ADP and [^{32}P]P_i. Furthermore, in our own experiment we observe immediate onset of ATP hydrolysis when anaerobiosis is allowed to occur in State 4 (result not shown). Moreover, the effect of protonophore on the $\Delta G_p/\Delta \tilde{\mu}_H$ ratio is observed independently of the direction from which State 4 is approached (cf. Fig. 2, start with ADP, to Fig. 3, start with AMP; starting with ATP yields similar results for the protonophore experiment). Other experiments (A. H. C. A. Wiechmann, J. C. Arents and K. Van Dam, personal communication) have shown that the $\Delta G_p/\Delta \tilde{\mu}_H$ ratio is essentially independent of time when we take our samples.

We conclude from the experiments presented here that neither localized chemiosmosis [including the Williams (1961, 1978) hypothesis], nor the chemical hypothesis, nor the conformational hypothesis, can explain the dependence of $\Delta G_p/\Delta \tilde{\mu}_H$ on $\Delta \tilde{\mu}_H$ essentially better than the chemiosmotic hypothesis itself. All these hypotheses need variability of the H^+ /ATP stoichiometry with $\Delta \tilde{\mu}_H$ to explain these results. The only remaining alternative coupling hypothesis which does not need a variable stoichiometry is the parallel-coupling hypothesis. This would, therefore, need reformulation in such terms that experimental tests are suggested. The variable stoichiometry of the proton-translocating ATPase can be tested. To avoid all the complications present

in intact rat liver mitochondria, probably the best test system would be the reconstituted, purified proton-translocating ATPase complex. Both ATP-pulse experiments (measuring the number of protons translocated per molecule of ATP hydrolysed) and 'static-head' experiments (measuring the ratio of $\Delta\mu_{\text{H}}$ to ΔG_{p} when the proton-translocating ATPase has reached its equilibrium) must be performed, since the stoichiometry may not be variable under all conditions. For the latter experiment a preparation with a second proton pump reconstituted into all ATPase-containing vesicles is needed (Ryrie *et al.*, 1979).

Finally, we should like to stress that we have only shown that localized chemiosmosis does not explain the $\Delta G_{\text{p}}/\Delta\mu_{\text{H}}$ variation with $\Delta\mu_{\text{H}}$. This does not necessarily mean that localized chemiosmosis is irrelevant. Under other conditions [submitochondrial particles, site one (Westerhoff *et al.*, 1981), kinetic rather than steady-state conditions; see, however, Junge *et al.*, 1978] the concept may still be valuable. Moreover, the most recent elaboration of the hypotheses of localized chemiosmosis and 'electrodics' (Kell, 1979) in terms of 'protoneural networks' (Kell & Morris, 1981) may turn out to be in keeping with the experimental results presented here (D. B. Kell & H. V. Westerhoff, unpublished work).

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