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Double inhibitor titrations of photophosphorylation are consistent with delocalized coupling

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The results of double inhibitor titrations have been interpreted as indicating that energy coupling in chromatophores from *Rhodopseudomonas capsulata* is direct (Hitchens, G.D. and Kell, D.B. (1983) Biochim. Biophys. Acta 723, 308–316). In this report a more detailed description of the steady-state response of a delocalized coupling system to double inhibitor titrations is presented. It is found that the results of the published titrations are consistent with delocalized coupling. Since they are also consistent with direct coupling, the responses of chromatophores to double inhibitor titrations do not distinguish between the two popular descriptions of coupling between electron transport and phosphorylation.

Introduction

Peter Mitchell proposed that a transmembrane gradient in proton electrochemical activity is the high energy intermediate linking phosphorylation to electron flow in the energy conserving membranes of bacteria, mitochondria and chloroplasts [1,2]. This chemiosmotic hypothesis is now generally accepted (e.g., Ref. 3) and interest is currently focused on the mechanistic and structural details of the energy-coupling process. One such detail, controversial from the start [4], is the nature of the path along which protons flow to the ATPases from their sites of release during electron flow. Mitchell suggested that the protons are released into and taken from the bulk aqueous phase. This requires that coupling be delocalized, since all the electron transport and ATPase complexes of a single vesicle interact with the same pool of high energy protons. Most modifications of this theory propose that protons are released into a more restricted phase of much more limited buffering capacity which conducts them directly to the ATPases. Leakage of protons into the surrounding bulk phases is slow, and the equilibration between the bulk and the limited phases may be imperfect. The proton conductor has been proposed to be a special ice-like layer of water and lipid head groups at the membrane surface [5], as well as actual channels of lipid and protein in the membrane [6]. Proton diffusion in the restricted phase is usually taken as being restricted, favoring interaction between adjacent, or in some way connected, electron transport and ATPase complexes. If this is the case, then coupling is localized, or direct.

Much of the evidence for localized gradients and direct coupling rests upon the observation of a poor correlation between the rates of phosphorylation and the size of $\Delta\mu_{H^+}$, the transmembrane proton activity gradient measured between the bulk

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Abbreviations: DCCD, *N*,*N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; P/e_2, twice the rate of phosphorylation divided by the rate of electron flow; SF 6847, 3,5-di-*t*-butyl-4-hydroxybenzylidene malononitrile.

phases (e.g., see Ref. 5). Most measurements of $\Delta\mu_{H^+}$ can be questioned on technical grounds, since it is difficult to relate the measured parameters to the size of the gradient in an absolutely convincing manner. Experiments which distinguish between localized and delocalized coupling without recourse to measurement of $\Delta\mu_{H^+}$ are thus of special interest. Along such lines a number of laboratories have titrated phosphorylation with an inhibitor under a variety of conditions to see if sensitivity to the inhibitor changes in a way consistent with either delocalized or direct coupling [7–9]. Recently, Hitchens and Kell have explicitly developed this approach into a double inhibitor titration technique which depends solely upon measurements of phosphorylation rates [10–12]. Experiments using this approach with chromatophores of *Rhodopseudomonas sphaeroides* were interpreted to indicate that the transfer energy from electron-transport chains to ATPases is direct in this organism. It is the contention of this paper that the descriptions of delocalized coupling used by Hitchens and Kell are too simple and that their data do not exclude a delocalized mechanism.

Experimental procedures

Chloroplasts were prepared from greenhouse spinach as described previously [13]. Reaction mixtures were 1 ml in volume and contained 50 mM Tricine-NaOH (pH 8.0), 50 mM KCl, 2 mM $K_3Fe(CN)_6$, 5 mM $MgCl_2$, 1 mM ADP, 2 mM KP_i (1 μCi ^{32}P/ml), 40 μM EDTA, chloroplasts equivalent to 50 μg chlorophyll and the indicated concentrations of inhibitors. Dicyclohexylcarbodiimide (DCCD) in ethanol was added to the stock solution of chloroplasts (2 mg chlorophyll/ml) at least 30 min before assay. Since DCCD inhibition increases with time, all tubes at a given DCCD concentration were illuminated at the same time. Uncouplers and dichlorophenyldimethyl urea (DCMU) were added to the reaction mixtures. Where appropriate, ethanol was added to the control chloroplast stock solutions or to the reaction mixtures. Illuminations were usually 2 min long. A bank of three 300 W bulbs provided light for the samples, which were illuminated in a water bath with shaking. Esterified phosphate was determined as by Avron [14].

Analysis

Two double inhibitor titrations will be discussed in detail to illustrate two different factors that received insufficient attention in the chromatophore experiments. The first titration will illustrate the concept that more than one factor can limit the rate or phosphorylation at a time. The second will emphasize the effect upon titrations of feedback loops inherent in delocalized descriptions of coupling. Finally, a mathematical model of delocalized coupling will be used to simulate titrations.

Titrations with antimycin A in the presence of dicyclohexylcarbodiimide

Hitchens and Kell titrated phosphorylation catalysed by chromatophores with the electron transport inhibitor antimycin A in both the presence and absence of the energy-transfer inhibitor DCCD [10]. When present, DCCD was added to a concentration giving roughly 50% inhibition of phosphorylation measured in the absence of antimycin. As antimycin was titrated in, the percent inhibition by a given concentration of DCCD remained constant. Thus, the effects of the two inhibitors were independent. This result is easy to understand in terms of direct coupling, which predicts that DCCD should inactivate some coupling units, but have no effect upon the antimycin A sensitivity of the remaining active ones.

Hitchens and Kell indicate that delocalized coupling cannot account for the results of these titrations. If coupling is delocalized, they argue, DCCD inactivation of ATPases will make the ATPase rate limiting for phosphorylation. Antimycin A induced inhibition of electron transport and thus of $\Delta\mu_{H^+}$ will not inhibit phosphorylation in such membranes until $\Delta\mu_{H^+}$ limits phosphorylation to rates even lower than those set by DCCD. As a result, DCCD-poisoned chromatophores should be more resistant to antimycin A than control membranes. The analysis of Hitchens and Kell is presented graphically in Fig. 1. DCMU replaces antimycin A as the electron flow inhibitor to make the figure directly comparable to Figs. 2 and 3.

There is an error in the analysis of delocalized coupling used by Hitchens and Kell [10]. This has been pointed out previously by Parsonage and

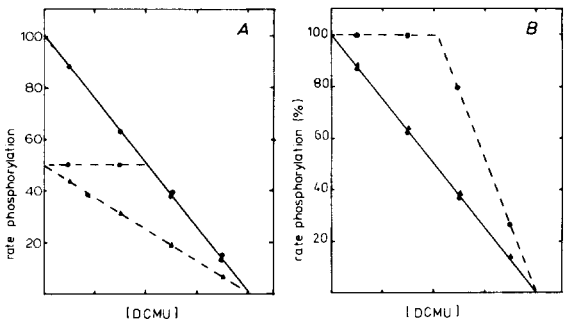

Fig. 1. Idealized titrations of photophosphorylation with DCMU according to the analysis of Hitchens and Kell. (A) The rates of phosphorylation catalyzed by control thylakoids (○) and thylakoids in which phosphorylation has been inhibited by DCCD (●, ▲) are plotted as a function of [DCMU]. The units are arbitrary. The predictions of both localized (▲) and delocalized (●) coupling are shown. (B) The same data plotted as percent activity relative to the appropriate rate measured in the absence of DCMU.

Ferguson [15] and Clark et al. [16]. The problem with the analysis can be seen by considering the rate of phosphorylation to be the product of the number of ATPases turning over multiplied by the turnover rate per active ATPase. In a delocalized model the second factor is a function of $\Delta\mu_{H^+}$ *. Addition of an energy-transfer inhibitor like DCCD reduces the number of ATPases that turn over, but the turnover rate per ATPase will still be a function of $\Delta\mu_{H^+}$. Addition of an electron transport inhibitor like antimycin A will reduce $\Delta\mu_{H^+}$ and thus the rate of phosphorylation, whether or not DCCD is present. There is no single limiting factor. The number of active ATPase complexes always limits phosphorylation, and the turnover rate is always a function of $\Delta\mu_{H^+}$ as long as $\Delta\mu_{H^+}$ is not saturating. There is no reason to expect that treatment with DCCD will have major effects upon the sensitivity of photophosphorylation to antimycin A, or vice versa. The two different coupling mechanisms predict similar responses.

Titration with SF 6847 in the presence of DCCD

Phosphorylation catalyzed by chromatophores was titrated with the uncoupler SF 6847 by Hitchens and Kell [11] both in the presence and absence of DCCD. As in the experiment discussed above, the DCCD concentration used gave roughly 50% inhibition of phosphorylation measured in the absence of the uncoupler. The inhibitions by DCCD and SF 6847 were not independent. The percent inhibition by DCCD increased as uncoupler concentration increased.

This synergism is not an obvious prediction of direct coupling. Hitchens and Kell suggest that inactivation of an ATPase by DCCD destroys its binding site for uncouplers, and thus increases the effective concentration of uncoupler [11,12]. They also suggest that delocalized mechanisms cannot account for the data. If coupling were delocalized, they argue, DCCD inhibition should increase the size of the transmembrane proton activity gradient by inhibiting proton efflux. Since uncouplers act by decreasing $\Delta\mu_{H^+}$, the two inhibitors have conflicting effects. DCCD-poisoned membranes will therefore be less, not more, sensitive to uncoupling.

The error committed in the analysis of the first type of experiment is not repeated here, but a subtle point is missed. In coupled membranes the inhibition of photophosphorylation by DCCD is not proportional to the fraction of ATPases that has reacted with and been inhibited by the reagent. This is the consequence of the fact that blocking proton efflux through the inhibited complexes increases $\Delta\mu_{H^+}$, thereby increasing the turnover rate of the remaining active ATPases. There is a feedback loop that masks or minimizes inhibition by energy-transfer inhibitors. This point was appreciated. However, the full increase in $\Delta\mu_{H^+}$ and thus the masking of the full extent of inhibition by DCCD is prevented if the membranes are uncoupled so that most of the protons exit through the uncoupler instead of through the ATPase (Ref. 9 and see also the Discussion section of this paper). Thus, if DCCD-poisoned chromatophores are titrated with an uncoupler, the full extent of inhibition by DCCD will become ap-

* Some delocalized descriptions of energy coupling suggest that the increase in the rate of ATP synthesis accompanying an increase in $\Delta\mu_{H^+}$ results from an increase in the fraction of active ATPases instead of from an increase in the turnover rate of individual ATPases [17]. Such an approach can be accommodated to the above discussion if "number of active ATPases" is replaced by "number of potentially active, i.e., non-DCCD inhibited, ATPases" and the "turnover rate per ATPase" is replaced by "turnover rate averaged over the potentially active ATPases".

parent as the uncoupler concentration rises. This will decrease the concentration of uncoupler needed to give 50% inhibition of phosphorylation, as observed by Hitchens and Kell [11,12]. In the complimentary experiment, uncoupled membranes will show full sensitivity to DCCD, while coupled controls will minimize sensitivity by the feedback mechanism. DCCD will appear to be a better inhibitor in the presence of uncouplers. Thus, while delocalized descriptions of coupling do indeed predict that uncouplers and energy-transfer inhibitors will have conflicting effects upon $\Delta\mu_{H^+}$, they also predict that the two classes of inhibitors will interact synergistically to inhibit phosphorylation. Once again, the two different coupling mechanisms predict similar responses.

Numerical model of delocalized coupling.

The arguments presented above are largely qualitative. In delocalized energy coupling the exact sensitivity of energy transfer inhibited vesicles to antimycin A or to uncouplers depends upon subtleties of the interaction between $\Delta\mu_{H^+}$ and the rate of phosphorylation and between $\Delta\mu_{H^+}$ and the rate of electron flow. To add plausibility to the revised predictions of the delocalized description, a crude mathematical model of delocalized coupling was constructed and the titrations mathematically simulated. The model is based upon the chloroplast thylakoid membrane because of the author's familiarity with this membrane. As a first approximation, which is all this claims to be, the general form and content of such a model should not depend upon the membrane to which it is applied.

In the steady state the rate at which protons are pumped into the thylakoid lumen by electron flow must equal the rate at which protons exit from the lumen, either through a passive leak or through an ATPase in a fashion coupled stoichiometrically to ATP synthesis. Exit through the leak may be thought of as being catalyzed by an uncoupler, either physiological or added. Given the rate of electron flow and the dependence of the rates of the leak and phosphorylation upon $\Delta\mu_{H^+}$, it is possible to calculate μ_{H^+} and the rates of proton efflux through the leak and through the ATPase. Electron transport is itself influenced by $\Delta\mu_{H^+}$, so a reasonable model must also formulate the rate of electron flow as a function of $\Delta\mu_{H^+}$, complicating the calculation somewhat. In the steady state in thylakoids $\Delta\mu_{H^+}$ is almost exclusively a proton concentration gradient, so $\Delta\mu_{H^+}$ can be equated with ΔpH. Since external pH is a constant in these experiments, $[H^+]_{in}$ is used as a more convenient variable than either ΔpH or $\Delta\mu_{H^+}$.

Portis and McCarty [18] found that between ΔpH values of 2 and 3 and at saturating light intensities the rate of electron transport from water to ferricyanide is inversely proportional to $[H^+]_{in}$. This cannot be strictly accurate as it predicts no upper limit to the rate of electron transport. Electron-transport inhibition can be mimicked by including a term giving the fraction of active electron transport chains, E:

$$R_e = E \cdot 3330/[H^+]_{in} \quad (1)$$

where R_e is the rate of electron flow expressed as μeq per h/mg chlorophyll, $[H^+]_{in}$ is expressed in units of μM and 3330 is an arbitrary constant chosen to fit the data presented by Portis and McCarty [18]. E varies between 1.0 (no inhibition) and 0 (all electron transport complexes inhibited). This equation has not been well tested.

At least for ΔpH values between 2 and 3 the rate of phosphorylation is nearly third order with respect to $[H^+]_{in}$ [13]. At very high ΔpH values saturation must occur, and at low ΔpH values the dependence may change. Energy-transfer inhibition can be modeled by including a term for the fraction of active ATPases, A:

$$R_p = A \cdot 6.7 \cdot [H^+]_{in}^3 \quad (2)$$

where R_p is the rate of phosphorylation, 6.7 is an arbitrary and convenient, though not unrealistic value for the proportionality constant, and where all units are as in Eqn. 1. A varies from 1.0 (no inhibition) to 0 (all ATPases inhibited). The exponent is rarely measured to be exactly 3, but for the purposes of this model 3 is a good fit and makes the final equation readily soluble.

Portis et al. [19] found that the rate of passive proton efflux is directly proportional to $[H^+]_{in}$ as long as ΔpH does not exceed three units. At high ΔpH values the linear relationship breaks down as a non-linear leak through the ATPase is intro-

duced [19]. The effect of uncouplers can be modeled by including the variable U in the constant of proportionality:

$$R_1 = (20+U)\cdot[H^+]_{in} \quad (3)$$

where R_1 is the rate of passive proton efflux, 20 is an arbitrary, if representative, value of the proportionality constant in the absence of added uncouplers, and where the units are as in Eqns. 1 and 2. U varies from 0 (no added uncoupler) to arbitrarily large values as more uncoupler is added.

Eqn. 4 is the steady-state requirement, written on the assumption that H^+/ATP is three and that H^+/e^- is two [20]:

$$2R_e = R_1 + 3R_p \quad (4)$$

Eqns. 1–3 can be substituted into Eqn. 4 tc give Eqn. 5:

$$6660E/[H^+]_{in} = (20+U)\cdot[H^+]_{in} + 20A\cdot[H^+]_{in}^3 \quad (5)$$

which can be rearranged to give:

$$E\cdot 6660 = (20+U)\cdot[H^+]_{in}^2 + A\cdot 20\cdot[H^+]_{in}^4 \quad (6)$$

Eqn. 6 can be solved for $[H^+]_{in}$ in terms of the various constants and parameters describing inhibition by use of the quadratic equation:

$$[H^+]_{in}^2 = \frac{-(20+U)\pm\sqrt{(20+U)^2+(E\cdot A\cdot 532800)}}{2A\cdot 20} \quad (7)$$

Only one root of the equation is a positive real number. Once $[H^+]_{in}$ is known, the rates of phosphorylation and electron flow can be calculated from Eqns. 1 and 2. Inhibitor titrations can be simulated by systematically varying E, A or U. Fig. 2A shows a simulated titration of phosphorylation with an electron transport inhibitor in both the absence ($A = 1.0$; at $E = 1.0$, $R_p = 492$ μmol/h per mg chlorophyll) and presence ($A = 0.1$; at $E = 1.0$; $R_p = 253$ μmol/h per mg chlorophyll) of energy-transfer inhibition. E was varied from 1.0 to 0.05. The data are plotted as a function of $-\log E$, which serves as a rough analog of the concentration of the electron-transport inhibitor. Fig. 2B shows a simulation of titration of phosphorylation with an energy-transfer inhibitor both

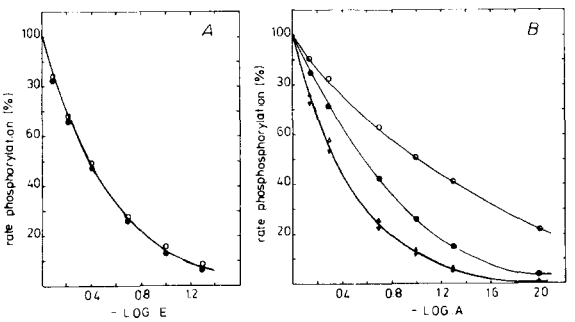

Fig. 2. Double inhibitor titrations simulated with a numerical model of delocalized coupling. (A) The effect of DCCD upon inhibition of phosphorylation by DCMU. Eqn. 7 was used with $U = 0$. In controls (○) $A = 1.0$, while in DCCD inhibited (●) $A = 0.1$. (B) The effect of uncoupler upon DCCD inhibition of phosphorylation. Eqn. 7 was used with $E = 1.0$. U was set at 0 (○), 180 (●), 580 (△), and 1180 (▼). Further increases in U did not result in further changes in the titration curve.

in the absence and in the presence of several concentrations of uncoupler (the rate of phosphorylation and P/e_2 were 492 and 1.24 and $U = 0$; 338 and 0.76 at $U = 180$; 165 and 0.29 at $U = 580$; and 75 and 0.10 at $U = 1180$). A was varied from 1.0 to 0.01. The data are plotted as a function of $-\log A$, which is a rough analog of the concentration of the energy-transfer inhibitor. The results of the simulations are not especially sensitive to the values chosen for the constants in Eqns. 1–4. The results are consistent with the qualitative arguments made in the previous sections. The figures are formulated for chloroplasts, so dichlorophenyldimethyl urea (DCMU) has been used instead of antimycin A. To emphasize small changes in the sensitivity of phosphorylation to inhibitors, the data are plotted as percent activity relative to the appropriate rate measured in the absence of DCMU.

Discussion

The equations used to model delocalized coupling in this paper are only approximations. It is possible to 'disprove' the equations by choosing inappropriate conditions. A more serious effort to quantitatively model delocalized coupling in thylakoid membranes has been made by Mills and Mitchell [21]. A very similar description of energy coupling in chromatophores is emerging [8,16], but

the details will be quantitatively different. However, the point is not that the equations give precise mathematical descriptions of delocalized uncoupling in either chloroplast thylakoids or *R. capsulata* chromatophores. Neither the accuracy nor the origin of the model are crucial. The critical point is that it is a reasonable model of delocalized coupling with which to simulate inhibitor titrations and that it can account for the results of published double inhibitor titrations which were thought to be incompatible with such models.

It has been assumed that $\Delta\mu_{H^+}$ is not saturating with respect to phosphorylation in these experiments. There is no particular reason to believe that it is saturating [8,15,16]. In the experiments discussed in this paper phosphorylation is titrated with a reagent which decreases $\Delta\mu_{H^+}$ so that if $\Delta\mu_{H^+}$ were initially saturating, it would not be saturating during the titration. Since DCCD increases $\Delta\mu_{H^+}$, it should take more SF 6847 or antimycin A to bring $\Delta\mu_{H^+}$ down to a point at which it is no longer saturating, i.e., at which inhibition becomes apparent. Thus a lag in the onset of inhibition by antimycin A or SF 6847 would be expected in the DCCD-poisoned chromatophores if coupling were delocalized and if $\Delta\mu_{H^+}$ were saturating in the controls. This was not seen, so if coupling is delocalized, the gradients are not saturating.

The inhibitor titrations were simulated with a steady state model, but the principles behind the behavior of the model can be formulated in terms of coupling or P/e_2 ratios, and may therefore be more widely applicable. The P/e_2 ratio may be thought of as a measure of the partitioning of proton efflux between the leak and ATPase pathways; if most protons exit through the ATPases, the P/e_2 will be high [9]. The presentation of the uncoupler titration is already cast in this vein. At high P/e_2 ratios most protons exit through the ATPase so blocking the ATPases with DCCD will substantially hinder proton efflux and thus will increase $\Delta\mu_{H^+}$. Disproportionality between inhibitor bound and inhibition will result, since the remaining ATPases will turn over more quickly. If uncoupler is present, the P/e_2 is low and most protons exit through the leak. In this case, blocking the ATPase with DCCD will not hinder proton efflux appreciably and will therefore not result in an increase in $\Delta\mu_{H^+}$. As a result, the masking of inhibition will not occur. The observation of synergism will depend upon the control being well coupled.

The considerations governing the antimycin A titrations are a little different. Inhibition by both antimycin A and DCCD are masked by feedback with $\Delta\mu_{H^+}$. The sensitivity of photophosphorylation to either inhibitor will depend upon the P/e_2, as described above. The lack of strong interactions between the two inhibitors depends upon the fact that the P/e_2 is much less sensitive to these inhibitors than is phosphorylation. This behavior is opposite to that of uncouplers. Because the P/e_2 is relatively high and constant, for every electron that does not flow a corresponding amount of ATP is not made, and vice versa. Since phosphorylation and electron flow are not directly coupled, each maintains its appropriate sensitivity to inhibitors. Since the P/e_2 is constant, the sensitivity of electron transport to antimycin A will be accurately transferred to phosphorylation, regardless of energy-transfer inhibition. The constant P/e_2 allows a delocalized model of energy coupling to mimic certain aspects of a localized model. At high concentrations of one inhibitor the sensitivity of phosphorylation to the other probably changes, but these effects might well be obscured by secondary effects of the inhibitors.

Venturoli and Melandri [8] drove electron flow in chromatophores with flashing light and titrated phosphorylation with DCCD. The inhibition curves were very similar at widely differing flash frequencies (i.e., at widely differing electron flow rates), seemingly at variance with the predictions of delocalized models of coupling [8]. However, there is relatively little change in the P/e_2 over the range of flash frequencies used [22]. This result is itself somewhat surprising, but if it is taken as a given, it means that the results of the DCCD titrations are not inconsistent with delocalized coupling.

The interactions between electron flow, phosphorylation, and $\Delta\mu_{H^+}$ are probably more complex than Eqns. 1–7 would indicate. More importantly, the model describes the effects of inhibitors in an extremely simple-minded fashion. More complicated patterns of inhibition are easy to imagine. For example, inhibition by energy trans-

fer (A) or electron transport (E) inhibitors may change with the size of the proton activity gradient, as might the proton conductivity of uncouplers (U). Complex patterns of inhibition and double inhibitor interaction are likely to be found in the real world. The interpretation of the results of such experiments should be made carefully.

Only two double inhibitor titrations have been discussed. This was done to raise two specific points about delocalized coupling. These were that no single factor limits phosphorylation, and that uncouplers can eliminate feedback effects that mask inhibition. Clark et al. [16] and Parsonage and Ferguson [15] have raised the first objection independently. These principles can be applied profitably to other titrations performed by Hitchens and Kell [10–12], and as mentioned above, to that performed by Venturoli and Melandri [8]. It should be borne in mind that inhibitor titrations tell us about the nature of coupling, and not about the nature of the gradients. A delocalized high-energy intermediate need not operate between bulk phases, and not everyone believes bulk phases to be uniform over vesicles [23].

Both direct and delocalized descriptions of coupling predict the phenomena found by Hitchens and Kell. The effects seen in their experiments

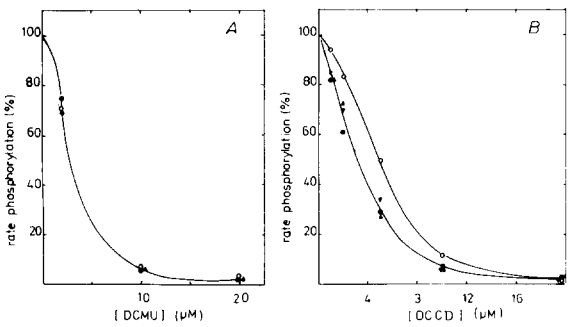

Fig. 3. Measured response of thylakoid membranes to double inhibitor titrations. (A) The effect of DCCD upon the inhibition of phosphorylation by DCMU. The rates of phosphorylation are given as percentages in the absence of DCMU, which were 63, 36 and 17 μmol/h per mg chlorophyll for the controls (○), 5 μM DCCD (●) and 10 μM DCCD (▲), respectively. (B) The effect of uncouplers upon the inhibition of phosphorylation by DCCD. Rates of phosphorylation in the absence of DCCD were 120, 77, 37 and 24 μmol/h per mg chlorophyll for control (○), 2 mM NH_4Cl (▼), 0.1 μg/ml nigericin (●) and 1 μM FCCP (▲), respectively.

may be more dramatic than those predicted by delocalized coupling. However, recent experiments by Cotton and Jackson [24] indicate that time dependent changes in the rate of phosphorylation in the presence of energy-transfer inhibitors exaggerate, if they do not create, some of the effects measured by Hitchens and Kell. Experiments with chloroplast thylakoids which should not be complicated by time-dependent changes in inhibition qualitatively confirm the results of the two types of titration examined in this paper, but are less dramatic (Fig. 3).

The imaginative mechanism of uncoupling advocated by Hitchens and Kell generates a response to uncouplers similar to that provided by a delocalized mechanism [12]. It may be liable to direct testing. Another difference between the two treatments is that the delocalized model predicts that in coupled vesicles the percent inhibition of phosphorylation by an energy-transfer inhibitor should be less than the percent of ATPase complexes inhibited. Unfortunately, inhibition of the hydrolytic activity of the ATPase complex by an energy-transfer inhibitor cannot be used to measure the fraction of reacted ATPases, since this activity is also likely to be masked by a feedback loop between $\Delta\mu_{H^+}$ and the rate of turnover. Addition of uncoupler may or may not remove the loop, but would certainly introduce inactivation phenomena. Thus, the similar titres of phosphorylation and ATPase activity for DCCD [8], while reassuring to advocates of direct coupling, does not provide a test of this point.

The inclusion of measurements of $\Delta\mu_{H^+}$ may allow the double-inhibitor titration technique to discriminate bteween direct and delocalized coupling. However, as mentioned in the Introduction, the interpretation of such measurements is controversial. It was the independence from such measurements that made the technique attractive. It is the conclusion of this paper that this independence is illusory.

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