# On the logic of the application of double-inhibitor titrations for the elucidation of the mechanisms of energy coupling

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Received 10 August 1984

In the last couple of years the chemiosmotic hypothesis has been severely criticised, such that many research groups now consider it to be a less than exact description of biological energy transduction. The most potent experimental support for this view is based upon the technique known as the double-inhibitor titration [e.g., (1982) Biochem. J. 206, 351–357]. The results of such experiments have been considered by many to exclude 'unequivocally' the chemiosmotic coupling model. It will be shown that such unequivocal statements are not possible. An argument is put forward which shows how the chemiosmotic model may explain these titrations without any further elaborations of the original hypothesis.

*Chemiosmotic hypothesis* *Double-inhibitor titration* *Proton conductivity* *Protonmotive force* *Energy coupling* *Oxidative phosphorylation*

## 1. INTRODUCTION

Much of the free energy made available by the redox reactions of biological electron transport chains is conserved as an electrochemical protonic potential difference ($\Delta p$) across a membrane [1]. The $\Delta p$ generated in this way is then utilised to promote the reversible synthesis of ATP. This description, originally introduced in [1], is known as the chemiosmotic hypothesis. It was envisaged that the $\Delta p$ was delocalised over the entire organelle or bacterium and thus could be 'tapped' wherever there was a sink [2]. The $\Delta p$ is, therefore, a macroscopic quantity and should obey a 'pool' type of behaviour.

During the period of the development of the more physiological aspects of the chemiosmotic hypothesis (1961–1966), another hypothesis was proposed [3] which advocated a more localised coupling of the 'energised' protons from the electron transport chains to the ATPase sinks. In fact, the author in [3] also contributed quite considerably to the development of the chemiosmotic hypothesis. However, a problem with the views of this author was that they were difficult to test.

More recently these proposals have been elaborated (see [5] for a review of the proposals), such that a coherent alternative to the classical chemiosmotic hypothesis now appears to exist, although alternative coupling hypotheses, by their nature, tend to be stated sufficiently vaguely as to be unfalsifiable and require for their verification the experimental falsification of the chemiosmotic hypothesis.

With these comments in mind and following the original approach of [6], authors in [7] and [8] have attempted to 'stringently' test the chemiosmotic hypothesis and further support their own interpretations of the mechanisms of energy coupling by the application of the so-called 'double-inhibitor titration' technique. The results of such experiments have been interpreted in [8] to be the most potent evidence against the chemiosmotic coupling mechanism of [1].

It would be useful, therefore, to reconsider the

*Abbreviations:* DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SF 6847, 3,5-di(*t*-butyl)-4-hydroxybenzylidene malononitrile

*Published by Elsevier Science Publishers B.V.*
00145793/84/$3.00 

logic of these double-inhibitor titrations before unequivocally accepting that localised energy coupling (in the manner advocated in [5]) is a feature of biological energy transduction.

## 2. WHAT ARE DOUBLE-INHIBITOR TITRATIONS?

The first such titrations were carried out in [6] and have been enthusiastically revisited by authors in [7–9]. The reader is referred to the cited references (and those therein) for a more complete rendition of the applied 'logics' and the interpretations. To aid our discussion, fig.1 has been included to indicate schematically the form of the experimental protocol applied and the results obtained with the double-inhibitor titration technique.

Let us consider a 'thought-experiment' such that we titrate a suspension of coupled mitochondria or bacteria under conditions of rapid (maximum)

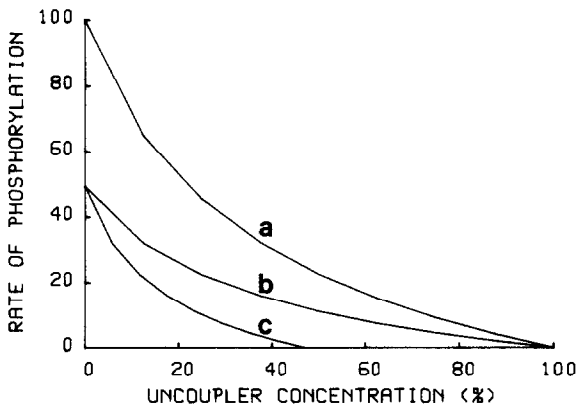


Fig.1. A schematic representation of a typical double-inhibitor titration. Curve (a) is a representation on a percent basis of the effect of an uncoupler titration on the rate of phosphorylation of a suspension of phosphorylating particles (e.g., mitochondria or bacteria). Curves (b) and (c) represent similar titrations after treatment of the particles with an $H^+$-ATPase inhibitor, e.g., venturicidin, oligomycin or DCCD, such that about half of the active $H^+$-ATPases are inhibited. Curve (c) is a representation of the actual experimental result. The form of these curves is taken from the experimental results of [8] (see fig.2 of [8]). Curve (b) indicates the behaviour expected by them (see their fig.1b) of such a treatment based on their interpretation of the chemiosmotic hypothesis.

phosphorylation with an uncoupler of oxidative phosphorylation (e.g., FCCP or SF 6847) and measure the effect upon the rate of phosphorylation ($J_p$). Curve (a) of fig.1 indicates the relationship between $J_p$ and the titre of the uncoupler which completely inhibits the phosphorylation of ADP. There is no question (i.e., all the proposed coupling mechanisms predict this behaviour) that this phenomenon is due to an increase of the rate of dissipation of the 'energised' protons which provide the driving force and the substrate for the phosphorylation events. The various coupling mechanisms, however, assign different mechanistic actions to the uncouplers.

The form of the illustrated curve (a) in fig.1 has been taken from the work of others (see fig.2 of [9]).

Now let us consider a similar experiment but this time with half of the active $H^+$-ATPases inhibited by treatment with a second inhibitor (e.g., DCCD or oligomycin) before commencing the titration with the uncoupler. Thus two inhibitors of oxidative phosphorylation are used together; hence the name of the technique.

Two possible results of this second titration are shown by curves (b) and (c) in fig.1, only one of which was experimentally found in [9] and is shown schematically by curve (c).

Another type of double-inhibitor titration has been applied to studies of oxidative phosphorylation [8]. The second approach is exactly analogous to the above-described protocol, however, instead of titrating with an uncoupler of oxidative phosphorylation, titrations were performed with an inhibitor of electron transport in the presence and absence of the $H^+$-ATPase inhibitor. Similar logics and interpretations were invoked [8].

## 3. THE APPLIED LOGIC OF THE DOUBLE-INHIBITOR TITRATION TECHNIQUE

To briefly summarise the foregoing, the first type of double-inhibitor titration, shown in fig.1, considers the relationship between the rate of dissipation of the $\Delta p$ and the $J_p$. In the second type (not shown) the rate of generation of the $\Delta p$ and the $J_p$ is studied.

The laboratories of Melandri and Kell have interpreted their observations with double-inhibitor

titrations to strongly support their contention of a localised coupling mechanism. We shall only consider the experiments described in fig.1 in detail but the same logics have been applied in the other type of experiments.

If we consider an experiment performed as in fig.1 curve (a): a relationship is obtained between $J_p$ and the added uncoupler concentration. On repeating the titration in the presence of the required concentration of an inhibitor which inactivates half the $H^+$-ATPases, a different uncoupler titre is obtained, i.e., curve (c). However, it has been suggested [9] that the chemiosmotically expected result is shown by the hypothetical curve (b) (see fig.1b of [9]). This line of reasoning is taken because these authors consider:

> "a delocalised coupling model cannot possibly expect that a diminuation of the output flux of the coupling system occasioned by decreasing the number of active $H^+$-ATPases can in some way decrease the magnitude of the proposed input force. Therefore, it is clear that for the same ionophorous uncoupler, the titre of the uncoupler for full uncoupling should be unchanged and certainly not decreased... Thus, the prediction of the delocalised chemiosmotic coupling model is that, for titrations with ionophorous uncouplers, the presence of a partially inhibitory titre of the $H^+$-ATPase inhibitor should not decrease the concentration required to effect full uncoupling".

These authors [9] thus propose that a titration curve similar to curve (b) of fig.1 should result for the double-inhibitor titration if the chemiosmotic (delocalised) model is the more exact description of energy coupling. However, the experimentally observed result is schematically illustrated by curve (c) which is apparently the predicted observation of the energy coupling model in [9].

## 4. DISCUSSION

Authors in [9] have suggested that their (and others, see [7]) observations obtained with the double-inhibitor titration technique indicate 'unequivocally' that the chemiosmotic mechanism cannot be correct. Such a conclusion, if correct, would mean a major revision of much of our understanding of bioenergetic systems. It must be worthwhile, therefore, to reconsider for the moment such titrations in terms of the chemiosmotic hypothesis:

It was originally implied [2] and recently reiterated [10] that for the efficient operation of oxidative phosphorylation, the phosphorylating $H^+$-ATPase must be the most able dissipator of the $\Delta p$ with respect to other dissipative processes. This proposal ostensibly implies that the $H^+$-ATPase allows the highest rate of proton conductance through the membrane, when phosphorylation is taking place. To further clarify this point, let us consider the factors which generate and maintain the $\Delta p$ together with those factors which dissipate it. Eq.1 indicates that a steady state $\Delta p$ the rate of generation of $\Delta p$ ($J_{gen}$) is given by the rate of electron transport ($J_{ox}$) multiplied by the number of protons ($n$) translocated or abstracted from the intra-mitochondrial space per oxygen atom consumed. The rate of dissipation of the $\Delta p$ is given by any device which catalyses proton leak through the membrane ($L$) and therefore is a function of the $\Delta p$ itself, i.e.,

$$J_{gen} = J_{dis}$$

where:

$$J_{gen} = J_{ox} \cdot n$$

and

$$J_{dis} = L \cdot \Delta p$$

therefore

$$J_{ox} \cdot n = L \cdot \Delta p \qquad (1)$$

Now let us consider which parameters might constitute the proton leakage term. Firstly there is the natural leak ($L_n$) of the phospholipid membrane, this factor must be fairly insignificant, however, as otherwise oxidative phosphorylation would be a very inefficient process. The natural leak, however, must be added to any other devices or processes which may catalyse the leakage of protons through the membrane. In the case of the above-described experiments, the latter devices are the reversible ATPases under phosphorylating conditions ($L_p$) and the proton ionophore, which catalyses proton movements across the membrane ($L_u$). The $L$ term, therefore, is a composite parameter described by any facility which allows a dissipation of the $\Delta p$. Under the usual circumstances of oxidative phosphorylation $L_p \gg L_n$

and $L_u$ may be considered to be absent.

With these latter comments in mind let us now consider again the predictions of a double-inhibitor titration of the type illustrated by fig.1. Curve (a) indicates that $J_p$ is decreasing as the uncoupler concentration is increasing. This means that the rate of proton conductance due to the uncoupler is becoming significant with respect to that catalysed by the $H^+$-ATPase. Or in other words, the $L_u$ term is becoming significant and is competing with the $L_p$ term. The consequence is, therefore, that $J_p$ decreases. The $J_p$ term may thus be considered to be equivalent to the $L_p$ term.

To extend our discussion let us consider the effect of inhibiting about half of the active $H^+$-ATPases as in fig.1 and in [9]. Under these circumstances the $L_p$ term is also decreased by about half. The high proton conductance associated with phosphorylation is thus considerably decreased. One would expect, therefore, that the $L_u$ term would have to increase by a much smaller amount to compete with the much depressed $L_p$ term. The consequence of this logical argument in terms of the double-inhibitor titration would be that the uncoupler titration of the half-inhibited $H^+$-ATPase system would exhibit a lower uncoupler titre than that of the uninhibited experiments. Indeed, we can be even more specific and anticipate that the percentage decrease in the uncoupler titre would be equivalent to the percentage inhibition of the active $H^+$-ATPases. In fact, this latter expectation is confirmed by the experiments in [9] (see their fig.2), and is shown schematically in the above fig.1, curve c.

It is abundantly clear from the above considerations that the application of the technique known as double-inhibitor titration is by no means unequivocal. In many ways it provides further support for a chemiosmotic interpretation of oxidative phosphorylation. Moreover, the technique may prove particularly useful for investigations of the chemiosmotic relationships between $\Delta p$ and $J_p$.

To conclude, we would like to stress that in principle we have no objection to the existence of the concept of a local coupling mechanism. In many ways we concur with earlier work [11] that a kinetic localisation exists within a chemiosmotic system. It is to the equivocal evidence erroneously cited to unequivocally support non-chemiosmotic mechanisms that we feel we must draw attention.

## ACKNOWLEDGEMENTS

We would like to thank Professor R.J. Williams, Dr P.D. Mitchell and Dr D.B. Kell for useful discussion during which their views have been further clarified. We would also like to thank Professor A. Azzi for continual support during the preparation of this work. The financial support of The Emil Barrel Stiftung and the Swiss National Science Foundation (grant no.3.501.083) is gratefully acknowledged.

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