

BBA 45915

WEAK-ACID UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION.

MECHANISM OF ACTION ON THIN LIPID MEMBRANES

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(Received December 4th, 1969)

SUMMARY

Weak-acid uncouplers of oxidative phosphorylation such as *p*-trifluoromethoxycarbonylcyanidephenylhydrazine, tetrachloro-2-trifluoromethylbenzimidazole, *m*-chlorocarbonylcyanidephenylhydrazine, and 2,4-dinitrophenol can increase the conductance of thin lipid membranes by several orders of magnitude. In this high conductance state these membranes appear to be ideally selectively permeable to H^+ or OH^- . We suggest, however, that the primary charge carrier in the membrane is neither H^+ or OH^- , but rather a dimer formed between the undissociated and dissociated form of the weak acid, and we show that all of the data on the action of these weak acids on thin lipid membranes are consistent with this picture.

INTRODUCTION

The effect on thin ($<100 \text{ \AA}$) lipid membranes of the weak-acid uncouplers of oxidative phosphorylation has recently been discussed in several papers¹⁻⁴. Examples of these acids are *p*-trifluoromethoxycarbonylcyanidephenylhydrazine (FCCP), tetrachloro-2-trifluoromethylbenzimidazole (TTFB), *m*-chlorocarbonylcyanidephenylhydrazine (CCCP), and 2,4-dinitrophenol (see Fig. 1). All of these agents have been reported to have the following three characteristic effects on the thin lipid membranes: (1) They increase the membrane conductance; (2) at a given uncoupler concentration, the conductance is maximal at a pH equal to the pK of the uncoupler; and (3) in the presence of uncoupler, a difference of pH across the membrane produces a potential difference, V , given by:

$$V = \frac{RT}{F} \ln \frac{[H^+]_1}{[H^+]_2}$$

where $[H^+]_1$ and $[H^+]_2$ are the H^+ activities on the two sides of the membrane, and R , T and F have their usual meaning.

These results have generally been interpreted to mean that the membranes are selectively permeable to H^+ (or OH^-) in the presence of the uncouplers, the uncouplers

Abbreviations: FCCP, *p*-trifluoromethoxycarbonylcyanidephenylhydrazine; TTFB, tetrachloro-2-trifluoromethylbenzimidazole; CCCP, *m*-chlorocarbonylcyanidephenylhydrazine.

somehow facilitating the transport of H^+ (or OH^-) (refs. 2-4). It is not clear, however, how these agents make the membrane more permeable to H^+ (or OH^-), nor why the conductance maximum occurs at a pH equal to the pK of the uncoupler. In particular, it does not appear reasonable from energetic considerations (see below)

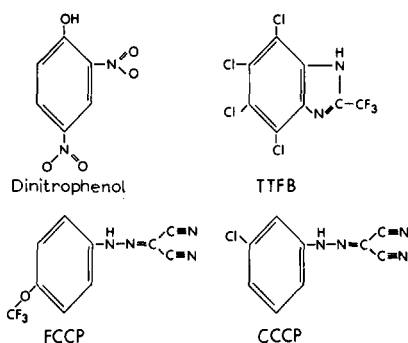


Fig. 1. The structures of some weak-acid uncouplers of oxidative phosphorylation.

that H^+ or OH^- can enter the hydrocarbon interior of these membranes to act as current carriers. We wish, in this paper, to offer an alternative explanation for the action of these uncouplers, and to suggest that the current carrier in the membrane is not H^+ or OH^+ , but rather a dimer of the ionized and unionized acid; that is, the membrane is not permselective to H^+ (or OH^-) but rather to a dimer of the uncoupler itself. We shall see that this model is consistent with all of the reported data and is physically reasonable in light of the presumed structure of the thin lipid films.

Before discussing the model, let us consider briefly the nature of the thin lipid membranes. These membranes are generally considered to be bilayer structures with the polar ends of the lipids in the aqueous phase and the hydrocarbon tails forming a hydrophobic interior. It appears that the major barrier to ion permeation is the hydrocarbon interior; that is, the large electrical resistance of these membranes in ordinary salt solutions is due to the very small partition coefficient of such ions as Na^+ , K^+ , Cl^- , *etc.* between water and the hydrocarbon interior⁵. This is a consequence of the enormous energy required to transfer a charge of approx. 2 \AA in radius from an aqueous phase of high dielectric constant (approx. 80) into a hydrocarbon region of low dielectric constant (approx. 2)^{5,6}. However, ions whose charge is either delocalized over a large area or buried in a large dielectric cage can have sufficient solubility in the hydrocarbon interior to significantly increase the membrane conductance^{5,6}. In this regard, it is significant that the charge on the ionized form of the uncouplers being considered is virtually completely delocalized, and that the order of effectiveness of these acids in increasing membrane conductance parallels their size^{2,4}; *e.g.* FCCP is effective at much lower concentrations than 2,4-dinitrophenol. With this brief introduction, let us now turn to the model.

Consider a thin lipid membrane separating two aqueous salt solutions (called Solutions 1 and 2) containing weak-acid uncoupler. Let AH represent the weak acid uncoupler and A^- the ionized form. Then, in addition to the equilibrium:



we assume that there also exists in solution the equilibrium



but the concentration of HA_2^- is small compared to HA and A^- , so that the total concentration of uncoupler, $[\text{A}_\text{T}]$, is effectively approximated by:

$$[\text{A}_\text{T}] = [\text{HA}] + [\text{A}^-] \quad (3)$$

(We shall attempt at the end of the derivations to justify physically the existence of HA_2^- .) We assume, (a) that the only ion significantly soluble in the membrane is HA_2^- (because of its large size), and that its concentration just within the membrane is directly proportional to its concentration in the aqueous phase at the interface. Thus, HA_2^- is the current carrier in the membrane and the only permeable ion; the membrane conductance, G , is directly proportional to the concentration within the membrane of this species, which in turn is proportional to its concentration in solution. That is,

$$G = k[\text{HA}_2^-] \quad (4)$$

where k is a constant proportional to the product of the mobility of HA_2^- in the membrane and the partition coefficient of HA_2^- between water and the membrane. (Eqn. 4 is a fair approximation for the small signal conductance of a membrane separating symmetrical solutions. We shall not attempt a more exact treatment involving the space-charge distribution existing in the membrane and at the aqueous interfaces; nor shall we take up the question of the non-linearities and time-variant behavior that can arise with large stimuli.) We further assume, (b) that the membrane is so permeable to the neutral species HA (because of its high solubility in hydrocarbon), that even if there is a difference in concentration of HA in Solutions 1 and 2 which the membrane separates, the concentrations of HA at the two membrane-solution interfaces are equal. (We shall justify this assumption at the end of the derivation.) From these assumptions, we can now derive all of the relevant properties of the system.

Consider first the situation in which the membrane separates identical solutions. Then combining Eqns. 1, 2, and 3 we have:

$$[\text{HA}_2^-] = \frac{K_1}{K_2} [\text{A}_\text{T}]^2 \frac{[\text{H}^+]}{(K_1 + [\text{H}^+])^2} \quad (5)$$

This is the expression for the concentration of HA_2^- in aqueous solution as a function of $[\text{H}^+]$. Substituting this into Eqn. 4 we have:

$$G = k \frac{K_1}{K_2} [\text{A}_\text{T}]^2 \frac{[\text{H}^+]}{(K_1 + [\text{H}^+])^2} \quad (6)$$

Eqn. 6 is the expression for conductance as a function of $[\text{H}^+]$ and is shown in Fig. 2 along with the experimental results reported for TTFB. Note that the conductance is a maximum when $[\text{H}^+] = K_1$; that is, the conductance is a maximum at a pH equal

to the pK of the acid, as is indeed experimentally observed with all the weak-acid uncouplers⁴. (HOPFER *et al.*³ report a conductance maximum for FCCP at pH 7.1, whereas LIBERMAN AND TOPALY⁴ report a maximum at pH 5.7, the pK of the acid.

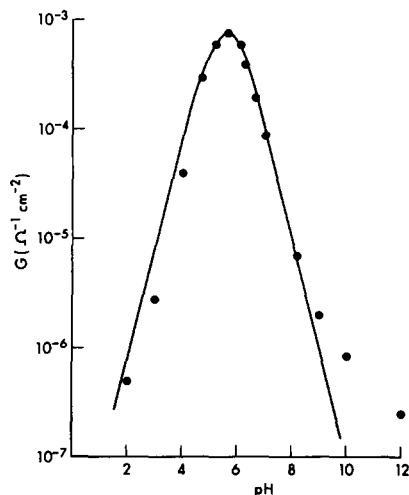


Fig. 2. Plot of conductance *vs.* pH for TTFB as given by Eqn. 6. $K_1 = 2.5 \cdot 10^{-6}$ (the dissociation constant for TTFB⁸); $[A_T] = 30 \mu\text{M}$; h/K_2 is arbitrarily set at $2.8 \cdot 10^8$ to fit the maximum in the experimental data; ●, experimental results in $30 \mu\text{M}$ TTFB⁴. The deviation from Eqn. 6 at $\text{pH} > 9$ can be attributed to the contribution to the conductance of TTFB⁻ itself⁴.

We are unable to account for this discrepancy.) Eqn. 6 also predicts that at a given pH the conductance should increase as the square of the total uncoupler concentration. In actual fact the dependence of conductance on uncoupler concentration varies between a first and second power relation, depending on the particular acid, the concentration range, and the pH (ref. 4). The deviation from a second power relation is probably due to the fact that A^- itself has finite solubility in the membrane (this would be especially true for the larger acids) and can therefore contribute to the conductance. This contribution of A^- to the conductance will be particularly manifest at low concentrations of uncoupler and high pH's. And indeed, LIBERMAN AND TOPALY⁴ have shown that at high pH, TTFB⁻ carries current and the conductance varies linearly with TTFB concentration, whereas at lower pH's the conductance varies as the square of TTFB concentration (see their Fig. 11).

Let us now consider the membrane potential (V) that develops when there is a difference of pH in the two solutions separated by the membrane. (For simplicity let us take the case when both solutions have high ionic strength and are well buffered, so that the pH at each interface is essentially the same as that in the bulk solution.) Since the membrane is permselective for HA_2^- , we have:

$$V = -\frac{RT}{F} \ln \frac{[\text{HA}_2^-]_{1i}}{[\text{HA}_2^-]_{2i}} \quad (7)$$

where the subscripts 1i and 2i refer to the concentrations in the aqueous phases at the interfaces of the membrane with Solutions 1 and 2, respectively. Combining Eqns. 1 and 2 and substituting into Eqn. 7 we have:

$$V = -\frac{RT}{F} \ln \frac{[\text{HA}]_{11}^2 [\text{H}^+]_{21}}{[\text{HA}]_{21}^2 [\text{H}^+]_{11}} \quad (8)$$

but from assumption (b): $[\text{HA}]_{11} = [\text{HA}]_{21}$ and because the solutions are well buffered: $[\text{H}^+]_{11} = [\text{H}^+]_1$ and $[\text{H}^+]_{21} = [\text{H}^+]_2$ so that Eqn. 8 becomes:

$$V = -\frac{RT}{F} \ln \frac{[\text{H}^+]_1}{[\text{H}^+]_2} \quad (9)$$

Thus, the membrane potential is the same as would be obtained with a membrane selectively permeable to H^+ . It should be remembered, however, that the model membrane is not permeable at all to H^+ but permeable only to HA_2^- . Eqn. 9 arises as an algebraic consequence of the equilibria between HA_2^- , HA , A^- , and H^+ in aqueous solution *plus* the fact that the HA concentrations at the two interfaces are equal.

This model adequately accounts for the results obtained with weak-acid uncouplers on thin lipid membranes and is consistent with the action of some other conductance-increasing agents. Thus, the high conductance of films in the presence of I^- and I_2 has been attributed to the formation of large polyiodides, whose charge is sufficiently delocalized to render them hydrocarbon soluble⁵. Similarly, the solubility of the valinomycin- K^+ complex in organic solvents results from the burying of the K^+ within the large complex⁷. It should be understood that although the size of HA_2^- is only approximately twice that of A^- , hence halving the electrostatic energy required to transfer the species from water into hydrocarbon and also increasing the "lipophilic" energy (resulting from short-range Van der Waal's forces) promoting transfer into hydrocarbon, this can result in an enormous increase of the water-hydrocarbon partition coefficient. This is a consequence of the fact that the energy terms appear in the exponent of the Boltzmann distribution, and hence a two-fold change in the energy terms will introduce a factor of two in the exponent⁵.

The assumption that a neutral lipophilic molecule such as HA will have essentially the same concentration at the two interfaces, even if there exists a difference in concentration in the two bulk phases, is simply a consequence of the fact that in any practical experimental arrangement, the resistance to diffusion offered by the unstirred aqueous layers is several orders of magnitude larger than the resistance offered by the film. The only quasi *ad hoc* assumption in the model is the existence of the dimer HA_2^- ; however, it is physically quite reasonable that molecules such as those shown in Fig. 1 should form such dimer, although their concentration in aqueous solution will probably be quite small. The combination of the strong Van der Waal's forces arising between such conjugated, planar molecules *plus* hydrogen bond forces between the undissociated and dissociated form should be sufficient to promote dimer formation. Note also that the charge will be delocalized over this entire dimer. Such an ion will be much more hydrocarbon-soluble than the simple A^- , for the reasons given above. Finally, it should be pointed out that if a membrane potential is involved in the scheme of oxidative phosphorylation (such as proposed in the chemiosmotic theory of MITCHELL⁹), the mechanism of action of these uncouplers on thin lipid membranes could be related to their mechanism of uncoupling oxidative phosphorylation. For these agents would create a parallel conductance pathway (with its own electromotive force) that would alter any pre-existing potential difference.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Grant NB 03356 and Grant No. 14-01-0001-1277 from the Office of Saline Water, U.S. Department of the Interior.

The author is a Career Development Awardee of the U.S. Public Health Service.

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