

*PROTONIC CONDUCTANCE ACROSS PHOSPHOLIPID
BILAYER MEMBRANES INDUCED BY UNCOUPLING
AGENTS FOR OXIDATIVE PHOSPHORYLATION*

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The chemiosmotic coupling hypothesis for oxidative phosphorylation proposed by Mitchell¹ postulates that electron transport along the respiratory chain in the mitochondrial membrane generates a transmembrane pH gradient, which becomes the immediate driving force for the enzymatic synthesis of adenosine 5'-triphosphate (ATP) from adenosine 5'-diphosphate (ADP) and phosphate through the reverse action of a vectorial ATPase system in the membrane. Mitchell has proposed that uncoupling agents such as 2,4-dinitrophenol, which uncouple phosphorylation of ADP from electron transport, are not inhibitors of specific enzymatic reactions *per se*, but act as lipid-soluble proton donor-acceptor systems that dissolve in the lipid phase of the membrane and conduct protons across it, thus preventing formation of pH gradients during electron transport. Experimental evidence for and against the Mitchell hypothesis has been discussed.²⁻⁴

The availability of phospholipid bilayer systems formed in apertures separating two aqueous phases⁵⁻⁷ has provided an opportunity to determine whether these simple model systems, which are devoid of proteins or enzymes, respond to uncoupling agents in the manner postulated by Mitchell. The thickness, high electrical resistance and capacitance, and high-water permeability of these membranes closely resemble those of natural membranes.⁸ In an earlier communication from this laboratory,⁹ it was shown that 2,4-dinitrophenol greatly increased the electrical conductance of such phospholipid bilayer membranes. The increase was sufficient to account for the uncoupling action of dinitrophenol in mitochondria, on the assumption that the chemi-osmotic coupling hypothesis is correct. However, the data did not allow a conclusion as to whether 2,4-dinitrophenol caused a specific protonic conductance or merely caused a generalized increase in permeability of the bilayer membrane to all cations (and anions) in the system studied.

In this communication data are presented showing that in addition to 2,4-dinitrophenol, the carbonylcyanide phenylhydrazone uncoupling agents also produce large increases in the specific conductance of phospholipid bilayer membranes, which are sufficient to account for their uncoupling activity. Each uncoupling agent was found to have a characteristic pH at which it is maximally effective. The increase in electrical conductance was found to be almost entirely the result of transport of protons across the membrane.

Experimental Methods.—The phospholipid bilayers were formed as described by Huang *et al.*¹¹⁻¹² The bulk lipid solutions employed consisted of 0.5–1.0% purified egg phosphatidyl choline and 0.25–0.50% cholesterol in *n*-decane. In some experiments 0.05–0.1% tocopheryl succinate was added. The aqueous phases consisted of 0.1 *M*

NaCl or KCl buffered with 1.0 mM Tris chloride, sodium or potassium phosphate, citrate, or glycylglycine buffer, depending on the pH range studied. The temperature was $25 \pm 1^\circ\text{C}$. The uncoupling agents were usually added to the aqueous compartments after formation of the membrane. The electrical circuits were those employed by Bielawski *et al.*⁹ The carbonylcyanide phenylhydrazones were gifts of Dr. P. G. Heytler, Central Research Department, Experimental Station, DuPont de Nemours and Co., Wilmington, Del.

Results.—The increase in specific conductance of the phospholipid bilayer membrane produced by uncoupling agents has been found to be highly dependent on the pH of the medium; the pH-conductance curves showed a maximum at a pH characteristic for each agent. Figure 1 shows the data for the agents

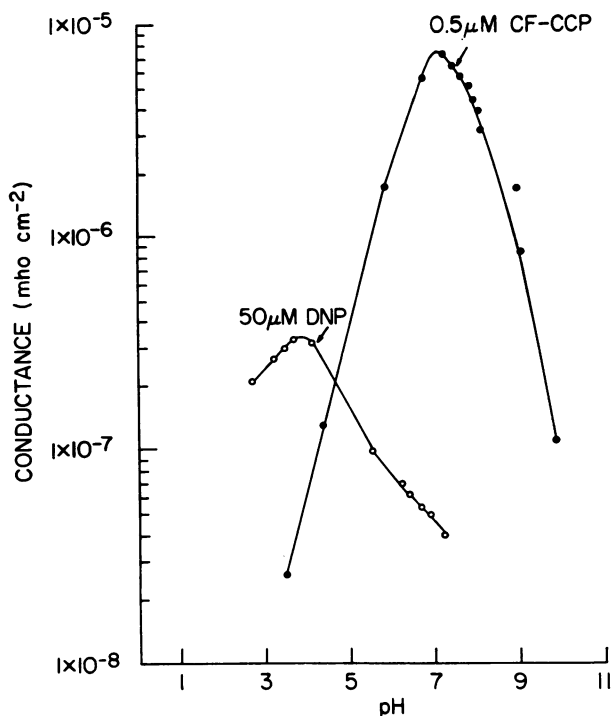


FIG. 1.—Effect of pH on the specific conductance of the phospholipid membrane in the presence of $0.5 \mu\text{M}$ CF-CCP or $50 \mu\text{M}$ DNP.

carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (CF-CCP) and 2,4-dinitrophenol (DNP). The maximum increase in specific conductance in the presence of CF-CCP occurred at about pH 7.1. At pH 4 and 10, respectively, the increase in conductance was only about 1/100 of that at pH 7.1. The optimum pH for carbonylcyanide *m*-chlorophenylhydrazone (Cl-CCP) is about 7.2, for 2,4-dinitrophenol, about 4.0, and for dicoumarol, about 5.5. The significance of the pH maxima is discussed below.

The uncoupling agents differ in their capacity to increase the specific conductance of the phospholipid bilayer (Table 1). CF-CCP is most active, Cl-CCP is slightly less active, and 2,4-dinitrophenol less potent. Dicoumarol produces qualitatively the same effect on the phospholipid membrane as the other uncouplers tested but it is less active. For reasons discussed earlier,⁹ it is not

TABLE 1. *Effect of uncoupling agents on specific electrical conductance of phospholipid bilayer membranes.*

Uncoupling agent	Concentration for half-maximal effect (μM)	Maximum specific conductance (mho cm^{-2})	pH of system
None	—	$1.7\text{--}6.7 \times 10^{-9}$	—
2,4-Dinitrophenol	340	1.4×10^{-6}	7.7
CF-CCP	0.9	5.6×10^{-5}	8.0
Cl-CCP	2.6	2.6×10^{-5}	7.8

expected that effects of the uncoupling agents on conductance of the phospholipid bilayer system should necessarily be proportional to their effects on mitochondria. In general, the different uncoupling agents increased the specific

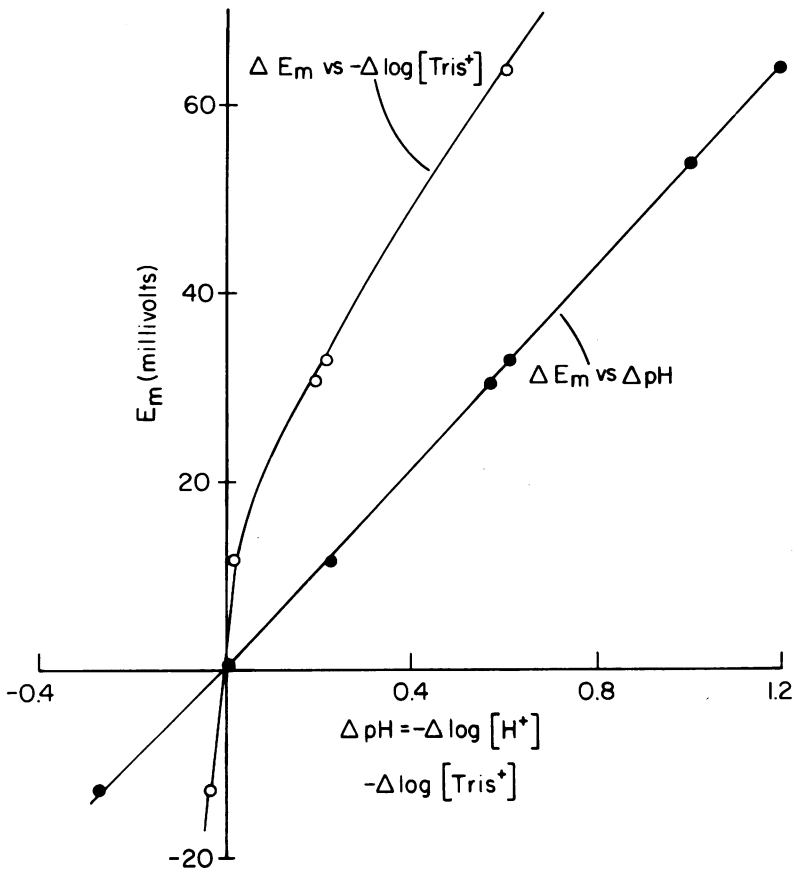


FIG. 2.—Effect of pH gradients across the phospholipid membrane on the transmembrane potential in the presence of $0.9 \mu\text{M}$ CF-CCP. The buffer was 1.0 mM Tris chloride; one compartment was held at $\text{pH} = 7.560$ and the pH of the other compartment varied by additions of KOH or HCl. The specific resistance of the membrane in the absence of a gradient (but in the presence of $0.9 \mu\text{M}$ CF-CCP) was $4 \times 10^6 \text{ ohm cm}^2$. The transmembrane potential is also plotted against $-\Delta \log [\text{Tris}^+]$, calculated from the observed pH values of the solutions on both sides of the membrane and the pK' for Tris = 8.14 with the Henderson-Hasselbach equation.

conductance from 10^3 - to 10^4 -fold when tested at concentrations giving maximal effects.

In order to determine whether H^+ ions are responsible for carrying the electrical current across the membrane in the presence of the uncoupling agents, membranes were formed between two aqueous compartments containing 0.1 *M* KCl and a small amount of buffer; the uncoupling agents were added to the aqueous compartments after the formation of the membrane. Each compartment contained a silver-silver chloride electrode connected to a potentiometer and a current source. No effect of the uncoupler on the silver-silver chloride electrodes could be observed. After formation of the membrane, the pH of one compartment was raised or lowered by addition of small volumes of HCl or KOH; the pH changes were monitored with a glass electrode and a recording system. Prior to the addition of acid or base, the ammeter was set to zero current. After bringing about the pH change, a current developed between the electrodes which was then opposed by the potentiometer so that the current flow was again zero. The voltage required to oppose the current flow was measured and taken as the transmembrane potential; the side of the membrane facing the higher pH is positive. The pH gradients so imposed varied from about 0.1 to about 1.2 pH units.

Figure 2 shows the results obtained from one of a number of such experiments. The transmembrane potential recorded is plotted against the pH gradient imposed. A rectilinear relationship was observed. In this experiment the slope of the line $\Delta E_m/\Delta pH$ was found to be equal to 54.0 mv per pH unit at 24°C, in fairly good agreement with the theoretical value of 59.3 if only H^+ (or OH^-) ions were transported across the membrane.

That other ions in the system can contribute very little to the total transport of charge across the membrane is shown by the following considerations. The transmembrane potential (E_m) can be described by the Goldman equation:^{18, 19}

$$E_m = \frac{2.303RT}{F} \log_{10} \frac{\Sigma P_{I^+}[I^+]_1 + \Sigma P_{I^-}[I^-]_2}{\Sigma P_{I^+}[I^+]_2 + \Sigma P_{I^-}[I^-]_1} \quad (1)$$

in which R is the gas constant, T is absolute temperature, F is the faraday, and P_{I^+} and P_{I^-} the permeability coefficients for all ions I^+ or I^- , whose subscripts 1 and 2 refer to sides 1 and 2, respectively. By separation of the terms for H^+ and OH^- , equation (1) becomes

$$E_m = \frac{2.303RT}{F} \log_{10} \frac{P_{H^+}[H^+]_1 + P_{OH^-}[OH^-]_2 + \Sigma P_{I^+}[I^+]_1 + \Sigma P_{I^-}[I^-]_2}{P_{H^+}[H^+]_2 + P_{OH^-}[OH^-]_1 + \Sigma P_{I^+}[I^+]_2 + \Sigma P_{I^-}[I^-]_1} \quad (2)$$

in which P_I and I now refer to all ions in the system except H^+ and OH^- ions. When $\Delta pH = 1.0$, the following relationships hold:

$$[H^+]_1 = 10[H^+]_2,$$

$$[OH^-]_2 = 10[OH^-]_1,$$

$$E_m = 54.0 \text{ mv.}$$

Following substitution of known terms, equation (2) simplifies to

$$54.0 = 59.3 \log_{10} \frac{10(P_{H^+}[H^+]_2 + P_{OH^-}[OH^-]_1) + \Sigma P_{I^+}[I^+]_1 + \Sigma P_{I^-}[I^-]_2}{(P_{H^+}[H^+]_2 + P_{OH^-}[OH^-]_1) + \Sigma P_{I^+}[I^+]_2 + \Sigma P_{I^-}[I^-]_1} \quad (3)$$

In order for this equality to hold, the numerator of the log term must be approximately ten times as great as the denominator. This condition is met if H^+ (or OH^-) is translocated across the membrane in very much larger amounts than all the other ions in the system, i.e., K^+ , Cl^- , $Tris^+$, and $CF-CCP^-$. Of these ions, K^+ , Cl^- , and $CF-CCP^-$ ($pK' = 5.8$) are present in equal concentrations across the membrane and thus contribute no gradient; moreover, it is likely that their permeability coefficients are independent of the direction of translocation. These ions can therefore be excluded as contributing significantly to the observed transmembrane potential; furthermore, they evidently do not collapse the potential difference due to the proton gradient. The remaining ion to be considered is $Tris^+$, which has a pK' value of 8.14 and which therefore is present in unequal concentrations across the membrane when a pH gradient is formed in the zone pH 7.5–8.5. However, it is seen (Fig. 2) that the plot of ΔE_m vs. $-\Delta \log [H^+]$ is precisely linear, whereas the plot of ΔE_m vs. $-\Delta \log [Tris^+]$ shows no simple linear relationship. Moreover, the concentration ratio of $Tris^+$ across the membrane does not exceed 3.0 when $\Delta pH = 1.0$, and becomes much smaller at the lower pH zone. It is clear then that the amount of $Tris^+$ translocated across the membrane must be relatively small compared to the amount of H^+ (or OH^-) to account for the observed value of $E_m = 54.0$, which is nearly equal to the expected value of 59.3 if all the terms $P_I[I]$ in the Goldman equation are zero except those for H^+ and OH^- .

It may be concluded from these considerations that in the presence of $CF-CCP$, protons or hydroxyl ions, or both, are the main ionic species which are translocated through the phospholipid membrane.

Discussion.—The optimum pH effect on the increase of membrane conductance in the presence of uncoupling agents and the fact that this optimum pH differs with the uncoupler used are predictable from Mitchell's hypothesis. The over-all increase in conductance produced by the uncoupler depends on the concentration of the undissociated uncoupling agent and its anion in the lipid phase and on the concentration of H^+ ions in the aqueous phase. At low pH values the concentration of the anionic form of the uncoupler in the lipid phase is rate-limiting for protonic conductance, whereas at high pH values, the low H^+ ion concentration in the aqueous phase becomes rate-limiting. The fact that the uncouplers differ in pK' and lipid solubility also contributes to the optimum pH values observed. Our findings on the membrane thus are similar to those of Hemker¹⁵ and Myers and Slater²⁰ on rat liver mitochondria. They observed a large decrease in the concentration of 2,4-dinitrophenol ($pK' = 4.0$) required for maximal stimulation of respiration and of ATPase activity in rat liver mitochondria on decreasing the pH from 8.0 to 5.0, consistent with our observation that the effect of 2,4-dinitrophenol on the phospholipid bilayer is maximal at pH = 4.0.

The high-resistance phospholipid bilayer membranes studied here are of the type that show a linear dependence of conductance on membrane area.¹⁴ The

lowering of membrane resistance by uncoupling agents observed in this and the preceding study⁹ was in all probability a generalized effect on the entire membrane surface. Membranes having a specific resistance less than 10^8 ohm cm^2 have been shown by Hanai *et al.*¹⁴ to undergo leakage of electrical current around the edge of the membrane. Nevertheless, even such leaky membranes showed a further and substantial lowering of electrical resistance in the presence of uncoupling agents. However, such already leaky membranes were found to yield transmembrane potentials that were considerably lower than those of high-resistance membranes when a pH gradient was applied in the presence of uncoupler.

The absolute values for the electrical conductance of phospholipid bilayers in the presence of uncouplers is in surprisingly good agreement with values calculated by Mitchell and Moyle for the protonic conductance of the inner mitochondrial membrane from the rate of absorption of H^+ under anaerobic conditions.¹⁶ They reported the value of 7.2×10^{-6} mho cm^{-2} for the mitochondrial membrane, which may be compared with the value of 6.7×10^{-6} mho cm^{-2} for the model membrane, at a concentration of $0.5 \mu\text{M}$ CF-CCP at pH 7.2.

The data reported here show that uncoupling agents of oxidative phosphorylation act on synthetic phospholipid bilayers in the manner postulated in the chemiosmotic hypothesis: they greatly increase the electrical conductance of the membrane to values comparable to the calculated conductance of the uncoupled mitochondrial membrane, they show an optimum pH for this effect as is predictable from the chemiosmotic hypothesis, and they increase the conductance by specifically allowing translocation of protons. While the bilayer system can only be regarded as a model of the mitochondrial membrane, the data reported here suggest that any natural lipid phase of about $70\text{-}\text{\AA}$ thickness can be expected to behave in a similar fashion. Although our data are consistent with the Mitchell hypothesis, they also are consistent with a mechanism in which a high-energy intermediate sensitive to protons is generated by electron transport in a lipid phase; the uncoupling agent can promote access of protons from the aqueous medium into the lipid phase and thus lead to discharge of the postulated high-energy intermediate.

While this work was under way, Skulachev reported similar results at the Seventh International Congress of Biochemistry in Tokyo in August 1967.¹⁷

Summary.—The mitochondrial uncoupling agents 2,4-dinitrophenol (DNP), carbonylcyanide *m*-chlorophenylhydrazone (Cl-CCP), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (CF-CCP), and dicoumarol increased the specific electrical conductance of model phospholipid bilayer membranes. Each uncoupling agent showed a pH optimum in increasing the transmembrane conductance. Measurement of the transmembrane potential in the presence of CF-CCP with a pH gradient of 1.0 unit across the membrane gave consistent values of 54 mv, in good agreement with the theoretical value of 59 mv expected for pure protonic conductance at 25° . It is concluded that the uncoupling agents increase the specific conductance of such model membranes by facilitating transport of H^+ or OH^- (or both) across the membrane, and that they do not facilitate significant transport of other ions in the system, such as K^+ , Cl^- , or

Tris⁺. These results are qualitatively in accord with the hypothesis proposed by Mitchell for the action of uncoupling agents on oxidative phosphorylation in mitochondria.

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