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Complexes Between Uncouplers of Oxidative Phosphorylation

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Summary. We have examined the effects of two weak acid uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and 5,6-dichloro-2-trifluoromethyl-benzimidazole, on the electrical properties of phospholipid bilayer membranes. All the effects they produce are consistent with the charged permeant species being a HA_2^- complex formed between the neutral acid HA and its anion A^- and the current in the aqueous phases being carried by buffered hydrogen ions. When both uncouplers are present simultaneously, all the evidence we have obtained is consistent with the charged permeant species being a HAB^- complex formed between the neutral acid HA of one uncoupler and the anion B^- of the other. It was necessary, however, to take into account interfacial processes and the unstirred layers adjacent to the membrane, the adsorption of anions to the bilayer and the buffer level in the aqueous phases to explain the results in terms of this model. The degree to which these factors will complicate a comparison of results obtained on artificial systems and mitochondria is also discussed.

The chemiosmotic hypothesis (Mitchell, 1961, 1966; Greville, 1969; Skulachev, 1971; Harold, 1972) predicts that there should be a direct correlation between the potency of uncouplers on artificial lipid bilayer systems and on mitochondria, the lipids of which also appear to be arranged primarily in the form of a bilayer (Blazyk & Steim, 1972; Hsia, Chen, Long, Wong & Kalow, 1972). Some experiments illustrate the expected correlation (Liberman, Topaly, Tsofina, Jasaitis & Skulachev, 1969; Bakker, Van den Heuvel, Wiechmann & Van Dam, 1973), and some do not (Ting, Wilson & Chance, 1970; Wilson, Ting & Koppelman, 1971). We believe that the question posed by these apparently contradictory results is important, but that it is unlikely to be resolved until the mode of action of the uncouplers on the simpler model systems is fully understood.

With respect to the artificial membranes, for example, there are two different types of weak acid uncouplers. For one class, the anion A^- is the

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charged permeant species (Le Blanc, 1971) whereas for the other class it is a HA_2^- complex formed between the anion A^- and the undissociated acid HA (Lea & Croghan, 1969; Finkelstein, 1970). For both classes, the current in the aqueous phases appears to be carried by buffered H^+ ions (Neumcke & Bamberg, 1974). When members of these two different classes are compared, their relative effectiveness in enhancing the conductance of a bilayer depends on the concentration at which the comparison is made, because conductance depends linearly on concentration for the A^- class but quadratically on concentration for the HA_2^- class. This does not facilitate a comparison between the artificial and biological systems.

A member of the A^- class of uncouplers, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), has been examined in some detail by Le Blanc (1971). We concentrate our attention on two weak acids believed to be members of the HA_2^- class and examine the effects of 5,6-dichloro-2-trifluoromethyl-benzimidazole (DTFB) and 2,4-dinitrophenol (DNP) on the electrical properties of phospholipid bilayer membranes. Our data for both DNP and DTFB and those of others for DNP are all consistent with the charged permeant species being the HA_2^- complex. It is necessary, however, to take into account the effects of unstirred layers, charge adsorption and buffer level to explain how the conductance depends on the uncoupler concentration and pH in symmetrical solutions and how the membrane potential depends on a difference of pH or uncoupler concentration across a membrane separating asymmetrical solutions. We also find that when both uncouplers are present together, membrane-permeant dimers are formed between the anion of one acid and the neutral form of the other. A preliminary account of this work has appeared (Foster, Harary & McLaughlin, 1973).

Materials and Methods

Bacterial phosphatidyl ethanolamine (PE), bacterial phosphatidyl glycerol (PG) and cholesterol (C) were obtained from Supelco, egg or dioleoyl phosphatidyl choline (PC) from Sylvana or Hormel, respectively, 7-dehydrocholesterol (7-DC) from Sigma, 2,4-dinitrophenol (DNP) from Fischer, tetrachloro-2-trifluoromethyl benzimidazole (TTFB) was a gift from Dr. K. H. Büchel and 5,6-dichloro-2-trifluoromethyl benzimidazole (DTFB) was synthesized by Ron Liotta, Dept. of Chemistry, SUNY, Stony Brook, using a modification of the procedure of Acheson, Taylor and Tomlinson (1958).

The aqueous solutions were prepared with 18 MΩ "super Q" (Millipore Corporation) water, and contained, in most cases, 10^{-1} M KCl buffered with 5×10^{-3} M citrate, phosphate and tris.

The experimental apparatus and the methods used were similar to those previously described (Szabo, Eisenman & Ciani, 1969). In brief, Teflon chambers were used which had two compartments, each of 25-ml capacity, separated by a thin wall having an aperture of 0.8 or 1.4 mm diameter. A small amount of lipid (1 to 3 μliters) dissolved

in decane (Eastman) to a concentration of 25 mg/ml was deposited on the aperture with a Pasteur pipette, and membranes were formed by passing a bubble over the aperture. The membrane was observed with a Wild M5 stereoscopic microscope and its area measured with a calibrated eyepiece reticule. The capacitance of the membrane was measured with a General Radio impedance bridge at 1,000 cycles/sec. The capacitance per unit area of the bilayer did not vary significantly with the area of the membrane, the nature of the polar head group of the lipid, or the concentration of uncoupler in the bathing solution. For both PE and PG bilayers it was 0.5 ± 0.05 μF/cm^2. Solutions were stirred with Teflon-coated magnetic stirrers and additions of microliter quantities of DTFB or DNP dissolved in ethanol were made in the immediate vicinity of the stirrers. The total amount of ethanol in the chamber never exceeded 1%, a concentration which had a neglible effect on the conductance of the membranes in control experiments. Similar results were obtained when aqueous solutions of DNP were prepared in advance from the crystalline acid. Experiments were performed at room temperature (24 ± 2 °C).

Conductance was routinely determined by measuring the current with a Keithley 602 electrometer when 10 mV were applied to the system, the membrane being in series with the high resistance of the electrometer. Current-voltage curves were obtained in some cases, and were always linear up to at least ± 30 mV across the membrane.

The potential difference between the two compartments was measured when the solutions were of different pH or contained different concentrations of uncoupler. The pH was varied by adding small aliquots of concentrated NaOH or HCl to the bathing solutions and was monitored directly in the experimental chamber with glass electrodes.

Theory

We summarize here the theoretically expected behavior for the HA_2^- class of uncouplers. In the model of Lea and Croghan (1969) and Finkelstein (1970) the following two reactions are assumed to occur in the aqueous phases:

$$HA \rightleftharpoons H^+ + A^-, \quad K_{HA} = \frac{[H^+][A^-]}{[HA]} \tag{1}$$

$$HA_2^- \rightleftharpoons HA + A^-, \quad K_{HA_2^-} = \frac{[HA][A^-]}{[HA_2^-]} \tag{2}$$

where A^- represents the anion of the weak acid HA. The concentration of HA_2^- is assumed to be much less than that of either HA or A^-, so the total concentration of uncoupler, $[A^{TOT}]$ may be approximated by:

$$[A^{TOT}] \cong [HA] + [A^-]. \tag{3}$$

By combining Eqs. (1)–(3) we obtain:

$$[HA_2^-] = \frac{K_{HA}}{K_{HA_2^-}} [A^{TOT}]^2 \frac{[H^+]}{(K_{HA} + [H^+])^2} \tag{4}$$

for the concentration of HA_2^- in the bulk aqueous phases.

Conductance

If there are charges or oriented dipoles at the membrane-solution interfaces there will be a potential ψ^* within the membrane relative to the bulk aqueous phases. The concentration of HA_2^- within the membrane at equilibrium will therefore be proportional to the Boltzmann expression, $\exp\left(+\frac{F\psi^*}{RT}\right)$, where F is the Faraday, R the gas constant and T is the temperature.

If we assume that only the HA_2^- complex carries current across the membrane and that the movement of the complex across the membrane is the rate-limiting step when a potential is applied, it follows that

$$G \sim [A^{TOT}]^2 \frac{[H^+]}{(K_{HA}+[H^+])^2} \exp\left(\frac{F\psi^*}{RT}\right). \tag{5}$$

McLaughlin (1972) may be consulted for a more detailed discussion of the assumptions implicit in this derivation. The simple model predicts that the conductance is proportional to the square of the total uncoupler concentration and that it has a maximum at a pH equal to the pK of the weak acid if ψ^* is a constant.

An equation of exactly the same form is obtained if we assume that the HA_2^- complex is formed at the interface via heterogeneous reactions, provided the interfacial reactions remain at equilibrium when a potential is applied to the membrane. There is, in fact, good experimental evidence that the current in aqueous phases is carried by (buffered) H^+ ions and not by the HA_2^- complex.[1] Neumcke and Bamberg (1974) may be consulted

1 At high concentrations of DTFB, the current measured after applying a voltage step (circles in Fig. 1) decays half-way towards a steady-state value (crosses in Fig. 1) in about 10 sec. We interpret this to mean that the steady-state current is limited not by the membrane but by diffusion through unstirred layers. The thickness of these aqueous layers is $\delta \sim 10^{-2}$ cm (Holz & Finkelstein, 1970; Le Blanc, 1971; Haydon & Hladky, 1972). Consistent with this interpretation we find that a plot of the "instantaneous" current *vs.* voltage is superlinear, as expected if the passage of ions through the membrane is the rate-limiting step (Neumcke & Läuger, 1969). A plot of the steady-state current *vs.* voltage at high concentrations is sublinear, curving toward the voltage axis, confirming that the passage of ions through the membrane is not the rate-limiting step for the steady-state current. The steady-state current, furthermore, is presumably limited by the diffusion of buffered H^+ ions through the unstirred layer, because when the buffer capacity was lowered, we observed a marked decrease in the steady state but not the instantaneous current. Neumcke and Bamberg (1974) discuss a second reason for believing that the current is carried by H^+ ions in the aqueous phases. They point out that the current produced by the benzimidazoles DTFB and TTFB is too high to be due to the movement of either the A^- or HA_2^- species through the aqueous phases. They may also be consulted for a more detailed discussion of diffusion polarization.

for a detailed discussion of this point and of the possible mechanisms by which the HA_2^- complex might be formed at the membrane-solution interface. They suggest that the HA_2^- complex is formed when a HA molecule in the membrane gives up a H^+ ion to the aqueous phase, then combines with a second HA molecule to form the HA_2^- species [*see* Eqs. (8) and (9)].

We derive, in a similar manner, an expression for the conductance when the membrane permeant species is a dimer (HAB^-) formed from the anion of one uncoupler (B^-) and the neutral form of the other uncoupler (HA):

$$G \sim [A^{TOT}][B^{TOT}] \frac{[H^+]}{(K_{HA}+[H^+])(K_{HB}+[H^+])} \exp\left(\frac{F\psi^*}{RT}\right) \tag{6}$$

where K_{HA} and K_{HB} are the aqueous dissociation constants for the two weak acids. Eq. (6) is similar to Eq. (5) but predicts a linear dependence of G on both $[A^{TOT}]$ and $[B^{TOT}]$, the total aqueous concentrations of the weak acid uncouplers, and a broad maximum in the G *vs.* pH curve at a pH midway between the two pK values if ψ^* is constant.

Potential

If we assume that the only charged permeant species is the HA_2^- complex, the potential difference ϕ between the two membrane-solution interfaces located at $x=0$ and $x=d$ is given by the Nernst expression:

$$\phi = \frac{RT}{F} \ln \frac{[HA_2^-]^*_{x=0}}{[HA_2^-]^*_{x=d}} \tag{7}$$

where the * denotes concentrations in the membrane phase.

The expression will reduce to a Nernst expression for H^+ ions under certain conditions. If the HA_2^- complex is formed in the aqueous phases, one assumes (*see* Finkelstein, 1970 for details) that the movement of HA is limited by the aqueous unstirred layers, that the solutions are well buffered and that reactions (1) and (2) are at equilibrium. We consider here the case where the complex is formed at an interface (e.g. $x=0$) by the mechanism suggested by Neumcke and Bamberg (1974).

$$HA^*_{x=0} \rightleftharpoons A^-_{x=0} + H^+_{x=0} \qquad K_1 = \frac{[A^-]^*_{x=0}[H^+]_{x=0}}{[HA]^*_{x=0}} \tag{8}$$

$$\underset{x=0}{HA_2^-} \rightleftharpoons A^-_{x=0} + HA^*_{x=0} \qquad K_2 = \frac{[A^-]^*_{x=0}[HA]^*_{x=0}}{[HA_2^-]^*_{x=0}} \tag{9}$$

where * denotes the membrane phase and identical reactions occur at the other interface situated at $x = d$.

The first requirement is that the concentrations of HA at the interfaces be identical:

$$[HA]^*_{x=0} = [HA]^*_{x=d}. \tag{10}$$

This will be true if the resistance to the movement of HA provided by either the aqueous unstirred layers or the interfaces is greater than the resistance provided by the membrane. (The possibilities are illustrated schematically in Fig. 2 of Eisenman, Szabo, Ciani, McLaughlin and Krasne, 1973). Although there is some evidence for DTFB that the interfaces limit the movement of the HA species (*see* Discussion), a sufficient condition is that the unstirred layers provide a greater resistance than the membrane; that is:

$$\frac{k\delta D^*}{dD} \gg 1 \tag{11}$$

where k is the partition coefficient of the neutral species into the membrane, δ is the thickness of the unstirred layers, d is the thickness of the membrane, D^* is the diffusion constant in the membrane and D is the diffusion constant in the aqueous solution. For an order of magnitude calculation, we take $d \sim 50$ Å, $\delta \sim 100$ μ (footnote 1), $D \sim 5 \times 10^{-6}$ cm^2/sec, $D^* \sim 5 \times 10^{-8}$ cm^2/sec, $k \sim 1$ (*see* Appendix). These values imply that $k\frac{\delta}{d}\frac{D^*}{D} \approx 10^2$ for both DNP and DTFB and the condition is thus satisfied for these two weak acids.

The second requirement is that the solution be well buffered. The H^+ concentration must remain constant through the unstirred layers ($\delta \sim 100$ μ) adjacent to the membrane and, if there are double layer potentials ψ_1 and ψ_2 due to charges at the interfaces $x = 0$ and $x = d$, vary according to the Boltzmann expression immediately adjacent (Debye length ~ 10 Å) to the membrane. (For the remainder of the derivation we ignore, for simplicity, any dipole potentials that may exist at the interfaces.) The Boltzmann relation states

$$\begin{aligned} [H^+]_{x=0} &= [H^+]_{x=-\infty} \cdot \exp -(F\psi_1/RT) \\ [H^+]_{x=d} &= [H^+]_{x=\infty} \cdot \exp -(F\psi_2/RT). \end{aligned} \tag{12}$$

The third requirement is that reactions (8) and (9) be at equilibrium at the interfaces. From Eqs. (9), (10) and (8) it follows in sequence that

$$\frac{[HA_2^-]^*_{x=0}}{[HA_2^-]^*_{x=d}} = \frac{[A^-]^*_{x=0}}{[A^-]^*_{x=d}} = \frac{[H^+]_{x=d}}{[H^+]_{x=0}}. \tag{13}$$

Noting that the total measurable potential V between the two aqueous phases is the sum of the diffusion potential ϕ within the membrane (Eq. 7) and the difference between the two surface potentials ψ_2 and ψ_1, $V=\phi+\psi_2-\psi_1$, we have from Eqs. (7), (13) and (12),

$$V=\frac{RT}{F}\ln\left\{\frac{[H^+]_{x=\infty}}{[H^+]_{x=-\infty}}\right\}. \tag{14}$$

That is, the membrane behaves as if it were selectively permeable to H^+, even though the only charged permeant species is the HA_2^- complex.

The model predicts that a difference between the uncoupler concentrations in the two aquous phases will produce no measurable potential ($V=0$) if the pH of the solutions is the same.

Results

DTFB

Conductance Experiments. The conductance of a bilayer membrane increases with the concentration of DTFB in the bathing solution as shown in Fig. 1. The circles, which designate the conductance measured within a millisecond of the application of a voltage step of 10 mV, lie on a line with a slope of 2 on the log-log plot, illustrating that the conductance depends on the square of the DTFB concentration.[1] This quadratic dependence of conductance on concentration is consistent with the model discussed above [Eq. (5)], which postulates that the permeant species is a charged complex formed between the undissociated and anionic forms of DTFB. In Fig. 2 the conductance in the presence of DTFB is plotted as a function of pH. The conductance has a maximum at a pH of about 7.3, the pK of DTFB in this solution.[2] The curve through the points in Fig. 2 illustrates the behavior predicted by Eq. (5) normalized to the conductance at pH 7. The data agree quite well with the theoretical prediction.

Figs. 3 and 4 illustrate the results of experiments with the negatively charged phospholipid, PG. The conductance again depends on the square of the DTFB concentration for a range of pH, as indicated in Fig. 3. At a given pH and DTFB concentration, however, the conductance on PG membranes is depressed 1.8 orders of magnitude with respect to that observed on the zwitterionic but net neutral PE membranes. This indicates that the

2 Using a spectroscopic method similar to that utilized by Le Blanc (1971), we determined the pK for DTFB to be 7.3 ± 0.1 in our 0.1 M salt solution. Büchel *et al.* (1965) report a pK of 7.6 for DTFB in 50% by volume aqueous ethanol.

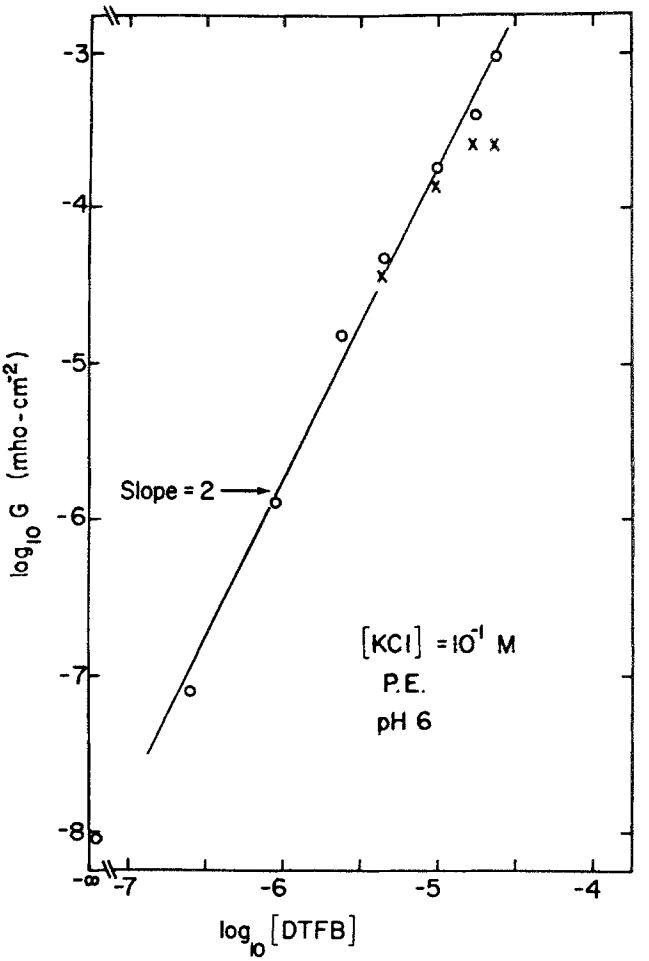

Fig. 1. Dependence of membrane conductance on aqueous concentration of DTFB with bilayers formed from the zwitterionic lipid phosphatidyl ethanolamine (PE). The 10^{-1} M KCl solution was buffered with 2×10^{-3} M phosphate to pH 6. Values from 3 experiments are averaged, the "instantaneous" values of the conductance being plotted with circles, and the steady-state[1] values with crosses when they differ from the "instantaneous" values. The straight line through the experimental points illustrates the theoretically expected quadratic dependence, $G \sim [\text{DTFB}]^2$, predicted by Eq. (5)

permeant species is indeed negative and that it responds to the negative surface potential ψ^* on the PG membrane, approximately[3] as predicted by Eq. (5). Fig. 4 shows the dependence of conductance on pH for a PG

3 The conductance produced by DTFB on bilayers was depressed 1.8 log units with respect to the value measured of PE bilayers. On bilayers formed from the same lipid samples, the conductance produced by nonactin-K^+ on PG was enhanced about 2.2 log

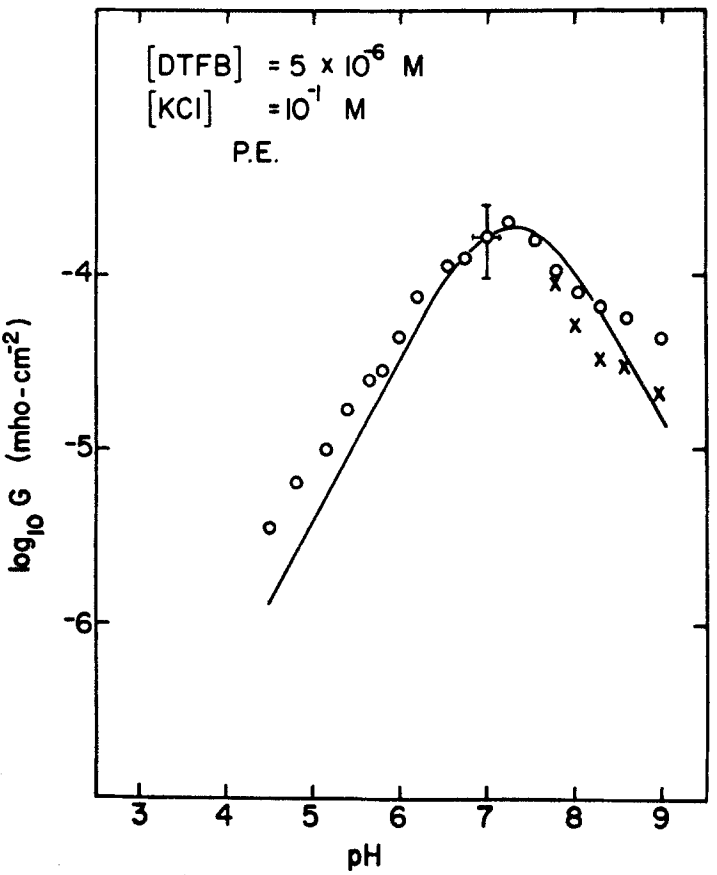

Fig. 2. Dependence of membrane conductance on pH in the presence of DTFB with bilayers formed from the zwitterionic lipid phosphatidyl ethanolamine (PE). The 10^{-1} M KCl solution was buffered with 5×10^{-3} M sodium citrate, 5×10^{-3} M potassium phosphate, and 5×10^{-3} tris. The points represent the average of 2 experiments, the "instantaneous" values being indicated by circles, and the steady-state values by crosses. The error bar represents the standard deviation for 6 measurements of the conductance at pH 7. The curve is the theoretically predicted behavior from the model discussed above [Eq. (5)], normalized to the conductance at pH 7

units with respect to PE. If DTFB was acting as a perfect "probe" (Szabo, Eisenman, McLaughlin & Krasne, 1972) of the surface potential the results would have been exactly symmetrical, as in fact they were with the polyiodide or I_5^- complex (McLaughlin, Szabo, Eisenman & Ciani, 1970; McLaughlin, Szabo & Eisenman, 1971). (It was necessary to make the comparisons on bilayers formed from the same lipid sample, because we have noted variations in the samples of PG we have obtained from Supelco over the last year with respect to the nonactin-K^+ and valinomycin-K^+ conductances. In a 10^{-1} M KCl solution the values obtained on PG with these carriers have ranged from 2.0 to 2.7 log units higher than that obtained on PE bilayers. We do not understand this variation, but stress that it affects none of the conclusions reached in this paper.)

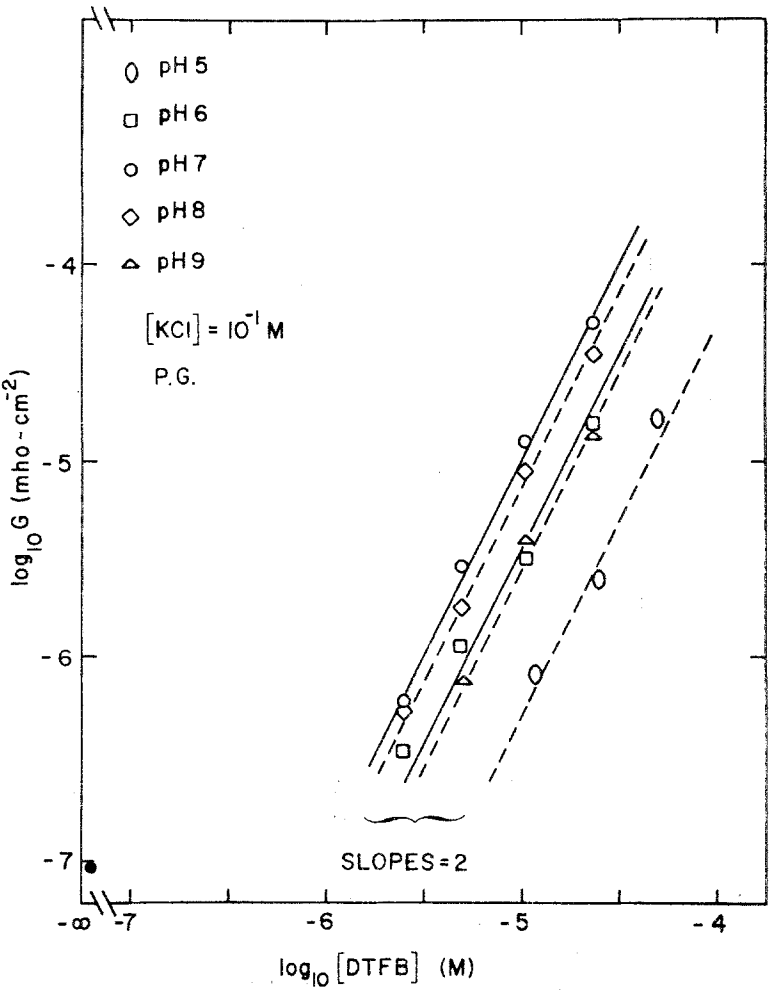

Fig. 3. Dependence of conductance on concentration of DTFB with bilayers formed from the negatively charged lipid phosphatidyl glycerol (PG). The 10^{-1} M KCl solution was buffered with 5×10^{-3} M sodium citrate, 5×10^{-3} M sodium phosphate, and 5×10^{-3} M tris. Only the instantaneous values of conductance are plotted. The values for pH 7 are the average of 2 experiments; the other values were obtained from single experiments. At each pH there is a quadratic dependence of conductance on concentration, as predicted by Eq. (5)

bilayer membrane bathed in solutions containing DTFB. The points are taken from the curves in Fig. 3, and they tend to lie along the theoretical expression of Eq. (5), normalized to the conductances at pH 7.

Potential Experiments. Potential experiments were performed in the presence of 5×10^{-6} M DTFB. The potential difference between the two

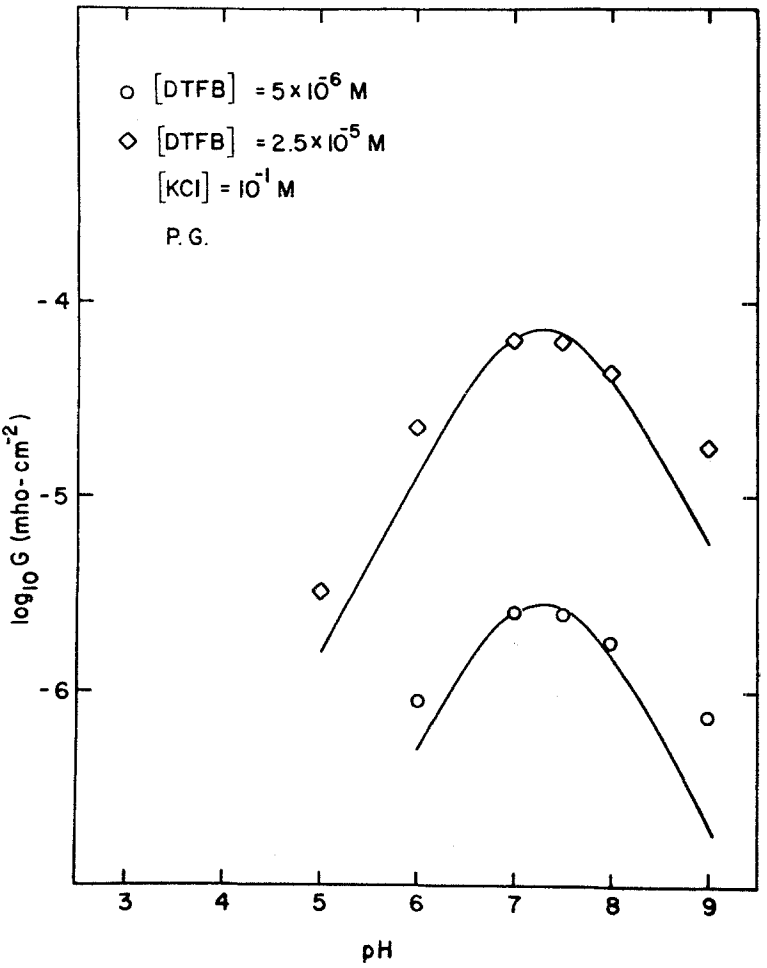

Fig. 4. Dependence of conductance on pH in the presence of DTFB with bilayers formed from the negatively charged lipid phosphatidyl glycerol, PG. Points are taken from the curves in Fig. 3. The curves in this figure indicate the behavior predicted from the model [Eq. (5)], normalized to the conductance at pH 7

solutions separated by a PE bilayer varied linearly with the difference in pH between the two solutions, the slope, $\Delta V/\Delta$ pH, being 59 mV over a range from pH 3.5 to pH 8.5. (Measurements were only made with pH gradients less than 2, because the membranes broke when the potential difference was greater than about 120 mV.) The potential was negative on the side having the lower pH. Less than 1 mV potential difference across the PE bilayer was observed when one solution contained 5×10^{-6} M DTFB and the other no DTFB. These results are consistent with the model discussed in the Theory section.

DNP

Conductance Experiments. The experimental data for the action of DNP on bilayer membranes are also consistent with the assumption that the permeant species is the HA_2^- complex. When the surface potential ψ^* produced by the adsorption of the anion of DNP to PE bilayers was taken into account, it was found that the conductance depends on the square of the concentration of DNP (McLaughlin, 1972). The surface potential was estimated by using the positively charged nonactin-potassium complex as a "probe" and measuring the increase in the carrier-mediated potassium conductance as a function of the concentration of DNP. In this study we obtained results similar to those illustrated in Figs. 1 and 2 of McLaughlin (1972) using bilayers formed from a 50% mixture by weight of egg phosphatidyl choline and cholesterol (PC-C). Specifically, the anion of DNP produced about the same change in surface potential on PC-C bilayers that it did on PE bilayers and the conductance due to DNP, when corrected for the surface potential, again depended on the square of the DNP concentration.[4]

4 We have measured the adsorption of the anion of DNP to bilayers formed from a variety of lipids. The shapes of the surface potential *vs.* [DNP] curves were identical to those measured previously for PE bilayers (McLaughlin, 1972) but DNP adsorbed most strongly to membranes formed from PE, the sequence being PE ~ PC-C ~ PC > 7-DC. Specifically, DNP adsorbed to PE bilayers at an order of magnitude lower concentration than to bilayers formed from oxidized 7-dehydrocholesterol (7-DC). The relative importance of electrostatic interactions with polar head groups (Rich, 1973), hydrophobic forces (McLaughlin, 1973), and charge transfer bonds (Rosenberg & Bhowmik, 1969) in the adsorption of DNP to bilayers is unknown at present, but as 7-DC is a neutral rather than a zwitterionic lipid we can say that electrostatic interactions with the polar head group should be minimal in this case.

The adsorption of the anion of DNP can also be demonstrated by measuring the electrophoretic mobility of phospholipid vesicles in a cylindrical microelectrophoresis apparatus and we have confirmed many of Rich's (1973) measurements on PC vesicles in the presence of DNP. We wish to stress, however, that there is a large difference between the surface potential ψ calculated from conductance measurements with nonactin on planar membranes of the Mueller-Rudin type and the zeta potential ζ calculated from the microelectrophoretic mobility measurements on vesicles of the Bangham type. A concentration of 10^{-3} M DNP, for example, produces a change in surface potential of about -60 mV on planar PC bilayers (from Hormel) bathed in 10^{-1} M KCl at pH 7 but produces a zeta potential of less than -10 mV on vesicles formed from the same lipid. The difference is probably due to two factors. First, the conductance but not the microelectrophoresis measurements monitor a change in dipole as well as double layer potential. Second, the ζ potential is not measured at the surface of the membrane but at a plane of shear some indeterminate distance from the membrane and is thus lower in magnitude than the surface potential. Lowering the ionic strength will have two effects. It will decrease the relative importance of the dipole *vs.* the double layer potential and will increase the Debye length (to about 30 *vs.* 10 Å in a 10^{-2} *vs.* 10^{-1} M salt solution). We thus expected, and indeed observed a better correlation between results from the two techniques at the lower salt concentration.

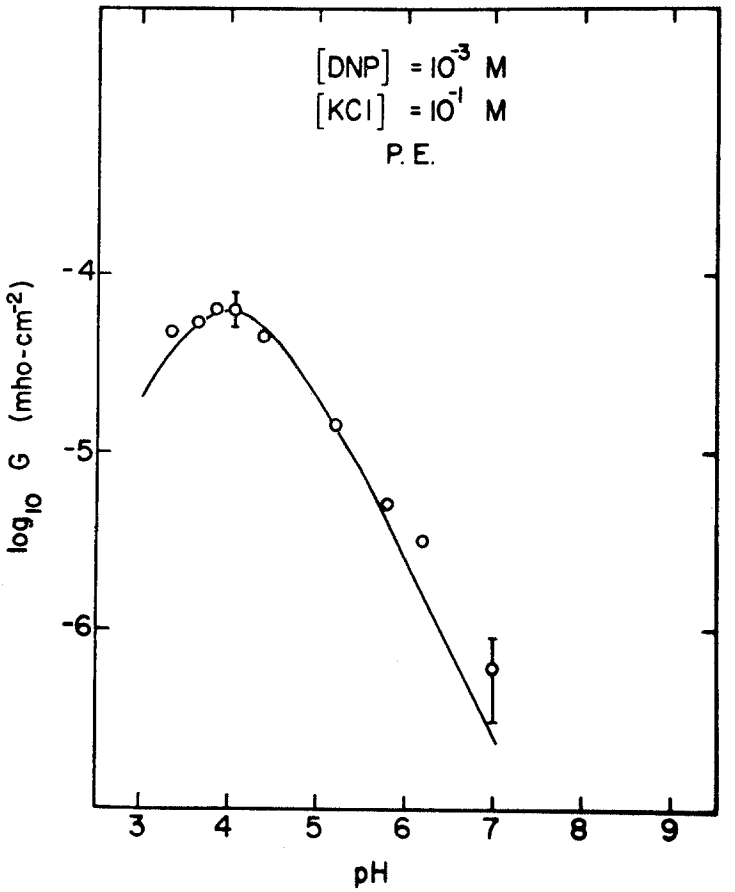

Fig. 5. Dependence of conductance on pH in the presence of DNP. Bilayers were formed from phosphatidyl ethanolamine, and the 10^{-1} M KCl solution was buffered with 5×10^{-3} M sodium citrate, 5×10^{-3} M potassium phosphate and 5×10^{-3} M tris. Values from at least 2 experiments were averaged. At pH 7, 10 measurements were made and the vertical bars represent the standard deviation. The curve is taken from Eq. (5), normalized to the conductance at pH 4

Fig. 5 is a plot of the conductance *vs.* pH for a PE bilayer in the presence of DNP. The conductance has a maximum at a pH of about 4, which is the pK of DNP.[5] The curve is drawn according to Eq. (5), normalized to the conductance at pH 4.

5 We note that the experimental points on the acid side of the pK for DNP would be expected to lie somewhat above the normalized theoretical curve due to the change in the number of the anions of DNP adsorbed to PE. The accuracy of the data obtained between pH 3.5 and 4.0 (*see* error bars) does not justify complicating the theoretical expression [Eq. (5)] to account for this phenomenon. The surface potential of PE bilayers is constant for $3.5 < \text{pH} < 8.5$ but below pH 3.5 the interpretation of the data is further complicated by the titration of the phosphate groups (Szabo *et al.*, 1972).

Potential Experiments. When potential experiments were performed in the presence of 10^{-3} M DNP and moderate concentrations of buffer (phosphate, citrate, and tris each present at 5×10^{-3} M), the potential resulting from a pH gradient across the membrane was only about two-thirds that of a Nernst potential for hydrogen ions, as previously noted by Lea and Croghan (1969). Furthermore, a potential difference of 10 to 20 mV was observed for a 10-fold difference in DNP concentration when the pH was the same on both sides. These results differed from those obtained with DTFB and disagreed with the predictions of the model. Before abandoning the model, however, we investigated the assumptions listed in the theory section above. We suspected that the above solution was not well-buffered through the unstirred layer and that the second assumption discussed in the theory section was not satisfied. When we increased the concentration of the phosphate and citrate buffers to 50×10^{-3} M each we observed a slope in the presence of 2×10^{-3} M DNP of $\Delta V/\Delta$ pH = 59 mV.[6] With these high concentrations of buffer there was no potential difference (< 3 mV) for a 10-fold concentration gradient of DNP when the pH was constant, as expected from the theoretical model.

DTFB and DNP

When both the DTFB and DNP are present, our data are consistent with the assumption that a permeant complex is formed between the anion of one acid and the undissociated form of the other. In Fig. 6, the conductance is plotted against concentration of DTFB, in the presence of DNP. In the absence of DNP (Fig. 1) we found a slope of two on such a log-log plot. In the presence of DNP (Fig. 6), however, we observed a slope of one, or a linear dependence of conductance on the concentration of DTFB, an observation which is consistent with the model discussed above [*see* Eq. (6)].

Fig. 7 illustrates the dependence of conductance on pH, in the presence of both DNP and DTFB. The conductance is not merely the sum of the individual conductances due to DNP and DTFB. The dashed line is the sum of the theoretical curves for DNP and for DTFB, as shown in Figs. 2 and 5. (The curve for DTFB has been depressed by the appropriate amount to take into account the response to the potential on the membrane due to the adsorption of anions of DNP.) The solid line is the theoretical curve

6 Liberman and Topaly (1968*b*), who used buffer concentrations of 2×10^{-2} M sodium citrate, 2×10^{-2} M sodium phosphate, and 2×10^{-2} M boric acid, found about a Nernst potential for H^+ in the presence of 2.5×10^{-3} M DNP for values of pH less than 6, and less than a Nernst potential for values of pH greater than 6.

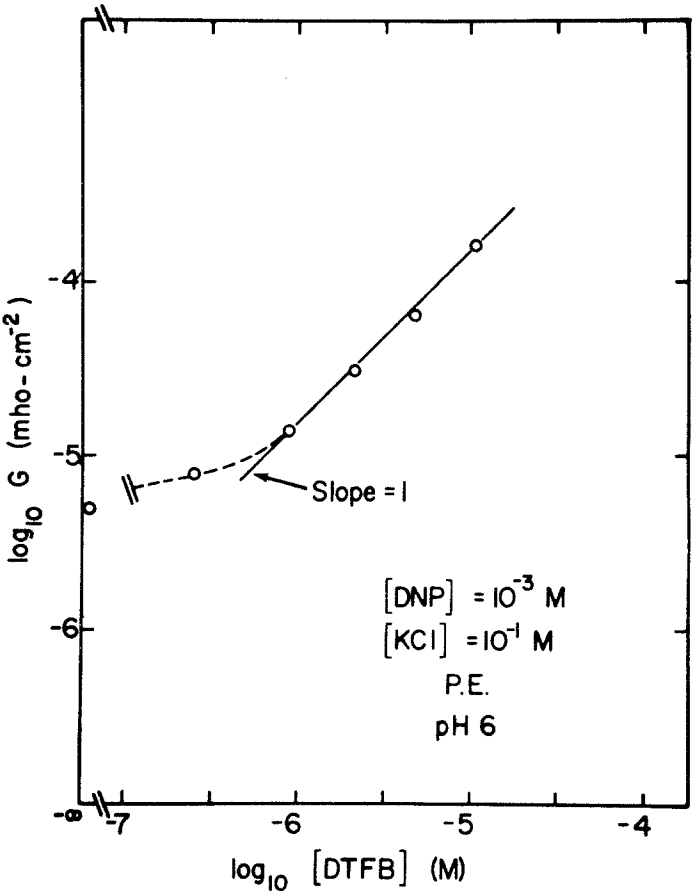

Fig. 6. Dependence of conductance on concentration of DTFB in the presence of DNP. Bilayers were formed from phosphatidyl ethanolamine, and the 10^{-1} M KCl solution was buffered with 2×10^{-3} M potassium phosphate to pH 6. Each point is the average from 2 experiments. In the concentration range shown, the instantaneous values of conductance did not differ significantly from the steady-state values. The solid line indicates the linear dependence of conductance on concentration predicted by Eq. (6)

for the conductance, under the assumption that the permeant species is a negatively charged complex involving one molecule of DTFB and one molecule of DNP [Eq. (6)]. This curve is normalized to the conductance at pH 7 and does agree reasonably well with the experimental data.

Fig. 8 shows the dependence of the conductance on the concentration of DNP in the presence of DTFB. The crosses indicate the measured conductance. The circles indicate the conductance after correction for the negative potential produced by the adsorption of anions of DNP to the bilayer (McLaughlin, 1972). The "corrected" conductance *vs.* concentra-

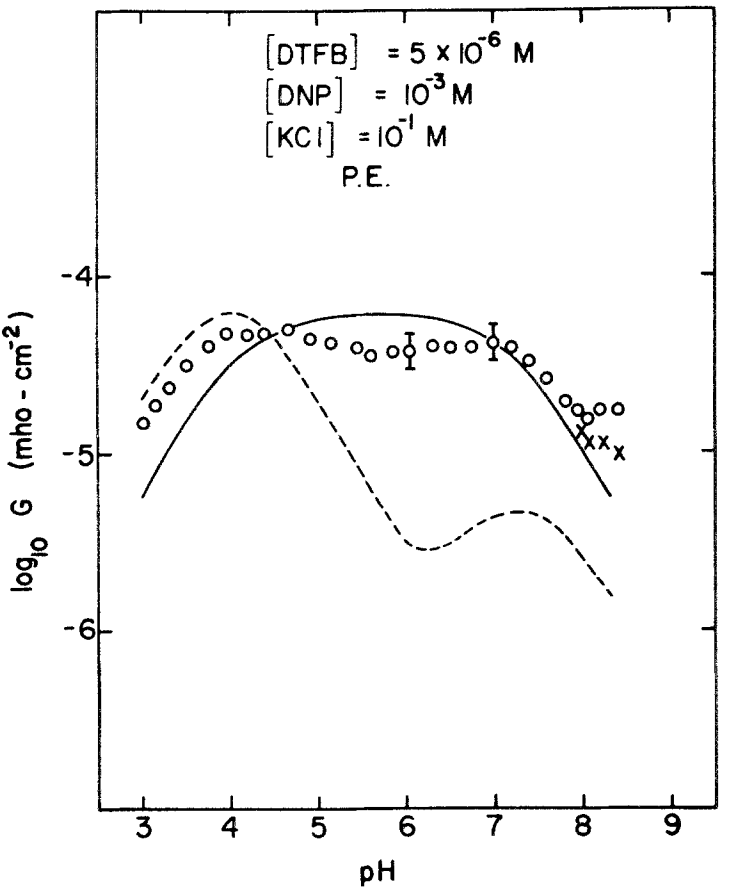

Fig. 7. Dependence of conductance on pH in the presence of both DTFB and DNP. Bilayers were formed from phosphatidyl ethanolamine, and the 10^{-1} M KCl solution was buffered with 5×10^{-3} M sodium citrate, 5×10^{-3} M potassium phosphate, and 5×10^{-3} M tris. The instantaneous values of conductance are represented by circles, and the steady-state values with crosses when they differ from the instantaneous values. The error bars illustrate the estimated 20% experimental uncertainty. The solid line is the theoretically predicted behavior from the model [Eq. (6)], normalized to the conductance at pH 7. *See text* for further details

tion data agree with the linear dependence of conductance on concentration of DNP in the presence of DTFB predicted by Eq. (6).

Discussion

The Substituted Benzimidazoles

DTFB. All the results we have obtained with DTFB are consistent with the simple model presented above. Specifically, the quadratic dependence

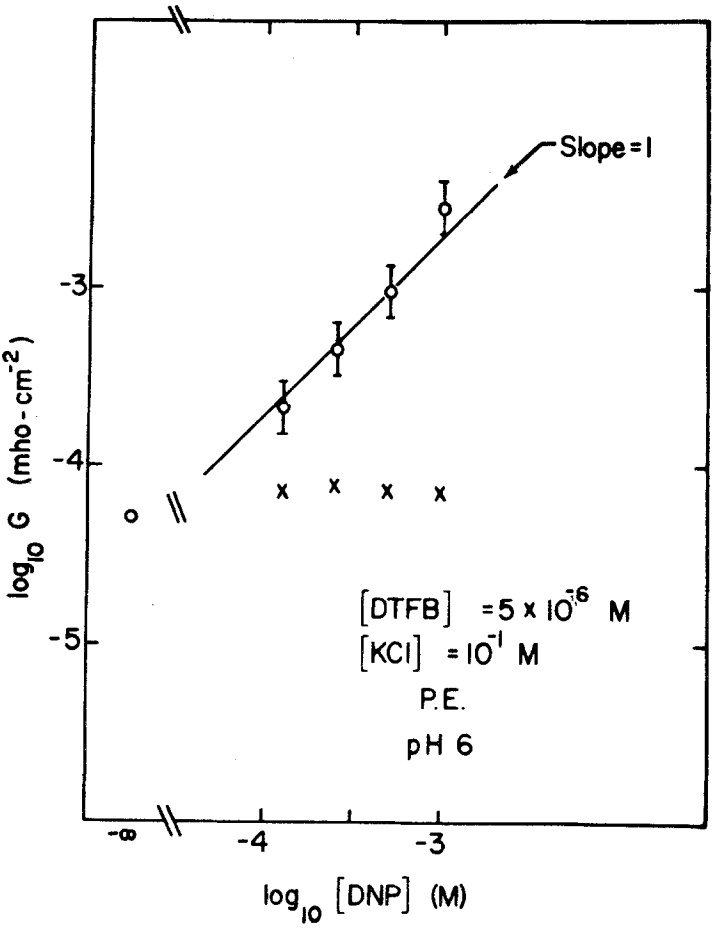

Fig. 8. Dependence of conductance on concentration of DNP in the presence of DTFB. Bilayers were formed from phosphatidyl ethanolamine, and the 10^{-1} M KCl solution was buffered with 5×10^{-3} M sodium citrate, 5×10^{-3} M sodium phosphate, and 5×10^{-3} M tris to pH 6. The crosses indicate the average of the measured values from 2 experiments, the instantaneous and steady-state values being identical. The circles indicate the conductance "corrected" for the effect of DNP on the surface potential of the bilayer membrane. *See text* for further details on this point. The vertical bars represent standard deviations, and the solid line illustrates the theoretically expected behavior predicted by Eq. (6)

of conductance on DTFB concentration (Figs. 1 and 3), the maxima in the conductance *vs.* pH curves (Figs. 2 and 4) and the depression in the conductance observed on negative (PG) *vs.* zwitterionic (PE) membranes (Figs. 1 and 3) are all predicted by Eq. (5) and are consistent with the permeant species being the HA_2^- complex. The importance of the unstirred layers or interfaces in determining the electrical properties of membranes

exposed to uncouplers is illustrated by two observations: bilayers in the presence of DTFB behave *as if* they were hydrogen electrodes and no potential is seen at constant pH when there is a gradient of DTFB concentration.

The model proposed by Bruner (1970) could also account for the quadratic dependence of conductance on concentration, but his model specifically predicts "a shift in the conductivity maximum towards lower pH with increasing uncoupler concentration". The shift is observed neither for DTFB (Fig. 4) nor for TTFB (Liberman & Topaly, 1968*b*). Furthermore, his model predicts that the conductance should vary inversely with the square of the proton concentration on the low pH side of the maximum whereas our data (Figs. 2 and 4) indicate the conductance depends inversely on the first power of the hydrogen ion concentration.

The available evidence implies that the anion of DTFB does not adsorb significantly to the bilayer membranes over the concentration range we have investigated. This is indicated by both the regular quadratic dependence of conductance on the concentration of DTFB (Figs. 1 and 3) and by the lack of any effect of DTFB on the conductance of the positively charged nonactin-potassium complex, which we have used as a "probe" of the surface potential. The conductance due to DTFB on the negative lipid PG is depressed, with respect to that observed on the zwitterionic lipid PE, by about the same factor that the conductance due to the positively charged nonactin complex is enhanced.[3] Since DTFB does not adsorb to the membrane, and since it does respond to the surface potential on the membrane it can be used as a negatively charged "probe" of this potential (McLaughlin, 1973).

TTFB. Since the substituted benzimidazoles DTFB and TTFB differ by only two chlorine atoms, it is not surprising that the bilayer data available in the literature for TTFB are also consistent with the model presented above. Liberman and his co-workers (Liberman, Mokhova, Skulachev & Topaly, 1968; Liberman & Topaly, 1968*a*) have demonstrated that the conductance depends approximately on the square of TTFB concentration. Deviations are observed only at high concentrations, when diffusion polarization is expected to be important (*see* our Fig. 1 and footnote 1), and at alkaline pH, when the anion might be expected to be the predominant permeant species, as discussed further by Neumcke and Bamberg (1974). Furthermore, the conductance *vs.* pH curves have a maximum at a pH of about the pK of TTFB. Liberman and Topaly (1968*a*, *b*) found a maximum at a pH of about 5.5; the pK of TTFB in an aqueous solution is 5.3 (Wilson *et al.*, 1971). Finally, bilayers in the presence of TTFB behaved as if they

were perfect hydrogen electrodes in the pH range between 4 and 7 (Markin, Pastushenko, Krishtalik, Liberman & Topaly, 1969) and we find that a gradient of TTFB produces no membrane potential, as expected from the model.

DNP

The anion of DNP adsorbs[4] to bilayers formed from PE (McLaughlin, 1972), PC, PC-C and 7-DC. The conductance *vs.* concentration of DNP curves were obtained on PE (McLaughlin, 1972) and PC-C bilayers and when these were corrected for the surface potential produced by anion adsorption, a quadratic relation was obtained in both cases, as predicted by Eq. (5). Our conductance *vs.* pH results with DNP on PE bilayers (Fig. 5) agree with those obtained by others on bilayers formed from other lipids. Hopfer, Lehninger and Thompson (1968), Liberman and Topaly (1968*a*), Lea and Croghan (1969) and Hopfer, Lehninger and Lennarz (1970), all found that the conductance produced by DNP on neutral or negative lipids has a maximum at a pH of about the pK for DNP. The shift in the maximum observed on a positive lipid (Hopfer *et al.*, 1970) is discussed elsewhere (McLaughlin, 1972). For the bilayer membrane to behave *as if* it were permeable only to hydrogen ions in the presence of DNP the system must be well buffered through the unstirred layers. We found that only in this case does the simplest form of the model presented above adequately describe the data. When comparisons are made of the relative effectiveness of uncouplers on mitochondria and model systems the buffer capacity of the systems must be taken into account, particularly for the less effective uncouplers.

DNP and DTFB Both Present

All the data we have obtained when both of these uncouplers are present simultaneously (Figs. 6–8) are compatible with the permeant species being a complex formed between the two different uncouplers, as predicted by Eq. 6. These observations strengthen the claim that such dimers really exist between the anion and undissociated acid of a single uncoupler. We have tried, with DNP, to isolate these dimers in organic solutions but failed, as discussed further in the Appendix.

Relevance of this Study to the Mitochondria

As oxidative phosphorylation takes place only in closed vesicles, the membrane would seem to be important for the coupling process. The

coupling process may be directly related to the gradient of potential and pH across the topologically closed surface (*see*, for example, Rottenberg, 1973, for a recent discussion of ion transport models), or the membrane may only provide the environment for certain chemical reactions. The following considerations are germane to a fair comparison of the effectiveness of uncouplers on mitochondria and artificial membranes.

The relative effectiveness of an uncoupler on a bilayer is usually defined in terms of the concentration required to produce a given conductance. For the two classes of weak acid uncouplers, this effectiveness obviously depends on the conductance level selected for the comparison. According to the level selected by Bakker *et al.* (1973), for example, TTFB (HA_2^- class, $G \sim [TTFB]^2$) is more effective than FCCP (carbonylcyanide *p* trifluoromethoxyphenylhydrazone) (A^- class, $G \sim [FCCP]$) whereas the level selected by Liberman *et al.* (1969) implies that FCCP is more effective than TTFB in enhancing the conductance.

The situation is further complicated because the nature of the dominant permeant species, A^- *vs.* HA_2^- can depend on the uncoupler concentration and pH as discussed further by Neumcke and Bamberg (1974). It is thus important that comparisons between the artificial membranes and the mitochondria be made at the same uncoupler concentration and pH. This might seem a trivial consideration, but the free concentration of uncoupler in a solution containing mitochondria appears never to have been measured in comparative studies and the implicit assumption that it is equal to the total concentration may be erroneous. Hemker (1962) has discussed this point with respect to certain dinitrophenols and Nicholls and Wenner (1970) have also found that for one of the more effective uncouplers, 3-*t*-butyl-5-chloro-2′-chloro-4-nitrosalicylanilide (S-13), the oxidation rate per mg of protein does depend on the mitochondrial concentration. Perhaps the dinitrophenols and S-13 are partitioning into or adsorbing onto the mitochondrial membrane and the free concentrations of uncouplers in the aqueous phases are being reduced? This possibility could easily be checked in future experiments on mitochondria by using a bilayer as an "uncoupler electrode", an idea which has already been utilized in a slightly different context (Grinius, Jasaitis, Kadziauskas, Liberman, Skulachev, Topali, Tsofina & Vladimirova, 1970).

The two different classes of uncouplers also seem to respond in a different way to surface potentials on bilayers. The conductance produced by the uncouplers of the HA_2^- class we have examined respond to the surface potential on the membrane as predicted by Eq. (5), whereas the conductance produced by an uncoupler of the A^- class (S-13) is much less responsive

to the surface potential (Hopfer *et al.*, 1970). The reason for this difference is not yet understood. It implies, however, that comparisons made between the effectiveness of uncouplers on artificial and biological systems should be made on lipid bilayers with the same surface potential as those of the mitochondria. The importance of lipid composition is discussed in a somewhat different context by Alkaitis, Merola and Lehninger (1972).

An important variable which appears to have been ignored in previous studies with uncouplers is the time required for the conductance of the bilayer to attain a steady-state value. The uncoupler, after being added to the aqueous phases must mix with the solutions, diffuse through the aqueous unstirred layers adjacent to the membrane, then partition into the membrane phase. Hladky (1973) has discussed these and other complicating processes (such as diffusional loss of the carrier from the bilayer into the torus, an artifact which was circumvented in these studies by simply making measurements on bilayers greater than 1 mm^2 in area with a relatively small torus) for the movement of carriers into membranes and may be consulted for a detailed theoretical analysis. We merely note here that for the well-characterized (e.g. Eisenman *et al.*, 1973) neutral carriers of alkali metal cations, valinomycin and nonactin, the conductance measured (McLaughlin & Foster, *unpublished data*) at a time t after mixing of the carrier in the bulk aqueous phase, $G(t)$, should and does increase towards the steady-state value $G(\infty)$ according to the expression:

$$\ln \{1 - G(t)/G(\infty)\} = -t/\tau.$$

The time constant τ, the time required for the conductance to increase to 63% its final value, is of the order of $\leqq 100$ sec for nonactin and 200 sec for valinomycin. This is in qualitative agreement with the prediction, assuming unstirred layer limitation, that the time constant should equal $d\delta k/2D$ where d is the thickness of the membrane, δ the thickness of the unstirred layers, D the diffusion coefficient in the aqueous phase and k the partition coefficient of the neutral carrier into the membrane. The partition coefficient of valinomycin into a bilayer has been estimated as 60,000 (Stark, Ketterer, Benz & Läuger, 1971), a number which included the valinomycin molecules adsorbed at the interface and is somewhat greater than the partition coefficient of 15,000 into a bulk solution of lipid in decane. If we take $k = 60{,}000$, $d = 50$ Å, $\delta = 200$ μ, $D = 5 \times 10^{-6}$ cm^2/sec the time constant calculated assuming no interfacial limitation is 60 sec for valinomycin. This order of magnitude calculation agrees about as well as could be expected with the experimental value of 200 sec and suggests that the interface does not provide a significant resistance to the movement of valinomycin into

the bilayer. Nonactin, which has a lower oil/water partition coefficient than valinomycin, has a lower time constant as expected. These and other (Hladky, 1973) results are consistent with the unstirred layers being the rate-limiting step for the movement of these carriers into the membrane, a hypothesis which was tested by more direct experiments and confirmed for a cyclic polyether (Eisenman *et al.*, 1973, *see* page 206).

For the uncouplers DNP and DTFB, plots of $\ln(1-\{G(t)/G(\infty)\}^{1/2})$ *vs.* t should yield straight lines, and indeed they do although DNP acts so rapidly we can only say that its time constant is $\leqq 100$ sec. For DTFB, however, the time constant was 500 sec. This is anomalously large: the partition coefficient of both DNP and DTFB into decane is of the order of unity (*see* Appendix) and the time constant expected on the basis of unstirred layer limitation is therefore of the order of milliseconds rather than hundreds of seconds. This suggests that the rate-limiting step for the movement of the neutral form of DTFB into the membrane occurs at the membrane-solution interface rather than through the unstirred layer. (The possibility that the partition coefficient of DTFB onto the membrane, including the interface, is $\sim 5\times 10^5$, is unlikely for several reasons, one being that an order of magnitude more DTFB than phospholipid molecules would be in the membrane at the highest aqueous concentrations of DTFB used in this study.) Irrespective of whether this suggestion is correct, we wish to stress that it takes DTFB significantly longer than DNP to enhance the conductance of a bilayer. In the swelling experiments of Bakker *et al.* (1973) the parameter measured was the swelling rate of a phospholipid vesicle 2 min after the addition of an uncoupler. It is thus not surprising that the relative potency of an uncoupler on this liposome system and its relative ability to enhance the steady-state conductance of a planar bilayer membrane are significantly different.

In previous studies with uncouplers on planar bilayer membranes the parameter generally measured was the steady-state current produced in response to an applied potential or the potential produced in response to a pH gradient. According to the chemiosmotic hypothesis, the important parameter for the action of an uncoupler on a mitochondrion is the rate at which it can transport H^+ down a concentration and/or a potential gradient. If this aspect of the chemiosmotic hypothesis is correct, these two parameters are related, but not necessarily in a simple manner, as discussed above. A lack of perfect correlation is not, therefore, a valid argument against the hypothesis. This point is also emphasized by Bakker *et al.* (1973), who exploited the more realistic model systems of Hinkel (1970), and of Singer and Bangham (1971) and Scarpa and de Gier (1971) to study un-

couplers. The correlation between the effectiveness of uncouplers on mitochondria and either the "Hinkel process" or the rate of swelling of liposomes was much better than that observed with the planar bilayers.

The experiments of Cunarro and Weiner (1973) provide perhaps the most convincing demonstration that there is a direct correlation between the ability of a weak acid to act as an uncoupler of oxidative phosphorylation and the ability of the weak acid to facilitate a passive flux of H^+ across a mitochondrial membrane. Utilizing poisoned mitochondria as their artificial system, they argued that the swelling induced in such mitochondria by uncouplers was a measure of net proton transport. They investigated the relation between the concentration of uncoupler required to induce passive swelling in poisoned mitochondria and the concentration required to release respiration in normal mitochondria. As the experiments were done under identical conditions, one expects all the variables discussed above (e.g. surface charge, buffer capacity, unstirred layers, interfacial limiting processes, adsorption of the uncoupler) to be identical and it is extremely gratifying that an excellent correlation was observed over some eight orders of magnitude of concentration.

Appendix

Bulk Phase Extraction Experiments

Using a method similar to that of Eisenman, Ciani and Szabo (1969), we attempted to isolate the HA_2^- complex of DNP by extracting it into a bulk hydrocarbon phase. A lipid-soluble cation must, of course, accompany the anionic form of DNP into the bulk phase to maintain electroneutrality. By vigorous shaking, 5 ml of decane were equilibrated with 2 ml of an aqueous solution containing various concentrations of DNP and of tetrabutylammonium (TBA^+) bromide at pH 6. The uptake of DNP into decane was monitored by measuring the UV absorbance with a Beckman Acta III spectrometer. (TBA^+ does not adsorb in the wavelength region of interest.) Some of the neutral form of DNP (HA) partitions into the decane, but there is an increase in absorbance in the presence of TBA^+. We found that the increase in uptake of DNP varied linearly with [DNP] and linearly with $[TBA^+]$.

The predicted relationship between the uptake of the HA_2^- species and the concentrations of DNP and TBA^+ was expressed by an equation similar to that of Eq. (27) in Eisenman *et al.* (1969). If the HA_2^- had existed in the decane in the dissociated form we would have seen a linear dependence on [DNP] and a dependence on the square root of $[TBA^+]$. If the HA_2^- were

associated with the tetrabutylammonium ion, we would have seen a dependence on the square of the [DNP] and on the first power of the [TBA^+].

Our results are consistent with the anionic or A^- form of DNP being associated with the cation TBA^+, and thus provide no information about the possible existence of the HA_2^- complex. Unless the relative uptake of HA_2^- to A^- can be greatly increased, it will not be possible to observe the HA_2^- by such a method of bulk phase extraction. A Born charging calculation indicates that increasing the dielectric constant of the hydrocarbon phase would decrease the ratio of partition coefficients and thus the relative uptake. At a pH closer to the pK, the uptake of HA_2^- would be increased, but the uptake of the neutral species would also be increased, and the change of absorbance would be too small to measure. It would appear that a colored, lipid-soluble cation which does not associate strongly with the anionic form of DNP is required, but we have not found such a cation. We mention our attempts at bulk phase extraction because it may be helpful to others to know that the postulated HA_2^- may be difficult to study except in thin membranes, where there is no requirement of electroneutrality because the thickness is less than the Debye length.

During the course of these experiments we have measured the partition coefficients for the neutral species of DNP and DTFB from 10^{-1} M KCl aqueous solution into decane. We found these to be 2.2 ± 0.7 for DNP and 1.25 ± 0.35 for DTFB, both in favor of the decane phase.

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