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The Kinetic Mechanism by which CCCP (Carbonyl Cyanide *m*-Chlorophenylhydrazone) Transports Protons across Membranes

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Summary. We demonstrate that a simple kinetic model describes the transport of protons across lipid bilayer membranes by the weak acid CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). Four parameters characterize this model: the adsorption coefficients of the anionic and neutral forms of the weak acid onto the interface (β_A and β_{HA}) and the rate constants for the movement of A^- and HA across the membrane (k_A and k_{HA}). These parameters were determined by equilibrium dialysis, electrophoretic mobility, membrane potential, membrane conductance, and spectrophotometric measurements. From these equilibrium and steady state measurements on diphytanoyl phosphatidylcholine/chlorodecane membranes we found that $\beta_A = \beta_{HA} = 1.4\ 10^{-3}$ cm, $k_A = 175\ s^{-1}$ and $k_{HA} = 12{,}000\ sec^{-1}$. These parameters and our model describe our kinetic experiments if we assume that the protonation reactions, which occur at the interfaces, remain at equilibrium. The model predicts a single exponential decay of the current in a voltage-clamp experiment. The model also predicts that the decay in the voltage across the membrane following an intense current pulse of short duration (~50 nsec) can be described by the sum of two exponentials. The magnitudes and time constants of the relaxations that we observed in both voltage-clamp and charge-pulse experiments agree well with the predictions of the model for all values of pH, voltage and [CCCP].

Key Words chemiosmotic theory · CCCP · uncouplers · protonophores · bilayer membranes

Introduction

Heytler and Prichard [15] and Heytler [14] first used CCCP to uncouple mitochondria and chloroplasts. According to the chemiosmotic theory [25], the uncoupling action of weak acids such as CCCP is due to their ability to transport protons across the electrically insulating lipid bilayer component of a biological membrane. Hopfer, Lehninger and Thompson [17] provided support for this interpretation by demonstrating that CCCP increases the conductance of phospholipid bilayers by several orders of magnitude. Skulachev and his coworkers [20, 31] demonstrated that the ability of several weak acids, including CCCP, to increase the conductance of bilayer membranes correlated well with their ability to uncouple oxidation from phosphorylation in mitochondria. LeBlanc [18] and O'Shaughnessy and Hladky [26] studied the mechanism by which CCCP transports protons across bilayer membranes. Our kinetic description of the protonophoric activity of CCCP confirms and extends these measurements. In an earlier study, we used a simple kinetic model to describe the proton ionophore activity of FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) [6]. In this study, we demonstrate that this model also describes the molecular mechanism by which CCCP transports protons across phospholipid bilayer membranes.

CCCP is also used extensively to study bacteria. For example, it has been used to investigate energy coupling [28] and to inhibit adenylate cyclase activity [27] in *Escherichia coli*, to block calcium transport in membrane vesicles from *Azobacter vinelandii* [7], and to study bacterial survival in aquatic environments [30]. Harold [13] has reviewed energy transduction in bacteria; McLaughlin and Dilger [24] and Terada [33] have reviewed the transport of protons across membranes by weak acids.

Theory

In a previous publication we described in detail a model for the transport of protons across a bilayer membrane by a weak acid protonophore such as FCCP [6]. We sketch here only the basic features of the model and list only the equations that relate the experimental data to the theoretical parameters. As illustrated in Fig. 1, the anion, A^-, is the only charged species that moves within the membrane in

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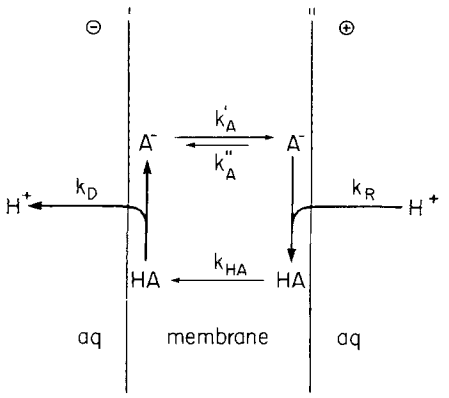

Fig. 1. The mechanism by which CCCP transports protons across lipid bilayer membranes. The circled positive and negative signs indicate there is a potential difference across the membrane. The rate constants k_R and k_D refer to the heterogenous recombination and dissociation of an aqueous proton with an anion adsorbed to the membrane. The rate constants k'_A and k''_A refer to the voltage-dependent translocation of the anion A from the ' to the " and from the " to the ' interfaces, respectively. The rate constant k_{HA} refers to the movement of HA between the two interfaces

our model: the rate constants for the movement of the anion are k'_A and k''_A. The anion adsorbed to the interface recombines with protons from the aqueous phase with a rate constant k_R. The adsorbed HA species dissociates with a rate constant k_D. The equilibrium association constant is $K = k_R/k_D$, and log K is the surface pK. The diffusion of HA across the membrane does not depend on voltage and proceeds with a rate constant k_{HA}. The number of CCCP molecules adsorbed to the membrane, N_o, does not change with time and is equal to the equilibrium value:

$$N_o \equiv 2N_A + 2N_{HA} \tag{1}$$

where N_A and N_{HA} are the surface concentrations of A^- and HA adsorbed to one interface. The adsorption coefficients are defined by

$$\beta_A \equiv N^o_A/[A^-] \tag{2}$$

and

$$\beta_{HA} \equiv N^o_{HA}/[HA] \tag{3}$$

where the square brackets denote the concentrations in the bulk aqueous phases and the superscript o denotes the equilibrium value.

Our kinetic measurements with the protonophore FCCP demonstrated that the interfacial reactions were always at equilibrium: $k_R[H^+]$, $k_D \gg k_A$, k_{HA}. We make the same assumption for CCCP, which greatly simplifies the kinetic equations. The assumption is also consistent with the experimental results.

Under voltage-clamp conditions the system is at equilibrium for times $t < 0$. At $t = 0$ a voltage of V is applied across the membrane. The current density, neglecting the capacitive transient, is given by [6]

$$\begin{aligned} I(t) &= -F(k'_A N''_A - k''_A N''_A) \\ &= I(\infty)[1 + \alpha \exp(-\lambda t)] \end{aligned} \tag{4}$$

where F is the Faraday, $I(\infty)$ is the steady-state current and

$$\alpha = k'_A + k''_A)/2K[H^+]k_{HA} \tag{5}$$

$$\lambda = (k'_A + k''_A + 2k_{HA}K[H^+])/(1 + K[H^+]). \tag{6}$$

The time constant of the relaxation is defined by $\tau \equiv 1/\lambda$. The dependence of k'_A and k''_A on voltage is a function of the potential energy barrier the A^- species encounters within the membrane. The barrier is due mainly to "image" forces and may be represented, to a good first approximation, by a trapezoid [12, 16]. If the minor base of the trapezoid spans a fraction b of the membrane, k'_A and k''_A depend on voltage in the following manner:

$$k'_A = k_A(bu/2) \exp(u/2)/\sinh(bu/2) \tag{7}$$

$$k''_A = k_A(bu/2) \exp(-u/2)/\sinh(bu/2) \tag{8}$$

where $u = FV/RT$ is the reduced voltage, F is the Faraday constant, R is the gas constant, and T is the absolute temperature. When $b \to 0$ the current $I(0)$ is proportional to $\sinh(u/2)$, as predicted by an Eyring model with a single barrier in the center of the membrane, and when $b \to 1$ the current is proportional to u, as predicted by a Nernst-Planck model with a square barrier. In the limit of small voltages, $u \ll 1$, k'_A and k''_A are independent of the barrier shape:

$$k'_A \simeq k_A(1 + u/2) \tag{9}$$

$$k''_A \simeq k_A(1 - u/2). \tag{10}$$

It follows that the conductance measured in the limit that voltage and time go to zero is

$$G(0,0) = F^2\beta_A[A^-]k_A/RT. \tag{11}$$

Under charge-pulse conditions it is assumed that the system is at equilibrium for $t < 0$ and that at $t = 0$ the membrane capacitance C_m is charged instantaneously to a voltage $V^o \ll 25$ mV. The subse-

quent decay in voltage has two exponential relaxations under the conditions described above [6]:

$$V(t) = V^o[a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t)] \quad (12)$$

where a_1, a_2, λ_1 and λ_2 are functions of the rate constants and the equilibrium constants and are given by Eqs. (13)–(15):

$$\lambda^2 - \left(\frac{(Bk_A N_o + 4k_A + 4k_{HA}K[H^+])\lambda}{2(1 + K[H^+])}\right) + \left(\frac{BK[H^+]k_A k_{HA} N_o}{(1 + K[H^+])^2}\right) = 0 \quad (13)$$

$$a_1 + a_2 = 1 \quad (14)$$

$$a_1\lambda_1 + a_2\lambda_2 = Bk_A N_o/2(1 + K[H^+]) \quad (15)$$

where $B = F^2/RTC_m$.

It is convenient to define the quantities K_A and K_{HA}, which are determined directly from the kinetic charge-pulse experiments [6]:

$$K_{HA} = k_{HA}K[H^+]/(1 + K[H^+]) \quad (16)$$

$$K_A = k_A/(1 + K[H^+]). \quad (17)$$

Materials and Methods

Sonicated unilamellar vesicles and multilamellar vesicles were formed from egg phosphatidylcholine (Avanti, Birmingham, AL). Planar bilayer membranes were formed from a 1 to 2% (wt/vol) solution of diphytanoyl phosphatidylcholine, bacterial phosphatidylethanolamine (both from Avanti, Birmingham, AL) or monoolein (Sigma, St. Louis, MO) in either *n*-decane (Supelco, Belefonte, PA) or 1-chlorodecane (Aldrich, Milwaukee, WI). The chlorodecane was purified by passing it through an aluminum oxide column (Baker, Phillipsburg, NJ).

Aqueous solutions were prepared with 18 MΩ-cm water (Millipore Super Q System, Bedford, MA) that was subsequently bi-distilled in a quartz apparatus.

CCCP (Calbiochem-Behring, La Jolla, CA) was prepared as a concentrated solution in ethanol. Small volumes of the solution were added to the aqueous phases. The final ethanol concentration never exceeded 1%; control experiments demonstrated that this concentration of ethanol had no significant effect on the parameters that we measured.

The vesicles used in the equilibrium dialysis experiment were sonicated and centrifuged [4]. The aqueous solutions contained 0.1 M NaCl, 0.001 M citric acid, and 0.001 M bicine. The pH of the aqueous solutions was adjusted to 3.9 to determine the adsorption coefficient of the neutral form (HA) or to 8.2 to determine the adsorption coefficient of the anionic form (A^-) of the protonophore. CCCP was added to the solution containing the sonicated vesicles (*cis* side) and this mixture was dialyzed in Teflon chambers against an aqueous solution (*trans* side) of identical [NaCl], [buffer] and pH. The equilibrium concentration of CCCP in the *trans* solution was determined spectrophotometrically; the lipid concentration was determined by phosphate analysis [21]. The area of the vesicles was estimated by assuming that each phospholipid molecule occupies 70 Å^2.

The aqueous pK of CCCP, pK^{aq}, was measured spectrophotometrically in the presence of 1 M NaCl and 5 mM buffer (citric acid, MOPS (3-N-morpholino-propanesulfonic acid) or AMP (2-amino-2-methyl-1-propanol) for pH = 3.7, 6 or 10.1, respectively). The surface pK of CCCP adsorbed onto egg phosphatidylcholine sonicated vesicles was determined in buffered 1 M NaCl and in 0.1 M NaCl solutions. The buffers for these experiments were citric acid, MES (2-N-morpholino-ethanesulfonic acid), or TRIS (tris-hydroxymethyl-aminomethane) for pH = 3.5, 6.2 or 9.5. The concentration of buffer was 25 mM for the 1 M NaCl, and 50 mM for the 0.1 M NaCl solutions. The total concentration of CCCP in the solution was 12.5 μM and the lipid concentration was 1.5 mg/ml. Most of the CCCP was bound to the lipid vesicles.

The permeability of bilayers to the neutral form of CCCP was deduced by the method of LeBlanc [18]: the potential across a planar bilayer membrane, ΔV, produced by a difference in pH, ΔpH, was measured. The membranes were formed from diphytanoyl phosphatidylcholine on a hole (1–2 mm diameter) in a Teflon partition that separated the two compartments of a Teflon chamber. The aqueous solutions on the two sides of the membrane were identical (0.1 M NaCl, 50 mM Na borate, 50 mM Na bicarbonate; [CCCP] = 10^{-5} M), except for the pH. The membrane potentials were measured with a matched pair of calomel electrodes and a Keithley model 602 electrometer (Cleveland, OH).

Microelectrophoresis experiments were performed to measure the adsorption of the anionic form of CCCP onto lipid bilayer membranes. The multilamellar vesicles were prepared from egg phosphatidylcholine [3]. The aqueous solutions contained either 0.1, 0.01 or 0.001 M NaCl with 0.001 or 0.0001 M MOPS buffer at pH 7.6.

Voltage-clamp and charge-pulse experiments were performed with planar bilayer membranes separating two well-stirred aqueous solutions of identical composition. The solutions for the voltage-clamp experiments contained 1 M NaCl, 0.1 M MES, 0.1 M MOPS and 0.1 M bicine, adjusted to pH 6.3, 7.3 or 8.3. The solutions for the charge-pulse experiments contained 1 M NaCl and one of the buffers above. The voltage-clamp experiments were performed by applying a voltage across the bilayer and observing the subsequent relaxations in the current [9], whereas the charge-pulse experiments were performed by applying an intense current pulse of short duration (about 50 nsec) across the membrane and observing the resultant voltage relaxations [5]. Details of the kinetic measurements and analysis can be found in Benz and McLaughlin [6].

The temperature for all of the experiments was 21–22°C, except for the electrophoresis measurements (T = 25°C).

Results

Equilibrium and Steady-State Measurements of β_A, β_{HA}, k_A, k_{HA}, pK and pK^{aq} of CCCP

The adsorption coefficients of both the neutral and anionic forms of CCCP to sonicated egg phosphatidylcholine vesicles were measured by equilibrium dialysis. The adsorption coefficients β_A and β_{HA} are $2.0 \pm 0.1 \times 10^{-3}$ cm and $2.2 \pm 0.2 \times 10^{-3}$ cm, respectively (±SD of three sets of three measurements).

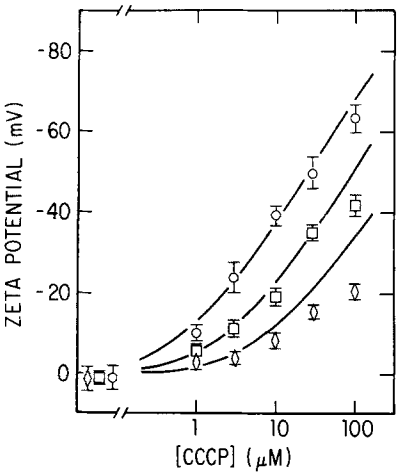

Fig. 2. A plot of the zeta potential of egg phosphatidylcholine multilamellar vesicles as a function of the aqueous concentration of CCCP. The zeta potentials of the vesicles were zero in the absence of CCCP. The adsorption of CCCP onto the vesicles produced a negative zeta potential. The curves are the predictions of a combination of the Gouy equation, the Boltzmann relation, and a Henry's law adsorption isotherm for 0.001 M NaCl (circles), 0.01 M NaCl (squares) and 0.1 M NaCl (diamonds) when $\beta_A = 2 \times 10^{-3}$ cm. The pH was 7.6 and $T = 25°C$

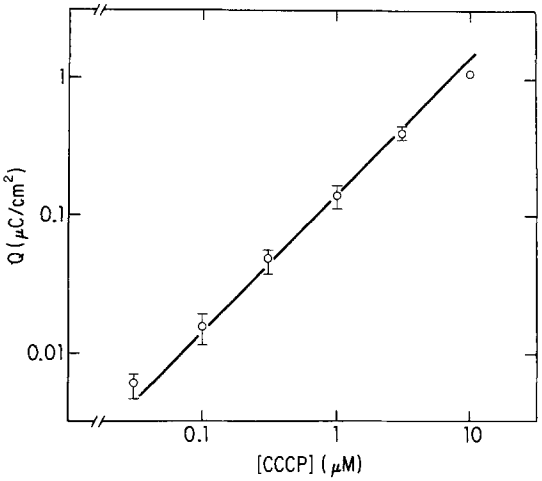

Fig. 3. The number of anions adsorbed onto one interface of a diphytanoyl phosphatidylcholine/chlorodecane bilayer plotted as a function of [CCCP] at pH 8.3. The charge adsorbed onto one interface, Q (C cm^{-2}), is obtained by integrating the current *vs.* time curves following the application of a 200-mV clamp across the membrane (ignoring the capacitance spike and subtracting the steady-state current). The line has a slope of 1 and represents an adsorption coefficient $\beta_A = 1.4\ 10^{-3}$ cm

We also made electrophoretic mobility measurements on multilamellar vesicles to determine the value of β_A. The zeta potential, ζ, is the electrostatic potential at the hydrodynamic plane of shear; it is determined from the electrophoretic mobility, u, using the Helmholtz-Smoluchowski equation:

$$\zeta = \frac{u\eta}{\varepsilon_o \varepsilon_r} \tag{18}$$

where η is the viscosity of the aqueous phase, ε_r is the dielectric constant of the aqueous phase and ε_o is the permittivity of free space. The results of mobility measurements are shown in Fig. 2. The adsorption of CCCP is described by a combination of the Gouy equation [23], the Boltzmann relation, and a Henry's law adsorption isotherm:

$$\sinh(F\psi_o/2RT) = A\sigma/\sqrt{C} \tag{19}$$

and

$$\sigma = 10^3 F\beta_A[A^-] \exp(F\psi_o/RT) \tag{20}$$

where ψ_o is the surface potential, C is the monovalent ion concentration in M, σ is the surface charge density in $C\ m^{-2}$, β_A is the adsorption coefficient of A^- in m, $[A^-]$ is the concentration of A^- in the aqueous phase in M and A is a constant = 8.6 $M^{1/2}$ $m^2\ C^{-1}$. The potential a distance x from the surface of the membrane, $\psi(x)$, can be described by the Gouy-Chapman theory [2]. The zeta potential can be related to the surface potential if we assume that the hydrodynamic plane of shear is 1, 0.4 or 0.2 nm from the surface of the membrane in 0.001, 0.01 or 0.1 M NaCl solutions, respectively [22]. A nonlinear least squares fit of Eqs. (19) and (20) to the 0.001 M NaCl data (upper curve) gave $\beta_A = 2 \times 10^{-3}$ cm. All of the curves are drawn with this value of β_A. The fits are reasonable except at high concentrations of CCCP[1]. The value of β_A obtained from the mobility measurements agrees well with the equilibrium dialysis measurements.

We can also determine β_A from voltage-clamp experiments. By applying a large voltage (200 mV) across the membrane and integrating the current, we obtain the charge adsorbed to one side of the membrane, Q (Fig. 3). The number of CCCP anions adsorbed is

$$N_A^o = Q/F = \beta_A[A^-]. \tag{21}$$

Measurements performed at pH 8.3 and 10^{-7} M $\leq$ [CCCP] $\leq 10^{-6}$ M, where Q depends linearly on

[1] The deviations of the zeta potentials from the predictions of the Gouy-Chapman theory observed in 0.1 M NaCl (Fig. 2) are presumably due to the adsorbed CCCP anions producing either a "boundary" potential within the membrane or a change in the dipole potential at the interface [1, 6, 23, 34]. These potentials hinder further adsorption of CCCP anions but do not affect the electrophoretic mobility.

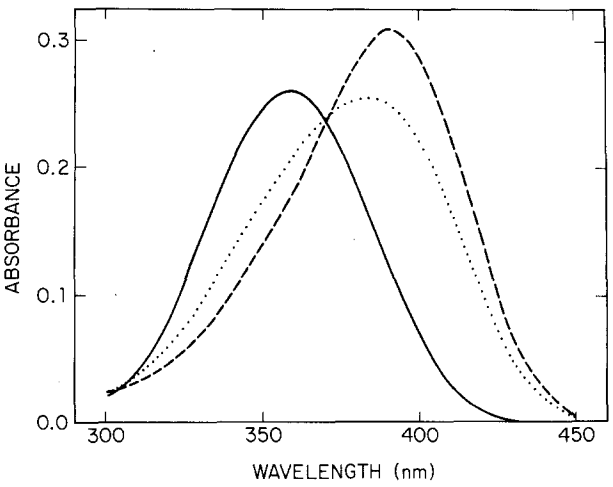

Fig. 4. Absorption spectra of CCCP in the presence of sonicated unilamellar vesicles. The solid line illustrates data obtained at pH 3.9, the dotted line pH 6, and the dashed line pH 10. The average of the results at 348 and 400 nm show that pK = 5.9. The solutions contained 1 M NaCl, 25 mM buffer (citric acid, MES, or TRIS), 12.5 μM CCCP and T = 22°C

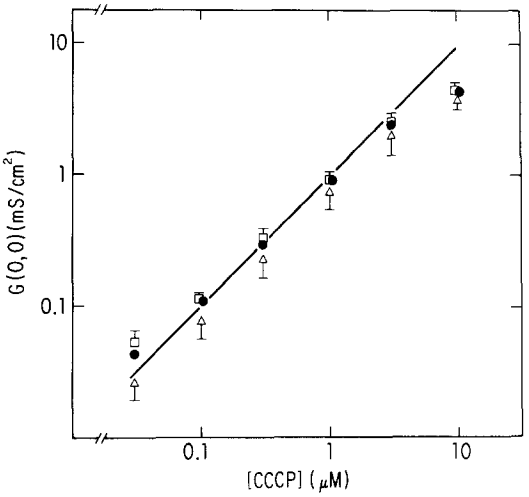

Fig. 5. A plot of the low voltage instantaneous conductance of diphytanoyl phosphatidylcholine/chlorodecane bilayers as a function of [CCCP]. The triangles, squares, and filled circles illustrate the data obtained at pH 6.3, 7.3, and 8.3, respectively. The straight line is the least squares best fit to the pH 8.3 data for 0.1 μM $\leq$ [CCCP] $\leq$ 1.0 μM. The value of β_A deduced from the Q *vs.* [CCCP] data illustrated in Fig. 3 allows us to calculate a value of k_A = 175 sec^{-1}, using Eq. (11)

[CCCP], yield a value of $\beta_A = 1.4 \times 10^{-3}$ cm. This value agrees within a factor of two with the results of the equilibrium dialysis and zeta potential measurements, which were made on sonicated and multilamellar egg phosphatidylcholine vesicles, respectively.

Our equilibrium dialysis measurements demonstrate that $\beta_A \simeq \beta_{HA}$; we expect the logarithm of the surface association constant to equal the logarithm of the aqueous constant, pK = pK^{aq}. Our spectrophotometric measurements of CCCP adsorbed to sonicated egg phosphatidylcholine vesicles demonstrate that pK = 5.9 (*see* Fig. 4). This value agrees, within experimental error, with the value we obtained spectrophotometrically, pK^{aq} = 6.0 (*data not shown*), and that obtained by LeBlanc [18], pK^{aq} = 6.1.

The rate constant for the movement of the anion across the membrane, k_A, can be deduced from the conductance data in Fig. 5 using Eq. (11). If $\beta_A = 1.4 \times 10^{-3}$ cm, the value we measure for diphytanoyl phosphatidylcholine/chlorodecane membranes, then k_A = 175 sec^{-1}.

The permeability of planar bilayer membranes to the neutral form of CCCP was deduced from membrane potential experiments [18, 24]: we measured the dependence of the membrane potential produced by a unit change in pH, $\Delta V/\Delta$pH, on pH (*see* Fig. 6). We assume that the A$^-$ form of CCCP is the sole charged permeant species, an assumption that is consistent with the results illustrated in Fig. 5. When the membrane separates two solutions of

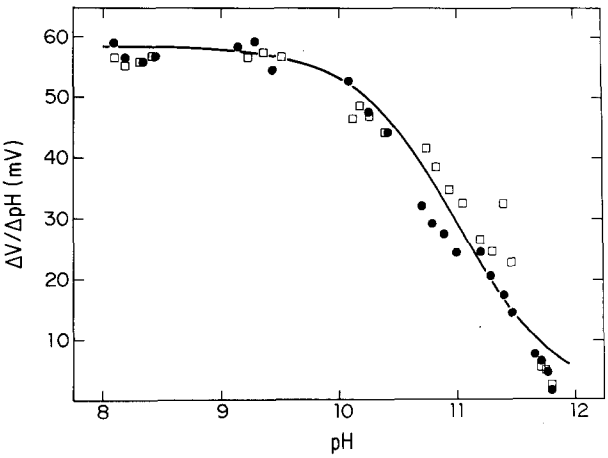

Fig. 6. A plot of the membrane potentials induced by pH gradients in the presence of 10^{-5} M CCCP on each side of the membrane. The theoretical curve is from Eq. (22) assuming that the permeability of the membrane to HA is P_{HA} = 20 cm sec^{-1}. The filled circles represent measurements on diphytanoyl phosphatidylcholine/decane membranes, and the open squares represent measurements on diphytanoyl phosphatidylcholine/chlorodecane membranes

different pH and CCCP behaves as a protonophore (Fig. 1), the potential should be Nernstian, $\Delta V/\Delta$pH = 2.303 (RT/F) = 58.5 mV at 22°C. As the pH increases, the concentration of HA at both interfaces decreases, the back diffusion of HA becomes rate limiting, and CCCP behaves like a lipid-soluble anion. It is apparent in Fig. 6 that the potential is Nernstian for pH $\leq$ 9 and that $\Delta V/\Delta$pH is sub-Nern-

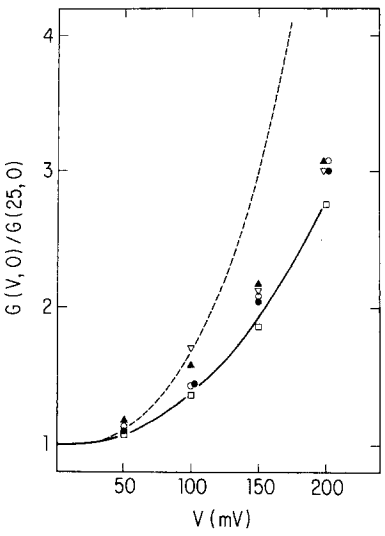

Fig. 7. Conductance-voltage curves for CCCP. The initial conductance, $G(V,0)$, of diphytanoyl phosphatidylcholine/chlorodecane membranes was measured as a function of voltage, V. $G(V,0)$ is divided by the initial conductance at 25 mV, $G(25,0)$, then plotted against the applied voltage: pH 6.3, [CCCP] = 10^{-7} M (inverted triangles); pH 6.3, [CCCP] = 3×10^{-7} M (filled triangles); pH 7.3, [CCCP] = 10^{-7} M (squares); pH 8.3, [CCCP] = 10^{-7} M (open circles) and pH 8.3, [CCCP] = 3×10^{-7} M (filled circles). The results demonstrate that the conductance-voltage relationship is independent of pH and [CCCP] as the model predicts. The data points represent average values for at least 5 membranes. The dashed line is the prediction of Eq. (23) in the limit that the width $b \to 0$, a single Eyring barrier. The solid line is the prediction of Eq. (23) assuming a trapezoidal barrier with fractional width of the minor base $b = 0.6$

stian for pH > 10. We obtain a value for the permeability of the neutral form, P_{HA}, by fitting Eq. (22) to the data. We assume that the aqueous diffusion coefficients of the anionic and neutral forms of CCCP are $D_A = D_{HA} = 5 \times 10^{-6}$ cm^2/sec, and that the thickness of the Nernstian unstirred layer is $\delta \simeq 100$ μm [8]. Note that $D/2\delta$ is the permeability of the two unstirred layers to HA. A reasonable fit to the data in Fig. 6 is obtained using Eq. (22) with P_{HA} = 20 cm sec^{-1}:

$$dV/dpH = 2.303(RT/F)\frac{([H^+]K^{aq})(1 + 2\delta P_{HA}/D)}{1 + ([H^+]K^{aq})(1 + 2\delta P_{HA}/D)}. \quad (22)$$

We obtained similar data with both decane and chlorodecane membranes (Fig. 6). Thus, the dielectric constant of the membrane has little effect on the permeability of the membrane to the neutral form of CCCP. We can use the permeability of the membrane to HA to deduce a value for the rate constant k_{HA}. P_{HA} represents the permeability of the entire membrane to HA. If the interfaces exert no resistance to HA, then $P_{HA} = \beta_{HA} k_{HA}$. From our equilibrium experiments, we found that the adsorption coefficients, β_A and β_{HA}, of CCCP are equal. In our conductance experiments we used diphytanoyl phosphatidylcholine/chlorodecane membranes and found that $\beta_A = 1.4 \times 10^{-3}$ cm. We will assume here, and throughout the remainder of the paper, that for diphytanoyl phosphatidylcholine/chlorodecane membranes $\beta_A = \beta_{HA} = 1.4 \times 10^{-3}$ cm. If $\beta_{HA} = 1.4 \times 10^{-3}$ cm, then $k_{HA} = 12{,}000$ sec^{-1}.

We determine the shape of the energy barrier the membrane presents to the anion A^- by measuring the instantaneous conductance as a function of voltage [12, 16]. If we assume the barrier is a trapezoid with a minor base that spans a fraction b of the membrane, we obtain a fit to the data in Fig. 7 assuming $b = 0.6$, which is similar to the value (b = 0.57) obtained by O'Shaughnessy and Hladky [26]. The solid line was drawn using Eq. (23), which is obtained by combining Eqs. (4), (7) and (8):

$$G(V,0)/G(0,0) = b \sinh(u/2)/\sinh(bu/2). \quad (23)$$

In our simple model, the shape of the barrier and the G-V curve should be independent of both the concentration of the uncoupler and the pH of the solution. The data in Fig. 7 confirm this prediction.

Kinetic Voltage-Clamp Experiments

When we applied a voltage across a bilayer membrane and measured the subsequent decay of the current we detected only one exponential relaxation at all voltages, all concentrations of CCCP, and all values of pH. Similar results were obtained with the protonophore FCCP, and typical experimental results can be seen in Benz and McLaughlin [6]. The model predicts that both the magnitude of the relaxation, α, and the time constant, τ, should be independent of [CCCP]. The data shown in Fig. 8*A* and *B* for diphytanoyl phosphatidylcholine/chlorodecane membranes when pH = 8.3 show α and τ are independent of [CCCP] for [CCCP] $\leq 10^{-6}$ M. Furthermore, the model predicts that with increasing voltage α should increase and τ should decrease. The lines illustrate the predictions of the model for V = 25, 100 and 200 mV using Eqs. (5) and (6) with the values of the model parameters determined from the independent equilibrium and steady-state measurements. The predictions for α (Fig. 8*A*) are satisfactory at low voltage. The predictions for τ (Fig. 8*B*) show excellent agreement at all voltages. For [CCCP] > 10^{-6} M, α decreases and τ increases with increasing concentration of CCCP, changes that are presumably due to CCCP producing an

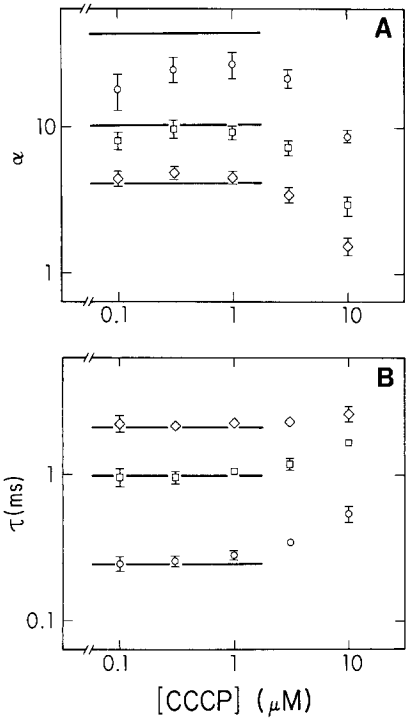

Fig. 8. The amplitude, α, and the time constant, τ, of the relaxations in the current observed in voltage-clamp experiments plotted as a function of [CCCP] on a log-log scale. The diamonds, squares and circles illustrate the results obtained at 25, 100 and 200 mV, respectively, for pH 8.3. The line through the 25, 100 and 200 mV data illustrate the model's predictions

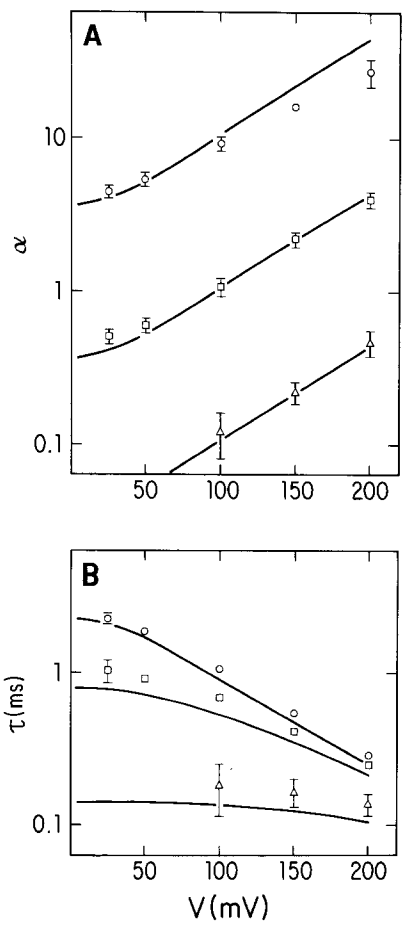

Fig. 9. Dependence of the amplitude, α, and the time constant, τ, on pH; the scales for α and τ are logarithmic. The planar bilayer membranes were formed from diphytanoyl phosphatidylcholine in chlorodecane. The triangles, squares and circles represent the results obtained at pH 6.3, 7.3 and 8.3, respectively. The lines illustrate the model's predictions, which are based on the values of the independently measured parameters

electrostatic "boundary" potential within the membrane [6].[2]

The model also predicts how α and τ should vary with pH. The model predicts [Eq. (5)] that the magnitude of the relaxation should increase by an order of magnitude for a unit increase in pH. The amplitudes of the relaxation at pH = 6.3, 7.3 and 8.3 are presented in Fig. 9*A*. The curves illustrate the predictions of the model, which are calculated from Eq. (5) using the results from the equilibrium and steady-state experiments. The predictions of the model fit the data well. The model also predicts that the time constant should decrease when either the pH decreases or the voltage increases. These predictions are illustrated by the lines in Fig. 9*B*. The experimental values of τ conform to the predictions of the model.

Charge Pulse Experiments

The results of the charge pulse experiments with CCCP were similar to those reported with FCCP [6]. The voltage decay following a charge pulse of about 50 nsec duration could always be described with two exponential relaxations. There was never any indication of an additional fast relaxation, even at times as short as 300 nsec. The result is consistent with our assumption that the heterogeneous reaction illustrated in Fig. 1 is approximately at equilibrium throughout the relaxation process. We analyzed the charge pulse data using Eqs. (13)–(17) and obtained estimates of K_{HA}, K_A and N_o [Eq. (1)].

We estimate the values of k_A and k_{HA} by measuring K_A and K_{HA} as a function of pH. Table 1 shows the results of charge-pulse experiments performed in the range $6.3 < \text{pH} < 8.3$. When the pH increases, K_{HA} decreases markedly, as predicted by Eq. (16). When the pH increases from 6.3 to 8.3, K_A increases by only a factor of two, as predicted by Eq. (17). Thus the pH dependence of the charge-pulse kinetic data is consistent with the predictions

[2] *See* footnote 1, p. 182.

Table 1. Charge pulse relaxation data from PC/chlorodecane or PE/chlorodecane bilayer membranes

pH	τ_1/μsec	τ_2/msec	a_2	K_{HA}/sec^{-1}	K_A/sec^{-1}	N_o/pmol cm^{-2}
			PC membranes			
6.3	—	0.68 ±0.064	1	—	—	—
6.7	250 ±52	0.65 ±0.046	0.89 ±0.069	1800 ±360	120 ±51	5.3 ±3.4
7.0	280 ±16	0.72 ±0.064	0.65 ±0.083	1200 ± 67	230 ±11	3.8 ±0.4
7.3	310 ±19	1.1 ±0.034	0.36 ±0.031	600 ± 23	260 ±16	3.9 ±0.2
7.7	280 ±18	2.3 ±0.13	0.21 ±0.018	270 ± 26	280 ±35	3.7 ±0.4
8.0	290 ±31	4.1 ±0.41	0.22 ±0.013	150 ± 17	310 ±30	3.3 ±0.2
8.3	310 ±24	7.9 ±0.34	0.23 ±0.014	80 ± 4	330 ±28	2.8 ±0.2
8.7	330 ±45	(12)	0.24 ±0.012		340 ±51	2.8 ±0.4
			PE membranes			
6.3	100 ±44	0.20 ±0.023	0.84 ±0.060	4200 ±520	230 ±86	10 ±2.8
7.3	180 ±12	0.55 ±0.026	0.44 ±0.037	1300 ± 50	470 ±32	3.1 ±0.1
7.5	200 ±22	0.81 ±0.084	0.30 ±0.021	810 ±110	420 ±30	4.0 ±0.7
8.3	230 ±18	4.6 ±0.25	0.27 ±0.009	150 ± 7	570 ±52	2.1 ±0.1

Relaxation data obtained from either diphytanoyl phosphatidylcholine/chlorodecane (PC) or bacterial phosphatidylethanolamine/chlorodecane (PE) bilayer membranes. The aqueous phases contained either 3 μM (pH 6.3, PE) or 1 μM (all other measurements) CCCP. The constants K_{HA}, K_A and N_o in Tables 1–3 were calculated from the relaxation data using Eqs. (1) and (13)–(15) and $C_m = 0.8$ μFcm^{-2}. The results (±SD) were obtained from at least four different membranes.

Table 2. Charge pulse relaxation data from PC/chlorodecane bilayer membranes

[CCCP]/μM	τ_1/μsec	τ_2/msec	a_2	K_{HA}/sec^{-1}	K_A/sec^{-1}	N_o/pmol cm^{-2}
0.001	—	700 ±100	1	—	—	—
0.01	—	90 ± 11	1	—	—	—
0.1	780 ±61	7.0 ± 1.3	0.87 ±0.032	320 ± 48	250 ± 4	0.45 ±0.07
1.0	270 ±33	1.4 ± 0.15	0.23 ±0.012	410 ± 19	270 ±40	4.0 ±0.3
10	110 ±19	0.45 ± 0.15	0.074 ±0.009	1300 ±340	170 ±28	22 ±1.0

Relaxation data obtained from diphytanoyl phosphatidylcholine/chlorodecane bilayer membranes at pH 7.5. The results (±SD) were obtained from at least four membranes.

of the simple model, using the surface pK determined from direct, independent spectroscopic measurements. Figure 10 illustrates the fit to the K_{HA} data in Table 1 using pK = 5.9 and k_{HA} = 15,000 sec^{-1} for phosphatidylcholine and 30,000 sec^{-1} for phosphatidylethanolamine membranes. The value of k_{HA} obtained from the charge-pulse measurements on phosphatidylcholine membranes agrees well with the value obtained from independent membrane potential measurements (k_{HA} = 12,000 sec^{-1}: Fig. 6) and voltage-clamp measurements on phosphatidylcholine membranes. On the other hand, the agreement between the values for k_A obtained from charge-pulse and voltage-clamp experi-

Table 3. Lipid dependence of relaxation data

Lipid	[CCCP]/μM	τ_1/μsec	τ_2/msec	a_2	K_{HA}/sec^{-1}	K_A/sec^{-1}	N_o/pmol cm^{-2}
Monoolein	1	—	3.1 ±0.42	1	—	—	—
	3	480 ±25	1.0 ±0.02	0.70 ±0.045	790 ±54	99 ± 10	4.9 ±0.5
Diphytanoyl Phosphatidylcholine	1	310 ±24	7.9 ±0.34	0.23 ±0.014	80 ± 4	330 ± 28	2.8 ±0.2
Phosphatidylethanolamine	1	230 ±18	4.6 ±0.25	0.29 ±0.009	150 ± 7	560 ± 52	2.1 ±0.1
	3	110 ±22	3.7 ±0.60	0.13 ±0.010	160 ±25	550 ±110	5.5 ±0.5

Relaxation data obtained from phospholipid/chlorodecane bilayer membranes. The aqueous phases were buffered to pH 8.3 and contained the indicated concentration of CCCP.

ments on phosphatidylcholine/chlorodecane membranes is not as good: the mean value is 280 sec^{-1} from charge pulse and 175 sec^{-1} from voltage-clamp measurements. The difference is presumably due to a difference in the age of the membranes. The charge-pulse experiments were performed about 20 min after the membranes became optically black. The voltage-clamp experiments were not performed until the membranes had aged for at least 1 hr; no further changes were apparent from 1–6 hr. Chlorodecane presumably moves out of the bilayer over time. This lowers the dielectric constant of the interior of the membrane and accounts for the lower value of k_A obtained with the voltage-clamp experiments. When the membranes were aged for only 20 min, higher values of k_A were observed with voltage-clamp experiments.

Table 2 illustrates the results of charge-pulse experiments performed with phosphatidylcholine/chlorodecane membranes at pH 7.5 and different concentrations of CCCP. Although the experimental results depend markedly on [CCCP], the values of K_{HA}, K_A and N_o/[CCCP] are essentially independent of protonophore concentration provided [CCCP] $< 10^{-6}$ M. This result is consistent with both the voltage-clamp results and our assumption that each CCCP molecule transports protons independently.

We performed some experiments with membranes formed with lipids other than diphytanoyl phosphatidylcholine. Table 3 compares the data obtained from charge-pulse experiments using three different lipids. K_{HA} is larger for membranes formed from monoolein than for membranes formed from phospholipids. On the other hand, K_A is largest for membranes formed from phosphatidylethanolamine and is smallest for monoolein membranes. The dipole potential is more positive for phosphatidylethanolamine membranes than for monoolein membranes [16]. This probably explains the high value of K_A observed with phosphatidylethanolamine membranes.

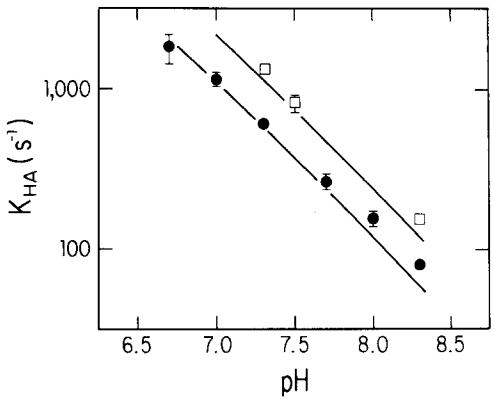

Fig. 10. Results of the charge-pulse experiments. The values of K_{HA} obtained with diphytanoyl phosphatidylcholine/chlorodecane and bacterial phosphatidylethanolamine/chlorodecane membranes are represented by filled circles and open squares, respectively. The data are plotted as a function of the aqueous pH. The solid lines illustrate the predictions of Eq. (16) with k_{HA} = 15,000 sec^{-1} (phosphatidylcholine) or 30,000 sec^{-1} (phosphatidylethanolamine) and the surface pK = 5.9

Discussion

Our main conclusion is that the model shown in Fig. 1 describes the transport of protons across artificial bilayers by CCCP. In our simple model we assumed that the interfacial reaction between the proton and the anion A^- is in equilibrium during the course of an experiment; our kinetic data are consistent with this assumption. A more detailed analysis [6], which considers the possibility that the interfacial

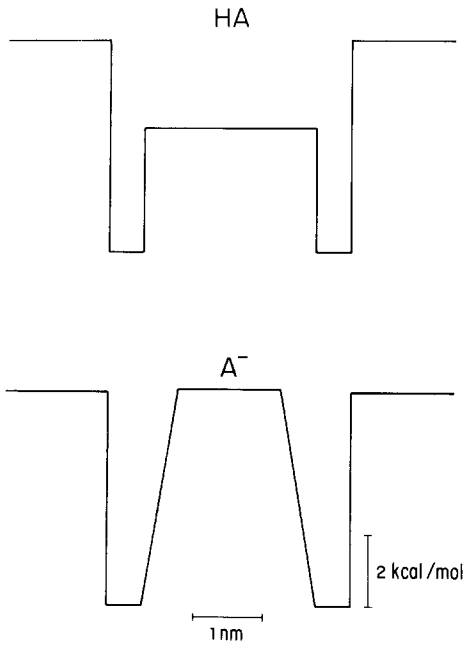

Fig. 11. A sketch of the Gibb's free energy profiles for the neutral (*HA*) and anionic (A^-) forms of CCCP in a diphytanoyl phosphatidylcholine/chlorodecane bilayer membrane. *See* text for details

reaction is not in equilibrium, demonstrates that our data are consistent with values of $k_R \geq 10^{11}$ M^{-1} sec^{-1}. The rate constant for the diffusion limited reaction between a proton and a weak acid in a bulk aqueous phase is $10^{10} - 10^{11}$ M^{-1} sec^{-1} [10]. Thus we must assume that the protons are combining with adsorbed anions at least as fast as they combine with anions in a bulk aqueous solution.[3] If we assume the interfacial reaction is in equilibrium, there are no adjustable parameters in the model: we measured each parameter (β_A, β_{HA}, k_A, k_{HA}, pK and pK^{aq}) independently. Using the results of these measurements, the model correctly predicts the results of the voltage-clamp and charge-pulse experiments for all [CCCP], pH values, and voltages (Figs. 8–10, Tables 1 and 2).[4]

In Fig. 11, we sketch the free energy profiles of the neutral and anionic forms of CCCP in a phosphatidylcholine/chlorodecane membrane. Consider first the free energy profile of the neutral form. We use the approach of Guggenheim rather than that of Gibbs [2] and consider the interface as a separate phase of thickness l. If we assume that $l = 0.5$ nm we calculate a dimensionless partition coefficient for HA into the surface phase: $\beta_{HA}/l = 1.4 \times 10^{-3}$ cm$/5 \times 10^{-8}$ cm $= 2.8 \times 10^4$. The drop in the free energy that occurs when HA crosses the membrane interface is $\Delta G = -RT\ln(\beta_{HA}/l) = -6.0$ kcal mol^{-1}. The free energy of HA within the membrane is estimated from $\Delta G = -RT\ln(P_{HA}d/D)$ where d is the thickness of the membrane and D is the diffusion coefficient for HA. Our value of $P_{HA} = 20$ cm sec^{-1} agrees, within a factor of two, with the value of 11 cm sec^{-1} deduced by LeBlanc [18] and the estimate of >27 cm sec^{-1} by O'Shaughnessy and Hladky [26]. We assume a diffusion coefficient $D = 10^{-7}$ cm^2 sec^{-1} and a membrane thickness $d = 3.5$ nm and find that $\Delta G = -2.5$ kcal mol^{-1}. The value of P_{HA} obtained from the membrane potential measurements (Fig. 6) represents the permeability of the entire membrane to the neutral form of CCCP. If we assume that there are no interfacial barriers to HA, then the permeability of the entire membrane can be equated with the permeability between the interfacial barriers: $P_{HA} = \beta_{HA}k_{HA}$. The value of k_{HA} that we obtain in this manner, 12,000 sec^{-1}, agrees well with the results of the kinetic measurements. Our conclusion that there are no appreciable interfacial barriers for the HA form of CCCP agrees with results obtained for FCCP [6] and for phloretin [35].

We next construct the energy barriers for the anionic form of CCCP. Our equilibrium dialysis experiments demonstrated that $\beta_A = \beta_{HA}$; thus the depth of the interfacial wells for both the A^- and HA species is 6.0 kcal mol^{-1}. The free energy difference between the aqueous phase and the interior of the membrane for the anionic form of CCCP is estimated from $\Delta G = -RT\ln\{RTG(0,0)d/F^2D[A^-]\}$. From Fig. 5, with pH = 8.3 and $[A^-] = 10^{-6}$ M, we find that $G(0,0) = 9.1 \times 10^{-4}$ S cm^{-2}. If we assume that $d = 3.5$ nm, then $\Delta G = +0.1$ kcal mol^{-1}. From the conductance-voltage curves for CCCP (Fig. 7), we deduced a value for the fractional width of the minor base ($b = 0.6$), which is illustrated in Fig. 11.

[3] The proton can also cross the membrane-solution interface by mechanisms more complicated than the heterogenous reaction illustrated in Fig. 1. For example, the protonated form of the buffer could transfer a proton directly to the adsorbed anion, A^-, or the adsorbed anion could move into the aqueous phase, receive a proton directly from the buffer, then adsorb to the interface again as the HA species. These reactions, in effect, keep the heterogenous reaction illustrated in Fig. 1 at equilibrium.

[4] Most of our results agree with the results of O'Shaughnessy and Hladky [26]. For example, our value for the adsorption coefficient β_A agrees, within a factor of two, with their result. However, our value for k_D/k_R at the surface of the membrane (and k_{HA}) is thirty times greater than their value. O'Shaughnessy and Hladky [26] were appropriately cautious about the values they deduced for these parameters, which were estimated from voltage-clamp experiments performed with large applied voltages at alkaline values of the pH. We determined the value of the surface pK directly from spectrophotometric measurements. We found that the surface pK and bulk aqueous pK were identical, a conclusion consistent with our observation that $\beta_A = \beta_{HA}$.

We have no information on the interfacial barriers for the A^- species.

Our measurements are consistent with the postulate that weak acids such as DTFB [8], TTFB [9], FCCP [6] and CCCP adsorb to membranes mainly due to hydrophobic interactions [32]. If the adsorption of the charged and neutral forms of a molecule is due to nonspecific hydrophobic interactions, we would expect the adsorption coefficients of the two forms to be identical [19, 24]. Our equilibrium dialysis experiments on sonicated vesicles indicate that the adsorption coefficients, β_A and β_{HA}, of CCCP are equal. We also predict from our model that the surface pK and aqueous pK should be identical; our spectroscopic measurements confirm this prediction.

We now consider three factors that affect the ability of a weak acid such as CCCP to act as an uncoupler in biological membranes: the surface area, dielectric constant, and surface charge of the membrane. CCCP adsorbs strongly to membranes, and the aqueous concentration of CCCP will become depleted if the total surface area of the biological membrane is large. For example, Grinius, Jasaitis, Kadziauskas and Liberman [11] used the conductance of a planar bilayer membrane to measure the aqueous concentration of the anion phenyl dicarbaundecaborane, demonstrating that submitochondrial particles decrease the aqueous concentration of the anion. The ability of CCCP to act as a proton ionophore depends markedly on the dielectric constant, ε_r. Although the adsorption coefficient, β_A, and the permeability of the membrane to HA, P_{HA}, are independent of the dielectric constant, the rate constant k_A should depend markedly on the dielectric constant. The Born charging energy required to move an ion from the aqueous phase into the interior of the membrane increases if the dielectric constant of the bilayer decreases. The dielectric constants of phosphatidylcholine/chlorodecane membranes and phosphatidylcholine/decane membranes are 2.7 and 2.1, respectively (J. Dilger, *personal communication*). If we replace chlorodecane by decane, the Born equation predicts the conductance due to CCCP should decrease by two orders of magnitude. Our kinetic model predicts the amplitude of the relaxation should decrease by two orders of magnitude [Eq. (5)]. A voltage-clamp experiment demonstrated that when we replaced chlorodecane with decane in diphytanoyl phosphatidylcholine bilayers both the conductance and the magnitude of the relaxation decreased by approximately two orders of magnitude (*data not shown*). This result agrees with the voltage-clamp measurements made by O'Shaughnessy and Hladky [26] on membranes formed from egg phosphatidylcholine and cholesterol in decane. A comparison of the protonophoric activity of CCCP on bilayers and mitochondria suggests that the ε_r of mitochondrial membranes is approximately 2.7, presumably because proteins increase the dielectric constant of biological membranes [24]. The surface charge density can also affect the protonophoric activity of CCCP. McLaughlin and Dilger [24] found that CCCP produced an order of magnitude higher conductance on bilayer membranes formed from phosphatidylethanolamine (a zwitterionic lipid) than on bilayer membranes formed from a mixture containing 20% cardiolipin (a negative lipid). The negatively charged lipids produce a diffuse double layer and an electrostatic potential in the aqueous phase adjacent to the surface of the membrane [23]. This potential decreases the surface concentration of A^-, decreasing the protonophoric activity of CCCP.

Finally, we consider the effect of high concentrations of CCCP on the electrostatic potentials adjacent to membranes. When [CCCP] $\geq 10^{-6}$ M, neither the conductance (Fig. 5) nor the adsorbed charge (Fig. 3) varied linearly with [CCCP], the relaxation magnitude decreased (Fig. 8*A*), the time constant increased (Fig. 8*B*), and the zeta potential measurements deviated from the Gouy-Chapman-Stern theory (Fig. 2). All these observations suggest that the adsorption of CCCP to bilayer membranes produces a boundary potential [23]. High concentrations of uncouplers (e.g., [CCCP] $\geq 10^{-6}$ M) inhibit oxygen consumption in mitochondria [29]. Reyes and Benos [29] speculate that the inhibition of mitochondrial oxygen consumption is due to the production of an electrostatic potential. Our results with CCCP are consistent with this hypothesis.

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