# THE MOLECULAR MECHANISM OF ACTION OF THE PROTON IONOPHORE FCCP (CARBONYLCYANIDE *p*-TRIFLUOROMETHOXYPHENYLHYDRAZONE)

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ABSTRACT We propose a simple model that accounts for the ability of the weak acid FCCP (Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) to both transport protons across phospholipid bilayer membranes and uncouple oxidation from phosphorylation in mitochondria. Four parameters are required to characterize this model: the rate constant for the movement of $A^-$ across the membrane, $k_A$, the rate constant for the movement of HA across the membrane, $k_{HA}$, the adsorption coefficient of $A^-$ onto the membrane-solution interface, $\beta_A$, and the surface p$K$. These four parameters were determined from kinetic measurements on planar bilayer membranes using the charge-pulse and voltage-clamp techniques. We confirmed the adequacy of the model by determining each of these parameters independently, utilizing equilibrium dialysis, zeta potential, membrane potential, spectrophotometric, and conductance measurements. For a phosphatidylethanolamine bilayer the values of the parameters are $k_{HA} = 10^4\ s^{-1}$, $\beta_A = 3\ 10^{-3}$ cm, and $6.0 < pK < 6.4$. As predicted theoretically, the value of $k_A$ depends on both the applied voltage, $V$, and dielectric constant of the membrane, $\epsilon_r$; when $V$ approaches zero and the membrane contains chlorodecane ($\epsilon_r \simeq 2.7$) $k_A = 700\ s^{-1}$. If oxidation is coupled to phosphorylation by means of a $\Delta\tilde{\mu}_H{}^+$, and $V \simeq 150$ mV, $\epsilon_r \simeq 2.7$ for the inner membrane of the mitochondrion, the model predicts that FCCP should exert maximal uncoupling activity at a pH $\simeq$ p$K$. This prediction agrees with the published experimental results.

## INTRODUCTION

Loomis and Lipmann (1948) demonstrated that 2,4-dinitrophenol stimulates respiration but inhibits ATP synthesis in mitochondria, and Mitchell (1961) proposed that weak acids uncouple oxidative phosphorylation by transporting protons across the phospholipid bilayer component of the inner mitochondrial membrane and dissipating a $\nabla\tilde{\mu}_H{}^+$. These proton ionophores, which are also termed protonophores, are used extensively to study energy coupling in mitochondria, chloroplasts, and bacteria (e.g., Hinkle and McCarty, 1978; Skulachev, 1981) and to investigate a variety of other transport phenomena associated with biological membranes (McLaughlin and Dilger, 1980).

In this study we determined the molecular mechanism by which a typical protonophore, FCCP (carbonyl-*p*-trifluoromethoxyphenylhydrazone), transports hydrogen ions across phospholipid bilayer membranes. We used both the voltage-clamp and charge-pulse techniques to obtain kinetic information. Under voltage-clamp conditions, a constant potential was applied across the membrane, and relaxations in the current were studied after the decay of the capacitance spike. For instance, if a 1 mm$^2$ membrane with a capacitance, $C \simeq 10^{-8}$ F, was in series with an external resistor, $R = 100\ \Omega$, the capacitance spike had a time constant of $RC \simeq 1\ \mu$s, and accurate data could be obtained after $\sim 10\ \mu$s. The voltage-clamp technique has been used extensively to investigate carrier mediated transport across planar bilayer membranes (Stark et al., 1971; Hladky, 1979; Läuger et al., 1981). Under charge-pulse conditions, the membrane capacitance was charged to an initial voltage by an intense current pulse of 10–100 ns duration; at the end of the pulse, the external charging circuit was switched to a resistance that was much higher than the resistance of the membrane. By measuring the decay of the potential across the membrane as a function of time, information was obtained about the mechanism of ion transport within the membrane (Benz and Läuger, 1976; Benz et al., 1976).

In principle, the voltage-clamp and charge-pulse techniques provide identical kinetic information. In practice, each technique has advantages. The charge-pulse technique has superior time resolution, whereas the main advantage of the voltage-clamp method is that the theoretical analysis of the data is simpler, particularly when voltages larger than $RT/F = 25$ mV are applied to the membrane.

It is possible to deduce all the parameters required to

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characterize a simple carrier model from kinetic measurements. For example, in the model presented below we assume that the reaction between a proton in the aqueous phase and an anion adsorbed to the interface occurs very rapidly. In this case the adsorption coefficients of the anionic, $A^-$, and acidic, HA, forms of the weak acid and the rate constants for the movement of the $A^-$ and HA species across the membrane can all be deduced from kinetic measurements. It is apparent, however, that the parameters deduced in this manner depend upon the assumptions inherent in the kinetic model. To test our simple model each of these four parameters was determined independently using zeta potential, equilibrium dialysis, spectrophotometric, membrane potential, and conductance measurements.

The reader who is interested in a chemiosmotic view of how FCCP functions as a protonophore and uncoupler, but who is not particularly interested in the details of kinetic measurements on bilayers, could peruse the next section, then skip both the Materials and Methods and Results sections, and proceed directly to the Discussion section.

## THE KINETIC MODEL

Fig. 1 illustrates the kinetic model we use to describe the transport of protons through a bilayer membrane. It is similar to the model used for neutral carriers such as valinomycin and nonactin (Läuger, 1972; Hladky, 1979; Läuger et al., 1981). In this section we describe the model and list our assumptions; the experimental basis for these assumptions will be considered in the Discussion section.

The aqueous phases contain well-buffered solutions of identical composition; specifically, the pH and the concentrations of FCCP in the bulk aqueous solutions are identical. The aqueous phases have a large volume and the strong adsorption of both the $A^-$ and HA forms of FCCP to the membrane-solution interface does not reduce the concentration of FCCP in the aqueous phase. The NaCl concentration in the aqueous phases is high and the potential applied to the electrodes in the aqueous phases drops only across the bilayer membrane. The following eight assumptions are common to the analysis of both the voltage-clamp and charge-pulse results.

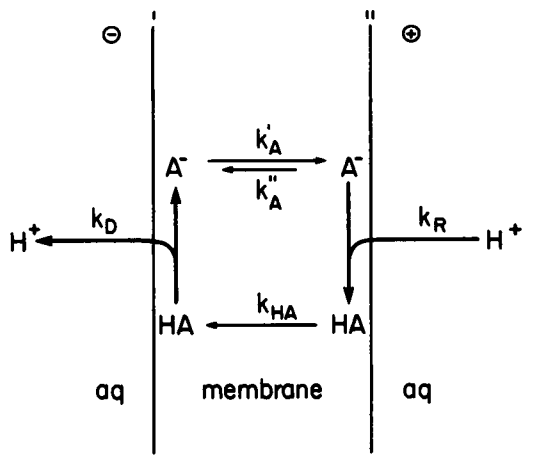


FIGURE 1 Diagram illustrating the mechanism by which FCCP transports protons across bilayer membranes. As indicated by the circled positive and negative signs, a voltage exists across the membrane. The rate constants $k_R$ and $k_D$ refer to the heterogeneous reactions whereby a proton from the aqueous phase either recombines with or dissociates from an anion adsorbed to the membrane, the voltage-dependent rate constants $k_A^{\prime}$ and $k_A^{\prime\prime}$ refer to the movement of $A^-$ from the ′ to the ″ and from the ″ to the ′ interfaces, respectively, and the rate constant $k_{HA}$ refers to the movement of HA between the two interfaces. The concentrations of $A^-$ and HA at the membrane-solution interfaces are much higher than the concentrations in either the aqueous phase or the interior of the membrane.

(*a*) We assume that the anionic form of FCCP, $A^-$, is the only charged species that moves across the interior of the membrane. The translocation is treated as a first-order reaction with a rate constant $k_A$. In either the Nernst-Planck or the Eyring models, the number of FCCP anions within the membrane is negligible compared with the number of anions adsorbed to the interfaces. Thus, we assume that a quasi–steady-state concentration profile is attained within the interior of the membrane instantaneously upon the application of a voltage pulse. The applied voltage causes $A^-$ to move through the membrane and to accumulate at the interface denoted as ″ in Fig. 1. The accumulation of $A^-$ disturbs the equilibrium between $A^-$ and HA at the ″ interface and causes an increase in the surface concentration of HA by means of the interfacial reaction illustrated in Fig. 1.

(*b*) We assume that a proton from the aqueous phase combines with an anion adsorbed to the interface by means of a heterogeneous reaction. Specifically, we assume that the so called "solution-complexation" mechanism, by which the proton combines with an anion in the aqueous phase and the HA complex then moves onto the interface, can be ignored (Neumcke and Bamberg, 1975). The rate constant for the heterogeneous reaction between the aqueous proton and the adsorbed anion is $k_R$. The rate constant for the reverse reaction is $k_D$. The association constant is $K \equiv k_R/k_D$ and $\log(K)$ is the surface p$K$. The heterogeneous reaction is assumed to be voltage independent and to occur at the membrane-solution interface.

(*c*) We assume that the proton is the only species that moves between the bulk aqueous phases. There are Nernstian unstirred layers, which have a thickness of ~100 μm, in the aqueous phase adjacent to the membrane (Helfferich, 1962; Vetter, 1967; McLaughlin and Eisenberg, 1975). The proton moves by a "buffer shuttle" mechanism through these unstirred layers (Gutknecht and Tosteson, 1973). The buffer species HB diffuses toward the ″ interface (Fig. 1) and the $B^-$ form of the buffer diffuses back towards the aqueous phase; the reaction $H^+ + B^- \rightleftarrows HB$ is at equilibrium through most of the unstirred layer. However, there is a region immediately adjacent to the membrane where equilibrium between the buffer and the proton cannot be maintained. This region is sometimes referred to as a "reaction layer" (Delahay, 1954; LeBlanc, 1971). It is also possible that the HB form of the buffer reacts directly with the adsorbed anion and donates a proton to it.

(*d*) We assume that the adsorption of FCCP produces no significant diffuse double layer, dipole, or boundary potentials (McLaughlin, 1977). Thus, when the $A^-$ and HA species move through the membrane between the two interfaces, we need consider only changes in concentration.

(*e*) We assume that the back diffusion of HA from the ″ to the ′ interface can be treated as a first-order reaction with a rate constant $k_{HA}$ that is independent of voltage.

The interfacial concentrations of $A^-$ and HA, $N_A$ and $N_{HA}$, respectively, change with time according to the following four differential equations:

$$\frac{dN_A^{\prime}}{dt} = -k_R[H^+]N_A^{\prime} + k_D N_{HA}^{\prime} - k_A^{\prime} N_A^{\prime} + k_A^{\prime\prime} N_A^{\prime\prime} \quad (1)$$

$$\frac{dN_A^{\prime\prime}}{dt} = -k_R[H^+]N_A^{\prime\prime} + k_D N_{HA}^{\prime\prime} + k_A^{\prime} N_A^{\prime} - k_A^{\prime\prime} N_A^{\prime\prime} \quad (2)$$

$$\frac{dN_{HA}^{\prime}}{dt} = -k_D N_{HA}^{\prime} + k_R[H^+]N_A^{\prime} - k_{HA}(N_{HA}^{\prime} - N_{HA}^{\prime\prime}) \quad (3)$$

$$\frac{dN^{II}_{HA}}{dt} = -k_D N^{II}_{HA} + k_R[H^+]N^{II}_A + k_{HA}(N^{I}_{HA} - N^{II}_{HA}) \quad (4)$$

where $[H^+]$ is the concentration of protons in the aqueous phase. The rate constants in Eqs. 1–4 are defined in Fig. 1.

(*f*) We assume that $N^{I}_A + N^{II}_A + N^{I}_{HA} + N^{II}_{HA} \equiv N_0$ remains constant for the time course of a relaxation experiment with FCCP, an assumption that is justified below.

If only the movement of $A^-$ depends on voltage (assumptions *b* and *e*), it is easy to demonstrate that

$$r \equiv N^{I}_A + N^{II}_A = N_0/(1 + K[H^+]) \quad (5)$$

$$s \equiv N^{I}_{HA} + N^{II}_{HA} = N_0 K[H^+]/(1 + K[H^+]). \quad (6)$$

In other words, both the total concentrations of $A^-$ and of HA adsorbed to the interfaces remain constant during a kinetic experiment with FCCP.

If the variables $X = N^{I}_A - N^{II}_A$ and $Y = N^{I}_{HA} - N^{II}_{HA}$ are introduced, Eqs. 1–4 reduce to the following two differential equations:

$$\frac{dX}{dt} = -(k_R[H^+] + k^{I}_A + k^{II}_A)X + k_D Y - (k^{I}_A - k^{II}_A)r \quad (7)$$

$$\frac{dY}{dt} = k_R[H^+]X - (k_D + 2k_{HA})Y. \quad (8)$$

The general solution to this system of two differential equations is presented in Appendix A for voltage-clamp and Appendix B for charge-pulse conditions. The analysis is greatly simplified if it is assumed that the reactions at the interface remain essentially at equilibrium throughout the relaxation process.

(*g*) We assume that $k_R[H^+], k_D \gg k_A, k_{HA}$, an assumption consistent with the experimental results presented below. In this case, Eq. 9 is approximately valid throughout the relaxation process:

$$N^{I}_{HA}/N^{I}_A = N^{II}_{HA}/N^{II}_A = k_R[H^+]/k_D = K[H^+]. \quad (9)$$

It follows from Eq. 9 that $Y \simeq K[H^+]X$. Thus Eqs. 7 and 8 reduce, via addition, to the following single differential equation:

$$\frac{dX}{dt} = [-1/(1 + K[H^+])] \cdot [(k^{I}_A + k^{II}_A + 2k_{HA}K[H^+])X + (k^{I}_A - k^{II}_A)r]. \quad (10)$$

Under voltage-clamp conditions it is assumed that the system is at equilibrium for times $t < 0$, and that at $t = 0$ a membrane voltage of $V$ is applied. The current density, $I$, neglecting the capacitance transient, is given by the net flux of charged particles within the membrane multiplied by the Faraday constant, $F$, and the valence:

$$I = F(-k^{I}_A N^{I}_A + k^{II}_A N^{II}_A). \quad (11)$$

It follows that

$$I(t) = I(\infty)[1 + \alpha\exp(-\lambda t)] \quad (12)$$

where

$$\alpha = (k^{I}_A + k^{II}_A)/2K[H^+]k_{HA} \quad (13)$$

and

$$\lambda = (k^{I}_A + k^{II}_A + 2k_{HA}K[H^+])/(1 + K[H^+]). \quad (14)$$

Our model predicts that the current will decay from an instantaneous value to a steady state value with a single time constant, $\tau \equiv 1/\lambda$. The magnitude of the relaxation is $\alpha = [I(0) - I(\infty)]/I(\infty)$. Eq. 13 predicts that $\alpha$ will increase tenfold for every unit increase in the pH. The dependence of $k^{I}_A$ and $k^{II}_A$ on voltage is a function of the potential energy barrier that the $A^-$ species encounters within the membrane. In the limit that the applied voltage approaches zero, $k^{I}_A = k^{II}_A = k_A$ for all barrier shapes and $\alpha = k_A/K[H^+]k_{HA}$. Both $\alpha$ and $\tau$ are predicted to be independent of [FCCP].

Under charge-pulse conditions, it is assumed that the system is at equilibrium for $t < 0$ and that at $t = 0$ the membrane capacitance is charged instantaneously to a voltage $V^\circ \ll 25$ mV. The rate of decay of the voltage after the initial charge pulse is given by the quotient of the current density (Eq. 11) and the specific membrane capacitance, $C_m$:

$$\frac{dV}{dt} = \frac{I(t)}{C_m} = (F/C_m)(-k^{I}_A N^{I}_A + k^{II}_A N^{II}_A). \quad (15)$$

When assumption *g* is valid, $V(t)$ has only two exponential relaxations:

$$V(t) = V^\circ[a_1\exp(-\lambda_1 t) + a_2\exp(-\lambda_2 t)] \quad (16)$$

where $a_1$, $a_2$, $\lambda_1$, $\lambda_2$ are constants that may be expressed in terms of the four parameters to be determined (Appendix B). In contrast to the results obtained under voltage-clamp conditions, the amplitudes and time constants of the relaxations measured under charge-pulse conditions are predicted to depend on [FCCP].

We can deduce the four parameters required to describe the model from either voltage-clamp or charge-pulse measurements made at low applied voltages. These four parameters are the rate constants, $k_A$ and $k_{HA}$, as well as the total number of adsorbed FCCP molecules, $N_0$, and the surface p$K$, log $K$. The latter two parameters are equivalent, under conditions that are discussed below, to the adsorption coefficients of both the $A^-$ and HA forms of FCCP. These adsorption coefficients are defined by

$$\beta_A = N^{\circ}_A/[A^-] \quad (17)$$

$$\beta_{HA} = N^{\circ}_{HA}/[HA] \quad (18)$$

where $N^{\circ}_A$ and $N^{\circ}_{HA}$ are the equilibrium surface concentrations of $A^-$ and HA and the square brackets denote the concentrations in the bulk aqueous phases.

The adsorption coefficient of the anion provides information about the depth of the energy well for $A^-$ at the membrane-solution interface. The value of $k_A$ provides information about the difference between the potential energies of $A^-$ in the interfacial well and the center of the membrane. We can obtain information about the shape of the potential energy profile encountered by the $A^-$ species as it moves through the membrane by measuring the conductance as a function of voltage in the limit that time goes to zero, $G(V, 0)$. If the ion encounters a high barrier in the center of the membrane, a conventional assumption in the Eyring model, the conductance depends strongly on voltage; $G(V, 0) \propto (1/u)\sinh(u/2)$ where $u = FV/RT$. If the ion encounters a square energy barrier, a conventional assumption in the Nernst-Planck model, the conductance is independent of voltage. An ion moving through the membrane almost certainly encounters a more complicated energy profile than predicted by these simple models, if only because of the ion-dipole (image charge) force it experiences (Neumcke and Läuger, 1969; Haydon and Hladky, 1972; Anderson and Fuchs, 1975). Hall et al. (1973) pointed out that a trapezoidal barrier is a good approximation to the image force barrier. Hladky (1974) showed that for a trapezoidal barrier the conductance depends on the voltage according to $G(V, 0) \propto \sinh(u/2)/\sinh(bu/2)$ if the minor base of the trapezoid spans a fraction $b$ of the membrane. Thus by measuring the dependence of the conductance on voltage, we can determine the value of $b$. In our model, the rate constants depend on voltage in the following manner:

$$k^{I}_A = k_A(bu/2)\exp(u/2)/\sinh(bu/2) \quad (19)$$

$$k^{II}_A = k_A(bu/2)\exp(-u/2)/\sinh(bu/2). \quad (20)$$

From Eqs. 11, 19, and 20 it follows that if $b \to 0$ the current is

proportional to $\sinh(u/2)$, as predicted by an Eyring model with a single barrier, and that if $b \rightarrow 1$ the current is proportional to $u$, as predicted by a Nernst-Planck model with a square barrier.

## MATERIALS AND METHODS

Optically black lipid bilayer membranes were formed from a 1–2% (wt/vol) solution of lipid in either 1-chlorodecane (Aldrich Chemical Co., Milwaukee, WI) or *n*-decane (Supelco, Inc., Belefonte, PA). The chlorodecane was purified by passage through an alumina column; other chemicals were used as supplied. Bacterial phosphatidylethanolamine (PE), egg- and diphytanoylphosphatidylcholine (PC) were obtained from Avanti Biochemicals, Inc. (Birmingham, AL), monoolein from Sigma Chemical Co. (St. Louis, MO).

The aqueous solutions were prepared with 18 MΩ cm water (Millipore Super Q, Millipore Corp., Bedford, MA) that was also double distilled in a quartz still. They contained, unless otherwise specified, 1 M NaCl buffered to the desired pH with 0.1 M phosphate plus either 0.1 M Tris or 0.1 M Bicine.

Experiments were performed at room temperature (20–22°C) in Teflon chambers with two compartments separated by a thin wall with an aperture of 1–2 mm diam. FCCP (Pierce Chemical Co., Rockford, IL) was added to the aqueous phase in the form of a concentrated solution in ethanol. The aqueous concentration of ethanol never exceeded 0.5% by volume, and control experiments demonstrated that this concentration of ethanol did not significantly affect either the conductance or the relaxations. The experimental procedures have been described in detail for both voltage-clamp (Dilger and McLaughlin, 1979) and charge-pulse (Benz et al., 1976) measurements. Briefly, the voltage-clamp experiments were performed by applying a single voltage pulse of amplitude 25–200 mV across the bilayer membrane with a Wavetek (San Diego, CA) 164 Sweep Generator connected to AgCl electrodes (Annex Instruments, Santa Ana, CA). The current flowing through the membrane was determined by measuring the voltage drop across a resistor (100–500 Ω) in series with the membrane using a Tektronix, Inc., (Beaverton, OR) 7A22 differential amplifier in a 7704A digital processing oscilloscope. A Tektronix CP4165 digital computer was used on-line to signal average and to analyze the relaxations for both the voltage-clamp and charge-pulse experiments. After the capacitance spike had subsided, the current transient in the voltage-clamp experiments could always be described by a single exponential. To analyze the transient, the stationary current was subtracted, the logarithm of the current plotted as a function of time, and a linear least-square best fit program used to determine the values of the time constant and the initial current.

The charge-pulse measurements were performed with one electrode connected to the pulse generator through a fast diode with a reverse voltage resistance of $10^{11}$ Ω and the other electrode grounded. The voltage between the electrodes was amplified with a fast voltage follower (Burr Brown Research Corp., Tuscon, AZ, Model 3551), and the amplified signal was monitored with a Tektronix 7A13 differential comparator in the digital processing oscilloscope. The time resolution of the system was ~300 ns. The voltage relaxations in the charge-pulse experiments could always be described by two exponentials.

The sonicated unilamellar vesicles used for the equilibrium dialysis measurements were formed from egg PC, following the procedure of Barenholz et al. (1977). The aqueous phases contained 0.1 M NaCl, 1 mM Tris, and 1 mM citrate. The pH of the solutions was 3.9 for the determination of the adsorption coefficient of the HA form of FCCP and 8.1 for the determination of the adsorption coefficient of the $A^-$ form. Dialysis was carried out in Teflon chambers for >12 h at 21°C. The lipid concentration was determined by phosphate analysis (Lowry and Tinsley, 1974) and the FCCP concentration by spectrophotometric analysis. It was necessary to equilibrate the dialysis membranes with the appropriate concentration of FCCP before the experiment to prevent a significant (20%) loss of FCCP from the chambers.

The multilamellar vesicles for the microelectrophoresis experiments were prepared from egg PC according to Bangham et al. (1974). The mobilities of the vesicles were measured in a Rank Bros. (Bottisham, Cambridge, U. K.) Mark I machine.

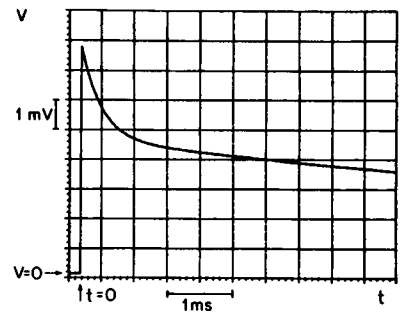


FIGURE 2 Record of a charge pulse experiment performed on a phosphatidylethanolamine/chlorodecane bilayer in a pH 8.4 solution containing $10^{-7}$ M FCCP. At time $t = 0$ the membrane capacitance was charged to a voltage $V^\circ = 7.84$ mV by a current pulse of 50 ns duration. The signal was averaged 20 times.

A matched pair of calomel electrodes and a Keithley Instruments, Inc. (Cleveland, OH) model 602 electrometer were used to measure the membrane potentials produced when the planar bilayer separated two solutions of different pH. The aqueous solutions contained 0.1 M NaCl, 0.1 M phosphate, 0.1 M borate, and 0.1 M bicarbonate. $T = 21$°C.

The $pK^{aq}$ of FCCP was determined spectrophotometrically to be 6.05 ± 0.05 in our buffered 1 M NaCl solutions at 21°C, which agrees with the value of 6.1 reported by Bakker et al. (1975). We also confirmed the spectrophotometric observation of Bakker et al. (1975) that the p$K$ of FCCP adsorbed to sonicated egg PC vesicles is 5.9 when the vesicles are suspended in buffered 0.1 M NaCl solutions. In our buffered 1 M NaCl

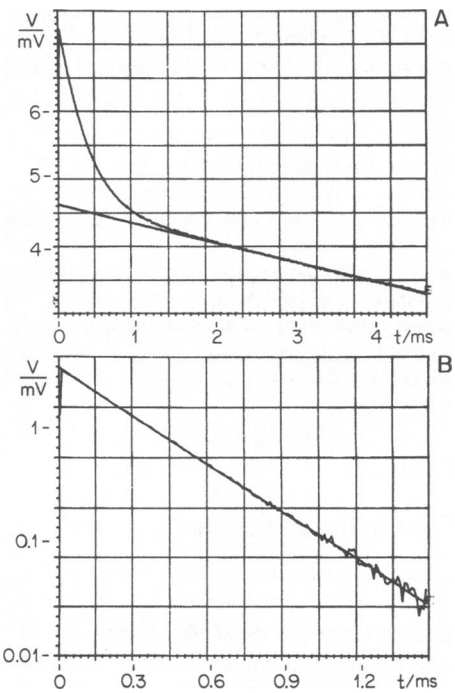


FIGURE 3 Analysis of the data in Fig. 2. The relaxation times, $\tau_1$ and $\tau_2$, and amplitudes, $V^\circ a_1$ and $V^\circ a_2$ (Eq. 16), were obtained from two successive plots, *A* and *B*, as described in the text. *A*, $V^\circ a_2 = 4.56$ mV, $\tau_2 = 17.4$ ms. *B*, $V^\circ a_1 = 3.27$ mV, $\tau_1 = 303$ μs. From Eqs. B20–B22 we obtain $K_{HA} = 67.1\ s^{-1}$, $K_A = 887\ s^{-1}$, and $N_o = 5.98\ 10^{-13}$ mol cm$^{-2}$.

TABLE I
CHARGE-PULSE DATA FROM PE/CHLORODECANE BILAYERS

| [FCCP]/$\mu$M | $\tau_1$/$\mu$s | $\tau_2$/ms | $a_2$ | $K_{HA}/10^2 s^{-1}$ | $K_A/10^2 s^{-1}$ | $N_o$/pmol cm$^{-2}$ | $\beta_o/10^{-3}$cm |
|---|---|---|---|---|---|---|---|
| | | | pH 6.4 | | | | |
| 0.001 | | 92 ± 12 | 1 | | | | |
| 0.01 | | 10 ± 0.2 | 1 | | | | |
| 0.1 | | 1.3 ± 0.17 | 1 | | | | |
| 0.3 | 110 ± 9 | 0.35 ± 0.01 | 0.91 ± 0.01 | 38 ± 3.2 | 4.5 ± 0.74 | 3.0 ± .50 | 5.0 |
| 1.0 | 71 ± 10 | 0.13 ± .01 | 0.67 ± 0.11 | 57 ± 4.0 | 4.8 ± 0.90 | 6.3 ± 1.3 | 3.2 |
| 3.0 | 42 ± 4 | 0.07 ± .009 | 0.68 ± 0.02 | 98 ± 13 | 6.3 ± 0.30 | 11 ± 1.0 | 1.8 |
| 10.0 | 40 | .060 | .73 | 110 | 5 | 15 | 0.8 |
| | | | pH 7.3 | | | | |
| 0.001 | | 34 ± 9 | 1 | | | | |
| 0.03 | 280 ± 14 | 12 ± 3 | 0.98 ± 0.01 | 9.8 ± 1.2 | 8.1 ± 0.90 | 0.072 ± 0.011 | 12.0 |
| 0.01 | 270 ± 37 | 5.2 ± 1.2 | 0.95 ± 0.04 | 9.1 ± 1.0 | 7.7 ± 0.85 | 0.18 ± 0.023 | 9.0 |
| 0.03 | 230 ± 25 | 2.3 ± 0.35 | 0.85 ± 0.09 | 8.8 ± 0.87 | 7.8 ± 0.45 | 0.46 ± 0.057 | 7.3 |
| 0.1 | 190 ± 14 | 0.98 ± 0.12 | 0.54 ± 0.10 | 9.0 ± 0.77 | 7.6 ± 0.71 | 1.3 ± 0.098 | 6.5 |
| 0.3 | 110 ± 12 | 0.65 ± 0.09 | 0.24 ± 0.03 | 9.6 ± 0.81 | 7.2 ± 0.56 | 3.6 ± 0.22 | 6.0 |
| 1.0 | 72 ± 8 | 0.58 ± 0.07 | 0.13 ± 0.04 | 9.5 ± 0.96 | 7.0 ± 0.72 | 6.9 ± 0.71 | 3.5 |
| 3.0 | 65 ± 7 | 0.42 ± 0.06 | 0.10 ± 0.02 | 13. ± 1.4 | 5.4 ± 0.62 | 11. ± 2.1 | 1.9 |
| | | | pH 8.4 | | | | |
| 0.001 | | 890 ± 130 | 1 | | | | |
| 0.003 | | 270 ± 30 | 1 | | | | |
| 0.01 | 480 ± 88 | 94 ± 22 | 0.94 ± .012 | 0.84 ± 0.19 | 9.2 ± 1.7 | 0.059 ± 0.003 | 3.0 |
| 0.03 | 440 ± 100 | 42 ± 10 | 0.85 ± 0.011 | 0.81 ± 0.11 | 9.4 ± 1.5 | 0.16 ± 0.030 | 2.7 |
| 0.1 | 320 ± 30 | 16 ± 4.6 | 0.60 ± 0.025 | 0.80 ± 0.24 | 8.8 ± 0.5 | 0.61 ± 0.052 | 3.2 |
| 0.3 | 160 ± 19 | 9.7 ± 1.1 | 0.32 ± 0.019 | 0.77 ± 0.08 | 9.5 ± 1.1 | 1.7 ± 0.28 | 2.8 |
| 1.0 | 74 ± 9 | 5.6 ± 0.49 | 0.14 ± 0.010 | 1.1 ± 0.08 | 9.2 ± 0.9 | 4.9 ± 0.41 | 2.5 |
| 3.0 | 53 ± 5 | 2.9 ± 0.27 | 0.06 ± 0.003 | 1.8 ± 0.17 | 5.7 ± 0.8 | 12 ± 0.81 | 2.0 |
| 10.0 | 87 ± 8 | 1.2 ± 0.20 | 0.03 ± 0.004 | 4.4 ± 0.80 | 1.7 ± 0.3 | 25 ± 3.1 | 1.3 |

Relaxation data derived from charge-pulse experiments on phosphatidylethanolamine/chlorodecane bilayers. The aqueous phases contained the concentration of FCCP indicated in the first column. The constants $K_{HA}$ (Eq. 21), $K_A$ (Eq. 22) and $N_o$ were calculated from Eqs. B20–B22, using the experimental value $C_m = 0.73\ \mu F\ cm^{-2}$. The results (±SD) were obtained from at least four membranes 30 min after the membranes became black.

solutions the surface p$K$ of FCCP adsorbed to egg PC vesicles, as determined spectrophotometrically (data not shown), is 6.0.

Control experiments demonstrated that the buffers, when present at concentrations >0.001 M, had a negligible effect on the kinetic parameters. For example, we obtained identical values for the magnitude and time constant of the relaxations, as well as the adsorbed charge and the conductance, using 0.1 and 0.001 M Bicine solutions (1 M NaCl, pH 8.4) in voltage-clamp experiments on PC/chlorodecane membranes. It is difficult to interpret kinetic experiments when the buffer concentration is <0.001 M because diffusion polarization of protons occurs in the aqueous unstirred layers.

## RESULTS

### Charge Pulse Results

Fig. 2 is an experimental record taken with a digital oscilloscope from a phosphatidylethanolamine/chlorodecane membrane in the presence of $10^{-7}$ M FCCP. The values of the membrane potential, $V(t)$, were plotted on a logarithmic scale (Fig. 3 *A*) and the amplitude and time constant of the slower relaxation were determined from a linear least-squares best fit to the data obtained at long times. The logarithm of the difference between the measured voltage and the voltage due to the slow relaxation was then plotted as a function of time (Fig. 3 *B*). The amplitude and time constant of the fast relaxation was determined from a linear least-squares best fit to all the data in Fig. 3 *B*. All the charge-pulse data could be fitted by assuming that there are only two relaxation processes. There was never any indication of an additional fast relaxation, corresponding to the heterogeneous reaction illustrated in Fig. 1, even at times as short as 300 ns. Thus, information about the surface p$K$ of FCCP could not be obtained from a kinetic experiment performed at a single pH. By analyzing the charge pulse data obtained at one pH using Eqs. B20, B21, and B22 we obtain an estimate of

$$K_{HA} \equiv k_{HA}K[H^+]/(1 + K[H^+]), \quad (21)$$

$$K_A \equiv k_A/(1 + K[H^+]) \quad (22)$$

and $N_o$.

Table I contains the experimental results obtained at three different values of the pH and several different concentrations of FCCP. Note that the relaxation time constants, $\tau_1$ and $\tau_2$, and the relaxation amplitudes, $a_1$ and $a_2$ ($a_1 + a_2 = 1$) depend on [FCCP] and pH. However, it is

TABLE II
CHARGE-PULSE DATA FROM PE/CHLORODECANE BILAYERS

| pH | $\tau_1/\mu s$ | $\tau_2$/ms | $a_2$ | $K_{HA}/10^2 s^{-1}$ | $K_A/10^2 s^{-1}$ | $N_o$/pmol cm$^{-2}$ | $\beta_o/10^{-3}$cm |
|---|---|---|---|---|---|---|---|
| 6.1 | 54 ± 10 | 0.093 ± 0.019 | 0.74 ± 0.11 | 83 ± 18 | 4.0 ± 1.1 | 12.3 ± 3.8 | 2.1 |
| 6.4 | 71 ± 10 | 0.13 ± 0.011 | 0.67 ± 0.11 | 57 ± 4 | 4.8 ± 0.90 | 6.3 ± 1.8 | 3.2 |
| 6.8 | 98 ± 11 | 0.25 ± 0.019 | 0.27 ± 0.056 | 24 ± 1.6 | 4.4 ± 0.71 | 7.3 ± 1.8 | 3.7 |
| 7.1 | 93 ± 14 | 0.42 ± 0.050 | 0.21 ± 0.020 | 14 ± 1.3 | 6.5 ± 0.50 | 5.1 ± 0.52 | 2.6 |
| 7.3 | 72 ± 8 | 0.58 ± 0.070 | 0.13 ± 0.040 | 9.5 ± 0.96 | 7.0 ± 0.72 | 6.9 ± 0.71 | 3.5 |
| 7.8 | 75 ± 12 | 1.39 ± 0.19 | 0.14 ± 0.01 | 4.9 ± 0.49 | 8.5 ± 1.2 | 5.1 ± 0.41 | 2.6 |
| 8.1 | 87 ± 5 | 2.9 ± 0.21 | 0.13 ± 0.007 | 2.0 ± 0.14 | 7.0 ± 0.53 | 5.3 ± 0.38 | 2.7 |
| 8.4 | 74 ± 9 | 5.6 ± 0.49 | 0.14 ± 0.010 | 1.1 ± 0.08 | 9.2 ± 0.89 | 4.9 ± 0.41 | 2.5 |
| 8.8 | 90 ± 4 | 12 ± 0.6 | 0.15 ± 0.009 | 0.49 ± 0.02 | 8.1 ± 0.63 | 4.3 ± 0.31 | 2.2 |
| 9.1 | 95 ± 5 | (20) | 0.14 ± 0.010 | — | 7.8 ± 0.51 | 5.2 ± 0.50 | 2.6 |

Relaxation data derived from charge-pulse experiments on phosphatidylethanolamine/chlorodecane bilayer membranes. pH is indicated in the first column. The aqueous phases contained either 3 $\mu$M (pH 6.1) or 1 $\mu$M (pH 6.4–9.1) FCCP.

apparent from Table I that the rate constants, $K_{HA}$ and $K_A$, and the overall partition coefficient, $\beta_o \equiv N_o/2[FCCP]$, are approximately independent of protonophore concentration for [FCCP] $\leq 10^{-6}$ M.[1] This result is consistent with our model, which assumes that the FCCP molecules act independently of each other.

Information about the surface p$K$ of FCCP can be obtained by measuring $K_A$ and $K_{HA}$ as a function of pH. Table II shows the results of charge-pulse measurements performed in the pH range 6.1–9.1 on PE/chlorodecane membranes. When the pH increases from 6.1 to 8.8, $K_{HA}$ decreases by a factor of ~200 and $K_A$ increases by a factor of ~2.[2] This result is consistent with the predictions of Eqs. 21 and 22 if the surface p$K$ is close to the value of the aqueous p$K$, which is 6.1. The result (Table II) that $\beta_o = N_o/2[FCCP]$ is independent of pH is also consistent with $\beta_A \simeq \beta_{HA}$.

To find the best fit of the kinetic parameters to the data presented in Tables I and II, a search over "parameter space" could be conducted to determine the minimum value of chi squared (Bevington, 1969). However, it is apparent that there is a broad minimum in parameter space for surface p$K$ values between 6.0 and 6.4 and a simpler procedure is adopted here. The sum of the relative standard deviations for $k_{HA}$ and $k_A$ was minimized for p$K$ values from 6.1 to 6.4 using the data in Table II. The best value for the surface p$K$ was 6.1. The fit to all the data in Table II with this value for the surface p$K$ is shown in Fig. 4. The agreement is satisfactory. However, other values of the surface p$K$ also provide reasonable fits to the data. When the surface pK varies between 6.0 and 6.4, $k_A$ changes by only 100 s$^{-1}$ and $k_{HA}$ changes by about a factor of two, being 2 $10^4$ s$^{-1}$ for p$K$ 6.0 and $10^4$ s$^{-1}$ for p$K$ 6.4.

## Independent Measurements

We made independent measurements of the four parameters that define our kinetic model: $\beta_A$, $\beta_{HA}$, $k_A$, and $k_{HA}$. The adsorption coefficient of the anion onto the membrane-solution interface was determined on the Mueller-Rudin planar bilayer membranes used for the kinetic studies. A large voltage, 200 mV, was applied to the PE/chlorodecane membranes and the current was measured after the capacitance transient had subsided. This large voltage moves essentially all the anions adsorbed to one interface across the bilayer to the other interface. At pH 8.3 the magnitude of the relaxation is large; for example, $\alpha = 69$ for $V = 200$ mV when [FCCP] = $10^{-6}$ M. Only a small number of neutral HA molecules back diffuse from the $^{II}$ to the $^{I}$ interface (Fig. 1) during times comparable with the time constant ($\tau = 78$ $\mu$s) under these conditions. Thus the integral of $I(t) - I(\infty)$ over time,

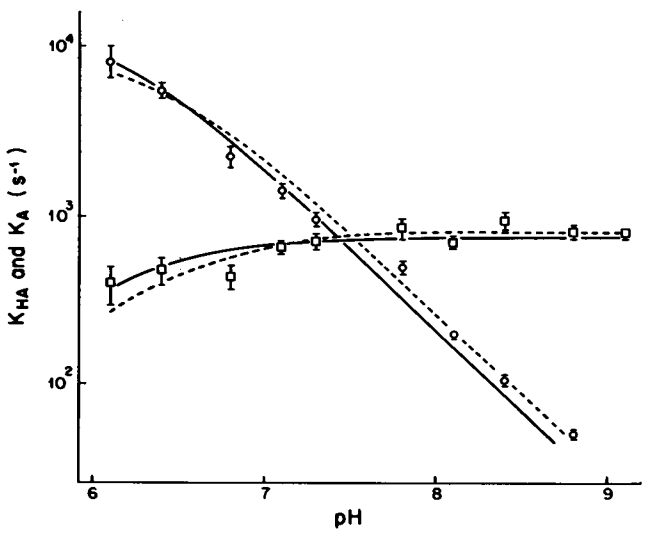


FIGURE 4 The values of $K_{HA}$ (O) and $K_A$ (□) plotted as a function of the pH of the aqueous phase. The solid curves (—) illustrate the predictions of Eqs. 21 and 22 with $k_{HA} = 1.7\ 10^4\ s^{-1}$, $k_A = 750\ s^{-1}$, and a surface pK = 6.1; whereas the dashed curves (---) were drawn with $k_{HA} = 1.1\ 10^4\ s^{-1}$, $k_A = 810\ s^{-1}$, and a surface pK = 6.4.

[1]When [FCCP] > $10^{-6}$ M, the electrostatic potential within the membrane changes, as discussed in Appendix C.

[2]At pH 9.1 it was not possible to determine $\tau_2$ accurately because of aqueous diffusion polarization. However, we could measure the fast relaxation and calculate $K_A$ from the data in a manner discussed previously for lipophilic ions (Benz et al., 1976).

where $I(t)$ is the current at time $t$ and $I(\infty)$ is the steady-state current, gives directly the number of charges per unit area adsorbed to one interface, $Q$ ($As/cm^2$ = $C/cm^2$). These values are plotted in Fig. 5. Note that at low concentrations the number of FCCP anions adsorbed to an interface, $N_A^o = Q/F$, increases linearly with the concentration of FCCP in the aqueous phase. For [FCCP] > $10^{-6}$ M, statistically significant deviations from linearity are observed; this saturation is discussed in Appendix C. We consider only the linear region of the curve and deduce the adsorption coefficient from Eq. 17, $\beta_A = N_A^o/[A^-]$. For [FCCP] = $10^{-6}$ M, $Q = 28.5 \pm 5\ 10^{-8}$ $C/cm^2$ (Fig. 5) at pH = 8.4, and $[A^-] \simeq$ [FCCP] so $\beta_A = 3.0\ 10^{-3}$ cm. This value agrees well with the value derived from the best fit of the model parameters to the charge pulse kinetic data obtained at pH 8.4 for [FCCP] $\leq$ $10^{-6}$ M, $\beta_A = 2.5\ 10^{-3}$ cm.

The adsorption of $A^-$ to zwitterionic phospholipid bilayer membranes was measured using two other techniques. We made zeta potential measurements with egg phosphatidylcholine (PC) multilamellar vesicles and equilibrium dialysis measurements with egg PC sonicated unilamellar vesicles. The zeta potential measurements are illustrated in Fig. 6. In the absence of FCCP the zeta potentials of the PC vesicles were zero in 0.1, 0.01, and 0.001 M NaCl. Addition of FCCP, more than 95% of which is in the anionic form at pH 7.5, produces a negative zeta potential (Fig. 6). The zeta potential is described theoretically by the Stern equation, which is a combination of the Langmuir adsorption isotherm, the Boltzmann relation, and the Gouy equation from the theory of the diffuse double layer (McLaughlin, 1977). The curves in Fig. 6 are the predictions of the Stern equation using the adsorption coefficient deduced from the 200 mV voltage-clamp measurements on the Mueller-Rudin black lipid membranes (Fig. 5). The fit of the theoretical curves of the data at low concentrations of FCCP and NaCl is quite acceptable. The deviations observed at high concentrations of FCCP and NaCl are presumably due to the production of an electrostatic boundary potential, as discussed in Appendix C. The adsorption coefficient of the anionic form of FCCP onto sonicated unilamellar vesicles of egg PC, determined by equilibrium dialysis, is $\beta_A = 3.9 \pm 1.1\ 10^{-3}$ cm (±SD of four sets of three measurements). This value agrees quite well with the value obtained using multilamellar egg PC vesicles (Fig. 6) and planar PE/chlorodecane bilayers (Fig. 5).

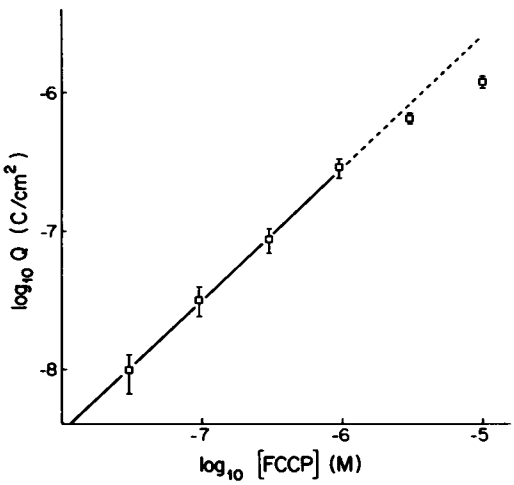


FIGURE 5 The number of FCCP anions adsorbed to one surface of a PE/chlorodecane bilayer membrane plotted as a function of the concentration of FCCP in the pH 8.4 solution. The charge adsorbed to one interface of the membrane, Q ($C/cm^2$ = $As/cm^2$), was determined by integrating the current $[I(t) - I(\infty)]$ vs. time curves obtained after a 200 mV pulse was applied to the membrane. The straight line is the linear least-squares best fit to the data obtained for [FCCP] $\leq$ $10^{-6}$ M; the slope is 0.99, which indicates that there is a linear relation between the aqueous concentration of FCCP and the number of adsorbed FCCP anions. The adsorption coefficient obtained from these data is $\beta_A = 3\ 10^{-3}$ cm.

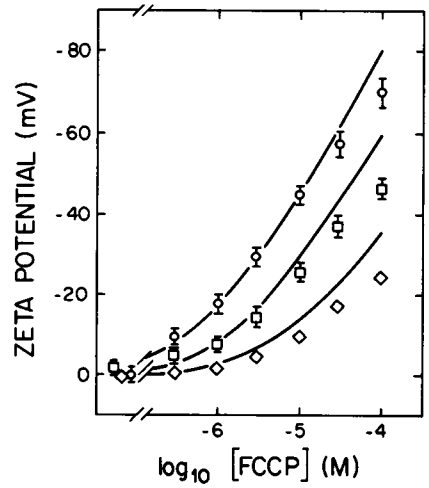


FIGURE 6 The zeta potential of egg phosphatidylcholine multilamellar vesicles measured as a function of the aqueous concentration of FCCP. The aqueous solutions contained 0.001 M NaCl (○), 0.01 M NaCl (□) or 0.1 M NaCl (◇) buffered to pH 7.5 with either 0.0001 or 0.001 M MOPS at 25°C. The vertical lines through the points represent the standard deviations of measurements made on 20 vesicles. The solid lines (—) are the predictions of the Stern equation, drawn for an intrinsic dissociation constant of $8\ 10^{-5}$ M. The density of binding sites was assumed to be 1/70 $Å^2$, about the area of a phosphatidylcholine molecule, and the plane of shear was assumed to be 2 Å from the surface of the membrane (Eisenberg et al., 1979). An intrinsic dissociation constant of $8\ 10^{-5}$ M is equivalent to an adsorption coefficient of $\beta_A = 3\ 10^{-3}$ cm, a value that agrees with the adsorption coefficient determined by the other independent measurements on planar bilayers and sonicated vesicles.

The adsorption coefficient of the neutral form of FCCP onto sonicated egg PC vesicles was also determined by means of equilibrium dialysis. The value is $\beta_{HA} = 3.3 \pm 0.7\ 10^{-3}$ cm (±SD of six sets of three measurements). The equilibrium dialysis results confirm the conclusion from charge pulse kinetic measurements that $\beta_{HA} \simeq \beta_A$.

The third parameter that was determined by independent measurements is the rate constant for the movement of the anion, $k_A$. Eq. 11 predicts that $I = F(-k_A^l N_A^l + k_A^{ll} N_A^{ll})$. In the limit that the time after the application of

the voltage approaches zero, the interfaces are not perturbed and $N^{I}_{A} = N^{II}_{A} = \beta_A[A^-]$ (Eq. 17). In the limit that the applied voltage, $u = FV/RT$, approaches zero, $k^{I}_{A} - k^{II}_{A} = k_A u$ (Eqs. 19, 20). Thus the conductance measured in the limit that both voltage and time approach zero, $G(0, 0)$, is given by

$$G(0, 0) = F^2\beta_A[A^-]k_A/RT. \quad (23)$$

We know the value of $\beta_A$, and we can determine the value of $k_A$ by measuring the conductance in the region where it depends linearly on the aqueous concentration of FCCP. These measurements are illustrated in Fig. 7. At pH 8.4, where essentially all the FCCP is in the $A^-$ form, the conductance produced by $3\ 10^{-7}$ M FCCP is $2.3 \pm 0.6\ 10^{-3}$ S/cm$^2$ (±SD 7 measurements). With this value of $G(0, 0)$ and $\beta_A = 3\ 10^{-3}$ cm, we obtain a value of $k_A = 700\ s^{-1}$ from Eq. 23. This value of $k_A$ agrees quite well with the value of $k_A = 750$–$810\ s^{-1}$ obtained from a best fit of the kinetic parameters to the charge-pulse data.

We know of no way to directly determine $k_{HA}$ for FCCP from independent measurements. However, we can measure $P_{HA}$, the permeability of the entire membrane to HA, by a technique developed by LeBlanc (1971). As discussed in more detail in McLaughlin and Dilger (1980), the membrane potential produced by a difference in the pH of the solutions on the two sides of the membrane will be Nernstian, $\Delta V/\Delta pH = 2.2303(RT/F) = 58.5$ mV at 22°C, when the mechanism illustrated in Fig. 1 is operative. However, as the pH of the aqueous solutions increases the concentration of HA decreases, the back diffusion of HA ultimately becomes rate limiting, and FCCP behaves as a simple lipid solution anion. In this limit $\Delta V/\Delta pH = 0$.

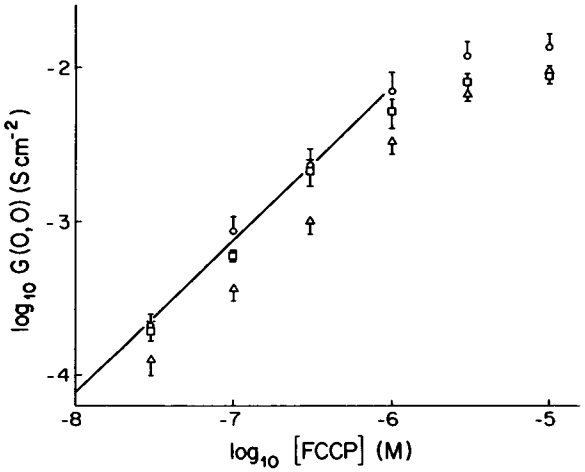


FIGURE 7 The conductance, $G(0, 0)$, of a PE/chlorodecane planar bilayer membrane plotted as a function of the aqueous concentration of FCCP. ○, pH 8.4; □, pH 7.4; △, pH 6.4. The solid line is the least-squares best fit to the pH 8.4 data obtained with [FCCP] $\leq 10^{-6}$ M; the slope of the log-log plot is 1.0, which indicates there is a linear relation between $G(0, 0)$ and [FCCP] at these concentrations. The deviations from linearity observed when [FCCP] $\geq 10^{-6}$ M are due to the production of an electrostatic potential (Appendix C).

Thus, by measuring $\Delta V/\Delta pH$ as a function of pH and fitting Eq. 24 to the data, we can obtain a value for $P_{HA}$:

$$dV/dpH = 2.303(RT/F)\frac{([H^+]K^{aq})(1 + 2\delta P_{HA}/D)}{1 + ([H^+]K^{aq})(1 + 2\delta P_{HA}/D)} \quad (24)$$

where $D/2\delta$ is the permeability of the two unstirred layers to HA, $\delta$ is the thickness of the Nernstian unstirred layers, which is ~100 μm (Cohen et al., 1977), $D$ is the diffusion coefficient of the HA and $A^-$ species of FCCP in the aqueous phases, which we assume to be $5\ 10^{-6}$ cm$^2$/s, and $K^{aq}$ is the association constant of FCCP in the bulk aqueous phase ($K^{aq} = 1.1\ 10^6\ M^{-1}$, or $pK^{aq} = 6.05$). As illustrated in Fig. 8, a reasonable fit to the data is obtained by assuming that the value of $P_{HA} = 50$ cm/s. The value of $P_{HA}$ for PC membranes formed with chlorodecane as a solvent may be slightly higher than the value of $P_{HA}$ for PC membranes formed with decane, but the difference is less than a factor of 2. (Increasing the value of $P_{HA}$ by a factor of two shifts the curve is Fig. 8 to the right by 0.3 pH units.) One conclusion that can be drawn immediately from the data of Fig. 8 is that the value of $P_{HA}$ does not depend markedly on the dielectric constant of the bilayer. In contrast, the permeability of a PC membrane to the anionic form of FCCP increases by more than two orders of magnitude when decane is replaced by chlorodecane (McLaughlin and Dilger, 1980), a procedure that increases the dielectric constant of the bilayer (Dilger et al., 1979).

The value of $P_{HA} = 50$ cm/s obtained from these experiments is the permeability of the entire membrane, including the interfaces, to HA. The "permeability" of the region between the two energy wells to HA may be calculated as $\beta_{HA} \cdot k_{HA} = (3.0\ 10^{-3}\ cm)\ (1\text{–}2\ 10^4\ s^{-1}) \simeq$ 30–60 cm/s. The approximate equality of these two num-

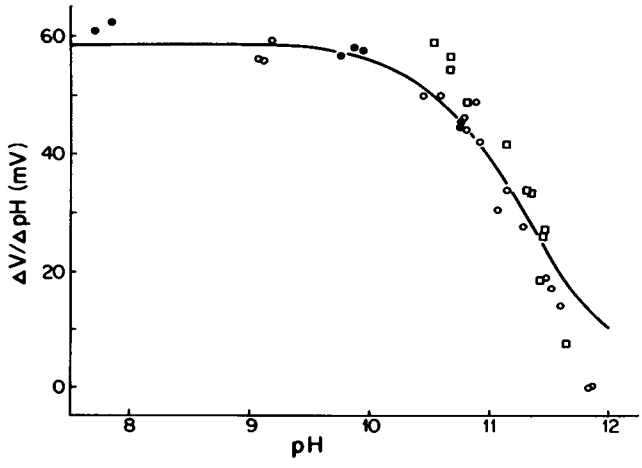


FIGURE 8 The membrane potential, $\Delta V$, produced by a small difference in pH, ΔpH, between the two aqueous solutions bathing a bilayer membrane formed from diphytanoyl phosphatidylcholine in chlorodecane (□) or decane (●, ○). The aqueous phases contained $10^{-5}$ M (open symbols) or $10^{-6}$ M (filled symbols) FCCP at the indicated pH. The solid line (—) is the prediction of Eq. 24, assuming that $P_{HA} = 50$ cm/s.

bers suggests that the HA form of FCCP encounters no large interfacial barriers that either limit its entry into or prevent its exit from the deep energy wells at the interfaces of a bilayer membrane.

## Voltage Clamp Results

We first examine how the conductance, $G(V, 0)$, depends on the applied voltage, $V$. It is apparent from Eqs. 11, 19, and 20 that

$$G(V, 0)/G(0, 0) = \sinh(u/2)/\sinh(bu/2) \qquad (25)$$

where $b$ is the fraction of the membrane spanned by the minor base of the trapezoidal energy barrier and $u \equiv FV/RT$. The model predicts that the shapes of the $G(V, 0)/G(0, 0)$ vs. $V$ curves should be independent of both [FCCP] and pH. Our experimental results agree with this prediction. Fig. 9 illustrates the results obtained at pH 6.4 for [FCCP] $\leq 10^{-6}$ M and at pH 8.4 for [FCCP] = $3\ 10^{-7}$ M. The data are well described by assuming that the $A^-$ species encounters a trapezoidal barrier whose minor base spans 0.65 of the membrane (solid line in Fig. 9).

Fig. 10 illustrates a voltage-clamp record made under the same conditions that were used to obtain the charge-pulse record illustrated in Fig. 2. Note, in the left-hand portion of Fig. 10, that the capacitance spike, which has a

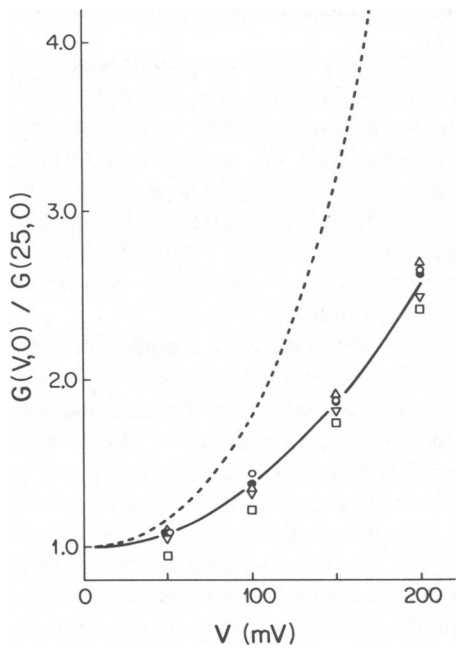


FIGURE 9 Conductance-voltage curves for FCCP. The initial conductance of a PE/chlorodecane bilayer measured upon application of a voltage $V$, $G(V, 0)$, was divided by the initial conductance measured on application of 25 mV, $G(25, 0)$, and plotted against the applied voltage. ○, pH = 6.4 and [FCCP] = $3\ 10^{-8}$ M; □, $10^{-7}$ M; △, $3\ 10^{-7}$ M; ▽, $10^{-6}$ M. ●, pH = 8.4 and [FCCP] = $3\ 10^{-7}$ M. The data points represent the averages of measurements on at least five different membranes. The solid line (—) is the prediction of Eq. 25 assuming $b = 0.65$. The dashed line (---) is the prediction of Eq. 25 in the limit that $b$ approaches zero, a single barrier Eyring model.

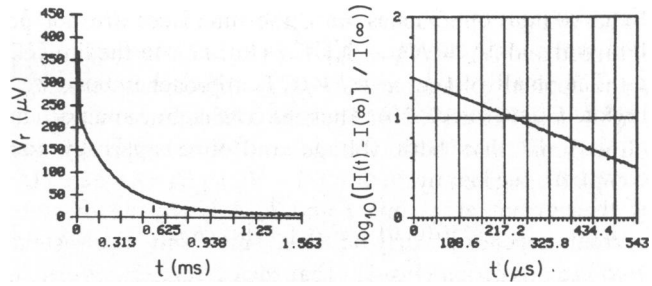


FIGURE 10 Voltage-clamp record obtained upon application of 100 mV to a PE/chlorodecane membrane in a pH 8.4 solution containing $10^{-7}$ M FCCP. *Left*, the voltage recorded across a 200 Ω resistor in series with the membrane, $V_r$, plotted as a function of time. The signal was averaged four times. *Right*, $\log_{10}\{[I(t) - I(\infty)]/I(\infty)\}$ plotted as a function of time, where $I(t)$ is the current at a time $t$ and $I(\infty)$ is the steady-state current.

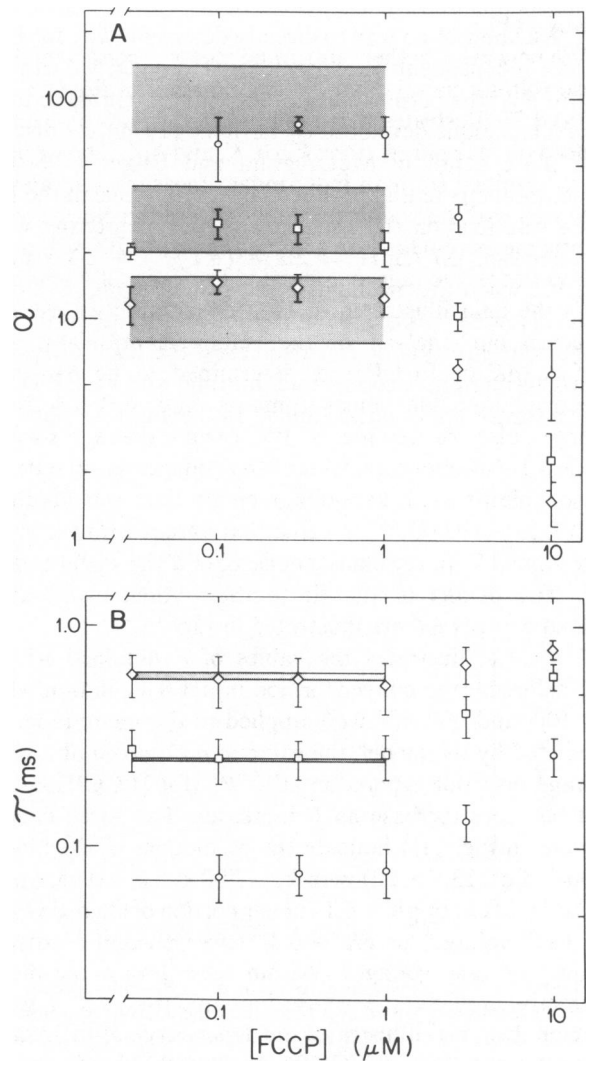


FIGURE 11 The average values of $\alpha$, the amplitude of the relaxation ($A$), and $\tau$, the time constant of the relaxation ($B$), both plotted as a function of the concentration of FCCP in the pH 8.4 solutions. ○, $V$ = 200 mV; □, $V$ = 100 mV; ◇, $V$ = 25 mV.

time constant of ~2 μs, is clearly distinct from the relaxation in the ionic current, which has a time constant of 262 μs. The small vertical bars near the abscissa indicate the region of data selected for analysis. The right-hand portion of Fig. 10 illustrates the data after analysis by the computer: the logarithm of $[I(t) - I(\infty)]/I(\infty)$, where $I(t)$ is the current at a time $t$ and $I(\infty)$ is the steady-state current, depends linearly on time. This result is consistent with the prediction (Eq. 12) that the current should decay with a single time constant. Only a single exponential was observed under voltage clamp conditions; this was true at all voltages, at all [FCCP] and at all values of the pH that we examined. In this example (Fig. 10), the magnitude of the relaxation was $\alpha = 25.0$ and the time constant of the relaxation was $\tau = 262$ μs. The values of $\alpha$ and $\tau$ were measured at [FCCP] between $3\ 10^{-8}$M, and $10^{-5}$M, at voltages between 25 and 200 mV and at pH 6.4, 7.4, and 8.4. Measurements were made on five or more membranes in separate experiments; representative results are presented in Fig. 11.

We now ask whether our simple model is consistent with these voltage clamp data. An examination of Eqs. 13, 14, 19, and 20 illustrates that the predicted values of $\alpha$ and $\tau$ depend on four parameters: $k_A$, $b$, $K$, and $k_{HA}$. The value of $k_A$ determined from an independent measurement agreed very well with the value determined from the charge pulse kinetic measurements; we assume that $k_A = 700\ s^{-1}$ in our analysis of the voltage clamp data. The value of $b$ obtained from the data illustrated in Fig. 9 was 0.65; we use this value in our analysis of the voltage-clamp data. The surface $pK$ of FCCP was determined to be 6.0 from spectrophotometric measurements and 6.0–6.4 from charge pulse measurements. We assume that the surface $pK$ is 6.1–6.4 in our analysis of the voltage clamp data. A reasonable fit to all the voltage clamp data was obtained with $k_{HA} = 10{,}000\ s^{-1}$, a value that agrees with the value determined from the charge pulse data if the surface $pK$ = 6.4. The details of the fit to the voltage clamp data obtained at pH 8.4 are illustrated in Fig. 11.

Fig. 11*A* illustrates the values of $\alpha$ obtained with a PE/chlorodecane bilayer formed in pH 8.4 solution when 25, 100, and 200 mV were applied to the membrane. As predicted by the model, the value of $\alpha$ observed at a given voltage does not depend on [FCCP] (for [FCCP] $\leq 10^{-6}$ M) but does increase as $V$ increases. The three banded regions in Fig. 11*A* indicate the predictions of the kinetic model (Eqs. 13, 19, 20) with $k_A = 700\ s^{-1}$, $b = 0.65$, $k_{HA} = 10^4\ s^{-1}$ and either $pK = 6.1$ (upper portion of shaded region for each voltage) or $pK = 6.4$ (lower portion of shaded region for each voltage). Within these limits, the model correctly predicts the relaxation amplitude at a given voltage. Fig. 11*B* illlustrates the dependence of the relaxation time constant on [FCCP]. As predicted by the model, $\tau$ is independent of [FCCP] for [FCCP] $\leq 10^{-6}$ M. The crosshatched regions represent the predictions of the model, Eq. 14, for the same choice of parameters as in Fig. 11*A*. The upper bound is given by $pK = 6.1$ and the lower bound by $pK = 6.4$. The fit is good. The marked decrease in the value of $\alpha$ (Fig. 11*A*) and the increase in $\tau$ (Fig. 11*B*) observed when [FCCP] increases above $10^{-6}$ M are also observed at pH 7.4. and 6.4. They are due to the production of an electrostatic potential at the surface of the membrane, as discussed in Appendix C.

Eq. 13 predicts that the amplitude of the relaxation measured at a given applied voltage should decrease by one order of magnitude as the pH decreases by one unit. The experimental data are consistent with this prediction. For example, when [FCCP] = $3\ 10^{-7}$ M and $V = 100$ mV, the values of $\alpha$ at pH 7.4 and 6.4 are 4.3 ± 0.4 and 0.4 ± 0.04, respectively. These values agree with the values of 4 and 0.4 predicted by Eq. 13 with $k_A = 700\ s^{-1}$, $b = 0.65$, $pK = 6.1$, and $k_{HA} = 10{,}000\ s^{-1}$. The dependence of the time constant on pH is also adequately described by the model. For example, when [FCCP] = $3\ 10^{-7}$ M and $V = 100$ mV the measured values of $\tau$ at pH 7.4 and 6.4 are 250 ± 40 and 145 ± 4 μs, respectively. The values predicted by Eq. 14 with $k_A = 700\ s^{-1}$, $b = 0.65$, $k_{HA} = 10^4 s^{-1}$, and $pK = 6.1$ are 212 and 107 μs.

In summary, the charge-pulse data, the independent measurements, and the voltage-clamp data are all qualitatively consistent with a simple kinetic model.

## Results with Other Lipids

Experiments were performed on membranes composed of lipids other than bacterial phosphatidylethanolamine to investigate the influence of membrane structure on protonophore kinetics. Table III presents the results obtained with diphytanoyl phosphatidylcholine/chlorodecane membranes when the aqueous phases are buffered to pH 7.4. The values of $K_{HA}$ and $K_A$ are smaller by factors of only 4 and 2, respectively, than the values obtained with PE/chlorodecane membranes.

Kinetic data obtained with membranes formed from different lipids are presented in Table IV. The value of $K_{HA}$ is largest for membranes formed from monoolein, about an order of magnitude larger than the values obtained with the phospholipids. On the other hand, $K_A$ is lowest for monoolein, about a factor of 2–5 lower than the value obtained with phospholipids. The partition coefficient of the anion onto the surface is also lower for the monoolein than for the phospholipid membranes. The lower values of the partition coefficient and the rate constant of the anion for monoolein membranes can be rationalized in terms of a less positive dipole potential in the interior of monoolein bilayer membranes (Hladky, 1974).

The values of $k_{HA}$, $k_A$, and $\beta_A$ for the three lipids that we have investigated are summarized in Table V. The protonophore activity of FCCP has also been examined on bilayer membranes formed from a mixture of lipids chosen to mimic the lipid composition of the inner mitochondrial membrane (McLaughlin and Dilger, 1980). Our main

TABLE III
CHARGE-PULSE DATA FROM PC/CHLORODECANE BILAYERS

| [FCCP]/$\mu$M | $\tau_1/\mu s$ | $\tau_2$/ms | $a_2$ | $K_{HA}/10^2 s^{-1}$ | $K_A/10^2 s^{-1}$ | $N_o$/pmol cm$^{-2}$ | $\beta_o/10^{-3}$cm |
|---|---|---|---|---|---|---|---|
| 0.001 | | 180 ± 12 | 1 | | | | |
| 0.01 | 860 ± 110 | 28 ± 5 | 0.94 ± 0.013 | 2.0 ± 0.09 | 3.5 ± 0.60 | 0.13 ± 0.021 | 6.5 |
| 0.03 | 640 ± 58 | 9.6 ± 0.53 | 0.86 ± 0.010 | 2.7 ± 0.08 | 4.2 ± 0.54 | 0.32 ± 0.010 | 5.3 |
| 0.1 | 490 ± 25 | 3.9 ± 0.36 | 0.56 ± 0.016 | 2.6 ± 0.16 | 3.8 ± 0.10 | 1.2 ± 0.060 | 6.0 |
| 0.3 | 270 ± 12 | 2.3 ± 0.16 | 0.30 ± 0.031 | 3.1 ± 0.25 | 4.2 ± 0.29 | 2.7 ± 0.15 | 4.5 |
| 1 | 130 ± 13 | 1.5 ± 0.09 | 0.12 ± 0.013 | 3.9 ± 0.27 | 3.9 ± 0.89 | 8.3 ± 1.1 | 4.2 |
| 3 | 86 ± 5 | 0.58 ± 0.10 | 0.05 ± 0.002 | 9.2 ± 1.6 | 2.1 ± 0.26 | 23 ± 2.1 | 3.8 |

Relaxation data obtained from charge pulse experiments on diphytanoyl phosphatidylcholine/chlorodecane bilayer membranes. The aqueous phases were buffered to pH 7.4 and contained the concentration of FCCP indicated in the first column. The values of $K_{HA}$, $K_A$, and $N_o$ were calculated from Eqs. B20–B22 using the experimentally determined value of $C_m = 0.83\ \mu F\ cm^{-2}$.

TABLE IV
LIPID DEPENDENCE OF RELAXATION DATA

| lipid | [FCCP]/$\mu$M | $\tau_1/\mu s$ | $\tau_2$/ms | $a_2$ | $K_{HA}/10^2 s^{-1}$ | $K_A/10^2 s^{-1}$ | $N_o$/pmol cm$^{-2}$ | $\beta_o/10^{-3}$cm |
|---|---|---|---|---|---|---|---|---|
| Monoolein | 3 | 320 ± 72 | 0.79 ± 0.13 | 0.40 ± 0.069 | 8.5 ± 1.3 | 1.7 ± 0.43 | 5.7 ± 0.59 | 0.55 |
| Diphytanoyl phosphatidylcholine | 1 | 150 ± 27 | 1.5 ± 1.1 | 0.12 ± 0.008 | 0.38 ± 0.02 | 4.0 ± 0.64 | 6.4 ± 0.60 | 3.2 |
| Phosphatidyl-ethanolamine | 1 | 74 ± 9 | 5.6 ± 0.49 | 0.14 ± 0.10 | 1.1 ± 0.08 | 9.2 ± 0.89 | 4.9 ± 0.41 | 2.5 |

Results of charge pulse experiments on bilayer membranes formed from different lipids dissolved in n-chlorodecane. The aqueous phases were buffered to pH 8.4 and contained the indicated concentration of FCCP. The values of $K_{HA}$, $K_A$, and $N_o$ were calculated from Eqs. B20–B22 using $C_m \simeq 0.8\ \mu F\ cm^{-2}$ for all membranes.

conclusion is that lipid structure has less effect on the protonophore activity of FCCP than do electrostatic potentials and the dielectric constant of the membrane.

## DISCUSSION

The model sketched in Fig. 1 illustrates the simplest possible mechanism by which a weak acid could carry protons across a membrane. The results obtained from both charge-pulse and voltage-clamp experiments with FCCP are all consistent with this model. The model is certainly not unique in this respect: the kinetic data are consistent with an infinite number of more complex models. For this reason, independent experiments were performed to provide more direct information about both the partitioning of the $A^-$ and HA forms of FCCP onto the membrane-solution interface and the permeability of the membrane to these species. The values deduced from the model using the kinetic data and the values determined by the more direct measurements agree within a factor of 2. This agreement suggests that the model adequately describes the ability of FCCP to transport protons across a bilayer membrane, and, by inference, across the bilayer component of a biological membrane.

TABLE V
LIPID DEPENDENCE OF RATE CONSTANTS

| lipid | $k_{HA}/s^{-1}$ | $k_A/s^{-1}$ | $\beta_A/10^{-3}$cm |
|---|---|---|---|
| Monoolein | $1.7\ 10^5$ | 170 | .55 |
| Diphytanoyl phosphatidylcholine | $7.6\ 10^3$ | 400 | 3.2 |
| Phosphatidylethanol-amine | $2.2\ 10^4$ | 920 | 2.6 |

Rate constants for FCCP-mediated $H^+$ transport through bilayer membranes formed from the indicated lipids dissolved in chlorodecane. The values of $k_{HA}$ and $k_A$ were calculated from the data presented in Table IV using Eqs. 21 and 22 and assuming that the surface pK = 6.1.

We now discuss the experimental evidence that supports each of the seven assumptions listed in the Kinetic Model section above. (*a*) The assumption that the anionic form of FCCP is the only charged species that moves within the membrane is consistent with the observation that the initial ohmic conductance increases linearly with the aqueous concentration of FCCP and has the expected dependence on pH (Fig. 7; McLaughlin and Dilger, 1980). (*b*) The assumption that a proton from the aqueous phase combines with an anion adsorbed to the membrane by means of a heterogeneous reaction, the interface-complexation mechanism, may be justified by the following argument. Neumcke and Bamberg (1975) have shown that the maximum conductance possible for the alternative solution-complexation mechanism, whereby a proton combines with an anion in the aqueous phase and the resultant HA complex crosses the interface, is

$$\bar{G}(0, 0) = (F^2 c/4RT)\ (Dk_D^{aq}/2)^{1/2} \qquad (26)$$

when pH = p$K$. In this equation, $D$ is the diffusion

coefficient of HA and $A^-$ in the aqueous phase, $k_D^{aq}$ is the aqueous dissociation rate constant, and $c$ is the aqueous concentration of the protonophore. For FCCP ($pK^{aq} = 6.05$), $k_D^{aq} = 10^4 s^{-1}$ if the forward reaction is diffusion controlled ($10^{10} M^{-1}s^{-1}$). If $D = 5\ 10^{-6}$ $cm^2s^{-1}$ and $c = 10^{-6}$ M, the maximum value of the conductance is $1.5\ 10^{-4}$ S $cm^{-2}$. The experimental value of the conductance at pH 6.4 is a factor of 20 larger than this predicted value (Fig. 7). Thus, <5% of the conductance could be due to the solution-complexation mechanism. (*c*) The Nernstian potential for protons that is observed when a pH difference exists across the membrane (Fig. 8, pH < 10) is consistent with the assumption that the proton is the only charged species that moves between the two aqueous phases. (*d*) The observation that both the conductance and the number of adsorbed anions increase linearly with the aqueous concentration of FCCP (Figs. 7 and 5, [FCCP] $\leq 10^{-6}$ M) is consistent with the assumption that the adsorption of FCCP to the membrane-solution interfaces does not significantly change the electrostatic potential. (*e*) The use of Eqs. 1–4 involves a number of assumptions about either the Nernst-Planck or the Eyring formalisms, assumptions that are discussed below. (*f*) The assumption that the total concentration of FCCP adsorbed to the membrane surfaces remains constant during a relaxation experiment is easy to justify. Most of our kinetic experiments require <1 ms. If the diffusion coefficient of $A^-$ and HA in the aqueous phase is $5\ 10^{-6}$ $cm^2s^{-1}$, these molecules can diffuse a distance of $(2Dt)^{1/2} \simeq 10^{-4}$ cm in 1 ms. If the aqueous concentration of FCCP is $10^{-6}$ M, a layer of thickness $10^{-4}$ cm adjacent to the membrane contains $10^{-13}$ mol $cm^{-2}$ The number of FCCP molecules adsorbed to half of the membrane is $3\ 10^{-12}$ mol $cm^{-2}$. Thus, the FCCP concentration in the membrane cannot change appreciably during the time course of a relaxation experiment. (*g*) The assumption that the interfacial reaction is essentially at equilibrium throughout the relaxation experiments implies that only two relaxations will be observed in a charge-pulse experiment, and only one relaxation will be observed in a voltage-clamp experiment. The converse statement is not true. The existence of only one relaxation in a voltage-clamp experiment does not imply that the interfacial reactions illustrated in Fig. 1 remain essentially at equilibrium. For the $k_{HA}$, $k_A$, $\beta_A$, and $K$ parameters characteristic of FCCP, a "slow" heterogeneous reaction of an aqueous $H^+$ with an adsorbed $A^-$ will not manifest itself as an additional relaxation but will cause $\alpha$ to increase above the value predicted by Eq. 13. This was demonstrated by solving numerically the complete voltage clamp equations presented in Appendix A. These calculations indicate that if $k_R = 10^{10}$ $M^{-1}s^{-1}$, the value of $\alpha$ would be four times larger than the value predicted by Eq. 13 because $I(\infty)$ would be reduced. If $k_R = 10^{11}$ $M^{-1}s^{-1}$, the interfacial reactions would remain essentially at equilibrium and $\alpha$ would be only 30% larger than the value predicted by Eq. 13. The agreement between the value of $\alpha$ measured in voltage-clamp experiments and the predictions of Eq. 13 (Fig. 11) suggest that $k_R \geq 10^{11}$ $M^{-1}s^{-1}$. A similar conclusion was reached from an analysis of the FCCP charge-pulse data using the complete equations presented in Appendix B. The rate constant for the diffusion limited reaction of a proton with a weak acid in a bulk aqueous solution is $10^{10} - 10^{11}$ $M^{-1}s^{-1}$ (Eigen et al., 1964). Thus the apparent rate constant for FCCP is slightly larger than the value that is characteristic of a diffusion limited reaction in a bulk aqueous phase. Similar experiments using the protonophores S-13 (Benz and McLaughlin, unpublished data) and TTFB (Cohen et al., 1977) indicate that the apparent rate constants for the combination of a proton with these adsorbed anions are $>10^{12}$ and $10^{13}$ $M^{-1}s^{-1}$, respectively. Those values are 1–2 orders of magnitude larger than the value for a diffusion-limited reaction. We cannot rule out the possibility that the protons diffuse more rapidly in the aqueous "reaction layer" immediately adjacent to a membrane than in a bulk solution. However, we consider it more likely that the proton is donated to the adsorbed anion by the acidic form of the buffer, which is present in relatively high concentration in the aqueous phase. There is a large body of experimental evidence indicating that protons can be rapidly transferred between weak acids in an aqueous solution (Bell, 1973). Jonsson et al. (1976) demonstrated that protons are transported to the active sites on carbonic anhydrase by the acidic form of buffers; the apparent rate constants for this transfer are $10^8$ $M^{-1}s^{-1}$ for six different buffers. All the data we have obtained with protonophores are consistent with the postulate that the proton is transferred directly from the acidic form of the buffer, which is in the aqueous phase, to the anionic form of the protonophore, which is adsorbed to the membrane, provided that the rate constant for the transfer is $10^8$ $M^{-1}s^{-1}$ or greater.

We now estimate the free energies of the neutral and charged forms of FCCP as they move through a PE/chlorodecane membrane. Consider first the HA species (Fig. 12). If we use the approach of Guggenheim rather than Gibbs (e.g., Aveyard and Haydon, 1973) and assume that the surface is a separate phase of finite thickness, we can calculate a dimensionless partition coefficient into the surface phase. For example, if the thickness, $l$, is assumed to be 0.5 nm, the dimensionless partition coefficient of HA into the surface phase is $\beta_{HA}/l = 3\ 10^{-3}$ cm/$5\ 10^{-8}$ cm = $6\ 10^4$. Thus the free energy, $\Delta G$, of HA drops by $-RT\ln(\beta_{HA}/1) = 6.4$ kcal/mol (27 kJ/mol) as it moves from the aqueous phase into the interfacial well. The free energy of HA within the membrane, which we represent as a constant in the absence of any additional information, is 3.0 kcal/mol (13 kJ/mol) lower than the free energy of HA in the aqueous phase. The free energy difference was estimated from the expression $\Delta G = -RT\ln(P_{HA}d/D)$ using the measured value of the membrane permeability, $P_{HA} = 50$ cm/s, assuming that the thickness of the membrane $d = 3.5$ nm, and assuming that the diffusion

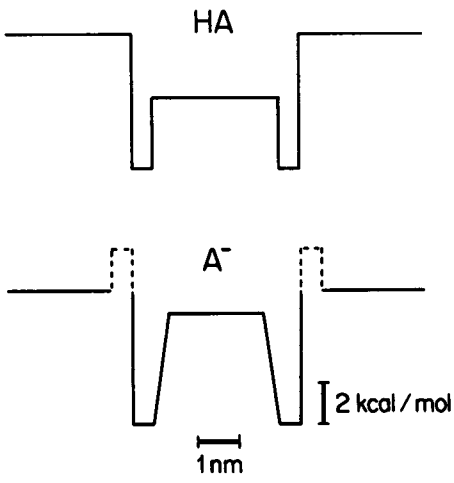


FIGURE 12 Profile of the free energy, $\Delta G$, of the neutral, HA, and anionic, $A^-$, forms of FCCP in a PE/chlorodecane bilayer membrane. See text for details.

coefficient of HA within the membrane $D = 10^{-7}$ cm$^2$/s. Our experimental evidence indicates that there is no interfacial barrier to the movement of HA from the aqueous phase into the interfacial well (see Results). Now consider the $A^-$ species. The depths of the interfacial wells are similar for the HA and $A^-$ species: $\beta_A = 3\ 10^{-3}$ cm and $\Delta G = 6.4$ kcal/mol (27 kJ/mol) if the width of the well is 0.5 nm.[3] We have no experimental information about the interfacial barrier in the case of the anion; the possibility that such a barrier might exist is indicated by the dotted lines in Fig. 12.[4] The free energy difference between the aqueous phase and the center of the membrane was calculated to be 1.1 kcal/mol (4.8 kJ/mol) from the equation $\Delta G = -RT \cdot \ln\{RTG(0,0)d/F^2D[A^-]\}$, using the experimental value for the initial ohmic conductance, $G(0,0) = 2.3\ 10^{-3}$ S/cm$^2$ when $[A^-] = 3\ 10^{-7}$M (Fig. 7, pH = 8.4), and assuming that $D = 10^{-7}$ cm$^2$/s and $d = 3.5$ nm. Note that the anion partitions favorably into the interior of the membrane.[5] From the shape of the current-voltage curves we deduce that $b = 0.65$ where $b$ is the fraction of the membrane spanned by the minor base of the trapezoid. If the thickness of the membrane is 3.5 nm, the width of the minor base of the trapezoid is 2.3 nm, as illustrated in Fig. 12.

The free energy profiles illustrated in Fig. 12 are only approximate values. Because our calculations are based on the Nernst-Planck formalism, we had to assume a value for the diffusion coefficient within the membrane to calculate the difference in free energy. If, for example, the diffusion coefficients of the HA and $A^-$ species in the membrane are actually $10^{-8}$ rather than $10^{-7}$ cm$^2$/s, as we assume, then the free energy differences between the aqueous phase and the center of the membrane will change by 1.3 kcal/mol (5.6 kJ/mol). There is little advantage to using the Eyring formalism because we would have to assume a value for the frequency factor (Zwolinsky et al., 1949). If we had used the Eyring formalism, we would have concluded either that $A^-$ experiences at least two barriers when it crosses the membrane or that only a fraction of the applied voltage drops across a single central barrier. In the Nernst-Planck formalism the shape of the energy barrier encountered by the anion can be represented by a trapezoid. It is our prejudice that the Nernst-Planck formalism provides a more realistic picture of how a carrier molecule traverses a bilayer membrane than does a one or two barrier Eyring model.

We now consider the roles that the lipid composition and dielectric constant of the bilayer might play in the transport of protons across membranes by FCCP. Neither the adsorption coefficients nor the rate constants are greatly affected by substitution of one zwitterionic phospholipid for another (Table V). The presence of negative lipids in the membrane decreases both the concentration of $A^-$ species at the membrane-solution interface and the ability of FCCP to act as a protonophore (McLaughlin and Dilger, 1980). This effect can be described by the electrostatic theory of the diffuse double layer (McLaughlin, 1977). The effect of dielectric constant is more interesting because it provides insight into the mechanism by which FCCP uncouples mitochondria. The partitioning of $A^-$ onto the membrane-solution interface does not depend upon the dielectric constant of the membrane interior: the value of the adsorption coefficient, $\beta_A$, is essentially the same for chlorodecane-containing planar bilayer mem-

[3]The interfacial adsorption coefficients of the HA and $A^-$ forms of the weak acid are approximately identical for FCCP and for a number of other weak acids (Cohen et al., 1977; Lee, 1978; Dilger and McLaughlin, 1979; McLaughlin and Dilger, 1980). These observations are consistent with the postulate that the free energy of adsorption is dominated by the hydrophobic tendency of the nonpolar regions of the molecule to escape from the aqueous phase. Microscopic reversibility and the assumptions inherent in our model imply that $K = (\beta_{HA}/\beta_A)K^{aq}$. Direct equilibrium dialysis measurements with egg PC demonstrate that $\beta_{HA} \simeq \beta_A$ for FCCP. If our simple model is adequate, $K$ should equal $K^{aq}$ for FCCP. The charge-pulse, voltage-clamp, and more direct spectroscopic measurements on membrane vesicles are all consistent with $6.0 \leq pK \leq 6.4$ for FCCP, values that agree reasonably well with $pK^{aq} = 6.1$. In a real system, $K$ need not equal $K^{aq}$ when $\beta_{HA} = \beta_A$ (Fernandez and Fromherz, 1977).

[4]The interfacial barrier for the $A^-$ form of FCCP should be small based on studies with other molecules. Specifically, there is no interfacial barrier to the movement of either the neutral form of FCCP (Results) or the neutral molecule phloretin into a bilayer membrane (Verkman and Solomon, 1981). Similarly, the interfacial barrier for the movement of the anion tetraphenylborate into the interfacial well is smaller than the barrier in the center of the membrane (Jordan and Stark, 1979; Brock et al., 1981).

[5]The Born energy required to move a charge into a low dielectric interior of the membrane is reduced because of the delocalization of the charge on the electrons of FCCP, a common feature of all known weak acid protonophores (McLaughlin and Dilger, 1980). In the case of FCCP, the Born energy must be less important than the terms favoring partitioning into the center of the membrane; these terms include the positive dipole potential in the interior of a phospholipid bilayer membrane and the hydrophobic interactions.

branes, solvent-free multilamellar vesicles and solvent-free sonicated vesicles. The available evidence also suggests that the partitioning of HA onto the membrane-solution interface does not depend on the dielectric constant: the value of $\beta_{HA}$ estimated from kinetic measurements on chlorodecane-containing bilayers is similar to the value obtained from equilibrium dialysis experiments with solvent-free sonicated vesicles. As one might expect, the permeability of a bilayer membrane to the neutral form of a weak acid is essentially independent of the dielectric constant of the membrane. Specifically, the permeabilities of the decane- and chlorodecane-containing bilayer membranes to the HA form of FCCP differ by less than a factor of 2 (Fig. 8). In contrast, the permeability of a bilayer membrane to the $A^-$ form of FCCP depends markedly on the dielectric constant: it increases by two orders of magnitude when decane is replaced by chlorodecane (McLaughlin and Dilger, 1980). Recent experiments by J. Dilger (personal communication) indicate that chlorodecane increases the dielectric constant of a phospholipid bilayer membrane from ~ 2.1 to 2.7. The increase in the dielectric constant of the membrane, which reduces the Born energy required to move the anion into the interior of the membrane, is the main cause of the observed increase in permeability (Dilger et al., 1979). Thus, the available evidence suggests that the model sketched in Fig. 1 and the analysis outlined above can be applied to membranes with different dielectric constants; only the value of $k_A$ need be changed. For example, steady-state conductance measurements indicate that the value of $k_A$ is reduced by about two orders of magnitude when chlorodecane is replaced by decane (McLaughlin and Dilger, 1980). Our kinetic model (Eq. 13) predicts that the magnitude of the relaxation observed in a voltage clamp experiment, $\alpha$, should be reduced by two orders of magnitude. Experiments confirm this prediction: at pH 8.3 the relaxations observed in voltage-clamp experiments with FCCP and PE/decane membranes are about two orders of magnitude smaller than the relaxations observed with PE/chlorodecane membranes (data not shown). In summary, we believe that we understand all the essential features of the mechanism by which FCCP transports protons across bilayer membranes.

We are now able to examine critically the postulate that weak acids such as FCCP act as uncouplers of oxidative phosphorylation simply by transporting protons across the bilayer component of the mitochondrial inner membrane and dissipating a $\Delta\tilde{\mu}_H+$, a postulate consistent with Mitchell's chemiosmotic hypothesis. If this postulate is correct, we should be able to calculate the pH dependence of the uncoupling activity of FCCP from the model presented above. To calculate the pH dependence of the ability of a weak acid to transport protons into mitochondria, it is first necessary to calculate the steady-state distribution of the weak acid between the bathing solution and the solution inside the mitochondria. In general, this distribution will be governed by both the difference in pH and the membrane potential that exist between the bathing solution and the solution inside the mitochondria (Roos, 1965; McLaughlin and Dilger, 1980). If the permeability of the membrane to the HA form of the weak acid, $P_{HA}$, is much greater than the permeability of the membrane to the $A^-$ form, $P_A$, the concentrations of the HA form will be essentially the same on both sides of the membrane in the steady state; under these circumstances the distribution of the less permeable $A^-$ form will not be influenced by the membrane potential, $V$. Wilson and Forman (1982) report that $V = 150$ mV, inside negative, for rat liver mitochondria. To simplify our calculations we assume that $P_{HA} \gg P_A$ and that $\Delta pH = 0$. It follows that the concentrations of HA in the bathing solution and in the mitochondria will be approximately the same. The concentrations of $A^-$ in the bathing solution and in the mitochondria will also be approximately the same and will be related to [HA] by the Henderson-Hasselbach equation. Thus, the ability of FCCP to uncouple mitochondria should depend on pH in the same way that the steady state current produced by a potential of 150 mV depends on pH in our bilayer experiments. The steady-state currents observed with FCCP upon application of 150 mV can be adequately described by our theoretical model. From Eqs. 11 and 12 the steady-state current is

$$I(\infty) = \frac{I(0)}{(1+\alpha)} \propto \frac{1}{(1 + K^{aq}[H^+])\,(1+\alpha)}. \qquad (27)$$

The first term in the denominator represents the manner in which $I(0)$ and the number of anions adsorbed to the surface depend on the pH. In the absence of any relaxation ($\alpha = 0$) the current is predicted to increase monotonically with the pH and to be half maximal at a pH equal to $pK^{aq}$. However, relaxations are observed with FCCP. By inserting Eq. 13 into 27 and noting that $k_A^I \gg k_A^{II}$ when $V = 150$ mV (Eqs. 19 and 20) we obtain

$$I(\infty) \propto \frac{[H^+]}{(1 + K^{aq}[H^+])\,(1 + 2K[H^+]k_{HA}/k_A^I)}. \qquad (28)$$

This proportionality predicts that the steady-state current (proton flux) produced by a weak acid uncoupler will attain a maximum value at a certain pH. If $K^{aq} \simeq K$ and $k_A^I \simeq 2k_{HA}$, a sharp maximum will occur at a pH $\simeq pK$. This is the case for FCCP-induced proton transport through a chlorodecane-containing bilayer membrane. If $k_A^I \ll k_{HA}$, a broad maximum will occur to the alkaline side of the $pK$. This is the case for FCCP-induced proton transport through decane-containing membranes. A detailed discussion of the pH dependence of the protonophore activity of FCCP is contained in Appendix D.

The curve in Fig. 13 illustrates the predicted uncoupling activity of FCCP. The curve was obtained from Eqs. 28 and 19, taking $pK^{aq} = 6.1$, using the values of the parameters obtained with chlorodecane-containing PE mem-

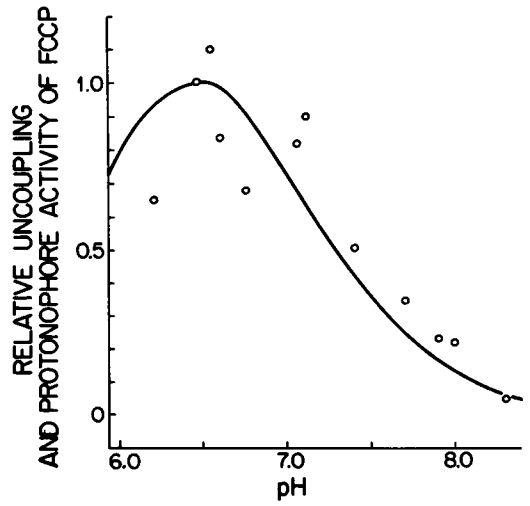


FIGURE 13 The pH dependence of the ability of FCCP to uncouple rat liver mitochondria (O) and to transport protons across a chlorodecane-containing bilayer membrane (curve). The mitochondrial data are taken from Wilson et al. (1971), and the curve is calculated from Eqs. 27 and 19. See text for details.

branes, $k_A = 700\ s^{-1}$, $k_{HA} = 10^4\ s^{-1}$, b = 0.65, p$K$ = 6.4, and assuming that $V = 150$ mV. The circles in Fig. 13 illustrate the experimentally determined uncoupling activity of FCCP (Wilson et al., 1971). It is apparent from Fig. 13 that the uncoupling activity of FCCP on mitochondria decreases with pH, for pH > 6.5, in much the same way that the protonophore activity of FCCP on chlorodecane-containing bilayer membranes decreases with pH.[6] When the biological data are compared to the data obtained from a decane-containing bilayer, the agreement is much less satisfactory. As discussed above, the values of $\beta_A$, pK, and $k_{HA}$ are similar for decane-containing and chlorodecane-containing bilayers, but $k_A$ is about two orders of magnitude smaller for the decane-containing membranes. Eq. 28 predicts, and experiments confirm (data not shown), that the steady state conductance of a decane-containing bilayer exposed to FCCP is essentially independent of pH for 6.5 < pH < 8.5. In the case of FCCP the pH dependence of the uncoupling activity can be accounted for by the pH dependence of the protonophore activity if the dielectric constants of the mitochondrial membrane and the chlorodecane-containing bilayer are similar. The skeptical reader might object that mitochondrial membranes do not contain chlorodecane. They do, however, contain a very high concentration of protein and three independent lines of evidence suggest that the effective dielectric constant of the inner mitochondrial membrane is similar to the dielectric constant of a chlorodecane-containing bilayer membrane (McLaughlin and Dilger, 1981).

[6]The agreement between the data obtained from bilayers and from mitochondria (Fig. 13) is probably fortuitous for a number of reasons. For example, we have ignored the Gouy-Chapman diffuse double layer potential, $\psi$, produced by the anionic lipids in the mitochondrial membrane (McLaughlin, 1977). This potential will increase $K$ by the factor exp $(-F\psi/RT)$ and shift the curve in Fig. 13 towards more alkaline values of the pH (see Eq. 28). If $-20 > \psi > -40$ mV, K will be increased by a factor of 2–5. Furthermore, the estimates of Berry and Hinkle (1983) indicate that the potential across the mitochondrial membrane may well be greater than the value of 150 mV estimated by Wilson and Forman (1982). A larger potential will increase $k_A$ and shift the curve in Fig. 13 to more acid values of the pH (see Eq. 28). If the membrane potential is actually 200 mV, $k^{I}_{A}$ in Eq. 28 will be increased by a factor of 2 (Eq. 19).

## APPENDIX A

The general solution to Eqs. 7 and 8 for voltage-clamp conditions is

$$I(t) = I(\infty)[1 + \alpha_1 \exp(-\lambda_1 t) + \alpha_2 \exp(-\lambda_2 t)] \quad \text{(A1)}$$

where

$$I(\infty) = \frac{-Fr(k^{I}_{A} - k^{II}_{A})k_R[H^+]k_{HA}}{(k^{I}_{A} + k^{II}_{A})(k_D + 2k_{HA}) + 2k_R[H^+]k_{HA}} \quad \text{(A2)}$$

$\alpha_1$

$$= \frac{-\{k^{I}_{A} + k^{II}_{A}\}\{[\lambda_2 - (k^{I}_{A} + k^{II}_{A})][k_D + 2k_{HA}] - 2k_R[H^+]k_{HA}\}}{2k_R[H^+]k_{HA}(\lambda_1 - \lambda_2)} \quad \text{(A3)}$$

$\alpha_2$

$$= \frac{-\{k^{I}_{A} + k^{II}_{A}\}\{[(k^{I}_{A} + k^{II}_{A}) - \lambda_1][k_D + 2k_{HA}] + 2k_R[H^+]k_{HA}\}}{2k_R[H^+]k_{HA}(\lambda_1 - \lambda_2)} \quad \text{(A4)}$$

$$\lambda_1 = P + Q \quad \text{(A5)}$$

$$\lambda_2 = P - Q \quad \text{(A6)}$$

$$P = (k_R[H^+] + k_D + k^{I}_{A} + k^{II}_{A} + 2k_{HA})/2 \quad \text{(A7)}$$

$$Q = [(k_R[H^+] - k_D + k^{I}_{A} + k^{II}_{A} - 2k_{HA})^2 + 4k_R[H^+]k_D]^{1/2}/2. \quad \text{(A8)}$$

Eq. A1 illustrates that there will be, in general, two exponential relaxations as the current decreases to its steady-state value. However, if the interfacial reactions are sufficiently rapid that $k_R[H^+], k_D \gg k_A, k_{HA}$ only one relaxation will be observed ($\alpha_1 \to 0$).

## APPENDIX B

We present here a solution to Eqs. 7, 8, and 15 under charge-pulse conditions. A solution to this system of equations can be given in closed form only if the analysis is restricted to small voltages, $V_m \ll 25$ mV or $u \equiv FV_m/RT \ll 1$. From Eqs. 19 and 20 we have

$$k^{I}_{A} \simeq k_A(1 + u/2) \quad \text{(B1)}$$

$$k^{II}_{A} \simeq k_A(1 - u/2). \quad \text{(B2)}$$

Inserting Eqs. B1, B2, and A10 into Eqs. 7, 8, and 15 yields the following set of three linear differential equations:

$$\frac{dX}{dt} = -(k_R[H^+] + 2k_A)X + k_D Y - rk_A u \quad \text{(B3)}$$

$$\frac{dY}{dt} = k_r[H^+]X - (k_D + 2k_{HA})Y \quad \text{(B4)}$$

$$\frac{du}{dt} = -Bk_A X - Brk_A u/2 \tag{B5}$$

where $B \equiv F^2/RTC_m$.

The solution for the reduced membrane voltage, $u(t)$, has the form

$$u(t) = u_o \sum_{i=1}^{3} a_i \exp(-\lambda_i t) \tag{B6}$$

where $u_o$ is the initial amplitude of the voltage and $\lambda_i$ are the roots of the characteristic equation

$$\lambda^3 - (2k_{HA} + k_D + 2k_A + k_R[H^+] + Bk_A r/2)\lambda^2 + (4k_A k_{HA} + 2k_A k_D + 2k_R[H^+]k_{HA} + Bk_A k_{HA} r + Bk_A k_D r/2 + Bk_R[H^+]k_A r/2)\lambda - Bk_A r k_R[H^+]k_{HA} = 0. \tag{B7}$$

The values of $a_i$ are given by the boundary conditions

$$\sum_{i=1}^{3} a_i = 1 \tag{B8}$$

$$\sum_{i=1}^{3} a_i \lambda_i = Bk_A r/2 \tag{B9}$$

$$\sum_{i=1}^{3} a_i \lambda_i^2 = (B^2 k_A^2 r^2/4 + Bk_A^2 r). \tag{B10}$$

In general, three exponential relaxations in the voltage will be observed under charge-pulse conditions.

When the reactions at the interface are rapid, $k_R[H^+]$, $k_D \gg k_A$, $k_{HA}$, Eqs. B3–B5 reduce to the following two equations upon insertion of the new variable $v \equiv (N_A^I + N_{HA}^I)/N_o - 1/2$.

$$\frac{dv}{dt} = \frac{-2(k_A + K[H^+]k_{HA})v}{(1 + K[H^+])} - \frac{k_A u}{2(1 + K[H^+])} \tag{B11}$$

$$\frac{du}{dt} = \frac{-2Bk_A N_0 v}{(1 + K[H^+])} - \frac{Bk_A N_0 u}{2(1 + [H^+])}. \tag{B12}$$

The solution for $u(t)$ has the form

$$u(t) = u_0[a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t)] \tag{B13}$$

where $\lambda_1$ and $\lambda_2$ are the roots of the characteristic equation

$$\lambda^2 - \frac{(Bk_A N_0 + 4k_A + 4K[H^+]k_{HA})\lambda}{2(1 + K[H^+])} + \frac{BK[H^+]k_A k_{HA} N_0}{(1 + K[H^+])^2} = 0 \tag{B14}$$

and the relaxation amplitudes $a_1$ and $a_2$, are given by the initial conditions $u(0) = u_o$ and $v(0) = 0$:

$$a_1 + a_2 = 1 \tag{B15}$$

$$a_1\lambda_1 + a_2\lambda_2 = \frac{Bk_A N_0}{2(1 + K[H^+])}. \tag{B16}$$

The relaxation amplitudes and time constants can be obtained from Eqs. B14–B16, but the expressions are rather cumbersome. Simpler expressions are obtained by defining

$$P_1 \equiv \lambda_1 + \lambda_2 = \frac{Bk_A N_0 + 4k_A + 4K[H^+]k_{HA}}{2(1 + K[H^+])} \tag{B17}$$

$$P_2 \equiv \lambda_1\lambda_2 = BK[H^+]k_A k_{HA} N_0/(1 + K[H^+])^2 \tag{B18}$$

$$P_3 \equiv a_1\lambda_1 + a_2\lambda_2 = Bk_A N_0/2(1 + K[H^+]). \tag{B19}$$

It follows from Eqs. B17–B19 that

$$k_{HA} = \frac{(1 + K[H^+])P_2}{2K[H^+]P_3} \tag{B20}$$

$$k_A = \frac{(1 + K[H^+])}{2}\left[(P_1 - P_3 - \frac{2K[H^+]k_{HA}}{(1 + K[H^+])}\right] \tag{B21}$$

$$N_o = \frac{2(1 + K[H^+])P_3}{Bk_A}. \tag{B22}$$

## APPENDIX C

We consider here the deviations from linearity observed in Figs. 5 and 7 when [FCCP] > $10^{-6}$ M. The saturation observed in both $Q$ and $G(0,0)$ when $10^{-6}$ < [FCCP] < $10^{-5}$ M cannot be due to a limited aqueous solubility of FCCP because Beer's law is valid in this region (data not shown). It is unlikely that the deviation from linearity is due to a saturation in the number of binding "sites" on the membrane because there is only one adsorbed FCCP anion for every ten phospholipids at a concentration of $10^{-5}$ M. Thus, the saturation is probably due to the production of a negative electrostatic potential that hinders the further adsorption of the anions. A potential of about −45 mV when [FCCP] = $10^{-5}$ M could account for the deviations observed in Fig. 7. This electrostatic potential is not due to the production of a Gouy-Chapman aqueous diffuse double layer by the adsorbed anions: Fig. 6 illustrates that the zeta potential produced by $10^{-5}$ M FCCP is about −10 mV in 0.1 M NaCl and the potential will be even less in 1 M NaCl. Thus, the electrostatic potential caused by the adsorbed anions must be due either to a change in the dipole potential at the interface or to the production of a boundary potential (McLaughlin, 1977; Andersen et al., 1978; Tsien and Hladky, 1982). By extending the arguments made for lipid soluble anions (Anderson et al., 1978) to protonophores, it can be shown that this electrostatic potential can account for the marked decrease in $\alpha$ and increase in $\tau$ observed in voltage-clamp experiments when [FCCP] increases above $10^{-6}$ M.

## APPENDIX D

To illustrate why the protonophore activity of FCCP decreases as the pH increases when pH > 6.4 we consider the changes in the concentrations of $A^-$ and HA that occur in the free energy wells (Fig. 12) at the membrane-solution interfaces when a voltage is applied to the membrane. We assume that the aqueous solutions are of identical composition and contain $10^{-8}$ M FCCP. Our measurements indicate that $\beta_A = 3\ 10^{-3}$ cm and that the surface p$K \simeq 6.4$. If the pH of the aqueous solutions is 7.4 and the thickness of the interfacial regions is ~ 0.5 nm, the concentrations of $A^-$ and HA in the interfacial wells are about $6\ 10^{-4}$ M and $6\ 10^{-5}$ M, respectively, when $V = 0$ (Fig. 14, upper left). On application of a voltage of 150 mV, the $A^-$ species is driven from the left hand ($^I$) to the right hand ($^{II}$) interface (Fig. 1). The interfacial reactions are essentially at equilibrium throughout the relaxation process (Eq. 9): The concentrations of $A^-$ and HA decrease proportionally at the left-hand interface and increase proportionally at the right-hand interface (Fig. 14, upper right). For these conditions it follows from Eqs. 11 and 12 that the ratio of the steady-state concentration of $A^-$ at the left-hand interface to the initial concentration is $N_A^I(\infty)/N_A^I(0) \simeq I(\infty)/I(0) = 1/(1 + \alpha)$. The value of $\alpha$ is given by Eq. 13 and, for these conditions, $\alpha = 3.9$. Thus, the steady-state concentrations of $A^-$ and HA are reduced by a factor of ~ 5 at the left hand interface (Fig. 14, upper right). Because the total concentrations of both species in the membrane remain unchanged, the concentrations of $A^-$ and HA are increased by almost a factor of 2 at the right hand interface (Fig. 14, upper right).

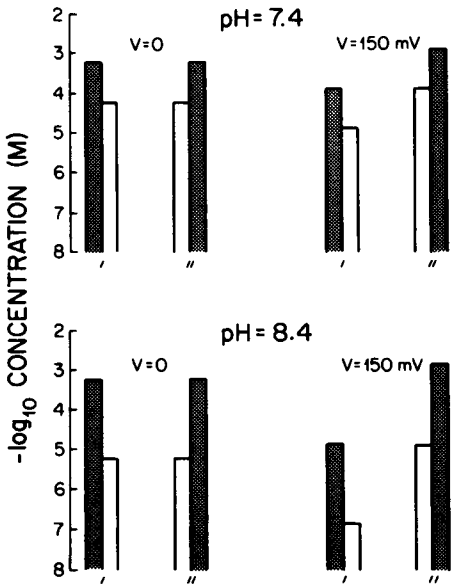


FIGURE 14 The initial and steady-state concentrations of the HA (open bars) and $A^-$ (filled bars) forms of FCCP in the free-energy wells at the interfaces of a chlorodecane-containing bilayer membrane. See text for details.

In the steady state the voltage-induced flux of $A^-$ from the left- to the right-hand interface, $J_A$, must be equal in magnitude to the diffusional flux of HA from the right- to the left-hand interface. For the conditions illustrated in the upper right-hand portion of Fig. 14, the equations adopt a simple limiting form: the flux of $A^-$ is proportional to the concentration of $A^-$ at the left-hand interface, $J_A = k_A^I N_A^I$, and the flux of HA is proportional to the concentration of HA at the right hand interface, $J_{HA} = k_{HA} N_{HA}^{II}$. The flux of protons through the membrane is also described by these equations. It is apparent that at pH 7.4 the flux of protons through the membrane is limited by the rate at which the HA species can diffuse from the right-hand interface to the left hand interface. For example, an increase in the voltage to $V \gg 150$ mV will produce a marked increase in the value of $k_A^I$ (Eq. 19), but will not significantly increase the flux of protons because the steady-state value of $N_{HA}^{II}$ cannot be greater than twice the value when $V = 0$. As illustrated in the upper right-hand portion of Fig. 14, a voltage of 150 mV caused the steady-state value of $N_{HA}^{II}$ to increase to 90% of its maximum value. Any increase in $k_A^I$ will simply reduce the steady-state value of $N_A^I$ without increasing the proton flux.

We now assume that the pH of the aqueous solutions is 8.4: the concentrations of HA and $A^-$ in the interfacial wells when $V = 0$ are illustrated in the lower left hand portions of Fig. 14. Note that there is 10-fold less HA in the interfacial wells at pH 8.4 then 7.4. Thus the maximum flux of HA, and consequently the proton flux, will be reduced by a factor of 10. The steady-state concentrations in the wells after the application of 150 mV are illustrated in the lower right hand portion of Fig. 14. The figure illustrates why the ability of FCCP to transport protons through a chlorodecane containing membrane is maximal at pH 6.4. We suggest that this analysis also explains why the ability of FCCP to uncouple mitochondria is maximal at pH 6.4.

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