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TRANSIENT CURRENTS CARRIED BY THE UNCOUPLER, CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE

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The weak acid uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, carries protons across lipid membranes. As predicted by the carrier model, at low pH, the current changes immediately following a jump in applied potential and then remains constant. By contrast at high pH, the currents relax from an initial value to a lower value as the carrier anions redistribute in the membrane. These relaxations are slower than those seen with other lipid-soluble anions which presumably explains why they had not been detected previously.

Introduction

The weak acid uncoupler, CCCP, allows a current to flow across lipid membranes. LeBlanc [1] found that as the pH was reduced below its p*K* (6.09) the conductance of the membrane fell proportionally with the concentration of the charged form $CCCP^-$. Thus, the actual charge transfer across the lipid core of the membrane appears to result from the movement of these anions. However, he also found that the reversal potential for the current in a gradient of CCCP was always small in this range of pH while the reversal potential for the current with a pH difference was that theoretically expected for a perfectly hydrogen-selective membrane. These results were taken to imply that at low pH the $CCCP^-$ which crosses the membrane returns as the CCCPH complex. Thus, the net effect is to transfer H^+ and not CCCP.

For high pH, e.g. 12, essentially all of the CCCP is present in the charged form, there is insufficient neutral complex to allow the hydrogen-carrying cycles to proceed, and the movement of $CCCP^-$ across the membrane corresponds to transport of CCCP from one solution to the other. Consistent with this view LeBlanc found at pH 12 less than 5 mV/unit reversal potential for pH differences, and nearly theoretical values in gradients of CCCP. In addition, the steady-state conductance approached the limit predicted from the rate of diffusion of $CCCP^-$ up to and away from the membrane in the aqueous phases. At high pH, but not at low pH, the currents displayed the time-dependent effects of aqueous concentration polarization.

LeBlanc calculated from his steady-state data that the permeability of the membrane to the protonated form, CCCPH, far exceeded that of the anion, $CCCP^-$; 11 and $2 \cdot 10^{-3}$ cm/s, respectively for his lecithin/cholesterol/*n*-decane membranes at 26°C. He also concluded that his results implied rapid protonation and deprotonation at the membrane surface. Neumcke [2] found it necessary to revise teh calculations and found that in the absence of such rapid reactions the maximum in

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

conductance would occur at a pH below the pK (see also Ref. 3). Since experimentally the maximum occurs 2 pH units above the pK, LeBlanc's conclusion thus becomes considerably more secure than he could have expected.

From these results it is predicted that at any pH for which there is adequate CCCPH present, i.e., for pH values less than about 8, the concentration of CCCPH will be the same on both sides of the membrane as a result of rapid transfer and the surface concentrations of $CCCP^-$ on each side will be determined locally by the protonation/deportonation equilibrium. Thus, when a potential is abruptly applied the initial current should be maintained with no relaxation to a lower value, since the current will not produce significant changes in the concentration of $CCCP^-$ on either side. By contrast at high pH, the current which flows after a potential is applied should deplete the supply of $CCCP^-$ available for transport. By analogy with the studies on tetraphenylboron and dipicrylamine this should be seen as an exponential decrease in current corresponding to redistribution of adsorbed anions, followed by a prolonged decline corresponding to concentration changes in the aqueous phases [4–8]. LeBlanc [1] observed the prolonged decline. This paper reports the existence of the redistribution transient at high pH and its progressive disappearance, as predicted, with decreasing pH. From these data it is possible to calculate the adsorption constant for the anions and the rate constant for transfer of the anions across the membrane.

Weak acid H^+ carriers are more widely known as uncouplers. Their properties and effects on artificial and natural systems have been studied extensively and this work has been reviewed [3,7,9–11].

Methods

The methods employed here have been described in detail elsewhere [12]. The membrane-forming solution was prepared fresh each day and consisted of 7 mg/ml each of egg phosphatidylcholine (Lipid Products, Redhill, Sussex) and cholesterol (BDH, Poole, Dorset) in *n*-decane (BDH). The stock phosphatidylcholine in chloroform/methanol was placed in a small flask together with an appropriate volume of cholesterol in chloroform solution. The solvents were then removed from the mixture using a rotary evaporator. The sample was resuspended and solvent removed using chloroform then diethyl ether twice and was finally resuspended in *n*-decane. The *n*-decane had been passed through an alumina column prior to use. Distilled water was obtained from a commercial still modified to exclude all plastics except polytetrafluoroethylene. All salts and buffers were Analar or equivalent grade.

TABLE I

BUFFERS

Buffers used to produce solutions with the pH values indicated in the figures.

pH range	Buffer solutions mixed
< 6.0	citric acid, Na_2HPO_4
6.1–7.5	NaH_2PO_4, Na_2HPO_4
7.6–9.5	H_3PO_4, Tris
9.6–10.9	$NaHCO_3$, NaOH
> 11.0	Na_2HPO_4, NaOH

Membranes were formed across a 1 mm hole in a conventional polytetrafluoroethylene and glass cell with a small inner compartment surrounded by the outer compartment. The electrolyte used consisted of a buffered 1 M aqueous sodium chloride solution. The buffer components were prepared as 50 mM solutions in 1 M sodium chloride and pairs, chosen as indicated in Table I, were mixed to give the desired pH. The electrolyte solutions were shaken with activated charcoal and filtered before use.

CCCP (Sigma) was added to the solution in the outer compartment from a 10 mM stock solution in ethanol. The inner and outer solutions were then mixed with each other using a Pasteur pipette. In control experiments ethanol alone had no effect. Step changes in the potential across the membrane were produced by connecting a pulse generator to one solution and a virtual earth circuit to the other via silver/silver chloride electrodes. The output of the virtual earth circuit can be made to be proportional to either the current or the charge transferred after a reference time by choosing either a resistor or a capacitor and a switch as the feedback element. Transient currents were mea-

sured as current and displayed on a storage oscilloscope. Capacitance was measured as the charge transferred in the charging transient during a change to 50 mV applied potential [13]. All experiments were performed at room temperature, 20–23°C.

Results

Bare membranes

More than 5 min after blackening, the conductances and capacitances of bare membranes were stable at $1 \pm 0.2\ 10^{-7}$ S · cm^{-2} and 0.582 ± 0.005 μF · cm^{-2}, respectively (mean ± S.E., $n = 10$). However, these membranes displayed a prominent current relaxation. For a step of 250 mV the initial current, somewhat less than 1 μA · cm^2, relaxed to the final value with a time constant of 9 ms. The transient was observable both at the beginning and the end of a potential pulse which excludes variation in membrane capacity as the explanation. The relaxation was also visible in the coloured film (the stage in the draining process when the thickness is somewhat greater than 100 nm). The time constant was insensitive to pH in the range from 7 to 11 and to the size of the applied potential step. The initial current was proportional to the applied potential. These last three properties contrast sharply with the additional relaxation seen in the presence of CCCP.

Membranes with CCCP

The concentration of CCCP (10 μM) was chosen so that the observed currents would contain a negligible contribution from the bare membrane. A tracing of a typical family of current records is shown in Fig. 1. When the first 60 ms of the traces for the higher potentials are plotted semilogarithmically (Fig. 2), very good straight lines are obtained ($r > 0.98$ in all cases) so that the relaxations are described by:

$$I(t) = I(\infty) + [I(0) - I(\infty)]e^{-t/\tau} \qquad (1)$$

where $I(0)$ is the initial current immediately after the change in potential, $I(\infty)$ the fitted estimate of the current after the initial redistribution but before aqueous depletion has progressed, and τ the time constant of the decay or relaxation. The reciprocal of τ is a measure of the rate of change

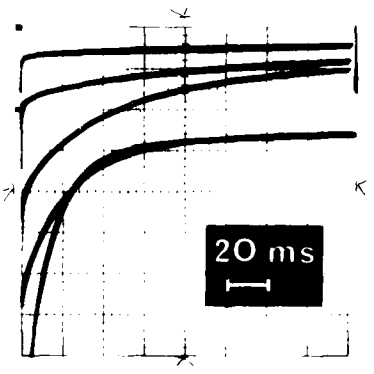

Fig. 1. Photograph of the current transients observed in the response to voltage steps of 50, 100, 150, 200 and 250 mV. The concentration of CCCP was 10 μM, pH 9.5 and membrane area = $1.72 \cdot 10^{-3}$ cm^2. The baseline for the upper three traces (5 nA/division) is at the top, for the lower two traces (10 nA/division) it is two divisions lower. The time scale is 20 ms/division.

of current. The relaxations become faster (Fig. 3) as the pH decreases. The values of $I(\infty)$ and $I(0)$ versus pH are shown in Fig. 4 and the relaxation amplitude, $[I(0) - I(\infty)]/I(\infty)$, in Fig. 5.

The possibility that a large, much faster relaxation escaped resolution was examined by measuring the apparent membrane capacitance at pH 9.5. The measured value, 0.577 ± 0.006 μF · cm^{-2}, was insignificantly different from that of a bare membrane. The transfer during the charging transient

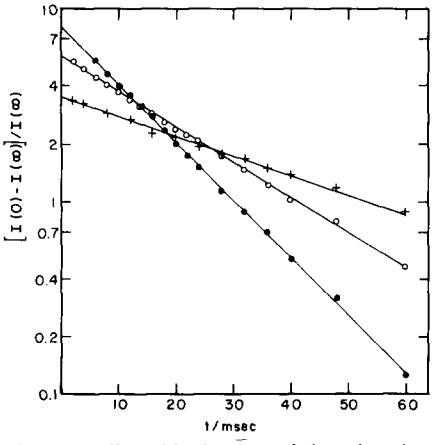

Fig. 2. Semilogarithmic plots of the relaxation currents shown in Fig. 1 for 150 mV (+), 200 mV (○) and 250 mV (●). The fitted amplitudes and time constants were 3.50, 5.70 and 8.75, and 43, 22 and 14 ms, respectively.

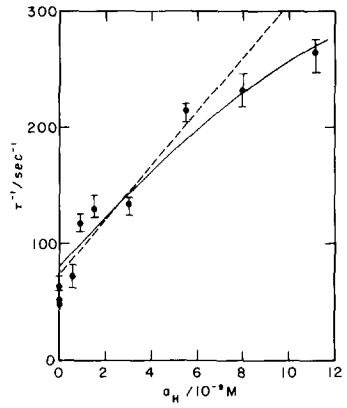

Fig. 3. The reciprocal time constant at 250 mV versus the H^+ activity (taken as equal to 10^{-pH}). The bars indicate the complete range of observations. (- - - - - -) The prediction if $k_{is} > k_D$: Eqn. 8 with $2k_s = 75\ s^{-1}$ and $\alpha = (3.1 \cdot 10^{-9}\ M/a_H)$. (———) The prediction if $k_D \gg k_{is}$: Eqn. 7 with $2k_s = 80\ s^{-1}$, α as above, and $k_R/k_D = 3 \cdot 10^7\ M^{-1}$.

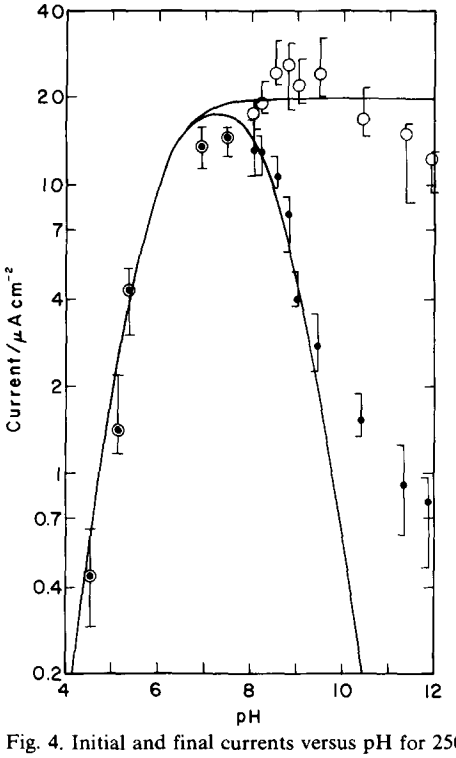

Fig. 4. Initial and final currents versus pH for 250 mV applied potential. The points are the means for five membranes with the complete range of observations indicated by the vertical bars. Initial current (○ and [), final current (● and]). Below pH 7.5 the initial and final currents could not be distinguished. The curves were calculated from Eqns. 3 and 4 with $\beta_s(k'_s - k''_s) = 2 \cdot 10^{-1}\ cm \cdot s^{-1}$ and $\alpha = (k'_s + k''_s)(k_D + 2k_{is})/(2k_{is}k_R a_H) = 3.1 \cdot 10^{-9}\ M/a_H$.

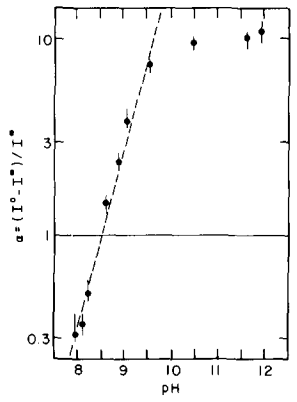

Fig. 5. The relaxation amplitude versus pH. The dot represents the mean of the observations, the line the complete range. The regression line for the data below pH 10 has a slope of 1.05. All observations are for 250 mV.

of the same amount of CCCP as transferred later would have more than doubled the apparent capacity.

It is immediately apparent from current records such as in Fig. 1 that the reciprocal time constant and the initial current increase rapidly with the applied potential while the 'final' current does not. Thus, the initial current represents the rate of transfer of anions across the hydrophobic core of the lipid membrane, since this is the region of greatest electric field strength. The final current is limited by steps which are insensitive to the applied potential.

The potential dependence of the initial current (Fig. 6) can be fitted using the assumption that transfer corresponds to migration across a high trapezoidal energy barrier. This (artificial) model predicts [14,15]:

$$I(0) = zF2k_sN_s(\gamma\Delta\psi/2)\sinh(\Delta\psi/2)/\sinh(\gamma\Delta\psi/2) \qquad (2)$$

where $\Delta\psi = zF\Delta V/RT$, ΔV is the applied potential, z the valence of the charged form of the carrier, F Faraday's constant, T the absolute temperature, N_s the concentration of free CCCP adsorbed on each side at equilibrium, and k_s the rate constant for transfer of the anions across the membrane in the limit of low applied potentials. At zero applied potential the symmetrical barrier stretches across the entire membrane, and has a top of which the width is a fraction γ of the total. For $\gamma = 0$, Eqn. 2

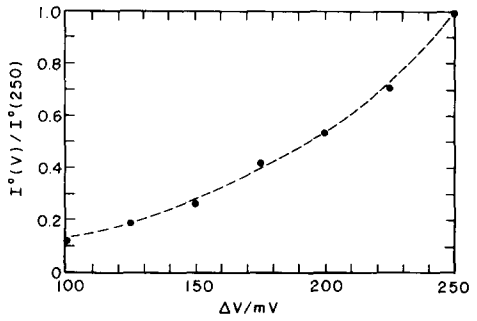

Fig. 6. Initial current relative to that at 250 mV versus the size of the potential step. The dashed curve is the prediction, Eqn. 2, for diffusion across a trapezoidal energy barrier with a top width, $\gamma = 0.57$. pH 9.5.

reduces to the usual expression for transfer across a steep high barrier, while for $\gamma = 1$, it implies a linear variation. Data were obtained between pH 7.9 and 9.5 (Fig. 6) and all were fitted by values of γ between 0.57 and 0.61.

Theory, Data Fitting and Calculation of Rate Constants

The data for lower pH can be interpreted using the conventional carrier model. In the general case (see Ref. 13) this model is complex, much more so than is warranted for the interpretation of the present data. The model can be simplified greatly by making two arbitrary but qualitatively reasonable assumptions. If the applied potential changes the rate of transfer of the free carrier and no other step in the carrier cycle, then the initial and final currents are given by:

$$I(0) = zF\beta_s c_s^T (k_s' - k_s'')/(1 + a_H/K_d) \quad (3)$$

and:

$$I(\infty) = I(0)/[1 + (k_s' + k_s'')(k_D + 2k_{is})/(2k_R a_H k_{is})] \quad (4)$$

and for high pH there will be a single exponential relaxation with reciprocal time constant:

$$\tau^{-1} = k_s' + k_s'' \quad (5)$$

If, in addition, the rate of deprotonation is much faster than the rate of transfer of the anion:

$$k_D \gg k_s' + k_s'' \quad (6)$$

then there will be only a single observable relaxation for any a_H and either:

$$\tau^{-1} = 2k_s(1 + 1/\alpha)/(1 + k_R a_H/k_D) \quad (7)$$

if $k_D \gg 2k_{is}$, or:

$$\tau^{-1} = 2k_s(1 + 1/\alpha) \quad (8)$$

if $k_{is} > k_D$. In these equations:

$$\alpha = \frac{I(0) - I(\infty)}{I(\infty)} = \frac{(k_s' + k_s'')(k_D + 2k_{is})}{2k_R a_H k_{is}} \quad (9)$$

a_H is the activity of H^+, K_d the aqueous dissociation constant for the carrier, k_s' and k_s'' the forward and reverse rate constants, respectively, for transfer of the free, charged carrier at the existing applied potential, k_R and k_D the rate constants of association and dissociation, respectively, for ion and carrier at the membrane surface, and k_{is} the rate constant for transfer of the neutral combined form of the carrier.

The so-called final currents measured in this study are the currents after the internal, strongly potential-dependent relaxation is complete but before aqueous concentration polarization has progressed. Thus, for high pH (above 9.5) where the true steady-state current is supported by diffusion of CCCP through the aqueous phases (see Ref. 1), these final currents and the relaxation amplitudes derived from them cannot be simply interpreted. For pH less than about 9.5 where the flux of $CCCP^-$ is supported by the return of CCCPH described in the model, Eqn. 4 should describe the results. The lines in Figs. 3–5 were calculated using Eqs. 3, 4 and 7–9 with the values shown in Table II. The fits are satisfactory except for pH > 10. For the final current this failure was anticipated, as the model does not allow for CCCP movements in the aqueous phases. For the initial current and the reciprocal time constant, however, this explanation of the discrepancy does not apply. LeBlanc noted that the application of the carrier model to the data was based on the assumption that membrane properties were constant at all pH values. The present relaxation data, which reflect the membrane transfer processes much more closely than did LeBlanc's steady-state data, suggest that such constancy fails above pH 10.

TABLE II

FITTED AND CALCULATED CONSTANTS

No.	Constants	Value	Source
(1)	c_s^T	10^{-9} mol/cm^3	chosen
(2)	K_d	$8 \cdot 10^{-10}$ mol/cm^3	Ref. 1
(3)	$\beta_s(k_s' - k_s'')$	$2 \cdot 10^{-1}$ cm/s (at 250 mV)	scaling factor in Fig. 4
(4)	$\{(k_s' + k_s'')(k_D + 2k_{is})\}/(2k_{is}k_R)$	$3.1 \cdot 10^{-9}$ M (at 250 mV)	α for $8 < pH < 9.5$
(5)	γ	0.59	potential dependence of $I(0)$
(6)	$k_s' + k_s''$	75 s^{-1}, $k_{is} > k_D$ 80 s^{-1}, $k_D \gg k_{is}$ (at 250 mV)	τ^{-1} at low a
(7)	β_s	$2.6 \cdot 10^{-3}$ cm	No. 3/No. 6
(8)	N_s	$2.6 \cdot 10^{-11}$ mol/cm^2	No. 7 × No. 1
(9)	k_s	1.7 s^{-1} (at 0 mV)	Nos. 5 and 6 and Eqn. 2
(10)	P_s	$4.4 \cdot 10^{-3}$ cm/s	No. 7 × No. 9
(11)	P_{is}	$27(1 + 2k_{is}/k_D)$	Nos. 2, 4 and 6
(12)	k_R	$2.4 \cdot 10^{13}$ cm$^3 \cdot$ mol$^{-1} \cdot$ s$^{-1} \times$ $(1 + k_D/2k_{is})$	Nos 4 and 6
(13)	k_R/k_D	$3 \cdot 10^{10}$ cm$^3 \cdot$ mol^{-1}, $k_D \gg 2k_{is}$ indeterminate, $k_{is} > k_D$	curvature and slope of τ^{-1} versus a and No. 4
(14)	k_{is}	440 s^{-1}, $k_D \gg k_{is}$ indeterminate, $k_{is} > k_D$	Nos. 4, 6 and 12

It has proved difficult to determine whether deprotonation or transfer of the protonated form of CCCP is more rapid. Since the interfacial region where the carrier adsorbs is more hydrophobic than an aqueous phase, the association constant, k_R/k_D, for adsorbed CCCP should exceed that for aqueous CCCP, K_d^{-1}. For the hypothetical case $k_D \gg k_{is}$, the data are consistent with this view, since $k_R/k_D = 3 \cdot 10^{10}$ cm$^3 \cdot$ mol^{-1} while $K_d^{-1} = 1.25 \cdot 10^9$ cm$^3 \cdot$ mol^{-1}. If, in addition, association is limited by H^+ movement through water, then $k_R > 10^{14}$ cm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}, and $k_D > 3000$ s^{-1} which is significantly greater than $2k_{is}$. Thus, deprotonation faster than transfer is qualitatively reasonable. For the alternative hypothetical case $k_{is} > k_D$, $k_R \simeq 2 \cdot 10^{13}$ cm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}, which is reasonable, and using again $k_R/k_D > K_d^{-1}$, $k_D < 16000$ s^{-1}. The condition that transfer is faster than deprotonation could be satisfied for any $k_{is} > 16000$ s^{-1}. Thus, a priori considerations are not adequate to distinguish these possibilities. There is, however, one feature of the data which suggests that $k_D \gg 2k_{is}$ is more nearly true; this is the curvature of the plot of reciprocal time constant versus H^+ activity *.

Discussion

The currents carried by CCCP across black lipid membranes have been shown to jump to a new value and then decline after a sudden increase in the applied potential. This initial transient is too large to represent depletion of CCCP in the aqueous phases and it must therefore represent redistribution of CCCP already adsorbed to the membrane. The potential dependence of the reciprocal time constant (not shown) and of the initial current can be fitted by assuming that $CCCP^-$ crosses the membrane by diffusing across a high trapezoidal energy barrier. Using this potential dependence as an extrapolation formula, the measured values of the reciprocal time constants at potentials between 100 and 250 mV correspond to a rate constant for transfer at zero applied potential of 1.7 s^{-1}. This constant cannot be measured

* The data for the time constants could be fitted more closely, with greater assurance that $k_D \gg 2k_{is}$, if $(k_s' + k_s'')(k_D + 2k_{is})/(k_R k_{is})$ were smaller, but a smaller value would be inconsistent with the measured amplitudes (Fig. 5).

directly using small applied potentials, since the transient then occurs slowly and is confused with the effects of changes in the aqueous phase concentrations.

The relaxation data also allow calculation of the adsorption constant for the charged form of the carrier, $\beta_s = 2.6 \cdot 10^{-3}$ cm, the permeability of the membrane for the charged form, $\beta_s k_s = 4.4 \cdot 10^{-3}$ cm $\cdot$ s^{-1} and a lower limit for the permeability to the neutral form, $k_{is} > 27$ cm $\cdot$ s^{-1}. These permeabilities are in reasonable agreement with those determined by LeBlanc [1] from analysis of the steady-state data. The adsorption constant for $CCCP^-$ is similar to those determined for other lipid-soluble anions. The rate constant for transfer of the anion is, however, lower. This observation would be explained if in $CCCP^-$ the charge were less well shielded from the environment and/or it were distributed over a smaller volume than in tetraphenylboron or dipicrylamine.

In conclusion, CCCP acts as expected of a weak acid carrier of protons.

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