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THE EFFECT OF STIRRING ON THE FLUX OF CARRIERS INTO BLACK LIPID MEMBRANES

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SUMMARY

Nonactin and valinomycin are small cyclic molecules which carry alkali cations across lipid membranes. If either is added in equilibrium proportions to both the solution from which a black lipid membrane is formed and the alkali chloride solution present on both sides of the membrane, then the conductance per unit area of the membrane is independent of membrane area and whether or not the aqueous phases are stirred. If, however, the carrier is added only to the aqueous phase, then the membrane conductance (and inferentially the concentration of carrier in the membrane) increases dramatically (2–7 times higher) whenever the aqueous phases are stirred and returns to the lower value when stirring is stopped. The conductance per unit area increases as the area increases. The probable explanation is that the carrier is diffusing across the unstirred layers into the membrane, then along the membrane into the Plateau border. Stirring reduces the resistance to transfer between the membrane and the aqueous phase and thus brings the concentration in the membrane closer to equilibrium with that in the aqueous phase. These effects can be demonstrated for hours. Thus the membrane concentration of carrier is not related to the concentration in the aqueous phase by an equilibrium partition within the time course of normal experiments. The pronounced effect of stirring clearly identifies the principal component of the observable resistance to exchange of free carrier between the bulk aqueous phases and the membrane as diffusion in the aqueous unstirred layers and not a process occurring at the interface.

INTRODUCTION

There is ample kinetic evidence that valinomycin and nonactin carry cations across black lipid membranes by forming one carrier–one ion complexes which cross from one side of the membrane to the other[1–4] (Hladky, S. B., unpublished) (reviewed in ref. 5). But while the steady-state ion fluxes have been investigated extensively, very little is known about the flux of the free carrier or even about the concentration of the carrier in the membrane. This should not be surprising since the flux is not measurable by electrical means. However, observation of the effects of stirring the aqueous phases and changing the membrane area can provide indirect information about the distribution and movements of the carrier.

METHODS

Conductances of glycerylmonooleate + *n*-decane or *n*-hexadecane membranes exposed to nonactin or valinomycin and of L-dioleoylphosphatidylcholine + *n*-decane membranes exposed to valinomycin have been measured using a standard black membrane apparatus. A round polytetrafluoroethylene cup with a 1 or 2 mm hole punched in the side was suspended inside a cubic, borosilicate cup. Stirring was accomplished with Pyrex or polytetrafluoroethylene-coated magnetic spin bars placed one above the other in the two compartments, and driven at 1 to 2 rev./s. In many experiments the carriers were added only to the outer compartment and in these experiments only this compartment was stirred, partially for convenience and partially to minimize convection in the membrane. The L-dioleoylphosphatidylcholine was purchased from the Hormel Institute (Austin, Minn.) and contained 0.01% nordihydroguaiaretic acid antioxidant. Nonactin, gifts of Dr K. Heckmann and of Miss B. Stern (The Squibb Institute, Princetown, N.J.), was added as a $1 \cdot 10^{-4}$ or $1 \cdot 10^{-5}$ M solution in ethanol. Valinomycin, purchased from Calbiochem (San Diego, Calif.) was added as a $9 \cdot 10^{-5}$ M solution in ethanol. The aqueous phase composition was normally in the range of 0.1–1 M KCl or sometimes in experiments with nonactin, NaCl. Further information about materials and experimental details are given by Hladky and Haydon[6].

RESULTS

According to the published versions of the carrier model, so long as the curren across the membrane does not actually deplete the supply of cations, the conductance per unit area of a black lipid membrane exposed to nonactin or valinomycin should not depend on the rate of stirring of the aqueous phases or on the membrane area[5,7]. If nonactin is equilibrated between the glycerylmonooleate + *n*-alkane used to form the membrane and the aqueous phases containing NaCl or KCl, then the experimental results are as predicted. If the nonactin is added only to the glycerylmonooleate + *n*-alkane, the conductance per unit area is slightly lower, decreases by 10 or 20% if both aqueous phases are stirred, and decreases as the membrane area is increased. By contrast for membranes formed from glycerylmonooleate + *n*-alkanes with no stirring, if the nonactin is added to one or both aqueous phases, the initial conductance per unit area is markedly lower (about 3–10 times), reversibly increases as much as a

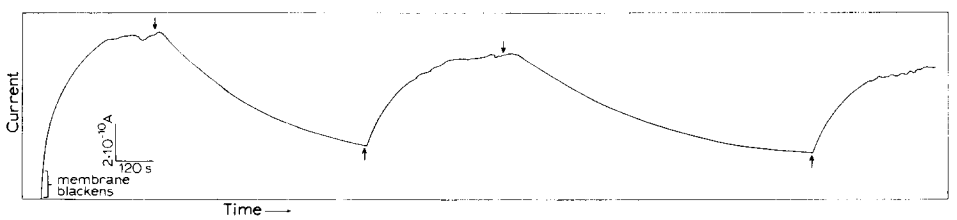

Fig. 1. The current *versus* time for a dioleoylphosphatidylcholine + *n*-decane membrane. The aqueous phases were both 0.1 M KCl with $1.8 \cdot 10^{-9}$ M valinomycin added to the outer compartment. At the times indicated by the upward arrows the stirrer in the outer compartment was turned on; at the downward arrows it was turned off. Temp. 23 °C. $\Delta V = 25$ mV. The area was $3.88 \cdot 10^{-3}$ cm² initially declining gradually to $2.94 \cdot 10^{-3}$ cm².

factor of 2 as the membrane area is increased from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-3}$ cm^2, and increases reversibly with stirring over a period of about 1 min to a new "steady" level 2–7 times higher than before. The conductances are lower and the effect of stirring greater if the nonactin is added to one rather than both aqueous phases. At least for glycerylmonooleate + *n*-hexadecane membranes, the size and time course of the conductance changes are not altered by changing the applied potential. Qualitatively similar results have been obtained for valinomycin in the aqueous phase using either glycerylmonooleate + *n*-hexadecane or L-dioleoylphosphatidylcholine + *n*-decane membranes (see Fig. 1).

THEORY AND DISCUSSION

It is known from studies on water permeability[8] and on the fluxes of tetraphenylboride[5] that there is an aqueous unstirred layer on each side of a black lipid membrane. In the experimental cell used in this study, the layers range from 300–600 μm thick in the absence of stirring to 50–100 μm with vigorous stirring (limited by the stability of the membrane). If carrier is added solely to the aqueous phases there will be a net flux of carrier across these unstirred layers and the lipid water interfaces into the membrane and Plateau border. The maximum possible flux of carrier is limited by the irreducible resistance of the unstirred layer, *i.e.* (see Fig. 2)

$$J < 2 \frac{D}{\delta} A_T c \tag{1}$$

where J is the flux, D the aqueous diffusion coefficient of the carrier, δ the thickness of the unstirred layer, A_T the area of the Plateau border and the membrane, approximately the area of the hole in which the membrane is formed, and c is the aqueous concentration of the carrier. Any additional resistance to the transfer of carrier, *e.g.* a slow interfacial step, would reduce the flux below this value. If $c^L(t)$ is the concentration of carrier in the Plateau border and V_b the volume of the border then the change in the total amount of carrier in the border is

$$\frac{dc^L(t)V_b}{dt} < 2 \frac{D}{\delta} A_T c \tag{2}$$

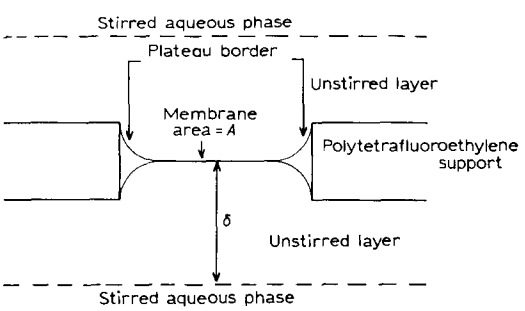

Fig. 2. The geometrical arrangement of the membrane, hole, Plateau border, support, and unstirred layers.

Eqn 2 implies that the time to reach equilibrium considerably exceeds

$$\Delta t = \Gamma V_b \delta / A_T D$$

where $\Gamma = c^L(\infty)/c$ is the oil–water partition coefficient for the free carrier. For the experimental cell used here (1 mm hole) reasonable values for the geometric parameters are $A_T \gtrsim 10^{-2}$ cm^2, $V_b \gtrsim 10^{-5}$ cm^3, and $\delta \gtrsim 10^{-2}$ cm. The diffusion constants for nonactin and valinomycin are likely to be less than $5 \cdot 10^{-6}$ cm^2/s while the oil–water partition coefficients have been estimated at 5000 for nonactin[1] and 25000 for valinomycin[4]. Thus Δt for nonactin is $\gtrsim 3$ h and for valinomycin $\gtrsim 15$ h. Thus if the carrier is added only to the aqueous phases, within the duration of normal experiments, the carrier is not at equilibrium between the Plateau border, the membrane, and the aqueous phases.

It may be possible to work with large membranes ($A > 5 \cdot 10^{-3}$ cm^2) and very small Plateau borders ($V_b < 10^{-6}$ cm^3), but there are no reports of attention being given to such a procedure in experiments on the actins or valinomycin. It will in any case be difficult to establish that such a small value of V_b has been obtained since the lipid solution in contact with the Plateau border is not always confined to the hole in the polytetrafluoroethylene support. A value of $V_b < 10^{-6}$ cm^3 represents one thousandth or less of the amount of lipid added initially in order to form a membrane.

The flux of ions, labelled water, or carrier molecules across the unstirred layers will of course change if the concentration on either side of the layer is changed or if δ is altered. The characteristic time for the rearrangement is given by

$$\tau = \frac{\bar{\delta}^2}{n^2 \pi^2 D} \tag{4}$$

where $1/2 \lesssim n \lesssim 1$ depending on whether the membrane is treated as an absorbing ($n=1$) or reflecting ($n=1/2$) barrier or some intermediate case[9]. In the reported experiments with tetraphenylboride$^-$, the ions were swept across the membrane as rapidly as they could be supplied, thus $n=1$. Using the known value of D ($5 \cdot 10^{-6}$ cm^2/s)[10] the thicknesses given above may be inferred from the ranges of values for τ, 0.5–2 s and 20–70 s for stirred and unstirred systems, respectively (in these experiments the abrupt change in conditions was a step change in the applied potential). In the experiments reported here the value of n is unknown particularly since the membrane will presumably act as an absorbing barrier near its boundary with the Plateau border and a reflecting barrier near its middle. In addition the changes in the unstirred layer are now brought about by changes in stirring which cannot be abrupt. In particular convection may persist for several minutes after stirring is stopped.

A full solution to the two phase, three-dimensional diffusion and adsorption problem (even ignoring the difficulty of changing convection) would be very complex mathematically. However, the general features of the process may be seen by means of a simple but crude approximation. For simplicity only the case where a negligible proportion of the carrier is complexed will be considered.

In Fig. 3 and the equations in the text k^{ma} is the rate constant for desorption of the carrier, β is the thickness of an aqueous layer which would contain the same amount of carrier as is adsorbed to one side of the membrane, α is the rate constant

for the exchange of carrier between the membrane and the Plateau border, ξ the analog of β for the hydrocarbon phase, $c(0)$ the aqueous concentration of carrier adjacent to the membrane, and N the concentration of carrier adsorbed to one side

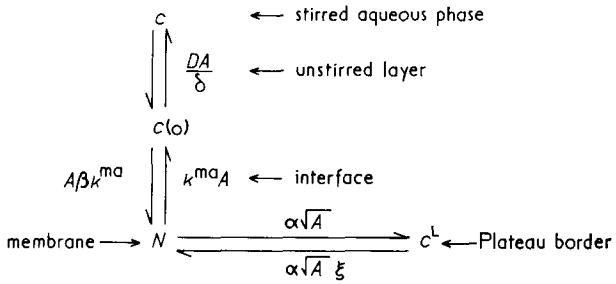

Fig. 3. Schematic diagram of the simplified model for the fluxes of the free carrier. The unidirectional flux along an arrow is given by the rate constant times the concentration at the base of the arrow.

of the membrane. Variations of N over the area of the membrane are specifically ignored with the result that the theory is much simpler but must be regarded as only a qualitative interpretation.

The flux per unit area of carrier into the membrane from an aqueous phase is given by both the flux across the unstirred layer

$$J = \frac{D}{\delta}[c - c(0)] \tag{5}$$

and by the flux across the interface

$$J = k^{\mathrm{ma}}[c0)\beta - N] \tag{6}$$

which together imply

$$J = Q[c - N/\beta] \tag{7}$$

where

$$Q = \frac{D}{\delta}\beta k^{\mathrm{ma}}/[\beta k^{\mathrm{ma}} + D/\delta] \tag{8}$$

The combination, Q, is just the series permeability of the unstirred layer and the interface. The flux per unit circumference of carrier from the Plateau border into the membrane is written as

$$Y = 2\alpha'[c^{\mathrm{L}} - N/\xi] = \frac{\alpha}{\sqrt{\pi}}[c^{\mathrm{L}} - N/\xi] \tag{9}$$

The rate of change of the quantity of carrier in the membrane will therefore (in the present crude approximation) vary as

$$\frac{\mathrm{d}2NA}{\mathrm{d}t} = 2AJ + Y(2\sqrt{\pi A}) = 2QA[c - N/\beta] + 2\alpha\sqrt{A}[c^{\mathrm{L}} - N/\xi] \tag{10}$$

which has as solution for N at constant c, c^L, A *etc.*

$$N = \frac{Qc + \frac{\alpha}{\sqrt{A}} c^L}{\frac{Q}{\beta} + \frac{\alpha}{\xi\sqrt{A}}} + \text{const} \cdot e^{-t/\tau_m} \tag{11}$$

where

$$\frac{1}{\tau_m} = \frac{Q}{\beta} + \frac{\alpha}{\xi\sqrt{A}} \tag{12}$$

If carrier is added only to the aqueous phases, *i.e.* $c^L = O$, then when stirring is started the conductance per unit area increases by 2–7-fold. From Eqn 11 this implies that Q increases by at least as much. Since D/δ alters by a similar factor it is apparent from Eqn 8 that

$$\beta k^{ma} \gtrsim D/\delta \tag{13}$$

and thus that $Q \sim D/\delta$. Therefore at least under the conditions of these experiments, the interfacial resistance to transfer of valinomycin or nonactin from the aqueous phase to the membrane is not larger than the resistance offered by a 50–100-μm layer of water. Upper limits for the value of Q/β are available from the time constants for the increase in G when stirring is started, which are 30–60 s for nonactin or valinomycin and glycerylmonooleate + *n*-alkane membranes and about 120 s for valinomycin and dioleoylphosphatidylcholine + *n*-decane membranes. Thus for glycerylmonooleate + *n*-alkane membranes

$$Q/\beta < 3 \cdot 10^{-2}\ s^{-1}$$

or assuming $D = 2 \cdot 10^{-6}$ cm^2/s, $\delta = 10^{-2}$ cm, and $Q > D/2\delta$

$$\beta > 3 \cdot 10^{-3}\ \text{cm}.$$

The inequality cannot be replaced by "much greater than" because of the observable effect of stirring when the carrier is added solely to the lipid and the size of the conductance for large membranes when the carrier is added solely to the stirred aqueous phases, each of which implies that α is not very much greater than $\xi\sqrt{A}D/\beta\delta$.

For nonactin, the value of $\Gamma = 5000$ implies that $\xi > 6 \cdot 10^{-7}$ cm. For a membrane of thickness d, the interpretation given above for the time course of the rise in conductance would require that the membrane contain more than $1.2 \cdot 10^{-6}/d \simeq 30$ times the amount of nonactin that would be contained in a layer of hydrocarbon of the same thickness. While the present line of reasoning is too tenuous to establish a value for ξ, it is not at all surprising that the carrier should adsorb to the membrane from the oil phase.

For valinomycin and L-dioleoyllecithin + *n*-decane membranes, the corresponding calculations yield $\beta > 1.2 \cdot 10^{-2}$ cm and $\xi > 5 \cdot 10^{-7}$ cm. In their investigation of the time response of the valinomycin–K^+ conductance across phosphatidylinositol

+n-decane membranes after an abrupt change in potential, Stark *et al.*[11] found $\Gamma = 15000$ and $\gamma = 2\beta/d = 60000$. In the present notation, for a membrane thickness of 50 Å, $\beta = 1.5 \cdot 10^{-2}$ cm and $\xi = 10^{-6}$ cm. These values are close to those estimated above. Nevertheless it is not clear if the membranes are sufficiently similar to justify the comparison.

The decline in conductance when stirring is stopped is more difficult to interpret in a definite manner because of the number of different processes which are occurring simultaneously, *viz.* decrease in convection in the membrane, decrease in convection in the aqueous phase, change of the concentration profile in the aqueous phase, and change in concentration of the carrier in the membrane. When stirring is started all of these processes except the last are complete within a few seconds, when stirring is stopped they may persist for minutes. There are, however, two curious effects which sometimes occur when nonactin is added only to the aqueous phases and for which it is possible to provide an explanation in terms of Eqn 11. Firstly, on many occasions the conductance initially rises when stirring is stopped. By direct observation of the trapped lenses in glycerylmonooleate+n-decane membranes[12], it is apparent that stirring causes considerable convection in the membrane which ceases within a few seconds when stirring is stopped. Thus the first effect to occur when the stirrer is turned off would be a decrease in α leading to an increase in N followed by the substantial but slower decrease in D/δ leading to a decrease in N. Secondly, there is occasionally a pronounced maximum in a record of the conductance *versus* time after membrane formation. Here there would be an abrupt decrease in α when the membrane blackens and a continuing slow relaxation of the concentration profile in the unstirred layer which is equivalent in the present notation to a slow decrease in D/δ. In both of these situations the conductance is always less than the equilibrium value.

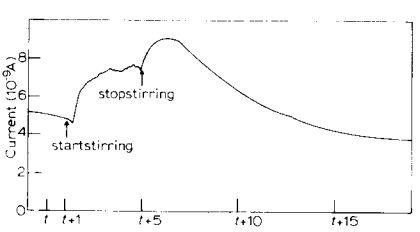

Fig. 4. The conductance *versus* time after starting and stopping the stirring. The membrane, area $1.6 \cdot 10^{-2}$ cm², was made from glycerylmonooleate+n-hexadecane. The aqueous phases, approx. $1 \cdot 10^{-8}$ M nonactin in 0.5 M NaCl, are both stirred or unstirred as indicated.

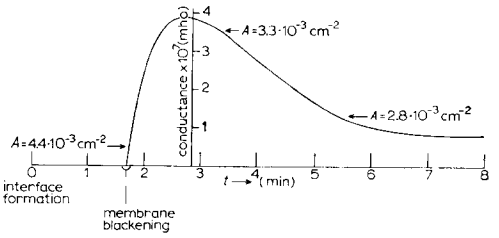

Fig. 5. The conductance *versus* time for a glycerylmonooleate+n-hexadecane membrane. The aqueous phases were both 0.5 M NaCl+$1 \cdot 10^{-7}$ M nonactin. Neither side is stirred. Temp. 23 °C. The applied potential is 100 mV.

The variations with area of the conductance per unit area may also be understood, qualitatively in terms of Eqn 11. When $c^L = 0$ and carrier is added to the aqueous phases, an increase in A would produce an increase in N, while for $c = 0$ and carrier in the Plateau border, there would be a much less pronounced decrease in N consistent with $\alpha/\xi\sqrt{A} > Q/\beta$ (unstirred).

CONCLUSION

Regardless of the validity of the more complicated aspects of these explanations, the dramatic changes in the conductance of the membrane as the stirring is changed clearly indicate that the principal resistance to the exchange of free carrier between the aqueous phase and the membrane is the aqueous unstirred layer and not an interfacial process. However, the resistance of these layers is already sufficiently high to complicate the interpretation of results when the carrier is added solely to the aqueous phase. Thus in such experiments, the longitudinal viscosity of the membrane (and extent of convection), area of the membrane, and thickness of the unstirred layer must all be constant from one experiment to the next in order for the results to be strictly comparable. Alternatively, if the area of the membrane is large enough, the unstirred layer thin enough, and the interfacial resistance still not rate limiting, then in practice the deviations from equilibrium will produce systematic errors which are small enough to be tolerated. For nonactin and glycerylmonooleate the latter conditions are reached for membranes with areas above $5 \cdot 10^{-3}$ cm^2 ($A_T = 8 \cdot 10^{-3}$ cm^2) and carrier present in both, stirred aqueous phases. In addition in the extensive work on carriers by Szabo *et al.*[1-3] it is likely that at least one of these sets of conditions has been met.

The experimental advantages of adding the carrier to the lipid phase were noted by Stark and Benz[4]. They, however, apparently thought that the unstirred layer could not provide a sufficient resistance to account for their results. This makes no difference to the rest of that paper, but the magnitude of Q is of crucial importance to the interpretation of the results in a subsequent paper by Stark *et al.*[13]. In this work, the authors have reverted to adding valinomycin to the aqueous phase. They then produce a change in the temperature while recording the time course of the conductance change. The conductance first increases as the temperature is increased, then decreases over a period of about 15 min. Stark *et al.*[13] interpret the rise as a change in rate constants at constant membrane concentration of carrier and the decline as the readjustment of the concentration in the membrane. Similar readjustments in membrane concentration have been shown here to start within seconds and to proceed with a time constant of the order of 2 min. Thus, while the explanation given by Stark *et al.* for the rise and fall may be correct, it would appear to be wrong to assume that the concentration of carrier in the membrane remains at its initial level during the period of several minutes required to change the temperature. If Stark *et al.*[13] have succeeded in reducing the volume of the Plateau border to less than 10^{-6} cm^3, part of the decrease occurring over a period of 15 min might represent the readjustment of the concentration of valinomycin in the border. The general decrease in the level of conductances which they noted to occur over a period of hours also occurred in the experiments reported here. It is probably a result of a loss of carrier from the aqueous phases onto the walls of the experimental cell and into the total volume of lipid solution added during the course of the experiments.

NOTE ADDED IN PROOF (Received March 26th, 1973)

$D/\beta\delta$ may also be estimated from the ratio of the rate of increase of conductance for a new membrane ($c^L = 0$) to the equilibrium conductance for the same aqueous concentration of carrier. For nonactin, M/2 NaCl and glycerylmonooleate+n-hexadecane membranes, the estimate for $D/\beta\delta$ is $1 \cdot 10^{-2}$ s^{-1}.

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