TRANSMEMBRANE LIPID MIGRATION IN PLANAR ASYMMETRIC BILAYER MEMBRANES

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ABSTRACT Planar asymmetric bilayer membranes, formed by apposing a monolayer of the neutral lipid glyceroldioleate (GDO) with one of the negatively charged lipid oleyl acid phosphate (OAP), were used to measure the rate of transmembrane OAP migration. The assay for this lipid flip-flop was the interaction of Ca^{2+} ions with negatively charged lipids which causes membranes to break: when Ca^{2+} is added to the compartment limited initially by the neutral lipid, flip-flop of the charged lipid eventually results in membrane breakdown. At 22 ± 2°C, in the absence of an externally applied electric field, an upper limit to the half time of OAP flip-flop was measured as 18.7 h, with a tentative lower limit of 14.4 h.

INTRODUCTION

There is now increasing evidence that many biological membranes show an asymmetric distribution, between the inner and outer membrane surfaces, not only with respect to proteins, but also with respect to lipids (1–9). This inside-outside lipid asymmetry is of interest as regards both the biological function, and the biosynthesis, of membranes (10). A particularly pertinent physicochemical parameter of such an asymmetric lipid distribution is the rate constant of lipid migration from one surface to the other, an event which has come to be known as lipid flip-flop (11, 12). If this rate constant is very small, then an initially synthesized asymmetric membrane would be expected to maintain its asymmetry for a relatively long period: on the other hand, if the rate constant is large, then there must exist biosynthetic mechanisms whereby the required asymmetry may be restored.

A model system especially appropriate to the study of asymmetric membranes is the planar asymmetric lipid bilayer (13, 14). Asymmetric lipid bilayers are formed by adjoining the hydrocarbon chains of two different lipid monolayers, originally at the air-water interface, through an aperture in a hydrophobic partition which separates two aqueous phases. Since the chemical compositions of the two monolayers may be independently controlled, this system permits the formation of lipid bilayers, the two monolayers of which may have any desired properties (15).

We report here the measurement of the rate constant of lipid flip-flop across a lipid bilayer formed originally from a monolayer of the neutral lipid glyceroldioleate

(GDO), and a monolayer of the negatively charged lipid oleyl acid phosphate (OAP). It has been shown previously (14, 16) that bilayers formed from neutral lipids are quite stable in the presence of various positively charged ions, such as Ca^{2+}. In contrast, such ions interact with bilayers formed from negatively charged lipids, causing under asymmetric conditions an increase in electrical conductance and finally, membrane breakdown. The mechanism of this calcium effect is likely to involve local ion-pair formation between the positively charged calcium ions and the negatively charged head groups of the lipids. The presence of such neutral ion-pairs would perturb the packing of the bilayer, thereby facilitating membrane breakdown. Since calcium ions can distinguish between neutral and negatively charged lipids, we may use the calcium effect as an assay for the presence of negatively charged lipids in a binary mixture (14).

The strategy of the flip-flop experiment is to form a bilayer from pure monolayers such that initially, one monolayer is neutral, and the other negative. We shall refer to the aqueous compartment originally limited by the negatively charged monolayer as "side 1"; that originally limited by the neutral monolayer as "side 2." After membrane formation, Ca^{2+}, as $CaCl_2$, is added to side 2. If lipid flip-flop occurs, the negative lipid will migrate from side 1 to side 2 so that, as time increases, the amount of negative lipid on side 2 increases from zero. At some stage, a critical density of negative lipid will be present on side 2 so as to interact with the Ca^{2+} and cause membrane breakdown. The time taken for membrane breakdown to occur under these conditions can be determined experimentally. This information, combined with an estimate of the minimum density of the negative lipid which shows the Ca^{2+} effect, allows the rate constant of lipid flip-flop to be determined.

THEORY

General Considerations

Consider a bilayer composed originally of, on side 1, a monolayer of N_A molecules of pure lipid A; and on side 2, N_B molecules of pure lipid B. This constitutes a non-equilibrium system, which will seek to return to equilibrium as the lipids flip from one side to the other, thereby randomizing the populations on both sides.

For a closed system in which the total numbers of molecules of both lipids in the bilayer is constant, the theoretical treatment of the flip-flop event is trivial. A problem arises, however, in that, in the membrane forming technique used here, the bilayer membrane is continuous with the two monolayers in either compartment. The bilayer membrane itself therefore does not constitute a closed system, but there exists the possibility of lateral diffusion of lipids between the bilayer membrane formed in the aperture and the two monolayers on either side, as shown in Fig. 1.

The possibility of lateral diffusion complicates matters considerably. For example, let us consider the flip-flop of molecules of lipid A from side 1 to side 2, such that, by time t, a total number $n_A(t)$ of molecules have appeared on side 2. In the absence of lateral diffusion on side 2, these $n_A(t)$ molecules will remain within the bilayer mem-

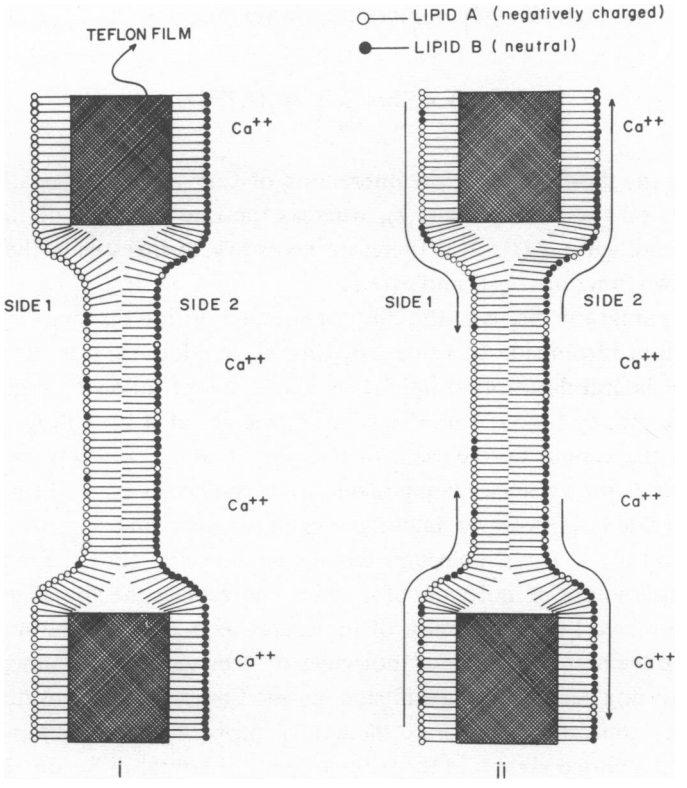

FIGURE 1 Schematic representation of the lipid flip-flop event in planar asymmetric bilayer membranes. (*i*) In the absence of lateral diffusion, all molecules in the bilayer in the aperture initially remain within the aperture at all times. (*ii*) Lateral diffusion on side 1 increases the density of molecules of lipid *A* on side 1 relative to figure *i*, and similarly decreases the density of lipid *A* on side 2. Not drawn to scale.

brane: furthermore, they will be distributed evenly throughout the aperture such that the density $d_A(t)$ of lipid *A* on side 2 is a function of time only, and will be given by

$$d_A(t) = n_A(t)/\pi r_0^2 \qquad 0 \leqslant r \leqslant r_0 \tag{1}$$

in which r_0 is the radius of the circular aperture.

In the presence of lateral diffusion on side 2, however, although by time t a total $n_A(t)$ of molecules have appeared on side 2, some of these will have diffused away from the bilayer into the monolayer, so that only a number $\nu_A(t) \leqslant n_A(t)$ of molecules of *A* actually remain in the bilayer membrane at time t. In addition, the $\nu_A(t)$ molecules remaining in the bilayer will not be distributed evenly over the aperture, but the distribution will be described by a function $\rho_A(r,t)$ which depends both on time t and also on the radial distance r from the center of the aperture. Since the effect of lateral diffusion is to deplete the periphery of the bilayer relative to the center, the density distribution function $\rho_A(r,t)$ will decrease radially from the center, and is

related to the total number $\nu_A(t)$ of molecules within the bilayer as

$$\nu_A(t) = 2\pi \int_0^{r_0} r\rho_A(r,t)\,dr. \tag{2}$$

The assay for the flip-flop event, the interaction of Ca^{2+} with the negatively charged lipid, depends on the function $\rho_A(r,t)$, whereas the rate constant of flip-flop determines $n_A(t)$, and hence $d_A(t)$. It is therefore necessary to investigate the relationship between the two functions $d_A(t)$ and $\rho_A(r,t)$.

The above paragraph discusses the effect of lateral diffusion on those molecules of A which have flipped from side 1 to side 2 by time t. In addition, it is necessary to take account of the lateral diffusion of lipid A on side 1, as the following argument shows. Let us assume that by time t, a total $n_A(t)$ of molecules of A have flipped from side 1 to side 2. If the system were closed, this implies that $N_A - n_A(t)$ molecules of A remain on side 1, the vacancies being taken up by molecules of B which have flipped from side 2 to side 1. If, however, lateral diffusion of lipids can occur on side 1, it is no longer true that $N_A - n_A(t)$ molecules remain on side 1 at time t, for the bilayer is in contact with a source of molecules of A which may replace the flipped molecules of A at a rate determined by the diffusion of molecules of A between the monolayer and bilayer. Since the actual number of molecules of A on side 1 determines the kinetics with which flip-flop occurs, lateral diffusion on side 1 must also be considered.

We are thus concerned with three theoretical problems: (*a*) The *flip-flop event* as would occur in a closed system in the total absence of lateral diffusion. (*b*) The *effect of lateral diffusion of lipid A on side 1* which replenishes the source of molecules of A which may flip from side 1 to side 2. (*c*) The *effect of lateral diffusion of lipid A on side 2* which drains molecules of A which have flipped from side 1 to side 2 away from the bilayer.

We shall discuss these three events, regarding the flip-flop event as the primary phenomenon, and the effects of lateral diffusion as perturbations.

The Flip-Flop Event

In the total absence of lateral diffusion, all molecules of A which flip from side 1 to side 2 remain in the bilayer on side 2, and furthermore, the population of A on side 1 is depleted simply by the number of molecules which have flipped to side 2. If N_A represents the original number of molecules of A on side 1 at time zero, and $n_A(t)$ the number of molecules of A which have flipped from side 1 to side 2 by time t, then the rate of appearance of A on side 2 may be expressed by assuming first order kinetics as

$$dn_A(t)/dt = k_{12}(N_A - n_A(t)) - k_{21}n_A(t), \tag{3}$$

in which k_{12} is the flip-flop rate constant from side 1 to side 2, and k_{21} that for flip-flop from side 2 to side 1. Since the areas of the two monolayers on each side of the circular planar bilayer are the same, Eq. 3 may be expressed in terms of the original

density D_A of A on side 1, and the density $d_A(t)$ at time t of A on side 2 as

$$\mathrm{d}\{d_A(t)\}/\mathrm{d}t = k_{12}(D_A - d_A(t)) - k_{21}d_A(t) \tag{4}$$

with solution

$$d_A(t) = (k_{12}/[k_{12} + k_{21}])\, D_A(1 - \exp - [k_{12} + k_{21}]t). \tag{5}$$

As shown theoretically by Israelachvili (17), in the absence of an externally applied electric field, the equilibrium distribution for a binary mixture of a neutral and a negatively charged lipid in a planar bilayer is such that the two lipids distribute themselves equally in the two monolayers, implying that

$$d_A(\infty) = d_A/2.$$

Hence

$$k_{12} = k_{21} = k,$$

giving

$$d_A(t) = (D_A/2)(1 - \exp - 2kt). \tag{6}$$

It should be noted that, for charged lipids in the presence of an externally applied electric field, the kinetic equation may be more complex than Eq. 6, and the rate constants will be field dependent. McLaughlin and Harary (18) have shown theoretically that the presence of a constant externally applied electric field imposes a steady-state asymmetric distribution of charged lipids across a bilayer, and so it must be stressed that Eq. 6 is valid only in the absence of externally applied electric fields.

We now calculate the relative weights of the two lipids A and B present in any unit area of the monolayer on side 2 of the bilayer at any time t. The density of molecules of A on side 2 is $d_A(t)$, and that of the molecules of B remaining on side 2 is $D_B - d_B(t)$. If the molecular weights of A and B are w_A and w_B, respectively, then the weight ratio $f(t)$ is

$$f(t) = d_A(t)w_A/(D_B - d_B(t))w_B. \tag{7}$$

Since the area of the bilayer is fixed, and the molecules are tightly packed, then if the molecular areas of A and B are a_A and a_B, we have at time zero,

$$D_A a_A = D_B a_B, \tag{8}$$

and at time t

$$(D_A - d_A(t))a_A + d_B(t)a_B = d_A(t)a_A + (D_B - d_B(t))a_B,$$

giving

$$d_A(t)a_A = d_B(t)a_B. \tag{9}$$

Substituting Eqs. 8 and 9 into Eq. 7, we derive

$$f(t) = [d_A(t)/(D_A - d_A(t))](w_A a_B/w_B a_A), \tag{10}$$

from which

$$d_A(t) = D_A[qf(t)/(1 + qf(t)], \tag{11}$$

where

$$q = w_B a_A/w_A a_B. \tag{12}$$

Substituting Eq. 11 into Eq. 6, we may derive an expression for the rate constant k as

$$k = (1/2t) \ln [(1 + qf(t))/(1 - qf(t))]. \tag{13}$$

At short times, $f(t)$ will be small, and if $qf(t)$ is much less than unity, Eq. 13 approximates to

$$k \simeq qf(t)/t. \tag{14}$$

In general, the weight fraction $f(t)$ will increase with time. If f_c represents the critical local weight fraction at which the density of lipid A is just sufficient to interact with Ca^{2+} to cause membrane breakdown, and t_c represents the time taken for $f(t)$ to become equal to f_c, then Eq. 13 implies

$$k = (1/2t_c) \ln [(1 + qf_c)/(1 - qf_c)] \tag{15}$$

$$k \simeq qf_c/t_c \qquad (qf_c \ll 1). \tag{16}$$

Measurement of the critical weight ratio f_c, and the critical time t_c therefore permits the estimation of the rate constant k under the assumption that no lateral diffusion can occur.

The Effect of Lateral Diffusion of Lipid A on Side 1

The effect of lateral diffusion of lipid A on side 1 is that, at any time t, diffusion of molecules of A from the monolayer into the bilayer will replenish the source of molecules of A which may then flip to side 2. In the absence of lateral diffusion on side 1, the number of molecules left on side 1 at time t is $N_A - n_A(t)$, whereas in the presence of lateral diffusion, the number left is greater than this. The kinetic equation 3 is accordingly affected.

The opposite extreme to the case of no lateral diffusion is that of infinitely fast lateral diffusion from an infinite source, such that the number of molecules of A left on side 1 is constant at all times at N_A, and the density is also constant at D_A. The kinetic equation appropriate to this case is therefore

$$\mathrm{d}\{d_A(t)\}/\mathrm{d}t = k_{12}D_A - k_{21}d_A(t),$$

with solution

$$d_A(t) = (k_{12}/k_{21})D_A(1 - \exp - k_{21}t),$$

which if $k_{12} = k_{21}$ becomes

$$d_A(t) = D_A(1 - \exp - kt). \tag{17}$$

Proceeding as before, we may express the rate constant k in terms of the weight ratio $f(t)$ on side 2 as

$$k = (1/t)\ln(1 + qf(t)). \tag{18}$$

Eq. 18 is the analogue of Eq. 13 for the case of infinitely fast lateral diffusion on side 1. It is particularly interesting to note that, at short times, when $f(t)$ is small, and the product $qf(t)$ is much less than unity, then Eq. 18 assumes the approximate form

$$k \simeq qf(t)/t, \tag{19}$$

which is identical to the approximate form, Eq. 14, of Eq. 13.

The significance of this is that, at short times when $f(t)$ is small, the value of the rate constant k obtained from Eqs. 13 or 14 will be the same as that obtained by Eqs. 18 or 19. These equations cover the two extreme cases of no lateral diffusion, and infinitely fast lateral diffusion, of molecules of lipid A on side 1, and so the real case of a finite rate of lateral diffusion must be bracketed between these two extremes. But since the results are the same for the two extremes when $f(t)$ is small, we may conclude that under this condition, the effect of lateral diffusion of lipid A on side 1 is negligible.

Physically, when $f(t)$ is small, relatively few molecules of A have flipped, and so the population of A on side 1 is hardly changed. Since the rate of flip-flop from side 1 to side 2 is determined by the population of A on side 1, at short times, the depletion of this population is negligible, and so the effect of diffusion in replacing flipped molecules would be insignificant.

The Effect of Lateral Diffusion of Molecules of Lipid A on Side 2

The effect of lateral diffusion of molecules of lipid A on side 2 is to deplete the number of A remaining in the bilayer membrane in the aperture to some number $\nu_A(t)$, which

is less than the number $n_A(t)$ of molecules of A which have flipped by time t. Also, the distribution of molecules of A on side 2 will be described by a function $\rho_A(r,t)$, which will depend on position as well as time. The local weight ratio of lipid A to lipid B will likewise depend on position and time, and may be expressed in terms of a function $\phi(r,t)$, which by analogy with Eq. 10, is given by

$$\phi(r,t) = [\rho_A(r,t)/(D_A - \rho_A(r,t))](w_A a_B/w_B a_A), \tag{20}$$

giving

$$\rho_A(r,t) = D_A \frac{q\phi(r,t)}{1 + q\phi(r,t)}, \tag{21}$$

in which q is defined in Eq. 12.

We now assume that the density distribution functions in the absence ($d_A(t)$) and presence ($\rho_A(r,t)$) of lateral diffusion are related by a function $p(r,t)$ such that

$$\rho_A(r,t) = p(r,t)d_A(r,t), \tag{22}$$

where, for all r and t,

$$p(r,t) \leqslant 1.$$

From Eq. 21, we now have

$$d_A(r,t) = (D_A/p(r,t))(q\phi(r,t)/[1 + q\phi(r,t)]). \tag{23}$$

Substituting Eq. 23 into Eq. 6 and rearranging

$$k = \frac{1}{2t} \ln\left\{\frac{1 + q\phi(r,t)}{1 - [(2 - p(r,t))/p(r,t)]q\phi(r,t)}\right\}, \tag{24}$$

which approximates to

$$k \simeq q\phi(r,t)/tp(r,t). \tag{25}$$

If, at some time t_c and position r_c, the weight ratio exceeds the critical value f_c, then

$$k = qf_c/t_c p(r_c,t_c). \tag{26}$$

If there is no lateral diffusion, $p(r,t)$ is unity for all r and t, and Eq. 26 reduces to Eq. 16, and likewise Eqs. 24 and 25 reduce to Eqs. 13 and 14. Thus Eqs. 24 and 25 are the appropriate forms for the calculation of the rate constant k when lateral diffusion on side 2 of flipped molecules of A may occur. Since the function $p(r,t)$ is necessarily less than unity, it should be noted that if Eqs. 13 or 14 are used when lateral diffusion is

present, then the value of the rate constant obtained will be *smaller* than that obtained from Eqs. 24 or 25.

Since lateral diffusion is physically possible in the present experiment, we wish to evaluate the function $p(r,t)$ so that the correct Eqs. 24 and 25 may be used. We present here an approximate means of evaluating the function $p(r,t)$.

In order to take account of the lateral diffusion of molecules of A on side 2, the kinetic equation 3 must be modified, in the region of the bilayer membrane in the aperture, to:

$$r \leqslant r_0, \quad \partial \rho_A(r,t)/\partial t = k_{12}(D_A - \rho_A(r,t)) - k_{21}\rho_A(r,t) + D\nabla^2\rho_A(r,t), \tag{27}$$

in which D is the lateral diffusion coefficient of lipid A. Eq. 27 is formally equivalent (19) to the neutron diffusion equation. In the domain away from the bilayer membrane, flip-flop cannot occur, and $\rho_A(r,t)$ obeys the simple diffusion equation

$$r > r_0, \quad \partial \rho_A(r,t)/\partial t = D\nabla^2\rho_A(r,t). \tag{28}$$

The general solution of Eqs. 27 and 28 for $\rho_A(r,t)$ is difficult, but we present the following argument as an approximation to the effect of lateral diffusion.

By time t, a total number $n_A(t)$ of molecules have flipped from side 1 to side 2, and simultaneously, some molecules have diffused away from the bilayer. We suggest that the true physical event of simultaneous flip-flop and lateral diffusion may be approximated as the instantaneous flip-flop of $n_A(t)$ molecules, followed by the diffusion, over time t, of these molecules in the absence of further flip-flop.

The nature of this approximation overestimates the effect of lateral diffusion, and is discussed further below.

The diffusion of these "instantaneously created" molecules may be described by the diffusion equation, applied to radial diffusion in an infinitely flat plane. The problem of the diffusion of N molecules in such a system may readily be solved (20). If at time $t = 0$ the N molecules were evenly distributed over a circular aperture of radius r_0, then the circularly symmetrical two dimensional diffusion Eq. 20

$$\frac{\partial \rho_A(r,t)}{\partial t} = D\left\{\frac{\partial^2 \rho_A(r,t)}{\partial r^2} + \frac{1}{r}\frac{\partial \rho_A(r,t)}{\partial r}\right\} \tag{29}$$

must be solved subject to the boundary conditions

$$\rho_A(r,0) = N/\pi r_0^2 \qquad r \leqslant r_0 \tag{30}$$

$$\rho_A(r,0) = 0 \qquad r > r_0. \tag{31}$$

The general solution (20) of Eq. 29 is a series of zero order Bessel functions:

$$\rho_A(r,t) = \sum_i a_i J_0(\lambda_i r) \exp - \lambda_i^2 Dt, \tag{32}$$

in which the values of the parameter λ_i are such that the boundary condition (31) is satisfied, implying for all λ_i

$$J_0(\lambda_i r_0) = 0. \tag{33}$$

Writing the zeros of the zero order Bessel function as z_i, Eq. 33 identifies

$$\lambda_i = z_i/r_0, \tag{34}$$

where (21)

$$z_i = 2.4048, 5.5201, 8.6537, \ldots. \tag{35}$$

The coefficients a_i may be identified using the boundary conditions 30 and 31 which imply

$$\rho_A(r,0) = \sum_i a_i J_0(z_i r/r_0). \tag{36}$$

On multiplying Eq. 36 by $rJ_0(z_j r/r_0)$ and integrating, we may use the orthogonality relations of Bessel functions (20) to derive

$$a_i = \frac{\int_0^{r_0} \rho_A(r,0) J_0(z_i r/r_0) r\,dr}{\int_0^{r_0} J_0^2(z_i r/r_0) r\,dr}$$

$$= \frac{N}{\pi r_0^2} \frac{\int_0^{r_0} J_0(z_i r/r_0) r\,dr}{\int_0^{r_0} J_0^2(z_i r/r_0) r\,dr}.$$

These integrals may be evaluated (21) to give

$$a_i = (N/\pi r_0^2)(2/z_i J_1(z_i)). \tag{37}$$

The complete solution is therefore

$$\rho_A(r,t) = \frac{N}{\pi r_0^2} \sum_i \frac{2}{z_i J_1(z_i)} J_0(z_i r/r_0) \exp - (z_i^2 Dt/r_0^2). \tag{38}$$

Identifying the original number N of molecules as the number $n_A(t)$ of flipped molecules, we recognize that $n_A(t)/\pi r_0^2$ is the density function $d_A(t)$ giving the final result

$$\rho_A(r,t) = d_A(t) \sum_i \frac{2}{z_i J_1(z_i)} J_0(z_i r/r_0) \exp - (z_i^2 Dt/r_0^2). \tag{39}$$

Comparison of Eqs. 22 and 39 identifies the correction factor $p(r,t)$ as

$$p(r,t) = \sum_i \frac{2}{z_i J_1(z_i)} J_0(z_i r/r_0) \exp - (z_i^2 Dt/r_0^2). \quad (40)$$

The use of Eq. 40 enables the effect of the lateral diffusion of molecules of lipid A on side 2 to be assessed quantitatively. The numbers z_i are constants, and the radius r_0 of the membrane may be measured. Recent electron spin resonance experiments (22, 23, cf. 24) have measured the lateral diffusion coefficient D of lipids in bilayer vesicles as $\sim 10^{-8}$ $cm^2 \cdot s^{-1}$, and although these measurements were not performed using OAP, the uncertainty associated with the published values suggests that this value may be taken as a meaningful approximation for the present study.

MATERIALS AND METHODS

GDO was obtained from Armak Chemicals, Philadelphia, Pa., and from Applied Science Laboratories, Inc., State College, Pa. An equimolar mixture of mono- and di-OAP was obtained from Hooker Industrial Chemicals, Niagara Falls, N.Y. The lipids were used without further purification.

The methods for the formation of the membranes, and the study of their electrical properties, have been previously reported in detail (13–15). The aqueous medium in each compartment of the membrane forming apparatus was 1 mM KCl (unbuffered, pH 5.5). Monolayers of a defined composition were spread from a hexane solution at the clean air-water interface in each compartment, and a membrane formed as described. The presence of the membrane was monitored by observing the capacitative current induced by a voltage pulse under conditions of voltage clamp (15). As shown theoretically by McLaughlin and Harary (18), the presence of a constant externally applied potential across a membrane containing charged lipids induces an asymmetric lipid distribution. In order to avoid any asymmetry as imposed by externally applied electric fields, the membrane was pulsed at 0.05 Hz by a 10 mV pulse of 1 ms duration. This allows for adequate testing of the presence of the membrane, but the electric field is of such a low frequency, magnitude, and duration that its effect on the lipid distribution is considered negligible. All experiments were carried out at 22 ± 2°C.

RESULTS

Flip-Flop Experiment

The flip-flop event was investigated as follows. An asymmetric membrane was formed by the apposition of a monolayer of pure GDO and a monolayer of pure OAP. This membrane was monitored as a function of time, and the time elapsing until spontaneous membrane breakdown occurred was measured. Routinely, membranes were stable for between 1 and 3 h. On the breakdown of this first membrane, a second membrane was formed, and at 5 min after formation, 0.05 ml of a solution of 100 mM Ca^{2+} as $CaCl_2$, was added to side 2 (the side of the original GDO monolayer) to give a final concentration in the 5 ml compartment of 1 mM Ca^{2+}. The time elapsing until membrane breakdown was measured, and the results are shown in Table I. The average lifetime of a membrane in the presence of Ca^{2+} was measured as about 20 min sig-

TABLE I

LIFETIMES OF ASYMMETRIC PLANAR BILAYER MEMBRANES OF, AT TIME ZERO, A MONOLAYER OF OAP ON SIDE 1 AND A MONOLAYER OF GDO ON SIDE 2, IN THE PRESENCE OF CA^{2+} ON SIDE 2

Membrane no.	Lifetime
	min
1	14
2	29
3	12
4	13
5	24
6	11
7	26
8	25
9	12
10	13
11	23
12	33
Mean	19
SD	8
Probable error in mean	3

The lifetime of a membrane in the absence of Ca^{2+} was at least 60 min (20 membranes).

nificantly shorter than the lifetime of an identically formed membrane in the absence of Ca^{2+}. The system was then cleaned, and the pair of experiments repeated.

This measurement, subject to the controls discussed below, identifies a critical time t_c beyond which the membrane is unstable in the presence of Ca^{2+}. If flip-flop is occurring, then by time t_c, there is a particular weight ratio f_c in the now mixed GDO-OAP monolayer on side 2, and this represents the minimum weight ratio of a mixed monolayer which shows sensitivity to Ca^{2+}. If this critical weight ratio can be measured, then the rate constant of flip-flop can be estimated.

How Sensitive are Mixed GDO-OAP Monolayers to Ca^{2+}?

A series of experiments to test the sensitivity of mixed GDO-OAP monolayers to Ca^{2+} were performed. Mixtures of $(100 - x)\%$ GDO and $x\%$ OAP (by weight) were prepared in hexane for $x = 0.5$, 1.0, 1.5, 2, and 5. Monolayers of a defined mixture were spread in both compartments, and a symmetric membrane of mixed monolayers of defined composition was formed. At 5 min after membrane formation, 0.05 ml of 100 mM Ca^{2+} was added to one compartment, and the time elapsing until membrane breakdown was measured. For $x = 0.5$ and 1.0, the membranes in the presence of Ca^{2+} lasted for times comparable to membranes in the absence of Ca^{2+}, which in all cases was at least 30 min. For $x = 1.5$, 2, and 5, however, the membranes in the presence of Ca^{2+} broke within 3 min of the Ca^{2+} addition. On the assumption that the composi-

tion of the monolayers in the bilayer is the same as the original binary lipid mixture in hexane, this series of experiments identifies the minimum weight percentage of OAP required in a mixed OAP – GDO monolayer to be sensitive to Ca^{2+} as 1.25 ± 0.25%.

Controls

A systematic error in the flip-flop experiment may be due to the fact that the test membrane was always the second membrane formed in a sequence of two. It is possible that lipid oxidation occurs during the lifetime of the first membrane, implying that the second membrane would have a systematically shorter lifetime. Control experiments in which a second membrane was formed without the addition of Ca^{2+} showed that these membranes also lasted for 1–3 h, suggesting that lipid oxidation does not account for the membrane lifetimes shown in Table I.

The flip-flop experiment identified the critical lifetime t_c of an asymmetric membrane in the presence of Ca^{2+} to be about 20 min. If flip-flop is indeed occurring, it would be expected that if Ca^{2+} is added to side 2 at any time $t < t_c$, then the membrane would remain stable until t_c. However, if Ca^{2+} is not added to side 2 until a time $t > t_c$, then the membrane should break essentially instantaneously. Membranes were formed as in the flip-flop experiment, and 0.05 ml of 100 mM Ca^{2+} was added at random times from 30 to 60 min after membrane formation. In all cases, the membrane broke within 3 min after the addition of the Ca^{2+}.

An alternative interpretation of the result of the flip-flop experiment is that, as opposed to the migration of OAP to the monolayer facing Ca^{2+}, what may be happening is the diffusion of Ca^{2+} across the bilayer so that membrane breakdown occurs by interaction of the Ca^{2+} with the pure OAP monolayer. The last-mentioned control argues against this in that, if the membrane were breaking due to Ca^{2+} diffusion, then the time interval between Ca^{2+} addition and membrane breakdown would be constant, no matter at what time after membrane formation the Ca^{2+} was added. This was not observed to be the case. However, to check this further, control experiments were performed to measure Ca^{2+} diffusion. Monolayers of 50% GDO and 50% OAP (by weight) were spread in both compartments, and a symmetric membrane formed. Ca^{2+} was then added to both sides such that the ratio of the concentrations on the two sides was from 10:1 to 100:1. If Ca^{2+} were freely permeable, the concentration ratio of 10:1 should give a membrane potential, at 22°C, of about 29 mV; and the ratio of 100:1, a potential of about 58 mV. In all cases, no membrane potential was observed, suggesting that the diffusion of Ca^{2+} through the bilayer is extremely slow. This experiment is technically difficult owing to the sensitivity of the membrane to Ca^{2+}, for the mixed monolayers lasted only some 2 or 3 min. Other experiments using pure GDO monolayers on both sides showed that no membrane potential appeared over 20–30 min for various Ca^{2+} concentration gradients.

These controls verify that membrane breakdown cannot be attributed either to lipid oxidation, or to Ca^{2+} diffusion through the bilayer. We therefore conclude that the observed membrane breakdown is induced by the presence of Ca^{2+}, and that this assay is indeed measuring transmembrane lipid migration.

The Flip-Flop Rate Constant

The critical time t_c for the breakdown of an asymmetric membrane is taken as 19 ± 3 min, and the critical weight ratio f_c at this time as 0.0125 ± 0.0025. For GDO, the molecular weight is $w_{GDO} = w_B = 620$, and the molecular area is given (25) by $a_{GDO} = a_B = 42$ Å^2; for an equimolar mixture of mono- and di-OAP, the average molecular weight is $w_{OAP} = w_A = 626$, and the average molecular area is (25) $a_{OAP} = a_A = 31.5$ Å^2. Using these figures, we calculate from Eq. 12

$$q = 0.94.$$

The correction factor $p(r,t)$ of Eq. 40 is a function of both distance r and time t. Since the assay concerns the time at which the weight ratio first exceeds a critical value, this event will occur at the point where the local density is highest. The density distribution function $\rho_A(r,t)$ of Eq. 38 is always maximal at the origin $r = 0$, and so the appropriate parameters to be used to evaluate the correction factor are $r = 0$ and $t = t_c = 19$ min. Using $D = 10^{-8}$ cm$^2 \cdot$ s^{-1} (22–24) and $r_0 = 9.5 \times 10^{-3}$ cm,

$$p(0,t_c) = 0.77.$$

Thus, the local density at the origin is depleted by 23% on account of lateral diffusion on side 2. Lateral diffusion on side 1 may be ignored since $qf_c = 0.012 \ll 1$.

From Eq. 26 we derive the value of the rate constant k as

$$k = (8.0 \pm 2.9) \times 10^{-4}\ \text{min}^{-1}$$
$$= (4.8 \pm 1.7) \times 10^{-2}\ \text{h}^{-1}.$$

The half time for OAP flip-flop is given by

$$t_{1/2} = \ln 2/k$$
$$= 14.4 \pm 5.2\ \text{h}.$$

It is interesting to compare the above value of the half time, in which lateral diffusion on side 2 is taken into account, with the value which would have been obtained if lateral diffusion had been ignored. Taking the value of the correction factor $p(r,t)$ as unity, we calculate $t_{1/2} = 18.7$ h, which is just as the upper limit of the error assessed above. Thus the effect of lateral diffusion is significant.

DISCUSSION

Two major assumptions have been made in the above calculation of the rate constant of lipid flip-flop. The first concerned the determination of the critical weight ratio of a mixed OAP-GDO monolayer for the system to be sensitive to calcium, in which it was assumed that the proportions of the lipids in the bilayer were the same as those in the

original lipid mixture in hexane. This we feel to be a valid assumption in view of the identity of the hydrocarbon chains of both lipids, and also since aggregation of the OAP is unlikely due to electrostatic repulsion between the head groups.

The second assumption concerns the value of the diffusion coefficient used in estimating the correction for lateral diffusion. As shown above, the correction for lateral diffusion is significant, and it depends markedly on the numerical value used for D. Available measurements (22–24) of lateral diffusion coefficients of lipids in bilayers have not been performed using OAP, but it is likely that the value of 10^{-8} $cm^2 \cdot s^{-1}$ is of the correct order for OAP in a bilayer. But perhaps more significant is to raise the question of whether the value of a lateral diffusion coefficient *in a bilayer* is in fact the value we require. The primary effect of the lateral diffusion is to drain OAP molecules from the periphery of the bilayer membrane into the adjacent monolayer. Thus the rate at which OAP molecules are drained from the bilayer is determined not so much by the diffusion coefficient within the bilayer itself, but rather by the diffusion coefficient across the boundary between the bilayer and the adjacent monolayer in contact with the Teflon partition. The molecular configuration at this boundary is ill defined, and it is possible that the bilayer-monolayer boundary presents a barrier to diffusion, in which case the bilayer in the aperture would act as a closed system, and no OAP molecules would diffuse away. In this case, the correct half time of flip-flop would be 18.7 h, and we suggest that this figure represents an upper limit. In the absence of measurements of the lateral diffusion of lipids in monolayers in contact with Teflon, we suggest that the half time of 14.4 h be taken as a tentative lower limit. In the light of the above considerations we feel that the approximation introduced in the calculation of the half time is acceptable.

Despite the problem concerning the translational diffusion coefficient, we feel that the method presented here for determining flip-flop rate constants offers significant advantages as compared to the techniques used hitherto: namely, the freeze fracture of bilayers composed of radioactively labeled fatty acids (26), and the analysis of electron spin resonance spectra of vesicles containing a lipid analogue with a nitroxide free radical (11, 12). In comparison with these techniques, the method described here is one of considerably greater generality and technical ease. Furthermore, the formation of planar bilayers from the corresponding monolayers (15) is the only currently available means whereby truly asymmetric bilayers may be prepared, implying that the asymmetry necessary for the measurement of flip-flop is an inherent feature of the experimental system. In contrast, in previous reports, the asymmetry is introduced artificially, as, for example, by the preferential reduction of the spin labeled lipid on the outer monolayer of a bilayer vesicle (11).

Direct comparison of the numerical value of the rate constant derived here with those of Kornberg and McConnell (11), McNamee and McConnell (12), and Deamer and Branton (26) is not considered appropriate in view of the differences between the systems and materials used. We use here a charged lipid with a single chain in planar asymmetric bilayers, whereas Kornberg and McConnell (11) and McNamee and Mc-

Connell (12), use a neutral lipid of two chains, in symmetric bilayer vesicles, and in symmetric membrane vesicles of the electroplax of the eel, *Electrophorus electricus*, respectively. However, according to a personal communication from McConnell cited in reference 18, measurements of the flip-flop rate of a charged lipid of two chains give a half time of about 24 h—rather greater, as expected, than our value for a charged lipid of one chain. Although Deamer and Branton (26) measured the flip-flop rate of stearate, a lipid similar to OAP, the methodology involved in freeze fracture is so far removed from the physical constraints used here, as well as those normally present in biological systems, that it is doubtful if a comparison of our result ($t_{1/2} \simeq 14.4$ h) with theirs ($t_{1/2} \simeq 50$ min) is meaningful.

The above discussion emphasises the importance of a systematic study of the behavior of the flip-flop rate constant in a single well-defined system. Asymmetric lipid bilayers are ideal for this task. Firstly, the system is initially truly asymmetric, so that the return to equilibrium may readily be monitored. It is very simple to repeat the protocol described here with lipids of systematically varying chain lengths, of different head groups, and of different overall structure, and the effect of lateral diffusion may be reduced by increasing the radius r_0 of the aperture. A very interesting study would be a comparison of the flip-flop rates of OAP (one chain), phosphatidyl serine (two chains), and cardiolipin (four chains). Furthermore, the dependence of flip-flop on physical parameters such as temperature and electric field strength would be readily measureable. The temperature dependence study would give information on the activation energy of flip-flop and would potentially provide insight as to the mechanism of the event, a problem first considered by Langmuir (27). Since biological membranes behave as energy transducers and as such are associated with membrane potentials (28, 29), the behavior with respect to electric fields is of extreme interest, especially in respect of possible field induced lipid rearrangements in response to changes in membrane potential.

A particularly interesting result from McConnell's laboratory is the difference between the half times of lipid flip-flop in the experimental system of sonicated lipid vesicles ($t_{1/2} \simeq 6.5$ h) (11), and in the natural system of the electroplax ($t_{1/2} \simeq 5$ min) (12). A possible explanation of this difference is that the presence of proteins in the natural system facilitates lipid flip-flop, perhaps as a result of the discontinuities introduced in the lipid bilayer (12). Recent results from this laboratory have shown that functional proteins may be incorporated into planar lipid bilayers (30–33), and so it is now possible to perturb an experimental lipid bilayer with native membrane proteins. This is an ideal system for studying both the effect of proteins on lipid flip-flop, and the role of lipid flip-flop in determining the specific short range lipid-protein interactions required by membrane proteins to express biological activity in functional reconstitution studies; such investigations are at present underway.

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