PHOSPHOLIPID FLIP-FLOP AND THE DISTRIBUTION OF SURFACE CHARGES IN EXCITABLE MEMBRANES

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ABSTRACT There is now good evidence that most of the lipids in a biological membrane are arranged in the form of a bilayer. Charged lipids in the membrane of an excitable cell are subject to a significant driving force, the gradient of the intramembrane potential, which will tend to redistribute the lipids between the two halves of the bilayer by a "phospholipid flip-flop" mechanism. We have calculated, by combining the Boltzmann relation from statistics and the Gouy equation from the theory of the diffuse double layer, the steady-state distribution of charged lipids in the bilayer. This distribution is completely determined, within the framework of the model, by three experimentally accessible variables; the percentage of charged lipid in the bilayer as a whole, the resting potential and the ionic strength. The known values for the percentage of anionic phospholipids in squid axons (10–15%), the membrane potential (50–100 mV) and ionic strength (0.5 M) imply that the charge density and double layer potential at the outer surface of the nerve will be substantially greater than the charge density and double layer potential at the inner surface, in agreement with the best available evidence from physiological measurements.

The available evidence suggests that there are negative charges in the immediate vicinity of both the sodium and potassium "channels" (Hille, 1970) in a variety of nerves. These charges give rise to an electrical "double layer" which extends for a few tens of angstroms into the aqueous phase (e.g., Grahame, 1947; Verwey and Overbeek, 1948; Haydon, 1964; Delahay, 1965; Barlow, 1970). A change in the potential at the membrane solution interface, produced by cations either screening the charges or binding to them, will manifest itself as a shift in the conductance voltage curves along the voltage axis. It is from studying such shifts that the potentials at the surfaces of nerves have been estimated. The magnitude of the potential at the outer surface of frog (Hille, 1968) and squid (Gilbert and Ehrenstein, 1969; Ehrenstein and Gilbert, 1973) axons in normal bathing solutions was estimated to be about 60–70 mV and is apparently even larger for crayfish axons

(D'Arrigo, 1973). This potential is defined as ψ_1 in Fig. 1. The inside of the squid axon, on the other hand, appears to have a substantially lower surface potential, defined as ψ_2 in Fig. 1. Chandler et al., (1965), who were the first to successfully apply the Gouy-Chapman theory of the diffuse double layer to excitable membranes, estimated that ψ_2 was about 17 mV in a physiological solution. Our report is concerned with a simple statistical mechanism which could produce and maintain this asymmetry of surface charge and potential in a biological membrane.

As the lipids in the axolemma are arranged in the form of a bilayer (e.g., Stoeckenius and Engelman, 1969; Singer and Nicolson, 1972), which constitutes an insulating framework for the rather widely separated channels of the nerve membrane, there is some justification for examining the simple model illustrated in Fig. 1. We consider a phospholipid bilayer consisting of a mixture of two species of lipids; one net neutral (e.g. phosphatidyl choline) and one bearing a negative charge (e.g. phosphatidyl serine). We define σ_1 as the charge density (in units of number of electronic charges per square angstrom) on the outer surface of the bilayer, σ_2 as the charge density on the inner surface and σ as the average charge density.

$$2\sigma = \sigma_1 + \sigma_2. \tag{1}$$

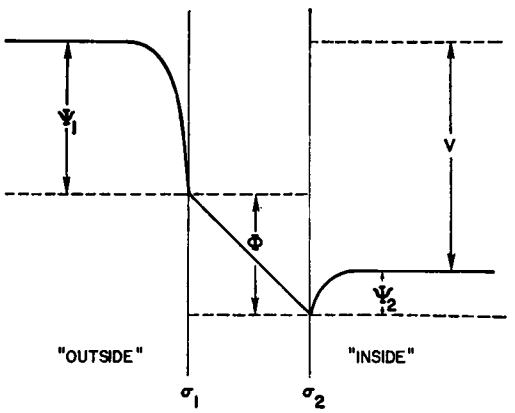

FIGURE 1 The profile of electric potential in the vicinity of a phospholipid bilayer when the charged lipids in the membrane are allowed to distribute themselves between the two interfaces according to the Boltzmann relation. The membrane is assumed to be homogeneous in the y–z plane, the charge per unit area at $x = 0$, σ_1, and at $x = d$, σ_2, is assumed to be distributed uniformly over the surface, and any potential due to dipoles is ignored for simplicity. V is the resting potential, ψ_1 the double layer potential at the outer surface, ψ_2 the double layer potential at the inner surface, and ϕ the potential difference between the two membrane solution interfaces. If the resting potential V is assumed to be 75 mV, the concentration of monovalent ions in the bathing solution 0.5 M and the percentage of negative lipid in the bilayer 15%, the calculations in the text indicate the surface potentials are $\psi_1 = 50$ mV and $\psi_2 = 15$ mV (see Table II). The potential profiles in Fig. 1 are drawn to scale for these values of V, ψ_1, ψ_2 and a membrane of thickness $d = 50$ Å. The profile in the membrane is assumed to be linear or the electric field a constant, which is equivalent to assuming that the concentration of ions in the membrane is less than $5 \cdot 10^{-5}$ M (Neumcke and Läuger, 1970).

The surface charge σ_1 produces a surface potential ψ_1. There is now good experimental evidence that the theory of the diffuse double layer describes adequately the potential produced by charges on a phospholipid bilayer (Lesslauer et al., 1967; McLaughlin et al., 1970; McLaughlin et al., 1971; Stark and Benz, 1971; MacDonald and Bangham, 1972; Muller and Finkelstein, 1972; Haydon and Meyers, 1973). If we assume for simplicity that the membrane is bathed in a symmetrical monovalent electrolyte solution of concentration C the surface potential is related to the charge density by the Gouy equation:

$$\sinh (F\psi_1/2RT) = 136\sigma_1/C^{1/2}, \tag{2}$$

where $RT/F = 25.3$ mV at 22°C and C is in units of moles per liter. In exactly the same manner,[1] the charge on the inner surface of the membrane produces a surface potential ψ_2:

$$\sinh (F\psi_2/2RT) = 136\sigma_2/C^{1/2}. \tag{3}$$

If V is defined as the resting potential, or potential that would be measured by two electrodes in the bulk aqueous solutions, and ϕ as the potential difference between the inner and outer surface of the membrane it follows from Fig. 1 that:

$$\phi = \psi_2 + V - \psi_1. \tag{4}$$

We have defined these four potentials and two charge densities as positive quantities for convenience.

To visualize the phenomenon, assume that σ_1 and σ_2 are initially identical. The surface potentials ψ_1 and ψ_2 will therefore also be identical and the resting potential V will equal the intramembrane potential ϕ. The gradient of ϕ, however, constitutes a driving force which will tend to move charged lipids from one surface of the membrane to the other. It is known from the spin label studies of Kornberg and McConnell (1971) that phospholipids do traverse bilayers and they refer to this transverse motion in a euphonious manner as "phospholipid flip-flop." If a steady state is attained, the distribution of charged lipids will satisfy the Boltzmann relation:

$$\sigma_2/\sigma_1 = \exp-(F\phi/RT). \tag{5}$$

Strictly speaking this is not correct because the asymmetric distribution of charged lipids produced by the potential will also produce an asymmetric distribution of neutral lipids. These neutral lipids will, in turn, tend to diffuse down their concen-

[1] The direct electrical coupling between the two double layers or surface potentials is negligible for normal values of the ionic strength, C. Chandler et al. (1965) or Muller and Finkelstein (1972) may be consulted for a further discussion of this point. The electrical diffuse layers produced by the resting potential are also negligible for normal values of the ionic strength (e.g., Walz et al., 1969).

tration gradient. We take this into account in the Appendix by using the formalism of irreversible thermodynamics and assuming that the flip-flop of the charged and neutral lipids are tightly coupled to obtain:

$$(\sigma_2/\sigma_1)\,([C_{tot} - \sigma_1]/[C_{tot} - \sigma_2]) = \exp-(F\phi/RT). \quad (6)$$

Eq. 6 reduces to Eq. 5 for small charge densities. If we specify the average charge density σ, the resting potential, V, the concentration of monovalent ions, C, and the total concentration of lipids on each side of the bilayer, $C_{tot} = 1/40$ (Å)$^{-2}$,

TABLE I

NUMERICAL SOLUTIONS OF EQS. 1–4 AND 6

(C = 0.15 M MONOVALENT SALT)

Percent negative lipid	V	ψ_1	ψ_2	ϕ	σ_1	σ_2	ψ_1^*
5	50	31.4	10.9	29.5	1.88	0.62	31.6
5	75	34.9	6.7	46.8	2.12	0.38	35.2
5	90	36.7	4.4	57.7	2.25	0.25	36.9
5	100	37.4	3.5	66.1	2.31	0.19	37.7
10	50	52.1	25.9	23.8	3.48	1.52	52.6
10	75	56.9	18.7	36.8	3.92	1.08	57.7
10	90	59.5	14.5	45.0	4.17	0.83	60.2
10	100	61.0	11.9	50.9	4.32	0.68	61.5
15	50	67.8	39.2	21.4	5.06	2.44	68.6
15	75	72.9	30.6	32.7	5.67	1.83	73.6
15	90	75.4	25.6	40.2	6.00	1.50	76.2
15	100	76.9	22.4	45.5	6.20	1.30	77.9
20	50	80.0	51.0	21.0	6.62	3.38	81.0
20	75	85.0	41.8	31.8	7.37	2.63	86.0
20	90	87.5	36.3	38.8	7.78	2.22	88.8
20	100	89.1	32.7	43.6	8.03	1.97	90.3
30	50	98.2	69.8	21.6	9.70	5.30	99.7
30	75	103.0	60.6	32.6	10.71	4.29	104.7
30	90	105.5	54.8	39.3	11.28	3.72	107.5
30	100	107.2	50.4	43.2	11.67	3.33	109.0

The values of V, ψ_1, ψ_2, and ϕ in Tables I and II are expressed in millivolts. The values of σ_1 and σ_2 are expressed in units of 10^{-3} electronic charges per square angstrom. ψ_1 was calculated to an accuracy of ±0.2 mV. ψ_1^* is the value of ψ_1 calculated by using Eq. 5 instead of 15, which is equivalent to ignoring any coupling between the flip-flop of the neutral and negatively charged lipids. Note that this coupling has little effect (<3%) on the value of ψ_1 for the charge densities and potentials under consideration.

TABLE II

NUMERICAL SOLUTIONS OF EQS. 1–4 AND 6
(C = 0.5 M MONOVALENT SALT)

Percent negative lipid	V	ψ_1	ψ_2	ϕ	σ_1	σ_2	ψ_1^*
5	50	18.9	5.0	36.1	1.99	0.51	19.1
5	75	21.2	2.6	56.4	2.24	0.26	21.3
5	90	22.0	1.6	69.6	2.34	0.16	22.1
5	100	22.4	1.2	78.8	2.38	0.12	22.5
10	50	33.4	12.7	29.3	3.69	1.31	33.9
10	75	37.4	7.7	45.3	4.21	0.79	38.0
10	90	39.2	5.4	56.2	4.45	0.55	39.5
10	100	40.0	4.4	64.4	4.55	0.45	40.5
15	50	45.3	20.8	25.5	5.30	2.20	46.0
15	75	49.8	14.5	39.7	5.99	1.51	50.6
15	90	52.1	11.0	48.9	6.35	1.15	52.9
15	100	53.4	9.1	55.7	6.56	0.94	54.1
20	50	54.9	29.3	24.4	6.82	3.18	56.2
20	75	59.7	22.1	37.4	7.66	2.34	61.0
20	90	62.2	17.8	45.6	8.13	1.87	63.5
20	100	63.8	15.1	51.3	8.43	1.57	65.0
30	50	70.6	44.3	23.7	9.85	5.15	72.4
30	75	75.4	36.2	35.8	10.95	4.05	77.4
30	90	77.9	31.4	43.5	11.57	3.43	80.2
30	100	79.7	27.6	47.9	12.00	3.00	81.7

See footnote to Table I.

Eqs. 1–4 and 6 may be solved numerically.[2] This was done for a range of values of σ (5–30% negatively charged lipid), V (50–100 mV), and C (0.15 and 0.5 M) and the solutions are given in Tables I and II.

Fig. 2 plots the difference between the two surface potentials, $\psi_1 - \psi_2$, as a function of the percent negative lipid in the membrane. To illustrate the magnitude of the phenomenon, we consider a particular value of σ. Direct analysis of the phospholipid composition of nerve membranes from both squid (Camejo et al., 1969; Zambrano et al., 1971) and garfish (Chacko et al., 1972) indicates that phosphatidyl serine alone comprises more than 10% of the phospholipids. If we assume that 15% of the lipids bear a negative charge, that the resting potential is 75 mV

[2]Eqs. 1–4 and 6 (or 1–5) were reduced to an expression in a single variable, $F(\psi_1) = 0$. The expression was then solved for its relevant zero using an iterative technique in which ψ_1 was incremented by 0.2 mV through the region in which a zero was expected. Once ψ_1 was determined, the values for σ_1, σ_2, ψ_2, and ϕ could be found directly from Eqs. 1–4. The values for the parameters in Tables I, II, and Fig. 2 are accurate to ±2%.

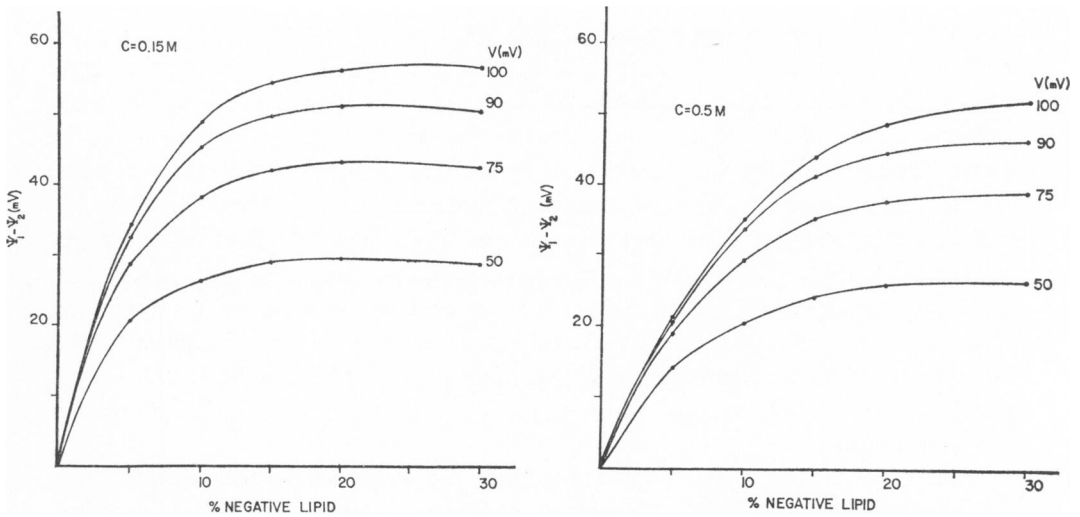

FIGURE 2 The difference between the two surface potentials, $\psi_1 - \psi_2$, illustrated as a function of the percent anionic lipid in the bilayer. The data in the left hand portion of Fig. 2 are taken from Table I ($C = 0.15$ M) and those in the right hand portion from Table II ($C = 0.5$ M). Curves are plotted for a number of different resting potentials, V, and illustrate that in many cases the intramembrane potential difference $\phi = V - (\psi_1 - \psi_2)$ is only about half the measurable resting potential. To consider the example discussed in the text and illustrated in Fig. 1 note from the right hand portion of Fig. 2 that a bilayer with 15% negative lipid and a resting potential of 75 mV will have $\psi_1 - \psi_2 = 35$ mV in the steady state. Of the applied resting potential, V = 75 mV, about 50%, $\phi = 75 - 35 = 40$ mV, actually drops across the two interfaces.

and the ionic strength is 0.5, reference to Fig. 2 indicates that the difference between the surface potentials is 35 mV. Thus, only about half the measurable resting potential actually drops across the membrane. The outer surface potential is $\psi_1 = 50$ mV and the inner surface potential is $\psi_2 = 15$ mV (Table II) for these values of σ, V, and C, as illustrated in Fig. 1. These values agree qualitatively with the estimates on squid axons of about 60 mV for ψ_1 from Gilbert and Ehrenstein's (1969) measurements and 17 mV for ψ_2 from the electrophysiological studies of Chandler et al. (1965).

The two major assumptions inherent in this theoretical analysis are both quite reasonable. Although the Boltzmann equation has not, to the best of our knowledge, been applied to the distribution of charged lipids between the two juxtaposed monolayers which constitute a bilayer, there is no reason to suspect its validity in this case. Many of the assumptions which enter in the Gouy-Chapman theory of the diffuse double layer, on the other hand, are admittedly quite questionable, but the theory does describe adequately the experimental data obtained with bilayers, as discussed in the references cited above.

The analysis then, is probably valid for a bilayer but could be irrelevant to the nerve. The charges near the "channels" could be associated with proteins, for

example, or on lipids which are intimately associated with proteins (e.g., Jost et al., 1973) and therefore relatively immobile. Even if we assume that the relevant charges are on lipids in the bilayer, the rate of biosynthesis of these lipids could be sufficiently rapid that the phenomenon under consideration is not applicable to the nerve. Germane to this point are Kornberg and McConnell's (1971) measurements of the half time (6.5 h) for the transverse movement of phosphatidyl choline in artificial bilayers. The movement of a charged lipid in an artificial bilayer membrane (>24 h; H. M. McConnell, personal communication) is slower, as might be expected, but the flip-flop rate for lipids in an excitable membrane, (4–7 min) on the other hand, appears to be significantly faster than in an artificial bilayer (McNamee and McConnell, 1973). These investigators have also suggested that "rapid flip-flop would allow charged phospholipids to respond to potential differences across the membrane."

More experimental evidence about the inner and outer surface potentials of axons would obviously be valuable. Conti et al. (1971) and Conti (personal communication) have shown that ANS, an anion which is known to adsorb to bilayers (McLaughlin et al., 1971) and produce a negative electrostatic potential, induces little change in the action potential of a squid axon when added extracellularly, but does produce marked changes in the shape, duration, amplitude and threshold of the action potential when perfused internally. This provides independent, albeit indirect support for the argument that the surface potential on the inside of a squid axon is less than the surface potential on the outside.

APPENDIX

We express the flux of charged and neutral lipids (J_1 and J_2, respectively) from interface to interface by the usual phenomenological equations (e.g., Katchalsky and Curran, 1965; see chapter 8):

$$J_1 = L_{11}X_1 + L_{12}X_2 \tag{7}$$

$$J_2 = L_{21}X_1 + L_{22}X_2. \tag{8}$$

The generalized forces, X_1 and X_2, are the gradients, or in this case the differences, of the electrochemical potentials of the charged and neutral lipids at the two interfaces:

$$X_1 = RT \ln (\sigma_1/\sigma_2) + zF\phi \tag{9}$$

$$X_2 = RT \ln (C_1/C_2). \tag{10}$$

R, T, and F have their usual significance; z, the valence, is -1; σ_1 and σ_2 are the concentrations of the charged lipids at the two interfaces in units of molecules per square angstrom; C_1 and C_2 are the concentrations of neutral lipids in the same units and ϕ is the potential defined in Fig. 1. L_{12} and L_{21} are the "coupling" or "cross-coefficients" and the Onsager reciprocal relation implies:

$$L_{12} = L_{21}. \tag{11}$$

We assume that the total number of lipids per unit area at each interface, C_{tot} remains constant. The assumption there are only two species of lipid present implies:

$$\sigma_1 + C_1 = \sigma_2 + C_2 = C_{tot}. \quad (12)$$

If we also assume that the number of lipids in the bilayer as a whole does not change it follows that the flux of negative lipids must be equal in magnitude and opposite in direction to the flux of neutral lipids. That is, the flip-flop is tightly coupled and $J_1 = -J_2$. From Eqs. 7, 8, and 11 this can only occur if:

$$L_{11} = -L_{12}. \quad (13)$$

In the steady state, the fluxes of charged and neutral lipids are zero, so we have from Eqs. 7, 9, 10, and 13 that:

$$O = RT \ln (\sigma_1/\sigma_2) + zF\phi - RT \ln (C_1/C_2). \quad (14)$$

Finally, by combining Eqs. 13 and 14 we arrive at the desired relation between the two charge densities and the membrane potential:

$$(\sigma_2/\sigma_1)\cdot([C_{tot} - \sigma_1]/[C_{tot} - \sigma_2]) = \exp-(F\phi/RT) \quad (15)$$

Note that Eq. 15 reduces to Eq. 5, the Boltzmann expression, when $\sigma_1, \sigma_2 \ll C_{tot}$. The minimal area a lipid could occupy in a bilayer is about 40 Å^2 and the calculations in the tables were made assuming that $C_{tot} = 1/40$ (Å)$^{-2}$. Eqs. 5 and 15 predict very similar results (compare the values of ψ_1^* and ψ_1 in the tables) as long as the percent negative lipid is less than 30% and the resting potential less than 100 mV. Over this range of values the calculations are thus relatively insensitive to the value chosen for the molecular area of the lipids in Eq. 15. The molecular area of the lipids also enters the calculations in relating the percent negative lipid to σ. If the molecular area is actually greater than 40 Å^2 by a factor f the column listed percent negative lipid in the tables need only be divided by f to obtain the appropriate value of ψ_1^*.

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