Transbilayer coupling mechanism for the formation of lipid asymmetry in biological membranes

Application to the photoreceptor disc membrane

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ABSTRACT An equilibrium transmembrane asymmetry in charged lipids is shown to arise as a result of oriented, bipolar proteins in the membrane. The basic interaction giving rise to the asymmetry is between a lipid molecule and a transbilayer potential generated by the asymmetric charge distribution in the protein. Thus, a protein can generate a lipid asymmetry without a direct binding interaction between lipid and protein. The generation of an asymmetry in charged lipid by this mechanism can also lead to a concomitant asymmetry in neutral lipids if deviations from ideality in the lipid mixture are taken into account. It is shown that regular solution theory applied to the lipid phase predicts an asymmetry in all components of a ternary mixture as long as one component is electrostatically oriented according to the mechanism mentioned above. The resulting asymmetry is not strongly salt dependent. The mechanism quantitatively accounts for the experimentally determined phospholipid asymmetry in the rod outer segment disc membrane of the vertebrate photoreceptor.

INTRODUCTION

An asymmetric distribution of phospholipids between the inner and outer monolayers is a feature of many membrane systems (see Op den Kamp, 1979 and Bishop and Bell, 1988 for reviews). The transmembrane distribution of phospholipids in photoreceptor disc membranes has been investigated by several groups using different experimental approaches (Smith et al., 1977; Crain et al., 1978; Drenthe et al., 1980*a* and *b*; Sklar and Dratz, 1980; Miljanich et al., 1981; Tsui et al., 1989). Miljanich et al. (1981), using chemical labeling methods, found 77–88% of the PS[1] and 73–87% of the PE in the outer monolayer of the disc membrane. The inner monolayer is then highly enriched in PC. Tsui et al. (1989) concluded that ~75% of the PS in the disc membrane must be located in the outer monolayer based on the electrostatic properties of the disc membrane. This asymmetry is consistent with the orientation found in other systems, i.e., with the aminolipids preferentially located in the monolayer facing the cytoplasm.

Although it is clearly established that an asymmetry in phospholipids exists in a number of membrane systems, it is less clear how the asymmetry arises, and whether or not it is an equilibrium property of the system. In the red cell, the most extensively studied system, there is evidence for an ATP-dependent process which maintains the asymmetry (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Van Deenen, 1981). This suggests that the asymmetry may not be an equilibrium property of the system, but is rather maintained against the entropy of mixing by a constant energy input (see, however, the critique by Williamson et al., 1987).

In other cases, lipid asymmetries appear to be stable states of the membrane system in the absence of ATP. In brush-border membranes from kidney cortex, a lipid asymmetry is maintained for at least 14 h in the absence of energy sources (Venien and LeGrimellec, 1988). In vesicles prepared from the ROS disc membrane, the asymmetry is stable for days in the absence of energy sources (Tsui et al., 1989) and apparently survives a freeze-thaw cycle, as well as sonication (Smith et al., 1977). Although the phospholipid flip-flop frequency has not yet been determined for this latter membrane, intrinsic half-lives for transmembrane migration are expected to be shorter than days based on its high content of integral membrane protein and polyunsaturated phosphatidylethanolamines (Van Zoelen et al., 1978; De Kruijff et al., 1985). Indeed, long-chain quarternary ammonium amphiphiles equilibrate across the membrane in ~60 s (Sundberg and Hubbell, 1986). These amphiphiles are rather similar to phospholipids in terms of their transmembrane migration in phospholipid vesicles (Castle and Hubbell, 1976). Taken together, the above evidence suggests that the lipid asymmetry in the ROS disc is an equilibrium feature of the system.

For cases where a phospholipid asymmetry is an equilibrium state of the system, it is of interest to consider possible origins of the asymmetry. "Flippase" proteins

[1] *Abbreviations used in this paper:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROS, rod outer segment.

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that catalyze the transmembrane movement of phospholipids have been described in membranes involved in lipid biosynthesis. A passive flippase cannot, however, contribute to equilibrium asymmetry; it can only increase the rate at which the equilibrium state is reached. Bishop and Bell (1988) mention that unidirectional transporter proteins might aid in maintaining asymmetry. This is possible for a steady-state asymmetry, but a transporter could not operate unidirectionally at equilibrium. Proposals that could account for an equilibrium asymmetry include lipid phase separations coupled to a surface curvature (Wu and McConnell, 1975), and interaction of lipids directly with integral membrane proteins or cytoskeletal elements (Haest et al., 1978; Bergmann et al., 1984; Dressler et al., 1984). For charged lipids, asymmetries can arise as a result of a potential difference between the bulk aqueous phases (McLaughlin and Harary, 1974) or asymmetric ionic solutions (McQuarrie and Mulas, 1977). Tenchov and Koynova (1984) have elaborated on the membrane potential as an orienting force, and have shown that nonideal interactions between lipids can significantly enhance the asymmetry of a charged component.

The ROS disc membrane lipid asymmetry is a particularly interesting case to consider in this regard, because (*a*) the asymmetry is stable for days in the absence of energy sources, (*b*) the asymmetry requires neither asymmetric salt solutions nor a potential difference between the bulk aqueous phases (Tsui et al., 1989), (*c*) the phospholipids that are asymmetric show no binding to rhodopsin, which accounts for 95% of the protein in the membrane (Watts et al., 1979), and (*d*) there is no cytoskeleton with which the lipids can interact. However, the rhodopsin molecule is highly oriented and bipolar, with a net positive charge on the cytoplasmic surface and a net negative charge on the intradiscal surface (Tsui et al., 1989). In the present paper it is shown that an oriented, bipolar protein like rhodopsin can create and stabilize a significant asymmetry of a charged phospholipid without direct interaction between the protein and lipid. The transbilayer coupling model presented here accounts reasonably well for the measured asymmetry of PS in the disc membrane. In addition, it is shown that the PS asymmetry thus created can give rise to spontaneous asymmetry of uncharged lipids like PE and PC as a result of nonideal mixing of the lipids.

Transbilayer coupling mechanism

Fig. 1 *a* shows an oriented bipolar protein such as rhodopsin in a bilayer of neutral lipid. This system will have an asymmetry in surface potentials. At equilibrium, this will result in a transbilayer potential equal in magnitude to the difference of the surface potentials, as shown in Fig. 1 *b*. The transbilayer potential is the potential drop across the bilayer from surface to surface, and is distinct from the experimentally measurable membrane potential difference which is the potential between the bulk aqueous phases on either side of the membrane. At equilibrium there is no potential difference between the bulk solution phases on either side of the membrane. If a charged lipid capable of flip-flop is introduced into this situation, it will redistribute under the influence of the transbilayer potential and a new equilibrium state with altered charge densities, surface potentials, and a reduced transbilayer potential will result. Fig. 1 *c* shows the situation for a negatively charged lipid. The charge densities at the membrane surfaces now have contributions from both the protein and lipid. The charged lipid will be in Boltzmann

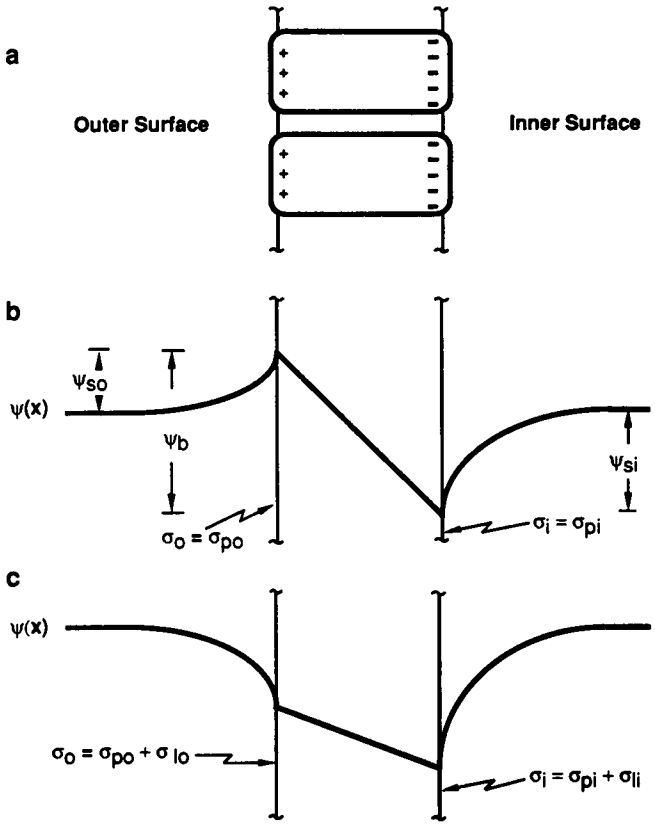

FIGURE 1 The transbilayer coupling mechanism. (*a*) An oriented bipolar protein in a membrane. (*b*) The equilibrium potential profile corresponding to the membrane in *a*, assuming a smeared charge model. ψ_{so} and ψ_{si} are the outer and inner surface potentials, respectively, and ψ_b is the transbilayer potential. The charge densities on the outer and inner surfaces (σ_o, σ_i) are due entirely to contributions from the protein (σ_{po}, σ_{pi}). (*c*) The equilibrium potential profile corresponding to the membrane in *a* after the addition of negatively charged lipid that is able to flip-flop. Now the surface charge densities have contributions from both protein (σ_{po}, σ_{pi}) and lipid (σ_{lo}, σ_{li}). Sufficient lipid has been added to result in a negative potential at both surfaces.

equilibrium with the transbilayer potential and will be asymmetrically distributed as long as there remains a transbilayer potential at equilibrium.

This latter statement contains a central point of the paper, i.e., that asymmetric protein components of the membrane can spontaneously give rise to asymmetric charged lipid distribution without direct interaction between the protein and lipid. The interaction is coupled through the transbilayer potential. The basic force giving rise to the asymmetry, i.e., an electrostatic potential gradient across the bilayer, is the same as that considered by McLaughlin and Harary (1976) and McQuarrie and Mulas (1977), but the origin of the gradient is different. For the case considered here, the asymmetry is as stable as the structure itself and requires neither a maintained potential difference between the bulk aqueous phases nor gradient of ionic composition, both of which are usually provided at the expense of metabolic energy in the cell. The only energy cost for creating the transbilayer potential is paid at the time of membrane synthesis in orienting the protein.

In analyzing this model, both the orienting electrostatic force and the effect of nonideal interaction between phospholipids will be considered as significant determinants for asymmetry creation. The importance of coupling between transmembrane interactions and lipid nonidealities was first pointed out by Tenchov and Koynova (1984). The ROS disc membrane is considered specifically because its electrostatics are well characterized, but the model should be generally applicable.

Rhodopsin in an ideal binary lipid bilayer

Before treating a multicomponent system with nonideal interactions, the lipid region of the disc membrane will first be treated as a two-component, ideal mixture of a charged lipid, PS, and a neutral lipid. This will serve to show the magnitude of the lipid asymmetry due to the electrostatic interaction alone. The aim of the following calculation is then to determine the distribution of PS in the membrane due to interaction with the transbilayer potential generated by the oriented rhodopsin.

Disc membranes are usually isolated in the form of large spherical vesicles. Redistributions of PS between the inner and outer vesicle surfaces is coupled to that of the other lipid species by the constraint that the total number of lipids must remain constant at each surface. McLaughlin and Harary (1974) have shown that for this coupled equilibrium the distribution of a charged lipid is related to the potential difference across the membrane according to (in slightly modified form):

$$X_i(1 - X_o)/X_o(1 - X_i) = \exp(-Z_1F(\psi_i - \psi_o)/RT), \quad (1)$$

where X_i and X_o are the mole fractions of charged lipid on the inner and outer surfaces of the vesicles, ψ_i and ψ_o are the inner and outer surface potentials, and Z_1 is the valence of the lipid. For the case treated here, the potentials ψ_i and ψ_o arise from the surface charge densities due to PS and rhodopsin. Tsui et al. (1989) have shown that the surface potentials at both the inner and outer surfaces of the disc vesicles are described adequately by the simple Gouy-Chapman theory (McLaughlin, 1977). Using this theory, relations between the total charge densities at the two surfaces and the potentials can be written as

$$(q_{lo} + q_{po})/A = \sigma_{lo} + \sigma_{po} = (X_oZ_1N_L/A) + \sigma_{po} = (c^{1/2}/136.6)\sinh(F\psi_o/2RT) \quad (2)$$

$$(q_{li} + q_{pi})/A = \sigma_{li} + \sigma_{pi} = (X_iZ_1N_L/A) + \sigma_{pi} = (c^{1/2}/136.6)\sinh(F\psi_i/2RT), \quad (3)$$

where q_{lo} and q_{li} are the net charges due to the lipid components, per rhodopsin, on the outer and inner surfaces, respectively, q_{po} and q_{pi} are the net charges due to rhodopsin on the outer and inner surfaces, respectively, σ_{li}, σ_{lo}, and σ_{pi}, σ_{po} are the corresponding "smeared" charge densities due to lipid and protein on the inner and outer surfaces, A is the area occupied on the membrane surface by a rhodopsin molecule and its associated lipid, N_L is the number of lipids per rhodopsin at one surface, and the other quantities are as defined above.

The conservation of the charged lipid gives the final needed expression:

$$X_i + X_o = 2X(\mathrm{PS}), \quad (4)$$

where $X(\mathrm{PS})$ is the mole fraction of PS in the binary mixture. To carry out a numerical solution of Eqs. 1–4, the rhodopsin contribution to the surface charge densities (σ_{po}, σ_{pi}) must be known. As discussed in detail by Tsui et al. (1989), this can be determined from the known distribution of ionizable residues on each surface of rhodopsin together with reasonable estimates for their intrinsic pK_as. In the calculations, their Eq. 1 is used to obtain the rhodopsin charge densities. This calculation properly accounts for the variation of charge on the protein with ionic strength and pH. The charge on each surface of the protein depends on any factor that effects the local electrostatic potential, i.e., salt concentration, pH, and lipid composition of the surrounding membrane. For example, at neutral pH the charge on the cytoplasmic surface of the protein in the disc membrane changes from +4.9 to +4.3 as the salt is changed from 0.01 to 0.1 M. Under the same conditions, the charge on the intradiscal surface changes from −3.7 to −4.5.

In the ROS disc membrane, there are a total of ~90 lipid molecules per rhodopsin, of which 72 are phospho-

lipids. Of these, ~11 are PS, and essentially all of the remaining are uncharged near neutral pH (Miljanich et al., 1981). The total area of a rhodopsin molecule and its associated lipid in the membrane surface is ~4,000 Å^2 (Amis et al., 1981; Liebman et al., 1987), and an average lipid molecule occupies ~70 Å^2 at the surface. Solution of Eqs. 1–4 with these values at 0.01 M NaCl, pH 7.2, gives equilibrium surface potentials of $\psi_i = -76$ mV, $\psi_o = -47$ mV, and a mole fraction of PS on the outer surface of $X_o = 0.18$. This corresponds to 75% of the PS on the outer surface of the vesicle. Thus a substantial equilibrium asymmetry is predicted to arise simply due to the presence of rhodopsin in the membrane. These values can be directly compared to experimental values reported by Tsui et al. (1989). These authors, using a spin label technique to estimate the inner and outer surface potentials of the disc membrane, found that at 0.01 M salt, $\psi_i = -76$ mV, $\psi_o = -49$ mV.[2] They concluded that to account for these potentials, ~75% of the PS had to be on the outer surface. Miljanich et al. (1981) found 77–87% of the PS on the outer surface using chemical labeling techniques. The agreement between the experimental values and those calculated on the basis of the transbilayer coupling mechanism is good, and indicates that the simple electrostatic model is sufficient to explain the asymmetry.

The asymmetry generated by the transbilayer coupling mechanism is salt dependent, because an increase in salt concentration screens the surface potentials. This effect, however, is not overwhelming. The fraction of PS on the outer surface is predicted to decrease from ~0.75 at 0.01 M to 0.68 at 0.1 M, still a substantial asymmetry. As will be shown below, this asymmetry can be greatly enhanced by including effects of lipid nonideal mixing. All further calculated results in this paper will refer to 0.1 M monovalent salt.

Rhodopsin in PS, PE, PC membranes with nonideal behavior

The ROS disc membrane, like most, contains three principle types of phospholipid: PS, PE, and PC. The above calculations suggest that the PS asymmetry can be understood on the basis of the electrostatic properties of rhodopsin. However, PE is also asymmetrically disposed in the membrane (Smith et al., 1977; Crain et al., 1978; Miljanich et al., 1981). Because PE is electrically neutral, its asymmetry cannot be accounted for on the basis of direct electrostatic interactions. In the following paragraphs, it is shown that nonideal interactions among the lipids are sufficient to account for the PE asymmetry, but only if electrostatic forces act to create an asymmetry in PS. These nonideal interactions act to further enhance the PS asymmetry.

The goal is to compute the distribution of all lipids in the potential gradient generated by the asymmetric protein. The framework of the calculation is similar to that above, except that the system now contains three lipid components that show nonideal mixing. For notational simplicity in the following, PS, PE, and PC will be referred to as S, E, and C, respectively. A subscript "i" (inner) or "o" (outer) refers to the membrane surface at which the lipid is located. As pointed out by McLaughlin and Harary (1974), the transbilayer movement of one lipid species is coupled to the movement of the others to maintain equivalent numbers on the two sides of the membrane. In other words, the "flip" of a lipid of one type must be accompanied by a "flop" of another type in the opposite direction. The possible flip-flop processes in this three component system are enumerated as:

$$S_i + E_o \rightleftharpoons S_o + E_i \quad (5)$$

$$S_i + C_o \rightleftharpoons S_o + C_i \quad (6)$$

$$E_i + C_o \rightleftharpoons E_o + C_i. \quad (7)$$

The equilibrium conditions are found from the differential of the total free energy of the lipid system, which is

$$dG = \mu_{Si}dn_{Si} + \mu_{So}dn_{So} + \mu_{Ei}dn_{Ei} + \mu_{Eo}dn_{Eo} + \mu_{Ci}dn_{Ci} + \mu_{Co}dn_{Co}, \quad (8)$$

where the μs are chemical potentials for the corresponding lipid components on the inside (i) or outside (o) of the vesicle. At internal equilibrium, $dG = 0$ for infinitesimal displacements of the system according to each of the three processes above. This gives rise to three conditions, of which two are independent. For process 5, $dn_{Ci} = dn_{Co} = 0$. From the stoichiometry of the process, it follows that $-dn_{Si} = -dn_{Eo} = dn_{So} = dn_{Ei} \equiv dn$, and for an equilibrium state, $dG = 0 = dn(-\mu_{Si} + \mu_{So} + \mu_{Ei} - \mu_{Eo})$. Therefore,

$$\mu_{Si} + \mu_{Eo} = \mu_{So} + \mu_{Ei}. \quad (9)$$

Two other equilibrium conditions follow similarly from processes 6 and 7:

$$\mu_{Si} + \mu_{Co} = \mu_{So} + \mu_{Ci} \quad (10)$$

$$\mu_{Ei} + \mu_{Co} = \mu_{Eo} + \mu_{Ci}. \quad (11)$$

[2]The experiments of Tsui et al. (1989) were done as a function of salt concentration. However, the disc vesicles were equilibrated for an extended period in 0.01 M salt before the measurements. When a measurement was to be made, NH_4Ac (a highly permeable salt) was added in the desired concentration and the surface potential determined within a few minutes. Unless the phospholipid flip-flop is very rapid, the phospholipid asymmetry would still correspond to that at 0.01 M salt. Thus, 0.01 M salt has been chosen to compare the calculated and experimental values.

To use conditions 9–11, expressions for the chemical potentials must be obtained. Nonideal interactions between the lipids have an important effect on the asymmetry, and will be accounted for on the basis of the Bragg-Williams approach to regular solutions (McClelland, 1973). This approach has been reasonably successful in accounting for phase diagrams of lipid mixtures (Lee, 1977). The assumptions and limitations of the model are discussed in McClelland (1973). Nonidealities are attributed to differences in pairwise interaction energies between different lipid species. If all lipids interact with each other with the same energy, the mixture is ideal. Expressions for the chemical potential of a lipid according to this theory thus involve a mean molecular interaction parameter, W, which characterizes the lipid interactions. For a three component mixture, there are three different parameters, one for each pairwise interaction. For the PS, PE, PC mixture they are:

$$W(\mathrm{SE}) = \alpha(\mathrm{SE}) - \tfrac{1}{2}[\alpha(\mathrm{SS}) + \alpha(\mathrm{EE})]$$

$$W(\mathrm{SC}) = \alpha(\mathrm{SC}) - \tfrac{1}{2}[\alpha(\mathrm{SS}) + \alpha(\mathrm{CC})]$$

$$W(\mathrm{EC}) = \alpha(\mathrm{EC}) - \tfrac{1}{2}[\alpha(\mathrm{EE}) + \alpha(\mathrm{CC})],$$

where the αs are the interaction energies between the pairs of molecules indicated in the subscript when they are nearest neighbors. Thus, the W parameter is just the difference between the pairwise interaction energy between unlike molecules and the mean of the interaction energies with the corresponding like molecules. Realistic ranges for the Ws are available from studies of lipid phase diagrams. The chemical potentials in terms of the Ws according to the Bragg-Williams formalism are:

$$\mu_S = \mu_S^\circ + RT \ln X_S + W(\mathrm{SE})X_E^2 + W(\mathrm{SC})X_C^2 + X_E X_C[W(\mathrm{SE}) + W(\mathrm{SC}) - W(\mathrm{EC})] \quad (12)$$

$$\mu_E = \mu_E^\circ + RT \ln X_E + W(\mathrm{SE})X_S^2 + W(\mathrm{EC})X_C^2 + X_S X_C[W(\mathrm{SE}) + W(\mathrm{EC}) - W(\mathrm{SC})] \quad (13)$$

$$\mu_C = \mu_C^\circ + RT \ln X_C + W(\mathrm{SC})X_S^2 + W(\mathrm{EC})X_E^2 + X_S X_E[W(\mathrm{SC}) + W(\mathrm{EC}) - W(\mathrm{SE})]. \quad (14)$$

Substitution of the above expressions for the chemical potentials into Eqs. 9–11 will give the equilibrium conditions in terms of the mole fractions, potentials, and Ws. Only two of the three resulting expressions are independent. Selecting Eqs. 9 and 11 for substitution, these are:

$$\begin{aligned} &RT[\ln (X_{Si}/X_{So}) + \ln (X_{Eo}/X_{Ei})] \\ &+ W(\mathrm{SE})[(X_{Ei}^2 - X_{Eo}^2) \\ &+ (X_{Ei}X_{Ci} - X_{Eo}X_{Co}) + (X_{So}X_{Co} - X_{Si}X_{Ci})] \\ &+ W(\mathrm{SC})[(X_{Ci}^2 - X_{Co}^2) + (X_{Ei}X_{Ci} - X_{Eo}X_{Co}) \\ &+ (X_{Si}X_{Ci} - X_{So}X_{Co})] + W(\mathrm{EC})[(X_{Co}^2 - X_{Ci}^2) \\ &+ (X_{So}X_{Co} - X_{Si}X_{Ci}) + (X_{Eo}X_{Co} - X_{Ei}X_{Ci})] \\ &- ZF(\psi_o - \psi_i) = 0 \end{aligned} \quad (15)$$

$$\begin{aligned} &RT[\ln (X_{Ei}/X_{Eo} + \ln X_{Co}/X_{Ci})] + W(\mathrm{SE})[(X_{Si}^2 - X_{So}^2) \\ &+ (X_{Si}X_{Ci} - X_{So}X_{Co}) + (X_{Si}X_{Ei} - X_{So}X_{Eo})] \\ &+ W(\mathrm{SC})[(X_{So}^2 - X_{Si}^2) + (X_{So}X_{Eo} - X_{Si}X_{Ei}) \\ &+ (X_{So}X_{Co} - X_{Si}X_{Ci})] + W(\mathrm{EC})[(X_{Ci}^2 - X_{Co}^2) \\ &+ (X_{Eo}^2 - X_{Ei}^2) + (X_{Si}X_{Ci} - X_{So}X_{Co})] \\ &+ (X_{So}X_{Eo} - X_{Si}X_{Ei}) = 0. \end{aligned} \quad (16)$$

There are eight unknowns in the above expressions: six mole fractions corresponding to the i and o locations of each of the three components and the two equilibrium surface potentials. Eqs. 15 and 16 provide two of the eight equations required for a solution. The remaining six relationships required for a solution are provided by the two Gouy-Chapman expressions relating the surface potentials to charge densities (Eqs. 2 and 3 above) and the four conservation statements given below. By definition,

$$X_{Si} + X_{Ei} + X_{Ci} = 1 \quad (17)$$

$$X_{So} + X_{Eo} + X_{Co} = 1. \quad (18)$$

Finally, there are two independent conservation conditions chosen as:

$$X_{Si} + X_{So} = 2f_S \quad (19)$$

$$X_{Ei} + X_{Eo} = 2f_E, \quad (20)$$

where f_S and f_E are the mole fractions of PS and PE, respectively, in the ternary mixture. A numerical solution of the eight equations enumerated above for the eight unknowns can now be carried out given values of W(SE), W(SC), and W(EC). These values can be estimated from the phase behavior of phospholipid mixtures.

It is generally found that the mixing behavior of phospholipids is dominated by the headgroup type, although it is certainly affected by the chain composition (see Lee, 1977 for review). Silvus and Gagne (1984*a*) found that synthetic dielaidoyl PS and PE formed ideal mixtures, and dimyristoyl PS and PE were close to ideal. This indicates that W(SE) is close to zero, and it will be taken as such in all cases considered below. PE and PC form nonideal mixtures (Shimshick and McConnell, 1973; Blume and Ackerman, 1974; Chapman et al., 1974; Wu and McConnell, 1975). Lee (1977) has fit phase diagrams for PE-PC mixtures to the Bragg-Williams model and finds values of W(EC) between 1.5 and 2.1 in units of RT. These rather large values indicate an unfavorable interaction of PE with PC compared to the interaction of either lipid with itself. PS and PC do not form ideal mixtures (Luna and McConnell, 1977; Stewart et al., 1979; Silvius and Gagne, 1984*b*), and values for W(SC) must be positive but have not been determined.

The qualitative effect of the nonideality can now be appreciated in terms of these interaction energies. As was shown above, PS will be asymmetrically disposed due to the interaction with the transbilayer potential. Now because PE shows a less favorable interaction with PC than with PS (W[ES] < W[EC]), PE will tend to follow PS and also distribute asymmetrically, biased towards the same surface. This asymmetry will be further enhanced because PS also shows an unfavorable interaction with PC. Thus it is anticipated that the electrostatic and the nonideal interactions together will lead to an asymmetry in which PS and PE are concentrated together on the surface containing the positive charge density from the protein (the cytoplasmic surface), and PC will be excluded to the opposite surface.

For convenience of discussion, the fraction of the total amount of a particular lipid that is located at the outer surface will be referred to as the "asymmetry" of that lipid with respect to the outer surface. Thus the asymmetry is defined as $X_o/(X_o + X_i)$. Asymmetries of 1, 0.5, and 0 correspond to a lipid localized entirely on the outer surface, equally distributed between the inner and outer surfaces and localized entirely on the inner surface, respectively. There are many parameters that could be defined as measures of asymmetry, but experimental results are often expressed in these terms. In addition, this parameter always has a finite, positive value. It has limited intuitive value in situations where $X_o + X_i > 1$, because even in the most asymmetric distribution possible, the asymmetry will be less than unity. This situation, however, is not encountered in the present discussion.

Fig. 2, *a–c*, show the effect of the interaction parameters on the asymmetry of PE, PS, and PC in a rhodopsin containing membrane at 0.1 M salt, with W(SE) = 0. The composition of the membrane is 12% PS, 35% PE, and 53% PC, which has the same content of PS and PE as the disc membrane but where all neutral lipids and PC are represented together as PC. For W(SC) = W(EC) = 0, the ideal mixing situation, ~69% of the charged PS resides on the outer surface due to the electrostatic potential created by the oriented rhodopsin. (It is less than the 75% quoted above because the salt concentration is now 0.1 M rather than 0.01 M.) Under these conditions, some 48% of both the PE and PC reside on the outer surface. (It is not 50%, because the asymmetry of PS leaves fewer sites for occupancy on the outer surface.)

As the interaction W(SC) increases while W(EC) = 0, little asymmetry increase is seen in any of the components. Notice in Fig. 2 *a* that the Z axis for PS is considerably expanded over those in Fig. 2, *b* and *c*, and the changes shown for PS are exaggerated in comparison to PE and PC. As discussed above, interaction parameters as high as $2RT$ can be expected for W(EC) which describes the PE-PC interaction. As W(EC) is increased

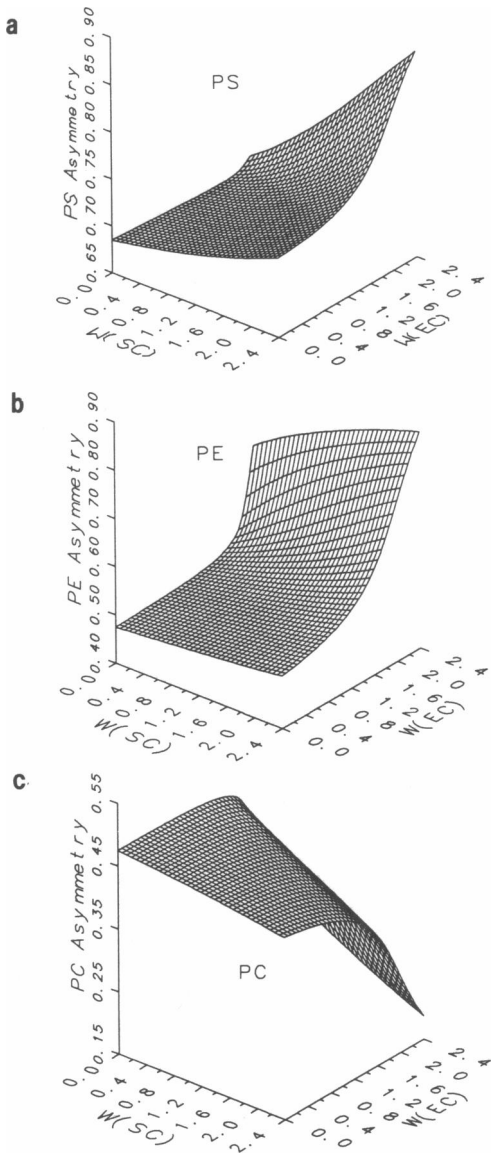

FIGURE 2 The asymmetries of PS, PE, and PC in a rhodopsin-containing bilayer as a function of the lipid interaction parameters, W(SC) and W(EC). The mole fraction composition of the membrane is: PS, 0.12; PE, 0.35; PC, 0.53. The salt concentration is 0.1 M. (*a*) PS. (*b*) PE. (*c*) PC.

from 0 with W(SC) = $2RT$, pronounced asymmetries arise in PE and PC, and a definite increase in the PS asymmetry is seen. The PE and PS are concentrated on the outer surface (asymmetry > 0.5), whereas PC is excluded to the inner surface (asymmetry < 0.5). However, the W(EC) interaction by itself does not generate a large asymmetry in PE or PC. For example, at W(EC) = $2RT$ with W(SC) = 0, ~51% of the PE and 45% of the PC reside on the outer surface, only about a +3% and −3% change, respectively, from the value in the ideal mixture.

However, at $W(SC) = W(EC) = 2RT$, ~80% of the PS and 70% of the PE reach the outer surface. At the same time, the percentage of the PC on the outer surface has decreased to only 30%, having been largely excluded to the inner surface.

Values of interaction parameters exceeding $2RT$ are not considered, because larger values lead to phase separation between components. This can indeed generate large asymmetries, but there is no experimental evidence for large-scale phase separations in the disc membrane lipids at room temperature (however, see below).

The above results show that appreciable asymmetries in all lipids, charged and neutral, can arise as the result of nonideal interactions between the molecules and the electrostatic orientation of one of them. It is important to realize that no asymmetries whatsoever would develop without the electrostatic orientation of one (charged) component. In the absence of a transbilayer potential, the surfaces in Fig. 2 would all be horizontal. In addition, a significant nonideality must exist between PC and both PS and PE, and $W(EC), W(SC) > W(ES)$. However, the interaction energies necessary are not so large as to result in a phase separation in the lipid mixture. Finally, it should be mentioned that the asymmetry generated by the transbilayer coupling mechanism depends on ionic strength, but the effect is remarkably slight in the presence of nonideal lipid interactions. For example, the asymmetry of PS and PE decreases only by ~3% and 1%, respectively, in going from 0.01 to 0.1 M salt at $W(SC) = W(EC) = 2RT$.

The degree of asymmetry achieved is a function of the membrane composition in the range relevant to most biological membranes, as shown in Fig. 3, *a–c*. In these figures the asymmetries of PS, PE, and PC are shown as a function of the mole fraction of PE and PS in the membrane for $W(SC) = W(EC) = 2RT$ and $W(SE) = 0$.

As shown in Fig. 3 a, the PS asymmetry continually increases as the amount of PS decreases at any PE content. This is due to the fact that as the PS content is reduced, it contributes less to the surface charge, so that the transbilayer potential remains higher at equilibrium. At very high PS, the surface charge is determined primarily by the PS and will be nearly symmetric. The PS asymmetry shows a relatively weak dependence on the PE content in the range explored.

The PE asymmetry (Fig. 3 *b*) reaches a broad maximum in the region 10–15% PS and 25–35% PE, roughly in the composition region of many membrane systems (Gennis, 1989), including the ROS disc membrane. The existence of the maximum with variation of PE and PS content can be understood in terms of the intermolecular interactions and the entropy of mixing. The PE asymmetry increases with PE content from 0 to the maximum due

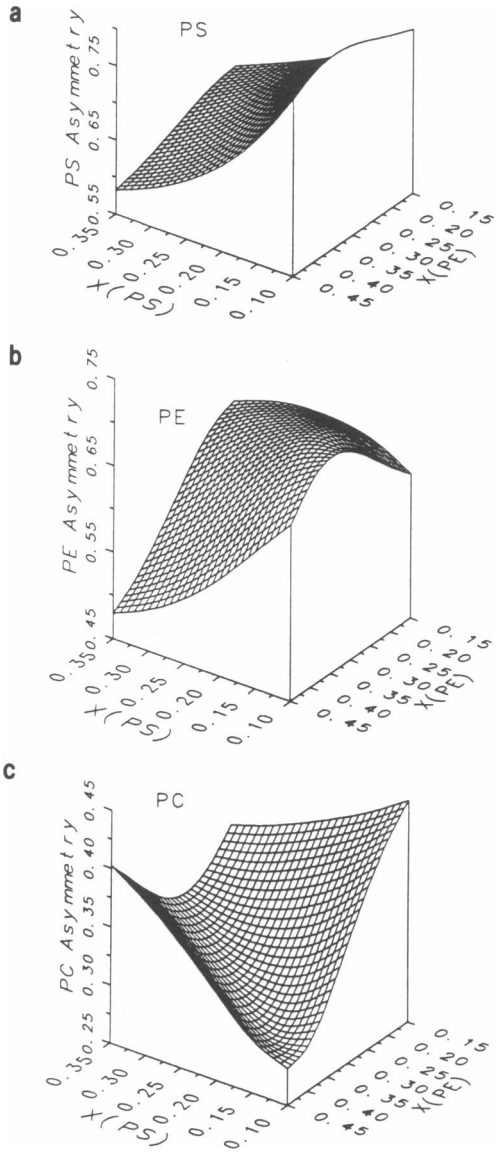

FIGURE 3 The asymmetries of PS, PE, and PC in a rhodopsin-containing bilayer as a function of lipid composition. $X(PS)$, $X(PE)$, and $X(PC)$ are the mole fractions of the lipids in the whole lipid mixture. $W(SE) = 0$ and $W(SC) = W(EC) = 2RT$. (*a*) PS. (*b*) PE. (*c*) PC.

to a decreasing probability of unfavorable PE-PC contacts on the outer surface because PE is replacing the PC. Near the maximum, the entropy loss of separating PC and PE across the membrane overcomes the lipid interaction energies and the PE asymmetry decreases with increasing PE. At very high PE, the asymmetry must decrease toward the symmetric value because all available sites on both surfaces become filled with PE.

For $X(PE) < \sim 0.3$, the PE asymmetry increases with increasing PS from 0 to a maximum, for the same reasons given above for the variation with PE (energetically, PS

and PE are indistinguishable in this calculation). At high PS, the PE asymmetry must decrease with increasing PS, because the PS asymmetry which drives the effect in the first place decreases. Thus a maximum is also expected with variation of PS.

The PC asymmetry shown in Fig. 3 *c* can be understood in similar terms. Because the shape of the surface is determined by that for PC, PE, and the requirement for conservation of lipid species, it will not be discussed in detail. Note that the asymmetry is everywhere <0.5, indicating that the PC is concentrated on the inner surface.

Information is now at hand to compare the experimental lipid asymmetries in the disc membrane with those calculated from the transbilayer coupling mechanism. The ROS disc membrane contains ~12% PS and 35% PE. This composition, with $W(SC) = W(EC) = 2RT$, could generate an asymmetry with ~80% of the PS and 70% of the PE on the outer surface at 0.1 M salt, as discussed above. Only ~30% of the PC would remain on the outer surface. These values are comparable to the experimental asymmetries of 77–87% for PS and 73–87% for PE reported by Miljanich et al. (1981).

There are approximations and omissions in these calculations that may cause an underestimation of the asymmetry. One approximation is that the disc membrane is treated as a ternary lipid system. Some 75% of the lipid is due to PS, PE, and PC, but the remaining 25% are distributed among at least six other species. These additional populations will decrease the entropy cost of separating a particular component to one surface (say PE) by providing additional configurations for generating the concomitant gradients of other lipids. This will lead to an increase in the asymmetry possible for a particular component. The binding of cations to PS in membrane surfaces is well known, and causes a significant reduction in surface potential at physiological salt concentrations (Eisenberg et al., 1979; Tsui et al., 1986). The present computation does not account for counter-ion binding to PS. Including this effect would reduce the contribution of PS to the surface potential and increase the calculated asymmetry of all lipids.

Additional effects of the other lipid components mentioned above depend on their mixing properties with PS, PC, and PE. Their influence could result in either a calculated over- or underestimation of the asymmetry, but too little information is available to draw any conclusions. However, Sklar et al. (1979) have described a solid-liquid phase separation in disc membrane lipids occurring near room temperature. This is apparently due to crystallization of a substantial amount of dipalmitoyl PC among the lipids of this otherwise highly unsaturated population. The phase transition involves ~10% of the PC. This shows an unusually large nonideality in at least a fraction of the lipid population that could significantly enhance the lipid asymmetry of the membrane.

The spontaneous generation of lipid asymmetry by the transbilayer coupling mechanism requires an asymmetric surface charge created by a stable structural element. In the disc membrane, the asymmetries are generated by the integral protein rhodopsin, which contributes ~0.001 electronic charges per A^2 of membrane surface. This is not a particularly high charge density, and corresponds to ~10 mol% of charged lipid. Fig. 4 shows the dependence of lipid asymmetry generated as a function of charge density due to a bipolar protein, taking the charge to be fixed and equal in magnitude but opposite in sign on the membrane surfaces. As can be seen, the PS asymmetry continues to increase with charge, but the PE asymmetry asymtotically approaches ~75% at the lipid composition of the disc membrane. Higher values than this would have to be created by larger interaction parameters with other lipids.

The asymmetry in surface charge can be produced by integral membrane proteins, as discussed here, or by cytoskeletal or peripheral membrane proteins that bring a net charge close to the membrane surface. Thus a direct binding of phospholipid to a surface protein is not necessarily required for it to generate a lipid asymmetry, but could greatly enhance it.

Membrane proteins are often bipolar, and in sufficient concentration for the transbilayer coupling mechanism to be effective. Whether or not this mechanism is the

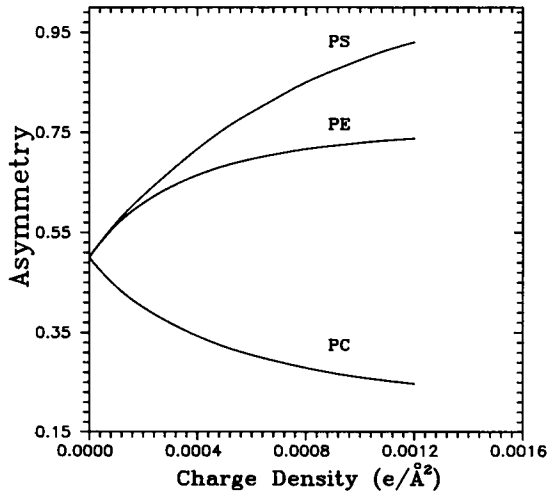

FIGURE 4 Asymmetry of phospholipids in a ternary mixture as a function of charge density due to an integral membrane protein. The asymmetry is the fraction of the lipid on the outer surface of a vesicle. The protein is assumed to have the same magnitude of charge on each surface, but of positive sign on the outer surface and negative sign on the inner surface. $W(SE) = 0$, $W(SC) = W(EC) = 2RT$. For reference, rhodopsin corresponds approximately to 0.0008 e/Å².

determinant of phospholipid asymmetry in other cell or organelle membranes, it is important to be aware of the effect in investigations of isolated membrane vesicles where it may be the only mechanism. As mentioned in the Introduction, the lipid asymmetry in the isolated ROS disc membrane appears to involve neither a cytoskeleton, an energy source, asymmetric salt solutions, direct interactions with rhodopsin, nor a potential difference between the bulk aqueous phases. If the transbilayer coupling mechanism is not responsible for the asymmetry, a new interaction must be sought along with a careful investigation of the points listed above.

Finally, it should be emphasized that the experimental measurements of asymmetry in the ROS disc membrane quoted above and the calculated values refer to osmotically shocked disc vesicles rather than the flattened structures found in the native ROS. This distinction is likely to be important, because in the native disc the intradiscal surfaces are very closely apposed (spacing of 20 Å; Chabre and Cavagionni, 1975) and the intradiscal space has a high local concentration of Ca^{++} (Kaupp et al., 1979; Shroder and Fain, 1984). In this state, the intradiscal surface potentials could be effectively screened and the asymmetry of charged lipid could be significantly altered (Schnetkamp, 1985).

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