Biochimica et Biophysica Acta 815 (1985) 380–391
Elsevier

BBA 72581

The effect of nonideal lateral mixing on the transmembrane lipid asymmetry

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(Received November 21st, 1984)

Key words: Lipid bilayer; Lipid asymmetry; Nonideal mixing; Gouy-Chapman theory; Bragg-Williams approximation

It is shown that the equilibrium transmembrane lipid asymmetry strongly depends on the degree of nonideality in the lateral mixing of the lipid components. In two-component bilayers the effect of nonideal lateral mixing is maximal for a given component at mole fractions of this component between 0.35 and 0.4. For asymmetry creating factors about 3 *kT* correcting for lateral nonidealities typical for lipids can increase as much as three times the transmembrane asymmetry. The relationship between lateral nonideality and transbilayer asymmetry is analysed in detail in the case of electrostatically induced asymmetry by using the Gouy-Chapman theory of electric double layers and the Bragg-Willliams (regular solutions) approximation of nonideal lateral mixing. Two representative models are studied: (a) a single flat bilayer with a transmembrane electric potential difference applied on it; (b) two parallel membranes at short separation. In case (a), for transmembrane potentials of about 50–100 mV the introduction of nonideality corrections increases up to 40% the transmembrane asymmetry. In case (b), at physiological electrolyte concentrations the lipid asymmetry and, consequently, the effect of lateral nonideality become significant only at unrealistically small separations between the membranes. The surprisingly great influence of the lateral nonideality on the equilibrium transmembrane asymmetry suggests a significant role for this effect in determining the membrane molecular organization. A restricted lateral lipid miscibility might serve as a peculiar, but rather strong 'amplifier' of the transmembrane asymmetry. The qualitatively different asymmetries found in small unilamellar phosphatidylcholine-phosphatidylethanolamine vesicles of different fatty acid composition (Lentz, B.R. and Litman, B.J. (1978) Biochemistry 17, 5537–5543) can be reasonably well explained as an effect of the lateral nonideality. A hypothesis considering the transmembrane distributions of the major phospholipid species in erythrocytes as evolving from their lateral miscibilities is proposed.

Introduction

Although there is ample evidence that the biological membranes are asymmetric [1–4], the nature of the forces maintaining the transbilayer molecular distribution is still unclear. The asymmetry-creating mechanisms can be conventionally divided into two groups: metabolical ones existing only in native membranes and unspecific 'physical' mechanisms acting both in native and model membranes. A number of experimental and theoretical studies on model bilayers have promoted some understanding of the unspecific lipid asymmetries [5–21]. The most extensively developed up to now theoretical approach of this kind makes use of the circumstance that, usually, about 10–20 of the membrane lipids are charged thus creating a significant net (negative) surface charge. For this reason the problem of lipid distribution in a lipid membrane can be treated, to a good approximation, as a problem of charge distribution between

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine,

the two surfaces of the membrane. As the surface charges are part of the electric double layers on both sides of the membranes, their symmetric equilibrium distribution will be affected by any factors inducing a difference between the two electric double layers. The effect of the most important of these factors has already been analysed: external electric potential applied across the membrane [5]; different ionic composition of the bathing solutions [6]; bilayer curvature which is especially important in small vesicles [7–9]; a system of two parallel membranes at a distance of the order of the Debye screening length [10,11].

It is noteworthy that in the cited above theoretical investigations of asymmetric lipid bilayers it is assumed that the lipids distribute between the two monolayers according to the Boltzmann law (usually modified by an additional requirement for a constant density of the two layers). In this way, it is implicitly assumed that the two components of the bilayer mix ideally in its plane. This implicit assumption is a consequence of the circumstance that the Boltzmann relation is written with concentrations, and not with activities. The same assumption about an ideal mixing in the membrane plane is involved also in studies of the lipid asymmetry induced by packing restrictions in curved membranes [21]. However, it is well known from studies on binary lipid mixtures that the ideal mixing is a highly unrealistic assumption [22–25]. All studied up to now phase diagrams show deviations from ideal mixing both in gel and liquid states of the membranes even for combinations of very similar lipids [24–26]. It is a matter of considerable interest with respect to membrane molecular organization to find out whether the mode of mixing of two lipid components in the plane of the membrane can influence their asymmetric transversal distribution. Here we study this problem and obtain the general result that the equilibrium lipid asymmetry strongly depends on the degree of nonideality in the lateral mixing of the lipid components. Besides this general conclusion, we develop in detail the theory of this effect in two representative models of electrostatically induced asymmetry, a single flat bilayer with an external transmembrane potential applied on it and a system of two parallel bilayers at short distance between them. The surprisingly great influence of the lateral nonideality on the transbilayer asymmetry suggests an important role for this effect in determining the membrane molecular organization.

Theoretical description of the relationship between lateral nonideality and transmembrane asymmetry

The physical model employed hereafter is a two-component lipid bilayer. The two lipids A and B (c and n for charged and electrically neutral lipids) are assumed to be approximately equal in size so that in a process of lipid rearrangement they exchange in a 1 : 1 ratio. Due to a requirement of constant membrane density the lipid rearrangement is tightly coupled [5].

The present theoretical considerations are confined to equilibrium lipid distributions both in lateral and transbilayer direction. However, a generalization including also nonequilibrium states is principally possible (see the last section of this paper for a brief discussion). An equilibrium lipid distribution might become asymmetric as a result of any physical interaction making one side of the bilayer energetically preferrable for only one of the lipids. Examples of such asymmetry-creating factors are given in the Introduction and also further in this section. In the general case we shall not specify the nature of these factors and shall define them only by their magnitude in energetical units in the expressions for the lipid chemical potentials.

Quite transparent and easily manageable are the electrostatic asymmetry-creating factors [5–11]. For this reason we formulate at first two models of electrostatically induced asymmetry and then consider the general case.

Formulation of the electrostatic models

The two models of electrostatically induced asymmetry are shown in Fig. 1. The bilayers consist of two lipids, a charged one and an electrically neutral one. With respect to the membrane electric double layers some standard assumptions are made which are commonly used in similar problems [27]. The dielectric permeability of the aqueous phase is held constant and equal to its bulk value up to the interface. The surface charge is treated as uniformly smeared on the membrane surface. Effects due to finite ion size and to ion adsorption to the

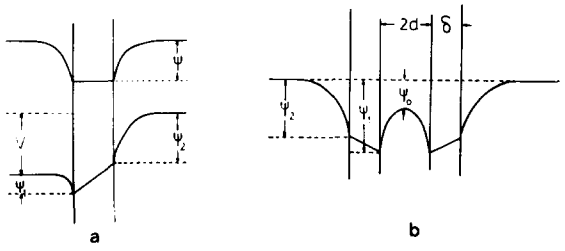

Fig. 1. The electric potential profile in charged bilayers according to the Gouy-Chapman theory: (a) potential difference V applied across a single flat bilayer; (b) two parallel membranes with overlapping electric double layers.

interface are neglected. All calculations are made for 1 : 1 electrolytes.

The electric potential outside the membranes is given by the Poisson-Boltzmann equation

$$d^2\psi/dx^2 = \sinh \psi \tag{1}$$

Here $\psi = e\varphi/kT$ is the reduced potential; $x \equiv x \cdot \mathscr{K}$ is the reduced distance; $\mathscr{K}^{-1} = \sqrt{\epsilon kT/8\pi e^2 c}$; e is the elementary charge; k is the Boltzmann constant; T is the absolute temperature; c is the electrolyte concentration far from the membrane where $\psi = 0$. In all calculations $T = 293.16$ K, $\epsilon = 80.36$ and $\epsilon_m = 2$.

For a single charged surface the solution of Eqn. 1 is (see, for example, Ref. 28):

$$\psi = 4 \tanh^{-1}\left(e^{-x} \tanh \frac{\psi_0}{4}\right) \tag{2}$$

The case of two parallel membranes was earlier studied using the linearized form of Eqn. 1 [10], valid only at low potentials ($\psi \ll kT/e$). Here we use an approximate analytical solution of Eqn. 1 obtained by taking into account the first nonlinear correction to the linear solution [11,29,30]. The solution of the Poisson-Boltzmann equation is expressed as power series in ψ_0:

$$\psi(x) = \sum_{k=1}^{\infty} \psi_0^k \cdot Y_k(x) \tag{3}$$

The substitution of Eqn. 3 into Eqn. 1 results in a system of differential equations for Y_k. Y_1 is the linear solution, $Y_2 = 0$, and Y_3 is the first nonlinear correction. For a flat gap between two membranes we obtain

$$Y_3^{in} = (\cosh 3x - \cosh x + 12x \sinh x)/192 \tag{4}$$

Thus, the approximate solution reads

$$\psi_{in} = \psi_0 \cosh x + \psi_0^3(\cosh 3x - \cosh x + 12x \sinh x)/192 \tag{5}$$

As shown elsewhere [11,29,30] approximations of this kind deviate from the exact solution of the Poisson-Boltzmann equation less than 10% for potentials up to 3 kT/e and less than 20% for potentials up to 4 kT/e. It is well known from studies on artificial lipid bilayers that their surface electric potential is often above 25 mV, but rarely above 70–80 mV [31,32]. Since at room temperature 3 kT/e ~ 75–80 mV, it might be expected that the easily manageable expression given by Eqn. 5 provides a sufficient description of the electric double layer in many practical cases.

A similar to Eqn. 5 expression for a single flat interface reads

$$\psi_{out} = \psi_2 e^{h-x} + \psi_2^3 e^{2(h-x)} \sinh(h-x)/24 \tag{6}$$

Here $h = d + \delta$; d and δ are defined in Fig. 1b. We used Eqn. 6 instead of the exact solution in Eqn. 2 for the case in Fig. 1b in order to maintain the same level of approximation over the whole system.

The electric field inside the membranes is assumed to be constant. The surface charge densities σ are defined in a standard way as the electric induction jumps at the interfaces:

$$\begin{aligned} \frac{\mathscr{K}}{2ec}\sigma_1 &= (d\psi_{in}/dx)_{x=d} - \frac{\epsilon_m}{\epsilon} \cdot \frac{\psi_2 - \psi_1}{\delta} \\ \frac{\mathscr{K}}{2ec}\sigma_2 &= \frac{\epsilon_m}{\epsilon} \cdot \frac{\psi_2 - \psi_1}{\delta} - (d\psi_{out}/dx)_{x=h} \end{aligned} \tag{7}$$

The subscripts in and out denote the electrolyte solutions adjacent to surfaces 1 and 2 of the membrane. The term $(\epsilon_m/\epsilon) \cdot (\psi_2 - \psi_1)/\delta$ accounts for the electrostatic coupling between the two surfaces. Since $\epsilon_m/\epsilon \sim 1/40$, its influence is negligible and it can be safely omitted from the calculations [33]. This point is additionally proved further in this paper by the numerical calculations about the system of two membranes.

Electrostatic equilibrium

Since the ions forming the diffuse layers are always in equilibrium with the reservoir far from the membranes, only the free energy of the surface charges should be taken into consideration. In equilibrium, the electrochemical potentials μ of the charged lipids on both membrane surfaces must be equal. Taking into account the tight coupling of the redistribution of charged and electrically neutral molecules, the equilibrium condition becomes:

$$\mu_1^{\text{charged}} - \mu_1^{\text{neutral}} = \mu_2^{\text{charged}} - \mu_2^{\text{neutral}} \tag{8}$$

The subscripts 1 and 2 denote the two membrane halflayers. As shown by McLaughlin and Harary [5], uncompensated lipid fluxes will exist if Eqn. 8 is not fulfilled.

(i) Ideal lateral mixing. If an ideal lateral mixing is assumed, Eqn. 8 can be written in the form of a Boltzmann distribution:

$$\ln \frac{X_1^c(1-X_2^c)}{X_2^c(1-X_1^c)} = \psi_2 - \psi_1 \tag{9}$$

X^c and $X^n = 1 - X^c$ are the mole fractions of the charged and electrically neutral molecules; $X^c = \sigma \cdot S/e$, where S is the surface area of one lipid molecule.

(ii) Nonideal lateral mixing. In order to introduce deviations from ideal mixing in the membrane plane, the concentrations in Eqn. 9 must be replaced by activities (activity $= c \cdot j$, where j is the activity coefficient). For determination of j we apply the Bragg-Williams approximation [34]. In its frame the chemical potentials obtain the form:

$$\begin{aligned} \mu^{\text{charged}} &= \mu_0^c + \ln X^c + \frac{\rho}{kT}(1-X^c)^2 + \psi \\ \mu^{\text{neutral}} &= \mu_0^n + \ln X^n + \frac{\rho}{kT}(1-X^n)^2 \end{aligned} \tag{10}$$

ρ is the nonideality parameter in the Bragg-Williams approximation:

$$\rho = Z\left[E_{nc} - \tfrac{1}{2}(E_{nn} + E_{cc})\right]$$

Here Z is the number of nearest neighbors; E_{cc}, E_{nn}, and E_{nc} are the interaction energies in the pairs of charged, neutral, and charged and neutral molecules, respectively; μ_0^c and μ_0^n are the standard chemical potentials of the pure components. The positive values of ρ reflect a tendency to separation of the two components in the membrane plane, while $\rho = 0$ corresponds to ideal mixing.

Substitution of Eqn. 10 into Eqn. 8 leads to the shown in Eqn. 11 modification of the equilibrium condition:

$$\ln \frac{X_1^c \cdot (1-X_2^c)}{X_2^c \cdot (1-X_1^c)} = \psi_2 - \psi_1 + \frac{2\rho}{kT}(X_1^c - X_2^c) \tag{11}$$

The Bragg-Williams approximation (theory of regular solutions, mean-field theory) has been frequently used to analyse the mixing in two-component membranes [25,35]. Its shortcomings are well known [34,36] but nevertheless it still remains a very widely used tool in various statistical problems mainly due to its simplicity and also to the lack of some very significant improvements in the more sophisticated approaches. Here we use it as our main purpose is to reveal in a simple manner the effect of nonideal lateral mixing on the transbilayer lipid distribution. It is necessary to assume also that the lipid clustering in the plane of the membrane caused by the nonideal mixing of the two components is not great enough as to invalidate the condition of smeared surface charge. In this connection it should be noted that a phase separation in the frame of the Bragg-Williams approximation occurs at $\rho \geqslant 2\ kT$ while in our calculations the effect of nonideality was determined at $\rho = 1\ kT$ (Fig. 2, 4, 8, 9, 11).

A generalization of the equilibrium condition

Besides the electrostatic factors mentioned in the Introduction there can be given also other examples of important factors making one side of the bilayer energetically favorable for only one of the lipids in the mixture. Among them might be the specific lipid-protein interactions with asymmetrically located proteins [3,42], selective adsorption of ions asymmetrically distributed between the two bathing solutions, packing constraints in curved membranes [21,44]. In order to account for such factors the equilibrium condition in Eqn. 11 can be rewritten in a more general form shown in

Eqn. 12

$$\ln\frac{X_1^A\cdot(1-X_2^A)}{X_2^A\cdot(1-X_1^A)}=\sum F+\frac{2\rho}{kT}(X_1^A-X_2^A) \qquad (12)$$

One of the terms of the sum in the right-hand part of Eqn. 12 might the electrostatic driving force $\psi_2-\psi_1$ acting only on charged lipids. The other terms account for the action of other possible driving forces leading to asymmetrical equilibrium states. However, specification of the shape and magnitude of all these forces is, even if possible, clearly out of the scope of the present paper so in the further considerations about the effect of lateral nonideality we shall treat the sum in Eqn. 12 as a single asymmetry-creating factor, F, defined only by its magnitude in kT units.

Equilibration of the bilayer

The redistribution of the lipid molecules to an asymmetric equilibrium state can be realised via two different mechanisms: (a) exchange by means of lateral diffusion with symmetrical reference areas of the membrane where the asymmetric external factor is not present (or it has another magnitude); (b) transbilayer redistribution with conservation of the total concentration of each component. In both cases the equilibrium state is determined by the same condition (Eqn. 11 or 12) but, since in case (a) there is no requirement for conservation of the total lipid concentrations, the mechanisms (a) and (b) lead to different equilibrium states. However, this difference is only quantitative. With respect to the physical results obtained the specific choice of any of the equilibration mechanisms is of no importance.

As the transbilayer redistribution is often a very slow process [3,37] while especially in fluid bilayers the lateral diffusion is a fast one (see, for example, Refs. 38 and 39), the first mechanism seems to be a more realistic one. At least, it does not require existence of low activation energy pathways between the two sides of the bilayer. However, there is evidence about a rapid transbilayer lipid translocation occuring under certain conditions [3,40,41,43].

Numerical evaluation in the nonideality correction to transbilayer asymmetry

In the numerical calculations we use the values 0.5, 1 and 2 of the nonideality parameter ρ as they cover the range typical for lipid mixtures [25]. The lipid asymmetry corresponding to a fixed value of ρ is denoted by $R_\rho=X_1^A/X_2^A$ (or $R_\rho=\sigma_1/\sigma_2$ for a mixture of a charged and an electrically neutral lipid). R_0 is the lipid asymmetry in case of ideal lateral mixing. The ratio $(R_1-R_0)/R_0$ is used as a quantitative measure of the effect of lateral nonideality on the lipid asymmetry.

General case

At first, we use the general equilibrium condition in Eqn. 12 and, as stated in the previous section, treat the sum ΣF as a single asymmetry-creating factor, F, of unspecified nature. In order to assess the effect of lateral nonideality the lipid asymmetries of $\rho=0$ and $\rho=1$ are calculated (Fig. 2, top). The nonideality corrections to the

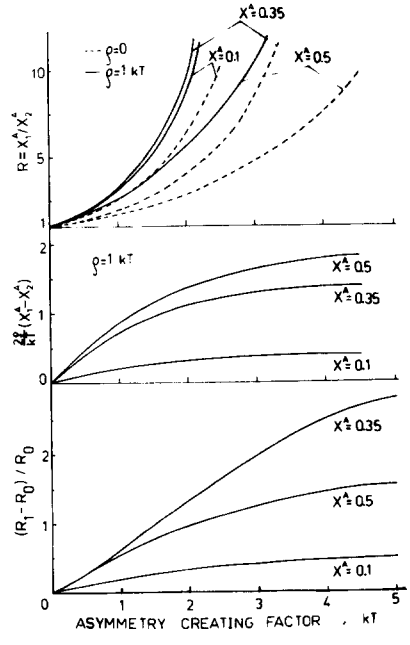

Fig. 2. The lipid asymmetries at $\rho=0$ and 1 kT (top), the nonideality correction to the right-hand side of Eqn. 12 (middle), and the effect of lateral nonideality on the lipid asymmetry (bottom) plotted against the magnitude of the asymmetry-creating factor F.

right-hand side of Eqn. 12 and to the lipid asymmetry are plotted against the magnitude of F in Fig. 2, middle and bottom, respectively. The increase of the transbilayer asymmetry with increase of ρ is shown in Fig. 3. The dependence of the effect of lateral nonideality on the lipid mole fraction is shown in Fig. 4. Fig. 5 can be used to determine the asymmetry increase due to nonideal mixing at different values of R_1. In all calculations shown in Figs. 2–5 the total concentrations of the two lipids were conserved assuming in this way a lipid redistribution by means of transbilayer translocation. Quite similar results can be obtained for a lateral redistribution, too.

The analysis of the general case cannot be carried further without specifiing the nature of F in Eqn. 12 and relating it to some other parameters of the membrane. For this reason the further numerical calculations are concerned with electrostatically induced asymmetries.

Electrostatically induced asymmetry

Instead of Eqn. 12 here we use Eqn. 11 as an equilibrium condition. According to it, the lipid asymmetry in a mixture of a charged and an electrically neutral lipid is created only by the electrostatic driving force $\psi_2 - \psi_1$. By using the Gouy-Chapman theory of electric double layers the quantity $\psi_2 - \psi_1$ can be related to some other, more reliable parameters of the bilayer such as the external potential V in Fig. 1a or the separation between membranes in Fig. 1b. The solutions of the Poisson-Boltzmann equation, the definition of the surface charge density in Eqn. 7, and the

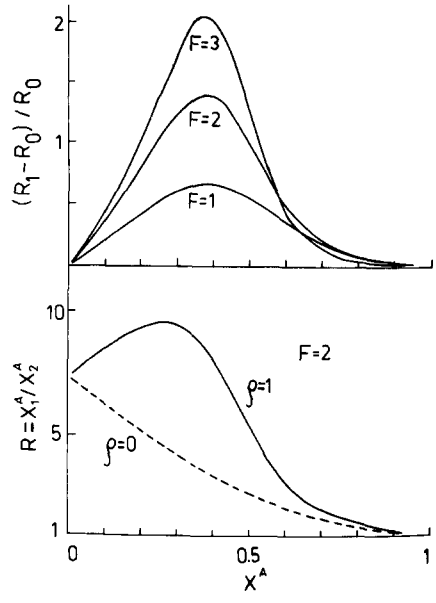

Fig. 4. The effect of lateral nonideality on the lipid asymmetry against the lipid mole fraction at some fixed values of the asymmetry creating factor F. The values of ρ and F are in kT units.

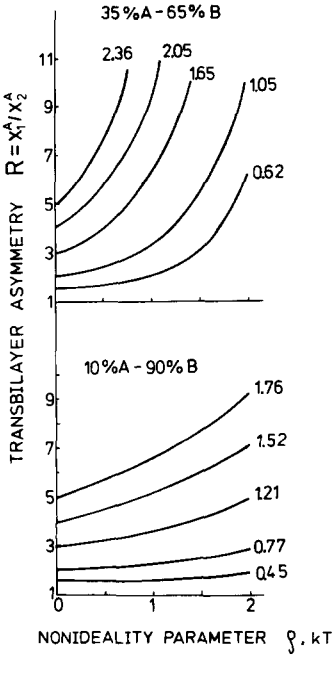

Fig. 3. The increase of the transbilayer asymmetry with increase of the nonideality parameter ρ. The different curves correspond to $R_0 = 1.5, 2, 3, 4, 5$. The values of F, in kT units, as calculated from Eqn. 12 are indicated against each curve.

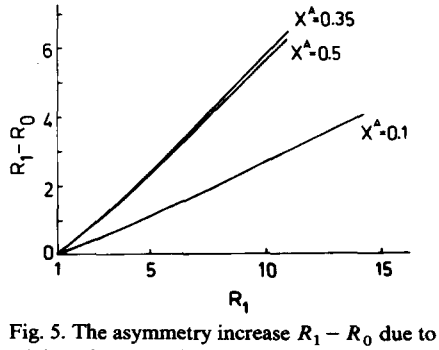

Fig. 5. The asymmetry increase $R_1 - R_0$ due to nonideal lateral mixing plotted against the lipid asymmetry at $\rho = 1$ kT.

condition of surface charge conservation $\sigma_1 + \sigma_2 =$ constant (for a lateral redistribution the latter condition is fulfilled only in the reference state) combined with Eqn. 11 constitute a self-consistent system with respect to σ_1 and σ_2. It can be solved in order to determine the equilibrium surface charge distribution and the effect of lateral nonideality as a function of the external potential V or the separation between the membranes for the cases shown in Figs. 1a and 1b, respectively.

Numerical calculations were made for 1, 10 and 100 mM 1 : 1 electrolyte and surface charge densities corresponding to 10 and 35% charged lipids in the membranes (0.7 nm^2 area per lipid molecule). The exact solution of the Poisson-Boltzmann equation was used for the case in Fig. 1a, the ap-

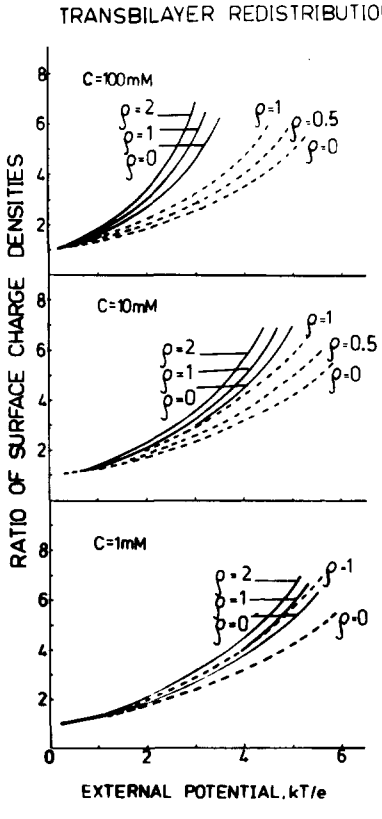

Fig. 6. The surface charge asymmetry σ_1/σ_2 in a single flat bilayer against the external potential V for different degrees of nonideal lateral mixing of the charged and neutral lipid components ($\rho = 0$, 0.5, 1, 2 kT). Only transbilayer lipid redistribution is allowed. The area per lipid molecule is 0.7 nm^2. ------ 10% charged lipids; ——— 35% charged lipids.

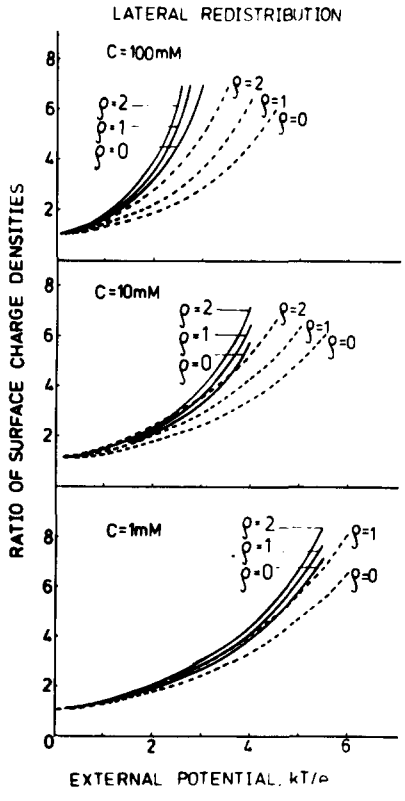

Fig. 7. The same as in Fig. 6 but for the case of a lateral redistribution. The amounts of charged lipids are assigned to the reference state.

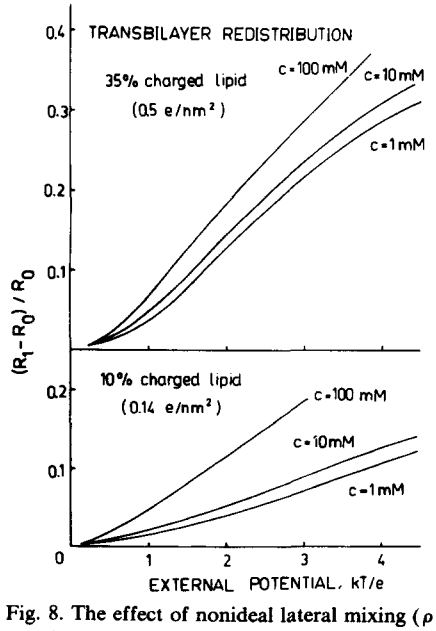

Fig. 8. The effect of nonideal lateral mixing ($\rho = 1$ kT) on the transbilayer surface charge asymmetry in a single flat bilayer in case of transbilayer lipid redistribution.

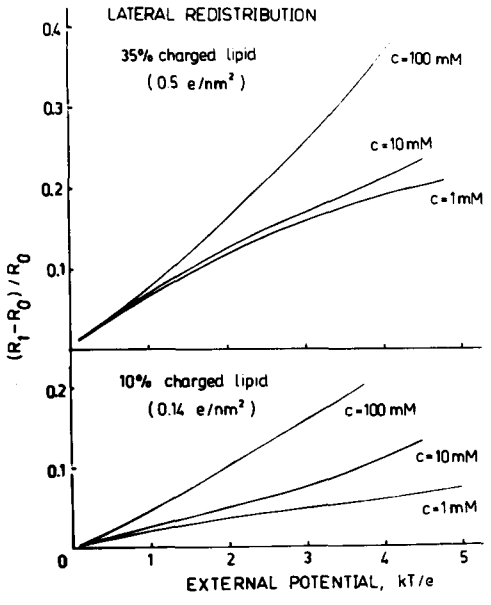

Fig. 9. The effect of nonideal lateral mixing ($\rho = 1\ kT$) on the transbilayer surface charge asymmetry in a single flat bilayer in case of lateral lipid redistribution.

proximate solutions given by Eqns. 5 and 6 were used for the case in Fig. 1b.

Figs. 6 and 7 show the surface charge asymmetry as a function of the external electric potential V for different values of ρ. The effect of lateral nonideality is shown in Figs. 8 and 9. In the calculations about the lateral redistribution in a single bilayer the electrostatic coupling between the two surfaces was neglected by omitting the term $\epsilon_m(\psi_2 - \psi_1)/\epsilon \cdot \delta$ in Eqn. 7.

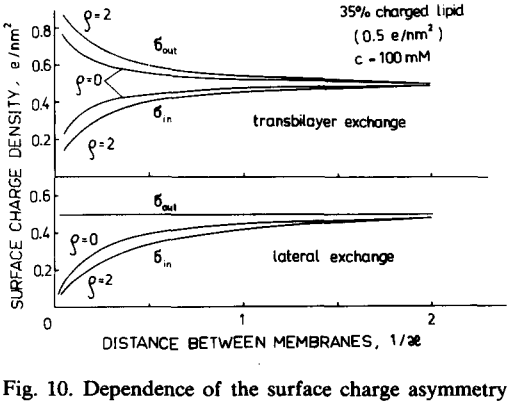

Fig. 10. Dependence of the surface charge asymmetry on the distance between two parallel membranes.

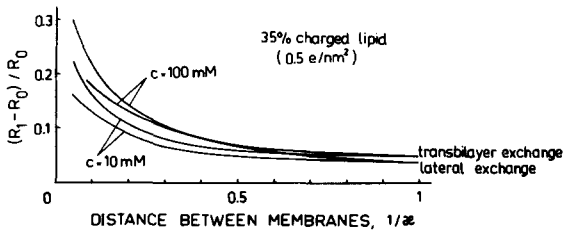

Fig. 11. The effect of nonideal lateral mixing ($\rho = 1\ kT$) on the transbilayer surface charge asymmetry in two parallel membranes.

By virtue of symmetry, in the case of two parallel membranes it is sufficient to consider only one half of the system which is qualitatively similar to the previous case. Fig. 10 shows the dependence of the surface charge densities on the distance between the membranes. The effect of nonideal lateral mixing is shown in Fig. 11. In all calculations shown in Figs. 10 and 11 the surface potentials do not exceed 75 mV so that the approximate expressions in Eqns. 5 and 6 remain valid. The electrostatic coupling was not neglected in this case in order to demonstrate its negligible effect on the equilibrium distribution.

Physical conclusions and biological significance

(1) It is evident from Eqns. 9, 11 and 12 that the nonideal mixing of the two lipids in the membrane plane affects their equilibrium transmembrane distribution. The physical explanation of this effect is quite clear. Positive values of the nonideality parameter ρ in Eqs. 11 and 12 indicate a tendency to clustering of the two components which is a result of a preference of the molecules for neighbors of the same kind ($|E_{AA}| + E_{BB}| > 2|E_{AB}|$ for $\rho > 0$). When a partial separation of the two lipid species across the membrane is created by some external asymmetric factor, the preference of the lipid molecules for neighbors of the same kind would result in an increase of the transmembrane separation. In the same way, negative values of ρ, which reflect a tendency to a 'chessboard' lateral arrangement of the two lipids, would result in a decrease of the transbilayer asymmetry (calculations of this kind are not shown here). It is

important to note that these conclusions do not depend on the specific assumption about the activity coefficient j. Obviously, the same effect can be obtained also in the frame of other statistical mechanical approximations, more precise than the Bragg-Williams model.

(2) The numerical calculations for a single bilayer show a very pronounced effect of the lateral mixing nonidealities on the transbilayer asymmetry (Figs. 2–9). An estimate based on Fig. 2, bottom, show that at $X^A = 0.35$ the values of R_1 are one and a half to three times greater than the values of R_0 for asymmetry-creating factors in the range of 1–3 kT. Similarly great increase is typical also for electrostatically induced asymmetries. For transmembrane potentials about 50–100 mV (2–4 kT/e) correcting for a moderate nonideal mixing increases up to 40% the surface charge asymmetry (Figs. 8 and 9). In addition, Fig. 5 explicitly shows that the portion of R_1 due to nonideal mixing is quite significant at any values of R_1. The surprisingly great nonideality effects become comprehensible by noticing that the nonideality correction in the right-hand side of Eqns. 11 and 12 is of the order of magnitude of the external driving force F (Fig. 2, middle). As a whole, the model calculations reveal a strong dependence of the equilibrium transmembrane asymmetry on the lateral lipid miscibility. This result provides theoretical support for the idea of Wu and McConnell that the lipid immiscibility in a binary mixture might result in a transmembrane separation of the two lipids [23].

(3) To our knowledge, all lipid pairs studied up to now are characterized by different but always positive values of ρ reflecting a lateral clustering and, sometimes, a phase separation of the like molecules (a single exception of this rule is the synthetic racemic modification of dipalmipoylphosphatidylethanolamine which can form a racemic compound at low hydration degrees [45]). Due to this the nonideality correction must always result in an amplification of the transmembrane asymmetry. Obviously, the magnitude of this effect will increase with increase of ρ (Fig. 3). It might be expected that equal in magnitude asymmetric forces would lead to different asymmetries when applied to lipid pairs of different miscibility. A practical conclusion following from here is that the lipid miscibilities must always be taken into account when considering the magnitude of a transbilayer lipid asymmetry. Although the restricted lateral miscibility is not by itself an asymmetry-creating factor, it might serve as a peculiar, but rather strong 'amplifier' of the action of some external asymmetric factors of moderate magnitude.

(4) Quite remarkable is the dependence of the nonideality correction on the lipid mole fraction (Fig. 4). It is clear that the nonideality effect must have a maximum as it is always positive but, obviously, tends to zero at $X^A = 0$ and 1. As shown numerically, for a given component this maximum is located at mole fractions between 0.35 and 0.4 (Fig. 4, top). The existence of a maximal nonideality effect results in a qualitatively different in comparison to R_0 dependence of the nonideal asymmetry on the lipid mole fraction. In case of ideal mixing the asymmetry monotonically decreases with increase of the mole fraction, while in case of a restricted lateral miscibility a maximum appears on the curve for R at mole fractions about 0.3 (Fig. 4). The existence of an optimal in this respect lipid molar ratio suggests certain possibilities for regulating the transmembrane asymmetry by changing the lipid concentrations within narrow limits. The qualitative difference predicted in the behavior of R_1 and R_0 provides a possibility for its verification by comparing it with abundant data about the lipid asymmetries at different lipid molar ratios in mixed small unilamellar vesicles [12–20]. However, a quantitative comparison cannot be made directly. As a preliminary step, it is necessary to modify the theoretical description of nonideality effect as to account for the effects of membrane curvature which are significant in small unilamellar vesicles. Due to its complexity, a modification of this kind cannot be made in a simple, unambiguous way and it certainly requires a separate investigation.

(5) In spite of the complication mentioned above, a qualitative comparison of the theoretical curves in Fig. 4 with experimental data can still be made and it provides interesting evidence in favour of a significant nonideality correction to the lipid asymmetry in PC-PE small unilamellar vesicles. As found by Lentz and Litman [16], small unilamellar vesicles prepared from dimiristoyl PC and PE are

characterized by a distinct maximum in the outer-to-inner ratio of PE at a mole fraction of this lipid about 0.25. The dependence of the PC asymmetry on its mole fraction closely resembles the curve for R_1 in Fig. 4. On the other side, the PE asymmetry in egg PC-egg PE small unilamellar vesicles monotonically decreases with increase of the PE mole fraction [13] in the same way as the dashed curve in Fig. 4. It is clear that egg PC and PE are better miscible than the dimiristoyl species due to their fatty acid composition which is inhomogeneous both in chain length and degree of unsaturation. In this way, the puzzling discrepancy between the two kinds of PC-PE small unilamellar vesicles can be reasonably well explained by taking into account the effect of lateral nonideality. However, it must be kept in mind that this explanation is a tentative one and in order to be positive about its correctness a number of experimental tests with small unilamellar vesicles of different composition must be carried out.

(6) The numerical results about the surface charge distributions controlled electrostatically are plotted in Figs. 6–11. Fig. 10 shows that the deviation of the charge distribution from the symmetrical state becomes significant when the distance between the membranes reduces to less than one half of the Debye length. For $c = 100$ mM such separations are unrealistically small since $1/\mathcal{H} = 0.96$ nm. It is clear that the effect of charge redistribution and, subsequently, the effect of lateral nonideality shown in Fig. 11 are not important in the range of the physiological electrolyte concentrations even at close approach of two membranes. Quite different is the situation in a single bilayer. For external potentials about 3–4 kT/e the surface charge ratio becomes as high as 4–6 (Figs. 6 and 7). The great difference in the magnitude of the effects is due to the simple fact that in the frame of the analogy between the two models the potential ψ_0 corresponds to the external potential V, and V is much greater that ψ_0.

When a lateral lipid redistribution is assumed, the outer surface charge in a system of two parallel membranes is practically insensitive to changes in the separation between the membranes (Fig. 10). This once more proves that the electrostatic coupling between the two membrane surfaces is negligibly small. The possibility to neglect the outer monolayers in electrostatic considerations suggests a close similarity with the electrostatic properties of the free foam films. The latter are composed of two monolayers separated by an aqueous gap of the order of several nanometers. Since the free foam films represent a convenient and very thoroughly studied model system [28,46], the electrostatic analogy between them and a system of two parallel membranes might be hepful in some respects.

(7) With a view to the properties of native membranes it makes sense to extend the present theoretical treatment in three principal directions as to include: (i) regions of phase separation; (ii) nonequilibrium lipid distributions; (iii) multicomponent lipid membranes. Some qualitative conclusions in these respects can be drawn on the basis of the results of the present work.

The main consequence of a lateral phase separation would be to increase the 'amplifiing effect' of the restricted lateral miscibility. It seems likely that in a phase separation region this effect might display itself as a abrupt increase of the transmembrane asymmetry triggered by a small change of the external asymmetric factor. However, the exact conditions of a transmembrane phase separation cannot be found without a sufficiently precise theoretical model. In this connection, it is worth noting that the Bragg-Williams approximation provides a simple and convenient formalism for describing phase separations in binary alloys [25,34].

(8) Although the present study deals with thermodynamic equilibrium only, it might be argued for a compatibility of the effect of lateral nonideality with at least some kinds of nonequilibrium states. A trivial inclusion of nonequilibrium states can be made by noticing that the equilibrium state defined by Eqn. 11 or 12 can be regarded also as a stationary state maintained by an asymmetric flow of one of lipids in and out of the membrane. Another type of nonequilibrium which might take place in cellular membranes is a slow dissipation of an initially highly asymmetric state. The restricted lateral miscibilities might be of importance in this case as they might reduce the rate of dissipation and maintain the nonequilibrium state for a longer period of time.

At the present time, nothing can be said about

how far are native membranes from thermodynamic equilibrium with respect to their transbilayer lipid distribution. Two extreme points of view oppose a complete equilibration to a very slow, practically absent transbilayer translocation hindering the dissipation of any nonequilibrium distribution induced metabolically or otherwise. In order to place the lipid distributions in the various membranes correctly on a scale between these two extreme cases much more data about lipid turnover, time constants of flip-flop, etc., are needed than available now. It is possible, however, that only part of the membrane lipids cannot equilibrate across the membrane, while the rest of them are in an equilibrium or stationary state. In that case the present analysis can be applied without modification to investigate the effects of lateral mixing on the transmembrane asymmetry.

(9) A very important role might play the lateral lipid interactions in determining the transversal lipid distributions in multicomponent bilayers. In a two-component bilayer, in the present of an asymmetric force acting only on one of the lipids, A, the distribution of the other lipid, B, is also completely determined due to the requirement of constant density (Eqn. 8). However, if a third lipid, C, is added to this mixture, then an additional relation will be needed in order to determine the transbilayer localization of both B and C. A simple and physically reasonable relation of this kind is provided by the lateral lipid-lipid interactions. For example, if lipid C is better miscible with A than with B, then its concentration in the membrane halflayer where lipid A is predominantly localised will be greater. The exact transbilayer distributions of the three lipids will be given by the minimum of the free energy of their lateral mixing. Exactly in the same way, in a multicomponent membrane in which only one of the lipids is influenced by some asymmetry-inducing force, the transbilayer distribution of the rest of the lipids will adjust automatically according to the preferences in their lateral contacts. This scheme of incorporation of the lateral lipid-lipid interactions in a concept about membrane asymmetry removes the necessity to invent an asymmetry-creating factor for any one of the numerous lipid species coexisting in a biological membrane. According to it, it is sufficient that such factors exist for only one (or very few) of the lipid components. In order to illustrate its application let us consider the phospholipid distribution in the erythrocyte membrane which is one of the most thoroughly studied native membranes. It is now firmly established that the major phospholipid components of the erythrocyte membrane (PC, PE, PS and sphingomyelin), which account for about 95 percent of the total phospholipid content, are distributed asymmetrically in such a way that the choline-containing lipids (PC and sphingomyelin) are located predominantly in the outer leaflet of the membrane while the lipids containing aminogroups (PE and PS) are in the inner halflayer [1–4]. Numerous studies on binary lipid mixtures have shown that the differences in the polar moieties of the phospholipids are a most important factor decreasing their miscibility (see, for example, Refs. 17, 22, 23, 25, 26). For example, mixtures of synthetic, fully saturated PC and PE or PS display a greater deviation from ideal mixing than PE-PS mixtures of the same fatty acid composition [25,47,48]. Although the mutual miscibilities of the erythrocyte phospholipids have not been specially investigated, it might be deduced from a number of studies on mixtures of similar lipids that the choline-containing lipids PC and sphingomyelin, on one side, and the aminolipids PE and PS, on the other side, must be better miscible between themselves than with a lipid of the other kind. According to the scheme proposed above, if one of these lipids is set to be distributed asymmetrically (for example, by means of a lipid-protein interaction) in a mixed bilayer comprising all of them in approximately equal amounts, then the other three lipids must arrange in a way minimizing the unfavorable lateral interactions. With account of their miscibilities an equilibrium arrangement will require a predominant localization of PC-sphingomyelin and PE-PS pairs in the opposite membrane monolayers. Of course, these considerations cannot explain the inside-outside orientation of the lipid asymmetry in erythrocytes but they strongly suggest that the transmembrane distributions of the various lipid species in them (including also cholesterol and glycolipids) are related to each other through the lateral lipid-lipid interactions in the membrane plane.

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