Biophysical Chemistry 14 (1981) 11–23
Elsevier/North-Holland Biomedical Press

STABILIZATION EFFECT OF PROTONS AND DIVALENT CATIONS ON MEMBRANE STRUCTURES OF LIPIDS

Kiyoshi TOKO and Kaoru YAMAFUJI

Department of Electronics, Kyushu University, Fukuoka 812, Japan

Received 17 November 1980
Revised manuscript received 16 February 1981

Ordered–fluid phase transitions of methylphosphatidic and phosphatidic acid bilayers are discussed theoretically to explain comprehensively various observed data in the presence of Ca^{2+} or protons. It is shown that the observed data can be explained reasonably by taking account of the interaction among neighboring head-groups with the aid of Ca^{2+} or protons. As a result, a quantitative explanation is given for the dependence of the phase-transition temperature on pH as well as Ca^{2+} concentration with the coexistence of monovalent cations. The dependence of the cooperativity of the transition on pH and monovalent cation concentration is also well explained. It is also pointed out that, in the ordered–fluid phase transitions, a hysteresis, metastable state and phase separation can be expected to appear under some conditions.

1. Introduction

Recently Eibl et al. observed a transition between fluid and ordered phases of lipid membranes for MPA (methylphosphatidic acid) and PA (phosphatidic acid) and found anomalous increases of both the transition temperature T_t and the cooperativity of the phase transition around pH 3 [1–3]. The anomalous increase of T_t is attributed to a change in the hydrogen-bonding energy between a protonated lipid and a single-charged lipid in the ordered–fluid phase transition [1–3]. The theory based on this mechanism [3] provides a good explanation of the observed increase of T_t at values of the degree of dissociation α near 0.5. It is, however, desirable to account for the observed dependence of T_t on pH for any value of α. Furthermore, a quantitative explanation has not been provided for the observed tendency that the cooperativity of the phase transition in PA decreases with increasing pH or the concentration of monovalent cations.

These works by Eibl et al. seem to show us a useful suggestion on the behavior of T_t in some lipid bilayers in the presence of divalent cations such as Ca^{2+}. So far, the observed increase of T_t due to the addition of divalent cations has been attributed mainly to two kinds of effects: an enhancement of the screening of repulsion among head groups and an adsorption of divalent cations on membrane surfaces resulting in a reduction of a surface charge [4–15]. These kinds of effects cannot cause resultant values of T_t to exceed T_t^0, which is defined as the value of T_t when the lipids have no net charge. In the presence of Ca^{2+}, however, the observed maximum value of T_t is 48°C for MPA [4,5,7], while the T_t^0 of MPA is 42°C [3]. As a result, an explanation of the above data due to electrostatic effects has been carried out by adopting trial values of 48°C for T_t^0 and 12.3 Å^2 for the increase of the molecular surface area at the transition [7], whereas the observed values are 42°C and 6 Å^2 [3,7]. It seems, therefore, to be necessary for a quantitative explanation of the behavior of T_t in the presence of Ca^{2+} [4,5,7] to assume some kind of strong binding between neighboring head groups with the aid of Ca^{2+}, as proposed by Eibl et al. [1–3] for the case of the presence of protons.

In addition, a phase separation was observed for some lipids either in the presence of Ca^{2+} or between pH 2 and 3 [1,2,4,5,7]. If an increase of

0301-4622/81/0000-0000/$02.50 © 1981 Elsevier/North-Holland Biomedical Press

electrostatic energy due to a charge inhomogeneity is only taken into account, however, a theoretical investigation leads to the definite conclusion of an impossibility of the phase separation [4,7]. This also suggests the possibility that there is some kind of strong binding of Ca^{2+} to head groups.

The purpose of this paper is to attempt to explain comprehensively the accumulated observed data for MPA and PA under the basic assumption that Ca^{2+} and protons form a bridging between neighboring head groups. Only the change in such a bridging energy contributes to the increase of T_t, as pointed out by Eibl and Woolley [3]. The remaining constant part of the bridging energy, on the other hand, can be incorporated into the usual adsorption energy, so that it does not contribute to T_t but to the degree of adsorption of ions. Furthermore, it may be possible to consider this constant part to be equivalent to the adsorption energy in itself in some cases, e.g., the interaction between divalent cations and lipids with a negative charge of unity. In this paper, therefore, we denote the changeable part of the bridging energy in the ordered–fluid phase transitions as "chelation energy" for convenience, and regard the constant one as a part or the whole of the adsorption energy.

With the aid of the same set of values of parameters, we get the following results. First, the dependence of T_t on pH and monovalent cation concentration as well as on Ca^{2+} concentration in the presence of monovalent cations is explained quantitatively. Secondly, the dependence of the cooperativity of the transition on pH and monovalent cation concentration is explained well. Finally, it is shown that the appearance of hysteresis, metastable state and phase separation can be interpreted in a fairly reasonable manner by introduction of the above chelation energy.

2. Free energy

To describe an ordered–fluid phase transition of lipid bilayers, let us assume that Ca^{2+}-chelated lipids or proton-chelated lipids are mixing randomly in free lipids, except for the case where a phase separation occurs. Then the phase transition can be described by the order parameter ξ defined as the fraction of hydrocarbon chains in the disordered or fluid state. When protons or divalent cations are adsorbed on polar head-groups of the lipids, the degrees of adsorption are defined as

$$\theta_i = n_{b_i}/[\mathrm{L}], \qquad \text{for } i = \mathrm{H} \text{ or } 2, \tag{1}$$

where n_{b_i} and [L] are the numbers of the bound ith cations and lipids, respectively. The equations for describing ξ and the θ_i can be derived by minimizing the free energy of the system with respect to ξ and the θ_i, together with the equations deduced from the Poisson–Boltzmann equation at a given temperature T.

In this paper, the Gibbs free energy of the system is assumed to be a function of ξ, θ_i and the temperature T, given by

$$G(\xi,\theta_i;T) = G_0(\xi;T) + G_{\mathrm{el}}(\xi,\theta_i;T) + E_{\mathrm{ch}}(\xi,\theta_i;T). \tag{2}$$

The first two terms on the right-hand side of eq. (2) correspond to the usual free energy in the absence of chelation, which have been used by Träuble et al. [4–6,16] and Payens [17]. The non-electrostatic part of the free energy is denoted by G_0, and G_{el} represents the electrochemical free energy which is given as the sum of the electrostatic and dissociation energies for a simple charging process [16,17]. The last term, E_{ch}, designates the chelation energy which is a changeable part of the bridging energy in the ordered–fluid phase transition.

Detailed expressions of these terms as functions of ξ and the θ_i will be discussed below.

2.1. Non-electrostatic free energy

Various theories have been proposed to describe the phase transitions of non-charged lipids (cf., e.g. refs. [18–20]). Expressions for the free energy used in these theories, however, seem to be unnecessarily complicated for describing the effects of both the electrochemical and chelation energies in terms of ξ and θ_i. In this paper, therefore, a mean-field theory [21,22] is applied for describing the non-electrostatic free energy.

Then the non-electrostatic Gibbs free energy per molecule may be given by

$$\begin{aligned} G_0 = {} & -T\Delta S \times -\left[\epsilon_{oo}(1-\xi)^2 + \epsilon_{ff}\xi^2 \right. \\ & \left. + 2\epsilon_{of}\xi(1-\xi)\right] \\ & + k_B T[\xi \ln \xi + (1-\xi)\ln(1-\xi)] + PAd, \end{aligned} \tag{3}$$

with k_B denoting the Boltzmann constant. In the above expression, the first term represents the thermal motion of hydrocarbon chains, where ΔS is the change in entropy in the ordered–fluid phase transition. The terms in the second part with the coefficients ϵ_{ij} represent the energies due to the van der Waals interaction among lipids, where i or j specifies one of the ordered and fluid states of lipid molecules. The third part is the entropy stemming from a random mixing of ordered and fluid lipids. The last term is the difference between the Helmholtz and Gibbs free energy, where P and d are pressure (1 atm) and the thickness of membrane, respectively, and A is the molecular surface area given by

$$A = A_o + \xi \Delta A, \tag{4}$$

with A_o representing the surface area for the ordered state and ΔA the increment due to the change from the ordered to the fluid state.

Eq. (3) can be reduced to a simpler form with the neglect of a trivial constant term as

$$\begin{aligned} G_0 = {} & (T_t^0 - T)\Delta S\xi - \tfrac{1}{2}\epsilon(\xi - \tfrac{1}{2})^2 \\ & + k_B T[\xi \ln \xi + (1-\xi)\ln(1-\xi)], \end{aligned} \tag{5}$$

with the definitions of T_t^0 and ϵ given by

$$T_t^0 \equiv (\Delta S)^{-1}(\epsilon_{oo} - \epsilon_{ff}) \tag{6}$$

and

$$\epsilon \equiv 2(\epsilon_{oo} + \epsilon_{ff} - 2\epsilon_{of}). \tag{7}$$

The contribution of the term PAd to T_t^0 is omitted in eq. (6) due to the negligibly small value of $Pd\Delta A$.

The ordered–fluid phase transition for non-charged lipids can be described by minimizing G_0 with respect to ξ, and the result gives T_t^0 as the transition temperature. Particularly for values of ϵ larger than 4 in units of $k_B T$, the phase transition is known to be of the first-order type [21]. Since sharp transition curves are obtained for many lipids in the absence of monovalent and divalent cations [3–8,12–16,20], the parameter ϵ is expected to be a positive quantity of about $4k_B T$.

2.2. *Electrochemical free energy*

(a) Proton-chelated lipids.

When Ca^{2+} is not contained in the sample, the electrochemical free energy per molecule for a charging process is given by [6,16,17]

$$\begin{aligned} G_{cl}^{H} = {} & k_B T \int_1^{\theta_H} \ln\left(\frac{K_d}{[H^+]} \cdot \frac{\theta_H}{1-\theta_H}\right) d\theta_H \\ & + A \int_0^{-(e/A)(1-\theta_H)} \psi(\sigma)\, d\sigma, \end{aligned} \tag{8a}$$

where K_d is the dissociation constant determined by the standard chemical potential containing the constant part of the hydrogen-bonding energy, $[H^+]$ the proton concentration far from the surfaces of lipid bilayers, e the positive elementary charge, σ the surface charge density, and ψ the surface potential. Integration of the Poisson–Boltzmann equation gives the following relation between ψ and σ [15,16,23]

$$\sigma = (2\epsilon_w k_B T/\pi)^{1/2}\sqrt{n_1}\, \sinh(\tfrac{1}{2}\phi); \tag{9a}$$

here ϵ_w is the dielectric permeability of water, n_1 the monovalent cation concentration far from the surfaces, and ϕ is defined by

$$\phi \equiv e\psi/k_B T. \tag{10}$$

(b) Ca^{2+}-chelated lipids.

In the presence of both monovalent and divalent cations, we are interested only in the circumstances near pH 8. In this situation, protons are fully dissociated and Ca^{2+} can bind simultaneously to two lipids with one negative charge [4,5,7,24]. Then the electrochemical free energy per molecule is given by

$$\begin{aligned} G_{cl}^{Ca} = {} & k_B T \int_{1/2}^{\theta_2} \ln\left(\frac{1}{K_2 n_2} \cdot \frac{2\theta}{1-2\theta_2}\right) d\theta_2 \\ & + A \int_0^{-(e/A)(1-2\theta_2)} \psi(\sigma)\, d\sigma, \end{aligned} \tag{8b}$$

where K_2 is the adsorption constant of Ca^{2+}, n_2 the Ca^{2+} concentration far from the surfaces and A is given by eq. (4). The relation between ψ and σ can be determined in the same way as in case (a) and is given by [23,25]

$$\sigma = -(\epsilon_w k_B T/2\pi)^{1/2} e^{\phi/2}(e^{-\phi}-1) \times \left[n_1 + n_2(e^{-\phi}+2)\right]^{1/2}. \tag{9b}$$

2.3. Chelation energy

(a) Chelation with the aid of protons.

The energy per molecule for the chelation process through protons is assumed to be

$$E^{H}_{chc} = -4\epsilon_H(1-\xi)^2\theta_H(1-\theta_H), \tag{11a}$$

where ϵ_H is the change in the hydrogen-bonding energy at $\theta_H = 1/2$ from the ordered to the fluid phase. In the above expression, the simple dependence of E^{H}_{ch} on θ_H is introduced according to the suggestion by Eibl and Woolley [3], since the structure of lipid membranes is known to be the most stable if half-protonated [1–3]. The simplest form is also assumed for the ξ-dependence according to the fact that the hydrogen bond is weakened with the increase of the spacing of neighboring head groups.

(b) Chelation with the aid of Ca^{2+}.

The chelation energy for this case is assumed to be given by

$$E^{Ca}_{ch} = -4\epsilon_2(1-\xi)^2\theta_2^2, \tag{11b}$$

where the parameter ϵ_2 expresses a change in the bridging energy at $\theta_2 = 1/2$ in the ordered–fluid phase transition. The above expression is derived from the idea that the chelation energy reaches the maximum value when the lipids are ordered thoroughly and half-bound by Ca^{2+}. The dependence on θ_2 reflects the cooperative nature of chelation [7].

3. Transition temperature and cooperativity

The equilibrium values of ξ and θ_i can be obtained by minimizing eq. (2) with eqs. (3)–(11). Let us, however, calculate at first a differential form of G_{el} with respect to ξ, since it is not easy to see immediately due to a coupling with the Poisson–Boltzmann equation. Differentiating G_{el} with respect to ξ leads to the following form:

$$-\partial G_{el}/\partial\xi = \Pi_{el}\,\Delta A. \tag{12}$$

In the above expression, the electric surface pressure Π_{el} [16,17] is given by

$$\Pi_{el} \equiv -\frac{\partial G_{el}}{\partial A} = \frac{k_B T}{e}\int_0^{\phi}\sigma(\phi)\,d\phi, \tag{13}$$

through the relation between A and ξ in eq. (4). If eq. (9a) or (9b) for the surface charge density σ is substituted into eq. (13), we get [23,25]

$$\Pi^{H}_{el} = 4k_B T q^{-1}\sqrt{n_1}\left[\cosh(\tfrac{1}{2}\phi) - 1\right], \tag{14a}$$

or

$$\Pi^{Ca}_{el} = k_B T q^{-1}\sqrt{n_2}\left[(e^{-\phi}+2)\eta(r,\phi) - 3\eta(r,0) + \frac{1}{2}r\ln\left(\frac{\eta(r,\phi)+1}{\eta(r,\phi)-1}\right) - \frac{1}{2}r\ln\left(\frac{\eta(r,0)+1}{\eta(r,0)-1}\right)\right], \tag{14b}$$

with the abbreviations

$$r = n_1/n_2, \tag{15}$$

$$\eta(r,\phi) \equiv \left[1 + (2+r)\,e^{\phi}\right]^{1/2},$$

$$q \equiv \left(\epsilon_w k_B T/2\pi e^2\right)^{-1/2}, \tag{16}$$

Eq. (14a) can also be derived by putting $r \to \infty$ in eq. (14b). In the above equations, ϕ is determined from eq. (19) given below describing the equilibrium.

Then the equilibrium values of ξ and the θ_i can be obtained by minimizing G given by eq. (2) with respect to them. The results for case (a) are given by

$$\xi^{-1} = 1 + \exp\left\{\left[(T_t^0 - T)\,\Delta S - \epsilon(\xi - \tfrac{1}{2}) - 8\epsilon_H(\xi-1)\theta_H(1-\theta_H) - \Pi^{H}_{el}\,\Delta A\right]/k_B T\right\}, \tag{17a}$$

$$\alpha \equiv 1 - \theta_H = \left\{1 + ([H^+]/K_d) \times \exp\left\{\left[-e\psi + 4\epsilon_H(1-\xi)^2(1-2\theta_H)\right]/k_B T\right\}\right\}^{-1}, \tag{18a}$$

where α ($\leqslant 1$) is the degree of dissociation. Similarly the results for case (b) are given by

$$\xi^{-1} = 1 + \exp\Big\{\Big[(T_t^0 - T)\,\Delta S - \epsilon(\xi - \tfrac{1}{2}) - 8\epsilon_2(\xi - 1)\theta_2^2 - \Pi_{\mathrm{cl}}^{\mathrm{Ca}}\,\Delta A\Big]/k_{\mathrm{B}}T\Big\}, \tag{17b}$$

$$\alpha \equiv 1 - 2\theta_2 = \Big\{1 + K_2 n_2 \times \exp\Big\{\Big[-2e\psi + 8\epsilon_2(1-\xi)^2\theta_2\Big]/k_{\mathrm{B}}T\Big\}\Big\}^{-1}. \tag{18b}$$

In the above expressions. $\Pi_{\mathrm{cl}}^{\mathrm{H}}$ and $\Pi_{\mathrm{cl}}^{\mathrm{Ca}}$ are given by eqs. (14a) and (14b), respectively. Expressions (18a) and (18b) are reduced to familiar forms [7,16] when ϵ_{H} and ϵ_2 are put to zero. Furthermore, the degree of dissociation α is connected with ϕ by the relation:

$$\alpha \equiv -(A/e)\sigma(\phi), \tag{19}$$

with σ given by eqs. (9a) and (9b). It is, therefore, possible to calculate ξ, θ_i and ϕ at each temperature for each case from eqs. (17), (18) and (19) with σ given by eq. (9).

To obtain the transition temperature T_t As a function of pH or n_2, we have only to put $\xi = 1/2$ in eqs. (17a) and (17b), and the results are given by

$$T_t = T_t^0 - (\Delta S)^{-1}\big[\hat{\Pi}_{\mathrm{cl}}^{\mathrm{H}}\,\Delta A - 4\epsilon_{\mathrm{H}}\hat{\theta}_{\mathrm{H}}(1 - \hat{\theta}_{\mathrm{H}})\big], \quad \text{for case (a)}, \tag{20a}$$

$$T_t = T_t^0 - (\Delta S)^{-1}\big[\hat{\Pi}_{\mathrm{cl}}^{\mathrm{Ca}}\,\Delta A - 4\epsilon_2\hat{\theta}_2^2\big], \quad \text{for case (b)}, \tag{20b}$$

where the hat specifies the quantity defined at $\xi = 1/2$. In the limits of $\epsilon_{\mathrm{H}} = 0$ and $\epsilon_2 = 0$, the above expressions coincide and equal that used frequently so far [1–7,16]. In these limits, however, T_t decreases with the increase of the ionization of head groups, contrary to the observed tendencies.

In figs. 1, 2, 3 and 4, the calculated results are compared with the experimental data for MPA and 1, 2-DMPA [3,7,16]. In fig. 1, the value of n_1 is specified only as lower than 1 mM [3]. We, therefore, regard these data as the ones performed for $n_1 = 4 \times 10^{-4}$ M, since they shift slightly more to lower pH than the data for $n_1 = 2 \times 10^{-4}$ M in

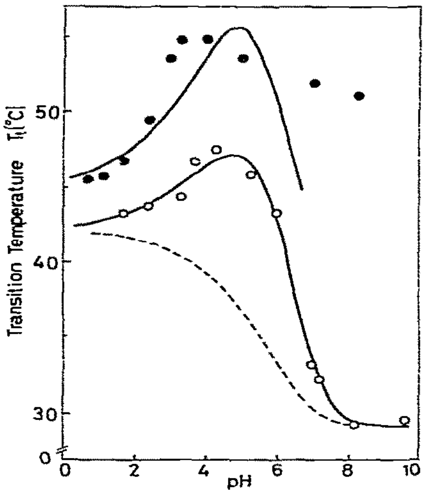

Fig. 1. The dependence of T_t on pH. Experimental data [3] are marked by ○ for MPA and ● for 1,2-DMPA. Theoretical curves are plotted by solid lines with $\Delta S = 17.5$ cal/mole deg [3], $\Delta A = 8$ Å^2, $A_0 = 40$ Å^2 [3,7], $T_t^0 = 42$°C [3], $K_d = 10^{-1}$ M and $\epsilon_{\mathrm{H}} = 185$ cal/mole [3] for MPA, and with $\Delta A = 5.1$ Å^2, $T_t^0 = 45$°C [1], $K_d = 10^{-0.8}$ M, $\epsilon_{\mathrm{H}} = 250$ cal/mole [3] and the same values of ΔS, A_0 as above for 1,2-DMPA. The theoretical curve without chelation is shown as a dashed curve.

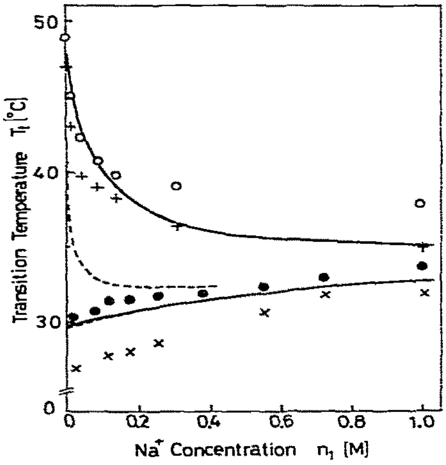

Fig. 2. The dependence of T_t on the Na^+ concentration. Experimental data for MPA [16] are marked by ○ and + for increasing and decreasing temperatures, respectively, at pH 4, and by ●, × at pH 8.5. The solid lines are theoretical curves obtained with the parameters of fig. 1, and the dashed lines are obtained without chelation.

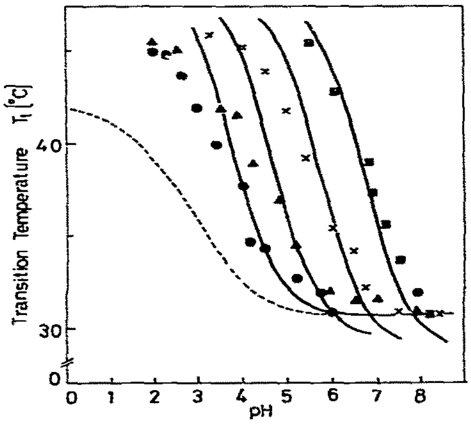

Fig. 3. The dependence of T_t on pH and the Na^+ concentration. Experiment data [16]: ■ for 2×10^{-4} M. × for 2×10^{-3} M. ▲ for 2×10^{-2} M and ● for 0.2 M. The parameters used for theoretical curves are those of fig. 1. The curve without chelation for 0.2 M is shown by a dashed line. Further decrease of pH for each Na^+ concentration will decrease T_t in a similar way as in fig. 1. since maximum T_t's are obtained at $\alpha\approx0.5$.

fig. 3, and furthermore even rough selection of n_1 does not lead to a serious change in the theoretical curves. The total concentration of Ca^{2+} is denoted by $[Ca^{2+}]$ in fig. 4, in distinction from the concentration of Ca^{2+} far from the surfaces of bilayers n_2. The difference between $[Ca^{2+}]$ and n_2 cannot be neglected when $[Ca^{2+}]$ is as low as the lipid concentration [L], due to the adsorption of Ca^{2+}. According to Woolley and Teubner [7], therefore, we estimate $[Ca^{2+}]$ by adding $[L]\theta_{2t}$ to n_2 in the range of $[Ca^{2+}]\leqslant 2\times10^{-4}$ M, with the aid of eq. (A1) for θ_{2t} in appendix 1. As for the monovalent cations like Na^+, on the other hand, they are scarcely adsorbed (cf., e.g. refs. [16,23]), and furthermore the cation concentration accumulated in the electric double layer may be neglected in the situation where Ca^{2+} or protons are adsorbed. We, therefore, do not distinguish between the total concentration of Na^+ and the concentration far from surfaces n_1, even for concentrations as low as $\approx 2\times10^{-4}$ M.

The parameters for MPA are chosen as $\Delta S=$ 17.5 cal/mole deg [3], $K_2=0.3\ \mathrm{M}^{-1}$ [7], $\Delta A=$ 8 Å^2, $A_o=40$ Å^2 [3,7], $T_t^0=42$°C [3], $K_d=$ 10^{-1} M, $\epsilon_H=185$ cal/mole [3], and $\epsilon_2=187.5$ cal/mole. Among the parameters for 1,2-DMPA, ΔS and A_o are chosen to have the same values as above, and the others are chosen as $\Delta A=5.1$ Å^2, $T_t^0=45$°C [1], $K_d=10^{-0.8}$ M and $\epsilon_H=250$ cal/mole [3]. The above values of ΔA are nearly the same as the observed value ≈ 6 A^2 for MPA [3]. The values of the non-electrostatic temperature T_t^0 are taken as the one at $\alpha\approx0$ observed in much lower pH regions [1,3]. The values of ϵ_H agree well with the rough estimate by Eibl and Woolley [3]. The dissociation constant for MPA is chosen so as to reproduce well the dependence of T_t on both pH and n_1 in fig. 3, in a similar way to Träuble et al. [16], besides the fact that maximum T_t corresponds to $\alpha\approx0.5$ [1–3]. For 1,2-DMPA, K_d is chosen to have almost the same values as for MPA. The value of ϵ_2 for Ca^{2+}-chelation does not differ from ϵ_H for proton-chelation, which seems to be reasonable since the maximum T_t is ≈ 48°C for both cases.

In figs. 1–4, the theoretical curves obtained in the absence of chelation are also shown as dashed curves for reference. The curves indicate clear deviations from the observed data, except at pH 8.5 where the effect of proton-chelation can be neglected because $\alpha\approx1$. The agreement between

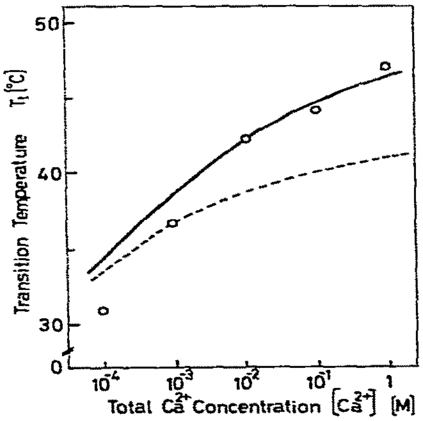

Fig. 4. The dependence of T_t on the Ca^{2+} concentration under the coexistence of $n_1=0.2$ M. Experimental data for MPA [7] are marked by ○. The solid line is a theoretical curve for $\epsilon_2=187.5$ cal/mole and values for the other parameters as in fig. 1. The theoretical curve for $\epsilon_2=0$ is shown as a dashed line.

our theory and the observed results, on the other hand, is satisfactory, except near neutral pH for 1,2-DMPA. The discrepancy in this region may be attributed to the tilting of hydrocarbon chains accompanying a further ionization [1,2].

Now let us discuss the cooperativity of the phase transition with the aid of the expressions given above*. Generally the cooperativity of the phase transition can be estimated from the average size of domains formed by like-molecules, which is termed the size of the cooperative unit in ref. [2]. As the cooperativity is enhanced, the phase-transition curve is expected to be sharper, and approaches the first-order type [20–22].

If the free energy of the system in the present theory is expanded in powers of ξ as

$$G+TS_{\mathrm{m}} = -\sum_{n=0}^{\infty} \frac{a_n}{n!}\xi n, \tag{21}$$

where S_{m} is the mixing entropy, a_n is defined by

$$a_n \equiv -\partial^n(G+TS_{\mathrm{m}})/\partial\xi^n\,|_{\xi=0}. \tag{22}$$

Expressions (5) and (11) contain no higher-order terms than ξ^3, and the electrochemical energy of eq. (8a) is composed of only negligibly small terms of the order of ξ^n for $n \geqslant 2$, as shown below. In the present case, therefore, it seems reasonable to define the value of α_2 as the cooperativity of the phase transition, since a more positive value of α_2 makes the transition curve sharper [21,22].

Let us first investigate the effect of the electrochemical energy without Ca^{2+}, or the effect of charge of head groups, on the cooperativity of the phase transition. Let a_2^0 and a_2^{el} denote the terms contributing to a_2 obtained from the differentiation of G_0 in eq. (5) and $G_{\mathrm{el}}^{\mathrm{H}}$ in eq. (8a), respectively. Under a high potential approximation [16,23] they are given by

$$a_2^0 = \epsilon, \tag{23}$$

$$a_2^{\mathrm{el}} = -\tfrac{2}{3}k_{\mathrm{B}}\left(K_{\mathrm{d}}/q^2A_{\mathrm{o}}^4[\mathrm{H}^+]\right)^{1/3}n_1^{1/3} \times\left\{1-\left(K_{\mathrm{d}}A_{\mathrm{o}}^2/27q^2[\mathrm{H}^+]\right)^{1/3}n_1^{1/3}\right\}(\Delta A)^2. \tag{24}$$

Since ϵ is positive as already mentioned, a_2^0 is positive and the phase transition is rather cooperative for non-charged lipids. The cooperativity a_2^{el} is negative, on the other hand, because the second term in the braces in eq. (24) is smaller than unity. This implies that the cooperativity decreases with an increase of the charge of head groups accompanying that of pH and n_1. As a result, the phase transition is expected to be gentler for charged lipids than for non-charged lipids, in accord with experiments [13] and theoretical explanations by Forsyth et al. [26]. The electrochemical effect, however, seems to be quite small, since a_2^{el} is only of the order of $10^{-2}\,k_{\mathrm{B}}T$. Higher-order terms such as $|a_3^{\mathrm{el}}|$ are negligibly small even compared with a_2^0, and hence do not affect the phase-transition curve very seriously.

Next, let us discuss the dependence of the cooperativity on pH and n_1 in the presence of chelation with the aid of protons. It has been observed that the cooperativity decreases drastically with an increase of pH and also with addition of monovalent cations [2]. A second differentiation of the free energy for this case is given by

$$a_2 = a_2^0 + a_2^{\mathrm{el}} + a_2^{\mathrm{ch}}, \tag{25}$$

where a_2^0 and a_2^{el} are expressed by eqs. (23) and (24), respectively, and a_2^{ch} is derived from eq. (11a) and given by

$$a_2^{\mathrm{ch}} = 8\epsilon_{\mathrm{H}}\theta_{\mathrm{H}}(1-\theta_{\mathrm{H}}). \tag{26}$$

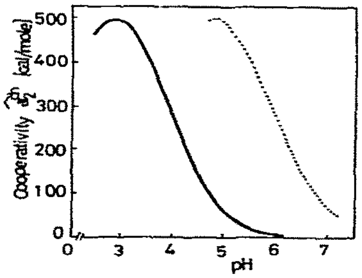

Fig. 5. The dependence of the cooperativity on pH and the Na^+ concentration. The curves for 1,2-DMPA are shown by a dotted line for $n_1 = 10^{-3}$ M and a solid line for $n_1 = 0.1$ M, with the parameters of fig. 1.

---

* According to Woolley, the cooperativity is very poorly reproducible because of some unknown factors.

The value of a_2^{ch} at $\xi = 1/2$ are plotted in fig. 5 against pH for two values of n_1, since a_2^0 is independent of both pH and n_1, and $|a_2^{\mathrm{el}}|$ is negligibly small compared with a_2^{ch}. It can be seen that the tendency agrees well with the observed data for the changes in both pH and n_1. In the present theory, therefore, the decrease of the number of hydrogen bonds is the main cause of the decrease of cooperativity with increasing pH and n_1.

Lastly, we mention qualitatively some causes of the hysteresis and metastable state observed in some lipid systems [1,5,6,10,13,16,27]. In doing so, the following three observations may be of interest: one is the observation that hysteresis does not occur in non-charged lipids but does occur in charged ones [16,27]. The second is the sigmoid relaxation of the order parameter in PG (phosphatidylglycerol) layers with T-jump in the presence of Ca^{2+} [10]. The third is an increase of the area of the hysteresis loop for MPA with an increase of the Mg^{2+} concentration at high pH [5,6]. Since the hysteresis and metastable state are known to occur as a result of an enhancement of cooperativity of the phase transition [21,22,28–30], the above-mentioned three phenomena are expected to be related strongly to an enhancement of cooperativity through some mechanisms.

From the present theory, phase-transition curves of 1,2-DMPA can easily be calculated with the aid of eqs. (9) and (17)–(19), e.g., in the case of the absence of Ca^{2+}. The result gives the largest hysteresis loop around pH 3 because of the formation of hydrogen bonds, but gives almost reversible curves around pH 1. This result agrees well with the experimental observations for 1,2-DMPA: the phase transition curve is reversible at pH 1.8 (cf. fig. 2 in ref. [1]) and widths of the hysteresis loop defined at $\xi = 1/2$ are $\approx 2°\mathrm{C}$ [5] and $\approx 1°\mathrm{C}$ [1,5] at pH 3 and 4–4.5, respectively. As pH is further increased, the area of the hysteresis loop again increases and reaches a maximum value at $\alpha \approx 1.5$ [4,5], which seems to be related to the tilting of hydrocarbon chains [1,2]. There is, therefore, a large possibility that the main origin of the hysteresis and metastability in the presence of divalent cations is the enhancement of cooperativity accompanying chelation with the aid of divalent cations.

4. Phase separation

It has been shown experimentally [4,5,7] that an excess step appears in the phase-transition curve when n_1 is lower than 10^{-2} M or so in the presence of Ca^{2+} concentrations lower than or comparable to the lipid concentration. When n_1 becomes higher, the excess step disappears for any concentration of coexistent Ca^{2+} [7]. From these observations, it has been suggested [7] that a mixed state of two kinds of domains appears between lower T_t and higher T_t, where one domain is composed mainly of fluid-like, Ca^{2+}-free lipids and the other consists mainly of ordered Ca^{2+}-bound lipids.

In spite of the above experimental facts, existing theories do not predict the appearance of such a phase separation [4,7,16]. In these theories, however, the excess entropy of the boundary energy resulting from the coexistence of two kinds of domains are omitted and, furthermore, the chelation energy is not taken into account. The purpose of this section is to show that the present theory taking account of the chelation energy does predict the appearance of the phase separation under the above experimental conditions. To do so, the value of the Gibbs free energy G_{h} describing a homogeneous state is compared with the value of G_{s} for the mixed state of Ca^{2+}-free and Ca^{2+}-rich domains. Then one can conclude that a phase separation occurs within the temperature range where G_{s} is lower than G_{h}.

As for G_{h}, the expression of the Gibbs free energy introduced in the previous sections can be used. Then the value of G_{h} can be estimated from eq. (2) together with eqs. (5), (8b) and (11b) by inserting equilibrium values of ξ_{h}, $\theta_{2\mathrm{h}}$ and ϕ_{h} obtained by solving eqs. (9), (17b), (18b) and (19). In this process, the integration in the second term of eq. (8b) is transformed to

$$\int_0^{-(e/A)(1-2\theta_{2\mathrm{h}})} \psi(\sigma)\, \mathrm{d}\sigma = \sigma\psi - \Pi_{\mathrm{el}}^{\mathrm{Ca}}, \qquad (27)$$

where $\Pi_{\mathrm{el}}^{\mathrm{Ca}}$ is given by eq. (14b).

The Gibbs free energy per molecule G_{s} describes the situation where a phase separation occurs between the Ca^{2+}-free domains with a small fraction of Ca^{2+}-chelated lipids and the Ca^{2+}-rich domains composed mainly of Ca^{2+}-chelated lipids, and hence is given by

$$G_s = \zeta G_r(\xi_r, \theta_{2r}; T) + (1-\zeta) G_{fr}(\xi_{fr}, \theta_{2fr}; T) + k_B T[\zeta \ln \zeta + (1-\zeta)\ln(1-\zeta)] + \Delta E_b(\xi_h, \zeta; T). \tag{28}$$

In this expression, the subscripts r, fr and b specify the Ca^{2+}-rich domains, Ca^{2+}-free domains and surface boundaries between neighboring domains, respectively. The fraction of lipids belonging to the Ca^{2+}-rich domains is denoted by ζ. The free energies per molecule of the Ca^{2+}-rich and Ca^{2+}-free domains, G_r and G_{fr}, are assumed to be given by the same form as eq. (2). The third term represents the excess entropy resulting from a random mixing of two kinds of domains. The last term represents the energy of surface boundaries between neighboring domains and is given by

$$\Delta E_b = \epsilon_s T \zeta^{-1/2} \lambda^{-2} [1 + (\Delta A / A_o) \xi_h]^{-1}, \tag{29}$$

$$\lambda^{-2} = 1 + (8\pi/\epsilon_w)(e^2/k_B T) A_t n_1, \tag{30}$$

where ϵ_s is a parameter measuring the strength of the boundary energy and A_t is the value of the molecular surface area A at T_t. A detailed derivation of eqs. (29) and (30) is given in appendix 2.

To estimate the value of G_s, equilibrium values of ζ, ξ_r, ξ_{fr}, θ_{2r} and θ_{2fr} should be evaluated. The equilibrium values of ξ_r and ξ_{fr} can be obtained by minimizing G_r and G_{fr}, respectively. The equilibrium value of ζ can be obtained by minimizing G_s as

$$\zeta^{-1} = 1 + \exp[(G_r - G_{fr} + \partial \Delta E_b / \partial \zeta)/k_B T]. \tag{31}$$

The degrees of adsorption like θ_{2r} and θ_{2fr}, on the other hand, cannot be derived from a usual condition representing an adsorption equilibrium which is related closely to the solution of the Poisson–Boltzmann equation for a homogeneous charge distribution on membrane surfaces. When a phase separation occurs, the charge distribution becomes quite inhomogeneous and it is not easy to derive expressions for θ_{2r} and θ_{2fr} by solving the Poisson–Boltzmann equation. In the present the-

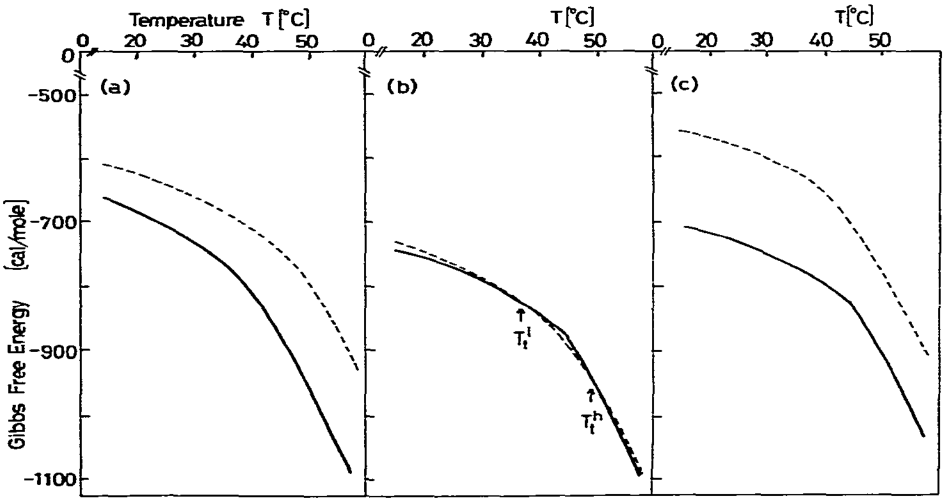

Fig. 6. A possibility of phase separation. The Gibbs free energy G_h is shown by a solid line and G_s by a dashed line in molar units. The conditions are chosen as $n_1 = 10^{-4}$ M, $n_2 = 2 \times 10^{-5}$ M for (a) and (b), and $n_1 = 0.2$ M, $n_2 = 0.1$ M for (c). The parameters ϵ_s, ϵ are taken as 0.457 cal/mole deg, 2376 cal/mole corresponding to $\approx 4\, k_B T$, respectively, with $\epsilon_2 = 0$ for (a) and 187.5 cal/mole for (b) and (c), and the values for the other parameters for MPA as in fig. 1. A theoretical lower transition temperature (≈ 36°C) and a higher one (≈ 49°C) are denoted by T_t^l and T_t^h, respectively, in (b).

ory, therefore, we assume that the equilibrium values of θ_{2r} and θ_{2fr} are nearly constant with respect to the temperature and determine these values so that the theoretical values of higher and lower transition temperatures agree with the observed values.

Even for an inhomogeneous charge distribution, Träuble [4] suggested that the average value of the degree of adsorption over the specimen may not differ much from θ_{2h} for a homogeneous charge distribution. In terms of the present notation, this requirement can be expressed as

$$\theta_{2h} \approx \theta_{2r}\zeta + \theta_{2fr}(1-\zeta), \tag{32}$$

with the aid of the equilibrium value of ζ given by eq. (31). As will be shown later, the values of θ_{2r} and θ_{2fr} determined from the present scheme always satisfy eq. (32), showing that our method of calculation is reasonable.

In fig. 6, the calculated results of G_h and G_s are shown in molar units for the cases of $n_1 = 10^{-4}$ M and 0.2 M, by choosing the value of a single adjustable parameter as $\epsilon_s = 0.457$ cal/mole deg. The values of degrees of adsorption θ_{2r} and θ_{2fr} are determined as 0.435 and zero, respectively, so as to reproduce the observed values of higher T_t ($\approx$ 49°C) and lower T_t ($\approx$ 33°C). For these values of θ_{2r} and θ_{2fr}, the requirement expressed by eq. (32) is fulfilled quite well, because the value of the right-hand side of eq. (32) is 0.299 against $\theta_{2h} = 0.304$ at $T = 47$°C for $n_1 = 10^{-4}$ M.

The present theory leads to the following three conclusions on the phase separation: (i) In the absence of chelation, the theory does not predict the appearance of any phase separation. (ii) In the presence of chelation with $n_1 = 10^{-4}$ M, the phase separation is expected to appear between the domains composed of fluid Ca^{2+}-free lipids and the ones consisting mainly of ordered Ca^{2+}-chelated lipids within the temperature range between lower T_t and higher T_t. (iii) When n_1 becomes as high as 0.2 M, on the other hand, the phase separation does not appear because of the increase of domain-boundary energy accompanying the decrease of the screening length.

5. Discussion

From the present theory, taking account of the chelation between neighboring head groups of lipids through protons and Ca^{2+}, many observed data can be explained comprehensively with the aid of known values of T_t^0, ΔA, ΔS and ϵ_H. The adjustable parameters used in the present theory are only two; one is ϵ_2 related to the chelating through Ca^{2+} and the other is ϵ_s related to the boundary energy among domains in discussing the possibility of a phase separation.

One of the reasons why chelation has not been taken into account in existing theories is that the observed data on the total degree of adsorption of protons or Ca^{2+} to lipids can be explained fairly well without taking account of chelation [7]. As can be seen from fig. 4, on the other hand, the observed data of T_t cannot be explained without introducing chelation through Ca^{2+}. This contradiction can be solved if one notices that the effect of chelation on the total degree of adsorption is quite small. A detailed discussion is given in appendix 1 and some results are shown in fig. A1, where solid and dashed curves indicate the cases with and without chelation, respectively. It can be seen clearly that there is little difference between these curves.

The results shown in fig. A1 may be understood intuitively from the following discussion. From eq. (18b), the effective adsorption constant of Ca^{2+} can be defined by

$$K_2^{\mathrm{eff}} = K_2 \exp(-2\phi) \exp\left[8\epsilon_2(1-\xi)^2\theta_2/k_BT\right]. \tag{33}$$

Under the conditions of, e.g., $n_1 = 10^{-2}$ M, $n_2 = 10^{-4}$ M at $T = 20$°C, the value of K_2^{eff} is estimated as

$$\begin{aligned} K_2^{\mathrm{eff}} &= K_2 \exp(10.37)\exp(0.75), && \text{for } \epsilon_2 = 187.5, \\ K_2^{\mathrm{eff}} &= K_2 \exp(10.81), && \text{for } \epsilon_2 = 0. \end{aligned} \tag{34}$$

Whereas there is only a slight increase of K_2^{eff} in the presence of chelation, the increment of T_t under the same conditions amounts to 4.8°C.

The possibility of the appearance of a phase separation at low n_1 is first predicted by the present theory with the aid of Ca^{2+}-chelation. The phase separation has been observed also between pH 2 and 3 for PA [1,2], and it is supposed to occur through proton-chelation in the same way as for Ca^{2+}. In biological systems, the phase separation is known to play an important role in membrane fusion [31], cell growth [32] and chemoreception [33]. Since various kinds of lipids and proteins are contained there, it may be expected that, in spite of high ion concentrations, a phase separation takes place more easily through protons or Ca^{2+} than in the one-component system investigated here.

In our theory, the phase transition is assumed to be a two-dimensional one and interactions among separate bilayers are not considered. But this simplification seems to be permitted even in the presence of Ca^{2+} from the following fact: With the aid of the observed data for PG bilayers in the presence of Ca^{2+} [10], one can estimate easily the Avrami exponent [34] as 2.5–2.8. As is well known [35], this value indicates that the phase transition occurs through a two-dimensional growth of nuclei of the ordered phase. It may be reasonable, therefore, to expect that a similar situation also occurs for the phase transition in MPA or PA.

Appendix 1

Here, we show that the total number of Ca^{2+} taken up by the membrane with intercalation does not differ much from that without intercalation. Let us denote the number of Ca^{2+} taken up by one lipid in the membrane by θ_{2t}; it may be composed of two parts [7]:

$$\theta_{2t} = \theta_2 + AS_2, \tag{A.1}$$

where S_2 is the number of Ca^{2+} per unit area trapped in the electric double layer. S_2 is given by

$$S_2 = n_2 \int_0^\infty \{\exp[-2e\psi(x)/k_B T] - 1\} \, dx. \tag{A.2}$$

where x is the spatial coordinate inside the water normal to the lipid membrane concerned, and $\psi(x)$ is the electric potential at x. By integrating the Poisson–Boltzmann equation, $d\psi/dx$ is given by [23,25]

$$\frac{d\psi}{dx} = \left(\frac{8\pi k_B T}{\epsilon_w} \right)^{1/2} \frac{1-u}{u} \left[(n_1 + 2n_2)u + n_2 \right]^{1/2}. \tag{A.3}$$

with the abbreviation

$$u \equiv \exp[e\psi(x)/k_B T]. \tag{A.4}$$

Straightforward integration in eq. (A.2) with the aid of eqs. (A.3) and (A.4) gives

$$S_2 = \left(\frac{k_B T \epsilon_w}{8\pi e^2} \right)^{1/2} \sqrt{n_2} \left\{ \eta(r,\phi)\, e^{-\phi} - \eta(r,0) - \frac{r}{2} \ln\left[\frac{\eta(r,\phi)+1}{\eta(r,\phi)-1} \right] + \frac{r}{2} \ln\left[\frac{\eta(r,0)+1}{\eta(r,0)-1} \right] \right\}. \tag{A.5}$$

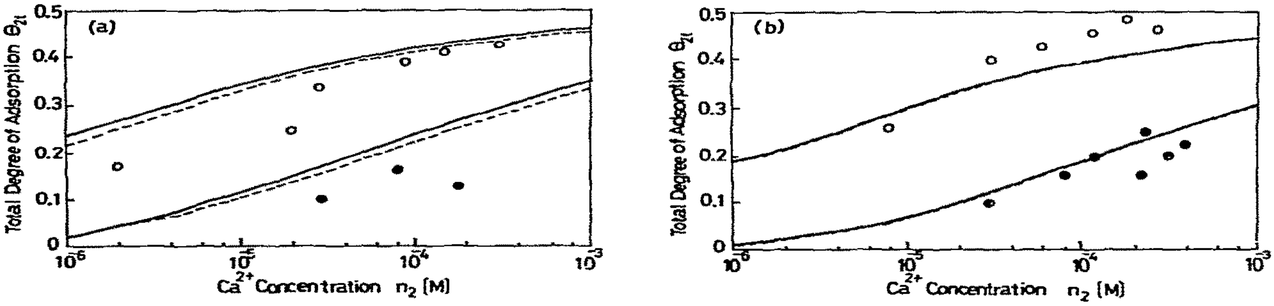

Fig. A1. The dependence of the total concentration of Ca^{2+} trapped by membranes on the Ca^{2+} concentration and temperature. Experimental data [7] are marked by ○ for $n_1 = 10^{-2}$ M and ● for 0.1 M, at $T = 20$°C (a) and 55°C (b). Theoretical curves are shown by a solid line with chelation and a dashed line without chelation. The parameters are those used for MPA in fig. 1.

where ϕ, r and η are defined by eqs. (10), (15) and (16).

Since ξ, θ_2 and ϕ are known by solving eqs. (9), (17b), (18b) and (19) at each temperature, θ_{2t} is obtained from eq. (A.1) with the aid of eqs. (4) and (A.5). For two cases of $\epsilon_2 = 0$ and 187.5 cal/mole, the calculated results of θ_{2t} for MPA are shown in fig. A1, where four conditions of $n_1 =$ 0.1 M and 0.01 M at $T = 20°C$ ($< T_t$) or 55°C ($> T_t$) are chosen for variations of n_2 from 10^{-6} M to 10^{-3} M. The theoretical curves in the case of $\epsilon_2 = 0$ have already been given only numerically by taking $A = 40$ Å^2 and 46 Å^2 at $T = 20°C$ and 55°C, respectively, by Woolley and Teubner [7]. From fig. A1, it can be seen that the values of θ_{2t} for the two cases with and without chelation do not differ very much, in spite of a large difference of T_t, e.g. as shown in fig. 4. This fact shows that the chelation does not contribute to the adsorption of Ca^{2+} but to the increase of T_t.

Appendix 2

The expression of ΔE_b given in eqs. (29) and (30) is obtained from the following scheme. In analogy with electrochemical free energy, the energy to form a domain boundary with area δA has the form:

$$\Delta E_b \propto (T/\tilde{A})\, \delta\alpha\, \delta A, \tag{A.6}$$

where $\delta\alpha$ denotes the change in α from one domain to the other and $\tilde{A}$ is the effective molecular surface area.

Among the quantities appearing in eq. (A.6), $\delta\alpha$ is nearly independent of temperature. The area of the boundary region, δA, is proportional to both the number of domains, n_d, and the number of lipids belonging to the domain boundaries. The number of domains n_d increases gradually with increasing ζ within the range where ζ is small and the nucleation of domains is the dominant process, but begins to decrease when ζ is increased further and larger domains grow at the expense of smaller ones [30,35]. This tendency of n_d may be expressed simply by

$$n_d = \beta_1 \zeta^{-1}[1 - \exp(-\beta_2 \zeta)], \tag{A.7}$$

with constants β_1 and β_2 determined by the following boundary conditions:

$$n_d = 1, \text{ at } \zeta = 0 \text{ and } \zeta = 1. \tag{A.8}$$

Since a phase separation can be observed when ζ becomes somewhat large, eq. (A.7) can safely be approximated by $n_d \approx \beta_1 \zeta^{-1}$. The number of lipids belonging to domain boundaries, on the other hand, is expected to be proportional to $\zeta^{1/2}$. As a result, δA would be proportional to $\zeta^{-1/2}$, which gives the net ζ-dependence of ΔE_b in eq. (29).

In the derivation of ΔE_b, the effect of the presence of a charge gradient must be taken into account. When the mean surface area of the domain boundaries per molecule, A_b, is of comparable order with A, the effect of the charge gradient is not serious and, hence, the effective area $\tilde{A}$ is nearly equal to A. When A_b becomes extremely small compared with A, the main contribution to ΔE_b comes from a drastic variation of the charge inside the boundaries. In this case ΔE_b may be inversely proportional to A_b instead of A. Since a macroscopic concept of domain boundary may lose its meaning when $A_b^{1/2}$ becomes smaller than the Debye screening length λ_D, the lowest limit of A_b can be taken as $(A/A_t)\lambda_D^2$, where A_t is the value of A at T_t. The factor A/A_t comes from the facts that A_b is proportional to A and also that the temperature range concerned is near T_t. Thus we can simply assume the following approximate relation for $\tilde{A}$:

$$1/\tilde{A} \equiv 1/\lambda^2 A = A_t/\lambda_D^2 A + 1/A. \tag{A.9}$$

If we use the well-known expressions for λ_D in the presence of monovalent cations for simplicity, eq. (A.9) is reduced to eq. (30). For A we adopt eq. (4) for a homogeneous charge distribution to avoid unnecessary complexity. Combination of all of these dependences leads to eq. (29) for ΔE_b with the parameter ϵ_s as a measure of the strength of the boundary energy.

References

[1] H. Eibl and A. Blume, Biochim. Biophys. Acta 553 (1979) 476.

[2] A. Blume and H. Eibl, Biochim. Biophys. Acta 558 (1979) 13.

[3] H. Eibl and P. Woolley, Biophys. Chem. 10 (1979) 261.
[4] H. Träuble, in: Structure of biological membranes, eds. S. Abrahamsson and I. Pascher (Plenum, New York, 1976) p. 509.
[5] H. Träuble and H. Eibl, in: Functional linkage in biomolecular systems, eds. F.O. Schmitt, D.M. Schneider and D.M. Crothers (Raven Press, New York, 1975) p. 59.
[6] H. Träuble and H. Eibl, Proc. Natl. Acad. Sci. USA 71 (1974) 214.
[7] P. Woolley and M. Teubner, Biophys. Chem. 10 (1979) 335.
[8] P.W.M. van Dijck, B. de Kruyff, A.J. Verkleij, L.L.M. van Deenen and J. de Gier, Biochim. Biophys. Acta 512 (1978) 84.
[9] J.F. Tocanne, P.H.J.Th. Ververgaert, A.J. Verkleij and L.L.M. van Deenen, Chem. Phys. Lipids 12 (1974) 201.
[10] M.M. Sacré, W. Hoffmann, M. Turner, J.F. Tocanne and D. Chapman, Chem. Phys. Lipids 25 (1979) 69.
[11] A.J. Verkleij, B. de Kruyff, P.H.J.Th. Ververgaert, J.F. Tocanne and L.L.M. van Deenen, Biochim. Biophys. Acta 339 (1974) 432.
[12] K. Jacobson and D. Papahadjopoulos, Biochemistry 14 (1975) 152.
[13] R.C. MacDonald, S.A. Simon and E. Baer, Biochemistry 15 (1976) 885.
[14] P.W.M. van Dijck, P.H.J.Th. Ververgaert, A.J. Verkleij, L.L.M. van Deenen and J. de Gier, Biochim. Biophys. Acta 406 (1975) 465.
[15] A.G. Lee, Biochim. Biophys. Acta 472 (1977) 237.
[16] H. Träuble, M. Teubner, P. Woolley and H. Eibl, Biophys. Chem. 4 (1976) 319.
[17] Th.A.J. Pavens, Philips Res. Rept. 10 (1955) 425.
[18] H.L. Scott and W.H. Cheng, Biophys. J. 28 (1979) 117.
[19] S. Marcelja, Biochim. Biophys. Acta 367 (1974) 165.
[20] D. Marsh, A. Watts and P.F. Knowles, Biochim. Biophys. Acta 465 (1977) 500.
[21] G. Weisbuch and E. Neumann, Biopolymers 12 (1973) 1479.
[22] T.L. Hill, in: Introduction to statistical thermodynamics (Addison-Wesley, Reading, MA, 1960) p. 235.
[23] K. Toko and K. Yamafuji, Chem. Phys. Lipids 26 (1980) 79.
[24] H. Hauser, D. Chapman and R.M.C. Dawson, Biochim. Biophys. Acta 183 (1969) 320.
[25] J.A. DeSimone, J. Colloid Interface Sci. 67 (1978) 381.
[26] P.A. Forsyth, S. Marcelja, D.J. Mitchell and B.W. Ninham, Biochim. Biophys. Acta 469 (1977) 335.
[27] E. Sackman, Ber. Bunsenges. Phys. Chem. 82 (1978) 891.
[28] G. Adam, Z. Phys. Chem. Neue Folge 68 (1969) 113.
[29] K. Binder, Phys. Rev. B8 (1973) 3423.
[30] M. Kalos, J.L. Lebowitz, O. Penrose and A. Sur, J. Stat. Phys. 18 (1978) 39.
[31] D. Papahadjopoulos, W.J. Vail, W.A. Pangborn and G. Poste, Biochim. Biophys. Acta 448 (1976) 265.
[32] N. Wakayama and T. Oshima, J. Biochem. 83 (1978) 1687.
[33] K. Kurihara, N. Kamo and Y. Kobatake, Adv. Biophys. 10 (1978) 27.
[34] F.W. Cope, Physiol. Chem. Phys. 9 (1977) 443.
[35] M. Avrami, J. Chem. Phys. 8 (1940) 212.