Surface Potential of Lipid Membrane Estimated from the Partitioning of Methylene Blue into Liposomes†

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ABSTRACT: The partition of methylene blue between negatively charged phospholipid membrane and the bulk aqueous phase was measured by using visible spectroscopy in very dilute aqueous membrane suspensions, 0.05–0.3 mg of dried phospholipids in 1 mL of buffer solution. Under these experimental conditions, the turbidities of liposome systems and the overlapping of the electrical double layers of different liposomes were negligible. The positively charged probe, methylene blue, forms dimers in membrane phase, resulting in a reduction of the absorbance intensity. The surface potential of the membranes (liposomes) was calculated from the partition coefficient of the dye between the membrane and the bulk phase. The effects of charge density of the membrane and of the ionic strength on the surface potential were also studied.

The charge density and the electrical potential of biological membranes have an important role in determining the structure and function of membranes. For the estimation of the potential, various kinds of spectral probes have been used. The surface potentials of charged lipid membranes have been obtained from the distribution coefficients of paramagnetic amphiphile (Castle & Hubbell, 1976) or a fluorescence probe (Eisenberg et al., 1979) between charged lipid membrane and the aqueous phase. Alternatively, absorbance or fluorescence spectra of solubilized pH indicator dyes have been employed to estimate surface potentials in micellar solutions (Mukerjee & Banerjee, 1964; Fernandez & Fromherz, 1977; Funasaki, 1977) and in aqueous suspensions of liposomes (Bakker et al., 1975; Mashimo et al., 1979). In the above studies, the amounts of lipids in aqueous solutions were sometimes large enough to result in excessive turbidities that interfered with absorbance and fluorescence measurements.

In this study, the partitioning of methylene blue, a positively charged metachromatic dye, between the membrane phase and the bulk aqueous phase was measured. The dye was concentrated in the negatively charged membrane phase and formed dimers, resulting in a reduction of the absorbance. The amounts of lipids used here were 0.05–0.3 mg of dried lipid in 1 mL of buffer solution. Such low concentrations of lipids did not give rise to turbidities appreciable enough to interfere with the spectroscopic measurements.

Experimental Procedures

Materials. L-α-Dipalmitoylphosphatidylcholine (L-α-DPPC)[1] and L-α-dipalmitoylphosphatidic acid (L-α-DPPA) were obtained from Sigma Chemical Co. The mixtures of L-α-DPPC and L-α-DPPA were prepared as follows: The lipids were dissolved in chloroform, the solvent was evaporated, and then the mixtures of lipids were dried under vacuum for 15 h. The complete removal of chloroform was essential for the stabilities of the buffer solutions and the pH and also for reproducible values of absorbance. Trace amounts of the solvent will also introduce errors in the estimation of the partitioning of the probe into membrane phases. Methylene blue (zinc free) was purchased from Nakarai Chemical Co. (Kyoto). This cationic dye has a single net charge in a molecule, and the charge is pH independent.

Preparation of Liposomes (Membranes). The lipid or the lipid mixtures were suspended in Hepes buffer of pH 6.0 (5 mM Hepes, 0.2 mM Na_2EDTA, and 0.2 mM Tris). The amount of lipid suspended ranged from 0.02 to 0.3 mg in 1 mL of the buffer solution. The amounts of lipid used here are much lower than those used in other studies (Castle & Hubbell, 1976; Bakker et al., 1975; Mashimo et al., 1979). The concentration of the probe (methylene blue, MB) in solution was 2×10^{-5} M. The lipids were dispersed in the buffer solutions containing MB by ultrasonication for 20 min. The solutions were cooled in ice during this process. In this way, the aqueous solutions inside and outside the liposomes (membranes) are the same. The ultrasonicator used was an UR-200P from Tomy Seiko Co. Ltd. The ultrasonication of the methylene blue solutions resulted in a small reduction of the absorbances, and a correction has been made for this. Phosphate buffer solutions of MB without lipids were also examined; serious reductions in the absorbances resulted after ultrasonication. These were considered to be caused from the binding of phosphate to titanium particles. Therefore, phosphate buffer was not employed here.

Absorbance Measurements. The spectra of MB in Hepes buffers with various amounts of liposomes and various compositions of the lipids were recorded with a Shimazu UV-180 spectrophotometer. The turbidities of the membrane systems increased the absorbances. This increase, however, was at most 2% for visible wavelength bands and was neglected in our experimental conditions. Membrane systems containing about 50 mg of dried phospholipids in 100 mL of buffer solution (0.5 mg in 1 mL) exhibited appreciable turbidity, and this prevented the use of sulfonphthalein dyes as pH indicators in liposomes since the latter dyes were found to require higher amounts of phospholipids to be solubilized in membranes completely.

Theory

Absorbance of the Probe in Membrane System. Methylene blue (MB) is one of the metachromatic dyes. This group of dyes undergoes dimerization in aqueous solution at concentrations greater than 10^{-4} M. The stacking interactions give

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[1] Abbreviations used: L-α-DPPC, L-α-dipalmitoylphosphatidylcholine; L-α-DPPA, L-α-dipalmitoylphosphatidic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; MB, methylene blue.

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not only dimers but also n-meric forms (Ghosh & Mukerjee, 1970). However, the extinction coefficients of dimer and n-meric form at the maximum absorbance of the longer wavelength band (664 nm) are almost the same (Massari & Pascolini, 1977; Vitagliano et al., 1973). Therefore, an n-mer is considered to be equivalent to $n/2$ dimers. Under the experimental conditions of this study (concentration of MB 2×10^{-5} M), MB molecules are in monomeric form in the bulk phase. On the other hand, in membrane phase, the molecules are considered to be in monomeric and dimeric forms. The absorbance of MB in the system, A, is given by

$$A = \frac{1}{V_B}[\epsilon_m(n_0 - n_d) + \epsilon_d n_d] \quad (1)$$

where n_0 and n_d are moles of the MB molecules in total and in dimeric form (gram equivalent of dimers), respectively, V_B is the volume of the buffer solution containing MB, the volume of membrane phase, V_M, is very small to be neglected in eq 1, and ϵ_m and ϵ_d are the extinction coefficients of monomer and dimer, respectively. ϵ_d in bulk and in membrane is considered to be almost the same (Massari & Pascolini, 1977). This will be discussed later in detail. The absorbance of the same solution without membranes, A_0, is given by

$$A_0 = \frac{1}{V_B}\epsilon_m n_0 \quad (2)$$

In this experiment, n_0/V_B was 2×10^{-5} M. From eq 1 and 2

$$\frac{n_d}{n_0} = \left(1 - \frac{A}{A_0}\right) \Big/ \left(1 - \frac{\epsilon_d}{\epsilon_m}\right) \quad (3)$$

n_d is calculated by eq 3 at the absorbance maximum of 664 nm. ϵ_d/ϵ_m at this wavelength is 0.1566 (Massari & Pascolini, 1977; Vitagliano et al., 1973).

Surface Potential of Membrane. The equilibrium of monomeric MB molecule, which has one positive charge, between membrane phase and bulk phase can be written as

$$\frac{C_{mM}}{C_{mB}} = P_0 \exp[-e\psi/(kT)] \quad (4)$$

where C_{mM} and C_{mB} are the concentrations of monomeric MB in membrane and bulk aqueous phases, respectively, and P_0 is the intrinsic partition coefficient of MB between membrane and bulk phases when the surface potential, ψ, is zero. Equation 4 can be rewritten as

$$\frac{n_{mM}}{n_{mB}} = \left(\frac{V_M}{V_B}\right) P_0 \exp[-e\psi/(kT)] \quad (5)$$

where n_{mM} and n_{mB} are the moles of monomeric MB in membrane and bulk phases, respectively.

The equilibrium between the monomeric and dimeric MB in the membrane phase is represented as

$$(C_d/2)/C_{mM}^2 = K_d \quad (6)$$

and, therefore, n_d is related to n_{mM} by

$$n_d = 2K_d n_{mM}^2/V_M \quad (7)$$

where C_d and n_d are the equivalent concentration and gram equivalent of dimer in the membrane phase. The dimerization constant was reported to be $K_d = 2.2 \times 10^3$ L mol^{-1} (Ghosh & Mukerjee, 1970). The volume of the membrane phase, V_M, is proportional to the weight of dried phospholipids in solutions, m (Castle & Hubbell, 1976; Cafiso & Hubbell, 1978), and given by

$$V_M = vm \quad (8)$$

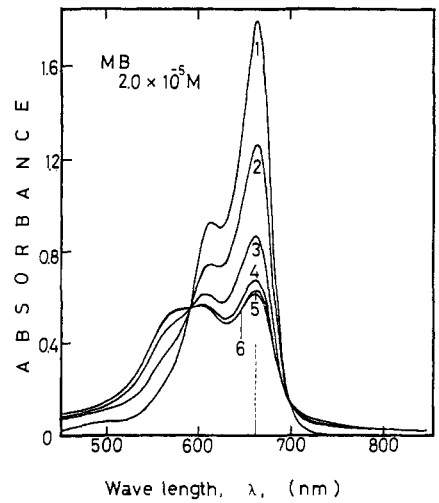

FIGURE 1: MB spectral change upon addition of phospholipid liposomes (membranes). The medium contains 5 mM Hepes, 0.2 mM EDTA–Tris, and 20 μM of MB at pH 6.0, 25 °C. The liposomes were formed by L-α-DPPC and L-α-DPPA in the mole ratio of 0.73:0.27. The amount of (dried) lipids added: (1) 0; (2) 4.00; (3) 8.12; (4) 12.12; (5) 16.08; (6) 20.22 (mg in 100 mL).

where v is the membrane volume per unit weight of phospholipid in the solution. Equation 7 can be rewritten as

$$n_{mM} = [n_d vm/(2K_d)]^{1/2} \quad (9)$$

On the other hand, the number of moles of monomeric MB in bulk phase is given by

$$n_{mB} = n_0 - n_d - n_{mM} \rightarrow n_0 - n_d \quad (10)$$

In eq 10, n_{mM} is neglected, because $V_B \gg V_M$ and $n_{mB} \gg n_{mM}$ if m is small. This point will be examined later in light of the results of this study.

From eq 5, 9, and 10, eq 11 and 12 are obtained

$$(n_d m)^{1/2}/(n_0 - n_d) = Km \quad (11)$$

$$K = K_0 \exp(-e\psi/(kT)) \quad (12)$$

where

$$K_0 = (P_0/V_B)(2K_d v)^{1/2} \quad (13)$$

Results

Absorbance of MB Solution Containing Membranes. The absorbances of MB solutions containing membranes were measured at 25 °C. The pH of the solutions were maintained at 6.0 with Hepes buffer. The membranes were composed of L-α-DPPC and L-α-DPPA. Absorbance measurements were carried out with varying composition and total amount of the membranes in the buffer solutions.

In Figure 1, the results for the membrane systems with a mole fraction of L-α-DPPA, $X_{PA} = 0.27$, are shown. It is seen that the absorbance maximum at the longer wavelength (664 nm) decreases with increasing amounts of the lipid membrane, m (milligram of dried lipids in 100 mL of buffer solution). Also, the wavelength of the maximum absorbance is not changed appreciably.

In Figure 2, the ratios of absorbances with and without membranes (A/A_0) at 664 nm for various compositions of the lipids as a function of the total amount of liposomes, m, are shown. It is found that the absorbance decreases more rapidly for membranes with a higher mole fraction of the acidic (negatively charged) phospholipid, L-α-DPPA. In the case of the membrane composed of only L-α-DPPA, A/A_0 decreased to 0.15 as shown in the figure. On the other hand, the absorbances were not changed appreciably by increasing

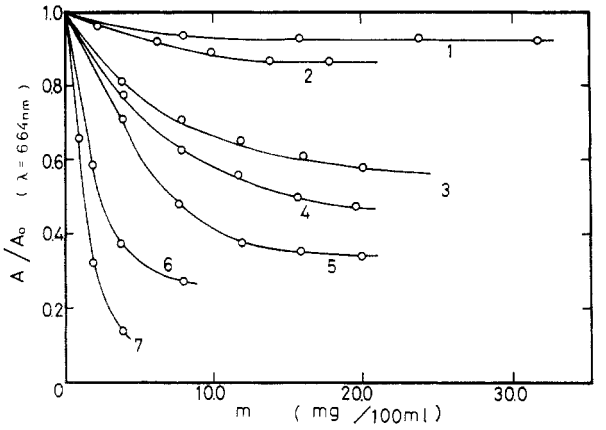

FIGURE 2: Ratio of absorbances with and without membranes, A/A_0, for various lipid compositions as a function of total amount of liposomes, m. X_{PA}: (1) 0.05, (2) 0.10, (3) 0.14, (4) 0.20, (5) 0.27, (6) 0.48, (7) 1.0.

Table I: Numerical Values of n_{mM}, n_{mB}, and the Ratio n_{mM}/n_{mB}[a]

$X_{PA} = 0.27$				$X_{PA} = 0.14$			
m (mg)	$n_{mM} \times 10^8$ (mol)	$n_{mB} \times 10^7$ (mol)	$n_{mM}/n_{mB} \times 10^2$	m (mg)	$n_{mM} \times 10^8$ (mol)	$n_{mB} \times 10^6$ (mol)	$n_{mM}/n_{mB} \times 10^2$
4.00	2.530	12.720	1.989	4.04	2.054	1.521	1.350
8.12	4.752	7.320	6.492	8.10	3.574	1.274	2.802
12.14	6.362	4.740	13.422	12.10	4.698	1.154	4.071
16.08	7.446	4.140	17.986	16.22	5.730	1.540	5.436
20.22	8.398	3.860	21.757	20.24	6.600	0.990	6.667
				31.96	8.528	0.916	9.310

[a] The values were calculated with eq 3, 9, and 10 where the membrane volume for unit weight of dried lipid, v, and the dimerization constant in membrane phase, K_d, were taken as 1 mL g^{-1} and 2.2×10^3 L mol^{-1}, respectively.

the total amount of liposomes composed of pure L-α-DPPC. Buffer solutions containing 50 mg of the membrane (dried lipid base) in 100 mL showed appreciable turbidity.

The decreases in A/A_0 with increasing membrane amounts are considered to be arising due to the partitioning of the positively charged MB molecules into the membrane phase and the formation of dimers (Massari & Pascolini, 1977). It is clear that the partitioning of the dye into the membrane phase increases for liposomes containing higher mole fraction of acidic lipid, L-α-DPPA. By use of eq 3, 9, and 10, the numerical values of the moles of monomeric MB in the membrane phase and the bulk phase, n_{mM} and n_{mB}, were calculated for membranes composed of $X_{PA} = 0.14$ and 0.27 of acidic phospholipid and are shown in Table I. In this calculation, the value of the membrane volume per unit weight of phospholipid, v, is taken as $v = 1$ cm^3/g (Huang, 1969). This value is the partial specific volume of the hydrated phospholipid. It is seen that the values of n_{mM}/n_{mB} change proportionately with m for a membrane of constant lipid composition.

Calculation of Surface Potential. In Figure 3, the values of $(n_d m)^{1/2}/(n_0 - n_d)$, calculated on the basis of the experimental results in Figure 2 and eq 3, are plotted against the total amount of membrane lipid, m, for various compositions of the membrane system. The slopes of these plots give the values of K in eq 11 and 12. It is found that K increases with X_{PA} and, hence, with the surface charge density of membranes.

In Figure 4, the logarithm of K is plotted against the mole fraction of the acidic lipid, L-α-DPPA, in the membrane, X_{PA}. The extrapolation of the logarithm of K to $X_{PA} = 0$ gives the

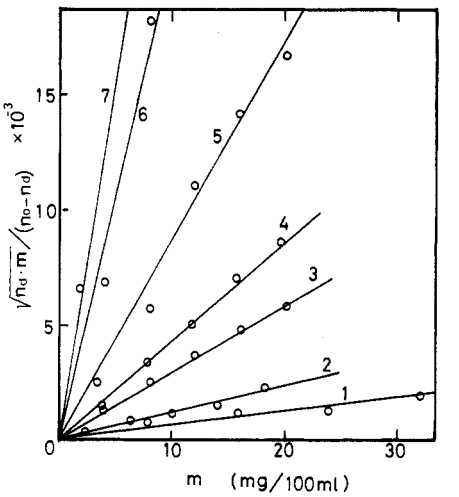

FIGURE 3: Variation of $(n_d m)^{1/2}/(n_0 - n_d)$ for various compositions of membrane, X_{PA}, as a function of total amount of lipid, m. See eq 11 and 12. X_{PA}: (1) 0.05, (2) 0.10, (3) 0.14, (4) 0.20, (5) 0.27, (6) 0.48, (7) 1.0.

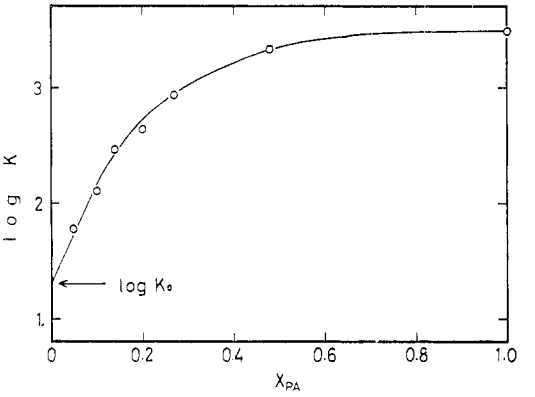

FIGURE 4: Dependence of logarithm of K on composition of membranes. X_{PA}: mole fraction of L-α-DPPA in membranes.

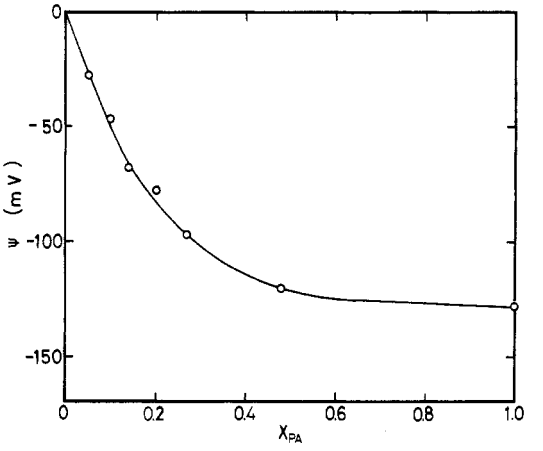

FIGURE 5: Variation of surface potential, ψ, with the membrane composition, X_{PA}.

value of the logarithm of K_0 as shown. The surface potential, ψ, can be calculated by using eq 12 as

$$\psi = 2.30\frac{kT}{e}(\log K_0 - \log K) \qquad (14)$$

where $2.30kT/e = 59.2$ mV at 25 °C. In Figure 5, the variation of surface potential, ψ (in millivolts), as a function of mole fraction of L-α-DPPA is shown.

Effect of Addition of Salt (NaCl). In Figure 6, the effect of the addition of increasing amounts of NaCl on the absorbance of a membrane system with $X_{PA} = 0.14$ and $m = 32$

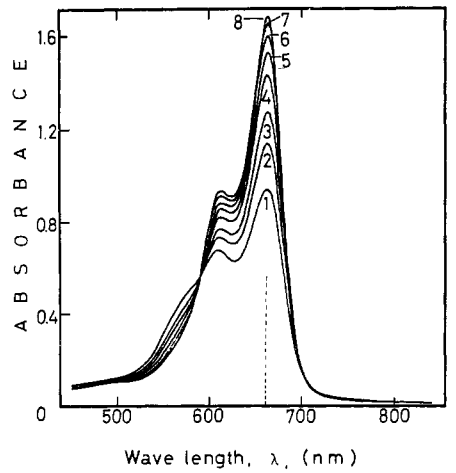

FIGURE 6: MB spectral change upon addition of NaCl. The membrane system consisted of $X_{PA} = 0.14$ and $m = 32$ (mg in 100 mL). The medium was the same as in Figure 2 except for the concentration of NaCl. [NaCl]: (1) 0, (2) 1.03, (3) 2.03, (4) 4.02, (5) 6.01, (6) 8.00, (7) 12.01, (8) 20.03 (mM).

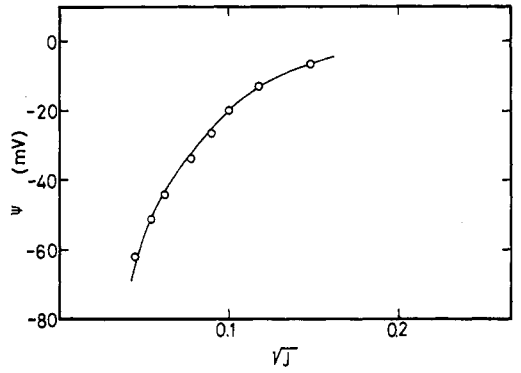

FIGURE 7: Variation of surface potential of membranes, ψ, with ionic strength, J. $J = 0.002 +$ [NaCl] (M), where 0.002 M is the buffer contribution.

mg is shown. It is found that upon increasing the concentration of NaCl the absorbance increased gradually, and, at 20 mM NaCl, 95% of the absorbance reduction due to the partitioning of the dye into the membrane phase is restored.

From eq 11 and 12, we get

$$\psi = \psi_i - 59.2 \log \frac{n_d^{1/2}/(n_0 - n_d)}{[n_d^{1/2}/(n_0 - n_d)]_i} \qquad (15)$$

where the subscript i denotes the values without NaCl. The changes in the surface potential, ψ, upon addition of NaCl were calculated on the basis of the experimental results in Figure 6 and eq 15.

Figure 7 shows the variation of surface potential, ψ, with ionic strength, J, for the membrane system composed of $X_{PA} = 0.14$ and $m = 32$ mg. The ionic strength, J, is given by $J = 0.002 +$ [NaCl] (M), where the buffer contribution is equal to 0.002 M.

Discussion

A phosphatidic acid such as L-α-DPPA carries a single net negative charge at pH 6.0 (Abramson et al., 1964). Phosphatidylcholine, L-α-DPPC, is zwitterionic and carries no net charge. The charge densities of mixed membranes (liposomes) containing L-α-DPPA and L-α-DPPC can be changed by changing the composition of the lipids. MB molecules which carry a single positive charge in a molecule would be attracted to the negatively charged membrane phase to form dimers.

The numerical values of the moles of monomeric MB were calculated and are shown in Table I. It is found that the values of n_{mB} are much higher than those of n_{mM}. These results prove that the approximation in eq 10 is valid. However, in the case of membranes composed of pure L-α-DPPA, the partitioning of MB molecules into membrane phases is so high that the values of n_{mB} are comparable with those of n_{mM}. The neglect of n_{mM} in eq 10 for this membrane system would give higher values of n_{mB} and higher values of surface potential, ψ. At 4 mg of L-α-DPPA, almost all MB molecules would be forming dimers in the membranes composed of the acidic lipid. The probe to lipid ratio is about 1:5, which modifies the charge densities significantly. This situation would also result in erroneous surface potential estimates.

The value of A/A_0 for the membrane system of 4 mg of DPPA is about 0.15, which is similar to the value of ϵ_m/ϵ_d in the bulk aqueous phase. This result indicates that the values of ϵ_d in the membrane phase and bulk phase are similar (Massari & Pascolini, 1977). An increase in the total membrane amount, m, beyond the amount where almost all dye molecules are in the membrane phase resulted in a gradual increase in the absorbance due to an increase in the available membrane volume and a decrease of concentration of MB in the membrane phase.

The experimental results of A/A_0 and eq 3, 11, and 12 are sufficient to calculate the surface potential. Therefore, the numerical values of K_d (in membrane phase) and v have no effect on the calculated values of surface potential. In eq 13, the value of K_0 was obtained experimentally. If the value of v is higher by a factor of 10, then the value of P_0 would be $10^{1/2}$ times lower. The uncertainty in v, therefore, affects only P_0 and does not alter or affect K or K_0, since these are experimentally determined. Subsequently, the surface potential, ψ, calculated by using K and K_0 is also independent of uncertainty in v.

The surface potential is found to be sensitive to changes in ionic strength. As shown in Figure 7, the addition of 20 mM NaCl results in a change of 60 mV in surface potential for the membrane system composed of $X_{PA} = 0.14$. If the value of X_{PA} is higher, the change in surface potential is expected to be larger.

In this study, the ionic strength inside and outside the liposome (membrane) is the same. If there is a difference in the ionic strength between aqueous phases inside and outside the membrane, the difference of potential across the membrane would be significant (the contribution to the transmembrane potential due to the membrane charge). This contribution should be added to the potential contribution due to diffusion of cation across the membrane (Waggoner, 1976; Cafiso & Hubbell, 1978) to give the total transmembrane potential. For membranes with negative charge, the two contributions have the same direction. On the other hand, for membranes with positive charge, the two contributions to the transmembrane potential oppose each other. Furthermore, the real availability of the transmembrane pH difference (Deamer et al., 1972; Cafiso & Hubbell, 1978), which is considered to have an important role in processes such as oxidative phosphorylation, will depend upon the transmembrane potential and the charge density of the membrane.

The concentration of lipids (membranes) used in this study ranges from 5 to 30 mg of dried lipid in 100 mL of the buffer solution. This range of the lipid concentration is much lower than the range used in other studies (Castle & Hubbell, 1976; Mashimo et al., 1979). In our experiments, 30 mg of dried lipid in 100 mL of the buffer solution is the maximum concentration where absorbance measurements in the visible wavelength region are not complicated by appreciable turbidity

(in the UV region, the turbidities are always more serious). Even fluorescent spectra were found to be not free from optical scattering due to the membrane system.

Smaller membrane volumes also simplify the calculation of the potentials to a great extent. For example, the volumes of membrane phase, V_M, are so small in this study that the values of $V_M + V_B$ are considered to be almost the same as the values of V_B.

When the lipid amount is 5–50 mg in 1 mL of aqueous solution, the available volume for a unilamellar liposome, which is composed of about 5000 lipid molecules and has a diameter of 250–300 Å, would be about 10^8–10^9 Å^3. For sodium dodecyl sulfate solutions (Mukerjee & Banerjee, 1964; Fernandez & Fromherz, 1977; Funasaki, 1977), a concentration 10 times the critical micellar concentration results in an available volume per micelle, composed of 60 surfactant molecules, of 10^6 Å^3. These values underline the difficulty of distinguishing the "distinct bulk aqueous phase" where the value of the potential should be taken as zero and also indicate the overlapping of electrical double layers of different liposomes or micelles (Mille & Vanderkooi, 1977; Huisman, 1964; Mukerjee, 1972; Winsor, 1953). The available volume per liposome is at least 3×10^{10} Å^3 in this study. Therefore, the theory of Guoy–Chapman or Donnan could be easily applied and examined. Further work on the theoretical aspects of our findings are in progress and shall be published later.

However, 2×10^{-5} M of MB has been used in this study, although a concentration of 2×10^{-6} M of MB is assumed to be satisfactory. This concentration corresponds to at most one probe for 25 lipid molecules.

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Stereochemical and Kinetic Investigation of ^{32}P-Labeled Inorganic Phosphate Exchange Reaction Catalyzed by Primer-Independent and Primer-Dependent Polynucleotide Phosphorylase from *Micrococcus luteus*†

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ABSTRACT: The S_P diastereomer of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS) is a substrate for the ^{32}P-labeled inorganic phosphate exchange reaction catalyzed by the T and I forms of polynucleotide phosphorylase. The exchange reaction occurs with retention of configuration. This exchange reaction is very slow when only ADPαS(S_P) is present but is greatly activated by dinucleotide primers and ADPαS(R_P), although the latter is not a substrate for the exchange reaction. Ap(S)A(R_P) is an ~50% better activator of the exchange than the S_P diastereomer. Furthermore, high levels of the ADPαS(S_P) eliminate the activation by primers and by ADPαS(R_P). A phosphatase activity is present with the I form of the enzyme which converts ADPαS(R_P) to AMPS. This activity may be responsible for the formation of the 5′-phosphate end group for de novo polymerization or for the processivity of this reaction.

Polynucleotide phosphorylase catalyzes the reversible polymerization of nucleoside diphosphates to polynucleotides and inorganic phosphate. Two forms of this enzyme have been described from the bacterial source *Micrococcus luteus* (Godefroy-Colburn & Grunberg-Manago, 1972). The native, or I form, catalyzes de novo polymerization of nucleoside diphosphates in the 5′ → 3′ direction via a processive mechanism. Only large molecular weight polymers are released from the enzyme (Moses & Singer, 1970), and they are terminated at the 5′ end by a phosphate group (Godefroy-Colburn & Grunberg-Manago, 1972). Polymerization is stimulated by oligonucleotides although they are not incorporated into the 5′ end of the polymer. The trypsinized form of the enzyme, form T, has a reduced de novo polymerization activity but

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