Activation Energy for Permeation of Phosphonium Cations through Phospholipid Bilayer Membrane[†]

Atsushi Ono,[‡] Seiji Miyauchi,[‡] Makoto Demura,[§] Tetsuo Asakura,[§] and Naoki Kamo[*,‡]

Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, and Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, Japan

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ABSTRACT: The conductance caused by translocation of various phosphonium cations across phospholipid (from soybean) bilayer membrane was measured. Phosphonium cations used were tetraphenylphosphonium (TPP^+) and triphenylalkylphosphonium cations formulated as $(Phe)_3\text{-}P^+\text{-}(CH_2)_nCH_3$ (n = 0–5). The conductance was dependent on voltage applied externally to the membrane in accordance with a theory developed by previous authors. Using the theory, values of βk_i were determined, where β and k_i are a linear partition coefficient and a rate constant of transmembrane ion transport, respectively. Since βk_i depended on the phosphonium ion concentration, values extrapolated to infinite dilution, $(\beta k_i)_0$, were determined. Temperature dependence of $(\beta k_i)_0$ allowed us to estimate the activation energy of transport, E_a. For TPP^+, thermodynamic values obtained were consistent with values calculated by Flewelling and Hubbell [(1986) *Biophys. J. 49*, 541–552]. When $(Phe)_3\text{-}P^+\text{-}(CH_2)_nCH_3$ (n = 0–5) were used, E_a depended on the odd or even of n. This "odd and even" pattern was observed in a variety of phenomena such as solubility in water, equivalent ionic conductivity in water, and ^{31}P NMR chemical shift.

Biological membranes are permeable to lipophilic or hydrophobic ions due to the unique character that they are soluble in both hydrophilic and hydrophobic solvents. This character is not found in small ions and most other charged molecules that cannot translocate through the membrane without a special device in the membrane called a channel, transporter, or ionophore.

The high permeability of membranes to lipophilic ions is used to estimate the membrane potential of cells or vesicles which are too small for microelectrodes to be impaled (Bakeeva et al., 1970; Harold & Papineau, 1972; Schuldiner & Kaback, 1975; Rottenberg, 1979; Kamo et al., 1982). At equilibrium, the following Nernst equation holds because the electrochemical potentials of lipophilic ions inside and outside the cells or vesicles should be equal.

$$E_m = \frac{RT}{z_iF}\ln\left(\frac{C_{out}}{C_{in}}\right)$$

Here, E_m is the membrane potential with reference to the outside, C_{out} the concentration (strictly, activity) of lipophilic ions outside the cell, C_{in}, the concentration (activity) of lipophilic ions inside the cell, z_i, the electronic charge of lipophilic ions, R, the gas constant, T, the absolute temperature, and F, the Faraday constant. This equation indicates that determination of the concentration ratio of C_{out} to C_{in} gives the membrane potential. Since ordinary cells have interior negative membrane potential, lipophilic cations are frequently used. Typical lipophilic cations are tetraphenylphosphonium (TPP^+),[1] triphenylmethylphosphonium ($TPMP^+$), or their homologues.

Kinetic analyses on the uptake of lipophilic cations have been done using liposomes or bacterial envelope vesicles (Shinbo et al., 1983; Demura et al., 1985a). The experiments using liposomes, vesicles, or intact cells have certain limitations. In this paper, we measure the transport rate of phosphonium ions as an electric current passing through phospholipid bilayer membranes.

Early studies (LeBlanc, 1969; Liberman & Topaly, 1969) showed that lipophilic anions have much greater permeability than cations: tetraphenylboron (TPB^-) has much greater value than tetraphenylphosphonium (TPP^+) in spite of structurally similar analogues. The first successful model accounting for the difference of the transport rate between lipophilic cations and anions was that of Ketterer, Nuemacke, and Läuger (1971), who developed charge–pulse experiments. They proposed that lipid bilayers possess a substantial membrane dipole potential (positive inside) which is responsible for the

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[*] To whom all correspondence should be addressed; tel/fax: 81-11-706-4984.

[‡] Hokkaido University.

[§] University of Agriculture and Technology.

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[1] Abbreviations: A, $2k_i/k$; C, phosphonium cation concentration in aqueous solutions separated by membrane; DMSO, dimethyl sulfoxide; DPH, diphenylhexatriene, E_a, activation energy; $G(V)$, steady-state conductance at the applied voltage V; $G(0)$, conductance at $V = 0$; h, Planck's constant, k, rate constant of interfacial ion transport; k_i, rate constant of transmembrane ion transport; N_A, Avogadro's number; NMR, nuclear magnetic resonance; S, solubility; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-3,5-triene; $TPAP^+$, triphenylamylphosphonium; $TPBP^+$, triphenylbutylphosphonium; $TPEP^+$, triphenylethylphosphonium; $TPMP^+$, triphenylmethylphosphonium; TPP^+, tetraphenylphosphonium; $TPPP^+$, triphenylpropylphosphonium; Tris, tris(hydroxymethyl)aminomethane; β, linear partition coefficient, a ratio of the surface density of adsorbed ions to their volume density in the aqueous solution; $(\beta k_i)_0$, βk_i value at infinity dilution; ΔH^*, ΔS^*, and ΔG^*, enthalpy, entropy, and Gibbs free-energy changes associated with the transfer of lipophilic cation from the adsorption site to the top of the energy barrier; ΔH_b, ΔS_b, and ΔG_b, enthalpy, entropy, and Gibbs free-energy changes associated with the transfer of lipophilic cation from the aqueous solution to the adsorption site in the bilayer; δ, binding-region thickness; Λ_0, equivalent conductivity at infinite dilution; λ_0, equivalent ionic conductivity; μ, reduced potential difference (FV/RT); ω, parameter originating from the membrane thickness.

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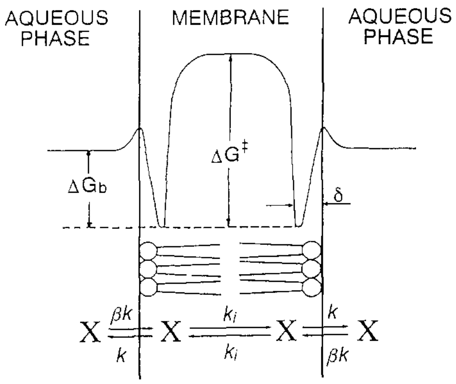

FIGURE 1: Potential profile for hydrophobic ions within the phospholipid bilayer membrane after Ketterer et al. (1971) and Flewelling and Hubbell (1986). An energy barrier is present in the middle of the membrane over which lipophilic ions must pass. Two binding sites of the lipophilic ions within the membrane are located near the two membrane interfaces, where the potential energy within the membrane is the lowest. Thus, these ions are present at the four sites shown by X in this figure. δ is the width of the adsorption potential energy well. k denotes the rate constant of the ion from the binding site at the interface to the bulk solution, and βk_i is the rate constant of the opposite direction. The meaning of β is a type of binding constant and is the ratio of the surface density of bound lipophilic ion molecules to that of free molecules in the solution; thus, β has unit of length. k_i is the rate constant of the translocation passing over the barrier in the absence of the external electric field. The Gibbs free-energy difference of lipophilic ions from the bulk aqueous solution to the binding site at the interface is denoted as ΔG_b and the energy difference between the binding site and the top of the barrier as ΔG^*.

difference in transport rate of anions and cations. Flewelling and Hubbell (1986b) calculated the potential energy profile within bilayer membranes for hydrophobic ion by combining four terms: the electrical Born, the image and dipole contributions, and a neutral energy term. The potential profile they deduced is essentially the same as that by Ketterer et al. The potential profile within the bilayer membrane is shown in Figure 1, where notations relevant to this paper are also given. Many experiments have since confirmed this basic potential profile (Andersen, 1978; Haydon & Hladky, 1972; Hladky, 1979). In addition, modification of surface dipole moment by addition of dipole moment modifiers is known to change the transport rate (Smejtek, 1987; Pickar & Benz, 1978).

Although there has been some discussion (Flewelling & Hubbell, 1986b) and experimental studies (Benz, 1988) on the potential profile within the membrane or on the activation energy of the translocation, we measured the activation energy of lipophilic ion translocation through bilayer membranes. The lipophilic ions used were TPP^+ and triphenylalkylphosphonium ions formulated as $(Phe)_3$-P^+-$(CH_2)_nCH_3$. We found that the activation energy depended on whether n was odd or even. In addition, a variety of phenomena such as solubility in water, equivalent ionic conductivity in water, and ^{31}P NMR chemical shift showed this "odd and even" pattern.

EXPERIMENTAL SECTION

Materials. The phospholipids used were partially purified soybean phospholipid by the method of Kagawa and Racker (1971). Analysis with two-dimensional thin-layer chromatography on silica gel (first developing solvent, $CHCl_3$:methanol:25% aqueous ammonia = 65:35:8, and second developing solvent, $CHCl_3$:acetone:methanol:acetic acid:H_2O = 10:4:2:2:1) gave the following contents: phosphatidylcholine, 37.9%; phosphatidylethanolamine, 30.3 %; cardiolipin, 5.3%; phosphatidylethanol, 3.4%; phosphatidylinositol, 3.5%; phosphatidylserine, 3.2%; phosphatidic acid, 15.2%; and others, 1.2%. No spot of free fatty acid was observed.

TPP^+ as Cl^- salt was obtained from Dojindo Chemical Co. (Kumamoto, Japan). Triphenylalkylphosphonium cations used were triphenylmethylphosphonium ($TPMP^+$), triphenylethylphosphonium ($TPEP^+$), triphenylpropylphosphonium ($TPPP^+$), triphenylbutylphosphonium ($TPBP^+$), triphenylamylphosphonium ($TPAP^+$), and triphenylhexylphosphonium ($TPHP^+$); all except $TPPP^+$ were purchased from Tokyo Kasei Co. (Tokyo), and $TPPP^+$ was from Aldrich (Milwaukee, WI). They were obtained as Br salt. In solubility experiments, the concentration of the phosphonium cation was estimated by absorbance at 267 nm.

Fluorescent probes of 1-[4-(trimethylamino)phenyl]-6-phenylhexa-3,5-triene (TMA-DPH) (Prendergast et al., 1981) and diphenylhexatriene (DPH) were obtained from Dojindo Chemical Co. (Kumamoto, Japan). Other chemicals were of highest quality.

Formation of Planar Bilayer Membrane and Measurement of I-V Curve. The planar lipid bilayers were formed by the folding method which was originally developed by Takagi et al. (1965) and modified by Montal and Mueller (1972). This procedure involves the use of a Teflon chamber with two compartments (each about 2.0 mL in internal volume) separated by a septum (membrane kit 5775; Yellow Springs Instruments, Yellow Springs, OH; 12.5 μm thick) with an aperture 100–200 μm in diameter. The chamber was placed in a laboratory-made shield box in which a temperature-controlling apparatus had been installed: temperature of the solution in the compartments was measured and kept constant at a desired temperature within ±0.05 °C. The shield box was fabricated to minimize the noise by mechanical vibration or sound. Electric current was passed through a pair of Ag–AgCl electrodes and measured with an amplifier (CEZ-2200; Nihon Kohden, Ltd., Tokyo) whose output signal was fed to a pen-writing recorder after filtration through a low-pass filter when necessary.

A small amount (ca. 5 μL) of lipid in normal hexane (2% w/v) was placed on the surface of a buffer solution (50 mM Tris-HCl, pH 7.4) whose level was below the aperture. When the activation energy was determined, pH was adjusted at 7.4 for each temperature employed. A lipid monolayer was formed spontaneously at the air/water interface after evaporation of the solvent within a few minutes. The water level in each compartment was then raised to above the aperture, where the lipid bilayer subsequently formed. Prior to experiments, the formed bilayer was allowed to stand for about 10 min so that it was stabilized.

To confirm that the formed membrane was a bimolecular membrane, electric capacitance was measured; a voltage (triangular-shaped wave of 50 Hz, 17 mVpp) was applied to the bilayer, and the capacitive current passed was measured. The capacitance was 500–700 nF/cm^2, and membrane resistance was more than 200 GΩ.

After the stabilization, a small volume of the lipophilic cation solution was added to each chamber so that both concentrations were the same. The steady transmembrane current was determined by incremental increases in voltage (10-mV increments).

Temperature Dependence of Membrane Viscosity. Temperature dependence of liposomal membrane viscosity was estimated from the steady-state fluorescence anisotropy using TMA-DPH, a cationic analogue of DPH. Liposomes (small unilamellar vesicle, SUV) from soybean phospholipid were prepared by the usual method. The anisotropy was measured with a Shimadzu spectrofluorophotometer (Model RF-540) with a thermostatic cell compartment. Anisotropy was obtained by the usual method. The membrane viscosity was estimated from the anisotropy according to an equation derived by Araiso and Koyama (1988). Their method was developed for DPH but is applicable to TMA-DPH data since physical quantities such as lifetime and effective volume of the fluorophore are almost equal to each other.

Equivalent Ionic Conductivities at Infinite Dilution. Equivalent conductivities of various phosphonium cations, Λ, in water and dimethyl sulfoxide (DMSO) were measured with an AC bridge (Model 4265B; Yokogawa-Hewlett Packard, Tokyo); the conductance cell was immersed in a silicon bath regulated at 25.0 °C. Values of Λ were extrapolated to zero concentration to obtain equivalent conductivity at infinite dilution, Λ_0, according to the Onsager equation (Harned & Owen, 1958a); we observed a linear equation in the plot of Λ against $\sqrt{C}$ below 5 mM. Since the equivalent ionic conductivity of Br^- is 78.1 Ω cm^2/mol in water and 23.6 Ω cm^2/mol in DMSO (Harned & Owen, 1958b), the equivalent ionic conductivity of phosphonium cations, λ_0, was calculated as λ_0 (in aqueous solution) = $\Lambda_0 - 78.1$ and λ_0 (in DMSO) = $\Lambda_0 - 23.6$.

^{31}P NMR Measurement. Measurements were performed with a JEOL FX-90Q NMR spectrometer (JEOL, Tokyo) operating at 36.25 MHz with gated proton decoupling at 25 °C using 85% H_3PO_4 as an external standard. A total of 16–32 free induction decays were accumulated with a 10.8-μs, 45° pulse, a 3.8-s interpulse time, 8K data points, and a 5K spectral width. For signal locking during the aquisition, the external ^7Li signal was used. The concentration of phosphonium cations was adjusted to 400 mM using D_2O, DMSO-d_6, and $CDCl_3$ as solvents.

RESULTS AND DISCUSSION

The conductance of bilayer membrane was measured in the presence of varying concentrations of TPP^+. Figure 2A shows the relationship between the current and the voltage in the presence of various concentrations, and Figure 2B shows the relationship between the conductance and the applied voltage, revealing that the conductance is voltage dependent. These results are consistent with the theory developed by Ketterer et al. (1971). The steady conductance at the applied voltage of V, $G(V)$, was calculated in accordance with the potential profile within the bilayer membrane and is given as follows:

$$\frac{G(V)}{G(0)} = \frac{2}{\mu}(A+1)\exp(-\omega\mu^2)\frac{\sinh\left(\frac{\mu}{2}\right)}{A\exp(-\omega\mu^2)\cosh\left(\frac{\mu}{2}\right)+1} \qquad (1)$$

where μ is the reduced potential difference ($\mu = FV/RT$), ω, a parameter originating from the membrane thickness, A, $2k_i/k$, k, the rate constant of interfacial ion transport, and k_i, the rate constant of transmembrane ion transport (also, see the legend to Figure 1).

In eq 1, $G(0)$ is the conductance at $V = 0$ and given by

$$G(0) = \frac{F^2}{RT}\beta k_i C \qquad (2)$$

where C is the concentration of lipophilic cation (C of both solutions separated by the membrane are the same). β in this equation is a linear partition coefficient, a ratio of the surface density of adsorbed ions to their volume density in the aqueous solution (see Figure 1).

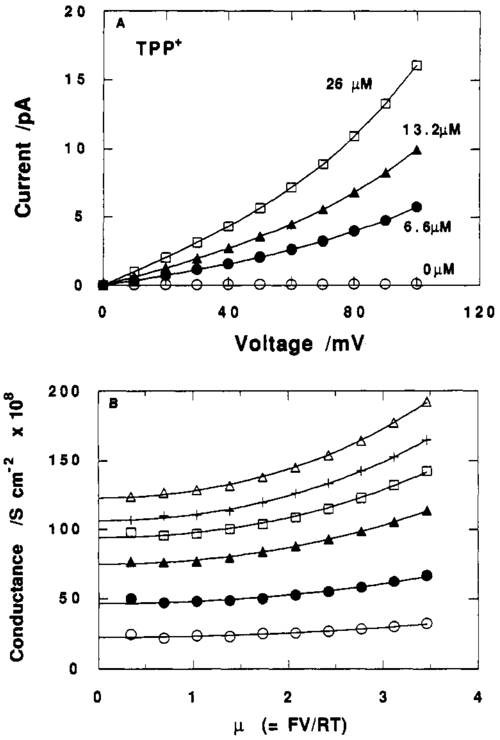

FIGURE 2: Plots of current (A) and conductance (B) against the voltage applied across the membrane. In panel A, TPP^+ concentrations were (○) 0 μM, (●) 6.6 μM, (▲) 13.2 μM, and (□) 26 μM. In panel B, the unit of the abscissa is the reduced potential difference μ. The TPP^+ concentrations used were (○) 3.3 μM, (●) 6.6 μM, (▲) 13.2 μM, (□) 19.6 μM, (+) 26.0 μM, and (△) 38.5 μM. Temperature was 25.5 °C. Bilayer membrane was formed with partially purified soybean phospholipid, and the aqueous solution contained 50 mM Tris-HCl, pH 7.4.

The regression of data obtained with eq 1 gives $A = 0$ and $\omega = 0.008$, and the solid lines in Figure 2B are the calculated curves. Ketterer et al. (1971) showed that for the transport of lipophilic cations the rate-determining step is the transport within the membrane, i.e., $k \gg k_i$, meaning that $A = 0$. According to a theory (Andersen & Fuchs, 1975), the value of $\omega = 0.008$ corresponds to a membrane thickness of 6 nm. If the dielectric constant of the hydrocarbon layer is assumed to be 4, the membrane capacitance of 500–700 μF/cm^2 gives a membrane thickness of 7–5 nm. Thus, eq 1 is simplified to eq 3.

$$\frac{G(V)}{G(0)} = \frac{2}{\mu}\exp(0.008\mu^2)\sinh\left(\frac{\mu}{2}\right) \qquad (3)$$

From the experimental relationship of $G(V)$ vs V obtained under various conditions, the values of $G(0)$ were determined by the nonlinear least-squares method using this equation, and the values of βk_i were calculated. We adapted the computer program from one by previous authors (Yamaoka et al., 1987).

To learn whether the electric current observed is due solely to the passage of lipophilic cations across the membrane, we examined the effect of NaCl addition in the chambers on the $G(0)$ under the condition of constant TPP^+ concentration (24 μM). The relationship between conductance and μ followed eq 3 well for all data obtained, and the values of $G(0)$ (S/cm^2) were as follows: 105 at 0 mM (i.e., buffer solution alone), 89.7 at 10 mM, 83.3 at 20 mM, and 78.6 × 10^{-8} at 30 mM. The results were essentially the same when KCl was used

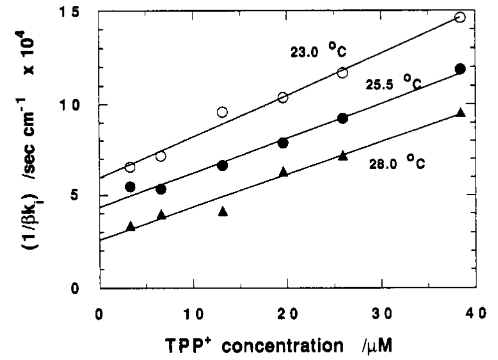

FIGURE 3: Dependence of the values of βk_i on the concentration of lipophilic cation. Under the condition of constant temperature (23.0, 25.5, and 28.0 °C), βk_i was measured at varying concentrations of TPP^+. Other experimental conditions were the same as those in Figure 2. The ordinate intercepts in this plot are denoted by $1/(\beta k_i)_0$; $(\beta k_i)_0$ means the value at infinite dilution.

instead of NaCl. The values of βk_i decreased as NaCl or KCl concentration increased, which is incompatible with the idea that small ions such as Na^+, K^+, or Tris base are responsible for the electric current. It is noted also that in the absence of lipophilic cations, we did not observe the electric current (see Figure 2A), and the observed relationship between current and applied voltage followed eq 3 even in the presence of added small salts. The reason for this decrease is interpreted as follows: the increase in the salt concentration reduces the negative surface potential of the membrane, which gives rise to a decrease in β. Actually, we obtained a rough linear relation when $G(0)$ was plotted against the square root of the ionic strength of the medium. Hence, we concluded that the electric current observed is due only to the translocation of lipophilic cations.

Values of βk_i were found to depend on the lipophilic cation concentration C. When the reciprocal of βk_i was plotted against C, a linear relation was found. Figure 3 shows results obtained with TPP^+, and fairly good linearity is obtained for all temperatures. The concentration dependence of βk_i was also reported previously (Miyauchi et al., 1993). The reason for the concentration dependence of βk_i may be due partially to the fact that the binding of various phosphonium cations to liposomal membrane follows the Langmuir-type equation (Demura et al., 1985b, 1987a,b; Kamo et al., 1986). The ordinate-intercept value in Figure 3 is the βk_i value at infinite dilution of C and is denoted as $(\beta k_i)_0$. The activation energy discussed below was estimated from $(\beta k_i)_0$.

First, we analyzed TPP^+ data because detailed data on β was published earlier, although the lipid used was egg-PC (Flewelling & Hubbell, 1986a). Typical Arrhenius plots are shown in Figure 4A, where $\ln(\beta k_i)_0$ is plotted against $1/T$, and $\ln(\beta k_i)$ values at 13.2 and 26.0 μM of TPP^+ are also plotted to show the concentration dependence on the activation energy. Figure 4B shows the plot of E_a against TPP^+ concentration. The activation energy for $(\beta k_i)_0$, E_a at the infinite dilution, is calculated to be 29.7 kcal/mol. Benz (1988) measured the activation energies for the translocation of various lipophilic anions through dioleoyl phosphatidylethanolamine or dioleoyl phosphatidylcholine membranes and found that values were in the range of 7.6–9.8 kcal/mol. The value is much smaller than 29.7 kcal/mol, E_a of TPP^+, which is consistent with the notion that lipophilic anions have much greater permeability than cations.

As shown in the Appendix, E_a estimated here is equal to $\Delta H_b + \Delta H^{\ddagger} + RT$, where ΔH_b and $\Delta H^{\ddagger}$ are the enthalpy change associated with the transfer of lipophilic cation from the aqueous solution to the adsorption site in the bilayer and the enthalpy change associated with the transfer of lipophilic cation from the adsorption site to the top of the energy barrier, respectively. The value of ΔH_b for TPP^+ binding to egg-PC liposomes was 3.5 ± 0.3 kcal/mol (Flewelling & Hubbell, 1986a). The value of $\Delta H^{\ddagger}$ was, thus, estimated to be 25.6 kcal/mol. Since $\Delta S_b = 21 \pm 1$ cal/mol/deg (Flewelling & Hubbell, 1986a), $\Delta S^{\ddagger}$ is estimated to be 29.8 cal/mol/deg and $\Delta G^{\ddagger}$ is 16.7 kcal/mol. Theoretical calculation by Flewelling and Hubbell (1986a) yielded that $\Delta G^{\ddagger} = 17.8$ kcal/mol. This value is very close to that obtained in our experiment if we neglect the difference of lipids used.

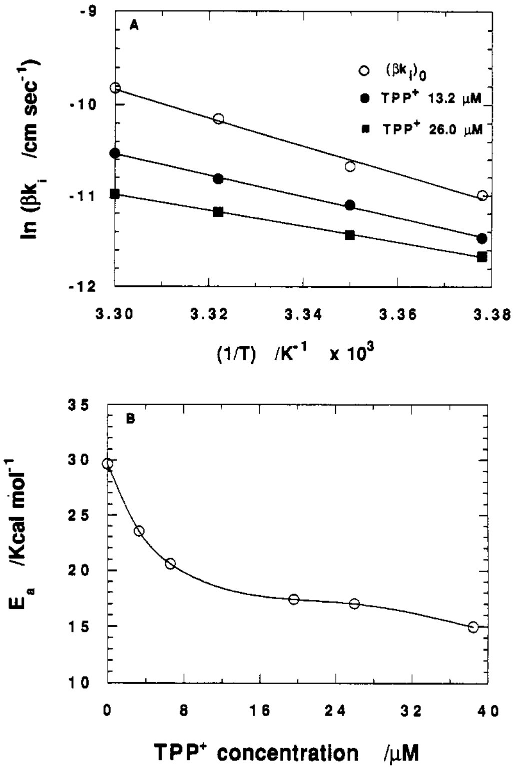

FIGURE 4: Dependence of Arrhenius-type plots (A) and the activation energy (B) on the concentration of lipophilic ions. In panel A, the TPP^+ concentrations were 13.2 μM (●) and 26.0 μM (■). The ordinate-intercept values at varying temperatures (see Figure 3) were plotted (○). In panel B, the activation energies at varying TPP^+ concentrations are calculated from panel A and plotted. The value plotted at zero TPP^+ concentration is from $(\beta k_i)_0$.

The membrane viscosity change may change k_i. From the Arrhenius plot of the membrane viscosity estimated from fluorescent anisotropy, we evaluated 3.2 kcal/mol as activation energy of the viscosity (data not shown). Hence, the value of $\Delta H^{\ddagger}$ calculated above may be overestimated by 3.2 kcal/mol.

The values of $(\beta k_i)_0$ for $TPMP^+$ homologues were determined by the same method as that used for TPP^+, and the results are shown in Figure 5, where the number in the abscissa stands for n of $(Phe)_3$-P-$(CH_2)_n$-CH_3. With increase of the chain length, $(\beta k_i)_0$ increased but with small irregularity. Previously, we showed (Kamo, 1991) that the logarithmic values of the binding constant of triphenylalkylphosphonium to liposomes, rat liver mitochondria, and envelope vesicles of *Halobacterium halobium* were linear with respect to n.

The activation energies of homologues of $TPMP^+$ were also determined, and the results are plotted in Figure 6, where E_a of various phosphonium ions is plotted against the lipophilic cation concentration. The data points plotted on the ordinate were values estimated from $(\beta k_i)_0$. This figure shows again

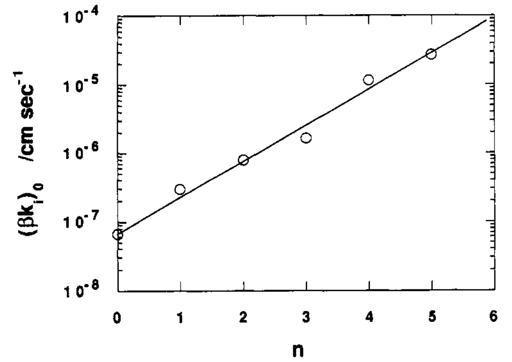

FIGURE 5: $(\beta k_i)_0$ for various triphenylalkylphosphonium cations formulated as $(Phe)_3$-P^+-$(CH_2)_n$-CH_3. The abscissa represents the n value of this formula. See Table 1 to correspond n with the abbreviation for each phosphonium cation. Temperature was 25.0 °C. Other experimental conditions were the same as those in Figure 2.

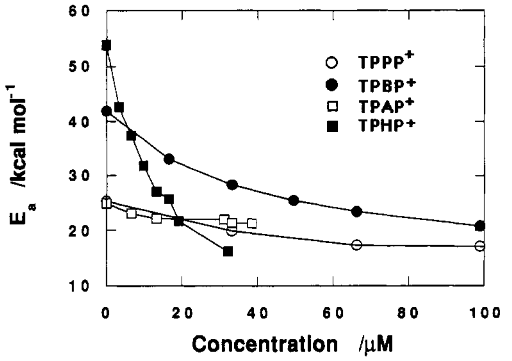

FIGURE 6: Activation energy of various phosphoniums as a function of concentration. Experimental conditions were the same as those in Figure 2, except for variations in temperature. Phosphoniums used were (○) TPPP$^+$, (●) TPBP$^+$, (□) TPAP$^+$ and (■) TPHP$^+$. The values plotted on the ordinate were obtained from $(\beta k_i)_0$, as shown in Figures 3 and 4.

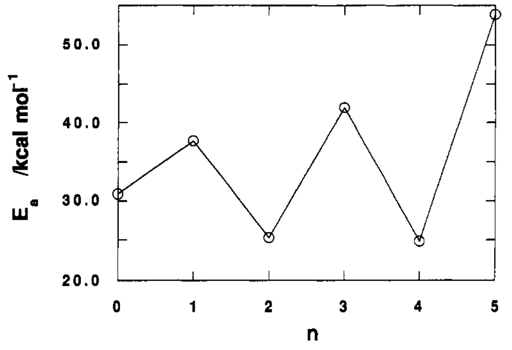

FIGURE 7: Plot of activation energies for various phosphonium cations against n value of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3. The odd and even pattern was observed.

the concentration dependence of E_a, and for TPHP$^+$, it was especially obvious. We hereafter discuss only E_a at infinite dilution, as plotted in Figure 7. It is very interesting that the activation energy depends on whether n is odd or even: TPMP$^+$, TPPP$^+$, and TPAP$^+$ have low activation energies (from 25 to 31 kcal/mol), and TPEP$^+$, TPBP$^+$, and TPHP$^+$ have high values (38, 42, and 54 kcal/mol, respectively). Table 1 summarizes the values of $\Delta H^\ddagger + \Delta H_b$, $\Delta S^\ddagger + \Delta S_b$, and $\Delta G^\ddagger + \Delta G_b$ for the phosphonium cations used. Here, the entropy was calculated by eq A6 and also showed the odd and even pattern. [After Flewelling and Hubbell (1986b), δ was taken as 0.4 nm]. We recognized a decrease in the free energy as n increased, which is consistent with the idea that more lipophilic ions may move faster within the membrane.

Table 1: Thermodynamic Parameters for Translocation of Tetraphenylphosphonium and Triphenylalkylphosphonium across Lipid Bilayer Membrane at 25 °C[a]

phosphonium	n	$\Delta H^\ddagger + \Delta H_b$ (kcal/mol)	$\Delta S^\ddagger + \Delta S_b$ (cal/mol/K)	$\Delta G^\ddagger + \Delta G_b$ (kcal/mol)
TPP$^+$		29.1	50.8	14.0
TPMP$^+$	0	30.3	44.1	17.2
TPEP$^+$	1	37.1	69.9	16.3
TPPP$^+$	2	24.6	30.2	15.6
TPBP$^+$	3	41.4	87.7	15.3
TPAP$^+$	4	24.5	30.5	14.3
TPHP$^+$	5	53.3	133.3	13.6

[a] The activation energy of the membrane viscosity was 3.2 kcal/mol. The value of $\Delta H^\ddagger$, hence, may be overestimated by 3.2 kcal/mol.

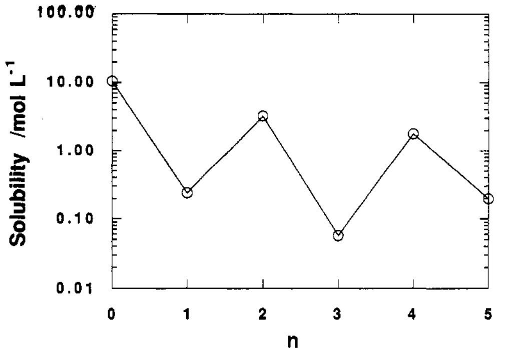

FIGURE 8: Plot of solubilities in water for various phosphonium cations against n value of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3. Temperature was 25.0 °C. The odd and even pattern was observed.

Why does the odd or even of n change the activation energy, $\Delta H^\ddagger + \Delta H_b$, or entropy? Efforts were made to make clear origins of this phenomena. First, we measured the solubility in water, S (see Figure 8), which depends on the odd or even of n: TPMP$^+$, TPPP$^+$, and TPAP$^+$ have relatively high solubility in water, and others have low. Second, we measured equivalent ionic conductivities at infinite dilution, λ_0: Figure 9A shows the values in water and Figure 9B those in DMSO. In water, phosphonium ions with odd n have large values of λ_0, while those with even n have small values. In DMSO, the values decrease smoothly with an increase of n, showing that in DMSO λ_0 depends only on the molecular size of phosphonium ions. Figures 8 and 9 suggest that triphenylalkylphosphonium cations whose alkyl chain is methyl, propyl, or amyl are relatively hydrophilic and have a large amount of hydrated water, while the cations whose alkyl chain is ethyl, butyl, or hexyl are less hydrophilic and have a little hydrated water.

The finding in Figures 6, 8, and 9 seems to indicate that the odd and even pattern in E_a cannot be accounted for simply by dehydration; in the hydrocarbon-chain moiety, phosphonium cations must be dehydrated. The entropy change ($\Delta S_b + \Delta S^\ddagger$, see Table 1) may also not be explained solely by the hydration. There are other factors which greatly affect the translocation rate.

^{31}P NMR chemical shifts of phosphonium cations are listed in Table 2. Chemical shifts of tetraalkyl-type phosphonium were also measured. Solvents used were D_2O, DMSO-d_6 and $CDCl_3$ with D_2O and $CDCl_3$, respectively, typical hydrophilic and hydrophobic solvents. In all solvents, TPEP$^+$ showed the largest chemical shift, and TPBP$^+$ in D_2O showed a chemical shift slightly larger than that of TPPP$^+$ and TPAP$^+$. The odd or even pattern was observed in the chemical shift. The presence of the benzene ring of triphenylalkylphosphonium increased the chemical shift as compared to that of tetraalkyl-

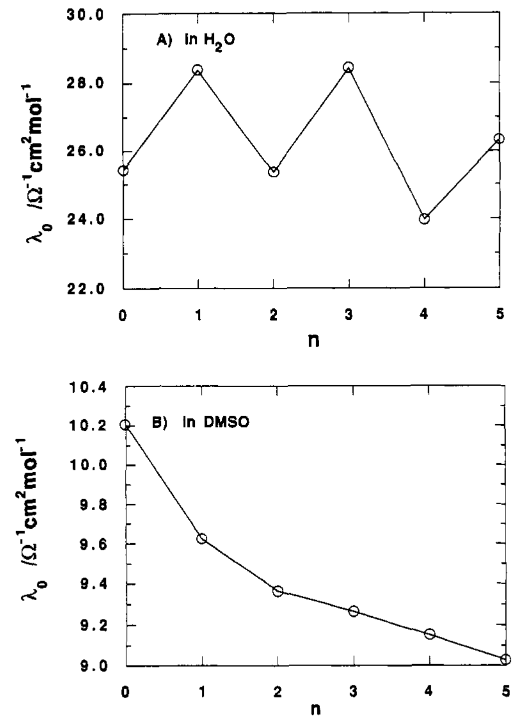

FIGURE 9: Plots of equivalent ionic conductivities at infinite dilution in water (panel A) and in dimethyl sulfoxide (panel B) for various phosphonium cations against n value of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3. Temperature was 25.0 °C. It is noted that the odd and even pattern was observed only in water.

Table 2: Chemical Shifts of ^{31}P NMR for $TPMP^+$ Homologues and Tetraalkylphosphonium in Various Solvents[a]

phosphonium	D_2O	DMSO-d_6	$CDCl_3$
triphenylalkyl			
$TPMP^+$	21.23	22.56	21.66
$TPEP^+$	25.65	25.98	26.17
$TPPP^+$	23.16	23.58	24.84
$TPBP^+$	23.99	24.03	25.19
$TPAP^+$	23.72	24.03	23.19
$TPHP^+$	23.72	24.01	25.18
tetraalkyl			
methyl	22.85		23.83
ethyl	40.18		40.05
butyl	33.32		32.95

[a] Unit is ppm. 85% H_3PO_4 was used as an external reference. The concentration of phosphonium was constant at 400 mM.

phosphonium. Although, unfortunately, tetrapropylphosphonium was not commercially available (so its shift could not be measured), the above described pattern seems to hold. Evans et al. (1990) measured ^{13}C and ^{31}P chemical shift of some tetraalkylphosphonium salts and described that ^{31}P chemical shifts were −116.52, −100.28, −108.99, and −107.36 ppm (relative to trimethyl phosphite) for tetramethyl-, tetraethyl-, tetrapropyl-, and tetrabutylphosphonium, respectively. Our data are consistent with their. The odd or even pattern may not come from the presence of the benzene ring but rather, from the property of quaternary phosphonium. According to their data, ^{13}C chemical shifts of the α-carbon of quaternary phosphonium also depend on the odd or even of n.

The hydration and activation energy seem to be much more affected by the odd or even pattern than the chemical shift: for cations with a longer alkyl chain, this pattern disappears in the chemical shift but is still visible in the hydration and the activation energy of the translocation through the lipid bilayer membrane.

Flewelling and Hubbell (1986b) succeeded in calculating theoretically the total potential energy profile for hydrophobic ion interactions with lipid bilayers that was assumed by Ketterer et al. (1971) (see Figure 1). The potential energy profile can be written as the sum of four terms: the electrical Born, the image and dipole contributions, and a neutral energy term. The neutral energy contribution is essentially the free energy of transfer to move a lipophilic ion from the aqueous phase to the hydrophobic membrane, apart from electrical contributions. It is anticipated that with increase of hydrocarbon-chain length, this energy may be reduced. This term cannot explain the odd and even pattern. The source of dipole energy contribution is the dipole organization of molecules at the lipid–water interface, with the most significant contribution from the ester linkages between the fatty acid chains, the glycerol backbone, and water organization. We observed the odd–even pattern even at infinite dilution of phosphonium cations. It is difficult to consider the dipole energy as a main reason, however, because at infinite dilution, the dipole energy cannot be changed by the exogenous phosphonium cations. The last energy is Born–image energy: it is governed by an effective ion radius of phosphonium ions or by the charge distribution within a molecule. The following may be possible; the electron distribution in the phosphonium molecule may be different, depending on the odd or even of n, and this may, in turn, affect the effective ion radius, which would change the activation energy. Further study on the molecular mechanism of the odd–even pattern found in this paper is necessary. In addition, using pure lipid, the correlation between lipid species and activation energies will be an interesting subject.

APPENDIX

According to the absolute rate theory (Johnson et al., 1954), the rate constant of k_i can be described as

$$k_i = \frac{RT}{hN_A}\exp\left(-\frac{\Delta G^*}{RT}\right)$$

Hence, we obtain

$$RT\ln k_i = RT\ln(RT/hN_A) - \Delta G^* = RT\ln(RT/hN_A) - \Delta H^* + T\Delta S^* \quad (A1)$$

where h and N_A stand for Planck's constant and Avogadro's number, respectively.

The parameter β is a kind of binding constant but has the unit of length. A thermodynamically defined binding constant should be the concentration ratio of bound to free molecules; thus, the binding-region thickness δ is necessary (see Figure 1) for thermodynamic treatment. After Flewelling and Hubbell (1986b), δ is taken as 0.4 nm. Since $K = \beta/\delta$ and $RT \ln K = -\Delta G_b$ (where ΔG_b is the standard free-energy change associated with the binding of phosphonium ions), we obtain eq A2:

$$RT\ln(\beta/\delta) = -\Delta G_b = T\Delta S_b - \Delta H_b \quad (A2)$$

where ΔS_b and ΔH_b are the standard entropy and enthalpy changes associated with the transfer of lipophilic cation from the aqueous solution to the adsorption site in the bilayer, respectively.

Combining eqs A1 and A2 leads to eq A3:

$$RT\ln(\beta k_i/\delta) = RT\ln(RT/hN_A) - (\Delta H^{\ddagger} + \Delta H_b) + T(\Delta S^{\ddagger} + \Delta S_b) \quad \text{(A3)}$$

Differentiation of this equation with respect to $1/T$ gives

$$\frac{\mathrm{d}\ln(\beta k_i)}{\mathrm{d}\left(\frac{1}{T}\right)} = -\frac{\Delta H^{\ddagger} + \Delta H_b + RT}{R} \quad \text{(A4)}$$

which means that

$$E_a = \Delta H^{\ddagger} + \Delta H_b + RT \quad \text{(A5)}$$

When eq A5 is substituted into eq A3, we obtain

$$\Delta S^{\ddagger} + \Delta S_b = R\left[\ln\left(\frac{\beta k_i h N_A}{RT\delta}\right) + \frac{E_a}{RT} - 1\right] \quad \text{(A6)}$$

Thus, we can calculate $\Delta H^{\ddagger} + \Delta H_b$ and $\Delta S^{\ddagger} + \Delta S_b$ from E_a and βk_i.

REFERENCES

Andersen, O. S. (1978) in *Membrane Transport in Biology* (Tosteson, D. C., Ed.) Vol. 1, pp 369–446, Springer-Verlag, New York.

Andersen, O. S., & Fuchs, M. (1975) *Biophys. J. 15*, 795–830.

Araiso, T., & Koyama, T. (1988) *Biorheology 25*, 253–259.

Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kadsiauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M., & Vladimirova, M. A. (1970) *Biochim. Biophys. Acta 216*, 1–12.

Benz, R. (1988) *Biophys. J. 54*, 25–33.

Demura, M., Kamo, N., & Kobatake, Y. (1985a) *Bioelectrochem. Bioenerg. 14*, 439–448.

Demura, M., Kamo, N., & Kobatake, Y. (1985b) *Biochim. Biophys. Acta 812*, 377–386.

Demura, M., Kamo, N., & Kobatake, Y. (1987a) *Biochim. Biophys. Acta 894*, 355–364.

Demura, M., Kamo, N., & Kobatake, Y. (1987b) *Biochim. Biophys. Acta 903*, 303–308.

Evans, D. J., Leigh, G. J., & MacDonald, C. J. (1990) *Magn. Reson. Chem. 28*, 711–714.

Flewelling, R. F., & Hubbell, W. L. (1986a) *Biophys. J. 49*, 531–540.

Flewelling, R. F., & Hubbell, W. L. (1986b) *Biophys. J. 49*, 541–552.

Harned, H. S., & Owen, B. B. (1958a) in *The Physcal Chemistry of Electrolyte Solutions*, p 105, Reinhold Book Co., New York.

Harned, H. S., & Owen, B. B. (1958b) in *The Physcal Chemistry of Electrolyte Solutions*, pp 699, 706, Reinhold Book Co., New York.

Harold, F. M., & Papineau, D. (1972) *J. Membr. Biol. 8*, 27–62.

Haydon, D. A., & Hladky, S. B. (1972) *Q. Rev. Biophys. 5*, 187–282.

Hladky, S. B. (1979) *Curr. Top. Membr. Transp. 12*, 53–164.

Johnson, F. H., Eyring, H., & Polissar, M. J. (1954) in *The Kinetic Basis of Molecular Biology*, John Wiley & Sons, Inc., New York.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kamo, N. (1991) in *Biophysical Technique* (Sugai, S., Ed.) p 220, IPC Publ., Tokyo.

Kamo, N., Racanelli, T., & Packer, L. (1982) *Methods Enzymol. 88*, 356–360.

Kamo, N., Demura, M., & Kobatake, Y. (1986) *J. Membr. Sci. 27*, 233–239.

Ketterer, B., Neumacke, B., & Läuger, P. (1971) *J. Membr. Biol. 5*, 225–245.

LeBlanc, O. H. (1969) *Biochim. Biophys. Acta 193*, 350–360.

Liberman, E. A., & Topaly, V. P. (1969) *Biophysics 14*, 477–487.

Miyauchi, S., Ono, A., Yoshimoto, M., & Kamo, N. (1993) *J. Pharm. Sci. 82*, 27–31.

Montal, M., & Mueller, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3561–3566.

Pickar, A. D., & Benz, R. (1978) *J. Membr. Biol. 44*, 353–376.

Prendergast, F. G., Haugland, R. P., & Callahan, P. J. (1981) *Biochemistry 20*, 7333–7338.

Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–569.

Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14*, 5451–5461.

Shinbo, T., Sugiura, M., Kamo, N., & Kobatake, Y. (1983) *Nihon Kagaku Zashi 6*, 917–923.

Smejtek, P. (1987) *J. Membr. Sci. 33*, 249–268.

Takagi, M., Azuma, K., & Kishimoto, U. (1965) *Annu. Rep. Biol. Works, Fac. Sci., Osaka Univ. 13*, 107–110.

Yamaoka, K., Nakagawa, T., & Uno, T. (1987) *J. Pharmacokinetic Biopharm. 6*, 165–175.