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# Determination of membrane potential with lipophilic cations: correction of probe binding

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**The binding of lipophilic ions to the membrane of envelope vesicles from *Halobacterium halobium* was examined in the absence and presence of membrane potential. The lipophilic ions used constitute a homologous series of $(Phe)_3\text{-}P^+\text{-}(CH_2)_n\text{-}CH_3$ ($n = 0$–4) and tetraphenylphosphonium ($TPP^+$). In the absence of membrane potential, the amounts of binding were proportional to the probe concentration in the medium when the concentration is dilute. Upon illumination, interior negative membrane potential is generated which induces the uptake of phosphonium cation probe. 2 $\mu$M were employed as the initial probe concentration. The real membrane potential was evaluated by means of extrapolation to the state of no binding: The values of $C_i^{app}/C_o$ for various probes are plotted against the binding coefficient. Here, $C_i^{app}$ is the apparent intra-vesicular concentration of the probes which is calculated without consideration of bound probes. The ordinate intercept of the plot gives the true concentration ratio, and from this the membrane potential is evaluated. The membrane potential-dependent binding was analysed with a model: the membrane is split into two halves, outer and inner half, and the amounts of bound probes in each region are governed by the concentration in the contiguous solution. We obtained a formula which describes amounts of binding as a function of the membrane potential.**

List of symbols and abbreviations:

$C_r$, the probe concentration of $r$ region ($r$ = o, mo, mi and i)
$C_i^{app}$, intravesicular concentration of probe which is calculated with neglection of bound probes
$f$, $K_{mo}V_{mo}/(K_{mo}V_{mo} + K_{mi}V_{mi})$
Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i, intra-vesicular aqueous space
$K_b$, binding coefficient ($= K_{mo}V_{mo} + K_{mi}V_{mi}$)
$K_{mo}$, $k_1/k_2$ in the absence of membrane potential
$K_{mi}$, $k_6/k_5$ in the absence of membrane potential
$k_r$, rate constants (see Fig. 1)
mi, inner half of membrane
mo, outer half of membrane
$n$, the fraction of the membrane potential that imposes effectively on mo region (Eqn. 6)
o, extra-vesicular space
$TPMP^+$, triphenylmethyl phosphonium
$TPEP^+$, triphenylethyl phosphonium
$TPPP^+$, triphenylpropyl phosphonium
$TPBP^+$, triphenylbutyl phosphonium
$TPAP^+$, triphenylamyl phosphonium
$TPP^+$, tetraphenyl phosphonium
$U$, uptake of probe by vesicles ($= V_i C_i^{app}$)
$U_b(0)$, amounts of binding of probe in the absence of $\Delta\varphi$
$U_b(\Delta\varphi)$, amounts of binding of probe in the presence of $\Delta\varphi$
$V_r$, volume of $r$ region ($r$ = o, mo, mi and i)
$X_r$, amounts of probe residing at $r$ region ($r$ = o, mo, mi and i)
$\Delta\varphi$, membrane potential with reference to the extra-vesicular space
$\Delta\varphi^{app}$, apparent membrane potential which is calculated with neglection of bound probe
$\mu^o$, standard chemical potential of probe in aqueous solution
$\mu^r$, standard chemical potential of probe in $r$ region ($r$ = mo, mi)

## Introduction

The chemiosmotic theory [1] states that, in oxidative phosphorylation, photophosphorylation and active transport, the electrochemical potential difference of proton across the membrane plays an essential role. Proton electrochemical potential is composed of both a concentration part ($\Delta$pH) and an electrical potential part ($\Delta\varphi$). Therefore, the exact measurements of these quantities are required for the analysis of the energy transduction in the membrane.

At present, no reliable method is available for direct measurement for the difference of proton electrochemical potential. Separate measurements of $\Delta$pH and $\Delta\varphi$ are required. Mitochondria and bacterial cells are too small to use microelectrodes and so indicator molecules (so-called probe) should be employed. For measurements of $\Delta\varphi$, one of methods is the use of lipophilic ions as probes [2–5]. These ions permeate the membrane freely and distribute in accordance with the Nernst equation:

$$\Delta\varphi = (RT/F)\ln(C_o/C_i)$$

Here, $C_i$ and $C_o$ stand for the intracellular and external concentrations of probes, and $R$, $T$ and $F$ are the gas constant, the absolute temperature and the Faraday constant, respectively. Then, determination of $C_i$ and $C_o$ enables us to evaluate the membrane potential. Several techniques [2,4,6] have been developed for determination of $C_i$ and $C_o$, and $\Delta\varphi$ for various small cells or organella have been determined to elucidate various membrane functions.

The principle of this method is clear and simple. However, less attention has been paid to the bound population of probes which overestimates the membrane potential, although several studies have been published [7–11]. Previously [12], we measured the uptake of various phosphonium ions by envelope vesicles of *Halobacterium halobium*. The membrane of *H. halobium* contains a light-driven pump which generates the interior negative membrane potential upon illumination. At the same light intensity, the membrane potentials estimated with various probes were compared: as the probe becomes more hydrophobic, the estimated value becomes larger. We proposed an extrapolation method to the state of no binding to evaluate the real membrane potential. The estimated value with various probes is plotted against the quantities of binding, such as binding coefficient and amounts of binding in the absence of membrane potential. The ordinate intercept of this plot gives the real membrane potential, since this value corresponds to that obtained with a hypothetical probe which does not bind.

In the present paper, we develop this method further and discuss the amount of binding of the probes which depends on the membrane potential. We give an equation which describes the binding well. Using this equation, we can estimate the membrane potential and the values estimated are strikingly independent of the probe species used.

## Materials and Methods

The strain of *H. halobium* used was KH-10 which contains halorhodopsin but not bacteriorhodopsin [13]. Halorhodopsin is a light-driven Cl pump [14]. Illumination induces inwardly directed $Cl^-$ transport which leads to interior negative membrane potential. The magnitude of membrane potential can be adjusted by changing the light intensity. Growth of bacteria and preparation of envelope vesicles were carried out as described previously [12,13]. The intravesicular volume ($V_i$) was 3.0 $\mu$l per mg of protein [15]. Protein was assayed by the method of Lowry et al. [16] with bovine serum albumin as reference standard. The medium was 4.0 M NaCl which was buffered at pH 7.0 with 10 mM Hepes/NaOH.

The phosphonium probes used were $TPMP^+$, $TPEP^+$, $TPPP^+$, $TPBP^+$, $TPAP^+$ and $TPP^+$ which were purchased from Tokyo Kasei (Tokyo) or from Aldrich (Milwaukee).

The uptake of these phosphonium cations by the vesicles was measured by means of an electrode selective for respective phosphonium ions [12,17]. The procedure for its construction and the apparatus used were the same as described previously [12]. The calculation for binding of probes and for amounts of uptake was the same as in the previous paper [12].

### *A model for lipophilic ion binding*

It is now generally accepted that for liposomes

and lipid bilayer membranes, the potential energy profile of a hydrophobic ion has a minimum near the solution-membrane interface and hence a hydrophobic ion reside near the interfaces [18]. Cafiso and Hubbell [20] used this concept to analyse membrane potential-dependent binding of spin-labeled phosphonium ions to liposome membranes. This profile comes from the regular arrangements of amphiphilic (lipid) molecules. Bio-membranes are constituted from lipids and proteins. Proteins are associated to or penetrate lipid bilayer membranes. Although the fundamental structure of biomembrane is lipid bilayer, the potential energy profile for a lipophilic ion is supposed not to be so simple as the lipid bilayer. For the sake of simplicity, therefore, we split the membrane phase into two parts, i.e., outer and inner halves of membrane (see Fig. 1). We consider following four regions where lipophilic ions reside: the extravesicular space (o), the outer half of the membrane (mo), the inner half of the membrane (mi), and the internal aqueous solution (i). The amounts (moles) of probes in each region are $X_o$, $X_{mo}$, $X_{mi}$, and $X_i$, respectively. The volume of each region is represented by $V_o$, $V_{mo}$, $V_{mi}$ and $V_i$, and the concentration of probe at each region are $C_o$, $C_{mo}$, $C_{mi}$ and $C_i$, respectively. Rate constants of each step in Fig. 1 are denoted as $k_r$ ($r = 1$–$6$). The kinetic equations with respect to $X_o$ and $X_i$ are written as follows:

$$\frac{dX_o}{dt} = -\frac{k_1}{V_o}X_o + \frac{k_2}{V_{mo}}X_{mo}$$
$$\frac{dX_i}{dt} = \frac{k_5}{V_{mi}}X_{mi} - \frac{k_6}{V_i}X_i \qquad (1)$$

At the steady state where $dX_r/dt = 0$ ($r =$ o and i), the ratio of $C_{mo}$ to $C_o$ and that of $C_{mi}$ to $C_i$ are readily derived from Eqn. 1 as follows:

$$C_{mo}/C_o = (X_{mo}/V_{mo})/(X_o/V_o) = k_1/k_2 \qquad (2)$$

and

$$C_{mi}/C_i = (X_{mi}/V_{mi})/(X_i/V_i) = k_6/k_5 \qquad (3)$$

Therefore, we obtain Eqn. 4 which describes the amount of probes bound to the membrane as a function of $C_i$ and $C_o$.

$$X_{mo} + X_{mi} = V_{mo}(k_1/k_2)C_o + V_{mi}(k_6/k_5)C_i \qquad (4)$$

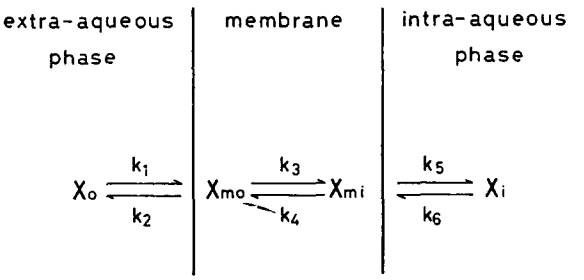


Fig. 1. Four-compartment model for the analysis of ion binding and uptake. They are the extra-aqueous phase (o), the outer half of the membrane (mo), the inner half of the membrane (mi) and the intra-aqueous phase (i). The amounts (moles) of probes in each region are $X_o$, $X_{mo}$, $X_{mi}$ and $X_i$, respectively. Rate constants of each step are represented by $k_r$ ($r = 1$–$6$).

Eqns. 2 and 3 mean that the amount of lipophilic ions which reside in the mo region is a function only of $C_o$ and that in the mi region is only of $C_i$. Note that the cross-term containing both $C_i$ and $C_o$ does not appear. In other words, amounts of binding can be described by the equation which suggests that there was no connection between the mo and mi regions, although lipophilic ions can easily permeate the membrane.

## Results and Discussion

It was shown previously [12] that in the dark, where $\Delta\varphi = 0$, the binding followed the Langmuir adsorption isotherm for $TPMP^+$, $TPEP^+$ and for $TPP^+$. For other probes, analysis revealed the presence of two, high and low affinity, binding sites. The Langmuir equation describes that amounts of binding increase proportionally with increase of the concentration when the concentration is dilute and levels off in a concentrated solution. Therefore, we focus on the binding where the concentration of phosphonium ions is dilute, since we will use Eqn. 4 for analysis of the probe binding. Results obtained are shown in Fig. 2, where amounts of binding at $\Delta\varphi = 0$, $U_b(0)$, are plotted against the free concentration of various phosphonium cations in the external medium. Here, 0 in $U_b(0)$ denotes the $\Delta\varphi = 0$. The figure indicates that $U_b(0)$ is proportional to $C_o$, except the case where concentrations of $TPAP^+$, $TPP^+$ and $TPBP^+$ exceed 10 $\mu$M.

Upon illumination, interior-negative membrane

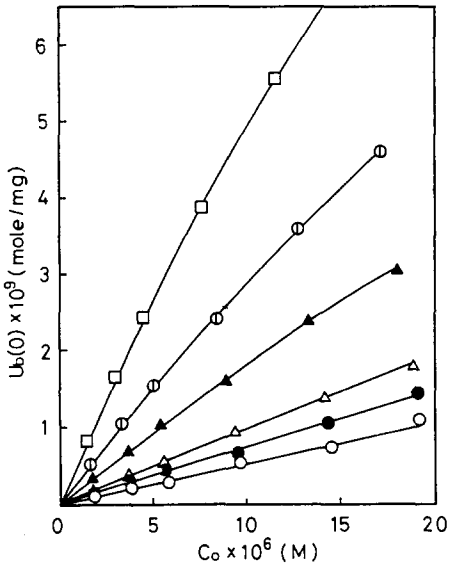


Fig. 2. Plots of the amounts of binding to membrane in the absence of membrane potential, $U_b(0)$, against the free concentration of various phosphonium cations, $C_o$. ○, $TPMP^+$; ●, $TPEP^+$; △, $TPPP^+$; ▲, $TPBP^+$; ⊕, $TPP^+$; □, $TPAP^+$.

potential is generated which gives rise to the uptake of phosphonium cation by envelope vesicles. The membrane potential was estimated with various phosphonium probes of varying concentrations. Results are shown in Fig. 3, where $\Delta\varphi^{app}$ is calculated without any correction of binding: the probe entrapped by vesicles is assumed to be present as free molecules within vesicles. The abscissa of Fig. 3A represents the extra-vesicular concentration of phosphonium probe ($C_o$) when the amount of uptake of probes becomes steady. With increase of probe concentration, the value of $\Delta\varphi^{app}$ becomes small, especially for $TPAP^+$, $TPP^+$ and $TPBP^+$ which are more hydrophobic than others. The passive electrophoretic transport of phosphonium probes results in the short circuit of the membrane potential and decreases it. In fact, the differences of estimated membrane potentials at initial probe concentration of 1 and 10 $\mu$M are roughly proportional to the permeability coefficient of various probes (data not shown). The value of membrane potential obtained by the extrapolation to $C_o = 0$ is, therefore, free from the collapse of the potential due to the probe transport. Moreover, we get rid of the ambiguity caused by the possibility that the binding of lipophilic ions inhibits the functional activity of the membrane proteins. When $\Delta\varphi^{app}$ is plotted against $C_i^{app}$, the values stay relatively constant when $C_i^{app}$ is dilute (Fig. 3B). $C_i^{app}$ represents the apparent intravesicular probe concentration which is calculated without considering the membrane-bound population. It is noted that the extrapolated values obtained from both Fig. 3A and B agree with each other. As the probe used becomes more hydro-

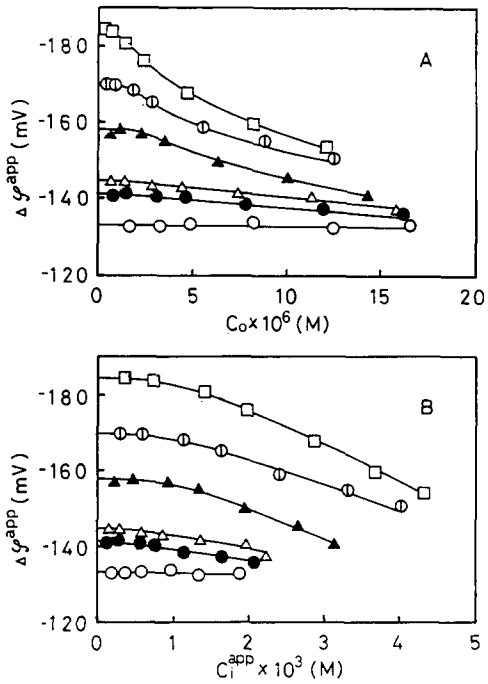


Fig. 3. Apparent membrane potential, $\Delta\varphi^{app}$ estimated with various phosphonium probes of varying concentrations. $\Delta\varphi^{app}$ was calculated without any correction of binding, i.e., under the assumption that the probe entrapped by vesicles was present as free molecules within vesicles. Initial concentration of probes (that before addition of vesicles) was changed from 1 to 20 $\mu$M (1, 2, 4, 6, 10, 15 and 20 $\mu$M from left to right). Membrane vesicles (0.61 mg protein/ml) were suspended in 4 M NaCl (pH 7.0 with 10 mM Hepes/NaOH). After the electrode potential became steady, the illumination was provided. The concentration of probes in extravesicular space at 'steady' under illumination is denoted as $C_o$. The uptake was calculated from the difference between the initial concentration and $C_o$ [12]. The temperature was kept constant at 30°C by circulating thermostated water around the cuvette. The actinic light intensity ($\lambda > 500$ nm) was 1120 W/m$^2$. (A) Plots of $\Delta\varphi^{app}$ vs. extravesicular concentration of probe, $C_o$. (B) Plots of $\Delta\varphi^{app}$ vs. apparent intravesicular concentration of probe, $C_i^{app}$, which was calculated without consideration of the probe binding. Notations for the probe used are the same as in Fig. 2.

phobic, the estimated membrane potential becomes larger. This indicates that the binding of the probe leads to the overestimation of the membrane potential, since more hydrophobic ions bind much to the membrane (see Fig. 2).

Fig. 3 shows that the value at initial concentration of 2 $\mu$M is approximately equal to the extrapolated value. Then, light-dependent membrane potentials were measured using a variety of phosphonium probes whose initial concentration was 2 $\mu$M, and the results are shown in Table I. As described above, the values estimated depended on the probe used. The value estimated with $TPMP^+$, the least hydrophobic probe, gave the smallest value, while $TPAP^+$, the most hydrophobic probe, gave the largest value. The difference of these two values amounts to as much as 60 mV, indicating that a correction should be made.

For correction of probe-binding, the potential-independent binding model [12] is frequently employed which assumes that the binding of the probe in the absence of membrane potential is saturated and that no additional probe binding occurs even when the probe is accumulated inside. The criterion for a valid correction is that the membrane potential estimated must be independent of the probe species used. The correction based on the potential-independent binding model, however, could not estimate the values which were independent of the probe used (data not shown). This correction appeared to exert its effect at low light intensity, i.e., small membrane potential, while no appreciable change was observed at large membrane potential. The exponential mean model [7] and other models [12] also failed to obtain the value of the membrane potential which was independent of the probe used. In the previous paper [12], we proposed a method for estimation of the 'real' membrane potential which has been called 'extrapolation to state of no binding'. This method implies that the values estimated with various probes are plotted against the quantity related to the probe-binding, ex. amount of binding in the absence of membrane potential or binding coefficients and that the ordinate intercept of the line connecting the data is 'real' membrane potential.

The apparent intravesicular concentration of the phosphonium probe, $C_i^{app}$ is expressed by

$$V_i C_i^{app} = X_{mo} + X_{mi} + X_i = U$$

and $V_i C_i^{app}$ is denoted as $U$. Insertion of Eqn. 4 and the relation that $\Delta\varphi = (RT/F)\ln(C_o/C)$ into above equation yields Eqn. 5.

$$\frac{U}{V_i C_o} = \frac{C_i^{app}}{C_o} = \frac{V_{mo}k_1}{V_i k_2} + \frac{V_{mi}k_6}{V_i k_5}\exp(-F\Delta\varphi/RT) + \exp(-F\Delta\varphi/RT) \quad (5)$$

TABLE I

APPARENT MEMBRANE POTENTIAL OBTAINED USING OF A VARIETY OF PHOSPHONIUM PROBES

Apparent membrane potential (mV) was calculated under the assumption that the probe entrapped by vesicles was presented as free molecules within vesicles. Initial concentration of probes was 2 $\mu$M. Membrane vesicles (0.61 mg protein/ml) were suspended in 4 M NaCl (pH 7.0 with 10 mM Hepes/NaOH). Experiments were performed at 30°C. The actinic light intensity ($\lambda > 500$ nm) was changed with an appropriate neutral density filter. 100% of light intensity corresponds to 1380 W/m$^2$. The values in the last column were determined with Eqn. 8.

| Light intensity (%) | $TPMP^+$ | $TPEP^+$ | $TPPP^+$ | $TPBP^+$ | $TPAP^+$ | $TPP^+$ | Extrapolated value |
|---|---|---|---|---|---|---|---|
| 4.1 | −78 | −88 | −98 | −110 | −139 | −124 | −36 |
| 9.5 | −83 | −93 | −103 | −117 | −145 | −130 | −13 |
| 17.8 | −95 | −99 | −111 | −125 | −152 | −139 | −32 |
| 23.6 | −99 | −110 | −116 | −129 | −155 | −146 | −70 |
| 32.2 | −105 | −115 | −122 | −136 | −162 | −151 | −65 |
| 40.0 | −109 | −121 | −128 | −139 | −166 | −156 | −74 |
| 51.6 | −119 | −128 | −135 | −146 | −173 | −163 | −97 |
| 64.9 | −122 | −130 | −139 | −150 | −176 | −167 | −94 |
| 74.6 | −125 | −134 | −141 | −154 | −179 | −169 | −93 |
| 81.9 | −127 | −137 | −144 | −156 | −181 | −171 | −101 |
| 90.6 | −128 | −138 | −145 | −158 | −183 | −173 | −90 |
| 100.0 | −131 | −140 | −147 | −160 | −186 | −175 | −100 |

When no potential difference is imposed, we write it as

$$K_{mo} = k_1/k_2,\ K_{mi} = k_6/k_5$$

In the presence of the membrane potential, however, it is considered that the potential difference may affect the binding coefficient: interior-negative membrane potential increases the cation binding at the mo region and decreases it at the mi region. The following discussion is instructive for an understanding of the membrane potential-dependent change in the binding coefficient or in rate constants.

At equilibrium, the following equation should hold between the o and mo regions.

$$\mu^{o} + RT \ln C_o = \mu^{mo} + RT \ln C_{mo} + nF\Delta\varphi \qquad (6)$$

where $n$ is the fraction of the membrane potential that imposes effectively on the mo region, and $\mu^{o}$ and $\mu^{mo}$ stand for the standard chemical potential of phosphonium cation at external aqueous solution and the membrane phase, respectively. Note that the potential is measured with reference to that in the extravesicular space (o). From this equation, we obtain

$$\frac{C_{mo}}{C_o} = \exp((\mu^{o} - \mu^{mo})RT)\exp(-nF\Delta\varphi/RT)$$

Comparing Eqn. 2, we conclude that

$$k_1/k_2 = K_{mo}\exp(-nF\Delta\varphi/RT) \text{ and}$$

$$K_{mo} = \exp((\mu^{o} - \mu^{mo})/RT)$$

Similarly, equilibrium at the opposite side leads to the following equation

$$\mu^{mi} + RT \ln C_{mi} + (1-n)F\Delta\varphi = \mu^{o} + RT \ln C_i + F\Delta\varphi \qquad (6a)$$

and we obtain

$$k_6/k_5 = K_{mi}\exp(nF\Delta\varphi/RT)$$

From these assumptions, Eqn. 5 is recast as

$$\frac{C_i^{app}}{C_o} = \frac{V_{mo}}{V_i}K_{mo}\exp(-nF\Delta\varphi/RT) + \frac{V_{mi}}{V_i}K_{mi}\exp((n-1)F\Delta\varphi/RT) + \exp(-F\Delta\varphi/RT) \qquad (7)$$

When $\Delta\varphi = 0$, the amount of binding, $U_b(0)$, can be expressed as

$$U_b(0) = X_{mo} + X_{mi} = (V_{mo}K_{mo} + V_{mi}K_{mi})C_o = K_bC_o$$

since $C_o = C_i$ in the absence of membrane potential. Fig. 2 shows that $U_b(0)$ is proportional to $C_o$ and proportionally constant is denoted $K_b$. Then, $K_b = V_{mo}K_{mo} + V_{mi}K_{mi}$. Defining $f = V_{mo}K_{mo}/(V_{mo}K_{mo} + V_{mi}K_{mi})$, we rewrite Eqn. 7 as follows:

$$\frac{C_i^{app}}{C_o} = [f\exp(-nF\Delta\varphi/RT) + (1-f)\exp((n-1)F\Delta\varphi/RT)]K_b/V_i + \exp(-F\Delta\varphi/RT) \qquad (8)$$

This equation indicates that when $C_i^{app}/C_o$ obtained for various phosphonium probes are plotted against its $K_b$ value, the ordinate intercept of the line connecting the data points gives the value of $\exp(-F\Delta\varphi/RT)$. This is what we proposed previously and called 'extrapolation to the state of no-binding'. The extrapolated value corresponds

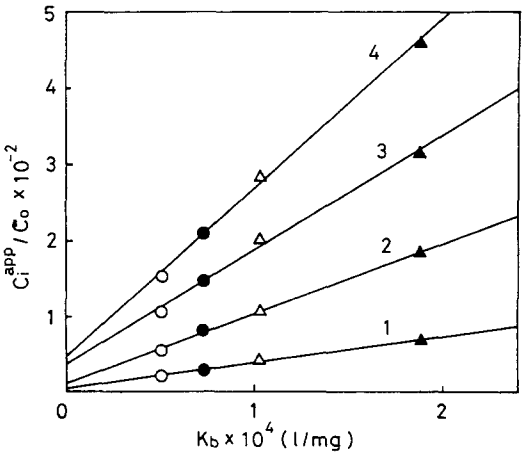


Fig. 4. Plots of $C_i^{app}/C_o$ vs. $K_b$ for various phosphonium probes. The ordinate intercept of the line connecting the data points gives the value of $\exp(-F\Delta\varphi/RT)$ (refer to Eqn. 8). The least-squares method was used for the data analysis. The light intensities from experiments 1 to 4 were 57, 442, 896 and 1380 W/m$^2$, respectively. Initial concentration of probes was 2 $\mu$M. Other experimental conditions were the same as in Fig. 3. Notations for the probe used were the same as in Fig. 2.

to the value measured with a hypothetical probe which does not bind to the membrane.

The results are plotted in Fig. 4, showing that fairly good straight lines are obtained. For probes whose $K_b$ are larger than those of the probes shown in this figure, positive deviation from the straight line was observed. As is seen in Fig. 2, for these phosphonium cations, the linear part is relatively narrow and saturation of binding appears at dilute solution. When $\Delta\varphi = -120$ mV and $C_o = 1$ $\mu$M, $C_i$ should be 100 $\mu$M where the amount of binding is not linear to the concentration as is assumed in Eqn. 8. On the other hand, for less hydrophobic probes which are shown in the figure, $C_i$ stays within the concentration range where binding is proportional to the concentration. This is the reason for the deviation. The values extrapolated in accordance with Eqn. 8 are listed in the last column of Table I. If the extrapolated value is taken as the real membrane potential, the difference between the value estimated with various phosphonium probes and this value comes from the probe binding. For $TPAP^+$, the difference at full light intensity amounts to 86 mV which means that more than 90% of the probes entrapped by vesicles exist as a bound population. The exact ratios of bound probes to total probes entrapped are 72% for $TPMP^+$, 78% for $TPEP^+$, 82% for $TPPP^+$, 90% for $TPBP^+$, 93% for $TPP^+$ and 95% for $TPAP^+$. This order is the hydrophobicity of probes. Shen et al. [19] showed that approx. 85% of $TPP^+$ is bound to the mitochondrial membranes. In their experiment, true membrane potential was estimated from $Rb^+$ uptake via valinomycin. Our value is slightly higher than theirs. This stems presumably from the high ionic strength of the medium used in our experimental system (4 M NaCl) where the phosphonium probe has relatively low solubility.

As light intensity increases, the membrane potential should increase. But, the extrapolated values listed in the column of Table I show a little irregularity, indicating that the method of the extrapolation does not afford the value of high accuracy. The range of variation in the ordinate of Fig. 4 is too large to determine the value of the ordinate intercept precisely. The value listed in the column, therefore, is regarded as a first approximation for the membrane potential. With this first approximation, we can calculate the amount of binding $X_{mo} + X_{mi}$ in the presence of membrane potential which is denoted as $U_b(\Delta\varphi)$. As shown in Eqn. 8, we obtain

$$U_b(\Delta\varphi) = X_{mo} + X_{mi}$$
$$= [f \exp(-nF\Delta\varphi/RT) + (1-f)$$
$$\times \exp((n-1)F\Delta\varphi/RT)]K_bC_o \quad (9)$$

Eqn. 9 indicates that when $U_b(\Delta\varphi)/(K_bC_o)$ is plotted against the membrane potential, the data obtained with various phosphonium ions follow a single curve. Such plotting is shown in Fig. 5 and the data points fall roughly on a composite curve.

As mentioned above (discussion of Fig. 4), for the more hydrophobic ion, Eqn. 9 does not strictly hold. However, the plot seems to indicate that the errors stemming from this are not significant. Eqn. 9 has two adjustable parameters, i.e., $n$ and $f$. We found that the best fit was obtained with a combination of $f = 0.85$ and $n = 0$ or with a combination of $f = 0.5$ and $n = 0.5$. The lines in this figure represent the calculated values of Eqn. 9 with two sets of parameters.

Cafiso and Hubbell [20] have studied the binding of spin-labeled phosphonium ions in liposomes in relation to the transmembrane potential. Rottenberg [11] measured the uptake of various lipophilic cations and of $Rb^+$ in the presence of valinomycin by energized mitochondria. They analysed the membrane potential-dependent probe binding and found that the binding coefficient is not influenced by the membrane potential, i.e. $n = 0$. Casadio et al. [8] and Lolkema et al. [10] also used the model which assumes the potential-independent binding coefficient. The value of $n$ is determined by the factor of the position where probe molecules mainly reside within the membrane and by the potential profile in the membrane. Eqn. 6 indicates that if the probe molecule in the membrane resides at the place where electrical potential is equal to that in the aqueous solution, the binding coefficient is not influenced by the membrane potential. When we assume that $n = 0$, Eqn. 8 yields

$$\Delta\varphi = -\frac{RT}{F}\ln\left(\frac{U/C_o - fK_b}{(1-f)K_b + V_i}\right) \quad (10)$$

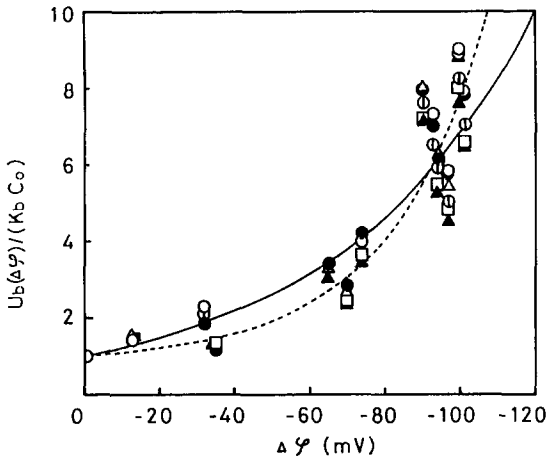


Fig. 5. Membrane-potential dependence of the amounts of binding in the presence of membrane potential, which is denoted $U_b(\Delta\varphi)$. The data of $U_b(\Delta\varphi)/(K_bC_o)$ are plotted against the membrane potentials which were calculated with the method described in Fig. 4. It is noted that the data points obtained for various probes approximately follow a single composite curve. Experimental conditions were the same as in Fig. 4. Notations for the probe used are the same as in Fig. 2. Two lines are values calculated with Eqn. 9. Solid lines, $n = 0.5$; broken lines, $n = 0$ and $f = 0.85$.

From Fig. 5, we obtained $f = 0.85$. Another method for determination of $f$ is as follows: when the membrane potential is enough large, $U_b(\Delta\varphi)$ may be approximately equal to $X_{mi}$, since $C_i$ is much larger than $C_o$. Then, $U_b(\Delta\varphi)/C_i$ in this region gives the appropriate value of the internal

TABLE II

DETERMINATION OF $f$ FOR THE CASE OF $n = 0$

$K_b$ and $U_b(\Delta\varphi)/C_i$ are expressed as l/mg protein. The light intensity was 1380 W/m$^2$ (100% light intensity in Table I), since the generation of a certain amount of large membrane potential is necessary for the determination with this method (see text). $C_i$ was calculated from $C_o$ and $\Delta\varphi$ (−100 mV see Table I). $f$ was calculated as $f = 1 - [U_b(\Delta\varphi)/C_i]/K_b$ (see Eqn. 9 with $n = 0$ without consideration of $X_{mo}$).

| Probe | $K_b$ | $U_b(\Delta\varphi)/C_i$ | $f$ |
|---|---|---|---|
| $TPMP^+$ | $5.0\cdot10^{-5}$ | $6.8\cdot10^{-6}$ | 0.86 |
| $TPEP^+$ | $7.2\cdot10^{-5}$ | $1.1\cdot10^{-5}$ | 0.85 |
| $TPPP^+$ | $1.03\cdot10^{-4}$ | $1.5\cdot10^{-5}$ | 0.85 |
| $TPBP^+$ | $1.9\cdot10^{-4}$ | $2.7\cdot10^{-5}$ | 0.86 |
| $TPAP^+$ | $4.7\cdot10^{-4}$ | $7.9\cdot10^{-5}$ | 0.83 |
| $TPP^+$ | $3.0\cdot10^{-4}$ | $5.1\cdot10^{-5}$ | 0.83 |

binding coefficient ($V_{mi}K_{mi}$) and the value of $f$ can be estimated. (See Eqn. 9 with $n = 0$. Note that $C_i = C_o \exp(-F\Delta\varphi/RT)$.) Table II shows the result thus calculated, revealing that $0.83 < f < 0.86$ which is very close to that determined from the non-linear square regression of results of Fig. 5. With these two methods, we concluded that $f = 0.85$. The deviation of $f$ from 0.5 shows that $K_{mo}V_{mo}$ differs from $K_{mi}V_{mi}$, which is maybe due to the asymmetry of membranes. Table IIIA shows the results obtained from Eqn. 10 with $f = 0.85$.

For the present study, we found another set of $n$ and $f$ with which Eqn. 9 fits the experimental

TABLE III

MEMBRANE POTENTIAL DETERMINED WITH VARIOUS PROBES AFTER CORRECTION OF BINDING

| Light intensity (%) | $TPMP^+$ | $TPEP^+$ | $TPPP^+$ | $TPBP^+$ | $TPAP^+$ | $TPP^+$ | Average ± S.D. |
|---|---|---|---|---|---|---|---|
| (A) | | | | | | | |
| 9.5 | −26 | −31 | −36 | −33 | −41 | −34 | −34 ± 4.6 |
| 23.6 | −56 | −61 | −59 | −56 | −59 | −62 | −59 ± 2.3 |
| 51.6 | −82 | −84 | −84 | −80 | −84 | −85 | −83 ± 1.7 |
| 81.9 | −91 | −93 | −95 | −91 | −93 | −95 | −93 ± 1.6 |
| 100.0 | −95 | −97 | −98 | −96 | −99 | −99 | −97 ± 1.5 |
| (B) | | | | | | | |
| 9.5 | −14 | −17 | −22 | −18 | −24 | −18 | −19 ± 3.3 |
| 23.6 | −44 | −50 | −47 | −42 | −45 | −49 | −46 ± 2.8 |
| 51.6 | −79 | −81 | −81 | −75 | −80 | −82 | −80 ± 2.3 |
| 81.9 | −92 | −95 | −98 | −93 | −95 | −99 | −95 ± 2.5 |
| 100.0 | −98 | −100 | −103 | −100 | −101 | −106 | −101 ± 2.6 |

(A) Results are from Eqn. 10, with $f = 0.85$. (B) Results are from Eqn. 11, with $n = 0.5$. Membrane potentials are in mV with reference to extravesicular space. The values in the last column are the averages of the value obtained with various probes, and standard deviation (S.D.). Initial concentration of probes was 2 μM. Experimental conditions were the same as in Table I.

data (i.e., $n = f = 0.5$). If $n = 0.5$, the parameter $f$ disappears in Eqn. 8. Assuming that $n = 0.5$, Eqn. 8 is recast to

$$\Delta\varphi = \frac{-2RT}{F} \ln\left( \frac{-K_b}{2V_i} + \sqrt{\frac{K_b^2}{4V_i^2} + \frac{U}{C_o V_i}} \right) \quad (11)$$

Table IIIB shows the results obtained with Eqn. 11.

The values listed in both Tables IIIA and B indicate that the estimated values are strikingly independent of the probe species used, even for the small membrane potential. In addition, when light intensity is equal, the average values (listed in the last column of Table III) are approximately equal to the value obtained from the extrapolation (listed in the last column of Table I), although the values calculated with $n = 0$ and $f = 0.85$ are larger in the small membrane potential than those with $n = 0.5$. The standard deviations (S.D.) listed in the last column in Table III shows that those obtained with a set of $n = 0$ and $f = 0.85$ are smaller than those obtained with another set of the parameters except for the weakest light intensity. In addition, the S.D. obtained with $n = 0$ and $f = 0.85$ for the fitting of Fig. 5 is somewhat smaller than that with another set of parameters. Thus, it is inferred that a set of parameters of $n = 0$ and $f = 0.85$ may be suitable to the present system. Further study is necessary to check the applicability of the present correction for another system.

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