Biochimica et Biophysica Acta 812 (1985) 377–386
Elsevier

BBA 72430

Determination of membrane potential with lipophilic cations. Comparison of estimated values with various phosphonium ions

Makoto Demura, Naoki Kamo and Yonosuke Kobatake

Department of Biophysics, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060 (Japan)

(Received April 3rd, 1984)
(Revised manuscript received August 6th, 1984)

Key words: Membrane potential, Lipophilic ions, Phosphonium ion, Tetraphenylboron, (*H. halobium*)

The binding of lipophilic ions to the membrane of envelope vesicles from *Halobacterium halobium* was examined. The lipophilic ions used constitute a homologous series of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3 ($n = 0$–5) and tetraphenylphosphonium (TPP^+). In the absence of membrane potential, the binding of probes to the membrane was measured. For the probes of $n = 0$ and $n = 1$, and for TPP^+, binding followed the Langmuir adsorption isotherm. For other probes, analysis revealed the presence of two, high- and low-affinity, binding sites. Upon illumination, which generated the membrane potential, the probe molecules were accumulated into the vesicles. If we ignore the membrane-potential-dependent binding of the probe molecules, the estimated values are larger when the probe used is more hydrophobic. We have tested some models describing the amount of probe bound on membranes in terms of concentration of free probe inside and outside the vesicles. No model has fulfilled the criterion of valid estimation that the membrane potentials estimated are independent of probes used. An experimental method for the estimation of true membrane potential is proposed. Effects of tetraphenylboron on the estimation of membrane potential and on the transport rate of phosphonium cations were examined.

Introduction

The electrical potential difference across cell membranes is called the membrane potential, which is measured directly by means of a microelectrode. Such measurement has been achieved only on the *Escherichia coli* cells enlarged by pharmacological interference of synthesis of the cell envelope [1], and generally the microelectrode technique is not applicable to bacterial cells or organelles. The importance of the membrane potential of bacterial cells or organelles is well recognized [2]. For such small cells that are too small to use the microelectrode, the probe method should be applied. One of probe methods is the use of lipophilic ions, which are considered to be freely permeable to the cell membranes [3–7]. Typical lipophilic cations are valinomycin + K^+ (Rb^+) and phosphonium cations such as triphenylmethylphosphonium ($TPMP^+$) and tetraphenylphosphonium (TPP^+). At equilibrium, these positively charged probes distribute between inside of cells and the external medium in accordance with the following Nernst equation:

$$\Delta\varphi = (RT/F)\ln(C_{out}/C_{in}) \quad (1)$$

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Mes, 4-morpholineethanesulfonic acid, TPP^+, tetraphenylphosphonium; $TPMP^+$, triphenylmethylphosphonium; $TPEP^+$, triphenylethylphosphonium; $TPPP^+$, triphenylpropylphosphonium; $TPBP^+$, triphenylbutylphosphonium, $TPAP^+$, triphenylamylphosphonium, $TPHP^+$, triphenylhexylphosphonium; TPB^-, tetraphenylboron anion

where $\Delta\varphi$, C_{out} and C_{in} stand for the membrane potential (with respect to the medium), and the concentrations of the probe in the external medium and in the cell, respectively.

The comparisons between the membrane potential obtained by direct measurement with use of a microelectrode and the value estimated from Eqn. 1 have been carried out. Some [1] reported excellent agreement and others [8] reported disagreement. The disagreement, however, does not necessarily make Eqn. 1 invalid, because the value obtained by impalement of a microelectrode in small cells is also ambiguous. A number of articles [9–16] deal with the comparison of the membrane potential estimated from the distribution of different lipophilic probes, such as valinomycin + Rb^+, TPP^+ or $TPMP^+$, and the value estimated with potential-dependent fluorescent dyes. Disagreement was reported [4,10,11,13,14,16], although qualitatively, the same results were obtained. It is considered that the discrepancy may stem from the binding of the probes to the membrane and/or to the intracellular constituents. Thus, the correction of the binding is a problem to be solved in order to evaluate the membrane potential accurately.

In the present research, we measured the uptake of various phosphonium probes by envelope vesicles derived from *Halobacterium halobium*. The phosphonium probes used were a homologous series of $(Phe)_3\text{-}P^+\text{-}(CH_2)_n\text{-}CH_3$ ($n = 0$–5) and TPP^+. The membrane of *H. halobium* contains a light-driven ion pump [17]. Illumination generates the interior-negative membrane potential which vanishes upon removal of the illumination. In addition, this process can be repeated and the size of the membrane potential generated upon illumination is the same each time insofar as the actinic light intensity is constant under the experimental conditions employed here. This enables us to measure the uptake of various phosphonium probes under the same conditions. First, in the dark, where no membrane potential is generated, the binding of the phosphonium probes to the membrane was measured and the isotherm was obtained. The membrane potential generated on illumination was estimated using various phosphonium cations. The estimated values depended on the probe used. As the probe becomes more hydrophobic, the estimated value becomes larger, indicating the necessity for making a correction for the binding of probe. The potential-depending binding is discussed. In addition, the effect of tetraphenylboron on the estimation of membrane potential and on the transport rate of phosphonium cations is examined.

Materials and Methods

A strain of *H. halobium* used was KH-10 which contains halorhodopsin but not bacteriorhodopsin [18]. Halorhodopsin is a light-dependent chloride pump [18–20]. Growth of bacteria and preparation of envelope vesicles were carried out as described previously [21]. The intravesicular volume was 3.0 μl per mg protein and more than 90% was right-side-out vesicle. Protein was assayed by the method of Lowry et al. [35] with bovine serum albumin as reference standard. The vesicles were suspended normally in 4 M NaCl whose pH was adjusted to 7.0 with 10 mM Hepes/NaOH. When the effect of pH on the binding of probes was examined, the medium employed was 3.5 M NaCl because the interior of the vesicle was exchanged by osmotic-dilution equilibrium method [21]. For the pH range between 5 and 7, 10 mM Mes/NaOH was used, and from 7 to 8, 10 mM Tris-HCl was used.

The phosphonium probes used and their abbreviations are: TPP^+, $TPMP^+$, $TPEP^+$, $TPPP^+$, $TPBP^+$, $TPAP^+$ and $TPHP^+$. TPP^+ was purchased from Dojindo Laboratories (Kumamoto, Japan) and others were from Tokyo Kasei Co. (Tokyo).

The uptake of these phosphonium cations by the vesicles was measured by means of an electrode selective for respective phosphonium ions. The procedure for electrode construction was essentially the same as that for TPP^+-selective electrode [22,23]. The stem of the electrode was electrically shielded with stainless steel tubing (personal communication from Y. Mukohata, Osaka University). This shielding enables us to construct a small electrode (approx. 5 mm in diameter) without increase of an electrical noise level. Before use, the electrodes were soaked in $1 \cdot 10^{-2}$ M of the respective phosphonium solutions for conditioning. The electrode potential follows the Nernst equation

over the concentration range of each probe between 1 μM and 0.1 M in 4 M NaCl. The reference electrode was Ag|AgCl electrode which was connected to the assay medium by a salt bridge. The bridge was made of 2% agar-agar dissolved in the same salt solution as the assay medium. Each time prior to the start of an experiment, a check as to whether the electrode could respond properly to the change in the probe concentration was carried out by adding a known amount of the probe into the assay medium. The assay medium (1.0 ml) was placed in a cuvette installed with water jacket and the temperature was kept constant at 30°C by circulating water. The potential difference between the selective electrode and the reference electrode was measured with an electrometer (Takeda Riken TR8651) and the signal was stored in a microcomputer (NEC PC-9801) through an A/D converter (A&D Electric Co. Tokyo). The calculations for binding and kinetic parameters were performed with the aid of a computer.

The illumination which generates the membrane potential across vesicle membranes was provided by a 1 kW projector lamp through a yellow cut-off filter (over 500 nm). No change in the potential was observed when the vesicles permeated by 1 mM octylglucoside were illuminated, suggesting that illumination caused no artefact in the electrode potential change observed. The actinic light intensity was changed with an appropriate neutral density filter, and measured with a radiometer (Model A26, YSI-Kettering).

Calculation for the binding of probes to the membrane was performed as follows. The electrode potential change was observed when V_1 ml (typical value of 0.03 ml) of the stock vesicle suspension (P mg protein/ml) was added to V_0 ml (1.0 ml as described above) of the medium containing a concentration C_0, of the probe. C_0 was varied between 10 μM and 1 mM. This change in the electrode potential, ΔE, has the following causes: (1) the dilution of the probe concentration by the addition of the stock vesicle suspension and (2) the binding of the probe to the membrane. Note that the membrane potential is not generated unless vesicles are illuminated. This was confirmed by the observation that addition of 1 μM gramicidin did not change the level of the probe uptake in the dark. The former can be calculated by $(RT/F)\ln((V_0 + V_1)/V_0)$, and then the binding of the probe per mg of protein, U_b, was evaluated as follows:

$$U_b = (C_0V_0/PV_1)[1-\exp(-F\Delta E_a/RT)] \quad (2)$$

where

$$\Delta E_a = \Delta E - (RT/F)\ln((V_0+V_1)/V_0)$$

The concentration of the free probe at equilibrium, C, can be evaluated as $C = C_0 \exp(-F\Delta E/RT)$. Preliminary experiment has shown that the total amounts of the binding, i.e., $C_0V_0(1 - \exp(-F\Delta E_a/RT)$ in the above equation, is proportional to the amount of vesicles, indicating applicability of the above equation describing that total amounts of binding are divided by the total amounts of vesicle protein, PV_1.

For the estimation of the light-induced membrane potential, the initial phosphonium probe concentration was 10 μM. When envelope vesicles were added into the medium containing 10 μM of the probe (in the dark), we observed the electrode potential change as described above. With the magnitude of this potential change and Eqn. 2, we could calculate the binding of the probe to the membrane in the absence of the membrane potential. This quantity is denoted as $U_b(0)$, where (0) signifies the value obtained under the condition of $\Delta\varphi = 0$. (This value is equal to U_b if the free concentrations of the probe are identical). Upon illumination, accumulation of the probe into the vesicles occurred, which led to a change in the electrode potential. The magnitudes of uptake of probes were calculated from the deflection of the electrode potential in a manner similar to Eqn. 2 [24].

Since the selective electrode can monitor continuously the change in the concentration of the probe in the external medium, we can analyse the kinetics of the transport of the probe into vesicles. The response time of the electrode was 4–7 s for doubling of the probe concentration. The kinetic constant, k, was calculated by the following first-order kinetic equations:

$$U(t) = U_e[1-\exp(-k^{on}t)] \quad \text{(on kinetics)} \quad (3)$$

$$U(t) = U_e \exp(-k^{off}t) \quad \text{(off kinetics)} \quad (4)$$

where $U(t)$ and U_e stand for the amount of probe accumulated at time t after the onset (on kinetics) and the removal (off kinetics) of the illumination and that at equilibrium, respectively.

Results and Discussion

Fig. 1 is the plot of U_b for various phosphonium probes as a function of the free concentration of the probe at equilibrium, C. U_b increases with the increase of hydrocarbon chain of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3 at the same value of C, which means that more hydrophobic probe binds to the membranes. As C increases, the amount of binding increases and approaches to a plateau level, except for $TPHP^+$. This finding suggests that the saturating binding model is applicable. Therefore, we analysed the binding data in terms of the Langmuir adsorption isotherm:

$$U_b = AC/(K + C) \tag{5}$$

where A and K stand for the maximum amount of binding and the dissociation constant, respectively. Rearrangement of Eqn. 5 yields the following equation:

$$C/U_b = K/A + C/A \tag{6}$$

This equation indicates that the values of K and A can be determined from the plot of C/U_b against C (Dixon plot [25]). Fig. 2A illustrates the plot for TPP^+, $TPEP^+$ and $TPMP^+$, showing that fairly good straight lines are obtained. A least-squares fit

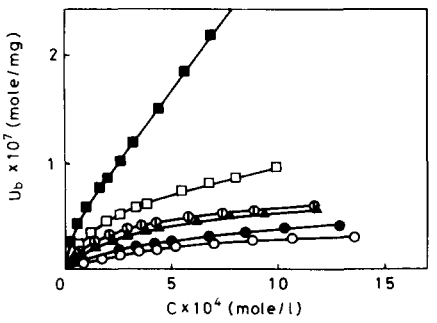

Fig 1 Plot of amounts of binding to membrane, U_b, against the free concentration of the probe, C. The experiments were performed under the condition that $\Delta\varphi = 0$ ○, $TPMP^+$, ●, $TPEP^+$, ▲, $TPBP^+$; ⊕, TPP^+; □, $TPAP^+$, ■, $TPHP^+$

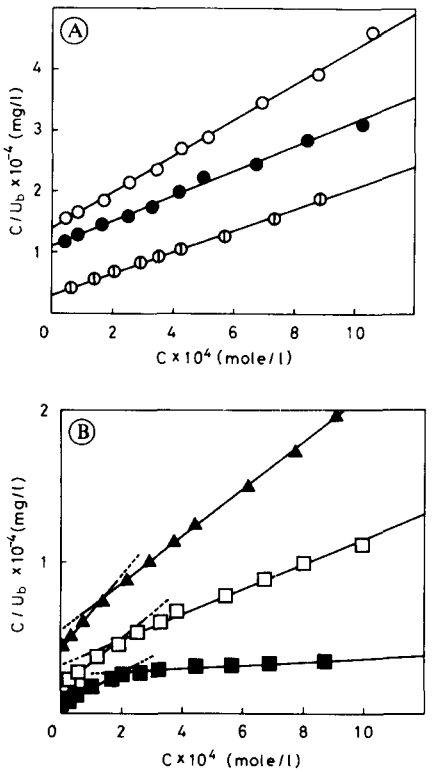

Fig 2 Analysis of the binding to the membrane in accordance with Eqn 6 Notations are the same as in Fig 1

of the data gave a coefficient of determination of 0.995 or more. When probes having longer a hydrocarbon chain, such as $TPBP^+$, $TPAP^+$ and $TPHP^+$, were tested, such plots exhibited the presence of two, high- and low-affinity, binding sites (see Fig. 2B). The concentration at which two straight lines intercept in the figure is denoted as C_t. In Table I, binding parameters in Eqn. 5 for various phosphonium cations are listed. The appreciable increase of amount of binding for probes having a longer hydrocarbon chain, especially for $TPHP^+$, as shown in Fig. 1, is attributed to an increase of the maximum amount of binding for the low-affinity sites.

Fig. 3 illustrates U_b of various probes under varying pH, showing that the binding is independent of pH in the solution. The independence of the binding on pH in the medium may be attributed to the high salt concentration of the medium (4 or 3.5 M NaCl), where the electrical force is

TABLE I

BINDING PARAMETERS OF VARIOUS PROBES TO *H HALOBIUM* MEMBRANES

Strain KH-10 vesicles were used Medium 4 M NaCl at pH 7 buffered with 10 mM Hepes/NaOH. Experiments were done at 30°C. The values of K and A were determined in accordance with Eqn 4

Probe	K (mol/l)		A(mol/mg)	
	Hihg affinity	Low affinity	High affinity	Low affinity
TPP^+	$1.95\ 10^{-4}$		$6.08 \cdot 10^{-8}$	
$TPMP^+$	$4\,48\ 10^{-4}$		$3.38 \cdot 10^{-8}$	
$TPEP^+$	$5\,40 \cdot 10^{-4}$		$4.92\ 10^{-8}$	
$TPBP^+$	$1\,61\ 10^{-4}$	$3.43 \cdot 10^{-4}$	$3.89\ 10^{-8}$	$6.45 \cdot 10^{-8}$
$TPAP^+$	$1\,01 \cdot 10^{-4}$	$4.51 \cdot 10^{-4}$	$5.99 \cdot 10^{-8}$	$1.29\ 10^{-7}$
$TPHP^+$	$4\,72\ 10^{-5}$	$4\,29\ 10^{-4}$	$8.26\ 10^{-8}$	$1.57 \cdot 10^{-6}$

shielded. Upon illumination of the vesicles, the interior-negative membrane potential is generated due to electrogenic chloride transport by halorhodopsin and proton is transported electrophoretically into the vesicles. Since the solutions inside and outside the vesicles are buffered, the pH change caused by H^+ uptake may not occur. Even if the pH change occurs, the results shown in the figure mean that it is not necessary to consider the effect of pH change on the binding parameters.

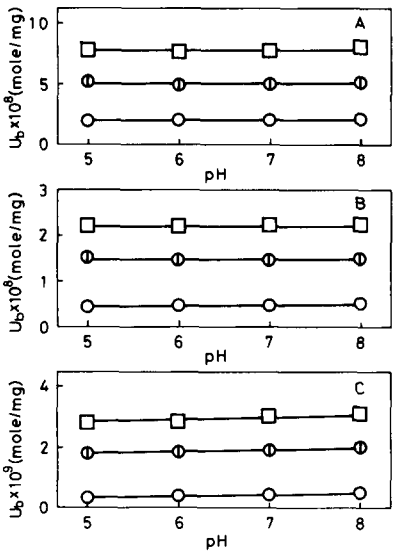

Fig. 3. Invariance of the amount of binding on pH in the medium. A, B and C illustrate the data obtained when the initial concentration C_0 (see Eqn. 2) is 1 mM, 100 μM and 10 μM, respectively The notations for probes are the same as in Fig. 1.

Potential-independent binding model

Upon actinic illumination, the interior-negative membrane potential is generated which causes the uptake of phosphonium probes. Assuming that no additional probe binding occurs even when the probe is accumulated inside, the membrane potential, $\Delta\varphi$, can be calculated and the values thus estimated with various probes are plotted against $U_b(0)$ in Fig. 4. This model is frequently employed for the correction of binding [9,15,16,26–28]. The definition of $U_b(0)$ was given in Materials and Methods. Experiments were performed under varying light intensities. The estimated values of

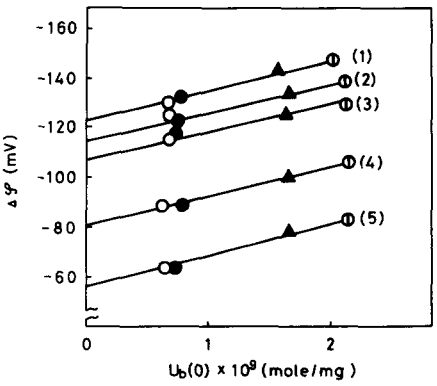

Fig. 4 The membrane potential estimated with the potential-independent binding model under varying light intensities. The abscissa is $U_b(0)$ which represents the amount of binding in the absence of membrane potential. The initial concentration of the probe was 10 μM. The notations for probes are the same as in Fig. 1. The light intensities from experiments (1) to (5) were 1120, 720, 465, 200 and 82 W/m^2, respectively.

$\Delta\varphi$ depended on the probe used. As an example, we compare the estimated values when light intensity is 1120 W/m^2. The largest value (−148 mV) was estimated when TPP^+ was used and the smallest value (−129 mV) was obtained with $TPMP^+$. It is noted that $U_b(0)$ for TPP^+ is larger than that of $TPMP^+$. Fig. 4 shows that the probe whose $U_b(0)$ is larger gave the larger estimated $\Delta\varphi$. Since a large value of $U_b(0)$ means that the probe is liable to bind, the additional binding due to accumulation of the probe should be taken into consideration. This conclusion is reasonable, because Fig. 1 shows that at 10 μM or less (the probe concentration inside the vesicle before illumination), the binding of the probe is not saturated.

Intravesicular binding model

When the membrane potential of −120 mV (interior negative) is generated, the concentration of positively charged probe inside the vesicle, C_{in}, is 100-times C_{out}. Therefore, it seems likely that the binding of the probe to the membrane is governed mainly by C_{in}. The binding is dependent on the membrane potential, but through C_{in}. The amount of binding in the presence of membrane potential, $U_b(\Delta\varphi)$, is given by:

$$U_b(\Delta\varphi) = (1/2)AC_{in}/(K + C_{in})$$

The factor of 1/2 means that the maximum amount of binding which is accessible from the intravesicular space may be half that of A in Eqn. 5.

In applying this equation of $U_b(\Delta\varphi)$ which depends on C_{in}, the difficulty arises that C_{in} is not known until the membrane potential has been calculated. We therefore adopted an iterative procedure:

$$C_{in}(\text{test}) \xrightarrow[\text{model}]{} U_b(\Delta\varphi) \xrightarrow[\text{total amounts}]{} C_{in}(\text{estimated})$$

First, neglecting the binding, we calculated the value of C_{in}, which is taken as C_{in}(test). With C_{in}(test) and the binding parameters (Table I), we calculated the amount of binding $U_b(\Delta\varphi)$ per mg of protein. In this calculation, the binding parameters were changed according to the conditions $C_{in}(\text{test}) > C_t$ or $C_{in}(\text{test}) < C_t$. Since the total amounts of the probe were known, C_{in}(estimated) was able to be calculated and used as a second C_{in}(test). This process was iterated until C_{in}(test) and C_{in}(estimated) agreed within 0.1%. At the same time, $U_b(\Delta\varphi)$ was estimated.

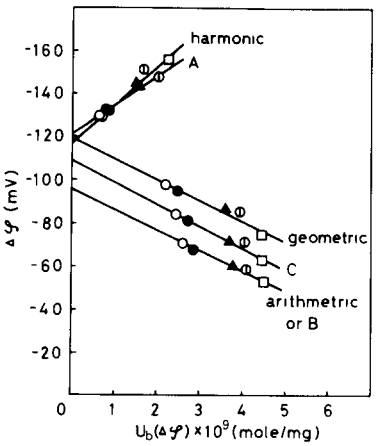

Fig 5 The membrane potentials estimated with the potential-independent model (A), intravesicular binding model (B), exponential mean model (C) and three kinds of mean against $U_b(\Delta\varphi)$ Arithmetic, geometric and harmonic in the figure represent the results obtained with use of arithmetic mean, geometric mean and harmonic mean of C_{in} and C_{out}, respectively For potential-independent model (A), the abscissa is $U_b(0)$ Light intensity was 1120 W/m^2 Notations for the probe used are the same as in Fig 1

The membrane potential which is calculated with C_{in} and C_{out} (see Eqn. 1) is plotted against $U_b(\Delta\varphi)$ in Fig. 5. The estimated $\Delta\varphi$ is not independent of the probe used. When the data points are connected, the straight line with negative slope is obtained. This may suggest that the binding is over-estimated (see below).

Analysis with a mean value of C_{in} and C_{out}

Next, we consider that the binding of the probe to the membrane depends both on C_{in} and on C_{out} [29,30]. Thus, we assume that the binding may be governed by some mean of C_{in} and C_{out}. We have examined the following means:

arithmetic $C_m = (C_{in} + C_{out})/2$

geometric. $C_m = (C_{in}C_{out})^{1/2}$

harmonic $C_m = 2C_{in}C_{out}/(C_{in} + C_{out})$

$$U_b = AC_m(C_m + K)$$

The geometric mean was used successfully for membrane phenomena of homogeneous ion-exchanger membranes. By this mean concentration, the values obtained from the experiment in which the membrane separates two different solutions are linked with the values obtained from the experiment in which both solutions separated by membrane are identical (unpublished data).

The estimated membrane potentials are plotted against $U_b(\Delta\varphi)$ in Fig. 5. None of these gave a value which was independent of the phosphonium probes used. When the harmonic mean was adopted, a rise in the hydrophobic probe which is liable to bind to the membrane gave a larger value. On the other hand, others gave the opposite dependence.

Exponential mean model

Zaritsky et al. [9] proposed a model involving a mean value of probe in the membrane, which is referred to as 'the exponential mean model'. (This mean was used previously by Schlögl [31].) This model is derived under the assumption that (1) the membrane is homogeneous, (2) the potential profile is linear within the membrane and (3) the amount of probe bound in the membrane at position x is proportional to $C_{out} \exp(-F\varphi(x)/RT)$, where $\varphi(x)$ is the electrical potential at x in the membrane.

The presence of the saturation in binding, as shown in Fig. 1 and Table I conflicts with the third assumption of the exponential mean model. This assumption is changed to the assumption that the amounts of binding at x is governed by the Langmuir equation and the other two assumptions are retained. Let us consider that the membrane fraction of x to $x + dx$ is immersed in a hypothetical solution whose probe concentration is $C_{out} \exp(-F\varphi(x)/RT)$. The binding at this stage is described by the Langmuir equation, and the total amount of binding is obtained by integration over the membrane thickness. The equation thus obtained is:

$$U_b(\Delta\varphi) = A \ln[(K + C_{in})/(K + C_{out})]/\ln(C_{in}/C_{out})$$

With this model of binding and the iteration procedure, we calculated $U_b(\Delta\varphi)$ and $\Delta\varphi$. Here, values of A and K were changed whether $C_{in} > C_t$ or $C_{in} < C_t$ (see Table I). The results obtained are plotted also in Fig. 5, showing that this model fails to yield a value independent of the probe used.

Extrapolation to the 'state of no binding'

Fortunately, we obtained a straight line for given light intensity when the values estimated with various probes are connected, as shown in Fig. 5. Moreover, it is interesting that the values extrapolated to the ordinate along with respective lines are approximately equal to each other except intravesicular binding model and arithmetic mean. The same results were obtained for all light intensities examined (data not shown). The value extrapo-lated to the ordinate corresponds to the value estimated with 'hypothetical' probes which never bind to the membrane. If the probes which do not bind to the membrane are accessible, we are able to determine the membrane potential without any correction. The membrane potential using this hypothetical probe is considered to be obtained by extrapolating the value to zero binding. The fact that the extrapolated values are approximately equal irrespective of the model used suggests that the extrapolated value is very close to the real membrane potential. For the sake of simplicity, the extrapolated value in the plot of the membrane potential estimated against $U_b(0)$ is taken as the 'real' membrane potential.

As shown in Fig. 4, the values estimated with $TPMP^+$ are closer to the real value than those with TPP^+. The rate of uptake of $TPMP^+$, however, is appreciably slower than that of TPP^+. It is well known that addition of tetraphenylboron (TPB^-) enhances greatly the rate of $TPMP^+$ transport [3,4]. The kinetic analysis in the presence of TPB^- and the effect on the membrane potential are treated next.

Effect of TPB^- on the binding and light-dependent uptake of phosphonium cations

The binding of the probe to the membrane was measured in the presence of TPB^-. Results are plotted in Fig. 6, showing that $U_b(0)$ does not depend on the concentration of TPB^- up to 10% of the molar ratio of TPB^- to the probe. Bakker [11] also has shown that TPB^- does not change the amount of $TPMP^+$ bound to *E. coli* membranes.

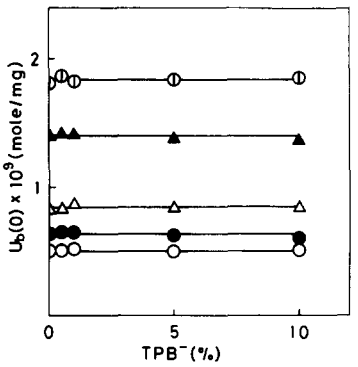

Fig 6. Independence of $U_b(0)$ by addition of TPB^- The initial concentration of phosphonium probes was 10 μM. KH-10 envelope vesicles (1 8 mg protein/ml) was suspended in the medium (4 M NaCl, pH 7 0 buffered with 10 mM Hepes/NaOH) Temperature was 30°C. 10% of TPB^- in the abscissa corresponds to the concentration of 1 μM ○, $TPMP^+$; ●, $TPEP^+$; △, $TPPP^+$; ▲, $TPBP^+$, ⊕, TPP^+

Table II shows the membrane potential estimated by the potential-independent binding model in the presence and absence of TPB^-. This table reveals that the magnitude of the light-dependent uptake of phosphonium probes was not affected by addition of TPB^- in the present system as far as the phosphonium probes used were concerned. This means that TPB^- does not change the estimated value of the membrane potential. The actinic light intensity for the measurement listed in this table was 1120 W/m^2. When weak actinic light intensities were employed, similar results were obtained.

Effect of TPB^- on the rate constant

Addition of TPB^- increased the kinetic constant appreciable. Table III shows the effect of TPB^- on the on- and off-kinetic constants of various probes. The on-kinetic constant of $TPMP^+$, for example, became about 3-times larger than that in the absence of TPB^- when $2 \cdot 10^{-7}$ M TPB^- (2% of $TPMP^+$) was added. Of the probes tested, $TPMP^+$ is most sensitive to the presence of TPB^-. The concentration of TPB^- which is required to give the half-maximum rate is smallest. On the other hand, TPP^+ is hardly influenced. The data of the off-kinetics were essentially the same (see Table III). It seems that the off-kinetics are slightly much affected by TPB^- in comparison with the on-kinetics.

The permeability coefficient is composed of two factors, i.e., the partition coefficient into the membrane phase and the mobilities in the membrane [32]. The parallelism between the binding constants (Table I) and the kinetic constants (Table III, data in the absence of TPB^-) stems from the former factor. The finding that TPB^- does not affect the binding of probes (Fig. 6) but accelerates the transport rate suggests that TPB^- affects only the mobility. Therefore, it is likely that TPB^- works as a carrier [33] for phosphonium probes in the present system rather than the changing the surface potential or intramembrane potential profiles [34], which would alter the amounts of binding. Why TPP^+ and $TPBP^+$, which are transported relatively quickly are not

TABLE II

EFFECT OF TPB^- ON THE MEMBRANE POTENTIAL-DEPENDENT UPTAKE OF VARIOUS PHOSPHONIUM CATIONS

KH-10 membrane vesicles (1 mg protein/ml) were suspended in 4 M NaCl (pH 7.0 with 10 mM Hepes/NaOH) Experiments were performed at 30°C Illumination (over 500 nm, 1120 W/m^2) induces the interior-negative membrane potential, which leads to the uptake of the phosphonium cations. Under the assumption that the membrane potential-dependent binding of probe is ignored (the binding in the absence of membrane potential is only subtracted) which has been called potential-independent binding model, the membrane potentials listed (interior negative) were calculated The initial concentrations of the probes were 10 μM

TPB^- (mole ratio, %)	Membrane potential estimated with Model A (mV)				
	$TPMP^+$	$TPEP^+$	$TPPP^+$	$TPBP^+$	TPP^+
0	122.6	126 6	128 6	139 8	144 1
0.5	125 9	128 2	128 5	140 4	144 5
1	125 7	129 2	130.9	139.7	145 1
5	120.3	125.1	131 4	138.1	144 7
10	121.0	125.5	130 7	137.9	145.4

TABLE III

EFFECT OF TPB^- ON THE ON-KINETICS (k^{on}) AND OFF-KINETICS (k^{off}) OF VARIOUS PHOSPHONIUM CATIONS

Experimental conditions were the same as that in Table II The kinetic constants were calculated in accordance with Eqns 3 and 4.

TPB^- (mol%)	k^{on} (s^{-1})					k^{off} (s^{-1})				
	$TPMP^+$	$TPEP^+$	$TPPP^+$	$TPBP^+$	TPP^+	$TPMP^+$	$TPEP^+$	$TPPP^+$	$TPBP^+$	TPP^+
0	0 022	0.045	0 074	0 131	0 16	0 040	0 079	0 13	0.17	0 16
0 1	0 031	n.d. *	n d	n d	n d	0 60	n.d	n d	n d	n d
0 5	0 059	0 053	0.081	0.14	0 16	0 14	0 098	0 16	0 19	0 16
1	0 065	0 079	0 10	0 14	0.17	0.14	0 20	0 21	0 20	0 17
2	0.75	n d	n d	n.d.	n d	0 20	n d	n d	n d	n d.
5	0 066	0 091	0 10	0.14	0 15	0 23	0.27	0.23	0 22	0 16
10	0 73	0 11	0 13	0 15	0 15	0 25	0.31	0 30	0 24	0 16

* n d means that the experiment was not done

affected by TPB^- is not clear at present. One possible explanation is that these phosphonium cations are more hydrophobic than $TPMP^+$ or $TPEP^+$ and then, the association with TPB^- in the hydrophobic moiety, such as in the membrane, may not occur.

Concluding remarks

In this paper, we have compared the membrane potential estimated with various phosphonium cations under identical conditions. The larger value was estimated when more hydrophobic probe, which binds more to the membrane, was used. We extrapolated the value to the 'state of no binding' and the extrapolated value was considered to be 'real' membrane potential. Further study is necessary to check the validity of this extrapolation. At present, one can say that the value with use of $TPMP^+$ is closest to the real membrane potential. The rate of permeation of $TPMP^+$ through membrane is, however, slow, or sometimes $TPMP^+$ does not permeate through membranes. TPB^- is added to increase the permeation rate. For the present system, at least, TPB^- removes partially the disadvantage of $TPMP^+$ without change of the membrane potential. But, the rate is still 46% of that of TPP^+.

The problem to be solved in future is to analyse quantitatively the membrane potential-dependent binding of the probe. If this succeeds, we can estimate the membrane potential with the probe which can permeate more quickly.

References

1 Felle, H , Porter, J.S , Slayman, C L and Kaback, H R (1980) Biochemistry 19, 3585–3590
2 Mitchell, P. (1979) Eur. J Biochem 95, 1–20
3 Grinius, L L , Jasaitis, A A , Kadziauskas, Yu P , Liberman, E A , Skukachev, V P., Topali, V P., Tsofina, L.M and Vlaoimirova, M.A. (1970) Biochim. Biophys Acta 216, 1–12
4 Harold, F M and Papineau, D. (1972) J Membrane Biol., 8, 27–44
5 Ramos, S , Schuldiner, S and Kaback, H R (1979) Methods Enzymol 55, 680–688
6 Heinz, E , Geck, P and Pietrzyk, C (1975) Ann. N Y Acad. Sci. 264, 428–441
7 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
8 Vacada, V., Kotyk, A and Sigler, K (1981) Biochim. Biophys. Acta 643, 265–268
9 Zaritsky, A., Kihara, M. and Macnab, R.M. (1981) J Membrane Biol. 63, 215–231
10 Casadio, R., Venturoli, G. and Melandri, B A. (1981) Photobiochem. Photobiophys 2, 245–253
11 Bakker, E P. (1982) Biochim Biophys. Acta 681, 474–483
12 Kuroki, M., Kamo, N , Kobatake, Y., Okimasu, E. and Utsumi, K. (1982) Biochim. Biophys. Acta 693, 326–334
13 Guffanti, A.A., Blumenfeld, H. and Krulwich, T A. (1981) J Biol. Chem. 256, 8416–8421
14 Bakker, E.P. and Harold, F M (1980) J Biol Chem 255, 433–440
15 Ghazi, A , Schechter, E , Letellier, L and Labedan, B (1981) FEBS Lett. 125, 197–200
16 Ahmed, S. and Booth, I.R. (1981) Biochem J 200, 573–581
17 Stoeckenius, W and Bogomolni, R A (1982) Annu Rev. Biochem. 52, 587–616
18 Hazemoto, N., Kamo, N., Tsuda, M , Kobatake, Y. and Terayama, Y. (1984) Biophys J 45, 1073–1077
19 Mukohata, Y. and Kaji, Y. (1981) Arch Biochem Biophys 206, 72–76
20 Schobert, B and Lanyi, J K (1982) J Biol. Chem. 257, 10306–10313

21 Lanyi, J K and MacDonald, R.E. (1979) Methods Enzymol 56, 398–407
22 Kamo, N., Muratsugu, M , Hongoh, R and Kobatake, Y (1979) J. Membrane Biol. 49, 105–121
23 Kamo, N., Racanelli, T. and Packer, L. (1982) Methods Enzymol 88, 356–360
24 Muratsugu, M., Kamo, N , Kobatake, Y and Kimura, K (1979) Bioelectrochem Bioenerg. 6, 477–491
25 Dixon, M (1953) Biochem J. 55, 170–171
26 Booth, I R , Mitchell, W.J and Hamilton, W A (1979) Biochem J. 182, 687–696
27 Kashket, E (1981) J Bacteriol 146, 377–384
28 Muratsugu, M , Kamo, N and Kobatake, Y (1982) Bioelectrochem Bioenerg 9, 325–331
29 Lolkema, J S., Hellingwerf, K J and Konnings, W N (1982) Biochim Biophys Acta 681, 85–94
30 Lolkema, J S , Abbing, A , Hellingwerf, K J and Konnings, W N (1983) Eur J. Biochem 130, 287–292
31 Schlogl, R. (1964) Fortschr Physik Chem , Band 9
32 Johnson, F M., Eyring, H and Polissar, M J (1954) Kinetics Basis of Molecular Biology, p 545, John Wiley and Sons, New York
33 Stark, F (1980) Biochim Biophys Acta 600, 233–237
34 Andersen, O.S., Feldberg, S , Nakadomari, H , Levy, S and McLaughlin, S (1978) Biophys J 21, 35–70
35 Lowry, O.H., Rosebrough, N J , Farr, A L and Randall, R J (1953) J. Biol Chem 195, 265–275