Biochimica et Biophysica Acta 894 (1987) 355–364
Elsevier

BBA 42634

Mitochondrial membrane potential estimated with the correction of probe binding

Makoto Demura, Naoki Kamo and Yonosuke Kobatake

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

(Received 21 July 1987)

Key words: Membrane potential; Lipophilic cation binding; Selective electrode; (Mitochondrion)

Lipophilic ions are widely used as the probe for estimation of the membrane potential. It is suggested that the correction of the probe binding to the membrane and/or intracellular constituents is a problem to be solved in order to evaluate the membrane potential accurately. Previously, we proposed a method for the correction of the probe binding (Demura, M., Kamo, N. and Kobatake, Y. (1985) Biochim. Biophys. Acta 820, 207–215). In this paper, the method was applied to the determination of the membrane potential of intact mitochondria. The probes used constitute a homologous series of $(Phe)_3$-P^+-$(CH_2)_n$-CH_3 ($n = 0$–4) and tetraphenylphosphonium (TPP^+). Binding of these probes to de-energized mitochondria followed the Langmuir isotherm. However, values of parameters determined at high (50–800 μM) and low (under 20 μM) probe concentrations were different, suggesting the existence at least two, high- and low-affinity, binding sites. With extrapolation to the 'state of no binding', the membrane potential of intact mitochondria was estimated to be −147 mV (interior-negative) when they were energized by 5 mM succinate in medium consisting of 125 mM KCl, 10 mM $MgCl_2$, 5 mM phosphate, 0.4 mM EDTA and 50 mM Tris-HCl (pH 7.5) at 25 °C. Parameters appearing in the equation for the correction of probe binding were determined with the use of this value of the membrane potential. The validity of the equation and the value of the parameters were revealed by the fact that after the correction, all probes used gave approximately the same value under the same conditions. We expanded the method so as to include the Langmuir adsorption isotherm. When the modified equation is used, the estimated membrane potentials were less dependent on a probe concentration less than 10 μM.

Introduction

Lipophilic cations such as tetraphenylphosphonium and triphenylmethylphosphonium are widely used as a probe for the interior-negative membrane potential of cells, organella and vesicles [1–4]. The principle is that at equilibrium, the probes distribute between internal and external spaces in accordance with the Nernst equation:

$$\Delta\psi = (RT/F)\ln(C_e/C_i) \quad (1)$$

Here, $\Delta\psi$, R, T and F stand for the membrane potential with respect to the outside, gas constant, absolute temperature and Faraday constant, respectively. C_i and C_e are the internal and external concentrations of the probe, respectively.

Abbreviations: TPP^+, tetraphenylphosphonium; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; $TPMP^+$, triphenylmethylphosphonium; $TPEP^+$, triphenylethylphosphonium; $TPPP^+$, triphenylpropylphosphonium; $TPBP^+$, triphenylbutylphosphonium; $TPAP^+$, triphenylamylphosphonium.

Correspondence: N. Kamo, Department of Biophysics, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.

Although the principle is simple, it is pointed out that the binding of the probe leads to an erroneous estimation [5–13]. Therefore, characterization of the probe binding should be studied and a reliable method for the binding correction should be developed. Hubbell and his colleages [14–17] synthesized spin-labeled phosphonium cations to investigate the binding of the spin probe to the liposomal membrane. They proposed a binding model, stating that the probe is located at the interfaces between the membrane and the aqueous phase. This model is derived from findings obtained by Ketterer et al. [18], who studied the transport of lipophilic anions through black lipid membranes with the method of charge-pulse relaxation. This binding model is consistent with the energetic calculation based on hydrophilic-hydrophobic profiles of membranes and on dipole interaction [19]. With the use of the spin-spin coupling method, we have presented the experimental evidence supporting this binding model [20].

Previously, we measured the binding and the uptake of various phosphonium cations by envelope vesicles of *Halobacterium halobium* [11] which generated the interior negative membrane potential upon illumination [22]. The probes used constituted triphenylmethylphosphonium, its homologues, and tetraphenylphosphonium. Apparent accumulation ratios by the cell of various probes are plotted against the binding coefficient of probes. The ordinate intercept of this plot gives the 'real' accumulation ratio because this corresponds to the value obtained with the hypothetical probes which do not bind. This is called extrapolation to the state of no-binding. The binding model described below was applied and the membrane potential obtained after the correction of probe-binding was constant, irrespective of the probe species used under various conditions [23]. In the present paper, we applied this model to the estimation of the membrane potential of intact mitochondria.

Binding model

Briefly, we describe the binding model employed in this paper; for details, refer to a previous paper of ours [23]. The binding sites of the lipophilic cations are the interfaces between the membrane and aqueous solutions. The total uptake of the probe by mitochondria, U, is the sum of free cations in the matrix, bound population at the external binding site, X_{me}, and that at the internal binding site, X_{mi}. Then,

$$U = C_i V_i + X_{me} + X_{mi}$$

where V_i stands for the internal volume of the mitochondria. We have derived that at steady-state, X_{me} is proportional only to C_e and that X_{mi} is proportional only to C_i by solving a set of kinetic equations [23]. We, therefore, obtain that

$$U = C_i V_i + K_e C_e + K_i C_i$$

where K_e is the binding coefficient at external surface multiplied by the volume of the external binding site and K_i is the same quantity of the internal site. It is noted that K_e and K_i are not necessarily equal to each other. Defining that $K_b = K_e + K_i$ and that $f = K_e/K_b$, we obtain that

$$U = C_i V_i + f K_b C_e + (1-f) K_b C_i \tag{2}$$

Combination of Eqns. 1 and 2 yields Eqn. 3.

$$\Delta\psi = -\frac{RT}{F} \ln \frac{U/C_e - fK_b}{(1-f)K_b + V_i} \tag{3}$$

K_b can be determined as follows: Even when $\Delta\psi = 0$, the probe binding occurs and the amount of binding in the absence of the membrane potential is written as $U_b(0)$. From Eqn. 2, $U_b(0)$ is expressed as

$$U_b(0) = K_b C_e = K_b C \tag{4}$$

since $C_e = C_i$ $(= C)$ under this condition. Note that for the calculation of $U_b(0)$, the amount of free probe in the matrix, $V_i C$, is subtracted from the total uptake by de-energized mitochondria. Rottenberg [8] employed essentially the same binding model to analyze the probe-binding and to estimate the mitochondrial membrane potential.

Materials and Methods

Materials

TPP^+ was purchased from Dojindo (Kumamo-

to) and other lipophilic ions were from Tokyo Kasei Co. (Tokyo). Gramicidin D and rotenone were purchased from Sigma, and FCCP was from Boehringer-Mannheim. Other chemicals were analytical grade.

Isolation of mitochondria

Mitochondria were isolated from rat liver by the standard method [24] and washed three times with 250 mM sucrose. Mitochondria were stored at 4 °C until use. Experiments were finished 3–4 h after the isolation. A respiratory control ratio with substrate of 5 mM succinate was 5–7. The medium contained 125 mM KCl, 10 mM $MgCl_2$, 5 mM phosphate, 0.4 mM EDTA and 50 mM Tris-HCl at pH 7.4. Protein was assayed by the method of Lowry et al. [25] with bovine serum albumin as a reference standard. The volume of the matrix was taken as 1.4 μl/mg protein, which was obtained previously in this laboratory [26].

Preparation of de-energized mitochondria

Mitochondria (2 mg protein/ml) were suspended in the medium which contained 2 μM gramicidin D and incubated at 25 °C for 10 min under enough aeration. The mitochondria were collected by centrifugation (6000 × g, 10 min) and washed two times with the medium without gramicidin D. De-energized mitochondria were prepared also with FCCP. The procedure was essentially the same as gramicidin-treated mitochondria, except that the concentration of the ionophore was 0.1 μM and washing was done three times. The probe binding to the de-energized mitochondria was measured in the presence of 2.5 μM rotenone.

Measurement of the uptake of lipophilic cations

The lipophilic cations used were $TPMP^+$ (triphenylmethylphosphonium), $TPEP^+$ (triphenylethylphosphonium), $TPPP^+$ (triphenylpropylphosphonium), $TPBP^+$ (triphenylbutylphosphonium), $TPAP^+$ (triphenylamylphosphonium) and TPP^+ (tetraphenylphosphonium). The amounts of uptake, U was calculated from the decrease in C_e using the following equation:

$$U = (V_i + V_e)C_0 - V_eC_e$$

$$\simeq V_e(C_0 - C_e)$$

where C_0 and V_e stand for the initial probe concentration and extramitochondrial volume, respectively. C_e was monitored with an electrode selective to these lipophilic cations. The construction of electrodes and the procedure and apparatus for measurements were the same as described previously [11,26,27]. All experiments were done at 25 °C. Mitochondrial concentration was typically 1–1.5 mg protein/ml when the respiration-dependent uptake of various phosphonium cations was examined. Mitochondria were energized on addition of 5 mM succinate in the presence of 2.5 μM rotenone. Rotenone was added as an ethanolic solution (ethanol volume was 0.5%).

Results and Discussion

Binding of lipophilic ions to the de-energized mitochondria

For the binding correction in accordance with the model described above, the value of K_b should be known. Eqn. 4 tells that this value is determined from the binding data for the mitochondria whose energy-dependent membrane potential is abolished. For this purpose, mitochondria permeated with lysophosphatidylcholine were prepared. The amounts of binding, however, were dependent on the concentration of lysophosphatidylcholine and it was due presumably to the change of the membrane composition. A high concentration of lysophosphatidylcholine-solubilized mitochondria gave a clear solution. With toluene-treated mitochondria, we could not obtain the reproducible results. Then, we employed the gramicidin D-treated mitochondria. The mitochondria de-energized with FCCP gave almost the same binding data as those of gramicidin de-energized mitochondria.

In Fig. 1A, $U_b(0)$, the amounts of binding of various phosphonium cations to the mitochondria whose membrane potential was abolished, are plotted against C_e. (We assume that $C_e = C_i$ under this condition.) Measurements were made on solutions ranging in concentration below 800 μM. On increasing the number of hydrocarbon chains, the amount of binding was increased. The saturation of binding was observed. Zaratti et al. [28] also reported the saturation of binding, but concentrations where the saturation was achieved were much

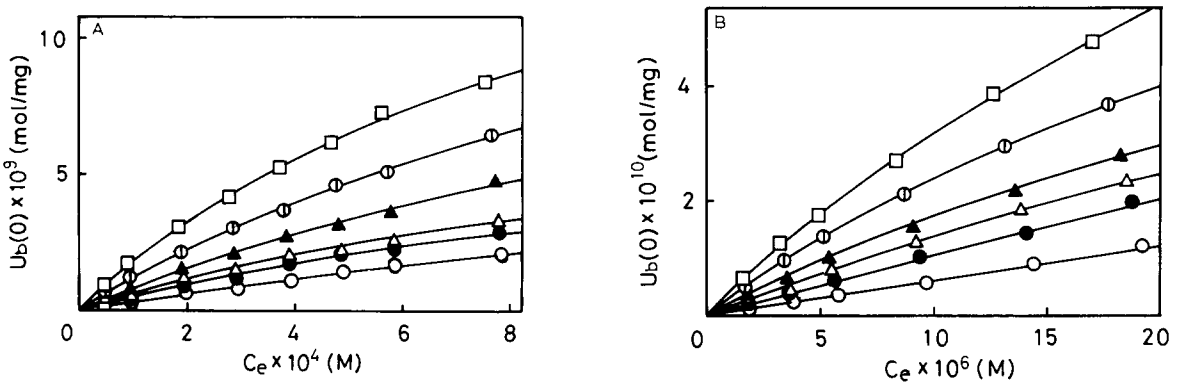

Fig. 1. Binding of various phosphonium cations to the de-energized mitochondria. The concentration range of the phosphonium cation in (A) is less than 0.8 mM. (B) is designed to study the binding at lower concentration of the cation, where the binding is expected to be proportional to the free concentration of the cation. ○, $TPMP^+$; ●, $TPEP^+$; △, $TPPP^+$; ▲, $TPBP^+$; □, $TPAP^+$; ⊕, TPP^+.

lower than those observed in the present paper. The saturation indicates that Langmuir adsorption isotherm is applicable. This isotherm was applied also for the binding to liposome [29] and envelope vesicles of *H. halobium* [11,30]. The Langmuir equation is written as

$$U_b(0) = AC/(K + C) \quad (5)$$

where A, K and C stand for maximum amounts of binding, dissociation constant and the free concentration of lipophilic cations, respectively. The values of the second and third column in Table I show K and A values of the concentration range (50–800 μM) of probes, respectively.

Fig. 1B shows the plot of $U_b(0)$ against C_e under the condition that $C < 20$ μM. Analysis of these data gives K and A values of respective probes and these values are listed in the fourth and fifth columns of the table. The difference in A and K values between the dilute (under 20 μM) and the concentrated (50–800 μM) range implies the existence of at least two binding sites. As this figure shows, the binding seems to be almost proportional to C in this dilute concentration range, except that the cation has a longer hydrocarbon chain. This linearity is also supported by the facts that A of the low concentration range listed in Table I is the order of 10^9 mol/mg, while that of the ordinate of Fig. 1B is 10^{10} mol/mg

TABLE I

BINDING PARAMETERS FOR BINDING OF VARIOUS PHOSPHONIUM CATIONS TO DE-ENERGIZED MITOCHONDRIA

Medium was 125 mM KCl, 10 mM $MgCl_2$, 5 mM phosphate, 0.4 mM EDTA and 50 mM Tris-HCl (pH 7.4). Temperature was 25 °C. De-energized mitochondria were prepared with 2 μM gramicidin D as described in Materials and Method. K, A and K_b are defined in Eqns. 5 and 6. The high concentration range is 50–800 μM of respective probes and the dilute concentration range is 2–20 μM. The right-most column shows the K_b value calculated by Eqn. 6 using the data of the low concentration range.

Probe	Langmuir equation				K_b
	high concentration range		low concentration range		
	K (mol/l)	A (mol/mg)	K (mol/l)	A (mol/mg)	
$TPMP^+$	$2.92 \cdot 10^{-3}$	$9.70 \cdot 10^{-9}$	$1.31 \cdot 10^{-1}$	$7.95 \cdot 10^{-7}$	$6.07 \cdot 10^{-6}$
$TPEP^+$	$2.53 \cdot 10^{-3}$	$1.23 \cdot 10^{-8}$	$8.93 \cdot 10^{-4}$	$9.53 \cdot 10^{-9}$	$1.07 \cdot 10^{-5}$
$TPPP^+$	$1.32 \cdot 10^{-3}$	$8.46 \cdot 10^{-9}$	$7.68 \cdot 10^{-5}$	$1.20 \cdot 10^{-9}$	$1.56 \cdot 10^{-5}$
$TPBP^+$	$2.19 \cdot 10^{-3}$	$1.78 \cdot 10^{-8}$	$6.09 \cdot 10^{-5}$	$1.21 \cdot 10^{-9}$	$1.98 \cdot 10^{-5}$
$TPAP^+$	$1.02 \cdot 10^{-3}$	$1.98 \cdot 10^{-8}$	$4.03 \cdot 10^{-5}$	$1.60 \cdot 10^{-9}$	$3.98 \cdot 10^{-5}$
TPP^+	$1.51 \cdot 10^{-3}$	$1.89 \cdot 10^{-8}$	$3.96 \cdot 10^{-5}$	$1.19 \cdot 10^{-9}$	$3.00 \cdot 10^{-5}$

and that K values of the low concentration range are larger than 20 μM. When C is dilute enough, Eqn. 5 is simplified to Eqn. 4, with the relation that

$$A/K = K_b \tag{6}$$

The values of K_b were determined from Eqn. 6 using the value of the low concentration range, and are listed in the right-most column of Table I. With the increase of the length of hydrocarbon chain, the value of K_b increases. These values of mitochondria are about 1/10 times as small as those of envelope vesicles of *H. halobium* for all cations examined [11]. The values of K and A for $TPMP^+$ of low concentration range are somewhat strange in comparison with those of others. As shown in Figs. 1A and B, its binding is almost linear up to as high as 0.8 mM. Then, the determination for the values of K and A are not easy. But K_b value is determined exactly.

Extrapolation to the 'state of no binding'

Fig. 2 shows the plot of value of C_i^{app}/C_e against C_e for various lipophilic cations, where C_i^{app} is the 'apparent' intramitochondrial concentration of lipophilic cations, which includes the free and bound populations of the ions inside mitochondria, and is calculated simply as $C_i^{app} = U/V_i$. The ordinate values depend both on the lipophilic cation used and on C_e. When the cation of high lipophilicity is used, C_i^{app}/C_e gives larger value and the dependence on C_e is obvious. The dependence on C_e will be discussed later. In a previous paper [11], we proposed that the ordinate intercept of the plot of C_i^{app}/C_e against K_b may be the 'real' accumulation ratio caused by the membrane potential because this value corresponds to the value obtained with the 'hypothetical' probe which does not bind. This plot was performed in Fig. 3, where the values extrapolated to $C_e = 0$ in Fig. 2 are plotted in order to remove the influence of the probe concentration. The binding coefficient, K_b, should be used because the probe concentrations are dilute enough.

The real membrane potential calculated from the ordinate intercept (see Eqn. 7) is -147 mV, which is comparable to those reported by previous authors, although $\Delta\psi$ may be changed depending on the experimental conditions. Rottenberg [8] reported the value of 150–118 mV by means of Rb^+ distribution in the presence of valinomycin and 157–123 mV using lipophilic cation after correction of binding. Azzone et al. [31] described that mitochondrial membrane potential is 143 mV when calculated on the assumption that only the

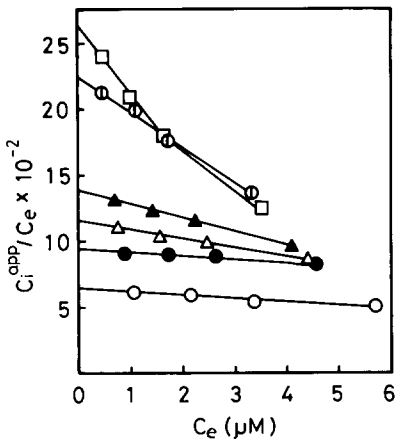

Fig. 2. Plot of C_i^{app}/C_e against C_e. C_i^{app} is the amount of uptake by energized mitochondria divided by the intramitochondrial volume, which means the apparent intramitochondrial concentration, including the bound populations of the probe. C_e is the free concentration of the probe in the solution. Notations are the same as in Fig. 1.

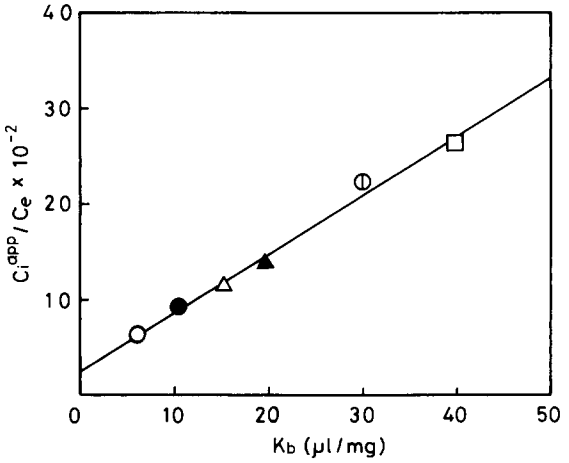

Fig. 3. Extrapolation to the 'state of no binding'. C_i^{app}/C_e values for various phosphonium cations are plotted against its K_b value. The ordinate value is taken from that extrapolated to $C_e = 0$ in Fig. 2 for the respective phosphonium cations. Notations are the same as in Fig. 1.

increase of cation uptake due to the presence of anions corresponds to cations free in the matrix. Scott and Nicholls [32] calculated 148 mV on the assumption that 60% of $TPMP^+$ accumulated is bound. From the value of C_i^{app}/C_e shown in Fig. 3, it is calculated that the values estimated with $TPMP^+$ and TPP^+ are overestimated by 22 and 57 mV, respectively. Wilson and Forman [33] showed that ($\Delta\psi$ with $TPMP^+$) = 0.96 · ($\Delta\psi$ with valinomycin + K^+) + 27.7 mV. If the value determined by means of K^+ distribution in the presence of valinomycin is taken as the true membrane potential, their data indicate that $TPMP^+$ gives the overestimation of 27.7 mV, because the slope of 0.98 is approximately equal to unity. This value of overestimation is close to ours. Duszynski et al. [34] obtained 169 mV using $TPMP^+$ without any binding correction. As is described above, the overestimation stemmed from $TPMP^+$ binding is 22 mV, and then this value may be corrected to 147 mV, which is equal to the value obtained in this paper. Wojtczak et al. [35] obtained a larger value of −190 mV after the binding correction.

If we used the value of C_i^{app}/C_e whose C_0 was larger than 4 μM instead of the extrapolated value in Fig. 2, the linearity of Fig. 3 was not good especially for $TPAP^+$. The deviation of the point of $TPAP^+$ may be caused from the fact that the binding is not proportional to the concentration, as shown in Fig. 1.

When −147 mV is taken as the membrane potential of mitochondria, we can calculate C_i and the amount of membrane potential-dependent binding (denoted as $U_b(\Delta\psi)$) can be also calculated by subtracting V_iC_i from U. In Table II, the values of U and $U_b(\Delta\psi)/U$ for various cations are listed. The value of $U_b(\Delta\psi)/U$ represents the ratio of the bound population to the total uptake of cations. For $TPAP^+$, this value amounts to as high as 0.97, meaning that 97% of the accumulated probe cation is bound. Shen et al. [36] reported that about 85% of TPP^+ accumulated by mitochondria is bound, indicating good agreement with our data. Rottenberg [8] and Wilson and Forman [33] reported a slightly smaller value of 77%. As mentioned above, Scott and Nicholls [32] assumed that 60% of $TPMP^+$ is bound and this value agrees well with the 59% of the present paper.

TABLE II

COMPARISON OF THE AMOUNTS OF BOUND POPULATION WITH THE TOTAL UPTAKE BY RESPIRING MITOCHONDRIA

Medium was the same as in Table I. Mitochondria were energized by 5 mM succinate in the presence of 2.5 μM rotenone. The temperature was 20 °C. The membrane potential was taken as −147 mV, which was calculated from the ordinate intercept of Fig. 2. The initial concentration of the probe was 2 μM.

Probe	U (nmol/mg)	$U_b(\Delta\psi)/U$
$TPMP^+$	0.98	0.59
$TPEP^+$	1.09	0.70
$TPPP^+$	1.18	0.73
$TPBP^+$	1.25	0.82
$TPAP^+$	1.48	0.97
TPP^+	1.44	0.87

Determination of the value of f

For the binding correction with use of Eqn. 3, we should know the value of f. The criterion for the valid estimation of f is that all probes should give the same membrane potential after a proper correction. We introduce the following R value: $R = \Sigma((\text{calculated } \Delta\psi \text{ with assumed } f) - (-147 \text{ mV}))^2$ where summation was taken for all lipophilic cations used. The most suitable value of f should give the smallest value of R. f was changed from zero to unity with a step of 0.1 to calculate the R value and the roughly fitted value was obtained. Around this value, f was changed with a step of 0.01 and we found that $f = 0.66$ as the best value. Table III shows the membrane potential estimated from Eqn. 3 with $f = 0.66$, revealing that the estimated values are very close to the value estimated from the ordinate intercept in Fig. 3. It is also noteworthy that all probes used give almost the same value (see top row). For this calculation, the data of $C_0 = 2$ μM were used (the left-most data of respective phosphonium cation in Fig. 2 whose abscissa is C_e).

Since $U = C_i^{app}V_i$, Eqn. 2 is rewritten with aid of Eqn. (1) to the following equation:

$$\frac{C_i^{app}}{C_e} = \{f + (1-f)e^{-F\Delta\psi/RT}\}\frac{K_b}{V_i} + e^{-F\Delta\psi/RT} \qquad (7)$$

TABLE III

MEMBRANE POTENTIAL OF RESPIRING MITOCHONDRIA AFTER THE CORRECTION OF PROBE BINDING

Values are mV. Correction was performed with Eqn. 3. f was taken as 0.66 and the values of K_b for the respective probe used is listed in the right-most column of Table I. The medium was the same as that of Table I. Mitochondria were energized by addition of 5 mM succinate in the presence of 2.5 μM rotenone. n.d., not determined. The averaged value, which is calculated over the values estimated by various probes, is shown by mean value ± S.D.

[Gramicidin] (nM)	$TPMP^+$	$TPEP^+$	$TPPP^+$	$TPBP^+$	$TPAP^+$	TPP^+	Average ± S.D.
0	−147	−145	−141	−141	−140	−145	−143 ± 2.6
0.2	−142	−146	−143	−140	−136	−143	−142 ± 3.1
2.0	−118	−116	−118	−115	−115	−121	−117 ± 2.4
20.0	−59	54	−61	−55	n.d.	n.d.	−57 ± 2.9

This equation indicates that we can calculate the value of f from the slope of Fig. 3, since $\Delta\psi$ is already known to be −147 mV from the ordinate intercept. The value of f thus estimated was also 0.66.

If this value is valid, the estimated values of membrane potential should be probe-independent under any conditions. We changed the membrane potential by addition of varying concentration of gramicidin D. The estimated values with $f = 0.66$ are listed also in Table 3, showing that the values obtained with various probes give almost the same value.

In a previous paper [23], we used the parameter n, which signified the effect of membrane potential on the binding coefficient. We analyzed our data to determine n by the method described previously [23] and found that $n = 0$ gave the best fit. If the bound probe is located at the interface between the membrane and aqueous solution, the value of n may be zero.

Comparison of a method of Lolkema et al.

Lolkema et al. [10,37] have proposed a model of the probe-binding. The average concentration within membranes was introduced. The flux equations of probes are composed of four unidirectional fluxes through the membrane/water interfaces. At steady-state, the net flux should be zero and the concentration within the membrane can be calculated. The final equation they obtained is as follows:

$$\Delta\psi = \frac{RT}{F} \ln \frac{\frac{C_0}{C_e} - 1 + \frac{V_i}{V_e}(1 - \frac{1}{2}f_{cm}K_{cm} - f_{om}K_{om})}{\frac{V_i}{V_e}(1 + K_i + \frac{1}{2}f_{cm}K_{cm})} \quad (8)$$

Here, definitions of parameters should be referred to Ref. 37. With the relation that $U = V_e(C_0 - C_e)$, Eqn. 8 is rewritten as

$$\Delta\psi = \frac{RT}{F} \ln \frac{\frac{U}{C_e} + V_i(1 - \frac{1}{2}f_{cm}K_{cm} - f_{om}K_{om})}{V_i(1 + K_i + \frac{1}{2}f_{cm}K_{cm})} \quad (9)$$

It may be possible to consider that the total binding coefficient in the present paper, K_b, corresponds to $V_i(K_i + f_{cm}K_{cm} + f_{om}K_{om})$ and that at outer surface, fK_b, to $V_i(1/2f_{cm}K_{cm} + f_{om}K_{om})$. Note that K_b has a dimension of volume. Therefore, Eqn. 9 is recast to Eqn. 10 with use of our notation.

$$\Delta\psi = \frac{RT}{F} \ln \frac{\frac{U}{C_e} + V_i - fK_b}{V_i + (1 - f)K_b} \quad (10)$$

The only difference between Eqns. 3 and 10 is the term of V_i in the numerator. Since $U > C_iV_i$ (note that U contains the amounts of bound probes) and since $C_i \gg C_e$ when interior negative membrane potential is enough large, we obtain that $U/C_e \gg V_i$. This means that Eqns. 3 and 10 are practically the same where the interior negative membrane potential is large enough.

On the dependence of the ordinate value in Fig. 2 on the probe concentration

As shown in Fig. 2, the value of C_i^{app}/C_e depends on C_e. The possible reason of this dependence may be as follows: Actual binding of lipophilic probe cations accords with the Langmuir equation, whereas in Eqn. 2, the binding is assumed to be proportional to the free concentra-

tion. Fig. 1(B) shows that even below 20 μM, the binding is not proportional to the free concentration for the probe having a longer hydrocarbon chain. At a relatively high concentration range of the probe, the binding may be saturated (note that the intramitochondrial free concentration is about 300-times as high as that of the outside) and this leads to the bound population contributing less to the total uptake. This may be the reason why the estimated membrane potential with high probe concentration is close to the true value. Lipophilic cations of higher concentration, however, may swell or do damage to membranes, which lowers the membrane potential.

We extend Eqn. 2 to the following equation:

$$U = C_i V_i + A_i C_i/(K_i + C_i) + A_e C_e/(K_e + C_e) \quad (11)$$

Here, subscripts i and e of the second and third terms in the right-hand side of Eqn. 11 denote the quantities of internal and external membrane surfaces. Since C_i is high, K_i is employed for the high concentration range and since C_e is dilute enough K_e is employed for the low concentration range. A_i and A_e stand for the maximum amounts of binding at internal and external surfaces of membrane. The problem for the value of A_i and A_e may arise: The maximum amounts of binding in high concentration range listed in Table I may not be equal to A_i, because only the inner surface of the membrane is contiguous to the solution of high probe concentration whereas the values in the table were determined when both solutions contiguous to the membrane were the same. A_i and A_e are assumed to be given by following expressions, respectively:

$$A_i = (1 - f)A \quad (12A)$$

where A is the maximum amounts of binding in the concentrated region and

$$A_e = fA \quad (12B)$$

where A is the maximum amounts of binding in the dilute region. It is noted that the values of A and K are determined so as to follow the data at low- and high-concentration ranges and that they are not those for high- and low-affinity binding sites. The value of f was determined by calculating R values described above and we obtained that $f = 0.63$. (It is noted that $f = 0.66$ for linear binding equation.) Then, only C_i is not known in Eqn. 11. Note that C_e is monitored with the electrode. Since this equation is a quadratic equation with respect to C_i, C_i is easily solved and the membrane potential can be calculated. Results are listed in Table IV, where the average values of those estimated with various probes of varying concentration are shown. It is noted that the dependence of the estimated potential on the probe concentrations (under 10 μM) becomes slight in comparison with those estimated with Eqn. 2. The values calculated with the linear binding equation are smaller than those with Langmuir equation, meaning that amounts of binding calculated with the linear binding equation are overestimated. This comes from the saturation of binding.

Further problems to be solved

There are several problems to be awaited for further studies. The first is the development of the method for determination of f. The present method requires the use of at least two kinds of probes because $\Delta\psi$ and f are unknown. If we develop the method of determination of f with only one probe, we can estimate the membrane potential with Eqn. 11 from the uptake of only one probe. On the other hand, all parameters which appear in the method of Lolkema et al. [10,37] can be determined experimentally. However, it is stressed that their model does not con-

TABLE IV

CORRECTION OF PROBE BINDING WITH Eqn. 11

The membrane potentials estimated with various probes whose initial concentration was listed in the first column are averaged and the S.D. is also shown. The value of f was 0.66 and 0.63 for linear and Langmuir binding equation, respectively. The experimental conditions were the same as those in Table II and III.

Probe concentration (μM)	Average ± S.D. estimated with Eqn. 11 (Langmuir) ($f = 0.63$)	Average ± S.D. estimated with Eqn. 2 (linear) ($f = 0.66$)
2	−152 ± 5.7	−143 ± 2.6
4	−151 ± 5.8	−140 ± 2.5
6	−151 ± 5.7	−138 ± 2.7
10	−147 ± 4.8	−133 ± 3.6

sider the asymmetry of membranes. If the asymmetry is incorporated into their theory, one additional parameter indicating the asymmetrical properties, such as f of the present paper, should be needed.

The second problem is the correction of Donnan potential of de-energized mitochondria. The present treatment ignores the Donnan potential of energy-depleted mitochondria, although the potential may be reduced in the high ionic solutions such as used in this work.

The third is the neglect of the change in V_i during energization. Fortunately, Eqn. 3 indicates that contribution of the volume change to the estimated $\Delta\psi$ may be small provided that $(1-f)K_b \gg V_i$. This condition is fulfilled for TPP^+, as is shown in Table I, since $V_i = 1.4$ μl/mg and $(1-f)K_b = 10$ μl/mg. But, for $TPMP^+$, the change in V_i is not negligible. When Eqn. 11 is used, the essential feature is also held.

The fourth is the question of whether binding parameters depend on the membrane potential or not. When the amounts of bound probes are increased, we cannot deny the possibility that some of probes are located in the middle part of membranes and that the binding parameters depend on $\Delta\psi$. Although our analysis gives the independence of the binding parameters on $\Delta\psi$, further study on this point seems to be needed.

The fifth is concerned with the use of the Langmuir equation, i.e., Eqns. 11 and 12. Since C_i is high, the values of K_i and A_i are used for those determined at the high concentration range. C_e is several μM and, then, A_e and K_e are used for those of low concentration range. This may be reasonable, but Eqn. 12 is just a simple assumption. Results shown in Table IV may support these assumptions. On the other hand, Eqn. 2 or 3 is valid as far as binding is linear to the probe concentration, even if the binding affinities are different at internal and external surfaces. The linearity of binding is held when the concentration of probe is dilute enough.

In spite of problems described above, the present paper shows one suitable method for the probe binding to estimate the membrane potential with the use of lipophilic ions.

References

1 Grinius, L.L., Jasaitis, A.A., Kadzianskas, Yu.-P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vlanoimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1–12

2 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 27–22

3 Ramos, S., Schuldiner, S. and Kaback, H.R. (1979) Methods Enzymol. 55, 680–688

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

5 Mehlhorn, R.J., Candau, P. and Packer, L. (1982) Methods Enzymol. 88, 751–762

6 Zaritsky, A., Kihara, M. and Macnab, R.M. (1981) J. Membrane Biol. 63, 215–231

7 Casadio, R., Venturoli, G. and Melandri, B.A. (1981) Photobiochem. Photobiophys. 2, 245–253

8 Rottenberg, H. (1984) J. Membrane Biol. 81, 127–138

9 Bakker, E.P. (1982) Biochim. Biophys. Acta 681, 474–483

10 Lolkema, J.S., Abbing, A., Heillingwerf, K.J. and Konnings, W.N. (1983) Eur. J. Biochem. 130, 287–292

11 Demura, M., Kamo, N. and Kobatake, Y. (1985) Biochim. Biophys. Acta 812, 377–386

12 Gibrat, R., Barbier-Brygoo, H., Guern, J. and Grignon, C. (1985) Biochiom. Biophys. Acta 819, 206–214

13 Muratsugu, M., Kamo, N., Kobatake, Y. and Kimura, K. (1979) Bioelectrochem. Bioenerget. 6, 477–491

14 Cafiso, D.S. and Hubbell, W.L. (1978) Biochemistry 17, 187–195

15 Cafiso, D.S. and Hubbell, W.L. (1981) Annu. Rev. Biophys. Bioeng. 10, 217–244

16 Cafiso, D.S. and Hubbell, W.L. (1982) Biophys. J. 39, 263–272

17 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 49, 531–540

18 Ketterer, B., Neumcke, B. and Lauger, P. (1971) J. Membrane Biol. 5, 225–245

19 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 49, 541–552

20 Demura, M. (1985) Ph.D. Thesis, Hokkaido University, Sapporo

21 Reference deleted

22 Hazemoto, N., Kamo, N., Tsuda, M., Kobatake, Y. and Terayama, Y. (1984) Biophys. J. 45, 1073–1077

23 Demura, M., Kamo, N. and Kobatake, Y. (1985) Biochim. Biophys. Acta 820, 207–215

24 Hogeboom, G. (1955) Method Enzymol. 1, 16–19

25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1953) J. Biol. Chem. 195, 265–275

26 Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membrane Biol. 49, 105–121

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

28 Zaratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. J. Biochem. 126, 443–451

29 Demura, M., Kamo, N. and Kobatake, Y. (1987) Biochim. Biophys. Acta, in the press

30 Kamo, N., Demura, M. and Kobatake, Y. (1986) J. Membrane Sci. 27, 233–239
31 Azzone, G.F., Bragadin, M., Pozzan, T. and Dell'Antone, P. (1976) Biochim. Biophys. Acta 459, 96–109
32 Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 186, 21–33
33 Wilson, D.F. and Forman, N.G. (1982) Biochemistry 21, 1438–1444
34 Duszynski, J., Bogucka, K. and Wojtczak, L. (1984) Biochim. Biophys. Acta 767, 540–547
35 Wojtczak, L., Zolkiewska, A. and Duszynski, J. (1986) Biochim. Biophys. Acta 851, 313–321
36 Shen, C., Boens, C.C. and Ogawa, S. (1980) Biochem. Biophys. Res. Commun. 93, 243–249
37 Lolkema, J.S., Hellingwerf, K.J. and Konnings, W.N. (1982) Biochim. Biophys. Acta 681, 85–94