

Calculation of Membrane Potential in Synaptosomes with Use of a Lipophilic Cation (Tetraphenylphosphonium)

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To estimate membrane potential in synaptosomes with the use of tetraphenylphosphonium (TPP^+), an equation relating the amount of TPP^+ accumulated in synaptosomes with membrane potential was derived from the following two assumptions. (1) TPP^+ molecules were distributed into plasma membranes, mitochondria and cytosol of synaptosomes. (2) TPP^+ achieves a Nernst equilibrium across both the synaptosomal and inner mitochondrial membranes. We propose three methods for calculation of membrane potential using this equation. The concentration of TPP^+ was measured under various controlled conditions with an electrode selective for TPP^+ . The amount of TPP^+ accumulated in synaptosomes was determined by measuring the difference between its initial concentration and the concentration after addition of synaptosomes, and membrane potential was estimated by the three methods. The resting potential of synaptosomes was estimated to be -75 to -90 mV by all of these methods. Membrane potentials under various controlled conditions were calculated, and the characteristics of the methods for estimation of membrane potential and those of membrane potential obtained by the methods are discussed.

Keywords membrane potential; tetraphenylphosphonium; lipophilic cation; synaptosome; energy metabolism

Neural cells are ordinarily too small to measure membrane potential directly with a microelectrode. There are several approaches available to measure membrane potential in small cells or vesicles. Permeable cations¹⁻¹²⁾ and many optical probes¹²⁻¹⁸⁾ have been widely used to estimate change in membrane potential in neural cells. The use of optical probes is experimentally easy, but some assumptions are needed to calculate membrane potential.^{12,14-17)} The direct measurement of the equilibrium distribution of K^+ (or Rb^+) allows us to determine a theoretically exact membrane potential.⁵⁾ Although the distribution of lipophilic cations is more complicated than that of K^+ , lipophilic cations labeled with an isotope have been widely used for measuring membrane potential. Several methods for calculation of membrane potentials using lipophilic cations were reported with synaptosomes and neural cells.^{1-2,5,12)}

In this report, we derive a new equation, which relates the distribution of a lipophilic cation, tetraphenylphosphonium, (TPP^+) in synaptosomes with membrane potential in synaptosomes. Using the equation, we propose three methods for estimation of membrane potential. The concentration of TPP^+ was measured with an electrode selective for TPP^+ under various controlled conditions, and membrane potential of synaptosomes was estimated from this value using the three methods. We discuss the obtained values and the characteristics of the methods for estimation of membrane potential.

Experimental

Solutions Incubation media used were: (1) high K^+ medium containing 137 mM KCl , 10 mM glucose and 10 mM imidazole-HCl (pH 7.4); (2) normal K^+ medium containing 132 mM NaCl , 5 mM KCl , 10 mM glucose and 10 mM imidazole-HCl (pH 7.4). In some experiments, we employed the medium without glucose. All solutions used in the present experiment were prepared with distilled water.

Preparation of Synaptosomes Synaptosomes were prepared from rat brains according to the method of Booth & Clark.¹⁹⁾ Some experiments were carried out using crude synaptosomes (P2 fractions).

Preparation of TPP^+ -Selective Electrode The membrane to be used for the electrode selective for TPP^+ was made from polyvinyl chloride (PVC), tetraphenylboron and dioctylphthalate essentially as described by Kamo

*et al.*²⁰⁻²²⁾ The membrane was glued on PVC tubing (diameter 1 cm) with tetrahydrofuran, and the internal filling solution was 10 mM TPP^+ . The electrode potential follows the Nernst equation above 500 nM TPP^+ .

Measurement of TPP^+ Accumulation by the Electrode The electrode was assembled into a glass chamber of 2.5 ml capacity containing a bridge to a KCl reference electrode. The temperature of the chamber was kept constant by circulating water. Synaptosomal suspension (0.25 ml) was added to incubation medium (2.5 ml) containing 0.005 mM TPP^+ . The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously. The signal was stored in a microcomputer (NEC PC-8001mk2, Japan) through an A/D converter, which was made of an ADC-0809 CCN (National Semiconductor, U.S.A.). Membrane potentials were calculated with the aid of the computer. The concentration of synaptosomal proteins was 2–4 mg protein/ml.

Reagents Tetraphenylboron was obtained from Nakarai Chemicals Co. (Kyoto). TPP^+ was purchased from Kanto Chemicals Co. (Tokyo). Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and valinomycin were from Sigma Chemicals Co. All other materials from commercial sources were of the highest purity available.

Results and Discussion

Synaptosomal and mitochondrial membranes are permeable to TPP^+ .^{2,5,12)} Synaptosomes accumulated TPP^+ in high K^+ medium where no electrical potential difference is thought to exist across the plasma membranes of synaptosomes. They also accumulated TPP^+ in the presence of CCCP in high K^+ medium which abolishes the membrane potential of mitochondria. This means that TPP^+ is distributed across plasma membranes of synaptosomes and intrasynaptosomal mitochondria. It was reported that TPP^+ is bound to biological membranes^{20,22)} and liposomes.²³⁾ When synaptosomal suspension (volume v) was added to saline containing TPP^+ (concentration C_0), the concentration of the cation was decreased to C_1 as the result of the uptake of the cation by synaptosomes. Assuming that TPP^+ molecules are distributed in the cytosol, plasma membrane and intracellular mitochondria of synaptosomes according to their electric potential, Eq. 1 can be obtained by the law of mass action:

$$V_0 C_0 = Q_s + (V_0 + v) C_1 + Ab \quad (1)$$

where V_0 is the initial volume of saline and Q_s and Ab are

the amount of TPP^+ incorporated into synaptosomes and that of the cation absorbed on synaptosomal membranes, respectively. Total uptake by synaptosomes (Q_t) is given by Eq. 2.

$$\begin{aligned} Q_t &= C_0 V_0 - C_1 (V_0 + v) \\ &= Q_s + Ab \end{aligned} \quad (2)$$

Since Q_s is the sum of the quantity of TPP^+ incorporated into the intracellular mitochondria (Q_m) and the amount present in synaptosomal cytosol, we obtain Eq. 3:

$$Q_s = Q_m + V_s C_s \quad (3)$$

where V_s and C_s are the volume of synaptosomal cytosol and the concentration of the cation in synaptosomal cytosol, respectively. When the distribution of the cation achieves a Nernst equilibrium across both the synaptosomal and intracellular mitochondrial membranes, the following Eqs. 4 and 5 can be obtained:

$$C_s = C_1 \exp(FE_s/RT) \quad (4)$$

$$Q_m = C_s V_m \exp(FE_m/RT) \quad (5)$$

where E_s and E_m are the membrane potential of synaptosomes and mitochondria, respectively, and V_m is the volume of mitochondria. F , R and T are thermodynamical constants.

Combining Eqs. 2–5, the membrane potential of synaptosomes (E_s) can be expressed by Eq. 6.

$$E_s = (RT/F) \ln(Q_t - Ab) / C_1 [V_s + V_m \exp(FE_m/RT)] \quad (6)$$

If T , C_1 , V_s , V_m , Ab and E_m are known, E_s can be estimated from Eq. 6. Mitochondrial electrical potential (E_m) has been estimated to range from -150 to -180 mV,^{5,21,24–27} and synaptosomal volume (V_s) from 3.2 to 4.0 $\mu\text{l}/\text{mg}$ of synaptosomal protein.^{4,5,8} For this paper, we adopted values of 80 nl/mg of synaptosomal protein for V_m , 3.2 $\mu\text{l}/\text{mg}$ of synaptosomal protein for V_s , and -160 mV for E_m . The values of V_m and V_s are the same as those used by Scott and Nicholls.⁵ We used the following three methods for estimation of Ab , and calculated membrane potentials. The initial concentration of TPP^+ was fixed at 0.005 mM, because the cation at high concentration affected membrane potential, as shown previously.²⁸

1) Method 1 The uptake of TPP^+ by synaptosomes in high K^+ medium, where membrane potential (E_s) is depolarized, must be related to that of TPP^+ by mitochondria in cytosol. In high K^+ medium, the uptake of the cation in mitochondria (Q_{mk}) can be expressed by Eq. 7, because C_s is equal to C_1 in Eq. 5.

$$Q_{mk} = C_1 V_m \exp(FE_m/RT) \quad (7)$$

Supposing that Ab is independent of change in membrane potential, Ab can be calculated using the result in high K^+ medium by means of Eq. 8, which was obtained from Eqs. 2, 3, 4 and 7 supposing E_s to be zero.

$$\begin{aligned} Ab &= Q_t - C_1 [V_s + V_m \exp(FE_m/RT)] \\ &= Q_t - C_1 V_s - Q_{mk} \end{aligned} \quad (8)$$

The partition of TPP^+ into the plasma membrane, cytosol and mitochondria in synaptosomes in high K^+ medium was estimated by using Eqs. 2, 3, 7 and 8 (Fig. 1). Table I shows

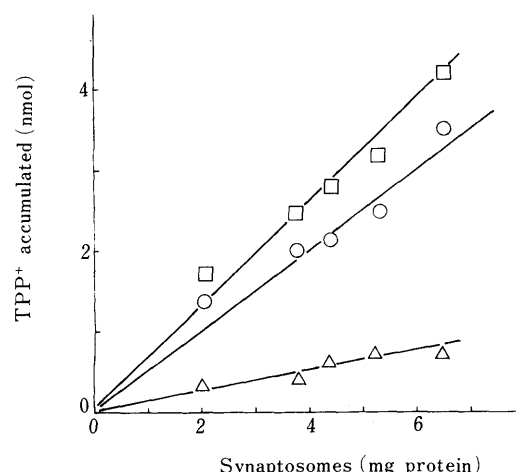


Fig. 1. TPP^+ Partition into Synaptosomal Plasma Membranes and Mitochondria in High K^+ Medium

TPP^+ accumulated was measured under various concentrations of synaptosomal protein. Total TPP^+ accumulated (\square), was calculated using Eq. 2. The amounts of TPP^+ accumulated in mitochondria (Δ) and bound to plasma membranes (\circ) were calculated using Eqs. 7 and 8, respectively.

TABLE I. Membrane Potentials of Purified and of Crude Synaptosomes and the Change in Membrane Potential (Hyperpolarization) with Glucose

Sample	Membrane potential (mV)	ΔE
Crude synaptosome	-67 ± 3	13 ± 1
Purified synaptosome	-71 ± 7	18 ± 3

Results are expressed as means \pm S.D. Membrane potentials were calculated by method 1 in the medium without glucose. Hyperpolarization by addition of glucose (ΔE) was measured.

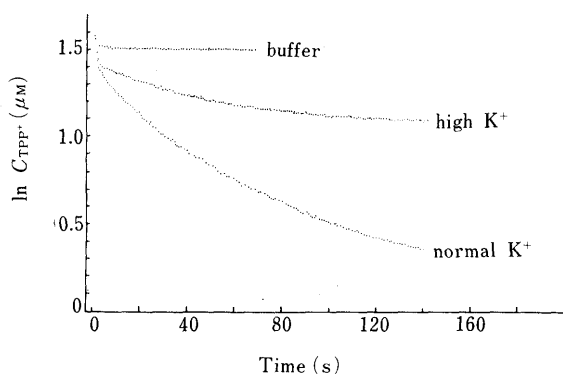
the calculated membrane potentials of synaptosomes (purified in a Ficoll gradient¹⁹) and of crude synaptosomes (P2 fraction). The membrane potential of synaptosomes was thought to be hyperpolarized by addition of glucose.¹⁷ Similar values were obtained for both purified and crude synaptosomes, and most of the TPP^+ taken up in a crude synaptosomal fraction seemed to be distributed to synaptosomes. Since the amount of TPP^+ bound to the plasma membranes was not the same among separate synaptosomal preparations (Table II), membrane potential was calculated from the value of Ab obtained from the same preparation (method 1 in Table II). Rotenone and pyruvate, which affect the membrane potential of mitochondria, altered the TPP^+ uptake in high K^+ medium. We supposed that only E_m alters, but Ab is independent of the presence of these chemicals.

2) Method 2 The absorption of TPP^+ on the surface of plasma membranes is thought to be faster than the transport across membranes. Three seconds after addition of buffer only (0.25 ml), the concentration of TPP^+ in the medium became constant (Fig. 2a). This means that mixing was completed in three seconds. The concentration of the cation in normal K^+ medium was measured, and the amount of absorption (Ab) was calculated from the intercept of extrapolation of the $\ln(\text{TPP}^+)$ vs. time plot between 5 and 20 s, with the aid of the microcomputer (Fig. 2c). Membrane potential (E_s) was calculated from Eq. 6 using the estimated Ab (method 2 in Table II). As shown in Fig. 2b, Ab estimated in high K^+ medium was almost the

TABLE II. Distribution of TPP^+ in Synaptosomes and Membrane Potential of Synaptosomes (E_s) Calculated by the Three Methods

No.	Medium	Q_t	Ab (10^{-10} mol/mg synaptosomal protein)	Q_m	$C_s V_s$	E_s (mV)
1	5K	12.7 ± 0.7		8.6 ± 0.7	0.74 ± 0.05	-80 ± 7 (9)
	(High K)	4.5 ± 0.8	3.3 ± 0.9	1.2 ± 0.2		(3)
2	5K	12.2 ± 0.6	3.0 ± 0.5	8.5 ± 0.6	0.73 ± 0.05	-77 ± 6 (4)
3	5K	12.7 ± 0.7		7.5 ± 0.6	0.64 ± 0.06	-76 ± 7 (9)
	(+Val)	6.3 ± 0.5	4.6 ± 0.7	0)		(4)
	5K	12.7 ± 0.7		8.8 ± 0.6	0.76 ± 0.05	-80 ± 6 (9)
	(+CCCP)	5.3 ± 0.8	3.1 ± 1.2	0)		(3)

The concentration of CCCP or valinomycin was $0.5 \mu\text{M}$. "No." means method number. Q_t (amount of total uptake) was calculated by using Eq. 2. Ab (amount of the ion bound to plasma membranes) was estimated by a separate method. Q_m (amount bound to mitochondria) and $C_s V_s$ (amount present in cytosol) were evaluated by using Eqs. 2–5. Values are mean \pm S.D. The numbers of experiments are shown in parentheses.

Fig. 2. Time Dependency of the Change in Concentration of TPP^+

An aliquot of buffer or of synaptosomal suspension (0.25 ml) was added to incubation medium containing $5 \times 10^{-6} \text{ M}$ TPP^+ and the electrical potential of an electrode selective for TPP^+ was recorded. The vertical axis shows the logarithm of concentration (micromolar). The concentration of TPP^+ was calculated with the aid of the computer as described in Experimental. a: The concentration of TPP^+ after addition of buffer was extrapolated to $4.6 \times 10^{-6} \text{ M}$. b: The concentration of the cation extrapolated after addition of synaptosomes in high K^+ medium was $4.1 \times 10^{-6} \text{ M}$. c: The concentration of the cation extrapolated after addition of synaptosomes in normal K^+ medium was $4.1 \times 10^{-6} \text{ M}$. Ab was estimated from the difference between the concentrations obtained in (a) and (c). This result shows that Ab in high K^+ medium and in normal K^+ medium were almost the same. The value after equilibrium distribution with synaptosomes (b or c) is not shown in the figure.

same as that obtained in normal K^+ medium.

3) Method 3 In synaptosomes, CCCP and valinomycin do not affect the uptake of K^+ ⁵⁾ and do not depolarize membrane potential. These chemicals are known to depolarize the membrane potential of mitochondria. The difference between the uptake of TPP^+ in the presence and absence of CCCP or valinomycin is thought to be attributable to the binding of the cation to mitochondria. If E_s and Ab are unchanged in the presence or absence of valinomycin or CCCP, Eq. 6 can be modified as follows:

$$E_s = (RT/F) \ln [Q_a - Ab] / C_{1a} (V_s + V_m) \quad (9)$$

where Q_a and C_{1a} are the total uptake and the concentration in the medium of the ion in the presence of these reagents, respectively. From Eqs. 6 and 9, Ab can be represented by Eq. 10:

$$Ab = (Q_t C_{1a} - Q_a Z C_1) / (C_{1a} - Z C_1) \quad (10)$$

where Q_t and C_1 are the values obtained in the absence of CCCP or valinomycin. In Eq. 10, Z is given by $[V_s + V_m \exp(FE_m/RT)] / (V_s + V_m)$, and calculated to be 12.5 using the values given above. Ab was calculated by using Eq. 10, and E_s was calculated by using Eq. 9 or 6 (method 3

in Table II).

Similar values of E_s for the same preparation were obtained by each method (Table II). They are also similar to those estimated with lipophilic ions, ^{1,4,8)} but larger than those estimated with optical probes. ^{14,15,17,18)} TPP^+ accumulated in synaptosomes was shown to be absorbed mostly into mitochondria in every method. The E_s values obtained seem to be reasonable for those of synaptosomes, indicating that the accumulation of TPP^+ by intracellular mitochondria is not neglected and the three methods are valuable to estimate E_s in synaptosomes. Since Ab is thought to be dependent on the concentration of the cation in external medium, ²²⁾ and since the final concentration of the cation in external medium must depend on the uptake of the cation by synaptosomes, the exact amount of Ab estimated by each method may change with wide variations in the concentration of synaptosomal protein. In method 2, the time interval to extrapolate (see Fig. 2c) was chosen somewhat arbitrarily, although it altered the values of Ab and membrane potential. It is, however, important to estimate the relative changes but not the absolute value of membrane potential. In addition, it was desirable to estimate Ab in a proper range of synaptosomal concentration, where the final concentration of TPP^+ would be above 0.001 mM in each method. The subsequent results, as shown below, were obtained by method 1. The values of Ab of various preparations were not constant, and were dependent on temperature and pH.

Membrane potential (E_s) was calculated by varying the ratio of the amount of synaptosomes to that of TPP^+ . E_s was almost constant within the concentration range of synaptosomes measured in this experiment. The amount of the cation bound to plasma membranes and that accumulated in mitochondria were dependent on protein concentration. These results (see above) are reasonable and the distribution of the cation in synaptosomal suspension is properly expressed by Eqs. 1–9.

TPP^+ uptake was measured in the presence of several substrates of energy metabolism in normal or in high K^+ medium without glucose. None of the substrates except pyruvate affected the uptake of TPP^+ in high K^+ medium. In high K^+ medium, we supposed that there is no electrical difference across plasma membranes, and the TPP^+ uptake by pyruvate was considered to change the membrane potential of mitochondria (E_m). When the change in TPP^+ taken up by pyruvate was considered in relation to the change in E_m , E_m was altered from -160 mV to

TABLE III. Effect of Various Substrates of Energy Metabolism on Membrane Potential (E_s) in Synaptosomes

	E_s (mV)	
	Without substrate	With substrate
+ Glucose	-72 ± 4	-86 ± 3 (5) ^{a)}
+ Mannose	-70 ± 5	-88 ± 5 (3) ^{a)}
+ Fructose	-73 ± 6	-74 ± 5 (3)
+ Pyruvate	-74 ± 7	-72 ± 6 (5)
+ Lactate	-70 ± 4	-77 ± 3 (3) ^{b)}
+ Glutamate	-70 ± 5	-69 ± 5 (3)
+ Succinate	-67 ± 3	-66 ± 5 (3)

Significantly different from without substrate, a) $p < 0.01$ and b) 0.1. All substrates added were 5.5 mM. E_s was estimated by method 1, where E_m (-160 mV) did not change on addition of glucose, lactate or succinate but hyperpolarized to -180 mV on addition of pyruvate (see the text). Values are means \pm S.D. The numbers of experiments are shown in parentheses.

-180 mV ± 12 (mean \pm S.D.) from Eqs. 7 and 8. We calculated E_s without addition of pyruvate by method 1 using the value of E_m equal to -160 mV (Table III). When pyruvate was added, E_m was considered to alter to -180 mV. Membrane potential was hyperpolarized by glucose and mannose, but not by pyruvate, fructose, glutamate or succinate. These results are parallel to the increase in lactate¹⁷⁾ and the change in adenosine triphosphate (ATP) content in synaptosomes (unpublished data). As shown in Fig. 1, most of the TPP^+ accumulated into synaptosomes was absorbed by mitochondria. Since succinate and pyruvate increased the amount of O_2 uptake into synaptosomes,^{20,29)} these substrates may result in hyperpolarization of the membrane potential of mitochondria (not of synaptosomes) and an increase in mitochondrial TPP^+ uptake (see Eq. 5). However, addition of succinate did not cause an increase in the uptake of TPP^+ by synaptosomes (Table III). The mechanism of the different effects of succinate and pyruvate on TPP^+ uptake in high K^+ medium (that is, uptake by mitochondria) is not clear. These results were essentially similar to those obtained using fluorescence of rhodamine 6G¹⁷⁾ except for pyruvate. Since rhodamine 6G inhibited the respiration of synaptosomes,²⁸⁾ rhodamine 6G can distribute to intrasynaptosomal mitochondria, like safranin⁷⁾ and TPP^+ . The difference in the results with pyruvate could be attributed to the fact that the distribution of rhodamine 6G to mitochondria was not considered for the calculation of membrane potential.^{15,17)}

Membrane potentials were calculated by method 1 at various pH values in normal K^+ medium (Fig. 3). The TPP^+ uptake of high K^+ medium is not dependent on the pH of the media (except at pH 4), and Ab is constant in the pH range measured (except at pH 4). As can be seen from the figure, membrane potential remained constant around neutral pH, as in reported results^{3,8)} and was depolarized by lowering the pH of the medium. This result is similar to that reported for the pH dependency of membrane potential in neurons^{30,31)}; the structure of the K^+ channel of synaptosomes is thought to be similar to that of axons. Synaptosomes and neurons show similar selectivity for cations.^{14,15)}

With decreasing temperature of the incubation medium, the rate of TPP^+ uptake by synaptosomes was decreased. At 5°C , TPP^+ was accumulated, indicating that a potential difference across the synaptosomal membrane exists at low

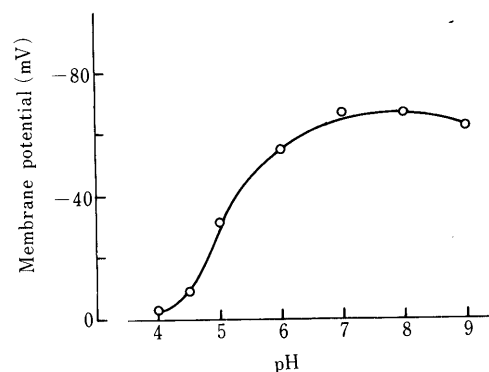


Fig. 3. Effect of pH of Medium on Membrane Potential in Synaptosomes

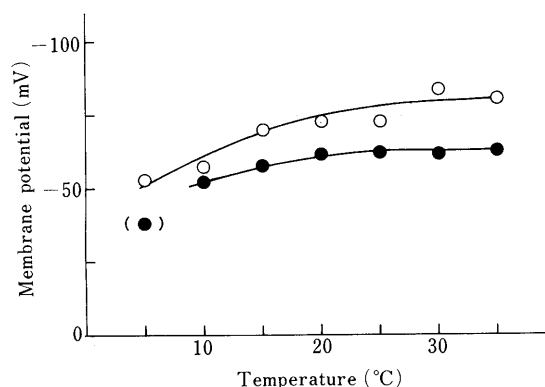


Fig. 4. Effect of Temperature on Membrane Potential in Synaptosomes in the Presence of 5.5 mM Glucose (○) or in Its Absence (●)

temperature. Figure 4 shows membrane potential plotted against temperature of the incubation medium. Below 15°C membrane potential was calculated from the value obtained 30 min after addition of synaptosomes, because TPP^+ uptake by synaptosomes below this temperature was very slow. As seen from the figure, membrane potentials above 20°C in the presence and absence of glucose are independent of incubation temperature, and at temperatures lower than 20°C , the hyperpolarization in membrane potential in the presence of glucose was diminished. Below 20°C , inhibition of hyperpolarization by glucose is thought to be due to that of glucose transport.^{17,32)}

In this calculation of membrane potential using TPP^+ , we supposed that TPP^+ is distributed into the plasma membrane and to cytosol of synaptosomes and into intrasynaptosomal mitochondria (Eq. 4). It should be noted that synaptic vesicles did not accumulate TPP^+ during this experimental period (data not shown). Although the synaptosomal fraction used in the experiment contained myelin fragments, free mitochondria and fragments of plasma and other membranes,^{11,33)} we assumed that these fractions were a part of the plasma membranes.

It was reported that membrane potential depolarized at 0°C .⁶⁾ However, the pH dependency of membrane potential shown in Fig. 3 was similar to the distribution of $^{42}\text{K}^+$ in media with different pH values at 2°C .³⁴⁾ This suggests that the membrane potential may not be depolarized at low temperature. Since the transport of TPP^+ across plasma membrane was very slow at low temperature, triphenylmethylphosphonium is also unlikely to be accumulated at

0 °C in the same period as that at 30 °C. We assumed the membrane potential of mitochondria to be -160 mV in the present study, but if the value of E_m was assumed to be smaller than -160 mV, the value of Ab would be increased and E_m would be estimated to be somewhat hyperpolarized by each method.

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