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SELECTIVE ELECTRODE FOR DIBENZYL DIMETHYL AMMONIUM CATION AS INDICATOR OF THE MEMBRANE POTENTIAL IN BIOLOGICAL SYSTEMS

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Summary

The electrode sensitive to dibenzyl dimethyl ammonium (DDA^+), which is considered to be an indicator of the membrane potential, was constructed by using tetraphenyl borone (TPB^-) embedded in dichloroethane. Rapid and Nernstian responses were exhibited against DDA^+ solutions ranging between 10^{-2} and $3 \cdot 10^{-6}$ M in concentration. High selectivity for DDA^+ was observed in the presence of various inorganic salts, ADP, ATP, oxidizable substrates and sugars. The electrode developed here was used to measure the DDA^+ uptake in *Streptococcus faecalis* and the results agreed with those reported by Harold, F.M. and Papineau, D. ((1972) J. Membrane Biol. 8, 27—44 and 45—62). While they determined the DDA^+ concentration in the medium by measuring the absorbance of the filtrate treated with the ion-exchangers, the electrode can measure directly the DDA^+ concentration in the bacterial suspension without any pretreatment. It was also shown that the electrode can measure the DDA^+ uptake in mitochondria during energization.

Introduction

The importance of the membrane potential is well appreciated by those who study energy-linked transport of mitochondria and bacteria and oxidative phosphorylation [1,2]. Due to the ingenious work by Skulachev, Liberman and their associates [3,4], lipid-soluble anions such as phenyl dicarbaundecaborane (PCB^-) or tetraphenyl borone (TPB^-) and cations such as dibenzyl dimethyl

Abbreviations: DDA^+, dibenzyl dimethyl ammonium cation; PCB^-, phenyl dicarbaundecarborane; TPB^-, tetraphenyl borone; Tris, tris(hydroxymethyl) aminomethane; EDTA, ethylenediamine tetraacetate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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ammonium (DDA^+) are used as indicators of the membrane potential of cells or organelles which are so small that a microelectrode cannot be inserted. These ions diffuse passively across membranes and are distributed between cells or organelles and their media in accordance with the Nernst equation. When the membrane potentials of cells or organelles are hyperpolarized, the DDA^+ added to the media is transferred electrophoretically to the cells or organelles and hence, the concentration of DDA^+ in the medium decreases, and vice versa.

The concentration of DDA^+ in the medium has been determined by measuring (1) the conductance change of bi-molecular phospholipid membrane as sensors [3—5], (2) the radioactivity of labeled DDA^+ [6—9], and (3) the ultraviolet absorbance of the sample solution [10—12]. In the first method, the preparation of stable and reproducible bimolecular membrane is necessary but difficult. It is troublesome to obtain the time course of the change in DDA^+ concentration by the second and third methods. Moreover, the radioactive DDA^+ is not easily accessible and the molar extinction coefficient of DDA^+ is not large.

In the present paper, we describe the preparation and properties of a selective-electrode for DDA^+ which can measure selectively and continously the concentration (activity) of DDA^+ in the medium. It is shown that the uptakes of DDA^+ in *Streptococcus faecalis* and in mitochondria are measured with use of the developed electrode.

Preparation of DDA^+ electrode

The DDA^+ electrode is composed of a sensor liquid membrane and an internal reference solution (see Fig. 1a). The sensor membrane is a water insoluble

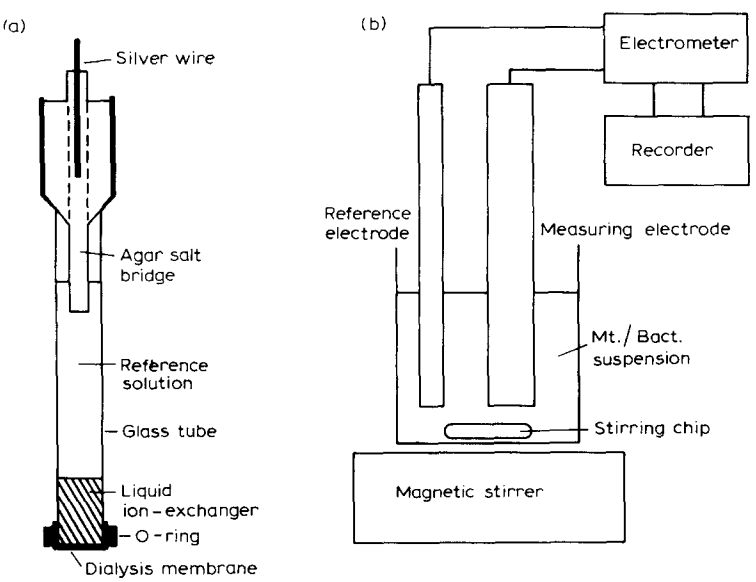

Fig. 1. Construction of DDA^+-electrode (a) and diagram of apparatus for measuring the DDA^+ uptake in bacteria or mitochondria (b).

organic solution which dissolves the associated DDA^+ and ion-exchanger. TPB^- was found to be the most suitable ion-exchanger of 10 compounds examined. Mixing equal volumes (50 ml) of 10^{-3} M DDA^+ and 10^{-3} M TPB^- produced a white precipitate. After the addition of 100 ml dichloroethane and vigorous stirring for about 3 h, the precipitate was completely solubilized and entered the organic phase which separated from the aqueous solution. The dichloroethane extract was, then, used as a liquid ion-exchanger. Fig. 1a illustrates the assembly of the DDA^+ electrode, where the end of the glass tube is sealed with a cellulose dialysis membrane and the liquid ion-exchanger is held in the glass tube. A 10^{-2} M DDA^+ reference solution is placed on top of the liquid membrane and a small glass tube filled with a saturated KCl agar (3%) and with a Ag-AgCl wire inserted is placed into the reference solution. The upper end of the glass tube is sealed airtight with a ground joint to prevent the loss of the liquid ion-exchanger.

The electromotive force (e.m.f.) between the DDA^+-electrode and a calomel reference electrode was measured by an electrometer (Model TR-8651, Takeda Riken, Tokyo) connected to a pen recorder (Fig. 1b). DDA^+ and TPB^- were obtained from Nakarai Chem. Co., Kyoto and Dojin Chem. Co., Kumamoto, Japan, respectively.

Properties of the electrode

As shown in Fig. 2, the response of the electrode was linear with the logarithm of the DDA^+ concentration with a slope of 59 mV per decade concentration until the concentration decreased to $3 \cdot 10^{-6}$ M. The electrode may also respond to certain substances other than DDA^+. When the sample solution contains both DDA^+ and other interfering substances, i, the e.m.f. is analyzed by the following equation:

$$E = (\text{const}) - (RT/F) \ln([DDA^+] + K_i[i])$$
$$= (\text{const}) - 59\ (\text{mV}) \log([DDA^+] + K_i[i]) \quad (1)$$

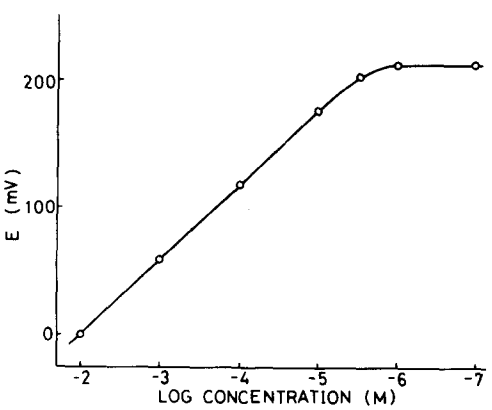

Fig. 2. Response of the electrode to DDA^+ solutions of varying concentrations.

where E, [DDA^+], [i], and K_i are the e.m.f. observed, the concentration of DDA^+, and that of the interfering substance i, and the selectivity coefficient, respectively, and (const) is a constant dependent only on the concentration of the internal reference solution. The method for evaluating K_i is noted briefly in the legend of Table I and details have been reported elsewhere [13]. The values of K_i for various substances are listed in Table I. As shown in Table I, the values of K_i for all substances examined are less than $5 \cdot 10^{-5}$. This means that the error due to the presence of such substance 10^3 times more concentrated than the DDA^+ concentration is only less than 2%. The electrode system respond in 10—15 s to a doubling of the DDA^+ concentration. This response time is short compared with the time course of DDA^+ uptake or release in bacteria and mitochondria as shown below.

Application of the electrode to bacteria and mitochondria

Harold and Papineau [10] measured the DDA^+ uptake in *S. faecalis* by determining the DDA^+ concentration in filtrates treated with an anion exchanger resin by means of the ultraviolet spectra. In order to demonstrate that the electrode developed here can be applied to biological systems, the DDA^+ uptake in *S. faecalis* was measured by the electrode. The cells were grown overnight in the following media: 1% K_2HPO_4, 1% bactotryptone, 1% glucose and 0.5% yeast extract. After the suspension was neutralized, the cells were centrifuged and washed twice with 2 mM $MgSO_4$. *S. faecalis* was supplied by the Laboratory of culture collection of microorganism, Hokkaido University.

The result is shown in Fig. 3. At the time indicated by the first arrow, the organisms were injected into 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-4} M DDA^+ and 10^{-6} M TPB^-, and the decrease of DDA^+ concentration in the medium was observed with the described electrode. Application of valinomycin brought about a further uptake of DDA^+, and subsequent addition of KCl led to a release of DDA^+. These results are essentially the same as those obtained

TABLE I

SELECTIVITY COEFFICIENTS OF VARIOUS SUBSTANCES FOR DDA^+ ELECTRODE

According to Eisenman et al. [15], the membrane potentials that arise between two solutions (denoted by ' and ''), E are represented by the following equation: $E = (RT/F)\ln\{([DDA^+]'' + K_i[i]'')/([DDA^+]' + K_i[i]')\}$. If the salt composition in solution ('') is kept constant, the above equation is rewritten as Eqn. 1. When the membrane potentials are measured under the bi-ionic condition the $[DDA^+]' = [i]' = 0$ and $[DDA^+]'' = [i]''$, the above equation is recast to give $E = (RT/F)\ln K_i$. Then, the values of K_i were evaluated from the bi-ionic potentials observed under the condition that $[DDA^+]'' = [i]'' = 10^{-2}$ M.

Substances	K_i	Substances	K_i
NaCl	$6 \cdot 10^{-6}$	Malate	$1 \cdot 10^{-5}$
KCl	$7 \cdot 10^{-6}$	Ascorbate plus TMPD *	$5 \cdot 10^{-5}$
$CaCl_2$	$5 \cdot 10^{-6}$	ATP	$7 \cdot 10^{-6}$
$MgCl_2$	$5 \cdot 10^{-6}$	ADP	$7 \cdot 10^{-6}$
Tris — HCl	$1 \cdot 10^{-5}$	AMP	$6 \cdot 10^{-6}$
Phosphate buffer	$2 \cdot 10^{-5}$	Sucrose	$<10^{-6}$
Succinate	$7 \cdot 10^{-6}$	Mannitol	$<10^{-6}$
Glutamate	$7 \cdot 10^{-6}$	Glucose	$<10^{-6}$

* The concentration of TMPD was 7% of that of ascorbate.

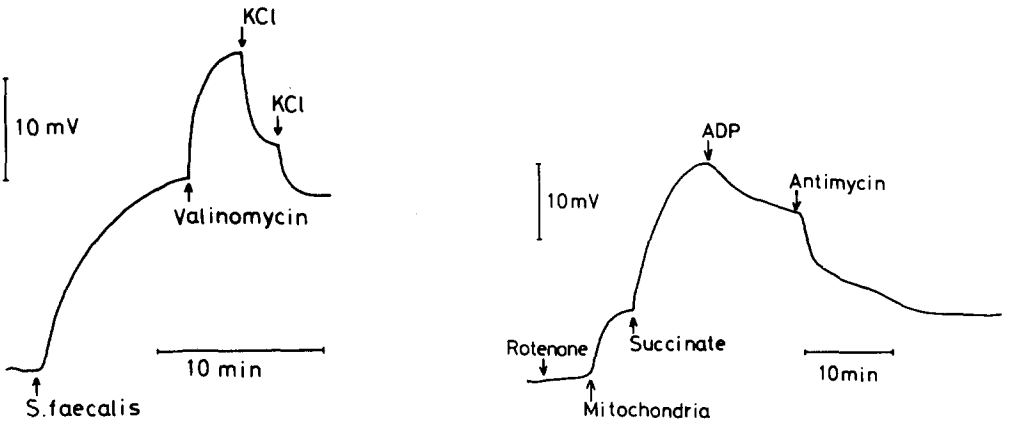

Fig. 3. Application of the electrode to the DDA^+ uptake of *S. faecalis*. Incubation mixture: 10 mM Tris · HCl (pH 7.5), 10^{-4} M DDA^+ and 10^{-6} M TPB^-. Additions: *S faecalis* (6.2 mg of protein/ml), 0.5 μg/ml valinomycin, 20 mM KCl. The vertical bar represents a change of 10 mV in the DDA^+ electrode.

Fig. 4. DDA^+ accumulation in mitochondria due to energization, and release due to phosphorylation and to addition of the inhibitor as measured with the described electrode. Incubation mixture: 10 mM Tris · HCl, 10^{-4} M DDA^+ and 10^{-6} M TPB^-. Additions: 0.35 μg/ml rotenone, mitochondria (3.1 mg of protein/ml), 3 mM succinate, 1 mM ADP, and 0.38 μg/ml antimycin A.

by Harold and Papineau [10]. It is apparent that the method developed here is much easier to use than that employed by them.

The electrode developed here was also used to measure the DDA^+ uptake in mitochondria. Mitochondria were isolated from rat liver with the standard method [14]. Sedimented mitochondria were suspended in 0.25 M sucrose, 10 mM Tris · HCl (pH 7.4), 0.2 mM EDTA, 10^{-4} M DDA^+ and 10^{-6} M TPB^-. Typical results obtained are shown in Figs. 4 and 5. When the mitochondria were injected into the medium in the presence of rotenone, a relatively small uptake of DDA^+ was observed. Subsequent addition of succinate led to a large uptake of DDA^+ in the mitochondria, and a small efflux of DDA^+ was observed after addition of ADP. Cessation of the energy by the addition of antimycin A led to an efflux of DDA^+, and the final concentration of DDA^+ in the medium was equal to that before the mitochondria were energized by addition of succinate. Similarly energization of mitochondria treated with antimycin A by addition of ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) led to uptake of DDA^+ and efflux of DDA^+ was observed by addition of KCN. Fig. 5 illustrates the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); DDA^+ accumulated by the energization was decreased rapidly after the addition of FCCP.

Although the assumption that DDA^+ is distributed passively in accordance with the membrane potential has not yet been fully proved, the following equation holds at the steady state if this assumption is valid.

$$\Delta\psi = \frac{RT}{F} \ln \frac{[DDA^+]_{out}}{[DDA^+]_{in}} = 59\ (mV) \log \frac{[DDA^+]_{out}}{[DDA^+]_{in}} \qquad (2)$$

Here, $\Delta\psi$ stands for the membrane potential with respect to the medium, and

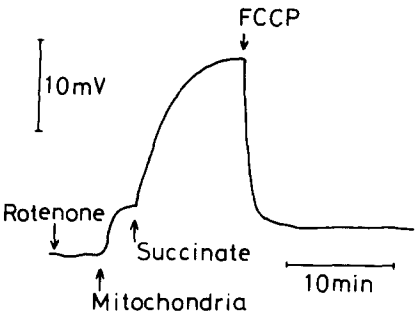

Fig. 5. Effect of FCCP on DDA^+ uptake in mitochondria. Incubation mixture is the same as that in Fig. 4. Additions: mitochondria (2.8 mg of protein/ml), 3 mM succinate, and 1 μM FCCP.

$[DDA^+]_{in}$ and $[DDA^+]_{out}$ are the concentrations of DDA^+ in the mitochondria and in the medium, respectively. The law of mass conservation requires the following equation under the assumption that $V \gg v$;

$$V[DDA^+]_{out} + v[DDA^+]_{in} = V[DDA^+]_o \quad (3)$$

where V and v represent the volume of the medium and of mitochondria, respectively, and $[DDA^+]_0$ is the initial concentration of DDA^+ before mitochondria are injected. Inserting Eqn. 3 into Eqn. 2 and using the relation between E and $[DDA^+]_{out}$ as shown in Fig. 2, we obtain

$$\Delta\psi = 59 \log(v/V) - 59 \log[10^{(E-E_0)/59} - 1] \quad (4)$$

where E_0 stands for the electrode potential observed before mitochondria are injected, i.e. the value of the base line in Figs. 4 and 5. According to Eqn. 4, the value of v/V is necessary to calculate the absolute value of $\Delta\psi$. If the swelling or shrinkage of mitochondria is ignored, the value of the term 59 log (v/V) remains constant and the magnitude of change in the membrane potential between the two different states can be calculated since the term is cancelled. Such calculation leads to the results that the energization by addition of substrate produces a negative-going potential change (40—50 mV) and the proceeding of phosphorylation decreases the membrane potential by about 10 mV. Uncouplers dissipate rapidly the membrane potential established by the energization.

As shown in this paper, the electrode described here is useful to monitor DDA^+ concentrations and may be applied to various biological systems. Further study may be necessary to prove that DDA^+ distributes passively in accordance with the membrane potential as assumed in the derivation of Eqn. 4.

References

1 Mitchell, P. (1966) Biol. Rev. 41, 445—502
2 Mitchell, P. (1973) in Mechanism in Bioenergetics, (Azzone, G.F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds.), pp. 177—201, Academic Press, New York
3 Grinius, L.L., Jasaitis, A.A., Kadsiauskas, Yu. P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vladimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1—12
4 Drachev, L.A., Kondrashin, A.A., Samuilov, V.D. and Skulachev, V.P. (1975) FEBS Lett. 50, 219—222

5 Liberman, E.A. and Topali, V.P. (1969) Biofizika 14, 452—461
6 Hirata, H., Altendorf, K. and Harold, F.M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1804—1808
7 Lombardi, F.J., Reeves, J.P. and Kaback, H.R. (1973) J. Biol. Chem 248, 3551—3565
8 Hoeberichts, J.A. and Borst-Pauwels, G.W.F.H. (1975) Biochim. Biophys. Acta 413, 248—251
9 de Cespedes, C. and Christensen, H.N. (1975) Biochim. Biophys. Acta 339, 139—145
10 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 27—62
11 Griniuviene, B., Chmieliauskaite, V. and Grinius, L., (1974) Biochem. Biophys. Res. Commun. 56, 206—213
12 Massari, S. and Pozzan, T. (1976) Arch. Biochem. Biophys. 173, 332—340
13 Kamo, N., Hazemoto, N. and Kobatake, Y. (1976) Talanta, in the press
14 Hogeboom, G. (1955) in Method in Enzymology, (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 1, pp. 16—19, Academic Press, New York
15 Sandblom, J., Eisenman, G. and Walker, Jr., J.L. (1967) J. Phys. Chem. 71, 3862—3870