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Determination of the pH difference across a cell membrane using a methylammonium-selective membrane electrode

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A method was developed for determining pH differences across cell membranes using a methylammonium-selective membrane electrode, based on monitoring of the pH gradient-induced uptake of methylammonium *in situ*. The methylammonium electrode was constructed using calix[6]arene–hexaacetic acid hexaethyl ester as a neutral carrier and bis(2-ethylhexyl) sebacate as a membrane solvent in a poly(vinyl chloride) membrane matrix. This electrode exhibited a near-Nernstian response to methylammonium in the concentration range 2×10^{-5}–1×10^{-2} M with a slope of 58 mV per concentration decade in a buffer solution of 150 mM choline chloride–10 mM TRIS–HCl (pH 7.5). The limit of detection was 5×10^{-6} M. In experiments using liposomes, the uptake of methylammonium into liposomes occurred effectively when the pH of the outside suspension medium was alkaline, and the determination of changes in methylammonium concentrations in the outer medium was quantitatively related to changes in the pH differences across the liposomal membrane. The transmembrane pH differences in *Escherichia coli* cells were also determined by this method.

Keywords: *Ion-selective electrode; methylamine; transmembrane pH difference measurement; ion distribution technique*

Methylamine is one of the most commonly utilized probes for determining pH differences across biological membranes.[1–4] This amine is highly permeable in its neutral form but impermeable in its charged form. Thus, at equilibrium, the concentration of the neutral form becomes identical on both sides of the membrane, leading to the following relationship:

$$[H^+]_{in}/[H^+]_{out} = [CH_3NH_3^+]_{in}/[CH_3NH_3^+]_{out} \quad (1)$$

where the subscripts in and out mean inside and outside the membrane, respectively. Hence the pH difference across the membrane (ΔpH), defined as $pH_{in} - pH_{out}$, can be expressed as follows:

$$\Delta pH = -\log\left(\frac{[CH_3NH_3^+]_{in}}{[CH_3NH_3^+]_{out}}\right) \quad (2)$$

This equation means that, under conditions where pH_{out} is higher than pH_{in} of cells, methylamine in the external medium is concentrated into the cell until the methylamine concentration ratio inside and outside the cell reaches ΔpH. Hence ΔpH can be determined by measuring the methylamine concentration ratio inside and outside the cell. The dissociation constant, pK_a, of methylamine (10.6 at 25 °C) is much higher than the physiological pH (around 7.5) and, therefore, most of the amine is in the charged form and the total methylamine concentration is for all practical purposes equal to that of the charged amine concentration.

The methylamine distribution ratio is commonly determined using radioactive ^{14}C-labelled methylamine.[1–4] However, the method requires a difficult separation of cells from the medium. We are particularly interested in the use of a methylammonium-selective electrode which allows the determination of charged methylamine *in situ*. In the screening of various electrode materials, we found that calix[6]arene–hexaacetic acid hexaethyl ester can be used as a neutral carrier for constructing a methylammonium electrode. Here we describe a potentiometric assay using the methylammonium electrode to estimate the ΔpH of liposomes and *Escherichia coli* cells.

Theoretical

ΔpH can be calculated by the evaluation of $[CH_3NH_3^+]_{in}$ in combination with the following mass conservation law:

$$(V + v)[CH_3NH_3^+]_0 = V[CH_3NH_3^+]_{out} + v[CH_3NH_3^+]_{in} \quad (3)$$

where $[CH_3NH_3^+]_0$ is the total concentration of methylammonium and V and v are the outer medium volume and intracellular volume, respectively. In the present experiment, $[CH_3NH_3^+]_0$ was set to 100 μM. Inserting eqn. (3) into eqn. (2) yields the following equation:

$$\Delta pH = -\log\left(\frac{V+v}{v} \times \frac{[CH_3NH_3^+]_0}{[CH_3NH_3^+]_{out}} - \frac{V}{v}\right) \quad (4)$$

$[CH_3NH_3^+]_0/[CH_3NH_3^+]_{out}$ can be correlated with a change in electric potential, ΔE, using the following Nernst equation:

$$\Delta E = E_0 - E_{out} = S \log\left(\frac{[CH_3NH_3^+]_0}{[CH_3NH_3^+]_{out}}\right) \quad (5)$$

where E_0 is the initial potential, corresponding to 100 μM methylamine in this experiment, E_{out} is electric potential corresponding to $[CH_3NH_3^+]_{out}$ in the outer medium and S is the slope of the electrode (58 mV in the present case). Combining eqns. (4) and (5), we obtain the following equation:

$$\Delta pH = -\log\left(\frac{V+v}{v} \times 10^{\Delta E/S} - \frac{V}{v}\right) \quad (6)$$

Calculation of ΔpH requires an accurate measure of the intracellular volume, in addition to the measurement of ΔE.

Experimental

Materials

The sources of the reagents used were as follows: calix[6]arene–hexaacetic acid hexaethyl ester (amine ionophore I) and bis(2-ethylhexyl) sebacate from Fluka (Buchs, Switzerland); poly(vinyl chloride) (PVC) (degree of polymerization, 1020) from Nacalai Tesque (Kyoto, Japan); egg phosphatidylcholine from Lipid Products (Red Hill, Surrey, UK); cholesterol and melittin (from bee venom, approximately 85% by high-performance liquid chromatography) from Sigma (St. Louis,

MO, USA); 2,2,6,6-tetramethylpiperidone-*N*-oxyl (TEMPONE) from Molecular Probes (Eugene, OR, USA); and choline hydroxide (50% m/m solution in water) and potassium tris(oxalate)chromate trihydrate from Aldrich (Milwaukee, WI, USA). All other chemicals were of analytical-reagent grade.

Electrode system

The methylammonium-selective electrode was constructed as reported previously.[5] The components of the sensor membrane were 1 mg of amine ionophore I, 60 μl of bis(2-ethylhexyl) sebacate and 30 mg of PVC. The materials were dissolved in tetrahydrofuran (about 1 ml), poured into a flat Petri dish (28 mm diameter) and the solvent was evaporated at room temperature. The resulting membrane was excised and attached to a PVC tube (4 mm od, 3 mm id) with tetrahydrofuran adhesive. The PVC tube was filled with an internal solution containing 10 mM methylamine hydrochloride and the sensor membrane was conditioned overnight. The electrochemical cell arrangement was Ag,AgCl/internal solution/sensor membrane/sample solution/1 M NH_4NO_3 (salt bridge)/10 mM KCl/Ag,AgCl. The electromotive force (emf) between the silver/silver chloride electrodes was measured using a voltmeter with high input impedance produced by a field-effect transistor operational amplifier (LF356; National Semiconductor, Sunnyvale, CA, USA; input resistance $> 10^{12}$ Ω) and was recorded. The detection limit was defined as the intersection of the extrapolated linear regions of the calibration graph.[6] The selectivity coefficients of the electrode, $k_{i,j}^{\mathrm{Pot}}$, were determined by the separate solution method[6,7] using the respective chloride salt at 10 mM and calculated from the equation $\log k_{i,j}^{\mathrm{Pot}} = (E_j - E_i)/S + \log c_i - \log c_j^{1/z_i}$, where E_i and E_j represent the emf readings measured for methylammonium and the interfering ion, respectively, S is the slope of the calibration graph for methylammonium, c_i and c_j are the concentrations of methylammonium and the interfering ion, respectively, and z_j is the charge of the interfering ion. The electrode was stored in 10 mM methylamine hydrochloride when not in use. All measurements were performed at room temperature (about 20 °C).

Preparation of liposomes and ΔpH *measurements*

Liposomes were prepared using the reversed-phase evaporation method.[8] Egg phosphatidylcholine (10 μmol, 7.7 mg) and cholesterol (7.5 μmol, 2.9 mg) were dissolved in 1.5 ml of diethyl ether, followed by addition of 1 ml of an aqueous solution of 120 mM choline chloride and 50 mM TRIS–HCl (pH 7.5). The mixture was sonicated (Model 5201 instrument; Ohtake Works, Tokyo, Japan) for 1 min at 0 °C to obtain a homogeneous emulsion. The diethyl ether solvent was then removed using a conventional rotary evaporator under reduced pressure (using an aspirator) at 25 °C. After the diethyl ether had been completely removed, a homogeneous suspension of liposomes formed. The liposomes were centrifuged (105 000*g*, 20 min) and washed once with 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5) to lower the buffer capacity of the outer medium of the liposomes. The final pellet was suspended in the above washing solution at 10 mg ml^{-1} of lipid. The osmotic pressures of the inner and outer aqueous solutions were measured with an OS osmometer (Fiske, Needham, MA, USA); both were approximately 290 mOsM.

The procedure used to evaluate ΔpH depending on the external pH is as follows. The liposome suspension (250 μl) was diluted in an assay solution (250 μl) containing 200 μM methylamine hydrochloride, 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5) in order to adjust the initial methylammonium concentration of the liposome suspension to 100 μM. The methylammonium and reference electrodes were then immersed in this solution, along with a miniaturized pH glass electrode (1826A-06T; Horiba, Kyoto, Japan) to monitor simultaneously the external pH of the solution. The solution was constantly stirred with a stir-bar. This electrode system, including the reference electrode,[9] was compact and, therefore, an assay solution volume as low as 500 μl could be measured. The pH of the outer medium was changed by addition of a small amount of 160 mM choline hydroxide or 160 mM hydrochloric acid.

Inner volume measurement

The internal volumes of liposomes were evaluated by a spin label method using a combination of a membrane permeable spin label (TEMPONE) and an impermeable broadening agent [potassium tris(oxalate)chromate].[10,11] The method involved measurement of ESR signal intensities of TEMPONE in (A) the presence and (B) the absence of potassium tris(oxalate)chromate, revealing the signal intensities inside liposomes and both inside and outside liposomes, respectively. Hence the internal volume of liposomes could be calculated from the A/B ratio. The procedure was as follows. A 20 μl volume of 10 mM TEMPONE solution in ethanol was pipetted into a test-tube (5 ml) and the ethanol was evaporated to leave TEMPONE in the walls of the tube. The liposome suspension (100 μl) was then added and dissolved the TEMPONE in the tube. An aliquot (25 μl) of this suspension was diluted with 25 μl of solution containing (a) 110 mM potassium tris(oxalate)chromate–choline hydroxide (pH 7.5) or (b) 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5). The osmotic pressures of solutions (a) and (b) were almost identical (about 290–300 mOsM). These samples were then transferred into 25 μl disposable micropipettes (Drummond, Broomall, PA, USA), capped with sealer clay (Critoseal; Sherwood, St. Louis, MO, USA) and the ESR signal intensities were determined using a JES-RE1X/HR spectrometer (JEOL, Tokyo, Japan). An ESR signal arising from membrane partitioning of TEMPONE, observed previously in the presence of potassium tris(oxalate)chromate,[10,11] was not observed in this experiment. This is because the liposomes prepared from the reversed-phase evaporation method possessed large internal volumes and emitted strong signals only from the aqueous part of the liposomes.

Preparation of E. coli *cells and* ΔpH *measurements*

E. coli W3133-2, a derivative of K-12, was used.[12] Cells were grown in a medium containing 100 mM TRIS–HCl (pH 7.6), 20 mM $(NH_4)_2SO_4$, 50 mM KCl, 1 mM K_2HPO_4, 0.3 mM $MgSO_4$ and 0.01 mM $CaCl_2$, supplemented with 40 mM potassium lactate, at 37 °C under aerobic conditions and harvested during the exponential phase of growth. After centrifugation, cells were suspended in an alkaline solution containing a high concentration of Li^+ [100 mM LiCl, 50 mM TRIS–HCl (pH 8.5) and 10 mM TRIS–lactate (pH 8.5)] and incubated for 10 min at 37 °C, in order to exchange K^+, which is largely contained in the cell cytoplasm, with Li^+. It has been shown that K^+ can be efficiently removed from the cytoplasm under alkaline conditions.[13] The cells were then cooled in ice, washed twice with buffer [150 mM choline chloride and 10 mM TRIS–lactate (pH 7.6)] and suspended in buffer at 5 mg ml^{-1} of protein. The protein content was determined by the method of Lowry *et al.*[14] The cell suspension (250 μl) was diluted in an assay solution (250 μl) containing 200 μM methylamine hydrochloride, 150 mM choline chloride and 10 mM TRIS–lactate (pH 7.6) to adjust the initial methylammonium concentration in the cell suspension to 100 μM, to create conditions similar to those in the liposome experiments. An initial external pH of 7.6 was regarded as the internal pH of *E. coli* cells.[12,15,16] An internal

volume of the *E. coli* cells of 3.7 μl mg^{-1} of protein[17] was used in this work.

Results and discussion

Response characteristics of the electrodes

An organic ammonium-ion selective electrode can be prepared using neutral carriers such as calix[6]arene–hexaacetic acid hexaethyl ester (amine ionophore I), dibenzo-18-crown-6 and phosphate esters, including tris(2-ethylhexyl) phosphate and tricresyl phosphate.[5] However, comparison among them[5] has shown that amine ionophore I is the most suitable carrier to prepare a methylammonium-selective electrode, because it does not exhibit interference from inorganic cations (especially Na^+ ions, which are contained in biological fluids in large amounts). Using this ionophore, we examined the effect of membrane solvents on methylammonium sensitivity, because membrane solvents have been shown to affect the electrode response. Calibration graphs were obtained by measuring known amounts of methylamine hydrochloride added to buffer solution of 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5) and plotting the concentrations against the corresponding emf values. An isotonic solution prepared from 150 mM choline chloride was preferentially used as a cell-suspension medium.[9,18] The membrane solvents tested were 2-fluoro-2′-nitrodiphenyl ether, *o*-nitrophenyl octyl ether, dioctyl phthalate, bis(2-ethylhexyl) sebacate, tris(2-ethylhexyl) phosphate and tricresyl phosphate. Of these, the electrode containing bis(2-ethylhexyl) sebacate was the most sensitive to methylamine hydrochloride. Fig. 1 shows the calibration graph for this electrode. The sensitivity of the electrode response was 58 mV per concentration decade and the limit of the detection was 5×10^{-6} M. The response time (90% of the final signal) of the electrode was below 10 s when the concentration of methylamine hydrochloride was changed from 10 to 100 μM. Further addition of lipophilic anionic salts such as sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (20 or 40 mol% of the ionophore) did not improve the electrode response, but rather deteriorated the electrode's sensitivity to methylamine. The selectivity coefficients of the electrode are given in Table 1. The electrode suffered no serious interference from Mg^{2+}, Ca^{2+}, Li^+, Na^+ and organic ammonium ions including lipophilic quaternary tetramethylammonium and tetraethylammonium ions, and also responded more strongly than ethylammonium, indicating that this ionophore acted primarily as a methylammonium ionophore. However, the electrode exhibited low selectivity to K^+ and NH_4^+, this being a significant disadvantage. The pH dependence of electrode response was below 0.5 mV in the pH range 7.5–8.5.

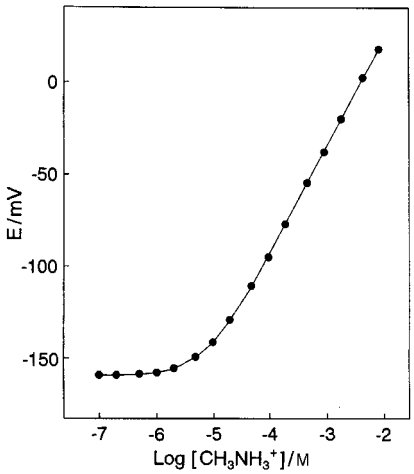

Fig. 1 Calibration graph for the electrode with methylammonium in buffer solution containing 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5).

Measurements of changes in the concentrations of methylammonium dependent on pH *gradients across cell membranes*

The electrode was used to determine the pH difference across liposomal membranes. Because the spontaneous diffusion rates of H^+ and/or OH^- across liposomal membranes are low,[19–21] it was assumed that the inner pH of liposomes would be constant during the short period of the experiments. Hence it was expected that when the outer pH was made more alkaline than the inner liposomal pH, the uptake of methylammonium in liposomes would be induced. As shown in Fig. 2, when choline hydroxide was added to the liposome suspension to make $pH_{in} < pH_{out}$, a significant decrease in electric potential and a corresponding decrease in methylammonium concentration in the outer medium were observed, and further addition of choline hydroxide caused further accumulation of methylamine inside the liposomes. The accumulated methylammonium in the liposomes was released when the outside pH was returned to the initial pH [Fig. 2(a)] or upon addition of the bee venom melittin [Fig. 2(b)]. Melittin disrupts the membrane structure of liposomes,[8,18] causing the pH difference between the inside and the outside of the liposomes to go to zero. These results clearly show that this methylammonium electrode can monitor changes in methylammonium concentrations caused by transmembrane pH differences.

We calculated the ΔpH from eqn. (6) and examined its dependence on the external pH. The intravesicular volume of

Table 1 Selectivity coefficients, log $k_{i,j}^{Pot}$ (i = methylammonium and j = interfering ion)

Interfering ion (j)	Log $k_{i,j}^{Pot}$
Mg^{2+}	−5.1
Ca^{2+}	−5.1
Li^+	−3.9
Na^+	−3.2
K^+	−1.1
NH_4^+	−1.3
Choline	−3.8
$(CH_3)_4N^+$	−3.2
$(C_2H_5)_4N^+$	−2.6
$C_2H_5NH_3^+$	−0.7

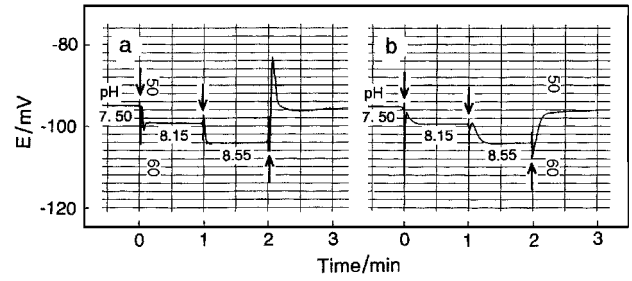

Fig. 2 Monitoring of changes in electric potential with variation in the external pH. Liposomes were suspended in solution (500 μl) containing 100 μM methylamine hydrochloride, 150 mM choline chloride and 10 mM TRIS–HCl (pH 7.5) at 2.5 mg ml^{-1} of lipid. At the times indicated by the first and second arrows in (a) and (b), 6 μl of 160 mM choline hydroxide were added; the third arrow in (a) indicates the time when 12 μl of 160 mM HCl were added to re-establish the initial pH, and that in (b) indicates the time of addition of 10 μl of 1 mM melittin to disrupt the liposomal membrane structure. The pH values of the solution after addition of choline hydroxide were monitored simultaneously using a miniaturized pH glass electrode and are given on the curves.

liposomes prepared in this study was estimated as 2.4 μl mg^{-1} lipid. This corresponds to an inner volume (v) of liposomes and an outer medium volume (V) of 6.0 and 494.0 μl, respectively. Using these values and electric potential changes, we calculated ΔpH and found a strong linear correlation between ΔpH and external pH, as shown in Fig. 3. We plotted −ΔpH as the ordinate, to highlight differences ($pH_{out} - pH_{in}$) and represent increasing external pH as a positive value. Linear regression analysis revealed a correlation coefficient (r) of 0.980 and a slope of 0.938. This result shows that the use of methylammonium is suitable for determining ΔpH across membranes and demonstrates experimentally that the internal pH of cells (pH_{in}) can be derived through the evaluation of the methylammonium concentration ratio inside and outside cells and by measurement of the external pH of the medium (pH_{out}) using a pH electrode.[1–4]

This method was used to examine the external pH dependence of ΔpH of *E. coli* cells. In this case, however, changes in methylammonium concentrations could not be measured because large amounts of K^+ efflux were induced from *E. coli* cells when the pH of the medium was made alkaline,[13] and this K^+ efflux seriously interfered with the electrode response. The selectivity coefficient of the present electrode against K^+ was insufficient to measure methylammonium uptake in the presence of large amounts of K^+. Therefore, prior to measurement, we removed a sufficient amount of K^+ from *E. coli* cells by treating the cells with high concentrations of Li^+ under alkaline conditions, as Li^+ interference was negligible, as shown in Table 1. Fig. 4 shows the external pH dependence of ΔpH using

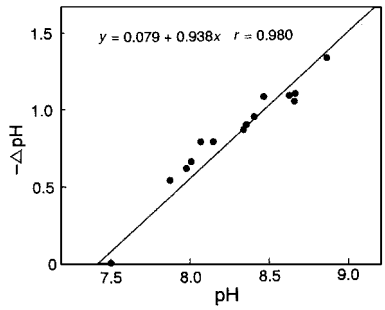

Fig. 3 Dependence of ΔpH across the liposomal membrane on external pH. The sign of ΔpH on the ordinate was changed to negative to illustrate the difference ($pH_{out} - pH_{in}$). The external pH shown on the abscissa was measured using a glass pH electrode.

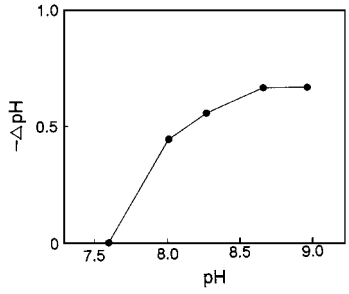

Fig. 4 Dependence of ΔpH across an *E. coli* membrane on external pH. As in Fig. 3, −ΔpH was ploted on the ordinate.

such cells. In this case, the value of −ΔpH increased with increasing external pH to around pH 8, but thereafter it deviated considerably from the theoretical slope of unity. A similar ΔpH profile for *E. coli* cells has been observed previously using tracer-labeled methylamine.[15] These results indicate that a significant amount of H^+ permeates through *E. coli* membranes at higher pH, which differs from artificial liposomal membranes. The *E. coli* membrane is an assemblage of lipids and proteins, and there are many ion pathways *via* membrane proteins. Ion movement through membrane proteins is important for the function of cell membranes. This membrane 'leakage' must be successfully controlled to keep the intracellular pH neutral even in alkaline environments. We have shown that *E. coli* intracellular pH regulation is successful up to around pH 8, and then it gradually diminishes at more alkaline pH, probably owing to retarded membrane functions.

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