

Dielectric Analysis of Outer Membrane Vesicles Isolated from Rat Liver Mitochondria

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Electrical properties of the outer membrane vesicles (OMV) isolated from rat liver mitochondria were studied by a dielectric method. The OMV's suspended in 130 mM KCl solution showed a dielectric relaxation around 50 MHz. The dielectric relaxation was analyzed by a dielectric theory based on interfacial polarization. The results were as follows: (1) The conductivity ratio of the outer membrane to the external medium was less than 3×10^{-4} . If the outer membrane contains large pores (1-2 nm in diameter) whose interior has the same conductivity as does the external medium, the number of the pores is less than a few hundreds per $1 \mu\text{m}^2$. (2) The membrane capacitance changed slightly from 1 to $0.7 \mu\text{F}/\text{cm}^2$ with increasing the KCl concentration in the external medium. (3) The conductivity ratio of the internal phase to the external medium depended on the KCl concentration of the external medium.

KEY WORDS: Mitochondria/Outer membrane/Membrane conductance/Dielectric relaxation/

INTRODUCTION

Mitochondrial outer membranes have high permeability to metabolites and small macromolecules, because the outer membranes contain large pores 2-3 nm in diameter. The pores were characterized by negative stain electron microscopy¹⁻⁵, X-ray diffraction techniques^{6,7} and reconstitution techniques with artificial membranes^{5,8-15}. These results are summarized in Table 1.

Negative-stain electron microscopy showed that mitochondrial outer membranes have stain filled pits, which are suggestive of pore orifices. The number of the pits per unit membrane area (or pore density) was estimated to be $4-6 \times 10^4$ pits/ μm^2 for higher plants (white potato and mung bean)^{1,6} and 4×10^4 pits/ μm^2 for fungus (*Neurospora crassa*)^{3,5}, whereas the pits were not detected for rat liver mitochondria¹.

Reconstitution experiments with planar bilayer lipid membranes and liposomes revealed that porin (pore forming protein) extracted from mitochondrial outer membranes forms pores across lipid bilayer membranes^{5,8-15}. The effective diameter of the pore was estimated to be 2-3 nm from the conductance increment that accounts for single pore formation¹⁰. The value of the pore diameter was consistent with that determined by negative stain electron microscopy¹⁻⁵. Porin was found in most of all mitochondrial outer membranes isolated from animals (rat liver^{9,11-13}, rat heart⁹ and beef heart⁹), higher plants (mung bean)^{6,16} yeast (*Saccharomyces cerevisiae*)⁹, protozoa (*Paramecium aurelia*)^{8,9} and fungus (*Neurospora crassa*)^{5,10,14-15}.

Comparing the results obtained for rat liver mitochondria by electron microscopy

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Dielectric Properties of Mitochondrial Outer Membrane

Table 1 A summary of properties of mitochondrial outer membranes isolated from various sources.

Sources	Negative stain filled "pits"	Channel forming activity	pore protein content
Rat liver	not detected	+	one molecule per 10000 nm ²
Plants			
white potato	diameter=2.8 nm	+	
mung bean	one pit/18 nm ²		
Fungus			
<i>Neurospora crassa</i>	diameter=2-3 nm one pit/22 nm ²	+	one molecule per 25 nm ²
Paramecium		+	

and reconstitution studies, we notice that electron microscopy failed to detect the pore structure in the outer membranes of rat liver mitochondria, in spite of the presence of porin. One possible explanation of this fact is that the pores distribute very sparsely over the outer membrane of rat liver mitochondria as pointed out by Fiek et al.¹³⁾. Our purpose of this study is, therefore, to test this idea from a dielectric standpoint. We isolated outer membrane vesicles from rat liver mitochondria and performed dielectric measurements of their suspensions to evaluate electrical properties of the outer membrane.

THEORY

For an understanding of dielectric behavior of outer membrane vesicle (OMV) suspensions, it is helpful to show its theoretical expectations in advance. If the OMV is represented by a "single-shell" model in which a sphere (of complex relative permittivity ϵ_i^*) is covered with a membrane (of ϵ_m^*) as shown in Fig. 1, the equivalent complex relative permittivity ϵ_s^* of the membrane-bounded sphere is given by

$$\epsilon_s^* = \epsilon_m^* \frac{2(1-v)\epsilon_m^* + (1+2v)\epsilon_s^*}{(2+v)\epsilon_m^* + (1-v)\epsilon_i^*} \quad (1)$$

where $v = [(R-d)/R]^3$, R is the outer radius of the shell-sphere and d is membrane thickness. Complex relative permittivities are defined as $\epsilon^* = \epsilon - j\kappa/\omega\epsilon_0$, ϵ is relative

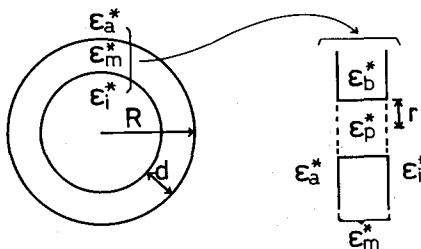


Fig. 1. An electrical model of OMV. A sphere (of complex relative permittivity ϵ_i^*) covered with a porous membrane (of ϵ_m^*) is suspended in a medium (of ϵ_a^*). The membrane contains pores (of ϵ_p^*) in the membrane matrix (of ϵ_b^*). R is the outer radius, d is membrane thickness, and r is pore radius.

permittivity, κ is conductivity, ϵ_v is permittivity of free space, $\omega = 2\pi f$, f is frequency and $j = \bullet - 1$. If the membrane-bounded spheres (of ϵ_s^*) are suspended in a continuous medium (of ϵ_a^*) at volume fraction Φ , the complex relative permittivity ϵ^* of the suspension is given by Hanai's mixture equation:

$$\frac{\epsilon^* - \epsilon_s^*}{\epsilon_a^* - \epsilon_s^*} \left(\frac{\epsilon_a^*}{\epsilon^*} \right)^{1/3} = 1 - \Phi. \tag{2}$$

The combination of Eqs. (1) and (2), which is termed Hanai-Asami-Koizumi's equation¹⁷⁾, has unequivocally reconstituted the dielectric relaxation curves of mitoplasts¹⁸⁾, erythrocytes²¹⁾, and microcapsules^{22,23)}.

If the membrane has N_p cylindrical pores per unit area and the complex relative permittivity of the pore interior is ϵ_p^* , the equivalent complex relative permittivity of the membrane ϵ_m^* are

$$\epsilon_m^* = \epsilon_b^* + P(\epsilon_p^* - \epsilon_b^*), \tag{3}$$

where ϵ_b^* is the complex relative permittivity of the membrane matrix without the pores and P is the porosity defined as $P = \pi r^2 N_p$, where r is the pore radius.

Substituting Eq. (3) for ϵ_m^* in Eq. (1), we can calculate the permittivity and conductivity of a suspension of vesicles with a porous membrane as a function of frequency. Numerical calculation was carried out using the following parameters: $\epsilon_a = \epsilon_i = \epsilon_p = 80$, $\kappa_a = \kappa_i = \kappa_p = 10$ mS/cm, $\epsilon_b = 10$, $\kappa_b = 10^{-5}$ mS/cm, $R = 0.25 \mu\text{m}$, $d = 7$ nm, $r = 1$ nm and $\Phi = 0.2$. A single dielectric relaxation is predicted as shown in Fig. 2a and the relaxation intensity decreases with increasing the pore density or the membrane

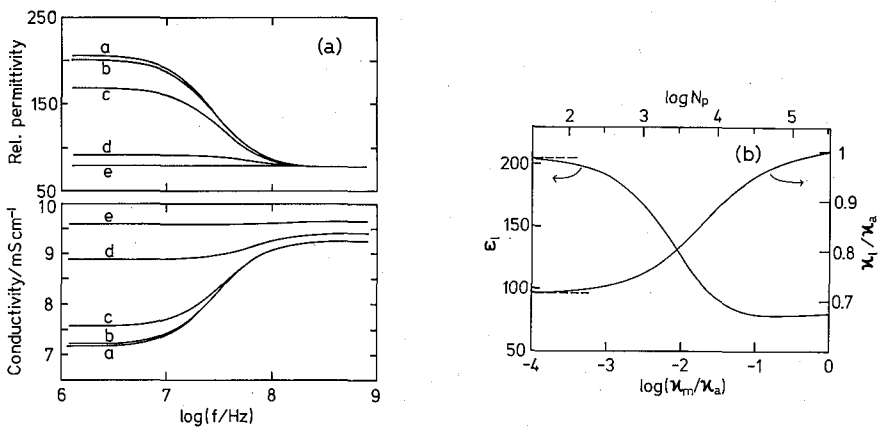


Fig. 2. Dielectric behavior simulated by the electrical model shown in Fig. 1. The following parameters were used in this calculation: $\epsilon_a = \epsilon_i = \epsilon_p = 80$, $\kappa_a = \kappa_i = \kappa_p = 10$ mS/cm, $\epsilon_b = 10$, $\kappa_b = 10^{-5}$ mS/cm, $R = 0.25 \mu\text{m}$, $d = 7$ nm, $r = 1$ nm and $\Phi = 0.2$. (a) Frequency dependence of relative permittivity ϵ and conductivity κ . The pore density N_p (number of pores/ $1 \mu\text{m}^2$) was varied as follows: curve a, 0; b, 100; c, 10000; e, 40000. (b) Plots of ϵ_i and κ_i/κ_a against N_p and κ_m/κ_a . ϵ_i and κ_i are the limiting relative permittivity and conductivity at low frequencies, respectively.

conductivity. Figure 2b shows the plots of ϵ_l (the limiting relative permittivity at low frequencies) and κ_l/κ_a (the ratio of the limiting conductivity at low frequencies to the medium conductivity) against N_p (the pore density) and κ_m/κ_a (the conductivity ratio of the membrane to the medium). The values of ϵ_l and κ_l/κ_a depend on the values of N_p and κ_m/κ_a as follows: (1) ϵ_l and κ_l/κ_a are unchanged with N_p below 100 (or $\kappa_m/\kappa_a < 3 \times 10^{-4}$), (2) ϵ_l and κ_l/κ_a change markedly when N_p is between 100 and 3×10^4 (or $3 \times 10^{-4} < \kappa_m/\kappa_a < 0.1$), (3) ϵ_l and κ_l/κ_a are close to ϵ_a and 1, respectively, with N_p above 3×10^4 (or $\kappa_m/\kappa_a < 0.1$). It is predictable from this calculation that a dielectric relaxation would be obtained for the OMV of rat liver mitochondria with $N_p \approx 100$ pores/ μm^2 , but not for the OMV of mung bean and *Neurospora crassa* mitochondria with $N_p \approx 5 \times 10^4$ pores/ μm^2 .

MATERIALS AND METHODS

Preparation of outer membrane vesicles

Rat liver mitochondria were prepared and purified by the method described in a previous paper¹⁷. The outer membranes were isolated from the mitochondria by controlled osmotic lysis followed by sucrose step density gradient centrifugation according to the method of Douce et al.¹⁹ and Mannella and Bonner¹⁶. The mitochondria suspended in 2 ml of 0.25M sucrose/5mM Tris-HCl (pH7.4) were diluted rapidly with 100ml of 10mM Tris-HCl (pH7.4). The osmolarity decrease from 280 mOsM to about 25 mOsM lysed the mitochondria to detach their outer membranes. The mitochondrial lysates were collected by centrifugation at $20000 \times g$ for 60min at 4°C. The pellet was resuspended in 5 ml of an isosmotic medium (0.25M sucrose/5mM Tris-HCl(pH7.4)) and then homogenized by three strokes with a Teflon homogenizer. The suspension was recentrifuged at $10000 \times g$ for 10 min. The pellet contained predominantly inner membranes (mitoplasts), which were served as a reference for comparison with the outer membranes. The mixture of the resulting turbid supernatant and fluffy layer, containing outer membranes and unbroken mitochondria, was layered atop 0.6/0.9/1.25M sucrose step gradient and centrifuged at $64000 \times g$ for 120 min in a Beckman SW 27 rotor. Three bands appeared at the interfaces of the sucrose layers. The band at the 0.6/0.9 M sucrose interface consisted of vesicles of outer membranes, termed outer membrane vesicles (OMV) (Fig. 3b). The band at the 0.9/1.25 M interface and the pellet contained unbroken mitochondria (Fig. 3c and 3d). Purity of the OMV fraction was routinely assessed by thin-section electron microscopy, which can distinguish the outer membranes from the mitoplasts and unbroken mitochondria.

Dielectric measurement

Dielectric measurements were performed exactly as described in a previous paper¹⁷. Briefly, the outer membrane vesicle (OMV) suspension was loaded into a measurement cell thermostated at 25°C. The equivalent capacitance and conductance were measured with a computer controlled RF Impedance Analyzer (HP model 4191A). Correction for the residual inductance and stray capacitance arising from the measure-

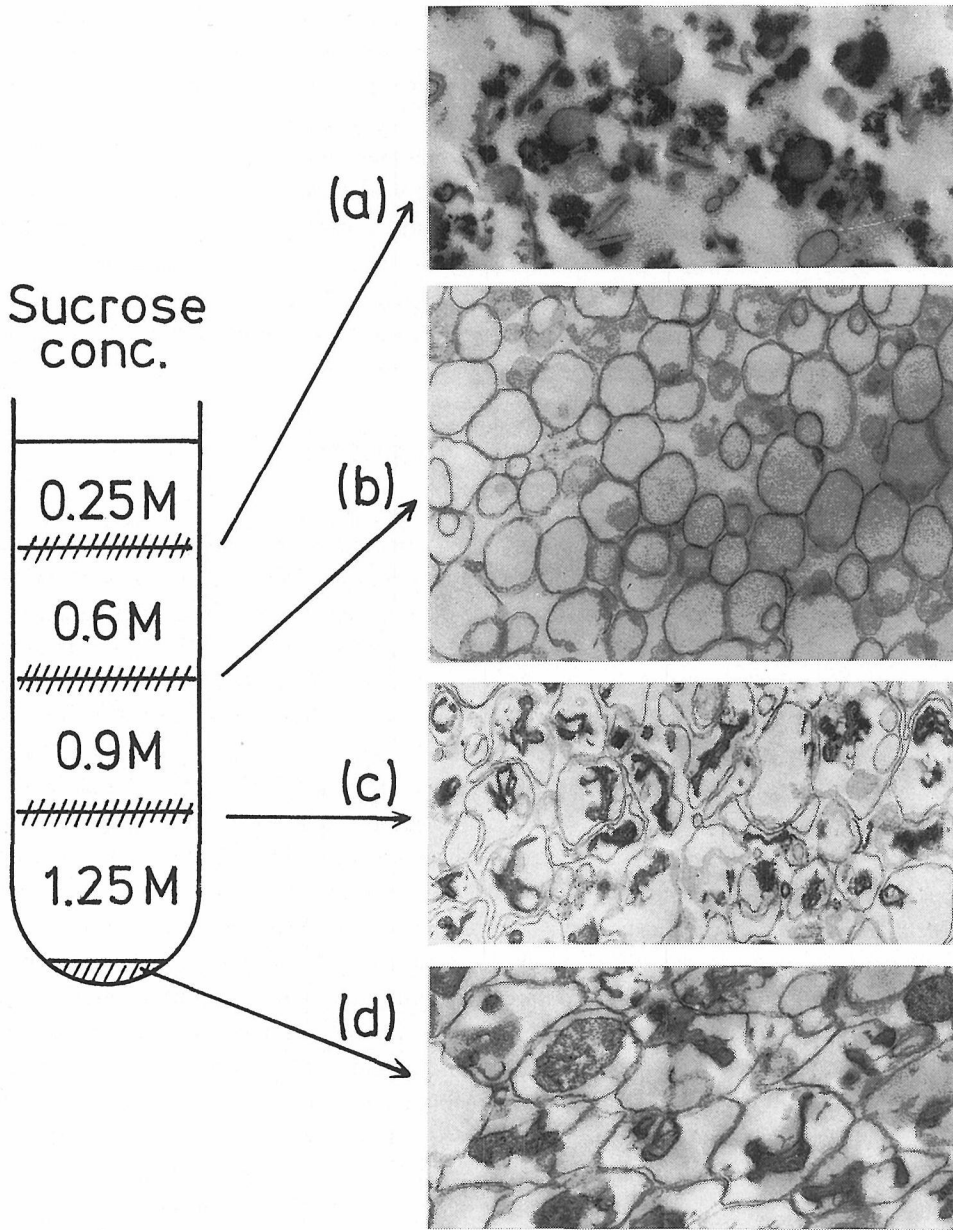


Fig. 3. Thin section electron micrographs of the fractions obtained from mitochondrial lysates by sucrose step density gradient centrifugation. (a) microsomes and unknown granules at the 0.25/0.6 M interface, (b) outer membrane vesicles at the 0.6/0.9 M interface, (c) unbroken mitochondria at the 0.9/1.25 M interface, (d) unbroken mitochondria at the bottom. Magnification, 15000.

parameter circuit model¹⁷).

Determination of volume fraction

Volume fraction of the OMV's in suspension was determined by electron micro-

Volume fraction of the OMV's in suspension was determined by electron microscopical morphometry. Immediately after dielectric measurements, the suspension was withdrawn from the measurement cell and then one part of the suspension (of volume fraction Φ) was mixed with one part of 1.75% glutaraldehyde containing 50 mM cacodylate buffer (pH7.4). The mixture (of volume fraction $\Phi/2$) was placed in a polyethylene tube of 1 mm inner diameter. The tube was sealed at one end, fitted in a stainless steel sleeve and set on a hematocrit centrifuge. Centrifugation at $10000 \times g$ for 1 hr separated the suspension into a clear supernatant and a closely packed pellet. Thus, the volume ratio of the pellet to the total suspension (R_p) was readily measured. The pellet, together with the tube, was cut into 3-4 pieces, which were further fixed with 1% OsO_4 containing 50 mM cacodylate buffer (pH7.4). The fixed pellets without removing the tubes were embedded in epoxy resin and the thin sections were observed under an electron microscope. The volume fraction of the OMV's in the pellet (Φ_p) was determined from the electron micrographs by morphometry²⁰. Finally, the volume fraction of the starting suspension is calculated from $\Phi = 2\Phi_p R_p$.

RESULTS AND DISCUSSION

Morphology

Figure 3b shows a thin-section electron micrograph of the purified outer membranes, which form resealed vesicles, termed outer membrane vesicles (OMV). The OMV was clearly distinguishable from mitoplasts and unbroken mitochondria because the OMV shows no osmotic volume change in sucrose solutions and has no inclusion of stained materials. Contamination of inner membranes and microsomes was less than 1%. The mean radius of the OMV was estimated to be $0.268 \mu\text{m}$ by morphometry.

Dielectric behavior

Figure 4a shows frequency dependence of relative permittivity and conductivity of the OMV's suspended in 130mM KCl buffered with 10mM Tris-HCl (pH7.4). As predicted from the simulation in Fig. 2, a typical dielectric dispersion was found around 50 MHz. The complex plane plots of this dielectric dispersion describe a semicircle with a depressed center (Fig. 4b and 4c).

Membrane conductance

The volume fraction of the OMV's in suspension can be determined by two methods, electrical and morphometrical methods. Comparing the obtained volume fractions between the two methods, we can estimate the outer membrane conductance. The volume fraction is given by the equation derived from Eqs. (1) and (2) as:

$$\Phi_e = 1 - \frac{RG_m(\kappa_l - \kappa_i) + \kappa_i \kappa_l}{RG_m(\kappa_a - \kappa_i) + \kappa_i \kappa_a} \left(\frac{\kappa_a}{\kappa_l} \right)^{1/3}, \quad (4)$$

where $G_m = \kappa_m/d$. On the assumption of $RG_m \ll \kappa_l, \kappa_i, \kappa_a$, which holds for most of

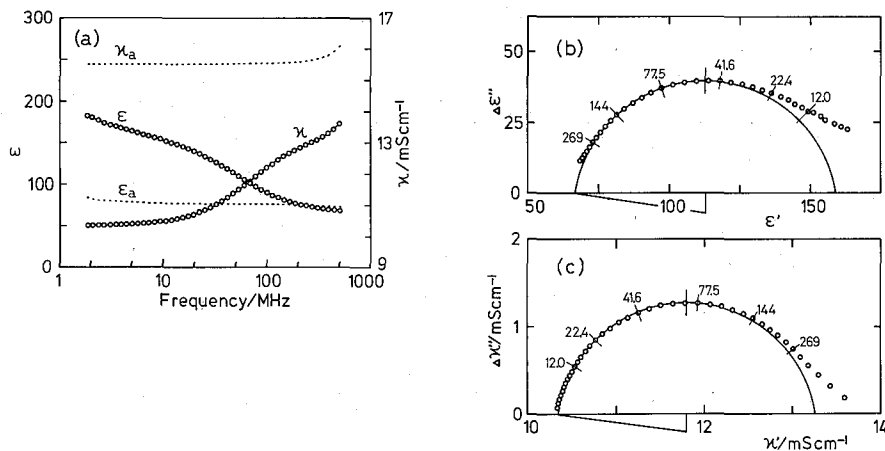


Fig. 4. Dielectric behavior of outer membrane vesicles suspended in 130 mM KCl/10 mM Tris-HCl (pH 7.4). (a) Frequency dependence of relative permittivity ϵ and conductivity κ . Open circles indicate the data of the outer membrane vesicle suspension; Dotted lines, the suspending medium. (b) Complex relative permittivity plane plots. Ordinate, $\Delta\epsilon'' = (\kappa - \kappa_l) / \omega\epsilon_v$, where κ_l is the limiting conductivity at low frequencies, ϵ_v is permittivity of free space and $\omega = 2\pi f$; abscissa, $\epsilon' = \epsilon$. (c) Complex conductivity plane plots. Ordinate, $\Delta\kappa'' = (\epsilon - \epsilon_h) \omega\epsilon_v$, where ϵ_h is the limiting relative permittivity at high frequencies; abscissa, $\kappa' = \kappa$. The number on each points refers to marker frequency in MHz.

plasma membranes and intracellular membranes, Eq. (4) becomes

$$\Phi_e = 1 - \left(\frac{\kappa_l}{\kappa_a} \right)^{2/3}. \quad (5)$$

In Table 2, we compare the volume fraction Φ_e of OMV's in suspension calculated from Eq. (5) with the volume fraction Φ_m determined by electron microscopical morphometry. In order to estimate the errors inherent in the morphometry, the comparison is also carried out for swollen mitoplasts, whose membranes, namely inner membranes, hold for the assumption that $RG_m \ll \kappa_l, \kappa_i, \kappa_a$. The value of the Φ_m/Φ_e ratio for the OMV's coincided with that for the mitoplasts within experimental errors, indicating that the outer membranes are also in the case of $RG_m \ll \kappa_l, \kappa_i, \kappa_a$. This also implies that the κ_m/κ_a ratio is less than 3×10^{-4} and the value of N_p is less than a few hundreds per $1 \mu\text{m}^2$ by reference to the simulation in Fig. 2b.

Analysis based on single-shell model

Table 3 shows the results obtained by fitting the single-shell model to the observed dielectric data under the conditions that $RG_m \ll \kappa_l, \kappa_i, \kappa_a$. The value of C_m changed from 1 to $0.7 \mu\text{F}/\text{cm}^2$ with increasing the KCl concentration of the external medium, being somewhat lower than that obtained with intact mitochondria using a double-shell model²⁴). The conductivity ratio of the interior to the external medium κ_i/κ_a is also dependent on the KCl concentration of the external medium. Since the OMV's prepared in 0.25M sucrose solution are expected to contain the same solution in the interior, it

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Table 2 The volume fractions estimated by the electrical and morphometrical method.

	Volume fraction		
	Morphometrical	Electrical	Φ_m/Φ_e
	Φ_m	Φ_e	
OMV	0.16	0.157	1.05
	0.26	0.240	
mitoplast	0.22	0.214	1.03
	0.29	0.280	

Table 3 The estimated phase parameters of outer membrane vesicles

Medium	No. of Exp.	$C_m/\mu\text{Fcm}^{-2}$	κ_i/κ_a
0.25M sucrose	2	1.00 (0.96-1.04)	1.02 (0.84-1.21)
13 mM KCl	4	0.87 ± 0.01	0.75 ± 0.05
130 mM KCl	7	0.74 ± 0.01	0.58 ± 0.01

is reasonable that the value of κ_i/κ_a is almost unity in 0.25M sucrose solution. On the other hand, the values of κ_i/κ_a was always below unity with the OMV's resuspended in 10 mM or 130 mM KCl solution even after an overnight incubation at 4°C. This fact is surprising because the solution inside and outside OMV's are expected to become uniform in concentration fairly rapidly owing to the high permeability of the outer membrane to sucrose molecules, K^+ and Cl^- ions. Although this phenomenon needs further investigation, it might be related to the property of the pore in the outer membrane, known as the voltage-gated anion sensitive channel²⁵. The opening and closure of the pore are controlled by the potential difference V_m across the membrane; the open state occurs at V_m below about 30 mV and the closed state at V_m more than about 30 mV.⁹ When the external medium (0.25 M sucrose) is replaced by KCl solutions, the membrane potential develops, thereby the pores are probably in the closed state. This might reduce the flux of sucrose molecules, K^+ and Cl^- ions, and keep a difference in conductivity between the internal and the external solution.

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