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Determination of Reflection Coefficients for Various Ions and Neutral Molecules in Sarcoplasmic Reticulum Vesicles through Osmotic Volume Change Studied by Stopped Flow Technique

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Summary. Osmotic volume change of sarcoplasmic reticulum vesicles was studied by following the change in light-scattering intensity using a stopped flow apparatus. From the analysis of the initial rate of scattering change, reflection coefficients for various ions and neutral molecules were determined. The following are typical results: K^+, 0.72; $Tris^+$, 0.98; choline, 1; NO_3^-, 0.32; Cl^-, 0.46; methanesulfonate, 0.62; gluconate, 0.96; glycerol, 0.86; and glucose, 1. When the K^+ permeability was increased in the presence of 10^{-6} g valinomycin/ml, the reflection coefficient for K^+ changed from 0.72 to 0.31. It was found that there was a close relationship between the reflection coefficients and the permeabilities of the solutes. Hydraulic conductivity was also determined from the initial rate of light scattering change and was not different for the different solutes. The water permeability was estimated to be 2.1×10^{-3} cm/sec at 23 °C.

Sarcoplasmic reticulum (SR) regulates the cytoplasmic calcium concentration of skeletal muscle cells and thereby controls muscular contraction and relaxation (Ebashi & Endo, 1968). In connection with these functions, release and uptake of Ca^{2+} by SR vesicles have been studied by many researchers (Weber, Herz & Reiss, 1966; Tonomura, 1972; Kasai & Miyamoto, 1976*a–b*; Meissner & McKinley, 1976). Studies of the permeabilities for ions through SR membrane are important to understand these functions on the molecular level. However, little is known about them because the permeability for important ions such as K^+, Na^+, and Cl^- is so high that it was difficult to follow their permeation by the usual tracer method (Kasai & Miyamoto, 1976*b*; Meissner & McKinley, 1976; Duggan & Martonosi, 1970; Jilka, Martonosi & Tillack, 1975).

In the previous paper (Kometani & Kasai, 1978), we have succeeded in measuring the passive permeabilities for various ions and neutral

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molecules by following the osmotic volume change through the light-scattering method. Now, more precise measurements are available.

According to irreversible thermodynamics (Kedem & Katchalsky, 1958) the permeability properties of solutes are described by three parameters, i.e., hydraulic conductivity L_p, permeability coefficient ω, and reflection coefficient σ. In this paper, the osmotic volume change of SR vesicles caused by various salts and neutral molecules was studied by following the light-scattering change with stopped flow technique similar to that used by Goldstein and Solomon (1960). At the same time, their permeabilities were also determined by analyzing the records of scattering change as described in the previous paper (Kometani & Kasai, 1978). As a result, L_p and σ for various ions and neutral molecules were determined. While L_p was constant through various solutes, σ changed depending on their permeability.

Materials and Methods

Theoretical

According to irreversible thermodynamics (Kedem & Katchalsky, 1958; Goldstein & Solomon, 1960), the volume change of vesicles is written by

$$\frac{dV}{dt} = -AL_p(\Delta\pi_i + \sigma\Delta\pi_s) \tag{1}$$

where V is the volume of the vesicle, A its outer area and L_p the hydraulic conductivity. π_i is the osmotic pressure of the impermeable solute, and π_s of the permeable solute. $\Delta\pi$ is the osmotic pressure difference between the outside and the inside of the vesicles, and σ the reflection coefficient for the permeable solute.

If we plot the initial rate of volume change $(dV/dt)_0$ against $\Delta\pi_s$ under the condition of constant $\Delta\pi_i$, the plot will give a straight line and σ can be obtained from

$$\sigma = -\Delta\pi_i/\Delta\pi_{so} \tag{2}$$

where $\Delta\pi_{so}$ is the osmotic pressure difference of the permeable solute which gives $(dV/dt)_0 = 0$ (Method I).

$AL_p\sigma$ can be obtained from the slope of the straight line (Method II).

Since the change in light-scattering intensity was approximately proportional to the vesicular volume in our case (Kometani & Kasai, 1978), (dV/dt) can be obtained from (dI/dt) where I is the scattering intensity.

Materials

SR vesicles were isolated from rabbit dorsal and hind leg muscle as a microsomal fraction by the method of Weber *et al.* (1966) with slight modifications (Kasai &

Miyamoto, 1976*a*) and stored in 0.1 M KCl, 5 mM Tris-maleate (pH 6.5), 20 mg SR protein/ml at 0 °C. SR vesicles were used within 3 days of isolation.

Valinomycin was purchased from Sigma Chemical Co. Other reagents were commercial products of analytical grade. Potassium 2 (N-morphorino) ethane sulfonate (K-MES), Tris-MES, potassium gluconate, and Tris-gluconate were prepared from base and acid solutions by neutralization.

Methods

Osmotic volume change of SR vesicles was monitored with scattered intensity of light (450 nm) at right angle to the incident beam using a stopped flow spectrophotometer (Union RA-401 and RA-450, Japan) as described in the previous paper (Kometani & Kasai, 1978). Temperature was maintained at 23 °C with a temperature controlling apparatus (Neslab RTA 8). In most experiments, SR vesicles were preincubated in a buffered solution at 0.4 mg SR protein/ml for an appropriate time at room temperature. This suspension was mixed with an equal volume of the solution containing salts or neutral molecules, and the change in scattered light intensity was followed. In most cases, the change in light scattering intensity was considered proportional to the volume change (Kometani & Kasai, 1978); but in the case of divalent cations, this relation is not always held, probably due to the binding to the vesicles. Detailed explanation will be given in Results. When valinomycin was used, it was added to the incubated suspension about 2 hr before the mixing.

Osmotic pressure of the solution was determined by an osmometer (Advance Model 3 W).

Protein concentration was determined by the biuret method, calibrated by nitrogen determination.

Results

Volume Change of SR Vesicles Monitored by the Change in Light Scattering

In Fig. 1, the change in light-scattering intensity is shown when SR vesicles incubated in a low osmotic pressure were mixed with an equal volume of a glycerol solution of high osmotic pressure. According to the previous paper (Kometani & Kasai, 1978), the initial increase in light scattering corresponds to the shrinkage of the vesicles due to the water efflux from the vesicles and the later decrease to their swelling due to the influx of glycerol accompanied by water. Since the change in scattering intensity was approximately proportional to that of the vesicular volume, the rate of the volume change due to the water movement can be determined from the initial rate of the scattering change. If we use various solutes instead of glycerol, we can measure the permeability properties for these solutes. When high concentrations of divalent cation such as Ca^{2+} or Mg^{2+} were used instead of glycerol, the scattering

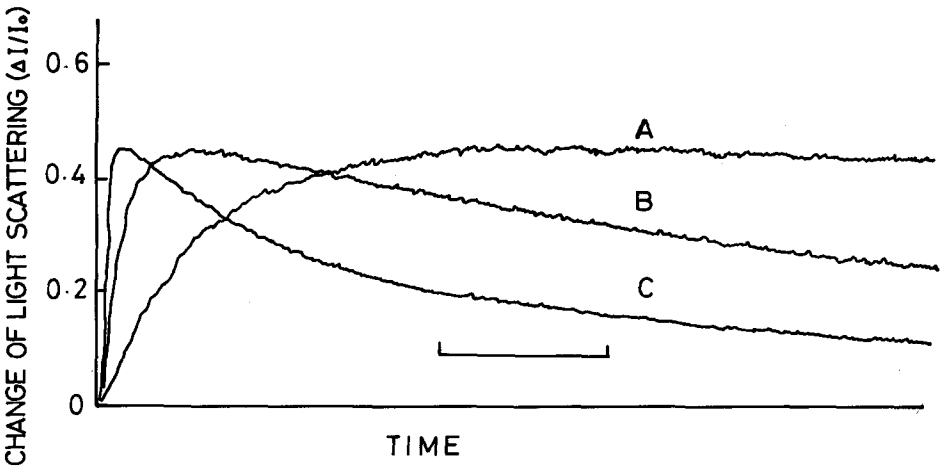

Fig. 1. Change in light-scattering intensity of SR vesicles caused by the volume change. SR vesicles incubated in 2 mM KCl, 0.1 mM Tris-maleate (pH 6.5), 0.4 mg SR protein/ml was mixed with an equal volume of 100 mM glycerol solution, and the change in scattering intensity was followed. $\Delta I/I_0$ is the relative change in light scattering intensity. Time calibration: *A*, 200 msec; *B*, 1 sec; *C*, 5 sec

intensity did not always return to the initial level even after the addition of the ionophore, A23187. This may be due to the aggregation of SR vesicles caused by the binding of divalent cations. Because of this phenomenon, the permeability properties for divalent cations could not be determined. In the case of monovalent cations, such effect could be neglected.

Permeabilities for the solutes were estimated from the decreasing phase of the records. The permeation time was defined as the time when the scattering intensity becomes a half of the maximal increment which was determined by extrapolating the decreasing phase to time zero, as described in the previous paper (Kometani *et al.*, 1978). This permeation time is considered to be inversely proportional to the permeability coefficient for the solutes.

On the other hand, no change in light-scattering intensity was observed when SR vesicles incubated in a high osmotic solution of glycerol were mixed with a solution of low osmolarity. This result suggests that SR vesicles exist in the most swollen state under the incubation condition.

Determination of σ by Method I

Since SR vesicles exist in the most expanded state in the usual condition, they must be shrunk to some extent beforehand in order to

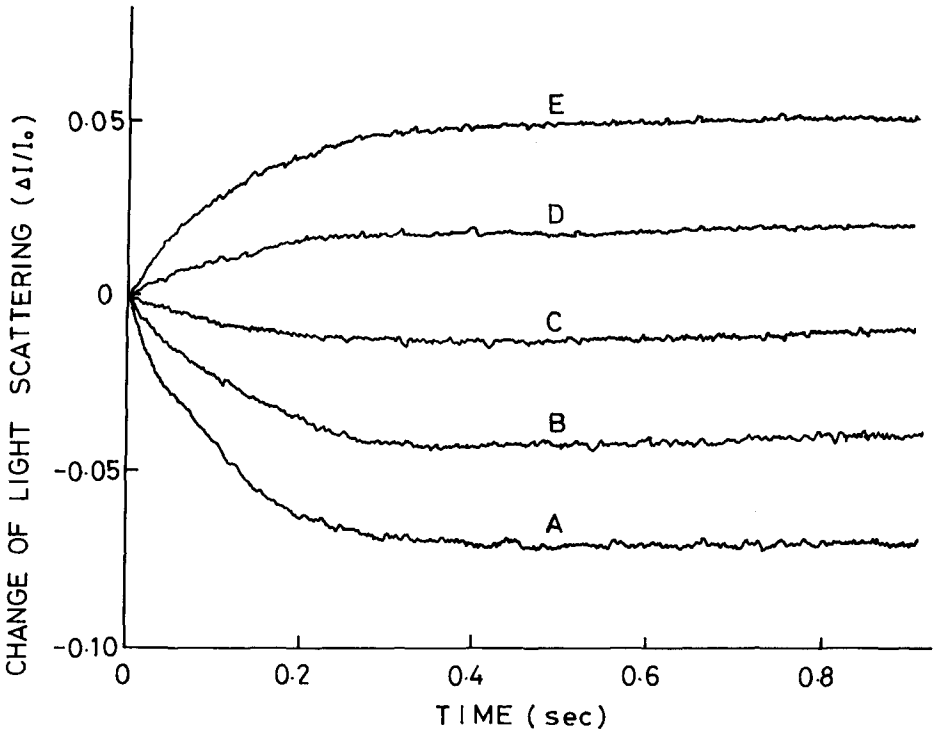

Fig. 2. Change in light scattering intensity of SR vesicles due to the volume change induced by glucose. After the incubation in 25 mM glucose and 0.8 mg SR protein/ml overnight at 0 °C, SR vesicles were first diluted twice by 175 mM glucose (conditioning). Three minutes after the first dilution, the suspension was secondly mixed with an equal volume of different concentrations of glucose by a stopped flow apparatus and the change in light scattering was followed. Time courses after the second mixing are shown. Concentration of the mixed glucose solution was as follows (the final concentration is half of these values): *A*, 0; *B*, 50 mM; *C*, 100 mM; *D*, 150 mM; *E*, 200 mM. In the case of *C*, both extravesicular and intravesicular glucose concentration became 100 mM

observe both their shrinkage and swelling due to the water movement. After incubation in 25 mM glucose overnight to attain an equilibrium, SR vesicles were diluted with an equal volume of 175 mM glucose. This treatment is referred to as conditioning. The final glucose concentration is 100 mM. Since the permeation of glucose is slow, the vesicles will be kept in a shrunken state for a while, and we can regard this glucose as the impermeable solute contributing to $\Delta\pi_i$ in Eq. (1). Three minutes after conditioning, this suspension was mixed with different concentrations of solute by a stopped flow apparatus and the change in light scattering was recorded. Figure 2 is a typical example which was obtained by using glucose. This latter glucose is regarded as the permeable

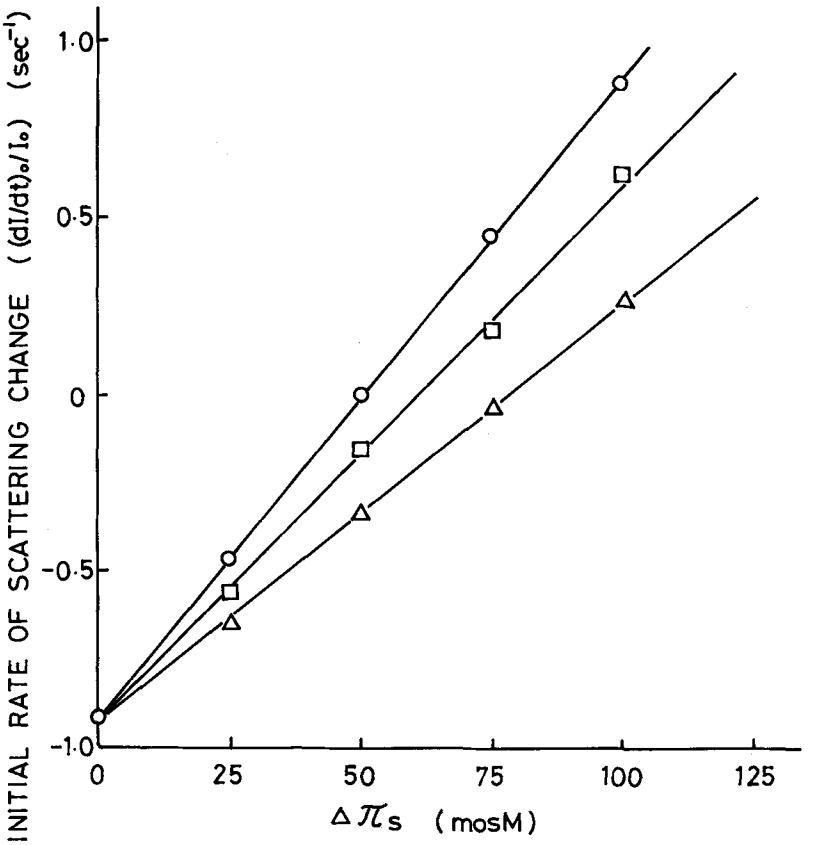

Fig. 3. Initial rate of the scattering change as a function of the osmotic pressure difference of the permeable solutes, $\Delta\pi_s$. Initial rates of the scattering change for glucose was taken from Fig. 2. Similar experiments to Fig. 2 were also carried out using glycerol and KMS. After conditioning with glucose by the same procedure as in Fig. 2, the suspension was mixed with an equal volume containing various concentrations of glycerol or KMS as permeable solute. ○, glucose; □, glycerol; △, KMS

solute contributing to $\Delta\pi_s$. When the osmotic pressure was decreased, an initial swelling of the vesicles was observed.

As seen in Fig. 2, a small decrease in light scattering was always observed when the vesicles were mixed with the same concentration of glucose used for the conditioning. This change is probably due to an artifact at the mixing. In the determination of the initial rate of scattering change in Fig. 3, this value was subtracted from the apparent initial rates. Similar experiments were carried out using glycerol and KMS as permeable solutes after the conditioning by glucose.

Table 1. Reflection coefficients and permeation times for various salts and neutral molecules[a]

Solutes	Reflection coefficient, σ		Permeation time (sec)
	Method I	Method II	
Glycerol	0.84 ± 0.10	0.86 ± 0.03	1.8
Urea		1.00 ± 0.03	1.2
Glucose	1[b]	1[b]	1500
KCl	0.55 ± 0.10	0.59 ± 0.02	10
KMS	0.68 ± 0.10	0.67 ± 0.03	50
KMES		0.78 ± 0.03	
KNO_3		0.52 ± 0.03	10
K gluconate		0.84 ± 0.03	100
K_2SO_4		0.80 ± 0.08	100
Tris-MES		0.91 ± 0.03	
Tris-gluconate		1.08 ± 0.08	1200
$CaCl_2$		(0.80 ± 0.10)	
Choline Cl		0.73 ± 0.03	360

[a] Reflection coefficients were obtained by the same experiments as described in Fig. 3 (Method I) and Fig. 5 (Method II). Permeation times were obtained from similar experiments in Fig. 1, where SR vesicles incubated in low osmotic solution were mixed with an equal volume of 200 mOsM solution

[b] σ for glucose was assumed to be 1.

Figure 3 shows the initial rate of the scattering change plotted against the osmotic pressure difference of the permeable solutes, $\Delta\pi_s$. As predicted from Eq. (1), the data give a straight line. If we read the osmolarity $\Delta\pi_{so}$, where $(dI/dt)_0 = 0$, σ can be calculated from Eq. (2). Although we do not know exact value of $\Delta\pi_i$ in Eq. (2), it was assumed equivalent to 50 mM glucose in this case, since glucose concentration inside the vesicles was equivalent to 100 mM and that outside was 50 mM just after the mixing. In this case reflection coefficient for glucose was assume to be 1 because its permeability is small. The data obtained by this procedure are summarized in Table 1.

This method is the best one to obtain σ directly. However, experimental error is not small, because the amount of the scattering change was small compared with that in the case of Method II and a small difference in the conditioning time probably affects the initial state of the vesicles. The evaluation of σ is also possible from the slope of the line in Fig. 3 since it is proportional to $L_p\sigma$. However, such an analysis was not done from this figure because the conditioning was not required for the determination of the slope only.

Determination of σ by Method II

Figure 4 shows the change in scattering intensity due to the shrinkage of the vesicles which was elicited by the mixing of the vesicles incubated in a low osmotic solution with different concentrations of glycerol. The records are less noisy than those observed in Fig. 2. Similar experiments were carried out using various kinds of solutes. The initial gradient of these curves are plotted against the osmotic pressure difference in Fig. 5. In this case a small increase in light scattering was observed without change in osmolarity of the solution, which might be due to an artifact (The record is not shown). This rate was also subtracted from the apparent rates in Fig. 5. The data show a straight line as predicted from Eq. (1) and the slopes must be proportional to $L_p\sigma$. If we assume $\sigma=1$ for glucose, $L_p\sigma/L_{po}$ can be obtained from the ratio of these slopes, where L_p and L_{po} are the hydraulic conductivities caused by the permeable solute and glucose, respectively, and σ is the reflection coefficient for the solute. When $L_p\sigma/L_{po}$ were compared with σ obtained by Method I for few solutes such as glycerol, KCl and KMS, there was

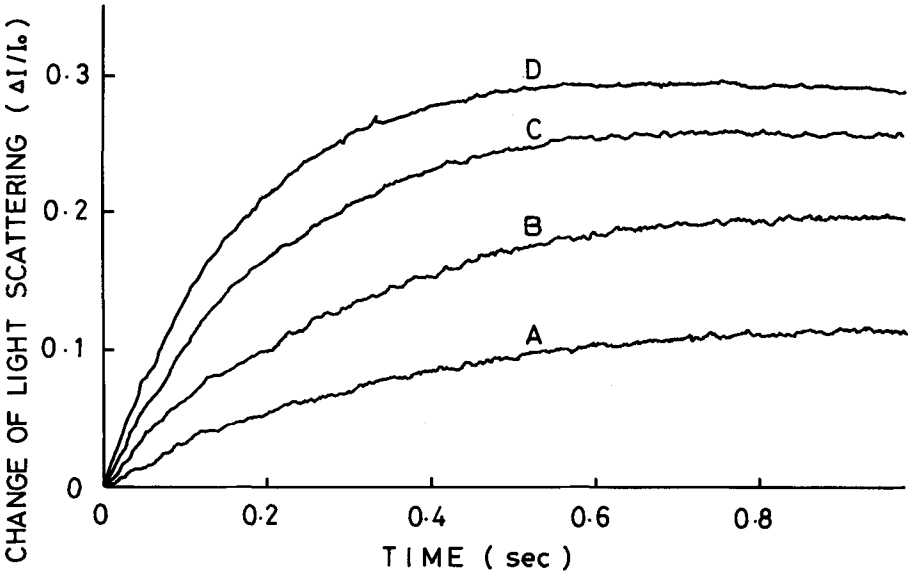

Fig. 4. Change in light-scattering intensity of SR vesicles due to the volume change induced by glycerol. After the incubation in 2 mM KCl, 0.1 mM Tris-maleate (pH 6.5) and 0.4 mg SR protein/ml for more than 3 hr at room temperature, SR vesicles were mixed with an equal volume of different concentrations of glycerol and change in light scattering was followed. Concentration of the mixed glycerol was as follows: *A*, 50 mM; *B*, 100 mM; *C*, 150 mM; *D*, 200 mM

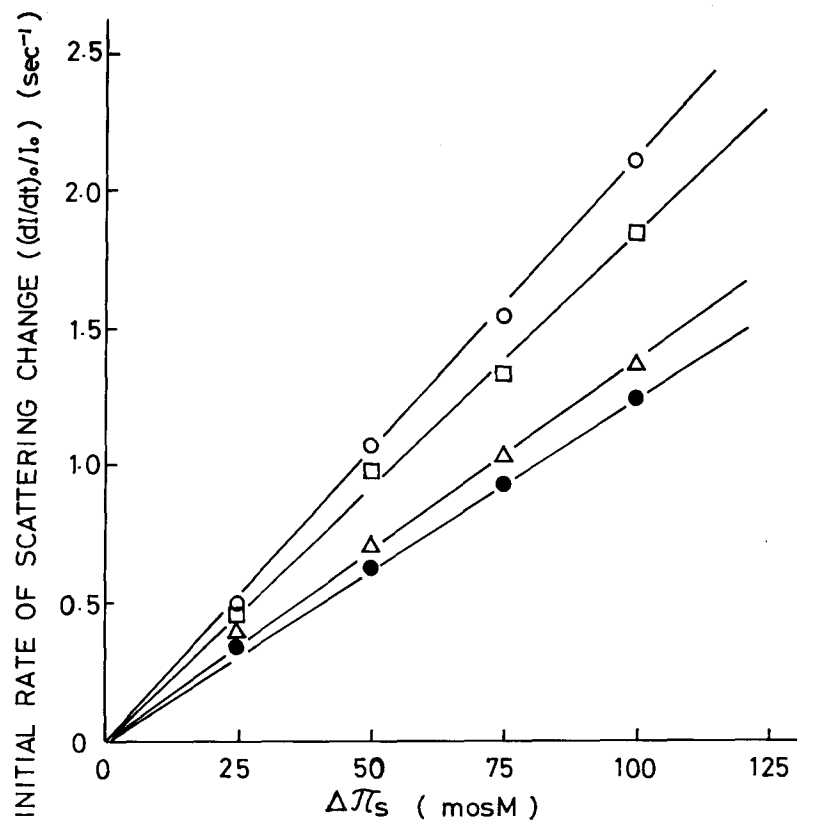

Fig. 5. Initial rate of the scattering change as a function of the osmotic pressure difference. Initial rates of the scattering change were obtained from experiments similar to Fig. 4. Abscissa shows the pressure difference of the mixed solutes after the mixing. ○, glucose; □, glycerol; △, KMS; ●, KCl

good agreement. This result indicates $L_p = L_{po}$ for these solutes. Hereafter, we assumed $L_p = L_{po}$ for all solutes which were examined. Accordingly, we can obtain σ by this procedure (Method II). The obtained values are shown in Table 1. Standard deviations for these two methods are also shown in Table 1. Since this method gave reproducible results, further experiments were carried out by this method.

Calculation of σ for Ions

In the above section we determined σ for various salts, i.e., ion pairs. These values are considered to be an average of σ over ion pairs as

Table 2. Reflection coefficients and permeation times for ions[a]

Ions	Reflection coefficient, σ	Permeation time (sec)
Cl^-	0.46	0.4
MS^-	0.62	20
MES^-	0.84	
NO_3^-	0.32	0.12
$Gluconate^-$	0.96	60
SO_4^{2-}	0.96	60
K^+	0.72	20
$Tris^+$	0.98	
$Choline^+$	1[b]	720

[a] Reflection coefficient for each ion was calculated from the data in Table 1 using Eq. (3). The permeation times for ions were obtained from Table 1 by similar analysis in the previous paper (Kometani & Kasai, 1978).

[b] σ for choline was assumed to be 1.

follows in analogy with nonelectrolytes

$$\sigma = \frac{\sigma^+ \Delta\pi^+ + \sigma^- \Delta\pi^-}{\Delta\pi^+ + \Delta\pi^-} \tag{3}$$

where σ^+ and σ^- are the reflection coefficient for cation and anion, respectively, $\Delta\pi^+$ and $\Delta\pi^-$ are the osmotic pressure difference of cation and anion, respectively. We assumed $\sigma = 1$ for choline since its permeability is small. The reflection coefficient for each ion was calculated from the values in Table 1 using Eq. (3) and shown in Table 2. In the case of divalent cations such as Ca^{2+}, the calculated σ became larger than 1. This may be an artifact due to the unusual increase in light scattering caused by the binding of Ca^{2+} to the vesicles. Then analysis was not done about divalent cations.

Effect of Valinomycin on σ for K^+

Valinomycin increases the K^+ permeability (Kometani & Kasai, 1978). The reflection coefficients for various kinds of potassium salts were determined by Method II. The results are shown in Table 3. From this data σ for K^+ in the presence of valinomycin was calculated under the assumption that valinomycin did not affect the permeability of

Table 3. Effect of valinomycin on reflection coefficient for K^+[a].

Salts	σ for salts	σ for K^+
KCl	0.38	0.30
KMS	0.46	0.30
KMES	0.59	0.34
KNO_3	0.30	0.28
K gluconate	0.64	0.32
K_2SO_4	0.52	0.30
Average		0.31

[a] After SR vesicles were incubated in 2×10^{-6} g valinomycin/ml for few hours, reflection coefficients were determined by Method II as in the case of Table 1. σ for K^+ was calculated using Eq. (3) under the assumption that σ for anions was not affected by valinomycin.

anions. The results are also shown in Table 3. In all cases σ for K^+ was around 0.31 in the presence of valinomycin. This result shows that the above assumption was valid and that σ for K^+ was changed from 0.72 to 0.31.

Relation between σ and Permeability

It was expected that σ is closely related to the permeability for the solutes. In order to elucidate the relationship between the reflection coefficient and the permeability, the permeation times for the solutes were determined as described in Fig. 1. SR vesicles incubated in low osmotic pressure were mixed with an equal volume containing various kinds of solutes of 200 mosM, and change in light scattering was followed. The permeation times were determined and are shown in Table 1. In the case of salts, the permeation times were separated into those of each ion according to the previous paper (Kometani & Kasai, 1978), which are shown in Table 2.

In Fig. 6, the permeation times were plotted against the reflection coefficients. Since the permeation time is considered inversely proportional to the permeability coefficient, it can be seen that σ increases with decreasing the permeability. Especially in the case of ions, σ are closely related to their permeability. However, neutral molecules such as glycerol and urea showed higher reflection coefficient than that expected from the relationship between σ and the permeability of ions. This difference may arise from the difference of permeation mechanism be-

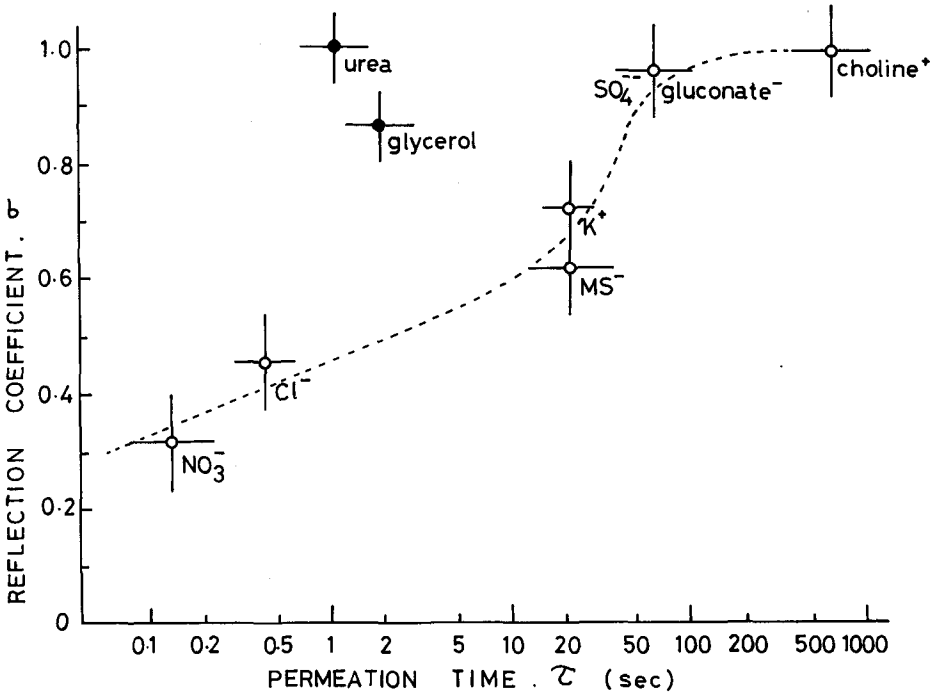

Fig. 6. Relation between reflection coefficients and permeation times for various solutes. The data were taken from Tables 1 and 2

tween ions and neutral molecules. It is considered that ions permeate through hydrated pores in the membrane, but neutral molecules do so through the solvation in the membrane phase. This difference may reflect the difference of σ between ions and neutral molecules, but further analysis has not yet been done to solve this problem.

Discussion

As shown in Results, the reflection coefficients for various ions and neutral molecules were determined. These results show that ions contribute independently to the reflection coefficient, which is consistent with the concept of van't Hoff. Obtained values of σ were correlated to the permeability coefficient as shown in Fig. 6. At present, however, further analysis has not yet been done.

Membrane vesicles of SR are not uniform in size, shape, and properties (McKinley & Meissner, 1978). σ obtained here must be regarded as

averages over these factors. At present, however, further analysis in detail has not yet been done.

The absolute value of hydraulic conductivity can be estimated from Eq. (1) as follows

$$L_p = -\frac{1}{A\Delta\pi}\left(\frac{dV}{dt}\right)_0 \tag{4}$$

for an impermeable solute such as glucose. Increase in scattering intensity, $\Delta I/I_0$, due to the shrinkage of the vesicle became about one when the vesicular volume became zero (Kometani & Kasai, 1978). Then we can put $(dV/dt)/V_0 = -(dI/dt)/I_0$ where V_0 is the initial volume. If we assume that the vesicle is a spherical shell of radius r and neglect the thickness, Eq. (4) can be written by

$$L_p = \frac{r}{3\Delta\pi I_o}\left(\frac{dI}{dt}\right)_0. \tag{5}$$

If we assume $r = 50$ nm (Arrio *et al.*, 1974) and that $(dI/dt)_o/I_o$ is about 2.1 $\sec^{-1}$ for $\Delta\pi = 100$ mosM from Fig. 5, L_p becomes 1.6×10^{-12} $\mathrm{cm^3/dyn \cdot sec}$ at 23 °C. L_p can be converted to the water permeability coefficient P_w (Kedem & Katchalsky, 1958) by

$$P_w = \frac{L_p RT}{V_w} \tag{6}$$

where v_w is the molar volume of water. P_w was 2.1×10^{-3} cm/sec. This is one order smaller than that of human red blood cell (1.7×10^{-2} cm/sec Sha'afi & Gary-Bobo, 1973) and the same order as that of liposomes (4.4×10^{-3} cm/sec, Jain, 1972).

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