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Changes in Potassium Permeability and Membrane Potential of Bovine Red Blood Cells estimated by the Use of a Dimerizing Fluorescence Probe

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The partition of 3,3′-dipropyl-2,2′-thiadicarbocyanine, diS-$C_3(5)$, between the i terior of bovine red cells and bulk aqueous medium was measured by using fluorescence spectroscopy. Inside the cells (concentrated hemoglobin phase), diS-$C_3(5)$ is intensely concentrated and consequently forms the nonfluorescent dimer. Valinomycin stimulates K^+ transport across the cell membrane and the resulting change in membrane potential induces an alteration of the partitioning and the dimerization of diS-$C_3(5)$. The changes in membrane potential and permeability of the red cell membrane to K^+ were quantitatively associated with this dimerizatio'. The permeability of the membrane to K^+ estimated here can be correlated with the characteristic properties of bovine red cell membrane, that is, the high content of sphingomyelin and the rigidity of this membrane.

Keywords——dimerizing fluorescence probe; bovine red blood cell; potassium permeability; membrane potential; rigidity of red cell membrane; valinomycin

Introduction

Some cyanine dyes are well known to exhibit remarkably fast and large changes in fluorescence in response to changes in membrane potential, which may play an important role in the widespread energy transducing and regulatory processes in cells.[1] However, the mechanism responsible for the potential-dependent fluorescence change has not been completely elucidated and the evaluation of the potential has, in practice, been based on empirical calibration.[1a,b]

Absorption and fluorescence spectra of 3,3′-dipropyl-2,2′-thiadicarbocyanine dis-$C_3(5)$, have been studied in aqueous media.[1b,2] The absorbance intensity of the probe in aqueous solution at 650 nm increases linearly with the concentration up to 1×10^{-6} M. The peak at 650 nm is assigned to the monomer.[1b] With increase of the concentration above 1×10^{-6} M, a new peak at 590 nm, corresponding to the dimer absorbance,[1b] becomes evident and the relative intensity of the monomer peak to that of the dimer decreases. The excitation spectra were measured and were superimposable on the monomer absorbances, which indicates that the dimer is nonfluorescent.[1b] It was also shown that the addition of human red blood cells to an aqueous solution of diS-$C_3(5)$ resulted in the appearance of a new dye absorption peak at 590 nm, accompanied by a decrease in monomer absorbance at 650 nm and monomer fluorescence at 665 nm; the contribution of white ghosts to these spectral changes was very small.[1b,c] Furthermore, the partitioning of dye into cells was revealed to be nonlinear in relation to external concentration of the dye.[1c,3] From these experimental results, it is concluded that diS-$C_3(5)$ permeates through the cell membranes and is preferentially partitioned into the cell and concentrated around the cell contents (mainly negatively charged hemoglobins). The resulting increase in concentration of the probe inside the cell leads to the formation of the nonfluorescent dimer of diS-$C_3(5)$.[1c] Dimerization of a positively charged membrane-impermeable probe, methylene blue, in negative charged phospholipid membrane was previously utilized to determine the surface potential of the lipid membrane.[4]

In this paper, the quantitative relation between the changes in membrane potential and permeability of the membrane to K^+ and the partition of a membrane-permeable positively charged dye, diS-$C_3(5)$, in bovine red blood cells are discussed. The membrane potential was

correlated with the permeability of membrane to ions and the relationship could be described by the Hodgkin–Katz equation. The permeability of the bovine red blood cell membrane to K^+ mediated by valinomycin is explained on the basis of the characteristic properties of this membrane, that is, the very high percentage of cerebroside (sphingomyelin) and the low permeability to ions.[5,6]

Experimental

Bovine Red Blood Cells——Fresh bovine blood was drawn into ACD solution (citrate buffer) from a normal adult cow and stored at 4°C. The plasma-free cells were prepared as follows. The blood was centrifuged at 2000 *g* for 10 min. The plasma and buffy coat were removed and the cells were washed 3 times at room temperature with a medium containing 145 mM NaCl, 5 mM KCl and 15 mM Tris-HCl (pH 7.3). The cationic composition of the inside of the bovine red blood cells was altered by the nystatin method.[1c,7] Nystatin is a cation-selective ionophore which forms pores across the membrane. The compositions of electrolytes in the nystatin treating solutions were (a) 150 mM KCl+15 mM Tris-HCl, (b) 75 mM KCl+75 mM NaCl+15 mM Tris-HCl, (c) 30 mM KCl+120 mM NaCl+15 mM Tris–HCl, (d) 1 mM KCl+149 mM NaCl+15 mM Tris-HCl. The pH of these solutions was maintained at 7.3. The cells with natural inner cationic composition (intact cells) were used within 24 h of collection, and the cells with artificial cationic composition were used within 10 h of nystatin treatment.

Measurements——The fluorescence intensities of a cationic probe, 3,3′-dipropyl-2,2′-thiadicarbocyanine, diS-$C_3(5)$, in aqueous suspensions of bovine red blood cells with and without valinomycin were measured with a Japan Spectroscopic FP-550 spectrofluorometer. The fluorescence probe, diS–$C_3(5)$, was purchased from Nippon Kanko Shikiso Kenkyusho. Valinomycin was obtained from Sigma Chemical Co. The excitation and emission wavelengths used in this study were 622 and 665 nm, respectively. DiS-$C_3(5)$ was added to the cell suspensions as a solution in ethanol. The final concentration of the probe was 0.738×10^{-6} M. Valinomycin was also added as an ethanol solution to give the final concentration of 2×10^{-6} M. An aliquot of ethanol solution of valinomycin or the same volume of ethanol was added to the medium and the final concentration of ethanol in the cell suspension was always kept constant at 0.28%. The stable fluorescence intensities at 665 nm were recorded 15 min after the addition of the probe to the red blood cell suspensions. The temperature was maintained constant at 25.5°C by water circulation through the cuvette holder of the spectrofluorometer. Correction was made for the slight increase in fluorescence arising from the turbidity of red blood cells.

Theory

Fluorescence Intensity of DiS-$C_3(5)$ in Bovine Red Blood Cell Suspension

Under the experimental conditions of this study (concentration of diS-$C_3(5)$: 0.738×10^{-6} M), the probe molecules are in monomeric form in the bulk medium phase. On the other hand, in the red blood cells, the probe molecules are considered to be in both monomeric and dimeric forms. The intensity of fluorescence of the probe at 665 nm in bovine red blood cell suspension, F, is given by

$$F = f\cdot n_m/V \qquad (1)$$

where n_m is the amount (mol) of probe in monomeric form, V is the volume of the buffer solution containing bovine red blood cells, and f is the molar fluorescence (arbitrary unit/M). The volume of the cells, v, is small enough to be neglected in equation 1. The fluorescence intensity of the same solution without the cells, F_o, is given by

$$F_o = f\cdot n_o/V \qquad (2)$$

where n_o is the total amount (mol) of probe molecules ($n_o/V=0.738\times10^{-6}$M) and $n_o=n_m+2n_d$; here n_d is the amount (mol) of dimer. From equations 1 and 2,

$$n_m = (F/F_o)n_o \quad \text{and} \quad n_d = \frac{1}{2}[1-(F/F_o)]n_o \qquad (3)$$

n_m and n_d can be calculated with equation 3 from fluorescence measurements at 665 nm.

Potential Profile across Bovine Red Blood Cell Membrane and Partitioning of a Cationic Probe, DiS-$C_3(5)$

The potential profile across the cell membrane is shown in Fig. 1. Both the outer and inner surfaces of membrane are loaded with electrical charges arising from lipids, proteins and glycoproteins. These surface charges result in potential differences between the outer surface and bulk medium phase (Donnan potential, ΔE_D^0) and between the inner surface and the cell interior (Donnan potential, ΔE_D^1). The interior phase can be regarded as a concentrated solution of polyelectrolyte (negatively charged hemoglobin). In addition to the Donnan potential outside and inside the membrane, a diffusion potential, ΔE_M, is generated by fluxes of ions through the cell membrane. The total (trans) membrane potential, ΔE, is therefore,

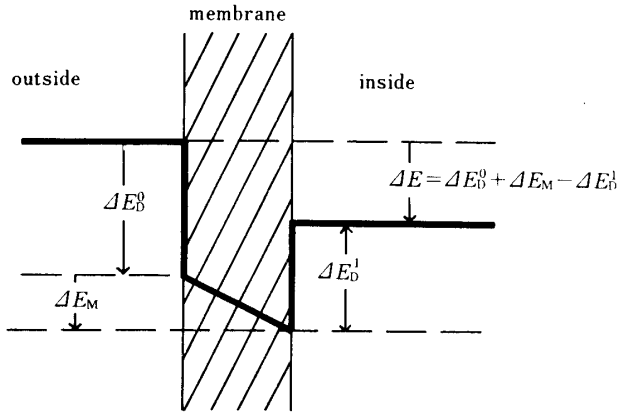

Fig. 1. Electrical Potential Profile across the Red Blood Cell Membrane

ΔE_D^0 is the Donnan potential between the outer surface and bulk medium phase, ΔE_M is the diffusion potential through the membrane, ΔE_D^1 is the Donnan potential between the inner surface and cell interior, and $\Delta E(=\Delta E_D^0+\Delta E_M-\Delta E_D^1$ is the (trans) membrane potential.

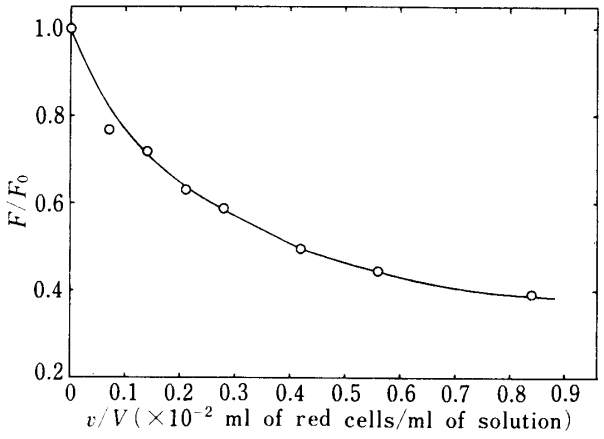

Fig. 2. Ratio of Fluorescence Intensities of DiS-$C_3(5)$ with and without (Intact) Bovine Red Blood Cell, F/F_0, as a Function of the Concentration of Cells, v/V

v is the pellet volume of cells and V is the volume of cell suspension. Excitation and emission wavelengths were 622 and 665 nm, respectively. The medium contained 15 mM Tris-HCl, 145 mM NaCl, 5 mM KCl, and 0.738×10^{-6} M diS-$C_3(5)$ at pH 7.3.

$$\Delta E = \Delta E_D^0 + \Delta E_M - \Delta E_D^1. \quad (4)$$

As discussed above, diS-$C_3(5)$ permeates through the cell membrane and is preferentially concentrated inside the cells (concentrated hemoglobin phase). The partition equilibrium between the monomeric probe in the bulk phase and the dimeric probe, which has two positive charges, inside the cell can be written as[4]

$$C_{dI}/C_{mo}^2 = P \exp(-2e\Delta E/kT) \quad (5)$$

where C_{dI} and C_{mo} are the concentrations of dimeric probe inside the cells (mol/l) and of monomeric probe in the bulk medium (mol/l) respectively, P is the intrinsic partition coefficient of diS-$C_3(5)$ and $P=(P_m^2K_d)=$constant. Here, P_m is the intrinsic partition coefficient of monomeric probe between the cell interior and the bulk medium, and K_d is the dimerization constant. Equation 5 can be rewritten as

$$(n_d/V_I) / (n_{mo}/V)^2 = P \exp(-2e\Delta E/kT) \quad (6)$$

where n_{mo} is the amount (mol) of monomeric probe outside the cell, and V_I is the effective volume inside the cell and is related to the cell pellet volume, v, by $V_I=\phi v$ (ϕ is constant). When the probe is highly concentrated inside the cells, n_{mI}, the amount (mol), of monomeric probe

inside the cells, is negligible[4] and

$$n_{mo} = n_m - n_{mI} \fallingdotseq n_m \quad (7)$$

From equations 6 and 7, equations 8, 9 and 10 are obtained.

$$V(n_d/n_m^2) = K(v/V) \quad (8)$$

$$K = K_o \exp(-2e\Delta E/kT) \quad (9)$$

where

$$K_o = P\phi = P_m^2 K_d \phi = \text{constant} \quad (10)$$

Results

Fluorescence Intensity of DiS-C_3(5) in Bovine Red Blood Cell Suspensions

The fluorescence intensity at 665 nm of diS-C_3(5) in bovine red blood cell suspension was measured at 25.5 °C. The excitation wavelength was 622 nm. The measurements were carried out with various cell pellet volumes and cationic compositions both inside and outside the cells. In Fig. 2, the results for intact bovine red blood cells in medium containing 145 mM NaCl+5 mM KCl+15 mM Tris-HCl are shown. It can be seen that the ratio of fluorescences with and without the cells, (F/F_o), decreases with increasing pellet volume of the cells in the medium. The decrease in (F/F_o) is considered to arise from the partitioning of positively charged probe, diS-C_3(5), into the cellular interiors, leading to the formation of dimers. Using equation 3, the amounts (mol) of monomer, n_m, and dimer n_d, were calculated.

In Fig. 3, the values of $V\cdot(n_d/n_m^2)$, calculated from the experimental results shown in Fig. 2, are plotted against the concentration of bovine red blood cell, (v/V). A clear linear relation was obtained and the slope gives the value of K in equation 8. This result is considered to provide further evidence for the formation of dimer of diS-C_3(5) inside the cells.

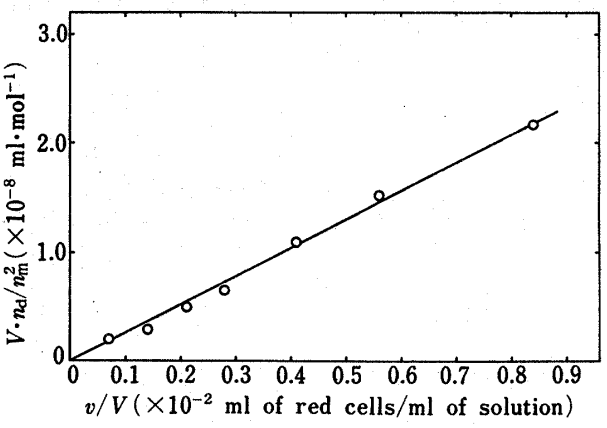

Fig. 3. Variation of Vn_d/n_m^2 for Intact Bovine Red Blood Cells as a Function of the Concentration of Cells, v/V

See equations 8, 9 and 10. The medium was the same as in Fig. 2.

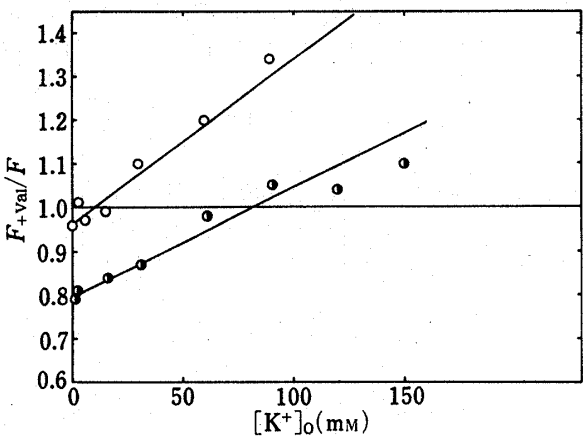

Fig. 4. Ratio of Fluorescence Intensities with and without Valinomycin, F_{+val}/F, as a Function of K^+ Concentration in the Bulk Medium, $[K^+]_o$

Intact bovine red blood cells (○), cells treated with nystatin with 150 mM KCl (◑). Medium: 15 mM Tris-HCl; the concentration of cells was kept constant as $(v/V)=3\times10^{-3}$; the total concentration of inorganic salts, [NaCl]+[KCl], was kept constant at 150 mM; pH 7.3.

It is well known that valinomycin stimulates the transport of K^+ across the cell membrane and then induces an alteration in (trans) membrane potential to hyperpolarization or to depolarization.[8] The effects of valinomycin were investigated with the intact and the nystatin-treated red blood cells. The ratio of fluorescence intensities of the cell suspensions with and without valinomycin, (F_{+val}/F), was measured as a function of K^+ concentration in the bulk medium, $[K^+]_O$. The subscript, +val indicates the values with valinomycin. The concentration of the cell was kept constant at $(v/V)=3\times10^{-3}$ ml of the cell/ml of solution. For example, the results for intact red blood cells and for the nystatin-treated cells with 150 mM KCl are shown in Fig. 4. The fluorescence intensities of cell suspensions without valinomycin were independent of K^+ concentration outside the cells, $[K^+]_O$. On the other hand, the fluorescence intensities with valinomycin were largely dependent on $[K^+]_O$. According to equations 3, 8 and 9, a decrease in the fluorescence upon addition of valinomycin, $(F_{+val}/F)<1$, could be correlated with the hyperpolarization of the cells, and an increase in the fluorescence, $(F_{+val}/F)>1$, with depolarization of the cells. Therefore, it was shown that the higher the concentration of K^+ outside the cells, the larger the change in membrane potential, ΔE, in the direction of depolarization.

Potential-dependent Dimerization

If the concentration of the cells, v/V, is constant, we can obtain from equations 8 and 9 a relation between the ratio of (n_d/n_m^2) with and without valinomycin, $(n_d/n_m^2)_{+val}/(n_d/n_m^2)$, and the change in membrane potential on addition of valinomycin, $\Delta\Delta E$, as follows:

$$[\,(n_d/n_m^2)_{+val}/(n_d/n_m^2)\,]^{1/2} = \exp[-e\,(\Delta E_{+val} - \Delta E)/kT] = \exp(-e\Delta\Delta E/kT) \quad (11)$$

In red blood cells, the anion transport is carried out by a membrane protein identified as band 3 on gel electrophoresis,[9] and it is well known that the permeability constant of Cl^-, P_{Cl^-}, is much greater than those of cations.[10] Thus, the resulting membrane potential is considered to be equivalent to the chloride determining potential. The addition of valinomycin to red cell suspension would alter the potential toward the potassium determining value. The latter value is necessarily dependent on the cationic compositions inside and outside the cells.

If the membrane potential of bovine red blood cells obeys the constant field assumption, the potential of the cells with valinomycin can be represented by equation 12:

$$\Delta E_{+val} = (kT/e)\ln\left(\frac{\alpha[K^+]_O + [Cl^-]_I}{\alpha[K^+]_I + [Cl^-]_O}\right) \quad (12)$$

α is the ratio of the permeability constant of K^+ to that of Cl^-, $\alpha=(P_{K^+}/P_{Cl^-})$. Here, the subscripts O and I indicate outside and inside the cells, respectively. For the cell suspension without valinomycin,

$$\Delta E = (kT/e)\ln\left(\frac{[Cl^-]_I}{[Cl^-]_O}\right) \quad (13)$$

From equations 11, 12 and 13, equation 14 is obtained:

$$[\,(n_d/n_m^2)_{+val}/(n_d/n_m^2)\,]^{-1/2} = A[K^+]_O + B \quad (14)$$

where

$$A = \frac{\alpha[Cl^-]_O}{[Cl^-]_I(\alpha[K^+]_I + [Cl^-]_O)} \quad (14a)$$

and

$$B = \frac{[Cl^-]_o}{(\alpha [K^+]_I + [Cl^-]_o)} \qquad (14b)$$

Here, $[Cl^-]_o$ is maintained at 165 mM, and $[Cl^-]_I$ is constant because nystatin does not exchange anions through the cell membrane. Therefore, A and B in equation 14 are constant for red blood cells of an arbitrary interior cationic composition.

In Fig. 5, values of $[(n_d/n_m^2)_{+val}/(n_d/n_m^2)]^{-1/2}$ calculated from the measured fluorescence intensities by means of equation 3 as

$$[(n_d/n_m^2)_{+val}/(n_d/n_m^2)]^{-1/2} = (F_{+val}/F)\,[(F_o-F)/(F_o-F_{+val})]^{-1/2} \qquad (15)$$

are plotted against $[K^+]_o$ for intact cells and nystatin-treated cells. The ratio of the slope to the intercept of the linear relation in Fig. 5. (A/B), gives the value of $(\alpha/[Cl^-]_I)$. Since there is some variation in reported values of $[Cl^-]_I$,[1a,11] an average value of $[Cl^-]_I$ (74 mM) was chosen to calculate α. The values of α calculated here are shown in Table 1. The errors in $[Cl^-]_I$ could result in 20% variation in the values of α. If the concentration of K^+ outside the cells at the null point of fluorescence ($F_{+val}=F$, $(n_d/n_m^2)_{+val}=(n_d/n_m^2)$ and $\Delta\Delta E=0$), is defined as $[K^+]_o^*$, the following equation is obtained from equations 14, 14a and 14b:

$$[K^+]_I = [K^+]_o^* [Cl^-]_o / [Cl^-]_I \qquad (16)$$

$[K^+]_I$ values calculated from equation 16 are shown in Table I. It is clear that the values of α lie in the narrow range of 0.33—0.64, and the calculated $[K^+]_I$ values are substantially in agreement with the individual concentrations of K^+ in nystatin treating solutions. $[K^+]_I$

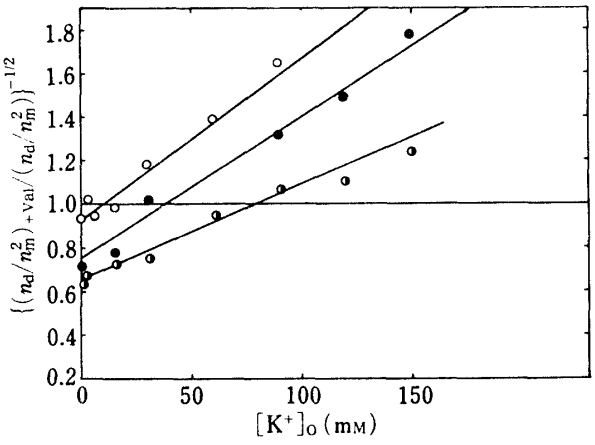

Fig. 5. Ratio of n_d/n_m^2 with and without Valinomycin as a Function of K^+ Concentration in the Bulk Medium, $[K^+]_o$

Intact cells (○); cells treated with nystatin with 75 mM KCl+75 mM NaCl+15 mM Tris-HCl (◎); and cells treated with nystatin with 150 mM KCl+15 mM Tris-HCl (◑). The medium was the same as in Fig. 4. The ratio of slope to intercept of the linear relations gives the value of $(\alpha/[Cl^-]_I)$. See Eq. 14, 14a and 14b.

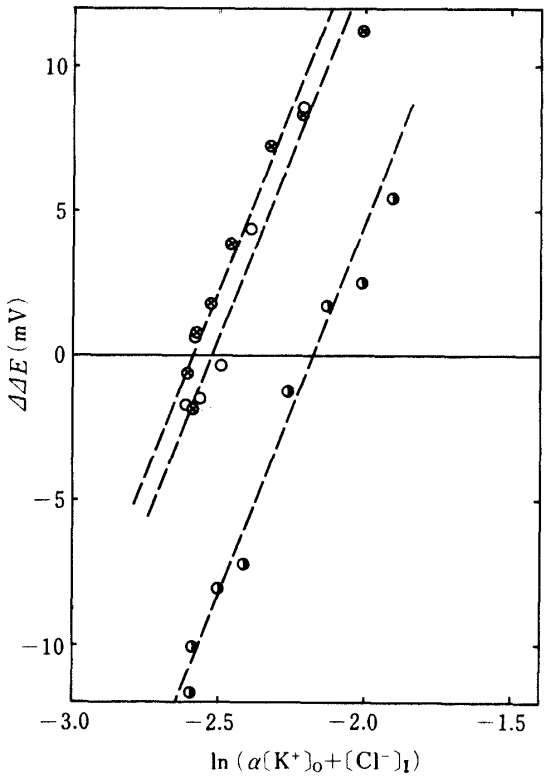

Fig. 6. Variation of $\Delta\Delta E\,(=\Delta E_{+val}-\Delta E)$ as a Function of $\ln(\alpha[K^+]_o+[Cl^-]_I)$

Intact cells (○); cells treated with nystatin with 1 mM KCl+149 mM NaCl+15 mM Tris-HCl (⊗); cells treated with nystatin with 150 mM KCl+15 mM Tris-HCl (◑). Broken lines in this figure are calculated from eq. 17 with the experimental values of α and $[K^+]_I$ (shown in Table I).

$$\Delta\Delta E = (kT/e) \ln(\alpha[K^+]_O + [Cl^-]_I) + C \quad (17)$$

$$C = (kT/e) \ln \frac{[Cl^-]_O}{[Cl^-]_I (\alpha[K^+]_I + [Cl^-]_O)} \quad (17a)$$

TABLE I. Calculated Numerical Values of $[K^+]_I$ and α

	$[K^+]$ in treating buffer (mM)	$[K^+]_I$ observed (mM)	α
Nystatin-treated cells	150	177.5	0.50
	75	84.0	0.64
	30	33.0	0.33
	1	7.2	0.40
Intact cells		23.4	0.60

The value of $(Cl^-)_I$ was chosen as 74 mM for the calculation with eq. 14a, 14b and 16.

estimated for the intact cells is also consistent with the reported value of 22±5 mM.[5] The uncertainty of $[Cl^-]_I$ in equations 14 and 16 would not seriously affect the above results.

From equations 12 and 13, $\Delta\Delta E$ can be represented as follows:
For an arbitrary cationic composition inside the cells, the value of C is constant and can be calculated by use of $[Cl^-]_O = 165$ m and α and $[K^+]_I$ shown in Table I. In Fig. 6, the changes in membrane potential, $\Delta\Delta E$, obtained from equation 11 are plotted for the intact and nystatin-treated cells as a function of $\ln(\alpha[K^+]_O + [Cl^-]_I)$. $\Delta\Delta E$ values calculated theoretically from equation 17 are also shown as a function of $\ln(\alpha[K^+]_O + [Cl^-]_I)$ as solid lines. From these results, it can be concluded that $\Delta\Delta E$, obtained from the measured partitioning equilibria between the dimer inside the cells and the monomer outside the cells, is in accord with the Hodgkin–Katz equations 12, 13 and 17.

Discussion

The sensitivity of the change in fluorescence of the dimerizing probe, diS-$C_3(5)$, depends on the total concentration of the probe in the cell suspension, (n_O/V). Although a higher concentration of the probe is often expected to give higher sensitivity, the dimerization in the bulk phase will complicate the equilibrium processes considerably. Also, the partitioning of a large amount of the probe into cells will disturb the function of the cells. Therefore, a compromise concentration should be selected.

As mentioned above, diS-$C_3(5)$ is strongly partitioned and is concentrated into the red cell interior to form a dimer, and the depletion of hemoglobin from the cell (red cell ghost) considerably decreases these effects. We observed that large amounts of red cell ghosts caused a change in the absorbance maximum of the dye from 650 to 665 nm which was not accompanied by a change in absorbance or fluorescence intensities or in the appearance of dimer absorption. As for negatively charged phospholipid membrane (in an aqueous suspension of phospholipid liposomes), we observed the same dimerization of the dye as in the red cell at very low ionic strength, but under isotonic conditions, no dimerization was detectable. From these results, we consider that strong partioning and concentration of the dye are essential for dimer formation. In ghost cells, dye partitioning into the membrane is too weak for dimer formation to occur, while in negatively charged phospholipid membrane at low ionic strength, electrostatic force attracts enough dye for the dimer formation.

Calculation with the dimerization constant, $K_d = 10^4 - 10^5$ ($mol^{-1} \cdot l$) showed that the amount (mol) of monomeric probe in the cell interiors, n_{mI}, went up to 20—10% of total monomer, n_m, at $v/V = 3 \times 10^{-3}$, and the approximation in equation 7 gives rise to errors of 5—2 mV in ΔE. However, in $\Delta\Delta E$ calculated from equation 11, the errors arising from ΔE_{+val} and ΔE could largely cancel out.

In this study, the change in membrane potential, $\Delta\Delta E$, was estimated for bovine red blood cells. To evaluate the membrane potential, ΔE, estimation of the intrinsic partition coefficient, P, in equation 5 is required. That is, the estimation of the partitioning equilibrium at ΔE $(=\Delta E_D^0+\Delta E_M-\Delta E_D^I)=0$ is necessary, although it is very difficult from the experimental standpoint. On the other hand, the membrane potential can be calculated theoretically from equations 12 and 13. For the intact bovine red blood cell ($\alpha=0.5$), in medium containing 1 mM KCl+149 mM NaCl+15 mM Tris-HCl, the membrane potentials are −23 mV with valinomycin and −21 mV without valinomycin, and in medium containing 150 mM KCl+15 mM Tris-HCl, 6 mV with valinomycin and −21 mV without valinomycin. As shown in Fig. 6, the change in membrane potential with the addition of valinomycin, calculated from our experimental results, fits well with that calculated from the Hodgkin–Katz equation (equations 12 and 13). The concentration of K^+ in the cells, $[K^+]_I$, calculated from our experimental results also fits well with that in nystatin treating buffer. From these results, we conclude that diS-C_3(5) is useful for the quantitative measurement of the change in membrane potential.

The Hodgkin–Katz equation for membrane potential is derived from the Goldman equation for diffusion potential with the assumption that ionic concentrations at membrane surfaces are directly proportional to the concentrations in bulk media.[12] Recent studies on the relationship between Goldman's diffusion potential, ΔE_M, and Donnan potentials, ΔE_D^0 and ΔE_D^I, reveals that the membrane potential calculated from the Hodgkin–Katz equation is really comprised of Donnan potentials as follows: $\Delta E=\Delta E_D^0+\Delta E_M-\Delta E_D^I$. In this case, α in equation 12 is a function of Donnan potentials.[13] Donnan potentials for bovine red blood cell are regarded as being highly dependent on the ionic strength of the medium and the charge density of the cell membrane, and the charge density is also dependent on pH. It is therefore reasonable that a narrow range of the value of α was obtained in this study, where the ionic strength and pH were maintained constant. In contrast in other studies using empirical calibration, great variation in the value of α with cationic composition has been reported for human red blood cells.[1a]

Bovine red blood cell membrane contains a very high percentage of sphingomyelin with saturated long chain fatty acid and this results in a reduced cationic permeability and a low level of potassium and a high level of sodium inside the cell, $[K^+]_I/[Na^+]_I=0.3$.[4] It is known that ion transport across a membrane by a carrier-type ionophore is sensitive to the fluidity of the membrane lipid bilayer,[14] while ion transport by a channel forming ionophore is independent of the fluidity.[15] The values of α obtained here for bovine red blood cells with a carrier-type ionophore, valinomycin, are very small compared with the values for human red blood cells, $\alpha=3-25$,[1a] or 16.6.[1c] These results are understandable in terms of the high rigidity of the bovine red blood cell membrane caused by the high content of sphingomyelin and the very low content of lecithin in comparison with pig, rabbit and human red blood cell membranes.[5]

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References

1) *a*) J.F. Hoffman and P.C. Laris, *J. Physiol.* (London), **239**, 519 (1974); *b*) P.J. Sims, A.S. Waggoner, C. Wang and J.F. Hoffman, *Biochemistry*, **16**, 3315 (1974); *c*) S.B. Hladky and T.J. Rink, *J. Physiol.* (London), **263**, 287 (1976); *d*) A. Waggoner, *J. Membr. Biol.*, **27**, 317 (1976).
2) W. West and S. Pearce, *J. Phys. Chem.*, **69**, 1894 (1965).
3) *a*) G. Burckhardt, *Biochim. Biophys. Acta*, **468**, 227 (1977); *b*) R.Y. Tsien and S.B. Hladky, *J. Membr. Biol.*, **38**, 73 (1978).
4) M. Nakagaki, I. Katoh and T. Handa, *Biochemistry*, **20**, 2208 (1981).
5) Y. Israel, A. MacDonald, J. Bernstein and E. Rosenman, *J. Gen. Phyiol.*, **59**, 270 (1972).
6) R. G. Kirk, *Biochim. Biophys. Acta*, **464**, 157 (1977).

7) A. Cass and M. Dalmark, *Nature, New Biol.*, **244**, 47 (1973).
8) D.C. Tosteson, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **26**, 1805 (1967); P. Mueller and D.O. Rudin, *Biochem. Biophys. Res. Commun.*, **26**, 398 (1967); P.J.F. Henderson, J.D. McGivan and J.B. Chappell, *Biochem. J.*, **111**, 521 (1969).
9) A. Rothstein, Z.I. Cabantchik, M. Balashin and R. Juliano, *Biochem. Biophys. Res. Commun.*, **64**, 144 (1975); A.H. Ross and H.M. McConnell, *Biochem. Biophys. Res. Commun.*, **74**, 1318 (1977); M.F. Lukacovic, M.B. Feinstein, R.I. Sháafi and S. Perrie, *Biochemistry*, **20**, 3145 (1981).
10) M.J. Hunter, *J. Physiol.* (London), **218** 49P (1971).
11) C.W. Duncan, C.F. Huffman and G.C. Gerritsen, "Biological Handbooks, Blood and Other Fluid," ed. by D. Dittmer, Fed. Am. Soc. Exp. Biol., Washington, 1961, pp. 29—31; H. Davson and M.B. Segel, "Introduction to Physiology," Vol. 1(1), Academic Press, London, 1975, Chapter 7.
12) A.L. Hodgkin and B. Katz, *J. Physiol.* (London), **108**, 37 (1949); G. Goldman, *J. Gen. Physiol.*, **27**, 37 (1943).
13) Y. Shinagawa, *J. Theor. Biol.*, **64**, 551 (1977); M. Nakagaki and R. Takagi, *Membrane* (Tokyo), **6**, 123 (1981).
14) R. Benz, O. Frohlich and P. Lauger, *Biochim. Biophys. Acta*, **464**, 465 (1977); G. Boheim, W. Hanke and H. Eibl, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 3403 (1980); N.R. Clement and J.M. Gould, *Biochemistry*, **20**, 1539 (1981).
15) S. Krasne, G. Eisenmam and G. Szabo, *Science*, **174**, 412 (1971); E. Racker and P.C. Hinkle, *J. Membr. Biol.*, **17**, 181 (1974).