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Estimation of Membrane Potential $\Delta\psi$ in Reconstituted Plasma Membrane Vesicles Using a Numerical Model of Oxonol VI Distribution

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A model of membrane potential-dependent distribution of oxonol VI to estimate the electrical potential difference $\Delta\psi$ across *Schizosaccharomyces pombe* plasma membrane vesicles (PMV) has been developed. $\Delta\psi$ was generated by the H^+-ATPase reconstituted in the PMV. The model treatment was necessary since the usual calibration of the dye fluorescence changes by diffusion potentials (K^+ + valinomycin) failed. The model allows for fitting of fluorescence changes at different vesicle and dye concentrations, yielding $\Delta\psi$ in ATP-energized PMV of 80 mV. The described model treatment to estimate $\Delta\psi$ may be applicable for other reconstituted membrane systems.

KEY WORDS: Membrane potential; reconstituted plasma membrane vesicles; oxonol VI fluorescence; yeast H^+-ATPase; *Schizosaccharomyces pombe*.

INTRODUCTION

Reconstituted vesicles offer the possibility to study electrogenic transport processes independent of cellular metabolism. They are a suitable tool for investigation of individual transport systems in a given membrane and their mutual coupling (Gläser and Höfer, 1987; Mair and Höfer, 1988; Villalobo, 1990; Höfer *et al.*, 1991). Reconstituted vesicles are mainly used to study active transport. When adding a source of metabolic energy (e.g., ATP) the incorporated transport proteins (such as membrane-bound ATPases) translocate specific ions across the vesicle membrane, generating an electrochemical ion gradient. For technical reasons the concentration gradient of ions (except for the proton gradient) cannot be measured. On the other hand, there are methods for continuous monitoring of membrane potential $\Delta\psi$ (the second component of the electrochemical gradient) based on optical fluorescence (Pratap *et al.*, 1990, Scherman and Henry, 1980; Bashford and Smith, 1979; Apell and Bersch, 1987; Smith *et al.*, 1981; Beeler *et al.*, 1981; Wolk and Höfer, 1987; Cooper *et al.*, 1990; Smith, 1990). However, the calibration of the probe signals for the absolute magnitude of the membrane potential is difficult. Until now, the generation of defined diffusion potentials, mostly by K^+ and valinomycin, was the common method to calibrate the changes of fluorescence.

For a precise determination of $\Delta\psi$ generated by an active transport system in reconstituted vesicles the relation of the fluorescence changes induced by diffusion potentials to those generated by an active transport system should be independent of the dye/vesicle ratio. Actually, this condition is only seldom met for one of the following reasons: (1) The calculations of $\Delta\psi$ according to the Nernst equation might be incorrect, $\Delta\psi$ being underestimated due to the transient nature of the diffusion potentials. (2) The signal changes induced by diffusion potentials are influenced by the formation of lipid-soluble complexes between the ionophore (valinomycin), the permeant ion (K^+), and the optical probe (Pratap *et al.*, 1990; Scherman and Henry, 1980; Bashford and Smith, 1979).

In this paper we describe a new method to estimate $\Delta\psi$ avoiding these effects. This method has been developed on the basis of a mathematical model which

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describes the distribution of the membrane potential-dependent dye oxonol VI in the vesicle population. The membrane potential generated by the reconstituted *Schizosaccharomyces pombe* plasma membrane H^+-ATPase was calculated by fitting the model to the measured fluorescence changes at various dye/vesicle ratios. Oxonol VI was chosen because its fluorescence spectra and kinetics are well characterized. Our novel method is particularly suitable for estimation of $\Delta\psi$ generated by membrane-bound ATPases in PMV when other calibration methods failed.

MATERIALS AND METHODS

The reconstituted plasma membrane vesicles (PMV) were prepared by a detergent/dialysis method as described by Mair and Höfer (1988). Fluorescence changes of oxonol VI at two different dye concentrations (0.031, 0.156 μM) with increasing PMV concentrations were measured with a fluorescence spectrophotometer (Hitachi, 650-10M) using an excitation wavelength of 580 nm (slit width 20 nm) and an emission wavelength of 660 nm (slit width 5 nm). The concentration of the stock PMV suspension is given by the amount of phospholipids (20 mg L-α-phosphatidylcholine, type XV-E) and the volume of buffer used during the reconstitution. Further dilutions of the stock PMV suspension were also done with reconstitution buffer. The reconstitution buffer consisted of 1 mM K_2SO_4, 5 mM $MgSO_4$, and 10 mM MES (pH 6 adjusted with LiOH). The density ρ of the phospholipids was 1 g/ml. Electron micrographs of reconstituted PMV show that the detergent/dialysis method yields a uniform-size population of PMV (100 nm in diameter; Mair and Höfer, 1988). $\Delta\psi$ generated by H^+-ATPase at each given oxonol VI and PMV concentration was measured after an addition of 5 mM Mg-ATP (pH 6 adjusted with LiOH). $\Delta\psi$ generated by the H^+-ATPase collapsed after addition of 5 μM FCCP, and the fluorescence returned back to its value at $\Delta\psi = 0$. Diffusion potentials of specific magnitude were induced by the addition of valinomycin and KCl. The corresponding potassium concentration $[K^+]_a$ was calculated according to the Nernst equation with $[K^+]_i = 2$ mM. All measurements were carried out at 20°C in a total volume of 2 ml.

Chemicals

Oxonol VI was from Molecular Probes Inc., USA. FCCP, valinomycin, and L-α-phosphatidylcholine were from Sigma. All other chemicals were of analytical grade from Merck.

RESULTS

The introduced model allows a quantitative estimate of the electrical potential difference across vesicle membranes from the measured fluorescence under two defined experimental conditions: F corresponds to the fluorescence measured at $\Delta\psi = 0$ and F_{pot} to that at $\Delta\psi > 0$ (steady-state fluorescence after the addition of ATP to the PMV, Fig. 1A, Inset). Comparing the fluorescence changes expressed as relative fluorescence $F_{pot}/F - 1$ (at different PMV concentrations) which were generated either by the plasma membrane-bound H^+-ATPase or by defined diffusion potentials (Fig. 1) revealed that the values for $F_{pot}/F - 1$ under the two experimental conditions are not related to each other, since otherwise the ratio of $\Delta\psi$-induced relative fluorescence should be independent of the dye and PMV concentrations. This prerequisite for successful calibration was not fulfilled, in particular, at low PMV concentrations (Fig. 1A). Figure 1B demonstrates another shortcoming of the common $\Delta\psi$ calibration method, i.e., the opposite effect of an increase of oxonol VI concentration on the relative fluorescence induced either by ATPase generated $\Delta\psi$ or by potassium diffusion potentials. Moreover, the relative fluorescence was affected by changing the concentration of valinomycin. Hence, the K^+ + valinomycin-induced relative fluorescence cannot be used for calibration of H^+-ATPase generated $\Delta\psi$ in PMV reconstituted from *S. pombe* plasma membrane fraction. We therefore developed a model which describes the potential-dependent distribution of oxonol VI in the membrane. This model is based on the assumptions that lipid bilayers are permeable to the anionic dye oxonol VI, and the dye distributes in the aqueous phases across the membrane according to the Nernst equation (Apell and Bersch, 1987; Plášek and Sigler, 1995):

$$\Delta\psi = \frac{RT}{F} \ln \frac{c'}{c''} \qquad (1)$$

In the equation c' is the concentration of the dye inside the vesicles and c'' is the concentration in the medium; $\Delta\psi$ is the membrane potential generated by the H^+-ATPase. R, T, and F have their usual meaning. The substantial difference between the present model and the previous treatment by Apell and Bersch (1987) consists

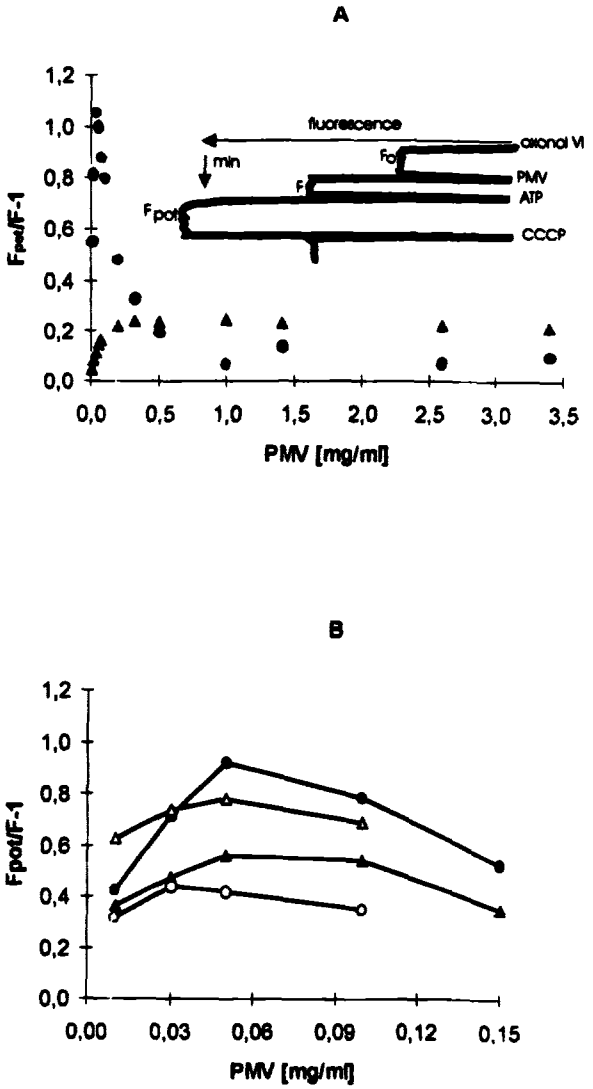

Fig. 1. Relative fluorescence $F_{pot}/F - 1$ of oxonol VI induced by a membrane potential Δψ. F—fluorescence in the presence of PMV at Δψ = 0; F_{pot}—fluorescence at Δψ ≠ 0. A: Effect of PMV concentration: ● relative fluorescence induced by Δψ generated by the plasma membrane ATPase; ▲ relative fluorescence induced by the addition of potassium in the presence of valinomycin ($[K^+]_i$ = 2 mM, $[K^+_o]$ = 32.6 mM, ATP = 5 mM, oxonol VI = 30 nM). B: Effect of oxonol VI concentration: closed and open symbols—31 nM and 156 nM, respectively; circles—relative fluorescence induced by the addition of 5 mM Mg-ATP; triangles—relative fluorescence induced by the addition of 126.4 mM potassium in the presence of 10 nM valinomycin ($[K^+]_i$ = 2 mM). Inset: A representative trace of fluorescence changes in a PMV suspension.

in the assumption that the number of binding sites for the dye is limited and the binding of the dye molecules obeys the Langmuir adsorption isotherm (see below).

The theoretical energy profile of a lipid-soluble ion in the membrane shows two minima (Kletterer *et al.*, 1971). Thus, the dye is assumed to bind to adsorption planes located symmetrically with respect to the center of the membrane, one in each monolayer of the phospholipid bilayer. The total amount of dye in the particular vesicle suspension is given by the equation

$$cV = c'V'L + n' + n'' + c''(V - V'L) \qquad (2)$$

where c is the total concentration of the dye in the vesicle suspension, V is the total volume, n' and n'' are the amounts of dye bound to the inner and outer adsorption planes of the vesicle membranes, respectively, V' is the inner volume of the vesicles at a lipid concentration of 1 mg/ml phospholipid, and L is the amount in mg of added phospholipids.

The relative fluorescence following the addition of ATP to a vesicle suspension depends on the dye concentration. The relative fluorescence decreased with increasing concentration of oxonol VI at each PMV concentration (Fig. 1B). To describe the fluorescence changes at different dye concentrations the Langmuir adsorption isotherm was used to calculate the amount of dye bound in the adsorption planes. According to the Langmuir adsorption isotherm the amount of binding sites per mg phospholipid n_∞ in each adsorption plane is limited. In addition, the amounts n' or n'' of dye bound to the membrane depend on the concentrations c' or c'' of the dye in the aqueous phases, and are expressed by Eqs. (3) and (4):

$$n' = n_\infty L \frac{c'K'}{1 + c'K'}, \qquad K' = K\, e^{-\alpha(\Delta\psi F/RT)} \qquad (3)$$

$$n'' = n_\infty L \frac{c''K''}{1 + c''K''}, \qquad K'' = K\, e^{\alpha(\Delta\psi F/RT)} \qquad (4)$$

where K is the equilibrium constant for the binding of oxonol VI to the inner adsorption planes at Δψ = 0. In the presence of a membrane potential the symmetrical energy barriers in the inner and outer layers of the vesicle membrane are modified by an electrostatic term (Zwolinsky *et al.*, 1949). On the assumption that Δψ drops linearly across the membrane, the change in the barrier height is αFΔψ, where αΔψ is the fraction of the membrane potential which drops across the energy barriers (Fig. 2).

To calculate the absolute fluorescence of the bound dye fraction and that of the dye in the two aqueous phases, the fluorescence changes due to the addition of PMV to the buffer solution at Δψ = 0 were measured at different dye concentrations. With Δψ = 0 the addition of PMV to a buffer containing oxonol VI led to an

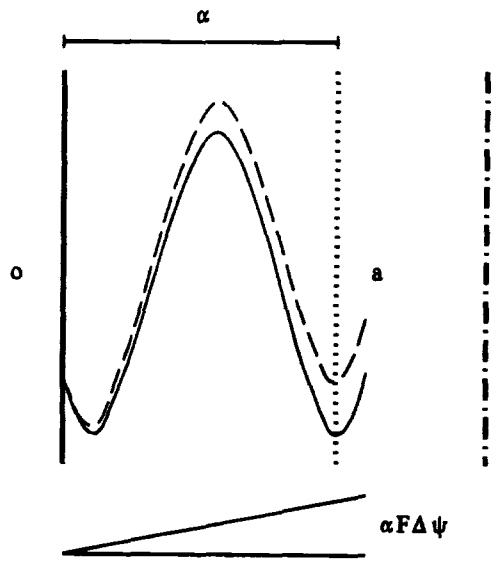

Fig. 2. Change of the energy barrier in the outer layer of the phospholipid bilayer. The change in the barrier hight is given by $\alpha F \Delta\psi$. o, outside; a, adsorption plane; the dash-and-dot line denotes middle of the membrane.

increase of fluorescence F as compared to the fluorescence of the dye in aqueous solutions F_0. This result is consistent with fluorescence lifetime measurements of Smith *et al.* (1981) who showed that the increase of fluorescence yield is due to an increase of the emission lifetime of the membrane-bound dye fraction. Our experiments show that the total fluorescence in the presence of PMV is increased by the fluorescence of the lipids. This effect is more pronounced at low dye concentrations. Figure 3 shows the relative fluorescence

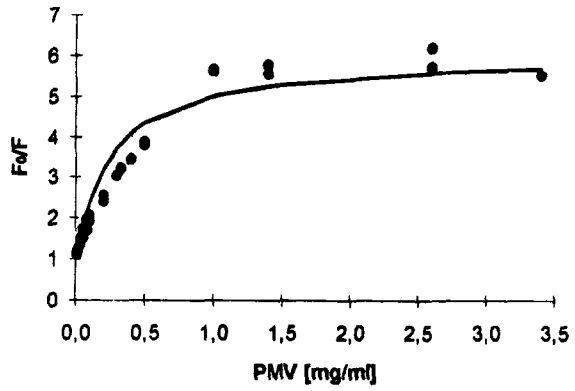

Fig. 3. Relative fluorescence F/F_0 as a function of plasma membrane vesicle (PMV) concentration for $\Delta\psi = 0$. F—as in Fig. 1 (values corrected for lipid fluorescence); F_0—fluorescence in the absence of PMV. The calculated curve resembles a saturation curve with a maximum value of relative fluorescence corresponding to the complete binding of oxonol VI to the PMV. The oxonol VI concentration was 31 nM.

F/F_0 as a function of the PMV concentration after subtracting the effect of lipid fluorescence. For all oxonol VI concentrations the F/F_0 ratio exhibited saturation behavior (data not shown). These results suggest that raising the PMV concentration at a constant total dye concentration leads to an increase in the membrane-bound dye fraction whereas the amount of dye in the aqueous phase decreases. At high PMV concentrations all dye is bound to the PMV and the F/F_0 ratio does not increase any further. The enhancement factor ϵ is the ratio of the dye fluorescence when it is bound within the membrane to that of the dye in aqueous solution. If the emission lifetime of oxonol VI in the lipid or aqueous phase is independent of the dye concentration, ϵ has the expected constant value for all dye concentrations.

The relative fluorescence F/F_0 for $\Delta\psi = 0$ is given by the following equation:

$$\frac{F}{F_0} - 1 = \frac{c''(V - V'L)}{cV} + \frac{2\epsilon}{cV} n'' \qquad (5)$$

using symbols explained in Eq. (2).

The fluorescence increase following an inside-positive membrane potential is caused by the enhanced binding of oxonol VI to the inner adsorption plane. According to the Nernst equation the dye accumulates in the inner aqueous vesicle phase and, consequently, more dye is bound to the inner adsorption plane of the vesicle membrane. For the relative fluorescence at $\Delta\psi > 0$ the following equation holds:

$$\frac{F_{pot}}{F_0} - 1 = \frac{\epsilon n'_{pot} + \epsilon n''_{pot} + c''_{pot}(V - V'L)}{cV} \qquad (6)$$

Equations (1), (3), and (4) can be used to determine c' from Eq. (2) using the Newton iteration method. With known c' the same equations can then be used to calculate the numerical values of n', n'', and c'', which enter in the equations for the relative fluorescence (5) and (6). To achieve this the values of the parameters $\Delta\psi$, n_∞, α, K, and ϵ of the model have to be determined by fitting the parameters to the experimental data (for further details see the Appendix). The measured relative fluorescence was therefore compared with the values of the relative fluorescence predicted by the model with the same total concentrations of the dye and the lipid, c and L, respectively, used in the experiment. As shown in Figs. 3 and 4 the fitted curves describe the experimental data under two different sets of condition, for $\Delta\psi = 0$, and for $\Delta\psi > 0$ fol-

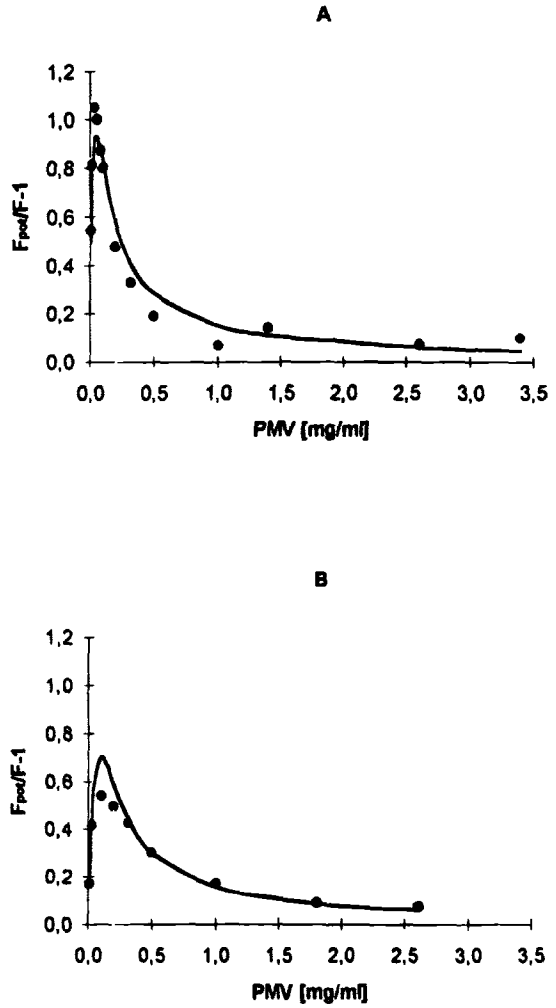

Fig. 4. Fitting of the model equations to experimental data of the relative fluorescence $F_{pot}/F - 1$ in energized PMV. The membrane potential Δψ was generated by the plasma membrane H^+-ATPase of *Schizosaccharomyces pombe*. Oxonol VI concentration was 31 nM in A and 156 nM in B. The parameter values giving the best fit were: $K = 2.2$ ml/nmol, $n_\infty = 0.95$ nmol/mg, $\alpha = 0.475$, $\epsilon = 6.00$, and Δψ corresponding to 0.080 V.

lowing the addition of ATP to the PMV suspensions (steady-state Δψ of PMV in the presence of 5 mM Mg-ATP). Under these conditions a membrane potential of 80 mV was assessed. The correlation coefficient between the experimental data and the calculated curves was 0.9726.

DISCUSSION

The Δψ value of 80 mV calculated from the data of Fig. 4 by the model fitting is consistent with the efficiency of yeast plasma membrane H^+-ATPases in reconstituted systems. Because the PMV were reconstituted in the presence of 2 mM K^+, proton pumping into the vesicles by the H^+-ATPase was electrically coupled to an K^+-efflux, which partially shortcut the generation of Δψ. In the steady state, the pump current was exactly balanced by the efflux of potassium and/or protons from the vesicles. Consequently, Δψ and the fluorescence signal were constant. This result is consistent with that of Perlin *et al.* (1984) who showed that for K^+ concentrations larger than 10 mM Δψ decreased significantly in favor of ΔpH. In addition, Δψ has an inhibitory effect on the rate constants of conformational changes of the H^+-ATPase itself.

The value for the enhancement factor ϵ is similar to that found by Apell and Bersch (1987) and is verified by the experimental data (Fig. 3). The predicted number of binding sites n_∞ corresponds to that calculated by the method of Bashford *et al.* (1979). Unfortunately, there are no comparable values for the equilibrium constant K. The value of α suggests that the binding sites for oxonol VI are located at the inner side of the membrane. The lower value of α, found by Apple and Bersch (1987), might be due to another type of phospholipids used during the reconstitution of the vesicles.

The relative fluorescence F_{pot}/F in energized vesicles ($\Delta\psi > 0$) shows a distinct maximum at low PMV concentrations. This is caused by two phenomena: (1) The amount of free oxonol VI permeating into energized vesicles ($\Delta\psi > 0$) depends on the concentration of the dye in the aqueous phase. With decreasing PMV concentration the amount of dye in the aqueous phases increases. Consequently more dye penetrates through the membrane and more dye is bound to the inner adsorption plane. This leads to the observed fluorescence increase. (2) According to the Langmuir adsorption isotherm the amount of binding sites for oxonol VI in the membrane is limited. At low concentrations of energized PMV ($\Delta\psi > 0$) all binding sites are occupied. Increasing the PMV concentration leads to more dye-binding sites so that beyond a certain PMV concentration there are more binding sites than free dye molecules. Hence, the relative fluorescence change decreases. To prevent self-quenching of the dye fluorescence the experiments were carried out with dye concentrations below 200 nM (Apell & Bersch, 1987; Smith *et al.*, 1981).

Fluorescence changes of oxonol VI caused by diffusion potentials could not, as expected, be fitted by the model (comparing Figs. 1A and 4). This may be due to the formation of lipid-soluble complexes of valinomycin, oxonol VI and K^+ in the apolar phase of the vesicle membrane (Pratap *et al.*, 1990; Scherman

and Henry, 1980; Bashford and Smith, 1979). This assumption is further supported by the observation that the addition of valinomycin to salt-free vesicle suspensions under steady-state conditions led to unspecific changes in oxonol VI fluorescence.

For a precise correlation of the observed fluorescence changes with a given $\Delta\psi$, irrespective of its generation, values of $\Delta\psi$-induced fluorescence changes have to be reproducible and independent of the dye/vesicle ratio. When this prerequisite is not met, the model fitting described here is a suitable method to assess $\Delta\psi$ generated by the H^+-ATPase in reconstituted plasma membrane vesicles.

ACKNOWLEDGMENTS

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APPENDIX

Parameter Fitting

The distribution of oxonol VI in the membrane is desribed by Eq. (2) after substituting for n', n'' from Eqs. (3) and (4), and for c'' from Eq. (1). Equation (2) can be then expressed as

$$f(c') = c'A + c'^2B + c'^3C - c \tag{A1}$$

In this equation A, B, and C are

$$\begin{aligned} A &= m + p(e^{-\alpha u} + e^{\alpha u+u}) + e^u - me^u \\ &\quad -cK(e^{-\alpha u} + e^{\alpha u+u}) \\ B &= mK(e^{-\alpha u} + e^{\alpha u+u}) + Ke^{-\alpha u+u} + 2Kpe^u \\ &\quad + cK^2e^u + Ke^{2u+\alpha u} - mK(e^{-\alpha u+u} + e^{2u+\alpha u}) \\ C &= mK^2(e^u + e^{2u}) \end{aligned} \tag{A2}$$

where

$$m = \frac{V'L}{V}, \quad p = \frac{n_\infty KL}{V}, \quad u = \frac{\Delta\psi F}{RT} \tag{A3}$$

The value of c' was determined using the Newton iteration method

$$c'_{i+1} = c'_i - \frac{f(c'_i)}{f'(c'_i)} \tag{A4}$$

For a suitable starting point the series c'_i converges to zero. As initial values for c'_i the concentration of the free dye for $\Delta\psi = 0$ was chosen.

For $\Delta\psi = 0$ and $c' = c''$ Eq. (A1) simplifies to

$$c' = \frac{c - 2n_\infty L/V - K^{-1}}{2} + \frac{1}{2}\sqrt{(c - 2n_\infty L/V)^2 + K^{-2} + 2\frac{2n_\infty L/V + c}{K}} \tag{A5}$$

With c' from Eqs. (A4) or (A5), the values of c'', n', and n'' can be calculated using Eqs. (1), (3), and (4), and the relative fluorescence can be determined for $\Delta\psi = 0$ using the equation

$$\frac{F}{F_0} - 1 = \frac{c''(V - V'L)}{cV} + \frac{2\epsilon}{cV}n'' \tag{A6}$$

Similarly, the relative fluorescence for $\Delta\psi > 0$ can be determined using the equation

$$\frac{F_{pot}}{F_0} - 1 = \frac{\epsilon n'_{pot} + \epsilon n''_{pot} + c''_{pot}(V - V'L)}{cV} \tag{A7}$$

Because the fluorescence from the inside of the vesicles cannot be measured, c' does not occur in the last two equations.

To determine the parameters K, n_∞, α, ϵ, and $\Delta\psi$ of the model we use the following procedure. The relative fluorescence of the experimental data $a_1, \ldots, a_n$ is measured in dependence on the parameters c (total dye concentration) and L (amount of lipids in mg). For each a_i, $F_p(v_i)$ is calculated using the equations of the model, and v_i is given by the parameters c and L of each experiment. The parameters K, n_∞, α, ϵ, and $\Delta\psi$ are expressed in terms of a series $p = (p_1, \ldots, p_m)$. The aim of the algorithm is to minimize the difference D_p between the experimental data a_i and the values given by the model $F_p(v_i)$. D_p is given by the equation

$$D_p = \sum_{i=1}^{n}(a_i - F_p(v_i))^2 \tag{A8}$$

For each parameter p_j Eqs. (A1) to (A7) are seeded with a starting value. For $j = 1$ to m the constants $p_1, \ldots, p_m$ are fitted. For each fit of p_j a new fitting of parameters p_1 to p_{j-1} is performed.

Calculation of V'

With a PMV diameter of 100 nm (Mair and Höfer, 1988), a membrane thickness of 4 nm, and a lipid density of 1 g/ml (Apell and Bersch, 1987) the amount of PMV can be calculated: The lipid volume per vesicle can be obtained by subtracting the inner from the total volume of the vesicle. The density equation gives the total number of reconstituted vesicles per 1 mg of lipids: $8.628 \cdot 10^{12}$. The inner volume of one vesicle is $4.077 \cdot 10^{-16}$ cm^3. The total inner volume V' of $8.628 \cdot 10^{12}$ vesicles is 0.00352 cm^3 per mg lipids.

REFERENCES

Apell, H.-J., and Bersch, B. (1987). "Oxonol VI as an optical indicator for membrane potentials in lipid vesicles," *Biochim. Biophys. Acta* **903**, 480–494.

Bashford, C. L., and Smith, J. C. (1979). "The use of optical probes to monitor membrane potential," *Methods Enzymol.* **55**, 569–586.

Bashford, C. L., Chance, B., and Smith, J. C. (1979). "The behavior of oxonol dyes in phospholipid dispersions," *Biophys. J.* **25**, 63–85.

Beeler, T. J., Farmen, R. H., and Martonosi, A. N. (1981). "The mechanism of voltage-sensitive dye responses on sarcoplasmatic reticulum," *J. Membr. Biol.* **62**, 3–137.

Cooper, C. E., Bruce, D., and Nicolls, P. (1990). "Use of oxonol V as a probe of membrane potential in proteoliposomes containing cytochrome oxidase in the submitochondrial orientation," *Biochem.* **29**, 3859–3865.

Gläser, H.-U., and Höfer, M. (1987). "Ion-dependent generation of the electrochemical proton gradient $\Delta\mu_H{}^+$ in reconstituted plasma membrane vesicles from the yeast *Metschnikowia reukaufii*," *Biochim. Biophys. Acta* **905**, 287–294.

Höfer, M., Mair, T., and Wernsdörfer, E. (1991). "Molecular biology and biotechnology," In: *Yeast* (Prasad, R. ed.), Omega Scientific Publishers, New Delhi, pp. 239–253.

Kletterer, B., Neumcke, B., and Läuger, P. (1971). "Transport mechanism of hydrophobic ions through lipid bilayer membranes," *J. Membr. Biol.* **5**, 225–245.

Mair, T., and Höfer, M. (1988). "ATP-induced generation of pH-gradient and/or membrane potential in reconstituted plasma membrane vesicles from *Schizosaccharomyces pombe*," *Biochem. Int.* **17**, 593–604.

Perlin, D. S., Kasamo, K., Brooker, R. J., and Slayman, C. W. (1984). "Electrogenic H^+ translocation by the plasma membrane ATPase of Neurospora," *J. Biol. Chem.* **259**, 7884–7892.

Plášek, J., and Sigler, K. (1995). "Slow fluorescent indicators of membrane potential: a survey of different approaches to probe response analysis," *J. Photochem. Photobiol.* in press.

Pratap, P. R., Novak, T. S., and Freedman, J. C. (1990). "Two mechanisms by which fluorescent oxonols indicate membrane potential in human red blood cells," *Biophys. J.* **57**, 835–849.

Scherman, D., and Henry, J. P. (1980). "Oxonol V as a probe of chromaffin granule membrane potentials," *Biochim. Biophys. Acta* **599**, 150–166.

Smith, J. C. (1990). "Potential-sensitive molecular probes in membranes of bioenergetic relevance," *Biochim. Biophys. Acta* **1016**, 1–28.

Smith, J. C., Hallidy, L., and Topp, M. R. (1981). "The behavior of the fluorescence lifetime and polarization of oxonol potential-sensitive extrinsic probes in solution and in beef heart submitochondrial particles," *J. Membr. Biol.* **60**, 173–185.

Villalobo, A. (1990). "Reconstitution of ion-motive transport ATPases in artificial lipid membranes," *Biochim. Biophys. Acta* **1017**, 1–48.

Wolk, U., and Höfer, M. (1987). "Interactions between cyanine dyes and yeast cells: do cyanine dyes act as membrane potential sensitive probes?" *Biochem. Int.* **14**, 501–509.

Zwolinsky, B. J., Eyring, H., and Reese, C. (1949). "Diffusion and membrane permeability," *J. Phys. Colloid Chem.* **53**, 1426–1453.