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Quantitative analysis of pump-mediated fluxes in reconstituted lipid vesicles

H.-J. Apell and P. Läuger

Department of Biology, University of Konstanz, D-7750 Constance (F.R.G.)

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Reconstituted vesicles with built-in ion pumps (or other transport proteins) are usually heterogeneous with respect to size and with respect to the number of pump molecules present in the membrane. In this paper a method is described for the analysis of ion flux experiments with reconstituted vesicles which is based on the statistical properties of the vesicle population. The method is based on the assumption that the fraction of vesicles containing $n = 0, 1, 2, \ldots$ pump molecules is given by a Poisson distribution; it further requires an estimate of the width of the distribution of vesicle radii. Under favourable conditions the intrinsic turnover rate of the pump and the density of functional pump molecules in the vesicle membrane can be determined separately. The method can be applied to isotope-flux experiments, to optimal measurements of active ion transport and to studies of pump-mediated charge transport.

Introduction

In recent years, methods have been developed for the isolation of active transport systems from biological membranes and for the subsequent incorporation of the purified transport protein into artificial lipid vesicles [1–3]. Such reconstitution experiments have been carried out, for instance, with the sodium-potassium pump from the plasma membrane of mammalian cells [4,5] and with the calcium pump from sarcoplasmic reticulum [6]. Reconstituted vesicles offer interesting possibilities for mechanistic studies of pump-mediated ion fluxes, since flux experiments can be carried out under well-defined conditions with respect to the nature of the lipid and to the composition of the internal and external aqueous media.

Since vesicle diameters are typically of the order of 100 nm or less (for vesicles prepared by detergent removal [7–10]), the intravesicular aqueous volume is extremely small. The analysis of flux experiments is thus faced with the problem that the ion concentration in the internal space of the vesicle changes rapidly after activation of the pump. With the limited time resolution of commonly used isotope-flux methods it is therefore difficult to determine initial transport rates accurately. A better time resolution can be achieved by measuring intravesicular ion concentration optically [11], but even in this case extrapolation to time zero may be uncertain unless rapid mixing techniques are used.

More reliable information on flux rates may be obtained by analyzing the time course of intravesicular concentration over the whole time-range of the experiment. Such an analysis requires that the heterogeneity of the vesicle population [11–13] is explicitly taken into account. Reconstituted vesicles exhibit a distribution of diameters, as show by electron microscopic analysis [5–7] and photon correlation spectroscopy [10,14]. In an experiment in which pump-mediated efflux of ions is measured, small vesicles lose their ion content faster than large vesicles and contribute only at short times to the overall transport process. Apart

Correspondence address: Department of Biology, University of Konstanz, D-7750 Konstanz, F.R.G.

from the variation in diameter, vesicles are heterogeneous with respect to the number of incorporated pump molecules. This results from the statistical nature of the process by which pump molecules are inserted into the vesicle membrane in the reconstitution experiment. Thus, if the average number of pump molecules per vesicle is, say, $\bar{n} = 2$, the vesicle population is composed of subpopulations of vesicles containing $n = 0$, 1, 2, 3, etc. pump molecules.

In the following, a method for the quantitative analysis of flux experiments is described which is based on the statistical properties of the vesicle population. It is shown that under suitable conditions the intrinsic turnover rate v of the pump and the number χ of functional pump molecules per unit area of the membrane can be determined separately. The usual analysis of flux experiments with vesicles yields only the product $\chi \cdot v$, i.e., the flux per unit membrane area. Estimates of χ based on total protein content of the vesicle suspension are uncertain, since in most cases the fraction of pump molecules which are functionally active and which are inserted with the correct orientation is not known. The separate determination of turnover rate v and pump density χ becomes possible when information on the size distribution of the vesicles is available and when the average number $\bar{n}$ of functional pump molecules per vesicle is small (e.g., $\bar{n} < 5$). This may be seen from the following hypothetical example. Consider a mixture of vesicles of uniform size containing $n = 0$, 1 or 2 functional pump molecules and assume that ATP-driven efflux of a substrate S is measured by adding ATP at time $t = 0$ to the vesicle suspension. The total amount of intravesicular substrate, $m(t)$, decreases steadily with time until the concentration of S in the vesicles with $n = 2$ has dropped to nearly zero. At this time the slope of $m(t)$ decreases steeply. A second transition (to zero slope) occurs when the vesicles with $n = 1$ have lost their trapped substrate. In this hypothetical experiment the turnover rate v is directly obtained from the time interval between successive transitions in the slope of $m(t)$ and from the known volume of intravesicular space. In a real experiment with vesicles of non-uniform size, the transitions of dm/dt are smoothed out, but $m(t)$ nevertheless contains information on v and χ.

Statistical model of the vesicle population

We consider a suspension of spherical vesicles with incorporated pump molecules. The ith vesicle is characterized by its internal radius r_i and the number n_i of functionally oriented pumps. (Reconstitution of an ATP-driven pump usually results in a mixture of both orientations with inward- and outward-facing ATP binding sites; when ATP is added to the medium, only pump molecules with the ATP site facing outward are activated.) The distribution of vesicle radii may be described by a probability density $\rho(r)$; the number $\Delta N(r)$ of vesicles with a radius between r and $r + \Delta r$ is then given by

$$\Delta N(r) = N\rho(r)\Delta r \tag{1}$$

N is the total number of vesicles in the suspension. We specifically assume that $\rho(r)$ may be approximated by a Gaussian distribution with mean value $\bar{r}$ and half-width σ:

$$\rho(r) = \frac{1}{\sqrt{2\pi}\,\sigma} \exp \frac{-(r-\bar{r})^2}{2\sigma^2} \tag{2}$$

$$\sigma^2 = \overline{(r-\bar{r})^2} \tag{3}$$

The assumption of a Gaussian distribution is an approximation which may be expected to hold in the vicinity of the average radius $\bar{r}$. For geometrical reasons a lower limit $r_0 \simeq 15$ nm exists for the vesicle radius [1], meaning that the distribution becomes non-Gaussian for small values of r. Since for vesicles prepared by detergent dialysis $\bar{r}$ is much larger than r_0, the size limitation ($r > r_0$) does not appreciably affect the analysis.

We further assume that the number n of functionally oriented pump molecules in a vesicle is described by a Poisson distribution and that the average value of n is proportional to the area of the vesicle membrane. The probability $P(n, r)$ that a vesicle of radius r contains n functionally oriented pumps is equal to

$$P(n, r) = \frac{(\bar{n})^n \exp(-\bar{n})}{n!} \tag{4}$$

$$\bar{n}(r) = 4\pi r^2 \chi \tag{5}$$

χ is the average surface density of oriented pump molecules (referred to the internal surface area of the vesicle).

The assumption of a Poisson distribution implies that pump molecules are inserted into the vesicle membrane independently of each other in the reconstitution process. In the case of ($Na^+ + K^+$)-ATPase, vesicles prepared by detergent dialysis statistical analysis of electron-microscopic pictures has shown that the experimental values of $P(n, r)$ approximately agree with a Poisson distribution [9,13].

For computational purposes it is convenient to divide the vesicle population into discrete classes, class (n, r_k) containing all vesicles which possess n pump molecules and have a radius between r_k and $r_k + r$. The number $\Delta N(n, r_k)$ of vesicles in class (n, r_k) is then given by

$$\Delta N(n, r_k) = NP(n, r_k)\rho(r_k)\Delta r \tag{6}$$

r_k is varied in discrete steps of length $\Delta r \ll \bar{r}$ between a lower limit $r' = \bar{r} - p\Delta r$ and an upper limit $r'' = \bar{r} + p\Delta r$:

$$r_k = \bar{r} + k\Delta r \quad (k = -p, -p+1, \ldots, p) \tag{7}$$

p is a positive integer chosen such that $\rho(r)$ is negligibly small outside the interval (r', r'').

Analysis of flux experiments

Isotope fluxes

In an experiment in which ATP-driven ion fluxes are studied, all pump molecules are simultaneously activated at time $t = 0$ by adding ATP to the medium, and the intravesicular concentration of the transported ionic species M is measured as a function of time. In the following we first treat experiments in which M is isotopically labeled. By taking samples from the vesicle suspension after certain time intervals, the total intravesicular amount, $m(t)$, of M may be determined. As a suitable experimental quantity we introduce the ratio $m(t)/m_0 \equiv S(t)$, where $m_0 \equiv m(0)$ is the initial value of m. Denoting the concentration of M in the ith vesicle by $c_i(t)$, the quantity $S(t)$ is obtained by summation over all vesicles:

$$S(t) \equiv \frac{m(t)}{m_0} = \frac{1}{m_0}\sum_{i=1}^{N} V_i c_i(t) = \frac{1}{c_0 V}\sum_i V_i c_i(t) \tag{8}$$

V_i is the volume of the internal aqueous space of the ith vesicle, V the total internal volume and c_0 the initial concentration of M which is common to all vesicles.

For a vesicle of class (n, r_k) which has an internal volume $V_k = (4\pi/3)r_k^3$, the time dependence of ion concentration c is given by

$$\frac{dc}{dt} = -\frac{\nu n v}{V_k} \tag{9}$$

ν is the number of ions transported in a single turnover and v the turnover rate of the pump molecule (v is taken to be positive if the pump promotes ion efflux). Implicit in Eqn 9 is the assumption that functional interactions between pump molecules in a vesicle are small. This assumption is justified when the average density of pumps in the vesicle membrane is low, as is the case in most reconstitution experiments. Furthermore, Eqn. 9 requires that leakage pathways are negligible; this has been shown to be true in experiments with reconstituted ($Na^+ + K^+$)-ATPase [11]. Under normal conditions the external volume of the vesicle suspension is much larger than the volume of the intravesicular space, so that the ion (and ATP) concentration in the medium remains nearly constant during the flux experiment. The turnover rate v is then a unique function of intravesicular ion concentration c (provided that electric field effects can be neglected; see below). If $v(c)$ is known, c is a well-defined function $c(n, r_k, t)$ of time for a vesicle of class (n, r_k) which, according to Eqn. 9, is determined by

$$\int_{c_0}^{c} \frac{dc}{v(c)} = -\frac{\nu n t}{V_k} \tag{10}$$

For a transport system which extrudes ions from the vesicle interior, $v(c)$ can usually be represented by a Michaelis-Menten equation:

$$v(c) = \frac{v_m c}{c + K} \tag{11}$$

v_m is the maximum turnover rate and K the half-saturation concentration. In this case $c(n, r_k, t)$ is given by the implicit equation

$$c + K \ln\frac{c}{c_0} = c_0 - \frac{\nu n v_m}{V_k} t \tag{12}$$

The summation in Eqn. 8 may be replaced by a summation over all vesicle classes (n, r_k). Since class (n, r_k) contains $\Delta N(n, r_k)$ vesicles (Eqn. 6), Eqn. 8 assumes the form

$$S(t) = \frac{1}{V} \sum_{n=0}^{\infty} \sum_{k=-p}^{p} V_k h(n, r_k, t) \Delta N(n, r_k) \tag{13}$$

$$h(n, r_k, t) \equiv c(n, r_k, t)/c_0 \tag{14}$$

Expressing the total intravesicular volume V by

$$V = N \sum_{k=-p}^{p} V_k \rho(r_k) \Delta r \tag{15}$$

one finally obtains (with $V_k = (4\pi/3) r_k^3$):

$$S(t) = \frac{\sum_{n=0}^{\infty} \sum_{k=-p}^{p} h(n, r_k, t) P(n, r_k) r_k^3 \rho(r_k) \Delta r}{\sum_{k=-p}^{p} r_k^3 \rho(r_k) \Delta r} \tag{16}$$

If the distribution function $\rho(r_k)$ has the form of Eqn. 2, the denominator D in Eqn. 16 may be replaced by

$$D \approx \bar{r}^3 + 3\bar{r}\sigma^2 \tag{17}$$

This relation is obtained by replacing the summation over r_k by an integration over r.

By fitting Eqn. 16, together with Eqns. 2 and 4, to the experimental values of $S(t)$, the turnover rate v and the mean density χ (Eqn. 5) of functionally active pump molecules can be evaluated separately. The analysis requires information on the average vesicle radius $\bar{r}$ and on the variance σ of the distribution of vesicle radii, which may be obtained from electron microscopy or light-scattering measurements. Application of the Michaelis-Menten relation (Eqn. 11) for the description of the concentration dependence of v yields the maximum transport rate v_m. Estimated values of the half-saturation concentration K are usually available form other sources. If the initial ion concentration c_0 is large enough ($c_0 \gg K$), the analysis is insensitive against variations of K. Alternatively, by carrying out flux measurements over a wide range of c_0, the constant K may be evaluated from the experiments. Examples of numerical fits are given in a later section.

From Eqns. 2, 9 and 16 a simple expression is derived for the initial rate of change of S:

$$\left(\frac{dS}{dt}\right)_{t=0} = -\frac{3\nu v_0 \chi}{c_0 \bar{r}} \cdot \frac{1+s}{1+3s} = -\frac{\nu v_0 \chi A}{c_0 V} \tag{18}$$

$$s \equiv \sigma^2/\bar{r}^2 \ll 1$$

v_0 is the initial value of turnover rate v, and A the total surface area of the vesicle suspension. Thus, $(dS/dt)_{t=0}$ is proportional to the product of turnover rate v_0 times the average pump density χ, as may be expected.

After long time periods $S(t)$ approaches a finite value $S(\infty)$ which depends on the fraction of vesicles with $n = 0$. Replacing the summation over r_k by an integration over r, the following relation for $S(\infty)$ is obtained from Eqn. 16:

$$S(\infty) = \frac{1+3s(1+y)}{(1+3s)(1+y)^3\sqrt{1+y}} \exp - \frac{y/2s}{1+y} \tag{19}$$

$$y \equiv 8\pi\sigma^2\chi$$

For vesicles of uniform radius r ($\sigma = 0$), Eqn. 19 reduces to $S(\infty) = \exp(-4\pi r^2 \chi) = \exp(-\bar{n}) = P(0, r)$, as expected. Using Eqns. 18 and 19, v_0 and χ may be evaluated, in principle, from the experimental values of $(dS/dt)_{t=0}$ and $S(\infty)$. In practice, however, it is often difficult to determine $(dS/dt)_{t=0}$ and $S(\infty)$ with sufficient accuracy. For this reason it is advantageous to use the whole time course of S for the analysis.

Optical measurement of ion fluxes

When an optical indicator for the transported ion species is added to the intravesicular aqueous space, ion fluxes may be detected by measuring the absorption or fluorescence of the vesicle suspension. For instance, fluorescence quenching by Tl^+ or Cs^+ has been used for monitoring intravesicular concentrations of these ions [16,17]. In the following, we specifically consider the fluorescence method; an analogous treatment is possible in the case of absorption measurements. We assume that the intravesicular medium contains a water-soluble fluorescent dye whose fluorescence depends on the concentration c of the transported ion. If $f_i(t)$ is the contribution of the ith vesicle to

the fluorescence intensity of the suspension and $f_{i0} \equiv f_i(0)$ the initial value of f_i, the ratio

$$\frac{f_i}{f_{i0}} \equiv q(c_i) \tag{20}$$

is a unique function $q(c)$ of intravesicular ion concentration c, which is independent of vesicle size and which can be determined by calibration experiments. The result of the fluorescence experiment may be represented by the ratio $F(t)/F_0 \equiv S(t)$ where $F(t)$ is the total fluorescence intensity of the suspension and $F_0 \equiv F(0)$ the initial value of F. $S(t)$ is obtained by summation over all vesicles:

$$S(t) \equiv \frac{F(t)}{F_0} = \frac{1}{F_0} \sum_{i=1}^{N} f_i(t) = \frac{1}{F_0} \sum_i f_{i0} q[c_i(t)] \tag{21}$$

Since the ratio f_{i0}/F_0 may be assumed to be equal to V_i/V (V_i is the internal volume of vesicle i and V the total internal volume), Eqn. 21 becomes

$$S(t) = \frac{1}{V} \sum_i V_i q[c_i(t)] \tag{22}$$

This equation is identical to Eqn. 8 when $c_i(t)/c_0$ is substituted by $q[c_i(t)]$. This means that Eqn. 16 may be directly applied to the fluorescence experiment, if $h(n, r_k, t)$ is defined by

$$h(n, r_k, t) \equiv q[c(n, r_k, t)] \tag{23}$$

A variant of the fluorescence method described above has recently been used in studies with reconstituted $(Na^+ + K^+)$-ATPase vesicles [11,18, 19]. A potentially sensitive fluorescent dye has been incorporated into the vesicle membrane and the intravesicular K^+ concentration has been measured via the Nernst potential in the presence of valinomycin. In this case $S(t)$ is defined as the fluorescence $F(t)$ at time t, divided by the fluorescence F^* for zero transmembrane voltage ($U = 0$); F^* is obtained under the condition that the intravesicular K^+ concentration c is equal to the external K^+ concentration c_{ext}:

$$S(t) \equiv \frac{F(t)}{F^*}; \quad F^* \equiv (F)_{U=0} = (F)_{c=c_{ext}} \tag{24}$$

For calibration of the fluorescence signal, separate experiments (without activation of the pump) are carried out with fixed c_{ext} and variable intravesicular K^+ concentration c. This yields a calibration function $g(c)$:

$$g(c) \equiv \frac{(F)_c}{F^*} \tag{25}$$

If $f_i(t)$ is the fluorescence of the ith vesicle in the flux experiment and f_i^* its fluorescence for $c_i = c_{ext}$ (or $U = 0$), then the relation

$$\frac{f_i(t)}{f_i^*} = g[c_i(t)] \tag{26}$$

holds. Since in this case the fluorescent dye is present in the vesicle membrane, the ratio f_i^*/F^* is equal to A_i/A, where A_i is the surface area of the ith vesicle and A the total surface area of the vesicle suspension. Thus,

$$S(t) = \frac{1}{F^*} \sum_i f_i(t) = \frac{1}{F^*} \sum_i f_i^* q[c_i(t)]$$

$$= \frac{1}{A} \sum_i A_i q[c_i(t)] \tag{27}$$

With

$$A = N \sum_{k=-p}^{p} A_k \rho(r_k) \Delta r \tag{28}$$

and $A_k = 4\pi r_k^2$ one obtains instead of Eqn. 16:

$$S(t) = \frac{\sum_{n=0}^{\infty} \sum_{k=-p}^{p} h(n, r_k, t) P(n, r_k) r_k^2 \rho(r_k) \Delta r}{\sum_{k=-p}^{p} r_k^2 \rho(r_k) \Delta r} \tag{29}$$

h is again defined by

$$h(n, r_k, t) \equiv g[c(n, r_k, t)] \tag{30}$$

If the distribution of vesicle radii is Gaussian (Eqn. 2), the denominator D of Eqn. 29 is given by

$$D \approx \bar{r}^2 + \sigma^2 \tag{31}$$

Charge translocation

Most ion pumps are electrogenic, i.e., they

translocate electric charge across the membrane. By measuring the time-course of transmembrane voltage, information on the turnover rate of the pump may be obtained. Since the electric field in the membrane acts back on the pump, the turnover rate v becomes a function of voltage. (The field effect on v may be eliminated by addition of a membrane-permeable ionic species such as SCN^-).

We now consider an experiment in which the transmembrane voltage U is measured using a potential-sensitive fluorescent dye. In this case the ratio f_i/f_i^* (Eqn. 26) is a function of U_i, which has to be determined by calibration:

$$\frac{f_i}{f_i^*} = w(U_i) \tag{32}$$

Accordingly, Eqn. 27 now assumes the form

$$S(t) \equiv \frac{F(t)}{F^*} = \frac{1}{A_i} A_i w[U_i(t)] \tag{33}$$

For a vesicle of class (n, r_k), the transmembrane voltage is a well-defined function $U(n, r_k, t)$ of time t. In a vesicle containing n pump molecules, the pump current is equal to $\nu_c e_0 n v(U) \equiv I_p$, where ν_c is the number of translocated charges per turnover and e_0 the elementary charge. $-I_p$ must be equal to the sum of the capacitive current, $A_k C_m dU/dt$, and the current $A_k G_m U$ through leakage pathways; C_m and G_m are the membrane capacitance and leakage conductance, respectively, referred to unit area, and $A_k = 4\pi r_k^2$ is the surface area of the vesicle. This leads to the following equation for $U(n, r_k, t)$:

$$\frac{dU}{dt} = -\frac{\nu_c e_0 n}{A_k C_m} v(U) - \frac{G_m}{C_m} U \tag{34}$$

When the leakage conductance G_m is small, the transmembrane voltage approaches the reversal potential U_r of the pump. $U(n, r_k, t)$ can be obtained by integration of Eqn. 34, if the voltage dependence of the turnover rate v is approximately known. Since Eqns. 33 and 27 are formally identical, Eqn. 29 can be applied also to the analysis of pump-mediated charge translocation, provided that the function h is now defined by:

$$h(n, r_k, t) \equiv w[U(n, r_k, t)] \tag{35}$$

Numerical examples

In the following we give a number of numerical examples in order to illustrate the above-described method of analysis of flux experiments. We specifically consider the case that the pump extrudes ions from the vesicle at a rate depending on intravesicular ion concentration according to the Michaelis-Menten relation (Eqn. 11). An average vesicle radius of $\bar{r} = 50$ nm is assumed throughout which approximately corresponds to the size of phosphatidylcholine vesicles prepared by cholate dialysis [5–8]. In Fig. 1 the quantity $S(t) = m(t)/m_0$ is plotted according to Eqn. 16 for a population of vesicles of uniform size ($\sigma = 0$), with the average number $\bar{n}$ of pumps per vesicle as a variable parameter. It is seen that $m(t)$ exhibits

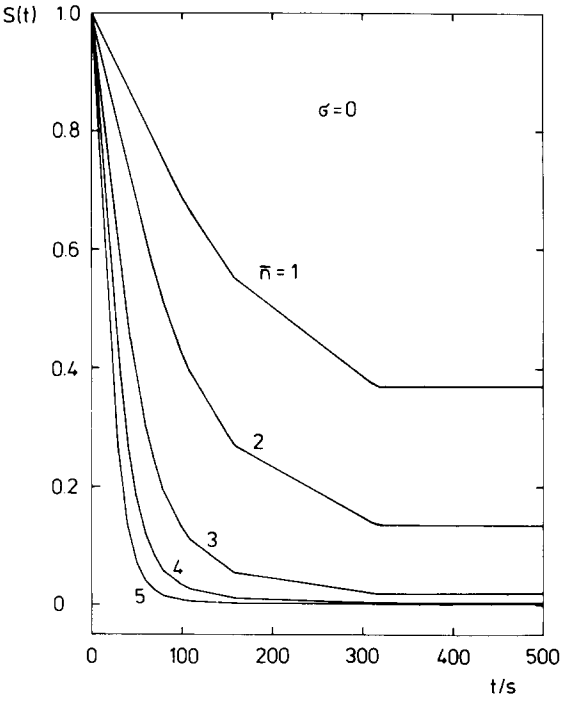

Fig. 1. Extrusion of ions from vesicles of uniform radius ($r = 50$ nm). $S(t) \equiv m(t)/m_0$ is the ratio of the total intravesicular content $m(t)$ of the transported ion species, divided by the initial value m_0. The turnover rate is assumed to depend on intravesicular ion concentration c according to the Michaelis-Menten relation (Eqn. 11) with $v_m = 100\ s^{-1}$ and $K = 1$ mM. The initial value of c was $c_0 = 100$ mM. $S(t)$ has been calculated for different values of the average number $\bar{n}$ of pumps per vesicle, using Eqns. 4, 12 and 16 ($\nu = 1$).

distinct transitions in slope. These transitions result from the heterogeneity of the vesicle population with respect to n. Vesicles with large n values lose their ion content after a short time and then no longer contribute to $m(t)$.

In Fig. 2, $S(t)$ is given for three vesicle populations differing in the width of the size distribution ($\sigma = 0$, $\sigma = 2$ nm, $\sigma = 5$ nm). As it may be expected, the discontinuities in the slope of $m(t)/m_0$ which occur with vesicles of uniform size ($\sigma = 0$) become smoothed out for $\sigma > 0$. (Note that the curves for $\sigma = 2$ nm and $\sigma = 5$ nm have been arbitrarily shifted along the S-axis for sake of clarity).

According to Eqn. 18 it is clear that from the slope of $S(t)$ after short time periods, t, only the product $v_0\chi$ can be obtained whereas a separate determination of turnover rate and pump density becomes possible by analyzing the whole time course of S. The question which then arises is to what extent the values of v_0 (or v_m) and χ are unique, which are determined by fitting Eqn. 16 (or Eqn. 29) to the experimental function $S(t)$. For an examination of this question we have plotted in Fig. 3A the function $S(t)$ for a certain combination of v_m and χ ($v_m = 100\ s^{-1}$, $\chi = 50\ \mu m^{-2}$). This curve, labeled with 'χ, v_m' is compared with $S(t)$ calculated with $v_m = 50\ s^{-1}$, $\chi = 100\ \mu m^{-2}$ (labeled '2χ, $v_m/2$') and with $v_m = 200\ s^{-1}$, $\chi = 25\ \mu m^{-2}$ (labeled '$\chi/2$, $2v_m$'). Since the product $v_m\chi$ is the same in all three cases, the curves coincide at $t \approx 0$. It is seen, however, that after longer time periods, $S(t)$ is sensitive to a variation of v_m and χ, meaning that a reliable determination of v_m and χ is possible. In Fig. 3B a similar comparison is carried out, but now with a much larger pump density ($v_m = 5\ s^{-1}$, $\chi = 1000\ \mu m^{-2}$), corresponding, at $r = \bar{r} = 50$ nm, to $\bar{n} = 31$. Under these conditions the three curves almost coincide, even after long time periods. This means that v_m and χ can no longer be evaluated unequivocally, if the average number of pumps per vesicle is large.

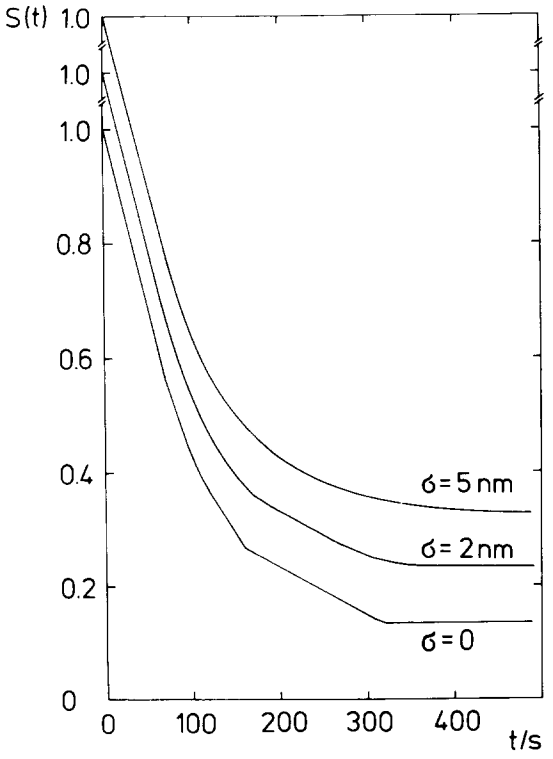

Fig. 2. $S \equiv m/m_0$ as a function of time t under the same conditions as in Fig. 1, for three vesicle populations differing in the half-width σ of the radius distribution (Eqn. 2). The average density of pump molecules was assumed to be $\chi = 64\ \mu m^{-2}$, corresponding to $\bar{n} = 2$ for $r = \bar{r} = 50$ nm. The length of the radius interval Δr was chosen to be $\sigma/4$. The curves for $\sigma = 2$ nm and $\sigma = 5$ nm have been shifted along the S-axis by 0.1 and 0.2 units, respectively.

Finally, we discuss an example from recent K^+-flux studies with ($Na^+ + K^+$)-ATPase vesicles using a voltage-sensitive fluorescent dye in the presence of valinomycin [11,18,19]. In the experiment represented in Fig. 4 the initial intravesicular K^+ concentration was $c_0 = 140$ mM and the external K^+ concentration $c_{ext} = 10$ mM, corresponding to a Nernst potential $U = -66$ mV (inside negative) and an initial fluorescence signal $F(0)/F^* \simeq 1.32$. (As above, F^* is the value of F for $U = 0$). After addition of ATP to the medium, K^+ is extruded and $F(t)/F^*$ starts to decrease. Using the calibration of F/F^* as a function of c ($q(c)$ from Eqn. 25), Eqn. 29 was fitted to the experimental $F(t)/F^*$ curve (heavy line in Fig. 4) with $\bar{r} = 45$ nm and $\sigma = 10$ nm (estimated from photon correlation spectroscopy). From the fit curve (dashed line) the maximum turnover rate v_m and the pump density χ were determined to be $v_m = 9.25\ s^{-1}$ and $\chi = 160\ \mu m^{-2}$. For comparison, Fig. 4 contains fit curves which have been calculated (by varying the value of $n \cdot v_m$) assuming a

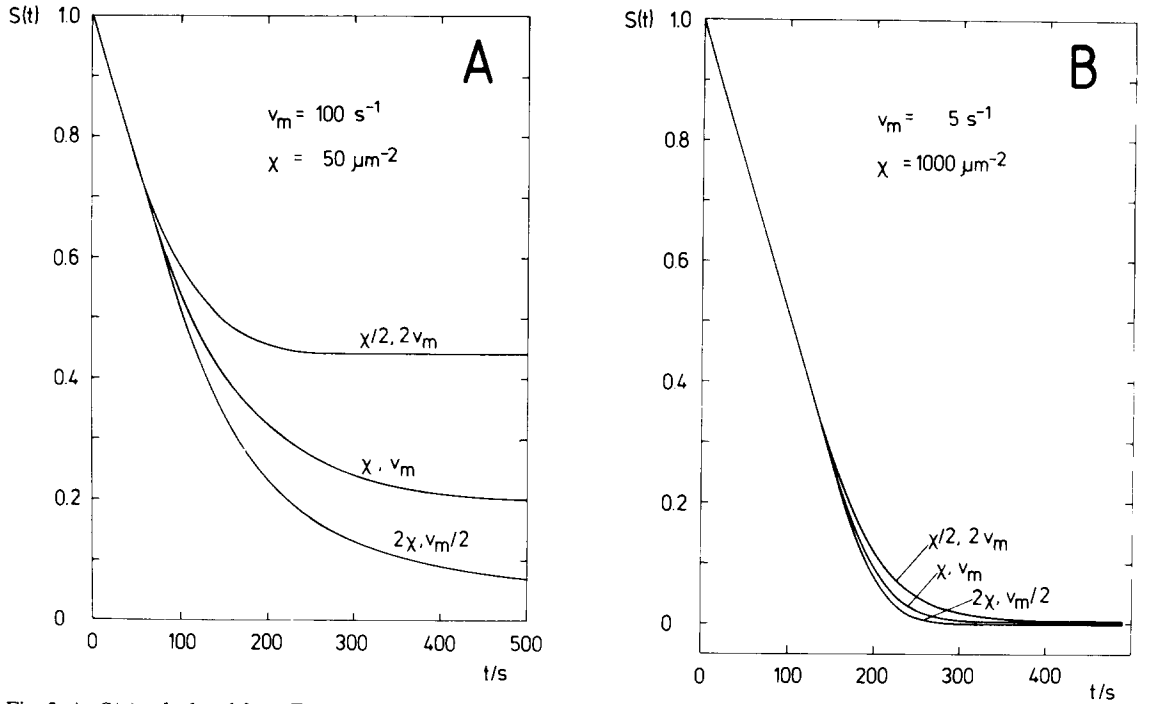

Fig. 3. A. $S(t)$ calculated from Eqn. 16 for three different sets of values of v_m and χ; v_m is the maximum turnover rate (Eqn. 11) and χ is the average density of pumps, referred to as unit area of the vesicle membrane. The value of $\chi = 50\ \mu m^{-2}$ corresponds, at $r = \bar{r} = 50$ nm, to an average of $\bar{n} = 1.6$ pumps per vesicle. The product $v_m\chi$ which determines the slope of $S(t)$ at $t = 0$ is the same in all three cases. $\bar{r} = 50$ nm, $\sigma = 5$ nm, $\Delta r = 1.25$ nm. The other conditions are the same as in Fig. 1. B. As A, but with a 20-fold higher pump density, corresponding to $\bar{n} = 31$ (at $r = \bar{r} = 50$ nm).

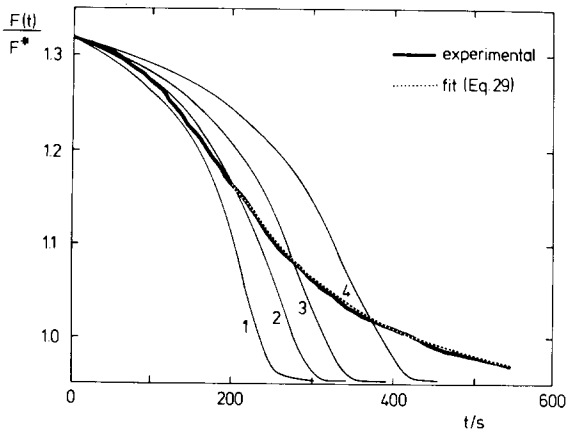

Fig. 4. ATP-driven extrusion of K^+ from $(Na^+ + K^+)$-ATPase vesicles at 13.9°C, detected by a voltage-sensitive indocyanine dye in the presence of valinomycin [11]. $F(t)$ is the fluorescence intensity at time t and F^* the fluorescence intensity at zero transmembrane voltage. The initial intravesicular K^+ concentration was $c_0 = 140$ mM and the external K^+ concentration $c_{ext} = 10$ mM, corresponding to a Nernst potential of -66 mV (inside negative). Assuming that voltage effects on pumping rate are small the experimental curve was fitted with $v_m = 9.25\ s^{-1}$ and $\chi = 160\ \mu m^2$ using Eqns. 29 and 11. The value of χ corresponds to $\bar{n} = 4\pi\bar{r}^2\chi \simeq 4$. $\bar{r} = 45$ nm, $\sigma = 10$ nm, $\Delta r = 2.5$ nm, $K = 0.1$ mM. Curves 1–4 have been calculated, by varying the value of $n \cdot v_m$, assuming a population of vesicles of uniform radius ($r = 45$ nm) and uniform number n of pumps, using the following values of $n \cdot v_m$: (1) 42.8 s^{-1}; (2) 37 s^{-1}; (3) 32 s^{-1}; (4) 27 s^{-1}.

population of vesicles of uniform radius ($\bar{r} = 45$ nm) and uniform number n of pumps. It is clearly seen that the assumption of uniform vesicles does not lead to an adequate fit of the experimentally observed time-course of F/F^*.

Discussion

The method for the analysis of flux experiments with heterogeneous vesicle populations which has been described above has been introduced recently in connection with optical studies

of active ion transport in reconstituted vesicles [11]. In this communication we have discussed the application of the method to other experiments, such as measurements of isotope fluxes, or studies of pump-mediated charge translocation. In the present context it has been assumed that the number of vesicles with $n = 0, 1, 2, \ldots$ pump molecules is given by a Poisson distribution and that the size distribution of the vesicles is Gaussian. The statistical method is more general, however, and can be used with other distributions as well. Under favourable conditions the intrinsic turnover rate of the single pump molecule and the density of functional pumps in the vesicle membrane can be determined separately. This is the case when the average number $\bar{n}$ of pump per vesicle is small, so that a non-vanishing fraction of vesicles with $n = 0$ exists. If necessary also leakage effects can be included [13]. The method is thus particularly suitable for experiments with reconstituted vesicles in which the protein content of the vesicles can be varied over a wide range.

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