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Liposome shrinkage and swelling under osmotic-diffusional stress: evaluation of kinetic parameters from spectrophotometric measurements

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Analyses of spectrophotometric measurements of shrinkage and swelling of liposomes under osmotic-diffusional stress tacitly assume an 'empirical' inverse relationship between the absorbance (A) and the liposomal volume (v). In this paper, we have proposed an alternate more explicit relationship:

$$A = E_c L\, e^{\frac{\alpha L v}{1 - L v}}$$

where L is liposomal concentration, E_c the extinction coefficient and α a dimensionless parameter. The exponential term, in essence, defines the partitioning of aqueous volume into intra- and extra-liposomal compartments. Experimental data obtained with glycerol as model compound are used to test the validity and internal consistency of the proposed formalism.

Introduction

Synthetic membrane vesicles (liposomes) offer a versatile model system to analyze the functional organization of multitude of integral membrane proteins in their native environments [1–3]. Several reports have appeared on the reconstitution of aqueous channel-forming proteins (such as gap junction proteins or porin like molecules) into liposomes [4–10]. Various techniques have been used to monitor the function of such aqueous channels in the reconstituted liposomes. These include direct measurements of influx/efflux of labelled (radiolabelled) molecules [4], biochemical methods involving chromogenic substrates [7], measurements of ionic conductance by electrophysiological techniques [8]. A relatively simple and convenient method exploited by several investigators may be traced back to earlier observations on the behaviour of liposomes as an 'ideal osmometer' [1,11–14] when subjected to hypo- and hyper-tonic environments.

Briefly, when liposomal suspensions are made hypertonic with respect to the test compounds, there is initially a sharp increase in the absorbance (at ≅ 500 nm) due to shrinkage of membrane vesicles caused by a rapid efflux of water driven by osmotic pressure. In case of diffusible molecules this is followed by an approximately exponential decrease in the absorbance with time as the liposomes swell to their initial size. The observed rate of decrease in the absorbance immediately after the maxima is taken as a measure of permeability of the compound. Or, to be more precise, the parameter generally used is given by:

$$-\frac{1}{A^2}\frac{dA}{dT}$$

where A is the absorbance value at the maximum and $-dA/dT$, the maximal decay rate as inferred from the slope of the tangent with highest steepness immediately after the peak. This follows directly from an 'empirical' reciprocal relationship between the volume (v) of membrane vesicles and the absorbance (A), i.e., $v \propto 1/A$. Although the expression described here has been widely used to obtain 'relative' estimates of permeability coefficients of a variety of compounds, it suffers from serious limitations. At the most, it can only give an approximate estimate of the desired parameter (permeability) under strictly controlled conditions, i.e., a consistent vesicular preparation (both with

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reference to the size and concentration), and a constant concentration gradient of the test solutes across the membrane. Even here, as the permeability of the test solute approaches that of water, the values obtained could be highly erroneous.

It is obvious from foregoing considerations that lack of a coherent theoretical frame work has severely impeded an optimal exploitation of the spectrophotometric method in the studies of transport across vesicular membrane. In this paper we propose an alternate expression for the relationship between liposomal volume and the absorbance, with particular reference to their variations under osmotic-diffusional stress. Kinetic data obtained with glycerol as a model solute under stringently controlled conditions are used to test the validity of the proposed formalism. Apart from demonstrating the internal consistency of the proposed relationship, the analysis provides an adequate rationale to obtain physiologically meaningful measures of permeability coefficients which can be readily compared with those derived from different methods.

Materials and Methods

Lipids. Egg phosphatidylcholine was purchased from Nippon Oils & Fats (Japan) and stored at −20°C under nitrogen as a stock solution of 0.1 g/ml. Cholesterol was purchased from Sigma (MO, USA).

Other chemicals. Tris and Dextran T10 were from Sigma (MO, USA). Sucrose was obtained from BDH (Poole, UK). All other chemicals used were of analytical grade.

Preparation of vesicles. In all these studies liposomes were prepared by the detergent dialysis method using the 'LIPOSOMAT' (Dianorm, Germany). This is often a method of choice as it gives a consistent and uniform population of liposomal vesicles [15]. Briefly, egg phosphatidylcholine, cholesterol and sodium cholate at a molar ratio of 7:3:0.052 in appropriate amounts were dissolved in chloroform. The solution was placed in a round bottomed flask and allowed to form a thin film on the walls following the evaporation of the solvent under vacuum. 1 ml of 5 mM Tris HCl buffer (pH 7.4) containing dextran T10 at a final concentration of 2% was added to the dried material and shaken thoroughly to obtain a clear micellar solution. This was then recycled through the 'LIPOSOMAT' machine as par manufacturer's instruction for 2–3 h, which permitted slow removal of detergent by dialysis against 5 mM Tris-HCl buffer (pH 7.4) leading to the formation of unilamellar vesicles with diameter in the order of 60 nm.

Experimental procedure. Our major objective in these studies has been to generate appropriate data under stringently controlled conditions to test the validity of the inferences derived in the 'Theoretical considerations' under Results. We selected glycerol as a model diffusible compound for this purpose; sucrose was used as non-diffusible (or very poorly diffusible) compound for the sake of comparison. Required amounts of test compounds dissolved in a minimal volume (20 μl) were added to the liposomal preparation at time zero, after ensuring that the liposomal preparation was stabilized in the steady state as inferred from a constant value of the absorbance at 500 nm. Variations in the absorbances at 500 nm arising from the shrinkage and swelling of vesicles were continuously monitored in a LKB Spectrophotometer (Ultrospec, Kinetic Spectrophotometer) over a period of 6–7 min. The instrument permits a print out of the absorbance values at appropriate intervals as well as a graphic output of the kinetic curves.

Results

Theoretical considerations

The rationale underlying the proposed relationship between A, L and v. In this section we briefly summarise the basic premises of the proposed relationship between the unit liposomal volume (v), concentration (L) and absorbance (A), and the inferences derived therefrom. We consider a total aqueous volume as 1 (say, in liter), which is partitioned between the two aqueous intra- and extra-liposomal compartments. Assuming v as volume per unit of the vesicular concentration L, the total volume of water in the intra-liposomal compartment may be taken as Lv. The volume of water in the extra-liposomal compartment is then given by $(1 - Lv)$.

Let I be the amount of light incident over a unit cross-sectional area at x. The decrease $(-\mathrm{d}I)$ in the transmitted light on passage through a distance $\mathrm{d}x$ in accordance with the Lambert-Beer's formalism is given by:

$$-\mathrm{d}I = E_c \phi I L \, \mathrm{d}x$$

where E_c is the extinction coefficient, and ϕ a factor having values in the range of 0 to 1. The rationale underlying the introduction of ϕ is based on the contention that the extra-liposomal aqueous compartment is optically transparent. A convenient expression for ϕ can be written as:

$$\phi = \mathrm{e}^{-\frac{\alpha Lv}{1 - Lv}}$$

Assuming $I = I_0$ at $x = 0$, on integration, we obtain an expression for the absorbance A:

$$\ln(I/I_0) = A = E_c L \, \mathrm{e}^{-\frac{\alpha Lv}{1 - Lv}} \qquad (1)$$

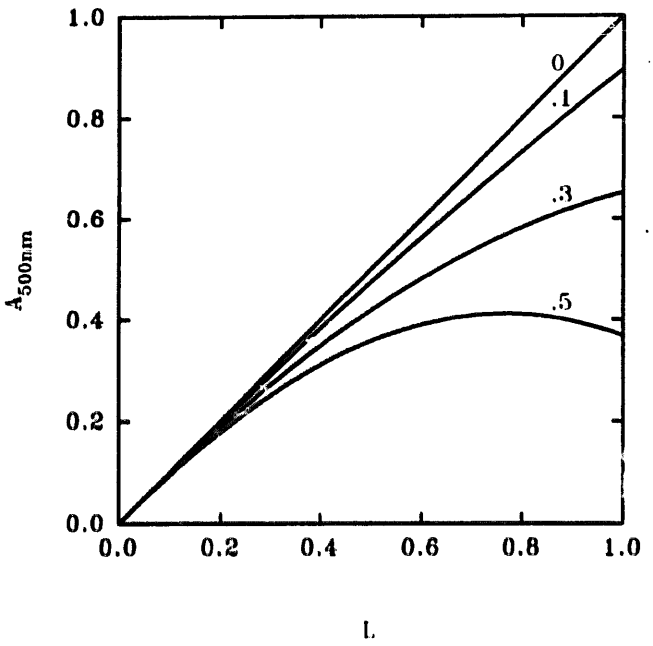


Fig. 1. In this figure, absorbance values (A) calculated from Eqn. 1 are plotted against liposomal concentration (L) for different unit liposomal volumes (v) as indicated. In these calculations both E_c and α are assumed to be 1.0. Note pronounced deviation from linearity with an increase in liposomal volume (v).

The value of x is taken as 1 in reference to the unit volume containing L liposomes. Note that the equation essentially follows Lambert-Beer's law except that the conventional extinction coefficient E_c is now multiplied by an exponential term related to the partitioning of water in the two compartments. α is a dimensionless constant. In essence, the apparent extinction coefficient comprising E_c and the exponential term is not an invariant in the strict sense, and depends on the size (v) and the concentration (L) of the membrane vesicles, which in turn define the partitioning of water into two optically distinct compartments. It is noticed that as $v \to 0$, Eqn. 1 is essentially reduced to the Lambert-Beer's equation depicting a linear relationship between the absorbance A and the concentration L. On the other hand, as Lv approaches 1, i.e., all the water is localized in the intra-liposomal compartment, the absorbance A approaches 0. It is instructive to examine the effect of liposomal volume v on the variation of A with L as described by Eqn. 1. This is shown in Fig. 1. Here E_c, L and α are assumed to be 1, and v is allowed to vary between 0 and 1 so as to ensure that $Lv < 1$. It must be emphasized that although the values assigned to these parameters may appear highly arbitrary, they do not in any way affect the salient features (as depicted by Eqn. 1). A more realistic evaluation of these features is presented in the 'Experimental' section.

Eqn. 1 may be conveniently written in a linear form,

$$\ln\left(\frac{A}{L}\right) = -\frac{\alpha Lv}{1 - Lv} + \ln(E_c)$$

For $Lv \ll 1$, we have

$$\ln\left(\frac{A}{L}\right) \cong -\alpha Lv + \ln(E_c) \tag{2}$$

Thus, $\ln(A/L)$ plotted against L will give a straight line, with the slope given by $-\alpha v$.

Kinetics of shrinkage-swelling of liposomes under osmotic-diffusional stress. The shrinkage-and-swelling behaviour of liposomes in presence of a diffusible solute is often described by a pair of coupled equations. Since here L remains constant, we may replace $E_c L$ by A_L and Lv by V, where V now represents the fractional volume of intra-liposomal compartment. The volume of the extra-liposomal compartment is given by $(1 - Lv)$. Let C_i and C_e be the amounts of non-diffusible molecules comprising the buffer components in the intra- and extra-liposomal compartments, respectively. The suspension is made hypertonic by adding S_0 moles of diffusible solute in the extra-liposomal compartment. We have the following two equations for variable V and S_i, the molar amount of the solute in the intra-liposomal compartment:

$$\frac{dV}{dT} = K_W\left(\frac{C_i + S_i}{V} - \frac{C_e + S_0 - S_i}{1 - V}\right) \tag{3}$$

$$\frac{dS_i}{dT} = K_S\left(-\frac{S_i}{V} + \frac{S_0 - S_i}{1 - V}\right) \tag{4}$$

where Eqn. 3 describes the changes in liposomal volume V, and Eqn. 4 follows directly from the Fick's 1st law of diffusion. V is related to absorbance by the equation:

$$A = A_L e^{-\frac{\alpha V}{1 - V}} \tag{5}$$

The reciprocal relationship between A and V is clearly brought out in Fig. 2, which includes two sets of A and V_i versus time plots. The curves were obtained from numerical solutions of Eqns. 3 and 4. In order to ensure that these simulations fall within a realistic range, the values assigned to various parameters (K_S, K_W, α and A_L) are kept within the range used in the quantitative evaluation of the experimental curves for glycerol (Figs. 5A and 7) presented in the 'Experimental' section

Maximal decay rate of absorbance at the peak as a measure of permeability coefficient. In order to appreciate the physiological significance of the initial rate of decrease in A (or that of increase in V), we consider the limiting situation where $K_W \gg K_S$, i.e., the perme-

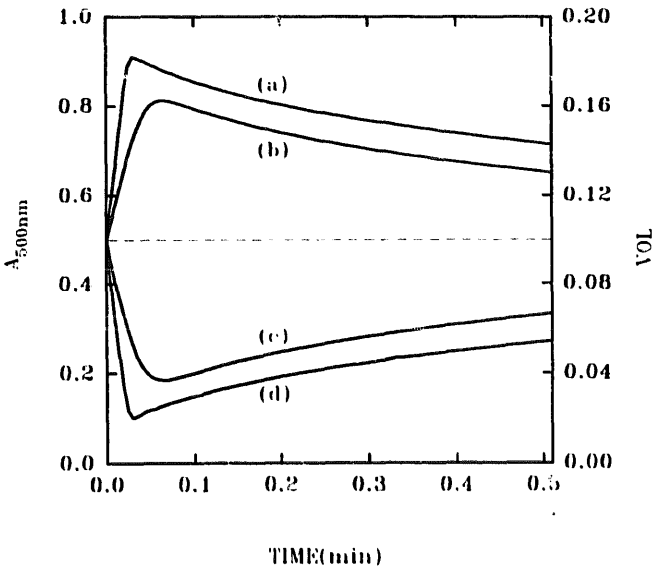


Fig. 2. The set of curves in the figure depict reciprocal relationship between absorbance (A) and intra-liposomal volume (V) as described by Eqns. 3–5. In these simulations liposomes in the steady state indicated by the dotted line for $A_0 = 0.5$ and $r_0 = 0.1$, are subjected to osmotic-diffusional stress by adding 0.4 M (a and d) and 0.2 M (b and c), of a diffusible compound (S_0). Other parameters used have the values: $A_1 = 1.05$, $\alpha = 6.6$, $K_W = 7$ and $K_S = 0.4$. Curves a, b and c, d refer to absorbance (A) and volume (V) plotted against time, respectively.

ability coefficient of water is much higher than that of test solute. Under such a constraint the volume V_m corresponding to the maximum in the absorbance versus time curve is readily obtained by equating $dV/dT = 0$ in the Eqn. 3, and is given by:

$$V_m = \frac{V_0 C_0}{C_0 + S_0} \tag{6}$$

where V_0 is the initial intra-liposomal volume in the steady state, C_0 the molar amount of essentially non-diffusible compounds comprising the buffered medium and S_0 is the amount of the test substance added at time zero. It can be further shown that the final volume (V_α) attained by the intra-liposomal aqueous compartment approaches the initial volume V_0, and S_α (the total amount of diffusible molecules incorporated into the liposomal compartment) is given by $S_0 V_0$. On differentiation of Eqn. 5 (for $V \ll 1$), we have:

$$\frac{dA}{dT} = -A_1 \alpha e^{-\alpha t} \cdot \frac{dV}{dT}$$

or

$$\frac{1}{A} \cdot \frac{dA}{dT} = -\alpha \frac{dV}{dS_i} \cdot \frac{dS_i}{dT} \tag{7}$$

Since

$$\frac{dV}{dS_i} \cong \frac{\Delta V}{\Delta S_i} = \frac{V_0 - V_m}{V_0 S_0}$$

and

$$\frac{dS_i}{dT} \cong K_S S_0$$

from Eqns. 6 and 7 we obtain:

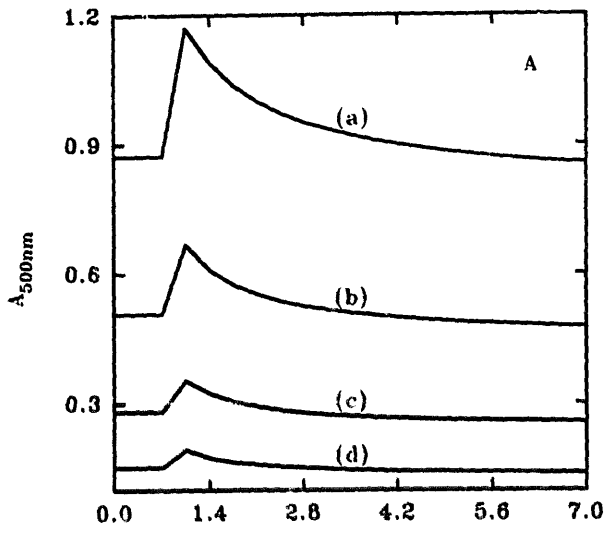
$$-\frac{1}{A} \cdot \frac{dA}{dT} = \alpha K_S \frac{S_0}{C_0 + S_0} \tag{8}$$

Thus, for a given amount of test soluble added to the extra-liposomal aqueous compartment at time zero the fractional rate constant $R_1(-(1/A) \cdot (dA/dT))$ may be taken as a measure of K_S, a parameter directly related to the permeability coefficient of the solute. It must be emphasized that Eqn. 8 is strictly valid only for solutes with $K_S \ll K_W$, i.e., the permeabilities of the solutes are much lower than that of water. As the values of K_S approach that of K_W the estimates obtained from Eqn. 8 may be fallacious, and indeed completely off the mark. These aspects are critically examined in detail in the Discussion.

Experimental

Validity of the proposed relationship between absorbance (A) and liposome concentration (L). Eqn. 1 in the preceding section brings out subtle deviations from the linear relationship between the absorbance and the concentration of a chromophoric compound as inferred from Lambert-Beer's law. Furthermore, such deviations from linearity are explicitly attributed to the size (r) of the membrane vesicles, which together with the concentration term L defines the partitioning of aqueous of volume into the intra- and extra-liposomal compartments. In essence, the liposomal suspension is considered as an optically inhomogeneous medium.

For a critical evaluation of the validity of the basic premises underlying the proposed relationship, we have carried out some simple measurements which, in essence, amount to little more than generating a standard curve. In these studies a concentrated liposomal preparation (with an absorbance value > 1) is serially diluted in isotonic buffer. We have arbitrarily defined the concentration (L) of liposomes in the original preparation as 1. (It may be mentioned that such an arbitrary choice of unit will not in any way affect the essential conclusions, as here we are only concerned with relative values). While the value of the coefficient E_c will depend on the choice of the concentration unit (L); α being a dimensionless parameter may be considered as an invariant. After the diluted preparations have achieved steady state, as judged from the con-



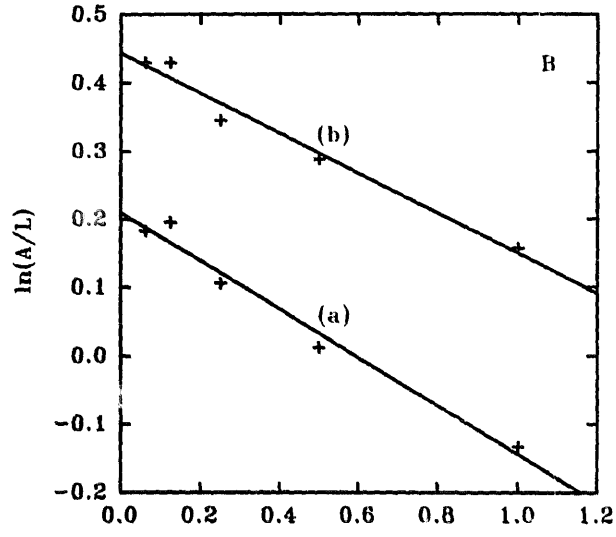


Fig. 3. (A) Original liposomal preparation ($L = 1$) is serially diluted in isotonic buffer. A constant volume of glycerol (0.02 M, final concentration) was added at the time indicated by arrow. Curves a, b, c and d refer to the liposomal concentration (L) of 1, 0.5, 0.25 and 0.125, respectively. (B) Linear plots ($\ln(A/L)$ vs. L) of the data, presented in Fig. 3A in accordance with Eqn. 2. Line (a) refers to the initial steady-state absorbances and (b) to the corresponding peak values (reduced liposomal size). From the slopes, αv_0 and αv_m are estimated to be 0.35 and 0.29, respectively, where v_0 and v_m are the unit liposomal volumes.

stancy of the absorbance values, appropriate amount of glycerol dissolved in a minimal volume ($\cong 20\ \mu l$) was added to a final concentration of 20 mM at time zero. The absorbance of the suspension was monitored over a period of 7 min or so.

A few representative kinetic curves are presented in Fig. 3A. Typically, a rapid rise in these curves is followed by a relatively slow decline as expected for a diffusible molecule. Fig. 3B includes linear plots of the data presented in Fig. 3A in accordance with Eqn. 2 for both the initial steady-state (a) and maximal (b) absorbance values. In Fig. 4, the absorbance values are plotted against the concentration (L). The lines drawn through the experimental points represent theoretical curves calculated from Eqn. 1 for $(1 - Lv) \cong 1$. The values of the parameter E_c and αv used in these calculations were obtained from the intercepts (on the ordinate) and the slopes of the linear plots presented in Fig. 3B. It is indeed gratifying to note an excellent correspondence between the calculated and experimental values.

Fractional decay rate ($-(1/A)(dA/dT)$) at the maximum as a measure of solute permeability. Eqn. 8 provides the necessary rationale for an estimation of the permeability of a diffusible solute from the observed decay rate of absorbance at the maxima in the kinetic curves. The term on the left-hand side of the Eqn. 8 may be viewed as fractional decay rate (R_f) of absorbance at the maximum, and is related to the concentration gradient across the membrane as determined by S_o, the amount of solute added at the time zero. Thus, for the same amount of solutes it provides a measure of their relative permeability coefficients as defined by the rate constant K_S.

In order to test the validity of the inferences derived from Eqn. 8 we have carried out a series of experi-

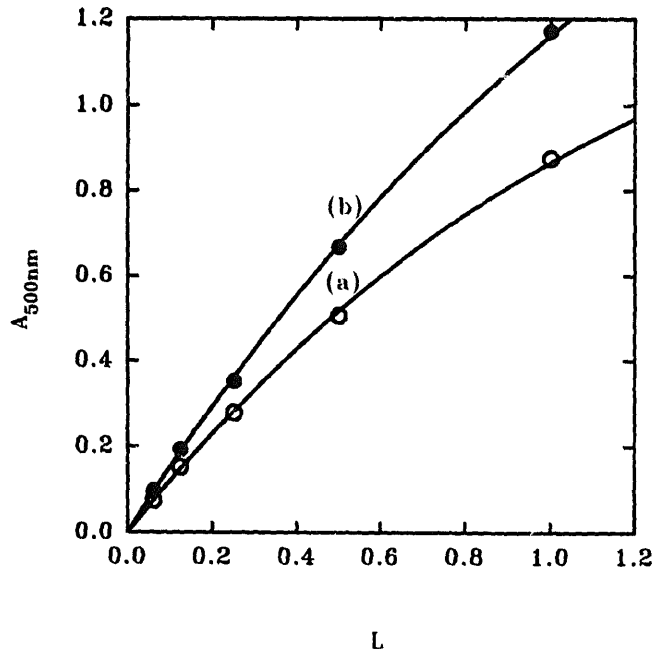


Fig. 4 Absorbances corresponding to the initial steady state (open circles) and the maxima (solid circles) in Fig. 3A are plotted against concentration L. The lines represent theoretical curves calculated from Eqn. 1 using the values of parameters derived from the linear plots in Fig. 3B.

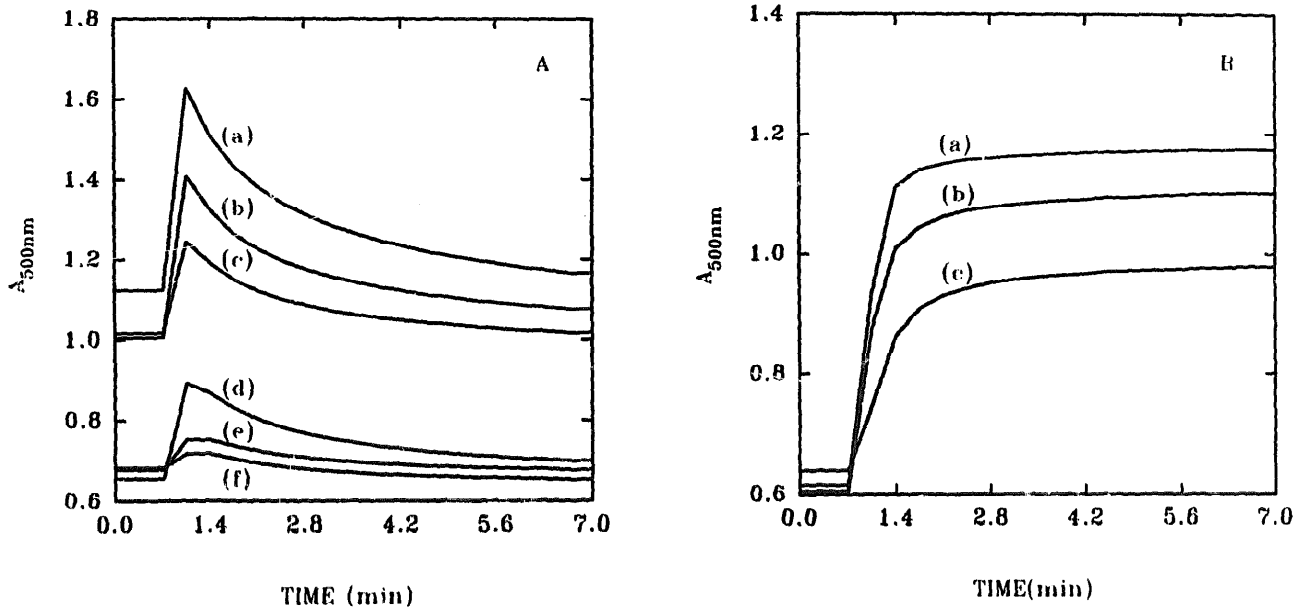


Fig. 5. Kinetics of liposomal shrinkage and swelling in the presence of varying amounts (S_o) of glycerol (a, 0.4 M; b, 0.2 M; c, 0.1 M; d, 0.2 M; e, 0.1 M; f, 0.05 M) for two independent preparations as indicated by the horizontal segments. Details of the experiments are described in the text. (B) Kinetics of liposomal shrinkage in the presence of varying amounts (S_o) of sucrose (a, 0.2 M; b, 0.1 M, c, 0.05 M) as indicated by the figure. Details of the experiments are described in the text.

ments using glycerol as a model diffusible solute. In these studies varying amounts (0.02–0.4 mol) of glycerol were added to the same liposomal preparations at time zero. A rapid initial shrinkage followed by a relatively slow swelling of liposomes was monitored by measuring the absorbance values over a period of 6–7 min. A few representative kinetic curves obtained with two independent preparations of liposomes are shown in Fig. 5A. A set of curves obtained with non-diffusible (or very poorly diffusible) compound like sucrose is included in Fig. 5B to emphasize the salient differences.

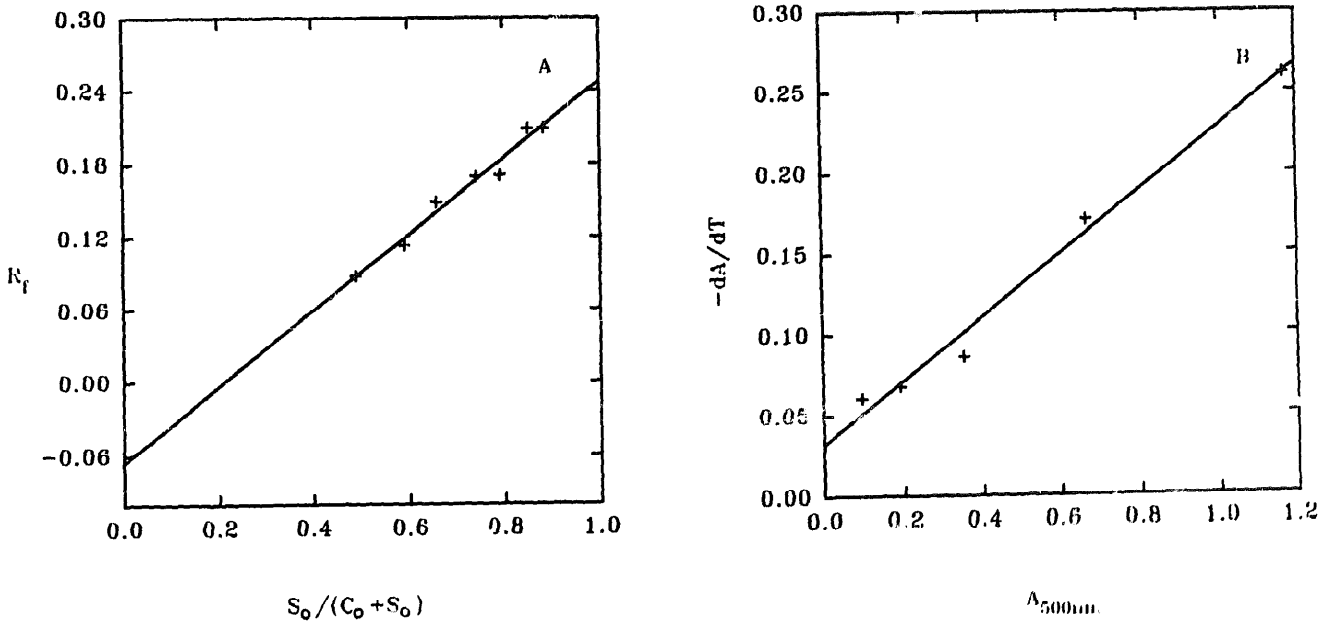


Fig. 6. (A) Fractional decay rates $R_f(=-(1/A)(dA/dT))$ estimated graphically from the data presented in the Fig. 5A are plotted against $S_o/(C_o+S_o)$ in accordance with Eqn. 8, where S_o is the molar amount of glycerol added at time zero and C_o (0.05 M) the osmolarity of the medium. The value of αK_S is estimated to be 0.31 from the slope of the straight line derived from linear regression analysis. (B) The decay rates $(-dA/dT)$ obtained graphically at the maxima in the curves presented in the fig. 3A are plotted against the absorbance values (A) at the peaks in accordance with Eqn. 8. The slope of the straight line is estimated to be 0.196 ($\cong 0.20$).

In Fig. 6A the absolute values of fractional decay rate $(-(1/A)(dA/dT) = R_f)$ are plotted against $S_0/(C_0 + S_0)$. The decay rates $(-(dA/dT))$ were graphically estimated from the slopes of the tangents at the maxima exhibiting highest steepness. C_0 is assumed to be 0.05 mol as inferred from the osmolarity of the media used in the preparation of liposomes. It is apparent from Eqn. 8 that fractional decay rate plotted against S_0 will give rise to a saturation-type curve, and will be approximately linear for $S_0 \ll C_0$.

For a constant value of S_0 Eqn. 8 further predicts that the slope $(-(dA/dT))$ should vary linearly with the absorbance A. The data presented in Fig. 3A are sufficiently explicit to test this prediction. In Fig. 6B, the decay rates $(-(dA/dT))$ at the maxima estimated graphically from the set of curves shown in Fig. 3A are plotted against A. The linear relationship as manifest in the straight line is again reassuring as regards the internal consistency of the formalism.

Quantitative simulation of the kinetic behaviour of liposomal shrinkage and swelling. In the preceding sections an attempt has been made to provide experimental justification for some of the inferences enumerated under 'Theoretical considerations'. In these analyses we have tacitly assumed that the permeability of the solute is much lower than that of the water, i.e., $K_S \ll K_W$. The differential Eqns. 3 and 4, on the other hand, being free from such constraints permit a more rigorous analysis of the shrinkage and swelling behaviour of liposomes in the presence of a diffusible solute (glycerol) over an extended time period. The solid lines in Fig. 7 represent theoretical curves obtained from numerical solutions of Eqns. 3 and 4; experimental points are indicated by the crosses. It is important to note that values of K_W (7.0) and K_S (0.42), basic invariants of the system related to the permeabilities of water and glycerol, respectively, have been kept constant in all these simulations. Values assigned to the 'extrinsic' parameters such as A_L, V and α governed by the liposomal preparations (vesicular size and concentration) are obtained by trial and error, and are indicated in the legend.

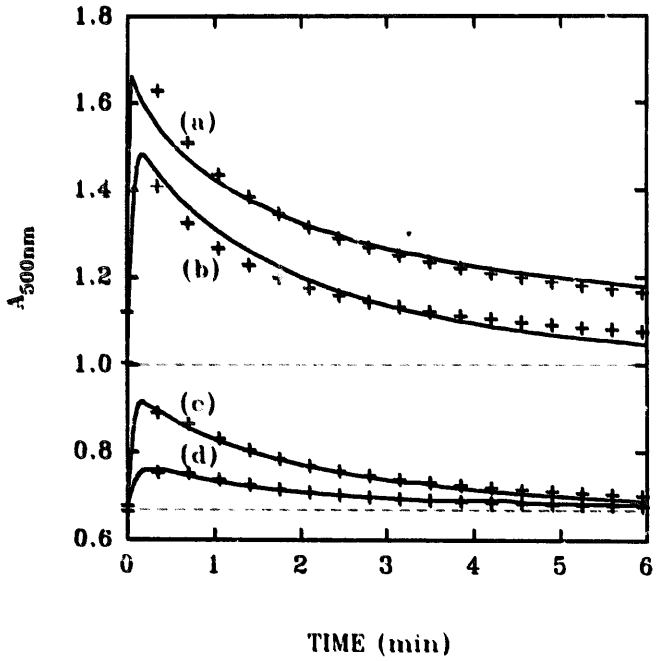


Fig. 7. A quantitative simulation of the kinetic curves presented in Fig. 5A. The values of 'extrinsic' parameters A_L, α and V_0 (initial liposomal volume) used in numerical solutions of Eqns. 3 and 4 were: a (1.8, 1.8, 0.3), b (1.75, 1.3, 0.3), c (1.05, 1.07, 0.2), d (0.85, 0.53, 0.2). Rate constant K_W and K_S related to the permeability coefficients of water and the solute (glycerol) were 7.0 and 0.42, respectively. Experimental values, indicated by the crosses, refer to different amounts (S_0) of glycerol used in these experiments, i.e., a (0.4 M), b (0.2 M), c (0.2 M) and d (0.1 M).

Discussion

The relationship between the absorbance (A), liposomal concentration (L) and the unit volume (v) as defined in Eqn. 1 is based on two basic postulates; (i) the absorbance varies linearly with the concentration of light scattering particulate material and (ii) the observed deviations from the linearity could be explicitly attributed to the optical inhomogeneity of the medium due to the partitioning of the aqueous volume into intra- and extra-liposomal compartments. In choosing an exponential function to define the optical inhomogeneity in (ii) we were admittedly guided by mathematical expediency. The only justification one could offer at this stage is that the inferences derived from such considerations are internally consistent. As regards the curves presented in Fig. 3B it must be emphasized that they are relevant only over a range where the absorbance could be ascribed to the Rayleigh-Debye scattering of light by particulate materials of appropriate sizes. Our objective here has been to indicate the general trend rather than to put an over-much emphasis on the limiting situations lying beyond the permissible domain.

The slopes of the straight lines (a) and (b) in Fig. 3B provide a measure of unit liposomal volumes (v_0) and (v_m) in the initial steady state and at the maxima (Fig. 3A), respectively. From the values of slopes the ratio v_m/v_0 is found to be 0.82. Assuming the osmolarity (C_0) of the suspension medium to be 0.05 M and S_0 as 0.02 M, from Eqn. 6 v_m/v_0 is estimated to be 0.72. It may be mentioned that the condition $K_S \ll K_W$ is reasonably justified for glycerol as a first approximation. We contend to note a fair agreement between the two estimates. Further, an enhanced scattering of light due to the reduced size of liposomes is manifest in a higher value of intercept in (b) as compared to that in (a). The values of the extinction coefficient (E_c) as

inferred from the intercepts in the two cases are found to be 1.56 and 1.23, respectively.

Fig. 6A includes a linear regression analysis of the data obtained for varying amounts (S_0) of glycerol in accordance with Eqn. 8. The slope (0.31) of the straight line provides a direct measure of αK_S. A similar analysis of the data included in Fig. 3A for varying concentrations (L) of liposomes and a constant amount (0.02 M) of glycerol is described in Fig. 6B. Note that in this case the absolute values of slopes are plotted against A, the absorbance at the maxima. Assuming the osmolarity (C_0) of the buffering medium (including dextran) to be 0.05 M and the slope of the straight line in Fig. 6B as 0.196 ($\cong 0.20$), the value of αK_S is found to be 0.7, which, though slightly on the higher side, is of the same order of magnitude as that (0.31) obtained from Fig. 6A. Keeping in mind the inherent complexity of the system and the theoretical constraints implicit in the derivation of Eqn. 8, the correspondence between the two estimates may be considered as fairly satisfactory, and is indeed highly reassuring as regards the internal consistency of the proposed formalism.

The rate constant K_S appearing in Eqn. 4 has a dimension of liter min^{-1}, and is related to the permeability coefficient (P_S) by the equation:

$$P_S = \frac{K_S v}{a}$$

where v and a refer to the volume and the area of liposomal vesicles, respectively. Assuming a spherical shape and a particle size (diameter) of 0.06 μm, P_S is estimated to be $0.42 \cdot 10^{-6}$ cm min^{-1} or $0.7 \cdot 10^{-8}$ cm s^{-1}.

The validity of the fractional decay rate ($-(1/A)(dA/dT)$) as suggested by the present analyses, or that ($-(1/A^2)(dA/dT)$) derived from an inverse relationship between the absorbance and liposomal volume as a measure of relative permeabilities of test solutes are subject to the same constraints, i.e., $K_S \ll K_W$. This implies that the amount of solute incorporated into liposomes during the rising phase of the curve, A versus time, is negligibly small. It is instructive to examine the nature and extent of discrepancies in such estimations due to a relaxation in the above constraint. For this purpose we generated a series of kinetic curves for different values of K_S; the values of all the other parameters including that (0.40 M) of S_0 were kept constant. A few selected curves are included in Fig. 8A. Values of fractional decay rate (R_f) are estimated graphically from these curves and are plotted against K_S in Fig. 8B. It is interesting to note that the curve in Fig. 8B is approximately linear over a limited range for $K_S < 0.5$. As the values of K_S approaches that of K_W the correspondence between R_f and K_S completely breaks down. The limiting value of K_S should be viewed in reference to that (7.0) used for K_W in these simulations. We have explored the possibility of using an appropriate correction factor, albeit an empirical one, in order to extend the range of its applicability. The absorbance values at the maxima varying systematically with K_S offer a convenient pa-

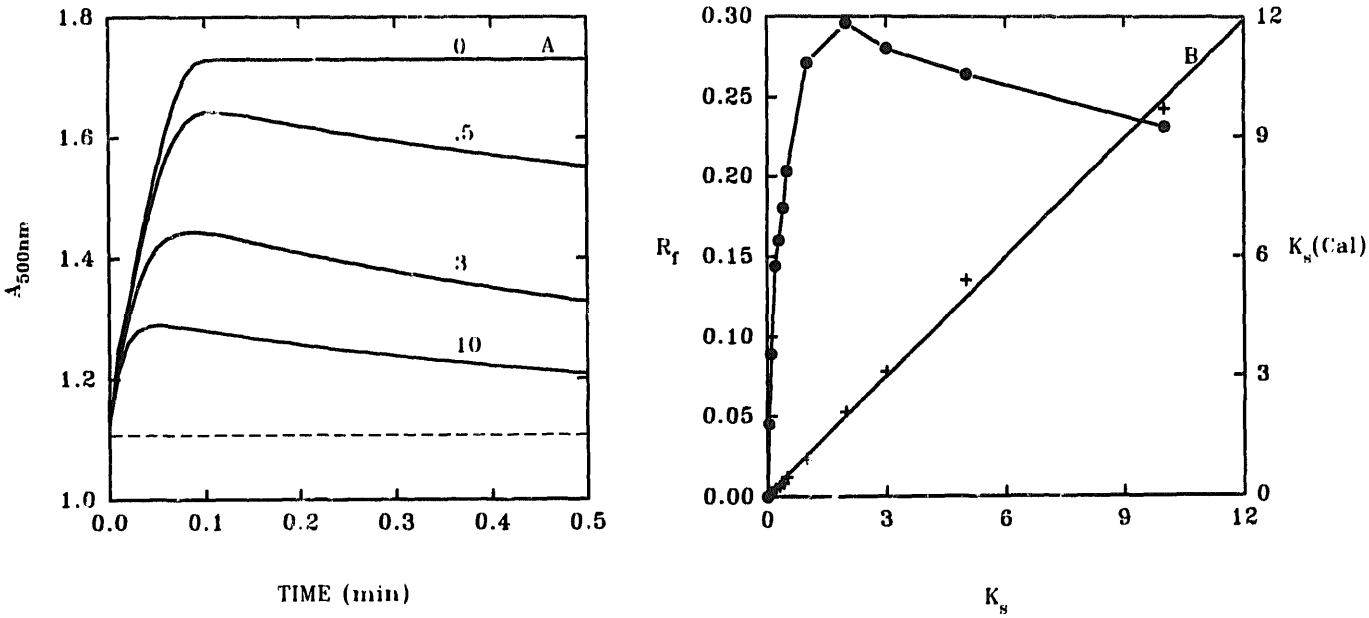


Fig. 8. (A) Effect of solute permeability (K_S) on the kinetic behaviour of liposomal shrinkage and swelling as described by Eqns. 3–5. The values of various parameters (A_L, α, V_0 and S_0) were same as those used for curve (a) in Fig. 7. The values of rate constant K_S used in these calculations are indicated in the figure. (B) Fractional decay rates R_f ($= -(1/A)(dA/dT)$) estimated graphically from the set of curves shown in Fig. 8A are plotted against rate constant K_S, and are indicated by solid circles. Note that only a few selected curves are included in fig. 8A for the sake of clarity. Values of K_S(cal) obtained from Eqn. 9 are indicated by crosses. The line passing through these points was obtained by least square analysis; the slope and the intercept are estimated to be 0.99 and 0.02, respectively.

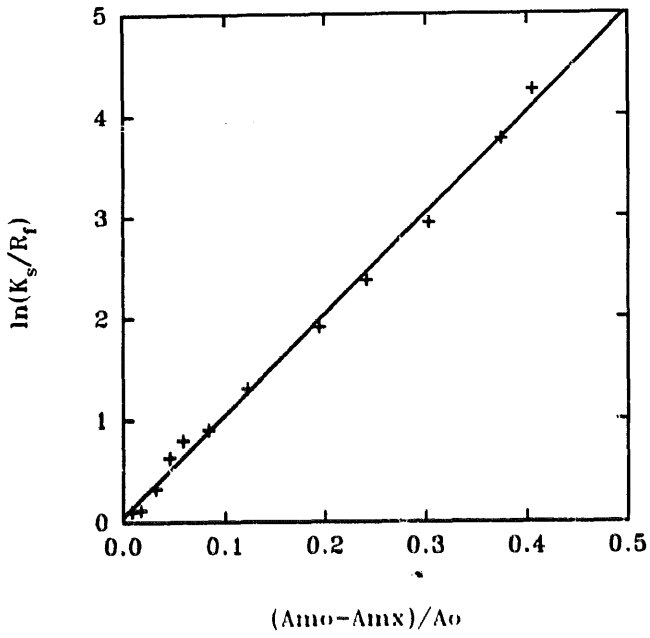


Fig. 9. Eqn. 9 in its linear form offers a convenient method to ascertain the validity of the relationship between kinetic parameters, i.e., R_f, K_S, A_{mo} and A_{mx}. The values estimated graphically from the theoretical curves for different rate constant, K_S presented in Fig. 8A are plotted as $\ln(K_S/R_f)$ vs. $(A_{mo} - A_{mx})/A_o$. δ is estimated to be 9.98 from the slope of the straight line.

rameter for this purpose. We consider the following relationship:

$$K_S(\text{cal}) = R_f\, e^{\frac{\delta(A_{mo} - A_{mx})}{A_o}} \qquad (9)$$

where R_f is the fractional decay rate $(-(1/A)(dA/dT))$ graphically estimated from the curves shown in Fig. 8A; A_{mo} and A_{mx} are the absorbance values at the maxima for a non-diffusible ($K_S = 0$) and the diffusible solutes for different values of K_S used in the simulation, respectively. A_o refers to the absorbance at time zero, and is assumed to be same for all the curves. The constant δ can be readily estimated from the slope of the linear plot, $\ln(K_S/R_f)$ versus $(A_{mo} - A_{mx})/A_o$ shown in Fig. 9. We have compared the values of K_S used in the generation of the curves shown in Fig. 8A with those (K_S(cal)) calculated from the values of R_f in accordance with Eqn. 9. The value (9.98) of δ used in these calculations is obtained from the slope of the straight line in Fig. 9. In Fig. 8B K_s is plotted against K_S(cal). The fact that the slope (0.99) of the straight line derived from least square analysis is close to 1 gives credence to the essential validity of the premises underlying Eqn. 9. We further note that the intercept (0.02) is again close to zero in accordance with Eqn. 9.

In conclusion, we have presented in this paper a viable alternative to the inverse relationship between the absorbance and the liposomal volume, which has been widely exploited by the investigators to analyse the shrinkage and swelling behaviour of liposomes subjected to osmotic-diffusional stress. Experimental data obtained with glycerol as a model solute have been used to test the validity of some of the inferences derived from the proposed formalism.

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