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A SIMPLE PROCEDURE FOR THE DETERMINATION OF THE TRAPPED VOLUME OF LIPOSOMES

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A new method is described for determining the volume of the aqueous compartment of liposomes. Liposomes are prepared in a solution of the fluorescent dye, calcein. The fraction of the total volume that is within the liposomes is obtained as the fraction of the fluorescence that remains after adding cobalt(II) ions which, when chelated by calcein, quench its fluorescence. The method is rapid, simple and accurate. Separation of the liposomes from the medium is not required. The procedure is equally well suited to the assay of permeability characteristics of liposomal membranes.

Introduction

Numerous methods are available for measuring the volume of the aqueous compartment of liposomes [1–3]. Most of these methods involve measurement of trapped marker inside liposomes after the removal of untrapped marker by dialysis, gel filtration, or centrifugation. It is, however, usually also necessary to measure the amount of residual marker present outside of the liposomes, since removal is often incomplete and leakage may occur during washing procedures. An exception is the ESR-based method of Vistnes and Puskin [4]. Here we describe a new method to measure the amount of trapped marker. Not only does it obviate the need for removal of external marker, but it is rapid, simple and utilizes commonly available instruments. The fluorescence of calcein (2′,7′-[(bis[carboxymethyl]-amino)-methyl]-fluorescein), a chelating derivative of fluorescein, is quenched by cations such as cobalt and copper [5]. Thus, when calcein is used as a marker of the aqueous compartment, that which is not trapped inside the liposome need not be removed, since the addition of a quenching cation suffices to eliminate the fluorescence of the external phase. The general procedure should also be useful as an assay for agents that may affect the barrier properties of liposomes. The appearance of fluorescence when the quenched chelate is dissociated with ethylenediamine-tetraacetic acid (EDTA) has been used as an assay for fusion of lipid vesicles [6].

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Chemicals

Chemicals were obtained from the following companies: Sigma Chemical Company, St. Louis, MO (egg yolk phosphatidylcholine, dicetyl phosphate, stearylamine, cholesterol and 4-methylumbelliferyl phosphate); Avanti Biochemicals Inc., Birmingham, AL (bovine brain phosphatidylserine); Radiochemical Centre, Amersham, U.K. (D-[U-^{14}C]glucose); Miles Laboratories, Elkhart, IN (alkaline phosphatase). All lipid pre-

parations were pure by normal silica gel thin-layer chromatography criteria. Calcein (2′,7′-[(bis[carboxymethyl]-amino)methyl]-fluorescein) was a gift from Drs. Harvey Diehl and John Furry, Iowa State University. Material suitable to the present procedure is obtainable from the Hach Chemical Co., Ames, IA.

Preparation of liposomes

Multilamellar liposomes were prepared using standard methods [1]. A solution of egg yolk phosphatidylcholine (5 μmol), dicetyl phosphate (0.5 μmol) and cholesterol (5 μmol) in $CHCl_3$ was dried in an evaporator. After at least 1 h in vacuo, the dried lipid film was swollen in 500 μl of Mops (4-morpholinepropanesulfonic acid)-buffered saline (pH 7.2) containing 50 nmol calcein.

Measurement of the amount of calcein trapped in liposomes

The amount of calcein was determined fluorometrically using a modified Farrand spectrophotometer (Farrand Optical Co., Inc., New York). High sensitivity with negligible interference from light scattering was obtained with the following procedure: All slits were removed and the empty slots were covered with black tape. This resulted in a band width of about 23 nm. 488 nm and 520 nm interference filters with 8 nm band widths were inserted in the excitation and emission paths, respectively. The excitation and emission monochrometers were set at 490 nm and 520 nm, respectively. For other instruments, the procedure would be to use the widest possible slits, insert the interference filters, and set the monochrometers for the largest difference between liposome suspensions with and without calcein.

12.5μl of liposomal suspension (125 nmol as phosphatidylcholine) was diluted to 500 μl with Mops-buffered saline and the fluorescence of the suspension was measured before (F_{tot}) and after (F_{in}) addition of 1.25 μl of $CoCl_2$ (10 mM). F_{tot} represents the fluorescence of all the calcein present. F_{in} is the fluoresecence from the internal compartment plus the unquenched fraction of the external compartment. Subsequently, 25 μl of 10% Triton X-100 was added and the fluorescence measured again. The latter addition destroys the integrity of the liposomes and the resultant fluorescence intensity (F_{totq}) represents the equilibrium concentration of free calcein. Thus, the extent of quenching is obtained directly from the sample itself as F_{totq}/F_{tot}. The trapped volume is calculated from:

$$\left[F_{in}-(F_{totq}\cdot r)\right]/\left[F_{tot}-(F_{totq}\cdot r)\right]\times 100$$

$$=\text{Trapping volume (\% of total)} \quad (1)$$

r is the dilution factor due to the Triton X-100 solution, in the present case, 1.05. The small dilution caused by cobalt addition can be neglected.

Measurement of the amount of glucose trapped in liposomes

The dried lipid film was swollen in 500 μl of Mops-buffered saline containing 2 μCi of [^{14}C]glucose and/or 50 nmol calcein. 100 μl of the liposomal suspension was applied to a Sephadex G-50 column (7.5 mm × 145 mm) and eluted with Mops-buffered saline. The liposome and free glucose fractions were collected and counted in a liquid scintillation counter. When checked, recovery of glucose counts after column chromatography was virtually complete.

Measurement of 4-methylumbelliferyl phosphate released from liposomes

Liposomes composed of egg yolk phosphatidylcholine, cholesterol and dicetyl phosphate (10:10:1, molar ratio) were prepared in a solution of Mops-buffered saline containing 4 mM 4-methylumbelliferyl phosphate. They were then dialyzed against 0.15 M NaCl solution for 4 h to eliminate untrapped marker. 2.5 μl of liposomal suspension (10 mM as phosphatidylcholine) was added to 500 μl of Mops-buffered saline containing 2 units of alkaline phosphatase. The amount of 4-methylumbelliferone generated from 4-methylumbelliferyl phosphate was measured fluorometrically using excitation and emission wavelengths of 340 nm and 448 nm, respectively [7].

Sonication and freeze-thaw treatment of liposomes

Liposomes composed of egg yolk phosphatidylcholine and dicetyl phosphate (10:1, molar ratio) were prepared in Mops-buffered saline containing 10^{-4} M calcein and sonicated at 25 mM

(as phosphatidylcholine) with a probe-type apparatus.

For freeze-thaw experiments, liposomes were frozen in a solid CO_2-acetone bath and thawed at room temperature.

Results

Reduction of fluorescence of calcein induced by cations

Fluorescein and some of its derivatives are known to undergo self-quenching by formation of non-fluorescent dimers (excimers) at high concentrations [8]. The measurements described below require a linear relationship between the amount of calcein and its fluorescence. It is therefore important to establish that self-quenching does not occur at concentrations we propose to use. That excimer formation does not occur to a significant extent at least up to concentrations of $4 \cdot 10^{-4}$ M is indicated by the data of Table I. Liposomes so loaded were passed over a gel filtration column to eliminate untrapped calcein. Addition of Co^{2+} has no effect on the fluorescence of these vesicles, verifying a lack of effect of Co^{2+} on vesicle integrity and that the fluorescence measured is due to entrapped calcein. Disruption of the vesicles with detergent has essentially no effect on fluorescence. Since the resultant dilution of the trapped solution is subtantial, self-quenching at this concentration would have been revealed by an increase in fluorescence. EDTA was included in some of the samples of Table I as a check for the adventitious presence of quenching cations.

TABLE I

FLUORESCENCE OF CALCEIN TRAPPED IN AND RELEASED FROM LIPOSOMES

Liposomes composed of egg yolk phosphatidylcholine, cholesterol and dicetyl phosphate (10:10:1, molar ratio) were formed in Mops-buffered saline containing $4 \cdot 10^{-4}$ M calcein. 200 μl of liposomes (10 mM as phosphatidylcholine) were applied to a Sephadex G-50 column and eluted with Mops-buffered saline. 100 μl of the liposome-containing fractions were diluted to 1.0 ml with Mops-buffered saline or diluted to 0.9 ml with Mops-buffered saline and 100 μl 10% Triton X-100 added. Fluorescence of all samples was measured wthout and with $2 \cdot 10^{-5}$ M Co^{2+}. The final concentration of calcein was about $2 \cdot 10^{-6}$ M.

Sample	Fluorescence (arbitrary units)
Mops-bufferd saline	1
Liposomes (+EDTA)	140
Liposomes + Co^{2+}	138
Liposomes (+EDTA) + Triton X-100	135
Liposomes + Co^{2+} + Triton X-100	2

Calcein is as fluorescent as the parent compound, fluorescein, which is one of the most fluorescent compounds known. Given that excimer formation occurs only at high concentrations of calcein, it is clear that liposomes can be loaded with sufficient calcein that their fluorescence is easily measured. Since the volume inside standard preparations of liposomes is quite small compared with the external volume, an efficient quenching agent is needed to reduce the external fluorescence to where it does not overwhelm the internal fluorescence.

The data of Fig. 1 reveal that Co^{2+} is indeed a very efficient quencher of calcein fluorescence. The open circles in that figure represent the fluorescence of a solution of calcein that has been titrated with $CoCl_2$. Extrapolation of the initial portion of the curve to zero fluorescence yields an intercept of $5 \cdot 10^{-6}$ M, indicating that, although there are two chelating groups on the calcein molecule, only one need bind a cobalt ion for the calcein fluoresence to be quenched. At equimolar concentrations of calcein and Co^{2+} fluorescence is quenched by about 90%. At a 2-fold excess of Co^{2+}, only a few percent of the initial fluorescence remains.

The other symbols in Fig. 1 represent titrations in which liposomes were present. Liposomes having no net charge, as well as both net positive and net negative charges, were tested. As is obvious from the figure, none of these liposomes interferes with the quenching of calcein fluorescence. Nevertheless, caution is necessary with vesicles having very high surface potential. This is especially true of positive vesicles, to which calcein binds (see below).

Copper ion, in addition to cobalt ion, was reported by Steck and Wallach [5] to quench calcein fluorescence. We also obtain results similar to those of Fig. 1 with $CuCl_2$ (data not shown). There are differences between Co^{2+} and Cu^{2+}, however (see below).

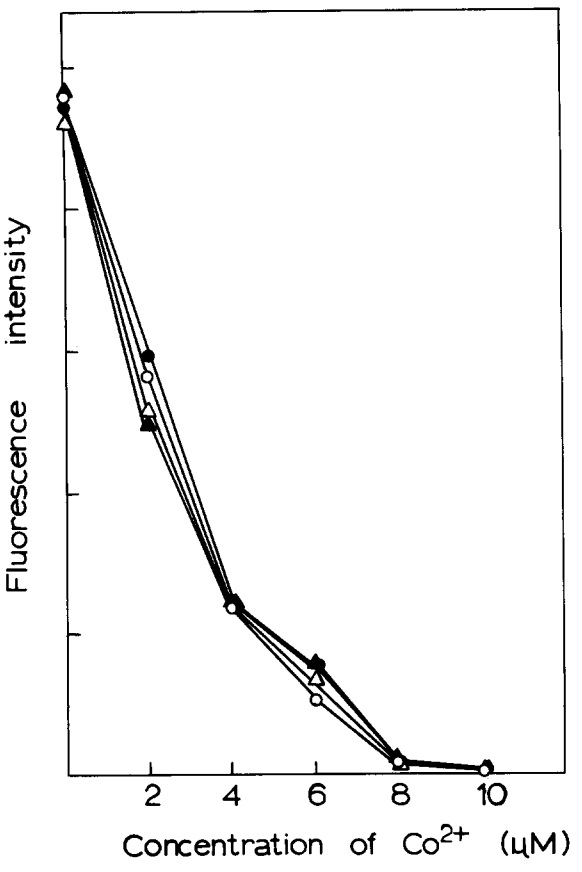

Fig. 1. Fluorescence quenching in the presence of different liposomes. The fluoresence of $5 \cdot 10^{-6}$ M calcein was quenched by various concentrations of Co^{2+} in the absence of liposomes (○) or in the presence of liposomes composed of equimolar phosphatidylcholine and cholesterol (▲), phosphatidylcholine, cholesterol and dicetyl phosphate (10:10:1, molar ratio) (△) or phosphatidylcholine, cholesterol and stearylamine (10:10:1, molar ratio) (●). Liposomes were preincubated with Co^{2+} for 10 min prior to calcein addition. All solutions were made in Mops-buffered saline at pH 7.2. Fluorescence is in arbitrary units.

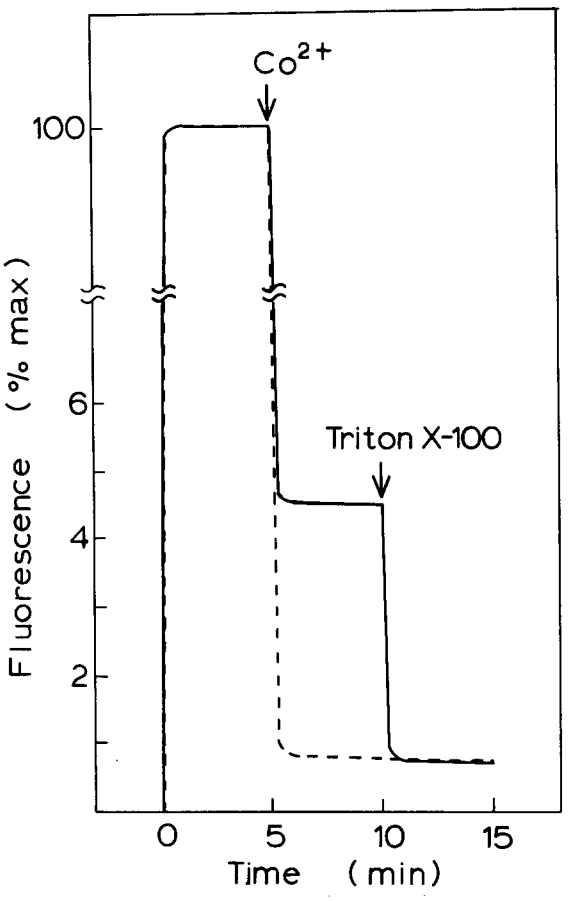

Fig. 2. Fluorescence of trapped calcein after quenching of fluorescence of external calcein. Liposomes composed of egg yolk phosphatidylcholine, cholesterol and dicetyl phosphate (molar ratio 10:10:1) formed in Mops-buffered saline (pH 7.2) containing 10^{-4} M calcein were added to 0.5 ml of Mops-buferd saline at zero time (final concentration of liposomes was $2.5 \cdot 10^{-4}$ M as phosphatidylcholine and final concentration of calcein was $2.5 \cdot 10^{-6}$ M). After 5 min preincubation at room temperature, 1.25μl of 10^{-2} M $CoCl_2$ was added (final concentration of Co^{2+} was $2.5 \cdot 10^{-5}$ M) incubation was continued 5 min at room temperature. 25 μl of 10% Triton X-100 was then added to the cuvette and fluorescence measured at each addition. The dashed line shows the behavior of a calcein solution having the same initial fluorescence as the liposome-containing suspension. The coincidence of the final fluorescence intensity indicates that the extent of quenching is independent of the presence or absence of liposomes.

Normally we use Co^{2+} at a 10-fold excess over calcein ($2.5 \cdot 10^{-6}$ M) to obtain complete quenching of calcein. The turbidity of liposomes, even at 10-times higher concentrations than those given above, does not affect the measurement of the fluorescence of calcein (data not shown).

Procedure for measurement of trapped volume

The data obtained in a determination of the trapped volume of liposomes is shown in Fig. 2. Commensurate with the intense fluorescence of calcein, the reading obtained from the liposomes formed in a calcein solution is very high, however, it is reduced substantially and rapidly by the addition of cobalt ion. Within a few seconds the quenching reaction is over and the attenuator con-

trol of the fluorometer can then be switched to a higher sensitivity. The remaining fluorescence is essentially unaffected by addition of more Co^{2+}. As revealed by the second drop in fluorescence, subsequent addition of detergent allows Co^{2+} access to the calcein that was sequestered within the liposomes. The remaining fluorescence represents the sum of the fluorescence of the small amount of free calcein and the very low fluorescence of the chelate. This can be reduced somewhat by using a very large excess of Co^{2+} or by using Cu^{2+}, which is a more potent quenching ion (see below), but the simplest procedure is to take as baseline, the fluorescence after the detergent addition. The fraction of the total volume that is trapped within the liposomes is given as the ratio of the change upon detergent addition to the difference between the initial and final values of fluorescence. Eqn. 1 is the algebraic form of the preceding statement. In the experiment of Fig. 2, the trapped volume was found to be 3.80%.

Validity of the method

Although the present method provides little opportunity for artifact, we felt it prudent to compare it with an established method for measuring trapped volume. One such method involves counting the amount of entrapped radioactive marker after removal of the external marker by gel filtration. Glucose is a suitable marker. Liposomes containing both radiolabelled glucose and calcein were prepared. Trapped glucose was determined by liquid scintillation counting of an aliquot of liposomes after gel filtration. The percent of the total volume that was trapped is given in Table II as the percent of the original radioactivity found in the total liposome fraction after gel filtration. Using portions of the same sample, the trapped volume was determined using the calcein-based procedure described above. As indicated in the table, the two methods give values that correspond well for three different liposome preparations. As indicated by the data of B, external calcein at 0.1 mM had no effect on the volume as measured by radioactive glucose. Since these data correspond closely to the volume of vesicles with the same composition prepared in the presence of calcein (A, first row), the latter has no effect on vesicle size.

To verify that the trapped volume varies in the expected way with a change in conditions, we sonicated multilamellar liposomes and determined trapped volume as a function of sonication time. Sonication is known to decrease the trapped volume of liposomes [9] due, presumably, to increasing numbers of small vesicles with high surface to volume ratios. Correspondingly, we find, as is shown in Fig. 3, a reduction in trapped volume with increasing sonication time. In accord with our experience that the first few minutes of sonication have the largest effect on average vesicle size [10], the trapped volume is reduced most in the initial sonication periods.

TABLE II

SIMULTANEOUS TRAPPING OF GLUCOSE AND CALCEIN IN LIPOSOMES

(A) Liposomes (10 mM as phosphatidylcholine (PC) or as phosphatidylserine (PS)) were formed in Mops-buffered saline containing 10^{-4} M calcein and a trace of [^{14}C]glucose. Measurement of calcein trapping was as described in the legend to Fig. 2. Glucose trapping was determined as follows. 100 μl liposome suspension was applied to a 7.5 mm × 145 mm column of Sephadex G-50. The column was eluted with Mops-buffered saline and the turbid void volume fractions collected. Continued elution yielded untrapped glucose. Sonication of liposomes was for 30 s with a probe-type sonicator. (B) Liposomes were prepared in Mops-buffered saline containing a trace of [^{14}C]glucose. For the second experiment (bottom), an aliquot was mixed well with 0.1 mM calcein before column chromatography. Glucose trapping was determined as described in (A). DCP, dicetyl phosphate; Chol, cholesterol.

Liposomes composed of	% calcein trapped	% glucose trapped
(A) Egg PC : Chol : DCP (10 : 10 : 1)	4.13	4.07
Bovine brain PS	5.74	5.90
Egg PC : DCP (10 : 1, briefly sonicated)	1.09	0.90
(B) Egg PC : Chol : DCP (10 : 10 : 1)		3.98
Egg PC : Chol : DCP (10 : 10 : 1) (plus external calcein)		3.98

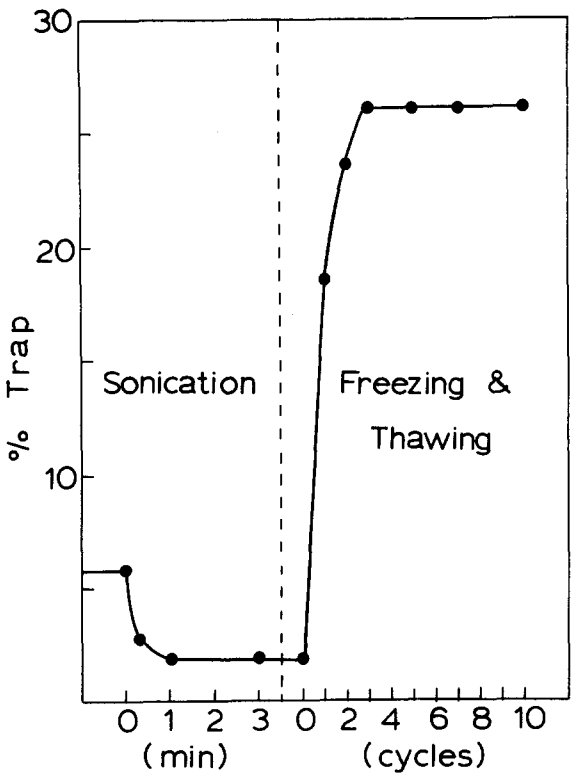

Fig. 3. Effect of sonication on trapped volume. Multilamellar liposomes (25 mM as phosphatidylcholine, prepared in Mops-buffered saline containing 10^{-4} M calcein) were prepared from egg yolk phosphatidylcholine and dicetyl phosphate (10:1, molar ratio). Liposomes were diluted with 1 ml of Mops-buffered saline (final concentration of liposome was $6.25 \cdot 10^{-4}$ M as phosphatidylcholine and final concentration of calcein was $2.5 \cdot 10^{-6}$ M). Fluorescence was measured without or with $2.5 \cdot 10^{-5}$ M Co^{2+}. Quenching of the total calcein was determined after addition of Triton X-100 to 0.5%. The trapped volume was calculated from Eqn. 1. The measurements were repeated with liposomes that had been sonicated with a probe-type apparatus for the times shown in the figure and subsequently subjected to the indicated number of freeze-thaw cycles.

The right side of Fig. 3 depicts the increase in amount of calcein trapped when the sonicated vesicles of the left half of that figure are frozen in dry ice and thawed. The amount of calcein trapped increases with up to three cycles of freezing and thawing and then remains constant. The increase in trapped calcein suggests that large vesicles were generated by freezing and thawing as reported elsewhere [11,12]. However, since a substantial amount of pure water may freeze out before the liposomes undergo rupture and resealing, it may be that the concentration of calcein entrapped in liposomes subjected to such a procedure actually increased over that found in the external solution. If this is the situation, the assay yields the amount of trapped solute rather than the trapped volume.

In addition to cobalt, copper ions also quench the fluorescence of calcein [5]. Since Cu^{2+} commonly forms tighter complexes than Co^{2+}, it could be more effective in the present assay, and we therefore tested both ions. Fig. 4 shows the changes of fluorescence when various amounts of cobalt and copper chlorides are added to liposomes containing calcein at $2.5 \cdot 10^{-6}$ M. The initial reduction in fluorescence induced by the ions is essentially the same. Subsequently, however, the effects of the ions differ, with Co^{2+} fluorescence dropping rapidly to a value that remains practically constant and independent of the concentration of the quenching ion. With Cu^{2+}, on the other hand, the fluorescence continues to fall after the initial decline, and at rate that depends upon the concentration of the ion.

It is evident from Fig. 4 that, in contrast to Co^{2+}, Cu^{2+} gains access to that portion of the total calcein that was trapped within the liposomes. This could occur either because copper ions permeate the bilayer membrane or because they induce disruption of the membrane. We have distinguished between these two possibilities by measuring the leakage rate of 4-methylumbelliferyl phosphate from liposomes. At $2.5 \cdot 10^{-5}$ M $CuCl_2$, the leakage of the umbelliferone derivative was less than 1% during a 20 min. incubation and essentially identical to the leakage rate in the absence of Cu^{2+}. It thus appears that copper ions penetrate lipid bilayer membranes quite rapidly, an unusual phenomenon, given the non-selective electrostatic barrier to movement of ions across media of low dielectric constant [13]. We considered the possibility that cupric ions form a complex with either the buffer or with chloride ions, which might generate a more permeant species. The pattern seen in Fig. 4 for $2.5 \cdot 10^{-5}$ M Cu^{2+} remains essentially the same, however, when Hepes or Tris buffers are substituted for Mops buffer or when chloride ions are entirely replaced with sulfate ions.

The explanations that remain appear to be either that cupric ion forms a complex with a compo-

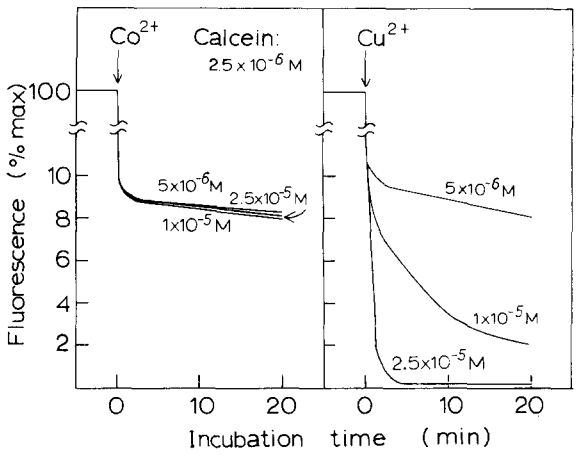

Fig. 4. Comparison of the effects of Co^{2+} and Cu^{2+} on the fluorescence of calcein trapped within the liposomes. Liposomes were prepared as described in the legend of Fig. 2. Various concentrations of Co^{2+} or Cu^{2+} were added to the samples. The concentrations of divalent cations exceeded by 2×, 4× and 10× the concentration of calcein which was $2.5 \cdot 10^{-6}$ M.

nent of the membrane which facilitates its transport or that it is carried by calcein itself. The latter complex would have to be two Cu per calcein in order to provide excess quenching capacity.

Although the calcein assay is generally flexible and accurate, trapped volume results are clearly invalid in the case of vesicles with a substantial positive charge. Experiments with positive vesicles (phosphatidylcholine/stearylamine, 10 : 1 molar ratio) gave anomalously high values of trapped volume, particularly for sonicated samples, where they were about an order of magnitude higher than expected. Such membranes, which were prepared in 10 mM Mops-buffer have surface potential of about 60 mV. While quite high, the corresponding space charge effects (see Discussion) could not account for the high trapped volume found. We therefore considered the possibility that calcein binds to the surface of positive vesicles and is hence effectively concentrated by them. This was verified by microelectrophoresis whereby it was found that the zeta potential was reduced by nearly 10-fold to about 7 mV in the presence of 0.1 mM calcein. Since this concentration of calcein is negligible compared to that of the buffer, a screening effect on the double layer (even given that calcein is multiply charged) is ruled out and binding must have occurred. It is thus clear that the calcein method cannot be used for trapped volume measurements of positively charged vesicles without correcting for binding. As shown by the data of Fig. 1, however, bound calcein is still available for quenching by cobalt ion, and the method is still suitable for applications in which membrane integrity is to be tested.

Discussion

A large proportion of membrane studies involves the preparation of membrane vesicles and subsequent measurement of the rate or extent of communication between the internal phase and the external phase, whether specific, i.e., involving a single species, or general, involving complete communication of the two phases. The former are represented primarily by transport studies [14,15], the latter by investigations of agents that cause disruption or perforation of membranes [16,17]. Recently, another application of membrane vesicles has emerged: the use of liposomes to deliver otherwise impermeant substances into cells by 'microinjection' [18,19]. All of these applications require, or, at least, are benefitted by routine measurements of the volume enclosed by the membranes of the vesicles. The assay described here can be applied to the determination of any membrane-enclosed volume that can be equilibrated with the moderately polar organic heterocyclic compound, calcein. Usually, but not necessarily, such equilibration is accomplished with liposomes by forming the vesicles in a solution of calcein.

Calcein, the molecule introduced here for enclosed volume determinations, has a fluorescence equal to that of the most fluorescent molecules known. The procedure thus has the high sensitivity generally associated with fluorometric procedures. The minimum detectable trapped volume is easily calculated. The lowest concentration of calcein that can be detected without taking special precautions in cleaning glassware and in obtaining purified solvents and other chemicals is about 10^{-8} M. Lipid vesicles can be loaded with calcein to a concentration of about 10^{-3} M without concern for quenching due to excimer formation. Were complete quenching possible, one could thus de-

termine a trapped volume equal to 10^{-5} that of the total volume. About 1 part in 10^3 remains unquenched, however, even in the presence of a several-fold excess of cobalt ion. It is preferable to avoid measurements in which the trapped fluorescence is less than about 10% of the total residual fluorescence after adding the quencher, so the practical limit is nearer 10^{-4} of the total volume. Given typical liposomes and minimal measurement volumes no smaller than 100μl, one could easily work with amounts of lipid on the order of 10 nmol. There are other fluorometric methods, as well as radioisotopic methods, that have comparably high sensitivities, but these require that untrapped material be removed. Because the trapped volume of almost all liposomes or other membrane vesicle preparations is invariably small compared with the total volume, the amount of untrapped marker is correspondingly large compared with the amount of trapped marker. This means that separation procedures must be capable of removing essentially all of the untrapped marker. Although dialysis or gel filtration is capable of good separation of small molecules from vesicles, verifying that complete separation has been obtained is usually tedious.

It should perhaps be emphasized that to accurately apply the method described here, the fraction of the fluorescence in the external compartment that is quenched need not necessarily be close to 1. It is required only that the residual fluorescence not be so much larger than the trapped fluorescence that measurement of the latter becomes uncertain. Since (see Fig. 2) well over 90% quenching can be obtained with micromolar concentrations, the concentrations of the reagents can be very low.

As shown by the data of Fig. 1, at the concentrations necessary for a typical assay, there is no interference of lipid, whether positively charged, negatively charged, or zwitterionic. Positively charged vesicles do bind calcein, however, and although this does not interfere with quenching by Co^{2+}, it does rule out accurate volume measurements on such vesicles. Co^{2+} is known to bind to phosphatidylcholine vesicles extremely weakly [20], and although interaction of Co^{2+} with bilayers would be expected to increase with increasing negative charge density, the integrity of vesicles of phosphatidylserine, a lipid with one full negative charge, is not affected by Co^{2+} at the concentration described here. Nevertheless, we recommend cobalt citrate over cobalt chloride for acidic lipids. Citrate buffers the free cobalt ion concentration and diminishes its potential influence on vesicle stability. The fluorescence of calcein is sufficiently intense and the binding of cobalt ion sufficiently strong between pH 4 and pH 10, that the assay can be used within that pH range, at least.

The quenching reaction is unaffected by NaCl (and presumably also other alkali metal chlorides) at concentrations up to at least 1 M, but multivalent cations may interfere. Since the affinity of calcein for cations can be expected to parallel that of EDTA, the transition metal ions would be expected to provide potential interference. Cobalt and, to an even greater extent, copper ions are generally among the most tightly chelated transition metal ions. Ca^{2+} and Mg^{2+} would thus not be expected to interfere except at very high concentrations and indeed, no competition has been observed from millimolar Ca^{2+}.

A potential disadvantage of the assay desribed here is that it involves a marker that is charged. This means that the amount trapped by vesicles composed of charged membranes can be influenced by the sign and magnitude of that charge [21]. Our results, particularly those with phosphatidylserine vesicles, indicate that electrostatic influences are insignificant at physiological concentration of electrolyte. Thus, the method should be quite adequate for most applications. There remains the potential for error, however, in special cases, for example, very small vesicles of highly charged lipids in solution of low electrolyte concentrations.

Although we have most experience with calcein, investigators who are interested in the procedures described here should be aware of the existence of a related chelating derivative of umbelliferone, namely calcein blue. A potential advantage of calcein blue over calcein is that the former absorbs in a region that encompasses one of the major peaks of mercury vapor lamps. In addition, umbelliferone bleaches much less rapidly than fluorescein. The latter two considerations would recommend calcein blue for investigations under the fluorescence microscope.

In conclusion, we would point out that the assay described here is applicable to the determination of the volume encapsulated by membrane-bounded vesicles and is equally well suited to the determination of changes in that volume. Thus, it could be used to assay loss of membrane integrity induced by lytic agents. A related application is in the assay of vesicle fusion [6].

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