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Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential

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A technique for the rapid production of large unilamellar vesicles by repeated extrusion under moderate pressures (≤ 500 lb/in^2) of multilamellar vesicles through polycarbonate filters (100 nm pore size) is demonstrated. In combination with freeze-thaw protocols where required, this procedure results in unilamellar vesicles with diameters in the range 60–100 nm and with trapped volumes in the region of 1–3 μl/μmol phospholipid. Advantages of this technique include the absence of organic solvents or detergents, the high lipid concentrations (up to 300 μmol/ml) that can be employed and the high trapping efficiencies (up to 30%) that can be achieved. Further, the procedure for generating these 'LUVET's' (large unilamellar vesicles by extrusion techniques) is rapid (≤ 10 min preparation time) and can be employed to generate large unilamellar vesicles from a wide variety of lipid species and mixtures. As a particular illustration of the utility of this vesicle preparation, LUVET systems exhibiting a membrane potential ($\Delta\psi$) in response to a transmembrane Na^+/K^+ gradient (K^+ inside) have been characterized. By employing the lipophilic cation methyltriphenylphosphonium ($MTPP^+$) it is shown that a K^+ diffusion potential ($\Delta\psi < -100$ mV) forms rapidly in the presence of the K^+ ionophore valinomycin for soya phosphatidylcholine (soya PC) LUVET's. The values of $\Delta\psi$ obtained correlate well with the K^+ concentration gradient across the membrane, and it is demonstrated that the decay of $\Delta\psi$ with time depends on the flux of Na^+ into the vesicles.

Introduction

Aqueous dispersions of phospholipid are commonly referred to as 'model membrane' systems. This designation is often optimistic, however, as such dispersions can exhibit a variety of forms which may have little relation to biological membranes. Different lipid species may assume different polymorphic phases such as the bilayer or hexagonal (H_{II}) organization [1], for example, whereas different modes of preparation may result in large or small unilamellar or multilamellar systems among other possibilities.

More faithful 'model membrane' systems must satisfy stringent criteria. Basic requirements are that such model vesicles be closed and unilamellar and that the vesicle be reasonably large to enclose an appreciable trapped volume and to avoid problems [2] associated with very small, highly curved systems. Given such large unilamellar vesicles, subsequent specifications include a transbilayer membrane potential and asymmetric distributions of protein and lipid to achieve a more realistic model of a biological membrane.

Abbreviation: $MTPP^+$, methyltriphenylphosphonium cation.

These demands have either proved difficult to fulfill or have not yet been attempted. A great deal of effort has been directed towards producing large unilamellar vesicle systems but significant problems remain (for review, see Ref. 3). A major problem involves the use of lipid solubilizing agents (organic solvents or detergents). These techniques often require lengthy dialysis procedures which can never completely remove the solvent or detergent employed. Further, a variety of protocols are required depending on the lipid species. For example, the limited solubility of certain lipids (e.g., cholesterol, phosphatidylethanolamine (PE)) in ether or ethanol requires modification of techniques employing these solvents. Alternatively, detergent dialysis procedures employing non-ionic detergents such as octylglucoside are tedious to apply as they can involve several days of dialysis.

A general and straightforward protocol for production of large unilamellar vesicles is therefore required. Preferably, such a technique would avoid the use of solubilizing agents, would produce vesicles of a relatively homogeneous size and would be relatively rapid. In this work we present a procedure which we believe satisfies most of these criteria and have employed these large unilamellar vesicles to model a basic feature of biological membrane systems, namely the presence of a transbilayer membrane potential ($\Delta\psi$). The procedure for generating large unilamellar vesicles involves an extension of the work of Olson et al. [4] who describe a technique whereby the trapped volume of large multilamellar vesicles can be increased by extrusion under relatively low pressures ($\leqslant 80$ lb/in^2) through polycarbonate filters of 0.2 μm pore size. These vesicles are multilamellar with a reasonably homogeneous size distribution centred about the pore size of the filter. It was reasoned that extrusion through smaller pores may result in vesicles sufficiently small that the presence of inner lamellae is unlikely. We show that repeated extrusion under moderate pressures ($\leqslant 500$ lb/in^2) of multilamellar vesicles through polycarbonate filters of 0.1 μm pore size results in a relatively homogeneous population of unilamellar vesicles. These LUVET's (large unilamellar vesicles by extrusion techniques) can be produced rapidly ($\leqslant 10$ min) in a manner which is relatively independent of lipid composition. Further, LUVET's can be generated at high lipid concentrations ($\leqslant 300$ μmol/ml) and can exhibit relatively high trapping efficiencies (up to 30%). These vesicles are employed to obtain a model system exhibiting a membrane potential, and certain aspects of the influence of phospholipid composition on maintenance of $\Delta\psi$ are investigated.

Materials and Methods

Lipids

Egg phosphatidylcholine (egg PC) and soya phosphatidylcholine (soya PC) were isolated employing standard procedures. Corresponding varieties of phosphatidylethanolamine (PE) and phosphatidylserine (PS) were prepared from egg PC and soya PC [5] to produce egg PE, soya PE, egg PS and soya PS. It may be noted that the lipids from the soya source are considerably more unsaturated than those derived from egg PC, due to the high content of linoleic acid in soya PC [6]. All lipids were more than 99% pure as determined by TLC. Acidic phospholipids (PS) were converted to the sodium salt form as described elsewhere [7]. Cholesterol (Sigma, St. Louis) was used without further purification.

Vesicle preparation

Large multilamellar vesicles were prepared by vortexing dry lipid in the presence of the appropriate aqueous buffer. The resulting large multilamellar vesicle dispersion was then transferred into a device (produced by Sciema Technical Services Ltd., Richmond, B.C.) which allowed the extrusion of the large multilamellar vesicles through standard 25 mm polycarbonate filters with 0.1 μm pore size (Nuclepore Corp., Pleasanton, CA). Briefly, large multilamellar vesicles (in 2–5 ml) were injected into a central chamber above the polycarbonate filters, and nitrogen pressure applied via a standard gas cylinder fitted with a high pressure (0–4000 lb/in^2) regulator. The vesicles were extruded through the filter employing pressures of 100–500 lb/in^2 resulting in flow rates of 20–60 ml/min, and were collected and re-injected. The majority of the LUVET preparations were passed through two (stacked) filters ten times. When the freeze-thaw procedure was utilized, the vesicles were sized as above, freeze-thawed (em-

ploying liquid nitrogen) and then sized again through new filters. A total of two freeze-thaw cycles were usually employed. All extrusion procedures were conducted at 20°C except for 16:0/16:0 PC (DPPC) which was extruded at 50°C, 10 deg. C above the gel-liquid crystal transition temperature.

Determination of trapped volumes

To determine trapped volumes, the multilamellar vesicles were prepared in the presence of 1 μCi of ^{22}Na or [^{14}C]inulin (NEN, Canada) and the LUVET's made as indicated above. An aliquot (100 μl) was then loaded onto a Sephadex G-50 column packed in a 1 ml disposable syringe [8], and vesicles eluted by centrifugation of the column at 500 × *g* for 3 min. In the case of ^{22}Na this was sufficient to remove all the untrapped material. However, to remove all the untrapped inulin this procedure was repeated once more. In some cases inulin was removed employing Ultrogel (LKB AcA-34) columns rather than Sephadex G-50. Aliquots of the eluted material were assayed for lipid phosphorus [9]; trapped ^{22}Na was determined employing a Beckman 8000 gamma counter and trapped [^{14}C]inulin was estimated using a Phillips PW-4700 liquid scintillation counter. Trapped volumes calculated are expressed as μl of trapped volume per μmol of phospholipid.

Freeze-fracture

Vesicle preparations were mixed with glycerol (25% by volume) and frozen in a freon slush. Samples were fractured and replicated employing a Balzers BAF 400D apparatus, and micrographs of replicas were obtained using a Phillips 400 electron microscope. Vesicle size distribution were determined by measuring the diameter of fractured vesicles that were 50% shadowed according to the procedure of Van Venetie et al. [10].

^{31}P-Nuclear magnetic resonance

^{31}P-NMR was employed to provide an indication of the extent to which the vesicle preparations were unilamellar. Briefly, Mn^{2+} was added to the vesicle dispersion (3 ml, 30–60 μmol phospholipid per ml in a 15 mm diameter NMR tube) at levels (5 mM) sufficient to a broaden beyond detection the ^{31}P-NMR signal from those phospholipids facing the external medium. If the vesicles are large and unilamellar then 50% of the signal should remain following the addition of Mn^{2+}. The impermeability of the vesicles to Mn^{2+} was straightforward to demonstrate by following the time-course of the signal intensity, which for the PC systems investigated here was stable over a period of days. Spectra were obtained employing a Bruker WP 200 NMR spectrometer operating at 81 MHz. Accumulated free induction decays corresponding to 1000 transients were collected using a 15 μs 90° radiofrequency pulse, gated proton decoupling and a 20 kHz sweep width. An exponential multiplication corresponding to a 50 Hz linebroadening was applied prior to Fourier transformation. Signal intensities were measured by cutting out and weighing spectra with triphenylphosphite (in a small central capillary in the NMR tube) as a reference.

Membrane potential and permeability studies

In order to produce the Na^+-K^+ chemical gradients required to establish a membrane potential, LUVET's (40–70 μmol phospholipid per ml) were made in a KCl buffer (150 mM KCl, 20 mM Hepes, pH 7.4). Subsequently, the untrapped buffer was exchanged for a NaCl buffer (150 mM NaCl, 20 mM Hepes, pH 7.4) by passage through a Sephadex G-50 column which was pre-equilibrated with the NaCl solution. Where employed, valinomycin (Sigma, St. Louis) was added in ethanol to a concentration of 1 μg/μmol phospholipid. The membrane potential generated was measured by determining the distribution of the lipophilic cation [^{3}H]methyltriphenylphosphonium iodide ([^{3}H]MTPPI, obtained from NEN, Canada). Routinely, 1 μCi of [^{3}H]MTPPI in 1 μl ethanol was added to 1–2 ml of a LUVET dispersion which was then incubated at 20°C for 20 min. An aliquot was withdrawn and the untrapped [^{3}H]$MTPP^+$ removed by passage through the 1 ml Sephadex G-50 columns described above. The trapped [^{3}H]$MTPP^+$ was determined by liquid scintillation counting and the phospholipid determined by phosphate assay. A potential difficulty with this procedure concerns the possibility that trapped [^{3}H]$MTPP^+$ leaks out while on the column. However, similar results were obtained employing the ultrafiltration technique detailed

below for ^{42}K in the presence of valinomycin. Knowledge of the trapped volume (see above) allows the concentrations of MTPP inside ($[MTPP^+]_i$) and outside ($[MTPP^+]_o$) the vesicles to be calculated, from which the membrane potential may be calculated employing the Nernst equation:

$$\Delta\psi(mV) = -59 \log \frac{[MTPP^+]_i}{[MTPP^+]_o} \quad (1)$$

The flux of Na^+, K^+ and Cl^- across the LUVET membranes subsequent to establishing the Na^+/K^+ distributions (K^+ inside) were determined employing the radioisotopes $^{22}Na^+$, $^{42}K^+$ and $^{36}Cl^-$ (NEN, Canada) in the absence and presence of valinomycin. Briefly, influx of Na^+ was determined by addition of $^{22}Na^+$ (2 μCi/ml) to the external medium. Aliquots (100 μl) were then removed at various time intervals, the untrapped Na^+ removed by passage over the 1 ml Sephadex columns, and the ^{22}Na influx determined by gamma counting. Efflux of K^+ in the absence of valinomycin was determined by a similar procedure where ^{42}K was initially trapped inside the vesicles. However, when valinomycin was added the column technique could not be employed due to efflux of K^+ while on the column, necessitating an alternative technique. In this case a 10 ml ultrafiltration unit (Amicon Corp.) operated at 5 lb/in^2 and fitted with a YM 10 Diaflo (Amicon Corp.) membrane was employed to separate the LUVET's from the external medium. Vesicles (40 μmol phospholipid per ml, 20 ml total volume) were made in the presence of $^{42}K^+$ and the outside medium exchanged for the NaCl buffer. Valinomycin was added and for each time point 1 ml of the vesicle dispersion was removed and placed in the ultrafiltration unit. Aliquots (100 μl) of the (untrapped) buffer were removed (within 30 s) and the ^{42}K content determined. Knowledge of the initial ^{42}K levels, the lipid concentration and trapped volume then allowed the distribution of K^+ to be calculated.

The Cl^- flux in and out of the LUVET systems was determined by similar procedures as the K^+ and Na^+ flux in the absence of valinomycin.

Results

The influence of repeated passages of (initially) large multilamellar egg PC vesicles through polycarbonate filters of 0.2 μm and 0.1 μm pore size on the amount of egg PC sequestered away from externally added Mn^{2+} is illustrated in Fig. 1. In the case of vesicles passed through the 0.2 μm filter the signal intensity drops to approx. 65% after five passes and then remains relatively constant. This suggests an appreciable population of multilamellar vesicles, a conclusion supported by freeze-fracture results (see Fig. 2(d) and related discussion). Extrusion of large multilamellar vesicles through the 0.1 μm filter, on the other hand, results in reduction in signal intensity to 50% after five or more passes, suggesting the presence of primarily unilamellar vesicles. This conclusion is supported by freeze-fracture studies for LUVET's formed from a variety of phospholipids as indicated in the micrograph of Fig. 2. Vesicles formed from soya PC, soya PC/soya PS (1 : 1) and soya PE/soya PS (1 : 1) (Fig. 2(a), (b) and (c), respectively) do not exhibit a significant number

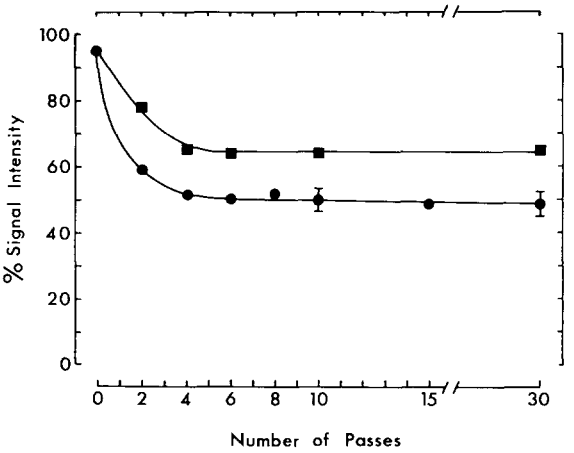

Fig. 1. ^{31}P-NMR signal intensity arising from egg PC multilamellar vesicles (in the presence of 5 mM $MnCl_2$) as a function of the number of extrusions through polycarbonate filters with 100 nm (●———●) and 200 nm (■———■) pore sizes. The error bars represent standard deviations ($n = 6$ for the point at 10 extrusions through the 100 nm filter; $n = 3$ for the point at 30 extrusions). All other experimental points represent the average obtained from two separate experiments. The lipid concentration was 30–60 μmol/ml. For other details, see Methods.

of cross-fractures (< 0.1%) indicating the absence of inner lamellae. This contrasts with the soya PC vesicle system formed employing a 0.2 μm filter (Fig. 2(d)) where such cross-fractures are readily observable.

The size distribution of soya PC LUVET's was determined from freeze-fracture micrographs according to the procedure of Van Venetië et al. [10] and is illustrated in Fig. 3. The half-tone columns represent soya PC LUVET's prepared by

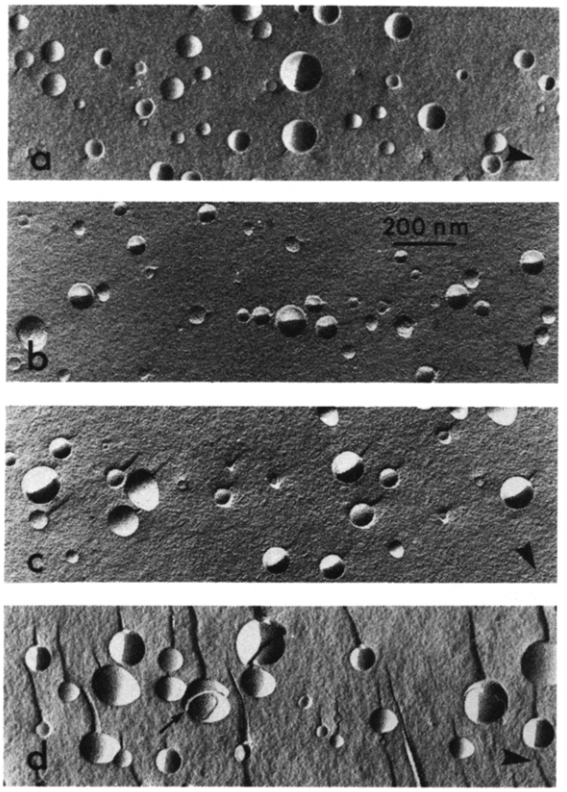

Fig. 2. Freeze-fracture micrographs of vesicles prepared by repeated extrusion of multilamellar vesicles (MLV's) of varying lipid composition through polycarbonate filters: (a) soya PC MLV's extruded through a 100 nm filter; (b) soya PC/soya PS (1 : 1) MLV's extruded through a 100 nm filter; (c) soya PE/soya PS (1 : 1) MLV's extruded through a 100 nm filter; (d) soya PC MLV's extruded through a filter with 200 nm pore size. The arrow in part (d) indicates a cross fracture revealing inner lamellae. All micrographs have the same magnification and the direction of shadowing is indicated by the arrowhead in the bottom right corner of each section. The extrusion procedure was repeated ten times on lipid systems containing 40–70 μmol/ml phospholipid.

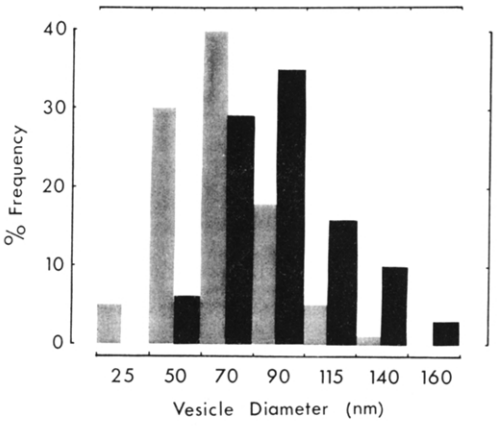

Fig. 3. Size distribution of soya PC vesicles obtained after 10 extrusions through a polycarbonate filter with 100 nm pore size. The vesicle diameters were measured from freeze-fracture micrographs employing the technique of Van Venetië et al. (1981). The half-tone columns represent vesicles that did not undergo a freeze-thaw cycle, the black columns represent vesicles subjected to the freeze-thaw protocol outlined in Materials and Methods.

passing large multilamellar vesicles through two (stacked) 0.1 μm pore size filters ten times. Assuming an area per phospholipid molecule of 0.6 nm^2 [11] and a bilayer thickness of 4 nm [12] and that the vesicles are unilamellar, the trapped volume and amount of inner monolayer phospholipid can be calculated for such a distribution. These estimates can be compared with the experimentally observed trapped volumes and amount of inner monolayer phospholipid to determine the proportion of unilamellar vesicles present. For the vesicle size distribution presented in Fig. 3 (half tone), it can be calculated that such a vesicle population (if unilamellar) would have an 'inner monolayer' signal intensity (after the addition of Mn^{2+}) of approx. 43% of the original intensity and that the trapped volume would be approx. 1.6 μl/μmol. This may be compared with the measured values of sequestered phospholipid (48%) and trapped volume (1.2 ± 0.2 μl/μmol). The agreement between measured and calculated results is perhaps reasonable given the number of assumptions, in particular the area per phospholipid molecule which can greatly affect the trapped volume expressed as μl trapped per μmole of phospholipid. However, as shown in Table I, LUVET's com-

posed of soya PC and egg PC consistently exhibit trapped volumes smaller than those expected from a unilamellar vesicle of the same diameter assuming an area/molecule of 0.6 nm^2 and a bilayer thickness of 4 nm. If a charged phospholipid species such as phosphatidylserine is present, the theoretical trapped volume is achieved. Two possible reasons for the low trapped volumes observed for egg PC and soya PC LUVET's are that there are a significant number of multilamellar vesicles present in the population, or that there are a greater proportion of small vesicles present than estimated from the freeze-fracture micrographs. Freeze-fracture results suggest that the number of multilamellar vesicles is very small (< 2%), even if it is assumed that only 5% of fractured multilamellar systems exhibit a cross-fracture [13]. However, an underestimation of the number of small vesicles is likely.

It has been noted [8] that sonicated vesicles increase in size, but remain unilamellar, following a freeze-thaw cycle. We therefore subjected soya PC and egg PC LUVET's to a freeze-thaw cycle followed by resizing through 0.1 μm pore size filters in order to see if we might reduce the proportion of smaller vesicles in our preparation. The resulting size distribution for freeze-thawed soya PC LUVET's is given in Fig. 3 (black columns). The mean diameter of the population increased by approx. 20 nm. The calculated trapped volume for this vesicle distribution is 2.3 μl/μmol which is in excellent agreement with the measured values for soya PC LUVET's following a freeze-thaw cycle, which fall within the range of 2.0–2.5 μl/μmol (Table I).

These observations lead us to conclude that the large majority of vesicles produced by repeated extrusion of PC large multilamellar vesicles through a filter with 0.1 μm pore size in the absence of a freeze-thaw step are unilamellar, even though the trapped volume is smaller than expected. In order to reinforce this conclusion, we prepared egg PC large unilamellar vesicles by two procedures which are widely accepted to produce unilamellar vesicles and subjected them to the same sizing procedure employed here. As indicated in Table I, the trapped volumes measured for egg PC large unilamellar vesicles produced by the octylglucoside detergent dialysis procedure [14] and the reverse phase evaporation procedure [3], which were subsequently extruded (10 times) through the filter with 0.1 μm pore size, are comparable to the trapped

TABLE I

PHYSICAL CHARACTERISTICS OF VESICLES PRODUCED BY EXTRUSION OF A VARIETY OF LIPID MIXTURES THROUGH FILTERS WITH A PORE SIZE OF 0.1 μm

Lipid	% Intensity [a]	Mean diameter ±S.D. (nm)	Mean trap volume [b] ±S.D. (μl/μmol)
Egg PC	48	71 ± 24	1.1 ± 0.1 (64)
Soya PC	48	70 ± 23	1.2 ± 0.2 (13)
Egg PC/egg PS (2:1)	46	73 ± 25	1.5 (2)
Soya PC/soya PS (2:1)	n.d. [c]	73 ± 20	2.4 (2)
Soya PE/soya PS (2:1)	n.d.	79 ± 36	2.0 (2)
Soya PS	n.d.	n.d.	2.3 (2)
Egg PS	n.d.	n.d.	2.2 (2)
Egg PC (freeze-thaw)	51	77 ± 16	2.2 ± 0.5 (17)
Soya PC (freeze-thaw)	48	94 ± 26	2.2 ± 0.1 (12)
Egg PC (octylglucoside)	49	n.d.	1.2 ± 0.1 (3)
Egg PC (REV)	50	n.d.	1.2 (2)

[a] Intensity of ^{31}P-NMR signal remaining in the presence of 5 mM Mn^{2+}.

[b] μl/μmol phospholipid (number of experiments in parenthesis)

[c] Not determined.

volumes obtained for the egg PC LUVET's. It is pertinent to note that when the octylglucoside procedure was employed to make vesicles consisting of egg PC/cholesterol (1 : 0.25), multilamellar vesicles were formed (results not shown).

Small (sonicated) unilamellar vesicles are a popular model system due, in part, to ease of preparation. However, aside from the small trapped volumes enclosed, these small unilamellar vesicle preparations have been criticized because of the highly curved nature of the membrane which results in increased disorder in the hydrocarbon region [2]. This is reflected in calorimetric studies of small unilamellar vesicles composed of saturated phospholipid by a reduction in the gel-liquid crystalline transition temperature (T_c) and a broadening of the transition [15]. In order to ascertain whether the LUVET systems are sufficiently large to avoid such complications, calorimetric studies were conducted on large multilamellar vesicles and LUVET's composed of 16 : 0/16 : 0 PC (DPPC). As shown in Fig. 4, the large multilamellar vesicles and LUVET's exhibit very similar values of T_c which are also consistent with literature values [16]. This contrasts to the behaviour observed for sonicated DPPC vesicles, which exhibit a broadened gel-liquid crystalline transition which occurs some 4°C below the melting temperature of the multilamellar systems [14]. The absence of a pretransition in the LUVET system may be attributed to the small vesicle size and is similar to behaviour observed elsewhere for large unilamellar vesicle systems of comparable size [17].

An important parameter of large unilamellar vesicle preparations is their trapping efficiency. This is particularly important when the agents to be trapped are either expensive, as is the case for many drugs, or have low solubilities. The LUVET preparations we have described above have a trapped volume of 1–3 μl/μmol phospholipid. Other preparations described in the literature have higher trapped volumes [3] and therefore might be expected to allow better trapping efficiency. However, an advantage of the LUVET protocol is that very high lipid concentrations can be employed. This is demonstrated in Fig. 5 where the percentage of aqueous volume trapped inside the LUVET's is plotted against lipid concentration. Preparation of LUVET's at lipid concentrations of up to 300 μmol/ml is quite possible, giving rise to a 30% trapping efficiency. It is interesting to note that the freeze-thaw cycle only gives rise to signifi-

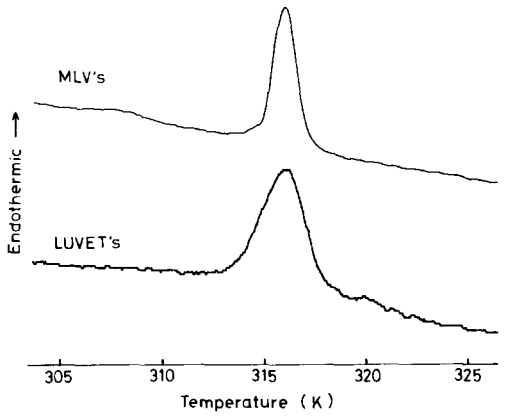

Fig. 4. Calorimetric behaviour of hydrated dipalmitoylphosphatidylcholine (DPPC) in large multilamellar vesicles (MLV) form and in LUVET form. The MLV's were formed by vortexing a dry lipid film in the bottom of a test tube in the presence of the NaCl buffer at 50°C (see Materials and Methods) whereas the LUVET's were formed by repetitive extrusion (10 times) of the MLV's (50 mg lipid/ml) through 0.1 nm pore size polycarbonate filters at 50°C. Scan rates of 2.0 K/min were employed.

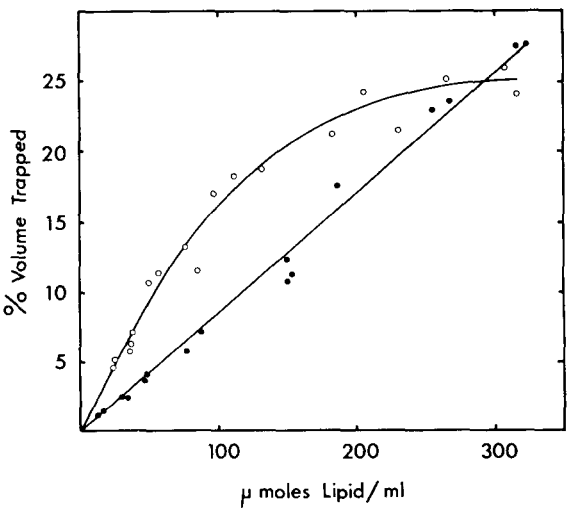

Fig. 5. The trapping efficiency of LUVET's prepared without (●———●) and with (○———○) freeze-thawing. [^{14}C]Inulin was used as a trap marker and LUVET's were prepared at various concentrations of egg PC as described in Materials and Methods.

cant increases in trapped volume per μmol of lipid at lipid concentrations below 200 μmol/ml. Similar types of observations have been reported elsewhere [8]. It should be noted that we have not characterized the unilamellar nature (employing NMR and freeze-fracture techniques) of vesicles prepared at lipid concentrations > 100 μmol/ml.

The LUVET system as characterized above would appear to have a wide range of application due to the absence of contaminants (detergents, organic solvents), the ease of preparation and the generality and reproducibility of the technique. As an illustration of this utility, we have chosen to develop and characterize a simple model membrane system exhibiting a transmembrane potential $\Delta\psi$. Reasons for such a choice include the demonstrated biological importance of $\Delta\psi$ in processes ranging from transbilayer transport to oxidative phosphorylation to protein insertion and the present lack of a well characterized unilamellar model system exhibiting a membrane potential, which would serve as a first step in modelling the more complicated biological situation. For example, the potentials exhibited by biological membranes correspond to very large electric field gradients (a typical value of 100 mV corresponds to a transbilayer electric field of 250 kV/cm), which may be expected to influence the transmembrane distributions of charged lipid and protein components. A simple model system incorporating a membrane potential would provide an appropriate system for studying these interesting possibilities.

The particular model system characterized is an soya PC LUVET preparation where Na^+/K^+ transmembrane chemical gradients (K^+ inside) are established by preparing the vesicles in a KCl buffer and then exchanging the untrapped KCl for an NaCl buffer by gel filtration. Questions addressed concern whether such systems exhibit a membrane potential in the absence of agents (valinomycin) to enhance K^+ permeability, what the influence of valinomycin is, how the magnitude of $\Delta\psi$ is related to transmembrane Na^+/K^+ distributions and how the stability of the potential may be influenced by lipid composition. In order to quantify $\Delta\psi$ we employed the radiolabelled lipophilic cation [^{3}H]MTPP$^+$ which has been used extensively to obtain measures of $\Delta\psi$ in energized biological systems (see, for example, Ref. 18).

As illustrated in Fig. 6, the LUVET preparations experiencing a transmembrane Na^+/K^+ chemical gradient (K^+ inside) do exhibit a membrane potential as detected by determination of outside and inside concentrations of [^{3}H]MTPP$^+$. A $\Delta\psi$ of -20 mV (negative inside) is apparent at the first time interval (30 min) which gradually decreases to -30 mV over eight hours. It may be noted that the equilibrium distribution of MTPP$^+$ across the vesicle membranes was achieved within 10 min (results not shown). As also indicated in Fig. 6, the Na^+/K^+ chemical gradients remain constant within the limits of experimental detection over the incubation period. The Na^+ influx into the soya PC LUVET's remained below detection limits even after a 24 h incubation at 20°C. These results indicate that a membrane potential is established in the soya PC LUVET system in the presence of a transmembrane concentration gradient of Na^+/K^+. This potential presumably arises

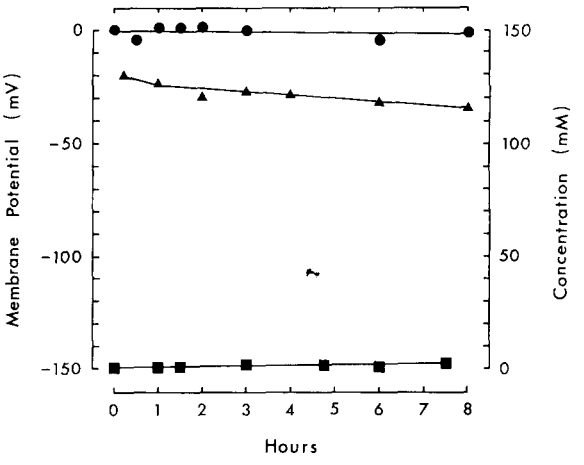

Fig. 6. Time-course at 20°C of the membrane potential (▲) and inside K^+ (●) and Na^+ (■) concentrations in soya PC LUVET's (20 μmol lipid/ml) on application of a Na^+/K^+ chemical gradient (KCl inside; NaCl outside). The gradient was established by preparing LUVET's in 150 mM KCl (pH = 7.0) and subsequent passage through a Sephadex G-50 desalting column equilibrated with 150 mM NaCl (pH = 7.0). The membrane potential was determined by measuring the transmembrane distribution of [^{3}H]MTPP (see Materials and Methods) whereas the interior Na^+ and K^+ concentrations were determined employing ^{22}Na and ^{42}K radioisotopes. Non-specific binding of the probe to LUVET's was measured in control experiments where K^+ was present inside and outside the vesicles, the experimental points were corrected accordingly.

from a difference in the permeability of soya PC LUVET's to Na^+ and K^+, which can create a diffusion potential if K^+ leaks out faster than Na^+ leaks in. This is consistent with the larger permeability coefficients observed for K^+ than for Na^+ in a variety of membrane systems [19,20].

When the K^+ ionophore valinomycin is added to the soya PC LUVET system with Na^+/K^+ chemical gradients, much different behaviour is observed as illustrated in Fig. 7. The (absolute) values of $\Delta\psi$ as reported by [^{3}H]MTPP$^+$ are much larger (120 mV after 30 min incubation in the presence of valinomycin at 20°C), and $\Delta\psi$ decays fairly rapidly for longer incubation periods. In addition, this decay is accompanied by significant Na^+ influx and K^+ efflux. These results indicate that, as expected, valinomycin enhances the K^+ permeability and thus gives rise to larger (absolute) values of $\Delta\psi$, but also that valinomycin enhances the Na^+ permeability which results in dissipation of the K^+ electrochemical gradient as Na^+ leaks in.

In order to demonstrate that the values of $\Delta\psi$ reported by MTPP reflect the K^+ diffusion potential generated, the potentials calculated from the measured interior and exterior concentrations of K^+ ($[K]_i$ and $[K]_o$) were determined employing

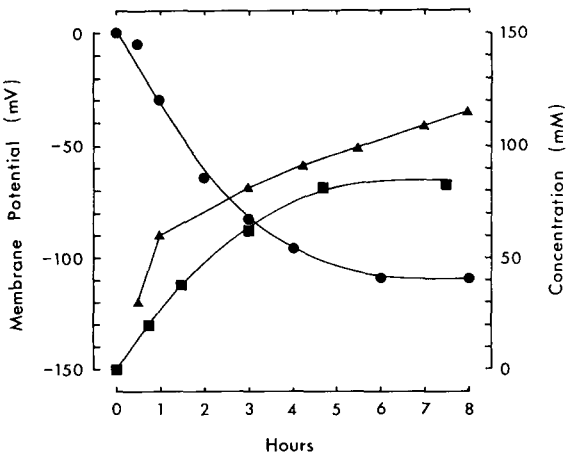

Fig. 7. Time-course at 20°C of the membrane potential (▲) and interior K^+ (●) and Na^+ (■) concentrations in soya PC LUVET's (20 μmol lipid/ml) on application of a Na^+/K^+ chemical gradient (KCl inside, NaCl outside) and in the presence of valinomycin (1 μg/μmol phospholipid). For details, see Fig. 5 legend and Materials and Methods.

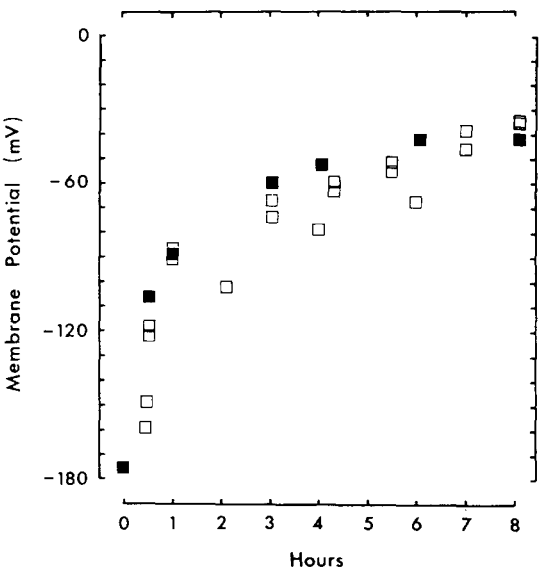

Fig. 8. Time-course of the membrane potential as determined by [^{3}H]MTPP$^+$ (□) and K^+ distribution (■) in soya PC LUVET's on application of a Na^+/K^+ transmembrane chemical gradient and subsequent introduction of valinomycin (see Fig. 5 legend and text for details).

^{42}K as indicated in Materials and Methods. As illustrated in Fig. 8, the values of $\Delta\psi$ obtained from both procedures are in good agreement and exhibit similar time dependent behaviour. For completeness, the inward and outward flux of Cl^- in these soya PC systems was also characterized employing ^{36}Cl (see Materials and Methods). The permeability coefficients calculated from the observed influx and efflux of Cl^- were identical within experimental error ($7.3 \cdot 10^{-11}$ cm · s^{-1} in the absence of valinomycin, $11 \cdot 10^{-11}$ cm · s^{-1} in the presence of valinomycin) indicating that the transmembrane diffusion of Cl^- is electrically silent. It may be noted these permeability coefficients for Cl^- are in close agreement with results obtained employing sonicated egg PC vesicles [21] and egg PC large unilamellar vesicles prepared by detergent dialysis employing octylglucoside [14].

Discussion

This work presents a new procedure for generating large unilamellar vesicles which we have employed to develop a simple unilamellar system

exhibiting a K^+ diffusion potential. We first discuss the LUVET procedure per se and subsequently the utility of a model membrane exhibiting a well defined $\Delta\psi$.

The LUVET systems obtained by extrusion of large multilamellar vesicles through polycarbonate filters of 100 nm pore size appear to possess significant advantages over large unilamellar vesicle systems produced by other means. One of the most important is the absence of residual organic solvent or detergent. While it is arguable whether the low levels of such contaminants present after extensive dialysis significantly perturbs such properties as permeability and dynamic behaviour of component lipids, the situation is clearly less ambiguous when such agents are not present at all. In addition, in applications such as the generation of drug carrier systems, the total absence of these potentially toxic agents is obviously beneficial.

A second advantage of the LUVET procedure concerns the generality of the technique. In our hands all lipid systems thus far investigated which give rise to large multilamellar vesicles on mechanical agitation (vortexing) in the presence of buffer can subsequently be converted to LUVET form employing the extrusion procedure. Further, these systems exhibit a relatively constant size distribution and associated trapped volume, allowing direct comparisons of the properties of unilamellar systems with differing lipid compositions. These features, coupled with the ease and speed of preparation, the range of lipid concentrations that can be employed, the high trapping efficiencies that can be achieved and the relatively gentle nature of the procedure establish it as a most attractive protocol.

One drawback of the LUVET systems as detailed here, however, concerns the relatively low trapped volumes (approx. 1–3 μl/μmol phospholipid) that can be achieved. Although high trapping efficiencies can be attained by increasing the lipid concentration, situations can be envisioned where it is desirable to employ unilamellar systems with increased trap to lipid ratios. In this regard preliminary studies on soya PC systems show that a freeze-thaw of LUVET's prepared by extrusion through the 100 nm pore size filters, followed by extrusion through filters with larger (200 nm) pore size results in larger systems with trapped volumes on the order of 5 μl/μmol phospholipid (Hope, M.J., unpublished data).

The LUVET systems exhibiting a large membrane potential are an important step in achieving more sophisticated and accurate models of biological membranes and understanding the functional roles of individual components. Aside from obvious applications in examining possible roles of various lipid species (such as cholesterol) in the maintenance of $\Delta\psi$ as well as a simple test and calibration system for development of fast and accurate probes of membrane potential, this system exhibiting a $\Delta\psi$ should allow several aspects of membrane structure and function to be examined in a direct manner. Three examples include the influence of $\Delta\psi$ on transbilayer distributions of charged lipid and protein components, the role of membrane potential in protein insertion [22,23] as well as relationships between $\Delta\psi$ and certain membrane transport processes. With regard to the latter area we have demonstrated massive uptake of safranine and other lipophilic cations into LUVET systems in the presence of a membrane potential as detailed in the following paper [24]. Finally, the application of a membrane potential or pH gradient to large unilamellar vesicle systems reconstituted with specific membrane transport proteins could also provide simple assay procedures for transport mechanisms relying on K^+ or H^+ symport or antiport processes.

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