Liposomes as Drug Carriers
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Novel procedures for generating and loading liposomal systems

MARCEL B. BALLY, MICHAL J. HOPE, LAWRENCE D. MAYER, THOMAS D. MADDEN and PIETER R. CULLIS
Department of Biochemistry, The University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia, V6T I W5, Canada

1. INTRODUCTION

Applications for lipid vesicles as carriers of biologically active agents have been frequently proposed. However, a clinically useful liposomal preparation has not yet been achieved. This is due, in part, to an inadequate understanding of the therapeutic advantage which can be obtained by utilizing liposome-drug carriers. Initial expectations regarding the use of targeting molecules to generate a liposomal 'magic bullet' have not been fulfilled. While vesicle preparations exhibiting covalently bound antibodies have applications for diagnostic assays (Martin and King, 1985), ***in vivo*** studies clearly indicate that liposomal deposition is primarily dictated by interaction with the reticuloendothelial system (RES) (Poste, 1983; Poste ***et al.***, 1985). Although circulation half-life and biodistribution is influenced by liposome size (Senior ***et al.***, 1985), composition (Juliano and Layton, 1980) and site of injection (Patel, 1985; Perez-Soler, ***et al.***, 1985), most vesicles are ultimately cleared by the phagocytic cells of the RES. This obstacle has led to a more realistic use of liposomal systems to deliver biologically active agents to the cells of the RES.

Employing this approach, investigators have utilized liposome-drug complexes to treat parasitic, bacterial and viral infections of the RES (Alving and Swartz, 1985; Koff and Fidler, 1985). In addition, immunomodulating agents entrapped in liposomes show therapeutic promise for potentiating macrophage-mediated destruction of cancer metastases (Fidler ***et al.***, 1982; Talmadge ***et al.***, 1986; Fidler

and Schroit, 1984; Deodhar et ***al.,*** 1982). Finally, several studies have demonstrated reduced toxicity and/or increased therapeutic efficacy for a variety of drugs delivered in a liposomally encapsulated form (Alving et ***al.,*** 1978; Lopez-Berestein ***et al.,*** 1984; 1985, Weinstein and Leserman, 1984; Shek and Barber, 1985; Juliano and Lopez-Berestein, 1985). The mechanism whereby liposomes buffer toxicity is not understood but may be related to changes in the biodistribution of entrapped drug, which will presumably be influenced by the RES.

Given these realistic therapeutic applications, the next problem concerns the appropriate liposomal system. The initial basis for liposome technology was established employing multilamellar vesicles which form spontaneously when phospholipids are dispersed in excess water. Since that time a variety of more sophisticated preparations have been developed. Currently, therefore, investigators can select a system which suits their particular application, according to the size, composition, charge, lamellarity, trapped volume and solute distribution desired (Hope ***et al.,*** 1986).

With respect to the liposomal carrier system, ease of preparation, reproducibility and efficient encapsulation and retention of the biologically active agent must be considered. Other considerations concern the desired biodistribution of liposomes, which is dependent on vesicle size and composition. In addition, other practical aspects must be considered, including stability, cost and industrial scale-up. This chapter will focus on techniques we have developed which address these problems. In particular, straightforward protocols for producing multilamellar and unilameilar vesicles and procedures for efficiently encapsulating bioactive agents will be presented. Finally, methods whereby these preparations can be stored in a dehydrated form with retention of their structural and permeability properties are described.

2. LIPOSOMAL PREPARATIONS

In current usage the term liposome is applied to any dispersion of lipid in water. Clearly, this fails to distinguish, between variables such as size, lamellarity and trapped volume. In the following sections we will discuss how these parameters can be defined and modulated. A discussion of the appropriate techniques to determine these parameters has been provided elsewhere (Hope ***et al.,*** 1986).

2.1. **Frozen and thawed multilamellar vesicles (FATMLVs)**

Phospholipids dispersed in water spontaneously form concentric bilayers separated by narrow aqueous compartments (Bangham ***et al.,*** **1965).** For zwitterionic phospholipids the resulting trapped volume (aqueous volume enclosed by the lipid bilayers) is small, less than $1\ \mu l\ \mu mol^{-1}$ lipid. While it was initially assumed that solutes entrapped within the aqueous space were in equilibrium across all the lamellae, recent studies have shown that the entrapped

solute concentration is less than that found in the exterior solution (Gruner et al., 1985; Mayer ***et al.,*** 1986d). Equilibrium solute distribution can, however, be promoted by procedures which optimize phospholipid hydration (Gruner ***et al.,*** 1985; Shew and Deamer, 1985; Szoka and Papahadjopoulos, 1980). These procedures result in preparations which exhibit larger trapped volumes (> 5 $\mu l\ \mu mol^{-1}$ lipid), but are technically difficult and often rely on the use of organic solvents. We have developed a simple procedure involving the repetitive freezing of MLVs in liquid nitrogen followed by thawing at40 **°C.** The resulting MLV preparation (FATMLVs) shows equilibrium solute distribution and exhibits a dramatic increase in trapped volume (Mayer ***et al.,*** 1986a). The morphological changes associated wth the freeze-thaw protocol are illustrated by freeze fracture micrographs shown in Fig. 1. In comparison to MLVs (Fig. l(a)), displaying the characteristic 'onion-ring' structure indicative of closely packed bilayers, FATMLVs (Fig. l(b)) give rise to cross-fractures which show large interlamellar spaces. The vesicle within a vesicle structure is characteristic of this preparation and is not observed with MLVs. As would be expected for these structural changes, there is a substantial increase in trapped volume for FATMLVs. At lipid concentrations of 100 mg ml^{-1} trapped volumes of greater than 5 $\mu l\ \mu mol^{-1}$ lipid are obtained (see Table 1). As a result, aqueous trapping efficiencies are in excess of 65 percent. At higher lipid concentrations (400 mg ml-r), one can achieve trapping efficiencies approaching

Table 1. Characteristics of FATMLV and VET preparations

Technique	Lipid composition	Lipid conc. (mg ml-r)	Mean diameter	Trapped vol. ($\mu l\ \mu mol^{-1}$)	% Trapping efficiency
MLV	EPC	100	> 1 μm	0.47	5.8
FATMLV	EPC	100	> 1 μm	5.30	65
		400	> 1 μm	1.80	89
	EPC/Chol (1: 1)	100	> 1 μm	5.54	
	EPC/EPS (1:l)	50	> 1 μm	14.1	95
	DMPG/DMPC (3:7)	40	> 1 μm	17.6	94
	EPC/EPE (1:l)	50	> 1 μm	3.2	22
	EPE/EPS (1: 1)	50	> 1 μm	7.20	48
VET_{100}	EPC	100	103 ± 20 nm	1.5	19
		400	N.D.	1.20	58
	EPC/EPS (1: 1)	100	73 ± 20 nm	1.5	20
	EPC/Chol (1: 1)	100	120 ± 20 nm	1.5	
	DSPC/Chol (1: 1)	50	N.D.	0.8	
VET_{400}	EPC	100	243 ± 91 nm	3.3	44
		400	N.D.	1.5	80
VET_{200}	EPC	100	151 ± 36 nm	2.1	28
VET,,	EPC	100	68 ± 19 nm	1.0	13
		400	N.D.	0.94	50
VET_{30}	EPC	100	56 ± 17 nm	1.0	13
	DSPC/Chol	50	N.D.	1.1	

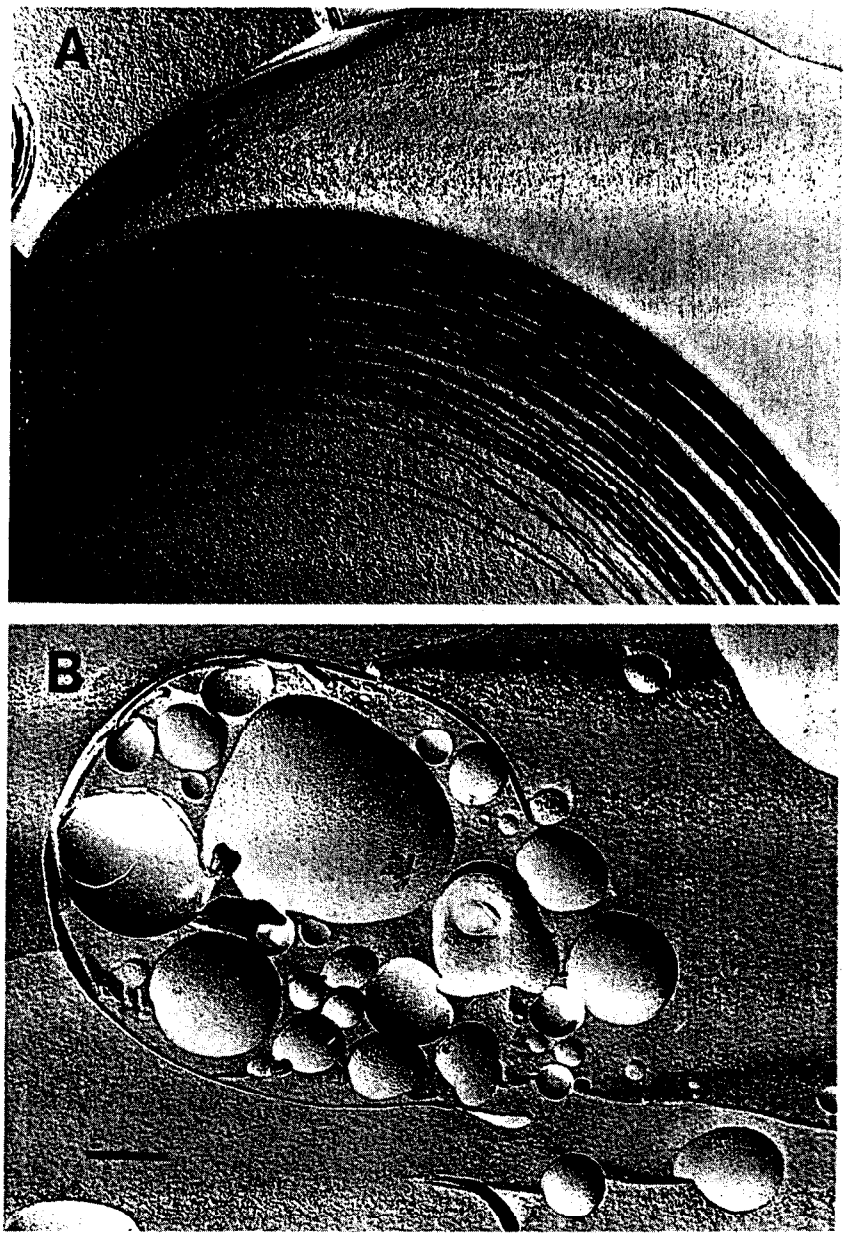

Fig. 1. Freeze-fracture electron micrographs of MLVs (a) and FATMLVs (b). Egg PC (100 mg ml^{-1}) was hydrated in 150 mM NaCl buffer. FATMLVs were obtained after subjecting the sample to five freeze-thaw cycles. The bar represents 200 nm

Table 2. Bioactive agents encapsulated using FATMLV and/or VET procedures

Technique	Lipid composition	Bioactive agent	Trapping efficiency (%)	T_{50} (h)
Passive				
FATMLV	EPC	DNA (pRSVCAT)	20	N.D.
VET_{100}	EPC	DNA (pRSVCAT)	22	N.D.
	EPC	Dopamine	30	<1
	EPC	Cytosine Arabinoside	33	18
	EPC	Methotrexate	33	50
	EPC	Adriamycin	30	1
	EPC/Chol (1: 1)	Adriamycin	30	1
Active				
FATMLV	EPC ($\Delta\psi$)	Adriamycin	up to 94	20
	EPC (Δ pH)	Adriamycin	up to 98	>24
VET_{100}	EPC ($\Delta\psi$)	Adriamycin	up to 99	16
	EPC/Chol (1:1, $\Delta\psi$)	Adriamycin	up to 95	30
	EPC/EPS (8:2, $\Delta\psi$)	Adriamycin	up to 95	6
	DPPC/Chol (1:1, $\Delta\psi$)	Adriamycin	up to 95	36
	EPC ($\Delta\psi$)	Vinblastine	up to 90	N.D.
	EPC ($\Delta\psi$ or ΔpH)	Dibucaine	up to 98	N.D.
	EPC ($\Delta\psi$ or ΔpH)	Chlorpromazine	up to 90	N.D.
	EPC ($\Delta\psi$)	Dopamine	up to 90	N.D.
	EPC (Δ pH)	Dopamine	up to 60	N.D.
	EPC ($\Delta\psi$ or ΔpH)	Serotonin	up to 40	N.D.
	EPC ($\Delta\psi$ or ΔpH)	Epinephrine	up to 40	N.D.

90 percent, although the measured trapped volume is reduced ($2\,\mu l\,\mu mol^{-1}$ lipid).

The mechanism whereby freezing and thawing converts MLVs into FATMLVs is not fully understood, but is presumably related to bilayer ruptures due to ice crystal formation. This procedure is effective for a variety of lipid compositions (Table 1). Preparations containing charged lipids exhibit trapped volumes in excess of $10\,\mu l\,\mu mol^{-1}$ lipid, even when present at only 2mol percent (Hope et *al.*, 1986). Further, the marked increases in trapped volume are observed even in the presence of cholesterol. The relative simplicity of this protocol and the absence of solubilizing agents constitutes a considerable advantage over other procedures.

2.2. **Vesicles produced by extrusion techniques (VETS)**

One drawback to the FATMLV procedure is the large and heterogeneous size distribution of vesicles produced. More to the point, therapeutic applications may require formulations of homogeneous size as well as an ability to obtain high aqueous trapping efficiencies. Recently we have shown that large

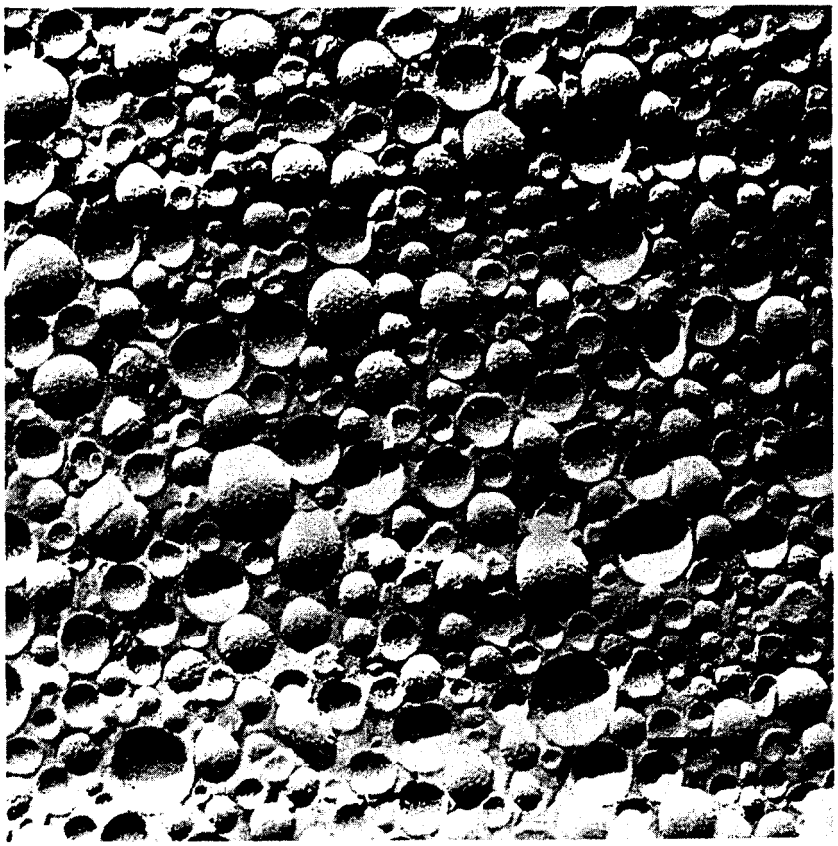

Fig. 2. Egg PC VET,, systems prepared at 400 mg lipid per ml of buffer. Frozen and thawed MLVs were passed through two stacked 100 nm pore size filters ten times. In preparation for freeze-fracture the sample was diluted 25 percent in glycerol. The bar represents 200 nm

unilamellar vesicles can be obtained by repeated extrusion of MLVs through two stacked polycarbonate filters of 100 nm pore size under moderate nitrogen pressure (Hope ***et al.,*** 1985). The resulting preparation consists of a homogeneous distribution of large unilamellar vesicles (diameter of approximately 90 nm) with trapped volumes of l-3 $\mu l\ \mu mol^{-1}$ lipid depending on lipid composition (Table 1). This procedure is applicable to a wide range of lipid compositions including samples with high cholesterol content and/or saturated lipid species (Sommerman ***et al.,*** 1986). A distinct advantage of the extrusion technique is that high lipid concentrations, up to 400 mg ml- **!,** can be used allowing for excellent trapping efficiencies (> 50 percent) (Mayer ***et al.,*** 1986c). This is impressively illustrated by the freeze-fracture micrograph in Fig. 2 which is of $\mathbf{VET_{100}}$ systems (vesicles

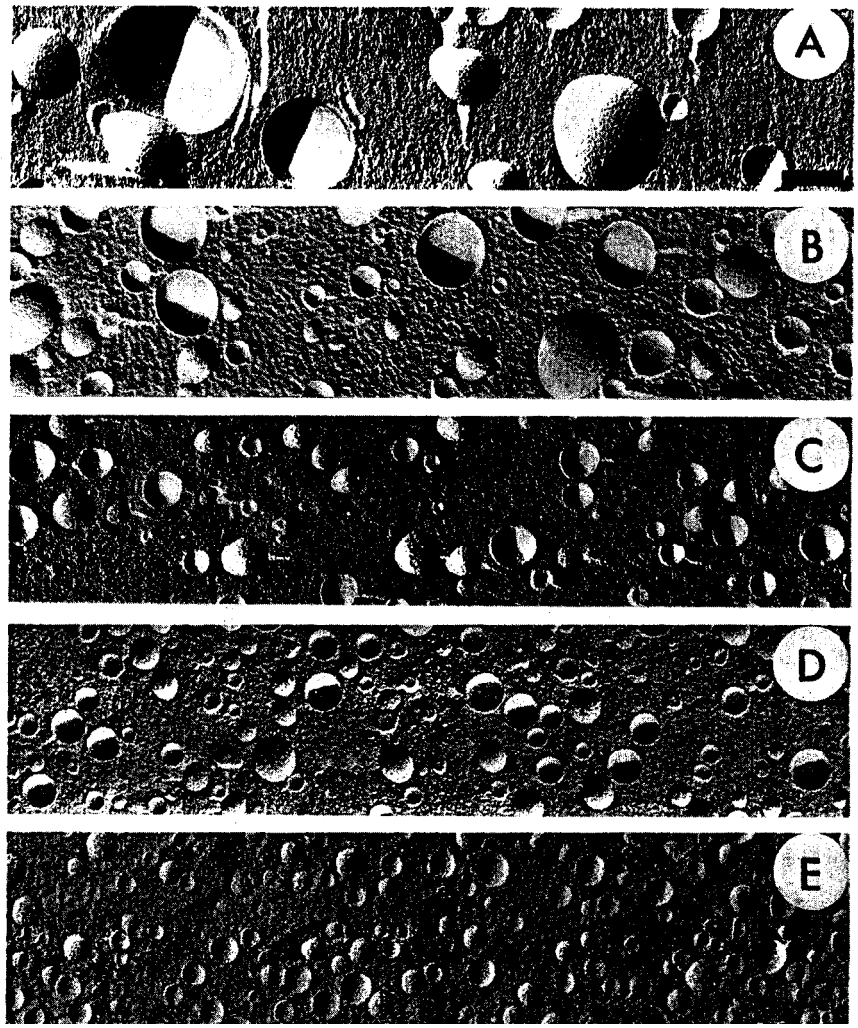

Fig. 3. Freeze-fracture electron micrographs of FATMLVs passed 20 times through filters of various pore sizes. Vesicles were prepared at 100 mg egg PC per ml of buffer and were subjected to five freeze-thaw cycles. Subsequently, the samples were extruded under pressure (100-1200 lb in^{-2}) through filters with pore sizes of 400 (A), 200 (B), 100 (C), 50 (D) and 30 (E) nm. The bar represents 150 nm and all panels exhibit the same magnification

sized through 100 nm pore size filters) prepared at 400 mg ml^{-1} and diluted 25 percent with the cryoprotectant glycerol.

In combination with the freeze-thaw protocol and by utilizing different pore size filters, a variety of vesicle preparations with different but homogeneous size distributions can be obtained (Mayer *et al.,* 1986c). As illustrated in Fig. 3,

vesicles can be prepared with mean diameters ranging from 50 to 250nm. Characteristics of these vesicle preparations are summarized in Table 1. Although VET.,,,,, systems are not unilamellar, their trapped volume (3.4 $\mu l\ \mu mol^{-1}$ lipid) allows for trapping efficiencies which approach 80 percent. The VET_{200} system is approximately 90 percent unilamellar and enables aqueous trapping efficiencies of 60 percent.

By using MLVs or FATMLVs as precursors the extrusion technique eliminates the need for solvents or detergents which may be difficult to remove. In addition, the procedure is rapid with preparation times typically less than 15 min. Further the range of vesicle sizes produced appears well suited for ***in vivo*** applications. For example, VET_{30} systems (liposomes prepared through 30nm pore size filters) appear to behave similarly to SUVs, demonstrating increased circulation half-life and significant deposition in the 'carcass', possibly in the bone marrow (Sommerman et ***al.,*** 1986).

3. DRUG ENCAPSULATION

Two approaches to drug encapsulation can be taken. Passive trapping of hydrophilic molecules occurs during vesicle formation, where the trapping efficiency is governed by the aqueous trapped volume of the vesicle system employed and the lipid concentration. Alternatively, passive encapsulation can result from specific drug-lipid interactions related to a drug's charge and/or hydrophobicity. A second entrapment procedure which we have developed relies on the ability of certain drugs to redistribute across the lipid bilayer in response to a transmembrane ion gradient. The following discussion will be limited to this active trapping technique and passive trapping procedures which utilize the liposomal preparations described above. Specific attention will be given to parameters such as trapping efficiencies, drug/lipid ratio, drug retention, stability, and ease of the procedure. A more comprehensive review of encapsulation techniques has been published recently (Mayer et ***al.,*** 1986d).

3.1. Passive trapping techniques

In passive trapping procedures for hydrophilic molecules the encapsulation efficiency is a reflection of the trapped volume and lipid concentration, and thus the expected trapping efficiency of FATMLVs and VET systems can easily be calculated from the data in Table 1. Both the freeze-thaw protocol and extrusion techniques are mild procedures and should not adversely affect bioactive agents, including antibiotics, DNA and certain proteins. In addition, harsh, potentially toxic solubilizing agents are not required. With respect to FATMLVs it may be noted that no other encapsulation procedure which relies on passive entrapment results in trapping efficiencies of almost 90 percent.

Homogeneous populations of unilamellar vesicles of 50-160 nm can be prepared readily using the extrusion procedure. **While the trapped volumes of these are significantly less than for FATMLVs, the high lipid concentrations which can be employed allows trapping efficiencies in excess of 50 percent. The ability of these vesicles to retain a passively trapped drug will, however, depend on its membrane permeability. This is indicated in Table 2 which shows the**

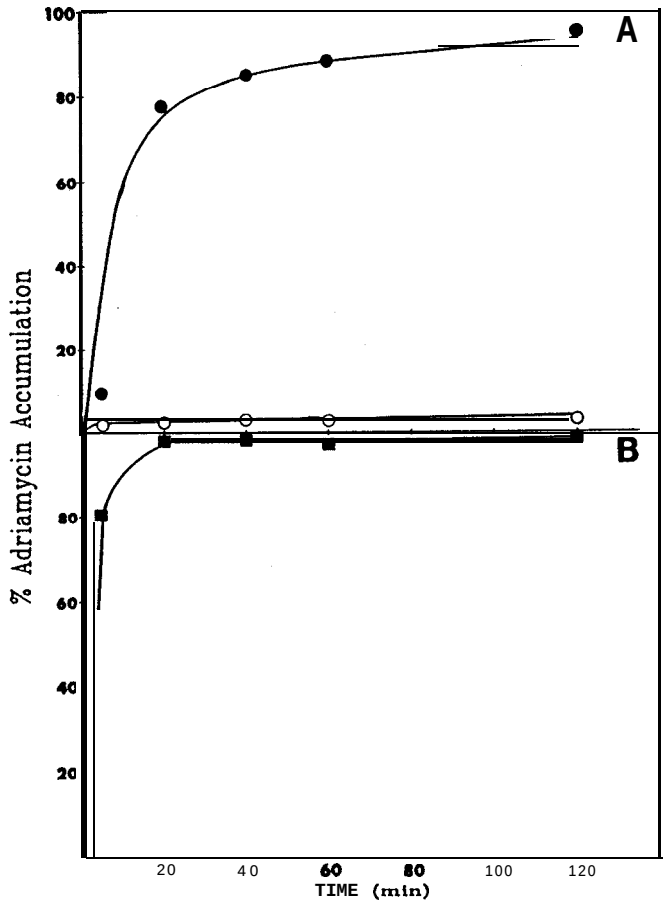

Fig. 4. Uptake of adriamycin into VET,, systems exhibiting a transmembrane $\Delta\psi$ **(A)** or ΔpH (B). The $\Delta\psi$ was created through a valinomycin-induced K^+ diffusion potential. Egg PC vesicles were prepared in K+-containing buffer which was subsequently exchanged for a Na+-containing buffer. Prior to addition of adriamycin (200 n mol μmol^{-1} lipid) valinomycin was added to achieve a final concentration of 0.5 $\mu g\ \mu mol^{-1}$ lipid. A transmembrane ΔpH was established by preparing the vesicles in **pH** 4.5 buffer then exchanging this for a buffer at pH 7.5. Adriamycin was added at a concentration of 100 nmol μmol^{-1} lipid

retention times for three antineoplastic agents. While methotrexate and cytosine arabinoside are relatively impermeable with retention half-times of 50 and 18 h, respectively, adriamycin is released much more rapidly from the same type of vesicles. Thus for many applications, passive entrapment of a drug will be inappropriate and we therefore developed techniques whereby the agent is 'actively' encapsulated.

3.2. **Active trapping procedures**

A variety of drugs, including adriamycin, have molecular characteristics similar to probes of membrane potentials in that they are charged and lipophilic. We have shown that in the presence of a transmembrane potential, these compounds can accumulate inside vesicle systems to achieve transmembrane distributions dictated by the magnitude of the ion gradient in a manner similar to that observed for probes of membrane potential (Bally et ***al.,*** 1985). The net result of this 'active' loading process is a large transbilayer drug gradient and an impressive encapsulation efficiency. Fig. 4 illustrates the results of this trapping protocol for the antineoplastic agent adriamycin in $\mathbf{VET}_{100}$ systems composed of egg PC. Both a transmembrane K+ and H+ ion gradient promote adriamycin accumulation, but unlike the $\mathbf{H}^+$ diffusion potential, an ionophore (valinomycin) is required to establish a $\mathbf{K}^+$ diffusion potential. Therefore, therapeutic applications for this trapping procedure will presumably be restricted to vesicle systems exhibiting a transmembrane H^+ (pH) gradient. By employing a A pH of 3, adriamycin can be actively loaded to achieve a trapping efficiency of 98 percent (Mayer et ***al.,*** 1986b) and drug:lipid molar ratios of 1:5. Further, when adriamycin is actively entrapped using this procedure, its subsequent retention characteristics are remarkably improved (see Table 2).

This active loading procedure has been employed to accumulate other anti-cancer agents (Bally et ***al., 1985), local*** anaesthetics (Mayer ***et al.,*** 1985a), &blockers and biogenic amines (Bally ***et al.,*** 1986) and is likely applicable to a variety of other drug classes. The procedure can be employed not only for loading VET systems of different sizes but also with MLV systems. Further, different lipid compositions, while influencing the rate of uptake, do not appear to affect the extent of accumulation. As indicated in Table 2, however, retention characteristics change drastically depending on vesicle composition. As a final indication of the general utility of this active loading procedure, it should be noted that drug 'cocktails' can be entrapped in vesicles by combining both active and passive trapping techniques. For example, we have shown that $\mathbf{VET}_{100}$ systems with passively entrapped methotrexate or cytosine arabinoside can actively accumulate adriamycin in response to an ion gradient (Mayer et ***al., 1985).*** In general, a particularly important advantage gained by this active trapping procedure is that drug encapsulation occurs after vesicle preparation. This 'remote' loading could allow pharmacists to encapsulate labile drugs just

prior to administration, thus minimizing many stability problems inherent in passive trapping procedures.

4. STORAGE AND STABILITY

As a final consideration, pharmaceutically acceptable and commercially viable drug carrier systems will have to be available in a stable from which is easily stored. In this regard, we have demonstrated that FATMLVs and VET systems can be dehydrated in the presence of certain disaccarides, the most effective being trehalose (Madden ***et al., 1985).*** Egg PC VET,, systems prepared in the presence of 250 mM trehalose which have undergone a dehydration-rehydration cycle display the same size and trapping characteristics with no apparent morphological changes. Further, greater than 90 percent of the entrapped contents are retained on rehydration.

The general utility of this protocol can be illustrated for the adriamycin liposome preparation. Vesicles actively loaded with adriamycin can be dried in the presence of trehalose and subsequently rehydrated with minimal drug loss. Further, the release characteristics of the drug are not altered in these rehydrated preparations. Remarkably, H+ and K+ ion gradients are maintained during the dehydration process allowing the accumulation of adriamycin subsequent to rehydration. Vesicles have been stored in powdered form for up to two years with no loss of ion gradients upon rehydration. With respect to 'remote' loading protocols discussed above, this dehydration procedure is particularly applicable.

5. CONCLUDING REMARKS

We have detailed procedures for generating liposome-drug carriers that satisfy many of the criteria necessary to establish them as pharmaceuticals. It is probable that the most immediate therapeutic applications will concern the ability of liposomes to buffer the toxicity of entrapped drugs. Even the most ardent skeptics (Poste, 1986) agree that this application holds significant promise.

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