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Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential

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Many drugs exhibit lipophilic and cationic (basic) characteristics. Previous studies have shown that lipophilic cations can be accumulated into model membrane 'liposomal' (vesicular) systems in response to establishing a membrane potential (inside negative) across the vesicle membrane. We demonstrate here that the anticancer drugs, adriamycin and vinblastine, can be rapidly accumulated into egg phosphatidylcholine large unilamellar vesicles in response to a valinomycin-dependent K^+ diffusion potential ($\Delta\psi$) to achieve high effective interior concentrations. Further, trapping efficiencies approaching 100% can be easily achieved. The influence of lipid composition and the requirement for valinomycin have been examined for adriamycin. Equimolar cholesterol levels inhibit the uptake process at 20°C. However, incubation at higher temperature results in enhanced uptake. Similarly, the presence of egg phosphatidylserine or incubation at elevated temperatures results in significant adriamycin uptake in the absence of valinomycin. It is shown that the adriamycin retention time in the vesicles is enhanced by an order of magnitude or more when actively trapped by the presence of a membrane potential in comparison to passive trapping procedures. It is suggested that such active trapping procedures may be of use for loading liposomal systems for drug delivery applications, and may provide avenues for controlled release of encapsulated material.

Introduction

Liposomal carrier systems have significant potential for the in vivo delivery of encapsulated drugs [1–3]. However, many difficulties remain before this potential can be realized. Particular problems include the efficient production of an appropriate liposomal carrier, efficient encapsulation and retention of drugs of biological interest, developing methods to avoid non-specific uptake by the reticuloendothelial system and mechanisms to target the carrier to specific tissue. These are all major problems and it is clear that a logical step-by-step development is required before successful delivery can be achieved.

A basic requirement for a liposomal carrier is that it can be generated rapidly and reproducibly and is of a size that can be efficiently endocytosed by target cells. Previous studies [4,5] indicate that vesicles of approx. 1000 Å diameter or less exhibit reduced endothelial adsorption and may be more readily accumulated in target tissues compared to larger liposomal systems. Many procedures exist for the generation of such vesicles. However, most procedures suffer significant drawbacks. These include low trapping efficiencies, the presence of

Abbreviations: LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; MLV, multilamellar vesicle; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

residual toxic agents (organic solvents, detergents) employed to generate the vesicles, as well as the often tedious and time-consuming protocols involved. However, in a recent work [6] we have demonstrated a procedure for the rapid generation of large unilamellar vesicles (LUVs) which involves extrusion of preformed MLVs through polycarbonate filters (1000 Å pore size) under moderate pressures. This procedure, which does not employ organic solvents or detergents, results in LUVs of approx. 90 nm diameter for which trapping efficiencies in excess of 30% can be easily achieved.

The next step in the generation of an appropriate carrier system involves the demonstration of an ability to encapsulate and retain drugs of clinical interest in lipid vesicle systems. This is not a trivial problem, as a large fraction of commonly employed drugs are weak bases with lipophilic characteristics (lipophilic cations). These lipophilic properties can allow relatively free permeation through lipid bilayers, resulting in rapid leakage of the drug from vesicular carrier systems.

In a previous study [7] we have demonstrated an ability of LUV systems to rapidly and efficiently accumulate certain species of lipophilic cations (commonly employed as membrane-potential probes) when a membrane potential ($\Delta\psi$, inside negative) is generated across the vesicle membrane. Preliminary investigations suggested that clinically important drugs with lipophilic and cationic properties can also be accumulated and retained by a similar mechanism. In the present study we characterize the ability of LUV systems exhibiting a membrane potential to actively accumulate and retain the cationic anticancer drugs, adriamycin and vinblastine. The ability of LUV systems to passively entrap drugs which do not have lipophilic or cationic characteristics (methotrexate, cytosine arabinoside) is also investigated. We show that the presence of a K^+ diffusion potential can result in the rapid accumulation of adriamycin or vinblastine into LUV systems with entrapment efficiencies approaching 100%. Further, this active trapping procedure results in retention times an order of magnitude (or more) longer than can be achieved by passive trapping procedures. Finally, an ability of LUVs containing passively entrapped methotrexate or cytosine arabinoside to actively accumulate the lipophilic, cationic drugs is demonstrated. This results in membrane encapsulated 'drug cocktails'.

Materials and Methods

Materials. Egg PC was purified from hen egg yolk using established procedures. Egg PS was prepared from egg PC as described previously [6]. Cholesterol, valinomycin, vinblastine, Hepes, dipalmitoylphosphatidylcholine and salts were obtained from Sigma Chemical Co. Tritiated methotrexate, DPPC and methyltriphenylphosphonium as well as ^{14}C-labelled inulin were purchased from New England Nuclear. Tritiated cytosine arabinoside was obtained from Amersham. Methotrexate, adriamycin and cytosine arabinoside were generous gifts of Dr. Alan Eaves (Cancer Research Centre, Vancouver, B.C.).

Reagents. Potassium glutamate and NaCl buffers were prepared in a 20 mM Hepes buffer adjusted to pH 7.5 with NaOH. The solutions were adjusted to a common osmolarity of 310 mosM/kg, which corresponded to NaCl and potassium glutamate concentrations of 150 and 169 mM, respectively.

Vesicle preparation. Vesicles were prepared according to the extrusion procedure described by Hope et al. [6]. Briefly, dry lipid films were hydrated with the appropriate buffers to produce large multilamellar vesicles at concentrations ranging between 25 and 200 μmol phospholipid/ml. These dispersions were subsequently extruded ten times through two (stacked) polycarbonate filters (Nuclepore) with a pore size of 0.1 μm. The resulting preparation was freeze-thawed twice and resized through the filters utilizing five additional passes. These LUVs had an average diameter of 90 nm and a trapped volume of approx. 1.5 μl/μmol phospholipid.

Generation of transmembrane potentials. Membrane potentials were generated by forming LUVs in the potassium glutamate buffer and subsequently exchanging the untrapped buffer for the NaCl buffer employing Sephadex G-50 desalting columns [6]. Where employed, the potassium ionophore, valinomycin (1 mg/ml ethanol), was added to achieve a concentration of 0.5 μg per μmol lipid. The membrane potential resulting in these

systems was determined utilizing the membrane potential probe methyltriphenylphosphonium as described previously [7].

Drug uptake employing membrane potentials. Adriamycin and vinblastine were added to LUV dispersions of defined concentration in the presence or absence of Na^+/K^+ gradients (K^+ inside) and valinomycin. At various times, the non-sequestered drug was removed by passing aliquots of the solution over 1 ml Sephadex G-50 columns. Lipid and drug concentrations were then assayed. Adriamycin was quantitated by mixing an aliquot of the column effluent with 0.5% Triton X-100 (which disrupted the vesicles and released the trapped drug) and monitoring the absorbance at 480 nm employing a Pye Unicam SP8-200 spectrophotometer. Vinblastine was assayed by determining the absorbance at 265 nm of the eluate dissolved in 94% ethanol. Lipid concentrations were determined by liquid scintillation counting to quantitate [^{3}H]DPPC (0.05 μCi/μmol lipid).

Passive drug entrapment. The antineoplastic agents, methotrexate and cytosine arabinoside, were trapped passively by preparing vesicles in a potassium glutamate buffer containing the drug. Tracer amounts of radiolabelled drug (2 μCi/ml) were included as required. Phospholipid concentrations were determined by established procedures [8].

Drug release experiments. Release of adriamycin from LUVs subsequent to active or passive trapping was assayed as follows: vesicles (10 mM phospholipid) containing adriamycin were first passed over a 15 ml gel filtration column equilibrated in the appropriate buffer to remove free drug. The eluate was then placed in a flow dialysis apparatus equilibrated at 37°C. Flow rates were adjusted to achieve total exchange of the sample compartment volume (50 ml) in 20 min. Aliquots (100 μl) were removed at various times and untrapped material was separated employing 1 ml gel filtration columns. The sample was then assayed for adriamycin and phospholipid.

Results

Previous work from this laboratory has shown that LUV systems exhibiting a K^+ diffusion potential (inside negative) can accumulate lipophilic cations such as safranine and methyltriphenylphosphonium, leading to high interior concentrations of the accumulated agent [7]. The K^+ diffusion potential is readily established by trapping a K^+ buffer in the LUV interior and then resuspending the LUVs in a Na^+ buffer to which the K^+ ionophore, valinomycin, is added subsequently. This leads to values of $\Delta\psi$ in the range -100 to -170 mV. Subsequent incubation of these LUVs with safranine, for example, results in accumulation of the cation to achieve interior concentrations in excess of 100 mM for initial exterior safranine concentrations of 2 mM.

Our initial experiments were aimed at determining whether similar $\Delta\psi$-dependent uptake processes can be observed for adriamycin and vinblastine. Two LUV systems were employed, the first exhibiting a transmembrane electrochemical Na^+/K^+ gradient (K^+ inside), whereas the second did not (K^+ buffer inside and outside). In the case of adriamycin, these vesicle systems (1 mM phospholipid) were incubated in the presence of 0.2 mM adriamycin ($\pm$ valinomycin) for various times, after which free drug was removed by gel filtration. The eluate was then assayed for phospholipid and adriamycin (see Materials and Methods). As shown in Fig. 1, in the absence of a Na^+/K^+ electrochemical gradient or valinomycin, low (≤ 6 nmol adriamycin/μmol phospholipid) LUV-associated adriamycin levels are observed over the 2 h incubation period. However, in the presence of a valinomycin-induced K^+ diffusion potential, a remarkable increase in the amount of vesicle-associated adriamycin is observed (Fig. 1). This uptake is more than 75% complete within 20 min and reaches an equilibrium level of 190 nmol adriamycin/μmol phospholipid. This indicates that 95% of the drug initially contained in the solution is taken up by the vesicles, reflecting a corresponding trapping efficiency of 95%. By analogy with previous studies [7,9] this uptake almost certainly reflects accumulation of adriamycin into the inner monolayer or interior aqueous compartment of the LUV systems, corresponding to interior concentrations of 127 mM adriamycin. This is comparable to uptake levels of safranine into egg PC LUVs in the presence of a K^+ diffusion potential [7], which appears to proceed by a K^+-lipophilic cation antiport exchange process.

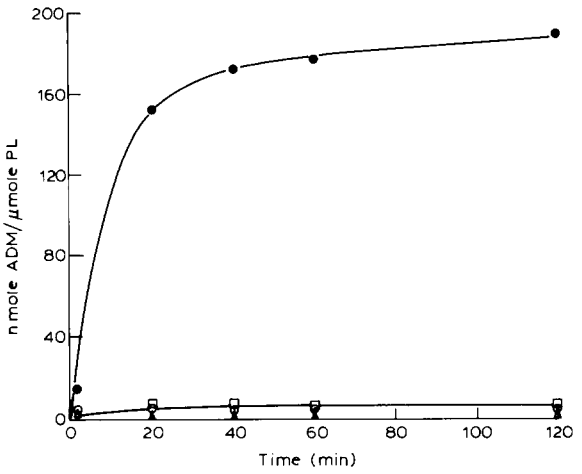

Fig. 1. Uptake of adriamycin (ADM) into large unilamellar vesicles in the presence and absence of K^+ diffusion potential. Egg-PC LUVs (1 μmol phospholipid/ml) were incubated at 20°C in the presence of 0.2 mM adriamycin. Drug uptake was quantitated as described in Materials and Methods. Experimental conditions were as follows: (▲) vesicles with 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) in the internal and external medium; (○) as (▲) plus valinomycin; (□) vesicles having 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) in the internal medium and 150 mM NaCl, 20 mM Hepes (pH 7.5) in the external medium; (●) as (□) plus valinomycin.

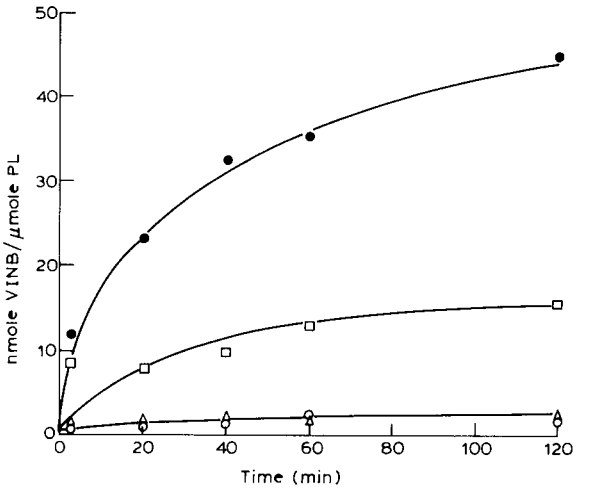

Fig. 2. Uptake of vinblastine (VINB) into large unilamellar vesicles in the presence and absence of a K^+ diffusion potential. Egg-PC LUVs (1 μmol phospholipid/ml) were incubated at 20°C in the presence of 0.2 mM vinblastine under the following conditions: (▲) 169 potassium glutamate, 20 mM Hepes (pH 7.5) as the interior and exterior buffers; (○) as (▲) but in the presence of valinomycin; (□) 150 mM NaCl, 20 mM Hepes (pH 7.5) in the exterior medium and 169 mM potassium glutamate in the vesicle interior; (●) as (□) but in the presence of valinomycin. Drug uptake was assayed as described in Materials and Methods.

Vinblastine can also be accumulated into LUV systems in response to a membrane potential, as illustrated in Fig. 2. In the presence of valinomycin and an Na^+/K^+ transmembrane chemical gradient, 40 nmol vinblastine/μmol phospholipid are accumulated within 2 h, as compared to little or no uptake in the absence of a Na^+/K^+ gradient. It may be noted that appreciable uptake is obtained in the presence of a Na^+/K^+ gradient and the absence of valinomycin. Similar behaviour has been observed for other lipophilic cationic drugs such as the local anaesthetics, dibucaine and chlorpromazine [7,9], and may be attributed to a drug-induced increase in K^+ permeability.

In summary, the results of Figs. 1 and 2 reveal a remarkable ability of LUV systems to sequester adriamycin and vinblastine in response to a K^+ diffusion potential. Under the conditions employed, this uptake corresponds to transmembrane drug concentration gradients of 200 and 20000 for vinblastine and adriamycin, respectively. Furthermore, these gradients were stable for 48 h or longer at 20°C.

The efficiency of the $\Delta\psi$-driven drug uptake process was further characterized by varying the amount of drug available. As shown in Fig. 3, increasing the initial adriamycin concentration between 0 and 10 mM, while maintaining a fixed vesicle concentration (1 mM phospholipid), revealed that the $\Delta\psi$-dependent adriamycin uptake process saturated at approx. 400 nmol adriamycin/μmol phospholipid. Between 0 and 0.2 mM adriamycin, the uptake was proportional to the initial free concentration and was nearly quantitative (95% or higher trapping efficiencies). Above 0.2 mM adriamycin, the trapping efficiency was reduced due to the saturation of the uptake process. However, high trapping efficiencies at these higher drug concentrations could be readily achieved by the simple expedient of increasing the vesicle concentration. For example, incubation of 10 mM adriamycin in the presence of LUVs corresponding to a 50 mM phospholipid concentration yielded uptake levels of 196 nmol adriamycin/μmol phospholipid, corresponding to a 98% trapping efficiency (data not shown).

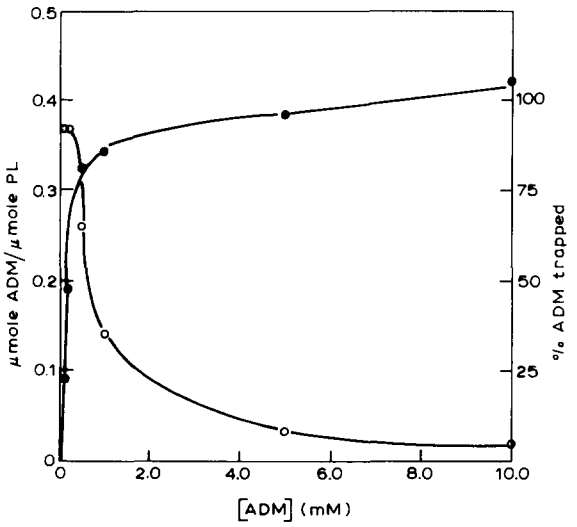

Fig. 3. (●), Uptake of adriamycin (ADM) into egg-PC LUVs systems exhibiting a K^+ diffusion potential as a function of the initial free adriamycin concentration. (○), The percentage of the total available drug which was accumulated. The LUV systems (1 mM phospholipid) exhibiting an Na^+/K^+ transmembrane chemical gradient were incubated at 20°C with the indicated concentrations of adriamycin for 2 h in the presence of valinomycin. For other details see Materials and Methods.

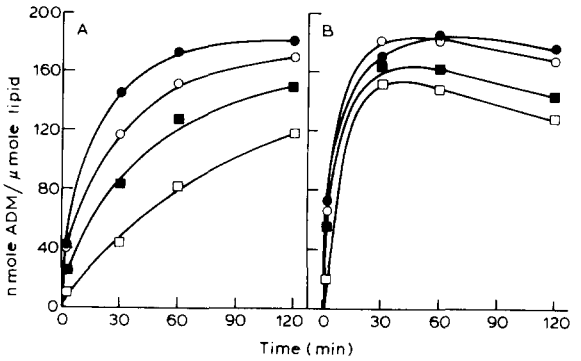

Fig. 4. Uptake of adriamycin (ADM) into egg-PC-cholesterol LUV systems in the presence of Na^+/K^+ transmembrane chemical gradients and valinomycin at (A) 20°C and (B) 37°C. The vesicles (1 µmol total lipid/ml) were incubated at 20 or 37°C for 2 h in the presence of 0.2 mM adriamycin. The molar ratios of egg-PC to cholesterol employed were (●) 1:0, (○) 9:1; (■) 3:1 and (□) 1:1.

Similar studies employing vinblastine revealed that uptake levels saturated at 40 nmol vinblastine/µmol phospholipid. Trapping efficiencies approaching 100% could be achieved on incubation of 0.2 mM vinblastine with a concentration of LUVs corresponding to 5 mM phospholipid. The reasons for the much lower saturation levels of sequestered vinblastine (as compared to adriamycin) are not understood, and may be related to the presence of two positively charged groups in vinblastine or surface potential effects [9].

Liposomal drug delivery systems commonly contain equimolar levels of cholesterol and more saturated phospholipid to reduce leakage of entrapped material induced by serum components [10–12]. We therefore investigated the influence of cholesterol on active trapping of adriamycin into egg-PC LUV systems. As shown in Fig. 4A, a stepwise increase in cholesterol content to achieve equimolar egg-PC/cholesterol levels results in a corresponding decrease in the rate of adriamycin accumulation. This may result from decreased efficiencies of valinomycin in the presence of cholesterol or a decreased permeability to adriamycin itself. However, rapid uptake could be achieved by incubating the vesicle-drug system at higher temperatures. As indicated in Fig. 4B equilibrium uptake levels are achieved within 30 min at 37°C. This effect was most pronounced for the egg-PC/cholesterol (1:1) system, where increasing the temperature from 20 to 37°C resulted in an increase of vesicle-associated adriamycin from 42 to 153 nmol/µmol lipid after a 30 min incubation.

Indications of the influence of increased saturation of the phospholipid acyl chains on $\Delta\psi$-driven uptake of adriamycin were obtained by monitoring uptake into DPPC-cholesterol (1:1) LUVs. As indicated in Fig. 5 no uptake of adriamycin could be observed over 4 h at 20°C. However, incubation at 60°C resulted in sequestered adriamycin levels of 150 nmol drug/µmol lipid within 2 h. Further, significant uptake (to approx. 60 nmol/µmol lipid) was observed for these systems incubated in the absence of valinomycin. This is to be compared with uptake levels observed in the absence of valinomycin employing egg-PC LUVs of 60 and 100 nmol drug/µmol lipid at 37 and 60°C, respectively (Fig. 5).

In addition to variations in acyl chain composition and cholesterol content, charged lipid species have also been incorporated into liposomal de-

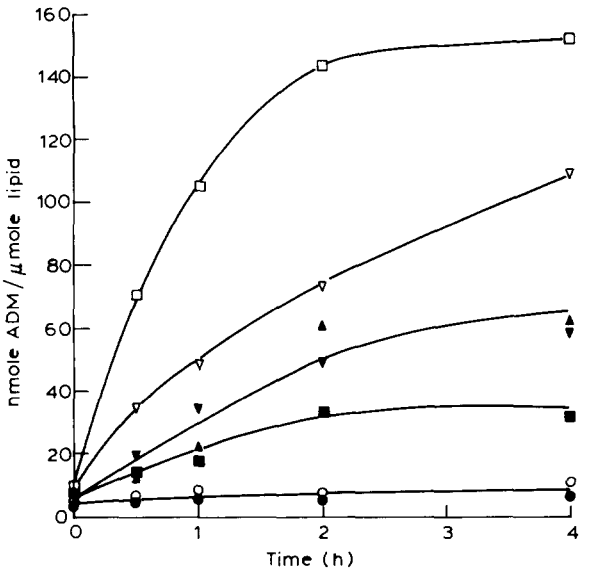

Fig. 5. Uptake of adriamycin (ADM) into DPPC-cholesterol (1:1) LUVs (1 mM lipid) in the presence of Na^+/K^+ transmembrane chemical gradients after incubations (in the presence of 0.2 mM adriamycin) at various temperatures: (○) 20°C, in the presence of valinomycin; (■) 37°C, in the presence of valinomycin; (●) 37°C, in the absence of valinomycin; (□) 60°C in the presence of valinomycin; (▼) 60°C, in the absence of valinomycin; (▲) Egg-PC LUVs (1 mM) at 37°C in the absence of valinomycin; (▽) Egg-PC LUVs at 60°C in the absence of valinomycin.

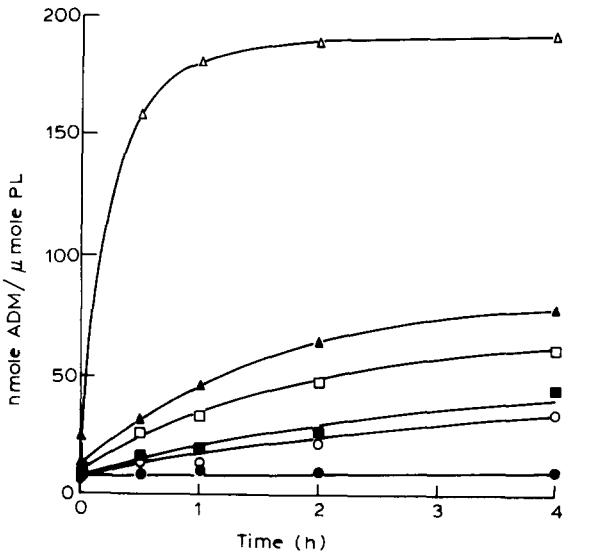

Fig. 6. Uptake of adriamycin (ADM) into egg-PC/egg-PS LUVs in the presence of Na^+/K^+ chemical gradients after incubation at 20°C in the presence of 0.2 mM adriamycin; (△) egg-PC/egg-PS (4:1), in the presence of valinomycin; (△) egg-PC/egg-PS (4:1), in the absence of valinomycin; (□) egg-PC/egg-PS (9:1), in the absence of valinomycin; (■) egg-PC/egg-PS (20:1) in the absence of valinomycin; (○) egg-PC/egg-PS (50:1) in the absence of valinomycin. (●) Uptake into the egg-PC/egg-PS (4:1) system in the absence of an Na^+/K^+ gradient (K^+ buffer inside and out).

livery systems, which influence in vivo distribution and uptake processes [13–15]. In order to demonstrate that $\Delta\psi$-driven uptake can also be observed in such systems, the influence of the acidic (negatively charged) phospholipid egg-PS was monitored in LUVs containing up to 20 mol% egg-PS. As shown in Fig. 6, systems containing 20 mol% egg-PS exhibited uptake behaviour in the presence of a valinomycin-induced K^+ diffusion potential which is virtually identical to that observed in the absence of egg-PS (Fig. 1). However, in contrast to the pure egg-PC systems, significant uptake is observed in egg-PS containing LUVs exhibiting Na^+/K^+ transmembrane gradients in the absence of valinomycin. Increasing the egg-PS content from 2 to 20 mol% increased such uptake (2 h incubation) from 30 to 78 nmol adriamycin/μmol phospholipid. This effect may arise from the negative surface charge due to egg-PS which increases the partition coefficient of adriamycin for membrane systems [10], or an increased K^+ permeability and correspondingly increased membrane potentials.

In previous work [6], an ability to passively entrap an aqueous marker ([^{14}C]inulin) to achieve a 30% trapping efficiency has been demonstrated employing the extrusion procedure. Similar trapping levels could also be achieved for the water-soluble anticancer drugs, methotrexate and cytosine arabinoside, which do not exhibit lipophilic or cationic characteristics. Briefly, egg-PC LUVs (150 μmol phospholipid/ml) were generated in a K^+ buffer containing 20 mg/ml cytosine arabinoside (2 μCi/ml [^{3}H]cytosine arabinoside) or 10 mg/ml methotrexate (2 μCi/ml [^{3}H]methotrexate). The vesicles were subsequently passed over a gel filtration column pre-equilibrated with a Na^+ buffer, which removed untrapped drug and also established an Na^+/K^+ chemical gradient. Analysis of LUVs prepared in this manner revealed that 33% of the available cytosine arabinoside or methotrexate was encapsulated (see Table I). Further, it was then possible to actively accumulate adriamy-

TABLE I

TRAPPING OF ANTINEOPLASTIC AGENTS IN LUVs COMPOSED OF EGG PC

Methotrexate was trapped passively, at a concentration of 20 mM, during preparation of vesicles (187 μmol lipid/ml). Adriamycin was trapped employing a transmembrane Na^+/K^+ gradient in the presence of valinomycin with a vesicle concentration corresponding to 1 mM phospholipid and a starting adriamycin concentration of 100 μM. Cytosine arabinoside was trapped passively, at a concentration of 25 mM, during preparation of vesicles (187 μmol lipid/ml), and vinblastine was trapped employing a transmembrane Na^+/K^+ gradient in the presence of valinomycin with a vesicle concentration corresponding to 5 mM phospholipid and a starting vinblastine concentration of 200 μM.

Drug	Trapping efficiency (%)	nmol drug per μmol lipid
Methotrexate	33	35.3
+ adriamycin	99	99
Cytosine arabinoside	33	44.5
+ adriamycin	98	98
Vinblastine	90	36
Adriamycin	95	95

cin into these vesicles employing the Na^+/K^+ gradient and valinomycin. In particular, as shown in Table I, incubation of an aliquot of the vesicles (1.0 mM phospholipid) in the presence of 0.2 mM adriamycin resulted in uptake of 98% of available adriamycin. Trapping efficiencies and uptake levels observed for adriamycin were almost identical to those observed in the absence of passively trapped drug (compare Table I and Fig. 2).

As a final stage of this investigation we chose to characterize the release characteristics at 37°C of drugs actively and passively trapped into LUV systems with varying lipid compositions. The passive trapping procedures were performed as indicated above for methotrexate and cytosine arabinoside. Inulin (containing [^{14}C]inulin) was entrapped as indicated elsewhere [6]. Leakage was assayed employing the flow dialysis procedure indicated in Materials and Methods. As noted in Table II, inulin exhibited slow release with less than 5% leakage after 72 h. The time required for 50% release (T_{50}) of methotrexate and cytosine arabinoside was 50 and 18 h, respectively, whereas passively trapped adriamycin (20 mM interior concentration) was rapidly released from both egg-PC and egg-PC/cholesterol (1 : 1) LUV systems (T_{50} approx. 1 h). This contrasts with the retention times observed for adriamycin actively sequestered in response to a valinomycin-induced K^+ diffusion potential, where T_{50} values of up to 36 h were obtained depending on the lipid composition (Ta-

TABLE II

DRUG RELEASE FROM LUVs OF VARYING LIPID COMPOSITION AT 37°C

After drug encapsulation (for details see Materials and Methods) the external buffer and untrapped drug were replaced with either a KCl or NaCl buffer as specified. T_{50} indicates the time needed for release of 50% of the trapped agent from the vesicles. Chol, cholesterol.

Lipid composition	Agent trapped	External buffer	T_{50} (h)
	Passively trapped agents		
Egg-PC	inulin	NaCl	$\gg 72$ [a]
Egg-PC	methotrexate	NaCl	50
Egg-PC	cytosine arabinoside	NaCl	18
Egg-PC	adriamycin	KCl	1
Egg-PC/Chol (1 : 1)	adriamycin	KCl	1
	Actively trapped agents		
Egg-PC	adriamycin	NaCl	16
Egg-PC/egg-PS (8 : 2)	adriamycin	NaCl	6
Egg-PC/Chol (1 : 1)	adriamycin	NaCl	30
DPPC/Chol (1 : 1)	adriamycin	NaCl	36

[a] 95% of the trapped inulin was remaining after 72 h.

ble II). Removal of the transmembrane Na^+/K^+ gradient employing gel filtration columns equilibrated in K^+-containing buffer subsequent to $\Delta\psi$-dependent adriamycin uptake yielded LUV systems which exhibited T_{50} values of approx. 1 h (data not shown), comparable to that observed for passively trapped adriamycin (Table II).

Discussion

The results presented in this work have important implications in three areas. These concern the mechanisms whereby lipophilic cationic drugs gain access to cytoplasmic components in vivo, mechanisms whereby these agents may be appropriately loaded into liposomal carrier systems, and procedures whereby the release characteristics of liposomally entrapped drugs may be regulated. We discuss these areas in turn.

A remarkably large fraction of commonly employed drugs are lipophilic cations. These include cholinergic alkaloids, antagonists for adrenergic receptors (α blockers, β blockers), certain antihypertensive agents, most anticonvulsants, local anaesthetics and antidepressants, to name but a few. Thus, the demonstration here that adriamycin and vinblastine can be rapidly accumulated into model membrane systems exhibiting a membrane potential, coupled with other observations that local anaesthetics (chlorpromazine, dibucaine) [7,9] and catecholamines (dopamine, epinephrine, norepinephrine, unpublished results) can be accumulated in a similar manner leads to the possibility that lipophilic cationic drugs can gain access to cells by a general $\Delta\psi$-dependent mechanism that does not depend on the presence of specific carrier proteins. Two additional points can be made. First, on purely intuitive grounds, such a mechanism appears reasonable. A lipophilic character will ensure a certain level of partitioning into a membrane lipid bilayer, with the result that the positive charge may experience the transmembrane electric field associated with the membrane potential. A $\Delta\psi$ of -100 mV, for example, corresponds to a transmembrane electric field gradient of $2.5 \cdot 10^5$ V/cm, which must be expected to influence the transbilayer distribution of charged compounds which fall within its domain. Alternatively, it is possible that neutral (deprotonated) species of the drugs move accross the membrane in coordination with a K^+–H^+ exchange arising due to high H^+ permeabilities [17]. The second point concerns the possibility that model membrane systems exhibiting a membrane potential may have some utility in drug design. In particular, chemical modifications of a biological agent resulting in lipophilic and cationic characteristics should facilitate its access to the cell cytoplasm. This could be assayed in such model systems as employed here.

The second and third areas of discussion deal with the utility of liposomal systems for drug delivery and concern drug loading and release properties. With regard to passive entrapment of drugs such as methotrexate and cytosine arabinoside, the relatively high passive trapping efficiencies demonstrated here (33%) are comparable with the most efficient procedures previously available (e.g., 35% trapping efficiency for the reverse-phase evaporation technique [16]). Further, higher efficiencies are likely to be obtained by employing higher lipid concentrations. The fact that the extrusion procedure avoids the use of organic solvents and is rapid and reproducible increases the appeal of this technique.

The ability to actively sequester drugs such as adriamycin and vinblastine into liposomal systems in response to a membrane potential is important for three reasons. First, higher drug levels can be loaded into the vesicle than could be achieved by passive trapping procedures. Levels of 400 nmol adriamycin/μmol phospholipid, for example, correspond to interior concentrations of approx. 260 mM. Second, as demonstrated here, this loading process can be highly efficient, exhibiting trapping efficiencies approaching 100%. This is obviously an extremely cost-effective method for entrapment. Third, this procedure can be employed to entrap drugs after the liposomal carrier system itself has been generated. This could be of utility for labile drugs where it may be important to load the carrier immediately prior to delivery.

A drawback of the active entrapment procedure detailed here, however, is that valinomycin is usually required to induce the K^+ diffusion potential required for uptake. It is not known whether the low levels of ionophore remaining after gel filtration would have toxic effects; however, it would clearly be more appropriate if valinomycin were

not employed. As indicated here, incubation at higher temperatures or inclusion of lipids such as PS partially circumvents this problem and results in appreciable uptake in the absence of ionophore. An alternative approach is to employ a H^+ diffusion potential (rather than a K^+ potential) which can be readily established in the absence of ionophore due to the high membrane permeability of H^+ in comparison to other cations [17].

A fundamental advantage of the $\Delta\psi$-driven loading process for lipophilic cationic drugs concerns the much improved drug retention and release characteristics. This is demonstrated here by an increase in 50% retention times (T_{50}) from approx. 1 h for passively entrapped adriamycin to 30 h for egg-PC/cholesterol (1 : 1) systems at 37°C, for example. This reduced leakage is likely due to the fact that drug efflux subsequent to uptake driven by a K^+ diffusion potential is coupled to influx of Na^+ counterions. This Na^+ influx is a much slower transmembrane diffusion process. It should be noted that the ability to regulate release according to the presence of $\Delta\psi$ allows possibilities for the engineering of controlled release systems which release their contents at different times according to the Na^+/K^+ or other transmembrane ion gradients present, as well as the lipid composition itself.

Acknowledgements

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