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Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient

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Previous work has shown that adriamycin can be accumulated into large unilamellar vesicle (LUV) systems in response to K^+ diffusion potential established by valinomycin. It is demonstrated here that adriamycin can also be rapidly and efficiently accumulated into egg phosphatidylcholine (egg PC) and egg PC-cholesterol (1:1) LUVs in response to a transmembrane pH gradient (interior acidic) in the absence of ionophores. This 'active' loading gives rise to trapping efficiencies as high as 98%, interior drug concentrations as high as 100 mM and significantly enhances drug retention within the vesicles. This procedure may be of general utility for loading liposomal systems for in vivo drug delivery.

There are growing indications that liposomal carrier systems can significantly buffer the toxicity of entrapped drugs without decreasing drug potency. Examples include liposomally entrapped amphotericin B and anticancer agents such as cytosine arabinoside [5,6] and adriamycin [7–11]. Current encapsulation protocols do not usually allow efficient entrapment of high concentrations of hydrophobic drugs such as adriamycin, which can also leak rapidly from the vesicle carrier. However, in recent reports we have shown that adriamycin [12] and other hydrophobic amines [13–15] can be efficiently loaded into large unilamellar vesicle (LUV) systems in response to a K^+ diffusion potential induced by valinomycin. The membrane potential also significantly reduces the rate of drug release. We show here that adriamycin can also be loaded into LUVs exhibiting pH gradients (inside acidic) in the absence of ionophores, resulting in trapping efficiencies of over 90%, interior adriamycin concentrations in excess of 50 mM and dramatic reductions in efflux rates.

The first set of experiments was aimed at showing that LUVs composed of egg-yolk phosphatidylcholine (egg PC) and egg PC-cholesterol (1 : 1) could efficiently accumulate adriamycin in response to a pH gradient. As shown in Fig. 1 for a ΔpH of 2.9 (interior pH 4.6), adriamycin is rapidly accumulated into both systems, in the absence of the proton ionophore CCCP, at both 20°C and 37°C. The rate of uptake at 37°C is somewhat faster, particularly for the cholesterol-containing systems. The uptake levels thus achieved (70–100 nmol adriamycin/μmol lipid) correspond to trapping efficiencies of 70–98%, as calculated from the percentage of available drug which is encapsulated into the vesicles.

The leakage of 'passively' entrapped adriamycin (i.e., adriamycin entrapped in the absence of K^+ or H^+ ion gradients) is relatively fast, leading to times for release of 50% of entrapped material (T_{50}) of 1 h or less at 37°C [12]. It has been shown [12] that retention times are significantly longer for adriamycin actively entrapped in response to a K^+ diffusion potential ($T_{50} \approx 30$ h for an egg PC-cholesterol 1 : 1 system at 37°C). Studies were therefore performed to determine the retention

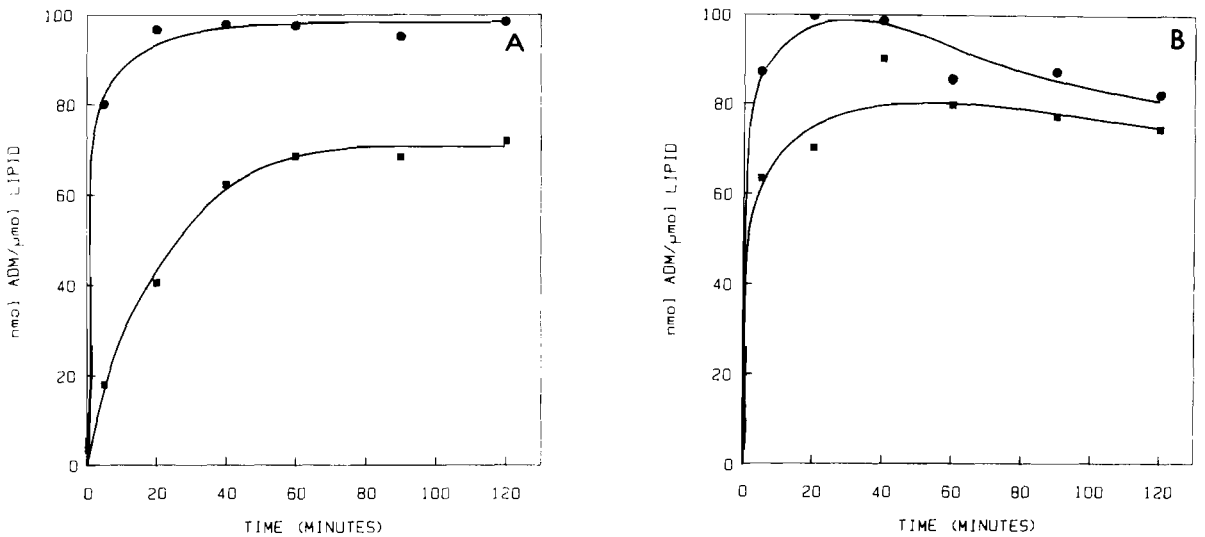

Fig. 1. Uptake of adriamycin (ADM) into LUVs exhibiting a transmembrane pH gradient at 20°C (A) and 37°C (B). Egg phosphatidylcholine was purified from egg yolks using established procedures and was greater than 99% pure as determined by thin-layer chromatography. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids. Cholesterol, CCCP and all salts were obtained from Sigma Chemical Co. Tritiated DPPC was purchased from New England Nuclear. Adriamycin was a generous gift from Dr. Alan Eaves (Cancer Research Center, Vancouver, B.C.). Vesicles were prepared according to the extrusion procedure described by Hope et al. [12] employing a freeze-thaw protocol [13] and 100 nm pore size polycarbonate filters. These LUVs had an average diameter of 103 nm and a trapped volume of approx. 1.5 μl/μmol phospholipid. Transmembrane pH gradients were generated by forming LUVs in 150 mM KOH/175 mM glutamic acid (pH 4.6) and subsequently exchanging the untrapped buffer for 150 KOH/125 mM glutamic acid/30 mM NaCl (pH 7.5) employing Sephadex G-50 desalting columns [11]. These buffer compositions yielded transmembrane pH gradients which were stable during the uptake process. Experimental conditions were: 2 mM lipid, 0.2 mM adriamycin. Lipid compositions were egg PC (●) and egg PC/cholesterol at a molar ratio of 1 : 1 (■). At various times, the untrapped 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. Lipid concentrations were determined by liquid scintillation counting to quantitate [^{3}H]DPPC.

times in the presence of the H^+ ion gradients. As shown in Fig. 2A, T_{50} values of approx. 24 h were observed for adriamycin sequestered into egg PC-cholesterol (1 : 1) LUVs, which were reduced to approx. 4 h when the H^+ ion gradient was eliminated (Fig. 2B). Similarly, retention times of 4 h for adriamycin actively entrapped into EPC vesicles were reduced to approx. 30 min when the ΔpH was eliminated. It may be noted that in some cases the presence of a K^+ ion gradient (K^+ buffer inside, Na^+ buffer outside), in addition to the ΔpH, results in extended adriamycin retention times. This is particularly noticeable for the egg PC systems, where the T_{50} values were increased from 3 to 16 h when the exterior K^+ buffer was exchanged for an Na^+ buffer prior to dialysis.

The mechanism whereby adriamycin is actively entrapped by vesicles exhibiting a transmembrane proton gradient is probably similar to the mechanism whereby dopamine and other biogenic amines are accumulated into LUVs exhibiting a pH gradient (inside acidic) [15]. According to generally held views, this results from rapid permeation of the neutral (deprotonated) form of the amine and subsequent transmembrane redistribution dictated by the Henderson-Hasselbach relation [16]. However, due to the relatively high proton permeability of phospholipid bilayers [17], these systems also exhibit substantial membrane potentials (negative inside) [15] which may also play a role in the uptake process.

In summary, the results presented here show

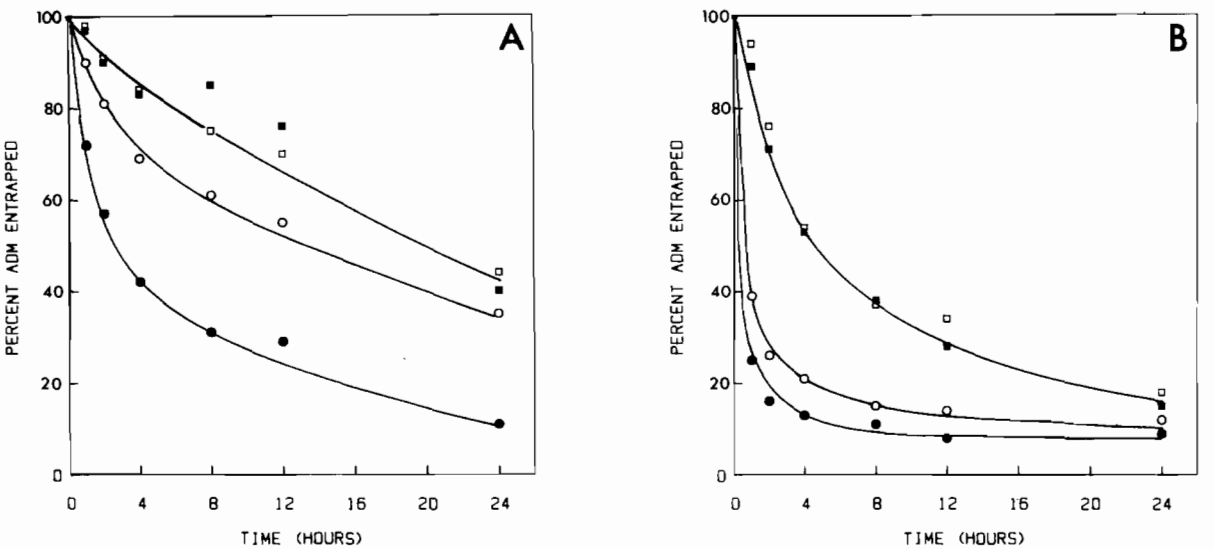

Fig. 2. Release of ΔpH-dependent accumulated adriamycin (ADM) from egg PC (●, ○) and egg PC/cholesterol at a molar ratio of 1:1 (■, □) LUVs at 37°C. Adriamycin was sequestered into vesicles in response to a pH gradient as described in the legend to Fig. 1 at lipid and drug concentrations of 5.0 and 0.5 mM, respectively. Free adriamycin was separated from vesicle-associated drug by gel filtration chromatography employing columns equilibrated in buffers adjusted to pH 7.5 (A) or pH 4.6 (B) which contained 150 mM K^+ (closed symbols) or 180 mM Na^+ (open symbols). Adriamycin release was monitored under flow dialysis conditions as follows. Vesicles (5 mM lipid) containing adriamycin were 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 10 min. Aliquots (0.15 ml) were removed at various times and untrapped material was removed employing 1 ml gel filtration columns. The sample was then assayed for adriamycin and lipid as described in the legend to Fig. 1.

that adriamycin can be efficiently and rapidly sequestered into LUV systems in response to transmembrane pH gradients (interior acidic), in the absence of any extraneous ionophores. The trapping efficiencies, drug retention times and concentrations of entrapped drug thus achieved are superior to those achievable by passive trapping procedures. Modulation of release kinetics can be conveniently achieved by variations in the lipid composition or ion gradients. Finally, as a large proportion of commonly used drugs are hydrophobic amines, it is likely that liposomal drug loading in response to pH gradients is of general utility.

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