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The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey

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We have shown previously that transmembrane proton gradients can be used to efficiently accumulate biogenic amines [M.B. Bally et al. (1988) Chem. Phys. Lipids 47, 97—107] and doxorubicin [L.D. Mayer, M.B. Bally and P.R. Cullis (1986) Biochim. Biophys. Acta 857, 123—126] to high concentrations within liposomes. To determine the generality of this loading procedure, representative drugs from a variety of different classes (antineoplastics, local anaesthetics, antihistamines, etc.) were examined as to their ability to redistribute in response to a proton gradient. While the majority of drugs examined, all of which are weak bases, were accumulated by large unilamellar vesicles exhibiting a pH gradient (interior acid) the extent of uptake varied considerably between different pharmaceuticals. These differences are discussed in the context of various factors which will likely influence drug accumulation including its membrane/water partition coefficient and its solubility in the intravesicular medium.

Keywords: liposomes; drugs; encapsulation; pH gradients.

Introduction

The therapeutic properties of many drugs can be dramatically improved by administration in a liposomally encapsulated form (for review see Ref. 1). In the case of amphotericin B [2] and doxorubicin [3] toxicity is reduced while efficacy is maintained or increased. This benefit is largely fortuitous and likely results from the altered pharmacokinetics and biodistribution of the entrapped drug [4]. These parameters will in turn be largely determined by the character of the carrier system and therefore optimization of a liposomal drug requires an examination of such variables as vesicle size, composition and drug to lipid ratio. Most drug loading protocols, however, do not permit independent variation of these parameters. Passively trapped drugs, for example, will exhibit differing drug to lipid ratios as size is varied due to the consequent changes in trapped volume. We have shown previously that several biogenic amines and antineoplastic agents can be accumulated by vesicles in response to an imposed proton gradient [5—7]. This "remote loading" technique allows independent variation of any liposomal parameter and in addition much higher drug to lipid ratios can be achieved in comparison to conventional techniques [8]. Further, as the transmembrane distribution of the drug is determined by the proton gradient, it may be possible to control the rate of drug leakage in the circulation by changes in the buffering capacity of the intravesicular medium.

Given the possibility that liposomal encapsulation would provide beneficial effects for a wide variety of drugs we examine here the generality

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of the remote loading technique. The ability of representative drugs from a variety of different classes (antineoplastics, local anaesthetics, antihistamines, etc.) to accumulate within large unilamellar vesicles in response to a pH gradient (interior acid) was compared. We show that the extent of uptake varies considerably between different drugs (all of which are weak bases) and discuss some of the factors that will influence accumulation.

Materials and methods

Egg phosphatidylcholine was purchased from Avanti Polar Lipids Inc. Cholesterol (standard for chromatography) propranolol, timolol, dibucaine, chlorpromazine, lidocaine, quinidine, pilocarpine, physostigmine, dopamine, imipramine, diphenhydramine, quinine, chloroquine, quinacrine, daunorubicin, vincristine and vinblastine were obtained from Sigma Chemicals, St. Louis, MO. Doxorubicin and epirubicin were obtained from Adria Laboratories of Canada, Mississauga, Ontario, while mitoxantrone was purchased from Cyanamid Canada Inc., Montreal, Quebec. Codeine was supplied by Abbott Laboratories Ltd, Downsview, Ontario. The radiolabels [*N*-methyl-^{3}H]-dipalmitoylphosphatidylcholine (58 Ci/mmol), phosphatidylcholine 1,2-di[1-^{14}C]palmitoyl (112 mCi/mmol), [7-^{14}C]dopamine (57 mCi/mmol) and [7,8-^{14}C]imipramine (52 mCi/mmol) were obtained from Amersham, while [benzene ring-^{3}H]chlorpromazine (23 Ci/mmol), [^{3}H]pilocarpine, [4-^{3}H]propranolol (19 Ci/mmol), [carboxyl-^{14}C]-lidocaine (48 mCi/mmol), [^{14}C]methylamine (46 mCi/mmol) came from NEN. The Liposome Company, Inc. N.J. kindly provided [^{14}C]timolol. All salts and reagents used were of analytical grade.

Lipid vesicle preparation

Unless otherwise stated all experiments were performed using egg phosphatidylcholine (EPC) vesicles. The dry lipid was hydrated with 300 mM citrate (pH 4.0) and the resulting MLVs subjected to five freeze-thaw cycles employing liquid nitrogen to enhance solute distribution [9]. Large unilamellar vesicles were then prepared using an Extruder (Lipex Biomembranes, Vancouver) employing the LUVET procedure [10] with 100 nm pore size polycarbonate filters (Nuclepore Inc.). These vesicles have a trapped volume of 1.5 μl/μmol phospholipid [7]. To establish a transmembrane pH gradient the vesicles were then passed down a Sephadex G-50 (fine) column (1.5 × 10 cm) preequilibrated with 300 mM NaCl, 20 mM HEPES (pH 7.5).

Drug uptake experiments

Large unilamellar vesicles (1 mM lipid) were incubated with the drug (0.2 mM) in 300 mM NaCl, 20 mM HEPES (pH 7.5) at 25°C unless otherwise stated. These ratios were selected such that redistribution in accordance with the Henderson-Hasselbach equation would result in approximately 50% of the drug being accumulated inside the vesicles. At various times up to 2 h, aliquots (100 μl) of the mixture were taken and vesicles separated from unentrapped drug by centrifugation through a 1 ml "minicolumn" of Sephadex G-50 (medium) [11]. Lipid and drug were quantified as described below.

Analytical procedures

Lipid concentrations were determined by liquid scintillation counting of [^{3}H]DPPC or [^{14}C]DPPC using a Packard 2000 CA instrument. Similarly, pilocarpine, chlorpromazine, timolol, propranolol, imipramine, lidocaine and dopamine were quantified using tracer quantities of the ^{3}H- or ^{14}C-radiolabel.

Physostigmine was assayed by fluorescence spectroscopy employing a SLM-Aminco SPF 500C spectrofluorometer following solubilization of the vesicles in 60% ethanol (v/v). The excitation and emission wavelengths used were 305 and 350 nm respectively. Quinacrine, chloroquine, quinine and quinidine were also quantified from their fluorescence using excitation and emission wavelengths of 420 nm and 505 nm; 335 nm and 375 nm; 335 nm and 365 nm; and 350 nm and 390 nm respectively.

Vinblastine and vincristine were assayed by U.V. spectroscopy from their absorbances at 262 nm and 297 nm, respectively, following solubili-

zation of the vesicles in 80% ethanol. Codeine was also measured by U.V. spectroscopy at 220 nm in this case after solubilization in 40 mM octyl-β-D-glucopyranoside. Mitoxantrone was quantified from its absorbance at 670 nm following solubilization of the vesicles in 2% Triton *X*-100.

Diphenhydramine was assayed by gas-liquid chromatography using a HP 9850 gas chromatograph fitted with a Chromatographic Specialties DB-225 (25% cyanopropylphenyl) capillary column. The helium carrier flow rate was 1 ml min^{-1} and detection was by flame ionization. An internal standard, methylpentadecanoate, was used to quantify diphenhydramine following its extraction from the aqueous sample in diethylether and its separation from egg phosphatidylcholine by thin layer chromatography.

Transbilayer pH gradients were quantified employing the weak base methylamine (^{14}C-labelled) as described previously [7].

Results

The response of the drugs examined to a transbilayer pH gradients is documented in Table I. The data presented represents the mean of triplicate determinations for single experiments. All of the drugs examined showed highly reproducible behaviour with uptake levels between different experiments agreeing closely. Essentially four drug categories can be defined on the basis of their uptake characteristics. First, those compounds which show essentially complete accumulation; second, drugs which show partial but stable uptake; and third partial uptake and then release. Finally some compounds do not redistribute in response to a proton gradient. While these four categories can be expected to encompass a continuous spectrum of uptake behaviour, in the majority of cases the assignment of a drug to a particular category was straightforward. Representative examples from these four classes of uptake will be discussed in turn.

The accumulation of mitoxantrone by EPC vesicles exhibiting a proton gradient is shown in Fig. 1. Accumulation was rapid and complete with little or no release observed over 2 h. In the absence of a pH gradient (vesicle interior and exterior are pH 4.0 or pH 7.5) only low levels of background binding are observed. Other drugs which showed similar uptake characteristics were daunorubicin, epirubicin, propranolol, dopamine, dibucaine, imipramine and doxorubicin.

Some of the pharmaceuticals tested were only partially accumulated by the vesicles but the level of uptake was stable. Examples of this type of response are shown in Fig. 2. Timolol is taken up to approximately 100 nmol/μmol lipid (50% of available drug Fig. 2A) while quinacrine reaches a level of approximately 80 nmol/μmol lipid after 30 min (Fig. 2B). While these levels are lower than for the drugs mentioned above, nevertheless they represent considerable concentration gradients. In the case of timolol, for example, an internal concentration of approximately 65 mM is achieved against an external concentration of 100 μM. Other drugs which exhibit partial but stable uptake are lidocaine, chlorpromazine, serotonin and chloroquine.

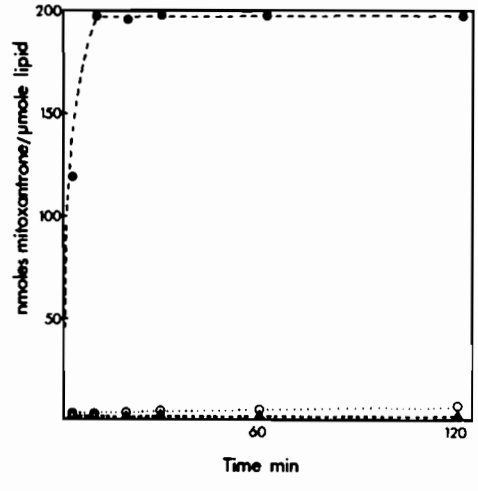

Fig. 1. Uptake of mitoxantrone by egg phosphatidylcholine vesicles. Mitoxantrone (200 μM) was incubated with vesicles exhibiting a proton gradient (pH 4.0 in/pH 7.5 out) --●--; or with control vesicles (pH 4.0 in/pH 4.0 out) ------△------, or (pH 7.5 in/pH 7.5 out) ·····○·····.

TABLE I

Extent and stability of accumulation of various drugs by vesicles exhibiting a pH gradient

Drug	Class	Uptake 15 min (nmol/μmol lipid)	Uptake 2 h (nmol/μmol lipid)
Mitoxantrone	Antineoplastic	200	198
Epirubicin	Antineoplastic	201	200
Daunorubicin	Antineoplastic	200	204
Doxorubicin	Antineoplastic	202	203
Vincristine	Antineoplastic	178	130
Vinblastine	Antineoplastic	175[a]	127
Lidocaine	Local anaesthetics	87	87
Chlorpromazine	Local anaesthetics	98	96
Dibucaine	Local anaesthetics	194	176
Propranolol	Adrenergic antagonists	198	187
Timolol	Adrenergic antagonists	95	97
Quinidine	Antiarrythmic agents	203	74
Pilocarpine	Cholinergic agents	<1	<1
Physostigmine	Cholinergic agents	<2	<1
Dopamine	Biogenic amines	190[b]	177
Serotonin	Biogenic amines	80[c]	78
Imipramine	Antidepressant	182	188
Diphenhydramine	Antihistamine	176[a]	87
Quinine	Antimalarial	148[a]	81
Chloroquine	Antimalarial	104[a]	88
Quinacrine	Antiprotozoan	73[b]	71
Codeine	Analgesic	<1	<1

[a]Maximum uptake taken at 5 min.
[b]Maximum uptake taken at 30 min.
[c]Maximum uptake taken at 90 min.

A third type of uptake response is illustrated in Fig. 3. While initially egg phosphatidylcholine vesicles rapidly accumulate virtually all available quinidine this uptake is unstable and within 30 min approximately 50% of the drug has been released from the vesicles (Fig. 3A). This release is not associated with any apparent structural change in the vesicles, for example fusion or aggregation, however an explanation is provided by measurements of the proton gradient. Prior to the addition of quinidine, [^{14}C]methylamine distribution indicates a gradient of approximately 3 pH units, however, rapid dissipation occurs upon addition of the drug. It seems reasonable to suggest that at the high intravesicular quinidine concentrations generated initially, sufficient drug partitions into the membrane to increase ion permeability leading to dissipation of the pH gradient. To test this hypothesis quinidine uptake into vesicles composed of egg phosphatidyl-

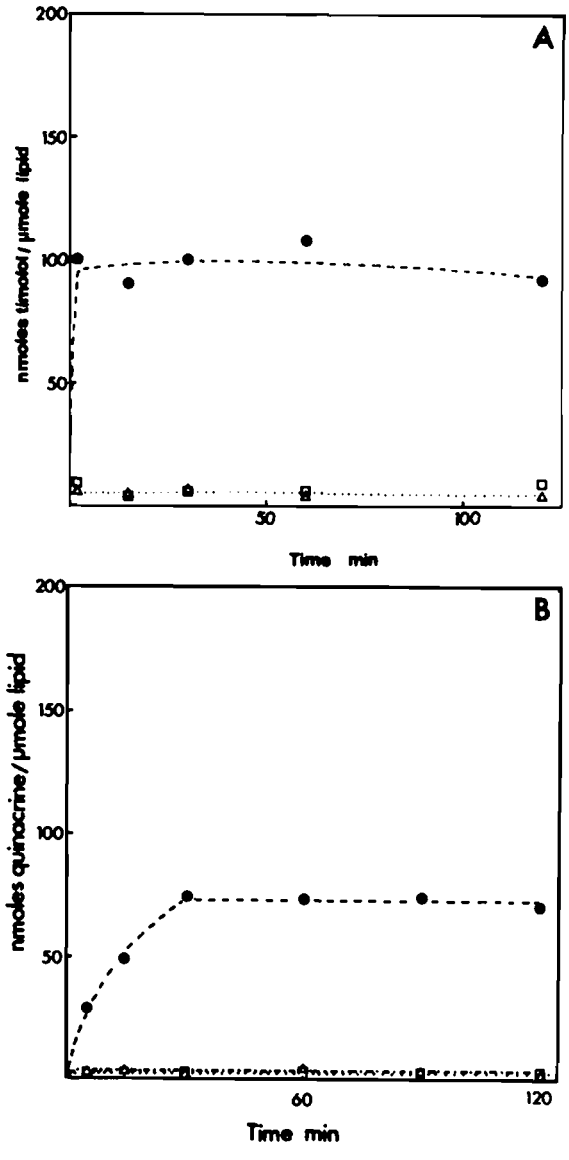

Fig. 2. Uptake of timolol and quinacrine by egg phosphatidylcholine vesicles. Timolol (A) or quinacrine (B) were incubated with vesicles exhibiting a proton gradient (pH 4.0 in/pH 7.5 out) – – -●– – –; or with control vesicles (pH 4.0 in/pH 4.0 out) ---△---, or (pH 7.5 in/pH 7.5 out) ·····□·····.

choline and cholesterol was examined. As a consequence of its condensing effect on membranes, cholesterol should reduce the membrane partition coefficient of quinidine and therefore its destabilizing effect on the pH gradient. Such proves to be the case as shown in Fig. 3B. Following quinidine uptake there is an initial decrease in the pH gradient (which is expected as uptake involves net proton binding by drug accumulated within the vesicles) but the level of drug accumulation and the residual pH gradient are then stable over the 2-h incubation. Other drugs which are released from egg phosphatidylcholine vesicles following uptake are quinine, diphenhydramine, vinblastine and vincristine. The leakage rates vary considerably with vincristine- and vinblastine-loaded vesicles losing only 27% of initially sequestered drug over 2 h. As would be expected, this loss is associated with a corresponding reduction in the residual pH gradient as determined using methylamine. A similar decrease in the proton gradient is observed as quinine and diphenhydramine are released from egg phosphatidylcholine vesicles.

Finally some drugs showed no measurable response to a transmembrane pH gradient, these include codeine, pilocarpine and physostigmine. One possibility is that these compounds cause a major increase in membrane permeability resulting in complete dissipation of the pH gradient. Such is not the case, however, as shown for physostigmine in Fig. 4. Under the conditions used to assess drug uptake (200 μM physostigmine) only a small decrease in the measured pH gradient is observed.

Discussion

It is clear from the results presented that the ability of pharmaceutical agents to accumulate within lipid vesicles exhibiting a proton gradient is not restricted to any particular drug class but is a fairly general phenomenon. Nevertheless, while the majority of drugs examined redistribute in response to such a transmembrane gradient the level and stability of uptake varies considerably. If we assume that the non-protonated drug species is membrane permeable and therefore present at the same concentration on both sides of the membrane, and the protonated form impermeable, and that the pK_a is the same on both sides of the membrane, then the influence of a transmembrane pH gradient on the intravesicular and external drug concentration can

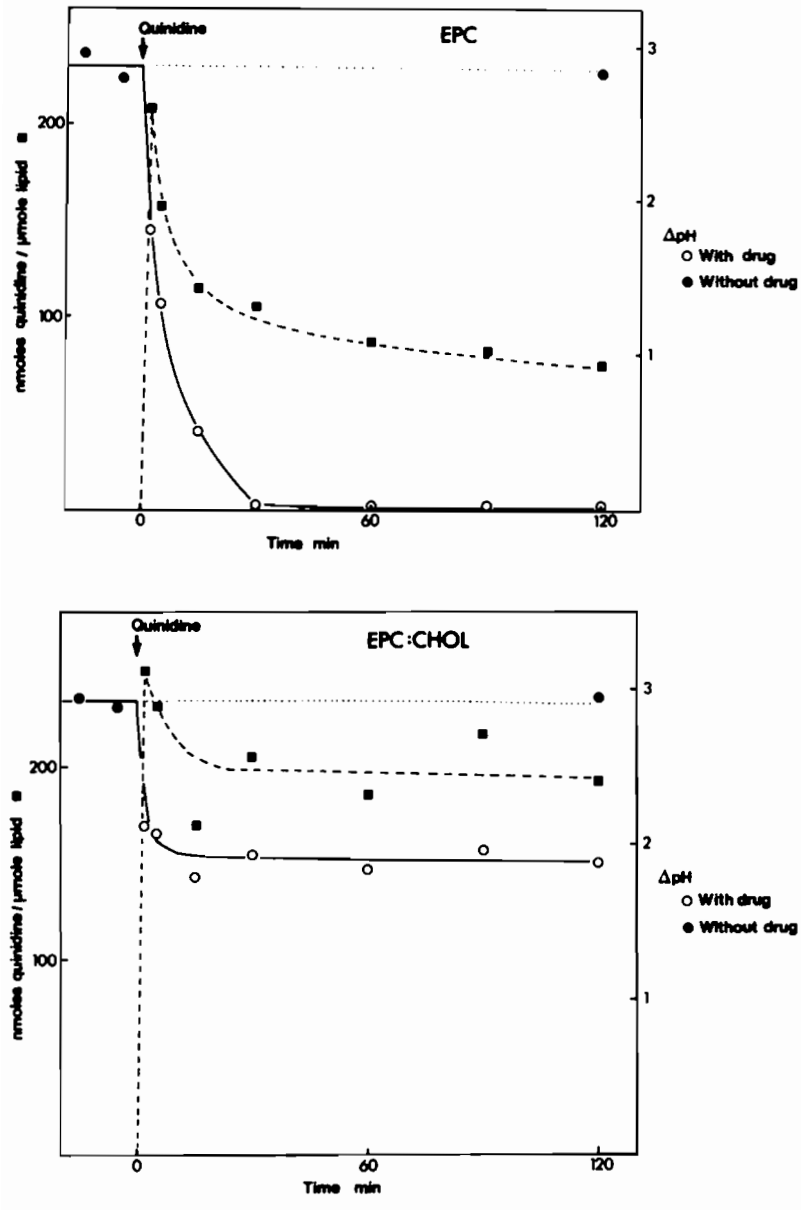

Fig. 3. Uptake of quinidine by egg phosphatidylcholine and egg phosphatidylcholine/cholesterol vesicles. Quinidine (200 μM) was incubated with egg phosphatidylcholine vesicles (A) or egg phosphatidylcholine:cholesterol (55:45 molar ratio) vesicles (B). The level of drug uptake is shown for both systems, – – -■- – -. The proton gradient as measured by [^{14}C]methylamine in the absence, ···●···, or presence —O—, of quinidine is also shown.

be derived from the Henderson-Hasselbach equation as:

$$[HA^+]_{in}/[HA^+]_{out} = [H^+]_{in}/[H^+]_{out} \quad (1)$$

where $[HA^+]$ is the concentration of the protonated drug inside or outside the vesicle and $[H^+]$ is the proton concentration inside or outside.

Using radiolabelled methylamine a 3.0 pH unit gradient across the vesicle membrane was measured in the absence of any drug which is in good agreement with the imposed gradient of 3.5 pH

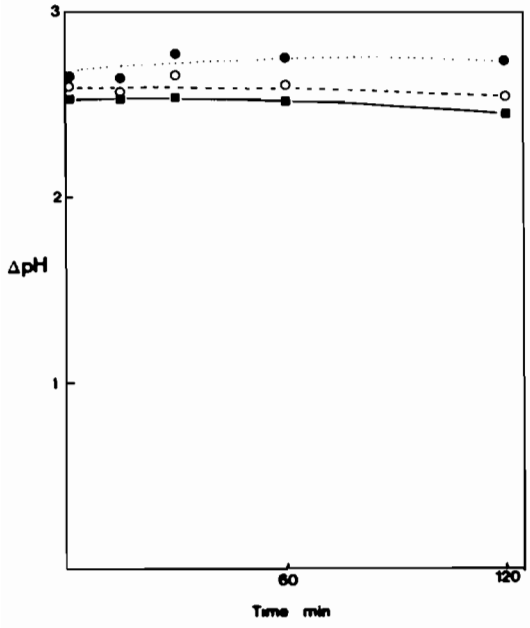

Fig. 4. Influence of physostigmine on transimembrane proton gradients. Egg phosphatidylcholine vesicles exhibiting a pH gradient (pH 4.0 in/pH 7.5 out) were incubated at room temperature in the absence of drug, ···●···; with 100 μM physostigmine, - - -○- - -; or with 200 μM physostigmine, ——■——. At various times the transmembrane proton gradient was quantified using [^{14}C]methylamine.

units. Drug uptake by the vesicles will be accompanied by a reduction in the transmembrane pH gradient as intravesicular protons are bound by the accumulated base. For molecules whose uptake does not result in a generalized increase in membrane permeability and corresponding decay in the proton gradient the residual pH gradient is between 2.4 and 2.8 pH units depending upon the extent of uptake. Given the trapped volume of the vesicles (1.5 μl/μmol phospholipid) we can calculate the drug concentration gradient following uptake. In the case of timolol a 650 fold concentration gradient (interior/exterior) is achieved which is in reasonable agreement with the measured residual proton gradient (2.7 pH units). Timolol, therefore, and other drugs which are accumulated to a similar extent, lidocaine, chlorpromazine, serotonin, chloroquine and quinacrine redistribute in response to the pH gradient in good agreement with the Henderson-Hasselbach equation. It naturally follows that drugs such as mitoxantrone, which are accumulated to a much greater extent than those mentioned above, exhibit concentration gradients which far exceed what would be expected based simply on the Henderson-Hasselbach equation. If 95% of the available drug is taken up, for example, this represents a concentration gradient which is an order of magnitude greater than the proton gradient. It can be seen from the results presented in Table I that many drugs are accumulated to an even greater extent than this. Three factors which may account for this anomalously high accumulation are considered.

The Henderson-Hasselbach relationship as written in Eqn. 1 assumes that the pK_as of the amines are the same on both sides of the vesicle membrane. If the pK_a of the amine group in the intravesicular compartment were higher than for the external medium a significant increase in vesicle uptake would be expected. That the properties of the internal aqueous medium differ from the external solution should not be surprising. Given the large membrane surface area a significant fraction of the intravesicular water will exist in the interfacial unstirred layer. The pK_a of membrane associated tetracaine, however, has been shown to be lower than the aqueous form due to the higher interfacial pH created by the positive surface potential [12]. This situation would be expected to decrease rather than increase the extent of uptake.

A second factor which will influence the degree of drug uptake is the partitioning of the protonated amine between the lipid bilayer and the aqueous medium. Following uptake into the vesicles any protonated drug which partitions into the bilayer will not contribute to the internal aqueous concentration. Given the high ratio of membrane to water in this compartment compared to the external medium any significant membrane solubility would result in drug uptake levels considerably above those predicted. In Table II are presented octanol/water partition coefficients for some of the drugs examined in this paper. It must be stated that while this data may not accurately reflect membrane/water partition coefficients it is useful on a comparative

TABLE II

A comparison between the level of drug uptake and its octanol/water partition coefficient.

Drug	Maximum uptake (nmol/μmol lipid)	Log. octanol/water partition coefficient [Ref.]
Daunorubicin	200	3.5 [14]
Doxorubicin	202	1.1 [14]
Vincristine	178	2.8 [14]
Chloropromazine	98	1.5 [14]
Dibucaine	194	4.4 [14]
Propranolol	198	1.3 [15]
Timolol	95	−0.1 [15]
Physostigmine	0	0.2 [14]
Imipramine	182	4.6 [14]
Diphenhydramine	176	3.4 [14]
Quinine	148	1.7 [14]
Codeine	0	1.2 [14]

basis [13]. Given this proviso, it is apparent that no clear relationship exists between drug uptake and its partition coefficient. Chlorpromazine and doxorubicin, for example, have similar partition coefficients yet display very different uptake levels. On the other hand timolol and chlorpromazine are accumulated by vesicles to a similar extent despite a large difference in their partition coefficients. While the membrane partition coefficient for the protonated amine must influence drug uptake, it alone cannot explain the differences observed.

A third factor which may influence the level of drug uptake is the solubility of the protonated species in the internal buffer. If the concentration of drug inside the vesicle exceeds its solubility product and precipitation occurs this will effectively reduce the transmembrane concentration gradient for the remaining soluble fraction. Precipitated drug is not described by the Henderson-Hasselbach equation and therefore does not influence the distribution of soluble protonated or non-protonated forms. As a result further accumulation (and subsequent precipitation) will occur until equilibrium is reached between the soluble protonated drug inside and outside the vesicles. In Table III are shown the maximum apparent solubilities in 300 mM citrate (pH 5.0) for most of the drugs whose proton gradient dependent uptake was examined. Interestingly, drugs such as mitoxantrone, epirubicin, doxorubicin and daunorubicin which show essentially complete and stable uptake are relatively insoluble in the intravesicular medium. This would suggest, therefore, that most of the accumulated drug is in the form of a precipitate and does not contribute to the concentration

TABLE III

Apparent maximum drug solubility in 300 mM citrate (pH 5.0).

Drug	Apparent maximum solubility (mM)
Mitoxantrone	<0.01
Epirubicin	0.26
Daunorubicin	9.10
Doxorubicin	0.24
Vincristine	>35
Vinblastine	19.1
Lidocaine	240
Dibucaine	>700
Propranolol	326
Timolol	135
Quinidine	5.83
Dopamine	1400
Imipramine	4.43
Quinine	1.05
Chloroquine	585
Quinacrine	90

gradient of the soluble protonated species thus accounting for the high levels of uptake observed. In addition, if most of the intravesicular drug is precipitated then the concentration of free drug available to partition into the membrane is correspondingly reduced which will contribute to the observed stability of the transmembrane proton gradient. As would be expected, drugs such as timolol, lidocaine, quinacrine and chloroquine, which exhibit concentration gradients in good agreement with the Henderson-Hasselbach equation, have maximum apparent solubilities which are in excess of the intravesicular concentrations achieved (Table III). Other drugs such as vincristine and vinblastine which are accumulated to levels intermediate between those first two groups have apparent solubilities which likewise fall between the two groups (data for vinblastine). A fraction of the accumulated drug would be expected to exist in a precipitated form therefore with the remainder in free solution. The higher intravesicular concentrations of soluble vinblastine and vincristine would be expected to contribute to the more rapid dissipation of the proton gradient observed for these systems compared to vesicles containing mitoxantrone, epirubicin or other of the more insoluble pharmaceuticals. Clearly, the solubility data, in conjunction with partition coefficients, can explain many of the observed differences in uptake characteristics for the various drugs examined. There are some drugs, however, whose uptake characteristics remain puzzling. Propranolol, dopamine and dibucaine all show stable accumulation to high levels despite the fact that their apparent solubilities far exceed their intravesicular concentrations suggesting that no precipitation occurs. In the case of dibucaine at least, this may result from its relatively high octanol/water partition coefficient (Table II). Another possibility is that the apparent solubilities measured represent either a drug complex, for example with citrate, or some form of micellar solution [16] and the concentration of protonated species in true solution is actually much lower than the intravesicular concentration.

Generally, it would be expected that those drugs which have higher solubilities in the intravesicular medium would show less stable uptake if membrane permeability was increased by drug partitioning into the inner leaflet of the vesicle bilayer resulting in dissipation of the proton gradient. The data indicates that this tendency is not particularly pronounced suggesting that the properties of the individual drugs and their partition coefficients are of prime consideration. In regard to the pH gradient it should be noted that membranes exhibit a relatively high proton permeability and that following the establishment of a transmembrane pH gradient protons will migrate down this gradient creating a membrane potential until the magnitude of the potential prevents any further net proton movement. At this point the rate of dissipation of the proton gradient is limited by the corresponding transmembrane flux of counterions such as sodium [17]. In the present situation, therefore, where drug accumulation results in dissipation of the pH gradient the permeability changes induced must result in enhanced flux of such counterions; an increased proton permeability alone would not result in faster proton flux.

In summary, the present paper demonstrates that the remote loading technique can be widely applied to encapsulate pharmaceutical agents inside liposomes. It should be noted that drugs which are only partially accumulated under the standard uptake conditions employed here can be entrapped to greater than 90% by appropriate adjustment of the initial drug-to-lipid ratio. In addition, as illustrated herein for quinidine, drug leakage following accumulation can be largely prevented, where necessary, by modifying the lipid composition of the vesicles.

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