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Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles

Pieter R. Cullis [a,b,*], Michael J. Hope [b], Marcel B. Bally [c], Thomas D. Madden [b,d], Lawrence D. Mayer [c], David B. Fenske [a]

[a] *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3*
[b] *Inex Pharmaceuticals Corporation, 1779 West 75th Avenue, Vancouver, B.C., Canada V6P 6P2*
[c] *British Columbia Cancer Agency, Division of Medical Oncology, Vancouver, B.C., Canada V5Z 4E6*
[d] *Department of Pharmacology and Therapeutics, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3*

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Abbreviations: 9-AA, 9-aminoacridine; Chol, cholesterol; DAPC, diarachidoylphosphatidylcholine; cryo-EM, cryo electron microscopy; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMO, 5,5-dimethyl-2,4-oxazolidedione; E_a, activation energy; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; ER, endoplasmic reticulum; ESR, electron spin resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; K_d, dissociation constant of weak base; K_p, drug membrane-water partition coefficient; LUVs, large unilamellar vesicles; Lys, lysine; MDR, multidrug resistance; MeAm, methylamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine ; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; ΔpH, transmembrane pH gradient; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; SPM, sphingomyelin; SRP, signal-recognition particle; SUVs, small unilamellar vesicles; T_c, gel-to-liquid crystalline phase transition temperature; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid; Trp, tryptophan

* Corresponding author. Fax: +1 (604) 822-4843; E-mail: pieterc@unixg.ubc.ca

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1. Introduction

It has long been recognized that the neutral forms of weak acids and weak bases can permeate through lipid bilayer membranes at much faster rates than the charged forms. Early studies in this area include the work of Jacobs [1] on the permeability properties of red cell membranes. This was followed by studies on mitochondria [2] and chloroplast [3,4] membranes which demonstrated the rapid transbilayer movement of the neutral form of carboxylic acids, such as acetate; or amines, such as ammonia. Permeation of the neutral form leads to large transmembrane concentration gradients of certain weak bases or weak acids when transmembrane pH gradients (ΔpH) are present, which led to their use to assay ΔpH in cells or organelles [5,6]. A variety of different probes have been utilized, ranging from radiolabelled amines ([^{14}C]DMO and [^{14}C]methylamine) to fluorescent amines such as 9-aminoacridine (9-AA).

These observations for cells and organelles were extended to model membrane liposomal systems by Deamer and colleagues [7]. Utilizing the quenching of 9-AA fluorescence, they demonstrated that gradients of 2–4 pH units (interior acidic) could be established and measured in small unilamellar vesicle (SUV) systems. Similar results employing spin-labelled weak acids as ΔpH probes were also obtained [8–10]. Nichols and Deamer [11] extended these results to demonstrate catecholamine (weak base) uptake into liposomes in response to ΔpH.

These early observations stimulated studies in our laboratory to investigate the transport of a variety of weak bases and weak acids into liposomes in response to ΔpH. As summarized in this review, this work has shown that the transbilayer distributions of a wide range of weak acids and bases are exquisitely sensitive to transmembrane pH gradients. This includes many commonly employed drugs, as well as certain lipids and peptides, leading to drug delivery systems of considerable therapeutic potential and deeper insight into transbilayer transport and biodistribution of ionizable compounds in vivo.

2. Transbilayer transport of weak bases and acids: theoretical aspects

2.1. Kinetic analysis of weak base uptake

2.1.1. Weak bases with one amino function

We first consider uptake of a weak base containing a single amine into an LUV with an acidic interior. Let $[A]_o$ ($[AH^+]_o$) and $[A]_i$ ($[AH^+]_i$) refer to the concentrations of the neutral (protonated) form(s) of the amine on the outside and inside of the vesicle, respectively. Then the total external and internal concentrations of the amine are given by:

$$[A]_o^{tot} = [A]_o + [AH^+]_o \quad (1)$$

$$[A]_i^{tot} = [A]_i + [AH^+]_i \quad (2)$$

We consider the case where the weak base is initially introduced in the external aqueous medium containing the LUVs with an acidic interior. Assuming that the neutral base is the only membrane permeable

form, the rate of uptake of the weak base into the LUVs is given by:

$$d[A]_o^{tot}/dt = -d[A]_o/dt \quad (3)$$

Let $N(A)$ be the number of molecules of the neutral form of the external weak base, P the permeability coefficient of the neutral form, A_m the surface area of the membrane, and V_o the external aqueous volume. Then

$$dN(A)/dt = -PA_m([A]_o - [A]_i) \quad (4)$$

Given that initially $[A]_i = 0$ and $N(A) = [A]_o^{tot} V_o$, Eq. (4) can be expressed as

$$d[A]_o^{tot}/dt = -(PA_m/V_o)[A]_o \quad (5)$$

By introducing the dissociation constant of the weak base K_d $(= [A]_o[H^+]_o/[AH^+]_o)$ we can rearrange Eq. (1) to express $[A]_o$ as a function of the external pH. Thus

$$[A]_o = [A]_o^{tot}/\{1 + [H^+]_o/K_d\} \quad (6)$$

We assume that $[H^+]_o \gg K_d$. At neutral pH values this is reasonable for a wide variety of weak bases, including drugs such as doxorubicin, for which $pK_a = 8.6$, for example. Eq. (6) then simplifies to

$$[A]_o \approx (K_d/[H^+]_o)[A]_o^{tot} \quad (7)$$

Substituting Eq. (7) into Eq. (5) gives

$$d[A]_o^{tot}/dt = -(PA_m K_d/V_o[H^+]_o)[A]_o^{tot} = -k[A]_o^{tot} \quad (8)$$

where $k = (PA_m K_d/V_o[H^+]_o)$ is the rate constant associated with uptake. By rearranging variables and integrating we then obtain

$$[A(t)]_o^{tot} = [A(0)]_o^{tot} e^{-kt} \quad (9)$$

where $[A(0)]_o^{tot}$ is the initial total external concentration of weak base and $[A(t)]_o^{tot}$ is the total external concentration at time t. As the interior concentration must obey the relation $[A(t)]_i^{tot} = ([A(0)]_o^{tot} - [A(t)]_o^{tot})V_o/V_i$, if all the drug goes in then $V_o[A(0)]_o^{tot} = V_i\ [A(max)]_i^{tot}$, where $[A(max)]_i^{tot}$ is the maximum possible interior drug concentration. Thus

$$[A(t)]_i^{tot} = [A(max)]_i^{tot}(1 - e^{-kt}) \quad (10)$$

2.1.2. Weak bases with two or more amino functions

Certain drugs, such as vincristine, contain two amino functions. In this case, the total concentration of the weak base can be written as

$$[A]_o^{tot} = [A]_o + [AH^+]_o + [AH_2^{2+}]_o \quad (11)$$

The dissociation constants K_1 and K_2 for the first and second amino groups, respectively, are given by

$$K_1 = [A]_o[H^+]_o/[AH^+]_o \quad (12)$$

$$K_2 = [AH^+]_o[H^+]_o/[AH_2^{2+}]_o \quad (13)$$

Which leads to

$$[A]_o^{tot} = [A]_o\{1 + [H^+]_o/K_1 + [H^+]_o^2/K_1K_2\} \quad (14)$$

It is usually straightforward to utilize pH_o values such that $[H^+]_o \gg K_1$, K_2 and that $[H^+]_o^2/K_1K_2 \gg [H^+]_o/K_1$. Eq. (14) then simplifies to

$$[A]_o^{tot} \approx [A]_o[H^+]_o^2/K_1K_2 \quad (15)$$

In general, for n amino groups

$$[A]_o^{tot} \approx [A]_o[H^+]_o^n/K_1K_2 \ldots K_n \quad (16)$$

The rate constants for uptake of drugs containing two or more amino groups can be obtained by substitution of $[A]_o$ values (obtained from Eq. (16)) into Eq. (5). The major point to note is that the rates of uptake of weak bases with more than one amino function are progressively more sensitive to the external pH as shown in Table 1.

Experimentally, it is most convenient to measure the amount of drug inside the vesicle. From Eq. (10) it is clear that a plot of $\ln\{([A(max)]_i^{tot} - [A(t)]_i^{tot})/[A(max)]_i^{tot}\}$ vs. time should give a straight line with a slope $-k$. In turn, a plot of log k vs. pH_o should result in a straight line with a slope of unity, 2 or n for a compound with 1, 2 or n amino groups, respectively.

2.1.3. Effects of membrane-water partitioning

Many drugs are lipophilic amines which partition strongly into the lipid bilayer in the region of the membrane-water interface. The model used in the previous sections did not include effects resulting from this partitioning, which can result in much higher drug concentrations at the lipid-water interface

than in the bulk aqueous phase, as illustrated in Fig. 2. In this case Eq. (4) can be rewritten in the form

$$d[A]_o^{tot}/dt = -(PA_m/V_o)\left([A]_o^m - [A]_i^m\right) \quad (17)$$

where $[A]_o^m$ and $[A]_i^m$ are the concentrations of the neutral forms of the drug located at the membrane interface in the exterior and interior of the vesicle, respectively. The total number of drug molecules $N(A)_o^{tot}$ in the external aqueous (w) and membrane (m) domains is given by

$$N(A)_o^{tot} = v_o[A]_o^w + v_o[AH^+]_o^w + v_m[A]_o^m + v_m[AH^+]_o^m \quad (18)$$

where v_o is the external aqueous volume and v_m is the volume of the vesicle membrane. Defining the drug membrane-water partition coefficient K_p as $K_p = [AH^+]_o^m/[AH^+]_o^w$ and assuming that the drug dissociation constant K_d is the same for the free and membrane associated drug, i.e., $K_d = [A]_o^w[H^+]_o/[AH^+]_o^w = [A]_o^m[H^+]_o/[AH^+]_o^m$, it follows that

$$[A]_o^{tot} = [A]_o^m\left\{1/K_p + [H^+]_o/K_dK_p + v_m/v_o + v_m[H^+]_o/v_oK_d\right\} \quad (19)$$

Under conditions where $[H^+]_o \gg K_d$ and $v_o \gg v_m$ this simplifies to

$$[A]_o^m \approx \left(K_dK_p/[H^+]_o\right)[A]_o^{tot} \quad (20)$$

Given that $[A]_i^m = 0$ under initial conditions, substitution of Eq. (20) into Eq. (17) yields

$$d[A]_o^{tot}/dt = -\left(PA_mK_dK_p/V_o[H^+]_o\right)[A]_o^{tot} = -k[A]_o^{tot} \quad (21)$$

The rate constant associated with drug uptake therefore increases in proportion to the membrane-water partition coefficient K_p. This can result in substantial increases in the rates of uptake, as K_p values of 10 or more are common.

2.2. Equilibrium transbilayer distribution of weak bases

In the aforegoing discussion, the assumption was made that uptake of weak bases into LUVs with an acidic interior would continue until all the drug was accumulated. This is obviously not the case – for example, as each (neutral) amine reaches the acidic interior of the vesicle, it will consume a proton, thus reducing the pH gradient which drives the uptake. Here we determine the transbilayer distribution of the weak base at equilibrium.

2.2.1. Elementary equilibrium analysis

We first consider the case where the aqueous interior of the LUV is sufficiently well-buffered that the accumulated weak base does not significantly influence the interior pH. Then, following the simple model illustrated in Fig. 1, uptake will continue until the internal and external concentrations of the neutral form of the weak base are equal, i.e., until $[A]_i = [A]_o$. Assuming that K_d (inside) $= K_d$ (outside) it follows that

$$[H^+]_o/[AH^+]_o = [H^+]_i/[AH^+]_i \quad (22)$$

Incorporation of Eq. (7) (see Table 1) into Eq. (22) results in the relation

$$[AH^+]_i/[AH^+]_o = [H^+]_i/[H^+]_o \approx [A]_i^{tot}/[A]_o^{tot} \quad (23)$$

Thus, at equilibrium, the concentration of the weak base inside compared to that outside mirrors the proton concentration gradient. For example, a pH gradient of 3 units is predicted to lead to a 1000-fold higher concentration of weak base within the vesicle as compared to the external environment.

Table 1
Rate constants for the uptake of drugs containing one and more amino groups into LUVs with an acidic interior

Amino groups	$[A]_o$	k
1	$(K_d/[H^+]_o)\,[A]_o^{tot}$	$PA_mK_d/V_o[H^+]_o$
2	$(K_1K_2/[H^+]_o^2)\,[A]_o^{tot}$	$PA_mK_1K_2/V_o[H^+]_o^2$
n	$(K_1K_2 \ldots K_n/[H^+]_o^n)\,[A]_o^{tot}$	$PA_mK_1K_2 \ldots K_n/V_o[H^+]_o^n$

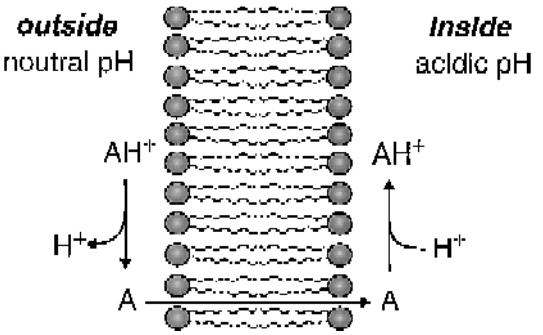

Fig. 1. Model for the uptake of weakly basic compounds into LUVs (inside acidic) in response to ΔpH. For compounds with appropriate pK_a values, a neutral exterior pH results in a mixture of both the protonated AH^+ (membrane impermeable) and unprotonated A (membrane permeable) forms of the compound. The unprotonated neutral form will tend to diffuse across the membrane until the interior and exterior concentrations are equal. However, an acidic interior results in protonation of the neutral form, thereby driving continued uptake of the compound. Depending on the quantity of the exterior weak base and the buffering capacity of the interior compartment, essentially complete uptake can usually be accomplished. The ratio of internal to external amine at equilibrium should equal the residual pH gradient, as described by Eq. (23). In much of the work from this laboratory, an exterior pH of 7.5 and an interior pH of 4.0 is employed with citrate (300 mM) as the internal buffer.

In the case of weak bases with two or more amino functions, it is straightforward to show that, for a weak base with two amino functions

$$[A]_i^{tot}/[A]_o^{tot} \approx [H^+]_i^2/[H^+]_o^2 \quad (24)$$

and that for a base with n amino functions

$$[A]_i^{tot}/[A]_o^{tot} \approx [H^+]_i^n/[H^+]_o^n \quad (25)$$

2.2.2. Effects of membrane-water partitioning or precipitation

We first consider the effects of partitioning into the inner monolayer. Utilizing the model shown in Fig. 2, it is possible, beginning from Eq. (19), to express the total external weak base concentration $[A]_o^{tot}$ in terms of the concentration of the neutral form of the weak base in the aqueous phase, $[A]_o^w$, rather than in terms of $[A]_o^m$ (as in Eq. (20)). A similar treatment can be performed for the interior weak base concentrations, giving

$$[A]_o^{tot} = [A]_o^w(1 + [H^+]_o/K_d)(1 + K_pV_m/V_o) \quad (26)$$

$$[A]_i^{tot} = [A]_i^w(1 + [H^+]_i/K_d)(1 + K_pV_m/V_i) \quad (27)$$

for $[H^+]_o$, $[H^+]_i \gg K_d$. At equilibrium, where $[A]_i^w = [A]_o^w$, the ratio of internal to external concentration of weak base is then

$$\frac{[A]_i^{tot}}{[A]_o^{tot}} = \frac{[H^+]_i(1 + K_pV_m/V_i)}{[H^+]_o(1 + K_pV_m/V_o)} \quad (28)$$

For most situations $K_p \ll V_o/V_m$ and thus Eq. (28) simplifies to

$$[A]_i^{tot}/[A]_o^{tot} = (1 + K_pV_m/V_i)[H^+]_i/[H^+]_o \quad (29)$$

For extremely high partition coefficients such as may be obtained with lipids that are weak bases, where $K_p \geq V_o/V_m$, V_i/V_m we obtain

$$[A]_i^{tot}/[A]_o^{tot} = ([H^+]_i/[H^+]_o)(V_o/V_i) \quad (30)$$

which can also be expressed as

$$N(A)_i/N(A)_o = [H^+]_i/[H^+]_o \quad (31)$$

where $N(A)_i$ and $N(A)_o$ are the membrane surface concentrations of the lipids at the inner and outer interfaces, respectively.

As can be observed from Eq. (29), the net effect of weak base partitioning into the inner monolayer is to increase the amount of drug accumulated in response to a given pH gradient. Similar effects would be expected if precipitation of the weak base was to occur after accumulation into the LUV. For example, doxorubicin can be accumulated into LUVs in re-

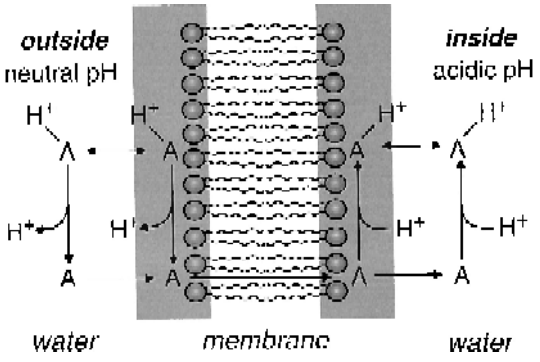

Fig. 2. Model for the uptake of weakly basic amines into LUVs, showing the effect of membrane-water partitioning. An amine possessing a high membrane-water partition coefficient $K_p = [AH^+]^m/[AH^+]^w$ (where the superscript m refers to the membrane-water interface (shaded area) and the superscript w refers to the bulk fluid phase) will accumulate to a greater extent than will an amine with a low partition coefficient (as described by Eq. (29)). See text for further details.

sponse to ΔpH to achieve apparent interior concentrations of 1 M or higher, which far exceeds the aqueous solubility of doxorubicin. In the case of doxorubicin there is evidence to suggest that the drug partitions into the inner monolayer, potentially reducing the aqueous drug concentration to soluble levels. However, if precipitation did occur the effect would be again to reduce the aqueous concentration of drug in the vesicle interior, thus leading to higher levels of accumulated drug than would be expected on the basis of the transmembrane pH gradient. As a result, it continues to be a challenge to experimentally distinguish between partitioning and precipitation phenomena.

2.2.3. Effect of internal buffering capacity

The equilibrium quantity of weak base that is accumulated in response to ΔpH is also dependent on the internal buffering capacity as each accumulated amine consumes a proton which will result in an increase in the interior pH if the interior aqueous volume is not adequately buffered. The total interior weak base concentration, $[A]_i^{tot}$, can be calculated from the value of $[H]_i^+$ after uptake and a knowledge of the interior buffering capacity, assuming that each neutral drug molecule that traverses the LUV membrane consumes a proton upon arrival in the vesicle interior, and non-specific leakage is avoided. In much of the work discussed here, citrate which has three ionizable groups ($pK_1 = 3.14$, $pK_2 = 4.78$, $pK_3 = 5.29$) was used as the internal buffer. Considering such a buffer 'B' with three ionizable groups, the relation between the total buffer concentration and the concentrations of neutral buffer [B] and the charged species ($[B^-]$, $[B^{2-}]$, $[B^{3-}]$) is given by

$$[B]^{tot} = [B] + [B^-] + [B^{2-}] + [B^{3-}] \quad (32)$$

$$[B] = [B]^{tot}/f(H^+) \quad (33)$$

$$[B^-] = K_1[B]^{tot}/([H^+]\,f(H^+)) \quad (34)$$

$$[B^{2-}] = K_1K_2[B]^{tot}/([H^+]^2 f(H^+)) \quad (35)$$

$$[B^{3-}] = K_1K_2K_3[B]^{tot}/([H^+]^3 f(H^+)) \quad (36)$$

where

$$f(H^+) = \left(1 + K_1/[H^+] + K_1K_2/[H^+]^2 + K_1K_2K_3/[H^+]^3\right) \quad (37)$$

where K_1, K_2, and K_3 are the dissociation constants for the ionizable groups of the buffer. Given that a single proton is consumed by each internalized drug molecule as it enters the vesicle interior, the total internal concentration of charged amine can be expressed by charge balance according to the relation:

$$[DH^+]_i = \left([B^-]^b - [B^-]^a\right) + 2\left([B^{2-}]^b - [B^{2-}]^a\right) + 3\left([B^{3-}]^b - [B^{3-}]^a\right) \quad (38)$$

where the superscripts b and a indicate the final and original concentrations of the charged forms of the buffer, respectively. It should be noted that if the interior pH is measured, Eq. (38) allows the theoretical concentration of internalized weak base to be calculated. This can be compared to the measured amount of drug accumulated to determine how well weak base accumulation is coupled to proton consumption.

2.3. Transbilayer transport of weak acids

The approach developed in Sections 2.1 and 2.2 for weak bases can also be applied to the uptake of simple weak acids, and to the transbilayer transport of acidic lipids such as fatty acids and some phospholipids. We consider uptake of a simple weak acid into an LUV with a basic interior. Let $[AH]_o$ ($[A^-]_o$) and $[AH]_i$ ($[A^-]_i$) denote the concentrations of the neutral (charged) form(s) of the weak acid on the outside and inside of the vesicle, respectively. Then the total external and internal concentrations of the weak acid are given by:

$$[A]_o^{tot} = [A^-]_o + [AH]_o \quad (39)$$

$$[A]_i^{tot} = [A^-]_i + [AH]_i \quad (40)$$

We consider the case where the weak acid is initially introduced in the external aqueous medium containing the LUVs with a basic interior. Assuming that the neutral (protonated) acid is the only membrane permeable form, it is then straightforward to show that the rate of uptake of the weak acid into the LUVs is given by:

$$d[A]_o^{tot}/dt = -(PA_m[H^+]_o/V_oK_a)[A]_o^{tot} = -k[A]_o^{tot} \quad (41)$$

where K_a ($= [A^-]_o[H^+]_o/[AH]_o$) is the dissociation

constant of the weak acid, and $k = (PA_m [H^+]_o/V_o K_a)$ is the rate constant associated with uptake. Aside from the terms which comprise the rate constant k, Eq. (41) is identical to Eq. (8), and thus the time dependence of the total external and internal weak acid concentrations is given by expressions analogous to Eqs. (9) and (10), respectively, where $[A(0)]_o^{tot}$ is now the initial total external concentration of weak acid, $[A(t)]_o^{tot}$ is the total external weak acid concentration at time t, and $[A(max)]_i^{tot}$ is the maximum possible interior weak acid concentration.

The equilibrium transbilayer distribution of weak acids can be derived using the approach given for weak bases in Section 2.2. We will consider only the case where the aqueous interior of the LUV is sufficiently well-buffered that the accumulated weak acid does not significantly influence the interior pH. Then, following a simple model similar to that illustrated in Fig. 1, uptake will continue until the internal and external concentrations of the neutral form of the weak acid are equal, i.e., until $[AH]_i = [AH]_o$. As K_a (inside) = K_a (outside) it follows that

$$[H^+]_o/[H^+]_i = [A^-]_i/[A^-]_o \quad (42)$$

Thus, at equilibrium, the transbilayer concentration gradient of the weak acid mirrors the inverse of the transbilayer concentration gradient of protons. For example, a ΔpH of 3 units (e.g., internal pH = 10, external pH = 7) should lead to a 1000-fold higher concentration of weak acid within the vesicle as compared to the external environment.

In closing this section, two points should be noted. First, factors such as surface potential which can significantly affect the interfacial pH have not been considered. For vesicle systems composed of PC/Chol mixtures, the membrane surface potential Ψ_o will be small, on the order of -10 mV for a bulk solute concentration of 10 mM [12]. For these conditions, the Gouy-Chapman-Stern theory [13,14] predicts a minor reduction in the pH of the membrane-water interface relative to the bulk solution (< 0.2 pH units), which becomes negligible (~ 0.03 pH units) when the bulk solute is increased to 300 mM [14]. However, the presence of high levels of positively charged lipophilic amines in the inner monolayer of a vesicle may be expected to raise the interfacial pH. An initial drug-to-lipid ratio of 0.2 (mol : mol) corresponds to a final drug concentration of 27 mol% in the inner monolayer, assuming complete drug uptake and partitioning of the drug into the inner monolayer. This corresponds to a surface potential of approximately 100 mV, leading to an inner monolayer interfacial pH which is approximately 0.5 pH units higher than the bulk (300 mM) solute [13,14]. For a trivalent solute such as citrate more effective shielding and thus smaller differences between bulk and interfacial pH would be expected.

The second point is that the treatment presented here is based on establishing the pH gradient by entrapping a buffer (such as citrate) and then adjusting the external pH. It should be noted that transmembrane pH gradients can also be established by other means. For example, entrapment of a weak base such as ammonium sulfate, followed by removal of external material, will result in a ΔpH (inside acidic). This is because the neutral (NH_3) form of ammonia is highly membrane permeable and is therefore able to leak out of the vesicle, leaving a proton behind. The pH gradient established, which obeys the relation $[H^+]_i/[H^+]_o = [NH_4^+]_i/[NH_4^+]_o$, can be three units or more. Drugs which are weak bases can then be accumulated in response to this ΔpH [15]. Generalized analyses for accumulation of drugs such as doxorubicin in response to pH gradients induced by ammonium sulfate gradients have been presented [16,17], with findings that are similar to those developed here.

3. Transbilayer transport of ΔpH probes, biogenic amines and drugs in response to transmembrane pH gradients

3.1. Probes of ΔpH

The first demonstration that transbilayer pH gradients can be generated, maintained, and measured in liposomal systems was provided by Deamer et al. [7]. These investigators examined the behaviour of small unilamellar vesicles (SUVs) subjected to a ΔpH employing the fluorescent probe molecules 9-aminoacridine and atebrin. They concluded that pH gradients as high as two units (inside acidic) could be generated in EPC SUVs employing an SUV with a transbilayer K^+ gradient, employing nigericin to mediate K^+ efflux in exchange for H^+ influx. This was

followed by elegant ESR spin label techniques developed by Cafiso and Hubbel [10] to determine ΔpH, again in EPC SUVs with an acidic interior. This study demonstrated excellent agreement with an equilibrium theory assuming that the permeating species is the neutral form.

Radiolabelled methylamine (MeAm), in combination with a spin column technique for the rapid separation of interior and exterior probe, has been applied extensively to determine ΔpH in large unilamellar vesicle (LUV) systems with an acidic interior [18,19]. This technique has been shown to give excellent agreement with results employing equilibrium filtration centrifugation techniques [19] and is the most readily employed procedure over reasonable ΔpH ranges. Certain cautions should be noted, however. First, as indicated in Fig. 3, MeAm does not necessarily provide an accurate measure of ΔpH in gel state lipid systems, and it may be necessary to briefly heat above the gel-to-liquid crystalline phase transition temperature T_c, before equilibrium redistributions of probe are observed [19]. This is presumably due to limited permeability of the neutral form of the probe through gel-state bilayers. As shown in Fig. 3, the presence of cholesterol, which eliminates the gel-to-liquid crystalline phase transition, also raises membrane permeability sufficiently to achieve equilibrium distributions of probe. Second, some buffering capacity inside the LUV is required to ensure that the probe itself does not dissipate the ΔpH. For example, to obtain an accurate estimation of a $\Delta pH > 2$ in 100 nm diameter LUVs with an acidic interior, an interior concentration of greater than 20 mM citrate or equivalent buffer is required [19].

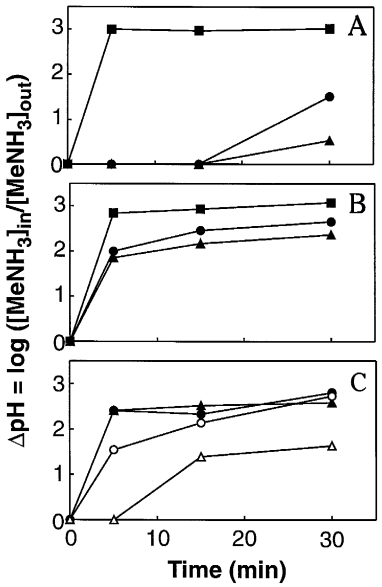

Fig. 3. (A) Apparent ΔpH as determined by $[^{14}C]MeNH_3^+$ distributions for vesicles with various lipid compositions in response to an imposed transmembrane pH gradient (inside acidic). Methylamine distributions were determined as described in Ref. [13] for vesicles containing 300 mM citrate (pH 4.0) extruded through 200 nm filters and subsequently incubated in 150 mM NaCl, 20 mM Hepes, pH 7.0 at 21°C. Vesicles were composed of EPC (■), DPPC (●), and DSPC (▲). (B) Methylamine response determined as in (A), but where vesicles contained 45 mol% cholesterol. (C) Methylamine response for vesicles (in the absence of cholesterol) incubated at 37°C (open symbols) or 60°C (closed symbols). The symbols have the same meaning as in (A). Modified from Harrigan et al. [19], with permission.

3.2. Drugs and biogenic amines

A large proportion of commonly employed pharmaceuticals are relatively lipophilic molecules containing primary, secondary, or tertiary amines. Indeed, the generality of this observation argues that such characteristics are important for function. A probable role concerns the ability of such molecules to traverse cell membranes to gain access to intracellular sites of action. As for MeAm, the presence of an ionizable amino function allows the compound to adopt a net neutral form which is considerably more membrane permeable than its charged (protonated) counterpart. In any event, the fact that a large proportion of drugs are lipophilic amines has two interesting consequences. First, by analogy with the response of methylamine, it would be expected that drugs which are lipophilic amines should be accumulated into liposomes with an acidic interior. Second, if the acidic interior is highly buffered, high interior concentrations of drug should be attained at equilibrium. As detailed in the following sections, these predictions are borne out for a great many drugs.

3.2.1. Doxorubicin

Doxorubicin, whose structure is shown in Fig. 4, is the most commonly employed chemotherapeutic agent, and is active against a variety of ascitic and solid tumours. Doxorubicin also induces a variety of toxic side effects including myelosuppression and a

cumulative dose-limiting cardiotoxicity [20]. As shown in Fig. 5, incubation of LUVs exhibiting a well-buffered (citrate) acidic interior ($pH_i = 4$, $pH_o = 7$) with doxorubicin at a drug-to-lipid ratio of 0.3 (w/w) results in the rapid uptake of the drug to achieve interior concentrations in excess of 300 mM [21–23]. This may be compared to initial exterior doxorubicin concentrations of 0.2–1 mM. These high interior to exterior concentration gradients can be stable for periods of weeks or longer and can be associated with 'trapping efficiencies', defined as the percentage of the drug which is entrapped in the LUVs, of 98% and higher [22,23]. Similar results can be achieved for a wide range of LUV sizes, drug-to-lipid ratios, and lipid compositions [23].

Several other important experimental features are illustrated in Fig. 5. First, the rate and extent of drug uptake is highly dependent on temperature [23] (Fig. 5A). For EPC/cholesterol LUVs, essentially 100% entrapment is achieved within 5 min at 60°C, and within 90 min at 37°C. However, at 21°C only 30% entrapment is achieved. Second, different lipid compositions lead to different uptake behaviour. For example, the presence of cholesterol reduces the rate and extent of uptake of doxorubicin into EPC LUVs (Fig. 5B). At 20°C, entrapment levels approaching 100% were achieved within 15 min in EPC LUVs. However, in the presence of 50 mol% cholesterol, uptake levels at 1 h were only 70% [21]. In general, factors which lead to increased order in the lipid bilayer, such as increased cholesterol content, acyl chain length or acyl chain saturation, result in slower uptake (and release) properties.

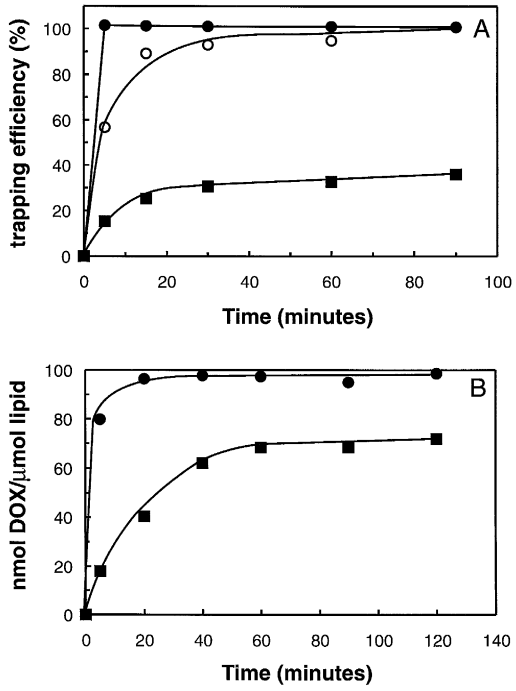

Fig. 5. (A) Effect of incubation temperature on uptake rates and trapping efficiencies of doxorubicin, accumulated into 200 nm EPC/cholesterol (55:45 mol/mol) LUVs exhibiting a transmembrane pH gradient (pH 4 inside, 7.8 outside). Doxorubicin was added to LUVs (drug-to-lipid ratio = 0.3, w/w) equilibrated at 21°C (■), 37°C (○), and 60°C (●). The trapping efficiency was calculated as the percentage of the drug initially in the exterior medium which was accumulated into the LUVs. Reproduced from Mayer et al. [23], with permission. (B) Effect of cholesterol on the uptake rates and trapping efficiencies of doxorubicin at 20°C into 100 nm LUVs exhibiting a transmembrane pH gradient (pH 4.6 inside, 7.5 outside). Lipid compositions were EPC (●) and EPC/cholesterol (1:1 mol/mol) (■). The initial drug-to-lipid ratio was 100 nmol/μmol lipid. Reproduced from Mayer et al. [21], with permission.

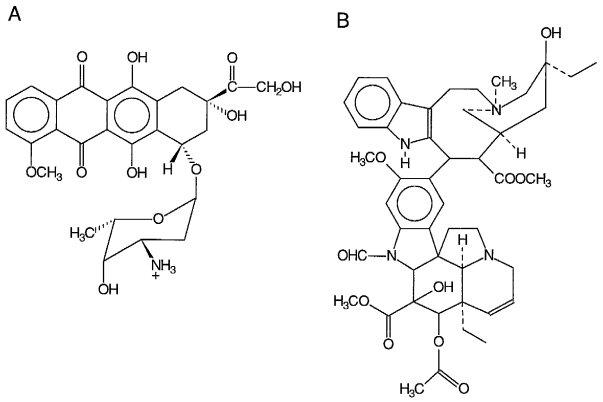

Fig. 4. The chemical structures of doxorubicin (A) and vincristine (B).

The strong temperature-dependence of doxorubicin uptake into LUVs reflects the high activation energies for transmembrane transport of the neutral form of the drug. Activation energies for the uptake of doxorubicin into EPC and EPC/Chol (55:45) LUVs were 28 kcal/mol and 38 kcal/mol, respectively [24]. These values are similar to those obtained for the transbilayer movement of other anticancer drugs such as vincristine [25], certain acidic phospholipids (phosphatidic acid and phosphatidylglycerol) [26,27],

and certain basic amino acids and peptides [28,29]. The increase in E_a upon addition of cholesterol to the membrane indicates that cholesterol, which increases the order of fluid membranes, also adds a considerable barrier to transport (see Fig. 5B).

There are a number of features of doxorubicin uptake into LUVs with an acidic interior which differ from the behaviour of methylamine. The primary difference is that more doxorubicin is accumulated in response to a given ΔpH than would be expected on the basis of the simple theory of Section 2.2.1. This is illustrated by the fact that the transbilayer concentration gradients of doxorubicin considerably exceed the concentration gradients of methylamine achieved in response to the same ΔpH, as shown in Fig. 6A. As indicated by Harrigan et al. [24], this behaviour can be accounted for by the high membrane-water partition coefficient of doxorubicin. The partition coefficient was calculated from the data in Fig. 6A using Eq. (30), where $[H^+]_i/[H^+]_o$ was obtained by determining $[MeAm]_i/[MeAm]_o$, and a value of $V_m/V_i = 0.33$ was assumed for a 100 nm LUV, giving $K_p = 70$. This value was found to be in excellent agreement with that obtained using the classical equilibrium filter centrifugation technique ($K_p = 74$) [24]. Thus the uptake of drugs into LUVs in response to transmembrane pH gradients provides a potentially useful methodology for measuring membrane-water partition coefficients. More importantly, drugs which exhibit high partition coefficients can be accumulated and retained in LUVs to much higher levels than drugs which exhibit small K_p values, in response to the same ΔpH.

It is of interest to determine if the systems are well 'coupled', so that each drug molecule taken up can be related to the consumption of one proton in the LUV interior. This can be determined by calculating the theoretical drug and pH gradients which are obtained for a given external drug concentration. From a knowledge of $[H^+]_i$ before and after uptake of the drug (obtained from methylamine uptake), the internal buffering capacity, and the doxorubicin partition coefficient, the final concentration of $[DH^+]_i$ can be calculated, with no adjustable parameters, from Eqs. (32)–(38), allowing construction of the theoretical plots shown in Fig. 6B [24]. The excellent agreement between actual and theoretical uptake demonstrates how well coupled the uptake process is, and

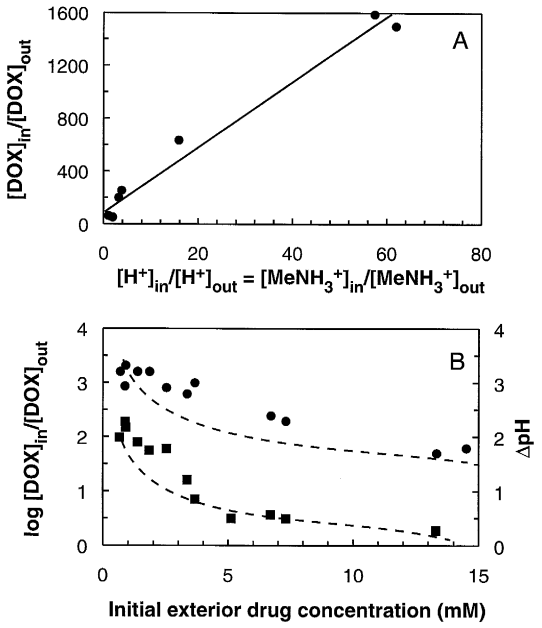

Fig. 6. Relationship between doxorubicin inside-outside concentration gradients and methylamine inside-outside concentration gradients. Doxorubicin and methylamine concentration gradients at equilibrium were determined by incubating EPC/cholesterol LUVs containing 300 mM citrate (pH 4.0) at 60°C for 20 min with the indicated concentrations of doxorubicin and trace concentrations of radiolabeled methylamine in Hepes-buffered saline (pH 7.0). (A) The relationship between the equilibrium concentration gradient of doxorubicin and the residual proton gradient, as determined by the concentration gradient of methylamine. The slope of the solid line (obtained by linear regression) is 24, allowing calculation of the partition coefficient, using Eq. (29), to be $K_p = 70$. (B) Coupling of doxorubicin uptake to internal buffering capacity. Experimental results presented are equilibrium doxorubicin (●) and methylamine (■) concentration gradients. The dotted lines were generated using Eqs. (29) and (38) as described in Section 2.2, given $K_p = 70$ and the initial interior pH and internal citrate concentration, and assuming a trapped volume of 0.84 litre/mol lipid and a bilayer thickness of 5 nm. For more details, see the text and Ref. [24]. Reproduced from Harrigan et al. [24], with permission.

that the permeability barrier of the LUV membrane stays intact even at extremely high levels of drug accumulation. For example, for initial exterior doxorubicin concentrations of 14 mM (Fig. 6B), internal doxorubicin concentrations of 4 M are reached.

Using Eq. (29), similar fits between drug uptake and transmembrane proton gradients can be generated using the partition coefficient K_p as the adjustable parameter. Reasonable fits were obtained for lidocaine, ethanolamine, timolol, and dopamine, using

K_p = 0, 5, 4, and 50, respectively. However, poor fits were obtained with imipramine and dibucaine, regardless of the value of K_p used, suggesting that accumulation of these drugs caused breakdown of the pH gradient by perturbing the bilayer permeability barrier [24].

The high membrane-water partition coefficient, in combination with the high levels of accumulated drug, can have potentially interesting consequences for the morphology of doxorubicin loaded LUVs. In particular, it is straightforward to show that a partition coefficient of 70, an initial drug-to-lipid ratio of 0.2 (mol : mol) in combination with a 99% trapping efficiency, and a V_m/V_i ratio of 0.3 leads to the conclusion that over 95% of the entrapped drug is associated with the inner monolayer of the LUV, which corresponds to a molar ratio of drug-to-lipid molecules in the inner monolayer of approximately 0.4. Assuming an area per molecule for doxorubicin at this interface to be 0.5 nm^2, this corresponds to an increase in the inner monolayer surface area of at least 30%. It would therefore be expected that at high entrapped drug levels the membrane associated drug would lead to marked increases in the area of the inner monolayer.

As indicated in Section 4.4 the morphological changes due to transbilayer surface area imbalances in LUVs can be dramatic. In this regard a constant feature observed by cryo-electron electron microscopy for doxorubicin loaded LUVs is illustrated in Fig. 7. The liposomes exhibit a characteristic 'coffee-bean' appearance due to the presence of an inner structure apparently separating the LUV into two sections. Two explanations of these structures have been proposed. The first proposes a role for transbilayer area imbalances due to the high levels of encapsulated drug. In this picture, the inner structure may represent lipid extrusions or blebbing off from the inner monolayer to relieve the excess inner monolayer surface area. This approach is supported by the ability to model the observed doxorubicin uptake on the basis of the known membrane-water partition coefficient [24]. It should be noted that the inner structure likely corresponds to a tube shape, as it is centrally located in a high proportion of vesicles containing doxorubicin.

A second possible explanation for the inner feature is that it corresponds to precipitated doxorubicin.

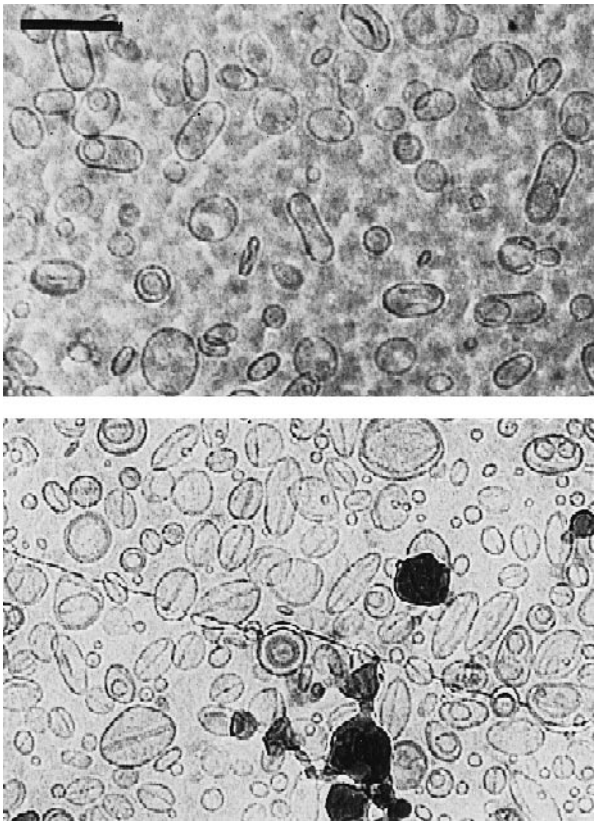

Fig. 7. Cryo-electron micrograph of 100 nm EPC/cholesterol LUVs (top) exhibiting a transmembrane pH gradient (pH_o = 7.0, pH_i = 4.0) (bottom) containing doxorubicin (drug-to-lipid ratio = 0.3 mol/mol) loaded in response to the transmembrane pH gradient (citrate internal buffer). The additional internal structure observed in the bottom figure, which gives the LUV the appearance of a 'coffee-bean', is thought to represent invaginated membrane segments or internal protrusions which bleb off from the inner monolayer as a result of the increased area of the inner monolayer caused by the high concentration of membrane-associated doxorubicin. Reproduced from Harrigan, P.R. (1994) Ph.D. Thesis, University of British Columbia, with permission.

This model is supported by the fact that if the doxorubicin is present in the aqueous interior of the vesicle, the aqueous concentration far exceeds its solubility. Barenholz and coworkers [15,30,31] have observed a similar coffee-bean morphology for uptake of doxorubicin induced by entrapped ammonium sulfate, and attribute it to a doxorubicin sulfate precipitate or gel. The fact that similar morphology is observed in the absence of sulfate (the vesicles in Fig. 7 contain citrate) indicates a more general phenomenon, and it should be noted that if 95% of the drug partitions into the inner monolayer, the concen-

tration of the remaining free drug in the vesicle interior will be reduced below the precipitation threshold.

The ability to entrap doxorubicin inside LUVs in response to ΔpH has proved to be of considerable practical utility in liposomal drug delivery systems. Early studies had shown (see [32] and references therein) that liposomal entrapment of doxorubicin could result in reduced toxicity (particularly cardiotoxicity) in animal studies, while maintaining or even increasing anticancer efficacy. However, methods for stably and efficiently entrapping doxorubicin in the liposome at high drug-to-lipid ratios were not available. The ΔpH methodology has solved these problems [32–37] and has led to liposomal doxorubicin preparations which are currently either in advanced clinical trials [38], or have been approved by the US FDA for clinical use [39].

3.2.2. Vincristine and other lipophilic amines

The observations made for doxorubicin can be extended to a large variety of drugs, as well as certain biogenic amines. For example, the anticancer drug vincristine (Fig. 4) can be efficiently trapped in LUVs in response to ΔpH, leading to liposomal vincristine preparations of potential therapeutic benefit [25,40–42]. It is interesting to note that because vincristine has two amino functions, the kinetics of uptake depend on the square of the external proton concentration [25], as predicted in Section 2.1.2. Further, possibly because the pK values of these amino groups are relatively low (pK_1 = 5.0, pK_2 = 7.4), the drug is not retained as easily within LUVs as doxorubicin. Improved retention can be obtained by increasing the chain length of the phosphatidylcholine and by increasing the internal buffering capacity [25]. However, the most significant improvements have been obtained by lowering the internal pH from 4.0 to 2.0, or by changing the lipid composition, with 90% retention obtained over 24 h in vitro for DSPC/Chol vesicles with an initial interior pH of 2.0 incubated in mouse serum [25].

In vivo, vincristine is released from the liposomes more rapidly than in vitro. However, the formulation with internal pH of 2 still exhibited 40% retention over 24 h, a 5-fold improvement over the formulation with an internal pH of 4 [25]. The importance of appropriate retention characteristics, which can result in dramatic improvements in therapeutic activity as measured in a murine P388 lymphocytic leukemia model [41], are shown in Fig. 8. More recently, it has been found that exchanging sphingomyelin for DSPC provides excellent retention characteristics combined with improved chemical stability [42], leading to a formulation with considerable pharmaceutical potential.

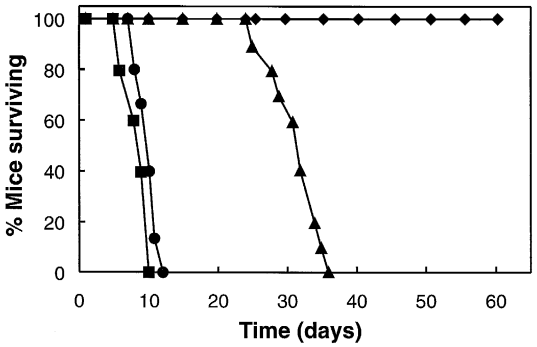

Fig. 8. Influence of intravesicular pH on the efficacy of vincristine encapsulated in DSPC/Chol LUVs against P388 tumors. BDF1 mice bearing peritoneal ascitic P388 tumors were untreated (■) or were treated with free vincristine (●) or LUVs composed of DSPC/Chol containing encapsulated vincristine and with an intravesicular pH of either 4.0 (▲) or 2.0 (◆). Reproduced from Boman et al. [123], with permission.

A summary of the uptake properties of a variety of drugs and biogenic amines into EPC LUVs experiencing a ΔpH of 3.5 units (pH$_o$ = 7.5, pH$_i$ = 4.0) is illustrated in Table 2 [43]. The experimental conditions were such that if the drug did not experience membrane partitioning effects, approximately half of the available drug should be accumulated. This corresponds to uptake levels of 100 nmol/μmol lipid. Higher levels of entrapment, such as are observed for the anticancer drugs (see Table 2) correspond to larger membrane-water partition coefficients as previously discussed for doxorubicin. It should be noted that essentially complete uptake and excellent drug retention can be achieved for these compounds by simply altering the initial drug-to-lipid ratio or the lipid composition.

In summary, the ability of drugs which are weak bases to accumulate into LUVs with an acidic interior is not restricted to any particular drug class, and different levels of uptake between drugs can be attributed to differences in the membrane-water partition coefficients. This procedure is now the preferred

Table 2
Extent and stability of accumulation of various drugs by vesicles exhibiting a pH gradient [a]

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 [b]	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
Dopamine	Biogenic amines	190 [c]	177
Serotonin	Biogenic amines	80 [d]	78
Imipramine	Antidepressant	182	188
Diphenhydramine	Antihistamine	176 [b]	87
Quinine	Antimalarial	148 [b]	81
Chloroquine	Antimalarial	104 [b]	88
Quinacrine	Antiprotozoan	73 [c]	71
Codeine	Analgesic	< 1	< 1

[a] Reproduced from Madden et al. [43], with permission.
[b] Maximum uptake taken at 5 min.
[c] Maximum uptake taken at 30 min.
[d] Maximum uptake taken at 90 min.

method of loading drugs into liposomes for drug delivery applications.

4. Transbilayer transport of lipids in response to ΔpH: lipid asymmetry

4.1. Simple amino lipids and fatty acids

The ability of lipophilic amino containing drugs such as doxorubicin and vincristine to accumulate into LUVs with an acidic interior suggests that aminolipids should also redistribute in a similar manner. This has been demonstrated for two lipids which are weak bases, namely sphingosine and stearylamine [44], when present as minority components of EPC LUVs. The imposition of a ΔpH (interior acidic) resulted in the immediate sequestration of sphingosine and stearylamine into the inner monolayer as detected by changes in surface charge employing column chromatography techniques. More recently, a series of six synthetic aminolipids, with structures based on 3-(*N*,*N*-dimethylamino)-1,2-propanediol, were shown to redistribute to the interior monolayer of EPC/Chol vesicles in response to a pH gradient [45]. As discussed more fully below, some of these aminolipids displayed fusogenic properties which allowed vesicle fusion to be regulated by lipid asymmetry established by the pH gradients.

As indicated in Section 2.3, the transbilayer distribution of fatty acids should also be sensitive to ΔpH, where a basic interior should result in sequestration to the inner monolayer. This behaviour has been observed for oleic acid, as illustrated in Fig. 9 [44].

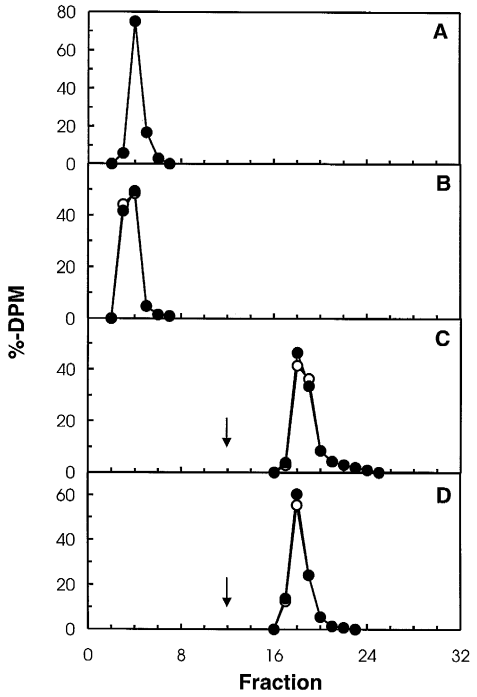

Fig. 9. Demonstration of ΔpH-dependent lipid asymmetry for LUVs composed of EPC:stearic acid (9:1 mol:mol). (A) Elution of EPC LUVs (neutral surface charge) in the void volume of DEAE-Sephacel using 10 mM Hepes pH 7.0. (B) EPC:stearic acid LUVs exhibiting ΔpH (interior pH = 10.0 and exterior pH = 7.0) eluted by 10 mM Hepes pH 7.0. The elution of these LUVs in the void volume, under conditions where negatively charged LUVs would bind to the positively charged column, indicates that the stearic acid has been localized to the inner monolayer. (C) EPC:stearic acid LUVs (interior and exterior pH = 7.0), exhibiting a negative surface charge due to the presence of stearic acid on both surfaces, do not elute in the void volume, but only after beginning elution with 0.5 M NaCl pH 8.5 (indicated by the arrow). (D) elution profile of EPC:stearic acid LUVs prepared as in (B) (interior pH = 10.0 and exterior pH = 7.0) and subsequently treated with valinomycin, nigericin, and KCl. This dissipates the pH gradient, resulting in a redistribution of the stearic acid to the outer monolayer, and retention of the LUVs on the column under low ionic strength conditions. Closed circles (●) represent [^{3}H]EPC and open circles (○) represent [^{14}C]stearic acid. Reproduced from Hope and Cullis [44], with permission.

The imposition of a ΔpH (interior basic) results in an immediate migration of oleic acid to the inner monolayer of EPC LUVs at 20°C. In addition, the ability of stearylamine and oleic acid to rapidly exchange between vesicles and the subsequent ability of vesicles with basic interiors to sequester fatty acids from other vesicles, as well as albumin, has also been demonstrated [46].

4.2. Phospholipids

The most likely phospholipids for which transmembrane pH gradients could induce net transbilayer movement are weak acids such as phosphatidylglycerol (PG) and phosphatidic acid (PA). By analogy with the behaviour of fatty acids, PA or PG containing LUVs with a basic interior should sequester the acidic phospholipids to the inner monolayer. This was first demonstrated for egg PG and DOPA [47], and more recently for DOPG [65]. Fig. 10 shows the time course of transport of DOPG (at 60°C) from the inner to outer monolayer in response to a transmembrane pH gradient (interior acidic) [65]. Fig. 11A shows the transport of DOPA from the outer to inner monolayer in response to ΔpH (interior basic). In contrast to the behaviour of fatty acids, detectable rates of egg PG, DOPG and DOPA transport only occur at elevated ($T > 30$°C) temperatures. Subsequent studies have demonstrated that the kinetics of transport of both egg PG [26] and DOPA [27] in response to ΔpH were consistent with the transport of the neutral (protonated) form, with half-times of 7 s and 25 s for egg PG and DOPA, respectively, at 45°C. This was determined from the dependence of the rate constant for transport on external pH. The transbilayer transport of both lipids was associated with a high activation energy, in the range of 30 kcal/mol, which may reflect requirements for dehydration of the phospholipid headgroup in order to cross the hydrocarbon region.

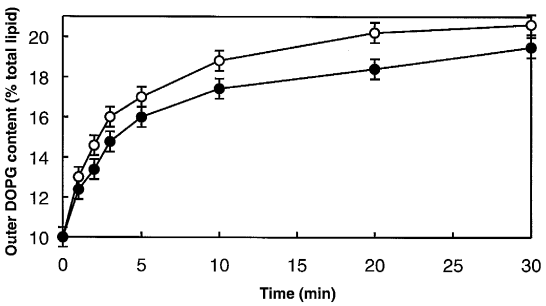

Fig. 10. Demonstration of ΔpH-dependent DOPG asymmetry for LUVs composed of DOPC:DOPG (9:1 mol:mol) (○) or DOPC:DOPG:Chol (6:1:3 mol:mol:mol) (●). The curves represent the transport of DOPG from the inner to the outer monolayer at 60°C in response to a transmembrane pH gradient (interior pH = 4.0 and exterior pH = 7.5). DOPG asymmetry was determined using the TNS assay as described in Ref. [65]. Reproduced from Mui et al. [65], with permission.

The ability to induce transbilayer lipid asymmetry in LUVs in response to pH gradients has allowed study of the influence of lipid asymmetry on membrane properties. Two important examples concern membrane fusion and membrane morphology, as discussed below.

4.3. Lipid asymmetry and membrane fusion

The ability of membranes to fuse is of fundamental importance in such intracellular vesicle transport phenomena as endocytosis and exocytosis, as well as in events such as fertilization and viral infection [48]. Studies on model systems have revealed that there is a strong correlation between fusogenicity and the ability of the lipid components to adopt non-bilayer phases, such as the hexagonal H_{II} phase and various cubic phases [49]. This has led to the view that transient non-bilayer lipid structures play an important role as intermediates in membrane fusion [50]. This, in turn, leads to the possibility that asymmetric transbilayer distributions of fusogenic lipids can regulate the fusogenicity of the membrane as a whole. A pertinent example is the transbilayer lipid asymmetry observed in the erythrocyte membrane, where the inner monolayer is composed largely of PE and PS, and the outer monolayer contains mainly PC and SPM. Vesicles composed of PE and PS fuse readily in the presence of Ca^{2+}, whereas those made from PC and SPM resist fusion [51]. Thus the sequestering of PE and PS to the inner monolayer may prevent extracellular fusion events which may lead to deleterious effects. It should be noted that erythrocytes which have lost lipid asymmetry fuse more readily than those which have not [52]. In addition, some cells, such as myoblasts, have a higher proportion of PE and PS in the external leaflets of their plasma membranes, consistent with the requirement of these cells to undergo fusion to form myotubules [53,54]. In fact, the levels of PE and PS in the myoblast outer monolayer have been observed to increase prior to fusion [55].

Until recently, the role of lipid asymmetry as a regulator of membrane fusion has proven difficult to investigate, due in part to the lack of appropriate model systems. For example, it is not yet possible to generate transbilayer phospholipid asymmetry in LUVs corresponding to that observed in erythrocytes, with PE and PS localized to the inner monolayer and PC and SPM to the outer. However, as previously indicated, transbilayer asymmetry of certain ionizable lipids can be induced in LUVs in response to transmembrane pH gradients [26,27,47], and this lipid asymmetry can regulate vesicle-vesicle fusion in certain systems [45,56,57].

The first example concerns LUVs composed of cardiolipin/DOPC/cholesterol (1:1:1) containing 20 mol% oleic acid, which were found to exhibit low fusion rates (as measured by a resonance energy transfer assay) in the presence of Ca^{2+} at pH 6.0, where the oleic acid is uncharged, but significant Ca^{2+}-dependent fusion at pH 7.5, where the oleic acid is partially charged [56]. In the presence of a pH gradient ($pH_i = 10.5$, $pH_o = 7.5$) the oleic acid was transported to the inner monolayer, and the extent of Ca^{2+}-stimulated fusion was reduced to minimal levels. Dissipation of the pH gradient, by addition of a proton ionophore, resulted in a rapid increase of the fusion rate, which can be attributed to re-equilibration of the oleic acid between the bilayer leaflets.

A second example of regulation of fusion by lipid asymmetry concerns LUVs containing PA. In particular, in LUVs with appropriate lipid compositions, as little as 10 mol% PA can render the LUVs sensitive to Ca^{2+}-induced fusion, and the extent of this fusion can be regulated by pH-gradient induced sequestration of the PA to the inner monolayer [57]. As shown in Fig. 11B, LUVs composed of DOPC/DOPE/PI/DOPA (25:60:5:10) undergo rapid and complete fusion (as assayed by resonance energy transfer techniques) in the presence of Ca^{2+}, but little or no fusion when the PA was sequestered in the inner monolayer in response to an applied pH gradient (interior basic). Furthermore, the extent of fusion was found to correlate with the amount of external PA, demonstrating that the fusogenic tendencies of lipid bilayers are determined by the properties of the monolayers proximate to the fusion interface.

Regulation of fusion by pH gradient-induced lipid asymmetry has also been demonstrated in LUVs containing synthetic aminolipids [45]. These systems differ from those discussed above in that Ca^{2+} is not a trigger for fusion. The LUVs were composed of EPC/DOPE/Chol (35:20:45) and contained 5 mol% of an aminolipid based on 3-(*N*,*N*-dimethylamino)-1,2-propanediol, with a pK_a value of 6.6.

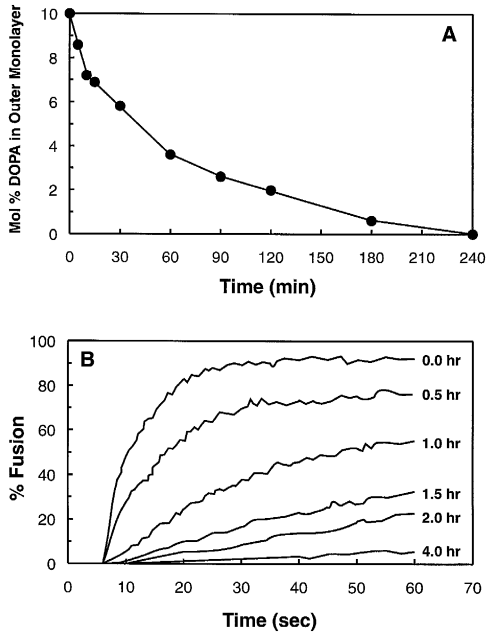

Fig. 11. (A) Demonstration of ΔpH-dependent DOPA asymmetry for LUVs composed of DOPC/DOPE/PI/DOPA (25:60:5:10). The curve represents the time course of DOPA transport from the outer to inner monolayer in response to a transmembrane pH gradient (interior pH = 7.5 and exterior pH = 4.0). The DOPA asymmetry was determined by employing a TNS fluorescent assay as described in Ref. [27]. (B) Effect of transbilayer lipid asymmetry on the Ca^{2+}-induced fusion of DOPC/DOPE/PI/DOPA (25:60:5:10) LUVs. LUV fusion, as assayed by lipid mixing, was monitored using resonance energy transfer (RET) as described in Ref. [57]. Briefly, unlabelled LUVs were mixed with LUVs containing 0.7 mol% each of NBD-PE and Rh-PE. NBD-PE fluorescence is quenched by the Rh-PE; upon lipid mixing, an increase in NBD-PE fluorescence is observed as lipid mixing dilutes the concentration of the Rh-PE. When DOPA is present in the outer leaflet of the LUV, the addition of Ca^{2+} causes LUV fusion. To assess the influence of lipid asymmetry on LUV fusion, LUVs were prepared exhibiting a ΔpH, and after the times indicated for each curve, fusion was monitored by the addition of Ca^{2+}. A marked decrease in both the rate and extent of fusion is observed as the DOPA partitions to the inner membrane, indicating that LUV fusion is dependent on the concentration of DOPA in the outer monolayer. Reproduced from Eastman et al. [57], with permission.

Thus at pH values of 7 or higher, this lipid is neutral and is similar to dioleoylglycerol, a very potent fusogen [58]. Fusion was not observed at pH 4.0, where the positively charged headgroup imparts bilayer-stabilizing character to the lipid, nor if the external pH was raised to 7.5, as this resulted in transport of the external aminolipid to the inner monolayer, analagous to the uptake observed with doxorubicin. However, when the pH-gradient was dissipated, allowing the internal aminolipid to redistribute to the exterior (pH 7.5) where the aminolipid was neutral, extensive fusion was observed. These results were corroborated by freeze fracture electron microscopy. Furthermore, the potential involvement of non-bilayer lipid structures in these fusion events were indicated by ^{31}P NMR and ^{2}H NMR studies demonstrating bilayer structure at pH 4.0, whereas at pH 7.5 partial hexagonal phase was observed [45].

4.4. Lipid asymmetry and membrane morphology

Dramatic variations in membrane morphology can be achieved in both biological membranes [59] and in lipid vesicles [60–64] simply by establishing an inequality between the surface areas of the two monolayers comprising the bilayer. For erythrocyte membranes, for example, the area of the outer monolayer can be increased by the addition of amphipathic compounds to the external medium, resulting in a transition from discocyte to echinocyte morphology [59]. The morphology of giant lipid vesicles can also be modulated by increasing the surface area of either monolayer relative to the other. Vesicles with larger surface areas of the inner monolayer as compared to the outer monolayer are associated with small vesicles inside larger vesicles [63,64]. Increases in the area of the external monolayer relative to the internal monolayer results in a well-characterized morphological progression. Initially, small vesicles are observed within larger vesicles, but this soon leads to the generation of discoid shapes, and then small vesicles outside of the large vesicles [60–62].

Several recent studies have shown that the induction of transbilayer area asymmetry also results in dramatic changes in membrane morphology in LUVs. As described above (Section 4.2), lipid asymmetry can be generated in mixtures of EPC containing either egg PG or DOPA in response to transmembrane pH gradients [26,27]. Depending on the direction of the pH gradient, net transport of egg PG or DOPA to the inner or the outer monolayer can be achieved. As available evidence [26] suggests that there is no compensatory redistribution of the EPC in response to the egg PG or DOPA movement, the transbilayer movement of the charged lipid gives rise

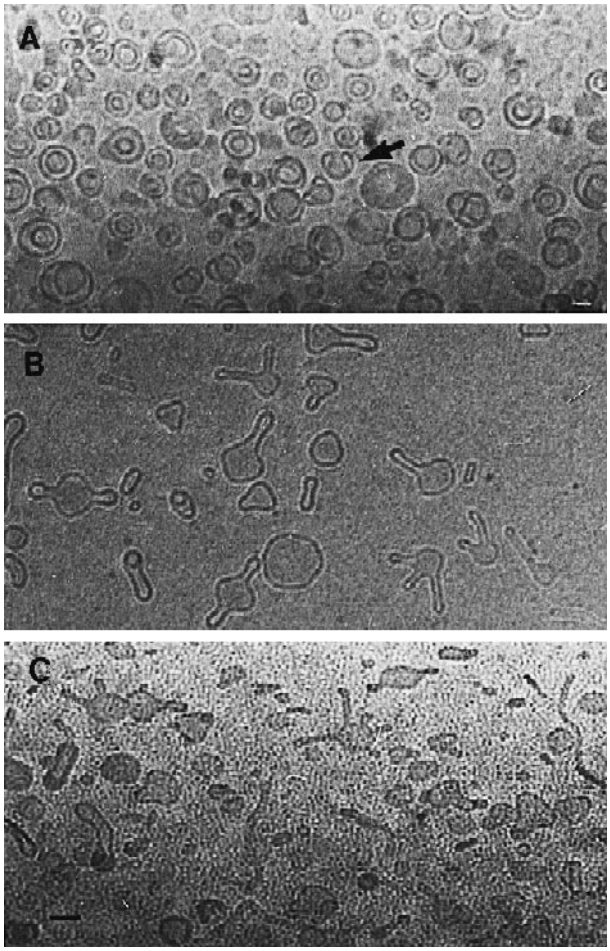

Fig. 12. Morphological changes in DOPC:DOPG (9:1 mol:mol) LUVs generated by the transport of DOPG to the outer monolayer. Cryo-electron micrographs were taken of DOPC:DOPG (9:1 mol:mol) LUVs, which have been exposed to a pH gradient ($pH_o = 7.5$, $pH_i = 4.0$) and incubated at 60°C for (A) 0 min; (B) 3 min; (C) 20 min. The bar (lower left of (C)) represents 200 nm. For other details, see Ref. [65]. Reproduced from Mui et al. [65], with permission.

to both lipid and surface area asymmetry. In LUVs composed of DOPC and DOPG this asymmetry results in dramatic morphological changes as revealed by cryo-electron microscopy. Transport of DOPG from the inner to outer monolayer transformed initially invaginated vesicles into either long narrow tubular structures, or spherical structures with one or more protrusions [65] (Fig. 12). This behaviour was also observed in the presence of cholesterol, supporting the surprising conclusion that cholesterol does not redistribute across the bilayer to relieve transbilayer area asymmetries. Transport of DOPG from the outer monolayer to the inner monolayer gave rise to invaginated structures. These results dramatically demonstrate the extent to which membrane morphology can be regulated by pH-gradient induced lipid asymmetry in vitro, and potentially in vivo.

5. Transbilayer transport of amino acids and peptides in response to ΔpH

The ability of a wide variety of weak acids and weak bases to accumulate into LUVs in response to pH gradients relies on the fact that the neutral forms are much more membrane permeable than the charged forms. This suggests that zwitterionic compounds such as amino acids would not be amenable to pH gradient-dependent uptake. However, if the carboxyl groups are modified to methyl ester or amide forms, the resulting basic amino acid derivatives can display the same behaviour as other amine-containing weak-base compounds [28,29]. One study examined the uptake of derivatives of lysine (two amino groups) and a pentapeptide (one amino group) into EPC LUVs displaying a pH gradient of 3.5 units ($pH_i = 4.0$, $pH_o = 7.5$) [28]. Both compounds displayed rapid uptake in the presence of a pH gradient but little or none in the absence of a pH gradient. Studies of the uptake of the peptide as a function of external pH (keeping the gradient at 3.5 units) allowed determination of the rate constants k (Table 1) as a function of pH. A plot of log k vs. external pH (see Table 1) yielded slopes close to 2 and 1 for the lysine and pentapeptide derivatives, respectively, consistent with permeation of the neutral forms.

These results have been recently extended to examine a number of di- and tripeptides composed exclusively of basic (lysine) and hydrophobic (tryptophan) amino acids [29]. Interestingly, in both di- and tripeptides in which the same amino acid residues were present, varying only in their order, significant differences were noted in the rate constants and activation energies for uptake. An example is given in Fig. 13, which describes the uptake of Lys-Trp-amide and Trp-Lys-amide into 100 nm EPC:chol vesicles. The rate constant for uptake of the Lys-Trp-amide was found to be 5×10^3 faster than for uptake of the Trp-Lys-amide. It was concluded that

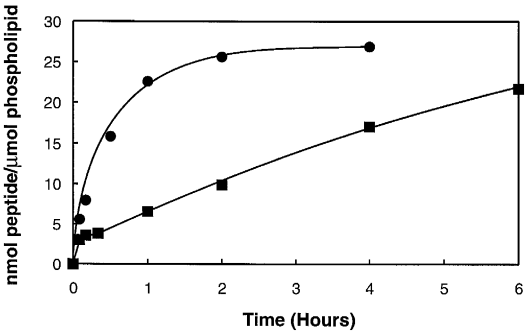

Fig. 13. Time course of uptake of Lys-Trp-amide (circles) and Trp-Lys-amide (squares) into 100 nm EPC:cholesterol (55:45) LUVs exhibiting a pH gradient ($pH_i = 3.0$, $pH_o = 5.5$, 21°C for Lys-Trp-amide; $pH_i = 4.0$, $pH_o = 8.0$, 55°C for Trp-Lys-amide). Reproduced from Chakrabarti et al. [29], with permission.

different charge distributions in short peptides of identical amino acid composition could strongly influence the ability of these groups to associate with and translocate across lipid bilayers [29].

6. Transbilayer transport of cations in the presence of ΔpH and ionophores

LUV systems possessing a pH gradient can also be used to accumulate cations such as Ca^{2+}, Fe^{2+}, and Ba^{2+} [66–68]. Since cations do not exist in a membrane-permeable neutral form, a carrier is required to permit uptake of the ion. The lipophilic ionophore antibiotic A23187 is useful in this regard as it is capable of transporting divalent cations across biological and model membranes, via an electroneutral transport arising from an M^{2+} for $2H^+$ exchange [69–72]. Thus it was hypothesized that a transmembrane pH gradient (inside acidic) should drive vesicular accumulation of Ca^{2+} via exchange of interior protons for exterior Ca^{2+} if A23187 was present. This was demonstrated by Veiro and Cullis [66] for EPC LUVs exhibiting a pH gradient of 3.5 units. An interior-to-exterior Ca^{2+} concentration gradient of over 400-fold was readily achieved, with an internal Ca^{2+} concentration in excess of 250 mM. The driving force for uptake was the presence of a pH gradient, and not a chelation process, as the internal citrate buffer was found not to be a sufficiently strong chelator to promote uptake.

Subsequent work by Wheeler et al. [68] demonstrated kinetic evidence for an electroneutral process whereby $2H^+$ are transported out for each Ca^{2+} transported in. The model employed also suggested that most of the accumulated Ca^{2+} exists in free, unchelated form. Two other Ca^{2+} ionophores, ionomycin and lasolocid A, were also examined in conjunction with A23187 for their Ca^{2+} uptake behaviour, and the stoichiometries of the ionophore:Ca^{2+} complexes were determined [68]. Both A23187 and ionomycin were found to transport Ca^{2+} in a 1:1 cation:ionophore complex, whereas a 1:2 complex was observed for lasolocid A. An example of pH-dependent Ca^{2+} uptake into DOPC LUVs mediated by A23187 is shown in Fig. 14, where the effect of ionophore concentration is shown [68].

These observations on Ca^{2+} were extended to the A23187-mediated pH-dependent uptake of other cations, namely Fe^{2+} and Ba^{2+} [67]. Both cations were accumulated in LUVs (interior acidic) composed of EPC and DSPC/Chol (55:45), with maximum uptake levels in the range of 300 nmol cation per μmol lipid. Better retention was observed for the DSPC systems. Both the Fe^{2+} and Ba^{2+} systems exhibited increased densities, as evidenced by enhanced gravimetric properties, as an increased proportion of the vesicles could be pelleted by low speed centrifugation. Cryo-transmission electron microscopy was used to study the influence of the accumulated Fe^{2+} or Ba^{2+} on the electron densities

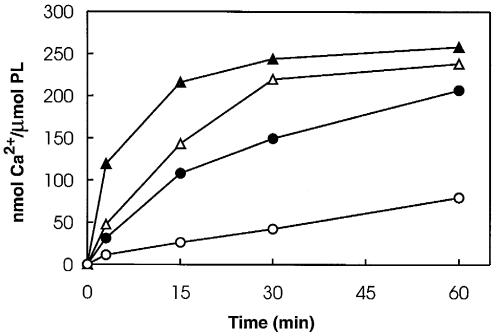

Fig. 14. Effect of A23187 concentration on Ca^{2+} uptake into DOPC LUVs (100 nm) experiencing a transmembrane pH gradient ($pH_i = 4.0$, $pH_o = 7.4$) in the presence of 0.5 mM Ca^{2+}. A23187 concentrations used were 0.02 (○), 0.05 (●), 0.1 (△), and 0.2 (▲) μg/ml. Reproduced from Wheeler et al. [68], with permission.

of the LUV systems. The Fe^{2+} systems exhibited only slightly improved contrast due to a slight darkening of the vesicle interior, whereas the Ba^{2+} systems displayed remarkably improved contrast due to the presence of electron dense particles within the LUVs. These were most likely crystalline barium citrate precipitates.

The ability to encapsulate contrast-enhancing and other cations within LUV preparations has many potential applications. The electron dense Fe^{2+} and Ba^{2+} preparations described here could be useful in cell separation protocols and as cell markers. The encapsulation of radionuclides such as $^{153}Gd^{3+}$ or $^{67}Ga^{3+}$ may prove useful for contrast enhanced magnetic resonance imaging, and for determining the biodistribution of LUVs in vivo [73–75].

7. Relevance of ΔpH to transbilayer transport in biological systems

Many biological membranes exhibit transmembrane pH gradients of 2–3 units. On the basis of the results summarized here, it would seem likely that transmembrane redistributions of weak acids and weak bases will occur in biological systems in response to these pH gradients. Here we focus on four areas where there is experimental evidence suggesting such a role.

Biological compounds which are weak bases, such as biogenic amines, are strong candidates for transmembrane accumulation in response to a pH gradient. Although the uptake of biogenic amines such as dopamine into secretory vesicles is usually thought to involve specific transport proteins [76,77], pH gradients (acidic interior) are present across these membranes [78,79]. Early studies showed that dopamine and other catecholamines can be accumulated within LUVs exhibiting such a gradient [11]. Thus at least a portion of the uptake into secretory vesicles would be expected to be protein-independent. This is further supported by later studies on the uptake of dopamine, serotonin, and epinephrine into LUVs exhibiting a well-defined ΔpH [80]. The extent and rate of catecholamine uptake into LUVs was found to be comparable to that observed in chromaffin granules [81,82].

The role of transbilayer pH gradients in transport is further indicated by the ability of certain drugs to interfere with the sequestration of neurotransmitters via a mechanism which seems to involve dissipation of the pH gradient in secretory vesicles. Examples include the lipophilic weak bases amphetamine and other related psychostimulants which accumulate within secretory vesicles, and thus dissipate ΔpH, resulting in release of sequestered dopamine and an inability to accumulate more [83,84]. Certain neuron blockers have been proposed to act in an analogous manner in synaptic vesicles [85].

An interesting illustration of the potential importance of intracellular pH gradients is found in the phenomenon of multidrug resistance (MDR). MDR occurs when tumour cells develop resistance, after long-term exposure to a chemotherapeutic drug, not only to the drug with which they are being treated, but also to many other drugs which may be of a different therapeutic class [86,87]. MDR is associated with increased expression of the MDR membrane protein and decreased retention of chemotherapeutic agents inside cells [88–91]. It is generally thought that the MDR protein acts as a molecular pump, actively pumping chemotherapeutic agents out of the cell. This is based on evidence that the decreased retention of chemotherapeutics is ATP-dependent [88,90,92], with direct interactions occurring between the MDR protein and a variety of compounds including some chemotherapeutics and ATP [93,94].

An alternative explanation concerns the roles of transmembrane pH gradients, and is based on two observations. First, MDR involves broad resistance to chemically unrelated compounds [91,95,96], and it seems unlikely that a single protein would be able to transport such a wide variety of structures. Second, tumour cells which are susceptible to chemotherapeutics often have a lower (acidic) intracellular pH [86], which would lead to enhanced accumulation of these drugs in response to the ΔpH. In contrast, the intracellular pH of resistant cells is significantly higher than that of non-resistant cells [91,97–99], giving rise to a reduced ΔpH which would lead to reduced intracellular accumulation of drugs. Thus, it is possible that the proteins responsible for MDR exert at least some of their effect by altering intracellular pH, which then reduces the ΔpH-induced uptake of a variety of chemotherapeutic agents leading to the multidrug resistance phenotype. Several studies support this view. In human myeloma cells, a near-linear

relationship has been observed between the intracellular pH and the efflux of doxorubicin [91]. Furthermore, studies involving systematic alterations in tumour cell intracellular pH reveal that acidification of the intracellular environment results in rapid accumulation of drug, whereas shifts to alkaline pH results in drug efflux [86,87]. An alternative role of pH gradients in drug resistance is suggested by a recent study demonstrating enhanced acidification of intracellular vesicular compartments of the exocytotic pathway in certain drug-resistant breast cancer cell lines [100]. This results in accumulation of the drug in these compartments for export from the cell. Drug-sensitive cells had reduced intracellular pH gradients, and thus were unable to sequester drugs away from their sites of action (cytoplasm and nucleus) for eventual export.

The above data suggests that ΔpH-dependent MDR mechanisms may operate in vivo, a view which is supported by studies on the efflux of daunorubicin from a variety of MDR cell lines. The efflux was found to occur by both P-glycoprotein-mediated drug pumping, as well as by passive drug permeation, with the relative proportion of the two mechanisms dependent on P-glycoprotein levels [101].

A third potential role of pH gradients in transbilayer transport is in the area of membrane lipid asymmetry. Simple lipids such as fatty acids can be sequestered to the inner monolayer of LUVs in response to ΔpH (inside basic) [44], which suggests that fatty acids will be preferentially located in the cytoplasmic monolayer of organelles such as the endoplasmic reticulum, which has an acidic interior [102,103]. ΔpH-dependent fatty acid transbilayer asymmetry has recently been observed in adipocytes, where raising the internal pH leads to accumulation of fatty acids in a manner consistent with passive diffusion of the neutral form [104], in agreement with results using model systems [44]. Another recent study has demonstrated that fatty acids can passively diffuse through the plasma membrane of adipocytes, and that this movement affects the internal pH of the cell in a manner consistent with permeation of the neutral form [105]. While these results are all consistent with transbilayer permeation of fatty acids (in the neutral form) in response to ΔpH, it should be noted that fatty acid transport proteins from adipocytes have recently been cloned [106,107]. This suggests the coexistence of specific and non-specific transport mechanisms, as for MDR.

Similar considerations also apply to other acidic lipids, including the phospholipids PA and PG, which will also preferentially locate in the monolayer experiencing the highest pH (Sections 4.1 and 4.2) [27,44]. Alternatively, amino-containing lipids such as sphingosine would be expected to locate in monolayers facing acidic environments. This ΔpH-dependent regulation of transbilayer distributions of lipids could regulate bioavailability and hence modulate metabolic processes.

Transmembrane pH gradients cannot account for lipid asymmetry such as is observed in the erythrocyte membrane, where PS and PE are concentrated in the inner leaflet, while PC and SPM are localized on the external leaflet [108], as the neutral form of these lipids is associated with a zwitterionic headgroup, resulting in low membrane permeability. There is strong evidence that asymmetric transbilayer distributions of PE and PS are maintained by an ATP- and Mg^{2+}-dependent aminophospholipid translocase [109,110].

A final process where transmembrane pH gradients may play a role is in protein translocation. The fact that signal sequences are usually lipophilic weak bases [111] raises the possibility of transbilayer movement across membranes in response to ΔpH towards the acidic environment [28,102,112,113]. In bacteria, for example, the export of proteins can require a pH gradient (exterior acidic) [114,115]. In thylakoid membranes, two mechanisms for protein translocation have been identified, one of which requires stromal factors and ATP, and the other which is completely reliant on the thylakoidal ΔpH [116]. The pathway utilized for translocation of a protein is dictated by the signal sequence, with signals for the ΔpH-dependent system containing a common twin-arginine motif immediately before the hydrophobic region [117]. In the pH-dependent pathway, the ΔpH is required for both the initiation and completion of translocation [118]. Finally, in some cases the ΔpH may be necessary for translocation to occur in the ATP-dependent pathway, with this requirement dictated by the passenger protein and concentration of ATP [119].

Although our understanding of protein translocation in the endoplasmic reticulum (ER) has greatly

increased over the past decade [120–122], little attention has been directed towards the possible role which may be played by transmembrane pH gradients. However, on the basis of the similarities between the eukaryotic and bacterial translocation systems [121], and the presence of an ATPase generating a pH gradient (acidic lumen) [102,103], a role for ΔpH in protein translocation in ER would seem a distinct possibility.

8. Concluding remarks

In summary, the studies reviewed here demonstrate that pH gradients can play a direct role in the transbilayer transport of a wide variety of weak bases and weak acids of biological interest, including drugs, lipids, and peptides and, in the presence of appropriate ionophores, metal ions. This has led to a variety of applications, ranging from drug loading of liposomes for drug delivery to the use of LUVs containing electron dense ions as potential contrast-agents in imaging protocols. Studies on lipid asymmetry induced by pH-gradients have led to new insights on regulation of such fundamental processes as membrane fusion and factors which regulate membrane morphology. In addition, pH gradients across organelle membranes may be expected to strongly influence the intracellular distribution and bioavailability of drugs and certain biological compounds. Transbilayer transport of ionizable compounds in response to transbilayer pH gradients clearly represents a general phenomenon of considerable practical and theoretical importance.

Acknowledgements

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