Biochimica et Biophysica Acta, 1149 (1993) 329–338

BBAMEM 76009

Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients

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(Received 25 November 1992)
(Revised manuscript received 2 March 1993)

Key words: Doxorubicin; Transmembrane pH gradient; pH gradient; Liposome; LUV

The uptake of the anticancer agent doxorubicin into large unilamellar vesicles (LUVs) exhibiting a transmembrane pH gradient (inside acidic) has been investigated using both kinetic and equilibrium approaches. It is shown that doxorubicin uptake into the vesicles proceeds via permeation of the neutral form and that uptake of the drug into LUVs with an acidic interior is associated with high activation energies (E_a) which are markedly sensitive to lipid composition. Doxorubicin uptake into egg-yolk phosphatidylcholine (EPC) LUVs exhibited an activation energy of 28 kcal/mol, whereas for uptake into EPC/cholesterol (55:45, mol/mol) LUVs $E_a = 38$ kcal/mol. The equilibrium uptake results obtained are analyzed in terms of a model which includes the buffering capacity of the interior medium and the effects of drug partitioning into the interior monolayer. From the equilibrium uptake behaviour, a doxorubicin partition coefficient of 70 can be estimated for EPC/cholesterol bilayers. For a 100 nm diameter LUV, this indicates that more than 95% of encapsulated doxorubicin is partitioned into the inner monolayer, presumably located at the lipid/water interface. This is consistent with ^{13}C-NMR behaviour as a large proportion of the drug appears membrane associated after accumulation as reflected by a broadening beyond detection of the ^{13}C-NMR spectrum. The equilibrium accumulation behaviour of a variety of other lipophilic amines is also examined in terms of the partitioning model.

Introduction

It is well known that transmembrane pH gradients (ΔpH) can strongly influence the equilibrium transbilayer distributions of certain weak acids and bases across biological membranes. Early work in this area includes that of Chappel and Crofts [1] on mitochondria and Crofts [2] on chloroplasts, demonstrating large transmembrane gradients of weak bases such as ammonia or carboxylic acids such as acetate in response to ΔpH. These large concentration gradients arise due to the highly permeable nature of the neutral form of these agents, resulting in transbilayer gradients which reflect the proton gradients. This has led to the use of weak acids and bases to assay ΔpH in cells and organelles [3] and has also stimulated studies in the uptake of other weak bases (such as catecholamines) into liposomes with an acidic interior [4,5].

Recent work from this laboratory has shown that transmembrane pH gradients in large unilamellar vesicle (LUV) systems can result in the transbilayer movement of a wide range of molecules and atoms of biological interest. These include phospholipids [6,7], metal ions [8], peptides [9] and a variety of drugs [10,11]. The anticancer drug doxorubicin has been a major focus of this work, due to the therapeutic properties of the liposomally encapsulated formulation of the drug which exhibits reduced toxicity (particularly cardiotoxicity) compared to the free form while anticancer efficiency is maintained or enhanced [12–14]. The technique of loading doxorubicin into liposomes in response to ΔpH (inside acidic) provides a dramatically improved method of drug loading, as trapping efficiencies approaching 100% can be readily achieved accompanied by drug-to-lipid ratios an order of magnitude or more larger than can be achieved with conven-

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Abbreviations: $MeNH_3^+$, methylamine; EPC, egg phosphatidylcholine; CHOL, cholesterol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LUVs, large unilamellar vesicles; NMR, nuclear magnetic resonance; TMS, tetramethylsilane.

tional procedures [14–16]. This procedure also markedly enhances drug retention properties. This has led to a liposomal doxorubicin preparation which is currently in advanced clinical trials [17].

The relation between the amount of doxorubicin (and other drugs) accumulated into LUVs in response to ΔpH, the internal buffering capacity and the residual pH gradient after accumulation, as well as the kinetics of uptake have not been adequately examined, however. In particular, it has been noted [11] that the transbilayer concentration gradients of doxorubicin achieved in response to ΔpH considerably exceed those expected on the basis of the residual transbilayer proton gradients, and that uptake is considerably more rapid at elevated temperatures and higher (exterior) pH values. In this work, we develop a kinetic and equilibrium model of the uptake process. It is shown that the uptake of doxorubicin into LUVs with an acidic interior proceeds via accumulation of the neutral form and that this uptake exhibits high activation energies. Further, the equilibrium behaviour can be explained quantitatively by a model which incorporates the ability of doxorubicin and other drugs to partition into the membrane and also takes into account the influence of internalized drug on the interior buffer capacity.

Materials and Methods

Vesicle preparation

Multilamellar vesicles (MLVs) were prepared by vortexing the (dry) lipid in an appropriate buffer (either 300 mM citrate (pH 4.0) or 150 mM NaCl, 20 mM Hepes (pH 7.0); Hepes-buffered saline, HBS) at 50 mg/ml total lipid. Cholesterol (45 mol% with respect to phospholipid) was incorporated by co-lyophilization from benzene/methanol (70:30, v/v). The lipid dispersions were frozen and thawed five times (the freezing step involved incubating the vial for more than 3 min in liquid nitrogen) to obtain equilibrium transmembrane buffer distributions [18]. These vesicles were extruded 10 times through two (stacked) Nuclepore polycarbonate filters with 100 nm pore size using an extrusion device (Lipex Biomembranes, Vancouver, B.C.) as described earlier [19]. The resulting vesicles are unilamellar, with average diameters of approx. 100 nm. Measurements of interior volumes by determining entrapped citrate buffer led to trapped volumes of 0.84 l/mol total lipid or 1.5 l/mol for EPC/cholesterol (55:45, mol/mol) or EPC LUVs, respectively [19].

Uptake of doxorubicin and radiolabelled probes into LUVs

Transmembrane pH gradients were created by preparing LUVs in the presence of 300 mM citrate (pH 4.0), and exchanging the untrapped buffer for 150 mM NaCl, 20 mM Hepes (pH 7.0) employing Sephadex G-50 gel filtration columns. These vesicles were then diluted to a final lipid concentration of approximately 2 mM into a solution containing the indicated concentration of drug dissolved in 150 mM NaCl, 20 mM Hepes (pH 7.0). In situations where a measure of the transmembrane pH gradient was required, the solution also contained 0.25 μCi/ml [^{14}C]methylamine [20]. For experiments in which the external pH was varied, the external medium was first exchanged for 150 mM NaCl, 3 mM citrate (pH 4.0) to reduce the external buffering capacity. Subsequently, these vesicles were diluted into a medium containing 200 μM doxorubicin in 150 mM NaCl, 20 mM Hepes, 20 mM Mes adjusted to the desired pH.

Exterior drug and probe was removed by gel filtration chromatography employing 1 ml Sephadex G-50 spin columns previously equilibrated with the appropriate buffer. Immediately after loading, the spin-columns were eluted by centrifugation at $2000 \times g$ for 3 min. Quantitation of vesicle associated [^{14}C]methylamine was performed by liquid scintillation counting and phospholipid analysis performed as previously described [20]. Phospholipid concentrations were determined by analysis of lipid phosphate as described previously [21]. Transmembrane pH gradients measured employing methylamine ($MeNH_3^+$) are calculated according to the relation:

$$\Delta pH = \log([MeNH_3]_i^+ / [MeNH_3]_o^+)$$

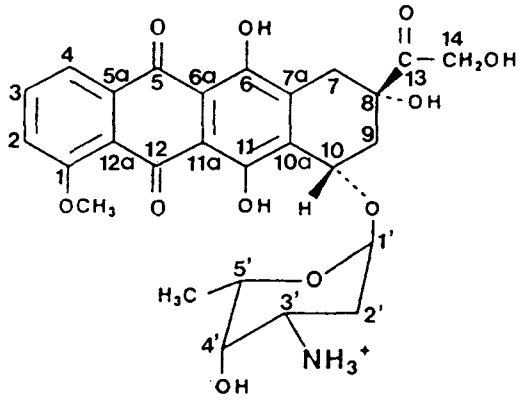
where the subscripts i and o indicate the interior and exterior environments, respectively [3,22].

Vesicle-associated doxorubicin was determined (after separation of untrapped drug) by measuring the absorbance at 480 nm in a 1% Triton X-100 solution which resulted in vesicle disruption and drug release. At extremely low drug concentrations, internal doxorubicin was determined using [^{14}C]doxorubicin (0.25 mCi/ml).

^{13}C-NMR studies

Doxorubicin was made up in solutions of 300 mM citrate (pH 4.0) or 150 mM HBS (pH 7.0). LUVs with or without a pH gradient were added to these solutions to achieve a final lipid concentration of 4 mM, and the final volume adjusted to 4.0 ml with D_2O. The proton decoupled ^{13}C-NMR spectra were obtained employing a Bruker MSL 200 spectrometer operating at 50.3 MHz. Free induction decays corresponding to 62000 transients were obtained by using a 10 μs 90° pulse, a 1 s interpulse delay and a 220 ppm sweep width. An exponential multiplication corresponding to 5 Hz was applied to the free induction decay prior to Fourier transformation. The chemical shift is referenced to external TMS.

a



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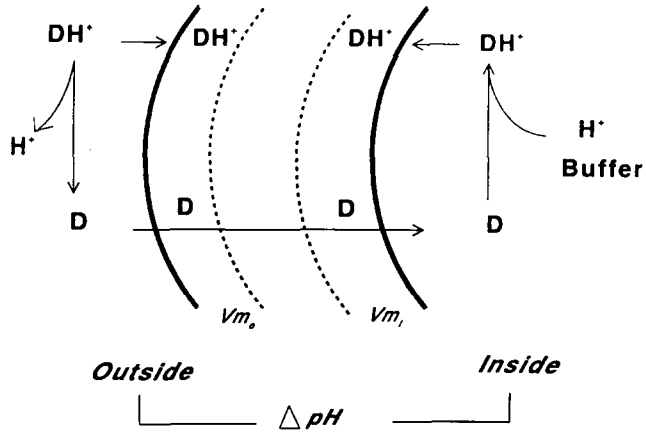

Fig. 1. (a) The structure of doxorubicin. The pK_a of the primary amine is 8.6. (b) Model of the interactions of doxorubicin with phospholipid LUVs exhibiting a transbilayer pH gradient (ΔpH). The drug assumes two forms, the protonated, membrane impermeable species (DH^+) and the deprotonated (neutral) membrane permeable species (D). The interfacial volumes V_i^m and V_o^m into which the drug partitions are exaggerated.

Kinetic analysis of drug uptake

Under the assumption that only the neutral form of the drug traverses the membrane and employing the model of Fig. 1, it follows that:

$$\frac{d[D]_o^{tot}}{dt} = -\frac{P \cdot A_m}{V_o}([D]_o^m - [D]_i^m) \quad (1)$$

where $[D]_o^{tot}$ is the total exterior concentration of drug (including charged, uncharged, free and membrane bound species), P is the permeability coefficient of the neutral form, A_m is the area of the membrane, V_o is the external aqueous volume and $[D]_o^m$, $[D]_i^m$ are the concentrations of the neutral form of the drug. Assuming that the drug dissociation constant K_a is the same for free and membrane associated drug, $[D]_o^m$ can be expressed as:

$$[D]_o^m = \frac{[D]_o^{tot}}{\frac{1}{K^*} + \frac{[H^+]_o}{K^* \cdot K_a} + \frac{V_m}{V_o} + \frac{[H^+]_o \cdot V_m}{K_a \cdot V_o}} \quad (2)$$

where $K^* = [DH^+]^m/[DH^+]^w$ is the apparent membrane–water partition coefficient for the charged form of the drug, $[H^+]_o$ is the exterior proton concentration and V_m is the volume of the membrane. Under the assumption that $[H^+]_o \gg K_a$ (the pK_a of doxorubicin is 8.6 and thus this condition is straightforward to observe) and that $V_o \gg V_m$, it follows from Eqn. 2 that $[D]_o^m = [D]_o^{tot} \cdot K^* \cdot K_a/[H^+]_o$. Thus:

$$\frac{d[D]_o^{tot}}{dt} = -\frac{P \cdot A_m \cdot K^* K_a}{V_o \cdot [H^+]_o}[D]_o^{tot} \quad (3)$$

where P is the permeability coefficient of the neutral form of the drug. In turn, this results in the relation:

$$[D(t)]_o^{tot} = [D(0)]_o^{tot} \cdot e^{-kt} \quad (4)$$

where the rate constant $k = PA_m \cdot K^* \cdot K_a/V_o[H^+]_o$. As the interior drug concentration must obey the relation $[D(t)]_i^{tot} = ([D(o)]_o^{tot} - [D(t)]_o^{tot})V_o/V_i$, we have that:

$$[D(t)]_i^{tot} = [D(eq)]_i^{tot}(1 - e^{-kt}) \quad (5)$$

where $[D(eq)]_i$ is the equilibrium interior drug concentration. A plot of $\ln(([D(eq)]_i - [D(t)]_i)/[D(eq)]_i)$ vs. time should therefore result in a straight line with a slope $-k$. In turn, a plot of the log of the rate constant k vs. the exterior pH should result in a straight line with a slope of unity.

Influence of membrane–water partition coefficient on equilibrium drug uptake

Our analysis is based on a model developed by Cafiso and Hubbell [23] to determine transmembrane pH gradients using spin-labelled electron paramagnetic resonance (EPR) probes and again employs the four compartment model shown in Fig. 1. The variables are the number of drug molecules in the outer and inner aqueous compartments (N_o^w and N_i^w, respectively) and the number of drug molecules in the outer and inner monolayers (N_o^m and N_i^m, respectively). From the definition of the membrane–water partition coefficient, we obtain:

$$K = \frac{N_o^m(+) \cdot V_o}{V_m^o \cdot N_o^w(+)} = \frac{N_i^m(+) \cdot V_i}{V_m^i \cdot N_i^w(+)} \quad (6)$$

Similarly, assuming that the drug dissociation constant (K_a) is the same, in both the aqueous and membrane phase:

$$K_a = \frac{(N_i^w/V_i)[H^+]_i}{(N_i^w(+)/V_i)} = \frac{(N_o^w/V_o)[H^+]_o}{(N_o^w(+)/V_o)} = \frac{(N_o^m/V_m^o)[H^+]_o}{N_o^m(+)/V_m^o}$$

$$= \frac{(N_i^m/V_m^o)[H^+]_i}{N_i^m(+)/V_m^i} \quad (7)$$

From the relations (6) and (7) and making the approximations that surface potentials due to the charged drug partitioning into the membrane are small due to the high ionic strength of the interior buffer and that $[H^+]_i \gg [H^+]_o \gg K_a$ (our usual parameters are $pH_i = 4$, $pH_o = 7$), it can be readily shown that the ratio of internal drug to external drug is given by:

$$\frac{N_i^{tot}}{N_o^{tot}} = \frac{N_i^m(+) + N_i^m + N_i^w(+) + N_i^w}{N_o^m(+) + N_o^m + N_o^w(+) + N_o^w}$$

$$= \frac{(1 + K \cdot V_m^i/V_i)[H^+]_i}{\left(\frac{V_o}{V_i}\right)\left(1 + K\frac{V_m^o}{V_o} + \frac{K_a \cdot K \cdot V_m^o}{[H^+]_o \cdot V_o}\right)[H^+]_o} \quad (8)$$

We define K^* as the apparent partition coefficient of the drug into the membrane where $K^* \equiv K \cdot V_m^i/V_m \equiv K \cdot V_m^o/V_m$. V_m is the total membrane volume. Working at low lipid concentrations where $V_m \ll V_o$, and for drugs with partition coefficients which obey the relation $K^* \ll V_o/V_m$, Eqn. 8 simplifies to:

$$\frac{[D]_i^{tot}}{[D]_o^{tot}} = \left(1 + \frac{K^* \cdot V_m}{V_i}\right)\frac{[H^+]_i}{[H^+]_o} \quad (9)$$

where $[D]_o^{tot}$ is the total exterior concentration of drug and $[D]_i^{tot}$ the effective vesicle associated 'concentration' of the drug (i.e., the total amount of drug associated with the LUVs divided by the LUV volume). The proton concentration $[H^+]_i/[H^+]_o$ can be readily determined by measuring the response of radiolabelled methylamine [24] and the amount of accumulated drug ($[D]_i^{tot}$) can be determined as indicated above, allowing the ratio $[D]_i^{tot}/[D]_o^{tot}$ to be calculated. In turn, this allows the partition coefficient K^* to be calculated employing Eqn. 9.

It is instructive to calculate the inside-outside concentration gradients for extremely high partition coefficients which lipids such as phospholipids may be expected to exhibit. Under the condition $K^* \gg V_o/V_m$, it is straightforward to show that Eqn. 8 reduces to:

$$\frac{N_i^m(+)}{N_o^m(+)} = \frac{[H^+]_i}{[H^+]_o} \quad (10)$$

as expected.

Equilibrium uptake and internal buffering capacity

If drug uptake proceeds via the neutral form and is well 'coupled' in the sense that each drug molecule is protonated on arrival in the LUV interior, and non-specific leakage is avoided, $[D]_i^{tot}$ can also be calculated from the value of $[H^+]_i$ after uptake in combination with a knowledge of the interior buffering capacity. In particular, for a buffer ('B') with three titratable groups, such as citrate, it can be shown that the concentration of the buffer in the neutral form ([B]), in the singly deprotonated form ($[B^-]$) and so on is related to the total buffer concentration $[B]^{tot}$ according to the relations:

$$[B] = [B]^{tot}/f(H^+) \quad (11)$$

$$[B^-] = K_1 \cdot [B]^{tot}/([H^+] \cdot f(H^+)) \quad (12)$$

$$[B^{2-}] = K_1 \cdot K_2 \cdot [B]^{tot}/([H^+]^2 \cdot f(H^+)) \quad (13)$$

$$[B^{3-}] = K_1 \cdot K_2 \cdot K_3 \cdot [B^{tot}]/([H^+]^3 \cdot f(H^+)) \quad (14)$$

where $f(H^+) = (1 + K_1/[H^+] + K_1 \cdot K_2/[H^+]^2 + K_1 \cdot K_2 \cdot K_3/[H^+]^3)$ and K_1, K_2, and K_3 are the dissociation constants of the titratable groups of the buffer. Given that each internalized drug molecule consumes a proton as it is reprotonated in the vesicle interior, the total concentration of charged amine in the vesicle interior ($[DH^+]_i$) can then be expressed by charge balance according to the relation:

$$[DH^+]_i = ([B^-]^b - [B^-]^a) + 2([B^{2-}]^b - [B^{2-}]^a) + 3([B^{3-}]^b - [B^{3-}]^a) \quad (15)$$

where the superscripts *b* and *a* indicate the final and original concentrations of the charged forms of the buffer, respectively.

Results

Kinetics of doxorubicin uptake as determined by fluorometric techniques

In previous studies of doxorubicin uptake into LUVs with an acidic interior [15,16], assays of entrapment have involved the separation of LUVs from untrapped drug and the subsequent assay of entrapped material. For kinetic studies, particularly those on systems exhibiting rapid uptake, a more convenient uptake assay is required. This was achieved by monitoring doxorubicin fluorescence during accumulation. This is illustrated in Fig. 2 for uptake into EPC/cholesterol (55:45) LUVs (100 nm diameter) in response to a transmembrane pH gradient ($pH_i = 4.0$, $pH_o = 7.0$). A small increase in fluorescence is observed on addition of the LUVs to the doxorubicin solution (200 μM

doxorubicin) which is followed by an exponential time-dependent decrease in fluorescence to an equilibrium value. (The small fluorescence increase was not detectable in cases where the drug is accumulated more rapidly.) The kinetics of the fluorescence decrease correlate well with doxorubicin uptake assayed by the chromatographic separation procedure (Fig. 2). Both data sets can be fitted using the first-order kinetic analysis indicated under Methods, leading to a rate constant $k = 3 \cdot 10^{-3}$ s^{-1}. The quenching of fluorescence on doxorubicin uptake is presumably related to membrane partitioning effects (see below).

pH dependence and activation energies associated with doxorubicin uptake into LUVs

The kinetic model developed in Methods predicts that $\log k = pH + c$ for transbilayer movement of the neutral form and thus a plot of log k vs. the external pH should reveal a straight line with a slope of unity. The rates of doxorubicin uptake into EPC and EPC/cholesterol LUVs were therefore determined over the range $pH_o = 5.7$ to $pH_o = 8.0$. As shown in Fig. 3(A), plots of the log of the rate constants derived from this data reveal the expected linear dependence with slope of 1.08 and 0.96 for EPC and EPC/cholesterol LUVs, respectively. This provides conclusive evidence for transbilayer movement of the neutral form.

Previous studies on the ΔpH dependent transport of phospholipids which are weak acids [7,26] and peptides

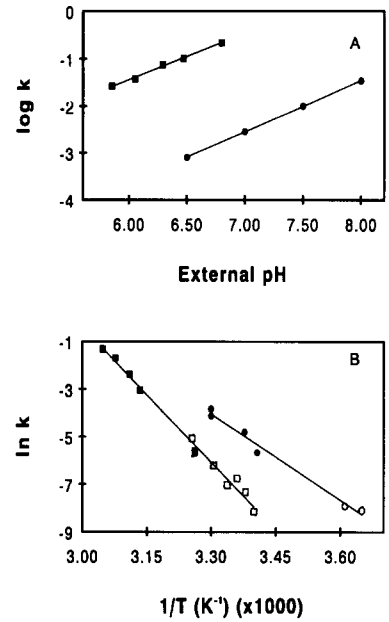

Fig. 3. Effects of external pH and temperature on the kinetics of doxorubicin accumulation. (A) EPC or EPC/cholesterol LUVs containing 300 mM citrate pH 4.0 were incubated with 200 μM doxorubicin at a variety of external pH values (see Materials and Methods) and the effects of the external pH on the rate constant of accumulation determined employing the fluorescence assay of Fig. 2. The slopes of the log k vs. pH plots were 1.08 for EPC LUVs (●) and 0.96 for EPC/cholesterol LUVs (■). The temperature was 21°C for the EPC vesicles, or 53°C for the EPC/cholesterol vesicles. (B) The effect of temperature on the rate constant of doxorubicin accumulation were monitored by fluorescence changes (closed symbols) or spin columns (open symbols) for EPC (●, ○) or EPC/cholesterol LUVs (■, □) as indicated in Materials and Methods.

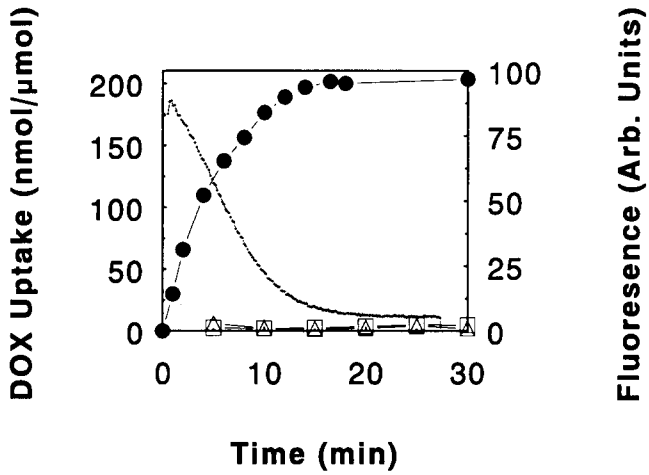

Fig. 2. Doxorubicin accumulation into LUVs in response to transmembrane pH gradients. EPC/cholesterol vesicles (100 nm diameter), 1.5 mM lipid were prepared with an interior buffer of 300 mM citrate (pH 4.0), and added to a doxorubicin solution (200 μM) in HBS (pH = 7.0), and the amount of vesicle associated doxorubicin (●) was determined as indicated in Materials and Methods. Doxorubicin association with vesicles without a transmembrane pH gradient is also shown at pH 4.0 (□) and pH 7 (Δ). Also shown is the fluorescence response of the doxorubicin incubated with EPC/cholesterol LUVs in the presence of a transmembrane pH gradient (---). Rate constants determined by both techniques were $3 \cdot 10^{-3}$ s^{-1}. The uptake temperature was 33.4°C.

which are weak bases [9] have demonstrated high activation energies in the range of 30 kcal/mol. Similar high activation energies may be expected for doxorubicin given the considerably enhanced uptake rate at 60°C as compared to 20°C [16]. Rate constants derived from uptake data obtained over the temperature range 5°C to 55°C demonstrate these high activation energies as shown in the Arrhenius plot of Fig. 3(B). It is interesting to note that different activation energies are observed for different lipid systems, where $E_a = 38$ kcal/mol for the EPC/cholesterol (55:45) LUV system whereas $E_a = 28$ kcal/mol for the EPC system.

Partition coefficients and coupling characteristics associated with doxorubicin uptake

As indicated under Methods, an ability of doxorubicin and other drugs which are lipophilic amines to partition into the lipid bilayer can result in inside/outside drug concentration ratios which significantly exceed the inside/outside proton concentration ratios. In particular, a plot of $[Drug]_i/[Drug]_o$ vs. $[H^+]_i/[H^+]_o$ should reveal a straight line with a slope K^*V_m/V_i. Doxorubicin uptake into 100 nm EPC/cholesterol LUVs (2 mM lipid) exhibiting a 3 unit pH gradient

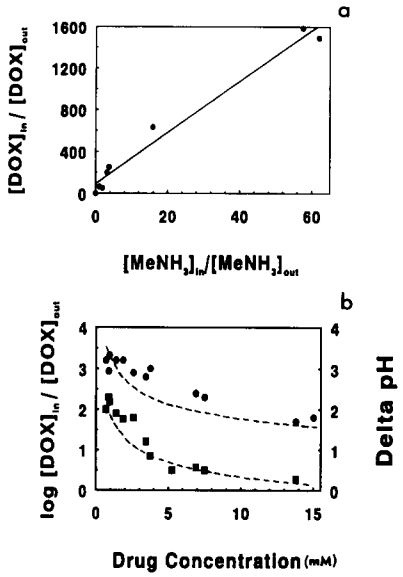

Fig. 4. Relationship between residual pH gradient and doxorubicin accumulation. Doxorubicin and methylamine concentration gradients at equilibrium were determined by incubating EPC/cholesterol vesicles containing 300 mM citrate (pH 4.0) at 60°C for 20 min with the indicated concentration of doxorubicin in HBS (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 linear regression (solid line) is 24. (B) The dotted lines were generated using the model presented in Methods, using trapped volumes of 0.84 litre per mol lipid and the apparent partition doxorubicin coefficient K^* determined from the slope of plot (A) above. Experimental results presented are equilibrium doxorubicin (●) or methylamine (■) concentration gradients.

($pH_i = 4.0$, $pH_o = 7.0$) and containing 300 mM entrapped citrate buffer was therefore monitored for a range of initial exterior doxorubicin concentrations up to 14 mM. Corresponding interior proton concentrations were determined employing [^{14}C]methylamine. A plot of the interior to exterior drug concentration ratios vs. the proton gradients is shown in Fig. 4(A), revealing a linear dependence with slope 24. For a 100 nm diameter LUV and assuming a bilayer thickness of 5 nm $V_m/V_i = 0.33$, leading to a doxorubicin partition coefficient $K^* = 70$ employing Eqn. 9.

It is of interest to compare this value of K^* with a determination of the partition coefficient employing more classical procedures. Thus, the equilibrium filter centrifugation technique (see Methods) was used to determine the partition coefficient of doxorubicin for EPC/cholesterol LUVs at pH 4.0 in 300 mM citrate by separating part of the exterior buffer from the LUVs. This was performed for a range of lipid concentrations (data not shown) and resulted in a K^* of 74.

The data of Fig. 4(A) can also be employed to determine how well doxorubicin uptake is coupled to the interior buffering capacity, providing a measure of the non-specific leakage which may be induced by drug accumulation. As indicated under Methods, a knowledge of the partition coefficient K^* can be employed to calculate the theoretical drug and proton (pH) concentration gradients at any external drug concentration, given the initial interior pH and the internal buffer (citrate) concentration. Plots of the theoretical doxorubicin uptake and residual pH gradient are shown in Fig. 4(B), as well as the observed drug uptake and pH gradients. It may be noted that the theoretical and actual accumulation levels agree closely, suggesting that little non-specific leakage has occurred from these vesicles.

^{13}C-NMR studies on doxorubicin uptake

It is straightforward to calculate that for a K^* value of 70, over 95% of the doxorubicin accumulated into

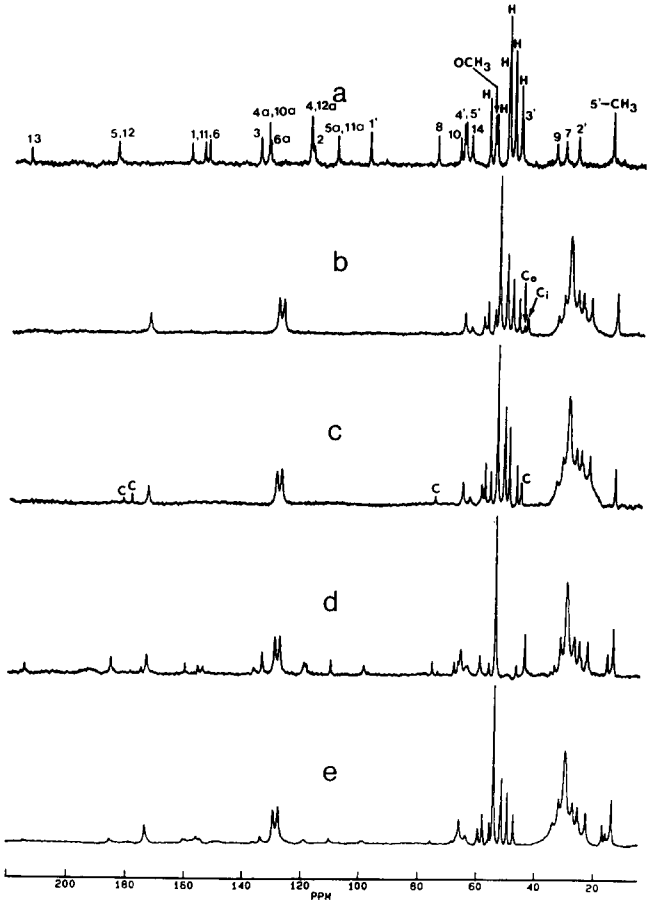

Fig. 5. Effect of transmembrane pH gradients on the proton decoupled ^{13}C-NMR spectra (50.3 MHz) of doxorubicin incubated with EPC LUVs (100 nm). Natural abundance ^{13}C-NMR spectra. (a) Free doxorubicin at pH 7.0 (150 mM NaCl, 20 mM Hepes). For the assignment of doxorubicin peaks, see Fig. 1. Peaks marked 'H' are those of Hepes buffer. (b) EPC vesicles with a pH gradient: 300 mM citrate (pH 4.0), inside, and 150 mM NaCl, 20 mM Hepes (pH 7.0), outside. The two peaks marked C_i and C_o refer to citrate inside, and the fraction of citrate that has leaked to the outside, respectively. Only the protonated carbons of citrate are detected. (c) Doxorubicin incubated with vesicles with an initial 3 units transmembrane pH gradient (pH 4.0 inside, pH 7.0 outside). Peaks indicated with C are those of citrate. (d) Doxorubicin incubated with vesicles without a pH gradient (pH 4 inside and outside). (e) As in (d), but at pH 7.0 (inside and outside).

100 nm LUVs will be partitioned into the inner monolayer of the LUV bilayer. As a result, it would be expected that the motional properties of accumulated drug would be restricted in comparison to exterior free drug. This was tested by monitoring the ^{13}C-NMR behaviour of doxorubicin before and after uptake. In order to detect the natural abundance ^{13}C-NMR spectrum arising from doxorubicin within a reasonable timeframe (24 h), relatively high drug concentrations (4 mM) must be employed. In turn, this requires higher LUV concentrations (12 mM lipid) in order that 90% or more of the available drug is accumulated.

The ^{13}C-NMR spectra arising from free doxorubicin and Hepes buffer (Fig. 5(a)) and doxorubicin in the presence of EPC LUVs without a pH gradient (Fig. 5(d,e)) reveal a broadening of the doxorubicin resonances, but this broadening does not result in signal disappearance. This is in contrast to the ^{13}C-NMR behaviour of doxorubicin incubated in the presence of LUVs exhibiting a ΔpH (Fig. 5(c)) where no reso-

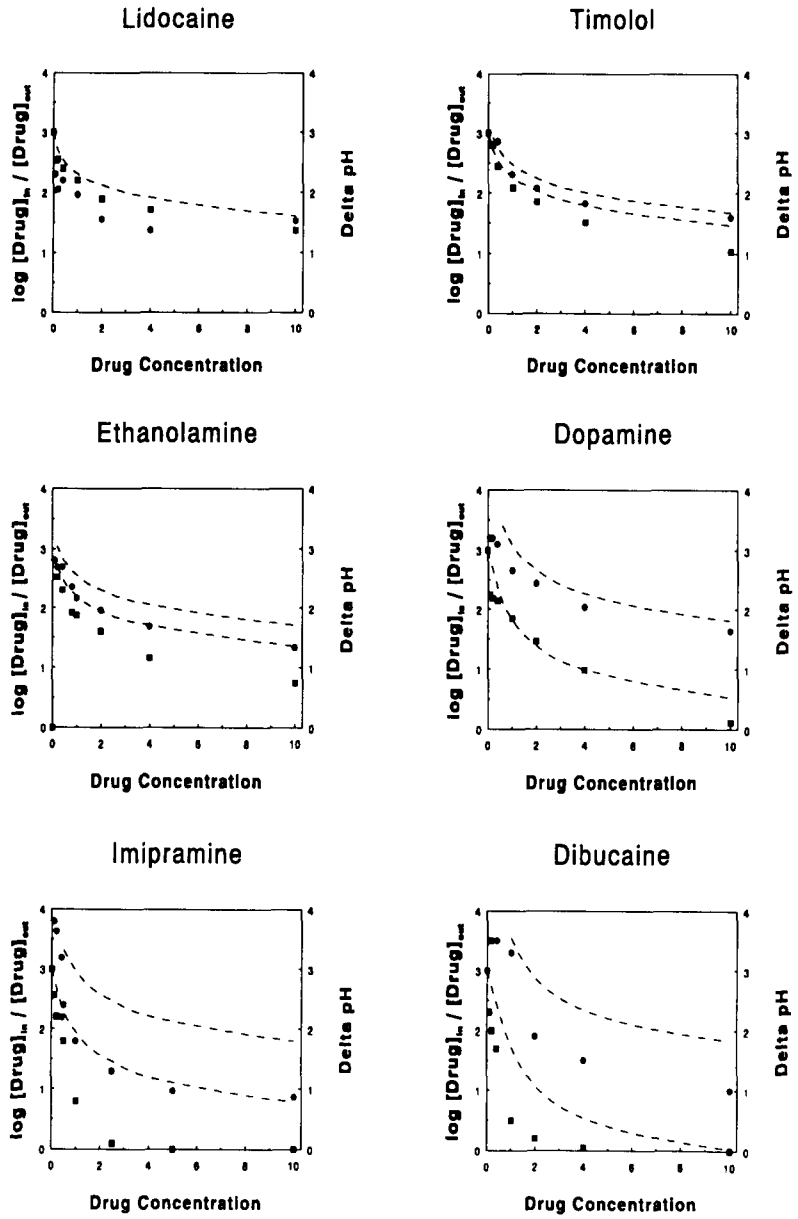

Fig. 6. Relationship between the residual pH gradient (■) and accumulation of a variety of amines (●) in LUVs (100 nm diameter) with a transmembrane pH gradient. EPC/cholesterol (55:45, mol%) vesicles prepared in 300 mM citrate (pH 4.0) were incubated in various concentrations of the indicated compounds in HBS at pH 7.0, and the equilibrium amounts of drug incorporation determined using the spin column procedure described in Materials and Methods. The dotted lines are simulations based on the model presented in Materials and Methods, using K^* values (partition coefficients) calculated from the slopes of the equilibrium transbilayer drug ratios vs. the equilibrium transbilayer proton ratios. These values were 0, 4, 5, 50, 30 and 170 for lidocaine, timolol, ethanolamine, dopamine, imipramine and dibucaine, respectively, assuming no non-specific proton leakage and that $V_m / V_i = 0.33$ (see Eqn. 9).

nances can be observed. It should be noted that the doxorubicin sample incubated at pH 7 (inside and out) was clumped and aggregated after 24 h of signal accumulation, which may account for some of the signal reduction in this sample.

Partition coefficients and coupling properties observed for uptake of other drugs which are lipophilic amines

In previous studies, it has been shown that a large variety of lipophilic drugs which are primary, secondary or tertiary amines can be accumulated in response to ΔpH, and that the extent of uptake in response to a given ΔpH varies considerably from drug to drug [11]. It is likely that this behaviour reflects different values of the partition coefficient K^*. The partition coefficients for lidocaine, ethanolamine, timolol, dopamine and imipramine were therefore determined employing the same procedures as for doxorubicin (Fig. 4) and plots of the $[Drug]_i/[Drug]_o$ and $[H^+]_i/[H^+]_o$ ratios for both the observed as well as the theoretical behaviour expected for 'well-coupled' systems (Fig. 6).

The theoretical and observed values agree reasonably for the cases of lidocaine, ethanolamine, timolol and dopamine, using K^* values of 0, 5, 4, and 50, respectively. The accumulation of imipramine and dibucaine are poorly modelled using K^* values of 30 and 170, respectively, suggesting that the accumulation of these drugs could cause disruption of the vesicles, resulting in 'uncoupling' of drug uptake from the interior buffering capacity. Alternative values of K^* did not improve the fits.

Discussion

The results presented here clarify the mechanism of accumulation of doxorubicin into LUVs with an acidic interior, demonstrate the high level of drug partitioning into the inner monolayer in the loaded systems, and explore the consequences of high levels of membrane associated drug. With regard to the mechanism of uptake, the kinetic studies demonstrating that the rate constant (k) associated with accumulation depends linearly on the external proton concentration unambiguously establish that the neutral form of doxorubicin is the transported species. This result is consistent with the large body of literature showing that the neutral forms of weak acids and bases are generally the membrane permeable species [3,22], and is important because in combination with the observed activation energies, the kinetics of drug accumulation and release properties can now be quantitatively described. Among other applications, this has utility for the design of liposomal drug delivery systems, particularly with regard to loading and regulated release properties. For example, for the loading conditions employed to obtain the data of Fig. 2, we obtain $k = 3 \cdot 10^{-3}$ s^{-1} or a half-time for uptake ($t_{1/2}$) of 3.8 min. Employing the relation $k = P \cdot A_m \cdot K^* \cdot K/V_o \cdot [H^+]_o$ and the activation energy of 28 kcal/mol, we can then obtain a generalized rate constant for doxorubicin uptake into EPC LUVs as:

$$k(T, pH_o, [PL]) = 2 \cdot 10^{pH_o - 7} \cdot [PL] \cdot \exp(62.3[1 - (307/T)])s^{-1}$$

From this relation, it can be calculated that at 60°C, $pH_o = 7$, uptake is extremely rapid as $k = 0.39$ s^{-1} ($t_{1/2} = 1.8$ s) for a phospholipid concentration ([PL]) of 1.5 mM. Alternatively, at 20°C uptake is considerably slower with a rate constant of $1.53 \cdot 10^{-4}$ s^{-1} ($t_{1/2} =$ 1.25 h). It is interesting to note that after rapid entrapment at an elevated temperature, the subsequent release at lower temperatures will be much slower, even though the effective lipid concentration for liposomally entrapped drug is considerably higher ($\sim$ 170 mM for a 100 nm diameter LUV assuming an area per phospholipid of 60 Å^2). Thus, a maximum rate constant for release of $1.7 \cdot 10^{-5}$ s^{-1} ($t_{1/2} = 11$ h) would be expected from fully loaded EPC LUVs at 20°C, $pH_i = 4.0$ under the assumption that all the doxorubicin which leaks out is immediately removed from the vicinity of the LUVs. The leakage rates will, of course, be considerably slower should the external drug concentrations be closer to equilibrium levels.

The high activation energies associated with the transbilayer permeation of the neutral form of doxorubicin, and the influence of cholesterol on these activation energies, are of interest. Briefly, these values are consistent with activation energies determined for a variety of other weak acids and bases, including phosphatidic acid and phosphatidylglycerol ($E_a \sim 30$ kcal/mol; see Ref. 7) as well as certain short (derivatized) peptides and amino acids which are weak bases [9]. Activation energies associated with diffusion across membranes have been rationalized on the basis of the need to break extramolecular hydrogen bonds to enter the hydrocarbon matrix and the ability to reform intramolecular hydrogen bonds in the hydrocarbon [26]. Activation energies can then be estimated according to the number of hydrogen bonds that must be broken (e.g., with water), less the number that can reform in the hydrocarbon, times the energy associated with hydrogen bond breaking ($\sim$ 5 kcal/mol). On this basis activation energies in the range of 30 kcal/mol could be achieved for doxorubicin, given the high hydrogen bond forming potential of this molecule. It should be noted, however, that the observed activation energies are likely overestimates. This is because the pK_a of doxorubicin is strongly temperature dependent [27]. If the pK_a of doxorubicin decreases by 0.5 pH units over the range 5°C to 37°C [28], the E_a determined would overestimate the actual activation energy by approx. 7 kcal/mol. The increase in E_a ($\sim$ 10 kcal/mol) ob-

served in EPC/cholesterol LUVs as compared to EPC LUVs implies that the presence of cholesterol in the bilayer adds a considerable barrier to doxorubicin movement.

The results of the equilibrium uptake studies presented show that the extremely high levels of doxorubicin accumulated into LUVs with an acidic interior, which result in effective internal/external concentration gradients which considerably exceed the proton gradients, can be accounted for by the ability of doxorubicin to partition into the LUV membrane. There are two aspects of this result which are surprising. The first concerns the ability of a lipid bilayer to maintain a permeability barrier when one monolayer is exposed to effective drug concentrations in the range of 300 mM. Such a concentration would be expected to be above the CMC of the drug, leading to detergent effects. Presumably, the partition coefficient of the drug for the bilayer is sufficiently high and the effective lipid concentration sufficiently large that the interior free drug concentration does not exceed the CMC. This leads into the second interesting feature of these results, which is that the high partition coefficients which are consistent with the uptake phenomena, and which imply high inner monolayer drug-to-lipid molar ratios, do not result in membrane disruption due to transbilayer packing differentials. In this regard, it is likely that monolayer associated doxorubicin is located at the lipid/water interface, with the amino function oriented towards the aqueous phase and the hydrophobic region oriented towards the hydrocarbon. Accumulation of drug to achieve internal concentrations of 300 nmol/μmol lipid corresponds to an internal surface concentration of approx. 0.6 mol drug per mol lipid for a 100 nm diameter LUV, assuming a membrane–water partition coefficient of 70. Even if membrane associated doxorubicins exhibited a cross-sectional area per molecule of only 30 Å^2 (half that of phosphatidylcholine), this would still correspond to an increase in surface area of 30%. It is probable that some morphological change occurs to compensate for this imbalance between inner and outer monolayer areas.

The accumulation of drugs other than doxorubicin into LUVs with an acidic interior can also be rationalized on the basis of the partitioning model employed here. Drugs such as lidocaine or timolol which have small partition coefficients dissipate the pH gradient at approximately the same external concentration as a comparable amount of the pH probe methylamine [24]. The transmembrane concentration gradients of these drugs therefore mirror the proton gradients. In contrast, drugs such as dopamine and doxorubicin which have larger partition coefficients are accumulated to a much greater extent (at the same intial exterior concentration). Thus their apparent transbilayer concentration gradients considerably exceed the residual proton gradient and the residual pH gradient of the vesicles is dissipated at a lower external concentration of drug than for drugs with smaller partition coefficients. The behaviour of imipramine and dibucaine, which was not well described in terms of the partitioning model presented, is most likely due to drug induced leakage. Under such circumstances the assumption that drug uptake and consumption of protons in the vesicle interior are well coupled would not be justified.

In summary, the results presented here demonstrate that doxorubicin is accumulated into LUVs with an acidic interior to achieve interior concentrations which can significantly exceed those expected on the basis of transmembrane proton gradients, and that this behaviour can be accounted for by a model which includes the ability of the drug to partition into the lipid bilayer. Accumulation proceeds via the neutral form and the rate constants for transbilayer movement exhibits high activation energies which are lipid dependent. Further, lipid bilayers are able to maintain an intact permeability barrier even at interior drug levels which approach equilimolar drug-to-lipid ratios in the inner monolayer.

Acknowledgements

This research was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.

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