Influence of Ion Gradients on the Transbilayer Distribution of Dibucaine in Large Unilamellar Vesicles†

Lawrence D. Mayer,* Kim F. Wong, Kusum Menon, Carolyn Chong, P. Richard Harrigan, and Pieter R. Cullis

Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1W5, Canada

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ABSTRACT: The uptake of dibucaine into large unilamellar vesicles in response to proton gradients (ΔpH; inside acidic) or membrane potentials (Δψ; inside negative) has been investigated. Dibucaine uptake in response to ΔpH proceeds rapidly in a manner consistent with permeation of the neutral (deprotonated) form of the drug, reaching a Henderson–Hasselbach equilibrium where $[\text{dibucaine}]_{in}/[\text{dibucaine}]_{out} = [H^+]_{in}/[H^+]_{out}$ and where the absolute amount of drug accumulated is sensitive to the buffering capacity of the interior environment. Under appropriate conditions, high absolute interior concentrations of the drug can be achieved (~120 mM) in combination with high trapping efficiencies (in excess of 90%). Dibucaine uptake in response to Δψ proceeds more than an order of magnitude more slowly and cannot be directly attributed to uptake in response to the ΔpH induced by Δψ. This induced ΔpH is too small (≤1.5 pH units) to account for the transmembrane dibucaine concentration gradients achieved and does not come to electrochemical equilibrium with Δψ. Results supporting the possibility that the charged (protonated) form of dibucaine can be accumulated in response to Δψ were obtained by employing a permanently positively charged dibucaine analogue (*N*-methyldibucaine). Further, the results suggest that Δψ-dependent uptake may depend on formation of a precipitate of the drug in the vesicle interior. The uptake of dibucaine into vesicles in response to ion gradients is of direct utility in drug delivery and controlled release applications and is related to processes of drug sequestration by cells and organelles in vivo.

The ability of ion gradients resulting in membrane potentials (Δψ) and pH gradients (ΔpH) to dramatically influence the transmembrane distributions of certain lipophilic cations and anions is well established. For example, the transmembrane distributions of probes of membrane potential (e.g., methyltriphenylphosphonium) or probes of pH gradients (e.g., methylamine) are used routinely in biological systems to determine Δψ and ΔpH (Waggoner, 1979; Lichtstein et al., 1979; Nicholls, 1979).

In recent work employing large unilamellar vesicles (LUVs),[1] we have pointed out that these ion gradients not only can result in large transmembrane concentration gradients of lipophilic cations but also can result in high absolute concentrations in the LUV interior. In the case of the membrane potential probe safranine, for example, Δψ-driven interior concentrations in excess of 100 mM can be achieved employing initial exterior concentrations of 2 mM (Bally et al., 1985). Similar Δψ-dependent effects have been observed for chlorpromazine (Bally et al., 1985), dibucaine (Mayer et al., 1985a), and doxorubicin (Mayer et al., 1985b) among others.

Two points of interest raised by these observations concern the utility of ion gradients for loading liposomal systems for drug delivery applications and the mechanisms whereby such redistributions proceed. For example, entrapping drugs in liposomal systems employing common passive trapping procedures usually results in relatively low drug to lipid ratios and trapping efficiencies which rarely exceed 50% (Mayer et al., 1986b; Cullis et al., 1987). Uptake in response to membrane potentials can result in significantly improved characteristics. In the case of doxorubicin, for example, Δψ-driven uptake can result in drug to lipid ratios an order of magnitude higher than available through other procedures, in combination with trapping efficiencies in excess of 95% (Mayer et al., 1986b; Cullis et al., 1987).

We have addressed the mechanism of ion gradient driven uptake employing lipophilic cations where the cationic character is provided by an amino function. Such lipophilic amines, which constitute a large proportion of commonly employed drugs (including doxorubicin and dibucaine), can exist in a neutral (deprotonated) form at higher pH values in addition to the positively charged form. It is commonly thought that the charged form is relatively impermeable, whereas the neutral form can readily permeate membranes (Yeagle et al., 1977). In systems exhibiting transmembrane pH gradients, this results in equilibrium transmembrane concentration gradients where $[AH^+]_{in}/[AH^+]_{out} = [H^+]_{in}/[H^+]_{out}$, where A indicates the lipophilic amine. The question therefore arises as to whether the protonated or the neutral form is accumulated by systems exhibiting a Δψ.

In the present work, we employ dibucaine as a model lipophilic amino-containing compound and characterize the uptake behavior in response to pH gradients and membrane potentials. In LUV systems exhibiting pH gradients, rapid uptake levels reaching interior concentrations in excess of 120 mM can be achieved, in combination with trapping efficiencies in excess of 90%. The transbilayer concentration gradients obtained and the influence of interior buffering capacity are

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[1] Abbreviations: LUV, large unilamellar vesicle; DIB, dibucaine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; P_i, inorganic phosphate; TPP, tetraphenylphosphonium; KGlu, potassium glutamate; TLC, thin-layer chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid.

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shown to be consistent with transport of the neutral form of the amine. Alternatively, uptake in response to $\Delta\psi$ proceeds more slowly and cannot be directly accounted for by $\Delta\psi$-induced pH gradients.

Materials and Methods

Materials. Dibucaine hydrochloride, valinomycin, and all salts were purchased from Sigma Chemicals. Tritiated DPPC, [^{14}C]tetraphenylphosphonium, [^{14}C]inulin, and [^{14}C]-methylamine were purchased from New England Nuclear. Egg PC was purified by standard procedures and was greater than 99% pure as detected by thin-layer chromatography (TLC).

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles (containing 0.04 μCi of [^{3}H]DPPC/μmol of phospholipid) were produced by extrusion of frozen and thawed MLVs (Mayer et al., 1985c) 10 times through 0.1-μm polycarbonate filters as previously described (Hope et al., 1985) employing an extrusion device (Lipex Biomembranes, Vancouver). Vesicles prepared in this manner had trapped volumes of 1.5 μL/μmol of phospholipid employing [^{14}C]inulin as an aqueous marker. Phospholipid concentrations and specific activities were determined by analysis of lipid phosphorus as described previously (Fiske & Subbarow, 1925).

Dibucaine Uptake Experiments. Transmembrane Na^+/K^+ gradients were created by preparing LUVs in the presence of a K^+-containing buffer and passing the vesicles over a Sephadex G-50 gel filtration column equilibrated in the appropriate Na^+-containing buffer of equal osmolarity. Transmembrane pH gradients were formed by preparing LUVs in 300 mM sodium citrate (pH 4.0–6.5) and passing the vesicles down Sephadex G-50 columns equilibrated in 20 mM Hepes/150 mM NaCl (pH 7.5). Experiments to monitor the uptake of dibucaine were initiated by adding a small volume of concentrated vesicles to buffer solutions containing the indicated dibucaine concentrations. Where employed, valinomycin (1 mg/mL in ethanol) was added to achieve levels of 1 μg/μmol of phospholipid. Vesicle-associated dibucaine was determined by passing 0.15-mL aliquots of the incubation mixtures over 1-mL Sephadex G-50 columns to separate free from vesicle-associated drug. The vesicle-containing eluant was then assayed for lipid by monitoring radioactivity and for dibucaine by monitoring the fluorescence at 410 nm of a Triton X-100 solubilized aliquot. Standard dibucaine solutions containing detergent and similar amounts of lipid were used to calibrate fluorescence intensity against actual amounts of anesthetic present. Unless otherwise indicated, uptake levels reflect values obtained under steady-state conditions. Membrane potentials and transmembrane pH gradients were determined by monitoring the transmembrane distributions of [^{14}C]TPP and [^{14}C]methylamine, respectively, employing 1-mL Sephadex G-50 columns to separate free from vesicle-bound material. As previously demonstrated for TPP (Bally et al., 1985), transmembrane distributions of methylamine closely reflected the actual pH gradient. For instance, vesicles containing pH 7.5 buffer in their external aqueous media and pH 4.6 or 6.6 buffers in their internal aqueous media yielded ΔpH values of 2.8 and 0.6 units employing [^{14}C]methylamine by this procedure.

NMR Investigations. The ^{31}P NMR spectra were obtained by employing a Bruker WP-200 spectrometer operating at 81 MHz. A free induction decay corresponding to 256 transients was obtained by using a 11-μs 47° pulse, a 1-s interpulse delay, and a 10-kHz sweep width. An exponential multiplication corresponding to 5 Hz was applied to the free induction decay prior to Fourier transformation. The pH of the vesicle interior

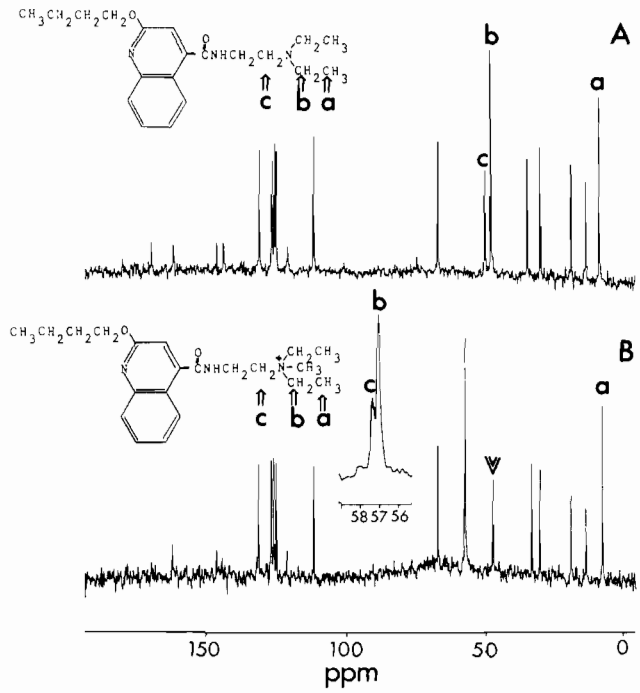

FIGURE 1: Natural-abundance ^{13}C NMR spectra of dibucaine hydrochloride (A) and *N*-methyldibucaine (B) dissolved in D_2O. The spectra were obtained by employing a Bruker WP-200 spectrometer operating at 50.3 MHz. The ^{13}C NMR assignments of the resonances were made on the basis of observed multiplet structure in the absence of nuclear Overhauser effects employing inverse ^{1}H decoupling and comparison with previously assigned ^{13}C NMR spectra (Yeagle et al., 1977). The double arrow in panel B designates the resonance arising from the *N*-methyl group added upon formation of the quaternary dibucaine derivative.

was determined by monitoring the chemical shift of P_i entrapped inside the vesicle and relating the chemical shift to those obtained for standard P_i solutions of known pH.

Potassium Release Experiments. The LUVs were prepared in 161 mM potassium glutamate/20 mM Hepes (pH 7.5) containing 20 μCi of ^{42}K. Subsequent to exchange of the external buffer for 150 mM NaCl/20 mM Hepes (pH 7.5), thereby removing untrapped ^{42}K, appropriate amounts of anesthetic and ionophore were added as indicated. At given times, 1-mL aliquots of the mixtures were placed in an Amicon apparatus containing a YM10 membrane and put under low pressure (5–10 psi). This allowed the recovery of vesicle-free exterior buffer. A 100-μL aliquot of the filtrate was analyzed for radioactivity utilizing a Packard 2000 CA scintillation counter. Conditions were such that leakage of 5% of the trapped potassium could be readily detected.

Synthesis of N-Methyldibucaine. Dibucaine (2.6 mmol) (free base) was dissolved in 20 mL of anhydrous ethanol. To this was added 5.2 mmol of methyl iodide with stirring. The reaction was allowed to proceed for 24 h at which time no dibucaine could be detected by TLC (the solvent system was $CHCl_3$/MeOH 4:1). The sample was then dried under vaccum and resuspended in 20 mL of $CHCl_3$. Petroleum ether was then added (200 mL) slowly with stirring followed by centrifugation. After removal of the supernatant, the precipitate was dissolved in 2 mL of $CHCl_3$, loaded onto a silica gel column, and eluted with $CHCl_3$/MeOH (4:1). The eluant containing the putative *N*-methyldibucaine was dried under vaccum, and the resulting material was recrystallized in H_2O. This material appeared greater than 95% pure as detected by TLC.

Characterization of N-Methyldibucaine. The structure of the *N*-methyldibucaine derivative was confirmed by natu-

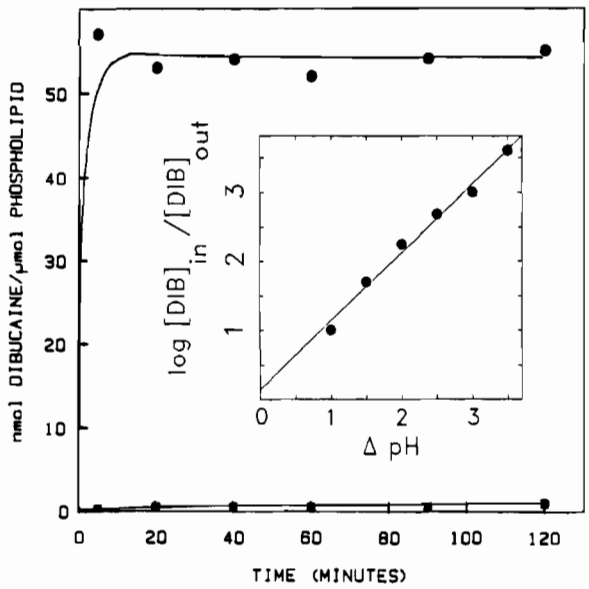

FIGURE 2: Uptake of dibucaine into LUVs exhibiting a pH gradient. LUVs (1 mM egg PC) containing 300 mM sodium citrate (pH 4.5) in the internal aqueous space and 150 mM NaCl/20 mM Hepes (pH 7.5) (●) or 250 mM sodium citrate (pH 4.5) (■) in the external medium were incubated in the presence of 0.1 mM dibucaine. Inset: Transmembrane dibucaine concentration gradients in LUVs as a function of the transmembrane ΔpH. Vesicles were prepared in the presence of 300 mM sodium citrate ranging in pH from 4.0 to 6.5. The external medium was exchanged for 250 mM sodium glutamate/20 mM Hepes (pH 7.5) to yield the indicated pH gradients. The LUVs (1 mM egg PC) were incubated in the presence of 0.1 mM dibucaine. Uptake levels reflect the values obtained after 2-h incubations.

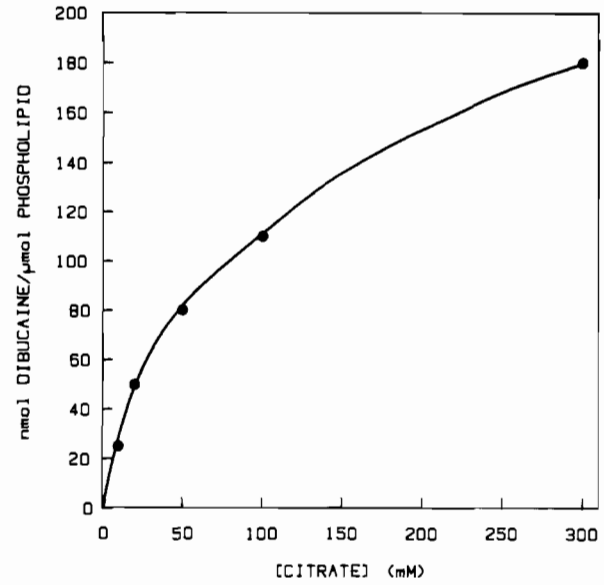

FIGURE 3: Effect of internal citrate concentration on the level of ΔpH-dependent dibucaine uptake. Egg PC vesicles were prepared in the presence of the indicated citrate concentrations (pH 4.0), and subsequently, the exterior medium was exchanged for 20 mM Hepes/150 mM NaCl (pH 7.5). Vesicles (1 mM phospholipid) were incubated in the presence of 0.2 mM dibucaine. Uptake levels reflect values obtained after 1 h.

ral-abundance ^{13}C NMR and mass spectroscopy. Figure 1 shows the ^{13}C NMR spectra obtained for dibucaine hydrochloride (Figure 1A) and the *N*-methyl derivative of dibucaine (Figure 1B). The assignments of particular resonances were made on the basis of observed multiplet structure in the absence of nuclear Overhauser enhancement effects employing inverse gated proton decoupling and comparison with previously assigned ^{13}C NMR spectra. The spectra of *N*-methyldibucaine (Figure 1B) appeared identical with those of dibucaine (Figure 1A) with the exception of two distinct alterations. Figure 1A identifies the resonance corresponding to the N-terminal methyl carbon of dibucaine. In the spectra of the methylated derivative, the terminal and internal *N*-methylene carbon resonances are shifted downfield (Figure 1B and inset) while the N-terminal methyl resonance remains unchanged. An additional resonance is also observed at 47.2 ppm (indicated by the double arrow). Spectra obtained in the absence of nuclear Overhauser enhancement effects indicated that this peak corresponded to a methyl carbon. These results are consistent with the presence of an N-methylated quaternary amine in the derivatized material. Mass spectra of dibucaine and *N*-methyldibucaine were obtained by employing fast atom bombardment techniques utilizing glycerol for sample application. Major ion peaks were observed as follows [molecular weight (relative intensity)]: dibucaine hydrochloride, 344 (100), 271 (22), 86 (94), and 72 (21); *N*-methyldibucaine, 359 (13), 358 (49), 272 (17), 271 (88), 215 (18), 86 (26), and 72 (100). These NMR and mass spectrometry results together confirm the structure of *N*-methyldibucaine given in Figure 1B.

RESULTS

The response of dibucaine to LUVs displaying pH gradients was examined to characterize the effects of a transmembrane ΔpH on dibucaine uptake. Figure 2 demonstrates that dibucaine is rapidly sequestered by LUVs with an interior pH of 4.5 and exterior pH of 7.5. In contrast, vesicle-associated drug levels are negligible in the absence of a pH gradient. ΔpH-dependent uptake levels of approximately 55 nmol of dibucaine/μmol of phospholipid are achieved within 2 min and remain stable over the 2-h time course. Under the conditions employed (0.1 mM dibucaine and 1 mM phospholipid, 21 °C), the rate of accumulation reflects an initial velocity of approximately 3.0×10^{-2} nmol/(min·cm^2) which is 27-fold greater than that observed for $\Delta\psi$-dependent dibucaine uptake employing Na^+/K^+ gradients under similar conditions [$\nu_0 = 1.1 \times 10^{-3}$ nmol/(min·cm^2); Mayer et al., 1985a]. This rapid uptake process is consistent with rapid transmembrane movement of the neutral form of dibucaine to the acidic vesicle interior where it is reprotonated. Such a process would suggest that the concentration gradients achieved should reflect the proton gradients (assuming a lack of influence of membrane partitioning effects) according to the Henderson–Hasselbach equilibrium $[AH^+]_{in}/[AH^+]_{out} = [H^+]_{in}/[H^+]_{out}$. As shown in the inset in Figure 2, the equilibrium dibucaine concentration gradients achieved for various transmembrane pH gradients closely follow those expected from the Henderson–Hasselbach relation. Further, reduced interior buffering capacities should result in less uptake for this type of process due to a rise in the interior pH as each neutral amine taken up sequesters a proton. As shown in Figure 3, dibucaine uptake is progressively reduced for lower interior buffering capacities. This is also reflected by reduced ΔpH values following uptake as assayed by methylamine (results not shown). It should be noted that the maximum uptake level observed for a ΔpH of 3.5 corresponds to internal dibucaine concentrations as high as 120 mM and more than 90% of the available drug is entrapped in this case.

Given that pH gradients can result in such rapid and efficient drug redistribution, it is important to reexamine the uptake of dibucaine in response to $\Delta\psi$, which has been suggested to involve transport of the protonated species of the drug (Mayer et al., 1985a). In particular, previous investigations

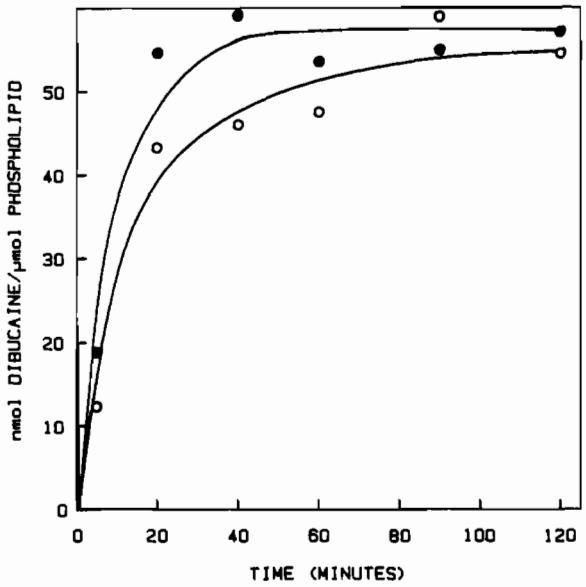

FIGURE 4: Uptake of dibucaine into LUVs containing 10 (●) and 200 (○) mM P_i in response to a K^+ diffusion potential. Vesicles were prepared in the presence of 125 mM K_2SO_4, the indicated concentrations of Na_2HPO_4 (pH 7.5), and Na_2SO_4 sufficient to maintain constant ionic strength. Na^+/K^+ gradients were established by exchanging the exterior buffer for 20 mM Na_2HPO_4/250 mM Na_2SO_4 (pH 7.5). The LUVs (1 mM egg PC) were incubated in the presence of 0.1 mM dibucaine and 1 μg of valinomycin.

employing sonicated (SUV) phospholipid vesicle systems (Cafiso & Hubbell, 1983; Garcia et al., 1984) have shown that K^+ diffusion potentials (inside negative) can give rise to transmembrane pH gradients (inside acidic). Since dibucaine is accumulated inside LUVs exhibiting transmembrane Na^+/K^+ gradients (Mayer et al., 1985a), it is of interest to determine the extent to which $\Delta\psi$ or an induced ΔpH dictates drug uptake.

If dibucaine accumulates inside vesicles experiencing Na^+/K^+ gradients according to induced proton gradients rather than the $\Delta\psi$ itself, then the rate and extent of drug uptake may be expected to be reduced for higher buffering capacities in the internal aqueous media of the vesicle. As shown in Figure 4, dibucaine uptake levels are unchanged while varying the internal phosphate buffer concentration between 10 and 200 mM. These levels of local anesthetic accumulation correspond to apparent $[\text{dibucaine}]_{in}/[\text{dibucaine}]_{out}$ ratios in excess of 800. The corresponding $\Delta\psi$ values as determined employing TPP are also comparable for all systems studied, ranging between −160 and −180 mV in the absence of dibucaine and between −120 and −160 mV in the presence of dibucaine (data not shown). In addition, the amount of buffering capacity had little effect on the rate of accumulation as shown in Figure 4.

^{31}P NMR experiments were performed to directly monitor $\Delta\psi$-induced pH gradients for LUVs containing varying amounts of P_i buffer by monitoring the ^{31}P NMR chemical shift of entrapped P_i. Figure 6 demonstrates that acidification of the vesicle interior increases with decreasing amounts of entrapped P_i. Incubating the vesicles (50 mM egg PC) with valinomycin alone (Figure 5A) results in pH decreases of 0.20, 0.35, and 0.71 units after 2 h for internal P_i concentrations of 200, 100, and 50 mM, respectively. The rate of the pH changes yields comparable initial velocities for proton uptake [1.4×10^{-4} nmol/(min·cm^2)] in all three systems. The addition of dibucaine, as well as valinomycin, results in increased acidification of the vesicle interior (Figure 5B). After incubation for 2 h, the respective interior pH decreases are 0.6,

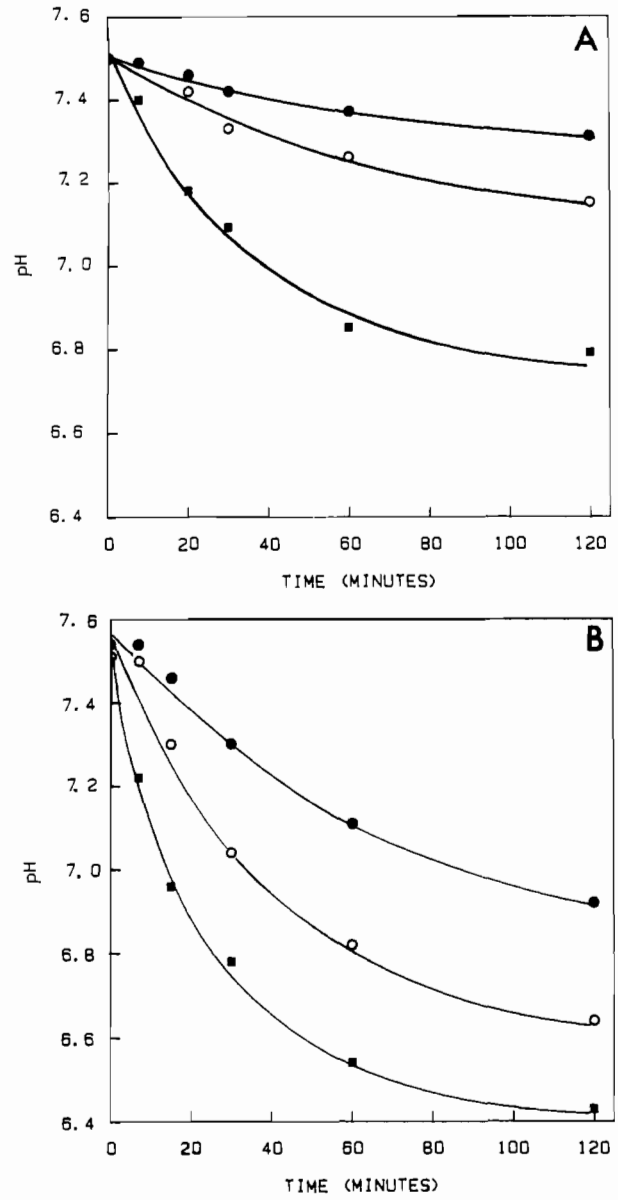

FIGURE 5: Effect of dibucaine uptake on the internal pH of LUVs. Vesicles were prepared in the presence of 200, 0 (●), 100, 100 (○), and 50, 125 (■) mM respectively Na_2HPO_4 (pH 7.5) and Na_2SO_4 and 125 mM K_2SO_4 and passed down gel filtration columns equilibrated in 20 mM Hepes/250 mM Na_2SO_4 (pH 7.5). The position of the ^{31}P NMR inorganic phosphate resonance was monitored to detect the pH of the vesicle interior. Egg PC LUVs (50 mM lipid) were incubated with 0.5 μg of valinomycin/μmol of PC in the absence (A) and presence (B) of 5 mM dibucaine.

0.8, and 1.1 units for P_i concentrations of 200, 100, and 50 mM, respectively. On the basis of Henderson–Hasselbach redistributions, these pH gradients would predict $[\text{DIB}]_{in}/[\text{DIB}]_{out}$ ratios of 4.0, 6.3, and 12.5, respectively. Under the conditions employed, however, a dibucaine concentration gradient of approximately 25 is obtained for all three P_i concentrations studied.

These results suggest not only that $\Delta\psi$-driven dibucaine uptake is insensitive to the internal buffering capacity but also that the induced pH gradients are insufficient to account for the transmembrane drug distributions achieved. However, the ^{31}P NMR studies require the use of higher lipid and drug concentrations than employed for the uptake experiments of Figures 2–4, which makes a direct correlation difficult. Therefore, the $\Delta\psi$ and ΔpH of LUVs in the presence of varying transmembrane K^+ gradients were determined em-

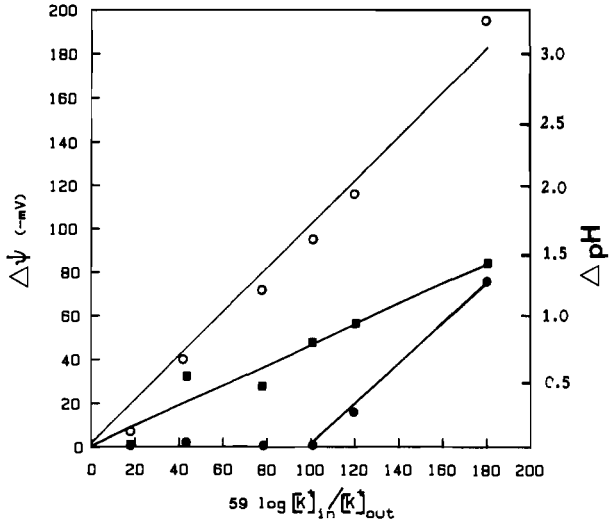

FIGURE 6: Δψ (○) and ΔpH (●, ■) as a function of the transmembrane K^+ gradient. Egg PC LUVs (1 mM lipid) having 169 mM KGlu/20 mM Hepes (pH 7.5) in the internal aqueous space were incubated in the presence of 1 μg of valinomycin/μmol of egg PC. [^{14}C]TPP (1 μCi/mL) or $^{14}CH_3NH_2$ (1 μCi/mL) was added to the samples for determination of Δψ and ΔpH, respectively. The indicated transmembrane K^+ gradients were achieved by passing the LUVs over Sephadex G-50 columns equilibrated with buffers consisting of 169 mM KGlu/20 mM Hepes (pH 7.5) and 150 mM NaCl/20 mM HEPES (pH 7.5) at ratios ranging from 50:50 to 0.1:99.9. Dibucaine was added (■) to achieve a final concentration of 0.1 mM.

ploying TPP and the pH probe methylamine for phospholipid and dibucaine concentrations of 1.0 and 0.1 mM, respectively. As shown in Figure 6, the ΔpH values obtained over a range of $[K^+]_{in}/[K^+]_{out}$ ratios do not correlate with measured Δψ values. This is consistent with the lack of equilibrium between Δψ and induced ΔpH gradients observed elsewhere (T. Redelmeier, unpublished observations). These results are also consistent with those obtained utilizing ^{31}P NMR techniques (Figure 5). Specifically, dibucaine increases the acidification of the vesicle interior over that observed in the presence of valinomycin alone, but in both cases, the resulting ΔpH is far smaller than predicted for electrochemical equilibrium of Δψ and ΔpH.

If the levels of Δψ-driven dibucaine uptake cannot be accounted for by the induced ΔpH, it is logical to suggest that the charged (protonated) species is accumulated. This possibility was tested by employing a permanently positively charged quaternary amine derivative of dibucaine (*N*-methyldibucaine). Figure 7 demonstrates that *N*-methyldibucaine is accumulated inside LUVs displaying a K^+ diffusion potential. The net Δψ-dependent uptake value of 20 nmol/μmol of phospholipid observed at 2 h is similar to dibucaine uptake at low pH (pH <5.5) where greater than 99% of the drug is in the protonated (charged) form (Mayer et al., 1985a). The initial rate of uptake [1.4×10^{-4} nmol/(min·cm^2)] is approximately 10- and 2-fold lower than that observed for dibucaine uptake at pH 7.5 and 5.5, respectively. Also, the Δψ-driven uptake of *N*-methyldibucaine is insensitive to the pH of the aqueous medium over the range 5.0–7.5 (data not shown).

The results to this point suggest that the Δψ-driven uptake process could be attributed to accumulation of a charged species in response to Δψ and a (smaller) sequestration of the neutral species in response to the induced ΔpH. Both of these processes would predict that uptake of dibucaine should be accompanied by a stoichiometric release of K^+. In the case of the uptake of the protonated species, this would be a direct (valinomycin-mediated) exchange whereas uptake of the

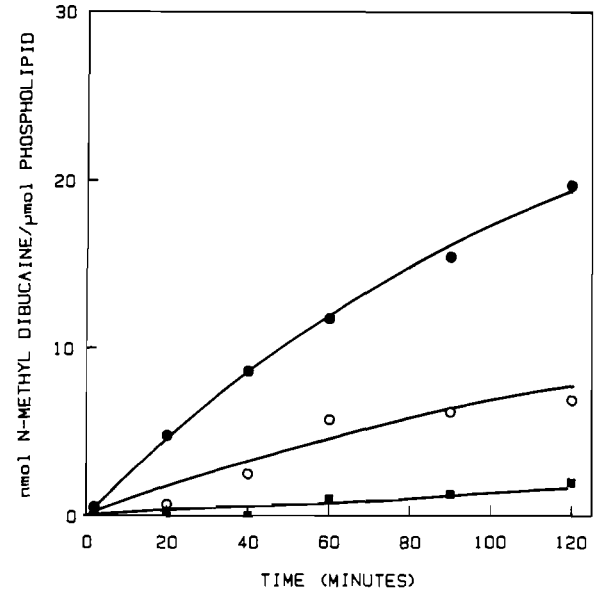

FIGURE 7: Uptake of *N*-methyldibucaine into LUVs at 22 °C. The LUVs (1 mM egg PC) were incubated in the presence of 0.2 mM *N*-methyldibucaine. The internal vesicle buffer was 169 mM KGlu/20 mM Hepes (pH 7.5). External buffers were (■) 169 mM KGlu/20 mM Hepes (pH 7.5) and (●, ○) 150 mM NaCl/20 mM Hepes (pH 7.5). Valinomycin was added (●, ■) to achieve 1 μg/μmol of egg PC.

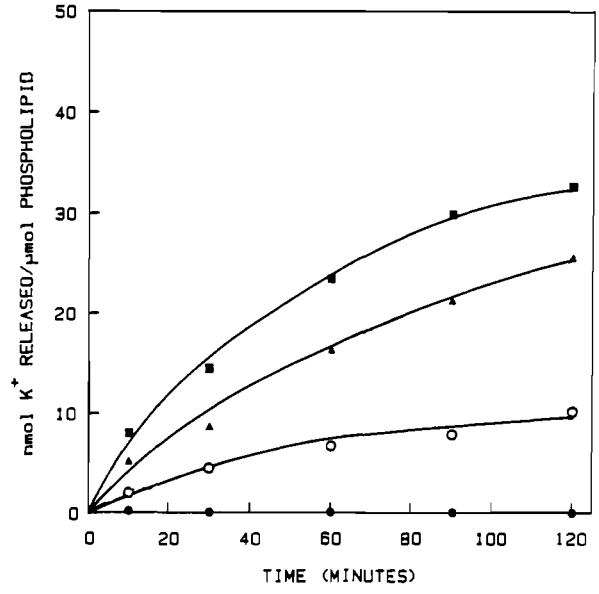

FIGURE 8: Potassium release from LUVs containing 169 mM KGlu/20 mM Hepes (pH 7.5) and 150 mM NaCl/20 mM Hepes (pH 7.5) in the internal and external media, respectively. Potassium release was determined by monitoring ^{42}K efflux as described under Materials and Methods. The vesicles (50 mM egg PC) were incubated in the presence (■, ▲, ●) and absence (○) of valinomycin (1 μg/μmol of egg PC). Dibucaine was added to achieve final concentrations of 5.0 (■) and 2.5 mM (▲).

neutral species (and subsequent sequestration of a proton) should result in a K^+/H^+ exchange to maintain the interior equilibrium proton concentration. However, as shown in Figure 8, the release of K^+ during Δψ-driven accumulation of dibucaine is much slower than dibucaine uptake (compare Figure 8 and Figure 4), and there is no obvious stoichiometric relationship between K^+ efflux and dibucaine influx. Specifically, for the 5 mM dibucaine/50 mM egg PC system, the initial rates of K^+ release [3.3×10^{-4} nmol/(min·cm^2)] are approximately 4-fold slower than dibucaine uptake, and after 2 h, the total K^+ release is 33 nmol of K^+/μmol of phospholipid

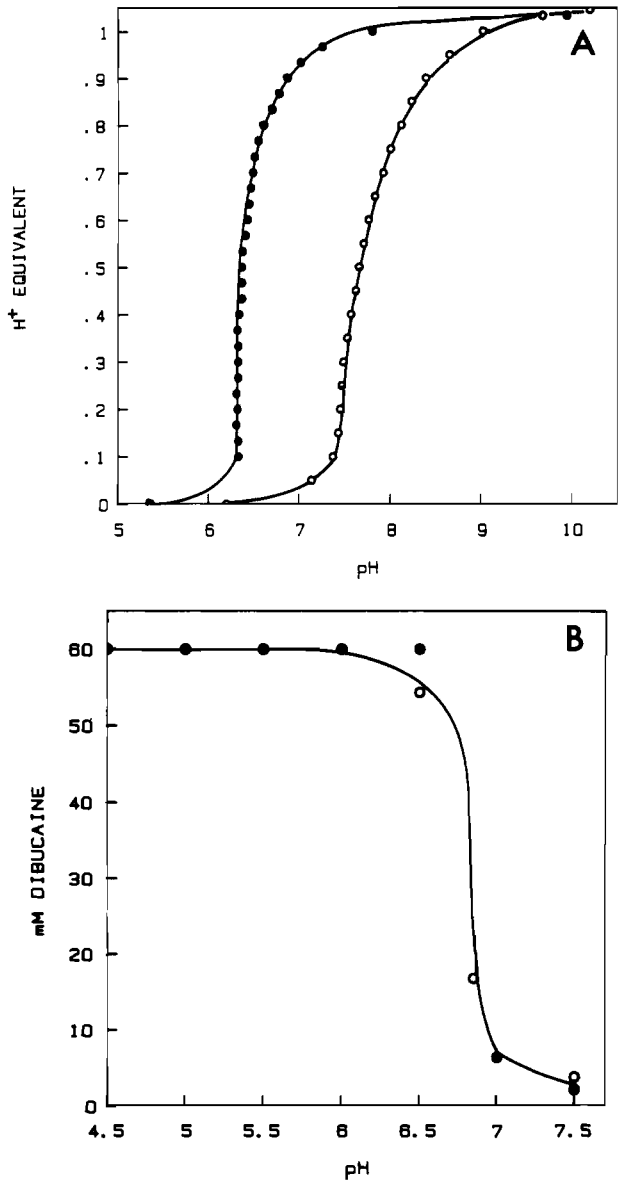

FIGURE 9: (A) pH titration of dibucaine dissolved in H_2O at a concentration of 60 (●) and 2 mM (○). The pH of the solution was continuously monitored while titrating in sodium hydroxide. Proton equivalents refer to moles NaOH per mole of dibucaine. (B) Solubility of dibucaine hydrochloride as a function of pH. Dibucaine hydrochloride was dissolved in H_2O at a concentration of 60 mM. Sodium hydroxide was added to increase the pH of the solution to the indicated values, and dibucaine concentrations were determined after removal of precipitated drug.

whereas dibucaine is accumulated to 45 nmol/μmol of phospholipid.

The results of Figure 8 clearly imply that a large proportion of the dibucaine sequestered in response to $\Delta\psi$ is not in the protonated (charged) form. In order for this to be the case, the apparent pK (pK_a) of the entrapped drug must be in the region of 7, much lower than that of the free form (pK = 8.5; Ritchie & Greengard, 1961). As noted elsewhere (Butler et al., 1973), the pK_a of the local anesthetic tetracaine is concentration dependent, where lower pK_a's are observed at higher drug concentrations. This has been attributed to formation of micelles containing the charged form of the drug, giving rise to lower interface proton concentrations than in the bulk phase due to surface charge effects (Westman et al., 1982). Similar effects are observed for dibucaine as shown in Figure 9A. Specifically, at 60 mM dibucaine, which corresponds to the maximum $\Delta\psi$-driven interior concentrations achieved, the apparent pK is 6.4. The corresponding pK for dibucaine at a concentration of 2 mM is 7.7. As shown in Figure 9B, titration through this pH region for a drug concentration of 60 mM dramatically reduces the solubility of the drug, presumably due to the relative insolubility of the neutral (deprotonated) form.

Discussion

The results presented here have employed dibucaine as a model lipophilic amine-containing drug to investigate the uptake of lipophilic amines into large unilamellar vesicles in response to transmembrane ion gradients. For the purpose of discussion, we cover in turn the topics of ΔpH-dependent uptake and $\Delta\psi$-dependent uptake, the implications for drug distributions in vivo, and the possible utility of these procedures for liposome loading in drug delivery applications.

The uptake observed in response to imposed pH gradients clearly reveals behavior consistent with accumulation via transmembrane movement of the neutral form of dibucaine. First, as indicated under Results, such a mechanism implies that equilibrium transmembrane concentrations of the protonated form of dibucaine should obey the relation $[AH^+]_{in}/[AH^+]_{out} = [H^+]_{in}/[H^+]_{out}$, as is observed experimentally. Second, the rate of uptake of dibucaine in response to ΔpH is much faster than observed for $\Delta\psi$, consistent with the relatively high membrane permeability of the neutral species. Third, the extent of uptake is reduced at lower interior buffering capacities, as would be expected for a process where neutral dibucaine sequesters protons from the vesicle interior after moving across the membrane. In summary, this uptake behavior is similar to that observed for other weak bases (dopamine, seratonin) in vesicle systems exhibiting transmembrane proton gradients (Nichols & Deamer, 1976; Sokol et al., 1985) with the additional feature that extremely high interior concentrations of the drug can be achieved for high interior buffering capacities [see also Bally et al. (1988)].

A surprising feature of the ΔpH uptake results concerns the accuracy with which the transmembrane distributions are described by the Henderson–Hasselbach relation. In particular, following the analysis of Westman et al. (1982), it is straightforward to show that the ratio of the total internal lipophilic amine concentration ($[A(T)]_i$) to that outside ($[A(T)]_o$), including the amounts partitioning into the interior and exterior vesicle monolayers, can be written as

$$\frac{[A(T)]_i}{[A(T)]_o} = \frac{1 + \frac{[H^+(B)]_i}{K_1(B)} + \frac{V(M)}{V_i(B)}K_2\left[1 + \frac{[H^+(B)]_i}{K_1(M)}\exp\left(-\frac{F\phi_i}{RT}\right)\right]}{1 + \frac{[H^+(B)]_o}{K_1(B)} + \frac{V(M)}{V_o(B)}K_2\left[1 + \frac{[H^+(B)]_o}{K_1(M)}\exp\left(-\frac{F\phi_o}{RT}\right)\right]} \quad (1)$$

where $K_1(B)$ is the dissociation constant of the lipophilic amine in the bulk (aqueous) phase, $K_1(M)$ is the dissociation constant in the membrane phase, $[H^+(B)]_i$ and $[H^+(B)]_o$ are the interior and exterior bulk proton concentrations, respectively, $V(M)$ is the volume of the membrane phase, $V_i(B)$ and $V_o(B)$ are the volumes of the interior and exterior aqueous phases, respectively, K_2 is the membrane partition coefficient for the neutral form, ϕ_i and ϕ_o are the surface potentials at the interior and exterior membrane interfaces, respectively, and F/RT = 38.9 V^{-1} where F is the Faraday constant, R is the molar gas constant, and T is the absolute temperature. Under the

conditions $[H^+(B)]_i$, $[H^+(B)]_o \gg K_1(B)$, eq 1 can be rewritten as

$$\frac{[A(T)]_i}{[A(T)]_o} \simeq \frac{[AH^+]_i}{[AH^+]_o} = \frac{[H^+(B)]_i\left[1 + \frac{V(M)}{V_i(B)}K_2\frac{K_1(B)}{K_1(M)}\exp\left(-\frac{F\phi_i}{RT}\right)\right]}{[H^+(B)]_o\left[1 + \frac{V(M)}{V_o(B)}K_2\frac{K_1(B)}{K_1(M)}\exp\left(-\frac{F\phi_i}{RT}\right)\right]} \quad (2)$$

Under the standard conditions employed in this work (vesicle diameter 100 nm, lipid concentration 1 mM) and assuming an area per lipid molecule of 60 Å^2 and a membrane thickness of 40 Å, it can be calculated that $V(M)/V_i(B) = 0.12$ whereas $V(M)/V_o(B) = 2.2 \times 10^{-5}$. Assuming $K_2 = 10^3$ for the neutral form of dibucaine and assuming that $K_1(B) \simeq K_1(M)$, it would therefore be expected that $[AH^+]_i/[AH^+]_o \simeq 120 \exp(-F\phi_i/RT)[H^+]_i/[H^+]_o$. Such a dependence is clearly not compatible with the measured $[AH^+]_i/[AH^+]_o$ ratios (Figure 3). The reasons for this discrepancy are not currently understood but could arise if $K_1(M) \gg K_1(B)$ as observed for procaine (Westman et al., 1982) or if effects corresponding to "membrane saturation" by the drug occur as noted by Frezatti et al. (1986), resulting in formation of a separate dibucaine-rich phase in the vesicle interior.

In the case of dibucaine uptake into LUVs in response to a K^+ diffusion potential, there are two major points of interest. These concern the mechanism by which the drug is accumulated and the physical form of the sequestered drug. With regard to mechanism, it is not clear whether the uptake process proceeds by permeation of the neutral form of the drug or the protonated (charged) species. First, the rate of $\Delta\psi$-dependent uptake is much slower than that observed for ΔpH-dependent uptake [initial rates of 1.1×10^{-3} nmol/(min·cm^2) as compared to 3.0×10^{-2} nmol/(min·cm^2)]. It may be suggested that this slower rate reflects the rate-limiting effects of H^+ ion movement to establish pH gradients in electrochemical equilibrium with $\Delta\psi$ but the induced proton gradients observed are too small (ΔpH <1.5) to account for the apparent transmembrane dibucaine concentration gradients observed (up to 1000) assuming that these reflect transmembrane gradients of the protonated species of the drug. The fact that the extent and rate of $\Delta\psi$-dependent dibucaine uptake are independent of the buffering capacity of the internal medium of the LUVs further suggests that uptake is not proceeding in response to pH gradient effects. In the case (Figure 5) where the internal pH is monitored by ^{31}P NMR, for example, the ΔpH values observed for different buffering capacities would suggest that accumulated levels of the protonated form of the drug should vary by a factor of 4, whereas no such variation is observed.

These effects could be rationalized to some extent on the basis of the observations that stoichiometric amounts of K^+ are not released during the uptake process and that lower apparent pK values occur as the dibucaine concentration is increased (Figures 8 and 9). As a result, a large proportion of the drug sequestered in response to $\Delta\psi$ may exist in the neutral form which would give rise to higher inside/outside concentration gradients than expected on the basis of the observed ΔpH. This implies transmembrane concentration gradients of the neutral form of the drug which conflicts with the $[AH^+]_i/[AH^+]_o = [H^+]_i/[H^+]_o$ equilibrium noted for the ΔpH uptake phenomena, which indicates that the interior and exterior concentrations of the neutral form of the drug are equivalent. This would suggest that the sequestered drug must exist in some alternate phase or precipitate in the vesicle interior, consistent with the limited solubility of the dibucaine at higher pH values. Formation of a precipitate would also be consistent with the abnormal retention characteristics noted for dibucaine and other lipophilic amines when the ion gradients giving rise to accumulation are removed (T. Redelmeier, unpublished observations).

In previous work, we have suggested that the protonated form of dibucaine is directly sequestered in response to $\Delta\psi$ and that such uptake proceeds to establish interior surface potentials which limit further uptake (Mayer et al., 1985a). Given the evidence provided here for accumulation of the neutral form, it would appear that a direct correlation between uptake levels and surface potentials may not be justified. With regard to uptake of the protonated drug species, the accumulation of the permanently positively charged dibucaine analogue *N*-methyldibucaine in response to $\Delta\psi$ clearly supports this possibility. Although the rate of uptake for this analogue is slower than for the native compound, the final $\Delta\psi$-dependent uptake levels are comparable to those obtained for dibucaine at low pH values where the drug is fully protonated [pH 5.5; see Mayer et al. (1985a)]. It should be noted that although it is normally viewed that local anesthetics only permeate through membranes in the neutral form (Hille, 1977a,b, 1980; Cahalan et al., 1980), the $\Delta\psi$-dependent uptake of dibucaine reflects a much slower accumulation process than is observed for uptake in response to ΔpH [1.1×10^{-3} nmol/(min·cm^2) as compared to 3.0×10^{-2} nmol/(min·cm^2)]. Thus, uptake of the protonated form may be sufficiently slow to escape detection in the previous studies. Further, the proposal that protonated weak bases can permeate through membranes is not novel. Studies by Boron and co-workers (Boron & De Weer, 1976; Boron & Roos, 1976; Boron, 1983) demonstrate that while squid axons are far more permeable to NH_3 than NH_4^+, influx of NH_4^+ does occur, resulting in intracellular acidification. Similar behavior has been observed for methylammonium (Boron & Roose, 1976), and more pertinent to this study, McLaughlin (1975) has shown that tetracaine can permeate membranes in the HA_2^+ form.

Difficulties with a simple HA^+/K^+ antiport mechanism include the fact that if a protonated form of dibucaine is accumulated in response to $\Delta\psi$, it would be expected that dibucaine could act as a proton ionophore, facilitating the inward flux of protons in the presence of $\Delta\psi$ (inside negative). There is some evidence for this [see Figure 5 and McLaughlin (1975)] as the interior pH observed in the presence of $\Delta\psi$ falls more quickly in the presence of dibucaine to lower equilibrium values, but other studies do not indicate that local anesthetics exhibit proton ionophore capacities (Garlid & Nakashima, 1983). It should also be noted that uptake of the protonated form in response to $\Delta\psi$ does not remove the problems concerning the form of the accumulated dibucaine. Specifically, as noted above, the lack of equivalence between amine uptake and K^+ release, which implies accumulation of a proportion of the neutral form of the drug, again suggests that accumulated drug exists in an alternate phase or precipitate by the reasoning presented above, even if a proportion of the drug is accumulated in the charged form. In addition, it may be expected that a certain fraction of the dibucaine will also be associated with the membrane.

To summarize, dibucaine can be rapidly accumulated into LUVs exhibiting ion gradients resulting in pH gradients (inside acidic) and membrane potentials (inside negative). In the case of uptake in response to pH gradients, the uptake characteristics are consistent with transport of the neutral form, resulting

in concentration gradients across the membrane which reflect the transmembrane proton gradients. The uptake of dibucaine in response to $\Delta\psi$ represents a more complicated process which could involve transport of a charged species of dibucaine or the neutral form. Both mechanisms appear to require formation of a precipitate in the vesicle interior and/or accumulation in the inner monolayer in order to account for the results presented here.

The results of this study have implications for the biodistributions of local anesthetics and other lipophilic amino-containing drugs and direct applications for loading liposomal systems for drug delivery applications. First, whatever the mechanism associated with the $\Delta\psi$-dependent uptake process, larger equilibrium concentrations of dibucaine (and presumably other lipophilic amines) would be expected at the inner monolayer of membranes exhibiting a $\Delta\psi$ than would be expected on the basis of their lipid–water partitioning behavior. This presumably extends to the nerve membrane and, as emphasized elsewhere (Mayer et al., 1985a), could result in the membrane perturbations leading to inhibition of the action potential. Second, the ΔpH uptake results would clearly suggest that organelles maintaining an acidic interior, such as secretory vesicles and lysosomes, would preferentially accumulate a large proportion of available drug in vivo (Casey et al., 1977).

With respect to liposomal drug delivery applications, the loading of lipophilic cationic drugs into liposomes in response to ion gradients has obvious utility. It should be noted, however, that ΔpH-dependent loading rather than loading in response to valinomycin-induced K^+ diffusion potentials would be the preferred procedure, due to the potentially toxic effects of valinomycin required for the latter case. As documented here, such ΔpH-dependent loading can result in extremely high entrapped dibucaine levels (up to 190 nmol/μmol of lipid) in conjunction with excellent trapping efficiencies (in excess of 90%). The additional advantage that the drug can be loaded into preformed liposomes immediately prior to use avoids many of the stability problems inherent in liposome–drug formulations. Such procedures may be of use in many applications including the development of long-lived liposomal anesthetics [see Djordjevich et al. (1986)] and liposomal formulations of anticancer drugs such as doxorubicin (Mayer et al., 1986a).

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REFERENCES

Bally, M. B., Hope, M. J., van Echteld, C. J. A., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 66.

Bally, M. B., Mayer, L. D., Loughrey, H. Redelmeier, T., Madden, T. D., Wong, K., Hope, M. J., & Cullis, P. R. (1988) *Chem. Phys. Lipids* (in press).

Boggs, J. M., Young, T., & Hsia, J. C. (1976) *Mol. Pharmacol. 12*, 127.

Boron, W. F. (1983) *J. Membr. Biol. 72*, 1.

Boron, W. F., & De Weer, P. (1976) *J. Gen. Physiol. 67*, 91.

Boron, W. F., & Roos, A. (1976) *Am. J. Physiol. 231*, 799.

Butler, K. W., Schneider, H., & Smith, I. C. P. (1973) *Arch. Biochem. Biophys. 154*, 548.

Cafiso, D. S., & Hubbell, W. L. (1983) *Biophys. J. 44*, 49.

Cahalan, M., Shapiro, B. I., & Almers, W. (1980) in *Molecular Mechanisms of Anesthesia* (Finley, B., Ed.) Vol. II, pp 17–33, Raven, New York.

Casey, R. P., Njus, D., Radda, G. K., & Sher, P. A. (1977) *Biochemistry 16*, 972.

Cullis, P. R., Hope, M. J., Bally, M. B., Madden, T. D., Mayer, L. D., & Janoff, A. S. (1977) in *Liposomes. From Biophysics to Therapeutics* (Ostro, M. J., Ed.) pp 39–71, Marcel Dekker, New York.

Djordievich, L., Ivankovich, A. D., Chigurupati, R., Woronowicz, A., & McCarthy, R. (1986) *Anesthesiology 63*, A185.

Fisk, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375.

Frezatti, W. A., Toselli, W. R., & Schreier, S. (1986) *Biochim. Biophys. Acta 860*, 531.

Garcia, M. L., Kitada, M., Eisenstein, H. C., & Kurlwich, T. A. (1984) *Biochim. Biophys. Acta 766*, 109.

Garlid, K. D., & Nakashima, R. A. (1983) *J. Biol. Chem. 258*, 7974.

Hille, B. (1977a) *J. Gen. Physiol. 69*, 475.

Hille, B. (1977b) *J. Gen. Physiol. 69*, 497.

Hille, B. (1980) in *Molecular Mechanisms of Anesthesia* (Fink, B., Ed.) Vol. II, pp 1–5, Raven, New York.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55.

Lichtstein, D., Kaback, H. R., & Blume, A. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 650.

Mayer, L. D., Bally, M. B., Hope, M. J., & Cullis, P. R. (1985a) *J. Biol. Chem. 260*, 802.

Mayer, L. D., Bally, M. B., Hope, M. J., & Cullis, P. R. (1985b) *Biochim. Biophys. Acta 816*, 294.

Mayer, L. D., Hope, M. J., Cullis, P. R., & Janoff, A. S. (1985c) *Biochim. Biophys. Acta 817*, 193.

Mayer, L. D., Bally, M. B., & Cullis, P. R. (1986a) *Biochim. Biophys. Acta 857*, 123.

Mayer, L. D., Bally, M. B., Hope, M. J., & Cullis, P. R. (1986b) *Chem. Phys. Lipids 40*, 333.

McLaughlin, S. (1975) in *Molecular Mechanism of Anesthesia* (Fink, B. R., Ed.) Vol. II, pp 193–221, Raven, New York.

Nicholls, D. G. (1979) *Biochim. Biophys. Acta 549*, 1.

Nicholls, D. G., & Deamer, D. W. (1976) *Biochim. Biophys. Acta 455*, 269.

Ritchie, J. M., & Greengard, P. (1961) *J. Pharmacol. Exp. Ther. 133*, 241.

Sokol, P. P., Holohan, P. D., & Ross, C. R. (1985) *J. Pharmacol. Exp. Ther. 233*, 694.

Waggoner, A. S. (1979) *Methods Enzymol. 55*, 689.

Westman, J., Boulanger, Y., Ehrenberg, A., & Smith, I. C. P. (1982) *Biochim. Biophys. Acta 685*, 315.

Yeagle, P. L., Hutton, W. C., & Martin, R. B. (1977) *Biochim. Biophys. Acta 465*, 173.