THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 2, Issue of January 25, pp. 802-808, 1985
Printed in U.S.A.

Uptake of Dibucaine into Large Unilamellar Vesicles in Response to a Membrane Potential*

(Received for publication, July 2, 1984)

Lawrence D. Mayer‡, Marcel B. Bally, Michael J. Hope, and Pieter R. Cullis§

From the Biochemistry Department, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

Local amine anesthetics appear to exert their effects in the charged (protonated) form on the cytoplasmic side of excitable membranes. Two features of interest are the mechanism whereby these drugs move across the membrane to the inner monolayer and the actual membrane concentrations achieved. In this work, we have investigated the influence of a K^+ diffusion potential, $\Delta\Psi$, on the transmembrane distribution and concentration of the local anesthetic dibucaine employing large unilamellar vesicle systems. It is demonstrated that egg phosphatidylcholine large unilamellar vesicles exhibiting a $\Delta\Psi$ (interior negative) actively accumulate dibucaine to achieve high interior concentrations. ^{31}P and ^{13}C nuclear magnetic resonance studies show that the internalized drug is localized to the vesicle inner monolayer, and suggest that the protonated form of the anesthetic is the species that is actively transported. The inner monolayer anesthetic concentrations thus achieved can be an order of magnitude or more larger than predicted on the basis of anesthetic lipid-water partition coefficients. It is suggested that these effects may be related to the mechanisms whereby local anesthetics are localized and concentrated at their sites of action in nerve membranes.

It is generally accepted that local amine anesthetics exert their effects when present in a charged (protonated) form on the cytoplasmic side of excitable membranes (1–8). Furthermore, the relative potencies of local anesthetics correlate strongly with their solubility in a lipid environment (9, 10). Taken together, these observations suggest that the anesthetic effects of these drugs involves a perturbation of the properties of the inner monolayer lipid environment. This perturbation may be related to changes in physical properties such as fluidity, permeability, or surface potential observed when local anesthetics partition into model membrane systems (11–15).

Two difficulties associated with such models are the amounts of the drug that partition into the inner monolayer and the mechanism whereby the anesthetic moves to this location. In particular, the concentrations of local anesthetic required to significantly perturb the physical properties of lipid systems are often 10-fold greater than the concentrations required to induce local anesthesia (14, 16–19). With regard to the transbilayer movement of the drug, available evidence suggests that the lipid bilayer is relatively impermeable to charged local anesthetics (20). As a result it is generally assumed that the drug permeates through the membrane in the neutral (uncharged) form, and is reprotonated on reaching the inner monolayer. This may be so, but the pK values of many local anesthetics are significantly higher than the physiological pH. Chlorpromazine, for example, has a pK of 9.3 (21) and thus at physiological pH the vast majority (>99%) would exist in the protonated, presumably impermeable, form.

In this work we have approached these problems from a point of view engendered by studies with the lipophilic cation safranin (22) which exists exclusively in a positively charged form (23). These investigations demonstrated that safranin can be rapidly sequestered into model membrane systems exhibiting a membrane potential (inside negative). As protonated, local anesthetics are essentially lipophilic cations, the possibility exists that uptake of the positively charged drug could proceed by a similar mechanism. We demonstrate here that this is indeed the case for dibucaine, a representative local anesthetic. In particular, the presence of a K^+ diffusion potential results in the efficient uptake of dibucaine into the inner monolayer of large unilamellar vesicle systems in a manner consistent with transport of the protonated species of the anesthetic. Furthermore, this uptake results in inner monolayer drug concentrations which are significantly higher than would be expected on the basis of classical partitioning properties.

MATERIALS AND METHODS

Dibucaine hydrochloride, valinomycin, and all buffers were purchased from Sigma. Tritiated dipalmitoylphosphatidylcholine, [^{14}C] inulin, and [^3H]methyltriphenylphosphonium were purchased from New England Nuclear. Egg phosphatidylcholine was purified by standard procedures and was greater than 99% pure as detected by thin-layer chromatography.

Preparation of Large Unilamellar Vesicles—Large unilamellar vesicles (containing 0.04 μCi of [^3H]dipalmitoylphosphatidylcholine/μmol of phospholipid) were produced by extrusion of a lipid dispersion through a 0.1-μm polycarbonate filter as previously described, including freeze-thaw steps (24). Vesicles prepared in this manner had trapped volumes of 1.5 ± 0.1 μl/μmol phospholipid employing [^{14}C] inulin as an aqueous marker. Phospholipid concentrations and specific activities were determined by analysis of lipid phosphorus as described previously (25).

Uptake of Dibucaine into Vesicles—Transmembrane Na^+/K^+ gradients were created by preparing LUV's[1] in the presence of 169 mM potassium glutamate, 20 mM Hepes, pH 7.5. These were then passed over a Sephadex G-50 gel filtration column equilibrated in the appropriate Na^+ buffer of equal osmolarity. 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

* This work was supported by the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Medical Research Council Postdoctoral Fellow.
§ Medical Research Council Scientist.

[1] The abbreviations used are: LUV, large unilamellar vesicle; EPC, egg phosphatidylcholine; MES, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ethanol) was added to achieve levels of 1 μg/μmol phospholipid. Quantitation of vesicle-associated dibucaine following these incubations was accomplished by passing 0.1-ml aliquots of the sample over a 1-ml Sephadex G-50 column (see Ref. 22) to separate free from vesicle-associated anesthetic. Half of the eluant was assayed for ^3H to determine the amount of phospholipid. The other half was brought to 1 ml with 0.2% Triton X-100 and the dibucaine was assayed by monitoring the fluorescence at 410 nm. Standard dibucaine solutions containing detergent and similar amounts of lipid were employed to calibrate the fluorescence intensity with actual amounts of the anesthetic present. Kinetics of dibucaine uptake were monitored over 2 h. Greater than 90% uptake was obtained after 1 h under all conditions (see "Results"). Where the incubation times are not indicated the values represent accumulations obtained for 2-h incubations.

NMR Investigations—The ^{13}C and ^{31}P NMR spectra were obtained employing a Bruker WP-200 spectrometer operating at 50.3 and 80.5 MHz, respectively. A free induction decay corresponding to 16,000 transients was obtained for ^{13}C spectra using a 15-μs 90°-radio frequency pulse, a 2-s interpulse delay, and a 10-kHz sweep width in the presence of broadband ^1H decoupling (power setting 20 db). An exponential multiplication corresponding to 5 Hz was applied to the free induction decay prior to Fourier transformation. The ^{13}C assignments of the dibucaine resonances were made on the basis of observed multiplet structure in the absence of nuclear Overhauser enhancement effects employing inverse gated ^1H decoupling and comparison with previously assigned ^{13}C NMR spectra (20).

The ^{31}P spectra were obtained by accumulating a free induction decay corresponding to 500 transients using a 10-μs 90°-pulse, a 4-s interpulse delay, and a 20-kHz sweep width in the presence of broadband ^1H decoupling (power setting 14 db). Relative intensities of ^{31}P NMR spectra were determined as previously described (24).

RESULTS

ΔΨ-Dependent Uptake of Dibucaine into Vesicles—Previous work from this laboratory has shown that LUV systems exhibiting a K^+ diffusion potential (inside negative) can accumulate lipophilic cations such as safranin and methyltriphenylphosphonium leading to high interior concentrations of the accumulated agent (22). The K^+ diffusion potential is readily established by trapping a K^+ buffer in the LUV interior and then resuspending the LUV's in a Na^+ buffer to which the K^+ ionophore valinomycin is added subsequently.

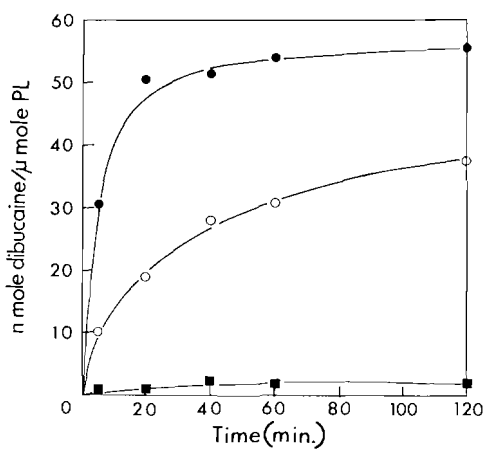

FIG. 1. **Time course of dibucaine uptake by LUV systems.** Egg phosphatidylcholine vesicles (1 mM phospholipid) were incubated in the presence of 0.1 mM dibucaine. Vesicle preparation and quantitation of vesicle-associated dibucaine was accomplished as described under "Materials and Methods." Conditions used were: ●, 169 mM potassium glutamate, 20 mM Hepes, pH 7.5, and 150 mM NaCl, 20 mM Hepes, pH 7.5, inside and outside the vesicles, respectively, in the presence of valinomycin (1 μg/μmol phospholipid); ○, 169 mM potassium glutamate, 20 mM Hepes, pH 7.5, and 150 mM NaCl, 20 mM Hepes, pH 7.5, inside and outside the vesicles, respectively, in the absence of valinomycin; ■, 169 mM potassium glutamate, 20 mM Hepes, pH 7.5, inside and outside the vesicles in the presence of valinomycin (1 μg/μmol of phospholipid).

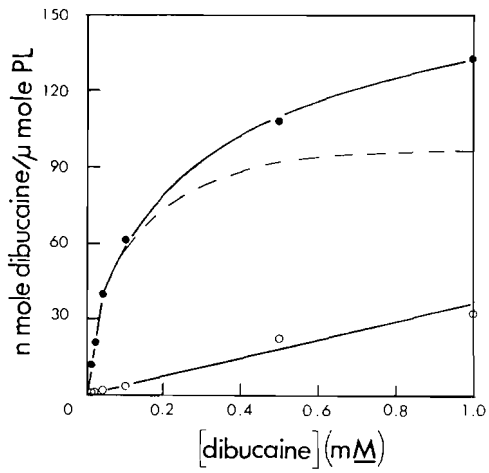

FIG. 2. **Uptake of dibucaine by EPC LUV's in the presence (●) and absence (○) of a membrane potential as a function of anesthetic concentration.** The LUV's (1 mM phospholipid) contained 169 mM potassium glutamate, 20 mM Hepes, pH 7.5, and the same buffer was employed in the external medium for the situation where $\Delta\Psi = 0$. To generate a K^+ diffusion potential, the external buffer contained 150 mM NaCl, 20 mM Hepes, pH 7.5, which yielded a $\Delta\Psi$ of −172 mV as detected employing [^3H]methyltriphenylphosphonium in the presence of valinomycin (1 μg/μmol phospholipid). The *dashed line* represents net $\Delta\Psi$ dependent uptake ($\Delta\Psi$-dependent minus $\Delta\Psi$-independent uptake).

This leads to values of $\Delta\Psi$ in the range −100 to −170 mV. Subsequent incubation of these LUV's with safranin, for example, results in accumulation of the cation to achieve interior concentrations in excess of 100 mM for initial exterior safranin concentrations of 2 mM (22).

Our initial experiments were aimed at establishing whether similar $\Delta\Psi$-dependent uptake processes can occur with dibucaine. Two LUV systems were employed, the first exhibiting a transmembrane electrochemical Na^+/K^+ gradient (K^+ inside) whereas the second did not (K^+ buffer inside and outside). These vesicle systems (1 mM phospholipid) were incubated in the presence of 0.1 mM dibucaine (±valinomycin) and LUV-associated dibucaine determined by a fluorescence assay (see "Materials and Methods") following gel filtration of the vesicles to remove free drug. As shown in Fig. 1, the LUV systems experiencing Na^+/K^+ chemical gradients exhibit an ability to accumulate dibucaine to high levels (~55 nmol of dibucaine/μmol of phospholipid) while LUV systems with the K^+ buffer inside and out exhibited much lower levels (<2 nmol of dibucaine/μmol of phospholipid). The presence of valinomycin is observed to increase the rate of uptake, but significant uptake is also obtained in the absence of ionophore. The latter process may be attributed to uptake of dibucaine in response to the $\Delta\Psi$ established by the passive efflux of K^+ (22) as well as altered K^+ permeabilities due to the presence of the anesthetic itself (26). Levels of vesicle-associated dibucaine in the absence of $\Delta\Psi$ were unaffected by valinomycin.

The data of Fig. 1 indicate that after 2 h, 55 nmol of dibucaine/μmol of phospholipid was accumulated by the vesicles exhibiting a valinomycin induced potential, corresponding to 55% of the available dibucaine. At lower dibucaine concentrations this trapping efficiency can be considerably higher as indicated in Fig. 2, which illustrates uptake levels (after 2 h) obtained as the initial (exterior) dibucaine concentrations are increased over the range 0–1 mM. For initial concentrations of 0.01, 0.02, and 0.04 mM the measured levels of LUV-associated dibucaine were 9.5, 21, and 41 nmol/μmol of phospholipid, respectively, indicating essentially complete

sequestration of the exterior dibucaine into the vesicles. The lipid-water partition coefficient of dibucaine for phosphatidylcholine vesicles is approximately 300 (13), indicating that for our experimental system approximately 25% of the dibucaine should partition into the LUV bilayers. Thus the presence of $\Delta\Psi$ results in a large increase in the effective lipid-water partition coefficient for dibucaine. This was also indicated by fluorescence measurements (results not shown) on a system containing 0.05 mM dibucaine, where the fluorescence polarization increased from 0.014 for the free drug to 0.04 and 0.168 in the presence of EPC LUV's (1 mM phospholipid) in the absence and presence of $\Delta\Psi$, respectively. These results suggest that the aromatic moiety of the accumulated dibucaine was buried in the lipid bilayer and indicated effective lipid-water partition coefficients in excess of 5000 for the systems exhibiting a potential under the conditions used.

At dibucaine concentrations above 0.04 mM the ability of the LUV systems to accumulate dibucaine in a $\Delta\Psi$-dependent manner becomes progressively saturated, leveling off at 95 nmol of dibucaine/μmol of phospholipid after subtraction of $\Delta\Psi$ independent uptake (*dashed line*, Fig. 2). The reason for this saturation is not understood in detail, but may be related to the surface potential effects noted under "Discussion."

Influence of $\Delta\Psi$ on Transbilayer Distributions of Dibucaine—The results to this stage show that the presence of a membrane potential results in a markedly enhanced association of dibucaine with LUV systems. Based on similar responses of other lipophilic cations to a membrane potential (22) we interpret this association as reflecting accumulation into the vesicle interior. In order to place this interpretation on a sound basis two experiments were performed. First, the paramagnetic cation Mn^{2+} can be employed as a "broadening" agent in ^{31}P NMR studies of phospholipid systems, effectively quenching the signal of available phospholipids. Local anesthetics can displace such paramagnetic ions from the lipid-water interface (20). Thus if LUV systems are prepared containing Mn^{2+}, and are subsequently incubated in the presence of dibucaine, the movement of dibucaine to the inner monolayer should be reflected by an increase in the intensity of the phospholipid ^{31}P NMR resonance. Thus, LUV's were prepared

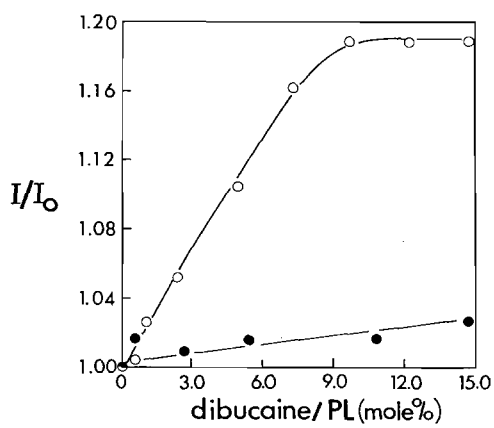

FIG. 3. **Influence of accumulated dibucaine on the ^{31}P NMR signal intensity arising from EPC LUV's containing entrapped Mn^{2+}.** The ratio I/I_o refers to the observed signal intensity I divided by the signal intensity I_o obtained prior to addition of dibucaine. The buffer in the LUV interior contained 20 mM Hepes, 169 mM potassium glutamate, pH 7.5, plus 0.5 mM $MnCl_2$. Exterior media in the presence and absence of a membrane potential were 150 mM NaCl, 1 mM EDTA, 20 mM Hepes, pH 7.5, (○) and 169 mM potassium glutamate, 1 mM EDTA, 20 mM Hepes, pH 7.5, (●), respectively. The phospholipid (*PL*) concentration was 25 mM for all samples. Valinomycin was added to achieve 1 μg/μmol phospholipid. For other details, see "Materials and Methods."

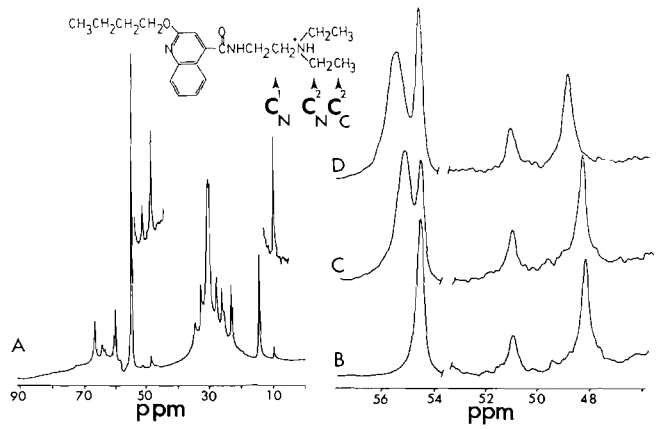

FIG. 4. **Natural abundance ^{13}C NMR spectra at 30 °C.** *a*, EPC LUV's (200 mM phospholipid) in the presence of dibucaine (20 mM), where the interior of the vesicles contained 50 mM K_2HPO_4, 225 mM K_2SO_4 and the exterior environment was composed of 50 mM Na_2HPO_4 and 225 mM Na_2SO_4. Valinomycin (100 μg) was added to develop a membrane potential. The *insets* represent vertical expansions (8 ×) of the spectral regions containing the dibucaine C^2_N *N*-methylene resonance (48.3 ppm) and the C^1_N *N*-methylene resonance (51.1 ppm) as well as the dibucaine tertiary amine terminal methyl resonance (9.8 ppm). *b* same as *a* displaying the expanded region between 46 and 58 ppm, where the interval between 46 and 53.5 ppm containing the dibucaine *N*-methyl and methylene resonances has been vertically expanded 16 × with respect to the spectral region containing the EPC choline resonance (54.5 ppm). *c*, EPC LUV's in the presence of 20 mM dibucaine, where the LUV interior contained 200 mM $K_3Fe(CN)_6$, 20 mM K_2HPO_4, pH 7.5, and the isoosmotic exterior aqueous buffer contained 50 mM K_2HPO_4, 225 mM K_2SO_4, pH 7.5. The spectral region between 46 and 53.5 ppm is expanded vertically by a factor of 4 with respect to the spectral region containing the EPC choline resonance. *d*, same as *c* but where the external buffer contained 225 mM Na_2SO_4, 50 mM Na_2HPO_4, pH 7.5, and valinomycin (100 μg).

in the presence of the potassium glutamate buffer containing 0.5 mM $MnCl_2$, and the exterior Mn^{2+} removed by passing the LUV's over a Sephadex G-50 column equilibrated in the appropriate buffer. The ^{31}P NMR signal intensity was then monitored as a function of externally added dibucaine, and as shown in Fig. 3 the LUV systems prepared with a Na^+/K^+ transmembrane electrochemical gradient (in the presence of valinomycin) exhibited a marked increase in signal intensity (to a maximum of ~20%) in comparison to LUV systems where a K^+ diffusion potential was not present. This maximum signal recovery was achieved at a dibucaine/phospholipid ratio of 0.09, comparable to the saturation level observed for drug uptake (Fig. 2). These observations provide strong indirect evidence for a $\Delta\Psi$-dependent movement of dibucaine to the inner monolayer.

More direct indications of the transmembrane distribution of accumulated dibucaine were obtained employing natural abundance ^{13}C NMR in conjunction with the anionic shift reagent ferricyanide ($Fe(CN)^{3-}_6$). As shown in Fig. 4: (*A*) the two *N*-methylene carbons (C^2_N) and the terminal methyl (C^2_C) groups of dibucaine give clearly resolved resonances (48.3 and 9.8 ppm, respectively) as does the *N*-methylene carbon (C^1_N) on the aromatic ring side of the tertiary amine (51.1 ppm). The C^2_N and C^1_N resonances lie adjacent to the EPC choline resonance at 54.5 ppm, and the spectral region containing these resonances (between 46 and 58 ppm) is shown in expanded form in Fig. 4*B*. Fig. 4*C* illustrates that incorporation of the anionic shift reagent $Fe(CN)^{3-}_6$ (K^+ salt) in the LUV interior, in the absence of a $\Delta\Psi$, results in a downfield shift of the choline methyl signal arising from EPC on the LUV interior, but has little or no influence on the chemical shift of

the C^2_N or C^1_N methylene resonances of externally added dibucaine. In contrast, in the presence of a membrane potential (≥180 mV as detected employing methyltriphenylphosphonium) the C^2_N resonance of externally added dibucaine is shifted downfield by 0.7 ppm (Fig. 4*D*) indicating a location in the vesicle interior. It may also be observed that the interior EPC choline methyl resonance is further shifted downfield, which is consistent with the presence of internalized dibucaine at the inner monolayer-H_2O interface. This would produce a positive surface potential and result in a high local concentration of the anionic ($Fe(CN)^{3-}_6$) shift reagent. It is of interest to note that neither the C^1_N nor the C^2_C dibucaine resonances was shifted under these conditions. The addition of ferricyanide solely to the external medium resulted in a significant shift of the dibucaine C^2_N resonance only in the absence of a $\Delta\Psi$ (data not shown).

The preceding NMR results demonstrate that the $\Delta\Psi$-dependent association of dibucaine with the LUV systems reflects a transbilayer movement of the drug to the inside of the vesicle. Due to the small interior aqueous volume of the LUV systems and the high lipid-water partition coefficient of dibucaine, it would be expected that the vast majority of this internalized dibucaine would reside in the inner monolayer. This is supported by three observations, the first of which concerns the large increase in fluorescence polarization of the drug on accumulation noted previously. Second, the ^{13}C NMR resonances of the aromatic and alkyl chain carbons of dibucaine were broadened beyond resolution on accumulation (data not shown). Finally, if the dibucaine partitions into the inner monolayer rather than the internal aqueous volume, the amount of dibucaine accumulated would be expected to be proportional to the amount of inner monolayer phospholipid present rather than the internal volume. That this was the case was indicated by the observation that variations in the vesicle trapped volume from 0.8 to 2.1 μl/μmol phospholipid did not affect dibucaine uptake as calculated per micromole of phospholipid present (data not shown).

Effect of pH on $\Delta\Psi$-Dependent Dibucaine Uptake—Previous work employing safranin indicated that the lipophilic cation is accumulated in the positively charged form in response to $\Delta\Psi$ (22). If the $\Delta\Psi$-dependent uptake of dibucaine proceeds via a similar mechanism, such accumulation should be sensitive to the pH of the aqueous medium, particularly in the region of the pK of the dibucaine amino group. This pK is not known precisely, as the free form of dibucaine exhibits a pK of 8.5 (21) which can decrease to as low as 7.5 for the membrane-associated form (27, 28). In any event, the extent of $\Delta\Psi$-dependent dibucaine uptake is markedly sensitive to pH as indicated in Fig. 5. The uptake levels are maximum in the region of pH 7.5 and decrease markedly at higher pH values. This decrease presumably reflects the effects of dibucaine deprotonation, and could arise if the neutral (deprotonated) form of the drug was readily removed by the gel filtration procedure. In addition, it is possible that the high membrane partition coefficients of unprotonated dibucaine (28) results in such high membrane anesthetic concentrations that the membrane permeability is increased to the extent that the potential is collapsed.

Decreasing the pH below 7.5 resulted in decreased $\Delta\Psi$-dependent dibucaine uptake to a plateau value of ~20 nmol of dibucaine/μmol of phospholipid at pH 6.0 and below (Fig. 5). Assuming a pK of 7.5, more than 95% of the dibucaine was protonated (positively charged) at pH 6.0 and below. The uptake kinetics at pH 5.5 were similar to those at pH 7.5, providing suggestive evidence that the protonated form of the drug is actively accumulated in response to $\Delta\Psi$.

In order to examine more definitively the form of dibucaine that is actively accumulated in response to the membrane potential, the internal pH of the LUV systems was monitored during the uptake process. The results of Fig. 5 suggest that the membrane-associated form of dibucaine is partially deprotonated at pH 7.5. Thus, if only the protonated form of the drug were actively accumulated, it would be expected that after it is transported into the inner monolayer, it would again assume the partially protonated form, resulting in a net acidification of the vesicle interior. Alternatively, if the neutral form is transported, this should result in an increase in the interior pH as the drug is protonated on arrival in the inner monolayer.

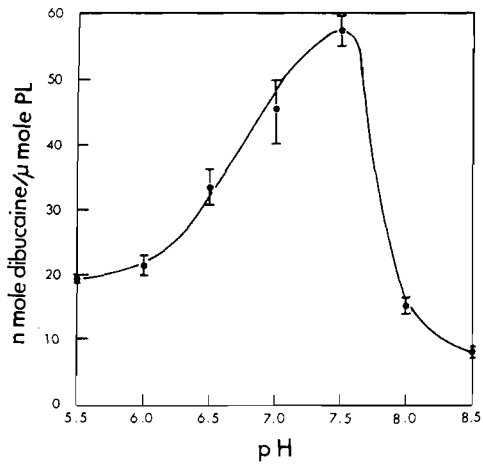

FIG. 5. **Effect of pH on dibucaine uptake into EPC LUV's exhibiting a membrane potential.** Vesicle preparation and quantitation of vesicle-associated dibucaine was accomplished as described under "Materials and Methods." Samples contained 1.0 mM phospholipid, valinomycin (1 μg/μmol phospholipid), and 100 μM dibucaine. The internal aqueous space of the LUV's contained 20 mM of the buffer and 169 mM potassium glutamate whereas the external media contained 20 mM of the buffer and 150 mM NaCl. The buffers employed were: MES, pH 5.5–6.5; Hepes, pH 7.0–7.5; Tris, pH 8.0–8.5. *Error bars* represent the standard deviation determined from three experimental points.

As noted elsewhere (29), the ^{31}P NMR chemical shift of inorganic phosphate is sensitive to pH in the region 5.5–8.5. Thus LUV systems were prepared with P_i trapped in the vesicle interior, and the chemical shift of the P_i resonance monitored on incubation of the vesicles with dibucaine, in the presence and absence of a membrane potential. As indicated in Fig. 6, the systems with a K^+ diffusion potential exhibited a 0.45 ppm upfield shift (corresponding to a decrease in the internal pH from 7.5 to approximately 6.8) of the P_i resonance after 90 min. No such effects were observed in the absence of a K^+ electrochemical gradient (Fig. 6*B*). Also, the P_i resonance upfield shift in the presence of $\Delta\Psi$ but in the absence of dibucaine was 0.06 ppm after 90 min (data not shown). The broadening of the P_i resonance at intermediate times (Fig. 6*A*) can be attributed to a distribution of vesicle sizes which would result in a corresponding distribution of internal pH values and P_i chemical shifts for a given proton uptake into each vesicle. The bottom spectra in Fig. 6*A* represents the subtraction of the spectra obtained at 90 min from that obtained before dibucaine addition (0 min) and vividly illustrates the upfield shift of the internal P_i resonance. In addition, the kinetics of the P_i resonance shift correlate closely with the kinetics of dibucaine uptake as shown in Fig. 1. This suggests that the acidification of the vesicle interior occurs concomitant with transport of dibucaine into the LUV inner monolayer. These observations are consistent with active uptake of the protonated species of dibucaine.

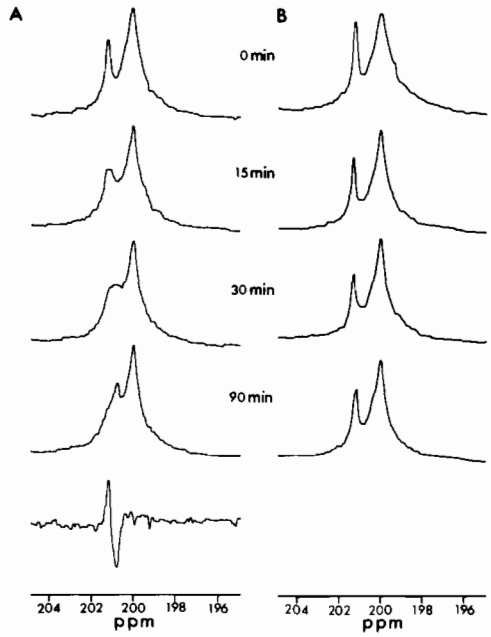

FIG. 6. **Influence of accumulated dibucaine on the ^{31}P NMR chemical shift of P_i entrapped in LUV systems.** The narrow peak in the region of 201 ppm arises from the internal P_i whereas the broader peak centered around 200 ppm arises from the phospholipid phosphate. Egg phosphatidylcholine LUV's were prepared in the presence of 200 mM potassium phosphate, pH 7.5. Exterior buffer was exchanged for 20 mM Hepes, 175 mM, Na_2SO_4, pH 7.5 (*column A*), or 20 mM Hepes, 175 mM K_2SO_4, pH 7.5 (*column B*). Valinomycin was incorporated into both samples by drying down 100 μl of a 1 mg/ml ethanol solution of valinomycin in a glass test tube, adding 4 ml of 25 mM egg phosphatidylcholine vesicles and vortexing the sample for 10 min. The zero time spectrum was obtained prior to dibucaine addition. After addition of dibucaine to achieve a concentration of 5 mM, sequential spectra were collected (200 transients, interpulse delay 2 s, total accumulation time 7.5 min). The *bottom spectra* in *column A* represents the subtraction of the spectra collected 90 min after dibucaine addition from that obtained prior to the addition of dibucaine.

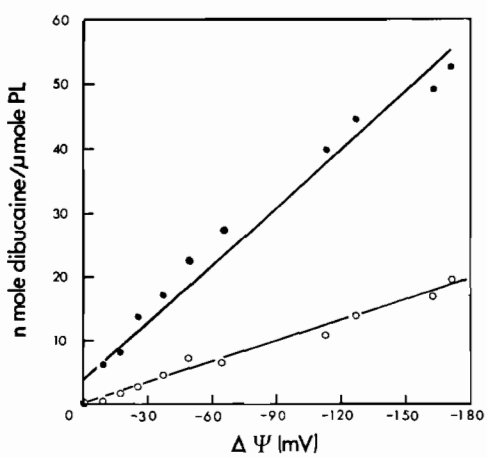

FIG. 7. **Dibucaine uptake into EPC LUV's as a function of the applied membrane potential.** Transmembrane Na^+/K^+ chemical gradients yielding the indicated ΔΨ values were created as described under "Materials and Methods." Hepes and MES (20 mM) were used as the internal and external buffers for vesicle systems at pH 7.5 (●) and 5.5 (○), respectively. Phospholipid and dibucaine were determined as described under "Materials and Methods." All samples contained 1.0 mM phospholipid, valinomycin (1 μg/μmol phospholipid), and 100 μM dibucaine. The indicated values represent active anesthetic uptake (after subtraction of ΔΨ-independent uptake).

Dependence of Local Anaesthetic Uptake on ΔΨ Magnitude—It is of interest to consider why the amount of dibucaine accumulated should be increased by a factor of two or more at pH values in the region of the pK of the anesthetic, pH 7.5, as compared to the pH values where the drug is fully protonated. One possibility is that the ΔΨ-dependent accumulation proceeds to achieve a fixed, ΔΨ-dependent charge density in the inner monolayer. This proposal, which is developed more fully under "Discussion," would predict that accumulated drug levels for pH values equal or greater to the dibucaine pK should be a factor of two or more larger than when the drug is fully charged, which is consistent with the results of Fig. 5. In addition, this theory (see "Discussion") would predict that at a given pH the amount of uptake should depend in a linear fashion on the applied potential. This was tested by preparing LUV systems with a variety of K^+ transmembrane electrochemical gradients corresponding to ΔΨ values in the range 0 to −172 mV, and monitoring the uptake observed. As shown in Fig. 7, levels of dibucaine uptake observed do indeed vary linearly with the applied potential both at pH 5.5 and 7.5.

DISCUSSION

The results presented in this work provide new insight into the interaction of local anesthetics with membrane systems which may be directly related to their mechanisms of action *in vivo*. There are three points of interest: the ΔΨ-dependent localization of dibucaine to the inner monolayer of LUV systems, the amount of dibucaine which is accumulated and the mechanism whereby this accumulation proceeds.

The results demonstrating that dibucaine is accumulated to the inner monolayer of LUV systems with a K^+ diffusion potential is consistent with studies indicating a biological site of action on the cytoplasmic side of nerve membranes (1–4). Such membranes exhibit potentials in excess of −70 mV (30), and thus an accumulation process similar to that observed here for LUV systems would be expected to occur. Furthermore, other considerations would suggest that nerve membranes may be able to accumulate dibucaine more efficiently than other plasma membrane systems, resulting in a measure of selectivity. In particular, we have noted elsewhere (22) that factors decreasing membrane fluidity, such as increased cholesterol content or increased acyl chain saturation reduce the rate of ΔΨ-dependent uptake of lipophilic cations. The nerve membrane exhibits lower cholesterol levels and a highly unsaturated lipid composition (31) in comparison to plasma membranes such as that of the erythrocyte, which may be expected to facilitate uptake.

The next area of discussion concerns the amount of local anesthetic sequestered in response to ΔΨ, which can result in markedly enhanced anesthetic concentrations in the inner monolayer over that expected on the basis of oil-water partition coefficients. For example, under our experimental conditions, essentially all of a 0.04 mM dibucaine solution can be sequestered into the inner monolayer of LUV systems, whereas only 12.5% or less could be expected to reside there assuming membrane-water partition coefficients of 300 and equilibrium transbilayer distributions of anesthetic. This suggests that the local anesthetic content of nerve membranes, in the presence of a ΔΨ, at "clinical" anesthetic concentrations (≤0.1 mM dibucaine) may be considerably higher than classical considerations would predict. In this regard it is interesting that such low concentrations of local amine anesthetics do not normally perturb the fluidity or permeability properties of phosphatidylcholine membrane systems significantly (13, 32). Such anesthetic concentrations can, however,

perturb the physical properties of lipid systems composed of acidic phospholipids such as phosphatidylserine (13, 32). This may be attributed to the enhanced lipid-water partition coefficients of local anesthetics for negatively charged phospholipid systems, which can be a factor of 20 larger than for uncharged lipid systems (13). Thus an enhanced $\Delta\Psi$-dependent partitioning of dibucaine into the inner monolayer may be sufficient to produce significant perturbations in the physical properties of the inner monolayer lipid matrix.

The next topic concerns the mechanism whereby dibucaine is translocated across the LUV bilayer. Studies on the effects of local amine anesthetics on sodium conductance of nerve membranes (5, 6) has led to the theory that it is the unprotonated (neutral) form of the drug that permeates the membrane. The results presented here, however, are consistent with a transbilayer movement of the protonated form of dibucaine in response to a K^+ diffusion potential as demonstrated by the decrease in interior pH observed on accumulation of dibucaine and the similar uptake kinetics observed at pH 7.5 and 5.5. It is, however, possible that an H^+/K^+ exchange occurs as neutral dibucaine moves to the vesicle interior. Alternatively, counterions (such as Cl^-) may be associated with the protonated drug during translocation. The possibility that dibucaine permeates the bilayer in the protonated form contradicts current assumptions that the large energy barrier presented by the hydrophobic core of the membrane would prevent passage of charged molecules. However, it must be noted that the electric field associated with a reasonable membrane potential provides a strong driving force encouraging such redistributions. A $\Delta\Psi$ of 100 mV, for example, corresponds to a transmembrane electric field gradient of $\sim 250 \times 10^3$ volts/cm which must be expected to influence the transbilayer distribution of charged lipophilic molecules.

The final topic of this discussion concerns the amount of dibucaine sequestered into the inner monolayer, which is sensitive to the ionization state of dibucaine and the absolute value of the membrane potential. In particular, more dibucaine is accumulated at pH values near the dibucaine pK, as compared to situations where dibucaine is fully protonated (Fig. 5). This implies a relation between the charge density due to internalized dibucaine and $\Delta\Psi$. A simple rationalization of this behavior is that dibucaine accumulation proceeds to a point at which the surface potential at the inner monolayer due to the charged dibucaine is equal and opposite to the K^+ diffusion potential driving the uptake. This admittedly simplified proposal neglects potentially important effects arising from factors such as the molecular packing of the dibucaine in the inner monolayer. However, it is instructive to calculate the surface charge densities such a relation would imply. Writing the inner monolayer surface potential as ϕ_d and the dibucaine charge density as σ_d, application of the Gouy-Chapman theory (33) gives:

$$\sigma_d = (8 \times 10^3\ kT\ c\ \epsilon\epsilon_0)^{1/2}\ sinh\left(\frac{e\phi_d}{2\ kT}\right) \quad (1)$$

where k is Boltzmann's constant, T is the absolute temperature, c is the ionic strength of the aqueous medium, ϵ is the dielectric constant, ϵ_0 is the permittivity of free space, and e is the unit of electron charge. For an aqueous buffer concentration of 150 mM at 20 °C, Equation 1 reduces to $\sigma_d = 4.5\ sinh\ (19.4\ \phi_d)$. For $\phi_d = -\Delta\Psi = 170$ mV this indicates a surface charge density $\sigma_d = 61$ μcoulomb/cm^2, which would require much higher levels of dibucaine uptake than are actually observed. For the fully protonated form of dibucaine (Fig. 7, pH 5.5), for example, a $\Delta\Psi$ of -170 mV gives rise to uptake levels of ~20 nmol of dibucaine/μmol of phospholipid. Assuming this is localized to the inner monolayer, such an uptake level corresponds to a surface charge density of 1.1 μcoulomb/cm^2.

This discrepancy can be removed on consideration of a fundamental assumption of the Gouy-Chapman theory, which is that aqueous ions can approach surface charges infinitely closely. This is not necessarily the case for membrane-associated dibucaine as indicated by the observation that the methylene carbon (C_N^1) immediately below the amino group, as well as the carbons of the amino methyl groups (C_C^2) were not influenced by the $Fe(CN)_6^{3-}$ shift reagent (see Fig. 4 and related text). If the charged amine does lie below the distance of closest approach of aqueous counterions, Stern's modification of the Gouy-Chapman theory must be employed, which holds that aqueous ions can only penetrate to within a distance x_2 of the charged interface. This gives (33)

$$\phi_d = \phi_{d2} + \frac{\sigma_d x_2}{\epsilon\epsilon_0} \quad (2)$$

where ϕ_{d2} is the (Gouy-Chapman) surface potential at $x = x_2$ as calculated from Equation 1. For a surface charge density of 1.1 μcoulomb/cm^2, $\phi_{d2} \simeq 12$ mV. Thus $\phi_d = 12 + 124\ (x_2/\epsilon)$ where x_2 is in angstroms and ϕ_d is in millivolts. Neither x_2 nor the dielectric constant ϵ is known. However, reasonable limits would suggest $0 \leq x_2 \leq 5$ Å whereas ϵ would fall between the dielectric constant appropriate to a hydrocarbon environment ($\epsilon \sim 2$) and that of water ($\epsilon = 80$). In any event, it appears that an x_2/ϵ ratio of 1.3 is possible, which is necessary to achieve the proposed equality $\phi_d = -\Delta\Psi = 170$ mV. It may also be noted that the Stern modification, under our conditions, results in the prediction that ϕ_d is proportional to σ_d (Equation 2), and thus the proposal that dibucaine accumulation proceeds to the point where $\phi_d = -\Delta\Psi$ predicts a linear relation between dibucaine uptake (which is proportional to σ_d) and $\Delta\Psi$ as is observed experimentally (Fig. 7).

In summary, the results presented here suggest that the membrane potential exhibited by excitable membranes could play an important role in local anesthetic action by actively transporting and concentrating the protonated form of the drug at the inner monolayer-water interface. The mechanism whereby this accumulation could result in anesthetic effects remains unknown. However, the fact that resulting inner monolayer anesthetic concentrations may be appreciably higher than predicted on the basis of their lipid-water partitioning behavior implies that perturbations observed in model membrane systems at high drug concentrations may be of more direct relevance than previously thought. In particular, physical properties of the lipid matrix (such as fluidity) may be perturbed, which may result in inhibition of Na^+ channel conductance (34). Other possibilities include the resulting changes in inner-monolayer surface potential which may influence Na^+ and K^+ conductance changes on depolarization, or an inhibition of neurotransmitter binding to postsynaptic membranes due to the presence of accumulated anesthetic. With regard to the latter point, recent studies[2] on a variety of neurotransmitters (dopamine, serotonin, adrenalin) indicate that these agents can also be accumulated into LUV systems via similar $\Delta\Psi$-dependent mechanisms as described here for dibucaine. The possibility therefore exists that local anesthetics such as dibucaine may effectively compete for neurotransmitter "binding" to the postsynaptic membrane.

[2] M. B. Bally, L. D. Mayer, H. Loughrey, M. J. Hope, and P. R. Cullis, manuscript in preparation.

Acknowledgment—We wish to thank Dr. R. Hancock (Microbiology Department, University of British Columbia) for use of his fluorometer.

REFERENCES

1. Narahashi, T., Frazier, D. T., and Moore, J. W. (1972) *J. Neurobiol.* **3,** 267–276
2. Frazier, D. T., Narahashi, T., and Yamada, M. (1970) *J. Pharmacol. Exp. Ther.* **171,** 45–51
3. Strichartz, G. R. (1973) *J. Gen. Physiol.* **62,** 37–57
4. Khodorov, B., Shishkova, L., Paganov, E., and Revenko, S. (1976) *Biochim. Biophys. Acta* **433,** 409–435
5. Hille, B. (1977) *J. Gen. Physiol.* **69,** 475–496
6. Hille, B. (1977) *J. Gen. Physiol.* **69,** 497–515
7. Hille, B. (1980) in *Molecular Mechanisms of Anesthesia* (Fink, B., ed) Vol. II, pp. 1–5, Raven Press, New York
8. Cahalan, M., Shapiro, B. I., and Almers, W. (1980) in *Molecular Mechanisms of Anesthesia* (Fink, B., ed) Vol. II, pp. 17–33, Raven Press, New York
9. Myer, K. H. (1937) *Trans. Faraday Soc.* **33,** 1062–1068
10. Seeman, P. (1972) *Pharmacol. Rev.* **24,** 583–655
11. Ohki, S. (1970) *Biochim. Biophys. Acta* **219,** 18–27
12. Singer, M. (1975) in *Molecular Mechanisms of Anesthesia* (Fink, B., ed) Vol. I, pp. 223–236, Raven Press, New York
13. Papahadjopoulos, D., Jacobson, K., Poste, G., and Shepherd, G. (1975) *Biochim. Biophys. Acta* **394,** 504–519
14. Ondriaš, K., Balgavý, P., Štolc, S., and Horráth, L. I. (1983) *Biochim. Biophys. Acta* **732,** 627–635
15. Schlieper, P., and Steiner, R. (1983) *Chem. Phys. Lipids* **34,** 81–92
16. Boggs, J. M., Young, T., and Hsia, J. C. (1976) *Mol. Pharmacol.* **12,** 127–135
17. Colley, C. M., and Metcalf, J. C. (1972) *FEBS Lett.* **24,** 241–246
18. Butley, K. W., Schneider, H., and Smith, I. C. P. (1983) *Arch. Biochem. Biophys.* **154,** 548–554
19. Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* **265,** 169–186
20. Yeagle, P. L., Hutton, W. C., and Martin, R. B. (1977) *Biochim. Biophys. Acta* **465,** 173–178
21. Lee, A. G. (1978) *Biochim. Biophys. Acta* **514,** 95–104
22. Bally, M. B., Hope, M. J., van Echteld, C. J. A., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** in press
23. Colonna, R., Massari, S., and Azzone, G. F. (1973) *Eur. J. Biochem.* **34,** 57–585
24. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** in press
25. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66,** 375–400
26. McLaughlin, S. (1975) in *Molecular Mechanisms of Anesthesia* (Fink, B., ed) Vol. I, pp. 193–221, Raven Press, New York
27. Rooney, E. K., and Lee, A. G. (1983) *Biochim. Biophys. Acta* **732,** 428–440
28. Schreier, S., Fregatti, W. A., Aranjo, P. S., Chaimovich, H., and Cuccovia, I. M. (1984) *Biochim. Biophys. Acta* **769,** 231–237
29. Hoult, D. I., Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E., and Seeley, P. J. (1974) *Nature (Lond.)* **252,** 285–287
30. Hodgkin, A. L., and Huxley, H. F. (1952) *J. Physiol.* **117,** 500–544
31. Zambiano, F., Cellino, M., and Cenessa-Fisher, M. (1971) *J. Membr. Biol.* **6,** 289–303
32. Davio, S. R., and Low, P. S. (1981) *Biochim. Biophys. Acta* **644,** 157–164
33. Bard, A. J., and Faulkner, L. R. (1980) *Electrochemical Methods,* John Wiley and Sons, New York
34. Lee, A. G. (1976) *Nature (Lond.)* **262,** 545–548