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EFFECTS OF LOCAL ANESTHETICS ON MEMBRANE PROPERTIES

I. CHANGES IN THE FLUIDITY OF PHOSPHOLIPID BILAYERS

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SUMMARY

The effect of the local anesthetic dibucaine on the solid to liquid-crystalline phase transition in phospholipid vesicles was studied by calorimetry and fluorescence polarization. The partition coefficient (> 3000) of dibucaine in the membranes of vesicles prepared from acidic phospholipids was more than 20 times higher than in neutral phospholipid membranes under the same conditions. Calorimetric measurements on vesicles prepared form acidic phospholipids (bovine brain phosphatidylserine; dipalmitoylphosphatidylglycerol) showed that dibucaine ($1 \cdot 10^{-4}$ M) produced a significant reduction in the gel-liquid crystalline transition temperature (T_c). This fluidizing effect of dibucaine on acidic phospholipid membranes was even more marked in the presence of Ca^{2+}. In contrast, dibucaine at the same concentration did not alter the T_c of neutral phospholipids (dipalmitoylphosphatidylcholine). Significant increase in the fluidity of neutral phospholipid membranes occurred only at higher dibucaine concentrations ($2 \cdot 10^{-3}$ M). Measurements of the fluorescence polarization and lifetime of the probe, 1,6-diphenylhexatriene, in acidic phospholipid vesicles revealed that dibucaine ($1 \cdot 10^{-4}$ M) caused an increase in the probe rotation rate indicating an increase in the fluidity of the phospholipid membranes. A good correlation was obtained between fluorescence polarization data on dibucaine-induced changes in membrane fluidity and calorimetric measurements on vesicles of the same type.

INTRODUCTION

Local anesthetics produce a wide variety of effects on biological membranes. In addition to their anesthetic action on excitable membranes [1–3] they have been shown to displace Ca^{2+} from membranes [4–6] to induce membrane expansion [5], to modify the osmotic fragility of erythrocytes [7], to inhibit cell fusion [8] and cell-

Abbreviations: TES, *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene.

to-cell adhesion [9–11] and to impair cell movement [8]. Although it is generally thought that local anesthetics produce such effects by interaction with membranes, the molecular events accompanying anesthetic-induced changes in membrane properties are still largely unknown. Studies on the interaction of local anesthetics with artificial lipid membranes, including monolayers [1, 12], black lipid films [13, 14] and phospholipid vesicles [15], have established that there is a good correlation between the ability of local anesthetics to interact with phospholipids and their ability to modify membrane properties. These observations, together with other studies reviewed by Seeman [5] and Papahadjopoulos [16], strongly suggest that the action of local anesthetics on membranes may be mediated via a specific interaction with acidic phospholipids. This is considered to involve both hydrophobic and ion-ion electrostatic interactions, and local anesthetic molecules are suggested to reside in membranes in close proximity to the polar groups of phospholipids (see ref. 16).

Recent studies using ESR and NMR techniques have shown that local anesthetics can induce significant molecular disordering and enhance the fluidity of phospholipids in natural [17] and model membranes [18, 19]. This "fluidizing" effect was only observed, however, at very high anesthetic concentrations (in the membrane lytic range), far above the concentration sufficient to induce anesthesia. In this communication we report the effect of the local anesthetic dibucaine on the fluidity of neutral and acidic phospholipids in vesicles. Calorimetric and fluorescence polarization measurements indicate that dibucaine has a significant fluidizing effect on acidic phospholipids even at low concentrations in the typical anesthetic range, but neutral phospholipids are unaffected. The ability of dibucaine to increase the fluidity of acidic phospholipids and displace Ca^{2+} from membranes may be an important mechanism underlaying the effects of dibucaine described in the accompanying paper [20] in which this drug was found to enhance the susceptibility of cultured cells to agglutination by plant lectins and to induce redistribution of "intramembranous particles" on the cell surface.

MATERIALS AND METHODS

Chemicals

Dibucaine hydrochloride (> 95 % pure) was obtained from K and K Laboratories (Plainview, N.Y.) and used without further purification. L-Histidine (Sigma grade) and *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) were obtained from the Sigma Chemical Company (St. Louis, Mo.). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was a gift from Dr M. Shinitzky of the Weizmann Institute and was also purchased from the Aldridge Chemical Company (Milwaukee, Wisconsin). Ethylene diaminotetracetic acid (EDTA) was purchased from Eastman Organic Chemicals (Rochester, N.Y.).

Lipids

The phospholipids used in this study were synthesized and characterized in this laboratory and were chromatographically pure. Phosphatidylserine was isolated from beef brain and phosphatidylcholine from egg yolk as described before [21]. Phosphatidylserine was rechromatographed on a silicic acid column, washed with EDTA and stored as the sodium salt in chloroform. Dipalmitoylphosphatidylcholine

and dipalmitoylphosphatidylglycerol were synthesized as described elsewhere [23]. Their fatty acid content was more than 99 % palmitic. Cholesterol (99 % pure) was purchased from Fluka (Buchs, Switzerland) and recrystallized twice from methanol. All lipids were stored under nitrogen in sealed ampoules at −50 °C at a concentration of approximately 10–20 μmol of phosphate per ml in chloroform. A newly opened ampoule was used for each experiment.

Phospholipid vesicles

Multilamellar vesicles were prepared by the method of Bangham et al. [22]. Unilamellar vesicles were made by sonication of multilamellar vesicles as described previously [15, 21]. The buffer used in all experiments contained 100 mM NaCl unless stated otherwise, L-histidine (2 mM), TES (2 mM), EDTA (0.1 mM) adjusted to pH 7.4. This will be referred to as NaCl buffer. The temperature of dispersion and sonication was 24 °C for phosphatidylserine and 45 °C for dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol. In experiments on vesicles containing the fluorescent probe, diphenylhexatriene, the probe was added in chloroform before dispersion of the lipids. The phospholipid content of vesicle preparations was measured by phosphate assay with molybdate and ELON following perchloric acid digestion as described previously [23].

Measurement of lipid transition temperatures

The transition temperature (T_c) of the phospholipids in vesicle preparations was determined with a Differential Scanning Calorimeter (Perkin-Elmer DSC-2) using a scanning rate of 5 °C/min and a sensitivity scale of 1 mcal/s for full-scale response as described elsewhere [23, 24]. Phospholipids were suspended in 100 mM NaCl buffer as described earlier at concentrations of 0.6 μmol/ml with the usual sample being 3 μmol in 5 ml of buffer. Local anesthetics were added at the specified concentration in buffer before dispersion of the phospholipid and the suspension dialyzed at room temperature for 2 h against buffer containing the same concentration of anesthetic. The suspension was then centrifuged at 100 000 × g for 30 min at 24 °C and the wet pellet transferred to the sample pan of the calorimeter. Each sample contained 0.5–1.0 μmol of phosphate in 15 μl. The enthalpy of the transition was calculated from the area under each peak (by weighing) and the amount of phosphate in the sample. The calorimeter was calibrated with an Indium standard (Perkin-Elmer: Norwalk, Conn.).

Measurement of partition coefficient of local anesthetics in phospholipids

The partitioning of dibucaine into phospholipid membranes was determined by incubation of dispersed phospholipids with 5 ml NaCl buffer containing different concentrations of dibucaine for periods of at least 4 h at 25 or 37 °C. The lipids were then separated from the aqueous medium (as judged by phosphate assay) by centrifugation at 100 000 × g for 30 min at 24 °C, and the supernatant assayed for dibucaine at the 325 nm absorption band in a Cary 15 or Beckman DB spectrophotometer. Partition coefficients (P) were calculated using the equation [25]:

$$P = \frac{C_T - C_S}{C_S m}(1 - m) \qquad (1)$$

where C_T is the optical density of control buffer with no lipid but containing dibucaine; C_s is the optical density of buffer depleted of dibucaine by the presence of lipid and m is the ratio of the weight of lipid to the weight of the entire suspension.

Fluorescence polarization measurements

Fluorescence polarization measurements were made in an Aminco-Bowman spectrophotofluorimeter as described previously [26] using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a polarization probe. DPH fluorescence was excited at 365 nm using a mercury-xenon lamp (American Instruments; Silver Spring, Maryland) and detected at 460 nm through 3 mm of 2 M $NaNO_2$ as a cut-off filter. One mm excitation and emission slits were used. To correct for variation in the intensity of the exciting beam, a mirror aligned at 45 ° was placed in the beam trap of the instrument. This served to deflect light going through the cuvette campartment upward through a condensing lens to an EMI 9524B phototube. Interposed between the lens and the phototube were an interference filter passing only the exciting wavelength, thin film neutral density filters providing a density of 6–7 and a ground quartz beam diffuser. Output from the phototube is fed to the second channel of a two-channel ratio averaging photometer (S and L Instruments, Champaign, Ill.); the first channel of this photometer measures the fluorescence emission. When the cuvette is lifted out of the light path an accurate measurement of the exciting beam intensity can be made. The logarithm of the ratio of this intensity to the value when the cuvette is in place gives the optical density. Direct ratio recording, however, can be achieved only with suspensions of constant optical density.

Lipid concentrations of approximately 0.1 μmol/ml were used and the ratio of DPH to lipid was between 1/800 to 1/200. Low concentrations of lipid were used in order to observe the effect of Ca^{2+} on acidic phospholipids without the optical complication of excessive turbidity caused by flocculation.

The use of DPH as a fluorescence probe is complicated by fading of its fluorescence after excitation. This is believed to result from reversible photoisomerization [27] from the all trans form to one or several of the less fluorescent cis isomers. In high viscosity phospholipid membranes the fading kinetics are not rapid enough to preclude polarization measurements by sequential measurements of vertical (I_{vv}) and horizontal polarized (I_{vH}) intensities, providing exposure for each intensity is limited to 4 s. The reverse sequence of intensities (I_{vH}, I_{vv}) is then measured to compensate for fading effects. The values from each set of intensities are averaged to give a typical standard deviation of the mean of less than $\pm$ 0.003.

Probe rotation rate (R), a kinetic coefficient describing rotation defined by Spencer and Weber [28], was calculated from the equation:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1+6R\tau\right) \tag{2}$$

where p is the measured polarization; p_0 is the limiting polarization and τ is the excited state lifetime of the fluorophore.

This equation has the same form as the Perrin equation when R is identified with the probe rotational diffusion coefficient. A rod-like molecule such as DPH has three modes of rotation; one about the long axis and two others (assumed to be

identical) about the short axes. Since the emission transition moment for polyene-type molecules is approximately parallel to the long axis, only the rotations about the short axes contribute significantly to the observed depolarization. Thus, our calculated rotation rates presumably correspond to "end over end" tumbling of the probe rather than the much more rapid rotations occurring about the long axis. We also acknowledge the possibility that only restricted angular motion [29] rather than true tumbling may occur. In this case the kinetic coefficient is related in an as yet undefined way to this alternate rotational mechanism.

The addition of dibucaine to DPH labelled dispersions produces several experimental problems. Firstly, dibucaine is a fluorophore having an excitation maximum at about 325 nm and a broad emission peaking at about 412 nm. However, it has little absorption at 365 nm so: (1) the inner filter effect was negligible (less than 2 %) and (2) dibucaine fluorescence was small (typically, less than 10 % of the total fluorescence at the highest concentrations of dibucaine employed). This dibucaine fluorescence had a smaller polarization than DPH and when subtracted produced less than a 7 % increase in the polarization calculated without considering this background fluorescence. It should be pointed out that since the dibucaine extinction rises sharply below 365 nm, the dibucaine contribution will depend strongly on the excitation source and optics.

A second complication in fluorescence polarization measurements on phospholipid membranes treated with dibucaine is that the drug may act as both a static and dynamic quencher of DPH fluorescence. The large membrane partition coefficient of dibucaine (see results section) gives rise to extremely high dibucaine concentrations in membranes (approximately 0.4 M) which means that both static and dynamic quenching are possible. The maximum static quenching effect can be estimated using the concept of an "active sphere" around each quencher [30]. At membrane dibucaine concentrations of 0.4 M (vide supra) the residual fluorescence for a sphere with a radius of 10 Å would be no more than 25–50 % of its original value. On the other hand, high concentrations of anesthetic in the membrane would also permit dynamic (lifetime shortening) quenching of DPH emission which would proceed via collisions between probe and quencher while the probe is in its excited state. This effect can be estimated using two dimensional encounter kinetics [31]. Assuming membrane diffusion coefficients of approximately 10^{-7} cm^2/s for both probe and quencher (since both are small molecules), a molecular radius of 5 Å for both, a lattice "jump" distance of 5 Å and a DPH lifetime of 10 ns, it can be calculated that a 75 % diminution in fluorescence could occur by dynamic quenching. Measurements of DPH fluorescence intensity in the presence of dibucaine indicated that quenching did occur. Therefore, we also measured DPH fluorescence lifetime in the presence of dibucaine in order to calculate the DPH rotation rate using Eqn 2.

Fluorescence lifetime measurements

The nanosecond fluorescence lifetime instrument is described in detail elsewhere [55]. The emitted light was analyzed through a three cavity interference filter having maximum transmission at 450 nm with a ±6.5 nm bandpass (Ditric Optics, Inc.). Measurements were made at 25 °C with dry nitrogen circulating through the sample chamber. The deconvolution routines used a method of moments analysis treating the spectra as if only a single exponential decay was present. All sample spectra

were accumulated at low counting rates for constant analysis time. Control spectra were acquired on unlabeled sonicated phosphatidylserine vesicles and with dibucaine present at various concentrations. Under these excitation conditions, there is significant dibucaine fluorescence. The lifetime and relative intensity of DPH emission when embedded in phosphatidylserine vesicles in the presence of dibucaine can be found by deconvoluting a difference spectrum produced by subtracting the dibucaine control spectrum from the DPH spectrum in the presence of the same concentration of dibucaine. This procedure also corrects for the scattering contribution of the phosphatidylserine vesicles ($< 5\%$ of the total intensity). The assumption is made that the presence of DPH does not affect the dibucaine lifetime. Steady state dibucaine intensity measurements, where the dibucaine in the presence of DPH labelled phosphatidylserine vesicles is excited at 334 nm and emission monitored at 370 nm, indicate that DPH could cause no more than a 10 % decrease of dibucaine lifetime at the highest DPH : dibucaine ratio employed.

RESULTS

Partition coefficient of local anesthetics in phospholipid membranes

The partition coefficient of dibucaine in acidic (phosphatidylserine) and neutral phospholipids (phosphatidylcholine) is summarized in Table I. The values of about 5500 obtained for the partition of dibucaine in phosphatidylserine membranes are among the highest reported to date for any anesthetic [5]. Ca^{2+} binding to phosphatidylserine membranes partially inhibits the binding of dibucaine at the concentration studied in these experiments. The partition coefficient of dibucaine in phosphatidylcholine membranes was much lower (Table I). The inclusion of cholesterol in phospholipid membranes significantly reduces dibucaine binding in both phosphatidylserine and phosphatidylcholine membranes (Table I). The very different partition coefficients obtained for dibucaine in phosphatidylserine membranes compared with mixed phosphatidylcholine/cholesterol membranes (Table I) would indicate a preference for occupation of negatively-charged domains in membranes by a factor of approximately 50.

Differential scanning calorimetry

Calorimetric measurements of the thermotropic properties of dipalmitoylphosphatidylcholine in NaCl buffer revealed that the mid-point of the main thermotropic transition, corresponding to the melting of the acyl chains, occurred at 42.5 °C (Fig. 1, curve a). This value is in agreement with previous studies [32, 33]. A minor transition ("premelt"), whose origin is still not understood, was detected at 37.5 °C (Fig. 1, curve a). This minor peak disappeared after incubation with dibucaine ($4 \cdot 10^{-4}$ M) and there was accompanying broadening of the main endothermic peak (Fig. 1, curve b). However, no significant shift was found in the mid-point temperature of the transition (T_c) which remained at 42.2 °C. Also, dibucaine at this concentration did not affect the total enthalpy of the transition ($\Delta H = 8.9 \pm 0.5$ kcal/mol) indicating a loss in cooperativity as the overall effect at that concentration. However, higher concentrations of dibucaine ($2 \cdot 10^{-3}$ M and $1 \cdot 10^{-2}$ M) produced a significant downward shift in the main endothermic peak (Fig. 1, curves c and d). These results indicate that relatively high concentrations of dibucaine are necessary to produce a

TABLE I

PARTITION COEFFICIENT OF DIBUCAINE BETWEEN PHOSPHOLIPID MEMBRANES AND AQUEOUS BUFFER

Lipid composition	Lipid concentration (mM)	Dibucaine concentration (M)	Partition coefficient[a]	Bound dibucaine per lipid (mol ratio)
Phosphatidylserine	0.1	$1 \cdot 10^{-4}$	5350 (2)	1:3.7
Phosphatidylserine	0.6	$2 \cdot 10^{-4}$	5700 (2)	1:4.6
Phosphatidylserine + 0.6 mM Ca^{2+}	0.6	$2 \cdot 10^{-4}$	2650 (1)	1:7.5
Phosphatidylserine/ cholesterol[b]	0.4/0.4	$2 \cdot 10^{-4}$	1150 (1)	1:13
Phosphatidylcholine[c, d]	0.6	$2 \cdot 10^{-4}$	235 (5)	1:30
Phosphatidylcholine/ cholesterol	0.4/0.4	$2 \cdot 10^{-4}$	46 (4)	1:220

[a] Mean values derived from number of experiments shown in parentheses; extreme values did not deviate from the mean by more than 5.5% for phosphatidylserine, 32% for phosphatidylcholine, and 50% for phosphatidylcholine/cholesterol.

[b] This concentration of an equimolar lipid cholesterol dispersion provides a suspension having the same weight as the 0.6 mM lipid dispersion.

[c] Phosphatidylcholine dispersions incubated with dibucaine consistently leave about 15% of the total phosphate in the supernatant under our centrifugation conditions. The partition coefficient is corrected for this effect under the assumption that the supernatant phospholipid binds dibucaine with the same affinity as the pellet phospholipid. The mean corrected P value is about 15% greater than the mean value calculated ignoring the supernatant phospholipid.

[d] Recently, bilayer binding studies of spin labelled analogs to the 2-(*N*, *N* diethylamine) ethyl-4-alkoxybenzoate series of local anesthetics have been reported [54]. Phosphatidylserine bilayers bind almost all the available anesthetic. Neutral phosphatidylcholine bilayers bind significantly lower amounts of these analogs but apparently still have a much higher affinity for these local anesthetics than for dibucaine.

"fluidizing" effect in dipalmitoylphosphatidylcholine membranes. Importantly, this drug has no significant fluidizing effect on dipalmitoylphosphatidylcholine at concentrations ($< 4 \cdot 10^{-4}$ M) which induce significant functional alterations in the membranes of living cells [20].

Since it is recognized that certain local anesthetics interact preferentially with acidic phospholipids (see ref. 16) the experiments were repeated using dipalmitoylphosphatidylglycerol membranes which are negatively charged at pH 7.4. The results are shown in Fig. 2. Pure dipalmitoylphosphatidylglycerol in 100 mM NaCl buffer without anesthetic displayed a thermotropic behaviour similar to dipalmitoylphosphatidylcholine. The main endothermic transition occurred at 41.5 °C and a minor transition occurred at 34.5 °C (Fig. 2, curve a). Several recent studies [23, 24, 34–37] have shown that the thermotropic properties of acidic phospholipids are substantially modified by divalent cations. Similarly, $6 \cdot 10^{-4}$ M Ca^{2+} increased the T_c of dipalmitoylphosphatidylglycerol to 51 °C (Fig. 2, curve b) and at even higher Ca^{2+} concentrations no transition was detectable up to 65 °C. This "stabilizing" effect of divalent cations on phospholipids has been discussed in detail elsewhere [34]. Addition of dibucaine to dipalmitoylphosphatidylglycerol membranes produced a large downward shift of the T_c. This "fluidizing" effect was very marked. The T_c was redu-

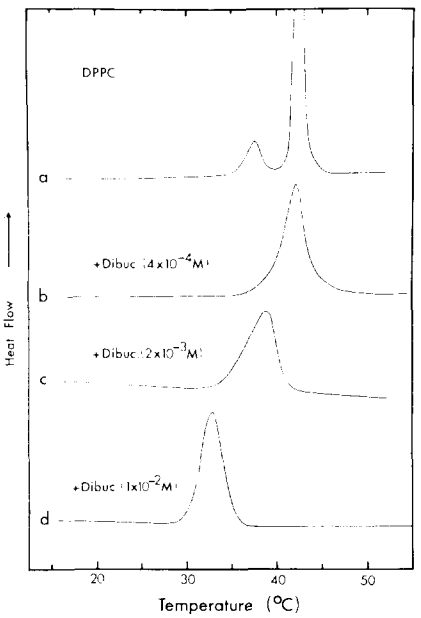

Fig. 1. Differential scanning calorimetry of dipalmitoylphosphatidylcholine (DPPC) vesicles in the presence of different concentrations of dibucaine hydrochloride: (a) no dibucaine; (b) $4 \cdot 10^{-4}$ M dibucaine; (c) $2 \cdot 10^{-3}$ M dibucaine and (d) $1 \cdot 10^{-2}$ M dibucaine. Vesicles were prepared in 100 mM NaCl buffer as described in the methods.

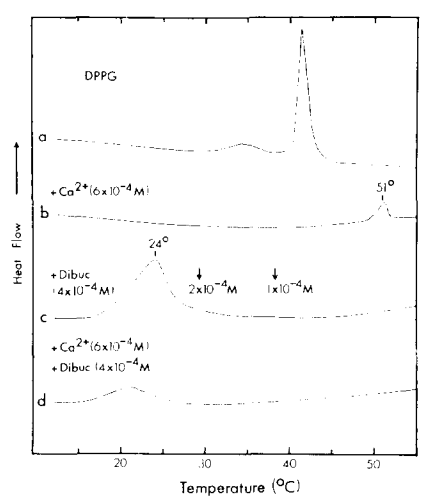

Fig. 2. Differential scanning calorimetry of dipalmitoylphosphatidylglycerol (DPPG) vesicles in the presence of different concentrations of dibucaine and Ca^{2+}: (a) vesicles in 100 mM NaCl buffer. The remaining curves were obtained with vesicle populations under the same conditions except for the presence of: (b) $6 \cdot 10^{-4}$ M $CaCl_2$; (c) $4 \cdot 10^{-4}$ M dibucaine. The arrows indicate the mid-point of the endothermic peak in the presence of $2 \cdot 10^{-4}$ and $1 \cdot 10^{-4}$ M dibucaine; (d) $6 \cdot 10^{-4}$ M Ca^{2+} and $4 \cdot 10^{-4}$ M dibucaine.

ced by as much as 18 °C (Fig. 2, curve c) by dibucaine concentrations ($4 \cdot 10^{-4}$ M) which failed to affect the T_c of dipalmitoylphosphatidylcholine membranes. Reduction of the T_c was also observed at even lower concentrations of dibucaine (13 and 3.2 °C downward shifts in the T_c at $2 \cdot 10^{-4}$ M and $1 \cdot 10^{-4}$ M dibucaine, respectively). More importantly, the striking "fluidizing" effect of dibucaine on dipalmitoylphosphatidylglycerol membranes was also displayed in the presence of $6 \cdot 10^{-4}$ M Ca^{2+} (Fig. 2, curve d), indicating that low concentrations of dibucaine ($\leqslant 4 \cdot 10^{-4}$ M) can produce a significant increase in the "fluidity" of acidic phospholipids membranes, even in the presence of divalent metals. It appears that dibucaine can displace Ca^{2+} from the membranes, a conclusion pertinent to the documented antagonism between Ca^{2+} and local anesthetics in both natural [3, 4, 38] and model membranes [15, 39].

Since the main acidic phospholipid in most mammalian cell membranes is phosphatidylserine, it was of interest to study the effect of dibucaine on this phospholipid. In contrast to synthetic dipalmitoylphosphatidylglycerol which contains more than 99 % palmitic acid residues, phosphatidylserine isolated from brain contains approximately 50 % unsaturated fatty acids [21]. This is reflected in the lower T_c of phosphatidylserine which occurs at 7.5 °C (Fig. 3, curve a). In the presence of Ca^{2+} ($6 \cdot 10^{-4}$ M) the endothermic peak for this lipid was broader, the heat of transition was diminished and the mid-point of the transition was displaced 13 °C upwards (Fig. 3, curve b). At higher Ca^{2+} concentrations, there was no observable transition between 0° and 70 °C. X-ray diffraction of similar phosphatidylserine samples in the presence of 2 mM Ca^{2+} has revealed a sharp 4.2 Å diffraction, indicating that the hydrocarbon chains are at least partly crystalline [34].

Addition of dibucaine ($1 \cdot 10^{-4}$ M) produced a 5 °C downwards shift of the endothermic transition of phosphatidylserine both in the absence (Fig. 3, curve c) and presence of Ca^{2+} ($6 \cdot 10^{-4}$ M) (Fig. 3, curve d). Thus, the "fluidizing" effect of

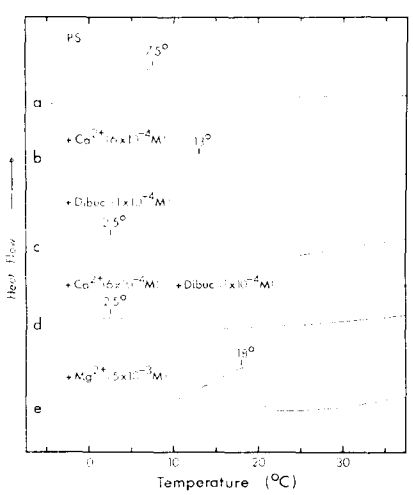

Fig. 3. Differential scanning calorimetry of phosphatidylserine (PS) vesicles in the presence of different concentrations of dibucaine and Ca^{2+}. (a) Vesicles in 100 mM NaCl buffer. The remaining curves were obtained with vesicle populations under the same conditions except for the presence of: (b) $6 \cdot 10^{-4}$ M $CaCl_2$; (c) $1 \cdot 10^{-4}$ M dibucaine; (d) $6 \cdot 10^{-4}$ M $CaCl_2$ and $1 \cdot 10^{-4}$ M dibucaine; (e) $5 \cdot 10^{-3}$ M $MgCl_2$.

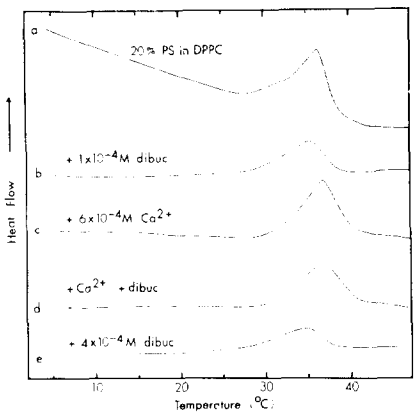

Fig. 4. Differential scanning calorimetry of mixed phospholipid vesicles composed of 20 % phosphatidylserine (PS) in dipalmitoylphosphatidylcholine (DPPC). (a) vesicles in 100 mM NaCl buffer. The remaining curves were obtained with vesicle populations under the same conditions except for the presence of (b) $1 \cdot 10^{-4}$ M dibucaine; (c) $6 \cdot 10^{-4}$ M $CaCl_2$; (d) $6 \cdot 10^{-4}$ M $CaCl_2$ and $1 \cdot 10^{-4}$ M dibucaine and (e) $4 \cdot 10^{-4}$ M dibucaine.

dibucaine observed earlier on synthetic dipalmitoylphosphatidylglycerol also occurs with the naturally occurring acidic phospholipid phosphatidylserine.

In addition to Ca^{2+}, the presence of Mg^{2+} can also stabilize acidic phospholipid membranes. As shown in Fig. 3 (curve e), Mg^{2+} ($5 \cdot 10^{-3}$ M) produced a 10 °C upward shift in the T_c of phosphatidylserine. This stabilizing effect was inhibited by dibucaine ($4 \cdot 10^{-4}$ M). The endothermic curve obtained under these conditions (not shown) was essentially similar to curve d in Fig. 3.

Calorimetric measurements on mixed lipid membranes composed of 20 % phosphatidylserine in dipalmitoylphosphatidylcholine revealed a broad endothermic peak with a mid-point at 36.2 °C (Fig. 4, curve a). Addition of dibucaine ($1 \cdot 10^{-4}$ M) had only a minimal eutectic effect on these membranes, shifting the T_c downward by 0.9 °C (Fig. 4, curve b). At higher dibucaine concentrations ($4 \cdot 10^{-4}$ M) the T_c was still only depressed by 1.7 °C (Fig. 4, curve e). The addition of Ca^{2+} ($6 \cdot 10^{-4}$ M) increased slightly the T_c of the mixed membranes (Fig. 4, curve c). However addition of dibucaine ($1 \cdot 10^{-4}$ M) to mixed membranes in the presence of Ca^{2+} ($6 \cdot 10^{-4}$ M) had little effect on the T_c (Fig. 4, curve d). Thus, dibucaine at low concentrations ($1 \cdot 10^{-4}$ M) does not substantially lower the T_c of the mixed membranes in the presence of Ca^{2+}. This contrasts with its marked "fluidizing" effect at the same concentration in membranes composed entirely of acidic phospholipids such as phosphophatidylserine or dipalmitoylphosphatidylglycerol.

The small but definite effect of dibucaine ($1 \cdot 10^{-4}$ M) on mixed phospholipid membranes (Fig. 4, curves a and b) is, however, compatible with an effect on the acidic phospholipid component of the mixed membranes. As shown earlier, dibucaine at this concentration has no effect on neutral phospholipids (Fig. 1) but lowered the T_c of phosphatidylserine membranes by 5 °C (Fig. 3, curve c). However, the dibucaine-induced depression of the T_c by approximately 1 °C in mixed membranes is approximately 20 % of the T_c depression occurring in pure phosphatidylserine

membranes and is thus directly proportional to the percentage of phosphatidylserine in the mixed membranes. Finally, the lack of effect of dibucaine on the thermotropic properties of the mixed membranes in the presence of Ca^{2+} (Fig. 4, curve d) could by explained by the previously observed requirement for a close-packed array of phosphatidylserine molecules in order for Ca^{2+}-mediated changes to occur in mixed membranes (see refs 23, 40). The dilution of phosphatidylserine below a 2 : 1 molar ratio with phosphatidylcholine would limit the occurrence of such close-packed arrays. The lack of effect of dibucaine on mixed membranes in the presence of Ca^{2+} is also potentially significant in relation to the proposal made in the accompanying paper that the physiological effects of dibucaine at low concentrations ($1 \cdot 10^{-4}$ M) might involve specific interaction of dibucaine with phosphatidylserine "domains" in natural membranes [20].

Fluorescence polarization measurements

Fluorescence polarization monitors phospholipid phase transitions as a sharp drop in polarization in the region of the T_c. The decrease in polarization corresponds to the marked increase in the rotational freedom of the probe upon melting of phospholipid acyl chains which occurs at the gel-liquid-crystalline transition [34, 41–43].

Fluorescence polarization measurements on sonicated dipalmitoylphosphatidylglycerol vesicles containing the fluorescence probe diphenylhexatriene revealed that dibucaine ($2 \cdot 10^{-4}$ M) produced a significant "fluidizing" effect on the vesicle membranes as indicated by an 18.5 °C downward shift in the T_c (Fig. 5). The magnitude of the shift produced by $2 \cdot 10^{-4}$ M dibucaine in Fig. 5 at a lipid concentration of 0.07 μmol/ml is in good agreement with that produced by $4 \cdot 10^{-4}$ M dibucaine in unsonicated dipalmitoylphosphatidylglycerol vesicles (0.6 μmol/ml). This suggests

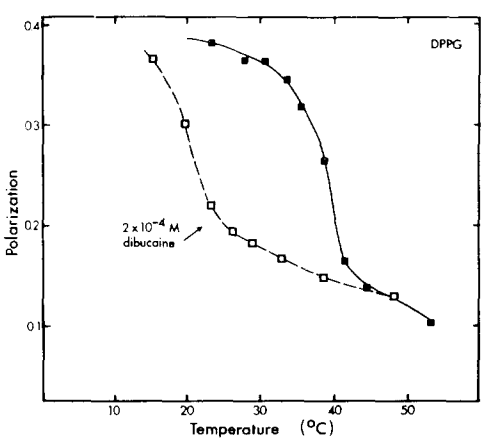

Fig. 5. Phase transition of sonicated dipalmitoylphosphatidylglycerol vesicles in the presence (□—□) and absence (■—■) of $2 \cdot 10^{-4}$ M dibucaine hydrochloride monitored by fluorescence polarization of 1,6-diphenylhexatriene. The lipid dispersion (15 ml) was made approximately $2 \cdot 10^{-4}$ M in unbound dibucaine by dialysis for 4 h at 45 °C against 100 mM NaCl buffer (200 ml) containing $2 \cdot 10^{-4}$ M dibucaine. The concentration of dipalmitoylphosphatidylglycerol was 0.07 μmol per ml; the diphenylhexatriene to lipid ratio was approximately 1/300. Polarization in the presence of dibucaine is not corrrected for the small amount of dibucaine fluorescence which contributes less than a 6 % increase in the observed values.

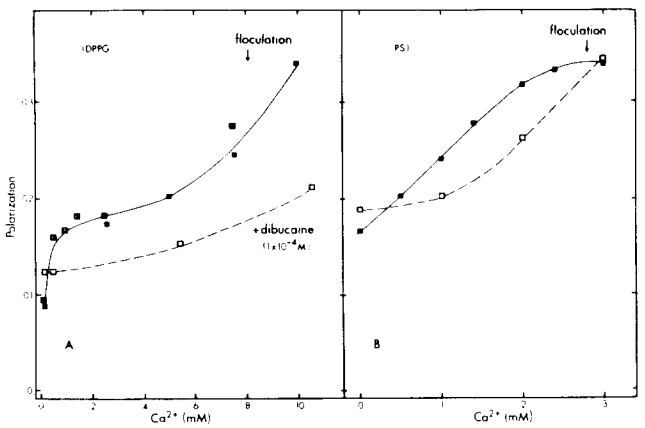

Fig. 6. The effect of Ca^{2+} on the polarization of 1,6-diphenylhexatriene in phospholipid vesicles in the presence (□—□) and absence (■—■) of $1 \cdot 10^{-4}$ M dibucaine hydrochloride. (A) Dipalmitoylphosphatidylglycerol (DPPG) vesicles at 48 °C. The experimental conditions were identical to those described in the legend to Fig. 5. (B) Phosphatidylserine (PS) vesicles at 25 °C. Vesicles were made approximately $1 \cdot 10^{-4}$ M in unbound dibucaine by dialysis for 4 h at room temperature (22 °C) against 100 mM NaCl buffer containing $1 \cdot 10^{-4}$ M dibucaine. The concentration of phosphatidylserine was 0.1 μmol per ml; the diphenylhexatriene to phospholipid ratio was less than 1/200. Polarization in the presence of dibucaine is not corrected for the small amount of dibucaine fluorescence which creates less than a 6 % increase in the observed values.

possible differences in the partition coefficient of dibucaine depending on sonication and concentration of phospholipid.

In order to demonstrate antagonism between Ca^{2+} and dibucaine in this experimental system, sonicated phosphatidylserine and dipalmitoylphosphatidylglycerol vesicles were titrated with Ca^{2+} in the presence and absence of dibucaine under conditions where both membranes were fluid ($T > T_c$). The results, shown in Fig. 6, indicate that addition of Ca^{2+} to both phosphatidylserine and dipalmitoylphosphatidylglycerol induced polarization values approaching those expected for phospholipids in the gel (solid) state. Flocculation of the vesicle preparations induced by Ca^{2+} hinders measurement of polarization at concentrations of Ca^{2+} required to induce the full effect. Similar titration of vesicles with Ca^{2+} in the presence of dibucaine ($1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ M) revealed that higher concentrations of Ca^{2+} were required to produce the same increase in polarization (Fig. 6). Competition between Ca^{2+} and dibucaine can be observed directly as a drop in polarization as the drug is added to vesicles previously titrated with Ca^{2+}. We interpret this as a fluidizing effect resulting from displacement of membrane-bound Ca^{2+}. With no Ca^{2+} present, the higher polarization values in the presence of dibucaine are compatible with a more "fluid" membrane state induced by the anesthetic rather than a more ordered state. This interpretation based on dibucaine quenching of DPH emission, is justified below (see Table II) using direct probe lifetime measurements.

Measurements on the effect of different concentrations of dibucaine on the rotation rate of DPH in sonicated phosphatidylserine vesicles at 24 °C revealed that the maximum membrane fluidizing effect (increased rotation rate) was produced by dibucaine at concentrations below $2 \cdot 10^{-4}$ M (Table II). The rotation rate of

TABLE II

EFFECT OF DIBUCAINE ON DPH LIFETIME[a], POLARIZATION AND ROTATION RATE IN PHOSPHATIDYLSERINE MEMBRANES[b]

Dibucaine concentration (M)	Lifetime of dibucaine emission (ns)	Lifetime of combined DPH and dibucaine emission (ns)	Lifetime of DPH emission alone (ns)	Polarization[c]	Rotation rate (mHz)
0.0			9.5	0.166	36.7
$1 \cdot 10^{-4}$	3.32	6.32	7.33	0.186	39.6
$2 \cdot 10^{-4}$	3.11	5.96	6.94	0.188	41.1
$4 \cdot 10^{-4}$	3.31	5.01	6.72	0.187	42.9

[a] Lifetimes measured by nanosecond fluorimetry as described in methods. Error estimated to be less than or equal to ± 0.1 ns.

[b] Sonicated phosphatidylserine dispersions measured at room temperature. Lipid concentration was 0.1 μmol/ml; probe to lipid ratio was less than 1/200.

[c] Polarized dibucaine fluorescence background subtracted through the use of identical lipid dispersions not labelled with DPH.

DPH could be calculated because both the polarization and the lifetime were measured. DPH lifetime measurements (Table II) indicated that dibucaine did act as a dynamic quenching agent since the lifetime decreased from 9.5 to 6.72 ns (a 29 % decrease) at at $4 \cdot 10^{-4}$ M dibucaine. Since the DPH intensity drops more rapidly than lifetime (a 56 % decrease in intensity at $4 \cdot 10^{-4}$ M dibucaine) a component of static quenching which increases with dibucaine concentration is also indicated. The value of 9.5 ns for the lifetime of DPH embedded in phosphatidylserine vesicles, at room temperature, is in good agreement with the values of the DPH lifetime in other lipid vesicles (estimated from intensity-temperature profiles [27]). Polarization of DPH emission actually increases by about 11 % on the addition of 0.1 mM dibucaine with no more increase on further additions (Table II). However, application of Eqn 2 using both lifetime and polarization measurements shows that dibucaine additions increase the DPH rotation rate presumably corresponding to an increase in membrane fluidity. In this somewhat unusual case the fluidity increase caused by dibucaine is accompanied by an increased DPH polarization because the anesthetic also markedly shortens the DPH lifetime.

DISCUSSION

Measurement of the freezing point depression of phospholipids (lowering of the temperature, T_c, for endothermic transition from solid to the liquid-crystalline state) has been used in the present study to monitor the effect of dibucaine on the fluidity of phospholipid membranes. Although this is an indirect method for evaluating changes in membrane fluidity, the results are in complete accord with direct measurement of the partition coefficient of dibucaine in different phospholipids. The recent work of Hill [44] on the effects of general anesthetics on the thermotropic properties of dipalmitoylphosphatidylcholine membranes has also demonstrated the value of freezing-point depression measurements as a tool for assessing anesthetic

potency. The eutectic effect of several morphine derivatives and antidepressant drugs on the phase transition of phospholipids has also been reported recently [45].

The present experiments indicate that the local anesthetic dibucaine produces a significant "fluidizing" effect on phospholipid membranes. The results obtained here with calorimetric and fluorescence polarization and lifetime techniques agree with previous observations using ESR [18, 19, 46] and NMR techniques [18] in which similar disordering and increased fluidity was detected in membranes treated with local and general anesthetics.

In addition the present results indicate that there is a marked difference in the affinity of dibucaine for acidic and neutral phospholipids. Both the calorimetric results, and differences in the partition coefficient of dibucaine in various lipids, indicate that dibucaine displays at least a 20-fold higher affinity for acidic phospholipids. This finding accords with earlier NMR observations [47] which indicated preferential immobilization of local anesthetic molecules by acidic phospholipids. Dibucaine is positively-charged at the experimental pH and the demonstrated preferential effect on acidic phospholipids reinforces the proposed importance of charge-charge interactions in the action of dibucaine and related drugs (see ref. 16).

At dibucaine concentrations ($2 \cdot 10^{-4}$ M) sufficient to induce changes in the topographical distribution of cell surface molecules reported in the accompanying paper [20], the fluidizing effect of dibucaine would be expected to be confined solely to acidic phospholipid species. The present results also indicate that the membrane partition coefficient of dibucaine is reduced in the presence of cholesterol, presumably as a result of the higher rigidity of such membranes. A similar finding has been described previously for benzyl alcohol [18].

The partition coefficient of dibucaine in phosphatidylserine membranes measured in the present experiments is very high. Calculation of the concentration of dibucaine in phosphatidylserine membranes exposed to an aqueous bulk concentration of $1 \cdot 10^{-4}$ M dibucaine yields a value of 0.4 molar. This corresponds to a molar ratio of membrane associated dibucaine to membrane lipid of approximately 0.25. This finding lends support to the proposals for lipid-anesthetic interaction suggested by Feinstein [48]. The membrane partition coefficient of dibucaine in phosphatidylserine is higher than any value reported to date for other positively-charged drugs (review ref. 5). For example, Seeman [5] reported that chlorpromazine achieved a concentration of 0.04 M in erythrocyte membranes in which acidic phospholipids represent approximately 10 % of the total membrane [49]. If, however, chlorpromazine were to bind preferentially to acidic lipids, then the drug concentration within such regions would approach the levels (0.4 M) obtained here with dibucaine in phosphatidylserine membranes. It seems likely therefore that the local concentration of dibucaine and other drugs in natural membranes may well vary widely, depending on the local charge and the fluidity of the bilayer region.

The results described here also indicate that dibucaine at physiologically relevant concentrations can compete effectively with Ca^{2+} and Mg^{2+} for negatively-charged groups in acidic phospholipids membranes. The ability of dibucaine to significantly lower the T_c of phospholipid membranes in the presence of divalent cations is directly opposite to that of divalent metals which have been shown to "stabilize" acidic phospholipid membranes (see refs 23, 24, 34, 35).

It is also important to note that dibucaine had no significant effect on the

thermotropic properties of membrane composed of 20% phosphatidylserine in phosphatidylcholine, a proportion of acidic to neutral phospholipid similar to that found in nerve membranes [50]. Consequently, it seems reasonable to suggest that if the biological effects of dibucaine and other local anesthetics at low concentrations are mediated via a specific interaction with lipids, then the interaction is more likely to occur in domains of acidic phospholipids. Such domains, if they exist, are probably associated with divalent cations and the basic groups of integral or peripheral membrane proteins. Domains of acid phospholipids have been induced in mixed phospholipid membranes by Ca^{2+} [23, 51, 52]. Furthermore, tetracaine has been reported to interfere with the Ca^{2+}-induced separation of phosphatidylserine domains from phosphatidylcholine (53). Binding of dibucaine would be expected to "fluidize" such domains or perhaps cause their dispersal (disintegration) by displacing Ca^{2+}. Inducement of such "domain dispersal" might then alter the binding and topographical distribution of other membrane components, notably proteins. This concept is elaborated in the following paper.

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