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NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC STUDIES OF PROCAINE HYDROCHLORIDE AND TETRACAINE HYDROCHLORIDE AT LIPID–WATER INTERFACES

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SUMMARY

High resolution NMR spectroscopy has been applied to the study of the interaction of two local anaesthetic molecules, procaine·HCl and tetracaine·HCl, with phospholipid dispersions. Interaction occurred with the acidic phospholipid phosphatidylserine but not with zwitterionic lecithin, indicating that the interaction was of an electrostatic nature. Van der Waals dispersion forces were also thought to be involved. Consistent with this idea was the observation of a differential increase in the line broadening of various portions of the local anaesthetic molecules.

An approximate value for the apparent equilibrium constant K for the interaction between phosphatidylserine and procaine·HCl has been determined. The interaction occurred in a molecular ratio of 1:1. It is concluded that the hydrophilic head groups of phosphatidylserine in a sonicated aqueous dispersion are readily accessible to the local anaesthetic molecule.

INTRODUCTION

It is often assumed that the locus of action of local anaesthetics is the lipoid-containing interface of the nerve axon[1–4]. Consequently, many investigators have studied the interaction of local anaesthetics with monolayers of lipids. Both SKOU[1] and SHANES[5] suggest a correlation between the lateral packing of the molecules and the permeability of a membrane. According to these authors, the permeability of a membrane is inversely proportional to the closeness of molecular packing in the membrane, which in turn will be affected by the degree of penetration of a foreign molecule such as a local anaesthetic.

BANGHAM *et al.*[6] investigated the effect of local anaesthetics on phospholipid liquid crystals. They established a relationship between the cation diffusion rate and the sign and magnitude of the lipid–water interfacial potential and showed that the ζ potential and cation diffusion rate of negatively charged phospholipid liquid-crystals are reduced in the presence of local anaesthetics.

The subject of this investigation is the study, using NMR spectroscopy, of the interaction between two local anaesthetic molecules, procaine·HCl and tetracaine·HCl, with phospholipids dispersed in a bulk aqueous phase.

EXPERIMENTAL

All reagents were A.R. grade. ^2H_2O was obtained from E. Merck, procaine·HCl from British Drug Houses Laboratory Chemicals, tetracaine·HCl from Jacobson van den Bergh and Co., and DEAE-cellulose DE-32 microgranular from Whatman.

Lecithin

Lecithin was extracted from egg yolks and was purified on alumina using methanol–benzene (1:10, v/v) as eluting solvent by Mr. M. Davis.

Phosphatidylserine

Crude ox-brain phosphatidylserine from Koch–Light was further fractionated on DEAE-cellulose following the method described by ROUSER *et al.*[7].

Thin-layer chromatography on silica-gel H (without binder) using chloroform–methanol–7 M aqueous ammonia (230:90:15, by vol.) as the solvent gave a single spot both with lecithin and phosphatidylserine.

Preparation of phospholipid dispersions

The phospholipid (12.5–100 μmoles for phosphatidylserine–procaine·HCl mixtures and 0.50–50 μmoles for phosphatidylserine–tetracaine·HCl mixtures) was coated on the glass surface of a test tube by evaporating a chloroform solution *in vacuo*. 1 ml of ^2H_2O was added to the test tube, and the tube was agitated on a mechanical shaker until no visible phospholipid was left adhering to the walls of the test tube. The aqueous dispersions of lecithin or phosphatidylserine were ultrasonicated with a soniprobe (Dawe Instruments) at 20 kcycles/sec for 10 min; the sample was surrounded by icewater. The pH of the lecithin and phosphatidylserine dispersions was betweeen 5 and 6.

The phospholipid–local anaesthetic mixtures were made up by mixing 1-ml ultrasonicated phospholipid dispersions and the requisite amount of procaine·HCl or tetracaine·HCl dissolved in 1 ml of ^2H_2O to give phospholipid–local anaesthetic mixtures as indicated in Figs. 3 and 4.

NMR spectrosoopy

The NMR spectra were obtained using a Perkin–Elmer Model R.10 spectrometer (60 Mcycles/sec). All spectra were run at 33.4°. Since the concentrations were low, a signal averager (Northern Scientific N.S. 544, digital memory oscilloscope) was used to increase the signal/noise ratio. Spectral accumulations were usually made for each concentration. An average of 64 accumulations was needed in the phosphatidylserine–procaine mixtures whereas about 1000 accumulations were needed for the tetracaine experiments.

RESULTS

The NMR spectra of a phosphatidylserine dispersion alone in ^2H_2O and of procaine·HCl alone in ^2H_2O are shown in Figs. 1A and 1B together with assignments. The NMR spectrum of tetracaine·HCl in ^2H_2O is shown in Fig. 2B. The NMR spectra of procaine·HCl and tetracaine·HCl mixed with phosphatidylserine in a molar ratio

of 1:1 are shown in Fig. 1C and 2C, respectively. The NMR signals arising from the alkyl chain protons of phosphatidylserine are overlapping. Signal f of procaine·HCl and Signals g, h and i of tetracaine·HCl; thus an examination of these signals is not

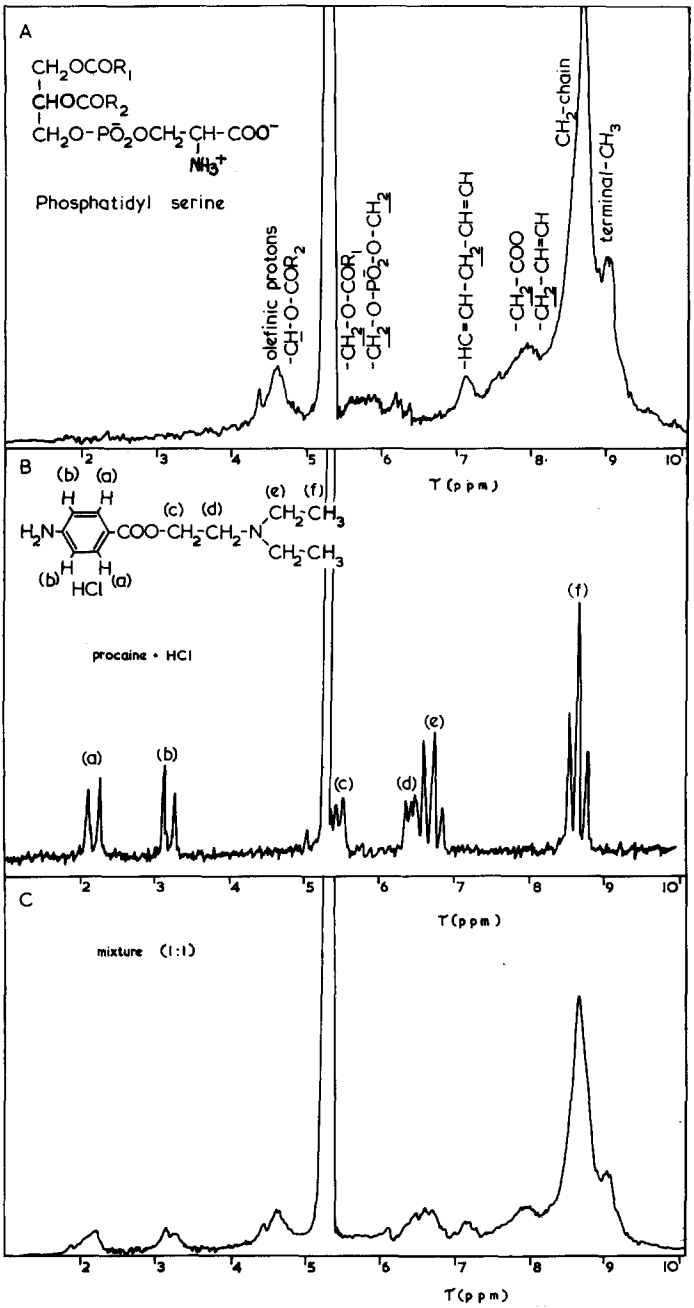

Fig. 1. The NMR spectra of (A) a sonicated phosphatidylserine dispersion (4% w/v) in ^2H_2O; of (B) procaine·HCl, 0.1 M in ^2H_2O; and of (C) a sonicated phosphatidylserine dispersion (2% w/v) in the presence of procaine·HCl in ^2H_2O (molar ratio 1:1); 33.5°.

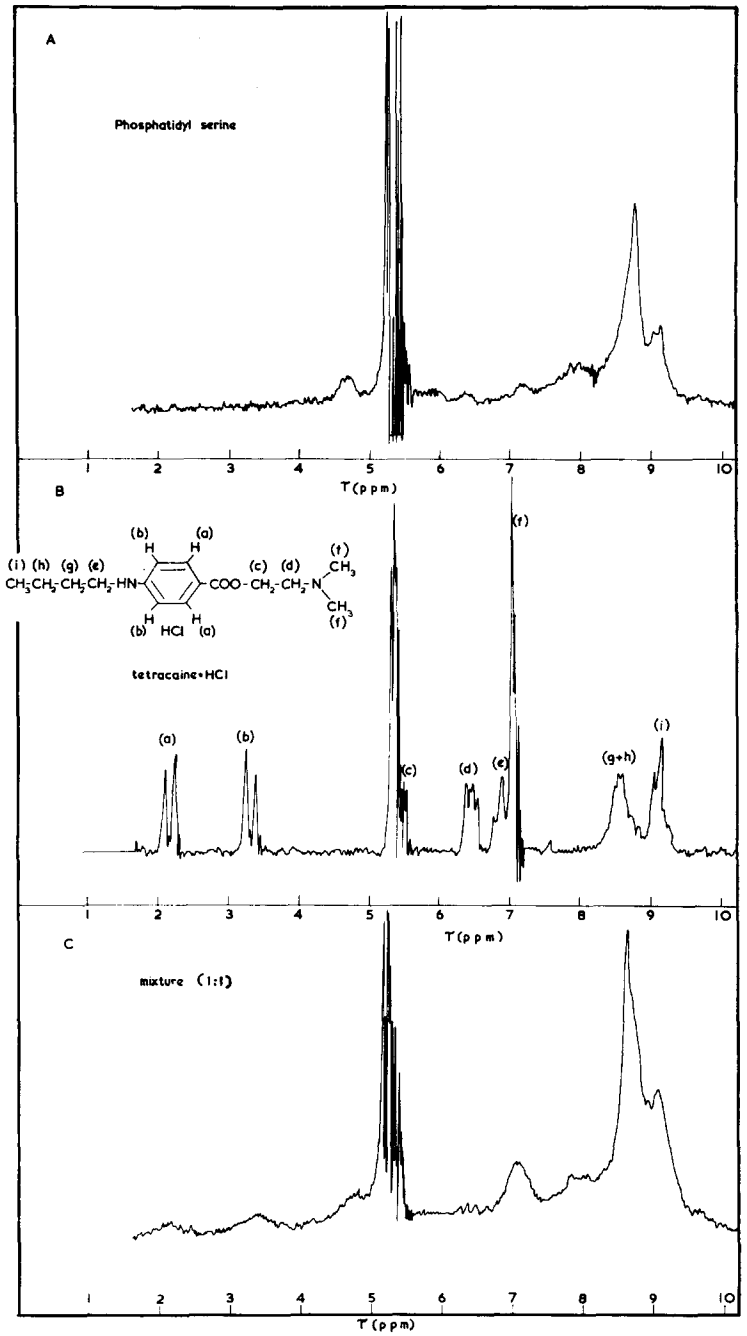

Fig. 2. The NMR spectra of (A) a sonicated phosphatidylserine dispersion (0.4 % w/v) in ^2H_2O; of (B) tetracaine·HCl, 0.1 M in ^2H_2O and of (C) a sonicated phosphatidylserine dispersion (0.4 % w/v) in the presence of tetracaine·HCl in ^2H_2O (mol. ratio 1:1); 33.5°.

possible. All other signals arising from the drug molecules are broadened when mixed with phosphatidylserine (see Fig. 1C and 2C). The signal from the alkyl chain protons of phosphatidylserine does not show broadening in the mixture. Broadening of all signals examined is greater with tetracaine·HCl than with procaine·HCl. No effect of phosphatidylserine on chemical shifts of the local anaesthetic molecules is observed, but in some mixtures with broad lines this is very difficult to detect.

In order to see whether broadening of the various signals occurs in a differential manner, the line width variation with the mole fraction is plotted for Signal a* (aromatic protons) and Signal e [$\rangle\overset{(+)}{N}$–(CH_2–CH_3)$_2$ protons] of procaine·HCl and Signal a (aromatic protons) and Signal f[$\rangle\overset{(+)}{N}$–(CH_3)$_2$ protons] of tetracaine·HCl. This is shown in Fig. 3 and 4, respectively. There is some difference in the degree of broadening experienced by the two signals of each local anaesthetic molecule. The differential broadening observed in the spectrum of tetracaine·HCl is more obvious than is observed with procaine·HCl (Figs. 3 and 4).

In both cases the signals from the aromatic nuclei are broadened to a greater extent than Signals e and f of procaine·HCl and tetracaine·HCl, respectively. A differential broadening is also detectable in the spectrum of a mixture of phosphatidylserine and tetracaine·HCl = (1:1) (Fig. 2c). Signal d is already lost in the base line

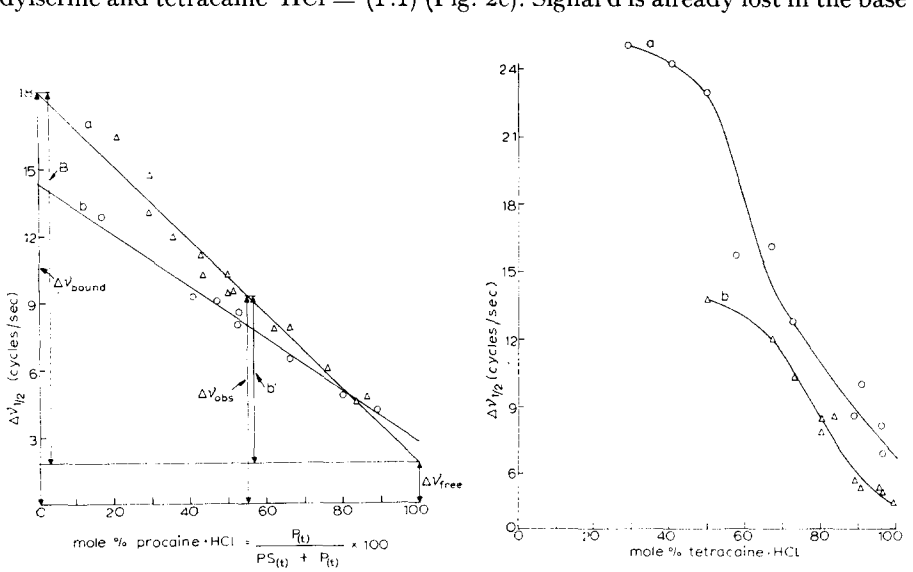

Fig. 3. Variation with mole % procaine·HCl of the line width in cycles/sec. Curve (a), the aromatic ring protons (Signal a); Curve (b), the methylene protons of the –$\overset{H}{\underset{|}{N}}$–($CH_2$-$CH_3$)$_2$ group (Signal e). Concentration of phosphatidylserine varying from 6 to 50 mM. The figure also contains a series of experiments in which the phosphatidylserine concn. (1.1 mM) was kept constant and the procaine·HCl concentration ranged from 0.7 to 56 mM.

Fig. 4. Variation with mole % tetracaine·HCl of the line width in cycles/sec. Curve a, the aromatic protons (Signal a); Curve b, the methyl protons of the –$\overset{H}{\underset{|}{N}}$–($CH_3$)$_2$ group (Signal f). Concn. of phosphatidylserine varying from 0.24 to 25 mM concn. of tetracaine·HCl = 5.4 mM.

* Both with procaine·HCl and with tetracaine·HCl the Signals a and b (Figs. 1B and 2B) arising from the aromatic protons are broadened to the same extent.

while Signals a and f are still noticeable. When the line width is plotted as a function of the mole fraction of procaine·HCl, a straight line relationship is obtained with both signals of procaine·HCl (Fig. 3). This is not observed with either signal of tetracaine·HCl (Fig. 4).

When procaine·HCl and tetracaine·HCl are mixed with lecithin neither the signals arising from the local anaesthetic molecules nor from the lecithin are broadened. If procaine·HCl is added to a mixture of phosphatidylserine and lecithin, all the procaine signals (except Signal f which cannot be examined because of overlapping with the alkyl chain signal) are broadened. The methyl signal of the choline group of lecithin is also broadened, whereas the signal arising from the $-CH_2$ chain protons shows no noticeable broadening. In a mixture of lecithin and phosphatidylserine (without procaine·HCl), neither the methyl signal of the choline group nor any other signal sufficiently resolved appears to be broadened. No line broadening is observed when acetylcholine chloride or tetramethylammonium chloride is added to phosphatidylserine.

Aqueous dispersions of phosphatidylserine (2%, w/v) are precipitated both by procaine·HCl and tetracaine·HCl, if the concentration is higher than 100 and 10 mM, respectively (Fig. 5). Lecithin dispersions (2%, w/v) cannot be precipitated by the addition of procaine·HCl or tetracaine·HCl. Both acetylcholine chloride and tetramethylammonium chloride do not at any concentration precipitate aqueous dispersions of lecithin or phosphatidylserine.

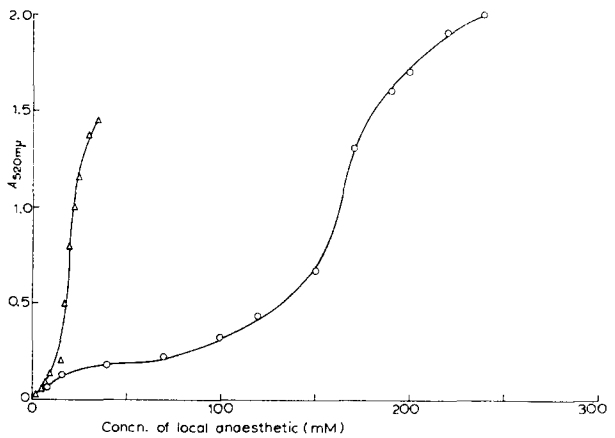

Fig. 5. The effect of procaine·HCl (⊙—⊙) and tetracaine·HCl (△—△) on the turbidity of phosphatidylserine dispersions (20 mg/ml) in water. The turbidity was measured at 520 mμ in a spectrophotometer.

The line widths of the individual anaesthetic molecules were measured at different concentrations from 0.005 M to 1 M and 0.005 M to a saturated solution for procaine·HCl and tetracaine·HCl, respectively. Over this concentration range, no difference was observed in the line widths of any of the signals.

Phosphatidylserine was precipitated by the addition of procaine·HCl to a concentration > 0.2 M. The precipitate was filtered off, and, on addition of an excess of $CaCl_2$ to an aqeuous suspension of the phosphatidylserine–procaine precipitate, procaine was released. An NMR spectrum was obtained from the supernatant in which the line width of the signals was identical with an aqueous solution of pure procaine·HCl.

DISCUSSION

The idea that both electrostatic and also Van der Waals dispersion forces may be involved in the mode of action of procaine and tetracaine molecules has been suggested by FEINSTEIN[8] and BLAUSTEIN AND GOLDMAN[4]. Furthermore, tetracaine·HCl with the same number of positive charges as procaine·HCl and with a chemical structure only slightly different from procaine is much more potent as a local anaesthetic[6,9].

Consistent with this idea are the results of the precipitation experiments. Phosphatidylserine dispersions (2 %) are precipitated by procaine·HCl or tetracaine·HCl solutions of concentrations higher than 100 and 10 mM, respectively (Fig. 5). The isoelectric point of phosphatidylserine[11] in 0.1 M NaCl is 1.2. Thus at a pH of 5.5, it would behave as an anion. The pK_a values of the tertiary aliphatic amino group and the aromatic amino group of procaine·HCl are 9.05 and 2.2, respectively. At a pH of 5.5 the alkyl tertiary amino group of the local anaesthetics would bear a positive charge. Lecithin[10] which has no net charge at pH 5.5 cannot be precipitated by procaine·HCl or tetracaine·HCl at this pH. It can therefore be concluded that electrostatic forces are involved in the interaction between the local anaesthetics and phosphatidylserine. In contrast to the local anaesthetics, small organic ions such as methylammonium and ethylammoniumchloride or the quaternary ions such as tetramethylammonium chloride or acetylcholine chloride at any concentration do not precipitate an aqueous dispersion of phosphatidylserine (2 %). Long chain quaternary ammonium salts such as cetyltrimethylammonium bromide or benzyldimethyl-*n*-hexadecylammonium chloride, however, precipitate 2 % dispersions of phosphatidylserine at pH 5.5; the precipitate redissolves in an excess of long chain quaternary ammonium salts. This indicates that the electrostatic forces between positively charged short chain ammonium salts and phosphatidylserine are not strong enough to cause interaction and precipitation. However in addition to electrostatic forces, the presence of other forces, as with long chain ammonium salts and probably with local anaesthetics, leads to an interaction and precipitation of phosphatidylserine. It seems that the strength of the electrostatic field at the positive charge must be important because the positive charge on the quaternary group of acetylcholine is less efficient than the tertiary ammonium group of procaine·HCl. Unpublished electrophoretic measurements of phosphatidylserine aggregates in aqueous dispersion show that the ζ potential of the phosphatidylserine particles in the presence of acetylcholine is much less changed than in the presence of an equivalent amount of procaine·HCl and still less changed than in the presence of an equivalent amount of NaCl. This indicates that the quaternary ammonium group of acetylcholine is less efficient as a counter ion than is Na^+.

Consistent with the precipitation experiments, the NMR spectra of the local anaesthetic molecules examined show a line broadening with phosphatidylserine but not with lecithin dispersions. In principle, line broadening can be due to various broadening mechanisms such as a local anaesthetic–local anaesthetic interaction, a bulk viscosity change and a magnetic inhomogeneity broadening. For the following reasons we conclude that the observed line broadening arises from an interaction between the local anaesthetic molecule and the phospholipid aggregate. (a) The line widths of the NMR spectra of procaine·HCl and tetracaine·HCl do not vary with concentration. (b) At a constant phospholipid concentration the line width from the

signals of the local anaesthetic molecules decreases as the concentration of the local anaesthetic molecule increases (Figs. 3 and 4). This is not consistent with the line broadening due to local anaesthetic–local anaesthetic interaction or to a general viscosity increase with an increasing local anaesthetic concentration or to a general inhomogeneity effect.

FISCHER AND JARDETZKY[12] have examined the interaction of small molecules with proteins using NMR spectroscopy and have shown that differential broadening sometimes occurs in the spectrum of the small molecules. We are concerned with the interaction of local anaesthetic molecules with large phospholipid aggregates. The molecular weight[11] of aggregates of the mono-sodium salt of phosphatidylserine in distilled water is $4 \cdot 10^6$; the molecular weight[13] of aggregates of lecithin in distilled water is $2 \cdot 10^6$. When one molecule interacts with another, the Brownian motion associated with the interacting part of the molecule will be slowed down, and consequently the magnetic relaxation rates of the interacting part will be increased. In liquids the spin–lattice relaxation rates are directly related to the NMR line widths. Thus an increase in line width will indicate a molecular interaction. A differential increase of one signal as compared to others will indicate that this part of the molecule from which the signal originates is more affected by the interaction than the rest of the molecule.

As a differential line broadening is observed with procaine·HCl and tetracaine·HCl, it could be considered that the anaesthetic molecule, whilst attached to the phospholipid by electrostatic forces, possesses because of steric hindrance a differential flexibility of the various parts of the molecule. We conclude that, in addition to the electrostatic interaction between the local anaesthetic molecule and phosphatidylserine, there are additional interaction forces present acting on different parts of the local anaesthetic molecules. In this case, line broadening reflects an inhibition of various parts of the local anaesthetic molecule in their movement due to these additional forces.

Precipitation experiments and NMR spectroscopy show that interaction only occurs when an electrostatic attraction can take place. Ion–ion interaction may be necessary to bring the local anaesthetic molecule in close vicinity to the phosphatidylserine molecule, enabling additional electrostatic forces of short range such as ion–dipole, dipole–dipole, ion–induced dipole, dipole–induced dipole forces and Van der Waals dispersion forces to become effective.

The additional interaction energy due to the butyl group on the aromatic amino group of tetracaine·HCl could account for the greater line broadening observed with tetracaine·HCl and its higher efficiency as a precipitant for phosphatidylserine dispersions and as a local anaesthetic.

Further evidence for additional forces besides the ion–ion attraction being effective in the interaction between local anaesthetics and acidic phospholipids was put forward by HAUSER AND DAWSON[14]. They investigated the displacement by local anaesthetics and other bases of Ca^{2+} adsorbed on monomolecular films of acidic phospholipids. These authors showed that tetracaine·HCl and procaine·HCl differ in their ability to displace Ca^{2+} by a factor of 7.5, and tetracaine·HCl was found to be several hundred times more effective than Na^+ or K^+ in displacing Ca^{2+}.

The nature of the differential line broadening should enable us to deduce which parts of the molecules are most affected by the additional forces. In the case of the anaesthetic molecules examined, the differential line broadening shown in Fig. 3 pro-

vides evidence for an interaction being in the region of the benzene nucleus. In this connection it should be mentioned that amines of similar chain lengths as local anaesthetics can interact electrostatically but do not have anaesthetic properties, unless a benzene ring is introduced into their structure[15].

It may be noted that in the spectra of mixtures of sonicated phosphatidylserine dispersions and local anaesthetics, there is no appreciable broadening of the lipid chain signal. These spectra can be compared with those of similar mixtures of phosphatidylcholine and cholesterol[17] or phosphatidylserine and cyclic polypeptides[18] where the chain signal is drastically broadened, indicating an hydrophobic interaction between cholesterol, *etc.* and the lipid chains. We conclude that the lipid–local anaesthetic interaction does not appreciably affect the motion of the lipid chains and is primarily in the polar region of the lipid rather than in the hydrophobic region.

In principle, provided the interaction is reversible*, the NMR data can be used to obtain the apparent equilibrium constant for the interaction between phosphatidylserine and local anaesthetic molecules.

In order to obtain the apparent equilibrium constant, the fraction F of the molecules which are bound must be determined. F can be obtained from the line width data (Fig. 3), using a treatment similar to that of FISCHER AND JARDETZKY[12].

$$F = \frac{\Delta\nu_{obs} - \Delta\nu_{free}}{\Delta\nu_{bound} - \Delta\nu_{free}}$$

where $\Delta\nu_{free}$ = line width of the free local anaesthetic molecule; $\Delta\nu_{bound}$ = line width of the bound molecule; $\Delta\nu_{obs}$ = line width of the local anaesthetic molecule observed at a certain mole fraction. With procaine·HCl a plot of the line width as a function of the mole fraction shows a straight line relationship (Fig. 3).

Analysis of the straight line relationship leads to the following expression for F (see Fig. 3).

$$F = \frac{P_{(b)}}{P_{(t)}} = \frac{PS_{(t)}}{PS_{(t)} + P_{(t)}} \qquad (1)$$

$P_{(t)}$ = total concentration of procaine·HCl; $P_{(b)}$ = concentration of bound procaine·HCl; $PS_{(t)}$ = total concentration of phosphatidylserine (PS).

Attempts to apply the mass equation in the form phosphatidylserine + drug $\rightleftharpoons$ (phosphatidylserine–drug) failed to give constant values for the apparent equilibrium constant K both in the case of procaine·HCl and tetracaine·HCl. The K values decreased as the concentration of phosphatidylserine or local anaesthetic molecules increased. Therefore we assume that the equilibrium was of the type

$$PS + \text{Procaine} \rightleftharpoons PS^1 + \text{Procaine}^1 \ldots \qquad (2)$$

where PS^1 and procaine^1 refer to molecules in a modified state.

$$K = \frac{(PS^1)\, P_{(b)}}{(PS)_{free}\, P_{free}} \qquad (3)$$

(PS^1) = concn. of occupied binding sites; $(PS)_{free}$ = concn. of free binding sites; P_{free} = concn. of free procaine·HCl. Since $P_{free} = P_{(t)} - P_{(b)}$

* An indication that the reaction is reversible is obtained by the behaviour of a procaine–phosphatidylserine precipitate in the presence of an excess of $CaCl_2$. Also, on dialysis of a procaine–phosphatidylserine precipitate against distilled water, procaine·HCl is found in the dialysate.

$$(PS)_{free} = n\,PS_{(t)} - P_{(b)}$$

where n = number of binding sites per phospholipid molecule and since $(PS^1) = P_{(b)}$ Eqn. 3 can be written

$$\frac{P_{(b)}}{P_{(t)}} = \frac{n\,PS_{(t)}}{n\,PS_{(t)} + P_{(t)}} + \frac{P_{(b)}^2\left[1 - \frac{1}{K}\right]}{P_{(t)}[P_{(t)} + n\,PS_{(t)}]} \qquad (4)$$

Eqn. 1 which is derived form the experimental relation between line width and mole fraction (Fig. 3) is a special case of the general Eqn. 4. Only if $n = 1$ and $K \longrightarrow 1$ does Eqn. 4 reduce to Eqn. 1.

Thus the experimental data in Fig. 3 are accounted for by the mass equilibrium applied to the phospholipid–local anaesthetic interaction in form of Eqn. 2 when $K \longrightarrow 1$, and the interaction takes place in a 1:1 ratio.

Experimental data shown in Fig. 4 do not suffice for the calculation of an n value by a similar application of the mass equation to the interaction between tetracaine·HCl and phosphatidylserine.

The finding of an interaction between phosphatidylserine molecules and procaine·HCl in the ratio of 1:1 is consistent with results obtained by SHANES AND GERSHFELD[16] who found that the penetration of stearic acid monolayers by procaine·HCl is limited to the formation of an equimolar mixed film of stearate–procaine. From the interaction between phosphatidylserine molecules and local anaesthetic in a ratio of 1:1, we conclude that the hydrophilic head groups of all phosphatidylserine molecules are accessible to the local anaesthetic molecule. This is consistent with results obtained by ABRAMSON *et al.*[11] on the titration of sonicated phosphatidylserine dispersions.

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