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# THE INTERACTION OF LOCAL ANAESTHETICS WITH SYNTHETIC PHOSPHOLIPID BILAYERS

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**Abstract**—The preferred position and orientation of two local anaesthetics in lipid bilayers has been determined using fluorescence quenching techniques. The aromatic amine of tetracaine and butesin quenches the fluorescence of a series of *n*-(9-anthroyloxy) fatty acids ($n = 2,6,9,12,16$) which place a fluorophore at a graded series of depths from the surface to the centre of a bilayer. A fluorescence method is used to resolve partition coefficients in the transverse plane of the membrane. The results show that the anaesthetics take up a distribution of positions about one or more preferred maxima. The aromatic amine group of tetracaine appears to be buried deep in the bilayer whereas the same group in butesin assumes positions at the surface as well as in the interior of the membrane.

The mechanism by which local anaesthetics block nerve conduction has been the subject of vigorous research and controversy (for review see Ref. 1). Classically, the lipid of the plasma membrane has been considered the site of action due to the correlation between oil–water partition coefficients and activity [2]. Proposed mechanisms have taken into account the lateral expansion of the lipid bilayer [3], increases in bilayer thickness [4], and changes in the fluidity of the bilayer [5]. All theories propose that a change in the physical properties of the lipid will indirectly affect membrane proteins through protein–lipid interactions. However, high lipid solubility might also be a prerequisite for the direct interaction of these drugs with hydrophobic sites on membrane proteins [6]. Direct theories of anaesthetic action must necessarily propose different sets of sites on membrane proteins to account for the diversity of structure of different classes of anaesthetic molecules. It seems likely that these sites will be in the highly lipophilic region of the protein–lipid interface.

Lipid solubility remains the primary event in most theories, and thus the orientation and position of the local anaesthetics within the bilayer becomes important for any mechanistic description of anaesthetic action. This paper describes the use of a series of *n*-(9-anthroyloxy) fatty acids ($n = 2,6,9,12,16$) to probe the nature of the anaesthetic–phospholipid interaction. The fluorophores of the probes locate at a graded series of depths from the surface to the centre of the bilayer [7–9]. Amine local anaesthetics are effective fluorescence quenchers, and we examine the possibility of defining the location of the quenching group and of thus defining the position and orientation of the local anaesthetic molecule within the bilayer. The quenching properties will also provide a means of determining quantitatively the partition of the local anaesthetics between lipid and aqueous phases.

## MATERIALS AND METHODS

*Materials.* Phosphatidylserine (monosodium) from bovine spinal cord was obtained from Lipid Products (U.K.). DL-α-Dipalmitoyl phosphatidylcholine was from Sigma as was tetracaine (free base and salt), procaine (free base and salt), butesin and benzocaine. *N*-methyl-*p*-amino benzoate was from Fluka. The anthroyloxy fatty acid probes were synthesized in this laboratory from anthracene-9-carboxylic acid and the appropriate *n*-hydroxy fatty acid by mixed anhydride synthesis [7, 10].

*Methods.* Liposomes were prepared by established procedures [7]. Briefly, phospholipid in 1:1 chloroform/methanol was dried as a film on the side of a sample vial and stored overnight under reduced pressure to remove residual solvent. Buffer (carbonate, $I = 0.01$ M, pH 9.5) or distilled water was added to give the required lipid concentration and the material was sonicated under nitrogen using an MSE 100 watt ultrasonic disintegrator. The suspensions were incubated for 30 min above the phase transition temperature to ensure annealling of vesicles and to prevent their aggregation [11]. Preparations were then centrifuged at 1000 *g* for 5 min to remove any contaminating titanium particles. The volume of the lipid phase was calculated using a value of 0.984 ml/g for the partial specific volume of the phospholipid.

The fluorescent fatty acid probes were added to vesicle suspensions as small aliquots (10–25 μl) of a 1 mM stock solution in methanol. A probe: phospholipid ratio of no more than 1:300 ensured that most of the probe was bound to the lipid bilayer [12]. The final probe concentration was about 3.3 μM. Uptake

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Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; 2AP, 16AP, 2- and 16-(9-anthroyloxy) palmitic acid; 6AS, 9AS, 12AS, 6-, 9- and 12-(9-anthroyloxy) stearic acid.

of the probe was accomplished by standing suspensions at room temperature for 1 hr in the dark. Most experiments were conducted with DPPC vesicles at 20° where the phospholipid is in its crystalline state.

Human erythrocyte ghosts were prepared by the method of Dodge *et al.* [13] and were suspended at 30 $\mu$g membrane protein/ml in 1 mM imidazole–6 mM KCl buffer (pH 7.6).

Equilibrium dialysis was carried out in Perspex cells possessing a 1.5 ml cavity on each side of a semipermeable membrane (Visking dialysis tubing). Anaesthetic was introduced into the lipid free compartment, and the cells were rotated at 0.5 rpm for 48 hr at 20°. At equilibrium, anaesthetic concentrations in the lipid free compartments were determined spectrophotometrically using extinction coefficients determined in the laboratory [tetracaine, $1.069 \times 10^4\,M^{-1}\,cm^{-1}$ (305 nm); butesin, $1.723 \times 10^4\,M^{-1}\,cm^{-1}$ (284 nm)]. The amount of anaesthetic in the lipid phase ($m_L$) relative to the aqueous phase ($m_A$) was expressed:

$$\frac{m_L}{m_A} = \frac{[Q_L]V_L}{[Q_A]V_A} = K_p \cdot \frac{V_L}{V_A} \quad (1)$$

where $[Q_L]$ and $[Q_A]$ are the anaesthetic concentrations in each phase and $V_L$ and $V_A$ are the volumes of each phase. Partition coefficients ($K_p$) were determined from plots of $m_L/m_A$ versus $V_L/V_A$.

Steady state fluorescence measurements were made with a Hitachi–Perkin Elmer MPF-3 spectrofluorometer equipped with a thermostatted cell block. Excitation was at 365 nm and emission at 440 nm for phospholipid dispersions and 450 nm for methanolic solvents. Quenching titrations were carried out by adding aliquots of anaesthetic stock solution directly into the fluorescence cuvette. Fluorescence lifetimes were measured by the technique of single photon counting as previously described [7–9].

## THEORY

Complete details of the theory have been described elsewhere [14] and only essential details are summarized below.

The dynamic quenching of a membrane bound probe is determined by the fluorescence lifetime of the probe in the absence of the quencher ($\tau$), and by the bimolecular quenching constant ($k_q$) as well as the local concentration of quencher as determined by its partition coefficient ($K_p$) into the membrane. The modified Stern–Volmer equation becomes:

$$\frac{I_0}{I} - 1 = k_q \cdot \tau \cdot K_p \cdot [Q]_T \cdot \quad (2)$$

where $[Q]_T$ is the total concentration of the quencher and $I$ and $I_0$ are the fluorescence intensities in the presence and absence of the quencher, respectively. Rearranging equation 2 and introducing the volume fraction of the lipid phase ($V_L/V_A$):

$$\frac{I}{I_0 - I}[Q]_T = \frac{1}{k_q \cdot \tau} \cdot \frac{V_L}{V_A} + \frac{1}{k_q \cdot K_p \cdot \tau} \cdot \quad (3)$$

Plotting the left hand term against $V_L/V_A$ (i.e. a range of lipid concentrations), allows $k_q$ to be determined from the slope and $K_p$ from intercept. Both $K_p$ and $k_q$ should be independent of lipid concentration provided that the lipid concentration is sufficiently high to ensure that practically all the probe is bound to the lipid phase and that the quencher does not exceed the solubility limit in either phase. In the case of an ionisable quencher where only the neutral form is capable of partitioning into the lipid phase, equation 3 becomes:

$$\frac{I_0}{I_0 - I}[Q]_T = \frac{1}{k_q \cdot \tau} \cdot \frac{V_L}{V_A} + \frac{1}{k_q \cdot K_p \cdot \tau}(1 + 10^{(pH - pK)}). \quad (4)$$

## RESULTS

### *The quenching moiety*

The use of strategies based on fluorescence quenching to determine the orientation of local anaesthetics in biomembranes requires knowledge of the precise location of the fluorophore as well as identification of the chemical group on the quencher which is responsible for the diminution of fluorescence. It has been shown previously that the fluorophore of the *n*-(9-anthroyloxy) fatty acids ($n$ = 2,6,9,12,16) senses a series of environments from the surface to the centre of the bilayer [7–9]. Several lines of evidence indicate that the aromatic amine, rather than the tertiary amine, is the quenching moeity (for structure of local anaesthetics, see Fig. 1). For example, benzocaine and butesin both lack the tertiary amine yet are still powerful quenching agents. Control experiments in methanol–water mixtures showed that the ionization of the tertiary amine had little effect on the intrinsic quenching efficiencies. Moreover, aromatic amines in general (e.g. aniline, dimethylaniline) are known to be powerful quenching agents [15, 16]. Thus the dominant quenching group on the local anaesthetics appears to be the aromatic amine.

### *pH dependence of quenching*

The pH titration of the quenching of 12AS by tetracaine in DPPC vesicles is shown in Fig. 2. Only the neutral form of the amine is capable of quenching the membrane bound probe. The apparent p$K$ value is 8.5 and, as has been observed by other investigators, the charged form of the anaesthetic is unable to interact with neutral bilayers [17]. As expected, butesin, which has no ionisable tertiary amine, showed little pH dependence of quenching in the DPPC vesicle system and in the pH range studied (Fig. 2).

The situation is different with negatively charged bilayers (e.g. phosphatidylserine vesicles) where considerable quenching by the charged amine is found due to electrostatic interactions with the phospholipid head groups (results not shown).

### *Relative quenching efficiencies*

Figure 3 shows the quenching of the fluorescent fatty acids by tetracaine and butesin in DPPC vesicles. The relative quenching efficiencies are in the order 12AS > 9AS > 6AS > 16AP > 2AP for tetracaine and 12AS > 2AP > 6AS > 9AS > 16AP for

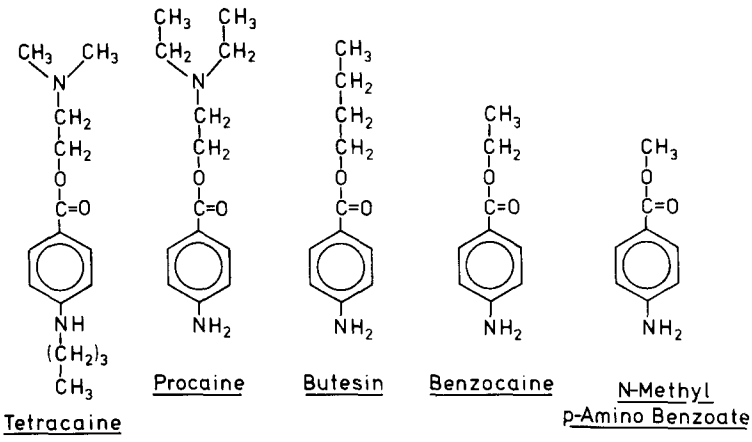


Fig. 1. Structure of the five compounds with local anaesthetic activity used in this study.

butesin. Quenching experiments in methanol–water mixtures showed that the relative quenching efficiencies were not due to differences in the intrinsic quenching efficiencies of the probes in free solution (i.e. intrinsic differences in $k_q$). The relative quenching efficiencies are therefore determined by the values of the partition coefficient and the bimolecular rate constant pertaining to each fluorophore–quencher pair. Of particular interest are the relative values of $K_p$ since it is possible that the local anaesthetics are not located at a single transverse position within the bilayer but take up a distribution of positions about one or two preferred maxima. The following experiments were designed to test this hypothesis by determining values of both $K_p$ and $k_q$ as a function of transverse position.

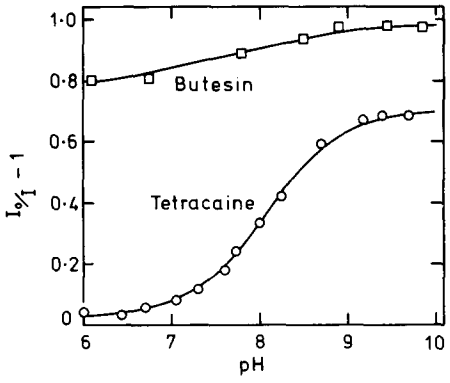


Fig. 2. pH titration of the quenching of 12AS by 0.2 mM tetracaine (○) and butesin (□) in 0.5 mM DPPC vesicles at 20°.

*Partition coefficients*

Figure 4 shows the dependence of quenching of 16AP by tetracaine on the concentration of phospholipid (DPPC). Values of the partition coefficient and the bimolecular quenching constant were obtained by plotting the data according to equation 3. Extending this analysis to five local anaesthetics provided the data summarized in Table 1. Tetracaine has a greater lipid solubility than procaine and the series of *n*-alkyl *p*-amino benzoate derivatives show partition coefficients which reflect relative hydrophobicity as determined by the size of their alkyl chain.

The possibility that there might be a concentration gradient of anaesthetic in the transverse plane of the bilayer was examined by comparing partition coefficients determined with each of the five fluorescent probes. The data are summarized in Fig. 5. The values of $K_p$ indicate that tetracaine and butesin occupy all transverse positions. However, the single

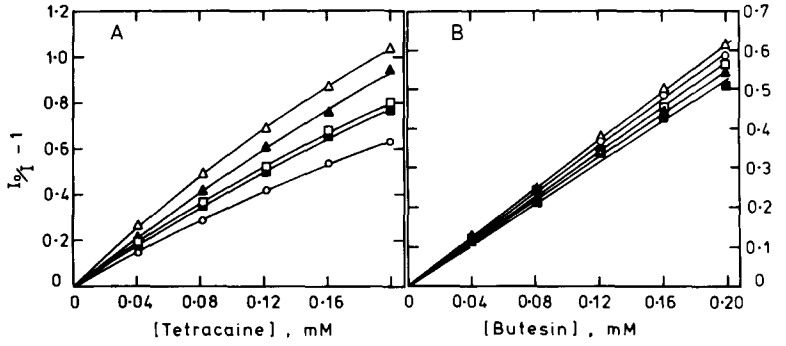


Fig. 3. Stern–Volmer plots of the quenching of each of the five fluorescent probes by tetracaine (A) and butesin (B) in 4 mM DPPC vesicles at 20° (pH 9.5). ○, 2AP; □, 6AS; ▲, 9AS; △, 12AS; ■, 16AP.

Table 1. Values of $K_p$ and $k_q$ obtained from the quenching of 16AP in DPPC vesicles by each of the five local anaesthetic compounds at pH 9.5, 20°

| Anaesthetic | $K_p$* | $k_q$* ($M^{-1}$ $nsec^{-1}$) |
|---|---|---|
| Tetracaine | 360 | 1.91 |
| Procaine | 224 | 0.60 |
| *N*-methyl *p*-amino benzoate ($n = 1$) | 210 | 0.21 |
| Benzocaine ($n = 2$) | 220 | 0.53 |
| Butesin ($n = 4$) | 455 | 1.00 |

* Calculated according to equation 3 assuming $\tau = 13.7$ nsec for 16AP. Values are ±4%.

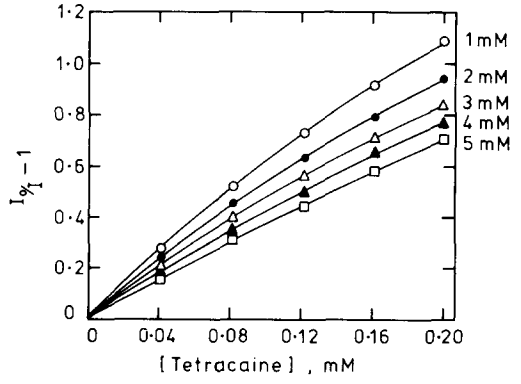


Fig. 4. Stern–Volmer plots for the quenching of 16AP by tetracaine at pH 9.5 (20°), and at several concentrations of DPPC vesicles.

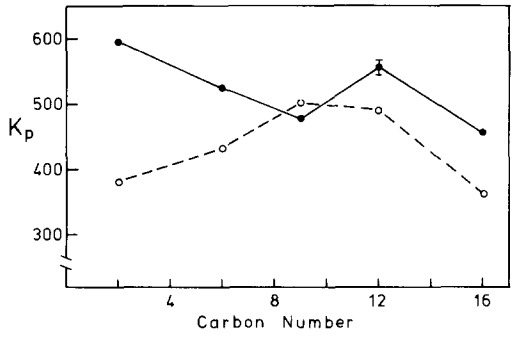


Fig. 5. Values of partition coefficients ($K_p$) for tetracaine (○-----○) and butesin (●——●) determined using the set of *n*-(9-anthroyloxy) fatty acids ($n = 2,6,9,12,16$). The error bar (±S.D.) was determined for triplicate samples of a single vesicle preparation in which 12AS was quenched by butesin. The bilayers were DPPC vesicles at 20°.

maximum in $K_p$ near position 9 for tetracaine suggests a preferred location of the aromatic amine group, and therefore of the tetracaine molecule in the bilayer. On the other hand, the maxima at positions 2 and 12 for butesin suggest that this anaesthetic is distributed between 2 positions or orientations. The corresponding values of $k_q$ ($M^{-1}$ $nsec^{-1}$) for the 2 through to the 16 positioned fluorophores were 1.94, 2.20, 2.17, 2.11, and 1.91 for tetracaine, and 1.07, 1.20, 1.28, 1.01, and 1.00 for butesin. Reconvolution of the data according to equation 3 showed that the relative quenching efficiencies depicted in Fig. 3 were determined predominantly by the differences in $K_p$ rather than those in $k_q$.

*Quenching in erythrocyte ghosts*

Erythrocyte ghosts were prepared to determine the relative quenching efficiencies in a natural membrane system. The results shown in Fig. 6 indicate that at pH 7.4 the quenching orders are 9AS > 6AS > 12AS > 2AP > 16AP for tetracaine, and 2AP > 6AS > 9AS > 12AS ≈ 16AP for butesin. The results also show that whereas the Stern–Volmer plots are linear for butesin, indicating a simple partitioning of butesin into the membrane, the plots for tetracaine are nonlinear in a manner which suggests saturable binding to the membrane. At pH 7.4 tetracaine exists predominantly in the charged form and will bind to acidic phospholipids in the natural membrane.

*Equilibrium dialysis*

The results of the equilibrium dialysis experiments at different volume fractions of the lipid phase were plotted according to equation 1. For DPPC vesicles the partition coefficients were 376 and 557 for tetracaine and butesin, respectively. The values agree

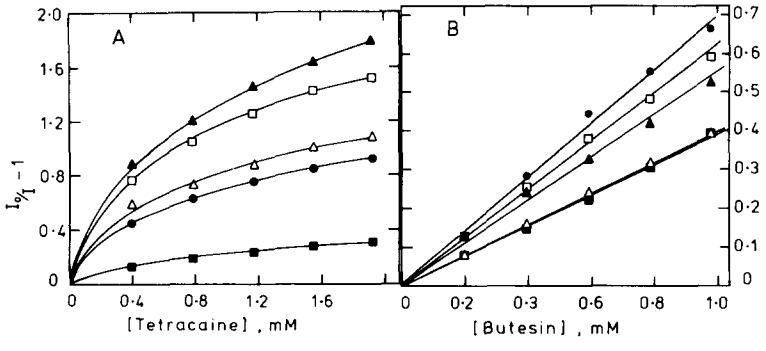


Fig. 6. Stern–Volmer plots of the quenching of each of the five fluorescent probes by tetracaine (A) and butesin (B) in erythrocyte ghosts (pH 7.4) at 20°. ●, 2AP: □, 6AS, ▲, 9AS; △, 12AS: ■, 16AP.

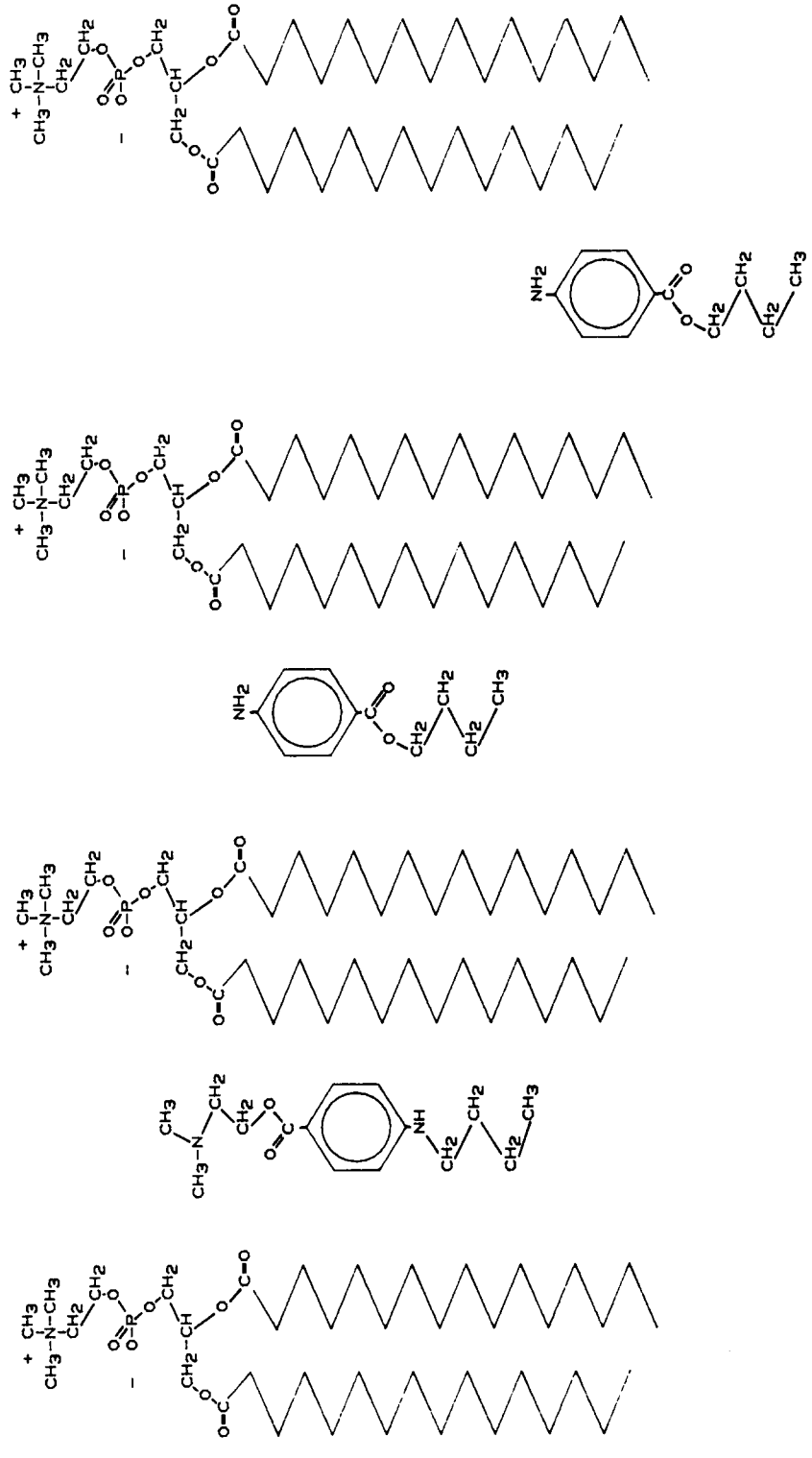

Fig. 7. Model for the interaction of neutral tetracaine and butesin with DPPC lipid bilayers. Note the quenching group (aromatic amine) lies near the 9 position of the phospholipid acyl chain for tetracaine. Two possible positions are shown for butesin.

well with those from the quenching experiments (Fig. 5), indicating that any perturbation of the bilayer structure by the fluorescent probe has little effect on the ability of anaesthetics to enter the membrane.

In both fluorescence and equilibrium dialysis determinations of the partition coefficients, considerable variation was observed between different vesicle suspensions, probably reflecting the difficulty of reproducing sonicating conditions from one preparation to another. Thus all comparisons between fluorescent probes or between local anaesthetics were made within single vesicle preparations.

## DISCUSSION

The orientation of an amphipathic molecule in a lipid bilayer depends on the magnitude, distribution and balance of the hydrophobic and hydrophilic forces involved. The problem has not been examined in detail except for simple molecules such as long chain alcohols and carboxylic acids where their effect on bilayer structure depends on the length of the acyl chain [18, 19]. Cholesterol is another class of amphipath which has been studied extensively [20].

The local anaesthetics such as procaine and tetracaine are unusual in that both the tertiary amine and the aromatic amine have some charge character, yet the methyl and methylene groups provide considerable hydrophobic character. The relative quenching efficiencies (Fig. 3) and the values of $K_p$ (Fig. 5) for tetracaine in its neutral state indicate that this anaesthetic is oriented in the bilayer so that its aromatic amine is near carbon 9 of the fatty acid. The two maxima seen for the corresponding data for butesin (Fig. 5) indicate that this molecule can occupy two positions or orientations in the bilayer. One such possibility is depicted in Fig. 7 where the aromatic amine of butesin is aligned with either carbon 2 or carbon 12 of the fatty acid. Alternatively, it is possible that a proportion of butesin molecules at a single transverse position is rotated 180° to bring about the same result. The distribution of a molecule between two transverse positions is not unusual. It has been seen previously with dimethylaniline in lipid bilayers [14] and detergent micelles [21]. For tetracaine, the amphipathicity is relatively high and only a single transverse position is favoured, whereas butesin has a more equal balance between hydrophilic and hydrophobic parts of the molecule so that 2 positions or orientations in the bilayer are more or less equally populated. A similar situation has been reported by Colley and Metcalfe [18] who found that the position of the hydroxyl group on *n*-aryl alcohols and on 1-phenyl alcohols could determine their orientation in the lipid bilayer.

At physiological pH the positively charged anaesthetics are unable to partition into the neutral bilayer. Such pH dependence is not seen for the neutral anaesthetics benzocaine and butesin. A similar conclusion was reached by Ueda *et al.* [22] on the basis of transition temperature depression for DPPC bilayers, and by Hille [23] on the basis of oil–buffer partition coefficients. The situation *in vivo* is different in that the membrane is negatively charged and may interact electrostatically with the positively charged anaesthetics. Such an interaction results in a nonlinear Stern–Volmer plot where the degree of quenching approaches a saturation level (Fig. 6A) and is better described as a binding process rather than a partition process [7, 12]. We further note that the orientation and position of a neutral anaesthetic such as butesin may differ in natural and synthetic membranes as indicated by the results in Figs. 3B and 6B where different orders of quenching efficiency are seen in the two systems. The effects on quenching of lipid heterogeneity, of lipid phase, and of the lateral segregation of lipid types are not presently understood.

Nevertheless studies with single nerve fibres have shown that the neutral base rapidly blocks the sodium channels (< 1 sec) while the charged anaesthetic may take several hundred seconds to do the same [24]. Again such pH dependence is not observed with benzocaine which lacks the tertiary amine. The data therefore imply that the neutral form is the more active species because of its ability to partition into the lipid phase and freely diffuse to a receptor whose binding site is oriented either towards the lipid phase or towards the aqueous phase at the inner surface of the membrane. Recent experiments by Ogden *et al.* [25] have also shown that charge *per se* does not have a critical role in the blockage of acetylcholine activated ionic current because both neutral and charged amine local anaesthetics can act at the same receptor.

Recently the binding of local anaesthetics to phospholipid bilayers has received considerable attention by many authors. X-ray diffraction studies by Coster *et al.* [26] and fluorescence studies on a procaine analogue by Trzos and Reed [27] have both reported results which agree with the model for tetracaine orientation put forward in Fig. 7. Furthermore results obtained by Boulanger *et al.* [28] using deuterium NMR support a strong binding site in the hydrophobic region of the bilayer. These authors also propose a second weak interaction with the phospholipid bilayer which may be similar to the model proposed by Feinstein [29] in the head group region of the bilayer.

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