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INTERACTION OF FATTY ACIDS WITH LIPID BILAYERS

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We present a method by which it is possible to describe the binding of fatty acids to phospholipid bilayers. Binding constants for oleic acid and a number of fatty acids used as spectroscopic probes are deduced from electrophoresis measurements. There is a large shift in pK value for the fatty acids on binding to the phospholipid bilayers, consistent with stronger binding of the uncharged form of the fatty acid. For dansylundecanoic acid, fluorescence titrations are consistent with the binding constants derived from the electrophoresis experiments. For 12-(9-anthroyloxy)stearic acid, fluorescence and electrophoresis data are inconsistent, and we attribute this to quenching of fluorescence at high molar ratios of 12-anthroylstearic acid to phospholipid in the bilayer.

Introduction

Fatty acids and their derivatives have been widely used as probes of membrane structure: nitroxide-labelled fatty acids in ESR studies, deuterium-labelled fatty acids in NMR studies, and anthroyl-labelled fatty acids and *cis*- and *trans*-parinaric acids in fluorescence studies. It is important, therefore, to understand how these probes interact with biological membranes. As hydrophobic molecules, the fatty acids could be expected to partition into the lipid component of the membrane but they could also bind to hydrophobic areas of membrane proteins. Elsewhere [1] we show how these two types of binding can be separated using dansyl-labelled fatty acids. Here we analyse the interactions of fatty acids with lipid bilayers by electrophoresis and by fluorescence.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid.

Materials and Methods

Lipids were obtained from Lipid Products and dansylundecanoic acid from Molecular Probes. Fluorescence spectra were recorded on Spex Fluorolog and Perkin Elmer MPF 44A fluorimeters. Lipids were dissolved in chloroform in 10 ml stoppered flasks and evaporated to dryness under a stream of nitrogen. Buffer (40 mM Hepes, 0.1 M NaCl, 0.1 mM EDTA, pH 7.2) was added and the mixture shaken on a vortex mixer. Fluorescence probes were generally added as methanol solutions, the final methanol concentration never exceeding 1%. Equilibration of 12-(9-anthroyloxy)stearic acid with pre-formed liposomes was very slow, so that in this case fatty acid was mixed with lipid in chloroform solutions before liposome formation. Liposomes were then left to equilibrate for 1 h at room temperature in the dark. Measurements of electrophoretic mobility were made on a Rank Bros. Mark I micro-electrophoresis apparatus. Care was taken to focus at the stationary layer. The buffer was 10 mM sodium phosphate,

10 mM NaCl, 0.1 mM EDTA.

Analysis of lipid binding data. In the analysis of fatty acid binding to lipid bilayers it is necessary to take account of the fact that fatty acids exist as mixtures of charged and uncharged species, the relative proportions of the two forms being given by the Henderson-Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K + \log([\mathrm{A}^-]/[\mathrm{HA}]) \tag{1}$$

where the pK of the fatty acid determines the relative amounts of the salt [A^-] and the acid [HA]. Both forms of the fatty acid can bind to the membrane, and we have shown elsewhere that binding can be described by Langmuir adsorption isotherms [2],

$$\sigma^{\mathrm{A}} = \frac{1}{K^{\mathrm{A}}}(\sigma^{\max} - \sigma^{\mathrm{A}} - \sigma^{\mathrm{HA}})[\mathrm{A}^-]_{x=0} \tag{2}$$

$$\sigma^{\mathrm{HA}} = \frac{1}{K^{\mathrm{HA}}}(\sigma^{\max} - \sigma^{\mathrm{A}} - \sigma^{\mathrm{HA}})[\mathrm{HA}]_{x=0} \tag{3}$$

where σ^{A} and σ^{HA} are, respectively, the number of molecules of A^- and HA adsorbed to the membrane per unit area, $\sigma^{\max}$ is the maximum number of molecules adsorbed per unit area, K^{A} and K^{HA} are dissociation constants for binding of A^- and HA, respectively, and $[A^-]_{x=0}$ and $[HA]_{x=0}$ are the aqueous concentrations of A^- and HA, respectively, at the membrane-solution interface, $x = 0$. The two dissociation constants are related by [2],

$$\frac{K^{\mathrm{A}}}{K^{\mathrm{HA}}} = \exp(2.303\Delta \mathrm{p}K) \tag{4}$$

where ΔpK is the shift in pK on binding. The concentration of HA close to the surface will be equal to the bulk concentration, but the concentration of A^- at the surface will be less than the bulk concentration of A^- because of the negative charge on the membrane that results from the binding of A^-. The charge effect can be described by the Boltzmann relationship,

$$[\mathrm{A}^-]_{x=0} = [\mathrm{A}^-]_{\mathrm{bulk}} \exp(F\psi_{\mathrm{o}}/RT) \tag{5}$$

where ψ_{o} is the electrostatic potential in the aqueous phase adjacent to the membrane. In turn, the potential depends on the surface concentration of bound A^-, according to the Grahame equation,

$$\sigma^{\mathrm{A}} = \left[2 \cdot 10^3 \cdot \varepsilon_{\mathrm{r}}\varepsilon_0 RT \sum_i C_i\{\exp(-Z_i F\psi_{\mathrm{o}}/RT) - 1\}\right]^{1/2} \tag{6}$$

where C_i is the concentration (mol/litre) of the ith ionic species of valence Z_i, ε_{r} is the relative permittivity of water (78.54 at 25°C), ε_0 is the permittivity of free space ($8.854 \cdot 10^{-12}$ F · m^{-1}), RT/F has the value 25.7 mV at 25°C, and the factor of 10^3 converts from a volume unit of litres to m^3.

We can write an equation analogous to the Henderson-Hasselbach equation (Eqn. 1) for the bound drug as,

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{b}} + \log(\sigma^{\mathrm{A}}/\sigma^{\mathrm{HA}}) \tag{7}$$

where pH is the pH of the bulk solution and pK_{b} is the apparent pK for the bound fatty acid. This apparent pK contains terms reflecting both the difference in binding constants for the charged and uncharged forms of the fatty acid (Eqn. 4) and the difference in pH values between the surface and the bulk solution, caused by the charge on the membrane. We therefore write pK_{b} as,

$$\mathrm{p}K_{\mathrm{b}} = \mathrm{p}K' + \Delta \mathrm{p}K \tag{8}$$

where pK' is an apparent pK accounting for the decrease in local pH in the interfacial region that results from the build-up of a negative surface potential. The proton concentration in the solution immediately adjacent to the membrane surface is related to the bulk concentration by,

$$[\mathrm{H}^+]_{x=0} = [\mathrm{H}^+]_{\mathrm{bulk}} \exp(-F\psi_{\mathrm{o}}/RT) \tag{9}$$

Thus, pK', which is expressed in terms of $[H^+]_{\mathrm{bulk}}$ will be related to pK, which is expressed in terms of the true local proton concentration $[H^+]_{x=0}$, by

$$\mathrm{p}K' = \mathrm{p}K - (F\psi_{\mathrm{o}}/2.303RT) \tag{10}$$

The apparent pK for the bound drug is therefore,

$$\mathrm{p}K_{\mathrm{b}} = \mathrm{p}K - (F\psi_{\mathrm{o}}/2.303RT) + \Delta \mathrm{p}K \tag{11}$$

The relative concentrations of the charged and uncharged forms of the fatty acid bound to the membrane are then given by,

$$\sigma^{\mathrm{HA}} = \sigma^{\mathrm{A}}/10^{(\mathrm{pH} - \mathrm{p}K_{\mathrm{b}})} \tag{12}$$

Since an appreciable fraction of the fatty acids are bound to the membrane, it is necessary to write,

$$[A]_{total} = [A]_{bnd} + [A]_{free} \quad (13)$$

where the subscripts denote fatty acid bound to the membrane and present in the bulk aqueous phase and $[A]_{total}$ is the total fatty acid concentration. The concentration of bound fatty acid expressed as mol/litre is related to the concentration in units of molecules/Å^2 by,

$$[A]_{bnd} = r(\sigma^A + \sigma^{HA}) \quad (14)$$

where r is the area of the membrane in units of mol · Å^2 per litre. The total area of the membrane is given by,

$$r = ([Lipid] r_{lipid} + [A]_{bnd} r_A) \quad (15)$$

where [Lipid] is the concentration of lipid and r_{lipid} and r_A are, respectively, the areas per molecule of a lipid and a fatty acid molecule in the membrane. These equations can be solved numerically, as follows. For a particular value of ψ_o, σ^A is calculated from the Grahame equation (Eqn. 6). From this, σ^{HA} can be calculated from Eqns. 11 and 12. $[A]_{bnd}$ can then be calculated from Eqn. 14, and $[A]_{free}$ from Eqn. 13. This allows the calculation of $[HA]_{free}$ from Eqn. 1 which, on substitution into Eqn. 3 gives a new value of σ^{HA}. The ratio of σ^A/σ^{HA} then gives a new value of ψ_o from Eqns. 11 and 12. The procedure is then repeated until the initial and final values for ψ_o agree to within the desired accuracy. The set of transcendental equations is solved by the Bolzano method [3].

The zeta potential, ζ, the potential at the hydrodynamic plane of shear, was calculated from the measured value of the electrophoretic mobility $\bar{u}$ by the Helmholtz-Smolouchowski equation:

$$\zeta = \frac{\bar{u}\eta}{\varepsilon_r \varepsilon_0} \quad (16)$$

where η is the viscosity of the aqueous phase, ε_0 is the permittivity of free space and ε_r is the dielectric constant. The relationship between ζ and the surface potential, ψ_o, is discussed by Aveyard and Haydon [4]. Eisenberg et al. [5] have concluded that ζ corresponds to the potential 2 Å from the surface of the membrane. This can be calculated from the surface potential ψ_o using the equation derived for the special case of a solution containing only monovalent and/or divalent ions [6]:

$$\psi_x = -\frac{RT}{F} \ln \left\{ \frac{(Q_x+1)^2 + 4\bar{\alpha}(Q_x - \alpha)}{(Q_x-1)^2 - 4\alpha(Q_x - \bar{\alpha})} \right\} \quad (17)$$

where $Q_x = A(y_o)\exp(\kappa x)$

$$A(y_o) = \frac{2\eta(\alpha y_o^2 + y_o + \bar{\alpha})^{1/2} + y_o(2\alpha+1) + 2\bar{\alpha} + 1}{(y_o - 1)}$$

$$y_o = \exp(-F\psi_o/RT)$$

$$\eta = (\alpha + 1 + \bar{\alpha})^{1/2}$$

$$\alpha = \frac{C_2(\infty)}{(C_1(\infty) + 2C_2(\infty))}$$

$$\bar{\alpha} = \frac{C_{-2}(\infty)}{(C_1(\infty) + 2C_2(\infty))}$$

$$\kappa = \left[\sum_{i=-2}^{+2} \frac{(i^2 C_i(\infty)) e^2 \cdot 10^3}{\varepsilon_r \varepsilon_0 RT} \right]^{1/2}$$

Here ψ_x is the potential at a distance x from the surface in a solution where the bulk concentration of the species of charge i is $C_i(\infty)$. For electrophoresis experiments as a function of pH in phosphate buffer systems, account was taken of the variation in concentrations of monovalent and divalent ionic species.

The measured zeta potentials were fitted to the theoretical equations developed above. The known parameters are the pK of the fatty acid in bulk solution, the pH and ionic composition of the bulk solution, and the total concentrations of fatty acid and phospholipid. From the measured zeta potentials we can calculate the surface potential, ψ_o (Eqn. 17). The unknown parameters required to fit the surface potential to the binding parameters are: the areas occupied by a lipid and a fatty acid molecule in the membrane; the maximum number of fatty acid molecules that can be adsorbed per unit area (σ^{max}); the dissociation constant for the binding of one form of the fatty acid chosen, for convenience, to be K^{HA} for the uncharged form HA; and the shift in pK on binding, ΔpK, which then gives the dissociation constant for binding of the other form (A^-) from Eqn. 4.

Fluorescence titrations. Under conditions where the effects of charge can be ignored, the fluorescence titration data should follow the equation:

$$F = \alpha(L_o - [EL]) + \beta[EL] \quad (18)$$

where α and β are coefficients describing the fluorescence intensity of probe in aqueous solution and bound to the membrane, respectively, L_o is the total probe concentration and [EL] is the concentration of bound probe, given by:

$$[EL] = \frac{A - \sqrt{A^2 - 4nE_oL_o}}{2} \quad (19)$$

with

$$A = K_d + nE_o + L_o \quad (20)$$

Here K_d is the dissociation constant, n is the number of binding sites and E_o is the phospholipid concentration. Fluorescence data was fitted directly to Eqn. 18 by a derivative-free, non-linear, least-squares technique [7]. The ratio of the square root of the residual sum of squares to the mean fluorescence value was taken as a measure of the coefficient of variation. The K_d value calculated in this way is an apparent value, being a weighted average of K^A and K^{HA} (Eqns. 2 and 3), depending on the pH of the medium.

Results

Particle electrophoresis

Since we wish to use electrophoresis data to obtain binding constants for fatty acids to lipid bilayers, it is important to show that the electrophoretic properties of the liposomes are explainable in terms of the simple Gouy-Chapman theory outlined above. In a recent paper, Hauser et al. [8] have reported relatively low electrophoretic mobilities for liposomes containing negatively charged phospholipids and fatty acids, which are inconsistent with simple theory. In contrast, Eisenberg et al. [5] report much higher mobilities for liposomes containing negatively charged phospholipids, which are consistent with theory.

Fig. 1 shows our data for the electrophoretic mobility of liposomes of phosphatidylcholine containing phosphatidylserine in buffer containing EDTA to complex any divalent metal ions present in the system. These mobilities agree with the data reported by Eisenberg et al. [5] and, as shown in Fig. 1B, zeta potentials calculated from the mobility data agree with zeta potentials calculated theoretically for the given molar ratios of negatively charged lipid, assuming an area per phospholipid molecule of 60 Å^2, and putting the hydrodynamic plane of shear 2 Å from the surface.

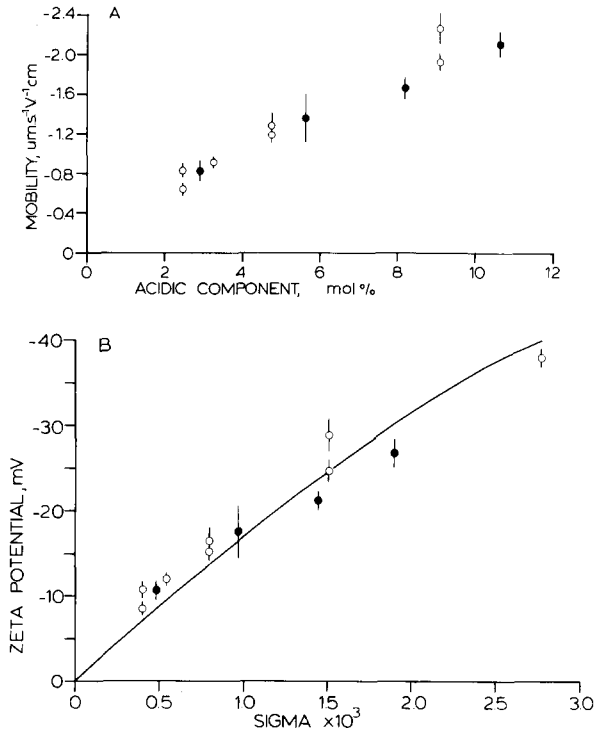

Fig. 1. (A) Electrophoretic mobilities of liposomes of egg phosphatidylcholine (0.275 mM) containing either brain phosphatidylserine (○, pH 7.4) or oleic acid (●, pH 11.0) at the given mol fraction. Buffer; 1 mM Mops, 0.1 M tetramethyl ammonium chloride, 1 mM disodium EDTA. Symbols represent mean ± standard deviation of 10 measurements. (B) Comparison of experimental and theoretical zeta potentials (mV) as a function of surface charge density sigma (electronic charges per Å^2) for liposomes of egg phosphatidylcholine (0.275 mM) containing either brain phosphatidylserine (○, pH 7.4) or oleic acid (●, pH 11.0). Buffer as above. Symbols represent values calculated from electrophoretic mobilities of Fig. 1A. The solid line shows the theoretically calculated dependence of zeta potential on surface charge density, assuming the place of shear to be located 2 Å from the surface. Surface charge densities were calculated assuming that lipids occupy an area of 60 Å^2 per molecule and that oleic acid occupies 20 Å^2 per molecule.

Electrophoretic mobilities in the presence of oleic acid at high pH (11.0) such that all of the acid will be in the ionised form, are also shown in Fig. 1A for the case where the concentration of phospholipid is sufficiently high that all the oleic acid will be bound. The concentration dependence of mobilities in the presence of oleic acid and phosphatidylserine are clearly very similar. Calculated zeta potentials agree closely with the measured values, assuming molecular areas of 20 $Å^2$ and 60 $Å^2$ for oleic acid and phosphatidylcholine, respectively (Fig. 1). When the experiments were repeated under the conditions reported by Hauser et al. [8], in the absence of any EDTA to complex divalent metal ions, we recorded electrophoretic mobilities very similar to those of Hauser et al. [8]. Thus, Hauser et al. [8] report an increment in electrophoretic mobility for stearic acid at pH 11.6 of -0.22 ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) per mol% and, under the same conditions, we find an increment of -0.24 for oleic acid at pH 11. We conclude that these measurements are artifactual due to the presence of contaminating Ca^{2+}.

In order to use electrophoretic data to calculate binding constants for fatty acids, we chose conditions under which only a fraction of the fatty acid will be bound to the liposomes. Under such conditions, electrophoretic mobilities as a function of fatty acid concentration and pH should be interpretable in terms of Eqns. 1–17. To produce a fit between experiment and theory, we need an estimate of σ^{max}, the maximum possible number of fatty acid molecules per $Å^2$ of the membrane surface. We can deduce a minimum possible value for σ^{max} by measuring electrophoretic mobilities at high fatty acid concentrations and at a pH where all the fatty acid will be ionised. Thus, in 10 mM sodium phosphate buffer containing 10 mM NaCl and 0.1 mM EDTA at pH 10.6 the zeta potential for liposomes in the presence of 1.1 mM oleic acid is -83 mV, corresponding to a surface potential of -99 mV (Eqn. 17). The surface concentration of fatty acid giving rise to this surface potential is, from Eqn. 6 $1/230$ $Å^2$, thus giving us a lower limit to σ^{max}. Differential scanning calorimetric data suggest that fatty acids can incorporate into phospholipid bilayers up to a molar ratio of at least 2 : 1 fatty acid to phospholipid [9]. Monolayer studies suggest an area per molecule of 20 $Å^2$ for oleic acid in monolayers at the air-water interface [10]. Taking 60 $Å^2$ for the area of a lipid molecule [11], a 2 : 1 molar ratio of fatty acid to phospholipid would correspond to a σ^{max} value of $1/50$ $Å^2$. As previously shown by McLaughlin and Harary [12] in a study of the binding of 2,6-toluidinylnaphthalene sulphonate to lipid bilayers, it is not possible to determine separately σ^{max} and the dissociation constant K from this type of experiment, although the quotient σ^{max}/K is well defined. Fig. 2 illustrates that the data can be well fitted to the equations derived above with a dissociation constant and shift in pK value given in Table I, taking the pK of the fatty acid in aqueous solution to be 5 [13], for a range of σ^{max} values greater than about $1/100$ $Å^2$.

The best fit to the data is obtained with $\Delta pK = 2.3$ and as illustrated in Fig. 2B, significantly worse fits are obtained with a ΔpK value of 2.0. Data for fatty acid probe molecules were calculated in the same way, using an area per molecule of 40 $Å^2$, the value for anthroyl stearic acid in a monolayer [10] (Table I).

Eisenberg et al. [5] demonstrated a significant degree of binding of sodium ions to the ionized carboxyl group of phosphatidylserine in liposomes composed of mixtures of phosphatidylserine and phosphatidylcholine, with a concomitant reduction in the observed zeta potential. Binding was characterised by an association constant of 0.6 M^{-1}. Using the same association constant to describe the possible interaction bteween Na^+ and the ionized forms of the bound fatty acids in our experiments, we calculate that Na^+ binding would make no significant contribution to the potentials we have observed, over the range of Na^+ concentration employed (10–30 mM).

Fluorescence titrations

Binding of the fatty acid dansyl undecanoic acid to lipid bilayers can also be studied using the increase in fluorescence emission for this molecule that follows from binding. Fig. 3 shows a fluorescence titration of dioleoylphosphatidylcholine with dansylundecanoic acid, in solutions containing 0.1 M NaCl. Using the binding constants derived from the electrophoresis data, we estimate that under the conditions of the fluorescence experiment, the surface potential on the liposomes that

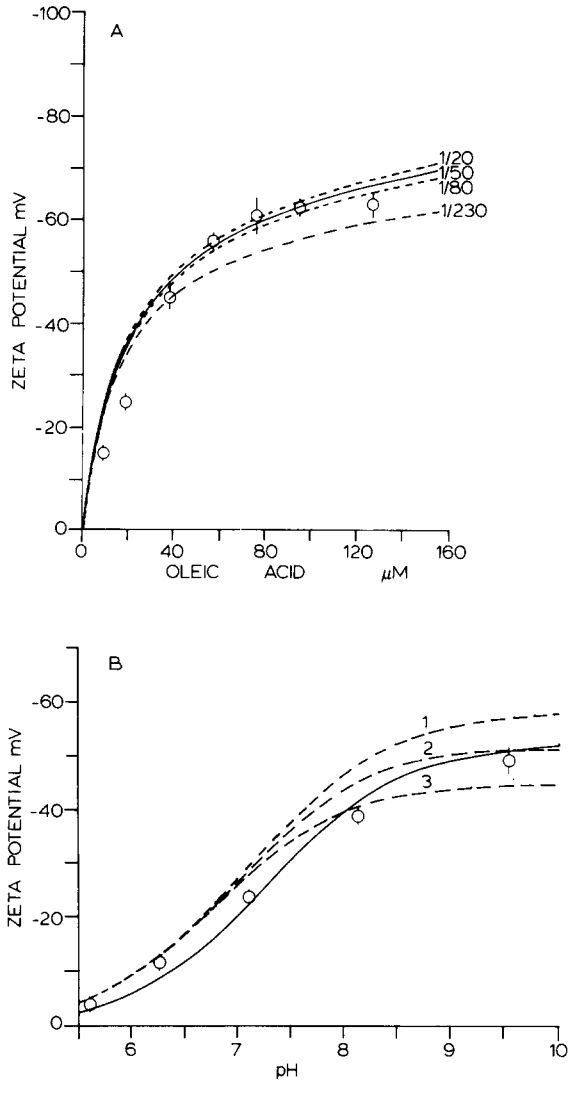

Fig. 2. (A) Zeta potentials for liposomes of egg phosphatidylcholine (0.277 mM) in the presence of the given total concentration of oleic acid at pH 10.1 in buffer (10 mM sodium phosphate, 10 mM NaCl, 0.1 mM disodium EDTA) at 25°C. Symbols represent the mean ± standard deviation for ten measurements. The solid line shows the predicted variation in zeta potential with oleate concentration calculated with the following parameters; $pK = 5.0$, $\Delta pK = 2.3$, $K_d = 1.5 \cdot 10^{-7}$, $\sigma^{max} = 1/50$ Å^2. The dotted lines were calculated with the same pK values but with other parameters as follows: $\sigma^{max} = 1/20$ Å^2, $K_d = 3.75 \cdot 10^{-7}$; $\sigma^{max} = 1/80$ Å^2, $K_d = 9.4 \cdot 10^{-8}$; $\sigma^{max} = 1/230$ Å^2, $K_d = 3.3 \cdot 10^{-8}$. (B) As for Fig. 2A, but at a fixed concentration of oleic acid (60 μM), varying pH. The solid line is the predicted variation in zeta potential, using the same parameters as used for the solid line in Fig. 2A. Parameters for broken lines are as follows; $pK = 5.0$, $\Delta pK = 2.0$, $\sigma^{max} = 1/50$ Å^2 and; 1, $K_d = 1.5 \cdot 10^{-7}$; 2, $K_d = 3 \cdot 10^{-7}$; 3, $K_d = 6 \cdot 10^{-7}$.

TABLE I

BINDING CONSTANTS FOR FATTY ACIDS TO EGG PHOSPHATIDYLCHOLINE CALCULATED FROM ELECTROPHORESIS DATA

Temperature, 25°C.

Fatty acid	σ^{max}	ΔpK	K_d (μM)
Oleic acid	1/20	2.3	0.38
	1/50	2.3	0.15
	1/80	2.3	0.09
Dansylundecanoic acid	1/40	2.1	7.0
	1/70	2.1	4.0
12-Nitroxystearic acid	1/40	2.2	1.75
	1/70	2.2	1.0
12-(9-Anthroyloxy)stearic acid	1/40	1.7	0.017
	1/70	1.7	0.010

results from fatty acid binding does not exceed −4 mV so that under these conditions the effects of charge can be ignored. The fluorescence titration can therefore be fitted directly to the binding equation, Eqn. 18.

The value of α describing the fluorescence intensity of dansylundecanoic acid in buffer was obtained from a linear least-squares fit of the

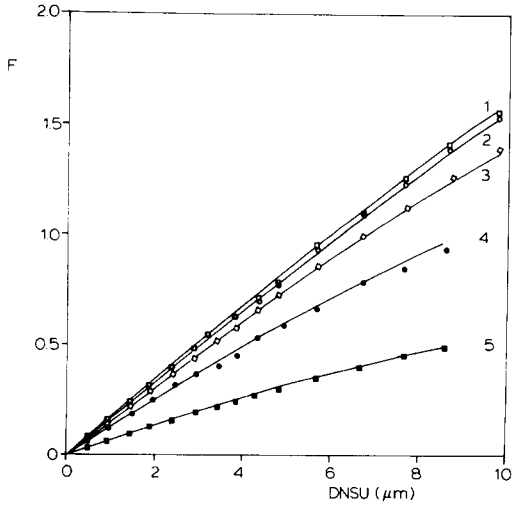

Fig. 3. Fluorescence titration of dansylundecanoic acid with dioleoylphosphatidylcholine at 23°C in buffer exciting fluorescence at 343 nm and measuring emission at 540 nm. Concentrations of lipid (μM): □ (1), 1332; ○ (2), 1066; ◇ (3), 533; ● (4), 266; ■ (5), 66.6. Solid lines, result of a non-linear least-squares fit.

fluorescence intensity in buffer versus concentration. Using this value of α, the results of a non-linear least-squares fit at 23°C are shown in Fig. 3, and the resulting parameters are given in Table II. Clearly the fit is good with a coefficient of variation of 0.24. Unfortunately, the values of the number of binding sites n and the dissociation constant, K_d, are highly correlated, with an estimated asymptotic correlation of 0.988. It is therefore possible to fix n at a wide range of values and obtain equally good fits. Thus, as shown in Table II, with n fixed at 2, an equally good fit occurs (coefficient of variation 0.27) but with a corresponding increase in K_d. At 37°C, a very different pair of values for n and K_d is obtained if all the parameters of the fit are left free, but with n fixed at 2 the K_d value obtained is very similar to that at 23°C. The fluorescence data agree well with electrophoresis data. Using the parameters derived from electrophoresis experiments (Table I), it is possible to calculate the concentration of dansylundecanoic acid bound under the conditions of the fluorescence experiment. Ignoring charge effects this data can then be expressed in the form of a binding equation:

$$K_{eff} = \frac{[\text{Lipid}][\text{free fatty acid}]}{[\text{bound fatty acid}]}$$

Taking $\sigma^{max} = 1/70$ Å^2, corresponding to two fatty acid binding sites per lipid, the value of K_{eff} calculated in this way varies between 540 and 570 μm over the course of the titration, agreeing well with the dissociation constant of 536 calculated from the fluorescence data for $n = 2$. The good agreement between the two types of experiment is also illustrated in Fig. 4 which compares the ratio of bound/total fatty acid as a function of phospholipid concentration as calculated from the electrophoresis data and as calculated from the fluorescence titration.

The fluorescence data obtained for anthroylstearic acid are harder to interpret. As shown in Fig. 5 a reasonable fit can be obtained to the simple binding equation (Eqn. 18) with the number of binding sites $n = 0.05$. In this case, however, the estimated asymptotic correlation between n and K_d is relatively low (0.876) and if n is fixed at 2, then agreement between calculated and experimental data becomes very poor (Table II). A large number of lipids per binding site for anthroylstearic acid ($n = 0.09$) has been reported previously by Haigh et al. [14]. Unfortunately, the fluorescence analysis is inconsistent with the electrophoresis data. A value of $n = 0.05$ corresponds to a value for the maximum number of bound fatty acids per Å^2 of $\sigma^{max} = 1/1200$. However, in

TABLE II

PARAMETERS OBTAINED BY NON-LINEAR LEAST SQUARES FITS TO FLUORESCENCE TITRATION DATA FOR THE BINDING OF FATTY ACIDS TO PHOSPHOLIPIDS

Conditions [a]	α [b]	β [c]	Number of binding sites per lipid, n	K_d (μM)	C.V. of fit
Dansylundecanoic acid to dioleoylphosphatidylcholine					
23°C					
All parameters free	0.027 ± 0.001	0.184 ± 0.002	0.070 ± 0.017	12.51 ± 4.51	0.24
n fixed at 2	0.027 ± 0.001	0.193 ± 0.002	$n = 2$	536.2 ± 25.2	0.27
37°C					
All parameters free	0.021 ± 0.001	0.189 ± 0.003	0.267 ± 0.257	63.54 ± 66.8	0.57
n fixed at 2	0.021 ± 0.001	0.190 ± 0.002	$n = 2$	510.5 ± 21.4	0.58
12-(9-Anthroyloxy)stearic acid to egg phosphatidylcholine					
37°C					
All parameters free	5.9 ± 0.1	162.0 ± 1.3	0.047 ± 0.003	0.765 ± 0.140	0.47
n fixed at 2	5.9 ± 0.1	166.5 ± 3.3	$n = 2$	149.0 ± 12.8	1.17

[a] Buffer, 40 mM Hepes, 0.1 M NaCl, 1 mM EGTA, pH 7.2.

[b] α is the fluorescence intensity of 1 μM fatty acid in buffer.

[c] β is the fluorescence intensity of 1 μM fatty acid fully bound to dioleoylphosphatidylcholine.

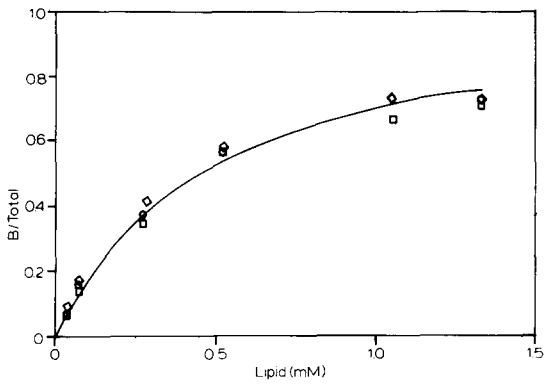

Fig. 4. Comparison of the calculated fraction of dansylundecanoic acid bound to liposomes (B/Total) as a function of lipid concentration (mM). Data from fluorescence experiments for dansylundecanoic acid concentrations of: □, 0.96 μM; ◇, 2.4 μM; ○, 6.7 μM. The solid line shows the curve calculated from the electrophoresis data for 2.4 μM dansylundecanoic acid; calculated curves for the other concentrations are almost superimposable.

our electrophoresis experiments with anthroylstearic acid we observed zeta potentials of up to −76 mV, corresponding to a surface potential of −90 mV and a σ value of 1/280 Å^2.

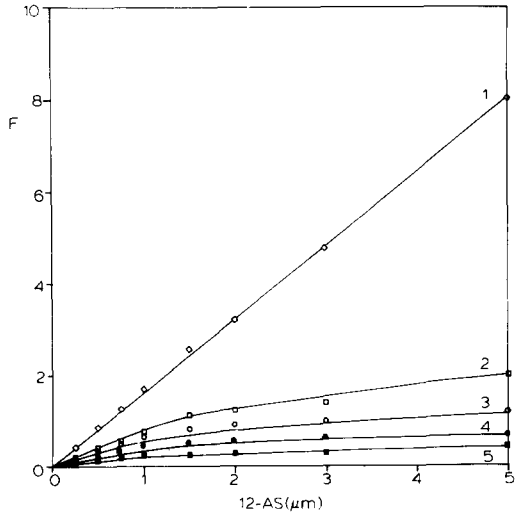

Fig. 5. Fluorescence titration of anthroyl stearic acid with egg phosphatidylcholine at 37°C in buffer (40 mM Hepes, 0.1 M NaCl, 1 mM EGTA, pH 7.2) exciting fluorescence at 365 nm and measuring emission at 455 nm. Concentrations of lipid (μM); ◇ (1), 2000; □ (2), 27.0; ○ (3), 13.5; ● (4), 6.9; ■ (5), 2.7. Solid lines, result of a non-linear least-squares fit.

We believe that the discrepancy is due to fluorescence quenching at high concentrations of anthroylstearic acid in the membrane. Thus, we find that a fit of all the data in Fig. 5 to the binding equation (Eqn. 18) gives a value of 162 for β, the parameter describing the fluorescence intensity of the bound fatty acid. However, if the fit is carried out only on the data for phospholipid concentrations of 27 μM and less, then a β value of 86 is obtained. This suggests fluorescence quenching is occurring in the system containing relatively little lipid and hence high molar ratios of fatty acid to phospholipid. Using the parameters deduced from the electrophoresis experiments we calculate that a large fraction of the 12-anthroylstearic acid will be bound throughout the titrations shown in Fig. 5 and thus that the profiles of fluorescence intensity are a complex function of binding and quenching. Similar problems are not observed with dansylundecanoic acid because its weaker binding ensures a low molar ratio of fatty acid to phospholipid throughout the titration.

Discussion

In general, hydrophobic molecules could be expected to bind to both the lipid and to the protein components of biological membranes. Here, we develop a formalism to describe binding to the lipid component of the membrane. For charged hydrophobic molecules, charge effects can be accounted for in terms of the Gouy-Chapman and Stern theories, as described previously by McLaughlin and Harary [12]. For hydrophobic fatty acids, it is necessary to consider binding of both charged and uncharged forms of the fatty acid to the membrane, and to take into account the possibility that the binding constants for the two forms may be different. We find that agreement between the experimental data for electrophoretic mobilities of liposomes in the presence of fatty acids and theory is good. In particular, and as previously found by McLaughlin and Harary [12], we see no evidence for discrete charge effects.

We find a large shift in pK on binding of fatty acids to liposomes, corresponding to a binding constant for the uncharged form of the drug about 100-times stronger than that for the charged form. This difference can be attributed to greater

penetration of the uncharged form into the bilayer with a consequent increase in hydrophobic interactions. Greater binding of the uncharged form seems to be common to a wide variety of drug molecules (Refs. 15, 16 and Rooney, E.K. and Lee, A.G., unpublished data). Hauser et al. [8] also found a higher than expected pK for fatty acids in lipid bilayers, but attributed this to a clustering of fatty acids in the membrane. Such clustering could produce a large shift in effective pK for fatty acids bound to the bilayer because the high local electrostatic field associated with such a cluster would produce a large increase in proton concentration close to the cluster. However, as shown in Appendix I and Table III, such a model appears to be inconsistent with experimental data for the oleic acid system.

Unfortunately, in the experimentally available range, the electrophoretic data is relatively insensitive to the value of σ^{max}, the maximum number of fatty acids that can be bound per Å^2, and σ^{max} can be varied over a small range as long as the quotient σ^{max}/K is maintained constant. In particular, σ^{max} values of 1/20, 1/40 and 1/80 all give good fits to the data for oleic acid, corresponding to unlimited binding of oleic acid and oleic acid/phospholipid stoichiometries of 2 : 1 and 1 : 1, respectively. Data obtained from differential scanning calorimetry suggest that fatty acids can be incorporated into lipid bilayers up to a molar ratio of 2 : 1 fatty acid/phospholipid but that beyond this some undefined change occurs [9]. A recent NMR study of the dipalmitoyl phosphatidylcholine/palmitic acid system at pH 4.0 has suggested that at a 2 : 1 fatty acid/phospholipid molar ratio, the system is no longer in a simple lamellar phase [18]. However, in preliminary studies we have shown that a variety of fatty acids do not affect the trapping of carboxyfluorescein in liposomes of egg phosphatidylcholine up to 4 : 1 molar ratios of fatty acids to phospholipid, arguing against any major change in membrane structure. Whether these apparent differences are due to differences in chemical structure, or whether they are due to the much higher concentrations employed in the NMR experiments is unclear.

For the fluorescent fatty acid dansylundecanoic acid we find that fluorescence titrations are consistent with data obtained by electrophoresis. Again, however, in the experimentally accessible range, it is not possible to define the stoichiometry of the interaction. Because of the close correlation between the number of binding sites and the binding constant, no meaning can be attached to the separate values of these two parameters but only to their ratio. For anthroyl stearic acid, we find that data from fluorescence and electrophoresis experiments are inconsistent. It seems likely that the fluorescence experiments are complicated by fluorescence quenching in the membrane, at the high molar ratios of fatty acid to phospholipid obtained with this fatty acid. Interaction between anthroylstearic acid molecules in lipid bilayers has previously been observed by Von Tscharner and Radda [17]. Unfortunately, such quenching could be a common limitation in the use of fluorescence techniques in determining stoichiometries of binding to phospholipid bilayers. In order to obtain numbers of binding sites it is necessary to extend fluorescence titrations into the region where a significant fraction of sites within the phospholipid bilayers are occupied, and, necessarily, it is at such concentrations that quenching of fluorescence is likely. Although it is also difficult to obtain stoichiometries of binding from electro-

TABLE III

OBSERVED AND PREDICTED ZETA POTENTIALS FOR PHOSPHATIDYLCHOLINE LIPOSOMES (1.33 mM) PLUS OLEIC ACID (70 mM) AT 25°C

Electrolyte concn. (mM) [a]	4	25	100
pH	7.4	7.1	7.2
Observed potential (± S.D.) [b] (mV)	−25.0	−9.1	−5.9
	±0.9	±0.8	±0.6
	−24.7	−8.7	−5.5
	±0.9	±0.4	±0.3
Predicted potential (clusters) (mV)	−15.0	−7.5	−7.8
Predicted potential [c] (random distribution) (mV)	−25.2	−9.0	−7.0

[a] Buffer was 5 mM Tris-HCl, with 0.1 mM Na_2EDTA and 0, 0.25 or 100 mM NaCl.

[b] Duplicate samples, using at least ten mobility measurements per sample.

[c] Binding constants for oleic acid taken from Table I.

phoresis data, binding constants can be readily obtained which adequately describe binding over the experimentally accessible range and which can be used to estimate the extent of binding of fatty acids to biological membranes.

Appendix I

Hauser et al. [8] interpreted the results of their micro-electrophoresis studies on the properties of fatty acids incorporated into liposomes of phosphatidylcholine as being due to lateral segregation of fatty acids from phospholipid to form clusters. The shift in apparent pK of the fatty acids on binding to liposomes was attributed to reduction of pH in the immediate vicinity of the fatty acid cluster(s) due to high surface potentials arising from the very high surface charge densities that might be attained in such clusters (up to 1 electronic charge per 20 Å^2). Differential binding of the two fatty acid species, charged and neutral, was not considered to be an important factor. If this is indeed the case, the variation of zeta potential with pH for liposomes bearing clusters of fatty acids is readily calculable under conditions where all the fatty acid is bound, as follows.

In a cluster of fatty acids, of sufficient size for 'edge effects' to be neglected, the surface concentration of fatty acids is known (1 molecule per 20 Å^2). The cluster is composed of a mixture of charged and uncharged species, the composition being given by the Henderson-Hasselbalch equation.

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{app}} + \log(\sigma^{A^-}/\sigma^{AH}) \tag{A1}$$

where σ^{A^-} and σ^{AH} are the surface concentrations of the deprotonated and neutral forms of the fatty acid, respectively, pH refers to the pH of the bulk aqueous phase and $\mathrm{p}K_{\mathrm{app}}$ is the apparent $\mathrm{p}K_a$ of membrane-bound fatty acid. The apparent $\mathrm{p}K_a$ is the intrinsic $\mathrm{p}K_a$ of fatty acid in the membrane (in this case, the same as the $\mathrm{p}K_a$ in the bulk phase, i.e. 5) modified by the effect of surface potential on the local ($x = 0$) proton concentration:

$$K = \frac{\sigma^{A^-}}{\sigma^{HA}}[H^+]_{x=0} \tag{A2}$$

where

$$[H^+]_{x=0} = [H^+]_{\mathrm{bulk}} \exp(-F\psi_o/RT) \tag{A3}$$

Therefore

$$\log K = \log(\sigma^{A^-}/\sigma^{HA}) + \log[H^+]_{\mathrm{bulk}} - (F\psi_o/2.303RT) \tag{A4}$$

For solutions containing only symmetrical monovalent electrolyte at 25°C, the surface potential ψ_o is related to the surface charge density σ^{A^-} by

$$\sinh(\psi_o/51.4) = 136.6\,\sigma^{A^-}/\sqrt{C} \tag{A5}$$

where ψ_o is expressed in millivolts, and the monovalent electrolyte concentration, C, in mol per litre.

A simple iterative routine is used to solve the system of Eqns. A1–5. An initial value for ψ_o is guessed, and the corresponding surface charge density calculated from Eqn. A5. The surface concentration of the neutral form of the fatty acid, σ^{HA}, is obtained from the relationship.

$$(\sigma^{A^-} + \sigma^{HA}) = 1/20 \tag{A6}$$

and the apparent pK is calculated from Eqn. A1. Rearrangement of Eqn. A4 then gives a new value for the surface potential, ψ_o. The cycle is repeated until there is no significant difference between successive approximations of ψ_o.

The above analysis gives us the surface charge density in the cluster of fatty acids. Electrophoretic mobility measurements depend on the average surface charge density over the whole particle (liposome). Assuming molecular areas of 60 Å^2 for phosphatidylcholine and 20 Å^2 for a fatty acid, the average surface charge density is

$$\sigma_{\mathrm{av}}^{A^-} = \sigma_{\mathrm{cluster}}^{A^-} \cdot \frac{20(n)}{60(1-n)+20(n)} \tag{A7}$$

where n is the mol fraction of fatty acid in the membrane.

From $\sigma_{\mathrm{av}}^{A^-}$ we calculate the zeta potential as the potential in the solution 2 Å from the membrane surface, as described previously.

Fig. 6 shows the predicted variation of zeta potential with pH for liposomes of egg phosphatidylcholine (1.33 mM) in the presence of oleic

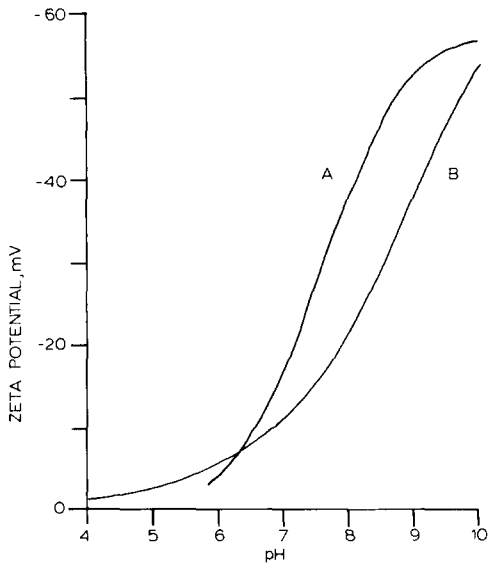

Fig. 6. Calculated zeta potentials for liposomes of egg phosphatidylcholine (1.33 mM) in the presence of oleic acid (70 μM) in a 5 mM monovalent electrolyte solution at 25°C. (A) Calculated curve assuming oleic acid molecules are randomly distributed in the membrane and binding constants as in Table I. (B) Calculated curve assuming that oleic acid is clustered in the membrane, as described in text.

acid (70 μM) in 0.005 M monovalent electrolyte solution. Curve A is calculated according to the theory outlined in the main section of this paper, using the binding constants given in Table I for oleic acid, almost all the fatty acid being bound under these conditions. Curve B was calculated assuming that the fatty acid was clustered in the membrane, as discussed above. Clearly the effect of fatty acid clustering is to broaden the ionization curve considerably. For the simple case, where fatty acid molecules are randomly distributed in the membrane, the dominant factor determining the apparent pK is the ΔpK term, reflecting preferential binding of the neutral form of the fatty acid. This will not be influenced by the surface potential, and we have attempted to distinguish between the two models of fatty acid distribution in this membrane by varying the electrolyte concentration, thus changing the surface potential whilst leaving the surface charge density constant. Simulations showed that for a given pH, the zeta potential of liposomes with clustered fatty acids would be less steeply dependent on electrolyte concentration than would be the case if the fatty acids were randomly distributed. Table III shows the results of duplicate experiments where zeta potentials were determined by micro-electrophoresis for liposomes of egg phosphatidylcholine (1.33 mM) in the presence of oleic acid (70 μM) in buffer containing 5 mM Tris HCl, 0.1 mM Na_2EDTA and 0, 25 or 100 mM NaCl. pH was measured in the liposome suspension after 20 min equilibration at room temperature. Calculated zeta potentials for the appropriate values of pH and electrolyte concentration are also shown, with good agreement between the observed values and those computed from the binding constants given in Table I assuming that the oleic acid molecules are randomly distributed in the membrane. We conclude that clustering of fatty acids cannot account for the electrophoresis data.

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