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The Hydrophobic Adsorption of Charged Molecules to Bilayer Membranes: A Test of the Applicability of the Stern Equation†

Stuart McLaughlin* and Howard Harary

ABSTRACT: To describe the hydrophobic adsorption of charged molecules to bilayer membranes, one must recognize that the adsorption produces a change in the electrostatic potential at the surface of the membrane. The surface potential produced by the adsorption of the charged molecules can be described most simply by the Gouy equation from the theory of the diffuse double layer. This potential will tend to lower the concentration of the adsorbing ions in the aqueous phase immediately adjacent to the membrane, a phenomenon which can be described by the Boltzmann relation. The number of adsorbed ions is, in turn, a function of the aqueous concentration of these ions at the membrane solution interface and can be described, in the simplest case, by a Langmuir adsorption isotherm. If the ions are regarded as point charges, the combination of the Gouy, Boltzmann, and Langmuir relations may be considered a simplified Stern equation. To test experimentally the applicability of this equation, one should measure both the charge density and surface potential as a function of the concentration of adsorbing molecules in the bulk aqueous phases. Direct, accurate measurements of one of these parameters, the number of moles of 2,6-toluidinylnaphthalenesulfonate ions bound to vesicles formed from phosphatidylcholine, are available in the literature (Huang, C., and Charlton, J. P. (1972), *Biochemistry 11,* 735). We estimated the change in the surface potential in two independent ways: by means of conductance measurements with "probe" molecules on planar black lipid membranes and by means of electrophoresis measurements on multilaminar unsonicated vesicles. The two estimates agreed with one another and all of the data could be adequately described by the Stern equation, assuming, at 25 °C, a dissociation constant of 2×10^{-4} M and a maximum number of binding sites of $1/70$ Å^2.

A variety of pharmacologically significant molecules are amphipathic in nature and adsorb "hydrophobically" to phospholipid bilayer membranes. The cationic local anesthetics, for example, adsorb to artificial bilayer membranes (Bangham et al., 1965; McLaughlin, 1975) at the same concentration at which they block nerves, but their mechanism of action on the biological membrane is unknown. Anions such as the salicylates enhance the cation and depress the anion conductances of *Navanax* neurons (Barker and Levitan, 1971) and black lipid membranes (McLaughlin, 1973) at identical concentrations, but the mechanism by which these molecules affect the electrical properties of neurons is a matter of debate (Levitan and Barker, 1972; McLaughlin, 1973). Fluorescent

† From the Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York, 11794. *Received November 13, 1975.* This investigation was supported by USPHS Grants NS 10485 and RR 05736.

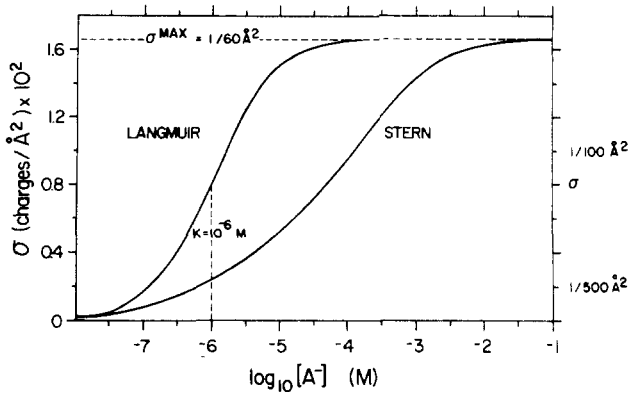

FIGURE 1: The binding curves relating the number of anions adsorbed per unit area of membrane, σ, to the concentration of these anions in the bulk aqueous phase, $[A^-]$, predicted by the Langmuir and Stern equations. In both cases the dissociation constant (K) was assumed to be 10^{-6} M and the maximum number of adsorbed anions (σ^{max}), $1/60$ Å^2. For the Stern equation the total concentration of monovalent electrolyte was assumed to be 10^{-1} M and the temperature 25 °C.

probes such as 1,8-anilinonaphthalenesulfonate (e.g., Azzi, 1975; McLaughlin, et al., 1971; Zingsheim and Haydon, 1973) and 2,6-toluidinylnaphthalenesulfonate (TNS[1]) (Huang and Charlton, 1972) adsorb hydrophobically to artificial bilayer membranes and respond with a change in fluorescence to a potential applied across the bilayer membrane (Conti and Malerba, 1972). These probes have also been used to follow action potentials in neurons (e.g., Conti, 1975) but would be of more value if the mechanism by which they responded to the change in membrane potential were known. Experimental investigations designed to reveal the mechanism by which local anesthetics block nerves and fluorescent probes respond to a change in membrane potential would obviously be facilitated if one could describe, in a quantitative manner, the adsorption of these molecules to the bilayer portion of nerves and other biological membranes.

A quantitative description of the adsorption would also aid investigators who are using these molecules as tools to perturb both artificial and biological membranes. Local anesthetics, for example, have recently been used to study the calcium-induced phase separation (Ohnishi and Ito, 1974) and temperature-induced phase transition (Papahadjopoulos et al., 1975) of lipids in bilayers, the conconavalin A induced clustering of intramembraneous particles (Ryan et al., 1974; Poste et al., 1975), the virus-mediated fusion of cell membranes (Poste and Reeve, 1972), and the discharge of mucocysts in *Tetrahymena* (Satir, 1975).

A variety of theoretical expressions have been used (e.g., Mohilner, 1966) to describe the adsorption of neutral and charged molecules to surfaces, one of the simplest expressions being the Langmuir adsorption isotherm (Appendix I):

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

where σ is the number of molecules adsorbed to the membrane per unit area, σ^{max} is the maximum number of molecules adsorbed per unit area, K is a dissociation constant, and $[A^-]_{x=0}$ is the aqueous concentration of the adsorbing species at the membrane solution interface, $x = 0$. We consider the adsorbing species to be an anion, A^-, for the remainder of this paper. If the membrane is initially neutral, and we ignore the change in surface potential produced by the adsorption of the anion, we can assume that the concentration at the membrane–solution interface, $[A^-]_{x=0}$ is equal to the concentration in the bulk aqueous phase, $[A^-]$. The curve in Figure 1 labeled "Langmuir" illustrates the dependence of σ on $[A^-]$ predicted by eq 1 when surface potential effects are ignored. Note that the shape of the curve is identical with that of a titration curve for the binding of H^+ to a weak acid or base. In this example the value of K was arbitrarily chosen as 10^{-6} M and the value of σ^{max} as $1/60$ Å^2, about the area of a phospholipid molecule in a bilayer membrane.

The simplest way one can take into account the surface potential produced by the adsorption of the charged A^- species is to relate the aqueous concentration at the surface of the membrane, $[A^-]_{x=0}$, to the bulk aqueous concentration, $[A^-]$, by the Boltzmann relation:

$$[A^-]_{x=0} = [A^-] \exp(F\psi_0/RT) \qquad (2)$$

where $\psi_0 = (\psi_{x=0} - \psi_{x=\infty})$ is the electrostatic potential in the *aqueous* phase immediately adjacent to the membrane located at $x = 0$. The Boltzmann relation follows from the equilibrium condition that the electrochemical potential of the anion must be independent of distance from the membrane, provided we assume that the charges are smeared uniformly over the surface, and that neither the standard chemical potential nor the activity coefficient varies with distance from the membrane. If we assume, for simplicity, that the membrane is initially neutral, the charge density on the surface of the membrane, σ, is equal to the surface concentration of adsorbed anions. The Gouy equation from the theory of the diffuse double layer has been shown by five independent experimental techniques to describe adequately the electrostatic potential produced by charges at the surface of a membrane (for a review and references, see McLaughlin and Eisenberg, 1975; McLaughlin, 1976). This equation predicts that the potential due to charges at the surface of a membrane, ψ_0 (mV), is related to the surface charge density, σ (electronic charges/Å^2), and the total concentration of monovalent electrolyte in the bulk aqueous solution, C (mol/l.), by:

$$\sinh(F\psi_0/2RT) = A\sigma/\sqrt{C} \qquad (3)$$

where R, T, and F have their usual significance and A is a constant which depends on temperature and dielectric constant. At 25 °C, $A = 136.6$ (mol/l.)$^{1/2}$(Å^2/electronic charge) and $RT/F = 25.7$ mV. Stern combined eq 1, 2, and 3 (e.g., Bockris and Reddy, 1970; Aveyard and Haydon, 1973), but he also took into account the finite size of the adsorbing ions. We will ignore this aspect of the phenomenon but will, nevertheless, refer to the combination of eq 1–3 as a Stern equation.

Equations 1 and 2 may be combined to eliminate $[A^-]_{x=0}$ and then the resulting expression substituted into eq 3 to eliminate either ψ_0 or σ. When ψ_0 is eliminated, one obtains a transcendental expression relating σ and $[A^-]$ which may be solved on a digital computer (Appendix II). The result for $K = 10^{-6}$ M and $\sigma^{max} = 1/60$ Å^2 is illustrated in Figure 1. Note that the Langmuir and Stern equations predict significantly different results.

A few other predictions of the Stern equation will also be considered. If the binding constant, K, is changed, then the curves predicted by both the Langmuir and Stern equations in Figure 1 merely shift along the abscissa. If the maximum number of binding sites per unit area of membrane (σ^{max}) is allowed to vary, the Langmuir expression predicts that the curves will all retain the same shape, in the sense that the

[1] Abbreviations used: TNS, 2,6-toludinylnaphthalenesulfonate; PC, phosphatidylcholine; DTFB, 5,6-dichloro-2-trifluoromethylbenzimidazole.

midpoint of the curves ($\sigma = \sigma^{max}/2$) will always occur at a concentration $[A^-] = K$. This can readily be seen by rewriting eq 1, assuming $[A^-]_{x=0} = [A^-]$, as $\sigma = \sigma^{max}[A^-]/(K + [A^-])$. The Stern equation predicts quite a different result. As illustrated in Figure 2, the midpoints of the curves, designated by the circles, shift back toward the value of the binding constant, $K = 10^{-6}$ M, as the maximum charge density (σ^{max}) decreases.

The total concentration of monovalent electrolyte in the bulk aqueous phases (C) also affects the curves, as illustrated in Figure 3. As the value of C decreases, the magnitude of the surface potential obtained for a given charge density increases (eq 3), which increases the deviation from the Langmuir expression. As the value of C increases, the curves approach the Langmuir expression asymptotically, but there remains a substantial difference between the predictions of the Langmuir (Figure 1) and Stern (Figure 3) expressions at salt concentration as high as 1 M.

Although the Gouy equation has been shown to provide an adequate description of the potential produced by charges at a membrane solution interface, we cannot assume that the Stern equation will provide an equally good description of the adsorption of charged molecules. As noted by Aveyard and Haydon (1973), "The surface charge has been assumed to be smeared over the surface rather than, as it actually is, in the form of discrete ions and electrons. The diffuse layer in reality consists of the overlapping ionic atmosphere of each individual surface charge and the potential in a plane parallel to the surface fluctuates from place to place according to the degree of overlap of these atmospheres. The potentials in the Gouy-Chapman theory are thus average potentials. As far as the properties of the diffuse layer are concerned this averaging probably does not introduce much error, but for the specific adsorption of ions, as in the Stern theory, the assumption of smeared charge is thought to be less valid." The object of this report is to test experimentally the ability of the Stern equation to describe the hydrophobic adsorption of a charged molecule to membranes in a concentration region where the discrete charge effect should be important.

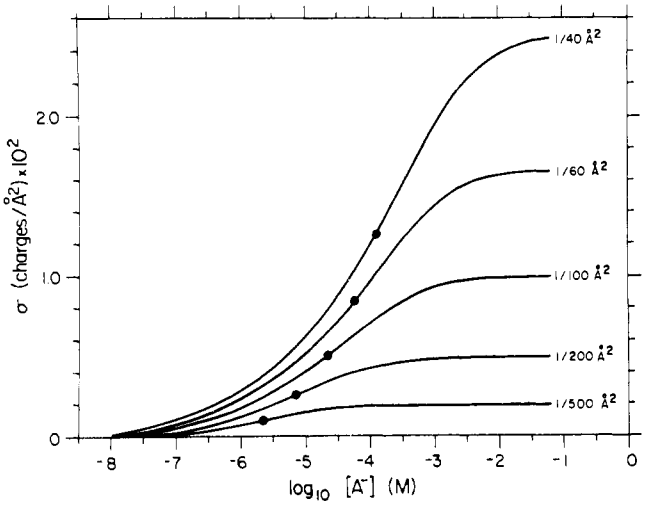

FIGURE 2: The effect of σ^{max}, the maximum number of adsorbed anions per unit area, on the binding curves predicted by the Stern equation. The Stern equation, in contrast to the Langmuir adsorption isotherm, predicts that the shape of the binding curves will change as the value of σ^{max} is varied. These curves were calculated from eq 1-3 assuming that $K = 10^{-6}$ M, $C = 10^{-1}$ M, and $T = 25$ °C. See text for details.

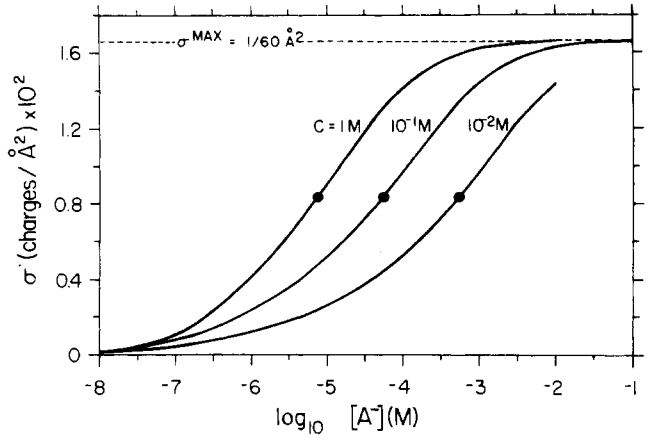

FIGURE 3: The effect of electrolyte concentration (C) on the binding curves predicted by the Stern equation. The filled circles denote the midpoints ($\sigma = \sigma^{max}/2$) of the curves. These curves were calculated from eq 1-3 assuming that $K = 10^{-6}$ M and $T = 25$ °C. See text for details.

Materials and Methods

Bacterial phosphatidylethanolamine and synthetic dioleoyl-L-α-phosphatidylcholine (PC) were obtained from Supelco, glycerol monooleate from Sigma, and egg PC prepared by the method of Singleton et al. (1965) was a gift of P. Ting and A. K. Solomon. Nonactin was a gift of Barbara Stearns (Squibb) and 5,6-dichloro-2-trifluoromethylbenzimidazole (DTFB) was prepared by a modification of the method of Acheson et al. (1958).

Planar black lipid membranes were formed by dissolving 25 mg of the lipid in 1 ml of n-decane (Eastman) and then applying a few microliters of the solution to an orifice in the wall of a chamber milled from a single piece of Teflon. The lipid forming solution was applied with a Pasteur pipet, which was flamed immediately prior to use to remove organic impurities. Small volumes of either nonactin or DTFB dissolved in ethanol were then added to the aqueous solutions. The volume of ethanol in the chamber never exceeded 0.5% and control experiments indicated that a concentration of 1% had no measurable effect on the electrical properties. The conductance was measured by applying ±10 mV, the current-voltage curves being linear to ±20 mV. The theoretical and experimental justifications for using the conductance produced by a neutral carrier to "probe" changes in the surface potential are discussed elsewhere (e.g., McLaughlin et al., 1970, 1971; Neumcke, 1970; Szabo et al., 1972; Eisenman et al., 1973; Haydon and Myers, 1973; Foster and McLaughlin, 1974; McLaughlin, 1976). The conductance was monitored as TNS was added to the aqueous solutions and the change in surface potential calculated from:

$$G_{non\text{-}K^+}'' = G_{non\text{-}K^+}' \exp(-F\Delta\psi_{0^-}/RT) \quad (4)$$

$$G_{HA_2^-}'' = G_{HA_2^-}' \exp(+F\Delta\psi_{0^-}/RT) \quad (5)$$

where $G_{non\text{-}K^+}'$ is the conductance of the zwitterionic or neutral membrane produced by the nonactin-K^+ complex, $G_{non\text{-}K^+}''$ is the conductance of the same membrane after the addition of TNS and $\Delta\psi_{0^-} = (\psi_{x=0^-}'' - \psi_{x=0^-}')$ is the *change* in electrostatic potential on the *membrane* side of the membrane solution interface, located at $x=0$, on addition of TNS. Similarly, $G_{HA_2^-}'$ is the conductance of the zwitterionic or neutral membrane produced by a HA_2^- complex formed between the neutral HA and ionic A^- species of the weak acid DTFB, and $G_{HA_2^-}''$ is the conductance after the addition of TNS to the aqueous solutions. If the changes in conductance produced by TNS are equal in magnitude but opposite in direction for the positive and negative membrane permeable species, we con-

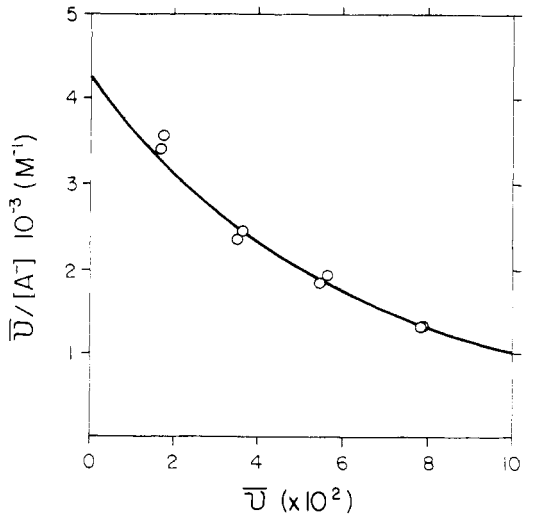

FIGURE 4: A Scatchard plot of the data (open circles) that Huang and Charlton (1972) obtained for the binding of TNS anions to bilayer membranes formed from phosphatidylcholine. The solid line is the prediction of the Stern equation for $K = 2 \times 10^{-4}$ M, $\sigma^{max} = 1/70$ Å^2, and $T = 25$ °C. $\bar{\nu}$ is the number of moles of TNS bound per mole of phospholipid.

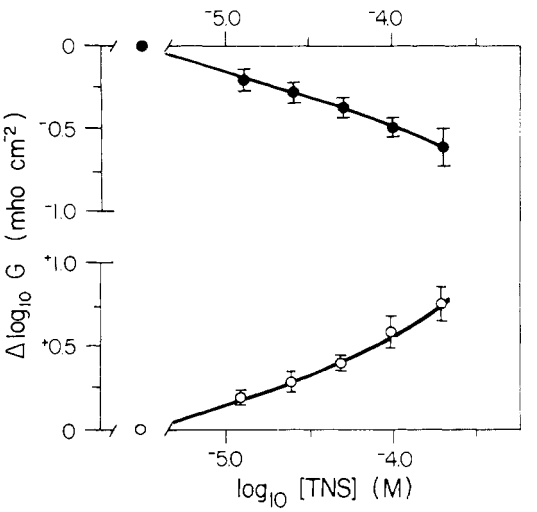

FIGURE 5: The effect of TNS on the conductance of a cation (open circles) and anion (filled circles) selective membrane. The black lipid membranes were formed from dioleoylphosphatidylcholine in a 10^{-1} M KCl, pH 7.0 solution at 25 °C. The heights of the vertical bars are twice the standard deviations of the measurements obtained from five separate experiments. The lines have no theoretical significance.

clude that only the surface potential has changed. Changes in any other parameter (e.g., the viscosity or dielectric constant) would produce changes in conductance in the same direction for both the positive and negative species. The changes in surface potential detected by these probes could result from either a change in charge density and the concomitant production of a diffuse double layer or from a change in dipole potential (e.g., Szabo et al., 1972; Haydon and Myers, 1973; McLaughlin, 1973; Szabo, 1975). The electrophoretic mobility, on the other hand, does not appear to respond to changes in dipole potential (Haydon and Myers, 1973; McLaughlin, 1976).

Vesicles for the microelectrophoresis experiments were prepared from either egg or dioleoylphosphatidylcholine (PC). The PC was dryed in a rotary evaporator, the desired aqueous solution and a few glass beads were added, and then multilaminar vesicles of the appropriate size (1-10 μm) for mobility measurements were prepared by gently shaking the flask (Bangham et al., 1974). Measurements of the electrophoretic mobility, u, were made at 25 ± 1 °C on a commercially available machine (Rank Bros., England) based on a design by Bangham et al. (1958). Care was taken to focus at the stationary layer, and results identical, within experimental error, with those in the literature for both erythrocytes (Seaman and Heard, 1960) and PC vesicles (Hanai et al., 1965) were obtained in preliminary experiments. The ζ potential or potential at the hydrodynamic plane of shear was calculated from the Smoluchowski equation:

$$\zeta = \eta u / \epsilon_r \epsilon_0 \qquad (6)$$

where η is the viscosity, ϵ_0 is the permittivity of free space, and ϵ_r is the dielectric constant. Shaw (1970) or Aveyard and Haydon (1973) may be consulted for a derivation of this equation. The relationship between the ζ and surface potentials is discussed in detail by Carroll and Haydon (1975). For low charge densities the plane of shear is thought to lie within an Angstrom of the envelope of the head group. The ζ potential should, therefore, closely approximate the surface potential, ψ_0, for the experimental conditions of this study.

Results

Vesicles prepared by the technique of Huang (1969) are better characterized with respect to weight, size, number of constituents, and molecular structure than are most macromolecules (Newman and Huang, 1975). Data obtained from a study of the binding of a ligand to a macromolecule are usually analyzed by means of either a Scatchard or a reciprocal plot, the main advantage of the former plot being that the data are more evenly weighted (e.g., Edsall and Wyman, 1958, p 617). In a Scatchard plot the ordinate is $\sigma/[A^-]$ and the abscissa is σ. If surface potential effects are ignored and it is assumed that $[A^-]_{x=0} = [A^-]$, then a Scatchard plot of the Langmuir adsorption isotherm (eq 1) yields a straight line with a slope of $-1/K$ and a y intercept of σ^{max}/K. The data obtained by Huang and Charlton (1972) were expressed in terms of $\bar{\nu}$, the number of moles of TNS bound per mole of phospholipid, but this may be converted into a charge density, σ, on the outer surface of the vesicle via the relation $\sigma = \bar{\nu}/60$ Å^2 (Appendix III). Figure 4 illustrates a Scatchard plot of the data Huang and Charlton (1972) obtained at 25 °C. In the absence of other information, Huang and Charlton (1972) described their data, reasonably enough, in the simplest manner possible; the best fit they obtained to a straight line (e.g., a Langmuir isotherm) yielded $K = 3 \times 10^{-5}$ M and $\sigma^{max} = 1/550$ Å^2. The Stern equation can also be used to describe the data. As discussed in Appendix II, a computer program was devised to search over "parameter space" and find the values of K and σ^{max} which provided the best fit of the Stern equation to the data of Figure 4. These values were $K = 2 \times 10^{-4}$ M and $\sigma^{max} = 1/70$ Å^2. It should be pointed out that, whereas the quotient σ^{max}/K is known accurately, neither the value of σ^{max} nor K can be determined with any great precision (Appendix IV). The curve predicted by inserting these numbers into the Stern equation is illustrated in Figure 4. Although both the Langmuir and Stern equations can provide a reasonable fit to the experimental data, there is a substantial difference between the interpretation of the data in the two cases. Both the Langmuir and Stern equations predict the same value for the y intercept, but the Langmuir isotherm predicts a slope of $-1/K$ whereas the limiting slope in the Stern equation can be shown to be $(-1/K)[1 + 273(\sigma^{max}/\sqrt{C})]$ at 25 °C. In the specific case under consideration it is apparent that the values of K and σ^{max}

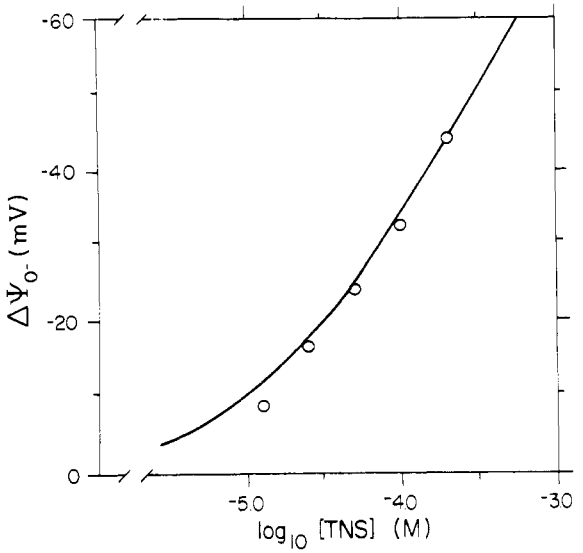

FIGURE 6: A comparison of the surface potentials (open circles) estimated from the data of Figure 5 with the prediction of the Stern equation (curve). The curve was calculated from eq 1-3 assuming that $K = 2 \times 10^{-4}$ M, $\sigma^{max} = 1/70$ Å^2, and $T = 25$ °C. See text for details.

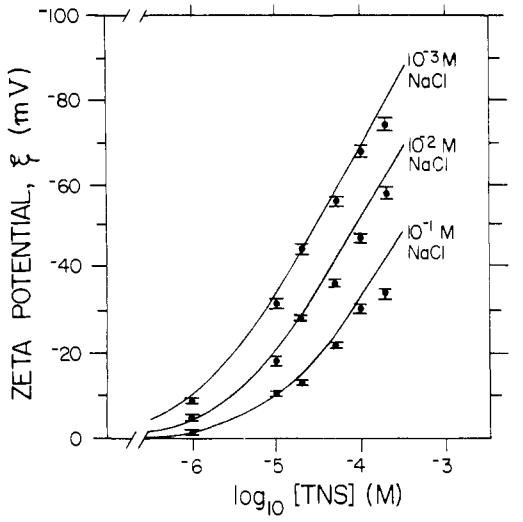

FIGURE 7: A comparison of the ζ potentials observed when phosphatidylcholine vesicles are exposed to TNS (filled circles) with the predictions of the Stern equation (curves) for the three indicated values of electrolyte concentration. The curves were calculated from eq 1-3 assuming that $K = 2 \times 10^{-4}$ M, $\sigma^{max} = 1/70$ Å^2, and $T = 25$ °C. The heights of the vertical bars through the points are twice the standard errors of the mean for measurements made on 80 different vesicles in 8 separate experiments.

derived from a best fit of the data to the Stern equation differ by about an order of magnitude from the values derived from a best fit of the same data to a Langmuir expression.

It is perhaps not surprising that the Stern equation is capable of fitting the σ, [A$^-$] data of Figure 4 because the equation contains two adjustable parameters. A test of the Stern equation for this molecule and membrane is thus reduced to the question: does the adsorption of TNS produce a measurable surface potential, ψ_0, and can one describe these ψ_0, [A$^-$] data with the Stern equation using the values of K and σ^{max} derived from a best fit of the σ, [A$^-$] data?

We estimated the change in surface potential by two independent techniques. Figure 5 illustrates the effect of TNS on the conductance of an anion and a cation selective membrane formed from dioleoylphosphatidylcholine. Note that the addition of TNS to the aqueous solution bathing the membrane produced an increase in the conductance when the permeant species was a cation and a decrease in the conductance when the permeant species was an anion. To the extent that the changes in conductance are equal in magnitude and opposite in direction, and they are within experimental error, we can interpret them via eq 4 and 5 as being due to a change in the electrostatic potential in the interior of the membrane, $\Delta\psi_{0^-}$. Similar results were obtained at pH 5 and 7, which proves that the anion, rather than the neutral form of TNS, produces the change in conductance. Results similar to those shown in Figure 4 were obtained on black lipid membranes formed from glycerol monooleate, phosphatidylethanolamine, and mixtures of phosphatidylcholine and cholesterol. This confirms the suggestion of Huang and Charlton (1972) that TNS adsorbs to membranes by essentially hydrophobic (e.g., Tanford, 1973) forces.

In Figure 6 the average values of the change in potential, $\Delta\psi_{0^-}$, derived from the data of Figure 5 via eq 4 and 5, are plotted as circles. The solid line is the prediction of the Stern equation for the values of K and σ^{max} which provided the best fit of the Stern equation to the σ, [A$^-$] data of Figure 4. The fit is satisfactory, but it could be argued that the agreement is fortuitous because these probes respond to a change in dipole as well as double layer potential. (Direct surface potential measurements on monolayers formed from the uncharged lipids, glycerol monooleate and phosphatidylethanolamine, indicate, for example, that the interior of a bilayer formed from glycerol monooleate should be about 180 mV less positive than the interior of a bilayer formed from phosphatidylethanolamine. The conductance produced by the nonactin-K$^+$ species is, as expected from an equation analogous to eq 4, two to three orders of magnitude higher on glycerol monooleate than on phosphatidylethanolamine bilayers (Hladky, 1974). As TNS binds equally well to membranes formed from glycerol monooleate and phosphatidylethanolamine, we conclude that the adsorption is independent of the dipole potential and is affected only by the diffuse double layer potential.) To check that the change in potential measured by the probes was in fact due to the production of a diffuse double layer (i.e., that $\Delta\psi_{0^-} = \psi_0$ for the case of TNS), we measured the effect of TNS on the electrophoretic mobility of unsonicated multilaminar vesicles formed from either egg or dioleoylphosphatidylcholine and then calculated the ζ potential from eq 6. The results are illustrated in Figure 7. The solid lines are the predictions of the Stern equation for the three indicated values of the indifferent electrolyte concentration. (As [TNS] $\leq$ 0.2 [NaCl], we made the approximation that the total electrolyte concentration $C \simeq$ [NaCl] in calculating these curves.) We stress that there are no adjustable parameters in these curves, the values of K and σ_{max} being determined from the fit of the σ, [A$^-$] data of Figure 4.

Discussion

The agreement between the experimental data obtained with TNS and the predictions of the Stern equation is suprisingly good in view of the many questionable assumptions which enter into the derivation of eq 1-3. Discrete charge effects, for example, could have manifested themselves at low charge densities and high ionic strengths (for references and a discussion of other effects, see Levine and Bell, 1966; Barlow, 1970; Brown, 1974; Nelson and McQuarrie, 1975).

The agreement between theory and experiment is not restricted, moreover, to one particular molecule and membrane. Haydon and Myers (1973) studied the hydrophobic binding of the anion dodecyl sulfate and the cation dodecyltrimethyl-

ammonium to membranes formed from glycerol monooleate. The data they obtained may be described by the Stern equation, the fit being about as good as that obtained with the TNS data presented above (McLaughlin, 1976).

Our conclusion is that, when one is studying the hydrophobic adsorption of charged molecules to either artificial bilayer membranes or the bilayer component of biological membranes, one must take into account the change in surface potential produced by the adsorption. The Stern equation (e.g., the combination of eq 1–3) is perhaps the simplest expression which explicitly considers the surface potential and it does describe adequately the hydrophobic adsorption of TNS and other molecules to bilayer membranes. Further, more detailed experimental studies may necessitate a modification of this equation (e.g., Appendix I) but it does not seem warranted at the present time to attempt to take into account phenomena such as the discrete charge effect.

The combination of eq 1–3 can also provide a starting point for examining the titration curves of lipids and other molecules in membranes. The changes in the surface potentials of both monolayers and bilayers formed from phosphatidylethanolamine (Szabo et al., 1972) are, for example, well described by the Stern equation if one assumes intrinsic pK values of ~1 for the phosphate and ~10 for the primary amine groups (McLaughlin, 1976). MacDonald et al. (1976) have recently discussed the titration behavior of phosphatidylserine bilayers in terms of these equations and the elegant studies of lipoid pH indicators in monolayers by Fromherz and Masters (1974) should also be mentioned. We note, finally, that there is a formal analogy between eq 1, which describes an equilibrium system, and the Michaelis–Menten equations which have been developed to describe enzyme kinetics. As stated by Edsall and Wyman, "any advance in the theoretical analysis of one [system] can be readily carried over to the other, provided that due account is taken of the physical significance of the mathematical symbols employed". Theuvenet and Borst-Pauwels (1976) have demonstrated that surface potential effects are indeed important in describing the kinetics of membrane-bound enzymes which translocate charged molecules.

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Appendix I: The Langmuir and Volmer Adsorption Isotherms

The Langmuir adsorption isotherm (e.g., Aveyard and Haydon, 1973, pp 25–27) is derived on the assumption that the adsorption sites are spatially fixed:

$$[A^-]/K_L = \sigma/[(\sigma_L{}^{max} - \sigma] \quad (1)$$

For the hydrophobic adsorption of a molecule to a fluid bilayer membrane, it is perhaps more appropriate to use the Volmer isotherm (e.g., Aveyard and Haydon, 1973, pp 22–24), which is derived on the assumption the adsorbed molecules are not localized in space:

$$\frac{[A^-]}{K_V} = \left(\frac{\sigma}{\sigma_V{}^{max} - \sigma}\right) \exp\left(\frac{\sigma}{\sigma_V{}^{max} - \sigma}\right) \quad (7)$$

It is easy to show, however, that the Volmer isotherm reduces to the same form as the Langmuir isotherm when $\sigma \ll \sigma_V{}^{max}$. We define $\sigma/\sigma_V{}^{max} = x$ and the right-hand side of eq 7 as $f(x)$ and then expand $f(x)$ in a Maclaurin series: $f(x) = a_0 + a_1x + a_2x^2 + \ldots$. For the particular function defined by the right-hand side of eq 7: $a_0 = 0$, $a_1 = 1$, $a_2 = 2$. The $[L,M]$ Padé approximant to $f(x)$ is given by $[L,M] = P_L(x)/Q_M(x)$ where $P_L(x)$ is a polynomial of degree at most L and $Q_M(x)$ is a polynomial of degree at most M (Baker, 1975). We desire the [1/1] Padé approximant to $f(x)$. That is, $P_1(x) = p_0 + p_1x$ and $Q_1(x) = 1 + q_1x$. The values of the coefficients of the polynomials are obtained from (Baker, 1975): $p_0 = a_0$, $p_1 = a_1 + a_0q_1$ and $p_2 = a_2 + a_1q_1 + a_0q_2$. It follows that $p_0 = 0$, $p_1 = 1$, $q_1 = -2$ and that:

$$[A^-]/(K_V/2) = \sigma/[(\sigma_V{}^{max}/2) - \sigma] \quad (8)$$

which is of the same form as eq 1, provided we equate $K_V/2 = K_L$ and $\sigma_V{}^{max}/2 = \sigma_L{}^{max}$. If one chooses to interpret the data presented in this report in terms of a combination of eq 7, 2, and 3 (e.g., Aveyard and Haydon, 1973, pp 112–116), the values of σ^{max} and K become respectively $1/35$ Å^2 and 4×10^{-4} M. A numerical calculation demonstrates that there is less than 0.5% difference between the predictions of the Langmuir isotherm (with $\sigma_L{}^{max} = 1/70$ Å^2 and $K = 2 \times 10^{-4}$ M) and the Volmer isotherm (with $\sigma_V{}^{max} = 1/35$ Å^2 and $K = 4 \times 10^{-4}$ M) for the entire experimental range investigated in this report. Equation 8 is thus an excellent approximation to eq 7 over this range. Only experiments conducted at higher concentrations of TNS will allow us to decide which of these two isotherms provides a better description of experimental reality.

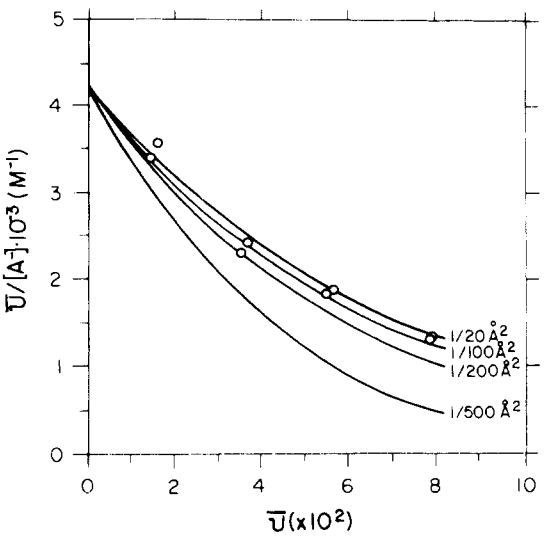

FIGURE 8: A demonstration that σ^{max} can be varied over a moderately wide range and a reasonable fit to the data of Figure 4 obtained with the Stern equation, provided the quotient of σ^{max}/K is maintained a constant. See text for details.

Appendix II: Fitting Experimental Data with the Stern Equation

The combination of eq 1–3 can be reduced to a 4th order polynomial in $\exp(F\psi_0/RT)$, provided only monovalent ions are present and the concentration of the adsorbing ion in the bulk aqueous phase does not significantly change the total concentration of electrolyte. The equation was solved by the standard Newton–Raphson iteration technique. A Fortran program is available in Bevington (1969) which enabled the computer to perform a grid search over "parameter space" and find the values of K and σ^{max} which provided the best fit (minimize χ^2) of the Stern equation to data points of the σ, $[A^-]$ form. Such a fit is illustrated in Figure 4. It should be noted that a plot of χ^2 vs. K and σ^{max} for the experimental data

illustrated in Figure 4 generates a surface in parameter space which does not have a sharply defined minimum. Rather, there is a "ravine", defined by the equation σ^{max}/K = constant, where the value of χ^2 is relatively low. This will presumably also be true for all other ions which adsorb hydrophobically to membranes. We also note that the computer program given by Bevington will often stop at a local minimum and that it is important to search along the ravine for the absolute minimum.

We originally attempted to fit the data by modifying a procedure discussed by Edsall and Wyman (1958, p 648) for fitting data obtained from the binding of ions to macromolecules. The procedure they discussed involves taking the raw data (e.g., pairs of σ, $[A^-]$ points), calculating the interfacial aqueous concentration, $[A^-]_{x=0}$, from an appropriate function (in this case a combination of eq 2 and 3), and then plotting $\sigma/[A^-]_{x=0}$ vs. σ as a corrected Scatchard plot. This method is obviously simpler in that a computer program is not required. An elementary error analysis reveals, however, that it compounds the errors inherent in the raw data. Fitting the raw data with the Stern equation via a computer program is, therefore, to be preferred.

Appendix III: Calculation of σ from $\bar{\nu}$

We converted the measured values of $\bar{\nu}$ (moles of TNS bound/mole of phospholipid) into values of σ (electronic charges/Å^2) via the expression $\sigma = \bar{\nu}/60$ Å^2. To derive this expression we note that TNS does not penetrate the PC vesicles within the time (<1 h) required to perform the Hummel and Dreyer (1962) type experiment. Huang (personal communication) came to this conclusion by studying the efflux of TNS from vesicles loaded with the anion, no significant efflux being observed in 24 h. The conclusion that TNS will not penetrate the vesicles in 1 h is consistent with our observation that TNS does not increase the conductance of a planar black lipid membrane at pH 7. The TNS bound to the vesicles must, therefore, be on the outer surface of the liposomes. These sonnicated vesicles have a uniform outer radius r_0 and contain n phospholipids. The charge density (σ) is, therefore:

$$\sigma = \bar{\nu}n/4\pi r_0^2$$

Inserting n = 2766 (Huang and Charlton, 1972) and r_0 = 115 Å, (Huang, 1969), one obtains the relation $\sigma = \bar{\nu}/60$ Å^2. More recent estimates of n = 2335 and r_0 = 105.6 Å (Newman and Huang, 1975) do not appreciably change this relation.

Appendix IV: Accuracy of the Data and Reliability of the Fit to the Stern Equation

The Stern equation is quite insensitive, at the low charge densities and surface potentials observed for TNS in these experiments, to changes in the value of σ^{max} provided the quotient σ^{max}/K is maintained a constant. This is illustrated in Figure 8 where the predictions of the Stern equation are shown for values of σ^{max} = $1/20$, $1/100$, $1/200$, and $1/500$ Å^2. The quotient σ^{max}/K is maintained a constant equal to the value obtained from a best fit to the data. Note that a not unreasonable fit to the experimental data is obtained when σ^{max} = $1/20$ and $1/100$, whereas the curve predicted by σ^{max} = $1/500$ provides a poor fit to the data.

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One-Step Purification and Properties of a Two-Peptide Fatty Acid Synthetase from the Uropygial Gland of the Goose†

J. S. Buckner and P. E. Kolattukudy*

ABSTRACT: Cell-free extracts from the uropygial gland of goose catalyzed the incorporation of malonyl-CoA into normal fatty acids and methylmalonyl-CoA into multimethyl branched acids with NADPH as the preferred reductant (J. S. Buckner and P. E. Kolattukudy (1975), *Biochemistry 14,* 1774). Purification of fatty acid synthetase from this extract was accomplished in one step by gel filtration with Sepharose 4B. Homogeneity of the fatty acid synthetase was shown by analytical ultracentrifugation, immunodiffusion assays, polyacrylamide disc gel electrophoresis, and sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. At a pH of 7.0, apparent K_m values of 3.6×10^{-5} M and 1.5×10^{-5} M were calculated for malonyl-CoA and NADPH, respectively. The major products synthesized by the enzyme from malonyl-CoA and methylmalonyl-CoA were free hexadecanoic acid and free 2,4,6,8-tetramethyldecanoic acid, respectively, with acetyl-CoA as primer. A molecular weight value of 547 000 was determined for the goose fatty acid synthetase by sedimentation equilibrium centrifugation. The purified enzyme had an $s_{20,w}$ of 13.5 S and was partially dissociated in low-ionic strength buffer into a 9.3S species, and this dissociation was accompanied by a corresponding partial inactivation of the enzymatic activity. Reassociation and reactivation of the partially dissociated fatty acid synthetase were accomplished in either 0.2 M KCl or 200 μM NADPH. These properties of the goose enzyme are similar to those of other animal fatty acid synthetases, as was the amino acid composition. Dissociation of the purified enzyme with sodium dodecyl sulfate resulted in only two equal molecular weight polypeptides (269 000), as determined by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. Injection of labeled pantothenic acid into the uropygial gland resulted in the synthesis of labeled fatty acid synthetase in which the label appeared to be located exclusively in the 4′-phosphopantotheine moiety. Analysis of the labeled enzyme by gel filtration and polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate showed that the labeled pantothenate was contained exclusively in the half molecular weight moiety. The enzyme contained one 4′-phosphopantetheine residue per subunit (269 000), as determined by measurement of the taurine generated by hydrolysis of performic acid-treated enzyme. Sodium dodecyl sulfate-activated proteolytic activity was shown to be associated with goose fatty acid synthetase, and this proteolysis was shown to result in the formation of small-molecular-weight protein fragments (<200 000) during treatment of the enzyme with sodium dodecyl sulfate. This proteolysis could be prevented by diisopropyl fluorophosphate and *p*-chloromercuribenzoate. These results strongly suggest that the goose uropygial gland fatty acid synthetase consists of two multifunctional polypeptide subunits, each containing one covalently linked 4′-phosphopantetheine.

Fatty acid synthetases isolated from bacteria (Lennarz et al., 1962; Goldman et al., 1963), plants (Overath and Stumpf, 1964), yeast (Lynen, 1961), and animals (Martin et al., 1961; Hsu et al., 1965; Smith and Abraham, 1970) have been studied extensively. The synthetase from *Escherichia coli* consists of several enzymes readily separable from one another by standard protein fractionation techniques (Vagelos et al., 1966). However, the fatty acid synthetases from yeast and animals do not dissociate into individual enzymes. The animal enzyme is obtained as a protein of approximately 500 000 molecular weight by conventional purification procedures (Smith and Abraham, 1970; Kumar et al., 1972; Yun and Hsu, 1972). Dissociation of this synthetase into 200 000–250 000 molecular weight subunits has been demonstrated with both low ionic strength buffers (Kumar et al., 1972; Yun and Hsu, 1972) and prolonged storage at 0–4 °C (Smith and Abraham, 1971; Muesing et al., 1975). The purified enzyme from pigeon liver has been separated into nonidentical subunits, each having a molecular weight half of that of the parent. The prosthetic

† From the Department of Agricultural Chemistry and the Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99163. *Received September 24, 1975.* Scientific Paper No. 4495, Project 2001, College of Agriculture Research Center, Washington State University. This work was supported in part by Grant GM-18278 from the U.S. Public Health Service.