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Characteristics of the Quenching of 9-Aminoacridine Fluorescence by Liposomes Made from Plant Lipids

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Abstract. Several laboratories have determined the surface charge density of membranes utilizing methods based on vesicle-induced quenching of the fluorescence of 9-aminoacridine and its relief by other cations. However, the computational methods by which surface charge density were calculated have not been verified in a model system. In this study, the quenching of 9-aminoacridine fluorescence by liposomes made from varying amounts of digalactosyldiacylglyceride and phosphatidic acid and relief of quenching by salts was examined. Quenching of 9-aminoacridine fluorescence increased with increasing amounts of phosphatidic acid added, independent of the composition of the added liposomes. In certain instances, the computational methods did not yield the surface charge density of the liposomes expected from their composition. However, when the effects of background ionic strength on surface potential were considered, there was a positive correlation between expected and calculated values. Therefore, the data support the contention that changes in the fluorescence of 9-aminoacridine can be used to calculate surface charge density of membranes.

Key words: Surface charge density — Electrostatic attraction — Sorption — Cations — 9-Aminoacridine — Liposome — Lipid composition

Introduction

Biological membranes have a net negative surface charge under most circumstances because of their composition. Positive surface charges can occur when biological membranes have been exposed to extremely low pH (Obi et al., 1989) or extremely high concentrations of divalent or trivalent cations (Abe & Takeda, 1988). The surface charge of biological membranes may have a profound effect on the activities of the enzymes localized within their matrix by effecting the binding of charged substrates and cofactors, and the ionization of charged side chains of amino acids of the membrane-bound enzymes and transport proteins (Wojtczak & Nalecz, 1979; Gilbert & Ehrenstein, 1991).

The consequences of a negative surface charge of biological membranes may not be limited to effects at the molecular level. There is substantial evidence that the negative surface charge of biological membranes affects ion transport processes at both membrane and tissue levels (McLaughlin & Whitaker, 1988; McLaughlin, 1989). Previous publications by Kinraide (1994) and Kinraide et al., (1992) support the contention that biological membranes alter the ionic composition immediately adjacent to the membrane surface in a fashion that can be predicted by Gouy-Chapman-Stern model. The solution immediately adjacent to the membrane surface tends to accumulate cations and exclude anions. Such a phenomenon has a profound effect on the toxicity of ions to roots. Kinraide (1994) and Kinraide et al., (1992) demonstrated that the degree of rhizotoxicity of a variety of ions is closely correlated with the surface concentrations of toxic ions as predicted by Gouy-Chapman-Stern model. In a similar view, Suhayda et al. (1990) and Matsumoto et al. (1992) have reported associations between electrostatic properties of plasma membranes from plant roots and tolerance to ionic stresses.

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The Gouy-Chapman-Stern model accounts for the interaction between the intrinsic charges of the membrane surface and the ions in the surrounding media while taking into account changes in ionic composition and SCD resulting from ion binding. Estimates of the intrinsic SCD of membranes are necessary to apply fully the Gouy-Chapman-Stern model to biological systems. Such determinations are not trivial. Electrokinetic measurements can provide information characterizing the zeta-potential of biological membranes. However, these values are usually lower than the surface potential because zeta-potential is the average electrical potential developed in the slipping plane where liquid and particles move with respect to each other, rather than reflecting the ionic composition immediately adjacent to the membrane's surface (Lewis-Russ, 1991).

The SCD of biological membranes can be calculated from data obtained by cation adsorption to membranes. However, several technical difficulties need to be overcome arising from small quantities of membranes available for such studies, and the presence of contaminating cations. SCD has been calculated by a variation of the ion adsorption method using changes in the fluorescence of the organic cation 9-AA (Chow & Barber, 1980*a*,*b;* Moller et al., 1981; Moller & Lundborg, 1985; Oka et al., 1988; Rugolo et al., 1991; Kicheva & Ivanov, 1992). Sorption of 9-AA into the diffusive layer surrounding a membrane vesicle was found to quench its fluorescence (Searle et al., 1977; Searle & Barber, 1978; Chow & Barber, 1980*a*,*b*). The sorbed 9-AA could be driven from the diffusive layer by the addition of increasing concentrations of cations, thus returning fluorescence to a value similar to that of 9-AA in solution without the vesicles. Qualitatively, changes in the fluorescence of 9-AA in various membrane systems are consistent with the contention that 9-AA is a probe for the electrostatic properties of biological membranes (Searle et al., 1977; Searle & Barber, 1978; Chow & Barber, 1980*a*,*b*). However, some characteristics of the quenching of 9-AA fluorescence by membranes are not consistent with the interaction of a monovalent cation with a charged surface (Moller et al., 1981).

Chow and Barber (1980*a*) used the Gouy-Chapman theory to derive a method to compute the SCD of the membranes by comparing abilities of divalent and monovalent cations to relieve membrane-induced quenching of 9-AA fluorescence as a starting point. Chow and Barber (1980*a*) reported a maximum SCD for phosphatidylserine liposomes of 1 charge per 100 $Å^2$ as compared to 1 charge per 60 to 70 $Å^2$ by other techniques (Jain & Wagner, 1980). Berczi and Moller (1993) recognized that the simplifying approximations of the earlier approach (Chow & Barber, 1980*a*) may have limited the utility of the method. Berczi and Moller (1993) modified the formulas for the calculation of SCD. However, neither computation method has not been tested in a model system. In this paper, the electrostatic properties of liposomes were varied by changing the proportions of DGDG and PA, and the SCD was calculated by several methods. Data from this study suggest that SCD of membranes can be determined from the quenching of 9-AA fluorescence if the certain analytical conditions and computational methods are employed.

Materials and Methods

Liposome Preparation

Small univesicular liposomes were prepared from DGDG and PA essentially as described previously (Brauer & Tu, 1988). DGDG purified from wheat flour and PA prepared from egg yolk lecithin were obtained from Sigma (St. Louis, MO). Most of the lipid in these preparations migrated through a gel filtration column with an apparent diameter of 200 to 500 Å, consistent with a classification as small univesicular liposomes (*data not shown*). A surface area of 60 $Å^2$ per lipid head, an average molecular weight of 800 daltons per lipid, and pK_{a2} for PA of 6.1 (Jain & Wagner, 1980) were assumed to calculate molarity of lipids and SCD of the resulting liposomes.

Measurement of 9-AA Fluorescence

Fluorescence measurements were modeled after those of Chow and Barber (1980*a*) but adopted to the conditions used previously in this laboratory to measure SCD of wheat plasma membranes (Yermiyahu et al., 1997). Emission spectra of the fluorescence of 20 μM 9-aminoacridine was determined from 445 to 480 nm in a solution of 0.25 M sucrose, 50 μM EDTA and 2 mM HEPES (titrated to pH 7 with 1.2 mM KOH) with a Perkin-Elmer LS-5B fluorometer (Norwalk, CT) using an excitation wavelength of 340 ± 3 nm and a scan rate of 120 nm min^{-1}. The emission silt was adjusted such that the maximum fluorescence with 9-AA, but without liposomes, had an approximate value of 900 ± 25 arbitrary units. After 5 min of incubation at 18 to 22°C, an emission spectrum was obtained as described above. Thereafter, the mixture was progressively supplemented with the chloride salt of the monovalent cation, TMA, or the divalent cation, HM. Organic cations, like TMA and HM, are preferred to that of inorganic cations for these types of studies because dissipation of the electrostatic potential of the membrane is due primarily to screening of the charges (McLaughlin et al., 1983). After each increase in salt concentration, the fluorescence emission was determined. Finally, the mixture was supplemented with 20 mM $MgCl_2$ to determine F_{max}. HM chloride was used instead of methyl viologen because Yermiyahu et al. (1997) reported previously that methyl viologen reduced the fluorescence of 9-AA in the absence of vesicles, necessitating a correction for changes in 9-AA fluorescence in the presence of methyl viologen. HM chloride had no such effect on the fluorescence of 9-AA (*data not shown*). HM has been used previously to study electrostatic properties of membranes (Alvarez et al., 1983).

The computer program of Rytwo (1994) was used to compute surface potential, and amount of cations sorbed to liposomes. Similar data could be calculated by an alternatively program described in Kinraide (1994). In such computation, the binding affinity for K, HM and TMA to negatively charged ligands were assumed to be 1, 2 and 0.1 M^{-1}, respectively (Yermiyahu et al., 1997). A binding affinity of 600 was estimated for 9-AA from trial and error computation using the

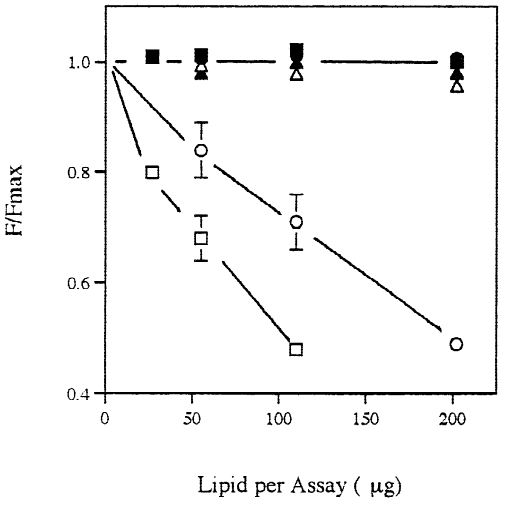

Fig. 1. Effects of varying lipid composition of liposomes and amount of lipid on the fluorescence of 9-AA. The fluorescence of 20 μM 9-AA was measured as described in Materials and Methods in the pH 7 Hepes buffer after the addition of various types and amounts of liposomes in the absence (open symbols) and presence of 20 mM $MgCl_2$ (closed symbols). Liposomes were made from DGDG (△) or with PA:DGDG ratio of 1:5 (□), or 1:10 (○). Fluorescence data are plotted relative to the fluorescence of 20 μM 9-AA in the absence of added liposomes. Each symbol represents the mean from triplicate determinations from three different preparations of liposomes. The SD is represented as bars where the value of the SD exceeds that of the data symbol.

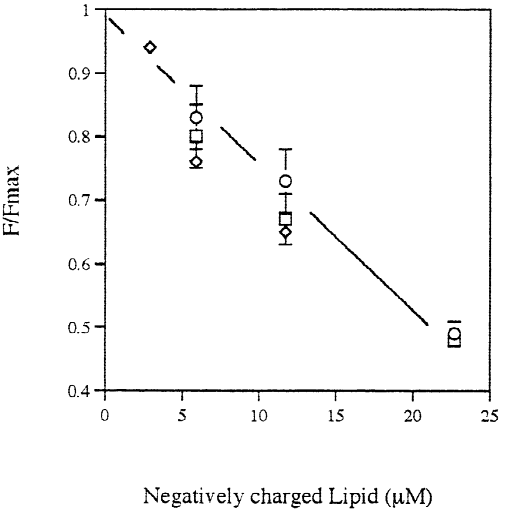

Fig. 2. Effect of the amount of negatively charged lipid as liposomes on the fluorescence of 9-AA. Liposomes were made with PA:DGDG ration of 1:5 (□), 1:10 (○) or 1:15 (◇). Fluorescence of 20 μM 9-AA in the pH 7 Hepes buffer was determined after liposomes were added as described in Materials and Methods. Fluorescence data are plotted relative to the fluorescence of 20 μM 9-AA in the absence of added liposomes. Each symbol represents the mean from triplicate determinations from three preparation of vesicles. The SD is represented as bars where the value of the SD exceeds that of the data symbol.

degree of fluorescence quenching as an indicator of sorbed 9-AA to varying amounts of liposomes of differing SCD (*data not shown*).

Abbreviations

9-AA, 9-aminoacridine; DGDG, digalactosyldiacylglyceride; F_{max}, maximum fluorescence intensity, usually determined by fluorescence of 9-AA in solution without liposomes; F/F_{max}, fluorescence intensity relative to maximum fluorescence intensity; HM, chloride salt of hexamethonium; PA, phosphatidic acid; 1PA:5DGDG, 1PA:10DGDG, 1PA:15DGDG, liposomes made from 1 part PA by weight and 5, 10, or 15 parts DGDG, respectively; SCD, surface charge density; TMA, chloride salt of tetramethylammonium.

Results

Characteristics of 9-Aminoacridine Fluorescence

In the basal assay medium, the addition of DGDG liposomes containing PA significantly decreased the fluorescence intensity of 9-AA (Fig. 1). Decreases in 9-AA fluorescence were dependent on the amount of lipid added and the PA content of the liposome. The fluorescence of 9-AA in the presence of 20 mM $MgCl_2$ was independent of lipid concentration and composition. There was a slight decrease in the fluorescence of 9-AA upon addition of DGDG liposomes. The magnitude of this change was only a few percent at 100 μg of lipid per assay as compared to over 50 and 20% with liposomes made of 1PA:5DGDG and 1PA:10DGDG, respectively.

The quenching of 9-AA fluorescence by liposomes containing three different proportions of PA and DGDG was closely related to the total PA concentration, but relatively independent of the lipid composition of the liposomes (Fig. 2). The results in Figs. 1 and 2 are consistent with the hypothesis that the quenching of the fluorescence of 9-AA by liposomes is due to an ionic interaction between the 9-AA and the negatively charged lipids of the liposomes that can be decreased by the addition of salts.

The effects of added cations on fluorescence quenching was investigated using liposomes of different compositions at a constant concentration of 12 μM PA. There was a substantial decrease in the fluorescence of 20 μM 9-AA when liposomes containing different proportions of PA were added to the assay medium (Fig. 3). Consistent with the results in Fig. 2, the fluorescence quenching caused by a total PA concentration was independent of the PA:DGDG ratio of the liposome. Data from liposomes of 1PA:DGDG, 1PA:10DGDG, and 1PA:15DGDG were not significantly different from each other. Quenching of 9-AA fluorescence could be progressively relieved by increasing the concentrations of either HM or TMA. At the same concentration, HM, a divalent cation, was much more effective in relieving the fluorescence quenching than TMA, a monovalent cation, as expected from the Gouy-Chapman model.

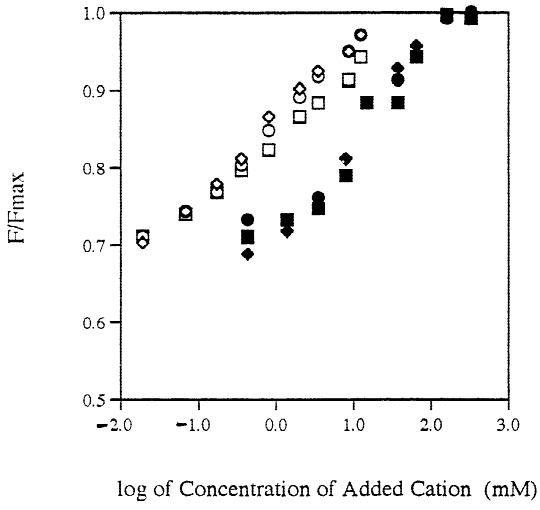

Fig. 3. The effects of various amounts of liposomes, lipid composition and chloride salts of either hexamethonium or tetramethylammonium on the fluorescence of 9-AA. Liposomes were made with PA:DGDG ratios of 1:5 (□), 1:10 (○) or 1:15 (◇) as described in Materials and Methods. Fluorescence of 20 μM 9-AA in the pH 7 Hepes buffer was determined after liposomes were added as described in Materials and Methods. After successive additions of either HM (open symbols) or TMA (closed symbols), fluorescence of 9-AA was determined. Data are plotted relative to the fluorescence of 9-AA in the presence of 20 mM $MgCl_2$ within a specified liposome composition and amount. These data are the mean of two duplicate determinations for two different liposome preparations ($n = 4$). The SD of these means are smaller than that of the data symbol.

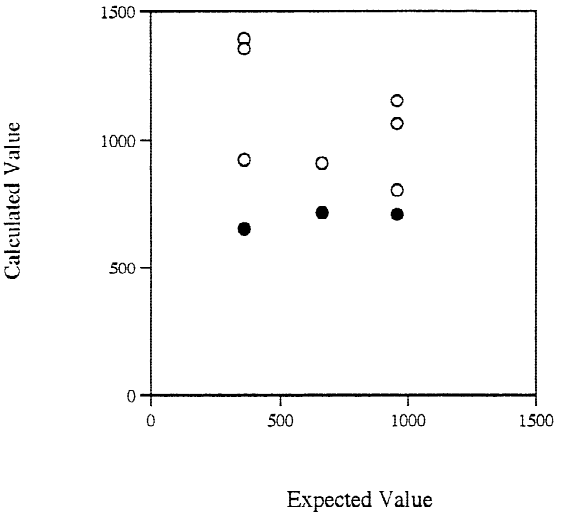

Fig. 4. Relationship between surface charge density (Å^2/charge) expected from lipid composition and that calculated from 9-AA fluorescence data. The SCD was calculated from changes in 9-AA fluorescence by equation [1] in text, original from Chow and Barber (1980*a*). Values calculated from F/F_{max} values between 0.6 and 0.85 are represented by open symbols and values calculated from F/F_{max} values of 0.9 are represented as closed symbols. The SD of these means are smaller than that of the data symbol.

Computation of Surface Charge Density

Chow and Barber (1980*a*) were the first to report a computational method to determine SCD from the quenching of 9-AA by membranes, using data similar to that in Fig. 3. Chow and Barber (1980*a*) made several simplifying assumptions to derive their computational method. First, the ratio F/F_{max} is assumed to be a measure of surface potential, E_o, and equal values of F/F_{max} are equal values of E_o, regardless of the salt milieu (Chow & Barber, 1980*a*). The second assumption was that the background level of K^+, (about 1 mM in that case) had no effect on the surface potential. The third assumption was that 9-AA did not bind to the membrane and thus had no effect on surface potential. Chow and Barber (1980*a*) applied the above assumptions to the Grahame equation to derive the following equation to compute SCD:

$$\sigma = (3.44 \times 10^{-6}(C' - 4C'C'')/C'')^{0.5} \quad (1)$$

where σ is SCD in C/m^2, C' and C'' are the concentration of TMA and HM in mM at the same value of F/F_{max}. The SCD of the PA:DGDG liposomes were computed using equation [1] over a range of F/F_{max} from 0.6 to 0.9. There was a very poor correlation between values calculated from using Eq. [1] and the expected values based on composition (Fig. 4). The relationship between expected and observed values was better when the data was limited to those obtained at F/F_{max} of 0.9.

Berczi and Moller (1993) demonstrated that ionic strength of the buffer must be incorporated into computational method when calculating surface charge density and that F/F_{max} must be between 0.85 and 0.9. The SCD of the liposomes was calculated by an approach similar to that of Berczi and Moller (1993). The computer program of Rytwo (1994) was used to find the SCD at which the surface potential was the same in the presence of concentrations of TMA and HM at F/F_{max} of 0.9 with a background K^+ concentration of 1.2 mM. There was a strong positive correlation between predicted and observed values using a computational approach similar to that of Berczi and Moller (1993) and data from F/F_{max} value of 0.9 (Fig. 5).

Discussion

For the last twenty years there has been interest in relating changes in the fluorescence of 9-AA to the surface charge properties of the membranes. Chow and Barber (1980*a*) were among the first to propose a method to calculate SCD of membranes from changes in 9-AA fluorescence. In this report, there was a very poor correlation between values calculates using Eq. [1] from

Chow and Barber (1980*a*) and the expected values based on composition (Fig. 4). There was a strong correlation between calculated SCD and that expected from lipid composition of liposomes made from PA and DGDG (Fig. 5) when SCD was calculated by a method similar to that recommended by Berczi and Moller (1993). The regression equation between SCD in Å/charge expected from lipid composition and that calculated from 9-AA fluorescence was: Calculated = 0.70 (expected) + 61.8 with r^2 of 0.99. The calculated values may have been closer to expected if the expected values were calculated to account for differences in the surface area of the headgroups of DGDG and PA, rather than using average values for surface area. Therefore, the results indicate that changes in 9-AA fluorescence can be used to calculate SCD when the appropriate assumptions and principles are considered.

The strong positive correlation between expected and calculated SCD may not have been anticipated. The calculations for SCD in this study are based on Gouy-Chapman theory in which charges are assumed to be uniformly smeared on the surface. However, the charges on a membrane surface occur as discrete sites, especially when the ratio of charged lipids to uncharged and/or zwitterionic lipids is greater than 1 to 5 (Langner et al., 1990). Most of the membrane preparations used in these experiments had SCD less than the above threshold. The observed electrostatic properties of membranes tend to deviate from those predicted by the Gouy-Chapman theory when the valence of the charges are greater (Langner et al., 1990) and when the charge is spatially distant from the membrane surface (McDaniel et al., 1984). Both of these conditions should have existed to some extent with the liposomes tested in these studies. Despite an incorrect assumption regarding the localization of charges on the membrane surface, Gouy-Chapman theory tends to predict the electrostatic interactions of membrane better than theories based on discreteness of charge effects (Winiski et al., 1986).

Results from Fig. 2 suggest that a direct and simple method can be used to determine SCD of membranes. The data in Fig. 2 indicate that the quenching of 9-AA fluorescence in basal media is dependent on the amount of PA present in the assay and thus the amount of negative charge. If one assumes that each PA molecule carries 1.8 negative charges at pH 7, then the amount of PA that gives a F/F_{max} of 0.5 corresponds to 41 nmol of membrane-bound, negative charges. Using data in Fig. 2 as a calibration curve, a SCD of 1.0 mmol(−) g^{-1} protein was calculated for the wheat root plasma membranes used in the studies of Yermiyahu et al. (1997) from the 9-AA fluorescence data (i.e., F/F_{max} of 0.5 induced by 40 μg of plasma membrane protein per assay), as compared to 1.2 mmol(−) g^{-1} protein as determined by Ca binding isotherms. The advantages of the 9-AA method, whether using a calibration similar to that in Fig. 2 or a computational method similar to that of Berczi and Moller (1993), is its ease and rapidity as compared to cation binding methods.

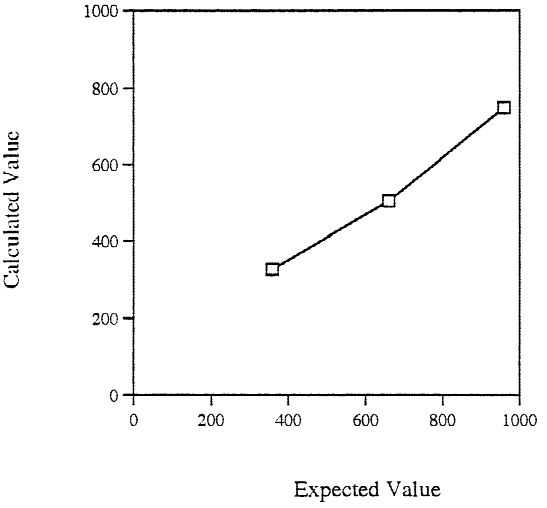

Fig. 5. Relationship between surface charge density (Å^2/charge) expected from lipid composition and that calculated from 9-AA fluorescence by an approach similar to that of Berczi and Moller (1993). Details for the computation of SCD from 9-AA fluorescence appear in the text. The SD of these means are smaller than that of the data symbol.

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