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Surface charge density estimation by 9-aminoacridine fluorescence titration: improvements and limitations

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Abstract. A large number of surface charge density (σ) and surface potential (ψ_o) estimations have been based on *1)* titrations of the fluorescence of 9-aminoacridine released from the diffuse double layer adjacent to negatively charged membrane surfaces by non-adsorbing monovalent and divalent cations, and *2)* calculations using experimental data from the titration curves and the Gouy-Chapman theory of the diffuse double layer. In this paper we discuss the different simplifying approximations employed in the earlier calculations and recommend modified formulas for the calculations. The latter have been derived without any simplifying approximation concerning the ionic (electrolyte) composition of the titration assays. We also show that σ depends, to some extent, on the concentrations of buffer and vesicles in the assays and present experimental evidence that decamethonium (decane-1,10-bis-trimethylammonium), a bulky organic divalent cation, can be satisfactorily used for the estimation of σ under well-defined conditions, despite its putative interaction with membranes.

Key words: 9-aminoacridine – Fluorescence – Membrane vesicles – Surface charge density – Surface potential

Introduction

The electrostatic potential at charged interfaces in contact with an electrolyte solution plays an important role in numerous interfacial phenomena where charged particles (i.e. ions, molecules) are involved (McLaughlin 1989). Biological membranes normally carry a net negative surface charge at neutral pH (Bérczi et al. 1984a; Chow and Barber 1980; Ivanov and Christov 1987; Møller et al. 1984a; Pethig et al. 1984; Searle et al. 1977; Stoicheva et al. 1987) and the size of the resulting negative surface potential depends on the charge density of the membrane surface and the concentration, as well as the valency, of the ions in the solution with which the membrane surface is in contact (Barber 1980; Kolber 1982; Searle et al. 1977). The potential distribution adjacent to the membrane surface determines the local concentration of charged particles, e.g. substrates for membrane-bound enzymes or ligands for membrane-bound receptors, and in this way it can strongly affect the rate or efficiency of biological processes (Barber 1982; Bérczi et al. 1984b; Borst-Pauwels and Severens 1984; Douzou and Maurel 1977; Gibrat et al. 1985; Itoh and Nishimura 1986; Møller et al. 1982, 1984b; Sjölin and Møller 1991; Theuvenet et al. 1984; Thibaud et al. 1984; Wojtczak and Nalecz 1979; Wojtczak et al. 1982). Electrostatic interactions also play a role in the association of soluble proteins with membranes (Karchikov et al. 1992; Nicholls 1974; Rogers et al. 1988; Telfer et al. 1980; Weinstein et al. 1982) as well as in the fusion of different membraneous structures (Attard et al. 1988; Evans and Needham 1987; Kjellander and Marcelja 1986; Marcelja 1992; Nir et al. 1983). Thus, for membrane studies, it is a distinct advantage to have information about the size of the surface potential and/or surface charge density.

There are numerous methods (i.e. electrophoretic mobility, ESR, NMR, fluorescence, conductivity measurements) for determining the surface potential and/or surface charge density of biological membranes (Alvarez et al. 1983; Castle and Hubbell 1976; Cherny et al. 1980; Chow and Barber 1980; Gibrat et al. 1983; Shin and Hubbell 1992; Winiski et al. 1986). However, the final step in all of these methods is a calculation of the surface poten-

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Abbreviations. 9-AA, 9-aminoacridine, $(DeM)^{2+}$, decamethonium; $(DiM)^{2+}$, dimethonium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glyol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, $(HeM)^{2+}$, hexamethonium; MES, 2-(N-morpholino) ethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; PM, plasma membrane; Tris, tris(hydroxymethyl)aminomethane; σ, surface charge density; ψ_o, surface potential

tial and/or surface charge density using the Gouy-Chapman-Stern theory and the Poisson-Boltzmann equations. At low ionic concentrations and surface charge densities many independent tests have shown this theory to describe accurately the electrostatic properties of biological membranes, especially those of purified membrane vesicles (Alvarez et al. 1983; Carnie and McLaughlin 1983; Henderson et al. 1985; Marcelja 1992; Searle et al. 1977; Shin and Hubbel 1992; Torrie and Valleau 1980; Winiski et al. 1986).

The simplest spectroscopic method for the measurement of surface charge density of membranes uses the relative fluorescence intensity values of 9-AA (a fluorescent monovalent cation below pH 8) which are obtained when the quenched fluorescence of 9-AA cations in the electrical double layer is released by replacing the 9-AA molecules with monovalent and/or divalent cations (Barber 1980; Chow and Barber 1980; Searle et al. 1977). It is assumed that the 9-AA cation distributes in the electrical double layer in such a way that the surface potential is the same when an equal increase in fluorescence (which is due to the release of the dye from the electrical double layer) has been detected, independently of whether the release of the dye has been achieved by the addition of a certain amount of monovalent cations or by the addition of another, and lower, amount of divalent cations. Sometimes, however, further simplications have been applied (e.g. neglecting the presence of buffer; replacement of 2:1 or 3:1 electrolytes actually used in the titration by 2:2 or 3:3 electrolytes, respectively, in the calculations; using divalent cations in the titration which bind to the surface of membranes, etc.). It has been reported that the calculated surface charge density depends on the relative fluorescence value at which the calculation was made (Chow and Barber 1980; Ivanov and Christov 1987; Møller et al. 1984a; Oka et al. 1988; Searle et al. 1977; Stoicheva et al. 1987). There were relative fluorescence values at which the equations given in the literature could not be used for obtaining a real value for the surface charge density of membranes. In the present paper we show that these problem disappear if the actual experimental conditions are used in the numerical calculations. We also show that the 9-AA fluorescence titration method is appropriate only for comparative studies.

Materials and methods

Plasma membrane vesicles were prepared from winter wheat (*Triticum aestivum* L. cv. Martonvásári-8) roots by aqueous polymer two-phase partitioning as detailed earlier (Bérczi and Møller 1986). The mitochondrial fraction used in some experiments was the 3 000–10 000 g (for 10 min) pellet. The 10 000–30 000 g (for 45 min) pellet (the microsomal fraction) was then used in the phase partitioning step. Membrane vesicles were prepared freshly, stored on ice in 25 mM MES-Tris buffer (pH 7) containing 250 mM sucrose (isoosmotic buffer), and used within 6 h. The protein concentration was determined according to Markwell et al. (1978) with minor modifications as given before (Bérczi and Møller 1986).

Ion-exchanged, double-distilled water (with a conductivity of <1 μS) was used for the preparation of all solutions.

The titration of 9-AA fluorescence by monovalent and divalent cations was performed as described earlier (Bérczi et al. 1984a) using a Perkin-Elmer spectrofluorimeter (model MPF-3, USA). Unless otherwise indicated, in most of the surface charge density determinations, the concentration of 9-AA was 20 μM and KCl (as 1:1 electrolyte) and decamethonium bromide (decane-1,10-bis-trimethylammonium bromide, $(DeM)Br_2$, as 2:1 electrolyte] were used in the 2.5 ml assay volume in a 1×1 cm quartz cuvette for the 9-AA fluorescence titrations. The excitation was at 400 nm with 3 nm band width while the emission was detected perpendicular to the excitation at 456 nm with 9 nm band width. There was always 40–50 μM EDTA present in the assays to remove all bound divalent cations left on the surface of the membrane vesicles (Møller and Lundborg 1985).

For an estimation of the surface charge density of membrane vesciles using experimental data obtained on the release of 9-AA flurorescence quenching after addition of different cations, we employ the Gouy-Chapman theory. The surface charge density (σ) is related to the surface potential (ψ_0) in this theory as

$$\sigma = \pm(2\varepsilon_r \varepsilon_0 R T)^{1/2} [\Sigma_i c_i (\exp\{-z_i F \psi_0/R T\} - 1)]^{1/2} \quad (1)$$

where ε_r and ε_0 are the relative dielectric constant and the permittivity of free space, respectively, R, T, and F are the gas constant, the temperature in K, and the Faraday constant, respectively, and c_i and z_i are the bulk concentration and valency of the i-th species (Barber 1980). There is always a background concentration of monovalent salts (mostly the buffer; c_b) present in the cuvette which, in combination with the added 1:1 electrolyte (c_m), results in the form of Eq. (1) as follows:

$$\sigma = \pm K[(c_b + c_m)(y + y^{-1} - 2)]^{1/2} \quad (2)$$

where

$$K = (2\varepsilon_r \varepsilon_0 R T)^{1/2} \quad (3)$$

and

$$y = \exp\{-F\psi_0/R T\}\,. \quad (4)$$

However, in the past, c_b has often used ignored compared with c_m in the calculations (e.g. Chow and Barber 1980) and then

$$\sigma = \pm K[c_m(y + y^{-1} - 2)]^{1/2} \quad (5)$$

was used instead of Eq. (2) in surface charge density calculations. When divalent cations are added to the background solution (the buffer; c_b), it can be done either in the form of a 2:2 or a 2:1 electrolyte. The two relevant expressions are:

$$\sigma = \pm K[c_b(y + y^{-1} - 2) + c_d(y^2 + y^{-2} - 2)]^{1/2}\,, \quad (6)$$

(for 2:2 electrolyte)

$$\sigma = \pm K[c_b(y + y^{-1} - 2) + c_d(y^2 + 2y^{-1} - 3)]^{1/2}, \quad (7)$$

(for 2:1 electrolyte)

where c_d is the concentration of divalent cation added.

Results and discussion

Theoretical considerations

Chow and Barber (1980) used the combination of Eqs. (5) and (6) for calculating ψ_0 and/or σ. Using the assumption that the relative fluorescence intensity of 9-AA is the same only if the surface potential is the same, independently of whether monovalent or divalent ions have been added, the combination of (5) and (6) yields the following expressions:

$$y+y^{-1}-2=c_m/c_d-4-c_b/c_d=F(c_i)\,, \tag{8}$$

$$\psi_0=-(2RT/F)\,\mathrm{arcsinh}[2\{F(c_i)\}^{1/2}], \tag{9}$$

$$\sigma=-K[c_m F(c_i)]^{1/2}\,. \tag{10}$$

In some cases, however, c_b was also neglected in (6) (see e.g. Ivanov and Christov 1987; Stoicheva et al. 1987) and the following expressions were derived for ψ_0 and σ:

$$\psi_0=-(2RT/F)\,\mathrm{arcsinh}[2\{G(c_i)\}^{1/2}], \tag{11}$$

$$\sigma=-K(c_m G(c_i)]^{1/2}\,, \tag{12}$$

where

$$G(c_i)=c_m/c_d-4=y+y^{-1}-2\,. \tag{13}$$

In reality, however, 1:1 and 2:1 electrolytes have always been used for the titration of 9-AA fluorescence intensity (Bérczi et al. 1984a; Chow and Barber 1980; Ivanov and Christov 1987; Møller et al. 1984a; Oka et al. 1988) in the presence of a background monovalent salt (buffer). Thus the combination of (2) and (7) gives us the exact solution to the problem. Consequently, the relevant expressions for ψ_0 and σ are:

$$\psi_0=-(2RT/F)\,\mathrm{arcsinh}[2\{H(c_i)\}^{1/2}], \tag{14}$$

$$\sigma=-K[(c_m+c_b)\,H(c_i)]^{1/2}\,, \tag{15}$$

where

$$H(c_i)=(c_m/c_d-3)^2/(c_m/c_d-2)=y+y^{-1}-2\,. \tag{16}$$

The surface charge density, whether calculated using (2) or (5), depends on the factor $(y+y^{-1}-2)$ [which is an implicit expression for ψ_0; see Eq. (4)], and $F(c_i)$, $G(c_i)$, and $H(c_i)$ are the expressions for this factor in the different approximations of the problem of calculating ψ_0 and σ. Therefore, the deviations between the three different approximations mentioned above can be visualized by showing the dependence of the expression $(y+y^{-1}-2)$ on c_m/c_d [in the cases of $G(c_i)$ and $H(c_i)$], and by showing it at different c_b/c_d [in the case of $F(c_i)$]; see Fig. 1. The implicit expression for ψ_0 is independent of c_b in (13) and (16) and the deviation between them is higher then 5% only if c_m/c_d is smaller than 7.6 (see the dashed line and the solid line closest to the dashed line in the main field of Fig. 1). Thus, using the 2:2 electrolyte approximation and neglecting the background electrolyte [i.e. using Eq. (12)], in spite of the fact that a 2:1 electrolyte is used and a background electrolyte is always present during the titrations, gives values almost as precise as those obtained by using

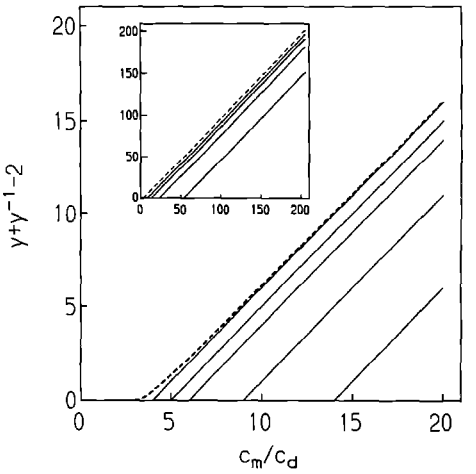

Fig. 1. The dependence of the factor $(y+y^{-1}-2)$ on c_m/c_d when different approximations are used for calculating ψ_0 and σ from the 9-AA fluorescence titrations. The insert has the same axes as the main figure but on a larger scale. *Dashed lines* represent the exact solution of the problem using Eq. (16). *Solid lines* were calculated using (8) with c_b/c_d ratios of 50, 20, 10, 5 (from right to left in the insert) and of 10, 5, 2, 1, and 0 (from right to left in the main figure). The last case, $c_b/c_d=0$, refers to the limiting case. It is equivalent to neglecting the presence of c_b in the assay: i.e. it is the case when $c_b=0$ and when (8) gives (13)

the exact solution [i.e. using (15)]. However, if one uses the 2:2 electrolyte approximation and takes into account the background electrolyte only in the case of the titration with divalent cations [i.e. using (10)] then: *1)* the deviation from the exact solution increases with increasing c_b/c_d, *2)* the deviation changes with changing c_d (c_b being constan during the titration), and *3)* ψ_0 is significantly underestimated. Furthermore, the reason for underestimating σ is not only the use of an underestimated value for ψ_0 but also the fact that c_b is not taken into account in the last step of the calculations; i.e. Eq. (5) is used instead of (2). For example, the set of experimental data of $c_m=2.6$ mM, $c_d=0.012$ mM, and $c_b=1.3$ mM (see Chow and Barber 1980) resulted in $\psi_0=-119$ mV and $\sigma=-30.7$ mC m^{-2} when $T=298$ K and approximations equivalent to (8)–(10) were used; the same set of data, however, results in $\psi_0=-137$ mV and $\sigma=-53.5$ mC m^{-2} when $T=298$ K and (14)–(16) are used. It is clearly seen that the percentage deviation between the appropriate values is more significant for σ (about 80%) than for ψ_0 (about 15%).

Since σ can be calculated only if $F(c_i)$ or $G(c_i)$ or $H(c_i)$ is not negative, this condition limits the applicability of (10), (12) and (15). This means that $c_m/c_d\geq 4$ has to be true in the case of $G(c_i)$, and $c_m/c_d\geq 2$ has to be true in the case of $H(c_i)$. However, $F(c_i)$ depends not only on c_m and c_d but also on c_b, and there are sometimes F/F_{max} values where the corresponding c_m and c_d values are so small in comparison to c_b that it is not the c_m/c_d ratio but the relation of c_m and c_d to c_b that limits the applicability of the approximation. This means that the calculation for σ can be performed only if

$$c_m-4c_d\geq c_b, \tag{17}$$

Table 1. Surface charge density values obtained from the fluorescence titration curves and with PM vesicles (as given in Fig. 2) by using (8)–(10) [columns A; it should be mentioned that Chow and Barber (1980) used equations similar to (8)–(10)] or (14)–(16) (columns B)

F/F_{max}	Surface charge density, mC m^{-2}					
	with $(DeM)Br_2$		with $(HeM)Br_2$		with $(DiM)Br_2$	
	A	B	A	B	A	B
0.76	–	−28.4	–	−25.6	–	−38.0
0.78	–	−31.2	–	−25.2	–	−39.0
0.80	−10.2	−32.1	−4.57	−23.6	−13.4	−38.3
0.82	−14.5	−32.0	−8.84	−23.5	−16.6	−35.5
0.84	−18.2	−32.4	−11.7	−23.4	−19.2	−33.9
0.86	−20.3	−31.9	−12.8	−22.5	−20.2	−31.9

Table 2. Surfache charge density of different membrane vesicles at pH 6 and 7. For the mitochondrial fraction, the results are from measurements performed in 250 mM sucrose, 50 μM EDTA, and 10 mM MES-KOH buffer with 90 μg ml^{-1} protein in the 2.5 ml assay volume and calculations at $F/F_{max}=0.85$. For the PM fraction, the results are from measurements performed in 250 mM sucrose, 0.1 mM EDTA, and 5 mM MES-KOH buffer (pH 6.0) or 5 mM MOPS-KOH buffer (pH 7.0) with 87 ± 19 μg ml^{-1} ($n=4$) protein in the 2.5 ml assay volume and calculations at $F/F_{max}=0.90$

Divalent salts	Surface charge density, mC m^{-2}				
	Mitochondrial function		Phase-partition-purified PM vesicles		
	pH 6 ($n=3$)	pH 7 ($n=3$)	pH 6 ($n=3$)	pH 7 ($n=4$)	pH 7[a] ($n=3$)
$(DiM)Br_2$	n.m.	−36.6 ± 1.8	n.m.	−37.3 ± 4.8	−30.9 ± 2.1
$(HeM)Br_2$	−16.6 ± 3.0	−20.2 ± 1.8	n.m	−18.8 ± 7.7	−22.1 ± 1.9
$(DeM)Br_2$	−21.3 ± 2.4	−37.1 ± 2.2	−18.6 ± 1.2	−28.7 ± 8.78	−30.3 ± 2.4

[a] In another series of experiments with PM vesicles, 10 mM MES-KOH buffer (pH 7), 250 mM sucrose, and 50 μM EDTA were used with 80 μg ml^{-1} protein in the assays and $F/F_{max}=0.85$ was used in calculations (see Fig. 2). n.m. – not measured. Data are averages ± s.d. of the number of independent experiments given in the parentheses

is true. Unfortunately, Eq. (17) is not always true (Møller et al. 1984a; Oka et al. 1988; see also Table 1 later in this paper). The higher the background (buffer) concentration in the assay and the smaller the F/F_{max} value, the more frequently this problem arises. The condition in Eq. (17) might result in almost always using solutions of low buffering capacity in the surface charge density determination experiments. This problem never arises if the exact solution is used.

Finally it should be noted that in the earlier studies (Chow and Barber 1980; Møller et al. 1984a, Oka et al. 1988) the surface charge density calculated at different F/F_{max} values increased with increasing F/F_{max} values. The surface charge density calculated with (14)–(16) does not depend on the F/F_{max} values (within experimental error) if those values are taken into account where the titrations with both the monovalent and divalent cations result in a close-to-linear dependence of F/F_{max} on the logarithm of cation concentration (see Table 1).

Methoniums: the bulky organic divalent cations

Practically all divalent metal and alkaline earth cations change the surface charge density of model and biological membranes by adsorbing or binding to the negative surface charges (Körner et al. 1985; McLaughlin et al. 1971, 1981; Møller et al. 1982; Obi et al. 1989; Ohki and Kurland 1981). Thus, their use in the 9-AA fluorescence titrations would have required a rather difficult iteration-type calculations to obtain the surface charge density and/or surface potential. To circumvent this difficulty, the bulky organic divalent cation, decamethonium, was introduced in the 9-AA fluorescence titrations (Barber 1980; Chow and Barber 1980). After applying five different experimental systems, however, Alvarez et al. (1983) concluded that decamethonium adsorbed (bound?) to the surface of membranes and affected the diffuse double layer potential by changing the surface charge density. They also introduced and recommended for biological applications another bulky organic divalent cation, namely hexamethonium [hexane-1,6-bis-trimethylammonium; $(HeM)^{2+}$], which exerted a smaller effect on the electrostatic potential of bilayer membranes than was predicted by the classical screening theory. Carnie and McLaughlin (1983) could explain the deviation from the classical screening theory by taking into account the shape and finite size of the hexamethonium cation. At the same time, McLaughlin et al. (1983) introduced dimethonium [ethane-bis-trimethylammonium; $(DiM)^{2+}$], a smaller bulky organic divalent cation, that exerted only a screening effect on the electrostatic potential adjacent to negatively charged surfaces. The results obtained with dimethonium were consistent with the predictions of the Gouy-Chapman theory.

We have compared the 9-AA fluorescence titrations and calculated the surface charge densities with all of the three methoniums mentioned above (Fig. 2, Tables 1 and 2). From the titration curves it is obvious that *1*) in the close-to-linear region of the titration curves a given F/F_{max} value was reached at lower $(DiM)^{2+}$ than $(HeM)^{2+}$ concentrations, *2*) the "slope" of the close-to-linear region of the titration curves was almost identical with $(DiM)^{2+}$ and $(HeM)^{2+}$ and significantly higher with $(DeM)^{2+}$, and *3*) at values of $0.85 \leq F/F_{max} \leq 0.90$ the 9-AA fluorescence titration with $(DeM)^{2+}$ was always very close to that with $(DiM)^{2+}$. The deviation between the curves obtained with $(DiM)^{2+}$ and $(HeM)^{2+}$ can be explained in the framework of the rod model that takes into account the different distances between the two positive charges inside the different methonium cations (Carnie and McLaughlin 1983). Titration curves obtained with $(DeM)^{2+}$, however, do not fit into the series (see Fig. 2) probably owing to the putative interaction of $(DeM)^{2+}$ with the surface of membranes (Alvarez et al. 1983). Consequently, the σ values, which were obtained by using *1*) the 9-AA titrations with

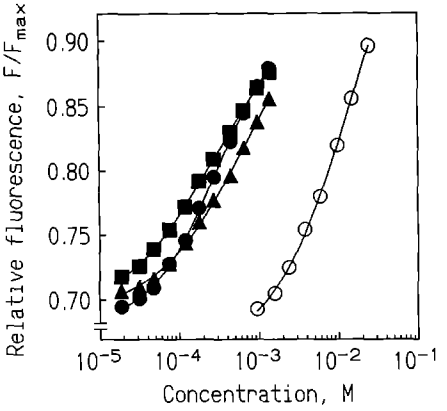

Fig. 2. Titration of 9-AA fluorescence by adding KCl (o), (DeM)Br_2 (●), (HeM)Br_2 (▲), and (DiM)Br_2 (■) into the cuvette to obtain the final concentrations of salts as given on the X axis. Assays were in 10 mM MES-KOH buffer (pH 7.0; hypoosmotic condition) with 80 μg ml^{-1} PM protein, 40 μM EDTA and 20 μM 9-AA. F_{max} was the fluorescence obtained after addition of $MgCl_2$ (its final concentration was 20 mM) at the end of each titration

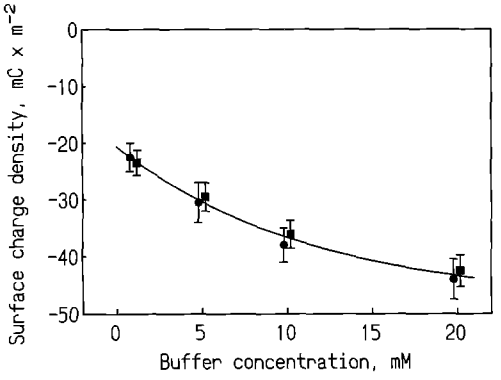

Fig. 3. Dependence of σ of the membrane vesicles in the mitochondrial fraction on the buffer concentration in the assays. Assay compositions: 20 μM 9-AA, 40 μM EDTA, 110 μg ml^{-1} protein in MES-KOH (●) and MES-Tris (■) buffers (pH 7.0; hypoosmotic condition). Surface charge density was calculated using (15) and (16). Results given are averages ±s.d. of three independent series of experiments. In each series of experiments at each buffer concentration, σ was obtained as the average of the three σ values obtained using the three c_m/c_d values determined by the relative fluorescence (F/F_{max}) values of 0.84, 0.85, and 0.86 on the fluorescence titration curves when KCl and (DeM)Br_2 were used for the titration (Møller et al. 1984a; see also Fig. 2)

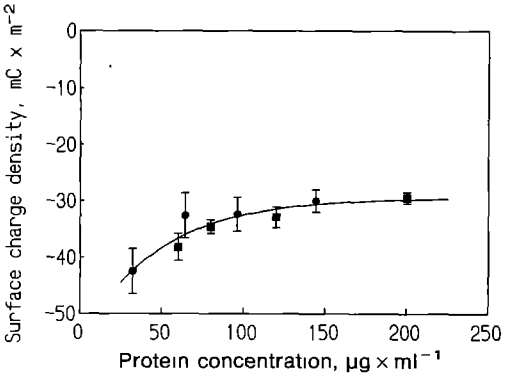

Fig. 4. Dependence of σ of the membrane vesicels in the mitochondrial fraction on the concentration of membrane proteins in 10 mM MES-KOH (●) or MES-Tris (■) buffer (pH 7.0; hypoosmotic condition). Otherwise as given in Fig. 3

$(DeM)^{2+}$, *2)* c_m and c_d values originating from the titratons where $0.85 \leq F/F_{max} \leq 0.90$ held, and *3)* the (14)–(16), seemed to be as high as those σ values which were obtained with $(DiM)^{2+}$ under similar conditions. Thus, $(DeM)^{2+}$ can be used cautiously if $(DiM)^{2+}$ is not available.

Effect of assay composition

There are at least five variables which may affect the surface charge density of membrane vesicles and/or the calculation of σ: the presence or absence of osmoticum and/or divalent cation chelator(s), the amount of the membrane vesicles added, the pH value and the concentration of the buffer used in the 9-AA fluorescence titrations. The influence of osmoticum (sucrose) and divalent cation chelator(s) (EDTA, EGTA) on σ have already been discussed (Bérczi et al. 1984a; Møller et al. 1984a; Møller and Lundborg 1985). There has not yet been a thorough study of this surface charge density estimation method, however, from the point of view of the stability and comparability of the σ values calculated when the buffer, the buffer concentration, or the amount of membrane vesicles is varied in the 9-AA fluorescence titrations.

The surface charge density of membrane vesicles calculated using Eqs. (14)–(16) has the following characteristics: *1)* It is not significantly affected by the type of buffer used (see Figs. 3 and 4 and the last two columns in Table 2); *2)* it increases with increasing concentration of buffer (Fig. 3); and *3)* it decreases with increasing amount of membrane vesicles (membranes protein; Fig. 4) in the assay. The non-linear dependence of 9-AA fluorescence on the concentration of membrane vesicles has already been established using membrane vesicles from several different species (Körner et al. 1985; Møller et al. 1981; Møller and Lundborg 1985). A possible explanation is that membrane vesicles in the stock solution are present in small clusters or aggregations (Lerche 1983; Nir et al. 1983; Ohshima et al. 1987). When they are added to the assay in different dilutions they separate but to a lesser extent at higher final protein concentrations (i.e. addition of two times more membrane protein may not result in the appearance of two times more vesicle surface in the same volume). Consequently the calculated σ decreases with increasing amount of vesciles. We cannot explain the dependence of σ on the concentration of buffer (Fig. 3).

Since the surface charge density of biological membranes is negative, one would expect it to becomes less negative at lower pH values. Our data in Table 2 verifies this expectation. This is, however, at variance with the results of Ivanov and Christov (1987) who studied the effect of plant hormones (indoleacetic acid; IAA, abscisic acid; ABA) on the surface charge density of pea mesophyll protoplasts. They found that not only the IAA- and ABA-treated protoplasts but als other control (untreated) protoplasts had higher surface charge densities at pH 6.3 than at pH 7.5. While the former phenomena were explained by the higher activity of the so-called "auxin-binding sites" at pH 6.3 than 7.5, the latter results were left unexplained. Using Eqs. (14)–(16) does not resolve this discrepancy either.

Conclusion

In spite of the fact that $(DeM)^{2+}$ interacts to some extent with biological and model membranes, it can be used for the titration of 9-AA fluorescence in order to obtain data for the calculation of the surface charge density of membranes. In the calculation of σ, the concentration of the background electrolyte (buffer) can never be neglected and the use of Eqs. (14)–(16) is recommended. The c_m and c_d values obtained between the relative fluorescence values of 0.85 and 0.90 (at the top end of the close-to-linear region of titration curves) should be used during the calculations. It should be kept in mind that, owing to the dependence of the size of σ on the actual composition of the assay (concentration of the buffer, the chelator(s), the vesicles; in general all charged substances), only results obtained under similar experimental condition can be compared.

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