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Influence of torus on the capacitance of asymmetrical phospholipid bilayers

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We have measured the specific capacitance of phosphatidylethanolamine/phosphatidylserine membranes formed from monolayers. The membranes were built across Teflon films whose thickness varied from 6 to 25 μm. The building up of the membranes was followed by recording the capacitance of the membranes during the entire process of their formation. It is observed that the specific capacitance increases by about 10% as the thickness of the film is increased. Furthermore, during formation of the membrane it is observed that the capacitance values increased markedly immediately before the membranes are completely formed and then suddenly decrease to their normal values when formed (closing-off phenomenon). These results have led us to propose that the transition region known to surround membranes formed from monolayers may affect the capacitance values measured for such membranes. However, this effect is indirect in the sense that the composition (most likely in the form of inverted micelles) of the transition region will ultimately modify the hydrophobic/hydrophilic barrier in the bilayer by affecting the lateral tension known to exist in such membranes and, as a consequence, the average molecular area occupied by a phospholipid molecule in the bilayer. By such a mechanism, one can rationalize our experimental finding that the membrane capacitance varies as a function of the partition thickness across which they are prepared. It also rationalizes the large fluctuations in capacitance values usually found in the literature for such membranes as well as, at least in part, the closing-off phenomenon.

1. Introduction

Studies on reconstituted planar membranes have deepened our understanding of a number of membrane phenomena to a large extent, particularly those related to transport processes. In this aspect, much is owed to the development of the black lipid membrane (BLM) methodology [1] or the more recent Montal-Mueller membrane reconstitution technique [2]. Indeed, these two techniques allow easy measurement of the electrical properties (e.g., specific capacitance and resistance) of the lipid bilayer, no restrictions being imposed on the accessibility of the aqueous phases on the two sides of the bilayer *. The Montal-Mueller technique, however, bears the additional advantage that planar asymmetrical bilayers can be formed, the bilayers being prepared by apposition of two monolayers of possibly different composition. This is actually an important improvement in biomembrane methodology if one is interested in studying, for example, the role of lipid asymmetry in the generation of transmembrane potential [3].

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* In this paper, we follow the convention proposed by Smith et al. [29] to refer to a membrane as designating the entire area of the lipid film across the hole in the septum, including any transition region linking the membrane to the septum. Bilayer refers to the regions of the membrane that are two molecules thick.

The present work deals more specifically with capacitance measurements of Montal-Mueller membranes. This type of measurement on reconstituted planar membranes has been used quite extensively in the past to study a number of membrane-related phenomena (e.g., refs. 4–10). However, there is an inherent weakness in measurements of the electrical properties of membranes. These measurements are indeed made on a system that is actually much larger than the bilayer of interest, comprising the transition zone (also called the torus or Plateau-Gibbs border) between the bilayer and the solid support (Teflon), the support itself, the troughs, the aqueous solutions on the two sides of the membrane and the electrodes. The electrical properties of the latter four components are easily measured but if the actual electrical characteristics of a given bilayer are to be determined, one must have a knowledge of how the transition zone affects the properties measured for the membrane. It is now indeed clear, as White et al. [11] have shown, that such a transition zone is present in Montal-Mueller membranes, although the overall volume of the transition is much less important than with BLM. Otherwise, the membranes would not be stable.

The aim of this work is to provide experimental evidence that this transition zone does influence the capacitance values measured for Montal-Mueller membranes. Phosphatidylethanolamine (PE)/phosphatidylserine (PS) membranes were formed across Teflon partitions of various thicknesses. It is found that the specific capacitance measured for the membranes depends significantly on the thickness of the partition. Furthermore, the capacitance values exhibit rather large fluctuations for a given set of experimental conditions. We show that these results are consistent with the existence of a transition zone linking the bilayer to its solid support and discuss the possible consequences of its presence.

2. Materials and methods

Montal-Mueller membranes were formed at room temperature ($22 \pm 1°C$) according to a technique described in detail by Tancrède et al. [12]. The membranes were formed on Teflon (Fluorocarbon, Dilectrix Division, Lockport, NY) partitions of various thicknesses, ranging from 6 to 25 μm. The aperture across the Teflon partition was punched using the perforating tool described by Robert et al. [13]. The quality of the hole punched was assessed by observation under a microscope (100 ×, Wild Heerbrugg M11, Basel, Switzerland) and the diameter was precisely measured, to within 3%, using a calibrated eyepiece graticle. Depending on the film thickness, the diameters ranged from 230 to 240 μm. The membrane area used to calculate the specific capacitance was taken as equal to the hole area. Prior to use, the apertures were pretreated with a solution (1 : 50, v/v) of squalene (Eastman Kodak, Rochester, NY) in *n*-hexane (BDH, Ville St-Laurent, Québec).

Asymmetrical PE/PS bilayers were formed. PE (beef heart) was obtained from Serdary Research Laboratories (London, Ontario) while two different PS samples were used. One PS sample (beef brain) was obtained from Sigma (St. Louis, MO), the other (hydrogenated bovine) being purchased from Pharmacia P-L Biochemicals (Milwaukee, WI). The two samples gave identical results within experimental error. All the samples showed only one spot when checked for purity by thin-layer chromatography and were used without further purification. The lipids were spread at a nitrogen/water interface using approx. 50 μl of a 2 mM solution of lipid dissolved in hexane/ethanol (9 : 1, v/v). The spreading area was 60 cm^2. Both hexane (BDH) and ethanol (Alcool de Commerce Limited, Gatineau, Québec) were distilled on a 30 cm high Vigreux column (Fisher, Montréal) prior to use and were found to be free of surface-active contaminants when checked on a classical Langmuir trough. The subphase on which the lipids were spread was 10^{-3} M phosphate buffer (pH = 7.0 ± 0.1) containing 0.1 M NaCl as additional electrolyte. The salts were Baker Analyzed Reagent grade (purity better than 99%, Baker, Phillipsburg, NJ) and were used without further purification.

The capacitance measurement is made by comparing two RC networks in parallel. A diagram of the electrical circuit and a detailed account of the technique used are described by Robert et al. [13]. The accuracy and precision of the capacitance

measurements are found to be better than 2%. Besides the capacitance measurements which have been used to follow the build up of the membranes, our set up was also equipped with a stereomicroscope (80 ×, M3, Wild Heerbrugg) focused on the hole area, which also allowed visual monitoring of the build up of the membrane. It was also useful in adjusting very precisely the water levels across the membrane, the two water interfaces being both visible due to the transparency of the Teflon film. The capacitance values presented below are therefore those corresponding to a membrane across which there is no difference in hydrostatic pressure.

Table 1

Specific capacitance of PE/PS membranes formed across partitions of various thicknesses

δ_p, Teflon partition thickness; C_M, specific capacitance (given as mean value ± S.D.); N, number of membranes formed.

δ_p (μm)	C_M (nF cm^{-2})	N
6.4	661 ± 50	54
9.5	665 ± 25	38
12.7	663 ± 44	55
19.0	694 ± 42	49
25.4	724 ± 48	25

3. Results and discussion

We have measured the specific capacitance of PE/PS membranes for five different Teflon partition thicknesses, ranging from 6.4 to 25.4 μm. The experimental results are presented in table 1. The specific capacitance has a mean value of about 660 nF cm^{-2} for partition thicknesses in the range 6.4–12.7 μm. This mean value rises to 694 and 724 nF cm^{-2} when the partition thickness is increased to 19.0 and 25.4 μm, respectively. Although the experimental error in the capacitance values is rather large (see below for a further discussion of this point), a statistical evaluation of these data using the Student criterion shows the increase in capacitance to be clearly significant. Considering the number of experimental measurements performed for each thickness studied, the Student criterion gives a probability of confidence of 99.98% for the difference in capacitance being significant between 12.7 and 19.0 μm and 99.2% for values between 19.0 and 25.4 μm.

Clues as to the possible origin of this 10% increase in the specific capacitance of membranes prepared on partitions of greater thicknesses may be provided by an analysis of the various parameters involved in calculation of the specific capacitance. Indeed, the capacitance, C_M, of a membrane is given by

$$C_M = \frac{\epsilon_0 \epsilon_M A_M}{\delta_M}$$

where ϵ_0 represents the permittivity of a vacuum (8.854×10^{-12} μF m^{-1}), ϵ_M the membrane dielectric constant (usually taken as 2.2, corresponding to the hydrophobic core dielectric constant), A_M the membrane area and δ_M the hydrophobic thickness. It is not a simple task to pinpoint more precisely which of the geometrical (A_M, δ_M) or electrical (ϵ_M) factors are involved in the observed increase of capacitance. However, we know that the specific capacitance we measure for Montal-Mueller membranes refers to both bilayer and non-bilayer parts. Although the transition zone for Montal-Mueller membranes appears to be of small dimensions with respect to the bilayer [7,14,15], no systematic study has yet been performed related to the electrical characteristics of this transition zone and their effect on the properties measured for this type of membrane. On black lipid films however, White [16] has shown that the capacitance of the transition zone accounts for less than 1% of the membrane capacitance, as expected considering the probable size and overall dielectric properties of this region.

In the case of Montal-Mueller membranes, clues pertinent to the importance of the transition region can be obtained if one considers in detail the mode of formation of Montal-Mueller membranes which has been followed here by measurements of the capacitance during the course of membrane formation. Fig. 1 shows a typical variation of the capacitance recorded during the various phases of membrane formation. The capacitance is given as a function of the approximate volume of aqueous

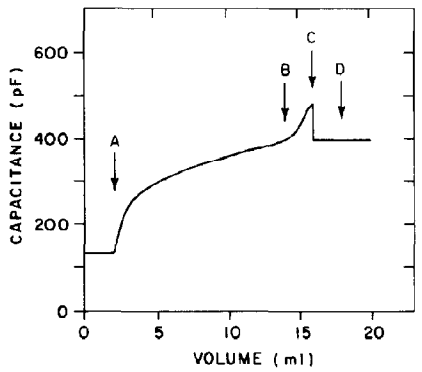

Fig. 1. Typical rise of capacitance during the formation of a membrane, plotted as a function of the approximate volume of aqueous solution added to the *cis* compartment to obtain a completely formed membrane. The various arrows (from A to D) refer to different time periods during the course of membrane formation, as described in the text.

solution added in one of the two troughs to obtain a completely formed membrane. The rather large volumes required reflect the overall dimensions of our troughs [12]. The *x*-axis is therefore a rough measure of the extent of membrane formation. Point A in fig. 1 corresponds to the situation where the water level in the *trans* compartment is raised over the aperture while that in the *cis* compartment is immediately below. The value of the capacitance at this point is related to the capacitance of the Teflon film separating the two aqueous compartments. When aqueous subphase is added to the *cis* compartment the capacitance begins to rise rapidly, subsequently slowing down as more solution is added. This rise in the capacitance corresponds to the value of the capacitance for a membrane of increasing area. When the bilayer is almost completed (point B) a rapid increase in capacitance (typically, about 10% of the capacitance measured) is observed (point C). Hansen [17] has also reported such a phenomenon for Montal-Mueller membranes. The shape of this overshoot in capacitance values between points B and D is extremely variable for different membranes prepared on any Teflon film thickness. Further discussion of this point will be presented below. Immediately after this overshoot, the final value for the bilayer is recorded (point D). This value is then stable, to within a few percent, as a function of time on a scale of about 3 h. Furthermore, it shows no variation upon a sudden change in the applied voltage thus indicating that the bilayer is virtually solvent-free [7].

The critical moment governing formation of the bilayer, its stability and subsequent behaviour is clearly when the membrane closes off. At this point, the interface in the *cis* compartment reaches the top part of the aperture on which squalene, lipid and water molecules from the *trans* compartment are already present. It is most likely that these components form a microemulsion (probably in the form of inverted micelles) in the transition region, which is thought to represent the nature of the material surrounding the bilayer. For thermodynamic reasons, these inverted micelles would remain mainly in the transition region [18]. This situation is quite clear inasmuch as BLM are concerned [19–21]. It may not be very different for Montal-Mueller membranes, since it has been shown [11] that these membranes cannot be formed if such a transition region is absent. In confirmation of this point, it has been observed that Montal-Mueller membranes, in a similarly way to BLM [15,22], have been shown to possess a measurable lateral tension of a few mN m^{-1} [23].

The existence of this lateral tension in Montal-Mueller membranes has important consequences, since it affects directly the surface pressure within the bilayer. Evans and Waugh [24] have related these two parameters explicitly by $\pi = \gamma - \sigma$, π being the surface pressure, γ, the surface tension and σ, the lateral tension. Therefore, increasing the lateral tension by 1 mN m^{-1} decreases the surface pressure by 1 mN m^{-1}. In turn, if the surface pressure within the bilayer is changed, so will the area per molecule of lipid in the bilayer. Actually, one can quantify this effect by taking into account the recent lateral compressibility data reported by White and King [25] for egg phosphatidylcholine multilayers, from which one can calculate that a change of 1 mN m^{-1} in the surface pressure of the bilayer can produce a change in area per molecule of the lipid of about 0.5–1.0 $Å^2$. This change does not seem large on an absolute basis but it has very im-

portant consequences inasmuch as capacitance measurements are involved. Indeed, this slight change in area per molecule of the lipid would be sufficient to affect the penetration of water molecules within the bilayer, close to its hydrophobic core (i.e., even within the carbonyl bonds of the lipid [26]), thereby modifying the position of the hydrophobic/hydrophilic barrier in the bilayer. This would have a large effect on the capacitance measurements. For example, a change of only 1.5 Å in the position of the barrier on each side of a bilayer 28 Å in thickness would change the capacitance values by about 11%. The argument presented here is similar, in principle, to that given by Bramhall [27] to rationalize the difference in fluorescence emission of probe molecules when present in the inner or outer monolayers of small lipid vesicles, or to that of Simon et al. [28] to explain the effect of cholesterol on the capacitance of bacterial PE Montal-Mueller bilayers.

If one now returns to table 1, bearing in mind the ideas put forward above, it becomes possible to rationalize the significant change in capacitance values observed when bilayers are prepared across apertures of different thicknesses. Indeed, we can reasonably assume that upon changing the thickness of the Teflon film across which the bilayers are formed, the volume of the transition region and, possibly also its composition will be different. Now, Gruen and Wolfe [20] have shown that the lateral tension in a BLM-type membrane is related to the composition as well as to the size of the micelles confined in the torus region. The change in lateral tension involved can reach a few mN m^{-1}, i.e., it corresponds to a large fraction of the actual tension measured in BLM or Montal-Mueller membranes. As explained above, this difference in tension would yield to measurable differences in capacitance values between membranes of the same lipid composition, but prepared on partitions differing in thickness.

It is also possible, by using the arguments presented above, to explain the rather large fluctuations in capacitance values measured for Montal-Mueller membranes. This is clearly observed in table 1, column 2, which shows the standard deviation of the capacitance with respect to the mean value for the five Teflon thicknesses studied. The fluctuations in most cases are about $\pm 8\%$ of the mean capacitance values, much beyond the negligible variations recorded when calibration capacitors are used. These large fluctuations are also evident in the capacitance values reported by various authors, for either phospholipid membranes (table 2) or monoglyceride membranes of well-defined chain composition (table 3). We believe that these fluctuations for a given type of membrane reflect the fact that upon preparing various membranes under a given set of conditions, the transition region may vary slightly in composition, thus changing the lateral tension in the membrane. As a consequence, the thickness of the hydrophobic portion of the bilayer will vary as more or less water molecules would be allowed to penetrate further into the region of the first methylene groups close to the carbonyls. Furthermore, it should be recognized that the large fluctuations observed in the capacitance measurements of Montal-Mueller membranes or of BLM are inherent to the mode of formation of such membranes. In particular, for Montal-Mueller membranes it is clear that these membranes are not, and cannot, be formed under completely reproducible conditions. For example, it is not possible to spread the squalene coating

Table 2

Literature data on the specific capacitance of Montal-Mueller phospholipid membranes

DPG, diphosphatidylglycerol; PC, phosphatidylcholine; δ_p, Teflon partition thickness; C_M, specific capacitance; values of C_M are means $\pm$ S.D., the number of membranes being given in parentheses when specified.

Composition	δ_p (μm)	C_M (nF cm^{-2})	Ref.
DPG/DPG		900 ± 20 (8)	30
PS/PS		700 ± 30 (12)	30
PC/PC	12.5	750 ± 50 (28)	31
PC/PC	12.5	760 ± 40	32
PE/PE	19.0	650 ± 10 (8)	7
PE/PS	19.0	720 ± 20 (11)	7
PE/PS	19.0	670 ± 30 (3)	7
PE/PE		630 ± 30	33
PE/PE		680 ± 10	14
PE/PS	12.5	700 ± 45 (32)	13

Table 3

Literature data on the specific capacitance of symmetrical Montal-Mueller monoglyceride membranes

MG, monoglyceride (chain length : unsaturation); δ_p, Teflon partition thickness; C_M, specific capacitance; values of C_M are means ± S.D., the number of membranes being given in parentheses when specified.

Composition	δ_p (μm)	C_M (nF cm^{-2})	Ref.
MG (16:1)	12.5	847 ± 26 (34)	23
MG (18:1)	12.5	745 ± 24 (33)	23
MG (20:1)	12.5	657 ± 26 (27)	23
MG (18:1)	19.0	750 ± 30 (26)	7
MG (18:1)		790 ± 10	14
MG (16:1)	20.0	985 ± 45	34
MG (18:1)	20.0	852 ± 43	34
MG (20:1)	20.0	788 ± 40	34

around the aperture in exactly the same way. It is not possible either to punch the aperture and produce a hole that is exactly identical, at the molecular level, to one which has been punched previously. The membrane formed at the end may retain more or less lipid, squalene and water molecules so that either the volume of the transition region or, more likely, the composition of this transition region in terms of the size and number of inverted micelles may be different from membrane to membrane.

It is also important to note that the mean capacitance and fluctuation values reported in table 1 represent the average values for a large number of membranes (column 4) prepared on different days of experimentation using different Teflon partitions each time. We have indeed noted that the capacitance values obtained on a given day (i.e., on a given Teflon partition) are rather reproducible (e.g., to within ± a few percent). However, the mean capacitance value for various days of experimentation may differ to a much greater extent (up to ± 8% as shown in table 1) than the fluctuations recorded during a given day. This is due to the fact that a new Teflon film is used each time. The general conditions being slightly different each time, the capacitance values may be rather different in response to the variability in composition of the transition region and the consequence of this variability on the position of the hydrophobic/hydrophilic barrier within the membrane. It is therefore most important to average out capacitance measurements over various film preparations in order to obtain significant results.

By using the same line of arguments, one can also rationalize the closing-off phenomenon, which was referred to as point C in fig. 1. As described above in brief, the rapid rise in capacitance values observed immediately before the final capacitance of the membrane is recorded can reach, typically, about 10% of the capacitance measured. However, this value is extremely variable and can be found to be much larger for some membranes, sometimes reaching values as high as 50% of the capacitance measured after the membrane is formed. This broad variability observed in the shape and amplitude of the closing-off phenomenon again reflects the fact that forming a Montal-Mueller membrane does not correspond to a true thermodynamic equilibrium process. In the very last step of formation, the membrane often builds up itself almost spontaneously, beyond the control of the experimenter. The large variability in the closing-off phenomenon may, at least in part, be related to the volume and composition (i.e., number and dimensions of the inverted micelles) of the transition region at the critical moment when the water level in the *cis* compartment reaches the top portion of the aperture. This particular composition may, to a large extent, affect the lateral tension of the almost completely formed bilayer and thus, via the mechanism described above, the capacitance values recorded at this point. It is also very likely, however, that in addition to this effect, other concurrent effects occur during the closing-off phenomenon (e.g., geometrical effects such as repositioning of the bilayer in the Teflon partition, which may affect the area of the bilayer).

In conclusion, the present work has explored the possibility that the transition region around the bilayer in Montal-Mueller membranes is likely to affect the capacitance values measured for such membranes. The effect is indirect in the sense that the composition of the transition region (which, for the reasons stated above, may be quite variable) will influence the lateral tension on the bilayer which, in turn, will ultimately result in re-

positioning of the hydrophobic/hydrophilic barrier, to which the capacitance values measured are so sensitively related. With the aid of such a mechanism, one can rationalize our experimental finding that the membrane capacitance varies as a function of the partition thickness across which they are prepared. It also rationalizes the large fluctuations in capacitance values usually found in the literature for such membranes as well as, at least in part, the closing-off phenomenon. Our results also draw attention to the fact that, for comparison of data between laboratories, the film thickness as well as the number of membranes and conditions prevailing should be given in detail.

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