LETTER TO THE EDITOR

Glyceryl Monooleate Black Lipid Membranes Obtained from Squalene Solutions

Dear Sir:

White (1978) measured the specific capacitance of black lipid films of glyceryl monooleate formed from squalene solution. The measured geometric specific capacitance of 0.78 μF/cm^2 corresponds to a thickness of 25 Å of the hydrocarbon layer, indicating that the bilayer is practically solvent-free. As bilayers formed from lipids dispersed in alkanes contain various amounts of solvent, the new technique developed by White (1978) gives a possibility to obtain solvent-free bilayers. It was of interest to see if another technique gives thickness comparable to that obtained by capacitance measurements. To reach this aim we measured the reflectivity of these bilayers. Previously, "solvent-poor" bilayers were prepared from glycerol monooleate in hexadecane (Fettiplace et al., 1971). They have specific capacitance of 0.58 μF/cm^2, and thickness of the hydrocarbon layer is 32 Å. Pagano et al. (1973) noted a discrepancy in the thickness of bilayers of glyceryl monooleate formed from tetradecane and hexadecane solutions, as determined by capacitance and by light reflectance methods. These authors concluded that this discrepancy stems from light reflecting microlenses which do not influence, however, the capacitance measurements. It has been suggested by one of us (Miller, 1979) that, when the cross-sectional areas of the polar groups of the bilayer-forming lipids are considerably larger than the cross-sectional areas of the hydrocarbon chains, the bilayer surface will not be strictly planar but will fluctuate, assuming a dynamic, wavy form with interchanging protrusions and indentations, which are formed at constant bilayer density. Thus, both methods measure an average thickness. However, the reflectance gives an average over the square of the thickness of the different regions while the capacitance averages over the reciprocal of the thicknesses.

The experimental set-up for measuring reflectance was as developed previously by Cherry and Chapman (1969), in which the membranes are illuminated at near normal incidence by a helium-neon laser. The membrane reflectance is obtained by calibrating the reflected intensity with a quartz plate placed in the same aqueous solution. The reflectivity of the membrane, R_m, is related to its thickness, by the following equation:

$$\frac{\lambda R_m^{1/2}}{2\pi d} = n_m - n_0 + \Delta. \tag{1}$$

$$\Delta = (n_m - n_0)^2/(n_m + n_0), \tag{2}$$

where λ is the wavelength of the incident light, d is the total membrane thickness, n_m is the effective refractive index of the membrane and n_0 is the refractive index of the aqueous phase. n_0 is varied by addition of sucrose to the aqueous phase. By extrapolating $R_m^{1/2}$ against n_0 to $R_m = 0$, an approximate value of n_m, used for evaluation of Δ, is obtained. The final values of d and n_m are calculated from the plot of $R_m^{1/2}$ against $(n_0 - \Delta)$.

Glyceryl monooleate (monoolein) was purchased from Sigma Chemical Co., St. Louis, Mo. Squalene, for synthesis, was purchased from Merck, Schuchardt, München, West Germany, and it was purified by passing through alumina column. The membranes were formed from 1.5% glyceryl monooleate dispersed either in *n*-decane or in squalene in unbuffered 10^{-3} M NaCl solutions (Analar, BDH Chemicals, Poole, England). Sucrose, used for varying the refractive index of the solutions, was also of analytical grade and it was purchased from BDH Chemicals. The experiments were performed at room

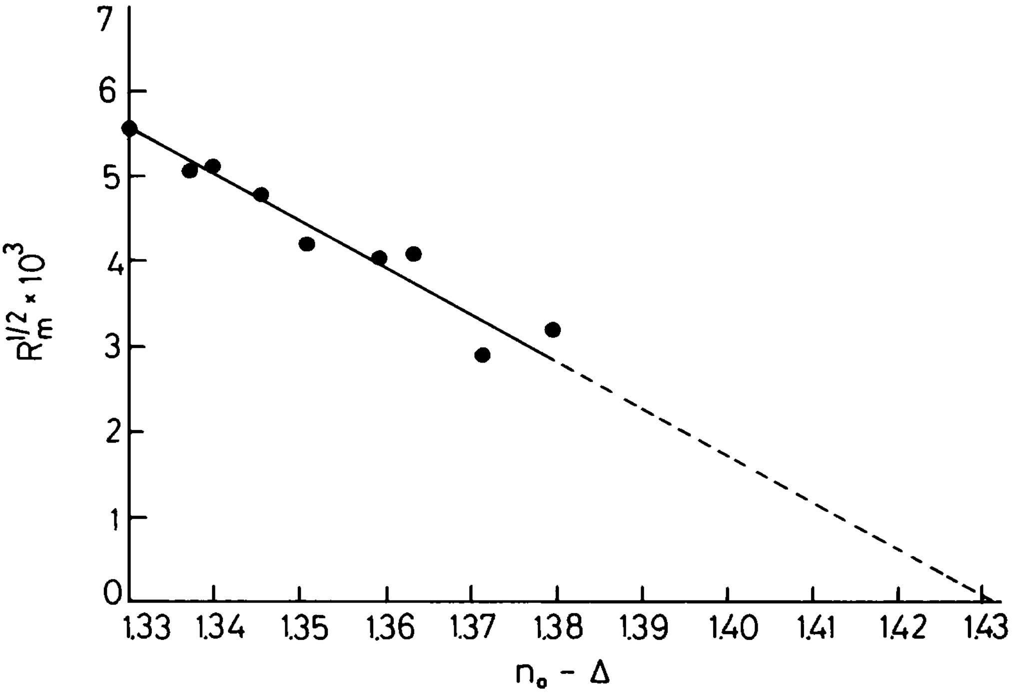

FIGURE 1 Variation of the square root of reflectivity (R_m) of bilayers formed from glyceryl monooleate in squalene with refractive index ($n_0 - \Delta$).

temperature. The performance of the experimental set-up was checked by obtaining the thickness of the glyceryl monooleate bilayers dispersed in decane; the value obtained, 67 Å, is quite in agreement with the previously published data (Pagano et al., 1973).

Fig. 1 presents square root of the reflectivity of glyceryl monooleate bilayers formed from squalene solution, as a function of the refractive index. The calculated thickness of the bilayers is 57 Å, and refractive index is 1.431. There may be some objection to the determination of the bilayer thickness and its refractive index by this method, mainly because of the possible influence of sucrose or of salt (used in other experiments) on the thickness and on the refractive index of the membrane. According to Andrews et al. (1970), saturated NaCl causes a reduction of thickness of glyceryl monooleate bilayers spread from hexadecane as inferred from the specific capacitance by ~10%. The effect of salt on the thickness seems to depend on the degree of solvation of the bilayer being relatively small in highly solvated bilayers, when spread from low molecular hydrocarbons. It reaches a maximum at some intermediate solvation when spread from tetradecane, and it decreases with further desolvation.

Changing the refractive index of the medium by NaCl, $CaCl_2$, or sucrose gives identical results for different systems (Cherry et al., 1972; Pagano et al., 1973). If the thinning is approximately proportional to the concentration of the additive $d = d_0 - \delta C_{ad}$ the relation between the reflectivity R_m and the thickness d_0 would assume the following form in water:

$$\frac{\lambda R^{1/2}}{2\pi} = d_0 - \delta C_{ad}(n_m - n_0 + \Delta)C_{ad} \qquad (3)$$

According to Eq. 3 the initial slope is still proportional to d_0. Moreover, no appreciable deflection from linearity, as required by Eq. 1, has been observed neither by us nor by other investigators. This could be explained by insufficient experimental resolution. However, even when fitting the reconstructed line to the experimental one under the assumption that there is a reduction in thickness with sucrose concentration up to 10% and the reflectivity would be adjusted to fit d_0, thus the recalculated thickness would be reduced by $<10\%$ to above 52 Å or to above 49 Å, respectively, if there is another systematic error of unknown source.

This thickness is much higher than the one determined by White (1978), even if one adds the thickness of the polar regions (2 × 5 Å) which are not measured by the capacitance method. The points given in Fig. 1 are based on more than 60 reflectivity measurements, and the validity of the straight line is better than 99% as obtained from the correlation coefficient. The reliability of the measurements inferred from the spread of the experimental points is better than ±2 Å. It is, however, possible that we may have a systematic error of +7% as our values for the glyceryl monooleate/decane bilayers (67 Å) are higher than those obtained by Pagano et al. (1973) (62 Å), which would reduce our measured value of the bilayers formed from squalene to 53 Å. Even this value is by ~18 Å larger than estimated from White's (1978) capacitance measurements after accounting for the polar layer not measured by the capacitance technique.

In the case of glyceryl monooleate bilayers formed from squalene, the discrepancy cannot be attributed only to microlenses for the following reasons: (*a*) the black layer forms abruptly and spontaneously from a thick-colored film, not allowing any entrapment of lenses, which usually occurs during the gradual thinning process. (*b*) The cross section of the laser beam on the bilayer could accomodate only a very small number of microlenses, even if they would exist. Since the fluctuation in the number equals to its square root, the fluctuation in reflectance due to fluctuating number of lenses should amount to several tens of percent, which was not observed.

We wish to propose that the main source of the difference of the thickness, as measured by the two methods, is the deviation from the planarity of the bilayer surfaces. The deviation may be due to fluctuating transients from planarity. The transient forms may be considered as transition state between two stable basic structures, namely the planar bilayer and the micelle. The reflectance measures an average on the square of the thickness δ over the area S:

$$\delta_r = \left(\frac{\int \delta^2 ds}{\int ds} \right)^{1/2}, \tag{4}$$

whereas the capacitance measures the average on the reciprocal of the thickness,

$$\frac{1}{\delta_c} = \frac{\int \frac{1}{\delta} ds}{\int ds}. \tag{5}$$

If we assume an oscillating wavy structure for the bilayer, as presented in Fig. 2, with swellings and indentations of equal sizes having forms of spherical caps, one obtains for the average capacitive thickness δ_c (Miller, 1979):

$$1/\delta_c = \frac{1}{\delta_{\max} - \delta_{\min}} \log \frac{\delta_{\max}}{\delta_{\min}}. \tag{6}$$

If White's (1978) capacitive thickness of 25 Å is obtained for a bilayer fluctuating between maximal chain extension of 55 Å and some minimal value, this minimal thickness is, according to Eq. 6, ~14 Å.

Is such a model energetically feasible? To answer this question, one has to estimate the energy invested during fluctuation per cooperative unit. A swelling and an indentation, not necessarily identical in shape but formed at constant volume, can be considered to be a cooperative unit. To maintain constant volume and constant macroscopic area of the membrane the radius of the swelling and of the indentation has to be of the order of the length of a lipid molecule. The area difference between a spherical cap and its cross section is $\pi(\Delta\delta_{\max})^2$, where $\Delta\delta_{\max}$ (see Fig. 2) is the height. The relative area increase is then

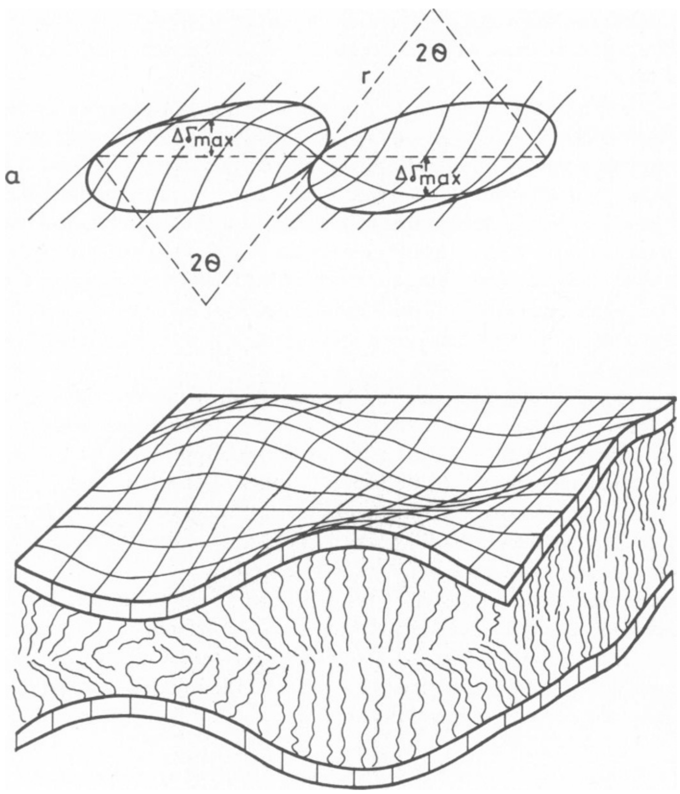

FIGURE 2 Breathing model of a lipid bilayer. (*a*) A swelling and an indentation, shaped like spherical cups.

$\Delta\delta^2_{max}/R^2$, where R is the radius of the cap. Taking $\Delta\delta_{max} = 9$ Å and $R = 30$ Å, the area will increase by ~9% during the cap formation. The cross section of the polar group is so much larger than that of the hydrocarbon chain that this relative increase in area does not need to reveal any appreciable high energy water hydrocarbon interface. Thus, the expansion is not likely to increase the local interfacial energy σ by more than 1–2 erg/cm^2, e.g., in *n*-decane with area per molecule 30–35% higher than in fully compressed state $-\sigma < 4$ erg/cm^2 is obtained (Andrews et al., 1970). The total interfacial fluctuation energy $\int \sigma ds$ per cooperative unit is not likely to exceed a fraction of kT or up to 1 or 2 kT U, which make it a very probable event.

The same model, presented in Fig. 2, gives for the average thickness, as measured by light reflectance (Eq. 4), the following relation:

$$\delta_r = \left[\left(\frac{\delta_{max} + \delta_{min}}{2}\right)^2 + \frac{1}{3}(\delta_{max} - \delta_{min})^2\right]^{1/2}. \qquad (7)$$

The thickness, δ_r, calculated from Eq. 4 for $\delta_{max} = 65$ Å and $\delta_{min} = 24$ Å (corresponding to the capacitive $\delta_{max} = 55$ Å and $\delta_{min} = 14$ Å adding only 10 Å for the polar layers), comes out to be 47 Å. This is by 6 Å lower than the lowest possible value inferred from our reflectance measurements. Presumably, some reflecting microlenses or other impurities could not be completely excluded. These impurities could come by adsorption from the aqueous solution containing dispersed lipid. There is still a possibility that the δ_{max} may be larger than the maximal chain extension since the chain ends of the lipids from the thin regions may contribute to δ_{max}. If we take $\delta_{max} = 70$ Å (δ_{max} hydrocarbon region $= 60$ Å), then the required $\delta_{min} =$

18 Å (hydrocarbon layer = 8 Å) for obtaining $\delta_c = 25$ Å, as measured by White (1978). The calculated value of δ_r, by Eq. 7, is for these values of δ_{max} and δ_{min} 53 Å, which is within the region of our measured values.

How should a solvent-free bilayer, visualized as a wavy structure, behave under the influence of an applied electrical field? The solvated bilayers undergo thinning, showing an increase in capacitance (Andrews et al., 1970). An electrical field across a wavy-shaped bilayer should effect more strongly the swellings rather than the indentations because of the steric repulsion contribution. Therefore, at constant membrane volume the planarity of the bilayer is bound to be enhanced by the field resulting in decrease of measured capacitance and of the reflectivity. Indeed, the relatively solvent-poor glyceryl monooleate bilayers formed from hexadecane show a decrease in capacitance upon application of an electrical field, which is contrary to the behavior of the more solvated glyceryl monooleate monolayers investigated by these authors.

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