LETTER TO THE EDITOR

Response to Energetics of Fluctuation in Lipid Bilayer Thickness by I. R. Miller

Dear Sir:

In response to Dr. Miller's letter we wish to make four points. We preface our comments by defining two terms. In discussing a wavy bilayer we will refer to "the area per molecule at the bilayer surface" and to "the area per molecule at the midplane." The former phrase means the area of the actual surface of the bilayer occupied by one molecule; the latter refers to the area of the image of the molecule projected onto the flat midplane. Of course, for a flat bilayer, the two are identical.

First, a large part of Miller's letter presents a calculation that demonstrates that a wavy bilayer structure can be generated with the same area per molecule at the bilayer surface and the same density of chain packing as a flat bilayer. In going from a flat to a wavy structure, the number of molecules per unit area of midplane is increased in such a way that the area per molecule at the surface remains constant. Since the density of chain packing remains constant, the average thickness of the bilayer increases in proportion to the increase in number of molecules per unit area of midplane. Equivalently,

$$\frac{\text{average thickness of wavy bilayer}}{\text{average thickness of flat bilayer}} = \frac{\text{area at bilayer surface}}{\text{area at bilayer midplane}},$$

as can be verified from Miller's Table I. We are in complete agreement with this section of Miller's letter.

We feel, however, that these geometrical arguments are not relevant to the prediction of the structure of a membrane with known capacitance per unit area of midplane. There is agreement between Miller and ourselves that a capacitance measurement leads to a value for the mean reciprocal thickness of the hydrophobic core, $\langle 1/d \rangle$. Hence, this quantity, $\langle 1/d \rangle$, can be taken as given. Any suggested structure for the bilayer must be consistent with the experimentally observed value for $\langle 1/d \rangle$. The structures displayed in Table I of Miller's letter do not have the same value for $\langle 1/d \rangle$ as a flat bilayer with the same area per molecule. Hence this table does not compare the various structures compatible with the experimental data. For constant $\langle 1/d \rangle$, the area per molecule is substantially larger for a wavy structure than for a flat structure for the bilayer.

It is also possible to argue, even without reference to the capacitance data, that a flat structure for a solventless membrane is strongly preferred over any of those considered in Miller's Table I. In all of these wavy structures, there are regions of the bilayer where the chains are required to be extended at least 90% of their fully extended length ($^{21}/_{23} \sim 0.9$). This imposes very severe constraints on the conformations available to these chains and hence is unfavorable in free energy terms. Although calculations have not been attempted for monoolein chains with the geometry envisaged by Miller, they have been undertaken for saturated C_{16} chains in flat bilayers (1). Examination of Fig. 4 from reference 1 suggests an estimate of 3–5 kT per chain as the free energy cost of requiring chains to have a 90% extension (equivalent for saturated C_{16} chains to an area per chain of ~ 23.5 Å^2). This estimate suggests that each of Miller's spherical caps would have a free energy cost of many kT and hence would be very unlikely.

Second, in the penultimate paragraph of his letter, Miller states, "...surface isotherms of different lipids indicate that close to the equilibrium spreading pressure there is a region of sizable compression without large variations in surface pressure. This suggests that some variations in area per molecule may be tolerated during fluctuations." Such regions of a surface pressure curve are most likely due either to a phase transition in the monolayer or to collapse of the monolayer, neither of which is occurring in the bilayer. Fortunately there is no need to rely on monolayer data. The effect of variations in bilayer area per molecule on bilayer surface tension have been directly measured (2). Thus, for egg lecithin, the bilayer surface tension (σ) satisfies $\sigma = 139\ \Delta A/A_0$ dyn/cm for a ΔA change in area from an unstressed area of A_0. On the basis of this measurement (admittedly with a different amphiphile), area changes are substantially more costly than suggested by Bach and Miller on page 186 of reference 3.

Third, in his final paragraph, Miller claims that the reflectance measurements of Bach and Miller (3) and of Dilger (4) give conflicting results. This is not the whole story. Comparison of Bach and Miller's Fig. 1 and Dilger's Fig. 2 reveals a substantial difference in the goodness of fit of the data to the respective linear regressions. A remark of Dilger's is apposite: "The reflectance measurements of Bach and Miller indicated that monoolein/squalene films in sucrose solutions had a thickness of 5.7 nm and a refractive index of 1.431, which is significantly different from our results (d = 4.23 nm, $n_\perp$ = 1.451). We note, however, that there is considerable scatter in their data and that d = 4.5 nm and $n_\perp$ = 1.45 appear to give an equally reasonable fit to their data. This is comparable with our results. If our measurements are correct, there is no need to postulate that monoolein/squalene bilayers have a structure which is not planar."

Thus, the measurements of Bach and Miller and of Dilger are consistent with a bilayer with small (or no) fluctuations from planarity. They are not both consistent with the bilayer structure suggested in reference 3 nor with any of the structures described in Table I of Miller's letter.

Fourth, there is general agreement between Miller and ourselves that fluctuations in shape must occur. We also agree on the geometrical consequences of such fluctuations. Our disagreement with Miller concerns the energetics of the fluctuations and thus their average size. Bach and Miller (3) proposed large fluctuations occupying most of the area of the membrane as the explanation of the difference between the thicknesses they calculated from measurements of capacitance and reflectance for glyceryl monooleate/squalene membranes. However, they did not evaluate the energy cost of such fluctuations. We attempted (5) to evaluate this energy cost and hence to estimate the size of the fluctuations theoretically. We concluded that for glyceryl monoolein/squalene membranes the root mean square (rms)

BIOPHYS. J. © Biophysical Society · 0006–3495/84/03/645/02 $1.00
Volume 45 March 1984 645–646

deviation in thickness was most likely to be <1 Å. If the fluctuations are of this size, different averages of thickness should give indistinguishable results (with present experimental accuracy). Here, we have presented reasons why we feel that the structures displayed in Table I of Miller's letter would occur only very infrequently.

Received for publication 13 July 1983 and in final form 9 August 1983.

REFERENCES

1. Gruen, D. W. R. 1982. Statistical thermodynamics of alkyl chain conformations in lipid bilayers. *Chem. Phys. Lipids.* 30:105–120.
2. Kwok, R., and E. Evans. 1981. Thermoelasticity of large lecithin bilayer vesicles. *Biophys. J.* 35:637–652.
3. Bach, D., and I. R. Miller. 1980. Glyceryl monooleate black lipid membranes obtained from squalene solutions. *Biophys. J.* 29:183–187.
4. Dilger, J. P. 1981. The thickness of monoolein lipid bilayers as determined from reflectance measurements. *Biochim. Biophys. Acta.* 645:357–363.
5. Hladky, S. B., and D. W. R. Gruen. 1982. Thickness fluctuations in black lipid membranes. *Biophys. J.* 38:251–258.

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