eralized polarization measurements of 2-dimethylamino-6-lauroylnaphthalene, which are ratiometric (Parasassi et al., 1995). Moreover, our data report a relatively small effect in comparison with that caused by phospholipid phase transition. In our interpretation, when regular molecular arrangements are formed because of cholesterol regular distribution, water concentration decreases rather than increases as suggested by the intensity quenching shown by Tang et al. (1995). DPH fluorescence quenching could also be due to DPH release to the solvent when regular arrangements occur. Given the hydrophobicity of DPH, this hypothesis is hard to believe. Other explanations for the decrease of DPH intensity are also difficult to believe, such as the proposed "geometrical quenching" by Tang et al. (1995). DPH molecules are far apart from each other, the molecular ratio with phospholipids being of 1:1000. A different packing of the bilayer due to regular arrangements at specific cholesterol concentrations cannot cause quenching of the DPH molecules, because at that distance, DPH molecules are noninteracting. Moreover, in a well packed membrane such as a single phospholipid in the gel phase, DPH is strongly fluorescent.

We suggest that the dips reported by Tang et al. (1995) are due to artifacts of the sample preparation. We believe that regular molecular arrangements due to guest molecules in the bilayer, such as pyrene-PC and cholesterol, can exist and can be of biological relevance, but their presence should not modify DPH fluorescence intensity to the extent reported by Tang et al. (1995).

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Response to Gratton and Parasassi

We published an article in the May 1995 issue of the *Biophysical Journal* showing evidence for a regular distribution of cholesterol in phospholipid bilayers (Tang et al., 1995). The phospholipid systems studied were mixtures of cholesterol and dimirystoylphosphatidylcholine (DMPC), and we used the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Drs. Parasassi and Gratton raised some questions concerning our paper (Parasassi and Gratton, 1996). Their concern refers to the anomalous quenching of DPH fluorescence we found at 20 mol % cholesterol. We also found dips at other cholesterol concentrations such as 25%, 40%, and so on. We do not know if they repeated these other results also. They state that this phenomenon is hard to explain. In their interpretation the formation of a regular molecular arrangement occurring at this 20 mol % should decrease the level of water penetration into the membrane, which would lead to an increase of fluorescence, not a quenching. They also reject our proposed "geometrical quenching." This point of criticism is well taken: At this moment, we do not think there is a satisfactory explanation for this quenching phenomenon. Our suggesting the term "geometric quenching" is not an explanation, it is just a way of saying that this strange quenching phenomenon—whatever its origin—is somehow related to the geometry of the regular distribution. Perhaps some sort of cooperativity is involved between the DPH molecules over distances of the order of the periodicity length of the superlattice. At this point, we do not know how to explain the phenomenon. We do know, however, that this quenching occurs. Drs. Parasassi and Gratton state that this anomalous DPH intensity behavior was not noticed by others. They are wrong about that: Lentz et al. (1980) observed an unusual drop of DPH fluorescence intensity in cholesterol/phospholipid systems near 20%. Recently Virtanen et al. (1995) found an anomaly in the excimer-to-monomer ratio of

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dipyrenylphosphatidylcholines at 20 mol % cholesterol. Chong (1994) reported a dip in the dehydroergosterol fluorescence at 20 mol % concentration of this cholesterol analog in phospholipids. Others have also observed drastic changes around 20 mol % using x-ray diffraction (Hui and He, 1983), differential scanning calorimetry (Estep et al., 1984; Mabrey et al., 1978), dilatometry (Melchior et al., 1980), spin-label ESR (Recktenwald and McConnell, 1981; Presti et al., 1982), freeze-fracture electron microscopy (Copeland and McConnell, 1980; Kleemann and McConnell, 1976; Lentz et al., 1980), and NMR (Opilla et al., 1976; Vist and Davis, 1990). Drs. Parasassi and Gratton claim that we overlooked the question of reproducibility of intensity-based measurements. They are wrong again. In our paper we stated: "... the unusual drops of fluorescence intensity at critical concentrations can be observed in different groups of independently prepared samples by comparing the intensities of neighboring concentrations." In our Fig. 3, we show standard deviations from three independent groups of samples. These standard deviations are of the order of 10%, in agreement with results of Drs. Parasassi and Gratton. In the legends of our other figures, we have indicated that the error bars are similar to the ones in Fig. 3. Drs. Parasassi and Gratton prefer ratiometric measurements. However, such experiments are directly related to intensity measurements. As long as the drop in intensity clearly exceeds the standard deviation as is the case in our results, there is no problem with intensity-based measurements. Drs. Parasassi and Gratton could not reproduce the dip at 20%. Why not? They claim that they reproduced all the experimental conditions described in our paper. However, they say that there are artifacts in our sample preparation. That means either that we made mistakes in our preparation or that their preparation is different from ours. Our sample preparation has been clearly described in our paper, we carefully checked our procedures, and we are convinced that we did not make any errors. We must conclude, therefore, that the reason Drs. Parasassi and Gratton could not reproduce the anomalous intensity behavior around 20% is that their sample preparation must have been different from ours. It is known that results concerning regular distributions are extremely sensitive to sample preparation. For example, Somerharju et al. (1985) found kinks in their excimer/monomer curve versus concentration in their bilayers containing pyrene lipids, whereas Tang and Chong (1992) found dips in the same system with a slightly different sample preparation. These discrepancies in the results were due to differences in sample preparation. For example, Somerharju et al. used a brief sonification that Tang et al. did not use, and Tang et al. applied a vortex, several heating and cooling cycles, and a long incubation before measuring. We believe that Drs. Parasassi and Gratton have some problems in their sample preparation. We had this impression also at the 1995 Biophysical Society Meeting, where Dr. Gratton asked one of us (D. Tang) to talk to one of his students about procedures for sample preparation. In conclusion, we agree with our opponents that our DPH quenching results are surprising. However, these results are certainly reproducible and are in agreement with results obtained by others in the literature. We suspect that the discrepancies between our results and those obtained by Drs. Parasassi and Gratton are due to differences in sample preparation. We are certainly willing to discuss these differences with them to obtain a better understanding of the underlying principles.

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