Letter to the Editor

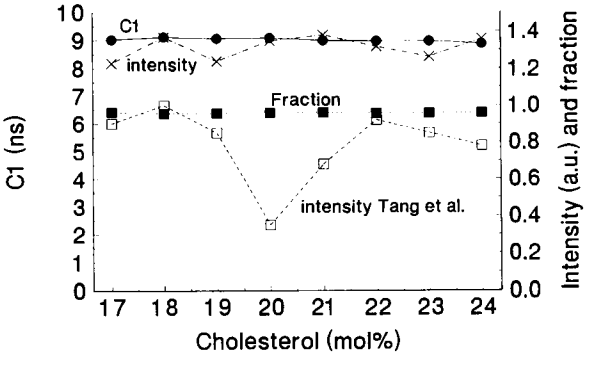
Comments on 1,6-diphenyl-1,3,5-hexatriene Fluorescence Decrease at Critical Cholesterol Concentration in Phospholipid Membranes

We were intrigued by the recent publication by Tang et al. (1995) describing unusual properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence intensity in dimirystoylphosphatidyl-choline (DMPC) as a function of cholesterol concentration in the phospholipid. A vast literature exists on phospholipid membrane properties viewed by DPH fluorescence parameters, reporting intensity, polarization, lifetime, and anisotropy data of the probe in phospholipids (for recent reviews see Lentz, 1995; Gratton and Parasassi, 1995). Among other researchers, we have performed several experiments using DPH to study the properties of lipid bilayers, also in the presence of cholesterol (Fiorini et al., 1988). Although the effect reported by Tang et al. occurs in a very narrow range of cholesterol concentration that can be easily missed, an anomalous DPH intensity behavior was not noticed by us or others.

To verify this inconsistency, we performed new experiments using DMPC vesicles labeled with DPH at cholesterol concentration from 17 to 24 mol %, where a profound dip of the DPH fluorescence intensity should occur according to Tang et al. (1995), and reproducing all the experimental conditions described by Tang et al. (1995). Our results are shown in Fig. 1, where the data from Tang et al. are also superimposed. Our results show a constant value of the DPH fluorescence intensity. We also measured DPH intensity decay in the same samples, and the data were analyzed using a model of two distributed lifetime components (Parasassi et al., 1991). The decay parameters do not show anomalous behavior versus cholesterol concentration (Fig. 1).

In our opinion, the hypothesis of a regular distribution of cholesterol in the bilayer matrix at peculiar concentrations is quite interesting. Several recent papers reported evidences for such superlattice organizations of guest molecules in phospholipid membranes (Tang and Chong, 1992; Chong, 1994). We reported intensity (Parasassi et al., 1994) and generalized polarization (Parasassi et al., 1995) measurements of 2-dimethylamino-6-lauroylnaphthalene in various phospholipids as a function of temperature and of cholesterol concentration, indicating the existence of regular molecular arrangements of cholesterol in the bilayer at peculiar guest molecule concentrations.

In the data of Tang et al., what is surprising to us is the magnitude of the variation of DPH fluorescence intensity. A decrease of fluorescence intensity of ~80% implies that ~80% of DPH molecules are totally quenched, because the fluorescence lifetime of the remaining 20% DPH molecules is not affected by the presence of cholesterol (Fig. 1). A membrane structure where most DPH molecules are quenched is difficult to imagine. Water is a well established quencher for DPH in the membrane and is the only possible quenching agent in the Tang et al. experiments. However, DPH in the presence of water develops a short lifetime component of ~1 ns, rather than being completely quenched (Parasassi and Gratton, 1995). Because of the magnitude of the effect reported, water should permeate the bilayer. To calibrate the magnitude of intensity changes, consider the variations of DPH intensity and lifetime values during the phospholipid phase transition, which are ~15% (Fiorini et al., 1988; Parasassi et al., 1984). The gel to liquid-crystal transition involves major changes in the membrane organization and water penetration. Based on the changes reported by Tang et al., the 20 mol % cholesterol concentration should introduce in the bilayer even greater perturbation (in regard to DPH location and water penetration) than the gel to liquid-crystal phase transition. From a methodological point of view, an important procedural question was, in our opinion, overlooked. To evaluate cholesterol effect, only the variation of fluorescence intensity in the individual samples was measured. Actually, other papers reporting studies on regular molecular arrangements are also based only on fluorescence intensity data (Chong, 1994). The excimer/monomer ratio measurements of pyrene-PC (Tang and Chong, 1992; Chong, 1994) also depend on absolute concentrations. The reproducibility of intensity-based measurements is questionable, because of individual variability in sample preparation. In the data reported in this letter (Fig. 1), our best care could not avoid a 10% variability of DPH intensity! To reduce variability, in our studies of regular molecular arrangements we use gen-



DPH fluorescence parameters in DMPC vesicles at 24°C as a function of cholesterol concentration. C1 (●) and F1 (■), center and fraction of the main distributed component of DPH continuous lifetime distribution. Also reported is the DPH total fluorescence intensity data in our samples (×) and in those reported by Tang et al. (1995) (□).

Received for publication 27 September 1995 and in final form 20 December 1995.

0006-3495/96/03/1560/03 $2.00

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

Received for publication 22 December 1995 and in final form 22 December 1995.

0006-3495/96/03/1561/03 $2.00