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On the use of partition coefficients to characterize the distribution of fluorescent membrane probes between coexisting gel and fluid lipid phases: an analysis of the partition behavior of 1,6-diphenyl-1,3,5-hexatriene

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The distribution of the fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene between coexisting gel and fluid phospholipid phases in multilamellar vesicles has been examined using fluorescence quenching by spin-labeled phosphatidylcholine. For both thermally-induced and Ca^{2+}-induced lipid phase separation, the ratio of probe concentration in the fluid liquid-crystal phase to that in the gel phase is found to be independent of either the probe concentration or the relative amounts of gel and fluid lipid phases, and hence is an equilibrium concentration ratio, or partition coefficient.

The distribution of fluorescent probe molecules between coexisting gel and fluid lipid phases has been described in terms of a partition coefficient by several authors [1–7]. However, the use of a partition coefficient is justified only if the system is at equilibrium, i.e. the chemical potential of the probe is the same in both lipid phases. If the probe distribution is influenced by kinetic effects, e.g. probe trapping in solid lipid domains, such that the equilibrium distribution is not reached during the course of the experiment, then the use of a partition coefficient to describe probe behavior is inappropriate. In addition, if the probe adsorbs significantly to the gel/fluid interface or is found in any location other than the bulk gel or fluid phase, then the use of a partition coefficient would also be inappropriate.

Using the contact quenching of probe fluorescence by spin-labeled phosphatidylcholine ((7,6)PC), we have previously determined the ratio of probe concentration, $R_{LC/G}$, of 1,6-diphenyl-1,3,5-hexatriene (DPH) between coexisting fluid liquid-crystal and thermally-induced [8] or Ca^{2+}-induced [9] gel phase in multilamellar vesicles composed of dipalmitoylphosphatidylcholine (DPPC)/(7,6)PC or dioleoylphosphatidylserine (DOPS)/(7,6)PC, respectively. In this report, we discuss the criteria by which the concentration ratio $R_{LC/G}$ can be called a partition coefficient, and we present experimental results which demonstrate that the distribution of DPH between

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Abbreviations: (7,6)PC, 1-acyl-2-[2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy]-*sn*-glycero-3-phosphocholine; $R_{LC/G}$, the ratio of probe concentrations in fluid liquid crystal and gel phases.

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coexisting gel and fluid lipid phases in DPPC/(7,6)PC multilayers, or DOPS/(7,6)PC in constant $[Ca^{2+}]$, is indeed described by a partition coefficient.

Multilamellar vesicles containing varying amounts of DPH were prepared, and measurements of fluorescence intensity performed, according to methods described previously [8,9].

The system of phase-separated phospholipid vesicles and probe molecules that partition exclusively into the lipid is described thermodynamically by four components (DPH, (7,6)PC, DPPC or DOPS, and H_2O or aqueous Ca^{2+}) and three phases (gel, liquid-crystal, and aqueous). For low probe : lipid ratios, the chemical potential of the probe in lipid gel or liquid-crystal phase is approximated by

$$\mu_p^{(G)} = \mu_p^{\circ(G)} + RT \ln C_p^{(G)} \tag{1}$$

or

$$\mu_p^{(LC)} = \mu_p^{\circ(LC)} + RT \ln C_p^{(LC)} \tag{2}$$

where $\mu_p^{\circ(G)}$ and $\mu_p^{\circ(LC)}$ are the chemical potential at unit activity in the gel and liquid-crystal phases, respectively, and $C_p^{(G)}$ and $C_p^{(LC)}$ are the respective probe concentrations in the two phases, using the convention that the activity coefficient γ_p approaches unity at infinite dilution of the probe. At equilibrium, the chemical potential of the probe must be the same in both lipid phases, i.e. $\mu_p^{(G)} = \mu_p^{(LC)}$. Therefore, from Eqns. 1 and 2,

$$\frac{C_p^{(LC)}}{C_p^{(G)}} = e^{\frac{\mu_p^{\circ(G)} - \mu_p^{\circ(LC)}}{RT}} \equiv K_p \tag{3}$$

The equilibrium ratio of probe concentrations in the two lipid phases, a constant, defines the partition coefficient K_p. Applying the phase rule to the system at equilibrium, the variance of the system at constant temperature and pressure is $v = c - p = 4 - 3 = 1$. The equilibrium thermodynamic state at constant temperature is thus fixed by specifying one concentration. For example, for a fixed vesicle lipid composition, the relative amounts of gel and liquid-crystal phases (determined from the phase diagram) are fixed. This in turn fixes the amount of probe in each phase since

$$\frac{\left(\begin{matrix}\text{fraction of probe in}\\ \text{liquid-crystal phase}\end{matrix}\right) \Big/ \left(\begin{matrix}\text{fraction of}\\ \text{liquid-crystal phase}\end{matrix}\right)}{\left(\begin{matrix}\text{fraction of probe}\\ \text{in gel phase}\end{matrix}\right) \Big/ \left(\begin{matrix}\text{fraction of}\\ \text{gel phase}\end{matrix}\right)} = \frac{C_p^{(LC)}}{C_p^{(G)}} = K_p \tag{4}$$

and K_p is a constant. The state of equilibrium would be verified experimentally by varying the overall probe concentration in the vesicles as well as the relative amounts of gel and fluid lipid phases and obtaining the same measured value of K_p.

Results of fluorescence quenching experiments to determine DPH partitioning are illustrated in Figs. 1 and 2 and summarized in Table I. For each

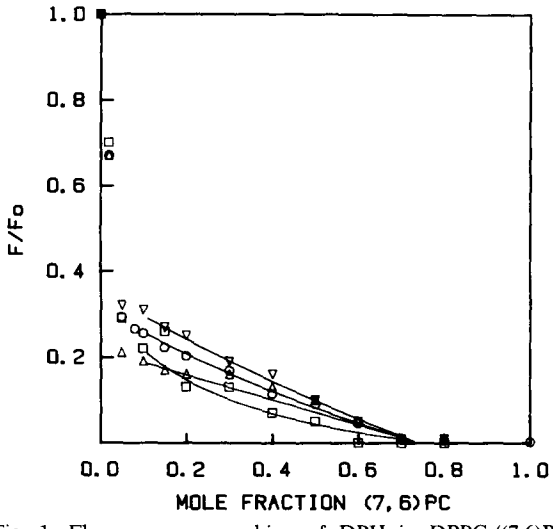

Fig. 1. Fluorescence quenching of DPH in DPPC/(7,6)PC multilamellar vesicles at 25 °C for probe : lipid ratios of 1 : 200 (○), 1 : 500 (▽), 1 : 2000 (△), and 1 : 10000 (□). The abscissa is the mole fraction of (7,6)PC in the vesicles and the ordinate is the ratio of fluorescence in the presence (F) and absence (F_0) of (7,6)PC. Data points are averages of triplicate samples of typically 5% fluorescence variation. Solid lines in the two-phase region are theoretical curves calculated from Eqn. 5 for $K_p = 1.3$ (1 : 200 data set), 1.2 (1 : 500 data set), 1.0 (1 : 2000 data set), and 2.5 (1 : 10000 data set), using $[(7,6)PC]_G = 0.11$ and $[(7,6)PC]_{LC} = 0.73$ [8]. For probe : lipid = 1 : 200, gel phase fluorescence has been corrected for the self-quenching which reduces the signal by 32% in pure DPPC and by 26% in 2% (7,6)PC.

TABLE I

PARTITION COEFFICIENTS (K_p) AND STANDARD FREE ENERGY CHANGES ($\Delta G°$) FOR DIPHENYLHEXATRIENE IN TWO-PHASE PHOSPHOLIPID SYSTEMS

Probe : lipid	Vesicle composition	T (°C)	K_p [a]	$\Delta G°$ (kcal/mol) [b]
1 : 200	DPPC/(7,6)PC	25	1.3 ± 0.2	−0.16
1 : 500	DPPC/(7,6)PC	25	1.2 ± 0.2	−0.11
1 : 1000	DPPC/(7,6)PC	25	1.5 ± 0.5 [c]	−0.24
1 : 1000	DPPC/(7,6)PC	21.5	1.4 ± 0.2	−0.20
1 : 2000	DPPC/(7,6)PC	25	1.0 ± 0.3	0.00
1 : 10000	DPPC/(7,6)PC	25	2.5 ± 1.0	−0.54
1 : 750	DOPS/(7,6)PC + Ca^{2+}	25	9 ± 1	−1.3
1 : 1000	DOPS/(7,6)PC + Ca^{2+}	25	12 ± 1 [c]	−1.5
1 : 10000	DOPS/(7,6)PC + Ca^{2+}	25	9 ± 2	−1.3

[a] Values represent the best theoretical fits to the experimental data, using Eqn. 5. Error indicates the range of K_p values which could reasonably fit the data.

[b] The standard Gibbs free energy change for probe transfer from gel to liquid-crystal phase at the indicated temperature and 1 atm pressure was calculated according to $\Delta G° = -RT \ln K_p$.

[c] Previously reported.

lipid system, the partition coefficient is found from the fluorescence quenching behavior of the probe in the two-phase region according to

$$F = F_{LC} + \frac{[G]}{K_p(1-[G])+[G]}(F_G - F_{LC}) \quad (5)$$

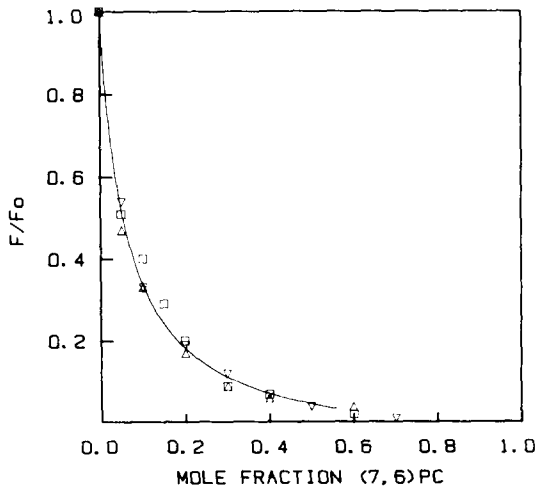

Fig. 2. Fluorescence quenching of DPH in DOPS/(7,6)PC multilamellar vesicles at 25 °C in the presence of 20 mM Ca^{2+} for probe : lipid ratios of 1 : 750 (▽), 1 : 1000 (△), and 1 : 10000 (□). Data points are averages of duplicate or triplicate samples of typically 5% fluorescence variation. The solid line is a theoretical curve calculated from Eqn. 5 for $K_p = 10$, using $[(7,6)PC]_G = 0.00$ and $[(7,6)PC]_{LC} = 0.56$ [9].

where F is the measured or calculated fluorescence intensity, $F_{G(LC)}$ is the fluorescence in a membrane of the composition at the gel (liquid-crystal) phase boundary, and

$$[G] = \frac{[(7,6)PC]_{LC} - [(7,6)PC]}{[(7,6)PC]_{LC} - [(7,6)PC]_G}$$

[4]. [(7,6)PC] is the overall mole fraction of (7,6)PC in the vesicles and $[(7,6)PC]_{G(LC)}$ is the mole fraction of (7,6)PC in the gel (liquid-crystal) phase at the boundary of the two-phase region, determined independently [8,9].

In DPPC/(7,6)PC at 25 °C, where thermotropic gel and fluid lipid phases coexist, the data show that over the entire two-phase region, and over a 50-fold probe concentration range, the measured DPH partition coefficient is constant within experimental error. Altering the temperature from 25 °C to 21.5 °C changes the phase boundaries from $[(7,6)PC]_G = 0.11$ to $[(7,6)PC]_G = 0.14$ and from $[(7,6)PC]_L = 0.73$ to $[(7,6)PC]_{LC} = 0.82$ [8], but does not alter K_p (Table I).

Ca^{2+}-induced phase separation in DOPS/(7,6)PC results in an 8-fold higher partition coefficient favoring the fluid phase, but in this system as well, the measured K_p is independent of probe concentration or the relative amounts of gel and fluid lipid phases. We note that the range of probe concentrations examined in DOPS/(7,6)PC is

smaller due to greater DPH aggregation and self-quenching in the $Ca(PS)_2$ phase compared to DPPC gel phase.

Kinetic and/or interfacial effects that could influence probe partition behavior include probe trapping in solid lipid domains and probe localization at domain boundaries where lipid packing is disrupted. Because the theoretical partition curves fit the experimental data equally well throughout the two-phase region, including the areas near the phase boundaries where domain interfaces first form, domain boundary effects are not important in these experiments. Probe trapping in the gel phase is also unlikely, since incubation of samples for 4 h before making fluorescence measurements resulted in no observed difference in K_p compared to samples used immediately after preparation. Longer sample incubation was not investigated, so the possibility of extremely slow probe diffusion in large solid lipid domains cannot be ruled out. However, it is reasonable to expect a probe concentration dependence of the fluorescence if this were the case, and none was observed.

An interesting and unexpected result obtained for DPH in DPPC(7,6)PC is that of somewhat steeper fluorescence quenching curves with decreasing probe concentration in the gel phase region, illustrated in Fig. 1. We conjecture that the preferred orientation of DPH in the lipid gel phase is along the bilayer normal, where probe fluorescence is efficiently quenched by (7,6)PC, the nitroxide moiety being located near the center of the acyl chain. If the preferred sites are filled, then as probe concentration increases, probe molecules would be forced into the center of the bilayer, oriented parallel to the plane of the bilayer, where fluorescence quenching is less efficient and hence the fluorescence decreases less strongly with increasing [(7,6)PC]. Using a probe : lipid ratio of 1 : 6500, Andrich and Vanderkooi [10] have shown, based on fluorescence polarization measurements of DPH in oriented DPPC multilayers, that below the lipid phase transition the probe is preferentially oriented perpendicular to the plane of the bilayer. Davenport et al. [11], using resonance energy transfer between DPH and fluoresceinthiocarbamylphosphatidylethanolamine in unilamellar DPPC vesicles, found a broad distribution of DPH about the center of the bilayer for probe : lipid ratios ranging from 1 : 350 to 1 : 1000, both above and below the phase transition temperature. Second- and fourth-rank order parameters obtained from time-resolved fluorescence anisotropy measurements [12–14] show evidence for a significant fraction of DPH molecules aligned parallel to the plane of DPPC bilayers, with that fraction increasing above the lipid phase transition temperature. Nonetheless, the chemical potential of DPH in the gel phase must be almost the same even for these putative different locations, because K_p is the same.

In conclusion, we have demonstrated that the distribution of DPH between coexisting gel and liquid-crystal phases in two very different phospholipid systems can be described in terms of a true partition coefficient, based on the lack of dependence of the experimentally determined K_p on probe concentration, on the relative amounts of gel and fluid lipid phase, or on sample incubation time. We emphasize that to describe probe partition behavior in terms of a partition coefficient is to describe the system as being at equilibrium, an assumption which may not always be valid but which can be tested as we have described here.

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