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PARTITION COEFFICIENTS OF FLUORESCENT PROBES WITH PHOSPHOLIPID MEMBRANES

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SUMMARY: A method for determination of membrane partition coefficients of five fluorescent membrane probes, 1,6-diphenyl-1,3,5-hexatriene(DPH), p-((6-phenyl)-1,3,5-hexatrienyl)benzoic acid (DPH carboxylic acid), 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH propionic acid), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene(TMA-DPH) and N-4-(4-didecylaminostyryl)-N-methylpyridinium iodide (4-di-10-ASP), was developed utilizing the fluorescence enhancement of a constant probe concentration by titration with excess phospholipid liposomes. The partition coefficients of DPH, DPH carboxylic acid, DPH propionic acid, TMA-DPH and 4-di-10-ASP into dipalmitoylphosphatidylcholine membranes were determined to be $1.3 \bullet 10^6$, $1.0 \bullet 10^6$, $6.5 \bullet 10^5$, $2.4 \bullet 10^5$ and $2.8 \bullet 10^6$ respectively. Knowledge of the partition coefficients may help select a lipid concentration for membrane studies that necessitate a probe's dominant incorporation into membranes.

1,6-Diphenyl-1,3,5-hexatriene (DPH), p-((6-phenyl)-1,3,5-hexatrienyl)benoic acid (DPH carboxylic acid), 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH propionic acid), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and lipophilic styryl dyes have been widely used as fluorescent membrane probes (1,2). The success of their applications relies on their ease of incorporation into either artificial or cellular membranes. It is generally accepted that presence of free probe in the aqueous phase should be minimized since its fluorescence or absorbance would interfere with spectroscopic measurements of the membrane-associated probe. However, the partition behaviors and partition coefficients that govern the distributions of commom probes between the aqueous phase and the membranous phase have not been previously determined. In this paper we described a simple method for determining the partition coefficients of five membrane probes between the aqueous phase and the phospholipid membranes.

METHODS

DPH, DPH carboxylic acid, DPH propionic acid, TMA-DPH and N-4-(4-didecylaminostyryl)-N-methylpyridinium iodide (4-di-10-ASP) from Molecular Probes, Inc. (Eugene, OR) were dissolved in N,N-dimethylformamide to make 1 mM stock solutions. The

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stock solutions were then diluted to the desired concentrations with phosphate-buffered saline (PBS). Dipalmitoylphosphatidylcholine (DPPC) from Avanti Polar Lipids, Inc. (Birmingham, AL) was mixed with PBS at a concentration of 2 mg / mL, and the mixture was then sonicated in a water-bath sonicator until a clear liposome dispersion was obtained. Mouse leukocytes harvested from D-MEM medium were washed three times in PBS by centrifugation (500 RPM x 5 minutes) and then suspended in PBS at a concentration of $1.6 \bullet 10^6$ / mL. Fluorescence measurements were carried out in a fluorescence plate reader, CytoFluor™ 2300 from Millipore Corporation (Bedford, MA), using a round bottom 96-well plate from Corning Glass Works (Corning, NY). For DPH-derived probes, an excitation of 360 nm, an emission of 460 nm and a sensitivity of 4 were set; for 4-di-10-ASP, however, an excitation of 485 nm, an emission of 620 nm and a sensitivity of 4 was set.

The partition coefficient of a membrane probe, K_p, is defined as

$$K_p = (P_b / L) / (P_f / W) \qquad (1)$$

where P_b, P_f, L and W refer to moles or molar concentrations of the membrane-associated probe, the free probe in aqueous phase, the phospholipid and water respectively. Since these particular probes are essentially nonfluorescent unless incorporated into membranes (1,2), the fluorescence, F, should be proportional to the concentration of membrane-associated probe, i.e.

$$F = \alpha \bullet P_b \qquad (2)$$

where α is simply a proportionality constant. Considering the total probe concentration $P = P_b + P_f$, insertion of equation (2) into equation (1) and rearrangement of equation (1) result in

$$F = F_0 \bullet L / (W/K_p + L) \qquad (3)$$

where F_0 ($= \alpha \bullet P$) is the maximum fluorescence resulting from total probe incorporation into membrane. Results of ultracentrifugation (100,000 g x 30 minutes) showed that 1 mg / mL DPPC liposome accounts for less than 0.2% fraction of total volume. Therefore the molar concentration of water in the liposome in this work may be treated as the same as pure water (i.e. 55.6 M), and equation (3) may be rewritten as

$$F = F_0 \bullet L / (55.6/K_p + L) \qquad (4)$$

or

$$1/F = [55.6/(K_p \bullet F_0)] \bullet (1/L) + 1/F_0 \qquad (5)$$

Equation (4) indicates that the fluorescence resulting from the DPPC liposome titration against a constant probe concentration should follow a saturation curve. The double reciprocal plot of the fluorescence and the lipid concentration should give a linear curve with an X-intercept of $0.018 \bullet K_p$. K_p can then be calculated as $55.6 \bullet$(X-intercept).

RESULTS AND DISCUSSION

As predicted by equation (4) and as shown in Fig. 1 (A), the fluorescence enhancement of 4.1 and 8.3 μM TMA-DPH by titration with DPPC liposomes exhibits a saturation tendency. Fig. 1 (B) gives the corresponding double reciprocal plots showing a good linearity and a

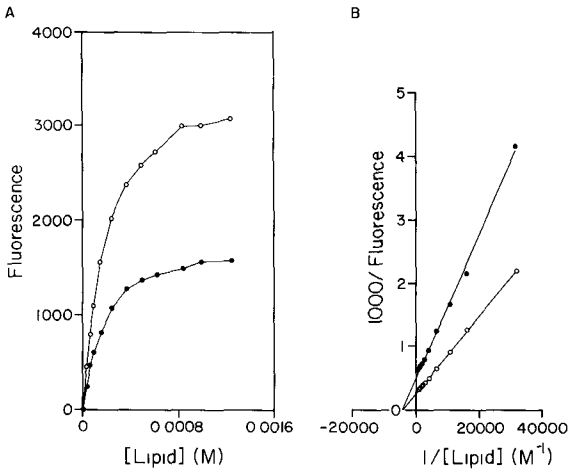


Fig. 1. (A) DPPC liposome titrations against 4.1 (•) and 8.3 (○) μM of TMA-DPH. In a Corning 96-well plate, 100 μL 8.2 or 16.6 μM TMA-DPH in PBS was added to 100 μL of various concentrations of DPPC liposomes in PBS. After 120 minutes incubation at room temperature, TMA-DPH fluorescence was measured in the CytoFluor instrument with excitation at 360 nm, emission of 460 nm and sensitivity setting of 4. The background reading of corresponding free probe in PBS (without lipid) was subtracted. (B) Double reciprocal plots of fluorescence versus lipid concentration for 4.1 (•) and 8.3 (○) μM TMA-DPH.

common X-intercept of 4370 M^{-1}. The TMA-DPH partition coefficient with DPPC membranes is then calculated to be $2.4 \bullet 10^5$. The probe fraction associated with the membrane is exclusively determined by the lipid concentration, *not* by the probe concentration. This is consistent with

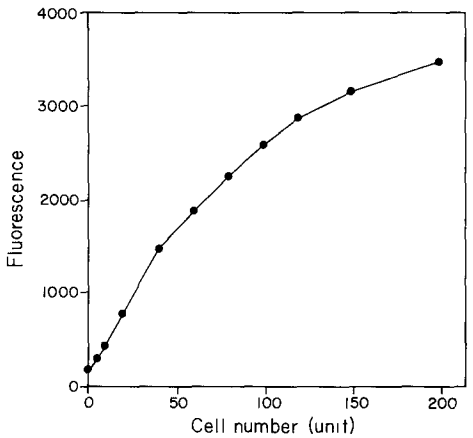


Fig. 2. Mouse leukocyte titration against 10 μM TMA-DPH. In a Corning 96-well plate, 100 μL 20 μM TMA-DPH in PBS was added to 100 μL of various number of mouse leukocytes in PBS (one unit of cells refers to approximately 800 cell counts). After 120 minutes incubation at room temperature, TMA-DPH fluorescence was measured in the CytoFluor instrument with the same conditions as in Fig. 1 (A).

the partition model where an unlimited membrane space for an infinite incorporation of the probe is assumed (3), although the minimum molar ratio of lipid to probe in Fig. 1 was only 4.

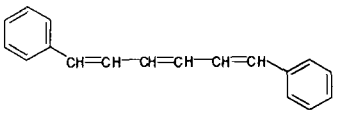
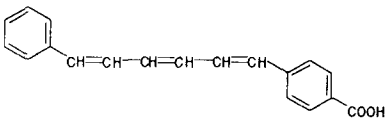
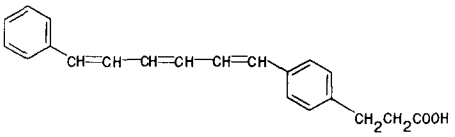
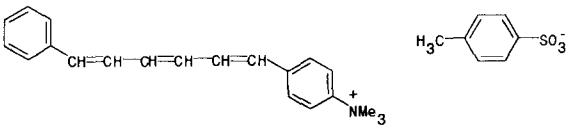
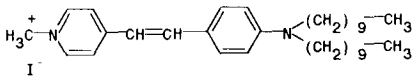
Titration of live cells, such as mouse leukocytes, against a constant concentration of TMA-DPH gives a fluorescence response as shown in Fig. 2 that has a saturation tendency similar to that in Fig. 1 (A). It appears that the lipid content of the cells constitutes a membrane phase that is similar to the DPPC artificial membranes and that the probe incorporation is in this phase. Comparing Fig. 1 (A) and Fig. 2 leads to the conclusion that the lipid content of 60 units of cells is approximately the equivalent of 37 μg DPPC, or the lipid content of the single cell is equal to approximately $4.6 \bullet 10^{11}$ DPPC molecules in terms of the incorporation efficiency of TMA-DPH.

The DPPC liposome titration against a constant concentration (3.3 μM) of DPH, DPH carboxylic acid, DPH propionic acid or 4-di-10-ASP also resulted in a saturation curve of fluorescence enhancement similar to Fig. 1 (A) (data not shown). The fluorescence enhancement of 4-di-10-ASP was measured using excitation at 485 nm while measuring the emission at 620 nm. Partition coefficients of DPH, DPH carboxylic acid, DPH propionic aicd and 4-di-10-ASP obtained from the double reciprocal plot according to equation (5) are given in Table I. It appears that the hydrophobicity of the membrane probes is a major factor determining the partition coefficients.

The DPPC liposome titration typified in Fig. 1 (A) may be a general means for determining the partition coefficient of membrane probes whose fluorescence is enhanced on incorporation into membranes. It should be mentioned that signals reflecting lipid titration or probe incorporation can be also obtained by absorbance differentiation (4), centrifugation (5), fluorescence polarization (6) or fluorescence quenching (7), if the fluorescence enhancement used in this work is not experimentally available. Obviously, lipid composition can be designed for a particular purpose too. Determination of a probe partition coefficient is useful not only for evaluating properties of a probe, but also for designing a lipid concentration which minimizes free probe in aqueous phase. For example, a DPPC liposome concentration of 232 μM (determined by $55.6/K_p$), is needed for a half incorporation of TMA-DPH. The greater the value of K_p, the lower the lipid concentration for half incorporation of a probe. Similarly, the lipid content of 60 units of mouse leukocytes should be met for a half incorporation of TMA-DPH into the cellular membranes.

Since most spectroscopic probes are generally used at concentrations below 1 μM, we expect that their incorporation into artificial and cellular membranes will follow a partition

TABLE I. Membrane Probe's Partition Coefficient

Probe	Structure	Kp (with DPPC)
DPH		1.3×10^6
DPH Carboxylic Acid		1.0×10^6
DPH Propionic Acid		6.5×10^5
TMA-DPH		2.4×10^5
4-Di-10-ASP		2.8×10^6

process. Because of the partition equilibrium for the probes between the membrane and water, the free probes in solution cannot be simply washed out by centrifugation. An excess lipid:probe ratio cannot ensure a dominant incorporation of a probe too. Use of a sufficient lipid concentration or content seems to be the exclusive way to reduce the concentration of free probe. The importance of lipid concentration in use of spectroscopic membrane probes in membrane study should be therefore recognized.

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