Biochimica et Biophysica Acta 812 (1985) 299–312
Elsevier

BBA 72351

Perturbation of egg phosphatidylcholine and dipalmitoylphosphatidylcholine multilamellar vesicles by *n*-alkanols. A fluorescent probe study

George B. Zavoico *, Laura Chandler and Howard Kutchai **

Department of Physiology, The University of Virginia Medical School, Charlottesville, VA 22908 (U.S.A.)

(Received July 11th, 1984)

Key words: Membrane fluidity, Multilamellar vesicle, *n*-Alkanol, Fluorescent probe

The perturbing effects of *n*-alkanols (pentanol, decanol and tetradecanol) in egg phosphatidylcholine and dipalmitoylphosphatidylcholine multilamellar vesicles were studied with five fluorescent probes, 1-(4′-trimethylaminophenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH), 1,6-diphenyl-1,3,5-hexatriene, and 2-, 7-, and 12-(9-anthroxyloxy)stearic acid (2-, 7-, and 12-AS). These probes localize at various depths in the membrane, enabling study of the membrane-order gradient. Phase-modulation fluorescence spectroscopy was used to measure steady-state anisotropies, excited-state lifetimes and differential polarized lifetimes from which the limiting hindered anisotropies (r_∞) and the logarithm of the rotational rate (log R) were calculated. The probes that localize at about the same depth in the membrane (TMA-DPH and 2-AS, diphenylhexatriene and 12-AS) generally, but not always, showed similar changes in r_∞ and log R with added alkanols. However, the absolute values of r_∞ and log R were usually different. The inconsistencies are attributed to differences in the probes' sizes, structures, photophysical properties and perturbing abilities. The perturbation of membranes by alkanols is chain-length-dependent. Pentanol disorders the membrane at all depths but is more effective in the membrane center than nearer to the polar headgroups of the phospholipids, tetradecanol can be accommodated into the membrane without effect or with increased order and the effects of decanol are intermediate between pentanol and tetradecanol. Our results with alkanols indicate that: (1) a single perturber can have different effects on membrane order at different depths in the bilayer; (2) the perturbation is observed at and distant from the perturbers' location in the membrane, and (3) the bilayer center is more susceptible to perturbation by alkanols than the region of the bilayer near the phospholipid headgroups.

Introduction

General and local anesthetics, and other small hydrophobic and amphipathic compounds, can perturb the structure of natural and artificial membranes [1–4]. We have previously reported that one class of local anesthetics, the normal alcohols or *n*-alkanols (henceforth referred to as alkanols) from butanol to octanol, increases the 'fluidity' of microsomal membranes from 20-day-old chick embryo hearts, as indicated by the decrease in the steady-state anisotropy of the fluo-

* Present address: Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032, U.S.A.
** To whom correspondence should be addressed.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; egg PC, egg phosphatidylcholine, DMPC, dimyristoylphosphatidylcholine, TMA-DPH, 1-(4′-trimethylaminophenyl)-6-phenylhexa-1,3,5-triene, (iodide), *n*-AS, *n*-(9-anthroxyloxy)stearic acid (n = 2,7,12), NMR, nuclear magnetic resonance, K_p, partition coefficient; τ_p, phase lifetime; τ_m, modulation lifetime; τ_{ave}, average lifetime; $\Delta\tau$, differential lifetime; tan Δ, differential tangent; r_0, limiting anisotropy; r_{ss}, steady-state anisotropy; r_∞, limiting hindered anisotropy; R, rotational rate.

rescent membrane probe 1,6-diphenyl-1,3,5-hexatriene [5]. The fluidizing effect of the alkanols was essentially equivalent when compared at the same membrane concentration. We suggested that the equipotent effect of the alkanols studied was due to the relative positions of the fluorescent probe and the alkanols in the membrane. Diphenylhexatriene is a hydrophobic molecule, partitioning into the bilayer parallel to the fatty acyl chains of the lipids [6–8]. The alkanols are amphipathic, partitioning into the bilayer with their hydroxyl moiety near the phospholipid polar headgroups and their alipathic chains intercalated among the fatty acyl chains of the phospholipids [3,9,10]. Since the longest of the alkanols we studied, octanol, penetrates slightly less than half way to the bilayer center, it maybe that the alkanols we studied and diphenylhexatriene were separated from one another within the bilayer. Consequently, diphenylhexatriene may have only been reporting the perturbations nearer the bilayer center which were equivalent for the alkanols we studied and distant from their location, and not the perturbations in the headgroup region which may have been different for each alkanol. The objective of this study was to determine the alkanol-induced perturbation in bilayer structure at different depths of the bilayer.

In this study, we used three normal alkanols of different chain-length, pentanol, decanol and tetradecanol, to perturb the structure and alter the dynamics of the acyl chains in egg phosphatidylcholine (egg PC) and dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles. We used fluorescence techniques to estimate the 'fluidity' or order of the multilamellar vesicles by calculating the rotational rate (R), which estimates the apparent rate of probe rotation, and the limiting hindered anisotropy (r_∞), a structural parameter that reflects the boundaries of the space within which the probe can rotate. Five fluorescent probes (diphenylhexatriene, 1-(4'-trimethylaminophenyl)-6-phenylhexa-1,3,5-triene iodide (TMA-DPH), and 2-, 7-, and 12-(9-anthroxyloxy)stearic acids (n-AS)) that localize at different depths in the bilayer were chosen enabling us to probe different segments of the order gradient. We found that alkanols have different effects at different depths in the bilayer and that the bilayer center is more likely to undergo larger perturbations than the headgroup region. Fluorescent probes that localize at similar depths generally result in the same qualitative conclusions, even though there may be large differences in the values of log R and r_∞.

Materials and Methods

Materials Pentanol was obtained from Fisher Scientific; hexanol, heptanol, octanol, decanol, dodecanol and tetradecanol from Sigma or from Applied Science. Gas chromatography (Silar 10C column in a Shimadzu GC-4BPT gas chromatograph) showed each alkanol to be free of volatile contaminants. All other organic solvents were of analytical grade from various sources. Egg PC and DPPC were obtained from Makor Chemicals, Calbiochem-Behring or Avanti Polar Lipids. Thin-layer chromatography of 1–5 μg lipid on silica-gel plates (Eastman Kodak or EM Laboratories) developed in chloroform/methanol/water (65 : 25 : 4, v/v) yielded one spot for each lipid. Diphenylhexatriene was obtained from Koch Light Laboratories and Aldrich Chemical. TMA-DPH and the n-AS probes (n = 2, 7 and 12) were obtained from Molecular Probes. Thin-layer chromatography of each probe of silica-gel plates developed in chloroform/methanol (95 : 5, v/v), chloroform/methanol/water (25 : 20 : 1, v/v), and hexane/ chloroform/ methanol (5 : 5 : 1, v/v) yielded only one spot as visualized by long ultraviolet light. Stock solutions of diphenylhexatriene in tetrahydrofuran, of the other probes in methanol, and of egg PC and DPPC in chloroform were stored under nitrogen in aluminum-foil-wrapped screw-capped tubes at −20°C. The deionized water used was free of fluorescent impurities.

Preparation of multilamellar vesicles and the addition of alkanols. Multilamellar vesicles [11] were prepared as follows: phospholipid stock solution was added to each of a group of 25 ml culture tubes with Teflon-lined caps containing two or three glass beads (3 mm diameter). Dodecanol and tetradecanol in chloroform were added to the lipid and mixed. The chloroform was evaporated at room temperature by a stream of nitrogen. Pentanol, hexanol, heptanol, octanol and decanol at the desired concentration in water or pure water was added to each tube of dried phospholipid which

TABLE I

PARTITION COEFFICIENTS (K_p) AND ALKANOL-TO-LIPID MOLE RATIOS

The concentration listed is of the total alkanol in the membrane suspension. K_p and mole ratio was estimated as described in Materials and Methods. K_p = [(mol alkanol in the lipid phase)/(mol lipid)]/[(mol alkanol in the aqueous phase)/(l)]

Alkanol	Melting pt (°C)	Concn (mM)	Egg PC		DPPC	
			K_p	Mole ratio	K_p	Mole ratio
Pentanol	−79	99	13.9	1.37	3 5	0 35
Heptanol	−34	8 6	131	1.11	37	0 32
Decanol	7	0.27	2940	0.55	2251	0 45
Dodecanol	26	0.075	25150	0.40	29110	0 41
Tetradecanol	39–40	0.075	215600	0.49	376300	0 49

were then flushed with nitrogen and capped. The phospholipid concentration was 0.15 mM. The alkanol concentrations are listed in Table I. To form multilamellar vesicles, the phospholipid suspensions were heated to 45°C (egg PC) or 50°C (DPPC) in a water-bath and vortexed three times for 1 min with intervals of at least a minute back in the water-bath. The multilamellar vesicle suspensions were then divided into a group to receive fluorescent probes and another to serve as light-scattering blanks. Fluorescent probes were added directly to the multilamellar vesicle suspensions to produce a lipid-to-probe mole ratio of about 300 : 1. The tubes were flushed with nitrogen, capped and incubated for 2 h with gentle shaking in a water-bath at 45°C (egg PC) or 50°C (DPPC). There was no difference in polarization values if the probes were added to the lipids before the chloroform was evaporated and the multilamellar vesicles were formed.

Calculation of alkanol-to-lipid mole ratios. the partition coefficients (K_p) of pentanol, hexanol, heptanol, octanol and nonanol in DPPC and egg PC multilamellar vesicles at 24°C measured by Jain and Wray [12] were used to estimate alkanol-to-lipid mole ratios. Their units (g alkanol/g of lipid phase)/(g alkanol/g of aqueous phase) were converted to units of (mol alkanol/mol lipid)/(mol alkanol/l) by using the molecular weight of DPPC (734.1) and egg PC (estimated to be 770) (Table I). Plots of log K_p versus the number of carbons in the alkanol chain which are linear for both DPPC and egg PC were used to estimate the K_p of the alkanols that were not directly measured. The alkanol-to-lipid mole ratio in the membrane was calculated from the total alkanol concentration, the lipid concentration and K_p (Table I). Although the K_p values listed in Table I were measured at 24°C, we used the same values at 38 and 47°C. Katz and Diamond [13] showed that K_p tends to decrease gradually with decreasing temperature, and that some solutes may be 'frozen out' of the membrane during a liquid-crystalline-to-gel phase transition. In effect, K_p decreases by 5–19% as the membrane enters the gel phase. From the magnitude of the changes observed by Katz and Diamond [13], we estimate that the K_p of the alkanols may increase by about 10% from 25°C to 38°C in egg PC vesicles, and by about 20% from 25°C to 47°C in DPPC vesicles. Katz and Diamond [13] did not observe the freezing-out phenomenon with the alkanols they studied (methanol to butanol) in DMPC vesicles, therefore, we did not expect this to occur with the alkanols in the membranes we used.

In DPPC vesicles, the concentrations of pentanol, decanol and tetradecanol used resulted in similar alkanol-to-lipid mole ratios (range: 0.35–0.49), whereas in egg PC vesicles, the concentrations resulted in similar mole ratios for decanol (0.55) and tetradecanol (0.49) but not pentanol (1.37) (Table I). For a satisfactory comparison of pentanol with the other alkanols in egg PC vesicles, the effects of pentanol must be estimated at a similar mole ratio. An adjusted value of r_{ss} was calculated from the linear relationship between r_{ss} and the pentanol-to-lipid mole ratio for the fluorescent probes used in this study [14] and adjusted values of r_∞ and log R were calculated from their relationship with r_{ss} (see Van Blitters-

wijk et al. [15]). The relationship of r_{ss} to r_∞ and log R is nonlinear over the possible range of r_{ss} (0–0.4). However, we can assume linearity for the short segment of the curve defined by the measured values of r_{ss} (control and with pentanol), and then calculate r_∞ and log R from the adjusted value of r_{ss}.

Fluorescence measurements and data analysis The methods described by Lakowicz et al. [16] were used to measure the steady-state anisotropies (r_{ss}), fluorescence lifetimes (τ_p and τ_m), and differential phase lifetimes ($\Delta\tau$) with an SLM 4800S spectrofluorometer (SLM Instruments), and to calculate the differential tangents (tan Δ), the logarithm of the rotational rate (log R) and the limiting hindered anisotropy (r_∞). The sample was contained in a quartz cuvette that had been flushed with nitrogen and fitted with a ground-glass cap. A small magnetic stirring bar in the cuvette prevented settling of the vesicles. The excitation wavelengths were 368 nm for diphenylhexatriene, 362 nm for TMA-DPH and 384 nm for the AS probes. Emitted light was detected after passing through a 418 nm sharp cut-off filter (Schott KV 418). Correction for light scattering was made by subtracting the measured emission intensity of appropriate blanks from the sample intensities.

Fluorescence lifetimes were measured at a modulation frequency of 18 MHz. The effects of Brownian motion on the lifetimes was eliminated by setting the emission polarizer to 55° [17]. The phase and modulation lifetimes (τ_p and τ_m) of the sample were measured relative to a reference of known lifetime (1,4-bis(5-phenyloxazol-2-yl) benzene (POPOP) in ethanol, $\tau = 1.35$ ns [18]) to minimize photomultiplier tube artifacts [19]. Since τ_p and τ_m are identical in a homogeneously emitting fluorophore population [20], and we estimate the experimental error of the lifetime measurements to be 0.5 ns, then we assume homogeneous emission when τ_p and τ_m differ by 0.5 ns or less, otherwise we assume heterogeneous emission. In both instances, log R and r_∞ were calculated from the arithmetic average of τ_p and τ_m. The limiting anisotropy (r_0) was measured in a Perkin-Elmer 650-10S spectrofluorometer. Each probe was dispersed in glycerol and the anisotropy was measured at -5 to -10°C [21]. The r_0 was 0.386 for diphenylhexatriene, 0.396 for TMA-DPH and 0.311 for the AS probes.

Other methods. Light scattering was measured with a Perkin-Elmer 650-10S spectrofluorometer. ^{31}P-NMR spectra were recorded with a JEOL NMR spectrometer Samples contained 10% ^2H_2O to decouple the protons and enhance the ^{31}P-NMR spectrum.

Results

Evidence of bilayer structure at high alkanol concentrations

The integrity of the bilayer in the presence of high concentrations of alkanols were assessed by light scattering and by ^{31}P-NMR. The results of the light-scattering experiments are presented in Table II. The scattered light intensity of the alkanol containing vesicles was calculated as a percent of the scattered light intensity of the alkanol-free vesicles. Measurements of four different lipid concentrations showed a linear relationship between scattered light intensity and lipid concentration. If we assume that the larger the particle, the greater the light scattering, our data suggests that pentanol and heptanol decrease vesicle size, while decanol, dodecanol and tetradecanol either (1) have no effect on vesicle size, or (2) cause aggregated or enlarged vesicles. In DPPC vesicles, dodecanol differs from decanol and tetradecanol be causing a reduction in light scattering.

TABLE II

SCATTERED LIGHT INTENSITIES OF EGG PC AND DPPC MULTILAMELLAR VESICLES WITH ALKANOLS

Vesicles without fluorescent probes were prepared as described in Materials and Methods with the alkanol concentrations given in Table I Excitation and emission monochrometers were set at 560 nm with a 3 5 nm bandpass The values of scattered light intensities are the means and standard deviations of several measurements of undiluted and 1 to 1, 2 to 1 and 3 to 1 dilutions of vesicles compared to control (100%)

Alkanol	Scattered light intensities (%)		
	Egg PC	DPPC	
	25°C	25°C	47°C
Pentanol	34 ± 5	49 ± 2	45 ± 5
Heptanol	59 ± 2	68 ± 3	78 ± 4
Decanol	102 ± 6	178 ± 4	229 ± 20
Dodecanol	133 ± 2	78 ± 2	97 ± 25
Tetradecanol	129 ± 1	176 ± 14	273 ± 37

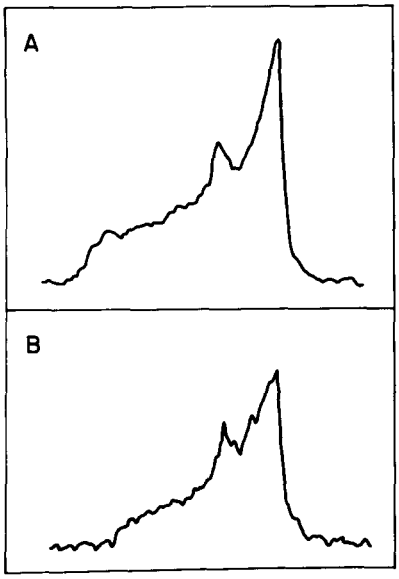

Fig. 1. ^{31}P-NMR spectra of DPPC multilamellar vesicles Concentration of DPPC is 12 mM, temperature is 46°C; assays contain 10% ^2H_2O, 5 kHz spectral width (A) Control, 100000 scans. (B) Containing pentanol at a mole ratio of 0 348, 40000 scans

The ^{31}P-NMR spectra of vesicles of pure DPPC and DPPC plus pentanol are representative of lipid bilayers [22]. The small mid-field peak indicates heterogeneous size distribution with the presence of smaller vesicular conformations (Fig. 1A). Compared to control, the mid-field peak is somewhat larger and the low-field shoulder is less pronounced with pentanol (Fig. 1B), suggesting a larger proportion of smaller vesicles, a result consistent with the light-scattering data. There is no evidence of DPPC structures other than a bilayer in the presence or absence of pentanol.

Egg phosphatidylcholine multilamellar vesicles

Membranes prepared from egg PC have a broad phase transition from −5 to about −15°C [23]. It was possible that tetradecanol, with a melting point of 39–40°C, could induce a phase transition at the temperatures used in our experiments. In vesicles containing tetradecanol and diphenylhexatriene, r_{ss} decreased linearly from 44 to 21°C. There was a break in the linearity at 21°C, possibly indicating the onset of a phase change (data not shown). We conclude that the egg PC vesicles are in a liquid-crystalline phase for our observations at 25 and 38°C, in the presence or absence of alkanols.

Measurements of r_{ss}, τ_p, τ_m and $\Delta\tau$ were made sequentially at 25 and 38°C. There was no difference in the data whether we first took measurements at 25 or 38°C. For each probe and alkanol, we made measurements on 3–6 different preparations. Table III lists the measured values of r_{ss}, τ_p, and τ_m, and the calculated values of τ_{ave}, tan Δ, log R and r_∞ in the presence and absence of the alkanols at 25 and 38°C for all probes used. Measurements made with each set of probes are considered in turn.

Of the two polyene probes, TMA-DPH localizes near the interfacial region due to its cationic trimethylammonium group being excluded from the hydrophobic domain of the bilayer interior. The fluorophore of TMA-DPH (which is diphenylhexatriene) extends parallel to the fatty acyl chains approximately to the level of the 8th to 10th carbon [8]. Diphenylhexatriene is hydrophobic, and is expected to localize parallel to the fatty acyl chains [6]. However, it is freely mobile, its orientation and location difficult to ascertain and variable with the phase state of the lipid. The effects of temperature and alkanols on the measured and calculated parameters of diphenylhexatriene and TMA-DPH (Table III) are similar at 25 and 38°C. At the lower temperature, r_{ss} and r_∞ are larger, while log R is smaller, consistent with increased order due to diminished thermal motion. Average lifetimes are 0.7–1.0 ns shorter at 38 than at 25°C. The small differences in τ_p and τ_m for TMA-DPH and diphenylhexatriene indicate relatively homogeneous emission both in the presence and absence of alkanols. The average lifetimes for TMA-DPH are less than for diphenylhexatriene, consistent with previous observations [8]. All the alkanols tested increased τ_{ave} of diphenylhexatriene by about a nanosecond. Pentanol decreased τ_{ave} of TMA-DPH by 0.6–0.8 ns, while decanol and tetradecanol had no effect. In the presence and absence of alkanols, TMA-DPH reports considerably larger value of r_{ss} and r_∞ and similar values of log R than diphenylhexatriene, suggesting that while the rotational rates for the two probes are similar, diphenylhexatriene has a greater extent of motion. The magnitudes of the changes in r_∞ due to the alkanols are greater with diphenylhexatriene than

TABLE III

FLUORESCENCE DATA FROM EGG PHOSPHATIDYLCHOLINE MULTILAMELLAR VESICLES

In egg PC, the pentanol-to-lipid mole ratio (1 37) was not the same as for decanol (0 55) and tetradecanol (0 49) The measured values for pentanol were adjusted as described in Materials and Methods to a similar mole ratio (0 52) and are given in parentheses Results are expressed as the means and standard deviations of 3–6 independent experiments. τ_{ave} is the arithmetic average of τ_p and τ_m

Probe	Alkanol	r_{ss}		τ_p (ns)	
		25°C	38°C	25°C	38°C
TMA-DPH	None	0 194±0.002	0 177±0 006	3 8±0 1	2 7±0.1
	Pentanol	0 182±0.002	0 165±0 004	3 2±0 1	2.2±0.2
		(0 189)	(0 172)		
	Decanol	0 187±0 002	0 178±0.005	3.8±0.1	2 7±0 2
	Tetradecanol	0.189±0 002	0 178±0 003	4.1±0.1	3.0±0 1
Diphenyl-hexatriene	None	0.086±0 001	0 060±0 001	8 1±0 2	7.2±0 1
	Pentanol	0.050±0 003	0 031±0 006	9 4±0 4	8 4±0 3
		(0.072)	(0 049)		
	Decanol	0 080±0 001	0 054±0.001	9 1±0 3	8 1±0 2
	Tetradecanol	0 094±0 001	0 062±0.001	9 0±0 1	8 1±0 1
2-AS	None	0 122±0.002	0 101±0 001	7 7±0 2	6 1±0 2
	Pentanol	0 099±0.002	0 081±0 006	6 5±0 1	4 9±0 1
		(0 113)	(0.093)		
	Decanol	0 112±0 001	0.093±0 001	6 9±0 1	5 4±0 1
	Tetradecanol	0 122±0 006	0 098±0 003	7 3±0 1	5 6±0 1
7-AS	None	0.092±0.001	0 071±0 003	10 3±0 1	8 4±0.1
	Pentanol	0.071±0.001	0 059±0 011	9 1±0 3	7 1±0.1
		(0.084)	(0 066)		
	Decanol	0.090±0.002	0 067±0 009	9 6±0.2	7.8±0 1
	Tetradecanol	0 096±0.004	0 076±0 005	9.9±0.1	7.9±0 2
12-AS	None	0.054±0 004	0 038±0.003	12.4±0.4	11.3±0 4
	Pentanol	0.035±0 004	0.025±0 005	11.8±0.3	10.3±0 8
		(0 047)	(0 033)		
	Decanol	0.046±0.003	0 031±0 003	12.2±0 3	10 9±0 4
	Tetradecanol	0.051±0.004	0 033±0 004	12.3±0 3	11 3±0 5

TMA-DPH. Correcting for the larger alkanol-to-lipid mole ratio of pentanol than of decanol and tetradecanol as described in Materials and Methods, pentanol decreases r_∞ about 26% with diphenylhexatriene and 4% with TMA-DPH. The effects of decanol on r_∞ with diphenylhexatriene and TMA-DPH are negligible, while tetradecanol increases r_∞ by 60% with diphenylhexatriene and only about 10% with TMA-DPH. These changes in r_∞ occur coincidentally with small increases in log R that are approximately equivalent with both probes and all the alkanols.

The AS probes have a stearic acid backbone that is intercalated parallel to the fatty acyl chains of the phospholipids with its charged carboxyl group anchored at the interfacial region of the bilayer [24–28]. The fluorophore is an anthracene group bonded to different carbons of the stearic acid chain via an ester linkage from the center ring and is long enough to span about six methylene groups of a polymethylene chain. In a fully saturated bilayer, with all carbon-carbon bonds in the *trans* configuration, 2-AS would probe the bilayer from the interfacial region to the 5th-6th carbon, 7-AS from the 2nd-3rd to the 10th-11th, and 12-AS from the 8th-9th to the 15th-16th.

Like the polyene probes, the effects of temperature and alkanols are similar at 25 and 38°C. At 25°C, r_{ss} and r_∞ are larger, log R are smaller and lifetimes are longer than at 38°C. Consistent with

τ_m (ns)		τ_{ave} (ns)		tan Δ		log R		r_∞	
25°C	38°C	25°C	38°C	25°C	38°C	25°C	38°C	25°C	38°C
4 2±0 1	3.2±0.1	4 0±0 2	3 0±0 3	0 066	0 049	8 05	8.23	0 119	0.106
3 3±0 1	2.7±0 3	3.2±0.1	2 4±0.4	0 057	0 038	8 14	8.37	0 101	0 097
						(8 08)	(8 29)	(0 112)	(0.103)
4 1±0 1	3.1±0.1	3 9±0 1	2 9±0 3	0 059	0 044	8 13	8 28	0 121	0 113
4 3±0.1	3 4±0 1	4 2±0 2	3 2±0 2	0 058	0 045	8 15	8 28	0 130	0 119
8.0±0.1	7 4±0 1	8 0±0.2	7 3±0 1	0 107	0.085	8.09	8 26	0 036	0 019
9.2±0 1	8 5±0 2	9 3±0 3	8 5±0 2	0 100	0.066	8 21	8 44	0 013	0.006
						(8 14)	(8.33)	(0.027)	(0 014)
9 0±0.2	8.3±0 1	9 1±0 2	8 2±0 2	0 106	0 079	8 12	8.31	0.037	0 021
8.9±0 1	8 2±0.1	9.0±0.1	8.1±0 1	0.093	0 075	8 16	8 32	0 057	0.030
8.6±0 5	6.8±0 3	8 2±0 6	6 5±0.4	0 104	0 087	7 84	7.99	0 067	0 046
7.2±0.5	5 5±0 2	6.9±0.5	5.2±0.4	0.094	0 068	7 96	8 16	0 043	0.030
						(7 89)	(8 05)	(0 058)	(0 040)
7 8±0.5	6 2±0.2	7 3±0 5	5 8±0 4	0 103	0 079	7 87	8 06	0 051	0 038
8.2±0.3	6 4±0 2	7 8±0 5	6 0±0 4	0 105	0 081	7 83	8.03	0 062	0 042
10.9±0 4	9 1±0 4	10.6±0 4	8 7±0 5	0 124	0.090	7.86	8 08	0 045	0 033
9 5±0 2	7 7±0.3	9 3±0 3	7 4±0 4	0 106	0 076	8 00	8 19	0 028	0 022
						(7 91)	(8.12)	(0 039)	(0 029)
10.1±0 3	8 4±0 4	9.8±0 3	8 1±0 4	0 120	0 093	7.88	8 07	0.041	0 024
10.5±0 5	8 6±0 5	10.2±0.4	8.3±0.5	0.127	0.098	7 84	8 07	0 045	0.034
13.6±0 5	12 1±0 7	13.0±0 7	11.7±0 7	0.129	0 103	7 96	8 11	0 018	0.008
12 8±0 9	11.1±0 6	12 3±0 8	10 7±0 8	0 112	0 080	8 07	8.25	0 003	0 0001
						(8 00)	(8 16)	(0 012)	(0 005)
13.2±0 4	11.8±0 6	12.7±0.6	11 4±0.7	0.130	0 090	7 97	8.19	0 009	0 004
13 6±0 4	12.1±0.6	12.9±0 8	11 7±0.8	0 126	0.101	7 98	8 13	0 016	0.004

previous reports [28,29], the lifetimes increase the deeper into the bilayer the probe is located. Unlike the polyene probes, lifetime heterogeneity is observed with the AS probes in the presence or absence of alkanols. There are differences of 0.8–1.3 ns between τ_p and τ_m with 12-AS, 0.4–0.7 ns with 7-AS, and 0.6–0.9 ns with 2-AS. The presence of alkanols generally decreases τ_{ave} with pentanol being most effective. Pentanol decreases τ_{ave} by 0.7–1.3 ns, decanol by 0.3–0.9 ns, and tetradecanol by only 0–0.5 ns. The decreases in τ_{ave} caused by the alkanols are largest with 2-AS and smallest with 12-AS. In the presence or absence of alkanols, r_{ss} and r_∞ decrease and log R slightly increases the closer the fluorophore is to the bilayer center. The largest decrease in r_∞ is between 7-AS and 12-AS. At 38°C, the low r_∞ value of 0.008 for 12-AS suggests nearly unhindered rotation of the fluorophore. Alkanols generally decrease r_∞ of all the AS probes. After correcting for the larger alkanol-to-lipid mole ratio of pentanol, pentanol and decanol generally decrease r_∞ of all three AS probes to an equivalent degree, while tetradecanol has a smaller effect, and no effect with 7-AS. The effects of alkanols on log R are not dramatic. All of the alkanols slightly increase log R, with pentanol and decanol being approximately equivalent and more effective than tetradecanol.

Dipalmitoylphosphatidylcholine multilamellar vesicles

The phase transition of DPPC vesicles we observed with diphenylhexatriene, TMA-DPH and 12-AS was centered between 40 and 42°C, consistent with previous observations [3,30–33]. As measured by diphenylhexatriene, the phase transition decreases to about 33°C without broadening with pentanol, decreases to about 38°C with slight broadening with decanol, and increases to about 49°C with slight broadening with tetradecanol (Fig. 2). There are differences in the profile and mid-point of the phase transition observed with diphenylhexatriene, TMA-DPH and 12-AS (Fig. 3). In alkanol-free vesicles, the decrease in r_{ss} from the gel-to-liquid-crystalline phase is much smaller and slightly broader with TMA-DPH and 12-AS than with diphenylhexatriene. In vesicles containing tetradecanol, the mid-point of the phase transition is different with all three probes: about 51–52°C with TMA-DPH, about 49°C with diphenylhexatriene and about 45°C with 12-AS. Note that at 47°C, the phase transition sensed with diphenylhexatriene and TMA-DPH is just beginning, whereas with 12-AS it is nearly over.

In our studies, DPPC vesicles are in the gel phase under all conditions at 25°C, and in the liquid-crystalline phase at 47°C for the control and with pentanol and decanol. In the presence of tetradecanol, the DPPC vesicles are in the midst of the phase transition at 47°C. Measurements of r_{ss}, τ_p, τ_m and $\Delta\tau$ were made sequentially at 25 and 47°C. As with egg PC vesicles, it did not matter at which temperature the measurements were first taken. For each probe and alkanol, we made measurements of 4–12 different preparations. Table IV lists the measured and calculated parameters at both temperatures for all the probes and alkanols used. Measurements made with each set of probes are considered in turn.

With TMA-DPH and diphenylhexatriene, there are large differences in nearly all the parameters

TABLE IV

FLUORESCENCE DATA FROM DIPALMITOYLPHOSPHATIDYLCHOLINE MULTILAMELLAR VESICLES

Results are expressed as the means and standard deviations of 4–12 independent experiments τ_{ave} is the arithmetic average of τ_p and τ_m. Values that could not be accurately measured or calculated are labeled n d

Probe	Alkanol	r_{ss}		τ_p (ns)	
		25°C	47°C	25°C	47°C
TMA-DPH	None	0.299 ± 0.031	0 165 ± 0 005	6.4 ± 0.6	3 6 ± 0 3
	Pentanol	0 318 ± 0 010	0 152 ± 0 004	5 6 ± 0 5	2 2 ± 0 1
	Decanol	0 258 ± 0 034	0 150 ± 0 007	7 5 ± 0 4	3 6 ± 0 1
	Tetradecanol	0 273 ± 0 041	0 167 ± 0 013	7 0 ± 0.8	4 2 ± 0 2
Diphenyl-hexatriene	None	0.313 ± 0 014	0.070 ± 0 003	10 5 ± 0.8	8 4 ± 0 7
	Pentanol	0.324 ± 0 006	0.034 ± 0.001	10.6 ± 0.4	8 6 ± 0 5
	Decanol	0.293 ± 0 019	0.076 ± 0.011	11 0 ± 0.5	8 7 ± 0 6
	Tetradecanol	0.312 ± 0.018	n.d	11 3 ± 0 6	10.5 ± 1 3
2-AS	None	0 182 ± 0 005	0 103 ± 0 004	9 7 ± 0 2	6.0 ± 0 1
	Pentanol	0.191 ± 0 009	0 078 ± 0 002	7 6 ± 0 1	4.4 ± 0 1
	Decanol	0.199 ± 0 012	0 090 ± 0 008	9 5 ± 0 4	5.2 ± 0 1
	Tetradecanol	0 163 ± 0 009	0 101 ± 0 004	9 2 ± 03	5 8 ± 0 1
7-AS	None	0 190 ± 0.011	0 075 ± 0 004	9 6 ± 0 3	8.3 ± 0 4
	Pentanol	0 156 ± 0.007	0 052 ± 0 002	8 9 ± 0 4	6.4 ± 0 2
	Decanol	0 231 ± 0 014	0 070 ± 0 003	10 0 ± 0.4	7 7 ± 0 1
	Tetradecanol	0 199 ± 0 019	0.080 ± 0.009	9 0 ± 0.4	8.3 ± 0 3
12-AS	None	0 164 ± 0.007	0 033 ± 0 002	10 2 ± 0.6	11.4 ± 0 2
	Pentanol	0 112 ± 0.007	0 017 ± 0 002	9 3 ± 0.4	9.9 ± 0 3
	Decanol	0 176 ± 0.004	0.028 ± 0 002	10.7 ± 0.6	11 1 ± 0 1
	Tetradecanol	0 170 ± 0.008	0 033 ± 0 005	9 7 ± 0.8	11 0 ± 0 3

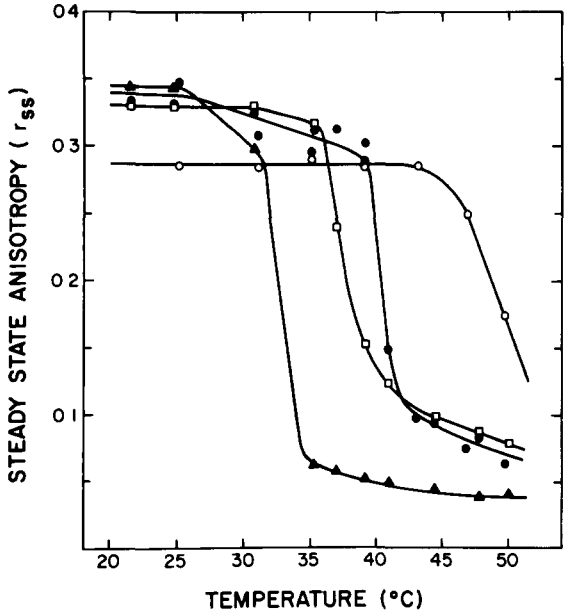

Fig 2 Steady-state anisotropies of DPPC multilamellar vesicles as a function of temperature measured with diphenylhexatriene in the absence of alkanols (●), and in the presence of pentanol (▲), decanol (□), or tetradecanol (○)

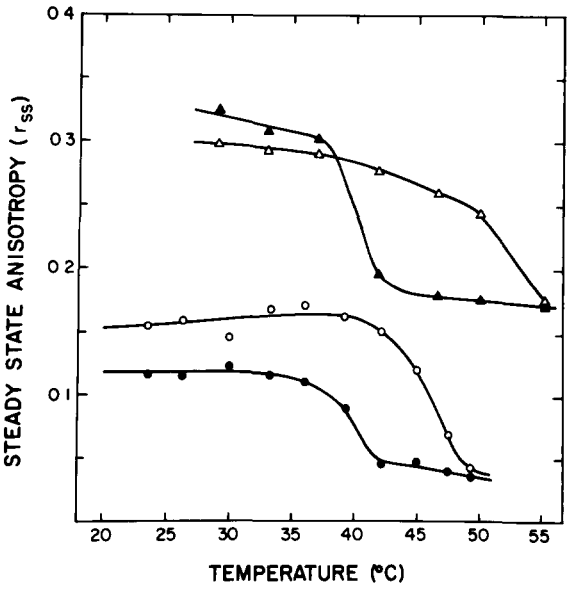

Fig. 3 Steady-state anisotropies of DPPC multilamellar vesicles as a function of temperature measured with TMA-DPH (▲,△) or 12-AS (●,○) Filled symbols are in the absence of alkanols, open symbols are in the presence of tetradecanol

τ_m (ns)		τ_{ave} (ns)		tan Δ		log R		r_∞	
25°C	47°C	25°C	47°C	25°C	47°C	25°C	47°C	25°C	47°C
6 9±0 4	3 7±0.2	6.7±0.5	3 6±0.3	0.001	0 049	9 52	8 29	0 298	0.110
7 4±0 2	2.5±0.5	6 5±1 0	2.4±0.2	−0 007	0.035	n.d	8.46	n d	0.093
8 0±0 3	3.6±0.2	7 8±0 7	3.6±0.1	0 003	0.036	9 31	8 48	0 257	0.112
7 5±0 4	4.4±0 3	7 2±0.7	4 3±0 3	0 0005	0.040	10.14	8.41	0.273	0.132
10 6±0.6	8 6±0.4	10.6±0.7	8.5±0 5	0.003	0.077	9.03	8.30	0.312	0 039
11 7±0 2	8 9±0 1	11.2±0.6	8 8±0.4	−0.009	0.055	n d.	8 52	n.d.	0.014
11.1±0 4	9 0±0 3	11 0±0.5	8.8±0.5	0.005	0.052	9 01	8 47	0 292	0.056
11 4±0 6	11 0±1 5	11 3±0.6	10.7±1.4	0.002	n.d	9.22	n d.	0 311	n d
10 7±0 3	6.0±0 3	10 2±0.6	6.0±0.2	0 045	0 085	8 10	7 99	0 165	0.044
8 1±0 3	4.3±0 5	7 8±0.3	4.3±0 3	0 051	0 061	7 98	8.21	0.164	0 022
10 3±0 5	5.1±0 4	9 9±0 6	5.2±0 3	0 035	0 072	8 15	8.10	0.186	0 034
10 7±0 4	5 8±0.5	10 0±0.8	5.8±0 3	0.036	0 079	8.28	8.03	0.150	0 045
10.6±0.2	8.8±0.5	10.1±0.6	8.5±0.5	0.033	0 104	8 23	7.99	0.178	0 028
10.4±0 3	6.7±0 4	9 7±0 9	6.6±0.3	0.057	0 061	8.08	8.31	0 134	0 020
10 4±0 3	8.0±0 6	10 2±0 4	7 9±0 4	0 028	0 085	8 09	8 11	0 220	0.030
10 3±0 4	8.6±0 4	9 6±0 8	8 4±0 4	0 016	0 093	8 53	8.04	0 193	0.038
12 7±0 3	12 1±0.5	11 4±1 3	11 7±0 5	0 069	0.100	7.98	8 14	0 140	0.004
11 6±0 4	10 5±0.5	10.5±1 3	10.2±0 5	0.096	0.069	7 95	8 34	0 076	−0.005
12.4±0 4	11.6±0.2	11.6±1 0	11 3±0.3	0.060	0 092	7 98	8.19	0.156	0.001
12.0±0.6	11 9±0.2	10.8±1.3	11 4±0.5	0.048	0 100	8.12	8.13	0.154	0 003

when DPPC vesicles in the gel phase are compared to liquid-crystalline phase. In the gel phase and in the absence of alkanols, r_{ss} and r_∞ are nearly identical with TMA-DPH and diphenylhexatriene, suggesting an ordered structure at all depths of the bilayer. The r_∞ values are almost identical to the r_{ss} values, consistent with the observation that r_∞ approaches r_{ss} in more ordered environments [15]. Identical values of τ_p and τ_m suggest a homogeneous probe population. The calculated values of log R for TMA-DPH are somewhat higher than for diphenylhexatriene. In the gel phase, the presence of alkanols have no effect on r_{ss} and r_∞. Pentanol induces heterogeneous emission with both probes, whereas homogeneity is maintained with decanol and tetradecanol. All the alkanols slightly increase the lifetimes to about the same degree. Pentanol decreases tan Δ to a negative value, preventing calculation of log R and r_∞, while decanol and tetradecanol have no effect. The values for log R with decanol and tetradecanol are larger with TMA-DPH than diphenylhexatriene. Decanol appears to cause a small decrease in log R, while tetradecanol causes an increase. Overall, the effects of alkanols on gel phase order and dynamics sensed by TMA-DPH and diphenylhexatriene are small and not dependent on the alkanol chain-length.

In contrast to the gel phase, there are large differences in the measured and calculated parameters of TMA-DPH and diphenylhexatriene, and the effects of the alkanols appear to be chain-length-dependent in the liquid-crystalline phase. In pure DPPC vesicles, the values of r_{ss} and r_∞ are smaller with diphenylhexatriene than with TMA DPH, consistent with the existence of an order gradient. Both probes exhibit homogeneous emission, the lifetimes of TMA-DPH are shorter than of diphenylhexatriene, and there are only small differences in log R. Alkanols do not affect lifetime homogeneity, however, pentanol decreases τ_{ave} of TMA-DPH by 1.2 ns, decanol has no effect, and tetradecanol increases τ_{ave} by 0.7 ns. In contrast, pentanol and decanol have almost no effect on τ_{ave} of diphenylhexatriene, while tetradecanol increases τ_{ave} by 2.2 ns. This large increase is due to the presence of gel-phase lipids since at 47°C DPPC vesicles with tetradecanol and diphenylhexatriene is in the middle of its phase transition (Fig. 3). The effects of alkanols on r_{ss} and r_∞ are different for each probe. Pentanol decreases r_∞ and r_{ss}, but the decrease is considerably larger with diphenylhexatriene than with TMA-DPH. Decanol has no effect on r_∞ of TMA-DPH, but it slightly decreases r_{ss}. In contrast, decanol causes a large increase in r_∞ of diphenylhexatriene, with virtually no effect on r_{ss}. Tetradecanol increases r_∞ of TMA-DPH, but has no effect on r_{ss}. With diphenylhexatriene and tetradecanol, log R and r_∞ could not be calculated. The absolute values of log R are similar with TMA-DPH and diphenylhexatriene in the presence or absence of alkanols. Alkanols slightly increase log R of both probes to about the same extent. Note that the absolute values of log R are substantially less at 47°C than at 25°C.

With the AS probes, like the polyene probes, there are large differences in nearly all the parameters between the bilayer in a gel phase and in a liquid-crystalline phase. In the gel phase, r_{ss} and r_∞ are nearly identical with 2-AS and 7-AS, decreasing slightly with 12-AS, suggesting disordering towards the bilayer center. Heterogeneous lifetimes of all the AS probes is observed. There is a small increase in log R from 2-AS to 7-AS, and then a decrease to 12-AS to a value less than that for 2-AS. The effects of the alkanols on all parameters are variable with each probe. Pentanol decreases r_{ss} and r_∞ with 7-AS and 12-AS but has no effect with 2-AS. Decanol slightly increases r_{ss} and r_∞ with all three probes, the largest increase being with 7-AS. Tetradecanol decreases r_{ss} and r_∞ of 2-AS, has no effect on r_{ss} but slightly increases r_∞ with 7-AS and 12-AS. Alkanols maintain lifetime heterogeneity while having no effect or slightly decreasing τ_p and τ_m of all AS probes. Pentanol slightly decreases log R for all probes, decanol has no effect on log R of 2-AS and 12-AS, but causes a small decrease with 7-AS, and tetradecanol increases log R for all probes.

Like the polyene probes, the AS probes in the liquid-crystalline phase bilayer report changes that can be correlated to the probes' depth in the bilayer and to alkanol chain-length. The data are consistent with increasing disorder from the phospholipid headgroup region towards the bilayer center. Specifically, r_{ss} and r_∞ decrease from 2-AS to 12-AS, with the largest decrease from 7-AS to

12-AS, while log R is not affected from 2-AS to 7-AS, then increases to 12-AS. The r_∞ value of 0.004 of 12-AS implies nearly unhindered rotation. Lifetime homogeneity is observed in the presence or absence of alkanols with 2-AS and 7-AS but not with 12-AS. Of the alkanols, pentanol causes the largest decreases in all the AS probes of r_{ss}, r_∞, and lifetimes, and the largest increases in log R. Pentanol decreases r_∞ of 12-AS to less than zero, suggesting unhindered rotation. Decanol has essentially the same effects as pentanol but of smaller magnitude on all parameters with all AS probes except 7-AS, with which decanol has no effect on r_∞. Tetradecanol has no effect on r_{ss}, lifetimes, log R and on r_∞ of 2-AS and 12-AS, but increases r_∞ of 7-AS.

Discussion

The effects of alkanols on membrane order have usually been measured with only a single probe [5,34–36]. In light of recent work with computer models of membrane structure [37] and in actual membranes showing that order varies with membrane depth (i.e., normal to the plane of the membrane) [38–40], we rationalized that the measurement of membrane order with a single probe was inadequate. Therefore, we studied in detail the changes in bilayer order and dynamics at different depths of the bilayer in the presence of alkanols of different chain-length with several fluorescent probes.

The phase transition of DPPC vesicles decreases with octanol or shorter alkanols and increases with dodecanol or longer alkanols [3,30,41]. Our results are essentially in agreement: pentanol decreases the phase transition of DPPC vesicles 8 K to 33°C, decanol decreases it 3 K to 38°C, and tetradecanol increases it 8 K to 49°C. Shifts in the phase transition reflect the overall effect a perturber has on bilayer order, therefore we may expect disordering by pentanol, slight disordering, if any, by decanol and ordering by tetradecanol. Although there was variability in the data that was due to which set of probes was used, our results generally bear out this prediction. Accordingly, the effects of each alkanol in liquid-crystalline phase bilayers were as follows: (1) Without exception, pentanol decreased r_∞ and increased log R. (2) Using AS probes, decanol was as potent as pentanol in decreasing r_∞ in egg PC vesicles, but less so in DPPC vesicles. In contrast, with polyene probes, decanol had no effect or increased r_∞ regardless of vesicle composition. With both sets of probes, decanol increased log R, but not as much as pentanol. (3) Tetradecanol increased r_∞ and log R as measured by the polyene probes, but had little effect when AS probes were used. In the gel-phase bilayer we studied, all three alkanols generally had little or no effect on r_∞. The effects on log R were a decrease by pentanol, no effect or a decrease by decanol and an increase by tetradecanol.

This study differs from others in that we were able to estimate the perturbing effects of alkanols at different depths of the bilayer by using several fluorescent probes that preferentially localize at fairly well-defined depths in the bilayers. In unperturbed bilayers of various composition, the variation in order with bilayer depth, termed the acyl chain order gradient, has been measured by NMR employing deuterium and ^{13}C-labelled phospholipids and by fluorescent probes. The NMR experiments show that in nearly all the bilayers studied, the order parameter, S, is greatest nearest the polar headgroups of the phospholipids, remains unchanged or decreases slightly to the level of the 9th to 10th carbon defining the plateau region, then decreases rapidly toward the terminal methyl group [38]. Studies utilizing fluorescent probes are essentially in agreement [8,39,40] even though these probes are relatively large and mobile in bilayers and do not have the advantage of being a fixed, nonperturbing part of the acyl chain as are the deuterium and ^{13}C-labelled phospholipids. Our results are in agreement with these studies, and show, in addition, that the order gradient is maintained in the presence of alkanols. In liquid-crystalline phase bilayers (egg PC at 25 and 38°C, and DPPC at 47°C) in the presence and absence of alkanols, r_∞ is largest for 2-AS, decreasing 9–37% to 7-AS and 73–100% * to 12-AS. The plateau region is defined by the smaller decrease in r_∞ from 2-AS to 7-AS compared to 7-AS to 12-AS. Similarly, r_∞ decreases 50–86% from TMA-DPH to diphenylhexatriene. That the order gradient in

* A decrease of 100% implies unhindered, isotropic motion ($r_\infty = 0$)

the liquid-crystalline phase is maintained in the presence of alkanols in concentrations that greatly affect membrane function supports the view that the order gradient is a ubiquitous feature of most membranes [38] and may not greatly influence function. Rather, it is the absolute change in order along the order gradient that affects membrane function. In gel-phase bilayers (DPPC at 25°C), the order gradient is not as steep; r_∞ increasing slightly (8%) from 2-AS to 7-AS, then decreasing moderately (15%) to 12-AS. There is no difference between r_∞ of TMA-DPH and diphenylhexatriene in the presence or absence of alkanols. If measured with the AS probes, the alkanols have a slight effect on the order gradient. From 2-AS to 7-AS pentanol decreases r_∞ 18% and decanol and tetradecanol increases r_∞ 18 and 29%, respectively. From 2-AS to 12-AS, pentanol and decanol decreases r_∞ 54 and 16%, respectively, and tetradecanol has no effect.

Examining the effects of the alkanols in more detail shows that the perturbations are not uniform at all depths of the bilayer. There is no a priori reason for the two structurally distinct regions of the bilayer, the plateau region of high order and the region of decreasing order towards the bilayer center, to be similarly affected by a perturber, especially one that preferentially localized in one or the other region. If r_∞ is normalized to the control value, and the fractional change in r_∞ due to the alkanols is calculated, then we observe that in liquid-crystalline bilayers, pentanol is 4–9-fold more effective in decreasing r_∞ of diphenylhexatriene than TMA-DPH, and 2.5–5-fold more effective in decreasing r_∞ of 12-AS than 2-AS and 7-AS, which are equally effective. As another example, tetradecanol is 5–6-fold more effective in increasing r_∞ of diphenylhexatriene than TMA-DPH, and 1.5–6-fold more effective in increasing r_∞ of 12-AS than 2-AS in egg PC vesicles. We have consistently observed that in liquid-crystalline bilayers, the greatest perturbations of both the ordering and disordering kind are at the bilayer center, and the least at the plateau region of the order gradient, suggesting that the plateau region is more structurally stable, and less susceptible to perturbation than the bilayer center. The efficacy of a perturber may not only depend on its general effect on bilayer order, but also on its effect at a specific depth of the bilayer. This raises the possibility that maintenance of normal order of some segments of the order gradient is more important for normal membrane function than of other segments. It is unclear whether the gel-phase bilayers is also differentially susceptible to perturbation, since the overall changes we observed are small and the large standard deviations of the r_{ss} measurements may mask any apparent differences.

Our measurements were made with only one set of perturbers, the alkanols. Can the effects of alkanols on bilayer order be generalized to all or most membrane perturbers? The hydroxyl group anchors the alkanol at the bilayer interface and the aliphatic chain intercalates between the acyl chains. Therefore, the packing density of the bilayer, or the number of molecules per unit of surface area, would increase (or remain the same if the surface area of the bilayer should expand to accommodate the alkanol) to about the depth of the end of the alkanol. It would decrease from that point to the bilayer center. Consequently, one may predict a slight disordering, if any, in the region where the alkanol overlaps the acyl chains, and significant disordering elsewhere in the bilayer. Our observations are consistent with this view. Long alkanols, such as tetradecanol, that overlap nearly the whole lipid acyl chain do not have a strong disordering effect, and behave almost like another acyl chain of a phospholipid. Shorter alkanols have a stronger disordering effect at the bilayer center than at the plateau region. Therefore, the effects of any set of perturbers may be partially dependent on their localization in the bilayer. In the region where they are localized there would be less disordering due to an increase in packing density than in the other regions of the bilayer. It is also important to consider the flexibility of the perturber. A rigid, multi-ringed perturber, or membrane component like cholesterol, is likely to order the membrane [42], whereas a flexible, chain-like perturber, like the alkanols, fatty acids and alkanes, would be more likely to disorder the bilayer.

Measurements made with fluorescent probes in membranes are not easily interpreted. The order and dynamics of the lipid acyl chains are inferred from the measured parameters that describe only the motion of the probe. Since different probes do

not have the same structure, photophysical properties, perturbing ability and localization in the bilayer, the estimation of the order and dynamics of the lipid acyl chains is dependent on the probe used. As a result, estimates of order and dynamics in the same bilayer under the same conditions are not likely to be the same with different probes. Accordingly, we found that probes localized at about the same depths of the membrane, TMA-DPH and 2-AS, diphenylhexatriene and 12-AS, do not always give the same results: (i) r_∞ and log R of the polyene probes are consistently greater. (ii) In DPPC vesicles r_∞/r_0, which is equal to the square of the order parameter, S [7,43,44], is greater for the polyene probes in the gel phase (0.74 ± 0.06 vs. 0.51 ± 0.11) and in the liquid-crystalline phase (0.20 ± 0.11 vs. 0.08 ± 0.05). (iii) In the gel phase of DPPC vesicles, $r_\infty = r_{ss}$ with TMA-DPH and diphenylhexatriene but not with the AS probes. (iv) The absolute values of r_{ss} and its change from the gel to the liquid-crystalline phase is larger for the polyene probes. (v) In the gel phase, the polyene probes report the same r_∞ whereas the AS probes show a slight order gradient. (vi) The polyene probes generally have homogeneous lifetimes whereas the AS probes do not.

Consider each set of probes in turn. The polyene probes are relatively small, rod-shaped and structurally symmetrical about their long axis, which is parallel to the excitation and emission dipole of the fluorophore [8]. Therefore, rotation about the long axis is invisible, while rotation about the two short axes are identical and visible. The polyene probes are oriented in the bilayer with their long axis normal to the plane of the bilayer [6–8], so only the out-of-plane rotations, or the end-over-end tumble of the probe is observed. Since the ordered array of acyl chains resists this rotational mode, large r_∞ values are observed. The unlikelihood of the cationic trimethylammonium moiety of TMA-DPH tumbling into the hydrophobic interior of the bilayer as well as the greater order nearer the headgroups is reflected in the higher r_∞ values of TMA-DPH relative to diphenylhexatriene. Their small size and elongated shape suggests that they may have negligible effects on the structural order and dynamics of the surrounding acyl chains. This is borne out by the observation that the order parameter, S, calculated from the equation $r_\infty/r_0 = S^2$ [7,43,44], is equivalent to the order parameter measured by NMR where the resonant nuclei are nonperturbing parts of the acyl chains themselves [7,43,45].

The AS probes are larger than the polyene probes, somewhat rod-shaped, but asymmetrical. Their excitation and emission dipoles are not parallel, and the emission dipole is parallel to one of the short axes [27]. Therefore, rotation about one of the short axes is invisible while rotation about the long axis and the other short axis are visible, but not identical. Since the AS probes are preferentially oriented with their long axis normal to the plane of the bilayer [24–28], both out-of-plane and in-plane rotations are observed. Therefore, the measured fluorescence parameters and calculated values of log R and r_∞ are weighed averages of unique values of both visible rotational modes. The factors that favor a larger r_∞ are the resistance to rotation about the long axis due to the ordered array of lipid acyl chains and the presence of the charged carboxyl group of the stearic acid. Favoring a smaller r_∞ are: (i) *trans-gauche* isomerizations of the stearic acid backbone of the probe to which the fluorescent moiety is attached. (ii) The increased frequency of *trans-gauche* isomerizations towards the terminal methyl group [37,46]. This would have a greater effect in decreasing r_∞ of 12-AS than of 2-AS. (iii) In-plane rotations about the visible short axis which are not likely to be strongly resisted by the ordered array of lipids. (iv) The size and shape of the probe may cause localized perturbations in the order of the surrounding lipids, especially in the gel phase, creating voids or vacancies [47]. Our data suggests that the factors favoring a smaller r_∞ outweight those favoring a larger r_∞.

We conclude that it is not advisable to compare the results from different types of probes, or to estimate lipid order and dynamics without considering the probes' structural and photophysical properties, its location in the bilayer and how its presence in the bilayer may perturb the actual order and dynamics of the lipid acyl chains. However, fluorescent probes can provide reliable qualitative data on overall changes in bilayer order. We advise caution in literally interpreting data obtained with fluorescent probes, since we have shown that changes in order due to perturbers are

not uniform at all segments of the acyl chain and that the probe itself strongly influences the data. The polyene probes appear to be more precise in estimating the actual order of the acyl chains as the calculated values of S are very similar to those obtained by NMR [43,45].

Acknowledgements

We thank the Biochemistry Department of the University of Virginia Medical School for permitting us to use their SLM 4800S Spectrofluorometer, Dr. Charles Schmidt for measuring the ^{31}P-NMR spectra, Dr. Peter Holloway for the use of his gas chromatograph, and Dr. Frank Prendergast of the Pharmacology Department at the Mayo Clinic for helpful discussions. This research was supported by grant R01 GM 24168 from the National Institutes of Health.

References

1 Seeman, P (1972) Pharmacol Rev 24, 583–655
2 Richards, C.D (1978) in Int. Rev Biochem Biochemistry of Cell Walls and Membranes II, Vol 19, (Metcalfe, J C, ed.), pp. 157–220, University Park Press, Baltimore
3 Eliasz, A.W, Chapman, D. and Ewing, D F (1976) Biochim Biophys Acta 448, 220–230
4 Lee, A G. (1977) Biochim. Biophys Acta 472, 285–344
5 Zavoico, G B and Kutchai, H. (1980) Biochim Biophys Acta 600, 263–269
6 Andrich, M P and Vanderkooi, J M (1976) Biochemistry 15, 1257–1261
7 Engel, L.W and Prendergast, F G (1981) Biochemistry 20, 7338–7345
8 Prendergast, F G, Haugland, R P and Callahan, P J (1981) Biochemistry 20, 7333–7338
9 Jain, M K., Gleeson, J, Upreti, A and Upreti, G C (1978) Biochim Biophys Acta 509, 1–8
10 Colley, C M. and Metcalfe, J C. (1972) FEBS Lett 24, 241–246
11 Bangham, A D, Standish, M.M and Watkins, J.C (1965) J Biol Chem 13, 238–252
12 Jain, M K. and Wray, L V (1978) Biochem Pharmacol 27, 1294–1296
13 Katz, Y. and Diamond, J (1974) J Membrane Biol. 17, 101–120
14 Zavoico, G B (1981) Ph.D Thesis, University of Virginia, Charlottesville
15 Van Blitterswijk, W.J., Van Hoeven, R.P and. Van der Meer, B.W (1981) Biochim. Biophys Acta 644, 323–332
16 Lakowicz, J R, Prendergast, F G and Hogen, D. (1979) Biochemistry 18, 508–519
17 Spencer, R D and Weber, G (1970) J Chem Phys. 52, 1654–1663
18 Lakowicz, J R, Cherek, H and Bevan, D R. (1980) J Biol Chem. 255, 4403–4406
19 Lakowicz, J R, Cherek, H. and Balter, A (1981) J Biochem. Biophys Methods 5, 131–146
20 Spencer, R D and Weber, G (1969) Ann N Y Acad Sci. 158, 361–376
21 Kawato, S, Kinosita, K and Ikegami, A (1977) Biochemistry 16, 2319–2324
22 Cullis, P R and De Kruijff, B. (1979) Biochim. Biophys Acta 507, 207–218
23 Huang, C and Thompson, T E (1974) Methods Enzymol 32, 485–488
24 Podo, F and Blasie, J K (1977) Proc Natl. Acad Sci USA 74, 1032–1036
25 Yguerabide, J and Stryer, L (1971) Proc Natl Acad Sci USA 68, 1217–1221
26 Lesslauer, W, Cain, J.E and Blasie, J.K (1972) Proc. Natl Acad Sci. USA 69, 1499–1503
27 Badley, R A., Martin, W G, and Schneider, H (1973) Biochemistry 12, 268–275
28 Chalpin, D B and Kleinfeld, A M (1983) Biochim Biophys Acta 731, 465–474
29 Thulborn, K R and Sawyer, W H (1978) Biochim Biophys Acta 511, 125–140
30 Lee, A G (1976) Biochemistry 15, 2448–2454
31 Mabrey, S and Sturtevant, J M (1976) Proc. Natl Acad Sci USA 73, 3862–3866
32 Suurkuusk, J, Lentz, B.R., Barenholz, Y, Biltonen, R L and Thompson, T E (1976) Biochemistry 15, 1393–1401
33 Lentz, B R, Barenholz, Y and Thompson, T E (1976) Biochemistry 15, 4521–4537
34 Grisham, C M and Barnett, R E (1973) Biochim Biophys Acta 311, 417–422
35 Chin, J H and Goldstein, D.B. (1977) Mol Pharmacol 13, 435–441
36 Lyon, R.C, McComb, J.A, Schreurs, J and Goldstein, D.B. (1981) J Pharmacol. Exp Ther 218, 669–675
37 Gruen, D W.R. (1980) Biochim. Biophys Acta 595, 161–183
38 Seelig, J and Seelig, A. (1980) Q. Rev Biophys. 13, 19–61
39 Thulborn, K.R, Tilley, L.M., Sawyer, W H. and Treloar, E. (1979) Biochim Biophys. Acta 558, 166–178
40 Tilley, L, Thulborn, K R. and Sawyer, W H (1979) J Biol Chem 254, 2592–2594
41 Hui, F K. and Barton, P G (1973) Biochim Biophys Acta 296, 510–517
42 Kutchai, H., Chandler, L H and Zavoico, G B (1983) Biochim. Biophys Acta 736, 137–149
43 Heyn, M.P (1979) FEBS Lett 108, 359–364
44 Lipari, G. and Szabo, A (1980) Biophys. J 30, 489–506
45 Jahnig, F (1979) Proc. Natl. Acad. Sci USA 76, 6361–6365
46 Schindler, H and Seelig, J (1975) Biochemistry 14, 2283–2287
47 Lee, A G (1977) Biochemistry 16, 835–841