Structure and Polarity of Mouse Brain Synaptic Plasma Membrane: Effects of Ethanol *in Vitro* and *in Vivo*[†]

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ABSTRACT: Structural and dielectric alteration by ethanol *in vitro* and chronic ethanol consumption were examined in synaptic plasma membranes (SPM) using diphenylhexatriene and charged diphenylhexatriene derivatives. These fluorophores, in combination with multifrequency phase and modulation fluorometry, allowed the examination of the surface and interior core of SPM. Limiting anisotropy and rotational relaxation time demonstrated that the synaptosomal plasma membrane surface domain was more rigid than the interior core domain. Ethanol *in vitro* fluidized the interior core and surface domains in SPM of the control, but not chronic ethanol-treated mice. Although the latter membranes were more rigid than control membranes, the intrinsic rigidity of the interior core of the synaptosomal plasma membrane did not strictly correlate with effects of ethanol *in vitro*. SPM of irradiated membranes were more rigid, but ethanol fluidized those membranes. Diphenylhexatriene lifetime and photoreactivity were sensitive to the range of dielectric constants in the SPM interior core. Ethanol *in vitro* increased both the surface and interior core range of dielectric constants of SPM from control but not chronic ethanol-treated animals. Thus, ethanol *in vitro* altered not only the fluidity but also the range of dielectric constants in both the surface and interior core domains in SPM of control but not chronic ethanol-treated mice.

Growing evidence since the early 1970s indicates that cell surface membrane lipids are organized into specific transbilayer domains [reviewed in Wood and Schroeder (1988, 1992), Schroeder and Nemecz (1990), Rothman and Lenard (1977), and Op den Kamp (1979)] and lateral domains [reviewed in Schroeder et al. (1991), Rothblat et al. (1992), Rodgers and Glaser (1993), Schroeder and Wood (1995), and Schroeder et al. (1995)]. Depending on the lipid composition, the lateral domains may even be transbilayer coupled (Haverstick & Glaser, 1988; Schroeder & Wood, 1995). Nevertheless, most early studies on the actions of ethanol *in vitro* and of chronic ethanol treatment on membrane structure concentrated on bulk membrane effects (Chin & Goldstein, 1977; Harris & Schroeder, 1981, 1982; Rottenberg et al., 1981; Stubbs et al., 1988; Wood et al., 1987, Beauge et al., 1985; Crews et al., 1983). More recently, new theories have evolved indicating that alcohols may exert specific effects on lateral lipid domains (Treistman et al., 1987; Harris et al., 1987; Wood et al., 1993), transbilayer lipid domains (Wood & Schroeder, 1988, 1992; Wood et al., 1988, 1989, 1990; Schroeder et al., 1988b; Hitzemann et al., 1986), membrane polar surface domains (Klemm, 1990; Chiou et al., 1990; Barchfeld & Deamer, 1988), and membrane polarity and hydration (Rottenberg, 1992; Ho & Stubbs, 1992).

Examination of the membrane surface effects of ethanol *in vitro* in neuronal membranes revealed a curious dichotomy. ^{1}H NMR demonstrated that ethanol disorders the terminal methyl chains in the interior core of the membrane lipid bilayer and orders the choline head groups in the polar surface of the membrane bilayer (Hitzemann et al., 1987). This is due to the fact that both the membrane interior and the membrane surface can accommodate polar molecules. In fact, the bilayer interior accommodates 2−30 mM H_2O (Meier et al., 1990). ^{2}H NMR showed that, in neuronal membranes as well as model membranes, ethanol at pharmacological doses partitioned into two membrane domains: an interior domain in which ethanol partitioned noncooperatively and a less mobile surface domain in which ethanol partitioned cooperatively (Hitzeman et al., 1986; Kreishman et al., 1985). The relative partition coefficients (K_p) of ethanol into the interior and surface domains indicated that, with increasing ethanol, partitioning of ethanol occurred more and more to the surface domain of the membranes. At 35 mM ethanol the K_p's were 0.0661 and 0.031, respectively; at 350 mM ethanol the K_p's were 0.059 and 0.163, respectively. Thus, ethanol would be expected to have a biphasic effect: preferentially partitioning into interior domains at low concentrations and increasingly into surface domains at high concentrations. Ethanol *in vitro* disorders (fluidizes) the bilayer interior [reviewed in Wood and Schroeder (1988, 1992)] and orders bilayer surface (Schueler et al., 1988) domains. Recent studies indicated that alcohols increased the relative dielectric constant in biological mem-

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branes (Rottenberg, 1992). Ethanol may weaken hydrogen bonding and replace water between interacting lipids or within the lipid−protein interface (Slater et al., 1993).

In the present work, differential polarized phase fluorometry and fluorescence lifetime analysis of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] and charged derivatives of DPH (TMA-DPH and PRO-DPH) were used to examine the range of dielectric constants sensed by the probe in SPM of control and chronic ethanol-treated mice. DPH partitions nearly equally into solid and fluid domains (Lentz et al., 1976). The membrane location of DPH appears to be the membrane interior (Harris & Schroeder, 1982; Harris et al., 1987). Since DPH lifetime is sensitive to the solvent dielectric constant (Zannoni et al., 1983), it should provide data for ethanol effects on the relative dielectric constants of the membrane interior core. Multifrequency (1−250 MHz) phase and modulation fluorometry and continuous distributional analysis of DPH provide two dielectric-sensitive parameters: center(s) of lifetime distribution and lifetime distributional width.

MATERIALS AND METHODS

Chemicals. DPH, obtained from Eastman (Rochester, NY), was stored as a stock solution in tetrahydrofuran at −20 °C in the dark. TMA-DPH and PRO-DPH were purchased from Molecular Probes Inc. (Eugene, OR). HEPES, Ficoll (70 000 MW), bovine serum albumin, and Tris-HCl were from Sigma Chemical Co. (St. Louis, MO). Dioxane was from Fluka Chemical Co. (Ronkonkoma, NY). Ethyl alcohol [200 proof dehydrated alcohol, U.S.P., punctilious (containing no denaturants and no fluorescent residues)] was obtained from Quantum Chemical Co. (Cincinnati, OH).

Animals. In the chronic alcohol studies, male C57BL/6NNIA mice, 4 months old, were fed Sustacal liquid diet with 6.6% (w/v) ethanol as described previously (Wood et al., 1989, 1990, 1993).

Synaptic Plasma Membrane Isolation. SPM of control and chronic ethanol-treated mice were obtained exactly as described previously (Wood et al., 1990, 1993; Schroeder et al., 1988b), except that trinitrobenzenesulfonic acid labeling was not performed.

Sample Preparation for Fluorescence Spectroscopy. All fluorescence parameters were determined with samples having absorbances less than 0.2 at the wavelength of excitation in order to avoid the inner filter effect. Light scattering was minimized by the use of dilute samples and sharp cutoff filters (Janos GG375, Townshend, VT). Cuvettes were rinsed with punctilious alcohol to avoid potential background fluorescence in commercial or grain alcohol preparations.

For all solvent studies, 0.1 μg of DPH, TMA-DPH, or PRO-DPH (0.1 mg/mL tetrahydrofuran) was added per milliliter of solvent and mixed, vortexed and the fluorescence parameters were determined at 37 °C.

For SPM studies, the probes dissolved in tetrahydrofuran (0.1 μg/mL) were added to SPM for incorporation as follows: In the case of DPH, 0.1 or 0.05 μg (0.429 or 0.214 nmol) was added per 25 μg protein/mL. On the basis of an SPM lipid to probe ratio of 1.33 μmol/mg protein for SPM (Wood et al., 1990), these concentrations represent probe to lipid ratios of 1:78 and 1:155, respectively. For TMA-DPH and PRO-DPH, 0.05 μg of probe was added per 50 μg of protein/mL to give a probe to lipid ratio of 1:310. These ratios were used to avoid self-quenching artifacts. Although the DPH probe to lipid ratio is higher than that usually used to avoid self-quenching artifacts, both fluorescence intensity and lifetime data indicated that, at the ratio used herein, self-quenching was not significant (see Results section). The established procedure for TMA-DPH is not less than 1:200. The probe to lipid ratio used herein was well within this limit. Consequently, probe−probe interactions or other potential probe-induced artifacts were avoided. The probes were treated differently to obtain optimal signal to noise parameters while at the same time avoiding potential inner filter artifacts. Each SPM sample was then incubated with DPH, TMA-DPH, or PRO-DPH at 37 °C for 30 min (with vortex mixing every 5 min) to maximally incorporate the probe into the membrane.

Spectral Properties of Diphenylhexatriene in Solvents and SPM. Excitation-corrected fluorescence excitation and emission spectra of DPH (0.5 μg/mL solvent) were obtained. Solvent dielectric constants were varied between 2 and 80 by using dioxane/water mixtures (Zannoni et al., 1983; Turner & Brand, 1968). A PC 1 photon-counting spectrofluorometer with ISSPC software for acquisition and photon-counting electronics was used for steady-state fluorescence measurements (ISS Inc., Champaign, IL). The light source was a Model K401.P 300 W xenon arc. Slit widths were 1 mm. Data were collected with a personal computer interfaced to the fluorometer through a K501 ADC interface card (ISS Inc.). Sample temperature was maintained at 37 °C with an external water bath (Model Exacal/Ex100/Endocal 850, Neslab, Portsmouth, NH). The excitation beam was split, and spectra were automatically corrected for wavelength- and source-dependent variables in the excitation system. All spectra were corrected for Raman scatter.

Photobleaching Experiments. For DPH photobleaching experiments in solvent or SPM, all sample conditions were the same as described earlier, except as follows: Irradiation was performed with a 7 W Innova 300 argon ion laser (Coherent Inc., Palo Alto, CA) using the fluorescence lifetime instrument described in the following. The polarizers were removed from the light beam. The ultraviolet multiline of the laser emission was used at 35 mW power to excite the DPH (0.05 μg/mL) in the solvents or SPM samples. The excitation intensity for photobleaching experiments was approximately 100-fold higher than used for other experiments described herein.

Irradiation Experiment. SPM were irradiated with Co-60 γ irradiation as described previously (Joseph et al., 1988).

Determination of the Fluorescence Lifetime of DPH, TMA-DPH, and PRO-DPH in SPM. Fluorescence lifetimes were determined at 14 frequencies logarithmically distributed between 10 and 140 MHz by using an ISS GREG MM phase and modulation fluorometer with a KOALA auto sample compartment (ISS Inc.). The light source was a either (a) a Model 4250NB He/Cd laser (Liconix, Sunnyvale, CA) with emission at the 325 nm line or (b) an Innova 306 argon ion laser (Coherent Inc.) with emission at the 331 nm line. Laser emission intensity was modulated sinusoidally with a Pockels

[1] Abbreviations: SPM, synaptosomal plasma membranes; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)-phenyl]-6-phenylhexa-1,3,5-triene; PRO-DPH, 3-[*p*-(1,6-diphenyl-1,3,5-hexatrienyl)phenyl]propionic acid; dimethyl POPOP, 1,4-bis[4-methyl-5-phenyl-2-oxazolyl]benzene.

cell. Sample emission was monitored in the L-format through a Janos GG-375 (Townshend, VT) sharp cutoff filter. Lifetimes were obtained using a reference solution of dimethyl POPOP[1] in punctilious ethanol: 1.45 ns at 24 °C (Lakowicz et al., 1981). Data were collected by a personal computer using a Model No. 1 IEEE-488 high-performance GPIB interface card with GPIB cables and No. 2 interface cards for two 250 mHz frequency synthesizers (Programmed Test Sources Inc., Littleton, MA) controlled through the GPIB card (ISS Inc.). Data were accumulated until standard deviations in phase and modulation values were below 0.2° and 0.004, respectively. These standard deviations are the default values provided by the data analysis program and have been used by this and other laboratories. The goal is to account for the sytematic deviations such that there be only random statistical uncertainties for each data point (Barbieri et al., 1990). Usually the systematic deviations are small for a "well behaved experiment": below 0.5° phase angle and 0.008 unit of modulation (Barbieri et al., 1990). Certainly, the settings of 0.2° and 0.004 modulation ratio used herein are well below this range. However, it should be noted that these deviations can become significant when the phase angle is a few degrees and the modulation value is below 0.05 (Barbieri et al., 1990). In the results reported herein, typical phase and modulation at 10 mHz were 30° and 0.854; at 140 mHz these values were 82° and 0.122. Again, the data collected herein were well within the parameters for a "well behaved experiment" (Barbieri et al., 1990). The sample temperature was maintained at 37 °C with an external Model 9101 water bath (Fisher Scientific Inc., Pittsburgh, PA).

Data were analyzed by both nonlinear least-squares and distributional analyses using ISS Lifetime Software (ISS, Champaign, IL) or Global Analysis Software (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). It should be noted that the values for χ^2 calculated by either method are relative values, depending on the standard deviations set for the data collection as well as the goodness of fit. With the preceding error limits, χ^2 values near 3 usually were considered acceptable, while values in excess of 10 were indicative of large discrepancies between the calculated theoretical curves and the experimentally determined ones (Schroeder et al., 1987). Therefore, relative changes in the χ^2 rather than only the absolute values are important in designating goodness of fit.

Nonlinear least-squares analysis, minimizing χ^2, was performed by a single- or multiexponential fit routine, using a 386/33i MHz personal computer (NEC Technologies Inc., Foxborough, MA), minimizing χ^2 according to

$$\chi^2 = \sum\{[(P_m - P_c)/S^P]^2 + [(M_m - M_c)/S^M]^2\}/[(2n - f - 1)] \quad (1)$$

where the subscripts c and m indicate the calculated and measured values, respectively, of phase (P) and modulation (M), n is the number of frequencies employed, f is the number of free parameters, and S^P and S^M are the standard deviations of each phase and modulation determination, respectively. The standard deviations are independent of modulation frequency and are constant for each determination in phase fluorometry (Gratton et al., 1984). Each term of a multiexponential fit was characterized by two parameters: lifetime and fractional intensity. The reduced χ^2 parameter was used to judge the quality of the fit (Lakowicz et al., 1984). The error of each parameter was determined from a covariance matrix of errors (Lakowicz et al., 1984).

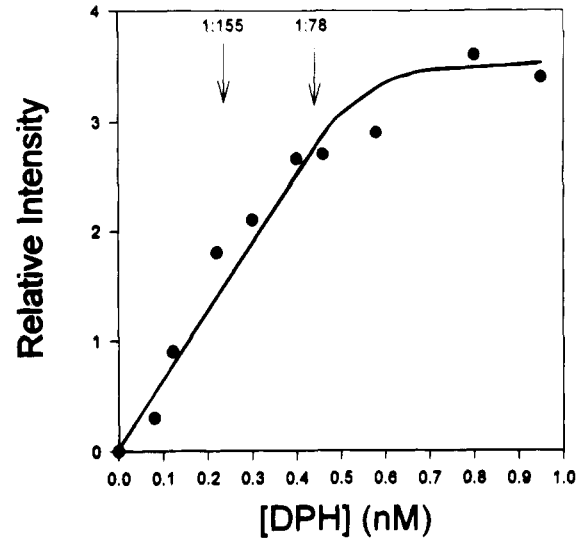

FIGURE 1: 1,6-Diphenyl-1,3,5-hexatriene fluorescence intensity in SPM from control mice. All conditions were as described in Materials and Methods. The arrow and ratio 1:78 indicate the molar ratio of DPH:lipid in the SPM used in most of the experiments described herein.

The phase and modulation data were also fit to distributional analysis according to Lorentzian distributions for each component (Caceri & Cacheris, 1980):

$$f(\tau) = A/\{1 - [(\tau - C)/(W/2)]^2\} \quad (2)$$

where A is a normalization constant, τ is the lifetime, C is the center of the distribution, W is the width of the distribution at one-half height, and $f(\tau)$ is the function that minimizes the reduced χ^2.

Differential Polarized Phase Fluorometry of DPH, TMA-DPH, and PRO-DPH in SPM. Differential polarized phase fluorometry according to the method of Weber (1978), as extended by Lakowicz et al. (1979), was used to resolve steady-state anisotropy into a static component (limiting anisotropy) and a dynamic component (rotational relaxation time, in nanoseconds), as described previously (Wood & Schroeder, 1988; Wood et al., 1989).

Miscellaneous Methods. Protein was determined by the method of Lowry et al. (1951). Statistical analysis was by the standard unpaired student's t-test.

RESULTS

Incorporation of Diphenylhexatriene into Synaptic Plasma Membranes. Because the partitioning of diphenylhexatriene into synaptic plasma membranes may differ from that observed with model membranes, it was essential to incorporate the probe such that self-quenching did not occur. Self-quenching would decrease the fluorescence intensity and lifetime of DPH.

Two independent experiments were conducted to examine the effect of diphenylhexatriene concentration on the relative fluorescence intensity of diphenylhexatriene in synaptic plasma membranes. In the first experiment, the diphenylhexatriene fluorescence intensity increased linearly with increasing probe concentrations up to a DPH:lipid ratio of 1:78 (Figure 1). Relative fluorescence intensity at DPH:

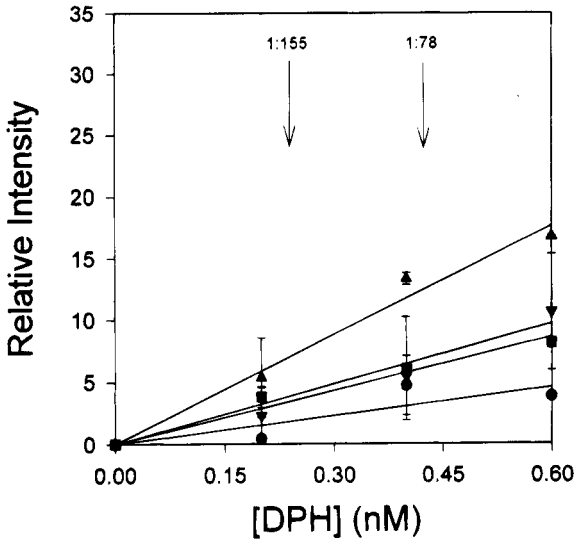

FIGURE 2: Relative fluorescence intensity of 1,6-diphenyl-1,3,5-hexatriene in SPM from control and chronic ethanol-treated mice: effect of acute ethanol. All conditions were as described in legend to Figure 1, except that SPM from chronic ethanol-treated and pair fed mice were used. The symbols are as follows: control, ●; control + 400 mM ethanol, ■; chronic ethanol, ▲; chronic ethanol + 400 mM ethanol, ▼.

lipid ratios of 1:155 and 1:310 was 2- and 4-fold less, respectively, than that at the 1:78 molar ratio. At the probe to lipid ratio 1:78 (arrow in Figure 1), the probe concentration was sufficiently high to be close to, but not in, the self-quenching range. In the second experiment, the possibility that ethanol (acute or chronic) shifted the partitioning of diphenylhexatriene into the synaptic plasma membranes was examined. Such shifts could induce diphenylhexatriene self-quenching and artifactually decrease the fluorescence intensity of DPH. The fluorescence intensity of diphenylhexatriene in control synaptic plasma membranes with or without acute ethanol (400 mM) increased linearly with increasing probe concentration (Figure 2). Likewise, diphenylhexatriene fluorescence intensity in synaptic plasma membranes from chronic ethanol-treated mice increased linearly with increasing diphenylhexatriene concentration, even in the presence of 400 mM acute ethanol (Figure 2). Thus, DPH self-quenching was absent at the DPH:lipid ratio of 1:78 used in the present studies.

Self-quenching of DPH decreases the DPH lifetime. Therefore, fluorescence lifetimes of DPH in SPM at two different DPH:lipid ratios, 1:78 and 1:155, were obtained (Table 1). Two lifetime components near 10.4 and 1.9 ns were observed in control SPM at a DPH:lipid ratio of 1:78. The major lifetime component near 10.4 ns did not significantly change at the lower DPH:lipid ratio of 1:155. Acute ethanol exposure at either ratio did not significantly alter this DPH lifetime (Table 1). Similar data were obtained with SPM from chronic ethanol-treated mice. The lack of changes in the main DPH lifetime near 10.4 ns is consistent with the absence of DPH self-quenching at the probe ratio of 1:78 used in these studies. With regard to the short lifetime component near 1.9 ns, there appeared to be some trend toward increasing lifetime at the 1:155 probe:lipid ratio in control SPM, but not in SPM exposed to acute or chronic ethanol (Table 1). However, none of these changes was statistically significant. The significance of the short lifetime component is dealt with further in the last section of the Results.

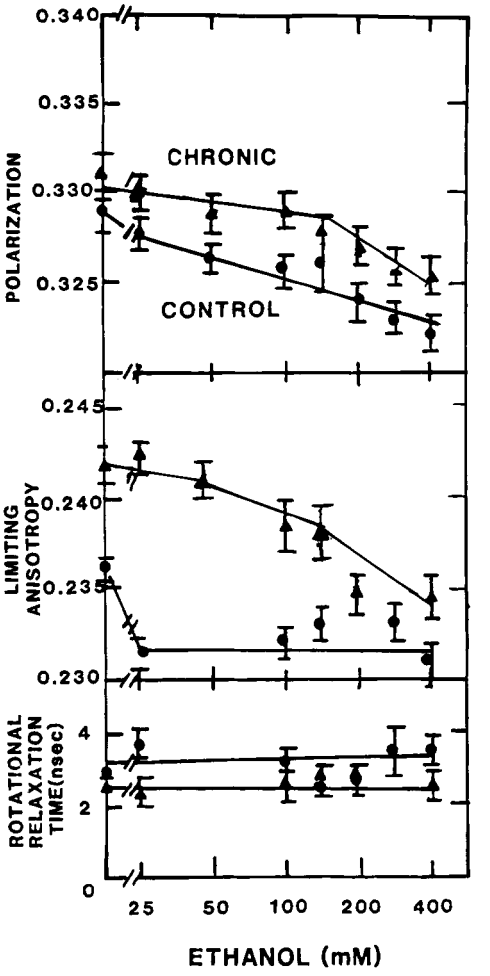

FIGURE 3: Effects of chronic and acute ethanol on 1,6-diphenyl-1,3,5-hexatriene fluorescence static and dynamic parameters in synaptosomal plasma membranes. DPH polarization, limiting anisotropy, and rotational relaxation time were determined in SPM as described in Materials and Methods. Synaptosomal membranes from control (●) and chronic (▲) ethanol-treated animals were exposed to acute ethanol as indicated. Values represent the mean ± SEM (n = 6).

In summary, the partitioning of DPH into SPM was not as high as that in model phospholipid membranes, and higher probe to SPM lipid ratios were needed to provide the equivalent intramembrane DPH concentration. Therefore, under conditions used in the subsequent studies (DPH:lipid ratio of 1:78), DPH self-quenching was not a complicating parameter according to data on fluorescence intensity and the major DPH lifetime component.

Relative Effects of Ethanol in Vitro on SPM Lipid Order: Surface and Interior Core-Sensitive Derivatives of Diphenylhexatriene. Fluorescence polarization of DPH is sensitive to the structure (fluidity, microviscosity) of lipids in membranes. As indicated by the decreasing fluorescence polarization of DPH, increasing concentrations of ethanol fluidized SPM of control animals (Figure 3, top panel). This effect of ethanol *in vitro* on DPH polarization in SPM was significant at the concentrations observed *in vivo* (50−100 mM). Since steady-state polarization measurements comprise both static and dynamic components, it is not known whether ethanol *in vitro*-induced changes in DPH polarization resulted from altered structural (order) or dynamic (rate of

Table 1: Fluorescence Lifetime Properties of DPH in Synaptosomal Plasma Membranes: Effects of DPH:Lipid Ratio and Acute and Chronic Ethanol[a]

ethanol (nM)	probe:lipid ratio	τ_1 (ns)	w_1 (ns)	f_1	τ_2 (ns)	w_2 (ns)	f_2
			Control				
0	1:78	10.4±0.3	0.06 ± 0.01	0.97 ± 0.03	1.9 ± 0.7	0.07 ± 0.01	0.03 ± 0.02
0	1:155	10.7 ± 0.1	0.05 ± 0.05	0.96 ± 0.03	3.8 ± 1.5	0.05 ± 0.01	0.04 ± 0.03
400	1:78	10.5 ± 0.5	0.34 ± 0.10*	0.85 ± 0.07	3.9 ± 1.9	0.05 ± 0.01	0.17 ± 0.07*
400	1:155	11.0 ± 1.0	0.41 ± 0.05*	0.83 ± 0.05	3.8 ± 1.6	0.05 ± 0.05	0.17 ± 0.05*
			Chronic Ethanol Treated				
0	1:78	10.7 ± 0.3	0.06 ± 0.05	0.90 ± 0.02	3.7 ± 0.5	0.63 ± 0.55	0.10 ± 0.03
0	1:155	10.5 ± 0.2	0.10 ± 0.05	0.89 ± 0.04	3.1 ± 1.2	1.28 ± 0.71	0.11 ± 0.04
400	1:78	10.4 ± 0.5	0.18 ± 0.06	0.86 ± 0.05	4.6 ± 0.6	0.17 ± 0.06	0.14 ± 0.05
400	1:155	10.8 ± 0.6	0.17 ± 0.08	0.79 ± 0.02	6.0 ± 1.1	3.19 ± 2.1	0.21 ± 0.02

[a] τ = center of lifetime distribution; w = width of lifetime distribution at peak half-height; f = fractional contribution of component. Values represent mean ± SEM (n = 3−8). An asterisk, *, refers to $p < 0.05$ as compared to no ethanol.

Table 2: Static and Dynamic Properties of DPH and Derivatives in Synaptic Plasma Membranes: Effects of Acute Ethanol[a]

probe	ethanol (nM)	polarization	limiting anisotropy	rotational relaxation time (ns)
DPH	0	0.329 ± 0.002	0.237 ± 0.002	3.0 ± 0.5
	400	0.322 ± 0.003*	0.231 ± 0.002*	3.2 ± 0.5
TMA-DPH	0	0.365 ± 0.001	0.265 ± 0.001	1.9 ± 1.0
	400	0.360 ± 0.002*	0.253 ± 0.003*	2.4 ± 1.2
PRO-DPH	0	0.358 ± 0.001	0.261 ± 0.002	1.4 ± 0.4
	400	0.353 ± 0.003	0.253 ± 0.002*	1.7 ± 0.3

[a] Values represent the mean ± SEM (n = 6). An asterisk refers to $p < 0.05$ by student's t-test.

rotation) parameters of DPH motion in SPM. Multifrequency phase and modulation fluorometry was used as described in Materials and Methods to resolve DPH steady-state polarization into limiting anisotropy and rotational relaxation time (nanosecond) components. The limiting anisotropy of DPH in SPM of control mice was near 0.237 (Figure 3, top panel, and Table 2). This limiting anisotropy was in the range of that reported for DPH in model membranes (Prendergast et al., 1981; Engel & Prendergast, 1984). Several early reports indicated that the steady-state polarization of DPH in SPM ranged from 0.269 at 37 °C (Harris et al., 1987) to 0.32−0.34 at 25 °C (Harris & Schroeder, 1981). When the latter steady-state polarization values are converted to steady-state anisotropy, it is possible to use the relationship between steady-state anisotropy and limiting anisotropy [Figure 9 of Engel and Prendergast (1981)] to estimate a limiting anisotropy for DPH in these SPM to be near 0.200 and 0.23−0.24 at 37 and 25 °C, respectively. The latter values are in close agreement with those determined experimentally herein. As shown in Figure 3 (middle panel), the DPH limiting anisotropy (but not the rotational relaxation time; Figure 3, bottom panel) in SPM was decreased when ethanol was added to the SPM of control mice. These observations confirm and extend those reported earlier (Schroeder et al., 1988b).

It has been reported previously that charged derivatives of diphenylhexatriene (TMA-DPH and PRO-DPH) anchor it closer to the membrane surface (Harris et al., 1987; Wood et al., 1988; Prendergast et al., 1981) As shown in Table 2, these derivatives had higher polarization and limiting anisotropy than did DPH. This indicated that in TMA-DPH or PRO-DPH the DPH moiety, anchored closer to the membrane surface in the charged derivative, sensed a more rigid domain. The limiting anisotropy of TMA-DPH, and that of PRO-DPH, in SPM was reduced by ethanol *in vitro*. Like DPH, the rotational relaxation times of the two charged probes were not altered by ethanol perturbation. With some exceptions, most of the subsequent studies were performed only with the interior core probe, DPH.

Diphenylhexatriene Static and Dynamic Properties in SPM: Response to Chronic Ethanol and Irradiation. As shown earlier, the interior core structural properties reported by DPH revealed that SPM of chronic ethanol-treated mice were more resistant to ethanol added *in vitro*. The steady-state polarization and limiting anisotropy (but not rotational relaxation time) of DPH in SPM of chronic ethanol-treated animals were higher than those in control membranes, indicating that baseline order was higher in SPM of the chronic ethanol-treated mice (Figure 3).

In order to test whether the resistance to ethanol perturbation in SPM of the chronic ethanol group was the result of a nonspecific increase in bulk membrane lipid order, SPM lipid order was altered without changing the temperature or other variables. SPM exposed to γ irradiation had higher DPH polarization, limiting anisotropy, and rotational relaxation time (Figure 4). However, the lipid structural parameters monitored by DPH in the more rigid irradiated SPM appeared just as susceptible to the actions of ethanol perturbation as the control SPM.

Diphenylhexatriene Spectral Properties in Solvents and SPM. In order to understand whether the effects of ethanol on SPM may in part be due to changes in membrane relative dielectric properties, the sensitivity of DPH spectral properties to range of dielectric constants was tested in solvents with known dielectric constants.

DPH had fluorescence excitation maxima in dioxane near 340, 360, and 374 nm (Figure 5A). Emission maxima occurred near 403, 425, and 452 nm (Figure 5B). Emission spectra of DPH in dioxane displayed a large Stokes shift of 65 nm and had almost no overlap with excitation spectra, making Forster energy transfer between DPH molecules very unlikely (Figure 5B). Increases in percent the water increased the dielectric constant (Turner & Brand, 1968) from that in 100% dioxane (dielectric constant of 2.2) to that in dioxane/water (10%/90%, dielectric constant of 76). Concomitantly, with an increasing solvent dielectric constant the following DPH spectral changes were observed: (1) excitation intensity decreased nearly exponentially with an increasing dielectric constant (Figure 6A), as did the ratio of excitation peaks 374/340 nm (Figure 6A, inset); (2) fluorescence emission intensity also decreased exponentially by

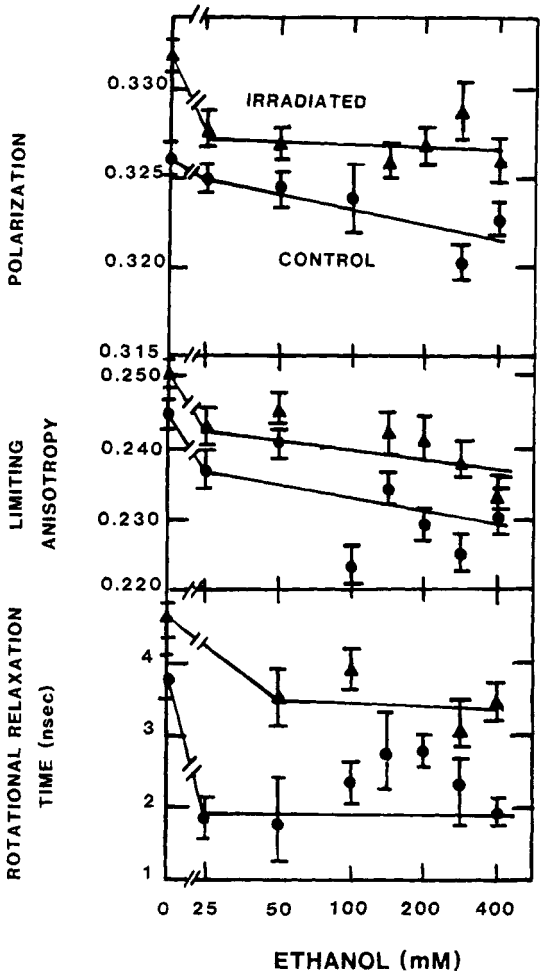

FIGURE 4: 1,6-Diphenyl-1,3,5-hexatriene static and dynamic parameters in synaptosomal plasmic membranes exposed to γ irradiation. DPH polarization, limiting anisotropy, and rotational relaxation time were determined in SPM as described in Materials and Methods. The control values in Figure 4 differ from those in Figure 3 because they were incubated as specified in the Materials and Methods with and without γ irradiation: ●, control; ▲, irradiated. Values are mean ± SEM (n = 6).

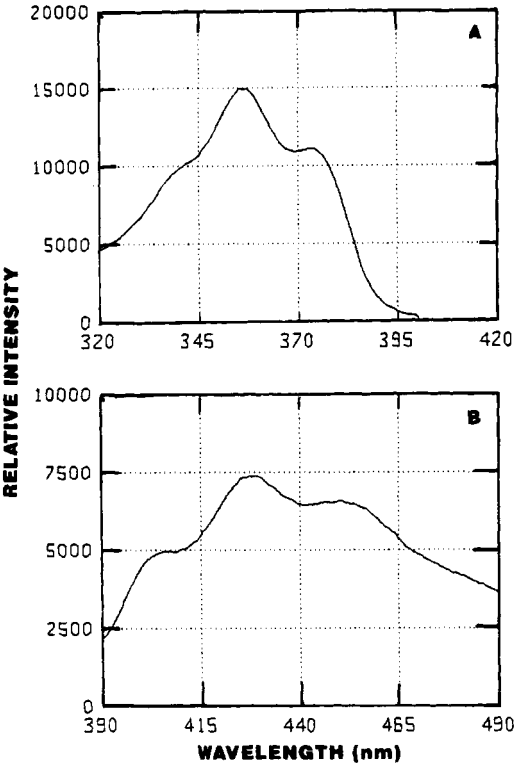

FIGURE 5: Fluorescence excitation and emission spectra of 1,6-diphenyl-1,3,5-hexatriene in dioxane (DPH, 0.05 μg/mL dioxane): (A) excitation spectra (emission = 430 nm); (B) emission spectra (excitation = 325 nm).

99.6% (Figure 6B), and the ratio of intensities of the emission peaks at 425/452 nm decreased (Figure 6B, insert); (3) the wavelengths of emission maxima were not changed; (4) the wavelengths of excitation maxima were slightly blue shifted, especially in the region with a dielectric constant less than 5 (Figure 7). The latter findings are consistent with those of Zannoni et al. (1983), who also observed an insensitivity of DPH emission maxima with alterations in polarity, viscosity, and temperature. Further, Zannoni et al. (1983) reported a blue shift in the DPH absorbance spectrum with increasing polarity, a finding consistent with the blue shift in the DPH excitation spectrum observed in Figure 7.

Although the DPH microenvironment in SPM may not be precisely reconstituted from observations in organic solvents, changes in SPM relative to the dielectric constant or range of constants in response to ethanol *in vivo* or *in vitro* may be inferred. Therefore, the preceding data with DPH in dioxane/water mixtures were used to estimate the dielectric properties of the DPH microenvironment sensed in the SPM interior core. The excitation and emission intensities of DPH in SPM were generally consistent with DPH being in a relatively hydrophobic environment. However, precise calibration from intensities alone is not possible since DPH partitioned between buffer (nonfluorescent DPH) and SPM (fluorescent DPH), while in dioxane all of the DPH was available for excitation and fluorescence. Two DPH fluorescence parameters in solvents provided useful calibrations for determining the relative effects of ethanol on dielectric properties of the SPM interior core sensed by DPH. First, the excitation peak ratio, and even more so the emission peak ratio, of DPH in SPM yielded a relative measure of the dielectric constant. When the DPH emission peak ratio was determined in SPM and compared to the inset in Figure 6B, the relative dielectric constant of the SPM interior core was 7 ± 3 and 10 ± 2 for control and chronic ethanol-treated mice, respectively. Thus, the SPM interior core had some polar character, but there was no significant difference between control and chronic ethanol-treated mice in these parameters. Upon the addition of acute ethanol (400 mM), the SPM interior core relative dielectric constant detected by DPH increased to 14 ± 3 for control mice, but remained unchanged near 10 ± 3 for chronic ethanol-treated mice. Second, DPH excitation peak maxima in SPM, in conjunction with the solvent data in Figure 7, were used to obtain the relative dielectric constant detected by DPH in SPM (Table 3): 2.20 ± 0.04 and 2.46 ± 0.01 for SPM from control and chronic ethanol-treated mice, respectively. Ethanol *in vitro* significantly increased ($p < 0.05$) the dielectric constant of DPH in SPM for control but not chronic ethanol-treated mice (Table 3). It should be noted that the relative dielectric constants obtained by these different methods do not reflect absolute values and therefore are not comparable between the different methods. However, comparisons of relative dielectric constants based on these spectral properties of DPH in solvents are consistent with the conclusion that ethanol *in vitro* increased the relative dielectric constant in the interior

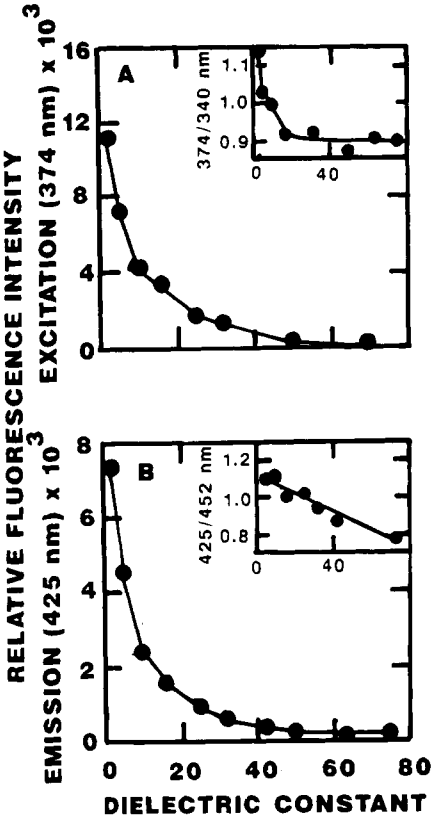

FIGURE 6: Fluorescence excitation and emission peak intensities and peak ratios from spectra of 1,6-diphenyl-1,3,5-hexatriene in dioxane/water mixtures of varying dielectric constant (DPH, 0.05 μg/mL solvent). Dielectric constants are 10% dioxane (76), 20% dioxane (64), 30% dioxane (50), 40% dioxane (42), 50% dioxane (32), 60% dioxane (25), 70% dioxane (16), 80% dioxane (10), 90% dioxane (5), and 100% dioxane (2) (Turner & Brand, 1968). (A) Excitation intensities at 374 nm and peak ratio at 374/340 nm (inset), emission = 430 nm. (B) Emission peak intensities at 425 nm and peak ratio at 425/452 nm (inset), excitation = 325 nm.

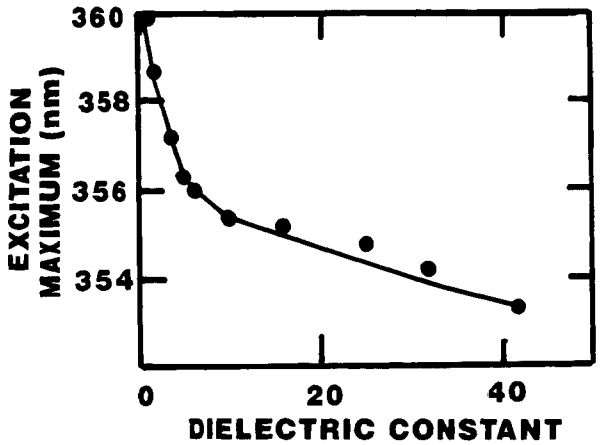

FIGURE 7: DPH excitation maximum response to solvent dielectric constant. All conditions were as described in the legend to Figure 6, except that the excitation maximum wavelength was determined.

core reported by DPH in SPM of control but not chronic ethanol-treated mice.

Lifetime of Diphenylhexatriene in Isotropic Solvents of Varying Dielectric Constant. Earlier work examining the effects of solvent viscosity demonstrated an insensitivity of diphenylhexatriene lifetime to solvent viscosity or solvent refractive index (Zannoni et al., 1983). However, since DPH has two excited singlet states that are very close in energy, their relative positions are dependent upon other factors such as solvent polarity (Zannoni et al., 1983). This multiplicity of excited states accounts for the unusual properties of DPH fluorescence decay: temperature independence in solvents of low dielectric constant, temperature dependence in solvents of high dielectric constant, and decrease in fluorescence lifetime in solvents of high dielectric constant (Zannoni et al., 1983). The latter property differs from those of most dielectric constant-sensitive probe molecules whose lifetimes increase with increasing dielectric constant.

In a series of dioxane/water mixtures with increasing dielectric constants (Figure 8A), the DPH lifetime determined by phase fluorometry decreased from 7.6 ns in pure dioxane (dielectric constant of 2.2) to 0.3 ns in 10% dioxane/water (dielectric constant of 76). It was not possible to construct similar curves for TMA-DPH or PRO-DPH since such molecules form micelles in polar solvents and reversed micelles in solvents with low dielectric constant (Prendergast et al., 1981). Micelle formation would result in interaction between the aromatic systems and either quenching or excimer formation (Prendergast et al., 1981). The formation

Table 3: Diphenylhexatriene Excitation Maximum in Synaptic Plasma Membranes: Effect of Ethanol *in Vitro* and *in Vivo*[a]

ethanol (nM)	excitation maximum		relative dielectric	
	control	chronic	control	chronic
0	359.8 ± 0.1	359.2 ± 0.1*	2.20 ± 0.04	2.46 ± 0.01*
75	358.9 ± 0.1†	359.4 ± 0.2	2.58 ± 0.08†	2.37 ± 0.08
400	359.1 ± 0.2†	358.8 ± 0.4	2.48 ± 0.10†	2.60 ± 0.17

[a] Values represent the mean ± SEM (n = 3–4). An asterisk refers to $p < 0.05$ as compared to control. A dagger refers to $p < 0.05$ versus no ethanol.

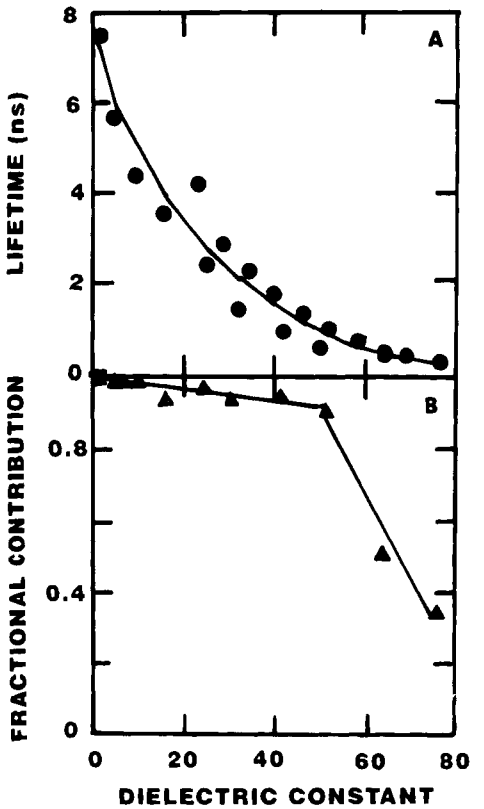

FIGURE 8: Effect of solvent dielectric constant on DPH lifetime. All conditions were as described for Figure 5, except that the lifetime center of distribution of component 1 (longer lifetime) is plotted versus dielectric constant (A), and the fractional contribution of component 1 (B) is plotted versus dielectric constant.

of micelles was also observed with DPH, but at much higher dielectric constant. With increasing dielectric constant, the fractional contribution of the DPH lifetime (Figure 8A) was 95% or more until the dielectric constant was greater than 50 (Figure 8B). Thereafter, the contribution of scattered light (0.01 ns lifetime) increased dramatically. This is interpreted as micelle formation. In order to determine whether the scatter component observed in lifetime experiments was due to solvent micelles or DPH micelles, steady-state right angle fluorescence measurements were made as described in Materials and Methods (data not shown). In the absence of DPH there was no increase in light scatter with increasing dielectric constant over the range shown in Figure 8. In contrast, in the presence of DPH, micelles formed in dioxane/water mixtures above a dielectric constant of 56.

Effect of Temperature on Diphenylhexatriene Lifetime Center of Distribution and Distributional Width in SPM. In contrast to the data observed in isotropic solvents such as ethanol (one DPH lifetime component), fitting of the phase and modulation data of DPH in SPM to multiple components improved the data fit. Four models available through the software were tested: Discrete, Lorentzian, Gaussian, and Uniform. For example, in a discrete analysis typical χ^2 values for one and two components were 18.7 and 2.89, respectively. For the corresponding Lorentzian analysis, the χ^2 values for one and two components were 2.73 and 2.66, respectively, for the Gaussian distribution the χ^2 values were 3.71 and 2.71, respectively, and for the Uniform fit model the χ^2 values were 4.81 and 2.93, respecively. The addition of a third component (due to light scatter) did not significantly improve the χ^2, indicating that light scattering was not significant. The Lorentzian analysis was chosen for two reasons: First, evaluation of the models indicated that two-component models had lower χ^2 values. Among the two-component fits, the Lorentzian two-component analysis had the lowest χ^2. Second, since the Lorentzian distributional analysis had already been used by this laboratory (Schroeder et al., 1988a; Nemecz & Schroeder, 1988; Schroeder & Nemecz, 1989; Jefferson et al., 1990; Nemecz et al., 1991) and others (Ho et al., 1992; Williams et al., 1990; Fiorini et al., 1987; Wratton et al., 1989) to examine the membrane environmental heterogeneity of the probe, it was chosen to simplify comparisons.

Fitting the DPH lifetime data in SPM to the two-component Lorentzian distributional analysis typically yielded χ^2 values between 2 and 5, with the major lifetime component having a fractional contribution near 0.97 (Table 1). DPH in SPM had two centers of lifetime distribution (Figure 9). The longer lifetime component near 11 ns is thought to arise from DPH residing in the interior of the lipid bilayer (Parasassi et al., 1984). Indeed, the lifetime of DPH in very nonpolar solvents (dielectric constant of 2.02) is 12−13 ns (Zannoni et al., 1983). The shorter lifetime component near 2−6 ns (Table 1) is not due to DPH localized in a more polar environment, as suggested in one study using model membranes (Fiorini et al., 1987). Instead, the short lifetime component appears to be due to an as yet uncharacterized photoproduct of DPH reacting with membrane phospholipids (Parasassi et al., 1984; Barrow & Lentz, 1985).

Although the longer DPH lifetime component was insensitive to viscosity in isotropic oils (Zannoni et al., 1983), it is not known whether this is also true for DPH in the anisotropic environment of the SPM lipid bilayer (Wood et al., 1990; Fontaine et al., 1979, 1980). Therefore, the effect of microviscosity on DPH lifetime in the anisotropic environment of the SPM lipid bilayer was determined by increasing the temperature from 10 to 42 °C (Figure 9). The center of lifetime distribution of the longer lifetime component was essentially unchanged by temperatures between 12 and 40 °C (Figure 9). If the increasing temperature were only to alter the microviscosity of the DPH microenvironment, then no change in the lifetime center of distribution would have been expected. However, if an increase in temperature were to significantly expand the membrane and allow large penetration and accommodation of water molecules into the bilayer (increased dielectric constant), the lifetime center of distribution of the longer lifetime component would be expected to decrease. Since the DPH lifetime center of distribution did not decrease significantly (Figure 10), the possibility of a massively increased relative dielectric constant sensed by DPH in the membrane with increased temperature and decreased microviscosity apparently did not occur.

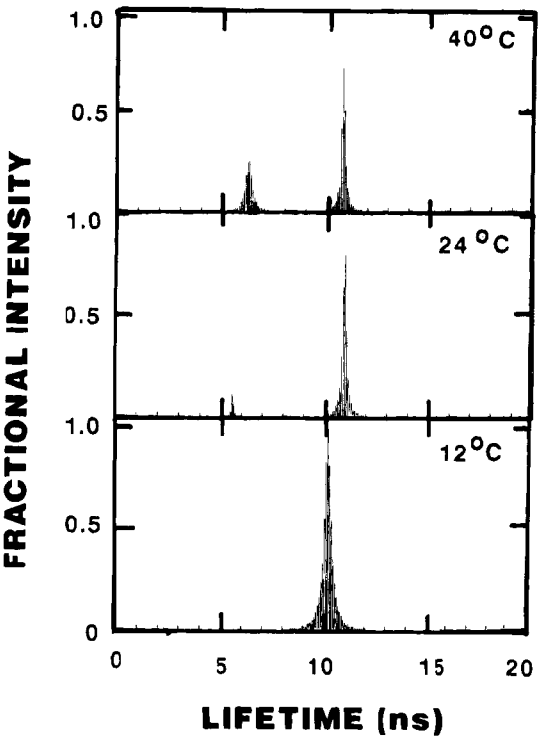

FIGURE 9: Effect of temperature on DPH two-component Lorentzian lifetime distributions in synaptosomal plasma membranes. DPH lifetime distributions were determined as described in Materials and Methods.

In contrast to the center of lifetime distribution, the lifetime distributional width of DPH in SPM appears to be a more sensitive parameter to small changes in heterogeneity of the DPH microenvironment microviscosity or DPH microenvironment heterogeneity/diversity/range of dielectric constants. Lifetime distributional width is not directly related to a single dielectric constant or microviscosity value, but rather reflects a range of values. The lifetime distributional width of DPH is affected by at least two factors: First, decreasing lipid order (microviscosity) appears to be associated with an increase in temperature and results in decreased DPH heterogeneity as indicated by the decreased width of the lifetime distribution (Fiorini et al., 1987; Williams & Stubbs, 1988). Secondly, increased penetration of solvent molecules

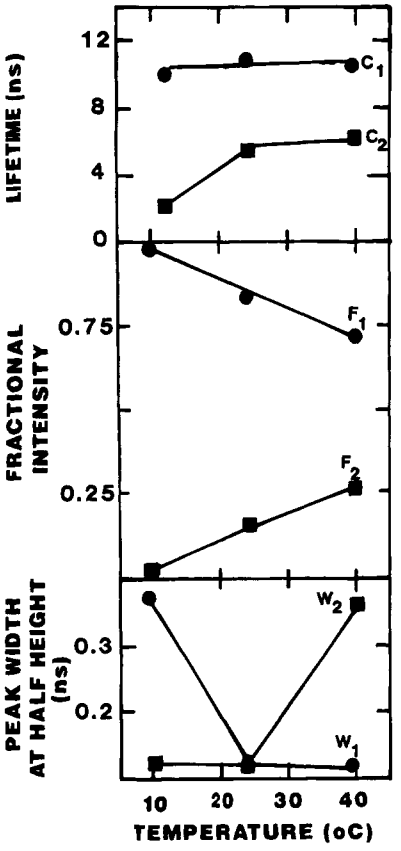

FIGURE 10: Effect of temperature on DPH Lorentzian lifetime distributional parameters. DPH lifetime distributions were determined in control SPM as described in Materials and Methods: ●, component 1; ■, component 2.

into the bilayer at higher temperatures would be expected to increase the diversity or range of dielectric constants. An increased range of dielectric constants increases DPH microenvironment heterogeneity, as determined by the increased distributional width (Parasassi et al., 1984; Fiorini et al., 1987). The effects of alterations in viscosity and the range of dielectric constants may be linked, but which predominates in a given situation must be determined experimentally. The data in Figure 10 show that, with increased temperature, SPM viscosity appears to be a more important determinant of DPH distributional width and more than offsets the potential effects of an increased range in dielectric constants if more water penetrated at higher temperature.

The sensitivity of the lifetime center of distribution and the distributional width (peak width at half-height) to the range of dielectric constants of the DPH microenvironment in SPM was further demonstrated by the use of γ-irradiated SPM (Figure 11). Irradiation increased the limiting anisotropy (increased order or viscosity) of DPH in SPM without increasing the temperature (Figure 4). Furthermore, irradiation increased the lifetime center of distribution of both lifetime components by more than 2 ns (Figure 11). Since the DPH lifetime is independent of viscosity, this observation is consistent with less water being accommodated in the more rigid (viscous) bilayer core of irradiated synaptosomal plasma membranes. The distributional width (peak width at half-height) of the DPH longer lifetime component was increased 2-fold in the more rigid irradiated synaptosomal plasma membranes. In more rigid membranes, the lifetime center of distributionand the distributional width are both expected to increase. Concomitantly, in the more rigid membranes,

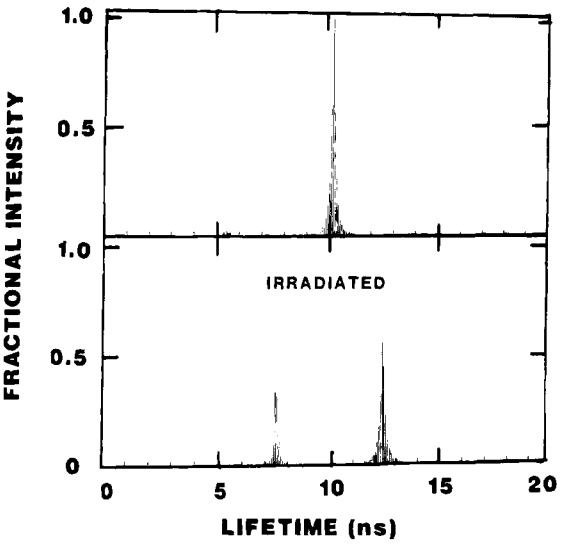

FIGURE 11: γ irradiation of synaptosomal plasma membranes alters subsequently incorporated DPH Lorentzian lifetime distributions: top panel, control; bottom panel, irradiated. Conditions for irradiation were as described in Materials and Methods.

as the polar solvent is displaced from the bilayer interior core, the range of dielectric constants sensed by DPH and distributional width would decrease. Thus, the viscosity contribution dominated over that of the range of dielectric constants in the irradiated SPM.

Effect of Ethanol in Vitro on DPH Lifetime Center of Distribution and Distributional Width in SPM: Surface and Interior Core Derivatives of DPH. The long lifetime centers of distribution of the TMA and PRO diphenylhexatriene derivatives (7.7 and 7.1 ns, respectively, Table 4) in SPM of control mice were significantly shorter than that of DPH (10.4 ns) (Table 1). Furthermore, the lifetime distributional widths of TMA-DPH and PRO-DPH (0.36 and 0.16 ns, respectively, Table 4) were considerably broader than that of DPH (0.06 ns) in SPM of control mice (Table 1). These findings are consistent with TMA-DPH and PRO-DPH being located closer to the SPM bilayer surface and sensing a microenvironment with a greater range of dielectric constants in the control mouse synaptosomal plasma membranes.

The DPH derivatives also provide information regarding the range of dielectric constants in the exofacial and cytofacial leaflets of the SPM bilayer. It was demonstrated previously that TMA-DPH and PRO-DPH preferentially localized in the inner and outer leaflets of the SPM bilayer, respectively (Schroeder et al., 1988b). As shown in Table 4, the center of lifetime distribution of TMA-DPH (inner leaflet selective) was not significantly different from that of PRO-DPH (outer leaflet selective). However, the distributional width of TMA-DPH, 0.36 ± 0.01 ns, was significantly longer than that of PRO-DPH, 0.15 ± 0.08 ns (Table 2). These results indicate that the range of dielectric constants sensed by TMA-DPH in the inner leaflet of the SPM was greater than that sensed by PRO-DPH in the outer leaflet. This might be expected on the basis of localization of anionic phospholipids in the inner leaflet of SPM.

Although the DPH long lifetime center of distribution was not significantly altered by ethanol *in vitro*, the distributional width of this component was significantly increased from 0.05−0.06 to 0.34−0.41 ns in synaptosomal plasma membranes from control mice (Table 1). Likewise, the distri-

Table 4: Fluorescence Lifetime Properties of DPH Derivatives in Synaptosomal Plasma Membranes: Effects of Acute Ethanol[a]

ethanol (nM)	probe	τ_1 (ns)	w_1 (ns)	f_1	τ_2 (ns)	w_2 (ns)	f_2
0	TMA-DPH	7.7 ± 0.3	0.36 ± 0.01	0.92 ± 0.05	2.4 ± 0.6	0.16 ± 0.10	0.08 ± 0.05
400		6.6 ± 0.1*	0.51 ± 0.04*	0.79 ± 0.06*	3.2 ± 1.5	0.18 ± 0.05	0.21 ± 0.06*
0	PRO-DPH	7.1 ± 0.5	0.15 ± 0.08†	0.76 ± 0.07	1.9 ± 0.7	0.26 ± 0.08	0.24 ± 0.05†
400		6.3 ± 0.3*	0.40 ± 0.04*	0.63 ± 0.06	3.9 ± 0.9	0.13 ± 0.10	0.37 ± 0.06*

[a] τ = center of lifetime distribution; w = width of lifetime distribution at peak half-height; f = fractional contribution of component. Values represent mean ± SEM ($n = 6$). An asterisk refers to $p < 0.05$ as compared to no ethanol. A dagger refers to $p < 0.05$ as compared to TMA-DPH.

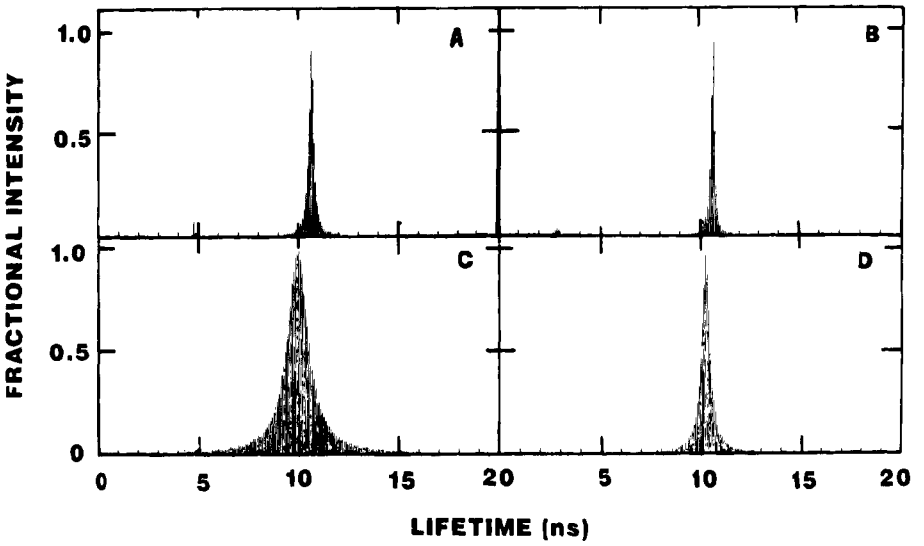

FIGURE 12: Sensitivity of DPH Lorentzian lifetime distributions in synaptosomal plasma membranes to acute and chronic ethanol: (A) control; (B) chronic ethanol-treated; (C) control + 400 nM ethanol; (D) ethanol treated + 400 mM ethanol.

butional widths of the longer lifetime components of TMA-DPH and PRO-DPH were increased by acute ethanol perturbation of SPM (Table 4). The fold increases in distributional width reported for DPH, TMA-DPH, and PRO-DPH in the presence of ethanol were 6.2−6.8-, 1.4-, and 2.7-fold, respectively. Thus, the effect of ethanol upon increasing the range of dielectric constants monitored by DPH in SPM was greater in the SPM outer leaflet than in the inner leaflet. Surprisingly, the fold effect of ethanol upon increasing the range of dielectric constants was greatest in the interior core monitored by DPH.

Thus, from both the centers of lifetime distribution and/or distributional widths of DPH, TMA-DPH, and PRO-DPH,, it appears that acute ethanol treatment increased the range of dielectric constants in both the SPM surface, preferentially the outer leaflet, and the SPM interior core domains in control mice.

Effect of Chronic Ethanol Consumption on DPH Lifetime Centers of Distribution and Distributional Widths in SPM of Chronic Ethanol-Treated Mice. The effect of ethanol *in vitro* on DPH lifetime distributions in the SPM of control (Figure 12A) and chronic ethanol-treated (Figure 12B) mice was determined. There were no significant differences in the centers of lifetime distribution, distributional widths, or fractional intensities of DPH in SPM from control and chronic ethanol-treated mice (Table 1). Thus, from the DPH long lifetime component center of distribution and long lifetime component distributional width, it appears that chronic ethanol treatment did not alter the intrinsic relative dielectric constant or the range of dielectric constants monitored by DPH in the SPM interior core. However, ethanol *in vitro* significantly increased the distributional width, w_1, in SPM from control but not chronic ethanol-treated mice (Figure 12C, Table 1). The range of dielectric constants monitored by DPH in the SPM of chronic ethanol-treated mice is resistant to the changes induced by ethanol *in vitro* (Figure 12D, Table 1). Thus, chronic ethanol treatment did make the SPM interior core, monitored by DPH, resistant to changes in the range of dielectric constants by ethanol *in vitro*.

Significance of the DPH Short Lifetime Component. Additional information can be obtained from the short lifetime component of DPH. The DPH short lifetime component is thought to arise from photochemical changes such as photobleaching or reaction with oxidized phospholipid species (Parasassi et al., 1984; Wratton et al., 1989). Several factors increase the rate of DPH photoreaction in the presence of strong ultraviolet radiation.

First, increasing temperature increased DPH photoreaction. For DPH in dioxane, an increase in the temperature from 20 to 37 °C doubled the rate of photobleaching when the sample was exposed to strong ultraviolet irradiation (data not shown). In SPM, the fraction of shorter lifetime component increased at the expense of the longer component with increasing temperature (Figure 10) and in the pre-irradiated sample (Figure 11). As the temperature increases, it is reasonable to expect the rate and/or accessibility of DPH to react with endogenous membrane-oxidized lipid components to increase.

Second, on the basis of the earlier observations with temperature, it might be expected that the fluidity of the SPM will determine the photoreactivity of DPH. DPH photoreaction is much more rapid in the more fluid dioleoylglycerophosphorylcholine vesicles than in dipalmitoylglycerophosphorylcholine vesicles (Duportail & Weinreb, 1983). However, the quantity of such reactive lipid species in the

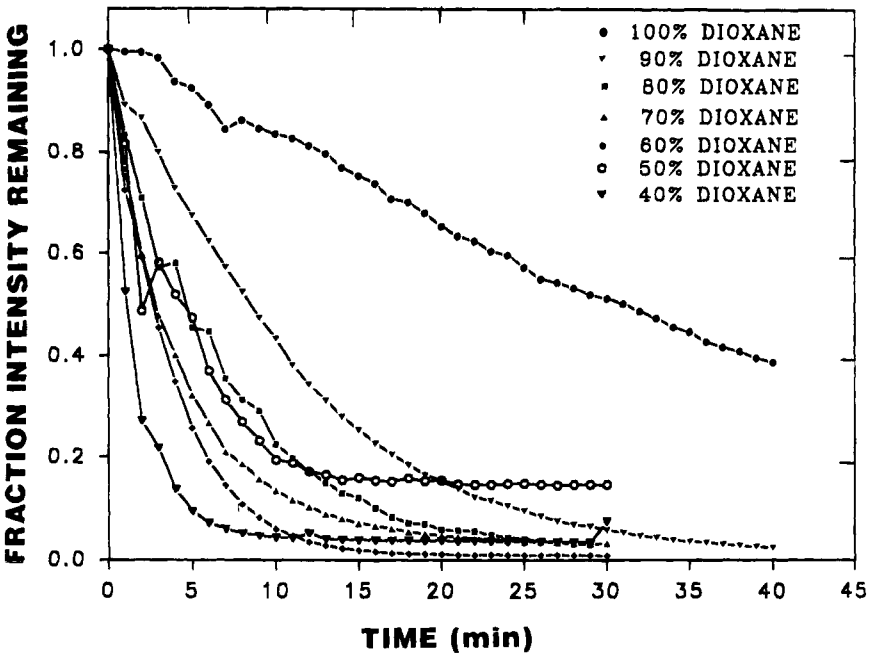

FIGURE 13: Dielectric sensitivity of DPH photobleaching to strong ultraviolet irradiation: DPH in dioxane/water mixtures (as indicated) at 37 °C.

SPM was not related to the intrinsic fluidity of the SPM from the control versus chronic alcoholic mice. In the absence of acute ethanol, the more rigid SPM from chronic ethanol-treated mice intrinsically did not differ significantly in the fraction of DPH shorter lifetime component from that of control mice (Table 1).

Third, the presence of oxidized membrane lipid enhances the rate of DPH photoreaction. Oxidized lipids themselves increase the DPH lifetime center of distribution and distributional width in model membranes through increasing the range of dielectric constants of the membrane (Wratton et al., 1989). In the case of SPM pre-irradiated with γ irradiation, more reactive lipid species are expected to be present, thereby resulting in increased formation of the fraction of the DPH short lifetime component.

Fourth, DPH photobleaching was dependent on the solvent dielectric constant. Studies were performed by exposing DPH in dioxane/water mixtures to strong ultraviolet irradiation. As shown in Figure 13, DPH photobleached much more rapidly with increasing polarity of the solvent. The rate of DPH photoreaction was 35-fold faster in ethanol (dielectric constant of 24) than in dioxane (dielectric constant of 2.2). Thus, DPH probes closer to the surface of the SPM bilayer would be expected to be in a more polar environment and demonstrate increased photoreactivity. Indeed, the intrinsic fraction of the short lifetime component, f_2, reported by the three DPH probes in SPM varied in the order PRO-DPH > TMA-DPH > DPH (Tables 1 and 4). As predicted, due to their closer proximity to the SPM bilayer surface and the higher dielectric constant environment (see preceding sections), both charged DPH derivatives showed more photoreaction than did the interior core probe, DPH. Further, the SPM outer leaflet surface (selectively probed by PRO-DPH) appeared to have more reactive lipid species than the inner leaflet surface (selectively probed by TMA-DPH). Nevertheless, the fold increase in the fraction of short lifetime component, f_2, in the presence of ethanol *in vitro* was 5.7–4.3-, 2.6-, and 1.5-fold for DPH, TMA-DPH, and PRO-DPH, respectively (Tables 1 and 4). Thus, the SPM interior core photoreactive species were the most sensitive to the actions of ethanol *in vitro*.

Finally, while ethanol *in vitro* increased the fraction of photoreacted probe in SPM from control mice ($P < 0.05$, Table 1), ethanol *in vitro* did not significantly increase the fraction of photoreacted probe in the interior core (DPH) in SPM of chronic ethanol-treated mice (Table 1). With regard to chronic effects of ethanol, the interior core relative dielectric properties and the fraction of phospholipid reactive with DPH were resistant to changes by additional ethanol *in vitro*. These data indicate that ethanol *in vitro* may enter the interior core, expand the polar molecule pool size (more ethanol and/or water molecules than original water molecules), and thereby stimulate the formation of DPH photoreaction products in SPM from susceptible but not resistant chronic ethanol-treated mice.

DISCUSSION

Diphenylhexatriene as a probe of SPM fluidity has been well accepted. In this regard, the SPM lipid domain sampled by DPH has been characterized as the interior core of the lipid bilayer. Four observations indicated that the charged derivatives of DPH, TMA-DPH and PRO-DPH, monitored the SPM in a domain closer to the surface of the bilayer than did DPH. First, the limiting anisotropy of the charged derivatives in SPM was higher than that of DPH, indicating that the domain near the surface of the bilayer was more ordered than the interior core. Ethanol *in vitro* altered the limiting anisotropy of the DPH probe in the exofacial leaflet, but not the cytofacial leaflet. In contrast, ethanol *in vitro* altered the limiting anisotropy of the two charged probes, TMA-DPH and PRO-DPH, in both leaflets (Table 2). The latter was due to ethanol reducing the leaflet selectivity of the charged DPH derivatives (Schroeder et al., 1988b). Moreover, SPM from chronic ethanol-treated mice were resistant to these fluidizing actions of ethanol *in vitro*. These findings are consistent with earlier reports from this labora-

tory (Wood et al., 1989; Schroeder et al., 1988a,b). Second, TMA-DPH and PRO-DPH had nearly 3 ns shorter lifetimes in SPM than did DPH. On the basis of observations of lifetime versus solvent dielectric constant, this lifetime difference is consistent with the interior core (sampled by DPH) having a lower relative dielectric constant than the microenvironment closer to the surface of the bilayer (sampled by TMA-DPH and PRO-DPH). Other investigators have shown that the relative dielectric constants for the hydrophobic core and the glycerol backbone of membrane bilayers were near 2 and 45, respectively (Perochan et al., 1992). Thus, the charged derivatives of DPH monitored SPM properties closer to the membrane surface, but not directly within the glycerol backbone/phospholipid polar head group region. Third, the lifetime distributional widths of TMA-DPH and PRO-DPH were much broader in SPM than that of DPH. Again, this indicates a greater range of dielectric constants of the microenvironment sensed by the charged DPH derivatives. Fourth, the more polar regions of the SPM sensed by TMA-DPH and PRO-DPH were more rigid (higher limiting anisotropy) when compared to DPH.

It was previously shown that TMA-DPH (positively charged) preferentially monitored the cytofacial leaflet while PRO-DPH (negatively charged) preferentially monitored the exofacial leaflet of synaptosomal plasma membranes (Schroeder et al., 1988a,b). As mentioned in the introduction, ethanol partitions into both the surface polar domain and the interior core of the SPM bilayer. The data presented herein indicate that the charged DPH derivative probes may be useful in investigating ethanol effects on SPM domain relative dielectric constants: first, to probe the relative range of dielectric constants of the SPM exofacial versus cytofacial leaflets, and second, to probe the surface (TMA-DPH and PRO-DPH) versus inner core (DPH) dielectric properties of the SPM bilayer. These were accomplished through the examination of ethanol effects *in vitro* and *in vivo* on the lifetime and spectral properties of both DPH and the charged DPH derivatives.

In the absence of ethanol *in vitro* or *in vivo*, the charged DPH derivatives provided information regarding the intrinsic relative dielectric constants of the exofacial and cytofacial leaflets. The cytofacial leaflet-selective TMA-DPH and the exofacial-selective PRO-DPH monitored very different relative ranges of dielectric constants, as indicated by the much broader lifetime distributional width of TMA-DPH. Thus, TMA-DPH appears to monitor a greater range of intrinsic dielectric constants in the cytofacial leaflet of the SPM of control mice. Ethanol *in vitro* in SPM of control mice increased the range of dielectric constants monitored by both probes. However, the fold increase in the range of dielectric constants was greater in the SPM exofacial leaflet monitored by the PRO-DPH. The magnitude of this effect was partly obscured by the tendency of ethanol *in vitro* to reduce the leaflet selectivity of the charged DPH derivatives (Schroeder et al., 1988b). These data are consistent with the results of others also indicating that ethanol acts selectively in specific domains (Wood & Schroeder, 1988, 1992; Treistman et al., 1987; Wood et al., 1989, 1990, 1993; Schroeder et al., 1988b; Hitzeman et al., 1986).

The surface and the interior core of the SPM differed significantly in their relative dielectric constants. The observation of shorter fluorescence lifetimes of both charged DPH derivatives compared to DPH was consistent with the SPM surface having a considerably higher relative dielectric constant than the interior core. In addition, in the absence of ethanol *in vitro*, the lifetime distributional widths of both charged DPH derivatives in SPM were broader than those of DPH. These data indicate that the range of surface dielectric constants is much higher than the interior core dielectric in the SPM. Ethanol *in vitro* increased the lifetime distributional widths of the charged DPH derivatives, indicating a potent hydration effect in the membrane surface. Indeed, ethanol *in vitro* at high concentrations (400 mM) preferentially partitioned into the SPM surface more than the interior core (Hitzeman et al., 1986; Kreishman et al., 1985). A unique observation, therefore, was the finding that the SPM interior core monitored by DPH was also sensitive to dielectric constant changes.

DPH lifetime is a parameter often overlooked as a monitor of membrane relative dielectric constant (Williams et al., 1990). DPH lifetime is insensitive to viscosity and to temperature in nonpolar solvents (Zannoni et al., 1983). In contrast, in polar solvents its lifetime is not only temperature sensitive (Zannoni et al., 1983; data presented herein) but also sensitive to the dielectric constant of the surrounding solvent. These observations allow refinement of the description of the DPH microenvironment in the SPM bilayer core. Several pieces of evidence indicate that although DPH lifetime (major lifetime component near 11 ns) is primarily due to the hydrophobic nature of the bilayer interior it also appears sensitive to parameters associated with dielectric constant alteration. For example, the DPH lifetime center of distribution was sensitive to membrane limiting anisotropy, a measure of order or viscosity. In irradiated SPM the lifetime center of distribution shifted to longer lifetimes. In the case of DPH, such shifts are interpreted to mean a shift toward a less polar/aqueous environment. The more rigid irradiated membrane thus allowed less penetration of water molecules into the SPM interior core, as monitored by DPH. In contrast, ethanol *in vitro* decreased the lifetimes of TMA-DPH and PRO-DPH but not that of DPH. These data might imply that the dielectric properties of the SPM interior core domain sensed by DPH were not altered by acute ethanol. On the contrary, data from increased distributional widths of DPH lifetime in SPM from control, but not chronic ethanol-treated mice, perturbed with ethanol *in vitro* indicate that the interior core range of dielectric constants also increases with acute ethanol, but that the change is less dramatic than at the surface. These data would indicate that ethanol (dielectric constant of 24) and water (dielectric constant of 80) must not only compete for binding sites within the SPM interior core but also increase the hydration therein. The significant fluidization of the SPM by ethanol *in vitro* appears to allow not only ethanol but also a larger amount of water to penetrate the interior core of the bilayer, the effect being observed as an increase in the dielectric constant. The latter observation is not consistent with the dehydration theory proposed by Klemm (1990).

These observations on the relative dielectric constants of the SPM interior core monitored by DPH were substantiated by DPH spectral properties. The majority of DPH is be expected to be localized in the lipid bilayer core away from the protein−lipid interface. This prediction is based on the following: (1) DPH is a very nonpolar molecule that partitions nearly equally into fluid and solid lipid domains (Lentz et al., 1976); (2) rotational mobility of proteins such

as concanavalin A receptors appears to be inversely proportional to lipid fluidity measured by DPH (Shinitzky & Inbar, 1976; thus, the mobility of lipids surrounding the protein might be expected to differ from that of the bulk lipid); (3) DPH displays very similar microviscosity values when incorporated into plasma membranes or lipid vesicles made from plasma membrane lipid extracts (Barenholz et al., 1976; Van Blitterswijk et al., 1977). It should be noted, however, that some DPH molecules appear localized in the protein−lipid interface: (1) DPH polarization is partly dependent on the protein content of the membrane (Lentz et al., 1979); (2) incorporation of gramicidin into lipid bilayers reduced the fluorescence lifetime of DPH (Ho & Stubbs, 1992); (3) Cytochrome b_5 induced the formation of an additional DPH center of lifetime distribution in model membranes (Ho et al., 1992); (4) cytochrome b_5 induced environmental heterogeneity in the protein−lipid interface monitored by DPH (Williams et al., 1990).

Previously, this and other laboratories utilized the spectral properties of fluorophores to determine the relative dielectric constant gradient between the surface and the interior of membrane lipids (Perochan et al., 1992; Schroeder et al., 1976) and in the lipid binding site of fatty acid binding proteins (Nemecz et al., 1991). Spectral peak ratios were used to localize the fluorophore of parinaric acid (carbons 9−15) deep in the bilayer interior of plasma membranes where it senses a relative dielectric constant near 2 (Schroeder et al., 1976), which is similar to that reported with other probes (relative dielectric constant of 2−4) (Perochan et al., 1992). In contrast, when bound to lipophilic proteins, i.e., fatty acid binding proteins from intestine and liver, the parinaric acids sensed relative dielectric constants of 16 and 30, respectively (Nemecz et al., 1991). X-ray crystallography indicates that the hydrophobic fatty acid binding pocket of intestinal fatty acid binding protein can accommodate 24 and 7 water molecules in the absence (Scapin et al., 1993) and presence (Sacchettini et al., 1989) of bound fatty acid, respectively. In the present work, spectral peak ratios and excitation maxima of DPH compared to standard curves were consistent with relative dielectric constants near 2−7 for SPM. The SPM relative dielectric constant sensed by DPH was higher than that in interior core lipids alone (dielectric constant of 2) and lower than the relative dielectric constant of lipid closely interacting with lipid binding proteins (dielectric constant of 16−30). Thus, it would seem that the majority of DPH would be localized in the bulk lipid interior core of the SPM, while a smaller fraction of the DPH in the SPM interior core might be located in the vicinity of the membrane protein−lipid interface. That the bulk lipid interior core is sensitive to the relative dielectric constant is based on the logic that the bulk lipid interior core of SPM is a microenvironment that must contain at least some polar groups sensed by DPH.

First, some of the DPH may be localized in the polar surface and therefore senses some polar groups. This is unlikely for the following reasons. Some investigators suggested that a shorter lifetime component near 2−3 ns was due to DPH in the polar environment of the model membrane surface (Fiorini et al., 1987). As stated earlier, the short lifetime component is a photoreaction product of DPH probes with lipids, not DPH in a more polar environment. Furthermore, the lifetime of DPH (11 ns) is much longer than that in polar microenvironments. In SPM bilayers, the major lifetime component of TMA-DPH (a probe located closer to the membrane surface than diphenylhexatriene) is significantly shorter than that of diphenylhexatriene (7 versus 11 ns, data herein; Schroeder et al., 1988b).

Second, some polar molecules are found in the hydrophobic core, and ethanol may partition there. Indeed it has been reported that the hydrophobic core of the membrane contains 2−30 mM water (Meier et al., 1990). Also, at low concentrations alcohol preferentially partitions to the interior of membrane bilayers, while at higher concentrations it partitions more to the membrane surface of both model membranes and SPM (Hitzeman et al., 1986; Kreishman et al., 1985).

Third, another measure that DPH monitors some polar (dielectric) properties in the SPM interior core is the second, longer lifetime component of DPH. The fractional contribution of the two diphenylhexatriene lifetime components in SPM appears to be informative in regard to the presence of highly reactive phospholipid species in the bilayer (Wratton et al., 1989). The fraction of the short lifetime component of DPH in SPM from control, but not chronic ethanol-treated, mice was increased in the presence of ethanol *in vitro* and with γ irradiation pretreatment. It has been shown by others that alcohol *in vitro* potentiates free radical formation in a number of tissues (Knecht et al., 1990; Reinke et al., 1991). Therefore, it appears that multifrequency phase and modulation lifetime analysis of DPH in SPM is sensitive to the presence of reactive phospholipid species induced by prooxidants, ethanol, or irradiation. By increasing the relative dielectric constant of the SPM interior core monitored by DPH, ethanol also increased the rate of photoreaction of DPH therein.

As pointed out earlier, the interior core of the SPM sensed by DPH comprises not only the lipid bilayer interior core but also the protein−lipid interface. Water can penetrate into the interface between protein and lipid in membranes, possibly more so than into the bulk lipid interior core (Ho & Stubbs, 1992). With few exceptions, most integral membrane proteins are located in cholesterol-poor, relatively fluid domains [reviewed in Houslay and Stanley (1982) and Schroeder and Wood (1993)]. The immediate layer of lipids surrounding these proteins is called the annulus or boundary lipid or interfacial lipid. Although the lipids in the protein−lipid interface are free to exchange with bulk lipid, this exchange of interfacial lipid with bulk lipid is 1−3 orders of magnitude slower than bulk lipid exchanges with bulk lipid. Consequently, the equilibrium composition of the lipids in the protein−lipid interface may differ significantly from that of the bulk lipid. There are some reports with erythrocyte membranes indicating that the anion transporter, band 3, may play a role in the formation of phosphatidylcholine domains (Rogers & Glaser, 1993). Interestingly, the band 3-induced formation of phosphatidylcholine domains was dependent on ionic strength, not temperature. However, in most cases the domains surrounding proteins have been reported to be enriched with anionic phospholipids (phosphatidylserine, phosphatidylinositol) and relatively devoid of cholesterol [reviewed in Houslay and Stanley (1982) and Schroeder and Wood (1993)]. In fact, studies of fluorescent sterol lifetime versus dielectric constant indicate an insensitivity of sterols to the relative dielectric constant of the microenvironment (Nemecz & Schroeder, 1988). Most importantly, the acidic phospholipids are often enriched with

specific fatty acyl groups that differ from those of nonannular phospholipids (Nichols & Roufogalis, 1991). For example, polyunsaturated fatty acids such as 22:6n3 are enriched in SPM. The 22:6n3 fatty acids are enriched in phospholipids such as phosphatidylserine that are more associated with the proteins in the membrane bilayer (Salem & Karanian, 1988). The 22:6n3 fatty acids have a helical structure that occupies considerably more space than other fatty acids (Salem & Karanian, 1988; Stubbs, 1992). Therefore, 22:6n3 fatty acids may accommodate higher levels of interstitial water than other fatty acids (Salem & Karanian, 1988).

The effects of ethanol on the interior core relative dielectric constant or the range of dielectric constants and the protein–lipid interface dielectric constant in SPM from control mice can be explained as follows. Recent evidence indicates that ethanol may disrupt hydrogen bonding at the membrane surface, thereby weakening such hydrogen bonds (Klemm, 1990; Ueda, 1991; Curatola et al., 1991; Urry & Sandorfy, 1991). Ethanol weakening of the hydrogen bonding between water and lipid at the membrane surface would increase the penetration of water into the interior core bulk lipid. Likewise, weakening of hydrogen bonding in the protein–lipid interface could result in increased penetration of water into the interface. Both effects would be consistent with the increased relative dielectric constant and photoreaction products detected by DPH in SPM of control mice. That the effect of ethanol *in vitro* on increasing relative dielectric constant may be more pronounced in the bulk lipid interior core is based on the distribution of 22:6n3 fatty acids. Since these water-rich fatty acids are enriched in anionic phospholipids, they are expected to accumulate in the protein–lipid interface. Consequently, the bulk lipid interior core of synaptosomal plasma membranes from control animals would be relatively deficient in 22:6n3 and thus have a low relative dielectric constant range. Indeed, the narrow lifetime distributional width and the spectral peak ratios of DPH therein are consistent with this observation. This distribution of 22:6n3 in the interior core protein–lipid interface versus the bulk lipid interior core of SPM of control mice may have made the interior core water more susceptible to the action of ethanol *in vitro*. The enrichment with readily oxidizable polyunsaturated fatty acids such as 22:6n3 might also account for the greater photoreactivity of this microenvironment than the bulk lipid.

The relative dielectric constants monitored by DPH in SPM from chronic ethanol-treated animals were unresponsive to perturbation by ethanol *in vitro*. SPM from chronic ethanol-treated animals had markedly lower levels of 22:6n3 fatty acids than those of control animals (Sun & Sun, 1985). If this resulted only in a lowering of the water in the protein-interface in SPM of chronic ethanol-treated animals, then a decrease in the interior core range of dielectric constants (decreased DPH lifetime distributional width) should have been sensed by DPH in SPM from the chronic alcohol-treated animals. Instead, the finding of no significantly increased range of dielectric constants (increased distributional width) by DPH in SPM from chronic alcohol-treated (lower 22:6n3) animals indicates that a major reorganization of 22:6n3-containing phospholipids and/or other lipids between the bilayer interior core and the protein–lipid interface may have occurred. Consistent with such a reorganization of lipids in SPM from chronic ethanol-treated animals was a report of altered transbilayer distribution of cholesterol (Wood et al., 1987). The net effect of this lipid reorganization in the synaptosomal plasma membranes from chronic ethanol-treated mice was an interior core lipid environment with a range of dielectric constants that apparently could not be increased significantly with the addition of ethanol *in vitro*.

In summary, ethanol can alter either membrane fluidity or dielectric properties or both in mouse brain SPM. These alterations occurred in both the interior core and the surface of the membranes from control mice. Hydration (increased relative dielectric constant) was observed by ethanol perturbation *in vitro* in SPM from control but not chronic ethanol-treated mice. In all cases, ethanol *in vitro* increased the fraction of DPH associated with photoreaction products with lipids from SPM of ethanol-sensitive mice.

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