Biochimica et Biophysica Acta 859 (1986) 189–197
Elsevier

BBA 73147

Ethanol-induced changes in neuronal membrane order. An NMR study

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(Received December 17th, 1985)

Key words: Ethanol; Membrane order; NMR; (Neuronal membrane)

The effects of ethanol-d_6 on the lipid matrix of rat brain neuronal membranes were investigated by delayed Fourier transform ^{1}H-NMR techniques. At 24°C, neither 0.1 nor 0.2% (v/v) ethanol-d_6 measurably affected the methylene resonance intensity. However, 0.4 and 1.0% ethanol-d_6 increased resonance intensity, 35 and 51%, respectively. With increasing temperature, a decrease in resonance intensity for 0.1% ethanol-d_6 was observed reaching a maximum of 20% at 42°C. Furthermore, increasing temperature attenuated the increases in resonance intensity seen with 0.4 and 1.0% ethanol-d_6. At 24°C, no concentration of ethanol-d_6 had a significant effect on the choline methyl resonance. However, with increasing temperature both 0.1 and 0.2% ethanol-d_6 decreased this resonance's intensity. The intensity of the terminal methyl resonance was increased in a dose related fashion by ethanol-d_6, reaching a maximum of +41% at 1.0% (24°C). Increasing temperature attenuated this effect, but no concentration of ethanol-d_6 significantly decreased resonance intensity. The increases and decreases in resonance intensity induced by ethanol-d_6 are interpreted in terms of a decrease and an increase in membrane order **, respectively. It is proposed that ethanol-d_6 exerts two effects on neuronal membranes, an ordering effect on the membrane surface and a disordering effect in the membrane interior. A higher enthalpy of ethanol binding to the surface as compared to the interior of the membrane leads to an attenuation of the ethanol disordering effect with increasing temperature.

Introduction

Ethanol-induced changes in membrane function are thought to result from the perturbation of either membrane lipid and/or membrane protein structure; changes in protein structure could result from a direct drug effect [1] or secondarily from changes on the lipid matrix [2,3]. The lipid perturbation hypothesis receives its strongest support at the molecular level from studies which show: (a) that pharmacologically relevant concentrations

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** Order (and disorder) are used here in the general sense and refer to the mobility of the membrane lipids. Since mobility in natural membranes is primarily a reflection of hindered or restricted movement and not viscosity, the term 'order' is the more appropriate designation. Increases in the spectral intensity of the membrane lipid NMR resonances are associated with a decrease in order. This is, however, not the only interpretation of such data (see James [30]).

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; ^{2}H-NMR, deuterium nuclear magnetic resonance; ethanol-d_6, deuterated ethanol; ESR, electron spin resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, tetramethylammonium-DPH.

of ethanol (< 100 mM) disorder nerve membranes as detected by changes in both electron spin resonance (ESR) and fluorescence polarization spectra [2,4,5], (b) that this ethanol effect is somewhat more marked in neuronal as compared to non-neuronal and artificial membranes [2,5,6], (c) that membranes in which the ethanol effect on membrane order should be attenuated (or augmented) on the basis of genetic selection [7] or chronic ethanol administration show the predicted response [8–13], and (d) that it is possible to form artificial membranes from certain neuronal membrane lipids which are exquisitely sensitive to the effects of ethanol [14]. Of the numerous arguments against the lipid perturbation hypothesis (see e.g. Ref. 1), perhaps the most persistent and cogent is that the effects of pharmacological concentrations (< 100 mM) of ethanol on membrane order are small and roughly equivalent to a 1 Cdeg or less increase in temperature.

In the present study we have re-examined the effects of ethanol on the neuronal membrane lipid matrix using ^{1}H-NMR techniques. The theoretical advantages of NMR over the two other biophysical techniques most widely used in ethanol research (ESR and fluorescence polarization) have been recently reviewed [15]. ^{1}H-NMR has been recently used to study the thermotropic behavior of rat brain synaptic plasma membranes [16]. With the use of delayed Fourier transform, it was possible to specifically monitor the effects of temperature on three aspects of the synaptic plasma membrane lipid matrix: the terminal methyl groups ($-CH_3$), the methylene moieties ($-CH_2-$), and the choline ($-N(CH_3)_3^+$) groups. The terminal methyl and methylene resonances are composite signals from all phospholipids present in the membrane and can be used to monitor order in interior membrane domains. Differently, the choline resonance arises primarily from only one phospholipid, phosphatidylcholine (PC). The amount of sphingomyelin present in our synaptic plasma membrane preparations is less than 2% of the total phospholipid present [20]. The choline resonance reflects order in a more superficial membrane domain than that reported by the methyl and methylene resonances. The ability of ^{1}H-NMR to simultaneously assess order in both the membrane surface and interior is of particular importance to the present study. Recently it was found by ^{2}H-NMR techniques that ethanol (at pharmacological concentrations) partitions in significant amounts to both the membrane surface and interior [17]. Rowe [6] has shown that at high concentrations (< 1 M), the surface effects of ethanol increase the T_m of DPPC liposomes by stabilizing the interdigetated gel phase. Evidence that ethanol effects on the membrane surface and interior differ at pharmacologically relevant concentrations comes from FPZ studies. 1,6-Diphenyl-1,3,5-hexatriene (DPH), a lipophilic fluorescent probe [17], reports that ethanol decreases fluorescence anisotropy [2,5], an effect now widely interpreted as a decrease in membrane order [18,19]. In contrast, tetramethylammonium-DPH (TMA-DPH), a DPH cogener which partitions to the membrane surface, is a poor reporter of the ethanol-induced membrane perturbations [5]. The present study demonstrates, by delayed Fourier transform techniques ,that the differential effects of ethanol on the membrane surface and interior are both concentration and temperature dependent.

Experimental Procedures

Membrane preparation. Synaptic membranes were prepared from the rat forebrain as described elsewhere [20]. The purity of this preparation from both a morphological and chemical perspective has been described [20]. In as much as not all of the membranes contain a clearly evident functional apparatus (pre- and post-synaptic thickenings), these membranes are probably best received as a neuronal membrane enriched fraction. The membranes reform as vesicles approx. 5000 Å in diameter.

The lipid composition of these membranes is routinely monitored (see Ref. 20 for technical details). For membranes used in this study, the sphingomyelin content was low (approx. 1% of total lipid-P) and no cerebrosides were detected. Ganglioside content was high and accounted for at least 10% of the total lipid present. The range of cholesterol/phospholipid ratios for all preparations used was 0.48 to 0.60. The average phospholipid composition was phosphatidylcholine (44%), phosphatidylethanolamine (22%), phosphatidylserine (14%), ethanolamine plasmalogen

(9%), phosphatidylinositol (4%), sphingomyelin (1%), and others (6%). The levels of long chain (> 22 carbons) saturated and mono-unsaturated fatty acids were below detection.

Membranes were washed three times in ^2H_2O phosphate buffered saline $p^2H = 8.2$ and then resuspended in this buffer at a concentration of approx. 10 mg protein/ml. A small aliquot of this suspension was used for protein determination [21]. On the basis of the protein determination, the sample was adjusted to 2 or 4 mg protein/ml, and 0.7 ml was transferred to a 5 mm NMR tube. The sample was purged with dry N_2 and frozen at −70°C until analysis.

Delayed Fourier transform-^{1}H-NMR measurements. The ^{1}H-NMR spectra were obtained using a Nicolet NMC 300 MHz FT-NMR spectrometer [16]. The delay between the excitation pulse and the start of acquisition was set at 240 μs. Under these conditions, less than 5% of the total spectral intensity is observed when the receiver is turned on. Thus, a small increase in the relaxation rates of the protons will lead to a marked increase in the observed spectral intensity. For example, with a 240 μs delay, an increase in the T_2 from 50 to 60 μs will result in a 122% increase in observed spectral intensity. Spectra were obtained over a temperature range of 24–42°C utilizing a NTC temperature control unit. The samples were allowed to equilibrate at each new temperature for 20 min prior to spectral acquisition. Chemical shifts are reported relative to an external capillary of 1% $CHCl_3$ in C^2HCl_3 at 7.25 ppm. This was confirmed by the fact that the resonance from the external capillary remained a constant. The spectra were processed with the same scaling factor so that direct comparisons of the intensities could be made. Due to the broad non-Lorenzian line shape of the resonances, base lines were drawn between minima around a resonance and spectral intensity was taken as the integrated area obtained by 'cutting and weighing'. From several trials, reproducibility in the area was ±5%. After each temperature run, a spectrum at 24°C was retaken. No changes were observed so factors like denaturation and vesicle size change can be ruled out as causes for the observed spectral behavior.

The residual proton resonance from the $-CH^2H_2$ group of the ethanol-d_6 was used to determine the membrane ethanol partitioning. As shown previously [17], the exchange rate of ethanol between the free and bound states is slow on the NMR time scale and thus only the free ethanol is observed in the spectra. The integrated intensity of the $-CH^2H_2$ resonance is thus proportional to the free ethanol concentration. It should be noted that in the NMR experiment, the [membrane] is not less than the [EtOH] as is the case in other techniques e.g., the fluorescence polarization experiment. Thus, increasing or decreasing the [membrane] will affect the apparent ethanol partitioning.

^{2}H-NMR measurements. In some experiments ethanol-d_6 partitioning was measured by ^{2}H-NMR techniques. The lock coil of the 5 mm ^{1}H proton probe was used as the observe coil using a frequency of 46.066 MHz for ^{2}H. The spectrometer was run in the unlocked mode. Peak intensities were obtained using minimum chi square residual fit of each resonance to a Lorentzian line, and peak area was taken as height times linewidth. The partition coefficients were calculated as described elsewhere [15].

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) techniques with resonance intensity serving as the dependent variable. Resonance intensity was determined either graphically (^{1}H-data) or by computer integration (^{2}H-data). All experiments were repeated at least three times.

Results

Effects of temperature on neuronal membrane NMR spectra

Under the spectral conditions employed, the resonances from the protein protons in the neuronal plasma membranes are not observed; relaxation for these protons is essentially complete at the time of acquisition (240 μs after the r.f. pulse). The residual spectral intensity observed is relatively simple and is primarily composed of lipid resonances. The terminal methyl resonance is seen at 0.6 to 0.8 ppm, the broad methylene resonance extends from 0.6 to 2.4 ppm and the choline resonance is seen at 3.2 ppm. The sharp resonance seen at 1.6 ppm is an impurity from the external standard. The two sharp resonances seen

at 3.6 and 3.8 ppm are probably derived from terminal carbohydrate, perhaps sialic acid resonances; model membranes formed from dipalmitoylphosphatidylcholine (DPPC) and cholesterol do not show these resonances, but they appear after the addition of ganglioside-G_{M1}.

Temperature was increased in 2°C increments over the range of 24 to 42°C (Fig. 1A). To analyze the effects of temperature the 24°C spectra were subtracted from the spectra at higher temperatures (Fig. 1B). As previously reported [16], the temperature effects are marked. From 24 to 42°C, spectral intensity increased 56% for the $-CH_2-$ resonance, 316% for the $-N(CH_3)_3^+$ resonance and 142% for the $-CH_3$ resonance. In some experiments, after acquiring the data at 42°C, samples were returned to 24°C and spectra re-acquired to check for potential sample deterioration. No deterioration was detected.

Effect of ethanol-d_6 on neuronal membrane NMR spectra

Ethanol-d_6 was added in concentrations of 0.1, 0.2, 0.4 and 1.0% (v/v) and delayed Fourier transform spectra were obtained at each concentration. Typical results are shown in Fig. 2 for the addition of 0.4% ethanol-d_6. The effects of all ethanol-d_6 concentrations are summarized in Figs. 3–5. Data for the $-CH_2-$ resonance are shown in Fig. 3. Collapsing data across temperatures reveals a significant difference among groups ($F_{4,45} = 26.9$, $p < 0.001$). 0.1% ethanol-d_6 significantly de-

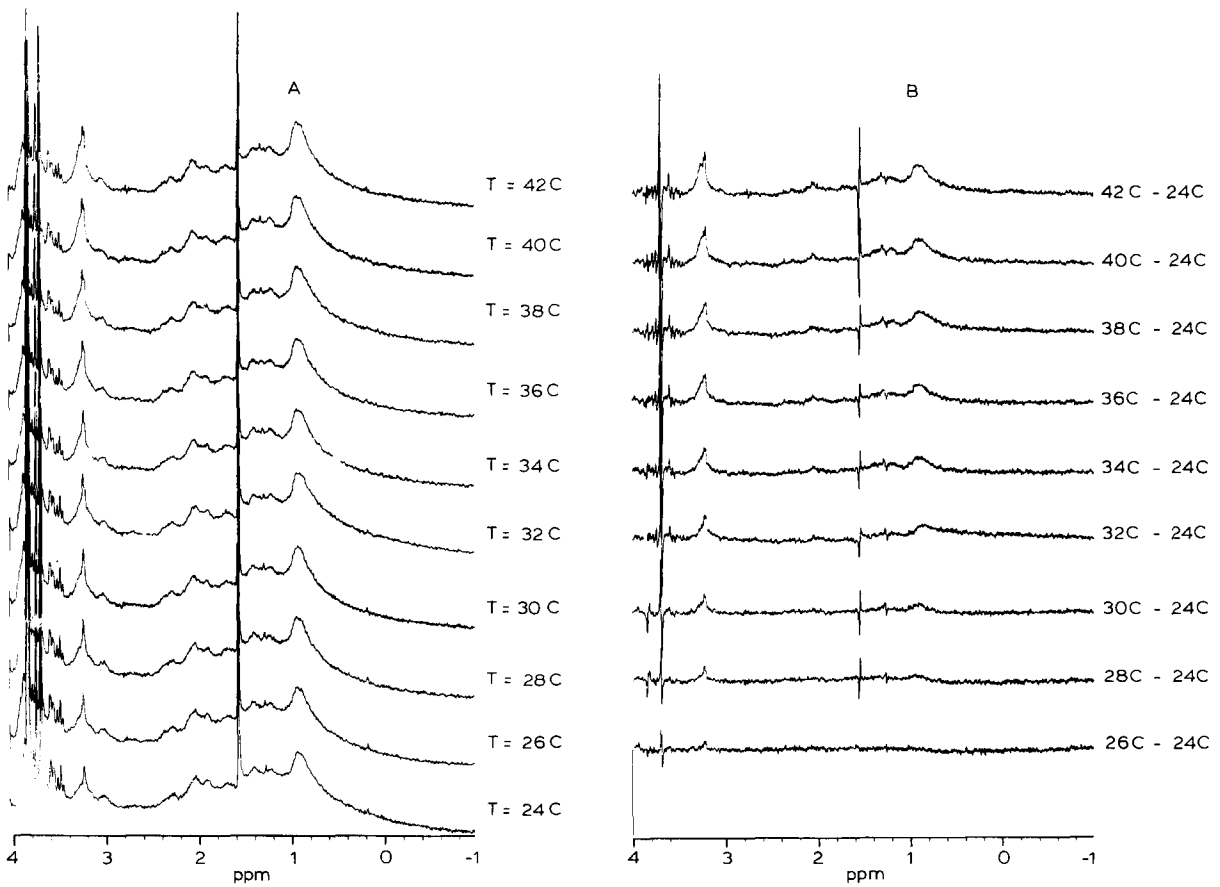

Fig. 1. Temperature dependence of the delayed Fourier transform spectra of rat synaptic plasma membranes (A). Difference spectra where the spectrum at 24°C is subtracted from the other temperatures (B).

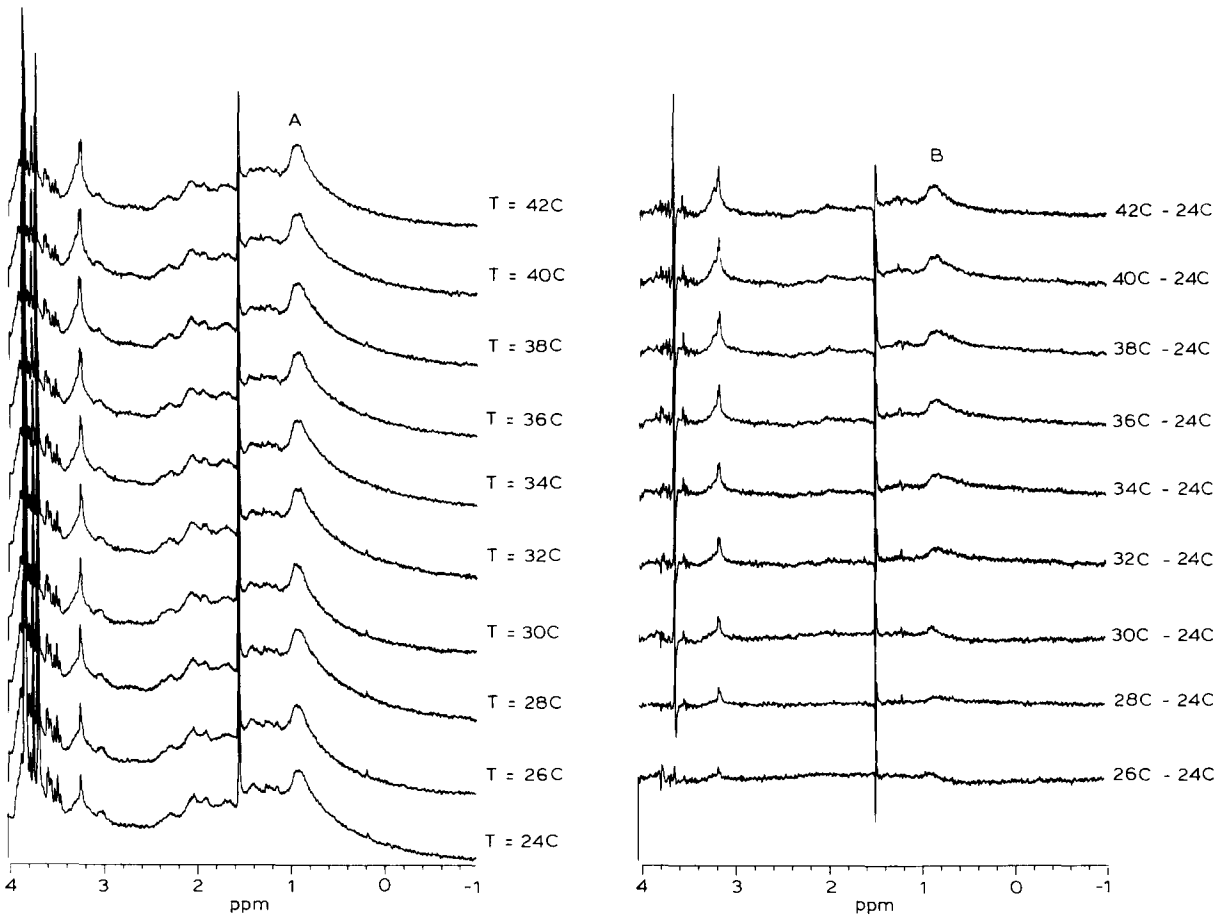

Fig. 2. Temperature dependence of the delayed Fourier transform spectra of rat synaptic plasma membranes with 0.4% ethanol-d_6 (A). Difference spectra where the spectrum at 24°C is subtracted from the other temperatures (B).

creased ($\Delta = -8.7\%$, $p < 0.05$) while 0.4% ($\Delta = +17.5\%$, $p < 0.01$) and 1.0% ($\Delta = +43.5\%$, $p < 0.001$) significantly increased average resonance intensity. These ethanol-d_6 effects were temperature dependent. Over the range of 24 to 42°C, 0.4 and 1.0% ethanol-d_6 increased the average resonance intensity 31.8% ($p < 0.001$) and 52.4% ($p < 0.001$), respectively. Differently, over the range of 38 to 42°C, 0.4% ethanol-d_6 had no significant effect on average resonance intensity ($\Delta = +5.6\%$, $p > 0.05$). 0.1% ethanol-d_6 significantly decreased resonance intensity ($\Delta = -18.6\%$, $p < 0.02$) while 1.0% increased intensity ($\Delta = +36.2\%$, $p < 0.01$) over this range, although the effect of 1.0% ethanol-d_6 was somewhat attenuated from that seen at lower temperatures (52.4 vs. 36.2%).

While the -CH_2- resonance data report from the membrane interior, the -$N(CH_3)_3^+$ resonance data (summarized in Fig. 4) report from the membrane surface, primarily the external membrane surface [22]. Collapsing data across all temperatures reveals a significant difference among groups ($F_{4,45} = 130$, $p < 0.001$). Both 0.1% and 0.2% ethanol-d_6 significantly decreased average resonance intensity (0.1%, $\Delta = -21.2\%$, $p < 0.01$) (0.2%, $\Delta = -20.0\%$, $p < 0.01$). The effects of both of these concentrations of ethanol-d_6 increased with increasing temperature. At 24°C neither concentration had a significant effect on resonance intensity, while at 42°C, 0.1% and 0.2% decreased resonance intensity 26.0% ($p < 0.01$) and 23.6% ($p < 0.01$), respectively. The pattern of effects for

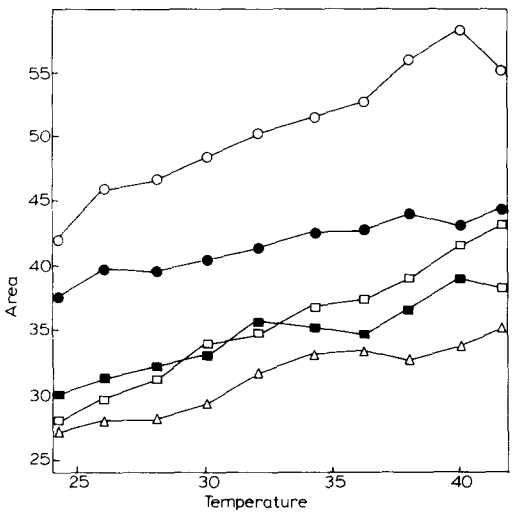

Fig. 3. Area, in arbitrary units, of the methylene resonance vs. temperature with 0.0% (□), 0.1% (△), 0.2% (■), 0.4% (●) and 1.0% (○) added ethanol-d_6.

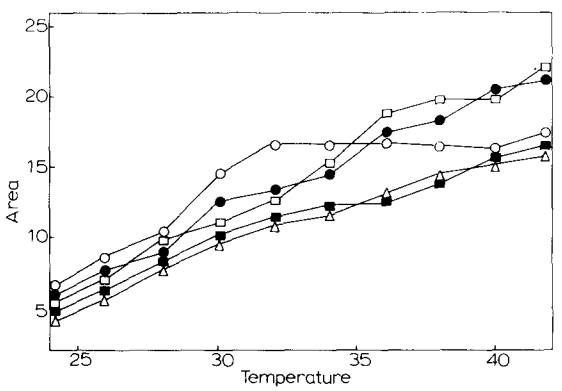

Fig. 4. Area, in arbitrary units, of the choline methyl resonances vs. temperature with 0.0% (□), 0.1% (△), 0.2% (■), 0.4% (●), and 1.0% (○) added ethanol-d_6.

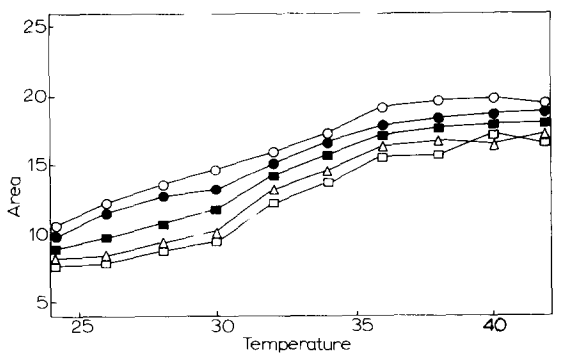

Fig. 5. Area, in arbitrary units, of the terminal methyl resonance vs. temperature with 0.0% (□), 0.1% (△), 0.2% (■), 0.4% (●) and 1.0% (○) added ethanol-d_6.

1.0% was somewhat complex, increasing resonance intensity at 30°C ($\Delta = +37\%$) and 32°C ($\Delta = +31\%$) but decreasing resonance intensity at higher temperatures e.g. $\Delta = -22.2\%$ at 42°C.

Data for the terminal $-CH_3$ resonances are shown in Fig. 5. 0.1% ethanol-d_6 had no significant effect on resonance intensity over the entire temperature range examined. Differently than the data for the $-CH_2-$ and $-N(CH_3)_3^+$ resonance, 0.2% ethanol-d_6 increased resonance intensity at 24°C ($\Delta = +19\%$, $p < 0.05$). Increasing the ethanol-d_6 concentration to 0.4% and 1.0% ethanol-d_6 further increased resonance intensity 30% ($p < 0.01$) and 41% ($p < 0.01$), respectively, at this temperature. Increasing temperature attenuated the increase in resonance intensity caused by 0.2, 0.4 and 1.0% ethanol-d_6.

Ethanol-d_6 partitioning to neuronal membranes

The residual $-CH^2H_2$ resonance from the ethanol-d_6 is present in the ^{1}H-spectra as a small peak approx. 0.2 ppm downfield from the fatty acid $-CH_3$ resonance. By decreasing the membrane concentration to 2 mg/ml, this peak is of sufficient spectral intensity for analysis. It should be noted that since the exchange rate of the ethanol between the free and bound states is slow on the

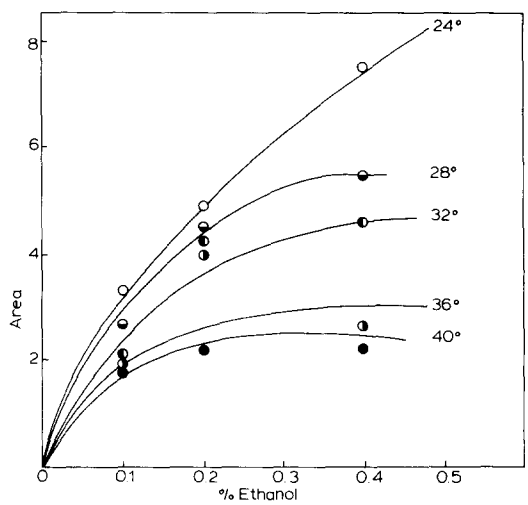

Fig. 6. Area, in arbitrary units, of the residual proton resonance of ethanol-d_6 vs. ethanol concentration at several temperatures.

NMR time scale, only the free state is observed in the ^1H spectra. The spectral intensity of the $-CH^2H_2$ group as a function of ethanol concentration at several temperatures is shown in Fig. 6. A nonlinear dependence is observed indicative of cooperative binding. With increasing temperature, the amount of free ethanol-d_6 decreases.

Previously it was demonstrated that ^2H-NMR spectra of ethanol-d_6 partitioning to liquid-crystalline DPPC multilamellar liposomes could be used to demonstrate two unique partitioning domains [15]. One domain is most certainly the hydrophobic membrane interior and partitioning to this domain is not cooperative. The other domain is characterized both by less mobile ethanol and a high degree of co-operativity and probably represents partitioning to the membrane surface. The ^2H-NMR technique was used to characterize the binding of ethanol-d_6 at 1.0% (v/v) to the neuronal membranes. Partitioning coefficients for K_1 (interior) and K_2 (surface) were determined over the range of 24 to 42°C. Data have been summarized in the form of van't Hoff plots. As shown in Fig. 7, ΔH for K_2 is 41.4 kJ/mol but only 15.5 kJ/mol for K_1.

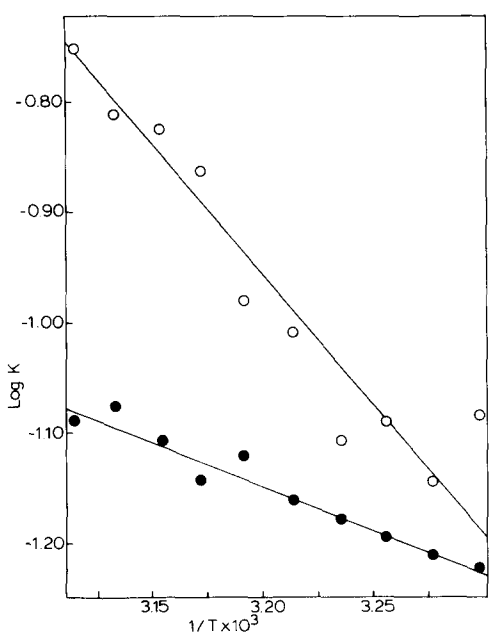

Fig. 7. Van't Hoff plot of K_1 (●) and K_2 (○) for the binding of ethanol-d_6 to the interior and surface, respectively, of rat synaptic plasma membranes.

Discussion

Current hypotheses regarding the mechanism(s) of action of alcohol and the general anesthetics are derived from the concept that these agents perturb the lipid matrix (see Hitzemann et al., 1984 [3]). After partitioning into the membrane, ethanol is thought to act (a) by decreasing membrane order [4,5,23] either throughout the membrane or in selective membrane domains [13,24], (b) by inducing changes in lipid phase behavior [6,25], or (c) by decreasing cooperative fluctuation between phases of initial clusters of lipid molecules [26]. The 'disordering' hypothesis has received considerable experimental support from both fluorescence polarization and ESR studies, despite the differences in molecular motions measured by these techniques. By using selective probes (e.g. TMA-DPH vs. DPH), it has been demonstrated that the disordering site(s) of ethanol action are within the hydrophobic membrane core [5].

Interestingly, ethanol has also been reported to order natural and artificial membranes under some conditions. For example, Rowe [6], using an absorbance technique, found that high (greater than 3% v/v) concentrations of ethanol increase the T_m of PC multilamellar liposomes by apparently stabilizing the interdigetated gel phase by surface bound ethanol. Michaelis et al. [27], detected an ordering effect of ethanol in neuronal membranes by ESR at low ethanol concentrations. The physiological significance of this ethanol ordering effect is unclear. Furthermore, the question of whether or not this ordering effect is shared by longer chain alcohols and/or anesthetics is not known.

Through the use of delayed Fourier transform NMR, it was possible to simultaneously assess ethanol's effects on the membrane surface as indicated by the choline resonance and the interior as indicated by the methylene and methyl resonances of the membrane lipids. As has been shown by Chan and co-workers [28] who applied delayed Fourier transform NMR to model membrane systems, the observed spectral intensities are governed by the relationship of T_2 to the preacquisition time. For lipids in a bilayer, the major contribution to the relaxation process is the dipolar coupling between neighboring nuclei and the ex-

tent of the relaxation process is determined by the degree of motional restriction and the time scale of the motion [29]. An increase or decrease in spectral intensity can therefore be regarded as a decrease or increase. respectively, in membrane order. Both the ethanol and temperature membrane effects were dependent on the membrane domain sampled. For example, under the spectral conditions employed, the $-N(CH_3)_3^+$ resonances showed the most marked temperature effect ($\Delta = +316\%$) while the $-CH_2-$ resonances showed the least temperature dependence ($\Delta = +56\%$). In lieu of knowing the actual T_2 relaxation times, our interpretation of this phenomenon (as well as the effects of ethanol) can only be qualitative. One interpretation of such data is that over the temperature range examined (24 to 42°C), the potential for increased mobility is greater for the choline as compared to the methylene resonances. Alternatively, one may simply view these data as a reflection of significantly more efficient relaxation mechanisms for the methylene as compared to choline moieties. The potential of technical artifacts in such ^{1}H-NMR spectra has been discussed elsewhere [16].

The effects of ethanol on the NMR spectra, like those of temperature, are complex and domain dependent. For example, ethanol-d_6 in a dose-dependent fashion increased the resonance intensity of the $-CH_3$ resonances. This apparent decrease in order was greatest at the lowest temperature examined (24°C) and diminished with increasing temperature. While 1% ethanol-d_6 had the equivalent effect of increasing temperature by 6°C at 24°C, at 42°C, the equivalent effect was only similar to a 2 Cdeg rise in temperature. Such data may suggest that ethanol and temperature both affect similar finite relaxation mechanisms; previously it was shown that the temperature induced increase in neuronal membrane methyl-methylene resonance intensity reaches a maximum at 44 to 45°C [16]. An additional explanation of these data is that ethanol also exerts an ordering effect in some membrane domain, an effect which increases with increasing temperature and which is transmitted throughout the membrane matrix. Non-NMR evidence supporting an ordering effect has been presented. The data in Fig. 4 show that concentrations of ethanol-d_6 (0.1 and 0.2%) which have little or no disordering effect on the membrane interior (Figs. 3 and 5), actually attenuate the temperature-induced increase in $-N(CH_3)_3^+$ resonance intensity. These data may reflect an ordering effect of ethanol-d_6 on the membrane surface, which enhances relaxation mechanisms. If this ordering effect is transmitted vectorally to the membrane interior it could compete with an outwardly transmitted ethanol-induced disordering vector originating from the membrane interior (Fig. 8). The $-CH_2-$ resonances appear to reflect this competing situation. Since the methylene resonance is a composite of all methylene groups of the fatty acid chain, some are near the surface of the membrane and their intensity upon ordering would decrease, while others are near the center of the membrane and their intensity would increase upon disordering. Hence, depending on the depth of the ordering vector, the methylene resonance can either decrease or increase in total intensity. A small but significant ordering effect is seen at 0.1% ethanol-d_6, and the disordering effects of the higher ethanol-d_6 concentrations are attenuated at higher temperatures for which the surface effects of ethanol-d_6 appear greatest.

The question arises as to why the ethanol-d_6 ordering effect is greatest at higher temperatures. The data in Fig. 7, clearly demonstrate that with increasing temperature ethanol partitioning to the membrane surface increases disproportionately in comparison to interior partitioning. While other factors may be involved such as temperature-in-

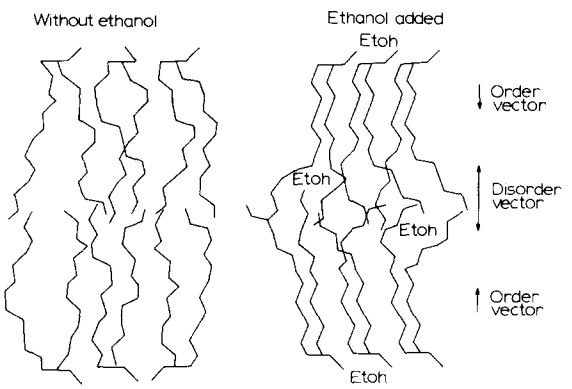

Fig. 8. Schematic representation of the diametrically opposing order and disorder vectors resulting from the binding of ethanol to the surface and interior of the bilayer.

duced changes in membrane structure, the differential partitioning most certainly contributes to the enhanced surface effects seen at high temperatures.

The apparent measure of the ordering and disordering gradients across the membrane lipid matrix using NMR clearly demonstrates that ethanol perturbations in neuronal membranes is a complex process. Ordering-disordering effects within differing membrane domains could selectively enhance and/or attenuate various membrane functions, the net result being the behavioral sequalae associated with ethanol intoxication.

Acknowledgments

Partial funding for purchase of the NMR spectrometer was provided by a grant from the National Science Foundation (CHE-8102974).

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