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Chronic ethanol intoxication induces adaptive changes at the membrane protein/lipid interface

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Abstract

Modifications were found to occur at the membrane protein/lipid interface of liver microsomes in animals that had been subjected to chronic ethanol ingestion. The effects were revealed by probing this region with 1,6-diphenyl-1,3,5-hexatriene (DPH), trimethylammonium-DPH (TMA-DPH) and DPH attached to the *sn*-2 chain of phosphatidylcholine (1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine, DPH-PC). In intact membranes, it was found that the decay of the excited state was heterogeneous, this being modeled by fitting the data to a fluorescence lifetime distribution. The full-width of the distribution at half-maximum, which relates to the degree of excited state environmental heterogeneity, increased for each fluorophore, as a result of chronic ethanol treatment. For TMA-DPH and DPH the excited state heterogeneity could have arisen from, (i) the protein/lipid interface and (ii) varied degrees of water penetration into the lipid, due to the ability of these fluorophores to sample along the bilayer normal. By contrast, the DPH in DPH-PC, due to its tethering, was only able to sample the heterogeneity at the protein/lipid interface, as confirmed by a homogeneous decay in vesicles of microsomal lipid extracts. The increased degree of DPH-PC fluorescence decay heterogeneity in microsomes from chronic ethanol-treated animals as compared to controls, was found to persist in vesicles of extracted lipids, when apocytochrome C was included in the vesicle preparations as a model protein. This effectively eliminated a protein modification from being responsible and indicated that a chronic-ethanol induced alteration in the lipids was being expressed in the form of a physico-chemical modification at the protein/lipid interface. The degree of DPH-PC environmental heterogeneity was also directly increased by ethanol, however, membranes from chronic ethanol-treated animals were resistant to this effect, showing that the phenomenon of 'membrane tolerance' extends to the membrane protein/lipid interface.

Key words: Ethanol; Protein–lipid interaction; Fluorescence lifetime; DPH

1. Introduction

Chronic ethanol ingestion leads to the development of a number of adaptive modifications to cell membranes. These include a resistance to lipid disordering by ethanol [1] (for reviews, see Refs. 2–6), a decreased membrane/buffer partition coefficient of hydrophobic compounds, including ethanol [7–9] and a decreased susceptibility to phospholipid hydrolysis by exogenous phospholipase A_2 [10,11], effects that are collectively termed 'membrane tolerance'. In liver cell membranes, membrane tolerance has been attributed to modifications in anionic phospholipids [11–14]. In the liver microsome, the major effect is to modify the molecular species profile of phosphatidylserine (PS) [11,14].

A key question, as yet unresolved, concerning the long term adaptive effects of chronic ethanol treatment to membrane lipids, is the mechanism by which such modifications might influence the functioning of membrane proteins. While there are many adaptive changes in membrane protein functions resulting from chronic ethanol treatment that have been described (reviewed

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Abbreviations: χ^2_R, reduced chi-squared; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethyl-ammonium)-6-phenyl-1,3,5-hexatriene; DPH-PC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-PC; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-PC; PC, phosphatidylcholine; D_w, full-width at half-maximum peak height of the major lifetime center of a bimodal Lorentzian lifetime distribution; ns, nanoseconds; PS, phosphatidylserine.

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in Refs. 2–6), the cause of these effects is obscure and the role of the lipid modifications unknown.

The mechanism(s) whereby altered membrane lipid composition and membrane physical properties in general may be expressed in terms of altered membrane protein function is still poorly understood. While alterations to *bulk* membrane fluidity properties may in certain circumstances affect membrane protein function, current consensus has moved away from this type of explanation and it appears more likely that effects on membranes are expressed in discrete regions of the membrane. Such regions include lipid asymmetry, for example, which has been shown to be subject to chronic ethanol-induced modifications [15–18]. Another is the protein/lipid interface, a potential site not only for the interaction of ethanol but of anesthetics in general [19,20]. Hydrophobic sites of interaction may potentially be independent of the lipid bilayer, as shown using the soluble enzyme fire fly luciferase as model [21,22]. Recently, evidence for an inhibitory site for alcohols and anesthetics on protein kinase C has been presented, here although the inhibition was not dependent on membrane association the potency of inhibition showed lipid dependency [23].

Recent studies from this laboratory have shown that apart from lipids, membrane proteins also contribute to fluorophore lifetime heterogeneity [24–26] and the potential of DPH fluorophores as probes of the protein/lipid interface has been described [27,28]. For a fluorophore in an environmentally homogeneous region the fluorescence decay will be a simple exponential decay, i.e., it has a single decay rate. Alternatively, if the fluorophore experiences heterogeneity in its excited state environment, then the decay is better described as a range of decay rates. The heterogeneity of decay rates can be modeled as a fluorescence lifetime continuous distribution, where the full-width of the distribution (at half-maximum) is proportional to the degree of fluorophore heterogeneity.

There are two major types of heterogeneity that can be sampled by an excited state fluorophore in a bilayer, 'vertically', along the bilayer normal, or in a lateral direction. TMA-DPH samples the bilayer normal, but due to surface tethering is restricted to that region, however, the degree of water penetration changes in the head group is large over a small vertical distance. Thus TMA-DPH is sensitive to small changes in water penetration, also its positive charge influences its ability to interact with other lipids and proteins. Free DPH also samples vertically, as for TMA-DPH except that being uncharged it is not restricted as is TMA-DPH and can sample right across the bilayer, including into the head group region. For DPH-PC, vertical sampling is severely restricted due to tethering of the DPH to the PC *sn*-2 position. Therefore its main sampling of heterogeneity is lateral. The region sampled is a band between the head group and bilayer central region, further restriction to regions where PC is located is also possible. Thus TMA-DPH and DPH can only be used to uniquely sample lateral heterogeneity in the *absence* of vertical heterogeneity, i.e., where there is little water penetration into the bilayer, and in a natural membrane the complex lipid mixture leads to considerable water penetration and vertical heterogeneity. DPH-PC being unable to sample vertically will only sense heterogeneity in a lateral direction and is therefore useful for that purpose.

Previous studies established that for the free probe DPH, environmental heterogeneity is detected in (protein-free) lipid bilayers for a mixture of molecular species (i.e., as obtained from a cell membrane lipid extract), or for gel-phase single species of phospholipids, but not for bilayers of (liquid crystalline phase) single species phospholipids, which were found to be environmentally homogeneous [24,29]. The environmental heterogeneity appears to be due to 'loose' lipid acyl chain packing allowing water to penetrate deep into the bilayer, inducing a 'dielectric constant gradient' across the membrane [24,29,30].

For *single* phospholipid species lipid vesicles, the DPH fluorescence decay is homogeneous, but inclusion of protein induces the appearance of environmental heterogeneity [24–28]. This effect may also be partly due to water at the protein/lipid interface [28] and other factors, such as amino-acid side chains, clefts in the protein hydrophobic surface etc. By contrast, for DPH-PC the fluorescence decay, while still heterogeneous in cell membranes, as for DPH, in protein-free lipid vesicles (e.g., made from a membrane lipid extract) the decay is homogeneous, opposite to DPH [28] (due to inability to vertically sample-see above). Thus analysis of the fluorescence decay of DPH-PC as a lifetime distribution, leads to a broad distributional width attributable to the protein/lipid interface alone and may be used to probe this region. Fluorophores in the excited state at the protein/lipid interface will have mostly decayed to the ground state by the time they laterally diffuse to the second lipid shell since the excited state decay rate ($\sim 10^9$ s^{-1}) is two orders of magnitude faster than the rate of exchange of phospholipids at the protein surface with the bulk lipids ($\sim 10^7$ s^{-1} [31–33]. Thus the fluorophores experiencing heterogeneity arise from the region that is identical to the so-called 'boundary (or annular) lipid' region. Thus our model for natural membranes, has two lipid regions, one being the bulk lipid region and the other the protein/lipid interface. In general the main factors that would contribute to the value of the distributional width, pertaining to the environmental heterogeneity at the protein/lipid interface, are the rate of exchange of bulk and boundary lipids, the fluorophore location and size etc., as previously discussed [24]. While this model

is still perhaps a simplification it provides a relatively simple fluorescence spectroscopic method for probing the protein/lipid interface in intact cell membranes.

In this work this approach was exploited to investigate the effects of chronic ethanol treatment on the membrane protein/lipid interface, using as a model, liver microsomal membranes prepared from ethanol-treated rats. When microsomes from chronic ethanol-treated animals were probed using DPH-PC and the fluorescence decay analyzed as a lifetime distribution, the width of the distribution (D_w), attributable to heterogeneity at the protein/lipid interface, was significantly increased over the value for control membranes. Upon addition of ethanol to microsomes from untreated animals, D_w for DPH-PC again increased, however, this did not occur in microsomes from chronic ethanol-treated animals. Thus this result is the first evidence for the expression of membrane tolerance at the level of the membrane protein/lipid interface.

2. Materials and methods

2.1. Materials

DPH-PC and DPH were obtained from Molecular Probes (Eugene, OR) and lipids were from Avanti Polar Lipids (Pelham, AL). Other chemicals were from Fisher Scientific (Pittsburgh, PA). Male Sprague-Dawley rats (Charles River Breeding Laboratories) were fed a liquid diet (Bio-serve, Frenchtown, NJ) for 35 days in which ethanol comprised 36% of total calories. Pair-fed littermate controls received the same diet, except that carbohydrates isocalorically replaced ethanol [34]. Ethanol consumption averaged 14–16 g/kg of body weight per day.

2.2. Preparation of microsomes and labelling with DPH-PC

Rat liver microsomes were prepared and characterized as previously described [35]. DPH and TMA-DPH were incorporated as previously detailed [27,36]. For DPH-PC, a sonicated vesicle preparation was incubated with microsomes for 2 h, then unincorporated probe was removed by a washing in buffer. Separate control experiments were performed to confirm that the DPH-PC had incorporated into the membranes and that non-incorporated probe had been removed [27].

2.3. Preparation of vesicles

Lipids were extracted from the isolated membranes [37] and the phospholipids were separated from the neutral lipids using silicic acid columns. The neutral lipids were first eluted by chloroform, followed by elution with methanol to obtain the phospholipids. The phospholipids were then quantified by the determination of lipid phosphorus [38].

Aliquots of lipids and DPH-PC were placed in a test tube and the solvent removed by a stream of nitrogen. The lipids were dispersed to form MLV by the addition of 10 mM Tris-HCl, 150 mM NaCl (pH 7.4), followed by vortexing. LUV (diameter ~ 100 nm) were then prepared by extrusion technique, using either a Lipex extruder (Lipex Biomembranes, Vancouver, British Colombia) as previously described [39] or an Avestin Liposofast Extruder (MM Developments, Ottawa, Canada) also as previously described [40], both techniques giving similar results. Apocytochrome C was prepared as described [41,42] and added to preformed vesicles into which it spontaneously inserts with the provision of a negative charge supplied by inclusion of the negatively charged PS.

2.4. Fluorescence measurements

Fluorescence lifetime data was obtained using an SLM 48000 multifrequency phase-modulation fluorimeter [43,44]. The excitation source was a Liconix Model 4210NB helium-cadmium laser emitting at 325 nm. The light was modulated by a Pockels cell to obtain a range of frequencies from 5 to 150 MHz. Emission was observed through a 420 nm red-pass filter and a Glan-Thompson polarizer, set at the magic angle. For a reference an aqueous solution of rabbit liver glycogen was used. With respect to the fluorescence background, by use of dilute vesicle systems and single unilamellar vesicles, the background signal (i.e., scattering, inner filter effect etc.) was kept to a negligible level.

2.5. Data analysis

The phase and modulation data were analyzed by the GLOBALS UNLIMITED software (Laboratory of Fluorescence Dynamics, University of Illinois, Department of Physics, Urbana, IL) as previously described [45,46], the data being fitted to minimal values of the reduced χ^2_R parameter. The experimental error used in the analyses was the standard deviation of averaged values for phase and modulation at each frequency (~ 0.002 and 0.2° in the modulation and phase, respectively). From the analyses, in addition to the major lifetime center(s), a minor lifetime component (< 10%) of ~ $(1–3) \cdot 10^{-9}$ s, was found. This is typical of DPH fluorophores and the basis of this is considered to be a combination of the photochemical properties of DPH and/or a photodegrative product [47–49].

3. Results

In order to assess the effect of chronic ethanol treatment on the protein/lipid interface in membranes, the fluorescence lifetime heterogeneity of DPH fluorophores in liver microsomes was examined. Analysis of the decay of the excited state as a bimodal Lorentzian distribution was found to be the most appropriate solution based on χ^2_R and residuals (see example data analyses for DPH-PC in microsomes in Fig. 1). This assumes a single centered, heterogeneous fluorophore environment in the membrane, apart from the minor lifetime component (see Methods). A triple exponential decay also gave a reasonable χ^2_R, but the fluorescence lifetimes recovered were unrealistic, indicating that this was not a useful solution. A further alternative, that we recently explored [27], is a trimodal Lorentzian distribution. This allows for two separate heterogeneous fluorophore environments, besides the minor component. In reality both the bimodal and trimodal Lorentzian solutions are at best only approximations to any 'real' environmental heterogeneity that exists in the membrane, the latter being more sophisticated. However, to examine the effects of ethanol, to reduce the error caused by the larger number and greater sensitivity of the recovered parameters in a trimodal analysis, the simpler bimodal Lorentzian solution was used. This has been widely used in other laboratories (e.g., see Refs. 29, 50–61).

For DPH, TMA-DPH and DPH-PC, D_w increased and the lifetime center decreased, as a result of chronic ethanol treatment (see Figs. 2 and 3 (A–C)). Only in the case of DPH-PC can the distributional width be attributed to environmental heterogeneity at the protein/lipid interface, as discussed above. This is because DPH and TMA-DPH are able to locate in a variety of positions along the bilayer normal. TMA-DPH being restricted to regions nearer the head group region, DPH being unrestricted. At different depths in the bilayer there are different levels of interstitial water, the amount decreasing with increased depth into the bilayer and this sets up a dielectric constant gradient, thus at any moment of time, each individual fluorophore in the ensemble is experiencing a different amount of water in the excited state solvent cages, dependent on its position. Thus a range of decay rates is recovered for both intact membranes and for vesicles made from extracted lipids. By contrast, the DPH in DPH-PC is fixed in a direction relative to the bilayer

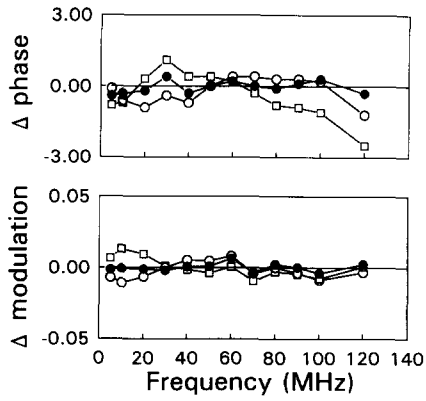

Fig. 1. Residuals from various analyses of the fluorescence decay of DPH-PC in liver microsomal membranes. Squares (unimodal Lorentzian distribution), χ^2_R 6.33; open circles (double exponential), χ^2_R 3.78; filled circles (bimodal Lorentzian distribution), χ^2_R 0.97. Recovered parameters from an example data set for a bimodal Lorentzian analysis (from a triplicate): lifetime centers 6.15, 0.53 ns, distributional widths 2.20, 0.00, fractional contributions 0.97, 0.03. Experimental and other details are as described under Materials and methods.

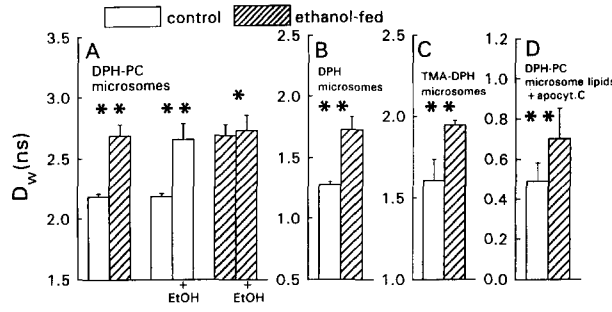

Fig. 2. Effect of chronic ethanol treatment on the D_w in microsomal membranes. ** Difference significant ($P < 0.05$); * not significantly different (paired t-test). (A) DPH-PC (χ^2_R: control, 0.896; ethanol-fed, 1.001; control+400 mM EtOH, 0.872; ethanol-fed+400 mM EtOH, 0.647). (B) DPH (χ^2_R: control, 1.505; ethanol-fed, 1.359). (C) TMA-DPH (χ^2_R: control, 1.284; ethanol-fed, 0.659). (D) DPH-PC in vesicles of extracted microsomal phospholipids with 10 mole% apocytochrome C (χ^2_R: control lipids, 1.063; ethanol-fed lipids, 0.724). In (A) the effect of ethanol even at the high level of 400 mM is shown to have no effect on D_w after chronic ethanol treatment. Details are as described under Materials and methods.

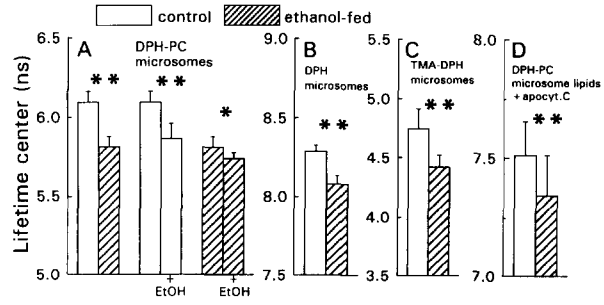

Fig. 3. Effect of chronic ethanol treatment on the major lifetime center in microsomal membranes. ** Difference significant ($P < 0.05$); * not significantly different (paired t-test). (A) DPH-PC, (B) DPH, (C) TMA-DPH and (D) DPH-PC in vesicles of extracted microsomal phospholipids with 10 mole% apocytochrome C. All χ^2_R are shown in legend of Fig. 2. In (A) the effect of ethanol even at the high level of 400 mM is shown to have no effect on the lifetime center after chronic ethanol treatment. Details are as described under Materials and methods.

normal, due to its tethering to PC and therefore does not sense the dielectric constant gradient. Thus the effects on D_w for DPH-PC (but not TMA-DPH and DPH) were considered to arise solely from a modification at the protein-lipid interfacial region.

The increased environmental heterogeneity at the protein/lipid interface, could have occurred either through a modification to the lipid or protein components, or both. To distinguish between these possibilities the protein apocytochrome C was added to vesicles made from extracted liver microsomal lipids from control and ethanol-fed animals. Thus, by keeping the protein component constant, the role of the lipid changes in the above effects could be assessed. Again a distributional width was recovered, due to the presence of the protein, although it was narrower than recovered for natural membranes, which have a much more complex protein composition. The D_w was again broader in the membranes from ethanol-treated animals as compared to controls (Fig. 2D) and the lifetime center lower (Fig. 3D), as found with intact membranes. Thus while the results do not rule out involvement of modifications to the protein component the effect must at least partially (if not entirely) be due to changes in the lipids.

A commonly found feature of cell membranes from animals subjected to chronic ethanol ingestion is that they exhibit membrane tolerance, in that there is a resistance to lipid disordering, by ethanol and other related lipophillic agents, as compared to control membranes (e.g., see Refs. 1, 9–11, 62, 63). To determine if this phenomenon extended to the protein/lipid interface, as probed by DPH-PC, the effect of the addition of ethanol was studied. It was found that membranes from chronic ethanol-treated animals were resistant to the effects of direct ethanol addition, in terms of D_w, even to 400 mM, which is well above the level of < 100 mM that is normally encountered, by contrast, D_w increased significantly in control membranes (see Figs. 2A and 3A). As a control experiment the effect of ethanol on the decay heterogeneity of DPH-PC was checked in vesicles of extracted microsomal lipids. The decay, however, remained homogeneous in that a bimodal Lorentzian fit returned a $\sim$zero D_w with no improvement of χ^2_R over a biexponential fit (data not shown), this confirming that the effect in intact membranes was protein associated.

4. Discussion

Chronic ethanol ingestion has been found to modify physico-chemical properties at the protein/lipid interface of rat liver microsomes. This was revealed as an increase in the excited state decay heterogeneity of DPH-PC, as reflected by an increased width of the continuous lifetime distribution (D_w) that was used to describe this process. Direct ethanol addition also increased D_w, but in membranes from chronic ethanol-treated animals there was a resistance to this effect, demonstrating the expression of membrane tolerance at the protein/lipid interface.

A broadened width of the major lifetime center for a bimodal Lorentzian distribution, defined here as an increased D_w, may be ascribed to several causes. A range of decay rates from fluorophores residing at the protein/lipid interface, together with a single decay rate from fluorophores in the bulk lipid region contribute to the major lifetime center. If the lifetime center of the bulk lipid fluorophores had separated from that of the protein/lipid interface fluorophores, then the width of the distribution encompassing both populations would have led to the broadened width. This should have been revealed in the trimodal Lorentzian fit to the data, where the two fluorophores populations are recovered separately. However, not withstanding, as discussed in the results section, that the lack of improved χ^2_R for the trimodal Lorentzian and the large number of fitting parameters led this solution to be disfavored compared to the simpler bimodal Lorentzian, a trimodal Lorentzian analysis did not reveal such a separation (results not shown). Also the relative contributions of the two populations may alter. Again a trimodal Lorentzian fit to the data did not reveal altered fractional contributions that would have supported this possibility. Thus the results point to a modification to either or both of the protein or lipid components of the membrane could potentially underlie the observed effects on the fluorescence lifetime parameters. The fact that the effect of the chronic ethanol treatment on the fluorescence lifetime center was found to persist in the vesicles made from extracted lipids would tend to argue that it is due to a lipid modification.

A heterogeneous fluorescence decay for DPH-PC is only found in the presence of protein, as demonstrated by a homogeneous decay in protein free lipid vesicle systems [27] and therefore differs from free DPH and TMA-DPH, which also sample environmental heterogeneity from other causes, principally from the dielectric constant gradient and in the case of TMA-DPH electrostatic interactions with protein [64]. Thus addition of a protein to a model membrane in essence re-organizes the membrane, establishing two regions, one at the protein/lipid interface and the other the bulk region, from a previously homogeneous region. From a consideration of the exchange rate of a lipid between the bulk region and the protein/lipid interface ($\sim 10^{-7}$ s, [31–33]), fluorophores in the excited state at the interface will have mostly decayed to the ground state before moving further than the second lipid shell from the protein surface [25]. Thus the decay

process may be used to examine the nature of the protein surface that is accessible to the fluorophore as in the present study. Environmental heterogeneity at the protein/lipid interface implies a molecular diversity of the excited state solvent cage. One obvious cause of this would be the different amino acid side chains that protrude into or interface with the lipid region. Thus if a given fluorophore locates in different regions on the protein/lipid interface, then each individual fluorophore excited state solvent cage will contain different amino acid side chains and this will lead to a heterogeneous fluorescence decay for the population of fluorophores as a whole. Also evidence has been presented for water molecules residing at the protein/lipid interface [28]. This water may be accommodated in packing defects at the surface and would again be a source of environmental heterogeneity. Both the amino acid side chain configuration at the protein/lipid interface and the accommodation of water molecules in this region are liable to be influenced by the packing constraints and properties of the phospholipid acyl chains.

Both a lipid or protein modification could result in the observed increase in D_w that was found to be a consequence of the chronic ethanol-treatment. In vesicles of extracted lipids, the decay of DPH-PC is environmentally homogeneous (i.e., very narrow or effectively zero D_w), as discussed above. Introduction of apocytochrome C to these vesicles, as a model protein, resulted in the reappearance of environmental heterogeneity and again a broad D_w was recovered which, however, was still greater in vesicles derived from lipids modified as a result of the chronic ethanol-treatment, as compared to the control. This provides evidence that the modification underlying the effect is primarily located in the lipids, but requires protein for 'expression', although this does not rule out a protein modification from also contributing to the effect. Lipid modifications that occur in microsomal lipids as a result of chronic ethanol-treatment include an increase in docosahexaenoate and a decrease of arachidonate in phosphatidylserine [11,14]. Although phosphatidylserine is a relatively minor microsomal lipid it tends to associate with proteins, an effect that would tend to concentrate the fatty acid changes at the protein/lipid interface. Docosahexaenoate contains six *cis*-double bonds along its entire length and tends to adopt a helical and hence bulky and relatively inflexible form in a lipid bilayer [65–70]. Thus it may not easily fit into the contours of the protein/lipid interface, as well as a more flexible fatty acyl chain. This will affect the distribution of any water molecules in this region and this may affect protein conformation, for example as shown recently in the lipid dependent structural studies of the gramicidin non-channel-to-channel conformational change [71]. Although the link between PS unsaturation changes and those detected by DPH-PC fluorescence properties are persuasive, at this stage whether the modifications to PS are in a region accessible to DPH-PC is not proven, in addition other microsomal phospholipids also change in composition as a result of the chronic ethanol-treatment.

According to previous studies of liver microsomes using EPR (e.g., see Refs. 12, 62, 63) and fluorescence spectroscopy [9] 'baseline' lipid order is not affected by chronic ethanol treatment. This contrasts with the liver plasma membrane which does show an alteration in lipid order (e.g., see Refs. 17, 72–74). Prior to the present study the only detectable 'baseline' changes in liver microsomes were as a response to some form of membrane perturbation such as the addition of ethanol or other external agent (e.g., see Refs. 9–12, 58, 59), even though evidence for lipid changes have been demonstrated [11,14]. Thus the results of the present study are important for two reasons. First it is the first report of a direct spectroscopically detectable change in liver microsomes resulting from chronic ethanol treatment. Thus the method may also be of use in the study of potential chronic-ethanol induced effects on membranes from other tissues. Secondly, the D_w parameter exhibits tolerance characteristics associated with the protein/lipid interface, thus offering a potential link between lipid changes and protein functional modifications.

In conclusion, the adaptive lipid modifications brought about as a result of chronic ethanol treatment and by the presence of ethanol itself, could potentially lead to modifications in protein conformation (and hence function) by interacting at the protein/lipid interface. These effects are detectable by placing fluorophores in this critical region of the membrane. Finally it is worth emphasizing that these studies are concerned with the averaged effects over a diverse range of protein hydrophobic surfaces in the microsome. Many regions may be minimally, if at all affected, reducing the overall average magnitude of any effect and masking other potentially larger effects in regions of other proteins.

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