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ESR Measurements on the Effects of Ethanol on the Lipid and Protein Conformation in Biological Membranes[1]

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LOGAN, B. J., R. LAVERTY AND B. M. PEAKE. *ESR measurements on the effects of ethanol on the lipid and protein conformation in biological membranes.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 31–35, 1983.—The effects of ethanol on the conformation of proteins or fluidity of lipids was studied in human erythrocyte ghosts and rat brain synaptosomal membranes. A maleimide nitroxide probe (MAL-6) was coupled to membrane protein, or 5-doxylstearic (5NS) was dissolved in the membrane lipid, and the electron spin resonance (ESR) spectra were recorded in the presence of increasing ethanol concentrations and at different temperatures from 4° to 37°. The lipid probe at all temperatures studied showed an increase in fluidity in the membrane lipid when ethanol was added in concentrations over 20 mM. The protein probe however required a high concentration of ethanol (200 mM) to produce an increase in the rigidity of the protein conformation at 4° in both erythrocyte and synaptosomal membranes. A decrease in protein rigidity was observed at high ethanol concentrations at 37° only in the erythrocytes. Thus an effect of ethanol on the conformation of membrane protein was observed using MAL-6 only at high ethanol concentrations and depended on the membrane used and the temperature of measurement.

Spin-labelled membrane ESR spectra Erythrocytes Synaptosomal membranes Ethanol

WHILE the behavioural effects of ethanol have been observed for centuries, the mechanism by which ethanol produces these effects remains to be discovered. Classically it has been assumed that ethanol, like many non-specific general anaesthetic molecules, dissolves in the membrane lipid causing some modification of the lipid structure, such as an increase in fluidity, that might account for its effects on neurotransmission and behaviour. Recently such an effect of ethanol on lipid fluidity has been shown [4,5] using electron spin resonance (ESR) techniques with fatty acid nitroxides as probe molecules to report on the lipid fluidity.

However, there has been increasing emphasis in recent years on the possible role of membrane protein in the mode of action of general anaesthetics [17,18]. We therefore investigated the effects of ethanol on the protein conformation of biological membranes by using a spin-labelled probe which binds to membrane protein. A commonly used protein probe is maleimide nitroxide compound (MAL-6) [1, 2, 9] which couples covalently to the sulphydryl groups of the protein in membranes [12].

In this paper we have compared the effects of ethanol on both lipid and protein ESR probes using two biological membranes, erythrocyte ghosts and synaptosomal plasma membranes. We have observed that the effects obtained depend on the chemical nature of the probe, the membrane source, and the temperature at which the ESR spectra are measured.

METHOD

Preparation of Membranes

Erythrocyte ghosts. Human blood (30 ml) fresh each day was collected into heparinized vacutainer tubes. The method of Dodge *et al.* [6] was used with some minor changes. The lysis buffer contained 1 mM EDTA, and after the final wash the ghosts were resuspended in 100 mM phosphate, pH 7.4, the buffer used throughout the labelling and washing procedures.

Synaptosomal plasma membranes. Synaptosomal membranes were isolated from whole rat brains by the combined floatation and sedimentation density gradient technique [11]. The synaptosomal fraction was collected and diluted 1:2 with ice cold buffer and centrifuged at 100,000 g for 30 minutes. The membranes were then resuspended in buffer for labelling.

Labelling

Protein label. The amount of protein was measured in each membrane sample [16].

The label, 2,2,6,6-tetramethyl-4-maleimide-piperidin-1-oxyl (MAL-6) (Syva) was dissolved in buffer, added to the membranes in a ratio of 1 mg MAL-6 to 50 mg protein, and left to incubate overnight at 4°C. The unbound label was

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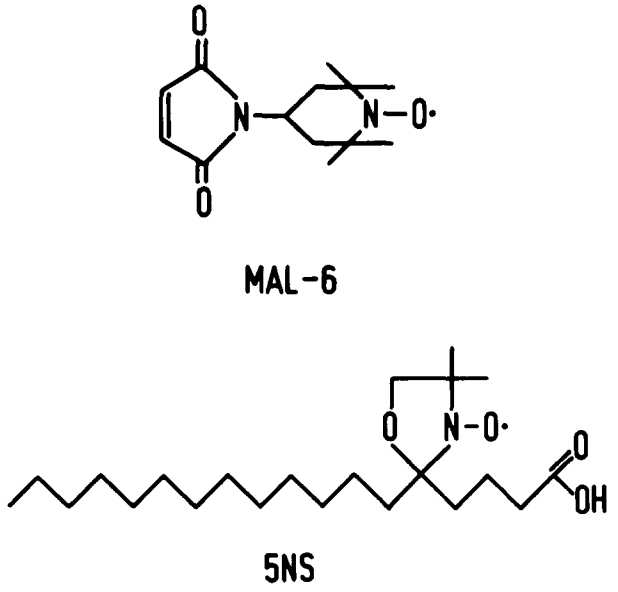

FIG. 1. Chemical structure of the ESR probe molecules.

removed by four successive washes in buffer. After each wash the membranes were centrifuged for 10 min at 40,000 g for erythrocyte ghosts, and at 25,000 g for synaptosomal plasma membranes.

Lipid label. Sufficient 5NS (N-oxyl-4',4'-dimethyloxolidine derivative of 5-ketostearic acid; Syva) for a ratio of 1 mg to 40 mg of membrane protein was dissolved in chloroform. The chloroform was evaporated under nitrogen and the membranes added and left to incubate overnight at 4°C.

Sample Preparation and ESR Measurement

Dilutions of ethanol (95% v/v) were added to the labelled erythrocyte ghost suspension to obtain the desired concentration, and aliquots were placed in 50 μl capillary tubes.

A similar procedure was used for the synaptosomal membranes: the tubes were sealed by heating and then centrifuged for 10 min, at 2000 g.

All ESR measurements were made using a Varian E-104 spectrometer with an E-231 cavity, and the temperature regulated by the E257 Variable temperature device calibrated with a laboratory built digital thermometer. Samples were equilibrated for at least 15 min at the temperature of measurement prior to insertion into the cavity.

The order parameter for 5NS was calculated by measuring the hyperfine splittings as described by Hubbell and McConnell [10]. The measurement of MAL-6 changes was taken as the ratio of the amplitude of the weakly immobilized (W) and strongly immobilized (S) peaks at low field. This has been found to provide an indication of changes in the protein conformation [2, 3, 9].

Each result unless otherwise states is the mean value and standard error of three separate membrane preparations, with ten replicates per sample. A two-way analysis of variance was used for the determination of statistical significance.

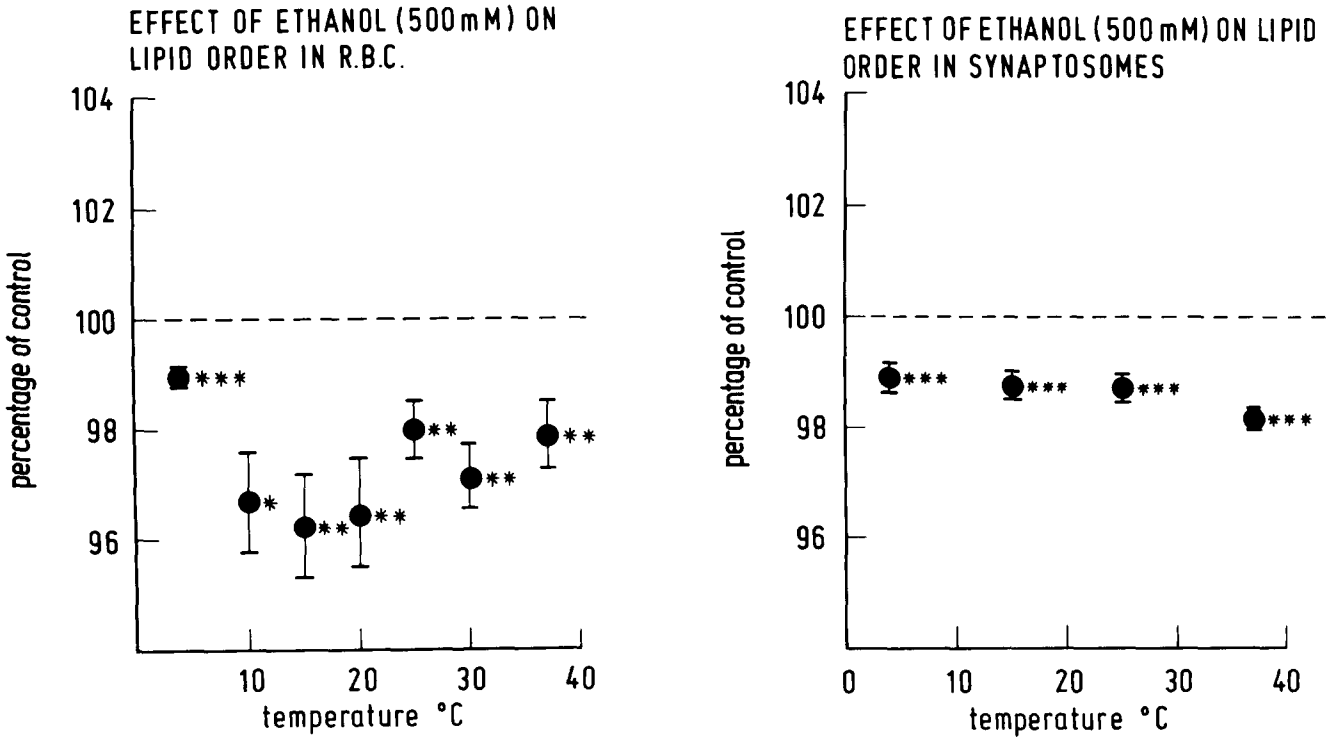

FIG. 2. The effect of membrane sample temperature on the change in lipid order parameter induced by ethanol (500 mM) in (A) erythrocyte ghost membrane (R.B.C.) and (B) synaptosomal membranes. Error bars represent the S.E. of the mean. The control value (100%) represents order parameter of each membrane sample replicate without the addition of ethanol. A decrease from control represents a decrease in order.

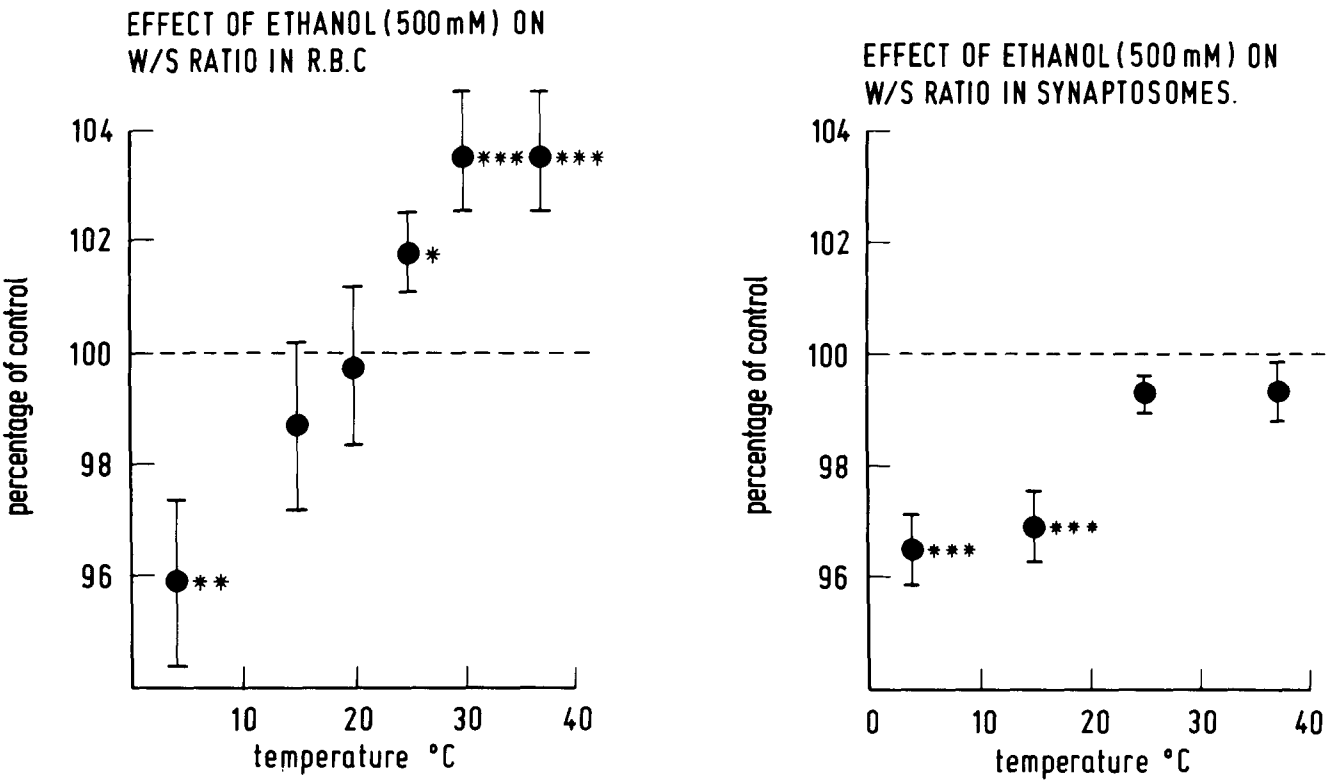

FIG. 3. The effect of membrane sample temperature on the change in protein conformation (expressed as the W/S ratio) induced by ethanol (500 mM) in (A) erythrocyte ghost membranes (R.B.C.) and (B) synaptosomal membranes. Error bars represent the S.E. of the mean. The control value (100%) represents the W/S ratio of each membrane sample replicate without the addition of ethanol. A decrease in W/S ratio represents an increase in order.

RESULTS

Earlier results [13] had indicated that an effect of ethanol on protein conformation could be measured but that this effect was variable and difficult to reproduce. An initial study was made of the effect of temperature on the changes due to ethanol in the ESR spectra from protein and lipid probes. The results of these experiments are shown in Figs. 2 and 3.

Ethanol (500 mM) increased the fluidity of the lipid probe region at all temperatures studied from 4° to 37° in both erythrocyte ghosts and synaptosomal membranes (Figs. 2A and 2B). In erythrocyte ghosts, ethanol (500 mM) caused a reduction in the W/S ratio i.e., an increase ordering of the protein conformation at temperatures below 20° but caused an increase in W/S at temperatures above 20° (Fig. 3A). By contrast, in synaptosomal membranes ethanol (500 mM) caused a reduction in the W/S ratio at 4° and 15° but had no effect on the protein conformation at 25° and 37° (Fig. 3B). Thus the effect of ethanol on the protein conformation in membranes depends on both the nature of the membrane and the temperature at which the spectrum is measured.

The effects of lower concentrations of ethanol were studied (Table 1). In both erythrocyte ghost and synaptosomal membranes, ethanol from 20 mM concentration upwards decreased the lipid probe order parameter. The effect of low concentrations of ethanol on the protein conformation as measured by the W/S ratio was examined in erythrocyte ghosts at both 4° and 37°; as before the ethanol caused an increased ordering at 4° and a decreased ordering at 37° but only at concentrations of ethanol of 200 mM or greater. In synaptosomal membranes measured at 4°C, ethanol caused an increased ordering of the protein conformation but only at concentrations of 200 mM or greater. Thus while ethanol produces observable changes in the lipid structure of membranes at low concentrations (20 mM), changes in protein conformation could only be observed with concentrations of 200 mM or more.

DISCUSSION

In these experiments we have confirmed the observation made earlier [4,5] that ethanol from low concentrations causes an increase in the fluidity of the lipid regions of membranes as measured by the freedom of rotation of a lipid-soluble nitroxide probe molecule dissolved in the lipid of the membrane. However the results using the protein probe were less consistent and more difficult to interpret.

The maleimide nitroxide (MAL-6) used as a protein probe is known to couple predominantly with protein sulphydryl groups [12] although there is a possibility of a reaction with amino groups [3]. MAL-6 has been used by a number of workers to measure the conformation or rigidity of membrane proteins [1, 2, 9, 12, 19, 20], though it has not been used as extensively as the lipid probes. Certainly much re-

TABLE 1

EFFECT OF VARYING THE ETHANOL CONCENTRATION ON THE LIPID ORDER PARAMETER OR THE PROTEIN CONFORMATION (EXPRESSED AS W/S RATIO)

Probe	Membrane and Temperature	Ethanol concentration (mM) 0	20	50	100	200	500
Lipid (5NS)	Erythrocyte 37°	0.6373 (±0.0018)	0.6335* (±0.0012)	0.6334* (±0.0005)	0.6301‡ (±0.0004)	0.6264‡ (±0.0055)	0.6254‡ (±0.0009)
	Synaptosome 37°	0.6068 (±0.0018)	0.5977† (±0.0015)	0.5939‡ (±0.0017)	0.5911‡ (±0.0008)	0.5851‡ (±0.0008)	0.5794‡ (±0.0020)
Protein (MAL-6)	Erythrocyte 4°	1.05 (±0.01)	1.04 (±0.01)	1.02 (±0.01)	1.02 (±0.01)	1.00* (±0.01)	0.92† (±0.05)
	Erythrocyte 37°	3.52 (±0.05)	3.52 (±0.04)	3.53 (±0.04)	3.51 (±0.04)	3.59 (±0.04)	3.82‡ (±0.05)
	Synaptosome 4°	4.97 (±0.12)	4.89 (±0.13)	4.93 (±0.12)	4.92 (±0.14)	4.77 (±0.12)	4.67‡ (±0.12)

*$p<0.05$; †$p<0.01$; ‡$p<0.001$.

Results are given as the mean ± S.E. of mean of 10 replicates from 3–4 separate membrane preparations. For the lipid order parameter an increasing value represents an increase in order. For the W/S ratio an increasing value represents a decrease in order.

mains to be understood concerning its use, particularly with regard to the site of its localization in the membrane. Studies on erythrocyte ghosts [2,8] suggest that the label attaches to spectrin-actin or band 3 complexes on the inner surface of the membrane; corresponding studies have not been made on the localization of the probe in the synaptosomal membranes. Our own studies [14,15] indicate that MAL-6 binds strongly to the membrane $Ca^{2+}Mg^{2+}$-ATPase and that ethanol could be exerting an effect through this enzyme. Even the interpretation of the W and S peaks in the ESR spectrum from membranes labelled with MAL-6 is open to debate. However, as the W peak increases in size with increasing temperature and disappears when labelled membranes are frozen (unpublished observations) it would appear that the usual assumption that the W peak arises from weakly immobilized probe molecules and the S peak from strongly immobilized probe molecules is supported by our studies.

The supposition that ethanol and other non-specific general anaesthetic molecules can alter the structure or fluidity of the lipid phase of membranes is now well accepted. The case of a role for membrane proteins as the site of action of anesthetics has only recently been put more strongly [7, 17, 18]. Our results might appear to support a lipid site of action more strongly than a protein site of action for ethanol, but the differences in the effects of ethanol on different membrane proteins or at different temperatures of measurement of the ESR spectra would suggest the effect of ethanol on membrane protein conformation is complex. Further work is required to establish whether the effects of ethanol on protein conformation are significant for its physiological actions. It is possible that the protein probe used attaches at a site too remote from the site of action of ethanol to be affected sufficiently by the concentrations of ethanol which produce behavioural effects. Alternatively it may bind at a site of ethanol action but in such a way that it prevents ethanol from having its perturbing effect except at high concentrations.

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