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ETHANOL-INDUCED CONFORMATIONAL CHANGES IN RAT BRAIN MICROSOMAL MEMBRANES

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Abstract—The conformation of membrane-bound proteins was monitored by the reaction of sulfhydryl (SH) groups with *N*-ethyl maleimide (NEM) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Brain microsomes isolated from rats chronically imbibing a 10% (v/v) ethanol solution were found to contain 12–16 per cent more DTNB-reactive SH groups than similarly isolated controls. [^{3}H]NEM reacted with 32 per cent more SH groups in the microsomes obtained from ethanol-treated rats than controls. Since no change in the protein composition was evident from disc electrophoresis, and no significant differences in the total number of SH groups was present between the two samples, these findings suggest a conformational change in the microsomal membrane. Microsomes isolated from ethanol-naïve animals, when treated with ethanol *in vitro*, showed a dose-dependent decrease in DTNB-reactive SH groups. The results indicate that the neural membrane may exist in different morphological states during acute and chronic exposure to ethanol.

Neural membranes are generally acknowledged to be ethanol's primary site of action on the brain [1–3], but considering the close association of membrane structure and function [4], it is surprising that little research has been devoted to ethanol-induced modification of neural membrane structure. The reports that have appeared in the literature have consisted of *in vitro* studies [5, 6] whereas the present work deals with changes in neural membrane structure in rats due to chronic ethanol consumption and therefore represents *in vivo* effects of ethanol.

A membrane in its normal state may be characterized by a unique optimal arrangement and conformation of its proteins, lipids and other constituents. Associated with this configuration of optimal function are a certain number of sulfhydryl (SH) groups that are available or "exposed" to reaction with SH-specific reagents. In the present study, we have chosen to use alterations in SH "exposure" as an index of conformational changes in the membrane proteins. Sulfhydryl groups were chosen because of their involvement in membrane processes from oxidative phosphorylation [7, 8] to the propagation of nerve impulses [9, 10] and because they can be readily monitored. We have examined the reactions of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and *N*-ethyl maleimide (NEM) with the SH groups present in rat brain microsomes in an effort to gain some understanding of ethanol's effect on neural membranes.

MATERIALS AND METHODS

Care and treatment of animals. Male, Sprague–Dawley rats 19 days old were purchased from Simonsen Laboratories (Gilroy, CA) and housed in a 12 hr light/12 hr dark environment and given rat chow *ad lib.* Following a short period of acclimatization, half of the rats were administered a 10% ethanol solution in water (v/v) for 6–8 weeks, while the other half received water *ad lib.* Twenty-four hr prior to sacrifice, the ethanol solution was replaced by water. These animals exhibited no withdrawal reaction.

Isolation of microsomal fractions. Brains were removed within 30 sec of decapitation, pooled according to group, and placed in ice-cold Buffer A (0.32 M sucrose, 0.01 M $MgCl_2$, 0.01 M sodium phosphate buffer, pH 7.0). The pooled brains were then rinsed three times and homogenized (1,000 rpm, 10–15 strokes) in 5 vol. of Buffer A using a size "C" Thomas glass homogenizer with a Teflon pestle. Fractionation of the brain homogenate followed the centrifugation scheme of Tewari and Baxter [11]. All procedures were performed at 4°. The microsomal pellet derived from 5 brains was suspended in 2–3 ml of Buffer B (0.001 M $MgCl_2$, 0.01 M solium phosphate buffer, pH 7.0) to yield a protein concentration of approximately 20 mg/ml. Samples were stored in glass vials at 4° for no more than 24 hr, with the exception of electrophoresis samples which were frozen at −70° until used.

Determination of SH groups using 5,5′-Dithiobis (2-Nitro-benzoic acid). The procedure for using DTNB as a quantitative SH reagent was based upon the method of Acharya and Moore [12]. A 100 μl aliquot of the microsomal suspension (approximately 1.5–2.0 mg protein) was added to 1.8 ml of Buffer B

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in a quartz cuvette adjusted to read zero absorbance. The baseline for the DTNB reaction was determined by the "absorbance" due to the optical dispersion of the microsomal suspension. No significant change in the turbidity of the solution occurred during the reaction time. A 100 μl aliquot of 0.025 M DTNB (Eastman) was added to the diluted microsomal suspension and mixed by inverting the cuvette. The absorbance was followed for approximately 10 min. The linear region of the absorbance vs time graph was extended back to the point of DTNB addition, and the vertical displacement from the baseline was taken to be the absorbance due to the thiol-DTNB reaction plus the absorbance of DTNB's dissociation into the thionitrobenzoate anion. Controls for DTNB dissociation consisted of 100 μl of the DTNB solution in 1.9 ml of Buffer B, and this absorbance was subtracted from all results yielding the net absorbance due to the "fast-reacting" SH groups. Total SH groups were estimated by DTNB reaction after denaturation of the microsomal sample in either 1% sodium dodecyl sulfate (SDS) or 8 M urea [12]. All absorbance measurements were made at 25° (room temperature) using a Gilford recording spectrophotometer, Model 2400, at a wavelength of 412 nm. All assays were done in quadruplicate.

Incubation of microsomal samples with ethanol in vitro. A 100 μl aliquot of the microsomal suspension (150–200 μg protein) was added to tubes containing 1.8 ml of 0, 25, 100, 200, 500, 1000 mg per dl-ethanol in Buffer B (final concentration in 2.0 ml) and incubated at 25° for 30 min. The contents of each tube were then transferred to a quartz photometric cuvette and reacted with DTNB (100 μl) as described above. Similar experiments were done using 2-mercaptoethanol (7×10^{-4} M) in place of the microsomes.

Protein estimation. The protein concentration of the microsomal samples was determined by the procedures of Lowry *et al.* [13]. Crystalline bovine serum albumin was used as a standard.

Treatment of data. From the DTNB absorbance data, we calculated the following: (1) the moles of fast-reacting SH groups/mg protein, (2) the total SH content, and (3) the percentage of the total SH groups that were exposed to DTNB reaction. The number of exposed SH groups was calculated using Beer's Law, using an extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ [14].

Determination of sulfhydryls using N-ethyl maleimide. Sulfhydryl groups were also determined by reacting microsomes with NEM using a modification of Acharya and Moore's procedure [12]. A 3.0 ml vol. of the microsomal suspension was added to an equal vol. of 0.17 mM NEM (ethyl-2-[^{3}H], specific activity 250 mCi/m-mole, New England Nuclear) in sodium phosphate buffer, pH 6.5, and reacted for 45 min at 25°. Controls were run by reacting the microsomes with an excess of non-radioactive NEM prior to the addition of [^{3}H]NEM. Labeled microsomes were purified by extraction on membrane filters (Schleicher and Schuell, No. B-6, 25 mm diameter, 0.45 μ pore size) washed once with 10 ml of 1 mM NEM (non-radioactive), 3 times with 10 ml of 0.1% 2-mercaptoethanol and twice with deionized water. The filters were then dissolved in 20 ml of Bray's solution and radioactivity was determined in a Beckman Liquid Scintillation Counter. The number of moles of exposed SH groups was calculated from the specific activity of the [^{3}H]NEM.

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed using the technique of Lim and Tadayyon [15] with the exception that a 5 per cent cross-link was used in place of the recommended 7 per cent to facilitate the protein entering the gel. Approximately 100 μg of protein was placed on the gel. Gels were stained with 1% napthol blue black (Eastman) and destained by soaking in a 7.5% acetic acid.

RESULTS

Figure 1 compares the amount of DTNB fast reacting SH groups and total SH content of microsomes isolated from the brains of ethanol and water drinking rats. The average number of DTNB-reactive thiols (per mg protein) was $2.00 \pm 0.02 \times 10^{-8}$ moles for the ethanol naïve and $2.32 \pm 0.02 \times 10^{-8}$ moles for the ethanol-imbibing rats ($P < 0.001$). This corresponds to an increase of 16.2 ± 1.4 per cent in fast reacting SH groups in the ethanol group as compared to controls. Total SH groups of microsomes were determined following either 1% SDS or 8 M urea treatment (Fig. 1). Brain microsomes of ethanol drinking animals contained essentially the same amount of total SH groups as the control fraction following both SDS and urea denaturation treatment.

In the undenatured microsomes (Fig. 2) 31.5 ± 0.5 per cent and 35.8 ± 0.5 per cent of the total SH content as determined by SDS treatment reacted with DTNB, for the control and ethanol-treated groups, respectively; an increase of 12.6 ± 2.2 per cent ($P < 0.01$). Also seen in Fig. 2, as determined by urea

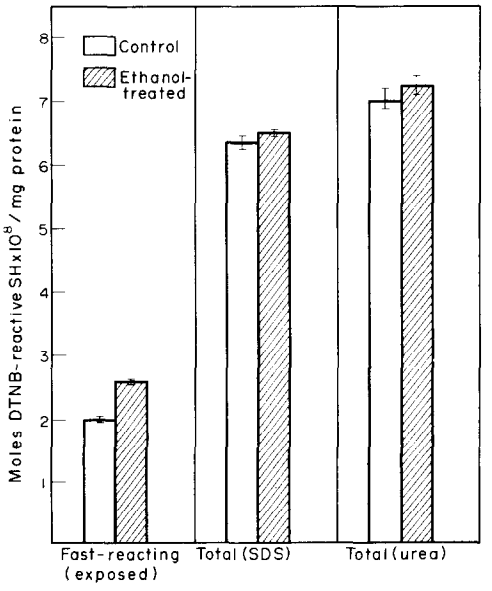

Fig. 1. Fast-reacting ("exposed") and total sulfhydryl groups of rat brain microsomes. Ethanol-treated animals imbibed a 10% ethanol–water solution for 6–8 weeks, while controls received water. SH groups were measured by reaction with DTNB as described in Methods. Mean ± standard error was determined from quadruplicate determinations from at least three separate experiments.

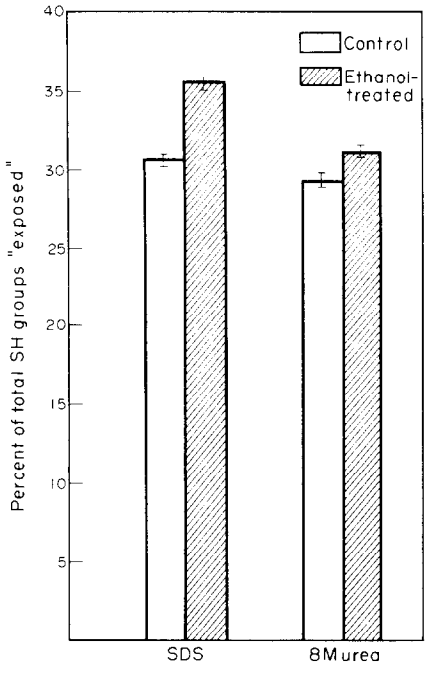

Fig. 2. *In vivo* effect of ethanol on the exposure of SH groups in rat brain microsomes. Results are expressed as the percentage of total SH groups as determined by denaturation in either sodium dodecyl sulfate or 8 M urea, and demonstrate an increase in sulfhydryl exposure in the ethanol-treated group.

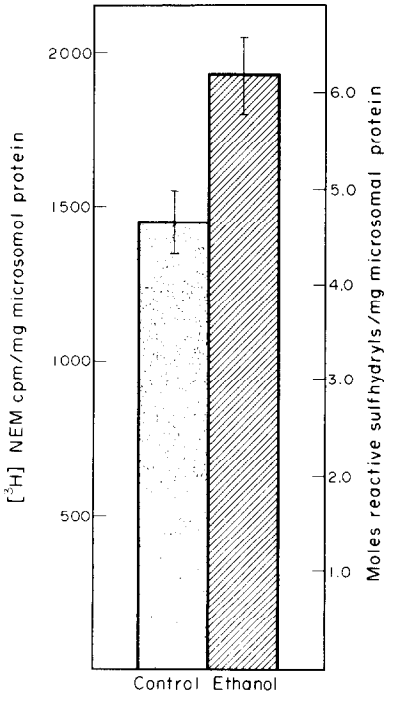

Fig. 3. Reaction of [^{3}H]*N*-ethyl maleimide with exposed sulfhydryl groups of brain microsomes isolated from control and ethanol-imbibing rats. Mean ± standard error.

denaturation, 28.9 ± 0.6 per cent and 32.3 ± 0.7 per cent of the total SH groups were "exposed" in the control and ethanol microsomes, respectively. This corresponds to ethanol-induced increase of 11.8 ± 3.2 per cent ($P < 0.025$). Even after adjusting for a very slight increase (not significant) in total SH content, a significant increase in the number of fast reacting SH groups was still evident. Although different procedures were used to estimate total SH groups, the increase in the ethanol-treated group was essentially the same.

The increase in SH exposure was substantiated by the results obtained from reaction with [^{3}H]NEM (Fig. 3). Here, also, a significant increase in SH exposure was found in the microsomes from ethanol-treated rats. Per mg protein, ethanol microsomes contained $6.17 \pm 0.41 \times 10^{-10}$ moles of reactive SH, whereas control microsomes contained $4.67 \pm 0.29 \times 10^{-10}$ moles, a difference significant at $P < 0.01$. The greater ethanol-induced increase of available SH groups using NEM (32 per cent), compared to the increase obtained with DTNB (16.2 per cent), and the fact that NEM reacted with only 3 per cent as many SH groups as did DTNB, may be explained by different chemical and electrical properties, molecular size and reacting concentrations of the two reagents. The same groups that were exposed to DTNB attack were not necessarily exposed to NEM. Nevertheless, an increase in the ethanol-treated fraction still prevailed.

Several explanations are possible for this increase in microsomal fast-reacting SH groups after chronic ethanol administration. One explanation would be that ethanol induces changes in the protein composition of the microsomes and that the increase in SH groups reflects this change in protein composition. To test this hypothesis, polyacrylamide gel electrophoresis was performed on control and ethanol microsomes using 8 M urea and Triton X-100 to solubilize membrane proteins (see Methods). No differences were observed in the protein banding patterns of the two samples. Furthermore, the data on the total SH groups per mg protein after urea or SDS denaturation (Fig. 1) show no significant difference between the control and ethanol microsomes. We believe that these data do not support a change in protein composition as the explanation for the observed increase in SH groups and argue in favor of conformational changes. Whether these conformational changes are directly or indirectly induced by ethanol remains to be determined.

To study the direct actions of ethanol on fast-reacting SH groups, *in vitro* studies were conducted on microsomes isolated from brains of ethanol-naïve rats. In contrast to chronic administration of the drug, where an increase in reactive SH groups was found, microsomes subjected to the acute effects of ethanol (30 min) revealed instead a dose-dependent decrease in fast-reacting SH groups as determined by the DTNB reaction. The results for a typical experiment are shown in Fig. 4. It is important to note that the decrease in SH exposure observed *in vitro* occurred at ethanol concentrations which are physiologically obtainable. To check the possibility of ethanol interfering with the kinetics of the thiol-DTNB reactions, 2-mercaptoethanol was reacted with DTNB following the same incubation procedure. No signifi-

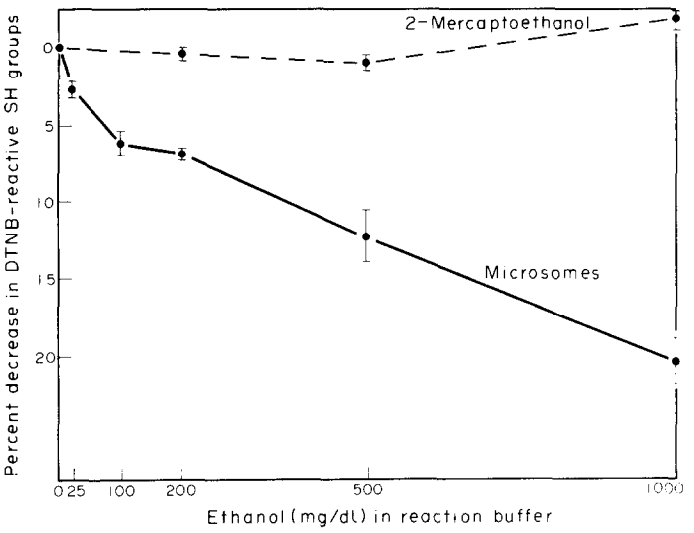

Fig. 4. Effect of *in vitro* addition of ethanol on the reaction of DTNB with sulfhydryl groups of brain microsomes isolated from ethanol-naïve rats. Samples were incubated at the designated ethanol concentration for 30 min at room temperature prior to DTNB reaction. Ethanol exhibited no significant effect on the reaction of DTNB with 2-mercaptoethanol. Values shown are mean ± standard error.

cant change in the reaction due to the presence of ethanol was observed (Fig. 4).

DISCUSSION

Most of the research to date concerning ethanol and neural membranes has dealt with active transport phenomena and ion permeability [16–18]. Although changes in neural function have been confirmed, a molecular or membrane model to explain such aberrations has yet to be established. The work presented in this paper is among the first to show that chronic ethanol consumption results in a structural change in membranes isolated from the CNS.

While we observed that chronic ethanol consumption results in an *increase* in SH exposure, acute *in vitro* treatment caused a *decrease* in the exposure of these groups. This evidence suggests that the neural membrane may exist in different morphological states during these two phases of ethanol administration. The results presented in the present study suggest the following model as a possible explanation of ethanol's effect on the structure and function of neural membranes.

In its normal state, the neural membrane exists in some optimum configuration consisting of a particular arrangement of proteins and lipids. This arrangement of proteins corresponds to the availability of a certain number of SH groups to reaction with DTNB or NEM. Under acute conditions when ethanol is first introduced to the system, this optimum arrangement is disturbed by the presence of the drug, resulting in a rearrangement or change in conformation of the membrane proteins. Incidental to this change is a decrease in SH exposure. Smith [10] and Komalahiranya and Volle [9] have reported that reacting exposed nerve fibers with NEM causes changes in nerve function such as depolarization, inexcitability and altered response to stimulation by acetylcholine. Ethanol, like NEM, may disturb the functional state of the membrane through a "blockage" of SH groups.

Upon chronic exposure to ethanol and continued depression of function, the homeostatic mechanisms of the animals induce other changes in the membrane in an effort to bring the system back to a functional norm. This arrangement of membrane proteins is characterized by an increase in DTNB and NEM reactive SH groups.

Although our results suggest conformational changes as the mechanism of these membrane alterations, it is possible that subtle changes in the lipid composition of the membrane may be responsible. It has been reported that chronic exposure to ethanol decreases the oxidation of palmitic acid [19] and that the incorporation of palmitate-1[^{14}C] into the lipids of mouse liver microsomes and mitochondria is also reduced [20]. Virtanen and Wallgren [21] have demonstrated an increase in the incorporation of [^{14}C]serine into brain microsomes in rats chronically treated with ethanol. That ethanol alters the nature of the lipid composition of the microsomal membrane with an indirect effect on protein conformation may therefore be an alternative explanation for the changes in SH exposure following chronic ethanol administration.

In a recent study [22] utilizing neural cells grown in culture, however, a conformational change in the cell membrane in the absence of a change in composition was reported. Noble *et al.* [22] have shown that the long-term presence of ethanol resulted in an enhanced "exposure" of surface sialic acid, without any change in the total amount of this component in the membranes of NN astroblasts.

That a change in SH exposure was observed after *in vitro* ethanol treatment further supports our contention that conformational changes are responsible for alterations in the number of reactive SH groups. The *in vitro* system used and the short time of incubation were probably not sufficient to result in a change

in the composition of the microsomal membrane. In addition, at the concentration of ethanol in the system, extraction of lipid membrane components into the ethanol-water phase is unlikely. It is probable that the presence of the ethanol molecule itself affected the membrane structure. Due to the molecule's simple chemical structure, its effect is most likely a non-specific one, affecting the membrane through steric restriction in the lipid phase or through free energy changes resulting from disturbed hydrogen bonding.

The study presented here has shown that ethanol affects the physical state of the membrane after both acute and chronic treatment. It is possible that the effects of other psychoactive drugs may be due to similar mechanisms, in addition to metabolic inhibition or receptor interference. Additional research must be done to determine the relative importance of these mechanisms in order to gain a further understanding of how drugs affect the central nervous system.

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