ALTERATIONS OF FAST-REACTING SULFHYDRYL GROUPS OF RAT BRAIN MICROSOMES BY ETHANOL

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SUMMARY. Brain microsomes isolated from rats chronically imbibing 10% ethanol contained 12-16% more 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) fast-reacting sulfhydryl (SH) groups than microsomes from control animals. (^{3}H)N-ethylmaleimide was also shown to react with more SH groups in the microsomes of ethanol imbibing rats than the controls. No changes were found in the total SH groups or in the disc gel electrophoresis protein banding profiles between the two fractions. However, the acute exposure of microsomes from ethanol-naive animals to ethanol resulted in a dose-dependent decrease in DTNB-reactive SH groups. These findings were interpreted as arising from time-dependent conformational changes in the membrane due to the presence of ethanol or compensatory response to such changes.

It is generally acknowledged that the neural membranes are the primary site of ethanol's actions on the brain (1-3). Although much research has been done concerning ethanol's effects on CNS enzymes and metabolism, studies involving ethanol-induced modification of biomembrane structure have been limited to a few *in vitro* investigations (4-6). To our knowledge, there are no reports in the literature on the chronic effects of ethanol administration on the conformation or structure of membranes isolated from nervous tissue.

An ethanol induced conformational change in membrane proteins may disturb finely tuned membrane processes and result in an arrangement whereby functional groups are either "buried" or "exposed" to attack by specific reagents. In this respect sulfhydryl (SH) groups were chosen for study because of their involvement in membrane processes from oxidative phosphorylation (6,7) to the propagation of nerve impulses (8).

METHODS

Ten Sprague-Dawley male rats (19 days old) were housed in a 12-hr light/12-hr dark environment and given rat chow *ad libitum*. 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 libitum*. Twenty-four hr prior to sacrifice, the

ethanol solution was replaced by water. Brains were removed within 30 sec of decapitation, pooled according to group, and placed in ice-cold Buffer A, containing 0.32 M sucrose, 0.001 M $MgCl_2$ and 0.01 M phosphate buffer, pH 7.0. Brain homogenization was done with a glass homogenizer and a Teflon pestle. Isolation of microsomes followed the procedure of Tewari and Baxter (9). The microsomal pellet derived from 5 brains was suspended in 2-3 ml of Buffer B, containing 0.001 M $MgCl_2$ and 0.1 M phosphate buffer, pH 7.0, and stored in glass vials at 4°C for no more than 48 hr with the exception of the samples for electrophoresis which were frozen at -40°C until used. Protein content was measured by the procedure of Lowry et al. (10).

The procedure for using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was based on the method of Acharya and Moore (11). A 100 µl aliquot of the microsomal suspension (approximately 1.5 - 2.0 mg protein) was added to 1.8 ml of Buffer B in a quartz cuvette adjusted to read zero absorbance, and followed by the addition of 100 µl of 0.025 M DTNB. After mixing, the absorbance was followed for 10 min at 412 mu in a Gilford recording spectrophotometer. Blanks were prepared by adding 100 µl of the DTNB solution to 1.9 ml of Buffer B, and their absorbance was subtracted to yield the net fast-reacting SH groups' absorbance. There was no significant difference in turbidity between control and ethanol microsomal suspensions during the 10 min period since nearly identical concentrations of protein were used. 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 (11).

Sulfhydryl groups were also determined by reacting microsomes with N-ethyl maleimide (NEM) using a modification of Acharya and Moore's procedure (11). A 3.0 ml volume of the microsomal suspension was added to an equal volume of 0.17 mM NEM (ethyl-2-^{3}H, specific activity 250 mCi/mmole, New England Nuclear) in sodium phosphate buffer, pH 6.5, and reacted for 45 min at room temperature (25°C). 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 washed on membrane filters (Schleicher and Schuell, no. B-6, 25 mm diameter, 0.45 µ pore size), once with 10 ml of 1 mM NEM (non-radioactive), 3 times with 10 ml 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.

Polyacrylamide disc gel electrophoresis was performed on the microsomes using the technique of Lim and Tadayyon (12) with the exception that a 5% cross-link was used in the running gel.

RESULTS AND DISCUSSION

Table 1 compares the amount of DTNB fast-reacting SH groups and total SH content of microsomes obtained from brains of ethanol and water-drinking rats. Using an extinction coefficient of 1.36×10^4 M^{-1} cm (13), the calculated average moles of fast-reacting SH groups/mg protein was $2.00 \pm 0.02 \times 10^{-8}$ for the ethanol naive and $2.32 \pm 0.02 \times 10^{-8}$ for the ethanol-imbibing rats ($p<0.001$). This corresponds to an increase of $16.2 \pm 1.4\%$ in fast-reacting SH groups in the ethanol fraction compared to control microsomes.

Total SH groups of microsomes were determined following either 1% SDS or

TABLE I

The Effect of Chronic Ethanol Consumption on DTNB Fast-Reacting and Total Sulfhydryl (SH) Groups of Rat Brain Microsomes

SH Groups	Control Microsomes	Chronic Ethanol Treated Microsomes	Significance
	Mean ± Standard Error		P
Moles of DTNB fast-reacting SH per mg protein (x 10^8)	2.00 ± 0.02	2.32 ± 0.02	< 0.001
Moles total SH(SDS) per per protein (x 10^8)	6.35 ± 0.09	6.49 ± 0.03	NS
Moles total SH(8 M urea) per mg protein (x 10^8)	7.02 ± 0.16	7.24 ± 0.12	NS
Reactive SH as % of total SH (SDS)	31.5 ± 0.5	35.8 ± 0.5	< 0.01
Reactive SH as % of total SH (8 M urea)	28.9 ± 0.6	32.3 ± 0.7	<0.025

Mean was derived from quadruplicate determinations from at least three different experiments.

NS = Non Significant

8 M urea treatment (Table 1). Brain microsomes of ethanol drinking animals contained essentially the same amount of total SH groups as the control fraction following the SDS or urea denaturation treatments. Of the total SH content following SDS denaturation, 31.5 $\pm$ 0.5% and 35.8 $\pm$ 0.5% for control and ethanol respectively, reacted with DTNB in the undenatured microsomes, an increase of 12.6 $\pm$ 2.2% ($p<0.01$). Similarly, after urea denaturation, 28.9 $\pm$ 0.6% and 32.3 $\pm$ 0.7% of the total SH groups reacted with DTNB in the control and ethanol microsomes respectively. This corresponds to an ethanol-induced increase of 11.8 $\pm$ 3.2% ($p<0.025$). Although different procedures were used in estimating total SH groups, the percent increase in the ethanol-treated group remained remarkably similar.

The 'exposed' SH groups of brain microsomes were also estimated by their reaction with NEM. The results using (^{3}H)NEM showed that, per mg protein, ethanol microsomes contained 6.10 $\pm$ 0.41 x 10^{-10} moles of reactive SH whereas control microsomes contained 4.67 $\pm$ 0.29 x 10^{-10} moles, a difference significant at $p<0.01$. The greater ethanol-induced increase of available SH groups using NEM (32%), compared to the increase obtained with DTNB (16.2%) may be explained by different chemical properties, molecular size, and reacting concentrations of the two reagents. Nevertheless, despite the fact that NEM reacted with only 3% as many SH groups as did DTNB, an increase in the ethanol 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 alcohol microsomes using 8M urea and Triton X-100 to solubilize membrane proteins (12). No differences were observed in the protein banding patterns. Further, the data on the total SH groups per mg protein after urea or SDS denaturation (Table I) show no significant difference between the control

and ethanol microsomes. We believe that these data rule out protein compositional changes as the explanation for the observed increase in SH groups and argue in favor of protein conformational changes induced by ethanol. Whether these conformational changes are directly or indirectly induced by ethanol remain 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-naive rats. The percent decreases in fast-reacting SH groups for a typical experiment were 2.6 ± 0.4, 6.1 ± 0.7, 6.7 ± 0.3, 12.2 ± 1.7, and 20.3 ± 1.5 for ethanol concentrations of 25, 100, 200, 500, and 1000 mg percent, respectively. Similar experiments using 2-mercaptoethanol (7×10^{-4}M) showed that the presence of ethanol in the reaction mixture did not interfere with the kinetics of the DTNB reaction. 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 of available SH groups as determined by the DTNB reaction.

Smith (14) and Komalahiryana and Volle (8) have reported that reacting NEM with exposed nerve fibers 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. In the acute response to ethanol, conformational changes occur resulting in a decrease in fast-reacting SH groups, as observed in our acute experiments, perhaps disturbing the functioning of the membrane. With the extended presence of the drug, compensatory modifications in membrane structure may occur, as evidenced by increased availability of SH groups following chronic ethanol consumption, perhaps reflecting responses of the membrane to reinstate its normal functional state. It has been reported that ethanol decreases the oxidation of palmitate (15) and that it reduces the incorporation of palmitate-1-^{14}C into total lipids of mouse liver microsomes and mitochondria (16). Therefore another possible explanation for the chronic effect of ethanol administration on fast-reacting

SH groups may be that ethanol alters the lipid composition of or amount in the microsomal membranes with an indirect effect on SH groups.

It is quite plausible that alterations in other components of membrane structure, as well, are induced by ethanol. Indeed, hypotheses have been proposed implicating either protein (3, 17) or the thermodynamic state of the lipids (18) as the critical determinant for the action of ethanol. Strong experimental support favoring either point of view is lacking, although in a recent study (19) utilizing neural cells grown in culture, the long-term presence of ethanol resulted in no change in total sialic acid but there occurred an enhanced 'exposure' of surface sialic acid, a component common to both gangliosides and glycoproteins.

The present study is among the first to show that chronic ethanol treatment affects the availability of a specific group in a neural membrane. By virtue of its biophysical characteristics, ethanol may also affect other aspects of membrane structure thus leading to changes in membrane fluidity and hence, function. How ethanol-induced alterations of specific structural elements affect various aspects of neural function remains a critical problem not only for alcoholism but for the field of neurobiology in general.

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REFERENCES

1. Kalant, H. (1971) in the Biology of Alcoholism, Vol. 1: Biochemistry, (Kissin, B. and Begleiter, H., eds.), pp. 1-62, Plenum Press, New York.
2. Wallgren, H. (1971) in the Biology of Alcoholism, Vol. 1: Biochemistry, (Kissin, B. and Begleiter, H. eds.), pp. 103-125, Plenum Press, New York.
3. Seeman, P. (1972) Pharmacol. Rev. 24, 583-655.
4. Spero, L. and Roth, S. (1970) Fed.Proc. 29, 474.
5. Seeman, P. and Roth, S. (1972) Biochim. Biophys Acta 225, 171-184.
6. Kurup, C.K.R. and Sanadi, D.R. (1968) Biochemistry 7, 4483-4491.
7. Miyahara, M. (1969) Arch.Biochem. Biophys. 134, 590-596.
8. Komalahiranya, A. and Volle, R.L. (1963) J. Pharm.Exp.Ther. 139, 304-311.
9. Tewari, S. and Baxter, C.F. (1969) J.Neurochem. 16, 171-180.

10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.
11. Acharya, A.S. and Moore, P.B. (1973) J.Mol. Biol. 76, 207-221.
12. Lim, R. and Tadayyon, E. (1970) Anal.Biochem. 34, 9-15.
13. Ellam, G.L. (1959) Arch.Biochem.Biophys. 82, 70-77.
14. Smith, H.M. (1958) J.Cell. Comp. Physiol. 51, 161-171.
15. Rebougas, G. and Isselbacker, K.T., (1961), J.Clin.Invest., 40, 1355-1361
16. Ferrell, J.J. and Miceli, J.N., (1970), Quart. J.Stud. Alc., 31, 810-815.
17. Seeman, P. (1974) Hospital Practice, Sept., 93-101.
18. Hill, M.W. and Bangham, A.D. (1975) Plenum Press, New York, in press.
19. Noble, E.P., Syapin, P.J., Vigran, R., Gombos, G., Vincendon, G., and Rosenberg, A. (1975) 5th International Meeting of the International Society for Neurochemistry, Sept. 2-6, Barcelona, Spain.