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# Differences in Susceptibility of Rat Liver and Brain Sialidases to Ethanol and Gangliosides

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MATHEW, J. AND W. R. KLEMM. *Differences in susceptibility of rat liver and brain sialidases to ethanol and gangliosides.* PHARMACOL BIOCHEM BEHAV **33**(4) 797–803, 1989.—Based on reports that ethanol can decrease the level of sialic acid (SA) (neuraminic acid) in several tissues, we tested the hypothesis that ethanol promotes SA cleavage by enhancing the activity of sialidases (neuraminidases). We also investigated whether brain and liver sialidases have the same response to ethanol and gangliosides, especially since our prior studies have demonstrated that gangliosides could antagonize ethanol-induced behavior. Experiments were conducted on homogenates of brain and liver and of liver slices of adult rats. In liver slices, cleavage of SA did not fall in proportion to the ethanol-induced inhibition of sialidase; in fact, at 0.1 M ethanol, free SA increased, even though sialidase was inhibited. Brain sialidase activity on endogenous sialoglycoconjugates was much more resistant to ethanol than liver sialidase and was fully active even in concentrations as high as 1 M. When gangliosides were incubated with liver slices in the absence of ethanol, sialidase was markedly stimulated. The ethanol-induced inhibition of sialdase in liver slices was mimicked by sorbitol, suggesting that the inhibition may be caused by a shift in redox state as a result of increased NADH. The ethanol metabolite, acetaldehyde, does not seem to be a factor, because sialidase inhibition still occurred when slices were incubated with ethanol containing pyrazole. The results indicate that ethanol promotes the accumulation of free SA in liver without stimulating sialdase; our other work suggests that the cause is an increase in accessibility to sialoglycoconjugates rather than decreased utilization of SA. Brain and liver sialidases clearly respond differently to both ethanol and gangliosides.

Alcohol Ethanol Sialidase Sialic acid Neuraminic acid Gangliosides Glycoproteins Sorbitol Pyrazole Brain Liver

SIALIC acid (SA) is a prominent constituent of many glycoproteins, which are well-known for various functions such as cell adhesion (7), cell-cell recognition and contact inhibition (13), and synaptic actions of certain neurotransmitters (15). SA is also a principal component of gangliosides, which are glycolipids that have a role in many functions, such as cell adhesion (4), binding of several toxins and hormones (9), certain antigen-antibody reactions (31), adenylate cyclase regulation (27), and synaptic transmission (34,40). They also have neuritogenic and neurono-trophic properties (9, 10, 24). SA of the liver is mostly of glycoprotein origin, while about ⅔ of the SA in brain is found in gangliosides (8).

Recent studies have indicated that ethanol, when given either acutely (16,17) or chronically (18, 30, 37–39) can decrease the level of SA in various mammalian systems. Furthermore, brain sialogangliosides were found to be decreased by acute alcohol exposure (19,20). The conditions under which SA decreases occur are not well defined, nor do we know the mechanism (e.g., increased cleavage or decreased synthesis). The functional significance of ethanol-induced SA decrements is also not known, but is potentially very relevant to ethanol abuse and alcoholism, given the fundamental role of sialoglycoconjugates in cellular function.

We postulated that ethanol would enhance the cleavage of SA, presumably because it enhances the activity of several forms of the sialidase. Another hypothesis was that added gangliosides might inhibit the sialidases and antagonize the ethanol effect. These hypotheses were tesed on sialidase of rat whole brain and liver homogenates as well as on homogenates of incubated liver slices. We also postulated that ethanol should affect brain and liver sialidases differently, because the brain has a much higher content of endogenous substrate and because brain and liver sialidases are different (33, 35, 36). Finally, we wished to test if the high lipid environment of brain would itself alter sialidase activity. We also examined whether the ethanol effect on sialidase involved redox shifts and acetaldehyde.

## METHOD

### *Experimental Design*

All experiments were performed on liver and brain of adult

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Sprague-Dawley rats. In addition to testing ethanol effects on the enzymatic hydrolysis of endogenous SA substrates (glycoproteins and gangliosides), parallel experiments were performed using an exogenous substrate, 2-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (4-MU-NANA) (26). Initial experiments were performed on tissue homogenates. However, because alcohol's action in vivo is commonly believed to involve reactions dependent upon alcohol-induced fluidization of intact membranes, we conducted additional experiments in slices of intact liver.

*Chemicals*

D-sorbitol, 4-MU-NANA (sodium salt), and pyrazole were purchased from Sigma Chemical Co., St. Louis, MO. Gangliosides that were added to homogenates were purified from rat brain as previously described (19, 20, 24). All other chemicals used were of the highest grade purity available.

*Incubation of Liver Slices*

Immediately after decapitation, livers were transferred into cold oxygenated Yamamoto solution (12) and slices of 1 mm thickness were prepared with a McIlwain tissue chopper. The incubation/perfusion apparatus consisted of 4 chambers, each with a nylon mesh that provided support for the slices. Chambers were surrounded by circulating water at 37°C. There was a sintered glass grid at the bottom of each chamber through which a mixture of 95% $O_2$ and 5% $CO_2$ continuously oxygenated the Yamamoto solution that was fed in at the inlet at the base of each chamber at a rate of 20–30 ml/hr. Slices from the same liver were divided into four equal portions and incubated with varying concentrations of the drug used. After five hours, the incubation medium was drained off and replaced with the control medium and the incubation was continued for another 30 min to remove chemicals from the system. Then slices were dried by pressing between filter papers, weighed and stored at −20°C.

*Preparation of Homogenates*

Slices were thawed and homogenized in ice in 5 volumes of 10 mM potassium phosphate buffer, pH 7.4, in an Ultra-Turrax (Tekmar Company, Cincinnati, OH) for 1 min. When whole rat brain or liver was homogenized, the tissue was frozen immediately after sacrifice and homogenized in a Potter-Elvehjem homogenizer, in ice, for 5 min in the same buffer, with a 3–5-min interval every 2 min.

*Assay of Sialidase*

Liver and brain sialidase activities were assayed under optimal conditions of pH and substrate concentration. The assay mixture consisted of 0.2 M sodium acetate, pH 5.0, and 0.2 ml of homogenate in a total volume of 0.5 ml. After incubation at 37°C for 1 hr, 0.5 ml 20% TCA was added, followed by centrifugation of the sample. The liberated sialic acid in the supernatant was measured according to Cassidy *et al.* (5). In controls, homogenate was added at the end of the incubation. Sialidase action on exogenous substrate was assayed as described (26) with minor modifications. The assay system contained 0.1 M sodium acetate (pH 5.5), 0.2 mM 4-MU-NANA, and 0.05 ml of homogenate in a total volume of 0.2 ml. Enzyme activity was stopped by the addition of 2 ml 95% ethanol; the sample was then centrifuged and the supernatant was measured for fluorescence. For the determination of free SA, a mixture of 0.5 ml of the homogenate and 0.5 ml 20% TCA was centrifuged, and the supernatant was used for assay.

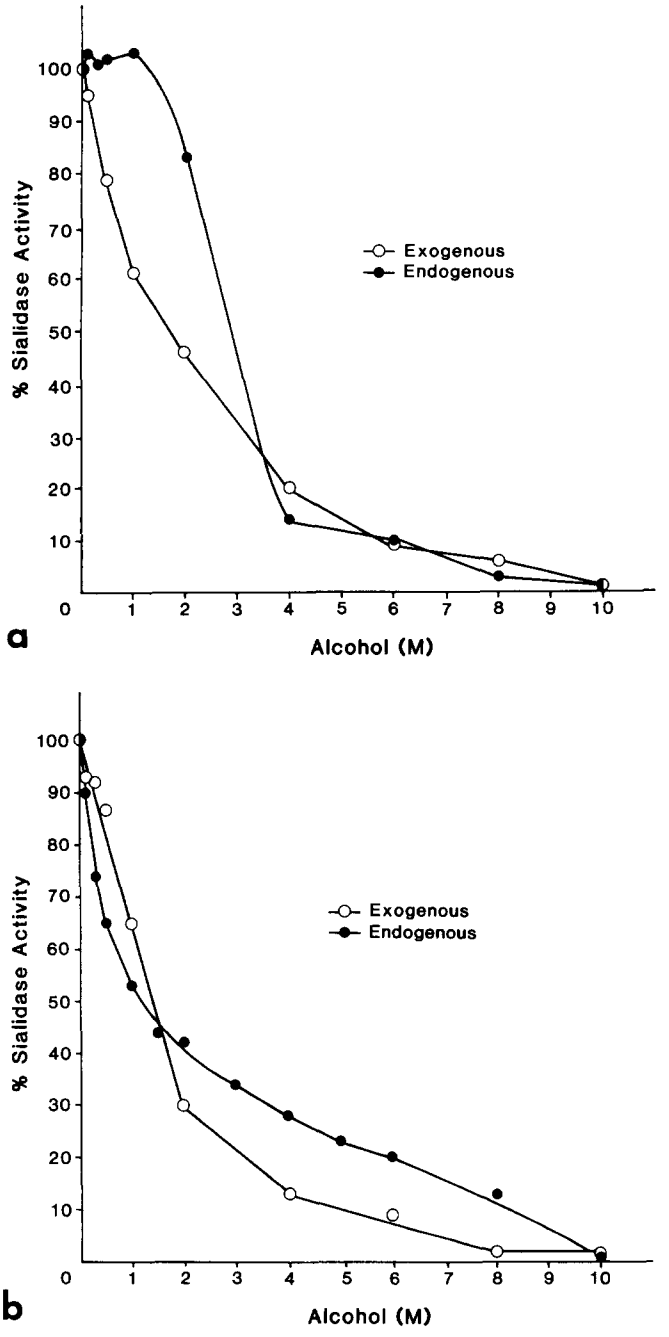


FIG. 1. (a) Ethanol effects on sialidase in rat brain homogenates. Ethanol slightly stimulated sialidase action on endogenous substrates at the lower concentrations (0.1, 0.3, 0.5, 1 M). With exogenous substrate, all concentrations inhibited the enzyme. At higher concentrations, sialidase activity on both substrates was markedly suppressed, but not completely abolished until >8 M was used. (b) Evidence that liver sialidase was more susceptible to ethanol than the brain enzyme. Sialidase was inhibited markedly at all concentrations on both endogenous and exogenous substrate.

Protein was estimated according to Bradford (2). As an index

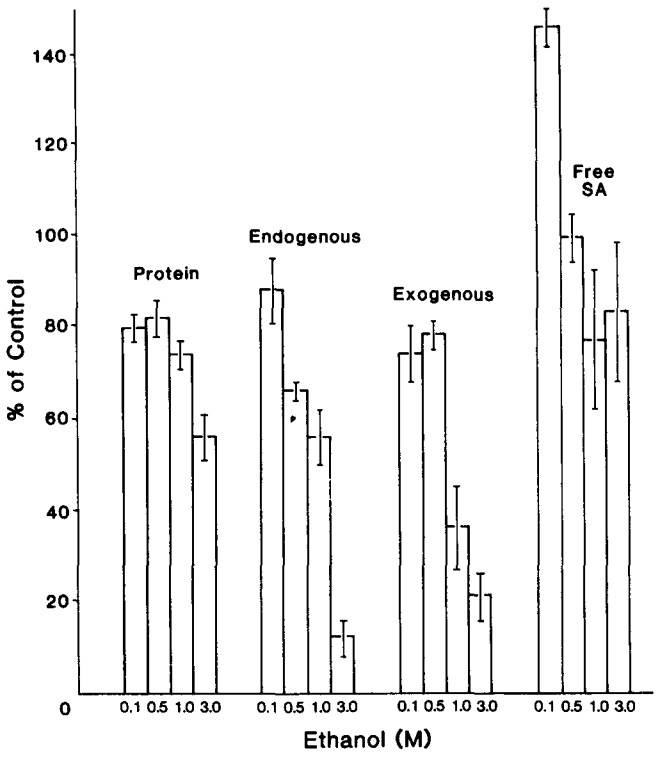


FIG. 2. Ethanol depression of sialidase in rat liver slices, with both endogenous and exogenous substrate. The depression occurs even at the lower concentrations, and is accompanied by a decrease in total protein. Free SA is actually elevated at 0.1 M ethanol, and is not decreased proportionately to sialidase activity at the higher ethanol concentrations. Sialidase activity is calculated as units/g tissue, while sialic acid is calculated as nmoles/g tissue. The values are mean ± SE of five experiments, expressed as % of control. The protein decrement at 1 and 3 g/kg is significant at $p<0.05$ and $p<0.01$ respectively. The decrement in sialidase activity on endogenous substrate at 0.5 and 1 M is significant at $p<0.002$, while that on the exogenous substrate at 1 M is significant at $p<0.01$. Free sialic acid increase at 0.1 M is significant at $p<0.001$.

of cell membrane viability, alkaline phosphatase released into the 100,000 × g supernatant was assayed as described (14) using p-nitrophenylphosphate as substrate.

*Gel Electrophoresis*

Samples of liver homogenate exposed to either ethanol, pyrazole, or sorbitol were run on 10% Laemmli gels (33) (0.75 mm thickness) approximately 5.5 cm high. The stacking gel was 5% acrylamide, approximately 2.5 cm high. Gels were run at 30 mA constant current for approximately 1 hr and stained with Coomassie Blue. Molecular weight standards were obtained from Sigma (MW-SDS-200 kit).

## RESULTS

*Sialidase Susceptibility Differences—Rat Brain and Liver*

When varying concentrations of ethanol were included in the homogenate, up to a concentration of 1 M, brain sialidase activity on endogenous substrate was unaffected, except perhaps for a mild degree of stimulation at lower concentrations (Fig. 1a). All the experiments were carried out 2–3 times and similar results were observed. Enzyme activity was linear with respect to time and amount of enzyme in all the assays. In both liver and brain homogenates, the lower concentrations of ethanol (0.1, 0.3, and 0.5 M) caused a marked, linear inhibition of sialidase activity in the presence of exogenous substrate (Fig. 1a and b). With both kinds of substrates, there was a steady ethanol concentration-dependent depression of sialidase above 1 M, and the activity was completely abolished only at 10 M.

*Sialidase Susceptibility Differences—Rat Liver Slices*

The viability of tissue slices was ascertained by incubating the slices in the presence of [$^3$H]-N-acetyl-D-mannosamine (25 μCi/g tissue). The incorporation of radioactivity into TCA-precipitable material was linear up to 4.5 hr (25). Further, there was an ethanol concentration-dependent increase in alkaline phosphatase activity in the 100,000 × g supernatant (18, 24, and 43% at 0.1, 1, and 3 M ethanol, respectively). No such increases could be demonstrated in liver homogenates, suggesting that alcohol was acting on intact, viable membranes in the slice preparation (14,41). When liver slices were incubated with varying amounts of ethanol, including the lower concentration of 0.1 M, both total protein and the enzyme activity decreased significantly (Table 1, Fig. 2). Total free sialic acid in the homogenate increased by 45% at 0.1 M concentration of ethanol ($p<0.001$), while 0.5 M ethanol did not affect free sialic acid. Although the total protein was 80, 74 and 56% of the control at ethanol concentrations of 0.1, 1.0 and 3.0 M, gel electrophoresis revealed no differential effect on any given protein at the concentrations of 0.1, 0.5, or 1 M. The corresponding values of sialidase acting on endogenous substrates were 88, 56 and 12% while those on exogenous substrates were 74, 36 and 21% respectively. Under similar incubation conditions (0.1 to 1 M ethanol), brain slices did not exhibit any decrement in protein (25).

*Effect of Pyrazole and Sorbitol on Sialidase in Rat Liver Slices*

When pyrazole, an inhibitor of alcohol dehydrogenase was used in presence of ethanol, the decrease in total protein was completely abolished, while there was no significant effect on the inactivation of sialidase (Fig. 3). When sorbitol was used instead of ethanol, the total protein was substantially decreased at all concentrations. Likewise, all concentrations of ethanol inactivated sialidase acting on both endogenous and exogenous substrates.

Gel electrophoresis was carried out to investigate whether the decrease in liver protein was in toto or whether there was a selective hydrolysis of any individual proteins. It failed to disclose any significant effect of either sorbitol or ethanol on any single protein (data not shown).

*Ganglioside Effect on Sialidase in Whole Tissue Homogenates*

Because ethanol was less potent in inhibiting brain sialidase action on endogenous substrates, as compared to liver, we hypothesized that the high concentration of gangliosides in brain serves to "protect" sialidase and that, therefore, the addition of ganglioside to liver homogenates should protect liver sialidase. Liver sialidase was actually stimulated by ganglioside, showing that liver and brain sialidases are different (Fig. 4a and b). Ganglioside produced a 3-fold stimulation of liver sialidase acting on both endogenous and exogenous substrate and also stimulated brain sialidase action on exogenous substrate by about 30%. However, brain sialidase activity on endogenous substrate was

TABLE 1

EFFECT OF ETHANOL ON RAT LIVER SIALIDASE AND FREE SIALIC ACID, EXPRESSED AS RELATIVE MEAN ± S.E.

| Ethanol (M) | Protein (mg/g tissue) | Sialidase Activity (units/g tissue) Endogenous | Exogenous | Free Sialic Acid (nmoles/g tissue) |
|---|---|---|---|---|
| 0 | 1.0 ± 0.10 | 1.0 ± 0.07 | 1.0 ± 0.12 | 1.0 ± 0.06 |
| 0.1 | 0.80 ± 0.03* | 0.88 ± 0.07 | 0.74 ± 0.06* | 1.45 ± 0.04¶ |
| 0.5 | 0.82 ± 0.04 | 0.66 ± 0.02§ | 0.78 ± 0.03 | 0.99 ± 0.05 |
| 1.0 | 0.74 ± 0.03† | 0.56 ± 0.06§ | 0.36 ± 0.09‡ | 0.77 ± 0.15 |

A unit is defined as nmoles SA liberated per hour.
*$p<0.1$, †$p<0.05$, ‡$p<0.01$, §$p<0.002$, ¶$p<0.001$.

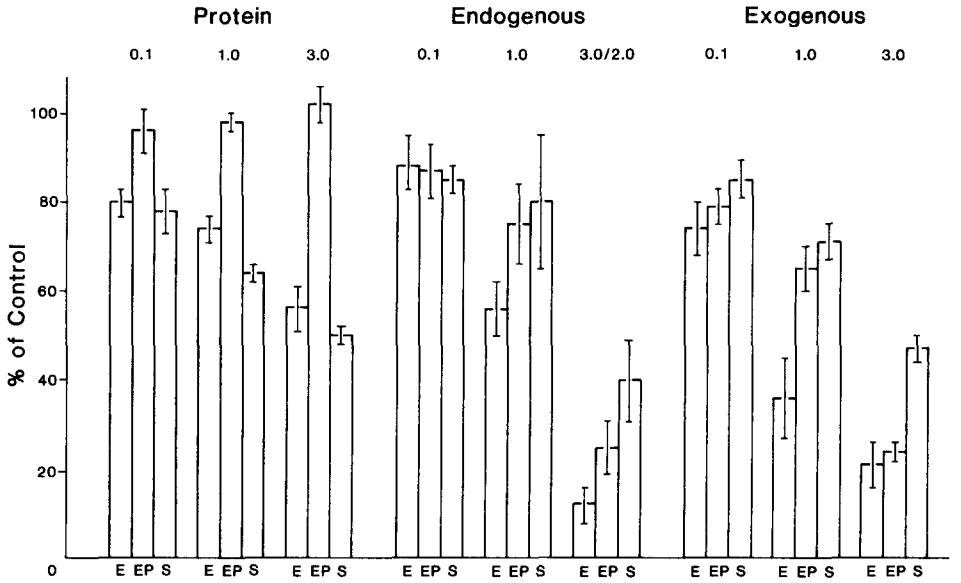


FIG. 3. Inactivation of sialidase in rat liver slices in the presence of varying amounts of ethanol (E) (0.1, 1 and 3 M), ethanol plus 2 mM pyrazole (EP), or sorbitol (S) (0.1, 1 and 2 M). Pyrazole prevents the depressive effect on protein, but not on sialidase. Sialidase depression occurs with both endogenous and exogenous substrates at all concentrations, and sorbitol mimics the ethanol effect (n = 4). Sialidase activity is calculated as units/g tissue. The values are mean ± S.E. of four experiments.

markedly inhibited by added gangliosides (Fig. 4a).

In the presence of ethanol (0.1, 0.3, and 0.5 M), added gangliosides still increased liver sialidase activity (on both endogenous and exogenous substrates), but the percentage inhibition remained the same with or without added gangliosides (data not shown).

## DISCUSSION

The major findings of these experiments were that: 1) cleavage of SA did not fall in proportion to ethanol-induced inhibition of sialidase, and, in fact, at 0.1 M ethanol free SA even increased even though sialidase was actually inhibited; 2) unlike liver sialidase, brain sialidase activity on endogenous substrates was fully active even when exposed to high concentrations of ethanol (0.3 to 1 M); 3) adding gangliosides to liver slices that were incubated without ethanol caused a marked stimulation of sialidase; and 4) inhibition by ethanol of sialidase in liver slices was mimicked by sorbitol and was not affected by concurrent incubation with pyrazole.

### *SA Cleavage Promoted Without Increase in Sialidase Activity*

Results did not support the original hypothesis that ethanol would stimulate sialidase. The important point is that low concentrations (0.1 M) of ethanol increased free SA (Fig. 2, Table 1), despite the fact that sialidase activities were not increased, and in fact were slightly inhibited, with both endogenous and exogenous substrate. Such an increase was demonstrable even though experiments were conducted with a perfused system in which the SA that was not "trapped" in the tissue would wash out with the perfusion fluid. The reason an increase in free SA has not been

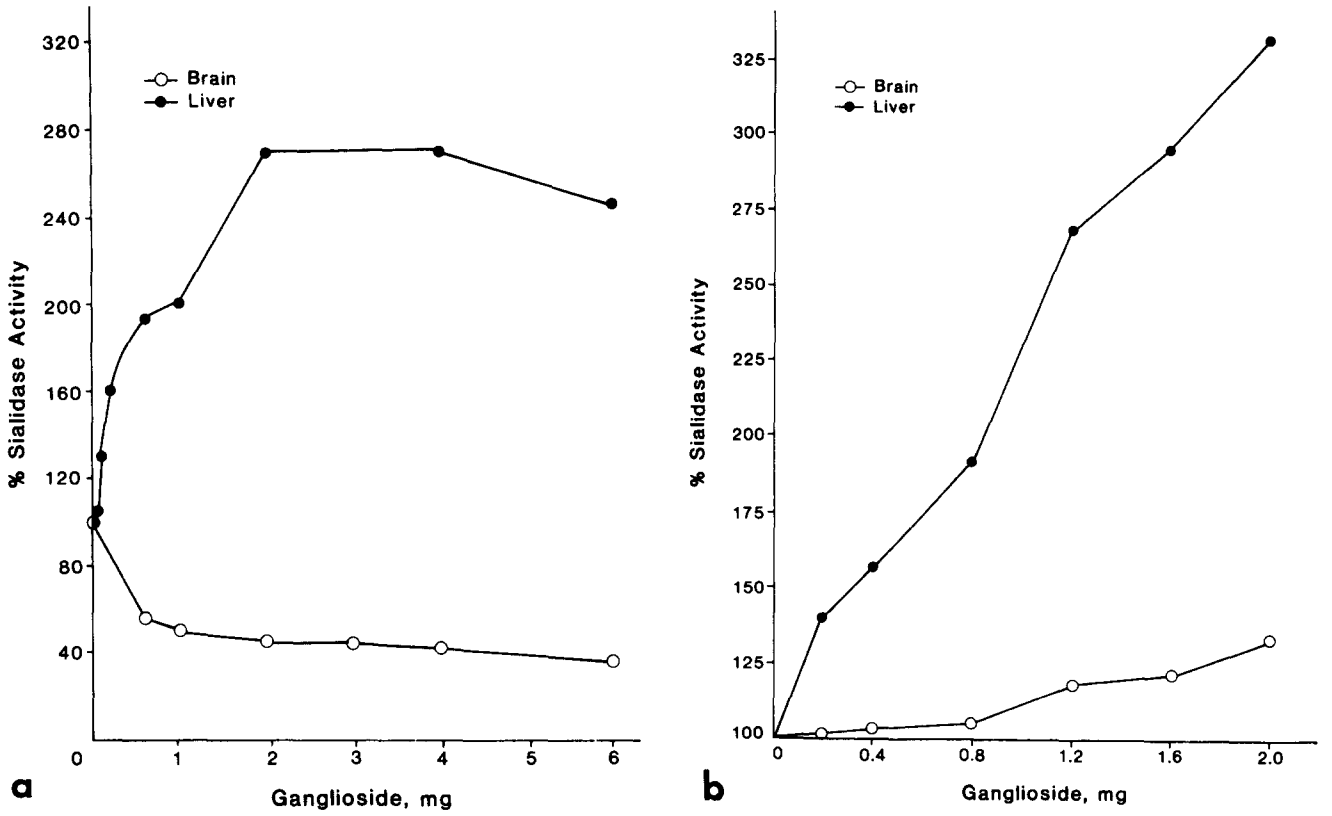


FIG. 4. (a) Gangliosides added to rat liver and brain homogenates, in the absence of ethanol, markedly stimulated sialidase action on endogenous substrates in the liver, while brain sialidase was markedly inhibited. Gangliosides (up to 6 mg/0.5 ml) were included in the assay mixture. (b) Same as a, except that exogenous substrate was used. Liver sialidase activity increased; and brain sialidase, unlike the case with endogenous substrate, was not inhibited and was even stimulated by larger concentrations of ganglioside (up to 2 mg/0.2 ml) included in the assay system.

seen in in vivo studies is presumably because in the intact animal free SA can escape into extracellular fluid, thereby creating a decrease in total tissue SA.

Note also that the free SA levels did not fall proportionately with the higher concentrations of ethanol, despite the marked inhibition of sialidase activity at these concentrations. Such observations suggest that ethanol, irrespective of concentration, increases the accessibility of sialoglycoconjugates to sialidase, compensating for any impairment of enzyme activity. The presence of SA-bearing glycolipids (23) and glycoproteins (3) that are inaccessible to membrane-bound sialidase has been demonstrated in neuronal membranes, and these sialoglycoconjugates can be made accessible to sialidase by sonication or detergents. It is possible that alcohol mediates its effect on sialoglycoconjugates in a similar way, with the destabilization/fluidization of intact membranes allowing sialidase to become more accessible to its substrates. A similar explanation has been offered for the observation that gas anesthetics promote cleavage of SA in brain (22,32). Also, clinical signs of genetic sialidase deficiency disease are improved by ethanol therapy (11), perhaps because the surplus sialoglycoconjugates are made more accessible to sialidase.

Although several lines of evidence suggest that enhanced accessibility for sialidase may be the basis for increased SA, we cannot rule out the possibility that under-utilization of SA might also contribute to at least part of the free SA pool. However, preliminary studies carried out in our laboratory both in vivo (3 g/kg) and in vitro (up to 0.5 M) suggested that ethanol does not affect incorporation of [$^3$H]-N-acetyl-D-mannosamine into the TCA-precipitable fraction in brain, supporting the idea of enhanced hydrolysis.

Note also that brain sialidase action on endogenous substrate (Fig. 1a) was fully active up to even 1 M ethanol, thus helping to explain why ethanol might promote cleavage of brain SA. In the case of liver, we report here for the first time evidence that ethanol at "physiological" concentrations (0.1 M) can promote cleavage of SA, even though sialidase activity was slightly inhibited (Table 1).

### *Differences Between Brain and Liver Sialidase*

Failure of the lower concentrations of ethanol to inhibit endogenous sialidase in brain could be interpreted to indicate that ethanol has no direct effect on the brain enzyme. However, low concentrations of ethanol did inhibit the sialidase activity on exogenous substrate in brain, while, in liver, sialidase activity was affected about equally for both endogenous and exogenous substrates (Fig. 1). The high concentration of gangliosides in brain may explain the differing response of brain and liver.

Brain enzyme action on endogenous substrates was inhibited by added gangliosides, while liver enzyme was stimulated to about 3-fold. The increased sialidase activity on exogenous substrate when gangliosides were added to brain and liver homogenate (Fig. 4b) is a true activation of sialidase, as opposed to cleavage of the added ganglioside, because the cleavage product measured is 4-methyl umbelliferone, not sialic acid. Another important influence may be that liver and brain have different kinds of sialidase

that have different substrate specificities (33, 35, 36).

Distinct ethanol inhibitory effects were seen in perfused liver slices (Fig. 2), which suggests that full expression of ethanol actions requires living cells with the enzyme operating in its normal, relatively unperturbed environment. This requirement for intact membranes seems to be supported by our observations that alkaline phosphatase can be released into 100,000 × g supernatant from liver slices by ethanol but not from homogenates. This supports an earlier report (41) that ethanol increases release of alkaline phosphatase from intact, isolated hepatocytes.

### *Protective Effect of Gangliosides*

The clear relative resistance of brain sialidase to ethanol (Fig. 1a) may be due to the fact that brain has a much higher concentration of gangliosides than does liver. We present evidence that gangliosides stimulate sialidase action on exogenous substrates in both brain and liver (Fig. 4b). Gangliosides do inhibit brain sialidase action on endogenous substrate (Fig. 4a), perhaps because ganglioside itself is a substrate. If ethanol-induced decrement of SA is part of the mechanism for intoxiation, ganglioside antagonism of intoxication (21) may occur in part because gangliosides inhibit brain sialidase (Fig. 4a) and thus interfere with SA cleavage. Gangliosides might also stabilize membranes and reduce the accessibility of substrates to sialidase.

### *Metabolic Mechanisms*

The fact that 0.1 M sorbitol mimicked the alcohol-induced inactivation of rat liver sialidase and depression of protein levels suggests that both effects may be caused by a shift in redox state as a result of high levels of NADH. Depression of protein synthesis reportedly occurs with a shift in the redox level (28,29).

Brain slices did not exhibit any protein loss (25) suggesting that liver is more vulnerable than brain, probably because brain slices are incapable of metabolizing ethanol (1). The gel electrophoretic tests indicated that all proteins were affected to the same degree. Thus, ethanol's well-known suppressant effect on protein synthesis (29) may be generalized and nonspecific.

The results obtained in the presence of pyrazole suggest that impairment of both liver and brain sialidase reflects a direct inhibition of the enzyme itself that is not due to the acetaldehyde metabolite. Because pyrazole prevented alcohol-induced decrement in protein, yet did not prevent the decrease in sialidase activity, the reduction in sialidase activity does not seem attributable to a decrease in the amount of enzyme protein.

To conclude, we would stress the importance of the observation here and in our previous work that ethanol promotes the accumulation of free SA from sialoglycoconjugates (25). If the ethanol effect is direct, as these in vitro experiments indicate, it would help to explain why gangliosides or SA can antagonize ethanol intoxication presumably by offsetting the ethanol induced release of SA as demonstrated in our previous experiments (6,21).

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