Effects of Ethanol and a Fat-Free Diet on Hepatic Mitochondrial Fragility and Fatty Acid Composition* (33654)

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Prolonged ethanol ingestion caused an increase in the fragility of rat liver mitochondria. This was demonstrated by succinic dehydrogenase (SD) assay and by electron microscopy examination (1). These studies showed that the loss of the membrane barrier to phenazine methosulfate was accompanied by the loss of the outer mitochondrial membrane. Electron microscopic examination of the livers of alcoholic patients and of rats fed ethanol revealed disruptions of the outer mitochondrial membrane in the intact liver cell (2–4), indicating an ethanol-induced increased *in vivo* fragility of the membrane.

In recent investigations into the pathogenesis of the above effect it was shown that ethanol feeding altered the fatty acid composition of mitochondrial total lipids and phosphatidyl choline (5). One of these changes was a reduction in the percentage of arachidonic acid. Turchetto, *et al.* (6) reported similar changes in total liver lipid after a single dose of ethanol. There is some evidence that an increased mitochondrial fragility could be related to the decreased proportion of arachidonic acid. In essential fatty acid (EFA) deficiency, for instance, a decrease in the percentage of arachidonic acid was associated with an increase in the permeability of mitochondria (7) together with an acceleration of mitochondrial aging (8). This could be explained by the suggestion that phospholipids containing arachidonic acid are bound more firmly to mitochondrial structural protein (9). To test this hypothesis we have correlated the level of mitochondrial fragility as measured by the SD assay technique with the proportion of arachidonic acid in these mitochondria.

Methods. Sixty Charles River male rats weighing an average of 225 g were divided into 5 groups of 12 each. Rats in group A were fed 30% ethanol (w/v) in their drinking water. They were fed a fat-free diet *ad libitum*. The composition of the fat-free diet used was that of a basal diet (10) which was modified so that 8% sucrose was substituted for the 8% fat removed from the diet. Group B was pair-fed the fat-free diet with group A and isocaloric sucrose was substituted for ethanol. Group C was pair-fed the basal diet (10) containing 2% corn oil and 6% hydrogenated cottonseed oil; isocaloric sucrose was substituted for the ethanol. Group D was pair-fed the basal diet plus 30% ethanol. Group E was fed the basal diet *ad libitum*.

Liver biopsy for SD activity (1) was done under ether anesthesia an average of 10 weeks after starting the diet. Nine animals in each group were studied. Three animals in group A lost weight on the diet. They were sacrificed along with their controls without assays being performed. At the time of biopsy group A rats averaged 335 g, group B 383 g, group C 448 g, group D 387 g, and group E 497 g. Rats on the ethanol-fat-free diet (group A) consumed an average of 21.5% of calories as protein, 45.5% as carbohydrate, and 34% as ethanol.

Liver mitochondria were isolated and purified by differential centrifugation (1). Mitochondrial SD activity was assayed manometrically with and without calcium added to the media and the percentage of maximum activity was calculated as a measure of mitochondrial fragility (1). Mitochondria for fatty acid analysis were obtained from 7 rats/group by repeat liver biopsy done an average of 3 weeks after SD assay. Mitochondria were isolated in the same way as for SD assay. Mitochondrial lipids were extracted and purified according to the method of Folch *et al.* (11). Methylation of the lipid extract was achieved by refluxing for 6 hr

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TABLE I. Effect of Fat-Free Diet and Ethanol on Rat Liver Mitochondrial Succinic Dehydrogenase (SD) Activity.

Group	Diet	SD activity[a]		
		No calcium added	Calcium added	Maximum activity (%)
A	Fat free + ethanol	89.1 ± 4.9[b]	205.3 ± 9.8	43.7 ± 2.0[d]
B	Fat free + sucrose	59.4 ± 5.0	189.3 ± 9.0	31.9 ± 2.9
C	Basal + sucrose	66.7 ± 4.4	196.9 ±10.4	34.3 ± 2.1
D	Basal + ethanol	104.9 ± 6.4[c]	196.3 ± 8.5	53.5 ± 2.8[e]
E	Basal + water	66.3 ± 4.9	184.4 ± 5.7	35.9 ± 2.3

[a] μl of O_2/mg of mitochondrial protein/hr; mean ± SE; 9 animals/group.

[b] $p < .01$ compared with groups B, C, and E.

[c] $p < .001$ compared with group B, and $< .01$ compared with groups C and E.

[d] $p < .01$ compared with group B, $< .02$ compared with groups C and D, and $< .05$ compared with group E.

[e] $p < .001$ compared with groups B and C, and $< .01$ compared with group E.

with 10 ml of methanolic sulfuric acid in benzene (12). After extraction in pentane the methyl esters were analyzed by gas-liquid chromatograph in a Barber-Coleman model 10 chromatograph at 185°C using 15% diethylene glycol succinate on Gaschrom P, 70-80 mesh. The peaks were identified by comparison with retention times of appropriate fatty acid standards.

Results and Discussion. Rats fed ethanol (groups A and D), developed a significant increase in percentage maximum activity of SD (Table I). Based on results reported previously this indicated an increase in the fragility of the outer mitochondrial membrane (1). The fat-free diet (EFA deficiency) partially reversed this effect of ethanol (group A compared to group D).

The EFA deficient diet (group B) did not cause an increase in fragility when compared with controls (groups C and E). These results differed from those of Hayashida and Portman (7). According to the data they obtained at 8 weeks on the test diets, the EFA-deficient rat liver mitochondria percentage maximum activity was 92% and the control was 56.5%. The assay method employed differed in several ways from that used in the present study and this may account for the difference in the data.

The EFA deficiency, with or without ethanol in the diet (groups A and B), caused a characteristic increase in 18:1, a decrease in 18:2 and 20:4 and the appearance of 20:3 ω 9 (5, 8, 11 eicosatrienoic acid) (Table II) (7, 12). Ethanol added to the fat-free diet (group A) resulted in a significant increase in the proportion of linoleic acid, and increase in arachidonic acid of borderline significance ($p<0.2$), and decreases in oleic acid and 5, 8, 11 eicosatrienoic acid which were also of borderline significance ($p<0.2$ for 18:1 and <0.06 for 20:3 ω (9) as compared to group B. Considered together, however, the changes reflect a partial reversal by ethanol of the characteristic EFA deficiency pattern produced by the fat-free diet. This effect could be a result of a stimulation of fatty acid synthesis by ethanol (13–15) or an inhibition of fatty acid oxidation (14, 16, 17). Either of these mechanisms could result in a sparing of the mitochondrial structural essential fatty acids.

Ethanol lessened the EFA-induced decrease in 18:2 more than that of 20:4, as indicated by the ratio of 20:4 to 18:2 in groups A and B (Table II). This is consistent with the ethanol induced increase in 18:2 (group D) when compared with sucrose (group C) ($p<0.05$). The net effect of ethanol was to decrease the ratio between 20:4 and 18:2 ($p<0.05$ when group D was compared to group C) (5).

Correlation of mitochondrial fragility with the mitochondrial fatty acid composition revealed no correlations between the percentage of 20:4 and the percentage maximum activity of SD. For example, 20:4 was lowest in

TABLE II. Effect of Fat-Free Diet and Ethanol on Rat Liver Mitochondria Fatty Acid Composition.

Group:	A	B	C	D	E
Diet:	Fat free + ethanol	Fat free + sucrose	Basal + sucrose	Basal + ethanol	Basal + water
Fatty acid					
16:0	20.8 ± 1.4[a]	21.3 ± 1.1	21.2 ± 1.3	21.5 ± 1.9	21.3 ± 0.9
16:1	5.8 ± 0.4	5.8 ± 0.4	4.0 ± 0.4	3.9 ± 0.5	2.7 ± 0.4
18:0	20.1 ± 0.6	19.7 ± 1.1	17.0 ± 0.8	15.8 ± 0.4[d]	17.8 ± 0.7
18:1	23.9 ± 1.1	26.2 ± 1.2	18.6 ± 0.7	17.3 ± 0.6[d]	16.0 ± 0.6
18:2	6.1 ± 0.5[b]	4.0 ± 0.4	12.9 ± 1.1	16.8 ± 1.4[d]	16.6 ± 1.0
20:3 ω 9	9.2 ± 0.6	11.9 ± 1.1	—	—	—
20:3 ω 6	—	—	2.4 ± 0.3	2.3 ± 0.4	—
20:4	14.8 ± 0.8	12.1 ± 0.8	23.8 ± 1.2	22.0 ± 1.5[d]	25.0 ± 0.7
20:4/18:2	2.4 ± 0.3[c]	3.1 ± 0.1	1.9 ± 0.1	1.4 ± 0.2	1.5 ± 0.1

[a] Percentage fatty acid; mean ± SE; 7 rats/group.
[b] $p < 0.01$ compared with group B and $p < 0.001$ compared with group D.
[c] $p < 0.05$ compared with groups B and D.
[d] $p < 0.001$ compared with group A.

group B where the percentage maximum SD activity was also lowest (group B), but where 20:4 was highest, the percentage maximum activity was still low (group E). Where the percentage maximum activity was highest, the 20:4 was either high or low (groups A and D). It was concluded that under the experimental conditions employed here, the level of arachidonic acid in mitochondria was not critical for maintaining outer membrane stability.

Summary. The effect of EFA deficiency and chronic ethanol ingestion on liver mitochondrial fragility and fatty acid composition was studied using young growing male rats. The results indicated that EFA deficiency (fat-free diet) partially protected the mitochondria from developing the increased fragility caused by ethanol ingestion. The mitochondrial fragility induced by chronic ethanol feeding was not related to changes in the proportions of arachidonic acid or other mitochondrial fatty acids. Additionally, it was found that chronic ethanol feeding partially prevented the changes in the mitochondrial fatty acid composition induced by EFA deficiency.

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1. French, S. W., Gastroenterology 54, 1106 (1968).
2. Edmondson, H. A., Peters, R. I., Frankel, H. H., and Borowsky, S., Medicine 46, 119 (1967).
3. Iseri, O. A., Lieber, C. S., and Gottlieb, L. S., Am. J. Pathol. 48, 535 (1966).
4. Klion, F. M. and Schaffner, F., Digestion 1, 2 (1968).
5. French, S. W. and Morin, R. J., *in* "Biochemical and clinical aspects of alcohol metabolism." Thomas, Springfield, Illinois, in press.
6. Turchetto, E., Ottani, V., Zanetti, P., and Weiss, H., Nutr. Dieta 12, 224 (1968).
7. Hayashida, T. and Portman, O. W., J. Nutr. 81, 103 (1963).
8. Johnson, R. M., J. Nutr. 81, 411 (1963).
9. De Purry, G. G. and Collins, F. D., Chem. Phys. Lipids 1, 1 (1966).
10. French, S. W., J. Nutr. 91, 292 (1967).
11. Folch, J., Lees, M., and Sloane-Stanley, G. H., J. Biol. Chem. 226, 497 (1957).
12. Morin, R. J., Bernick, S., Mead, J. F., and Aflin-Slater, R. B., J. Lipid Res. 3, 432 (1962).
13. Lieber, C. S. and Schmid, R., J. Clin. Invest. 40, 394 (1961).
14. Reboucas, G. and Isselbacher, K. J., J. Clin. Invest. 40, 1355 (1961).
15. Alexander, N. M., Scheig, R., and Klatskin, G., J. Lipid Res. 7, 197 (1966).
16. Lieber, C. S., Lefèvre, A., Spritz, N., Feinman, L., and De Carli, L. M., J. Clin. Invest. 46, 1451 (1967).
17. Zakim, D. and Green, J., Proc. Soc. Exptl. Biol. Med. 127, 138 (1968).

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