Biochemical Pharmacology, Vol. 31, No. 15, pp. 2447–2452, 1982.
Printed in Great Britain.

0006-2952/82/152447-06 $03.00/0

EFFECTS OF ETHANOL ON FATTY ACID COMPOSITION OF MUSCLE PHOSPHOLIPIDS OF RATS FED NUTRITIONALLY COMPLETE LIQUID DIETS

ROSEANN L. SHOREY,* BOB PYLE, MARGIE MCALLISTER, STIRLING S. MILLER, CARLTON K. ERICKSON and GUY A. THOMPSON, JR.

Divisions of Nutrition and Biological Sciences, College of Natural Sciences; Division of Pharmacology, College of Pharmacy; and Clayton Foundation Biochemical Institute, University of Texas at Austin, Austin, TX 78712, U.S.A.

(*Received* 27 *July* 1981; *accepted* 29 *October* 1981)

Abstract—Although the effects of ethanol on phospholipid fatty acid composition have been examined in single cell organisms and in rodents given ethanol as a vapor or in solution as sole drinking fluid, there are no reports of analogous experiments with nutritionally adequate liquid diets as the source of ethanol. In this experiment, young, 88 ± 5 g, male Sprague–Dawley rats given a nutritionally adequate liquid diet containing ethanol voluntarily consumed 12–18 g ethanol per kg body weight per day after 23 days. In 27 days with food intakes 64% of control, weight gains of ethanol-fed animals (group 2) were 50% of *ad lib.*-fed control animals (group 1) but 88% of isoenergetically pair-fed animals (group 3). Thus, energy in the ethanol diet was utilized for growth 88% as efficiently as isoenergetic diets containing dextrin. Liver and gastrocnemius muscle weights of group 2 were significantly lower than group 3 but brain weights were not similarly affected. Blood ethanol levels determined on days 14 and 24 were above 200 mg/dl at 10:00 p.m. and 7:00 a.m. but decreased to 50 mg/dl at 4:00 p.m. on a feeding schedule that began at 5:00 p.m. Dependence was confirmed by withdrawal symptomology. Compared to group 1, phospholipids isolated from gastrocnemius muscles of group 2 exhibited significant modifications in fatty acid composition. In ethanol-fed animals, 18:0 and 20:4 were lower, and 18:1 and 18:2 were higher, than *ad lib.*-fed controls. However, when group 2 was compared to group 3, the pair-fed control, there was no significant difference in fatty acid composition. The observed changes in fatty acid composition appear to have been due to the reduced food consumption that accompanied the model rather than to ethanol *per se*. These findings underline the importance of appropriate controls in liquid diet animal models of alcoholism.

A convenient model for human alcoholism involves administration of ethanol to animals as a component of a total liquid dietary formulation [1]. Such a model was developed in this laboratory [2] and was updated to meet recommendations of the American Institute of Nutrition [3]. This nutritionally adequate liquid diet containing ethanol has been shown to produce dependence and tolerance in rats [4].

The effect of ethanol on membrane fatty acid composition has been studied in single cell organisms, in rodents given ethanol in drinking fluid, and in mice exposed to ethanol vapor. Ingram [5] found that long chain alcohols produced a decrease in unsaturated fatty acids in *Escherichia coli* and that short chain alcohols, including ethanol, produced an increase in unsaturated fatty acids. Nandini-Kishore *et al.* [6] reported that *Tetrahymena* changed its membrane composition upon exposure to ethanol by decreasing 16:1 and 16:2, while markedly increasing 18:2. Ihrig *et al.* [7] reported increased 16:0 and 18:2 and decreased 20:4 and 20:4/18:2 ratio in liver mitochondria and erythrocyte ghosts of mice given 30% ethanol (w/v) in drinking water. Similarly, Reitz *et al.* [8] found the percentage of 18:2 to be increased significantly and of 20:4 to be decreased significantly compared to controls in rats given 25% (v/v) ethanol as drinking fluid. Littleton and John [9] noted a significant increase in 18:0, and a decrease in 20:4, in synaptosomes of mice after 10 days of exposure to ethanol vapor.

The effects of ethanol on fatty acid composition of skeletal muscle membranes in animals chronically fed ethanol in the form of a nutritionally complete liquid diet have not been reported. The purpose of this study was to determine the effects of ethanol on growth, organ weights, and fatty acid composition of muscle phospholipids in young rats fed a nutritionally complete liquid diet formulation containing 35% of energy as ethanol. Additionally, the effect of the food deprivation which often accompanies the model was distinguished from the effect of ethanol *per se* by including two control groups in the experiment—one given *ad lib.* access to control diet and one isoenergetically pair-fed control diet at the level of intake of the ethanol group. A preliminary report of these data has been published [10].

MATERIALS AND METHODS

Animal treatment. Male Sprague–Dawley rats were obtained from Sprague–Dawley (Madison, WI) and housed separately in stainless steel mesh-bottomed cages. Standard laboratory conditions and a

* Address all correspondence to: Dr. RoseAnn L. Shorey, Department of Home Economics, Graduate Nutrition Division, The University of Texas at Austin, Austin, TX 78712.

12-hr light/dark cycle were maintained. Animals were given continuous access to stock diet (Purina Chow) and deionized water for 3 days. Rats were then divided into three groups of ten animals each and were given control liquid diet without ethanol for 2 days. Following this adjustment period, animals in group 1 were given *ad lib.* access to a control diet. Animals in group 2 received liquid formulation containing ethanol in increasing concentrations from 20% of energy [2.5% (w/v) ethanol] for 2 days, 30% of energy [4% (w/v) ethanol] for the next 2 days, and 35% of energy [4.5% (w/v) ethanol] for the remainder of the 27-day experiment. Animals in group 3 were fed isoenergetic diets in which dextrin replaced ethanol in amounts equal to the previous day's consumption of group 2. The diet contained 19% of energy as protein (casein + methionine), 67.5% as carbohydrate (dextrin and sucrose), 12% as fat (corn oil) plus AIN vitamin and mineral mixtures [3] and choline bitartrate. Ethanol replaced dextrin at 20, 30 or 35% of calories. The liquid diet (Shorey–AIN) was prepared and administered as described by Miller *et al.* [4]. The procedure was modified to allow feeding from 5:00 p.m. to 2:00 p.m. daily.

All animals were killed at 2:00 p.m. by decapitation on day 27 following the withdrawal protocol. Livers, brains and gastrocnemius muscles were excised, blotted, and weighed immediately after the animals were killed.

Blood ethanol. Blood ethanol levels were determined from samples (50 μl) taken from the tails of chronically ethanol-treated rats. Samples were collected at 10:00 p.m., 7:00 a.m., and 4:00 p.m. on days 8, 14, and 24 and were analyzed directly with a Perkin–Elmer F-40 gas chromatograph by the micro-method described by LeBlanc [11] modified as reported by Erickson [12].

Withdrawal. Animals in group 2 were rated for withdrawal from ethanol using a protocol previously described [4, 13]. On the night before the tremor–rigidity scoring session, ethanol-treated rats were intubated at 8:00 p.m. with 10% ethanol (w/w) made in the same proportions as the ethanol diet. The dose of ethanol varied with the existing state of intoxication of the animals so as to produce similar blood ethanol concentrations in all animals. The method of intubation was similar to that described by Majchrowicz [14]. Animals considered normal received doses of 5 g/kg ethanol. Following intubation, liquid diets were removed and replaced with deionized water. Determination of severity of withdrawal symptoms was made using a behavioral scoring system adapted from that described by Majchrowicz [14] and modified by Goldman *et al.* [13]. The withdrawal protocol was conducted on day 27. Rats were scored beginning 11 hr and ending 19 hr, after withdrawal of ethanol.

Fatty acid analysis. Gastrocnemius muscles from both legs were combined, weighed, frozen quickly and stored at −70° for subsequent analysis. Total lipids were extracted by a modification of the method of Bligh and Dyer [15] and stored either in chloroform or in chloroform–methanol (6:1). Neutral lipids and phospholipids were separated by silicic acid column chromatography. Neutral lipids were eluted with 60 ml of chloroform, and phospholipids were eluted with 85 ml of chloroform–methanol (1:1). Samples were evaporated to dryness and stored in 2 ml of chloroform–methanol (6:1). Methyl esters of fatty acids were prepared with boron trifluoride–methanol reagent by the method of Morrison and Smith [16], and fatty acid composition was determined on a Gas-Varian 3700 chromatograph equipped with a 1/4in. stainless steel column packed with 10% diethylene-glycol succinate (DEGS) on 100 mesh chromosorb support. NIH fatty acid standards were obtained from Applied Science Laboratories (Waltham, MA).

Statistical analysis. Data from ten animals in group 1, nine in group 2, and eight in group 3 were analyzed by the use of a computerized statistical package, Omnitab [17], maintained on file at the computation center of the University of Texas. One-way analysis with pair-wise multiple comparison of means was used to determine significant differences between groups.

RESULTS

Energy intake and growth. Table 1 indicates the effects of ethanol on energy intake and growth. Ethanol-treated rats (group 2) voluntarily consumed 65% of the energy intake of the *ad lib.* control group (group 1). The adequacy of the pair-feeding protocol was confirmed by the measured intake of the pair-fed group (group 3). Total mean ethanol consumption was 45 g in the 27-day experiment. By day 6 group 2 was consuming ethanol in excess of 12 g/kg body weight, a level maintained or exceeded throughout the study. No animal in group 2 refrained from consuming the diet on any day.

The gain in weight of the ethanol-treated group was 86 g or 50% of group 1; that of the pair-fed group 3 was 98 g or 58% of control. This difference was not significant but it has been seen repeatedly in other experiments. Of the 12 g differential in weight gain between ethanol- and pair-fed animals, 9 g was present by day 15 of the experiment. The difference persisted and was slightly augmented by the 3 g greater growth rate of pair-fed animals over the next 7 days (data not shown).

Figure 1 illustrates the efficiency of utilization of food energy for growth, calculated as g gained per kcal consumed over the 27-day experiment. The calculation indicates the efficiency of utilization of ethanol diet versus control diet for growth. For several days following the introduction of ethanol, growth efficiencies in all groups declined and then increased until day 11. After this rise, efficiency ratios of ethanol- and pair-fed animals stabilized, whereas the ratio of the *ad lib.* control rats declined due to their more rapid growth, as shown in Table 1. The growth efficiency of ethanol-fed animals was significantly less ($P < 0.001$) than pair-fed and *ad lib.*-fed animals from day 13 to 27. For the entire experimental period, growth efficiencies for all groups were significantly different ($P < 0.001$) with ethanol-fed being lowest, 0.092 ± 0.0024 g/kcal consumed; pair-fed animals intermediate, 0.108 ± 0.0036; and *ad lib.* control highest, 0.119 ± 0.0012.

Table 1. Food consumption and weight gain

Group	Treatment	N	Food consumption† (kcal)	Percent of control	Final body wt*‡ (g)	Total weight gain* (g)	Percent of control	Percent of pair-fed
1	Control diet (*ad lib.*)	10	1429 ± 49[b]§		258 ± 9[b]	171 ± 7[b]		
2	Ethanol diet	9	931 ± 25[a]	65	174 ± 6[a]	86 ± 4[a]	50	88
3	Control diet (pair-fed to group 2)	8	912 ± 3[a]	64	187 ± 2[a]	98 ± 3[a]	58	

* Values are means ± S.E.
† Food consumption for 27 days ± S.E.
‡ Initial body weight was 88 ± 5 g for all animals.
§ In this and subsequent tables, the significance of difference between group means is shown as a value for F probability (F) obtained from the F ratio of analysis of variance. The Newman–Keuls technique, Hartley modification, was used for pairwise multiple comparison of means. Means within columns not followed by a common superscript are significantly different at the 0.05 level of confidence [17]. Significance was not determined for percentages.

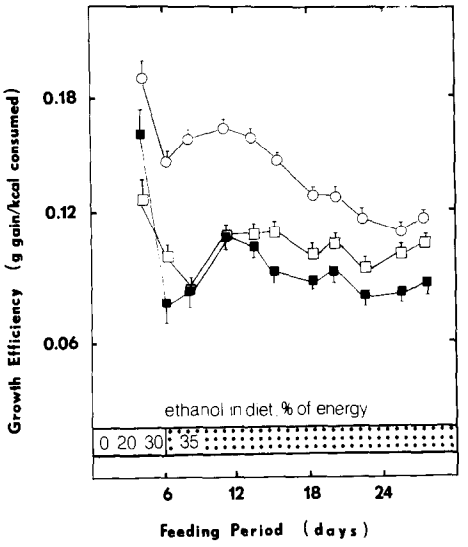

Fig. 1. Growth efficiency ratios (± S.E.) of rats fed control diet *ad lib.* (○—○), diets containing 35% of energy as ethanol *ad lib.* (■—■), or control diet pair-fed to the ethanol group (□—□).

Organ weights. In Table 2, organ weights are shown as total wet weight and relative weight, calculated as g per 100 g body weight. Muscle weight of ethanol-fed animals was significantly less than groups 1 and 3. This difference persisted when relative muscle weight was calculated. Liver weights were similarly affected, being significantly depressed in ethanol-fed animals not only as absolute wet weight, 5.9 vs 7.28 g, but also as a function of a total body size. In contrast, brain weights of ethanol-fed animals were identical to pair-fed.

Blood ethanol concentrations. Ethanol consumption and blood ethanol concentrations of ethanol-treated rats are shown in Table 3. Consumption figures are for the day preceding blood sampling because the sampling protocol resulted in lower food intake for the day. Blood ethanol concentrations exceeded 200 mg/dl at 10:00 p.m. and 7:00 a.m. but declined to about 50 mg/dl prior to feeding, 4:00 p.m.

Withdrawal scores. Mean total tremor–rigidity scores following withdrawal of ethanol indicated that, from a uniformly intoxicated state, rats exhibited increasingly more severe tremors and rigidity. Mean total scores were 6.4 ± 1 after 11 hr and progressed 18 hr after intubation to 11.2 ± 0 (maximum possible score = 12). The withdrawal curve obtained in this experiment (data not shown) was virtually identical to that previously published by Miller *et al.* [4]. In that paper [4], it was shown that animals fed the nutritionally adequate liquid diet developed dependence as indicated by the more severe withdrawal signs of ethanol-fed versus similarly treated control-fed animals.

Fatty acid composition. The fatty acid composition of phospholipids isolated from pooled gastrocnemius muscle is shown in Table 4. There were no significant differences in 16:0 or 16:1 between the three groups. Both 18:0 and 20:4 were decreased significantly in groups 2 and 3 compared to group 1; 18:1 and 18:2 were significantly increased in the same groups. In

Table 2. Mean organ weights of rats

Group	Description	Muscle*		Liver		Brain	
		Absolute wet weight (g ± S.E.)	Relative weight (g/100 g body wt)	Absolute wet weight (g ± S.E.)	Relative weight (g/100 g body wt)	Absolute wet weight (g ± S.E.)	Relative weight (g/100 g body wt)
1	Control diet (*ad lib.*)	3.06 ± 0.01[c]†	1.23 ± 0.03[b]	10.27 ± 0.28[c]	4.12 ± 0.10[c]	1.72 ± 0.02[b]	0.70 ± 0.02[a]
2	Ethanol diet (*ad lib.*)	1.73 ± 0.10[a]	1.06 ± 0.04[a]	5.90 ± 0.26[a]	3.65 ± 0.10[ab]	1.62 ± 0.03[a]	1.01 ± 0.04[c]
3	Control diet (pair-fed to group 2)	2.33 ± 0.07[b]	1.27 ± 0.03[b]	7.28 ± 0.28[b]	3.98 ± 0.13[bc]	1.63 ± 0.03[a]	0.89 ± 0.02[b]

* Total gastrocnemius muscle from both legs.
† Means within columns are significantly different, $P < 0.05$, if followed by a different superscript letter as described in Table 1.

no variable was there a significant difference between animals fed 35% of energy as ethanol and isoenergetically pair-fed animals.

DISCUSSION

Young Sprague–Dawley rats were given *ad lib.* access to nutritionally adequate liquid diets containing increasing concentrations of ethanol. Ethanol levels reached 35% of total energy by day 6. As is often noted in animal models [1], food consumption and the rate of growth of ethanol-fed rats were depressed when compared to *ad lib.* control animals. However, with the exception of the first day of introduction of high ethanol concentrations in formulas, animals fed the Shorey–AIN liquid diet protocol gained weight throughout the 4-week experiment. In a previous experiment [13], older rats on a similar protocol showed greater inhibition in weight gain.

Ethanol-fed animals gained slightly less weight than the isoenergetically pair-fed animals. Comparison of weight gains of ethanol-treated and pair-fed animals is indicative of the efficiency of energy extraction from ethanol and, hence, is suggestive of the predominant mechanism of the metabolism of ethanol in this model. It has been reported that alcohol dehydrogenase (ADH) is the predominant enzyme system, but that the concentration of ethanol and pretreatment affect the extent of peroxide-generating systems that utilize $NADPH_2$ [18]. Since such systems consume rather than generate energy, it would be predicted that, if $NADPH_2$ were extensively used under the conditions of this experiment, growth efficiency ratios, i.e. the g gained per kcal consumed of ethanol-fed and isoenergetically pair-fed animals, would differ by the extent of wastage. In the calculation of growth efficiency ratio, body composition of the animals is assumed to be the same, the absorption of nutrients is presumed identical, 1 g of ethanol is set equal to 7.1 kcal, and the amount of ethanol lost through evaporation from the very small surface area exposed in the Richter feeding tubes is deemed insignificant. As shown in Fig. 1, growth efficiency ratios of pair-fed rats were significantly greater than the ethanol-fed groups on days 13–27. The difference in overall efficiency ratios was 15%. The difference would be even less if ethanol-fed animals had a greater carcass fat composition. We conclude that at least two-thirds, or most, of the ethanol is metabolized via the ADH pathway in this model. This is similar to the extent determined by Wendell and Thurman [18] from ethanol elimination rates.

Selective effects of ethanol on components of lean body mass are suggested by the lower liver and muscle weights of ethanol-fed animals. When calculated as a function of body weight, ethanol-fed animals had greater relative brain weights, suggesting that brain weight was conserved or grew under conditions that inhibited liver and muscle growth. Carcass analysis or densiometric measurement would be required to substantiate this observation.

Ethanol consumption of young rats after 13 days ranged from 10.1 to 18.0 g/kg body weight, and mean consumption ranged from 12.8 to 15.6 g/kg. This is

Table 3. Ethanol consumption and blood ethanol levels

	Ethanol consumption (g/kg)			Blood ethanol (mg/dl) time		
				10:00 p.m.	7:00 a.m.	4:00 p.m.
Day 13*	14.2 ± 0.2†	13.4–15.0‡	Day 14	208 ± 11†	239 ± 21†	54 ± 6†
Day 23	15.6 ± 0.6	11.9–18.0	Day 24	218 ± 17	254 ± 29	48 ± 1

* Differences between days were not significant.
† Mean ± S.E.
‡ Range.

Table 4. Fatty acid composition of muscle phospholipids*

Fatty acid	Percent of total fatty acids Group 1 (*ab lib.* control)	Group 2 (ethanol-fed)	Group 3 (pair-fed)
16:0	26.93 ± 0.41[a]†	28.05 ± 0.27[a]	28.18 ± 0.51[a]
16:1	1.32 ± 0.07[a]	1.55 ± 0.07[a]	1.50 ± 0.06[a]
18:0	15.37 ± 0.18[b]	14.33 ± 0.26[a]	14.65 ± 0.17[a]
18:1	8.56 ± 0.20[a]	9.42 ± 0.21[b]	9.44 ± 0.13[b]
18:2	22.09 ± 0.44[a]	24.70 ± 0.37[b]	23.80 ± 0.56[b]
20:4	20.80 ± 0.45[b]	16.63 ± 0.37[a]	17.53 ± 0.54[a]

* Gastrocnemius muscle from both legs was pooled for determination. Values are means ± S.E.
† Significantly different, $P < 0.05$, across rows if followed by a different superscript letter as described in Table 1.

a greater ethanol intake than that previously reported from this laboratory for older animals [2, 4]. The ethanol intake is similar to that reported by other investigators [1, 19–21]. High ethanol intakes were accompanied by blood ethanol levels (BEL) of 200–250 mg/dl. We observed that ethanol-fed animals decreased their average intake on the 2 nights when tail vein blood samples were taken for BEL determination. BEL fell to about 50 mg/ml prior to the offering of fresh diet late in the afternoon. The observation of similar low levels in the afternoons of days 15 and 23 suggests that the animals were metabolizing almost all of the 12–15 g/kg ethanol voluntarily ingested during the time interval and that there was little or no carry over of ethanol.

In this analog of chronic alcoholism, a liquid diet was used and both *ad lib.*- and pair-fed controls were included. Confirming previous reports [4], substantial elevations of BEL were observed and the animals exhibited severe withdrawal signs.

In this experiment, elevated BEL were produced by a liquid diet over a 27-day period. The results obtained in this study are similar to other reports of the effects of ethanol on fatty acid composition of membranes of rodents. The percentages of 18:2 and 18:0 were increased and that of 20:4 decreased compared to *ad lib.*-fed controls [8, 9]. This study differs in one very important way, however. In none of the fatty acid variables was the ethanol group distinct from the isoenergetically pair-fed group, indicating that the changes in fatty acid composition in muscle of chronically ethanol-fed animals mimicked changes in energy-deprived animals. Our conclusion is that the observed changes in fatty acid composition toward more saturated moieties cannot be attributed to the direct effect of ethanol. Other studies are necessary to determine the mechanism by which ethanol alters membrane fluidity and if there are alterations in variables other than cholesterol [22].

REFERENCES

1. L. A. Pohorecky, *Fedn Proc.* **40**, 2056 (1981).
2. R. L. Shorey, P. Terranella and W. Shive, *J. Nutr.* **107**, 614 (1977).
3. J. G. Bieri, G. S. Stoewsand, G. M. Briggs, R. W. Philips, J. C. Woodard and J. J. Knapka, *J. Nutr.* **107**, 1340 (1977).
4. S. S. Miller, M. E. Goldman, R. L. Shorey and C. K. Erickson, *Psychopharmacology* **68**, 55 (1980).
5. I. O. Ingram, *J. Bact.* **125**, 670 (1976).
6. S. G. Nandini-Kishore, S. M. Mattox, C. E. Martin and G. A. Thompson, *Biochim. biophys. Acta* **551**, 315 (1979).
7. T. J. Ihrig, S. W. French and R. J. Morin, *Fedn. Proc.* **28**, 626 (1969).
8. R. C. Reitz, E. Helsabeck and D. P. Mason, *Lipids* **8**, 80 (1973).
9. J. M. Littleton and G. John, *J. Pharm. Pharmac.* **29**, 579 (1977).
10. R. L. Shorey, B. Pyle and G. A. Thompson, Jr., *Fedn Proc.* **40**, 800 (1981).
11. A. E. LeBlanc, *Can. J. Physiol. Pharmac.* **46**, 665 (1968).
12. C. K. Erickson, K. I. Koch and J. W. McGinity, *Pharmac. Biochem. Behav.* **13**, 781 (1980).
13. M. E. Goldman, S. S. Miller, R. L. Shorey and C. K. Erickson, *Pharmac. Biochem. Behav.* **12**, 503 (1980).

14. E. Majchrowicz, *Psychopharmacologia* **43**, 245 (1975).
15. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
16. W. R. Morrison and L. M. Smith, *J. Lipid Res.* **5**, 600 (1964).
17. D. Hogben, S. T. Peavy and R. V. Varner, *Omnitab II, User's Reference Manual*, Technical Note 552, National Bureau of Standards, Washington, DC (1971).
18. G. D. Wendell and R. G. Thurman, *Biochem. Pharmac.* **28**, 273 (1979).
19. R. F. Geisler, B. E. Hunter and D. W. Walker, *Psychopharmacology* **56**, 287 (1978).
20. L. A. Pohorecky, *Psychopharmacology* **50**, 125 (1976).
21. C. S. Lieber and L. M. DeCarli, *Res. Commun. Chem. Path. Pharmac.* **6**, 983 (1973).
22. D. B. Goldstein and J. H. Chin, *Fedn Proc.* **40**, 2073 (1981).