Pharmacology Biochemistry & Behavior, Vol. 13, Suppl. 1, pp. 191–195. Printed in the U.S.A.

Alcohol Tolerance in *Escherichia coli*[1]

L. O. INGRAM, N. S. VREELAND AND L. C. EATON

Department of Microbiology and Cell Science and Department of Immunology and Medical Microbiology
University of Florida, Gainesville, FL 32611

INGRAM, L. O., N. S. VREELAND AND L. C. EATON. *Alcohol tolerance in* Escherichia coli. PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 191–195, 1980.—During growth with ethanol, the proportion of 18:1 fatty acid in the lipids of *E. coli* increases at the expense of saturated fatty acids. The significance of these changes was investigated in terms of growth and survival in the presence of ethanol. Two approaches were used: (1) A comparison of alcohol tolerance among strains of *E. coli* with different fatty acid compositions; (2) A comparison of alcohol tolerance using a lipid mutant in which the fatty acid composition was controlled by exogenous supplements. An increase in unsaturated fatty acid content was beneficial for both growth and survival. We conclude that the alcohol-induced changes in the fatty acid composition of *E. coli* are part of an adaptive response, compensating for some of the harmful effects of this drug.

Ethanol Alcohol Bacteria *Escherichia coli* Lipids Membranes Fatty acids Alcohol tolerance

MICROBIAL cells are remarkable in their ability to adapt to environmental changes. These adaptive processes are particularly well-documented in the bacterium, *Escherichia coli*, during shifts in levels of nutrition [17] and during shifts in growth temperature [5,14]. During growth in the presence of ethanol, a major fermentation product of *E. coli* [4], numerous changes occur in membrane composition. These changes include an increase in 18:1 fatty acid at the expense of 16:0 [10], an increase in phospholipid molecules containing two unsaturated fatty acids [1], an increase in anionic phospholipids [9] and numerous changes in the proportions of membrane proteins (unpublished observations). Some or all of these changes could be involved in adaptation to ethanol. In this study, we have investigated the significance of the changes in fatty acid composition for growth and survival in the presence of ethanol.

METHOD

Organisms and Growth Conditions

Three strains of *Escherichia coli* K-12 were used in this study. All contained a defect in fatty acid degradation (*fad* E^-). Strain TB4 is wild type for fatty acid synthesis [2]. Strain WN1 and a derivative, strain BB3, contain a defect in fatty acid synthesis (*cvc*$^-$) and are unable to synthesize vaccenic acid [16]. All three strains were grown in complex medium, Luria broth [13], without added carbohydrate. Cultures were supplemented with the detergent, Brij 58 (1 g/L), with and without fatty acids (20 mg/L). Cells were grown at 37° in a reciprocating water bath. Growth was monitored by measuring optical density at 550 nm. Generation times were computed using least squares regression analysis to determine the slope of the growth curve.

Cell Survival in Buffered Ethanol

Cells were grown with various additives, harvested in log phase by centrifugation, and washed twice with 0.1 M phosphate buffer (7.2). The resulting cell suspension was diluted into phosphate buffer containing various concentrations of ethanol and incubated at 37°. Cell survival was determined by plating appropriate dilutions in triplicate on Bacto antibiotic medium 2. Viable cells were scored as those forming colonies during overnight incubation at 37°.

Lipid Analysis

Cells were harvested by centrifugation, washed and extracted overnight with a mixture of chloroform and methanol as previously described [10]. For fatty acid analysis, methyl esters were prepared by transesterification and were analysed by gas chromatography [10].

Fluorescence Depolarization

The fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a comparative measure of membrane fluidity [7]. Cell membranes were prepared using a freeze/thaw lysis procedure and were harvested by centrifugation [6]. Membranes were resuspended in Tris buffer (0.01 M, pH 7.5), mixed with an equal volume of a microcrystalline dispersion of DPH (2×10^{-6} M) [6], and incubated in the dark for 2 hr in a reciprocating water bath (37°) to allow probe insertion. Samples were measured using an SLM series 4000 polarization fluorimeter with excitation at 360 nm and emission above 470 nm. Sample temperature was controlled using a Neslab circulator and was monitored within the cuvette. Ethanol was added by syringe and was allowed to equilibrate for 2 minutes prior to measurement. Polarization was computed as described by Chen and Bowman [3].

Chemicals

The complex nutrients for growth medium (antibiotic medium 2, yeast extract, tryptone) were products of Difco

[1]This investigation was supported by Grant No. 1 R01 GM 24059-19 from the National Institute of Health, by Grant No. 1 R01 AA 3816-01 from the National Institute of Alcohol Abuse and Alcoholism, and by the Florida Argicultural Experiment Station (Publication Number 1916). L.O.I. is the recipient of a Career Development Award from the National Institute of Alcohol Abuse and Alcoholism (1 K02 00036-01).

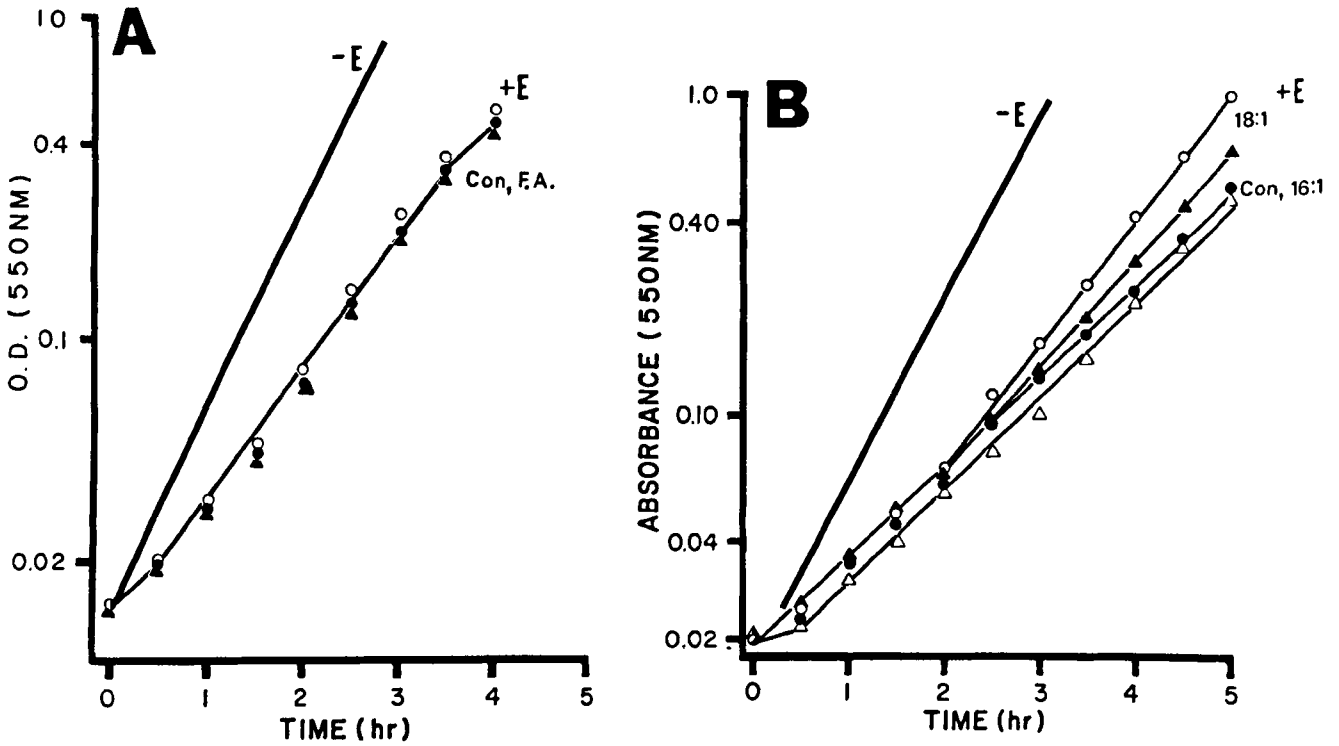

FIG. 1. The effects of ethanol and fatty acids on growth. An overnight culture was diluted 1:300 into fresh medium containing ethanol (0.67 M). This was subdivided into flasks and various fatty acid supplements (20 μg/L) added. Growth was measured as optical density. The solid lines represent the rate of growth of cells in the absence of ethanol and were included for comparison. (A) Strain TB4: ●, no fatty acid; ○, vaccenic acid; ▲, palmitic acid. (B) Strain WN1: ●, no fatty acid; ○, vaccenic acid; ▲, oleic acid; △, palmitic acid. Cultures supplemented with palmitoleic acid were identical to those lacking fatty acid supplements.

Laboratories (Detroit, MI). Brij 58 and all fatty acids were obtained from the Sigma Chemical Co. (St. Louis, MO). DPH was obtained from the Aldrich Chemical Company (Milwaukee, WI). Radioactive chemicals were purchased from Amersham Corporation (Arlington Heights, IL).

RESULTS

Effects of Fatty Acid Composition on Growth in the Presence of Ethanol

The growth of an organism provides a useful index of its physiological activity. To investigate the effect of membrane fatty acid composition on ethanol tolerance, we have compared the effects of ethanol on the growth of two strains of *E. coli* with different lipid compositions (Fig. 1). Strain TB4 contained high levels of unsaturated fatty acids (16:1 + 18:1) which increased further during growth with ethanol (Table 1). Strain WN1 is defective in the synthesis of 18:1 fatty acid. It contained a lower proportion of unsaturated fatty acids and was unable to change its fatty acid composition during growth with ethanol. The growth of strain WN1 was more strongly inhibited by ethanol (0.67 M) than that of strain TB4 (Table 1). The inclusion of fatty acid supplements did not alter the rate of growth of strain TB4 in medium containing ethanol (0.67 M). Unlike strain TB4, some fatty acid supplements had a dramatic effect on the rate of growth of strain WN1 in the presence of ethanol (Fig. 1, Table 1). Two *cis*-18:1 fatty acids, vaccenic acid and oleic acid, partially relieved the growth inhibition by ethanol after approximately 2 hr. This delay probably represents the time required for sufficient fatty acid incorporation. Both of these fatty acids were incorporated into the lipids of strain WN1 and reduced the level of saturated fatty acids present (Table 1). Other fatty acids such as *trans*-18:1 fatty acids (elaidic), palmitoleic acid (16:1) and palmitic acid (16:0) did not affect the growth of strain WN1 in the presence of ethanol.

Effects of Fatty Acid Composition on Survival in the Presence of Ethanol

The survival of cells during incubation in phosphate buffer (0.1 M, pH 7.2, 37°) containing ethanol was used as a comparative measure of alcohol tolerance. Three strains were grown in the presence and absence of ethanol and their subsequent survival in buffered ethanol was compared (Fig. 2). During the incubation in buffered ethanol, no growth or changes in fatty acid composition occurred. Each of the strains tested contained a different fatty acid composition (Table 1). Strain TB4 contained the highest proportion of unsaturated fatty acids and was the most resistant to killing by ethanol (Fig. 2, Table 1). During prior growth with ethanol, the proportion of unsaturated fatty acids increased further in strain TB4 and this was accompanied by a further increase in resistance to ethanol. Strain BB3 contained the lowest proportion of unsaturated fatty acids and was the most sensitive to killing by ethanol (Fig. 2, Table 1). During prior growth with ethanol, the proportion of unsaturated fatty acids in strain BB3 increased and was accompanied by an increase in resistance to killing by ethanol. Strain WN1 contained an intermediate level of unsaturated fatty acids and was intermediate in its tolerance to ethanol (Fig. 2, Table 1). Strain WN1 did not alter its fatty acid composition during

TABLE 1

SUMMARY OF LIPID COMPOSITION, GENERATION TIME, AND PHYSICAL PROPERITES BY VARIOUS *E. coli* STRAINS

Strain	Growth supplement*		Fatty acid composition (%)			Generation time‡	Membrane fluidity§	
	Fatty acid	Ethanol	Sat. F.A.†	16:1	18:1	(min)	− ethanol	ΔP + ethanol
TB4	−	−	35	40	25	28	0.276 ± 0.001	−0.001 ± 0.001
TB4	−	+	17	42	40	44	0.266 ± 0.001	−0.003 ± 0.001
BB3	−	−	75¶	25	tr	33	0.319 ± 0.001	−0.004 ± 0.001
BB3	−	+	68#	32	tr	52	0.318 ± 0.001	−0.003 ± 0.002
WN1	−	−	53	46	tr	32	0.290 ± 0.001	−0.004 ± 0.001
WN1	−	+	52	48	tr	63	0.295 ± 0.002	−0.003 ± 0.001
WN1	palmitic	+	54	46	tr	62	0.308 ± 0.001	−0.004 ± 0.002
WN1	palmitoleic	+	51	50	tr	62	n.d.	n.d.
WN1	vaccenic	+	47	35	18	62,45**	0.290 ± 0.001	−0.004 ± 0.002
WN1	oleic	+	51	33	16	62,55**	n.d.	n.d.
WN1	elaidic	+	46	40	15	62	n.d.	n.d.
WN1	palmitic	−	77	24	tr	32	0.337 ± 0.001	−0.008 ± 0.001
WN1	palmitoleic	−	48	52	tr	32	n.d.	n.d.
WN1	vaccenic	−	33	17	40	32	0.245 ± 0.001	−0.003 ± 0.001
WN1	oleic	−	43	19	37	32	n.d.	n.d.
WN1	elaidic	−	35	29	37	32	n.d.	n.d.

*fatty acid, 20 mg/l; ethanol, 0.67 M.
†Sat. F.A., saturated fatty acid; primarily 16:0.
‡time required for one mass doubling.
§average ± standard deviation; + ethanol, 0.67 M.
¶39% 14:0.
#20% 14:0.
**biphasic growth.
n.d., not determined.

growth with ethanol. Unlike the other strains, strain WN1 did not acquire increased resistance to ethanol during prior growth with ethanol.

The fatty acid composition of strain WN1 was extensively modified by growth with fatty acid supplements in the absence of ethanol (Table 1). These changes altered the sensitivity of strain WN1 to killing by ethanol (Figs. 3 and 4). Growth with vaccenic acid resulted in an increase in the proportion of unsaturated fatty acids and an increased resistance to killing by ethanol. Growth with palmitic acid resulted in a decrease in the proportion of unsaturated fatty acids and an increase in sensitivity to killing by ethanol. Growth with palmitoleic acid had little effect on the proportion of unsaturated fatty acids and did not alter survival.

Figure 5 summarizes the relationship between *cis*-unsaturated fatty acid content and survival in ethanol for all three strains and for strain WN1 grown with fatty acid supplements. Alcohol tolerance appears to be directly related to the proportion of unsaturated fatty acids present in membrane lipids.

Relationship Between Alcohol Tolerance and Membrane Fluidity

The steady state fluorescence depolarization of DPH was used as a comparative measure of membrane fluidity. These results are reported in units of polarization (P) which are inversely related to bulk membrane fluidity. Thus fluid membranes exhibit low P values while rigid membranes exhibit high P values. Fatty acid composition is a major determinant of membrane fluidity. In general, the measured fluidity in isolated membranes agreed well with the fatty acid composition. That is, membranes containing high levels of saturated fatty acids were relatively rigid and exhibited high P values while membranes with high proportions of *cis*-unsaturated fatty acids were more fluid and exhibited lower P values. As with fatty acid composition, P values can be correlated with survival. A decrease in P was directly related to an increase in survival in most cases. For growth, this relationship was less clear.

The addition of ethanol (0.67 M) to isolated membranes caused a decrease in polarization (ΔP, ethanol, Table 1). This change in fluidity is equivalent to that caused by a 1° increase in temperature. The magnitude of this change did not correlate with either alcohol resistance or with fatty acid composition. In most cases, the degree of disturbance caused by ethanol was similar. The changes in lipid composition which accompanied an increase in alcohol tolerance also resulted in a decrease in polarization. Thus, the alcohol-induced increase in the fluidity of isolated membranes does not appear to be causally involved in the killing effects of ethanol.

DISCUSSION

The increase in unsaturated fatty acids (primarily vaccenic acid) which occurs in *E. coli* during growth in the presence of ethanol is beneficial for cell growth and survival. A

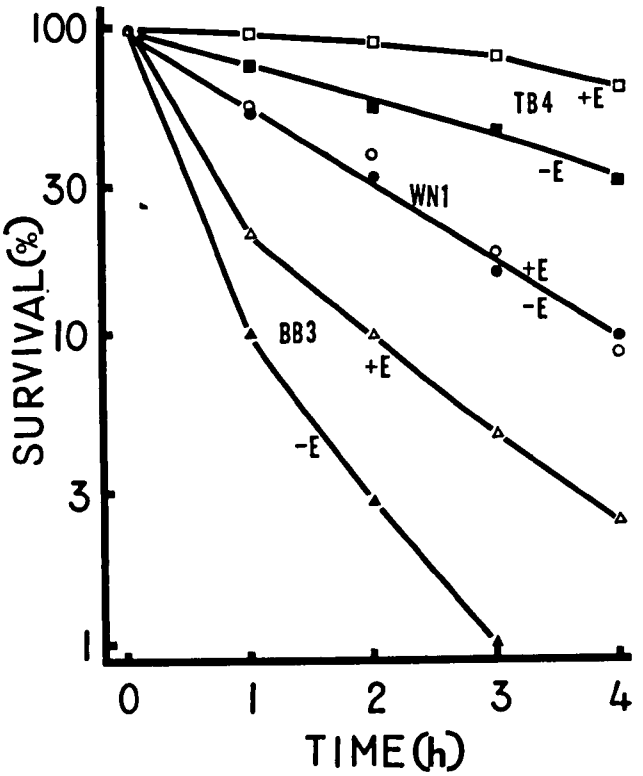

FIG. 2. Survival of different strains in buffered ethanol (0.67 M). Symbols: □, strain TB4 grown in the presence of 0.67 M ethanol; ■, strain TB4 grown in the absence of ethanol; ○, strain WN1 grown in the presence of ethanol (0.67 M); ●, strain WN1 grown in the absence of ethanol; △, strain BB3 grown in the presence of ethanol (0.67 M); ▲, strain BB3 grown in the absence of ethanol.

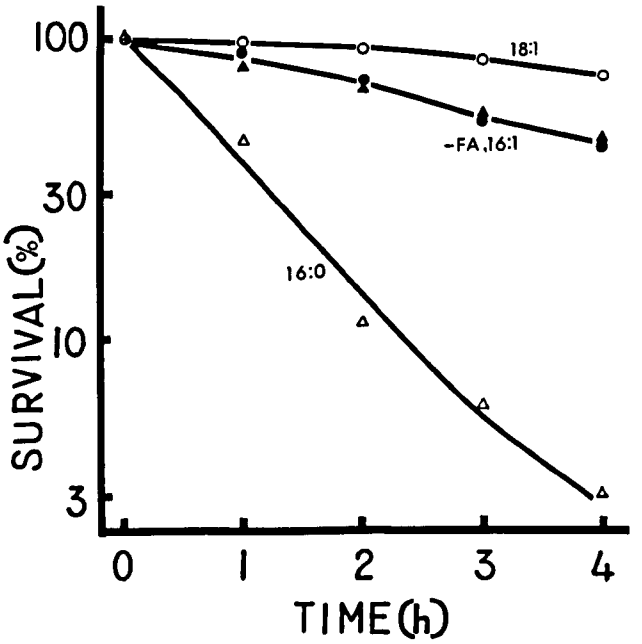

FIG. 4. Effects of fatty acid supplements on survival of strain WN1 in buffered ethanol (0.45 M). Symbols: ○, cells grown with vaccenic acid; ●, cells grown without added fatty acid; ▲, cells grown with palmitoleic acid; △, cells grown with palmitic acid.

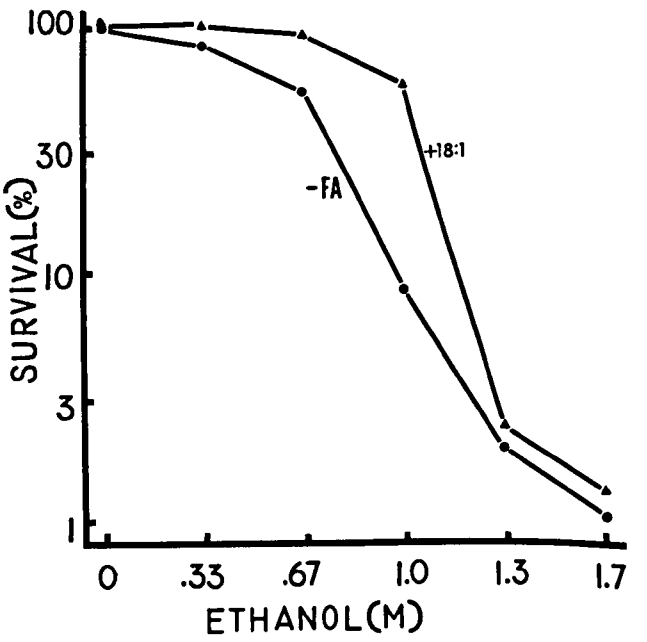

FIG. 3. Effects of alcohol concentration on the survival of strain WN1 grown with and without vaccenic acid (2 hr, 37°, various alcohol concentrations). Symbols: ●, cells grown without added fatty acid; ▲, cells grown with vaccenic acid (20 mg/L).

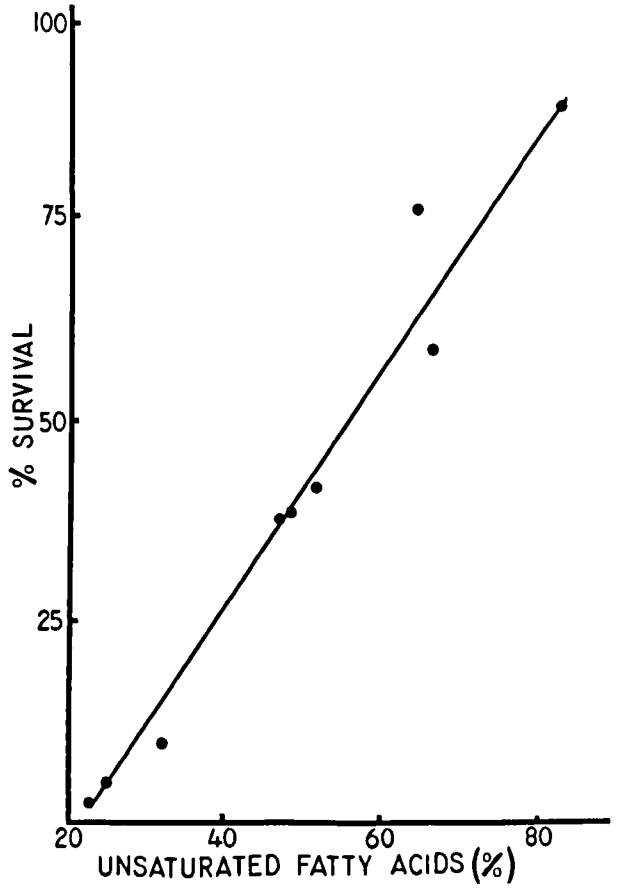

FIG. 5. Relationship between cell survival (2 hr) in buffered ethanol (0.67M) and lipid composition. Symbols: ●, all values from cells containing mixture of *cis*-unsaturated and saturated fatty acid.

mutant unable to make vaccenic acid and unable to increase its proportion of unsaturated fatty acids during growth with ethanol was hypersensitive to growth inhibition and killing by ethanol. This hypersensitivity was relieved by supplementing cultures with vaccenic or oleic acid. The total amount of unsaturated fatty acid present was directly related to cell survival in buffered ethanol solutions. A lower level of unsaturated fatty acid was required to relieve hypersensitivity to growth inhibition by ethanol. Our results indicate that the changes in fatty acid composition induced by the presence of ethanol are part of an adaptive response, preventing some of the harmful effects of this drug.

Fatty acid composition appears to be the principal determinant of alcohol tolerance in *E. coli*. Other changes such as the increase in anionic phospholipids and the changes in membrane proteins appear secondary in importance. The hypersensitivity of strain WN1 results from a specific mutation in fatty acid synthesis which is relieved by supplementing these cells with the deficient fatty acid. This mutation did not prevent other changes in the cell envelope composition. Supplementing strain WN1 with fatty acids did not itself alter the level of anionic phospholipids.

An increase in the proportion of unsaturated fatty acids in membrane lipids during growth in the presence of ethanol may be a general phenomenon. Analogous changes have been observed with the protozoan, *Tetrahymena pyriformis* [15] and in liver tissues [11,18]. The alcohol-resistant organism known, *Lactobacillus heterohiochii*, grows in alcohol concentrations above 20% and also contains high levels of unsaturated fatty acids [21]. In the yeast, *Saccharomyces cerivisiae*, an increase in membrane unsaturation has been demonstrated to increase tolerance [8,20]. In neural tissues, however, conflicting changes have been reported to occur during the development of alcohol dependence. Synaptosomal membranes have been reported to increase in unsaturation [19], to decrease in unsaturation [12] and to exhibit little change in unsaturation (H. Weiner, this meeting) during the development of alcohol dependence. At present, it appears unlikely that large changes in bulk fatty acid composition analogous to those observed in *E. coli* are involved in CNS alcohol tolerance in mammalian systems.

The mechanism by which an increase in level of unsaturated fatty acids in membrane lipids ameliorates some of the harmful effects of ethanol in microorganisms is unclear. Both the increase in unsaturation and the intercalation of ethanol into membranes result in an increase in bulk membrane fluidity. Thus it is unlikely that the fluidizing effect of ethanol is itself causally involved in the deleterious effects of ethanol on *E. coli*. A similar increase in fluidity was induced in the absence of ethanol by an increase in temperature of 1°. This change neither killed, nor impaired the growth of, *E. coli*.

ACKNOWLEDGEMENT

We wish to thank Ms. M. G. Pate for her excellent technical assistance.

REFERENCES

1. Berger, B., C. E. Carty and L. O. Ingram. Alcohol-induced changes in phospholipid molecular species of *Escherichia coli*. *J. Bacteriol.* **142**: 1040–1048, 1980.
2. Buttke, T. M. and L. O. Ingram. Mechanism of ethanol-induced changes in lipid composition of *Escherichia coli*: Inhibition of saturated fatty acid synthesis *in vivo*. *Biochemistry* **17**: 637–644, 1978.
3. Chen, R. F. and R. L. Bowman. Fluorescence polarization: Measurement with ultraviolet polarizing filters in a spectrophotofluorimeter. *Science* **147**: 729–732, 1965.
4. Chesbro, W., T. Evans and R. Eifert. Very slow growth of *Escherichia coli*. *J. Bacteriol.* **139**: 625–638, 1979.
5. Cronan, J. E., Jr. Physical properties of membrane lipids: Biological relevance and regulation. *Ann. Rev. Biochem.* **47**: 163–189, 1978.
6. DiRienzo, J. M. and M. Inouye. Lipid fluidity-dependent biosynthesis and assembly of the outer membrane proteins of *E. coli*. *Cell* **17**: 155–161, 1979.
7. Esko, J. D., J. R. Gilmore and M. Glaser. Use of a fluorescent probe to determine the viscosity of LM membranes with altered phospholipid compositions. *Biochemistry* **16**: 1881–1896, 1977.
8. Hayashida, S., D. D. Feng and M. Hongo. Function of the high concentration alcohol-producing factor. *Agr. Biol. Chem.* **38**: 2001–2006, 1974.
9. Ingram, L. O. Preferential inhibition of phosphatidylethanolamine synthesis in *E. coli* by alcohols. *Can. J. Microbiol.* **23**: 779–789, 1977.
10. Ingram, L. O. Adaptation of membrane lipids to alcohols. *J. Bacteriol.* **125**: 670–678, 1976.
11. French, S. W., T. J. Ihrig, G. P. Shaw, T. T. Tanaka and M. L. Norum. The effect of ethanol on the fatty acid composition of hepatic microsomes and inner and outer mitochondrial membranes. *Res. communs chem. pathol. Pharmac.* **2**: 567–585, 1971.
12. Littleton, J. M., G. R. John and S. J. Grieve. Alterations in phospholipid composition in ethanol tolerance and dependence. *Alcoholism: Clin. exp. Res.* **3**: 50–56, 1979.
13. Luria, S. E. and M. Belbruck. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**: 491–511, 1943.
14. Marr, A. G. and J. L. Ingraham. Effect of temperature on the composition of fatty acids in *Escherichia coli*. *J. Bacteriol.* **84**: 1260–1267, 1962.
15. Nandini-Kishore, S. G., S. M. Mattox, C. E. Martin and G. A. Thompson, Jr. Membrane changes during the growth of *Tetrahymena* in the presence of ethanol. *Biochim. biophys. Acta* **551**: 315–327, 1979.
16. Nunn, W. D. and J. E. Cronan, Jr. Unsaturated fatty acid synthesis is not required for induction of lactose transport in *Escherichia coli*. *J. Biol. Chem.* **249**: 724–731, 1974.
17. Pastan, I. and S. Adhya. Cyclic adenosine 5'-monophosphate in *Escherichia coli*. *Bacteriol. Proc.* **40**: 527–551, 1976.
18. Spach, P. I., J. W. Parce and C. C. Cunningham. Effect of chronic ethanol administration on energy metabolism and phospholipase A_2 activity in rat liver. *Biochem. J.* **178**: 23–33, 1979.
19. Sun, G. Y. and A. Y. Sun. Effects of chronic ethanol administration on phospholipid acyl groups of synaptosomal membrane fractions isolated from guinea-pig brain. *Res. communs chem. pathol. Pharmac.* **24**: 405–408, 1979.
20. Thomas, D. S., J. A. Hossack and A. H. Rose. Plasma membrane lipid composition and ethanol tolerance in *Saccharomyces cerivisiae*. *Archs Microbiol.* **117**: 239–245, 1978.
21. Uchida, K. Lipids of alcoholophilic lactobacilli. II. Occurrence of polar lipids with unusually long acyl chains in *Lactobacillus heterohiochii*. *Biochim. biophys. Acta* **369**: 146–155, 1974.