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Mechanism of Ethanol-Induced Changes in Lipid Composition of *Escherichia coli:* Inhibition of Saturated Fatty Acid Synthesis in Vivo†

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ABSTRACT: The in vivo effects of ethanol on lipid synthesis in *Escherichia coli* have been examined. Under conditions which uncoupled fatty acid synthesis from phospholipid synthesis, ethanol decreased the amount of saturated fatty acids synthesized but had little effect on the selectivity of their incorporation into phospholipids. In the absence of fatty acid degradation and unsaturated fatty acid synthesis, *E. coli* was still able to adapt its membrane lipids to ethanol, while the inhibition of total fatty acid synthesis eliminated this response. During growth in the presence of ethanol, strain K1060 (an unsaturated fatty acid auxotroph) incorporated an increased amount of exogenous heptadecanoic acid (17:0) to compensate for the reduction in palmitic acid (16:0) available from biosynthesis. Thus, our results indicate that the reduced levels of saturated fatty acids observed in the phospholipids of *E. coli* following growth in the presence of ethanol result primarily from a decrease in the amounts of saturated fatty acids available for phospholipid synthesis.

In a previous report from our laboratory it was shown that *Escherichia coli* will alter its membrane fatty acid composition when grown in the presence of straight chain alcohols (Ingram, 1976). These alcohol-induced fatty acid changes are similar to those induced by changes in growth temperature (Marr & Ingraham, 1962). Short chain alcohols such as ethanol cause a decrease in the amounts of saturated fatty acids, similar to a shift down in growth temperature. Long chain alcohols such as hexanol induce changes analogous to a shift up in temperature, an increase in the proportion of saturated fatty acids (Ingram, 1976). Like temperature adaptation, these changes in fatty acid composition are independent of major changes in phospholipid composition (Ingram, 1977a). These results with alcohols have been extended to show that a wide variety of other lipophilic agents (organic solvents and food preservatives) also induce fatty acid changes in *E. coli* (Ingram, 1976). The diverse structures of these compounds as well as their lipophilic nature support the theory that these changes in fatty acid composition result from a general interaction with a hydrophobic site, rather than a result of specific catabolic processes (Ingram, 1977b). The intercalation of these agents into the membrane would be expected to alter membrane fluidity. The

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TABLE I: Bacterial Strains.

Strain	Relevant genotype	Relevant phenotype	Source
CSH2	Wild type	Wild type for fatty acid synthesis and fatty acid degradation	Cold Spring Harbor Laboratory
TB4	*fadE*$^-$	Prototrophic for fatty acid synthesis; defective in fatty acid degradation	This study
K1060	*fabB*$^-$*fadE*$^-$	Defective in unsaturated fatty acid synthesis; defective in fatty acid degradation (Overath et al., 1970)	D. F. Silbert & B. J. Bachmann
L43	*fadE*$^-$	Prototrophic for fatty acid synthesis; defective in fatty acid degradation (Silbert et al., 1973)	D. F. Silbert
LW1	*fadE*$^-$*plsA*$^-$ *glpD*$^-$	Defective in *sn*-glycerol 3-phosphate utilization; defective in fatty acid degradation; defective in glycerol catabolism (Cronan et al., 1975)	J. E. Cronan, Jr.
LW3	*fadE*$^-$*gpsA*$^-$ *glpD*$^-$	Defective in *sn*-glycerol 3-phosphate synthesis; defective in fatty acid degradation; defective in glycerol catabolism (Cronan et al., 1975)	J. E. Cronan, Jr.
L8	*fabE*$^-$	Temperature sensitive for fatty acid synthesis (Semple & Silbert, 1975)	B. J. Bachmann
L48	*fabD*$^-$	Temperature sensitive for fatty acid synthesis (Silbert et al., 1976)	B. J. Bachmann

resulting changes in fatty acid composition suggest that the cell may be able to detect changes in membrane fluidity, per se, and compensate for the direct actions of drugs.

In view of the importance of ethanol, ethanol tolerance, and withdrawal syndrome in society, the ability of ethanol to induce dramatic changes in the fatty acid composition is of particular interest. A membrane whose lipid composition has been altered in response to ethanol could be considered as being adapted to the presence of the alcohol (tolerance), much as membrane lipid changes allow a membrane to adapt to growth temperature (Shaw & Ingraham, 1965). The removal of ethanol would cause additional changes in membrane organization which would be compensated for by a return to the original composition (withdrawal). Thus, an understanding of the mechanisms responsible for ethanol-induced changes in fatty acid composition in *E. coli* would provide a model for the development of experiments in mammalian systems.

The similarity of the changes in fatty acid composition induced by ethanol and temperature adaptation suggests that these processes may share a common mechanism. This regulation of fatty acid composition could be carried out at any of three levels: (1) fatty acid degradation; (2) fatty acid synthesis; and (3) phospholipid synthesis (Cronan & Vagelos, 1972). *E. coli* mutants blocked in one or more of these processes have proven useful in studying the control of thermal regulation of membrane lipid composition (Cronan & Gelmann, 1975). We have used these same mutants to elucidate the mechanisms by which ethanol induces membrane fatty acid changes. The results of our experiments indicate that the ethanol-induced changes in fatty acid composition in vivo result primarily from the inhibition of saturated fatty acid synthesis.

Experimental Procedure

Bacterial Strains. The *E. coli* K12 derivatives used in this study are listed in Table I. Strain TB4 is derived from strain CSH2 and is defective in fatty acid degradation (*fadE*$^-$). Strain CSH2 was made *proB*$^-$ by mutagenesis with ultraviolet light followed by penicillin selection (Miller, 1972). A *proB*$^+$ *fadE*$^-$ lysate was made from strain L43 by the method of Marsh & Duggan (1972) using the Plvir (Cold Spring Harbor Laboratory), and this was used to introduce the *fadE*$^-$ gene into strain CSH2. Potential *fadE*$^-$ transductants were selected by their ability to grow without proline supplementation.

Growth Conditions. Unless otherwise indicated, all bacterial strains were grown in a gyrotory shaker at 30 °C. The standard medium used was E medium (Vogel & Bonner, 1956). Supplements differed for each strain. Strains CSH2, TB4, and L43 were grown with sodium succinate (0.2%), thiamine (0.001%), and unsaturated fatty acid supplements (0.005%). Strains LW1 and LW3 were supplemented with sodium succinate (0.2%), thiamine (0.001%), casein hydrolysate (vitamin free) (0.05%), and *sn*-glycerol 3-phosphate (0.01%). Glycerol 3-phosphate deprivation was performed by filtration as described by Cronan et al. (1975). For strains L8 and L48, the media was supplemented with glycerol (0.4%), thiamine (0.001%), monosodium glutamate (0.1%), yeast extract (0.0001%), Brij 58 (0.4%), potassium palmitate (0.001%), and potassium oleate (0.001%). Cell growth was monitored by measuring the absorbance at 550 nm using a Beckman Model 25 spectrophotometer. Cultures were routinely used in the experiments at a cell density of 2×10^8 cells/mL (0.35 absorbance at 550 nm) to ensure that cells were in log phase.

Analysis of Fatty Acids by Gas Chromatography. Cells were harvested by centrifugation, inactivated by the addition of 5% trichloroacetic acid, and extracted into chloroform–methanol as described by Kanfer & Kennedy (1973). For the separation of neutral lipids (includes free fatty acids) from phospholipids, washed lipid extracts were chromatographed on unactivated silica gel G impregnated glass-fiber paper (Gelman ITLC-SG, Fisher Scientific Co., Pittsburgh, Pa.) using solvent 1 as described by Freeman & West (1966). Phospholipids were eluted with chloroform–methanol (2:1), and transesterified using 2% H_2SO_4 in methanol, as described by Silbert et al. (1973a,b). The methyl esters were extracted into pentane and concentrated under N_2 prior to analysis. The conditions for gas chromatography have been described (Ingram, 1976).

Analysis of Radioactive Lipids. The incorporation of labeled acetate into fatty acids was assayed essentially by the procedure of Cronan & Wulff (1969). One-milliliter samples of cells were added to tubes containing 10 μCi of [^{14}C]acetate (61 mCi/mmol) and incubated for 10 min. The reaction was stopped by the addition of chloroform–methanol (1:2) and extracted overnight. The resulting lipid extract was separated into two phases by the addition of chloroform and water. Radioactive lipids were present in the lower (chloroform) phase. An aliquot (0.2 mL) was removed from the chloroform phase and counted to determine total lipid synthesis. The remaining radioactive lipids were transesterified as described above, and the radioactive methyl esters were analyzed by argentation chromatography. When the incorporation of [^{14}C]acetate into free (nonesterified) fatty acids was measured, the lipid extract was separated into phospholipids and neutral lipids by thin-layer chromatography using either the solvent system of Klein et al. (1971) or the two-step procedure described by Cronan et al. (1975). Both the phospholipid and the neutral lipid

TABLE II: Effect of Fatty Acid Degradation on Ethanol-Induced Changes in Fatty Acid Composition.[a]

Strain	Conditions	Fatty acid composition (%) 14:0	16:0	16:1(Δ17)	*cis*-Δ[11]-18:1	UFA/SFA ratio[b]
CSH2 (*fadE*$^+$)	No EtOH	3.1 (0.5)	32.9 (0.2)	36.6 (0.8)	26.8 (1.3)	1.7
	3% EtOH	1.9 (0.6)	21.9 (0.5)	37.1 (1.1)	38.1 (0.6)	3.0
TB4 (*fadE*$^-$)	No EtOH	3.3 (0.5)	31.5 (0.4)	35.5 (0.5)	28.5 (0.9)	1.8
	3% EtOH	1.9 (0.5)	20.9 (1.2)	37.4 (0.8)	39.1 (1.4)	3.2

[a] Cells were grown in the presence and absence of alcohol, harvested, and analyzed by gas chromatography. These results represent an average of four separate experiments and are expressed as a percentage of total fatty acids with the standard deviation in parentheses. [b] UFA, unsaturated fatty acid; SFA, saturated fatty acid.

fractions were eluted, transesterified, and analyzed by argentation chromatography. Incorporation of exogenously supplied [^{14}C]- or [^{3}H]palmitic acid and [^{3}H]oleic acids was performed in essentially the same fashion, except phospholipids were separated from neutral lipids by thin-layer chromatography using the solvent diethyl ether–acetic acid (100:1). The phospholipid region was scraped into vials containing toluene scintillation solution and counted on a Beckman Model 133 liquid scintillation counter adjusted for dual label counting.

Argentation Chromatography. Thin-layer chromatography plates coated with silica gel G were impregnated with 10% aqueous silver nitrate and activated as described by Cubero & Mangold (1965). The labeled methyl esters were applied to the argentation plate in pentane and developed twice in toluene at −17 °C (Morris et al., 1967). Radioactive regions were scraped into vials and counted.

Chemicals. Heptadecanoic acid was purchased from Applied Science Laboratories Inc. (State College, Pa.). All other unlabeled fatty acids were obtained from the Sigma Chemical Co. (St. Louis, Mo.). [1-^{14}C]Acetate, [9,10(*n*)-^{3}H]oleic acid, and [1-^{14}C]palmitic acid are products of Amersham Corp. (Arlington Heights, Ill.). [9,10(*n*)-^{3}H]Palmitic acid was purchased from New England Nuclear (Boston, Mass.).

Results

Effect of Fatty Acid Degradation (fadE) on Ethanol-Induced Changes in Fatty Acid Composition. E. coli possesses a multienzyme complex capable of fatty acid degradation (Binstock et al., 1977). This enzyme complex may partially determine the fatty acid composition of membrane lipids by altering the relative amounts of fatty acid species available for incorporation into phospholipids. Thus, the ethanol-induced reduction in palmitic acid previously reported in *E. coli* (Ingram, 1976) could result from a preferential degradation of endogenously synthesized palmitic acid. To determine if this was indeed the case, the effects of ethanol on strain CSH2, proficient in fatty acid degradation (*fadE*$^+$), and strain TB4, defective in fatty acid degradation (*fadE*$^-$), were compared (Table II). In both strains the proportion of palmitic acid (16:0)[1] was reduced by 10%, with an equal increase in the proportion of *cis*-vaccenic acid during growth in the presence of ethanol. Neither strain showed a significant change in the level of palmitoleic acid (16:1). These results indicate that preferential fatty acid degradation is not responsible for ethanol-induced changes in fatty acid composition.

Effect of Ethanol on the Fatty Acid Composition of Newly Synthesized Phospholipids. Previous experiments have shown that growth in the presence of ethanol results in the synthesis of lipids rich in unsaturated fatty acids (Ingram, 1976), and that this is not due to selective fatty acid degradation (Table II). To determine if these changes represent a direct effect of ethanol on lipid synthesis (fatty acid synthesis + utilization) or a secondary effect mediated through such processes as enzyme induction or other alternatives in metabolism, we have examined the immediate effects of ethanol addition on newly synthesized lipids. Strain TB4 (*fadE*$^-$) was pulse labeled with [^{14}C]acetate to measure the effects of various concentrations of ethanol on the incorporation of newly synthesized fatty acids into phospholipids. The addition of ethanol caused an immediate increase in the abundance of newly synthesized unsaturated fatty acids which were incorporated into phospholipids (Table III). This resulted in a dose-related increase in the UFA/SFA ratios and is analogous to the changes observed in the bulk lipid composition (Table II; Ingram, 1976). High concentrations of ethanol (4% and 6%) strongly inhibited the synthesis and utilization of both unsaturated and saturated fatty acids, and this inhibition may be related to the "lipid poor" cells described earlier (Ingram, 1977). The ethanol-induced increase in the UFA/SFA ratio was immediately reversed by ethanol removal (washing), even after 16 h of growth in the presence of 3% ethanol. The immediacy of action of ethanol on the UFA/SFA ratio of newly synthesized lipids coupled with its equally rapid reversibility provide evidence for the direct action of ethanol on lipid synthesis. Since fatty acid synthesis is tightly coupled to phospholipid synthesis, this

TABLE III: Effect of Ethanol on the Fatty Acid Composition of Newly Synthesized Phospholipids in Strain TB4.

EtOH (%)	[^{14}C]Acetate incorp. (cpm) Total	Saturated	Unsaturated	UFA/SFA ratio[c]
0[a]	14 400	4100	10 300	2.5
1	8 800	1700	7 200	4.2
2	9 300	1400	7 900	5.6
3	8 600	1100	7 500	6.8
4	2 600	360	2 300	6.4
6	1 600	350	1 300	3.7
3%, 16 h[b]	4 800	900	3 900	4.3
Removed	10 800	2800	8 000	2.9

[a] Cells were grown in the absence of ethanol. Samples were removed and added to tubes containing 10 μCi of [1-^{14}C]acetate (61 mCi/mmol) with various concentrations of ethanol. The samples were incubated for 10 min. Fatty acids were separated using argentation chromatography. [b] Cells were grown in the presence of 3% ethanol for 16 h (with appropriate dilutions). The culture was split into two parts. One part was rapidly washed using membrane filtration and resuspended to its original volume. Samples were removed from both parts, pulsed with [^{14}C]acetate, and analyzed by argentation chromatography. [c] UFA, unsaturated fatty acid; SFA, saturated fatty acid.

[1] Abbreviations used: UFA, unsaturated fatty acid; SFA, saturated fatty acid; ACP, acyl carrier protein; 16:0, palmitic acid; 16:1, palmitoleic acid; 17:0, heptadecanoic acid; *cis*-Δ^9-18:1, oleic acid; *cis*-Δ^{11}-18:1, *cis*-vaccenic acid; Δ17; *cis*-9,10-methylenehexadecanoic acid; 20:2, eicosadienoic acid; Δ19; *cis*-11,12-methyleneoctadecanoic acid.

TABLE IV: Effect of Incubation Temperature on Ethanol-Induced Changes in the Fatty Acid Composition of Newly Synthesized Phospholipids.[a]

Incubation temp	Conditions	Fatty acids (cpm) Saturated	16:1	*cis*-Δ^{11}-18:1	UFA/SFA ratio[b]
23 °C	No EtOH	1600	3600	5300	5.6
	3% EtOH	520	2900	2800	11.0
30 °C	No EtOH	2500	2700	3800	2.6
	3% EtOH	580	1900	1700	6.2
37 °C	No EtOH	580	580	480	1.8
	3% EtOH	300	540	420	3.2

[a] Samples from an exponential culture of strain TB4 were pulse labeled with 10 μCi [1-^{14}C]acetate (61 mCi/mmol) at various temperatures in the presence and absence of 3% ethanol. Fatty acids from purified phospholipids were separated by argentation chromatography. [b] UFA, unsaturated fatty acid; SFA, saturated fatty acid.

change in UFA/SFA ratio could be due to the direct action of ethanol on fatty acid synthesis, fatty acid utilization (acyltransferase system), or both.

Pulse-labeling experiments typically yielded UFA/SFA ratios which were higher than those of bulk lipids. This probably resulted from the incorporation of newly synthesized fatty acids into phosphatidylglycerol (rich in unsaturated fatty acids) during de novo phospholipid synthesis as well as during the turnover of phosphatidylglycerol. However, the UFA/SFA ratios following the initial addition of ethanol (Table III) were much higher than expected based upon determinations of bulk lipid composition (Table II). During growth in the presence of ethanol, the ratios observed in log phase cells pulsed with [^{14}C]acetate approached that of bulk lipid, 7.1, 6.1, 5.8, and 4.4 after 0, 1, 2, and 16 h, respectively. Thus the initial increase in the UFA/SFA ratio following the addition of ethanol represented an overcompensation in newly synthesized lipids which was subsequently decreased as the cells adjusted to the presence of ethanol.

Effect of Incubation Temperature on Ethanol-Induced Changes in the Fatty Acid Composition of Newly Synthesized Lipids. E. coli alters its membrane fatty acid composition in response to changes in growth temperature (Marr & Ingraham, 1962). A shift to a higher growth temperature leads to an increase in the proportion of palmitic acid while a shift to a lower growth temperature leads to an increase in the proportion of unsaturated fatty acids. These changes in lipid composition are regulated both at the level of fatty acid synthesis (Cronan, 1975) and fatty acid utilization for phospholipid synthesis (Esfahani et al., 1969; Sinensky, 1971). We have shown that the addition of ethanol causes changes in fatty acid composition similar to a reduction in growth temperature. To further explore this relationship, we have investigated the simultaneous effect of ethanol addition and a shift in incubation temperature on the fatty acid composition of newly synthesized lipids. Samples from an exponential culture of strain TB4 grown at 30 °C were incubated at 23, 30, and 37 °C and pulse labeled with [^{14}C]acetate in the presence and absence of 3% ethanol. Esterified fatty acids were separated by argentation chromatography, scraped and counted (Table IV). Strain TB4 altered its fatty acid composition (UFA/SFA ratio) in response to temperature as previously reported (Marr & Ingraham, 1962). This same trend was reflected among samples containing ethanol indicating that ethanol did not disrupt the normal regulatory systems. The addition of ethanol resulted in an increase in the proportion of unsaturated fatty acids at all incubation temperatures. Thus, the effects of ethanol and a shift down in temperature were roughly additive while the effects of ethanol and a shift up in temperature were opposing.

The increases in the proportion of unsaturated fatty acids in response to ethanol addition and a shift to lower temperature were primarily the result of an absolute decrease in esterified saturated fatty acids. Unlike ethanol addition, a shift to lower temperature was also accompanied by an absolute increase in esterified unsaturated fatty acids. A shift to higher temperature caused an inhibition of acetate incorporation into both saturated and unsaturated fatty acids which was further inhibited by the inclusion of ethanol.

The stimulation of lipid synthesis by a shift to lower temperature and its inhibition by a shift to higher temperature are difficult to explain. These changes may be related to changes in the levels of guanosine 3′,5′-bis(diphosphate). High levels of ppGpp inhibit both phospholipid synthesis (Merlie & Pizer, 1973) and fatty acid synthesis (Nunn & Cronan, 1976). Recent studies by Gallant et al. (1977) have shown that *E. coli* rapidly accumulates this nucleotide upon shifting to a higher temperature.

Effect of Ethanol on the Fatty Acid Composition of an Unsaturated Fatty Acid Auxotroph. Our results thus far demonstrate that the addition of ethanol causes a reduction in the proportion of saturated fatty acids incorporated into phospholipids. This reduction could reflect an increased availability of unsaturated fatty acids for phospholipid synthesis resulting from a stimulation of unsaturated fatty acid synthesis by ethanol. To test this, we used an unsaturated fatty acid auxotroph, strain K1060, which derives all its esterified unsaturated fatty acids from exogenous supplements (Overath et al., 1970). Strain K1060 was grown in the presence and absence of ethanol with several unsaturated fatty acid supplements (Table V). When supplemented with either palmi-

TABLE V: Effect of Ethanol on the Fatty Acid Composition of an Unsaturated Fatty Acid Auxotroph, Strain K1060.[a]

Unsaturated supplement	Conditions	Fatty acid composition (%) 14:0	16:0	16:1	*cis*-Δ^9-18:1	20:2	UFA/SFA ratio[b]
16:1	No EtOH	5.7	44.1	48.9			0.96
	3% EtOH	4.3	38.4	56.1			1.28
cis-Δ^9-18:1	No EtOH	6.1	31.6		62.3		1.65
	3% EtOH	3.5	27.0		69.5		2.28
20:2	No EtOH	14.4	46.0			39.6	0.66
	3% EtOH	9.2	40.8			49.9	1.00

[a] Cells were grown in the presence of 3% ethanol with different fatty acid supplements. Phospholipids were separated by thin-layer chromatography prior to fatty acid analysis by gas chromatography. Results are expressed as a percentage of total fatty acids. [b] UFA, unsaturated fatty acids; SFA, saturated fatty acids.

toleic acid (16:1) or oleic acid (*cis*-Δ^9-18:1), the addition of ethanol resulted in an increase in the proportion of esterified unsaturated fatty acids. Further, this ethanol-induced change was not dependent upon specific unsaturated acyl chains. *E. coli* does not normally synthesize diunsaturated fatty acids or fatty acids of chain lengths greater than 18 carbons. However, strain K1060 supplemented with eicosadienoic acid (20:2) still underwent significant changes in the proportion of unsaturated fatty acids in response to ethanol (Table V). These results demonstrate that ethanol does not act by stimulating unsaturated fatty acid synthesis, and that the regulation of unsaturated fatty acid synthesis is not essential for the ethanol response.

Effect of Ethanol on Free Fatty Acid Synthesis. Fatty acid synthesis in *E. coli* is normally tightly coupled with phospholipid synthesis (Cronan, 1974) and prevents an evaluation of the effect of ethanol on either of these systems independent of the other. In order to determine if ethanol directly affects fatty acid synthesis, we have used two mutants in which fatty acid synthesis can be uncoupled from phospholipid synthesis in vivo by glycerol 3-phosphate starvation. The removal of exogenous glycerol 3-phosphate from growing cells of strains LW1 and LW3 causes an inhibition of phospholipid synthesis and an accumulation of free (nonesterified) fatty acids (Cronan et al., 1975). These are easily labeled by pulsing the cells with [^{14}C]acetate during starvation. Subsequent analysis of the free fatty acids provides an in vivo method for examining the regulation of fatty acid synthesis, per se, independent of the acyltransferase system.

The addition of ethanol to strains LW1 and LW3 altered the proportions of saturated and unsaturated free fatty acids which accumulated (Figures 1A and 1C). Although the synthesis of both saturated as well as unsaturated fatty acids was inhibited by ethanol ([^{14}C]acetate incorporation), 3% ethanol caused only a 23% decrease in the synthesis of unsaturated fatty acids while causing a 73% reduction in the synthesis of saturated fatty acids. Thus the ethanol-induced increase in the UFA/SFA ratio during free fatty acid synthesis results from the preferential inhibition of saturated fatty acid synthesis.

In order to determine whether ethanol affects the incorporation of fatty acids into newly synthesized phospholipids in strains LW1 and LW3, we also examined the fatty acid composition of the phospholipids. In the absence of exogenous glycerol 3-phosphate, a small amount of residual phospholipid synthesis continues in these strains (Cronan et al., 1975). The ratio of unsaturated to saturated fatty acids either in these residual phospholipids or in the phospholipids of cells supplemented with glycerol 3-phosphate can be compared with the ratios in the accumulated free fatty acids to determine the effect of ethanol on the selectivity of the phospholipid synthesizing enzymes. Figure 1 (B and D) demonstrates that phospholipids extracted from glycerol 3-phosphate supplemented cells as well as from glycerol 3-phosphate starved cells contained reduced levels of saturated fatty acids as a result of ethanol addition. Further, the dose-related reductions of saturated fatty acids incorporated into phospholipids are very similar to the dose dependent inhibition of de novo saturated fatty acid biosynthesis. This suggests that the ethanol-induced changes in the fatty acid composition of newly synthesized phospholipids arise prior to phospholipid synthesis and that ethanol may have little effect upon the selectivity of fatty acid utilization for phospholipids. Our results with strains LW1 and LW3 demonstrate that ethanol acts directly at the level of fatty acid synthesis, preferentially inhibiting the synthesis of saturated fatty acids.

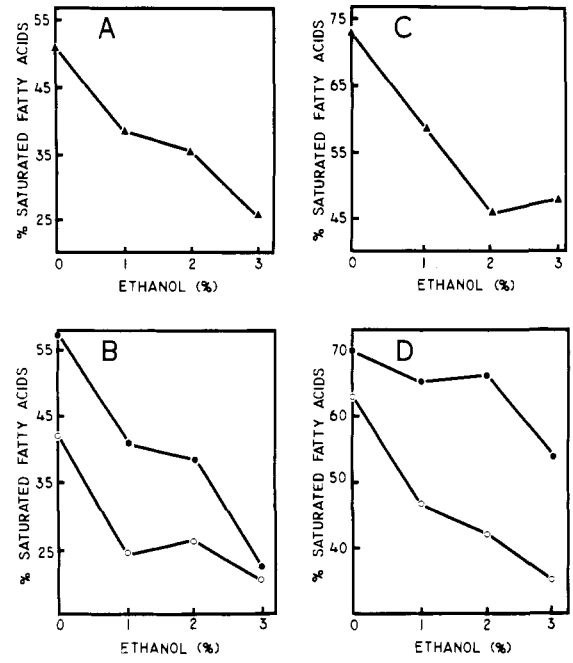

FIGURE 1: Effect of ethanol on free fatty acid synthesis and total lipid synthesis in strains LW1 and LW3. Exponential cultures grown with glycerol 3-phosphate supplementation were filtered, washed, and resuspended in fresh media minus glycerol 3-phosphate. Cultures were split and one-half of each culture was supplemented with glycerol 3-phosphate. Growth was continued for 3 h at which time 1-mL samples were removed from the cultures and added to tubes containing 10 μCi of [1-^{14}C]acetate (61 mCi/mmol) and various concentrations of ethanol. After 10 min the reaction was stopped by the addition of chloroform–methanol (1:2). Lipids were extracted and analyzed by argentation chromatography as described in the Experimental Procedure section. (A) Free fatty acid synthesis in strain LW3 starved for glycerol 3-phosphate. (B) Incorporation of fatty acids into phospholipids of strain LW3 starved for glycerol 3-phosphate (—●—) and supplemented with glycerol 3-phosphate (—○—). (C) Free fatty acid synthesis in strain LW1 starved for glycerol 3-phosphate. (D) Incorporation of fatty acids into phospholipids of strain LW1 starved for glycerol 3-phosphate (—●—) and supplemented with glycerol 3-phosphate (—○—).

Effect of Ethanol on the Incorporation of Exogenous Fatty Acids into Phospholipids in the Absence of de Novo Fatty Acid Synthesis. We have shown that ethanol increases the UFA/SFA ratio produced during de novo fatty acid synthesis by preferentially inhibiting the synthesis of saturated fatty acids. The dose-dependent shift in this ratio is almost identical with that observed following incorporation into phospholipids, suggesting that the acyltransferase system may not be affected directly by ethanol (Figure 1). This differs from temperature adaptation of fatty acid composition, which has been shown by other workers to be regulated by both fatty acid synthesis (Cronan, 1975) and the selectivity of the acyltransferase system (Esfahani et al., 1969; Sinensky, 1971). However, it is possible that in vivo the selectivity of the acyltransferase enzymes is only a secondary means of regulating fatty acid composition. Cells which can regulate their fatty acid composition at the level of fatty acid synthesis may not require or exhibit additional changes in selectivity of incorporation of fatty acids into phospholipids. Indeed, the similarity between synthesized fatty acids and phospholipid composition in *E. coli* in response to growth temperature (Cronan, 1975) would seem to support this theory. Thus the effect of ethanol on fatty acid synthesis may obscure any effect of the alcohol on the acyltransferase enzymes. To test this possibility, the effect of ethanol on the incorporation of exogenous fatty acids into phospholipids in vivo was examined under conditions which minimize de novo fatty acid synthesis. Strains L8 and L48 synthesize fatty acids at one-fourth the normal rate when

TABLE VI: Effect of Ethanol on the Incorporation of Fatty Acids into Phospholipids.[a]

	Fatty acids incorp.[b]					
	No EtOH			3% EtOH		
Strain	Total	Saturated	Unsaturated	Total	Saturated	Unsaturated
L8	298	216	82	266	193	73
L48	283	204	79	225	161	64

[a] Cells were grown with palmitic acid and oleic acid supplements at 37 °C, to a density of 10^8 cells/mL. The cells were harvested by centrifugation and resuspended in 0.5 volume of fresh media. Samples (1 mL) were removed and added to tubes containing 1 mL of media, 50 μCi of [9,10(*n*)-^{3}H]oleic acid (2.2 Ci/mmol), and 1.3 μCi of [1-^{14}C]palmitic acid (56 mCi/mmol) in the presence and absence of ethanol. Phospholipids were separated by thin-layer chromatography and analyzed by liquid scintillation counting. [b] Picomoles of fatty acid/sample.

TABLE VII: Effect of Exogenous 16:0 on Ethanol-Induced Changes in Fatty Acid Composition in Strain TB4.

	Fatty acid composition (%)							
	No EtOH				3% EtOH			
Supplement (mg/L)	14:0	16:0	16:1(Δ17)	*cis*-Δ^{11}-18:1	14:0	16:0	16:1(Δ17)	*cis*-Δ^{11}-18:1
0	3.3	28.0	38.1	30.1	1.7	20.8	35.4	41.9
5	4.3	54.6	32.3	7.9	1.0	45.0	41.6	11.5
10	2.9	58.1	30.0	8.1	0.9	47.6	39.3	11.9
50	5.9	62.7	23.9	6.9	1.0	51.5	33.4	13.5
100	2.3	51.8	34.7	10.6	1.1	45.5	37.5	15.9

[a] Cells were grown in the presence and absence of ethanol with various concentrations of palmitic acid (supplement), harvested at a density of 2 × 10^8 cells/mL, and analyzed by gas chromatography. Results are expressed as a percentage of total fatty acids.

shifted to 37 °C, and therefore require supplementation with both saturated and unsaturated fatty acids for phospholipid synthesis (Harder et al., 1972). The results in Table VI show that ethanol caused a slight inhibition of the incorporation of exogenous 16:0 and *cis*-Δ^9-18:1 fatty acids into phospholipids, but did not alter the ratio of their incorporation. These results demonstrate that ethanol has little direct effect upon the selectivity of the acyltransferase enzymes.

Effect of Exogenous Fatty Acids on Ethanol-Induced Changes in Fatty Acid Composition. Our results have shown that ethanol preferentially inhibits the synthesis of saturated fatty acids. The ethanol-induced reduction in the proportion of saturated fatty acids in cellular phospholipids could simply result from a reduced availability. To examine this possibility, we have determined the fatty acid composition of phospholipids from strain TB4 grown with a series of concentrations of 16:0 in the presence and absence of ethanol (Table VII). Supplementation with exogenous palmitic acid increased the proportion of esterified 16:0 with decreases in both *cis*-Δ^{11}-18:1 and 16:1 fatty acids. A similar pleiotropic effect of 16:0 was previously reported by Silbert et al. (1973a). The increase in abundance of esterified 16:0 was nearly constant at all levels of supplementation. Further, supplementation with palmitic acid did not prevent the ethanol-induced decrease in esterified 16:0. This decrease in esterified palmitic acid was compensated for primarily by an increase in 16:1 fatty acid rather than *cis*-Δ^{11}-18:1, in contrast to the previous experiments without supplements. These results suggest that the ethanol-induced reduction in esterified 16:0 is not due solely to a limitation of 16:0 available to the cell. However, it is not clear to what extent the activated form of 16:0 is available to the acyltransferase system.

The apparent failure of excess 16:0 to eliminate the ethanol-induced reduction in esterified palmitic acid could result from a limited ability to transport or activate exogenous 16:0, consistent with the lack of a dose-dependent response to 16:0 supplement. Alternatively, ethanol could directly inhibit the transport or activation of exogenously supplied 16:0. Either

TABLE VIII: Effect of Ethanol on the Incorporation of Exogenous 16:0 (^{3}H) and Endogenous 16:0 (^{14}C) into the Phospholipids of Strain TB4.[a]

	Palmitic acid incorp. into phospholipids (cpm)		
Conditions	Endogenous ^{14}C	Exogenous ^{3}H	Ratio[b]
No EtOH	2500	18 200	7.3
3% EtOH	310	20 800	67.1

[a] Samples from an exponential culture were pulsed for 10 minutes with 10 μCi of [1-^{14}C]acetate (61 mCi/mmol) and 10 μCi/mL of [9,10(*n*)-^{3}H]palmitic acid (442.5 mCi/mmol). Lipids were extracted and the phospholipids separated using thin-layer chromatography. [b] Ratio of the ^{3}H cpm to ^{14}C cpm.

of these coupled with a high affinity of the acyltransferase enzymes for 16:0 would lead to changes in fatty acid composition which still predominantly reflect the biosynthetic production of 16:0. To investigate these possibilities, we have examined the effect of ethanol on the contribution of de novo biosynthesis and of exogenously supplied 16:0 to esterified lipids in both pulse-label experiments and also in bulk lipid composition.

Double-label experiments were performed with strain TB4 using [^{14}C]acetate to label esterified palmitic acid derived from biosynthesis and [^{3}H]palmitic acid to label that derived from supplements (Table VIII). In strain TB4, the incorporation of exogenously supplied 16:0 (^{3}H) was slightly stimulated by the addition of ethanol while the incorporation from biosynthesis (^{14}C) was strongly inhibited. These results confirm that ethanol inhibits saturated fatty acid synthesis and demonstrate that ethanol does not decrease the availability of exogenously supplied 16:0 to the acyltransferase enzymes.

The results of the pulse-label experiments were further confirmed in a second organism, strain K1060, using exogenously supplied 17:0 and *cis*-Δ^9-18:1 fatty acids. Heptadecanoic acid (17:0) is used by *E. coli* as a 16:0 equivalent (Silbert et

TABLE IX: Effect of Ethanol on the Incorporation of 17:0 into the Phospholipids of an Unsaturated Fatty Acid Auxotroph, Strain K1060.[a]

	Fatty acid composition (%)				Ratio
Conditions	14:0	16:0	17:0	*cis*-Δ^9-18:1(Δ19)	(14:0 + 16:0)/17:0
No ethanol	7.0	40.7	25.4	26.9	1.9
3% ethanol	3.4	25.8	41.2	29.6	0.7

[a] Cells were grown with both 17:0 and 18:1 fatty acids in the presence and absence of ethanol. Phospholipids were purified by thin-layer chromatography and analyzed by gas chromatography. Results are expressed as a percentage of total fatty acids.

al., 1973; Ingram, 1977c). In the absence of a direct effect of ethanol on the utilization of exogenous fatty acids for esterification, the ratio of esterified (14:0 + 16:0)/17:0 provides a measure of saturated fatty acid biosynthesis (Table IX). These fatty acids are easily resolved by gas chromatography. As observed in pulse-label experiments, the utilization of saturated fatty acids derived from biosynthesis (16:0 + 14:0) and of that derived from exogenous supplements (17:0) did not follow the same trends in response to ethanol addition. The addition of ethanol resulted in a dramatic increase in the proportion of exogenously supplied saturated fatty acid (17:0) and a simultaneous decrease in the proportion of saturated fatty acids derived from biosynthesis (16:0 + 14:0). Further, the ethanol-induced increase in the proportion of unsaturated fatty acid (18:1) was also reduced in strain K1060 as compared to strain TB4 grown with a saturated fatty acid supplement (Table VII). This may be due to the more efficient transport or utilization of exogenous fatty acids by strain K1060. Spontaneous secondary mutations appear to be selected for in fatty acid auxotrophs (Fox et al., 1970). Thus the exogenous supply of saturated fatty acids to this mutant partially compensates for the direct inhibition of saturated fatty acid synthesis by ethanol. These results provide further evidence that the ethanol-induced changes in fatty acid composition do not result from a direct effect of ethanol on the selectivity of the acyltransferase system in vivo.

Discussion

We have previously shown that the addition of ethanol to *E. coli* caused a reduction in the proportion of palmitic acid (16:0) esterified into membrane lipids (Ingram, 1976). This decrease in palmitic acid was subsequently found to occur in both phosphatidylglycerol and phosphatidylethanolamine (Ingram, 1977a), the major phospholipids present in *E. coli* (Cronan & Vagelos, 1972). The experiments described in this paper represent an extension of these previous reports and were designed to determine the in vivo mechanisms by which ethanol induces fatty acid changes. Fatty acid degradation, fatty acid synthesis, and fatty acid utilization (incorporation into phospholipids) were considered as possible sites for the interaction with ethanol. The results presented in this paper provide evidence that these changes in fatty acid composition result primarily from the preferential inhibition of saturated fatty acid synthesis by ethanol.

Several observations support our conclusion: (1) mutants deficient in fatty acid degradation showed an ethanol-induced change in their fatty acid composition identical to that observed in wild-type cells (Table II). Thus, the addition of ethanol does not lead to reduced incorporation of palmitic acid into phospholipids as a result of preferential degradation of the saturated fatty acids. (2) The immediacy of the ethanol-induced changes in the fatty acid composition of newly synthesized phospholipids, as well as their equally rapid reversal upon ethanol removal suggest that ethanol is acting directly at the level of lipid synthesis (Table III). (3) In an unsaturated fatty acid auxotroph, the ratio of unsaturated to saturated fatty acids incorporated into phospholipids was elevated in response to ethanol. This elevation occurred regardless of the unsaturated fatty acid supplement provided (Table V). Thus, the increase in the proportion of unsaturated fatty acids did not result from a stimulation of their synthesis by ethanol. (4) When fatty acid synthesis was uncoupled from phospholipid synthesis, ethanol caused a dose-related reduction in the proportion of saturated fatty acids synthesized (Figures 1A and 1C). This indicates that ethanol is acting directly at the level of fatty acid synthesis. In addition, the similarity between the ratios of free fatty acids and esterified fatty acids suggests that the effect of ethanol on fatty acid synthesis is responsible for most, if not all, of the observed reduction in esterified palmitic acid. (5) In the absence of endogenous fatty acid synthesis, ethanol did not alter the ratios of fatty acids incorporated into phospholipids (Table VI). Unlike temperature adaptation, adaptation to ethanol is not mediated by altering the selectivity of the acyltransferase enzymes. These experiments demonstrate that the endogenous synthesis of saturated fatty acids is essential for ethanol-induced fatty acid changes to occur. The ability to eliminate several lipid synthesizing and degradative processes without eradicating the ethanol response is in agreement with our conclusion that ethanol preferentially inhibits saturated fatty acid synthesis.

In view of our present understanding of lipid synthesis in *E. coli*, the inability to overcome the ethanol response with exogenous 16:0 is surprising. If the inhibition of saturated fatty acid synthesis simply results in decreased amounts of saturated acyl chains available to the acyltransferase enzymes, exogenously supplied 16:0 would be expected to overcome this limitation. However, it is possible that *E. coli* has a limited capacity for activating or transporting exogenous fatty acids. This could explain why we did not observe a dose-related incorporation of exogenous palmitic acid when strain TB4 was grown with a wide range of palmitic acid supplements (Table VII). Thus, in the presence of ethanol, strain TB4 may not have been able to incorporate the increased levels of palmitic acid necessary to compensate for the inhibition of saturated fatty acid biosynthesis. The ability of strain K1060 to incorporate higher levels of 17:0 in response to ethanol (Table IX) may result from a secondary mutation relating to the transport of exogenous fatty acids. Indeed, several strains defective in unsaturated fatty acid synthesis, such as strain K1060, have been shown to acquire secondary mutations enabling them to utilize unusual fatty acids for phospholipid synthesis (Fox et al., 1970). Although we cannot entirely exclude the possibility that ethanol inhibits the transport or activation of saturated fatty acids, our results with [^{3}H]palmitic acid (Table VIII) make this possibility unlikely.

Recent studies by Nunn (1977) have shown that the addition of phenethyl alcohol also results in a decreased incorporation of saturated fatty acid into newly synthesized lipids, analogous to our results with ethanol. Unlike ethanol, however, phenethyl alcohol strongly inhibits the incorporation of exogenous fatty acids into phospholipids and had little effect on fatty acid synthesis per se. Thus, Nunn concluded that phenethyl alcohol affects lipid synthesis primarily at the level of fatty acid utilization, while ethanol primarily affects the synthesis of saturated fatty acids. During the accumulation of free fatty acids in the absence of phospholipid synthesis, however, both phenethyl alcohol and ethanol cause similar increases in the

ratio of unsaturated fatty acids to saturated fatty acids. It is believed that the same enzymes are responsible for elongating both saturated and unsaturated intermediates except for the dehydrases which form the double bonds in β-hydroxydecanoyl-ACP (Volpe & Vagelos, 1976). β-Hydroxyacyl-ACP dehydrase catalyzes the dehydration of β-hydroxydecanoyl-ACP to form *trans*-2-enoyl-ACP thioesters which are utilized in the synthesis of long chain saturated fatty acids. Unsaturated fatty acids, however, are formed as a result of the β-hydroxydecanoyl-ACP dehydrase which catalyzes the formation of *cis*-3-decanoyl-ACP. Therefore, competition between these two enzymes for the β-hydroxydecanoyl-ACP could determine the ratio of saturated to unsaturated fatty acids synthesized by the cell (Volpe & Vagelos, 1976). In this regard, it is of particular interest that Rando & Bloch (1968) have reported the ability of the β-hydroxydecanoyl-ACP dehydrase to form *trans*-2-decanoyl-ACP as well as the *cis*-3-decanoyl-ACP. A preferential inhibition of the β-hydroxyacyl-ACP dehydrase by ethanol could result in the fatty acid changes observed, providing the β-hydroxydecanoyl-ACP dehydrase was not inhibited. In vitro experiments are now in progress to determine the specific site of ethanol interaction.

Our observation that *E. coli* initially over-compensates in its response to ethanol is in accord with our view that the bacteria are adapting to the presence of alcohol. Such an adaptation of membrane lipids may occur in mammalian cells, leading to the conditions of tolerance and withdrawal (upon removal). A recent report by Chin & Goldstein (1977) demonstrated that membranes isolated from mice subjected to long-term ethanol treatment were relatively resistant to the effects of alcohol, suggesting a direct membrane adaptation. Since we previously hypothesized that changes in membrane lipids may be responsible for the tolerance of humans and animals to ethanol (Ingram, 1976), it would be of interest to determine if the adapted membranes isolated from mice also displayed specific membrane lipid changes.

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