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EFFECTS OF CHANGING THE CARBON SOURCE ON THE
PHOSPHOLIPIDS COMPOSITION OF E. COLI

by

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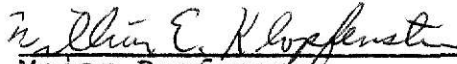
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INTRODUCTION

The cell membrane is one of the most important cellular structures. Phospholipids are the major structural component of cell membranes throughout nature and are also thought to play an important functional role in certain membrane localized process.

Escherichia coli has been chosen frequently for membranes studies because it is the best characterized cell in nature.

The phospholipids of E. coli have been found to consist of five classes: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylserine (PS) and phosphatidic acid (PA) with PE being the major phospholipid in both wild type and its unsaturated fatty acid auxotrophic mutant 29.

The effect of changing the carbon source on the amount of the total phospholipids and PE fraction and on their constituents fatty acids were examined.

The changes in the composition of phospholipids have been associated with their effect on membrane functions. One such relationship between composition and function which has been suggested for animal cells links alterations in the cell membrane composition with malignancy.

ABBREVIATIONS

Some common abbreviations used throughout this thesis are the following:

- PE - Phosphatidylethanolamine
- PG - Phosphatidylglycerol
- CL - Cardiolipin
- PS - Phosphatidylserine
- PA - Phosphatidic Acid
- TLC - Thin Layer Chromatography

LITERATURE REVIEW

The bacteria possess many advantages as organisms in which to study membranes (1) and their lipid compositions are much less complex than those of eukaryotic cells. The obvious choice among the eubacteria is Escherichia coli, due to the wealth of genetic, biochemical and physiological data concerning this organism. The lipid composition of this organism is at least as simple as that of other bacteria and the bulk of the data concerning lipid metabolism in bacteria has employed this organism (1).

Intracellular Location of the Phospholipids

The cell envelope of E. coli consists mainly of an inner (cytoplasmic) and an outer membrane (2). The inner membrane contains cytochromes, the enzymes of electron transport system, the enzymes of phospholipid biosynthesis, and various proteins involved in active transport. In contrast, the outer membrane is almost devoid of known enzyme activities and contains the lipopolysaccharide component of the envelope. The envelope of E. coli contains all the cellular phospholipid, which is distributed between the inner and outer membranes (2). In addition to phospholipid, the outer membrane has two other lipid components, the lipid A moiety of the lipopolysaccharide and the murein lipoprotein (2). The role of these structures in the function of the

outer membrane is unknown, although lipid A has recently been shown to be required for cell growth (c.f. 2). The site of synthesis of the phospholipids appears to be within the cell envelope rather than in the cytoplasm of the cell (1).

Membrane phospholipids have separate structural and nonstructural roles separately (3). The structural roles of membranes are currently considered to be (1) subdivision of the cell and (2) formation of a structural framework upon which enzyme systems may be assembled in a spatially ordered way. The nonstructural roles are phospholipids are required for the maximal activity of many membrane bound enzymes.

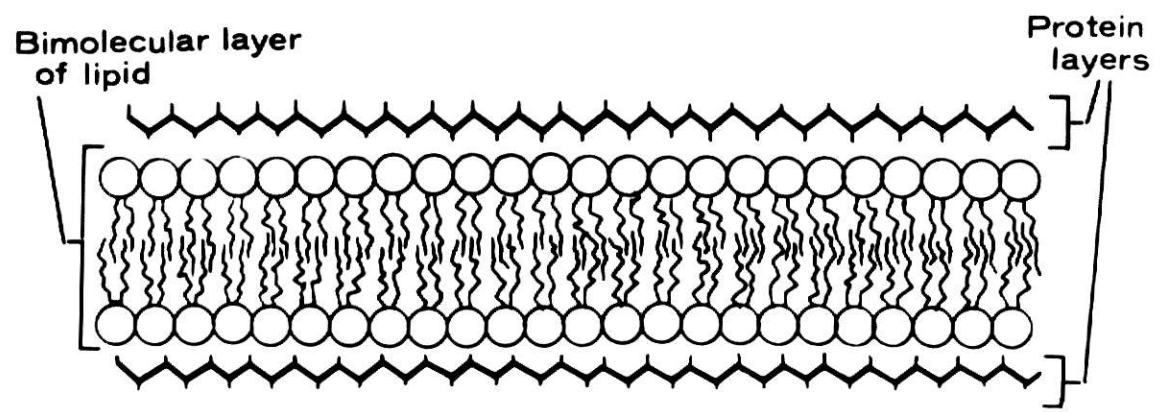
The phospholipid molecule is ideally suited to a role in membrane formation by virtue of the possession of both a long chain hydrophobic region (which is responsible for the insolubility of these molecules in aqueous solutions) and a hydrophilic, charged, polar grouping. When dispersed in aqueous solutions, these molecules spontaneously form micells or bilayers in which the hydrophobic regions are oriented toward one another and the polar groups are presented to the aqueous phase. Such a bilayer arrangement may occur in vivo in biological membranes, similar to what was proposed by Danielli and Davson in 1935 (4). This hypothesis said that the membrane exists as a bilayer of mixed lipids sandwiched between two layers of adsorbed protein as shown in Figure 1 (5). The state of the acyl moieties of membrane

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Figure 1. The Davson-Danielli Membrane Model

(Drawing taken from Bloom and Fawcett, A Textbook of Histology, 10th ed., W. B. Saunders, Philadelphia, PA, 1975)



phospholipids can change depending on the temperature. It can change from a solid, hexagonal close-packing to a fluid, more random array. This has been termed a phase transition (c.f. 2).

Phospholipid Composition and Structure

Phospholipid Species

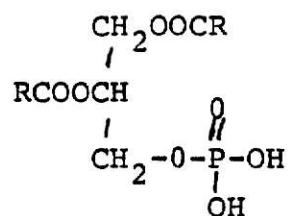
The phospholipids commonly encountered in E. coli are all derivatives of phosphatidic acid. The structures of the five species of phospholipid are shown in Figure 2. The data establishing these structures are given below. All of the lipids found in E. coli are found in cells grown in defined medium, thus establishing their synthesis by the bacterium (1).

Phosphatidylethanolamine. This is the major phospholipid found in E. coli and comprises 70-80% of the cellular phospholipid (c.f. 1). The structure of this lipid is identical to that shown on Figure 2 as has been established by Cronan and Vagelos via (1) (i) cochromatography with authentic PE of both synthetic and natural origin in many chromatographic systems; (ii) the presence of an amino group, phosphate, glycerol and acyl ester in molar ratio of 1:1:1:2; (iii) the production of glycerol phosphory-ethanolamine upon mild alkaline hydrolysis; (iv) the degradation with specific phospholipase to the expected products and finally (v) infrared spectral analysis.

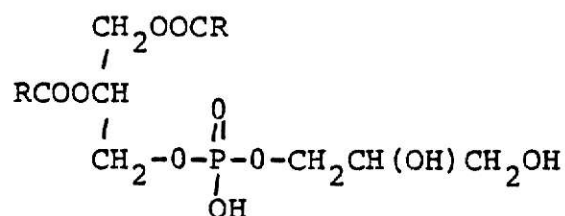
Phosphatidylglycerol. Cronan and Vagelos (1) reported a value of 5-15% of PG in the total cellular phospholipid while Ames (6) reported a value of 19%. PG structure is shown in Figure 2. The structure of this lipid was identified by the following criteria as cited from Cronan and Vagelos (1) (i) cochromatography with authentic PG; (ii) presence of glycerol, phosphate, and acyl ester in a 2:1:2 ratio; (iii) presence of vicinal hydroxyl groups in the intact lipid; (iv) formation of one mole of formaldehyde per mole of phosphate upon periodate oxidation of the intact purified lipid; (v) formation of PG by mild alkaline hydrolysis of the lipid; and production of the predicted products upon degradation by specific phospholipases.

Cardiolipin. This lipid comprises about 5-15% of E. coli phospholipids. It has a structure identical with that of diphosphatidylglycerol and bovine heart CL (1). The CL structure of the E. coli lipid was demonstrated by (c.f. 1). (i) cochromatography with bovine heart CL; (ii) the finding of glycerol, phosphate, and acyl ester in a 3:2:4 ratio; (iii) the formation of di (glycerolphosphoryl) glycerol upon mild alkaline hydrolysis; and (v) the production of predicted products upon degradation by specific phospholipases.

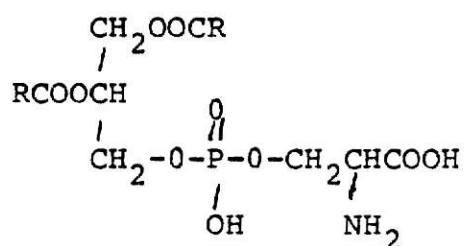
Phosphatidic Acid. This is found in trace amount (less than 1% of the total phospholipid phosphorus) in E. coli (c.f. 1),



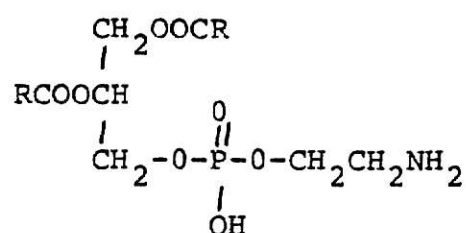
Phosphatidic Acid



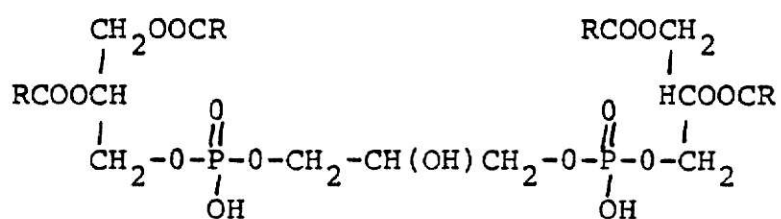
Phosphatidylglycerol



Phosphatidylserine



Phosphatidylethanolamine



Cardiolipin (Diphosphatidylglycerol)

Figure 2. The Structure of the Phospholipids of E. coli.

although a value of 5% of the total phospholipid was reported by Randle et al. (7). This result was based on the analysis of the products of mild alkaline hydrolysis of the mixture of lipids extracted from the cell. Another report from the same laboratory didn't report phosphatidic acid in an analysis of E. coli lipid (c.f. 1).

Phosphatidylserine. Very small amounts of this lipid are found in E. coli (1). The identification processes used were (i) cochromatography with authentic PS; (ii) labelling with radioactive phosphate and L-serine; (iii) production of glycerol phosphorylserine by mild alkaline hydrolysis of the lipid and finally (iv) reaction with ninhydrin (c.f. 1).

Other Phospholipids. Lysophosphatidylethanolamine has often been reported in trace amounts in E. coli (c.f. 1). Cronan and Vagelos (1) claimed that the main reason for the presence of this lipid in many of these cases is that enough care was not taken to distinguish this lipid from PS.

Phosphatidylglycerol phosphate has been reported twice in E. coli but the authors did not present conclusive evidence (1).

One report mentioned the presence of phosphatidylinositol in E. coli (c.f. 1). Cronan and Vagelos (1) said that this lipid was probably PG, since the test used to identify inositol would also react with glycerol.

Cytidine diphosphate diglyceride has been reported in E. coli (c.f. 1). The evidence for identification again was not conclusive.

The following lipids have not been detected in E. coli, although they were searched for by sensitive methods (c.f. 1): phosphatidylcholine, N-methylated derivatives of PE, and phosphatidylinositol. No plasmalogens have yet been detected in E. coli.

A minor phospholipid designated Y was reported by Ames (6) in both *Salmonella typhimurium* and E. coli. It contains phosphate and fatty acid in the ratio expected for a phosphatide (8). The identification of this lipid has been hampered by the elusiveness of its production; it didn't give any of the common hydrolysis products.

The Fatty Acids of the Phospholipids

Saturated Fatty Acids. Palmitic (hexadecanoic) and myristic (tetradecanoic) are the main saturated fatty acids of E. coli phospholipids. Stearic (octadecanoic) and lauric (dodecanoic) acid are present in trace amounts (c.f. 1). The structures of these fatty acids together with the other fatty acids are given in Table 1. The identification of these acids is based on their gas chromatographic behavior. Palmitic acid comprises about half of the total fatty acid of the cell, and is found mostly esterified to position 1 of the glycerol

backbone of the phospholipid, while the other saturated fatty acids are distributed between both positions (c.f. 1).

Unsaturated Fatty Acids. The unsaturated fatty acids found in E. coli are all monoenes of the cis conformations (c.f. 1). Their structures were determined by gas-liquid and argentation chromatographic behavior, and on the infrared spectra of the acids. These acids all contained sixteen or eighteen carbons. The hexadecenoic acid has been identified as palmitoleic acid (c.f. 1). While the octadecenoic acid fraction has now been shown by several techniques to consist solely of cis vaccenic (cis-11-octadecenoic) acid. A. G. Marr (9) was the first one who analysed the composition of octadecenoic acid and reported that it consisted of a mixture of cis-vaccenic and oleic (cis-9-octadecenoic). These workers used a strain of bacteria isolated and identified by their laboratory rather than a standard laboratory strain.

But the workers approached the conclusion that cis-vaccenic is the only octadecenoic acid found in E. coli depending on the old finding of Bloch et al. (c.f. 9) and other workers on the mechanism of fatty acid synthesis in E. coli. The pathway elucidated by these workers produces only unsaturated fatty acid molecules with the double bond between the seventh and eighth carbon atoms from the methyl end of the molecule (e.g. palmitoleic and cis-vaccenic acid) (c.f. 9). The use of silver nitrate-impregnated thin layer

Table 1

The Structure of the Fatty Acids of E. coli

Structural Type	General Structure	Systematic Names	Trivial Names
Saturated	$\text{CH}_3 - (\text{CH}_2)_x - \text{COOH}$	Dodecanoic acid (x=10) Tetradecanoic acid (x=12) Hexadecanoic acid (x=14) Octadecanoic acid (x=16)	Lauric acid Myristic acid Palmitic acid Stearic acid
Unsaturated	$\text{CH}_3 - (\text{CH}_2)_5 \overset{\text{H}}{\underset{\text{H}}{\text{C}}} = \text{C} - (\text{CH}_2)_x - \text{COOH}$	cis-9-Hexadecenoic acid (x=7) cis-11-Octadecenoic acid (x=9)	Palmitoleic acid cis-Vaccenic acid
Cyclopropane	$\text{CH}_3 - (\text{CH}_2)_5 \overset{\text{CH}_2}{\underset{\text{H}}{\text{C}}} - \overset{\text{H}}{\underset{\text{H}}{\text{C}}} - (\text{CH}_2)_x - \text{COOH}$	cis-9, 10-Methylene hexadecanoic acid (x=7) cis-11, 12-Methylene octadecanoic acid (x=9)	None Lactobacillic acid
Hydroxy	$\text{CH}_3 - (\text{CH}_2)_x - \text{CHOH} - \text{CH}_2 - \text{COOH}$	D(--) - 3-Hydroxytetradecanoic acid (x=10)	~~3~~ -Hydroxymyristic acid

chromatography also helped them in solving the problem of oleic acid existence since it resolves the methyl esters of oleic and cis-vaccenic acid (c.f. 9).

Another report for the presence of oleic acid in E. coli was by Weinbaum and Panos (10), but a later report from the same laboratory using the same techniques demonstrated that oleic acid was not present (11).

Scheuerbraandt and Bloch (12) were the first to report that cis-vaccenic acid is the sole octadecenoic acid found in E. coli. Many other workers later reached the same conclusion, that cis-vaccenic is the sole octadecenoic acid in different strains of E. coli. From these reports plus the support given by the genetic and enzymological analyses it has been established that oleic acid is not synthesized by E. coli (1). Crowe and Urban (13) did not observe oleic acid as a fatty acid component in two strains of E. coli 15T⁻. Cronan and Vagelos (1) reported that palmitoleic and cis-vaccenic acids are found esterified predominately to position 2 of the sn-glycerol-3-phosphate backbone of the phospholipids.

Cyclopropane Fatty Acids

The cyclopropane fatty acids of E. coli are formed by the methylation of the unsaturated fatty acids of the phospholipids (14), (15) and (16). The methylene group is donated by S-adenosyl methionine and the addition is

believed to occur at the level of the phospholipids. These fatty acids consist of cis-9, 10-methylenehexadecanoic acid (c.f. 1) and cis-11, 12-methyleneoctadecanoic (lactobacillic) acid $\text{H}_3\text{C}-(\text{CH}_2)_5-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-\overset{\text{H}}{\underset{\text{H}}{\text{C}}}-(\text{CH}_2)_9 \text{COOH}$ (15) and (16). The structures of these acids were determined based on (i) the structures of the monoenoic acid from which they are derived, (ii) chromatographic analysis of the acids before and after degradation (c.f. 1) of the cyclopropane ring, and (iii) mass and infrared spectroscopy (c.f. 1). Indirect data indicate the configuration is cis (1).

Miscellaneous Fatty Acids

An acid possessing a gas chromatographic behavior of a fifteen-carbon cyclopropane fatty acid has been mentioned in several papers (c.f. 1). To date this acid has not been identified (1). DeSiervo (17) has reported the presence of a large number of fatty acids (identified as gas-liquid chromatography peaks) which have not been recorded by other workers. Many of these peaks are due to impurities in the unusual solvents he used for extraction and chromatography according to Cronan and Vagelos (1).

Other fatty acids that are present in the cell are the acyl moieties of the "Lipid A" component of the cell wall. Lipid A consists of a mixture of lauric, myristic, palmitic and β -hydroxymyristic acids (1). β -hydroxymyristic acid

is a specific marker for lipid A and is not found in phospholipid (c.f. 1).

Effect of Culture Conditions

1. Effect of Temperature. Escherichia coli was known to adjust the fatty acid composition of its phospholipids in response to growth temperature as noted by Marr and Ingraham (18). Many other workers have noted the same effect (1). As the temperature of growth is lowered the proportion of unsaturated fatty acids (chiefly cis-vaccenic acid) in the membrane increases. The work of Crowe and Urban (13) showed that the saturated/unsaturated fatty acid ratio of Escherichia coli 15T⁻ decreases almost three-fold as growth temperature decreases from 43 to 27⁰ C, whereas this ratio in a fast-growing mutant derived from 15T⁻ changes only half as much.

2. Effect of Growth Phase. Four pronounced changes in the phospholipid composition occur during the transition of E. coli cultures from exponential growth to the stationary growth phase. These changes are an increase in CL, a decrease in PG, an increase in cyclopropane fatty acids, and a decrease in unsaturated fatty acids (c.f. 1). The first two changes are thought to be related as PG is a precursor of CL, and the decrease in PG phosphorus is almost stoichiometric with the increase in CL phosphorus (c.f. 1).

The increase in cyclopropane fatty acids is accompanied by a concomitant stoichiometric loss of unsaturated fatty acids, as once again the former is produced from the latter.

3. Changes in Energy Metabolism. The changes in the proportions of PG and CL which occur during the transition from exponential to stationary phase also occur during the following manipulations: addition of colicin K, dinitrophenol, penicillin, or cyanide, and infection with bacteriophage T₄ (c.f. 1).

Changes in the amount of cellular cyclopropane fatty acid can be engendered by changes in the medium, although the data are not explained by a simple hypothesis (c.f. 1). Decreased oxygen tension dramatically increases the amount of cyclopropane fatty acid (19) and (20). In addition to the variables listed, limitation of carbon source decreases cyclopropane acid content while phosphate starvation has no effect (21).

Many investigators have studied the effect of changing the growth medium on the composition of E. coli lipids. Dagley and Johnson (22) reviewed the work of Stephenson and Whetham (c.f. 22) who showed that the addition of acetate to an inorganic salt medium increased the lipid content of the Timothy grass bacillus but did not affect protein formation. Glucose additions increased both protein and lipid contents, the former more than the latter. Dagley

and Dawes 1949 (c.f. 22) demonstrated increased polysaccharide contents for *bact. coli* when glucose and other sugars were added to the growth medium. Dagley and Johnson (22) estimated both lipid and polysaccharide in the same batches of *bact. coli*, harvested from media containing varying amounts of glucose and acetate 90 minutes after the end of the logarithmic phase when polysaccharide storage is maximal (from Dagley and Dawes 1949).

Polysaccharide and lipid contents were determined for the fully-grown cultures and for the inocula. The results showed that the increase in polysaccharide provided by added glucose accompanies a fall in lipid content. Additional lipid storage resulting from the presence of acetate in glucose media is associated with a decrease in stored polysaccharide. Marr and Ingraham (18) showed that E. coli cells grown in a chemostat, limited by the concentration of ammonium salts, show a much higher content of saturated fatty acids (principally palmitic acid) than do cells harvested from an exponentially-growing batch culture in the same medium. Cells grown in a chemostat, limited by the concentration of glucose, show a slightly higher content of unsaturated fatty acids than cells from the corresponding batch culture. Their results suggested a significant influence of nutrition on the proportions of fatty acids produced by E. coli.

The influence of nutrition and of reduced growth rate was tested further by cultivation of E. coli at 30° C in a chemostat, limited by the concentration either of the carbon source or of the nitrogen source. The flow rate was adjusted to provide a specific growth rate (division per hour) of 0.2 hour⁻¹. Cells harvested from the glucose-limited culture differ only slightly in fatty acid composition from cells grown in batch culture. Thus, a reduction in specific growth rate from 0.65 to 0.2 hour⁻¹ does not significantly alter the proportions of fatty acids. The fatty acid composition of the cells from the nitrogen-limited chemostat is strikingly different. The amounts of palmitic and methylene hexadecanoic acids are increased, and the amount of unsaturated fatty acid is decreased. The effect of nitrogen limitation mimics the effect of growth at high temperature. Damoglou and Dawes (23) studied the lipid content and phosphate requirement of glucose and acetate-grown Escherichia coli as follows:

1. The phosphate requirement, i.e., the concentration of inorganic orthophosphate that just ceases to be limiting for growth of Escherichia coli N.C.T.C. 5928 was determined for growth in ammonium-salts media containing glucose or acetate as the carbon and energy source, and compared with that of six other strains of E. coli.

2. The phosphate requirement for E. coli N.C.T.C. 5928 growing on acetate was about ten times that for growth on glucose, but this difference was not observed with any of the other strains.

3. After about 40 generation's growth on acetate with phosphate limitation in a chemostat, the phosphate requirement of the cells gradually decreased until it was equivalent to that of the glucose-grown organism; a single passage through glucose batch culture sufficed to restore the original high phosphate requirement, indicating a permeability phenomenon.

4. The lipid content of E. coli N.C.T.C. 5928 grown on glucose or acetate was measured isotopically by fractionation of cells grown on inorganic ^{32}P orthophosphate and gravimetrically after extraction from the cells by three different methods; change of carbon source from glucose to acetate did not affect the lipid content, which remained constant at 8-9% of the bacterial dry weight. This finding is in a conflict with that of Dagley and Johnson (22).

Many studies have been done in the last ten years about the effects of changing the media on the phospholipid composition of E. coli mutants. Esfahani et al., 1969 (24) examined the effect of exogenous unsaturated fatty acids on the fatty acid composition of phospholipids of a mutant of E. coli. These exogenous acids serve as growth factors for

an auxotroph requiring unsaturated fatty acids. When each member of a structurally homologous series of cis-unsaturated fatty acids serves as a growth factor, the percentage of unsaturated fatty acids present in phospholipids increases with increasing chain length or decreasing number of double bonds in the apolar chain of the supplement. At 37° trans-octadecenoic acids support growth and are incorporated into phospholipids at levels higher than the corresponding cis-acids. However, a temperature shift to 27° with trans-acids results in loss of viability and lysis. Utilization of cis-acids as supplements at decreasing temperatures between 42° and 27° results in increasing amounts of unsaturated fatty acids in phospholipids with decreasing temperature. These observations suggest the operation of a regulatory mechanism which controls the composition of saturated versus unsaturated acids in order to maintain the physical properties of phospholipids with narrow limits.

Silbert et al. 1972 (25) investigated several different Escherichia coli K-12 strains for evidence of control of fatty acid metabolism in response to exogenous fatty acid supplementation. [¹⁴C]-acetate incorporation into lipid was used to identify the synthesized fatty acids recovered from the cell and the culture fluid. The identity and quantities (micrograms) of these fatty acids were determined by gas-liquid chromatography. When oleic acid is

present in the growth medium as the supplement, synthesized (unsaturated fatty acids) decrease in the cultures of two strains; furthermore, turnover of synthesized fatty acids via β oxidation can not occur in one of these strains. Taken together, these observations show that there is regulation of unsaturated fatty acid synthesis in these two strains. A third strain shows a less well defined response to oleate supplementation while an unsaturated fatty acid auxotroph from this strain clearly accumulated saturated and β -hydroxy fatty acids. The difference between strains with respect to regulation of synthesis may be explained partly in terms of differences in the β oxidation activities of the cells.

Silbert et al. (25) showed that the composition of the synthesized fatty acids recovered from the medium is consistent with the release of lipopolysaccharide and phospholipid from the outer membrane of the cell envelope.

Two studies were run in Silbert et al. laboratories which examined the incorporation of radioactive fatty acids and of fatty acid precursors into E. coli cellular phospholipid. One study suggested that previous growth of an unsaturated fatty acid auxotroph on different unsaturated fatty acid supplements resulted in regulation of subsequent fatty acid synthetase activity in resting cells, such that incorporation of radioactive acetate into saturated fatty acids was geared to the amount of saturated fatty acid in

the phospholipid. The second study showed a selective decrease in the specific radioactivity of fatty acid in the phospholipid associated with fatty acid supplementation of wild type cultures, together with an apparent constancy in the fatty acid composition of the phospholipid.

A study on temperature dependence and total replacement by branched-chain fatty acids was done by Silbert et al. (26). They evaluate the unsaturated fatty acid requirement in Escherichia coli. A derivative of a double mutant defective both in unsaturated fatty acid biosynthesis and in fatty acid degradation has been selected which grows equally well on anteisopentadecanoate (12-Me-14:0) or cis- Δ^9 octadecenoate (cis- Δ^9 -18:1). When this strain is grown for many generations on 12-Me-14:0, there is extensive incorporation of this analogue into the membrane phospholipid and essentially no detectable unsaturated fatty acids residues in any lipid-containing structures of the cell envelope. Secondly, as the maximal growth temperature of E. coli is approached, the minimum content of unsaturated fatty acid required by this strain for growth decreases to a few per cent and is associated with the appearance of substantial amounts of 12:0 (8%) and 14:0 (50%) in the phospholipid. These experiments demonstrate that the cis unsaturated fatty acids of E. coli phospholipids can be replaced by residues which possess no special electronic configuration. Hence, the unsaturated

fatty acids do not participate in specific interactions with other membrane components but serve a general role of controlling the packing of paraffin chains in the membrane bilayer.

Silbert went further in his study on E. coli and together with other workers, they studied the utilization of exogenous fatty acids by wild type E. coli K-12 using [^{14}C]-acetate incorporation to monitor fatty acid synthesis and unusual fatty acid analogs as a nonradioactive supplement to distinguish acyl groups in the phospholipid derived from the exogenous fatty acid supplements. This effect is associated with incorporation of the exogenous supplement into phospholipid. Even and odd chain-length saturated, trans and cis unsaturated fatty acids were included among the fatty acid supplements examined. Although a principal result of incorporation of the exogenous fatty acid is a replacement of structurally related acyl groups, the effect is pleotropic in many instances. For example, 16:0 replaces 18:1 and vice versa. These observations can be rationalized in terms of the positional distribution in the phospholipid of the acyl residues derived from synthesis and the pattern of incorporation of the exogenous fatty acids. β -OH-14:0 and -12:0, added to cultures as supplements, are not detectably incorporated into complex lipid (predominantly lipid A) and show little or no influence on fatty acid synthesis.

The incorporation of fatty acids containing photo-sensitive groups into phospholipids of Escherichia coli was studied by G. R. Greenberg et al. (27). They used a series of new fatty acids containing photosensitive groups at different positions on the paraffin chains. These acids supported the growth of an auxotroph of E. coli requiring unsaturated fatty acids. The derivatives were 6-, 9-, 11-, and 12-azido-stearic acids, 12-azido-oleic acid, 16-azido palmitelaidic acid, and 12-(4-azido-2-nitro Phenoxy)-stearic and oleic acids. Analyses of the phospholipids from cultures grown in the presence of the first six compounds showed that these derivatives accounted for 16-43% of the total fatty acids. Further analysis of phospholipids from cultures grown with 12-azido-oleic acid, 11-azidostearic acid, or 16-azido palmitelaidic acid indicated that the azido fatty acids were at the 2-position of the glycerol moieties. The incorporation of these fatty acid derivatives offers a new approach to the study of membrane structure and, in particular, phospholipid-protein interactions by photolysis-induced crosslinking of the fatty acids to the structures in their immediate vicinity.

More recent work in this area has been done by W. L. Olsen et al. (28). They found that E. coli K1060-B5 fab (E fab E thi) incorporates into phospholipids several new synthetic fatty acid analogs including those containing phenyl, phenoxy, and azidophenoxy groups at the ω position.

Thin Layer Chromatography of Lipids

Many thin layer techniques are known which give good separation of animal and bacterial lipids.

Skidmore and Entenmann (29) developed a system of two-dimensional TLC that separated rat liver phosphatides into several phosphate-positive spots in about two hour developing time. Characteristic hydrolysis products derived from PS, PE, phosphatidyl inositol, phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline were identified. The hydrolytic products of phosphatidic acid were not definitely characterized.

The application of TLC as described for rat liver phosphatides can be extended to phosphatide extracts of other tissues.

Skidmore and Entenmann (29) tried many solvents for TLC. The more important two solvents were: Solvent I which contain chloroform-methanol-7N ammonium hydroxide mixed in the ratio of 60:35:5. Solvent II which contains chloroform-methanol-7N ammonium hydroxide mixed in the ratio of 35:60:5.

The most useful solvent system for lipid extraction is the chloroform:methanol:water system (30). The polarity of the solvent plays an important role in the separation procedure. The polarity of the solvent is indicated by its dielectric constant. The smaller the dielectric constant the less polar the solvent, conversely the higher the value

the more polar the solvent (30). Polar lipids require non-polar solvents for separation while nonpolar require nonpolar solvents. The migration of lipid components in TLC will be increased as solvent polarity increases and as a result of that the R_f will be higher as the polarity increases.

Many detecting reagents for phospholipids and their hydrolytic products have been described. In 1964 Dittmer and Lester (31) described a spray reagent specific for phosphate esters on thin layer plates. The spray was a modification of the molybdenum blue reagent of Zinzadze (c.f. 31) which, when used as a spray, gives an instantaneous, specific reaction with phospholipids on silica gel or alumina plates.

Molybdenum blue reagent was prepared as follows:
Solution I: To 1 liter of 25N H_2SO_4 , 40.11 g of M_0O_3 is added and the mixture is boiled gently until the M_0O_3 is dissolved. Solution II: To 500 ml. of soln. I 1.78 g of powdered molybdenum is added and the mixture is boiled gently for 15 minutes. The soln. is cooled and decanted from any residue that may be present.

Molybdenum spray. Equal volumes of soln. I and II are mixed and the combined solution is mixed with two volumes of water. The final solution is greenish yellow in color. If too little water is added it will be blue; if too much, yellow. The spray is stable for months. Plates are sprayed lightly until the adsorbent is uniformly damp. Compounds containing

phosphate ester show up immediately as blue spots on a white or light blue-grey background. The intensity of the color increases on standing.

The molybdenum spray is very sensitive. It can detect as little as 0.005 μ mole of both phosphatidyl ethanolamine and phosphatidyl choline.

Regarding the specificity of the molybdenum spray a wide range of compounds including PA, Cl, sphingomyelin, -serine, -choline, -inositol, and -inositol diphosphate were all found to give positive reaction with the spray (31).

The major problem with the molybdenum spray is the colored background. In 1979 Ryu and MacCoss (32) tried to solve this problem. They described a simple modification of the Dittmer-Lester reagent that allows the detection of phospholipid derivates at very low concentration on silica gel and reversed-phase thin-layer plates. This modification, which involves the addition of acetic acid to the mixture, permits the observation of sharp blue spots on a white background.

The existence of good spraying reagent is very important in the quantitative analysis of lipid by TLC. Many procedures have been described concerning this quantitative estimation.

One of the better procedures mentioned was that by Blank et al. 1964 (33) employing densitometry. The chromatoplates are prepared with the usual solvent systems. The spots are

charred under standard conditions and analyzed with a Photovolt Corp. densitometer equipped with a special stage designed for holding 20 x 20 cm. chromatoplates. Each spot on the chromatoplate gives a peak of density values which is used for quantitative analysis. Charring of the spots was carried out by heating the plates in an oven for 25 minutes at 180° C, after spraying them lightly with a saturated solution of $K_2Cr_2O_7$ in 70% by volume of aqueous sulfuric acid. Analysis of the charred spots of a large number of lipids showed that the peak area vs. the amount of sample gave a linear relationship which passed through the origin (33). Regardless of the technique employed, poorly developed chromatoplates should not be used for quantitative analysis.

Quantitative studies on E. coli phospholipid composition and identification were done in 1967 by Kanemasa et al. (34) using thin layer and modified silicic acid. One-dimensional systems were developed with chloroform-methanol-water (70:25:4 by vol.). For two-dimensional separations, the first solvent was the one described above and the second, chloroform-methanol-7N NH_4OH (60:35:5, by vol.). Ninhydrin spray was used after development. The plate was then treated with molybdenum reagent. The results of the two-dimensional TLC of E. coli phospholipids identified the following phospholipids CL, PG, PE. One spot was negative for phosphorus, one showed that it is a phosphatidyl derivative, and the

last one was identified on the basis of its R_f value to be either PS or lyophosphatidyl ethanalamine. Kanemasa et al. (34) reported the anomalous behavior of PG on two-dimensional TLC using silica gel G. They found that PG isolated by column chromatography gave only one spot when applied alone, but it was separated into two spots when applied with PE. They said that the separation of PG into two spots occurs in the neutral solvent and not in the alkaline solvent. This suggests that in the neutral solvent PE and PG might interact to form a "salt" complex which is dissociated in the basic solvent. They further studied the fatty acid composition of the individual phospholipids and found no marked difference in fatty acid composition of the various lipids.

GioVanna F. Ames later quantitatively (6) studied the composition of E. coli K-12 phospholipids using thin layer chromatography. Four different developing solvents were used. Solvent 1; chloroform-methanol-water (65:25:4 v/v). Solvent 2; chloroform-methanol-7N ammonia (60:35:5 v/v). Solvent 3; chloroform-methanol-concentrated ammonia (70:21:1 v/v). Solvent 4; chloroform-methanol-glacial acetic acid (65:25:8 v/v). Their results are tabulated as follows:

E. Coli K-12

<u>Lipid</u>	<u>u moles/g (dry weight)</u>	<u>Per Cent of Total Phospholipid</u>
PE	33	69
PG	9	19
CL	3.1	6.5
PS	--	--
Y	2.9	6
PG-X	Undetectable	Undetectable
PA	--	--

MATERIALS AND METHODS

Growth of Cells

Wild type Escherichia coli 15T⁻ (555-7) arg⁻, try⁻, met⁻, and thy⁻ and its slow growing mutant (which is deficient in unsaturated fatty acid synthesis) referred to as 29 (Urban) were used. The growth medium consisted of M9 (c.f. 13) salts which, after autoclaving, were aseptically supplemented with L-arginine (100 ug/ml), L-methionine (50 ug/ml), L-tryptophan (50 ug/ml) and thymin (4 ug/ml). The mutant and its wild type parent were individually cultivated in M9-salts containing either 1) fatty acids (at 0.1% w/v) or 2) fatty acids (at 0.1% w/v) plus 0.1% (w/v) aspartate; 0.45% (w/v) of succinate or 0.40% (w/v) glucose as the carbon source. Cells used were the following:

<u>Wild Type (15T⁻) Cell</u>	<u>Mutant 29</u>
15 Aspartic (1 + 2)	29 Oleic (A + B)
15 Oleic + Aspartic (1 + 2)	29 Oleic (A + A)
15 Succinic (A + B)	29 Oleic (B + B)
15 Glucose (A + B)	29 Oleic + Aspartic (A + B)

Overnight cultures were used to inoculate 1000-1500 ml. of correspondings media at cell density of $\leq 2 \times 10^6$ cells/ml. Newly inoculated cultures were split into approximately 500 ml. aliquots and shake-incubated at 37^o C. Each aliquot was monitored for cell numbers at about 1/3 doubling time intervals.

Cells were harvested for fatty acid analysis only if doubling times were equal ($\pm 5\%$) to the standard doubling time in that medium. The standard doubling time is shown in Table 2.

<u>Cell</u>	<u>Wild Type</u>	<u>Mutant</u>
Media type	15T ⁻	#29
M-9 oleic	119	125
M-9 oleic + aspartic	65	106
(doubling time in minute)		

The standard doubling times were the mean doubling measurements resulting from 3-5 individual cultures, each of which experienced balanced growth. Cells were centrifuged at 5875 X g for 15 minutes, washed with fresh medium, recentrifuged and kept frozen. Cells were grown by Dr. James Urban of the Division of Biology (Kansas State University).

Phospholipid Extraction

Phospholipids were extracted from the frozen samples by the method of Bligh and Dyer (35). All extraction procedures were performed at 0° C using ice. Each cell pellet was weighed (generally pellets weighed 0.2 to 0.5 gm) and enough distilled water was added to bring the weight to about 0.8 gm. The resulting paste was stirred gently for five minutes using a glass rod. Two milliliters of methanol and 1.0 ml. of chloroform were added to the paste to keep the volumes of

chloroform, methanol, and water in the proportions 1:2:0.8. Phospholipids were extracted for 15 minutes by vigorous mixing with a glass rod. The resultant solution was filtered through Fisher filter paper (qualitative) on a Coors No. 3 "Buchner funnel with vacuum suction. Extraction was repeated twice with another 2 ml. methanol and 1 ml. chloroform. The filtrates were combined in a glass centrifuge tube [15 ml. Cortex (R) No. 8441]. The extracted phospholipids were separated from water soluble material by adding 1.0 ml. chloroform and 1.0 ml. of 0.1M KCl with vigorous mixing following each addition (30). The mixture was vortexed for one minute and centrifuged at 65,000 RPM at 5° C for 15 minutes. Centrifugation separated the mixture into an upper layer of methanol and water and a lower layer of chloroform (containing the lipids) with the cell debris as a third layer at the interface. The chloroform layer was transferred to a small vial by serological pipette. Another 1 ml. of chloroform was added twice following exactly the same procedure, the chloroform layers were combined and kept in a freezer under a nitrogen atmosphere.

Thin Layer Chromatography

Thin layer chromatography was used for the:

Quantitative Determination of Phospholipids Using Densitometry.

Thin layer plates were (commercial pre-coated TLC plates of E. M. Laboratories, Inc., Elmsford, N. Y.) silica gel 60

(without fluorescent indicator). Gel thickness was 0.25 mm. Plates were 20 x 20 cm. Five of these plates were spotted quantitatively with 1, 2, 3, 4, 5, 8 and 10 uL of PE, PS, PA, PG and CL of concentration 5 mg/ml. All plates were developed in paper lined tanks. The solvent was added just before insertions of the plates. The solvent system used was 65/25/4 chloroform, methanol and water except for PA which was developed in 65/25/8 chloroform, methanol and glacial acetic acid. One and one fourth ml of the following extracts (15 aspartic, 15 oleic + aspartic, 29 oleic, 29 oleic A and 29 oleic B) were evaporated to dryness under a stream of N₂ gas. One tenth mls. of chloroform were added to the dried samples and these were applied to two plates in a volume of 5 uL. The two plates were developed separately in the above mentioned solvent for 1 1/2 hours. The dried plates were sprayed lightly with molybdenum blue spray reagent (prepared as described in the literature review) until the adsorbent was uniformly damp. Compounds containing phosphate ester showed up immediately as blue spots on a light blue-grey background. The plates were scanned (Densicord, Photovolt Corp., N. Y.) to provide quantitation of the lipid components. The peaks produced by the recorder had areas proportional to the size and color density of the spots on the chromatograms. Standard curves were prepared by determining the peak areas for the standards spotted with known quantities of lipids.

Lines for the standard curves were determined by the method of least squares using intensity units (IU) versus the micrograms of phospholipids. Quantities of lipids in the samples were determined by comparing the IU's to the standard curves.

Thin Layer Chromatography for Methyl Ester Preparation

Thin layer chromatography was done using the thin layer plates previously mentioned. One and a half ml. of the chloroform extract was evaporated to dryness under a stream of N_2 gas. One tenth ml. of chloroform was added to the dried sample and this applied to the plate by a micropipette as a line. Five uL of PE was applied as a spot on one end of the plate and the plate was developed in chloroform:methanol:water (65:25:4 v/v). PE spot was developed with molybdenum blue spray. The area corresponding to the PE in the sample was recovered using a sample recovery tube.

Thin Layer Chromatography for Phosphorus Determination

Concentrated samples of the chloroform extracts from cell preparations 15 glucose, 15 aspartic, 15 aspartic + oleic, 29 oleic + aspartic, 29 oleic and 15 succinic were applied to the Brinkman precoated plates in two positions. Standards of PE, PG, PA, PS and CL were spotted on the same plate. A solvent system of 60:35:5 chloroform:methanol:5N NH_3 was added to paper-lined tanks just prior to adding the plates. The spots were located by exposing the plates

to an atmosphere of iodine vapors (solid iodine crystals in the bottom of a TLC tank). The iodine-positive spots were outlined and the iodine allowed to evaporate.

The spots were then separately recovered from the plates using a chromoflex sample recovery tube (size 22 T.M. Kontes Glass Co.) and transferred to centrifuge tubes. Two ml. of the developing solvent were added to each tube. The mixture was then vortexed, centrifuged at 2500 RPM for 10 minutes, and the supernatant fluid was transferred to another tube. The silica gel residue was extracted two more times with 2 ml., 1 ml. methanol. All supernatants were combined.

Phosphorus Determination

The solvents were evaporated in an oven at 120° C. Subsequently, 0.25 ml. of 70% perchloric acid was added to each tube. Each tube was covered with a marble, inserted into a 220° heat block and allowed to digest overnight. The inorganic phosphorus resulting was then determined by the method of Fiske and Subbarow (36). To each tube 4 ml. of 0.25% (w/v) ammonium molybdate in 5N sulfuric acid and 2 ml. of 15% (w/v) sodium metabisulfite were added. The mixture was vortexed for thorough mixing. A standard curve was prepared by taking 0.5, 0.8, 1.0, 3.0, 6.0 and 8.0 ug of phosphorus equivalent (monobasic potassium phosphate). The molybdenum and bisulfite reagents were added to each tube and all tubes were vortexed. All the tubes were placed in a

wire basket and incubated in a boiling water bath for 30 minutes. After the samples were cooled, absorbances were measured at 830 nm on a Beckman DU-2 double beam spectrophotometer [Beckman Instruments, Inc., Fullerton (A)]. Standard curves were prepared by least squares analyses of absorbance at 830 nm vs. micrograms of phosphorus. Unknowns were calculated by comparing their absorbance to that of the standard.

Fatty Acid Methylation

Methyl esters were prepared from the fatty acids of the lipids in either a portion of the chloroform extract or from the phosphatidylethanolamine band of the preparative thin layer chromatograms.

Methylation of Chloroform Extracts

One ml. of the chloroform extract was evaporated to dryness under a stream of N_2 gas. The dried phospholipid was incubated with 5 ml. 1% KOH-methanol for 30 minutes at $120^\circ C$. After incubation and cooling methyl esters were extracted by addition of 0.5-1 ml. of hexane with vigorous shaking, followed by addition of H_2O to reduce the solubility of the esters in the alcohol layer. Hexane extraction was repeated twice. Hexane extracts were combined and washed with 0.5 ml. distilled water. The water was drawn off and the hexanes were dried of residual water with anhydrous sodium sulfate.

The resulting solvent was concentrated to approximately 50uL prior to analysis by the gas-liquid chromatography.

Methylation of Silica Gel-Lipid

To the extracted silica gel containing PE, 10 ml. of 1% KOH-methanol was added, vortex gently and incubated for 30 minutes at 120° C. Extraction of phospholipids from the gel was exactly as described for the chloroform extract.

Gas Liquid Chromatography of Methyl Ester

Fatty acid methyl esters were separated on a series 5000 Barber-Colman gas chromatograph (Barber Colman Co., Rockford, IL). A glass U-tube column 2.5 meters long and 3 mm. inside diameter was packed with 7.5% diethylene glycol succinate on Chromosorb G 80/100 mesh. Column temperature was maintained at 175° C. Injector temperature was 220° C. and detector temperature 230° C. The carrier gas was nitrogen at a flow rate of 30-35 ml/min. The detector was flame ionization. The recorder was a Honeywell (Minneapolis-Honeywell Reg. Co., Brown Instrument Division, Philadelphia, PA) with a range of 1 mv. full scale and a chart speed of 20 inches/hour. Methyl esters of lauric, myristic, palmitic, palmitoleic, stearic, oleic and C₁₇ cyclopropane acids were used as authentic standards (Applied Science Laboratories, State College, PA).

CALCULATIONS

Gas-Liquid Chromatography Peak Areas

The fatty acid compositions of the total phospholipid and the isolated phosphatidylethanolamine were analyzed by gas-liquid chromatography. The relative composition of the samples were determined by measuring the peaks produced on the recorder chart. Peak areas were determined as shown in Equation 1. The areas were then summed and the ester percentages were calculated as shown in Equation 2.

$$\text{Equation 1} \quad \text{AREA} = \text{PEAK HEIGHT} \times \text{PEAK WIDTH AT HALF HEIGHT}$$

$$\text{Equation 2} \quad \% \text{ composition} = \frac{\text{area of peak}}{\text{sum of peak areas}} \times 100$$

Weight Per Cent Phospholipids

The different classes of phospholipids were separated by TLC. The phospholipid spots were removed from the plates, the phospholipids recovered from the silica gel and analyzed for phosphorus as described in Materials and Methods. The slopes of the lines for the standard curves were determined by the method of least squares. Since each of the common phospholipid, except for cardiolipid, contains one phosphorus per mole, the mole percentages of each may be calculated as shown in Equation 3.

$$\text{Equation 3} \quad \text{Mole \%} = \frac{\text{ug P from individual phospholipid spot}}{\text{ug P from total phospholipid in sample}} \times 100$$

Since the molecular weights of the various phospholipids do not differ greatly, the mole percentage is a close approximation of the weight percentage.

Cardiolipin is an exception since it has two moles of phosphorus per mole. Therefore its mole percentage is half of the percentage of the total lipid which is made up of cardiolipin, it is calculated as in Equation 4.

$$\text{Equation 4} \quad \text{Mole \% cardiolipin} = \frac{\frac{\text{ug P from cardiolipin}}{2}}{\text{ug P from total phospholipid in sample}} \times 100$$

However since cardiolipin contains four fatty acids, two phosphates and three glycerols per mole compared to the ratio of 2:1:1 for the other phospholipids, its weight percentage will be approximately twice its mole percentage. Therefore, Equation 3 will produce an approximation of weight percentage for any phospholipid in the sample.

Phospholipid per Gram of Cells

An approximation of the amounts of the phospholipids present in a gram of cell may be obtained as follows: First, the fraction of the extract which the spot analyzed represents of the total extract is calculated. The phosphorus content of the individual spot is divided by that fraction. This gives the micrograms of phosphorus in that phospholipid in the total extract. Dividing that value by the weight of

the cells extracted provides the micrograms of phosphorus in that phospholipid in a gram of cells. A sample calculation is shown below.

$$\frac{15 \text{ aspartic} + \text{oleic}}{\text{wt of cells (gm)}} = 0.2438$$

$$\text{Total volume of extract (ml)} = 2.5$$

$$\text{Total ug phosphorus/spot} = 3.5$$

$$1.0 \text{ ml of the extract} \xrightarrow[\text{chloroform}]{\text{dryness 0.02 ml}} \text{divided}$$

equally into two spots

$$\text{The fraction of the original volume used} = 0.2 \text{ ml}$$

$$\frac{3.5}{0.2} \approx 17 \text{ total ug phosphorus in total extract}$$

$$\frac{17}{0.2438} \approx 71 \text{ total ug phosphorus/gm cells}$$

Densitometry Peak Area

PE and PG were the only phospholipids which were present in high enough concentrations to be detected by the densitometer. The areas of the peaks obtained from the densitometer were measured by a planimeter (K and E 4236 compensating polar planimeter). These peaks were not uniform which made their measurement by the usual triangulation method impossible. The peaks' areas represent their intensity units. A standard curve was prepared by least squares analysis of the peak areas. The ug of phospholipid in the individual spots were determined from this standard curve.

RESULTS AND DISCUSSION

It is generally believed that the physicochemical properties of membrane lipids play an important role in membrane structure and function. Studies with both natural and synthetic phospholipids (37) have suggested that the lipids of the biological membrane are normally in a liquid expanded state. If such an arrangement is necessary for a functional membrane, it is natural to assume that the organism must maintain the physical character of the lipids within definite limits.

One approach to the study of specific lipid functions is to isolate mutant organisms defective in a single step of the biogenesis of membrane lipids, thus giving rise to cells with a specifically altered pattern of membrane lipids (38).

A brown waxy material (0.2-0.5 gm) was obtained as the net weight from frozen E. coli (15T⁻ and its mutant 29) cells in the log phase of growth. A total of five phospholipids could be extracted by the Bligh-Dyer method (35). This method was simple and rapid. The extraction was at neutrality and at 0° C, conditions which are unlikely to cause any breakdown of lipid. The addition of 0.1M potassium chloride instead of distilled water aids in the breaking of lipid-water-organic solvent emulsions and facilitating phase separation (30). The phospholipids were separated and identified by TLC. Color reactions and R_f values are shown in Table 3.

Table 3. Color Reactions and R_f Values of Phospholipids

Phospholipids	R_f Values	Color Reactions†		
	Solvent*	I ₂	Nin.	Mo.
Cardiolipin	0.84	+	-	+
Phosphatidyl Glycerol	0.75	+	-	+
Phosphatidyl Ethanolamine	0.55	+	+	+
Phosphatidyl Serine	0.28	+	+	+
Phosphatidic Acid	0.12	+	-	+

*Solvent used was chloroform-methanol-7N ammonium.

Hydroxide in the ratio of 60:35:5.

† abbreviations were I₂--Iodine; Nin--Ninhydrin; Mo--

Molybdenum blue reagent for phosphorus. The R_f values were determined from Figure 6.

A total of five phospholipids were detected in E. coli. Figures 3 and 4 for mutant and Figures 5 and 6 for wild type show representative one-dimensional separation of phospholipids by TLC. The spots were identified by the following criteria: (i) all spots gave positive iodine test; (ii) their R_f 's agreed with those of Table 3; (iii) presence of phosphorus as detected both by molybdenum spray and colorimetric determination of phosphorus. Only two spots PS and PE gave positive ninhydrin tests. For the wild type E. coli, as can be seen from Table 4, the total phosphorus (ug) per gm cells varies from one medium to the other. On comparison of the total amount of phosphorus in the aspartic acid medium with that in aspartic + oleic, it can be seen that in the later case the phosphorus content was approximately double that in the former. The only difference between the two media is the presence of oleic in addition to aspartic in the second one. Possibly this increase results from increased synthesis of phospholipids due to the availability of the oleic acid.

Succinic acid in the growth medium results in a slightly higher level of total lipid phosphorus per gram of cells (42 ug/gm) than when aspartic acid was the carbon source. Thus these two carbon sources have similar effects on the levels of phospholipids in E. coli. Perhaps this is because of their involvement in or close association with the citric acid cycle.

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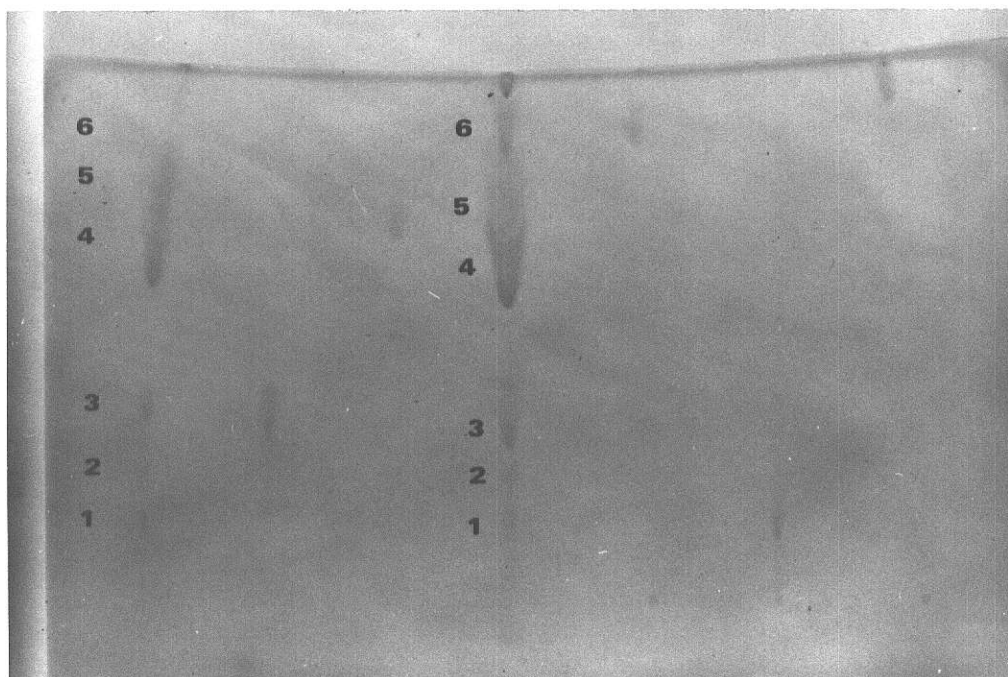
K

Figure 3. A Representative One Dimensional Separation
of Phospholipids by TLC From a Mutant of
E. coli 29 Grown on Oleic + Aspartic Medium

Figure 4. A Representative One Dimensional Separation
of Phospholipids by TLC From a Mutant of
E. coli 29 Grown on Oleic Medium

S refers to the sample which is E. coli mutant 29.

S PS PE S PG PA CL



S PS PE S PG PA CL

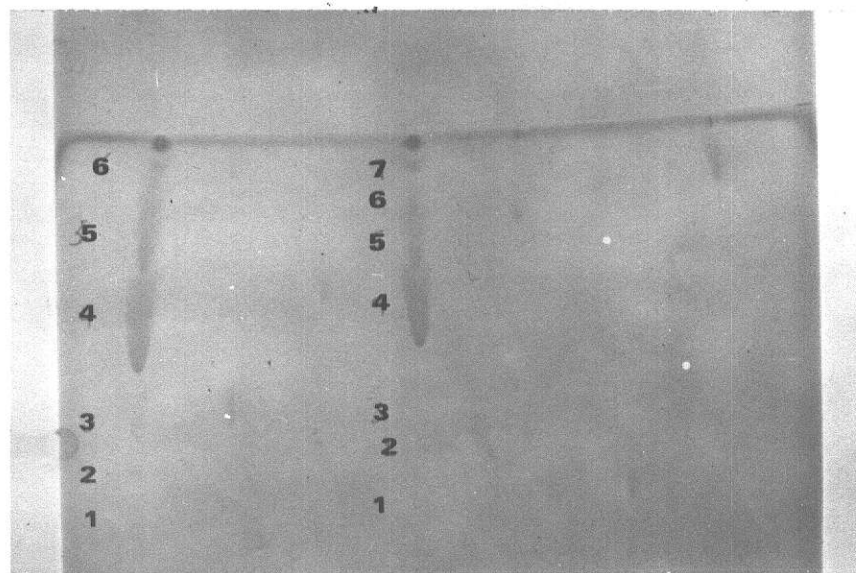
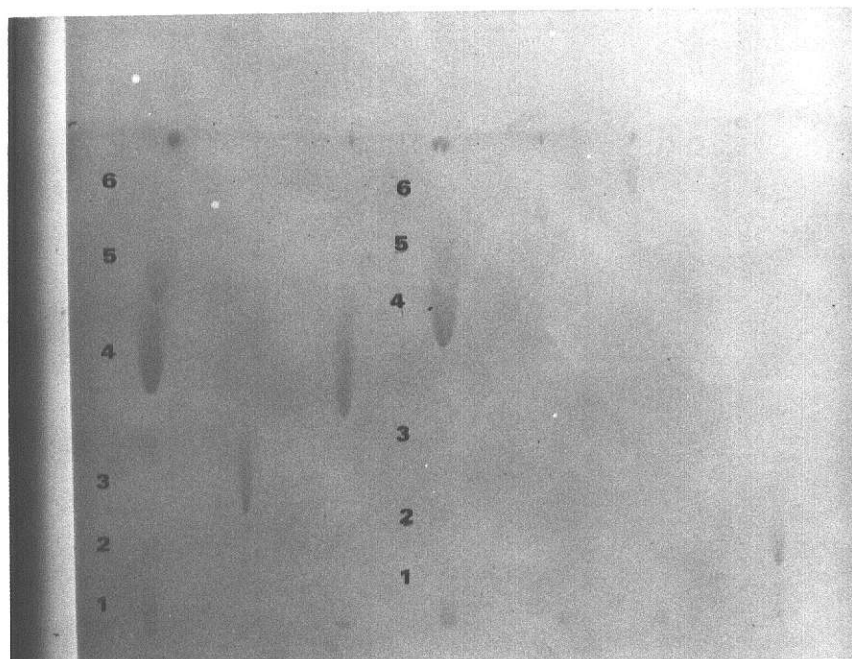


Figure 5. A Representative One Dimensional Separation of Phospholipids by TLC From E. coli Grown on Aspartic + Oleic Medium

Figure 6. A Representative One Dimensional Separation of Phospholipids by TLC From E. coli Grown on Glucose Medium

S refers to the sample which is the E. coli 15T⁻.

S PS PE S PG CL PA



S PS PE S PG PA CL

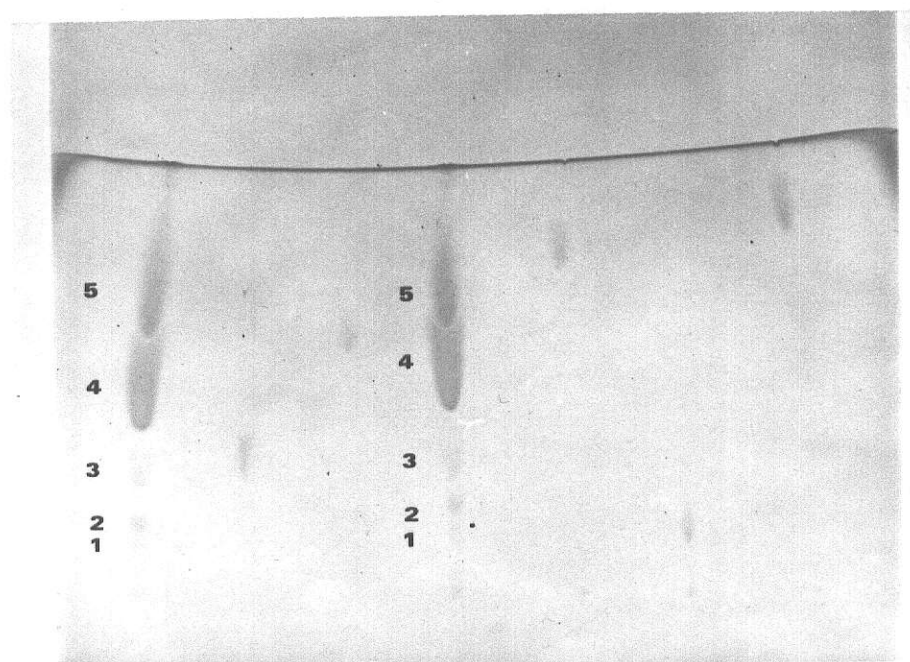


Table 4

Analytical Data of the Phospholipids From E. coli

Cell Type and Growth Medium	Wt of Cells (gm)	PA	PS	PG	PE	CL	Total
15 Aspartic	0.2375						
	ug phosphorus/spot	Trace	Trace	0.3	0.8	0.1	1.2
	ug phosphorus/gm cells (approximately)	Trace	Trace	9	24	3	36
	weight % phospholipid	Trace	Trace	25.0	66.0	4.1	
15 Aspartic + Oleic	0.2438						
	ug phosphorus/spot	Trace	Trace	0.3	3.2	Trace	3.5
	ug phosphorus/gm cells (approximately)	Trace	Trace	6	65	Trace	71
	weight % phospholipid	Trace	Trace	8.5	91.4	Trace	
15 Succinic	0.4046						
	ug phosphorus/spot	Trace	0.05	1.0	2.5	Trace	3.6
	ug phosphorus/gm cells (approximately)	Trace	0.5	12	29	Trace	42
	weight % phospholipid	Trace	1.4	28.3	70.2	Trace	

Table 4 (continued)

Cell Type and Growth Medium	Wt of Cells (gm)	PA	PS	PG	PE	CL	Total
15 Glucose	0.4544						
		ug phosphorus/spot	Trace	0.02	1.08	5.3	Trace 6.4
		ug phosphorus/gm cells (approximately)	Trace	0.3	14	70	Trace 85
		weight % phospholipid	Trace	0.3	16.9	82.3	Trace
29 Oleic	0.2778						
		ug phosphorus/spot	Trace	Trace	0.5	1.57	0.05 2.12
		ug phosphorus/gm cells (approximately)	Trace	Trace	18	57	2 77
		weight % phospholipid	Trace	Trace	23.5	65.1	1.1
29 Oleic + Aspartic	0.2177						
		ug phosphorus/spot	1.47	0.6	5.0	15.7	1.4 24.2
		ug phosphorus/gm cells (approximately)	20	8	69	216	19 333
		weight % phospholipid	6.0	2.5	20.2	63.6	2.8

Glucose gave the highest amount of the total phosphorus per gm cells; it was approximately 85 ug per gm cells. This might be due to the relative ease of metabolizing the substrate into fatty acids. These differences in the total amount of phosphorus per gm cells in the different carbon sources suggests that the amount of phospholipids in E. coli is not stable and it is medium dependent.

For the mutant 29, Table 4 shows the data from two different media which were oleic and oleic + aspartic. The total amount of phosphorus per cell weight in oleic was just 77 while that in oleic + aspartic was 333 ug/gm which is about four times higher than that in oleic only. Comparing the amount of total phosphorus in the mutant in oleic + aspartic with that of its parent in the same medium, it is obvious that the rate of incorporation of the external carbon source into phospholipid in the mutant was much more than that in its parent. The increased rate of incorporation of the external carbon source might be due to activation of other sites of phospholipid synthesis which might be more active in the mutant than in its parent. Thus, it is apparent that variations in the total amounts of phospholipid phosphorus in E. coli arise from changing the growth media. My findings are similar to those of Stephenson and Whetham (c.f. 22), Dagley and Johnson (22) and Ames (6) who mentioned that growth conditions could affect lipid composition at least quantitatively. Silbert et al. (25) found that the micromoles

of lipid phosphate per mg of cellular protein averaged about 50% higher in L010 than in Hfr 139 cultures. From that finding they could not confirm whether the amount of lipid in the cell membrane is tightly controlled.

The more recent study in this regard in E. coli was that of O. Pierucci (39). She found that the changes in the rate of incorporation of radioactive lipid precursors and of [$3\text{-}^3\text{H}$] serine into methanol-chloroform extracts of E. coli cultures were considered to be consequences of changes in the rate of lipid and of PE synthesis respectively. There was an increase in the rate of synthesis of the two other phospholipid components, PG and CL. The increase in PG and CL synthesis could be a consequence of the activation of new sites of envelope growth.

The amount of lipid present also responds to the external carbon source in higher organisms. Spector et al. (40) findings indicate that the human fibroblast can tolerate considerable variability in fatty acid composition, even in membrane phospholipids. The triglyceride content of the cells increased when they were grown in the presence of added fatty acids, but the phospholipid and cholesterol content remained unchanged.

In contrast to the results cited above, Cronan and Vagelos (1) have found that the amount of lipid present in E. coli is independent of the growth medium applied.

The increase in the total lipid phosphorus will appear as increases in the individual phospholipids as we will see in the following section.

Amount of Phospholipid Present

The weight percentages of phospholipids were calculated as mentioned in the section of calculations. The results are shown in Table 4.

For the wild type (15T⁻) PE was the most abundant phospholipid in the four different media. This is in good agreement with many other studies. The weight percentages of PE ranged from 66-91.4% of the total phospholipids. Cronan and Vagelos (1) mentioned a value of 70-80%, while DeSiervo (17) mentioned a value of 70-90% of the total phospholipids.

As can be seen from Table 4, the effect of media on the total amount of phosphorus per cell weight is reflected in the weight percentage of PE. This is an expected result since PE is the most abundant phospholipid in E. coli. On comparison of PE amount in aspartic with that in aspartic + oleic, PE was much more higher in the second medium. The only difference between the two media is that oleic is present as a carbon source in addition to the aspartic. The increase in PE in presence of oleic might be due to the probability of incorporation of oleic acid into PE.

PE percentage in glucose media was higher than that in succinic but not as high as in the aspartic + oleic media.

It appears that presence of a second carbon source in the medium increases the amount of PE in E. coli.

PG was the second most abundant phospholipid after PE (Table 4) in wild type E. coli in all media. Its weight percentages ranged from 8.5-28.3. Cronan and Vagelos (1) reported value of 5-15% while Ames (6) reported 19%. Since the range of PG fluctuated from one study to the other, it may be that PG mimics PE amount in being media dependent.

CL comprises 5-15% of the total phospholipids in E. coli as reported by Cronan and Vagelos (1) while Kanemasa et al. (34) reported a value of 5-12%. Unfortunately, I could not detect CL quantitatively in cells grown on most of the media that were used. This might be due to many possibilities (i) the solvent system might not have completely extracted the CL; (ii) the CL spot in TLC migrated fairly fast and might not have been resolved from the solvent front; (iii) PG gets converted, in vitro, to CL by addition of a new PA moiety (41). Since PG was relatively high, this means that the conversion process might be very low.

PS is present in very small amount in E. coli (1). Succinic acid and glucose media showed detectable amounts of PS while the other two media showed only trace amounts. The spot corresponding to PS gave a positive ninhydrin test in addition to the other color tests.

PA was detected in cells grown in all four media but its amount was too small to be detected colorimetrically. The

PA amount as reported by Cronan and Vagelos was less than 1% of the total phospholipids. Although Randle et al. (7) reported value of 5% of the total phospholipids, they could not detect its presence in another report.

For the mutant 29, PE was the most abundant phospholipid as in the parent strain (Table 4). Its weight percentage ranges from 63-65, somewhat lower than the parent strain on most of the media.

PG followed PE in its amount in the mutant and its weight percentages were from 20.2-23.5, similar to its occurrence in the parent strain on several of the media.

CL percentage in oleic media was 1.1 while it raised to 2.8 in oleic + aspartic media, in contrast to the parent strain where it was generally present in trace quantities.

PA and PS were present in trace amounts in oleic media but their percentages were 6 and 2.5, respectively, in the oleic + aspartic medium. As can be seen from the above and Table 4 the mutant behaved to certain extent similarly to its parent. There was a general increase in the percentages of all the five phospholipids upon the addition of aspartic to the oleic acid medium for both strains. As shown in Table 4, the amount of the minor phospholipids (PA and PS) could not be detected quantitatively in oleic media while they were detectable after aspartic addition. For example, the CL percentage more than doubled in the presence of aspartic and oleic.

All the previous mentioned data were from colorimetric determination of phosphorus from the individual lipids. Densitometry studies gave a general idea about phospholipid compositions of E. coli. It was in agreement with much of the literature in that PE was found to be the main phospholipid. PG followed PE in its amount in E. coli as illustrated in Figure 7. The method was not sufficiently reliable because the reagent produced blue spots on a blue-grey background. Therefore the spots were difficult to detect.

The solvent system used for this study was chloroform-methanol-water in the ratio of 60:25:4. The detecting spray was molybdenum blue. From Figure 7 it is clear that the solvent system used did not adequately separate the phospholipids. Thus I shifted to the chloroform-methanol-7N ammonium hydroxide solvent which gave better resolution. The second problem with this study was the detecting reagent. As mentioned above, the background of the TLC plate was also colored which made the detection of trace components very hard.

We determined the fatty acid compositions of the phospholipid fractions to determine whether fatty acid compositions were altered by the various growth media.

It is obvious from Figures 8 and 9 for the wild type (15T⁻) and its mutant 29, the fatty acid compositions were quite similar which might be expected since they have the same genotype.

Figure 7. The Structure of the Phospholipids of E. coli
and its Mutant 29 From Densitometry Study

The upper dark spots represent PE.
The lower faint spots represent PG.

29 oleic
B₁

29 oleic
A₁

29 oleic
B

15 aspartic

15 oleic +
aspartic

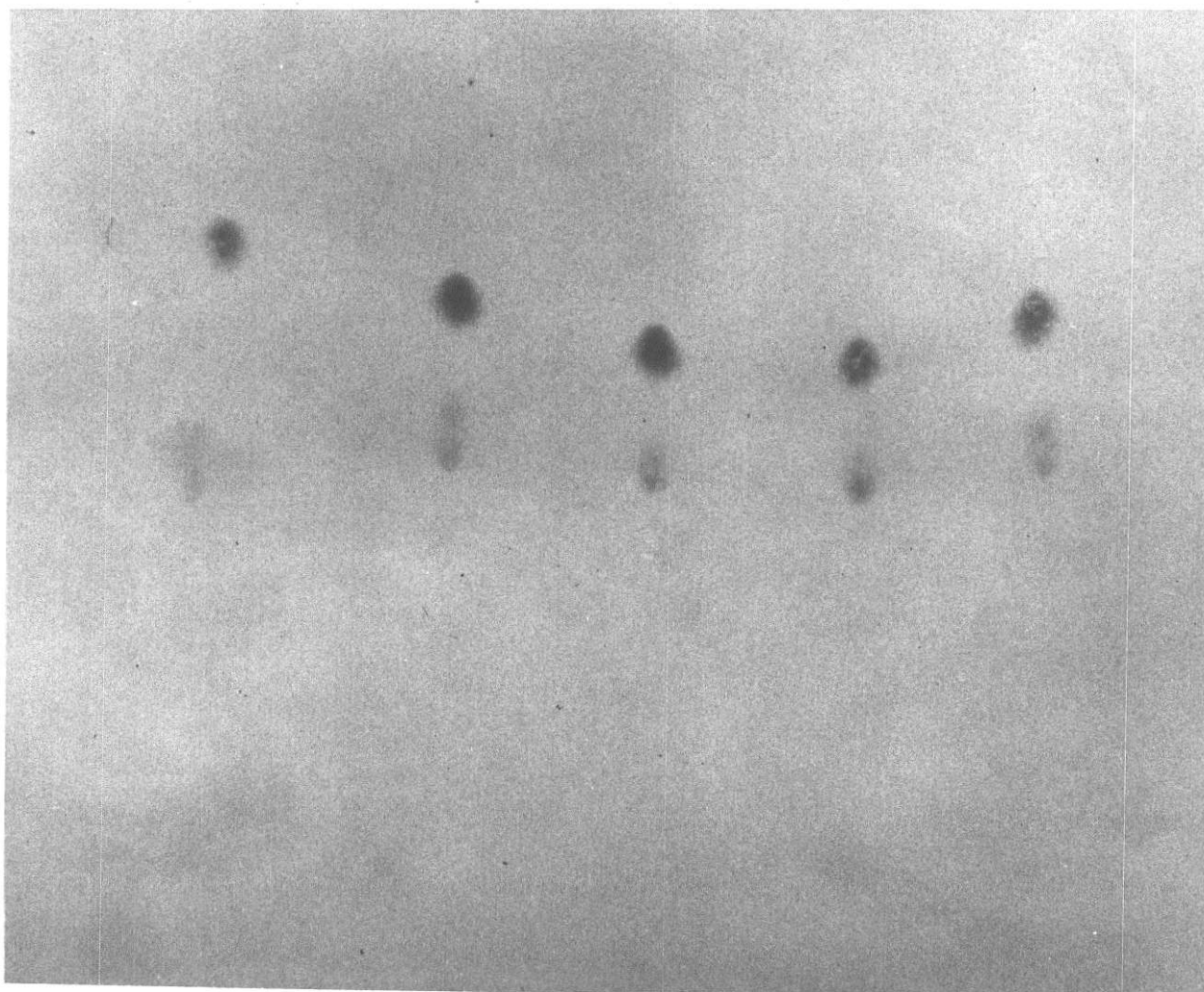


Figure 8. The Fatty Acid Compositions of the E. coli
Grown on Aspartic Medium

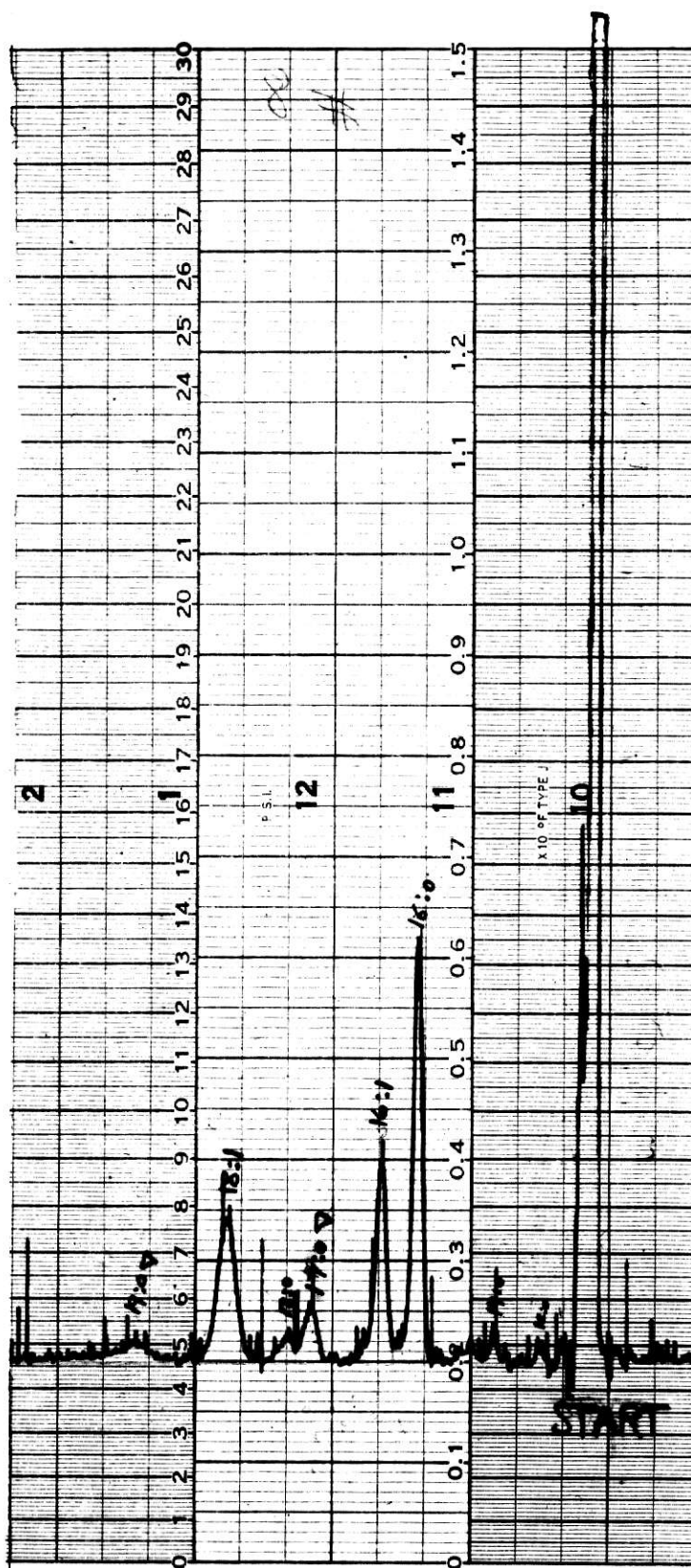
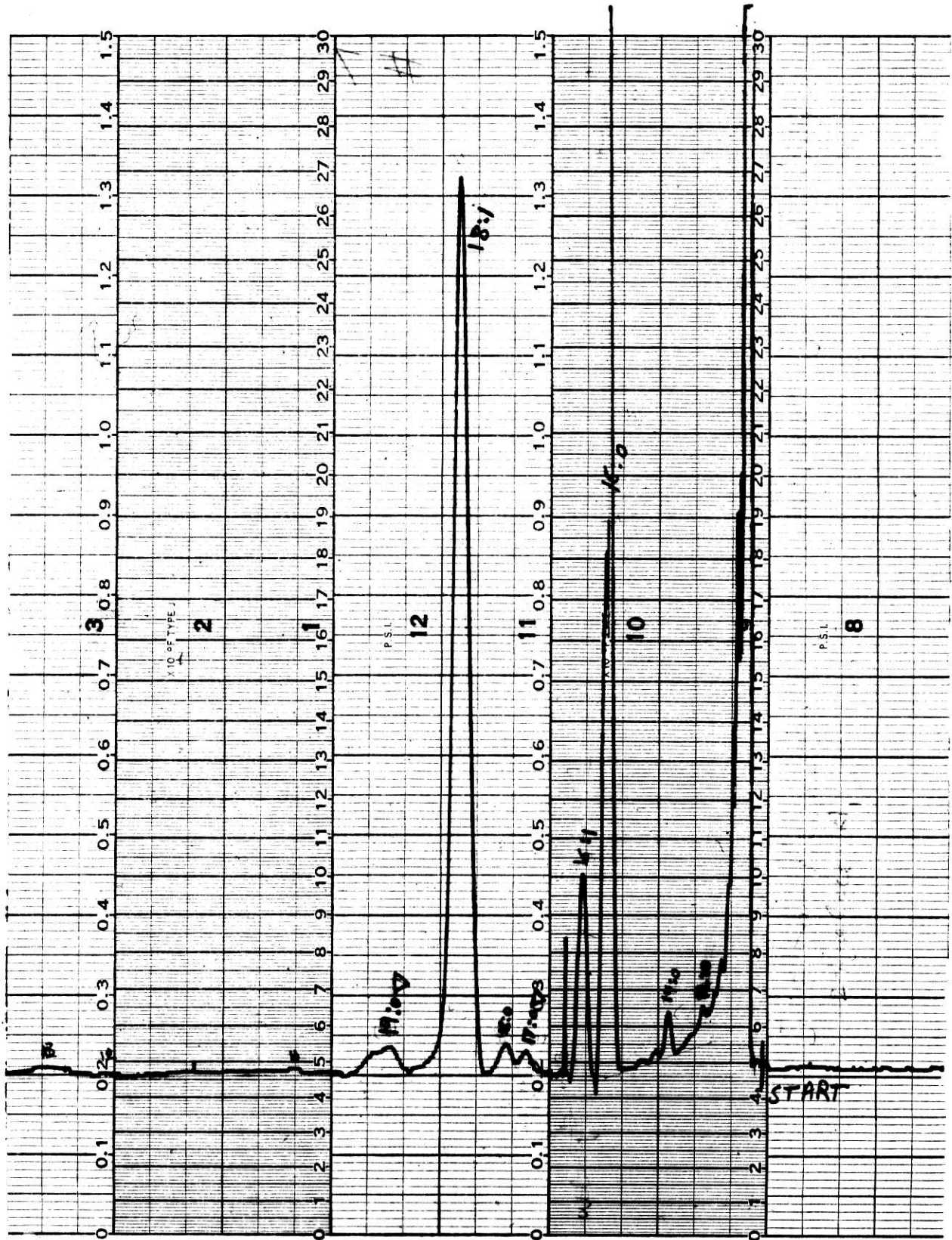


Figure 9. The Fatty Acid Compositions of the E. coli
Mutant 29 Grown on Oleic Medium



From Table 5, it can be seen that the main fatty acids in the wild type (15T⁻) in the four media were myristic, palmitic, palmitoleic, methylenehexadecanoic, cis-vaccenic and lactobacillic acid. In all kinds of the media, palmitic acid was the most abundant acid as is expected for E. coli. Lauric acid was found in trace amounts, except in the case of aspartic and succinic acid media. Several minor components were also evident in the chromatogram between the peaks of the main fatty acids. Stearic acid, which is usually present in trace amounts (1) was present in a fairly high concentration in the aspartic-acid grown cells.

The quantities of the two cyclic fatty acids (17:0 Δ +19:0 Δ) were generally low, but the quantity of 17:0 Δ in most cases exceeded that of 19:0 Δ . Crowe and Urban (13) reported values of 8 and 0% for 17:0 Δ and 19:0 Δ respectively for 15T⁻ grown in M9-0.1% aspartate at 37° C in the log phase while they reported a value of 1.2% for 19:0 Δ grown under the same conditions but the cells were collected in the stationary phase. The low content of the two cyclic fatty acids in our bacterial cells might be due to the fact that cyclopropane fatty acids were found mainly in CL with less in PE and none in PG (17). Since CL was found to be present in trace amounts in 15T⁻ and its mutant in different media, this will give rise to trace amounts of the cyclic fatty acids.

The fatty acid designated 18:1 refers to cis-vaccenic or oleic acids because in our laboratory we could not

differentiate between them. They only differ in the position of the unsaturation and our chromatographic system could not resolve them.

As can be seen from Table 5 the proportion of various fatty acids is somewhat dependent on the medium. The major difference is that addition of oleic to the aspartic acid media led to a significant increase in the 18:1 peak. This would be expected to result from direct uptake and incorporation of oleic acid into the lipids of the cell.

Table 5

Fatty Acid Composition of Phospholipids From
E. coli Grown on Various Media

Fatty Acids in Phospholipids	Carbon Source			
	Glucose	Aspartic	Oleic + Aspartic	Succinic
Unidentified	--	--	--	0.9
9:1	--	--	--	0.3
12:0	0.2	1.8	0.7	1.2
Unidentified	--	--	--	0.6
14:0	2.3	2.2	0.7	1.8
14:1	0.1	--	--	1.1
Unidentified	0.5	--	--	--
16:0	38.3	35.7	28.5	35.9
16:1	32.6	18.6	20.2	28.3
17:0 Δ^*	2.9	8.5	Trace	5.5
18:0	--	4.8	--	0.3
18:1	22.0	19.5	46.3	23.2
19:0 Δ^*	0.9	--	1.7	0.8
Unidentified	0.2	--	--	--
Unidentified	0.4	--	--	--
Unidentified	--	--	1.0	--
Unidentified	--	--	0.9	--

*The Δ sign is used to designate the presence of cyclopropane ring.

From Table 6 it can be seen that addition of oleic to the media increased the ratio of cis-vaccenic or oleic to palmitic. The presence of oleic did not affect the ratios of the cyclopropane acids or palmitoleic to palmitic acid.

Table 6

Ratios of the Main Fatty Acids to Palmitic Acid of the Phospholipids From E. coli Grown on Various Media

Carbon Source	$\frac{\text{Palmitoleic}}{\text{Palmitic}}$	$\frac{17:0\Delta + 19:0\Delta}{\text{Palmitic}}$	$\frac{\text{Cis-Vaccenic or Oleic}}{\text{Palmitic}}$
Glucose	0.9	0.08	0.6
Aspartic	0.5	0.4	0.5
Oleic + Aspartic	0.7	0.06	1.6
Succinic	0.8	0.2	0.6

Table 7 shows the fatty acid compositions of the PE fraction from the wild type in the same four media. From this table the fatty acid composition of PE is similar to that reported by several investigators (c.f. 17) for the total fatty acid composition of E. coli. This would be expected since PE does constitute 70-90% of the total phospholipids. The ratios of the main fatty acids to palmitic of PE are shown in Table 8.

Table 7

Fatty Acid Composition of Phosphatidylethanolamine
From E. coli Grown on Various Media

Fatty Acids in PE	Carbon Source			
	Glucose	Aspartic	Aspartic + Oleic	Succinic
Unidentified	--	--	--	1.1
9:1	--	--	--	0.9
12:0	3.1	--	0.1	0.2
Unidentified	--	--	--	1.0
14:0	3.2	6.9	0.4	2.4
14:1	0.9	0.3	--	2.6
Unidentified	--	0.2	--	--
15:2	--	0.4	--	--
16:0	29.5	44.4	35.2	38.9
16:1	34.1	20.6	18.3	24.9
17:0 Δ	0.2	--	--	6.9
18:0	3.8	9.5	--	1.7
6t-18:1 or 16t-18:1	0.1	--	--	--
12t-18:1	0.1	--	--	--
18:1	24.5	21.2	45.0	18.1
19:0 Δ	0.1	3.2	0.3	1.4
Unidentified	0.9	--	0.7	--
Unidentified	0.1	--	--	--

Table 8

Ratios of the Main Fatty Acids to Palmitic
Acid of PE From E. coli Grown on Various Media

Carbon Source	<u>Palmitoleic</u> <u>Palmitic</u>	<u>17:0Δ + 19:0Δ</u> <u>Palmitic</u>	<u>Oleic or</u> <u>Cis-Vaccenic</u> <u>Palmitic</u>
Glucose	1.2	0.01	0.8
Aspartic	0.5	0.1	0.5
Oleic + Aspartic	0.5	0.01	1.3
Succinic	0.6	0.2	0.5

The results of this table are in a good agreement with those of Table 6.

For the mutant 29 the fatty acid compositions of the total phospholipid and for the PE fraction are summarized in Tables 9 and 10. The first two columns of these tables represent replicate samples grown on oleic acid at different times and are labelled A and B. The third column contains the results from cells grown on oleic + aspartic acids as carbon sources.

It is apparent from Tables 9 and 10 that addition of aspartic acid to the media increased the amounts of the major fatty acids (16:0 and 18:1) in total phospholipids but not in PE fraction. In the PE fraction the content of 16:0 was quite high in cells grown on oleic acid alone. Addition of aspartic acid brought about a decrease in the content of 16:0 to the levels in wild type accompanied by a slight

Table 9

Fatty Acid Composition of the Phospholipids From
a Mutant of E. coli 29 Grown on Various Media

Fatty Acids in Phospholipids	Carbon Source		
	Oleic A	Oleic B	Oleic + Aspartic
Unidentified	--	--	2.1
12:0	0.03	--	0.4
Unidentified	--	--	0.8
Unidentified	--	--	0.3
14:0	0.8	0.4	1.7
14:1	--	--	0.01
16:0	26.4	23.9	32.8
16:1	12.1	10.2	13.2
17:0 Δ	--	--	1.9
18:0	2.3	5.6	1.1
18:1	31.2	32.2	44.1
19:0 Δ	2.0	1.2	0.3
Unidentified	3.8	5.0	1.3
Unidentified	2.8	1.1	--
Unidentified	18.0	20.5	--

Table 10

Fatty Acid Composition of the Phosphatidylethanolamine
From an E. coli Mutant 29 Grown on Various Media

Fatty Acids in PE	Carbon Source		
	Oleic A	Oleic B	Oleic + Aspartic
Unidentified	--	--	1.2
12:0	--	--	0.5
Unidentified	--	--	0.2
14:0	1.9	1.4	6.6
14:1	--	--	0.03
16:0	42.6	41.8	28.5
16:1	16.8	17.1	14.7
17:0 Δ	--	--	0.2
18:0	2.2	3.3	9.5
18:1	32.7	32.4	36.1
19:0 Δ	1.3	2.0	2.1
Unidentified	3.2	2.1	--

decrease in 16:1 and increases in myristic, stearic and oleic or cis-vaccenic acids.

Tables 11 and 12 show the ratios of the main fatty acids to palmitic in total phospholipids and in PE fraction for the mutant 29.

Table 11

Ratios of the Main Fatty Acids to Palmitic
Acid of Phospholipid From a Mutant E. coli 29
Grown on Various Media

Growth Cell	$\frac{\text{Palmitoleic}}{\text{Palmitic}}$	$\frac{17:0\Delta + 19:0\Delta}{\text{Palmitic}}$	$\frac{\text{Oleic}}{\text{Palmitic}}$
Oleic (B)	0.3	0.17	1.3
Oleic (A)	0.5	0.07	1.2
Oleic + Aspartic (A)	0.4	0.06	1.3

Table 12

Ratios of the Main Fatty Acids to Palmitic
Acid of PE Fraction From a Mutant E. coli 29
Grown on Various Media

Growth Cell	$\frac{\text{Palmitoleic}}{\text{Palmitic}}$	$\frac{17:0\Delta + 19:0\Delta}{\text{Palmitic}}$	$\frac{\text{Oleic}}{\text{Palmitic}}$
Oleic (A)	0.4	0.03	0.8
Oleic (B)	0.4	0.05	0.8
Oleic + Aspartic (A)	0.5	0.08	1.3

Combining the data of the wild type with that of the mutant it is quite clear that significant incorporation of the external carbon source into E. coli fatty acids did occur. This finding is in agreement with those of many investigators. Marr and Ingraham (18) showed that the fatty acid composition of cells grown in glucose-minimal medium at 10° C differs markedly from that of cells grown at the same temperature if this medium is supplemented with yeast extract.

Esfahani et al. (24) observed that the effect of oleate, cis-vaccenate, or palmitoleate as growth supporting of E. coli mutant is similar to a decrease in growth temperature. In both cases, the amounts of unsaturated fatty acids in phospholipids increased.

Robert E. Williams et al. (42) in their study on a cultured animal cell indicated that the degree of unsaturation in phospholipids of animal cells grown in culture can be manipulated experimentally. Animal cells cultured in serum-free minimal essential medium (Eagle) containing a biotin antagonist can effectively utilize fatty acids from Tweens (Sorbitan-fatty acid esters) provided as medium supplements. The extent of utilization of unsaturated fatty acids from Tween supplements was approximately half that observed with saturated fatty acid Tween supplements. Cells treated in this fashion provide an ideal laboratory system for studying structure-function relationships in animal cell membranes.

Many mechanisms have been proposed to explain the process of incorporation of an external carbon source into lipids. One mechanism has suggested that the condensing effect of lower temperature, which energetically favors closer packing of fatty acid chains may be overcome by fatty acyl groups that structurally favor a more expanded form, namely, unsaturated fatty acids (c.f. 24).

My results with variations of fatty acid composition in wild type (15T⁻) and its mutant 29 with differences in structures of the carbon sources and the supplement suggest that mechanisms are operative in E. coli which control the fatty acid composition of the phospholipids, which in turn affects their physiochemical states.

It has been shown that mechanisms which maintain the physical character of membrane lipids are operative in higher organisms. Meyer and Bloch (43) found that in anaerobically-grown yeast, the decrease of unsaturated fatty acids was compensated for by an appearance of saturated fatty acids of shorter chain length. Van Golde and Van Deenen (44) have shown that the effect of variations in membrane fatty acids of rat liver due to dietary alteration may be compensated for by replacement of fatty acids of related unsaturation and shifts in the relative proportions of different lecithin species.

The significance of this study might lie in the fact that the origin of chromosome replication and the replication

complex itself are membrane associated (Urban, Personal Communication) and the DNA-bearing portion of the membrane has a distinct phospholipid composition. Consistent with these reports, it was found that unsaturated fatty acids were required for chromosome initiation in E. coli and it was proposed that membrane growth was involved in timing of initiation (Urban, Personal Communication). In addition, membrane fatty acid composition influences the rate of chromosome replication.

If unsaturated fatty acids and/or particular phospholipids are important in initiation and replication of chromosomes (Urban, Personal Communication) and if, as is almost universally accepted, chromosome initiation and replication are highly regulated events in the bacterial cell cycle, Urban's data suggest that a bacterial cell may regulate its growth by regulating which fatty acids (or which phospholipids) it inserts into its membrane.

This study might be useful in the future to understand or link the alterations in the animal cell membrane with malignancy.

SUMMARY

The nature and quantity of the phospholipids of Escherichia coli (15T⁻) and its unsaturated fatty acid auxotroph 29 have been examined.

PE and PG were found to be the main classes of phospholipids. Three minor compounds have been detected: PS, PA and CL. With no exception PE was the major phospholipid present in all cells regardless of the culture media in which they were grown.

The fatty acid compositions in the total phospholipid as well as that in the PE fraction were determined from cells grown on different carbon source. Oleic acid, aspartic acid, glucose and succinic acid were the supplemental carbon sources.

For 15T⁻, when oleic is present in the medium in addition to aspartic, the amount of PE present (whether expressed in micrograms PE per gram of cells or as per cent of PE is much higher than when aspartic only is included in the medium. For the mutant in oleic + aspartic medium, the amounts (microgram per gram of cells) of PE, PG and CL were all much greater than in oleic only but the relative composition remained nearly the same.

The fatty acid composition of the total phospholipid as well as that of the PE fraction shows an apparent response to presence of oleic in the growth medium. The ratio of 18:1/16:0 rises considerably in the wild type 15T⁻ when

oleic is present. The effect is not as clear in the mutant because it derives all of its unsaturated fatty acids from the growth media.

These data indicate that the amount of phospholipids and the degree of unsaturation in phospholipids in E. coli 15T⁻ can be manipulated experimentally to a certain extent. Cells treated in this fashion might provide an ideal laboratory system for studying structure-function relationships in cell membranes.

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EFFECTS OF CHANGING THE CARBON SOURCE ON THE
PHOSPHOLIPIDS COMPOSITION OF E. COLI

by

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ABSTRACT

A brown waxy material (0.2-0.5 gm) was obtained as the net weight from frozen cells in the log phase of growth of *E. coli* 15T⁻ and its unsaturated fatty acid mutant 29. The phospholipids were extracted according to the Bligh-Dyer method (34). A total of five phospholipids could be detected in this extract. The phospholipids were identified after complete separation on one-dimensional TLC using chloroform-methanol-7N NH₃ in the ratio of 60:35:5 as the solvent system. PE, PG were found to be the major phospholipids present while PS, PA and CL were the minor phospholipids.

The fatty acid compositions in the total phospholipid as well as that in PE fraction were determined after cells were grown on different carbon sources. Oleic, aspartic acid, succinic acid and glucose were the supplemental carbon sources.

The amount of PE in the 15T⁻ was found to be much higher when cells were grown in oleic + aspartic than aspartic only. For the mutant in oleic + aspartic medium, the amounts (microgram per gram of cells) of PE, PG and CL were much more than in oleic only but the weight per cent were similar in the two media.

The ratio of 18:1/16:0 rises considerably in 15T⁻ when oleic was added to the growth media in both the total phospholipids as well as the PE fraction. This effect was not

clear in the mutant since it has derived all of its unsaturated fatty acids from the growth media.

These data suggest that E. coli can tolerate considerable variability in fatty acid composition, even in membrane phospholipids.