IN VIVO METABOLISM OF THE PHOSPHOGLYCERIDES OF ESCHERICHIA COLI 015

A thesis submitted by

Elizabeth Bright-Gaertner

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Department of Biochemistry

Faculty of Medicine

University of Ottawa

Ottawa, Ontario

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SUMMARY

We have investigated the *in vivo* metabolism of phospholipids of *Escherichia coli* 015 under normal growth conditions at 37° C and in stationary phase induced either by low environmental conditions (10° C) or by carbon starvation.

At 37° C, the levels of phosphatidyl ethanolamine, doubly labeled with ^{32}P and ^{14}C increased slightly during a three hour chase. This change was accompanied by a moderate and parallel turnover of both of these labels in phosphatidylglycerol and cardiolipin.

Analysis of the turnover of the acyl groups of the phospholipids revealed that a phospholipase A_1: lysophosphoglyceride acyltransferase cycle was not operative at either 37° or 10° C, although such enzyme activities were demonstrated *in vitro* at 37° C.

Turnover and synthesis of phosphatide species were examined simultaneously at 37° and at 10° C. In cells incubated at 37° C the metabolism of phosphatidylethanolamine and phosphatidylglycerol, containing ^{3}H-labeled acyl groups, was typified by a conversion of the monoenoic fatty acids to their cyclopropane fatty acid derivatives; this conversion was impaired in cells incubated at 10° C. During the incubation at 37° C, synthesis of phosphoglycerides from ^{14}C-acetate favored saturated and cyclopropane species of phosphatidylethanolamine and phosphatidylglycerol whereas at 10° C unsaturated species formation was predominant.

In cells incubated in medium containing no carbon source, ^{32}P- or ^{14}C-labeled phosphatidylethanolamine was stable; labeled phosphatidyl-

glycerol and cardiolipin decreased for 60-90 minutes. Phosphatidyl-glycerol continued to decrease for another 90 minutes but during this time there was a net increase in cardiolipin with no de novo phospholipid synthesis.

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INTRODUCTION

As phospholipids of Escherichia coli, like those of other microorganisms, are almost exclusively localized in the cell membrane, any alteration in their composition due to environmental factors will have a direct effect on the anatomy of the membrane. In this regard, the fatty acid composition of the phospholipids, rather than the relative amounts of the individual phospholipids, may be the more important factor. Numerous studies have illustrated the influence of various fatty acid components of phospholipids on the relative compactness of artificial membranes (1). In general, the shorter the chain length and the greater the degree of unsaturation of the fatty acid, the greater the area of the phospholipid molecule and the more expanded the membrane. The force area curves of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine with identical fatty acid compositions are much the same (2). Thus far no such studies of phosphatidylglycerol and cardiolipin have been reported.

Membranes serve as permeability barriers and, theoretically, a change in membrane lipid composition would be reflected in this characteristic. This has been much studied with artificial membranes. For example, the penetration of glycerol into liposomes prepared from Escherichia coli lipids increased as the temperature at which the cells were grown decreased (3). The increased permeability was directly related to the increased percent of unsaturated fatty acids in the phospholipids of the cells grown at the lower temperatures.

It is likely then that in natural membranes, the lipid composition influences permeability but the major role of protein relating to membrane properties cannot be overlooked.

Phospholipids may also have functions not directly related to their influence on membrane permeability. Specific phospholipids are associated with various enzymes and transport processes, for example, phosphatidyglycerol with the vectoral phosphorylation of α-methyl glycoside in isolated membrane preparations of Escherichia coli (4) and cardiolipin with cytochrome oxidase (5,6) and cytochrome c (7) of mitochondria. Even in such instances the fatty acid composition may be significant. Hydrogenation of cardiolipin has been found to reduce its capacity to bind cytochdrome c in vitro (7).

Environmentally induced changes in the phospholipids of Escherichia coli (8-12), and indeed the turnover of individual phospholipids during normal growth (13,14), illustrate the dynamic nature of the cell membrane. Enzymes capable of facilitating such changes in membrane lipids are present in Escherichia coli. However, the data available on the in vivo metabolism of Escherichia coli phospholipids, especially phosphatidylglycerol and cardiolipin, are very limited. The following work deals with the metabolism of the phospholipids of Escherichia coli 015 during normal growth and during stationary phase induced either by low environmental temperature or by carbon starvation.

REVIEW OF THE LITERATURE

Microorganisms, and Escherichia coli in particular, have proven to be extensively useful tools in the study of diverse metabolic pathways. In the case of lipids, this Gram negative facultative anaerobe has been valuable in the elucidation of the pathways of fatty acid and phospholipid metabolism.

Microorganisms are very responsive to changes in their environment, such as the composition of the medium in which they are grown (14-16) and the temperature of incubation (16,17). Therefore, one can observe changes in cell metabolism due to external factors which could not so readily be done with higher organisms.

Wild strains of E. coli have a growth range extending from 7.5° (17) to 45° C (18). The usual growth pattern includes a lag phase, a period of exponential growth and finally a stationary phase. The actual rate of growth, i.e., the doubling times per hour, can be influenced by many factors. For example, at 37° C growth is progressively greater with succinate, glycerol, and glucose as the source of carbon in the medium (16). A lag phase can be induced in cells growing exponentially at 37° C by lowering the temperature of the incubation to 10° C (8, 19,20). However, no lag phase results if the cells are grown anaerobically at 37° C and maintained anaerobically at 10° C (20).

Phospholipids of Escherichia coli

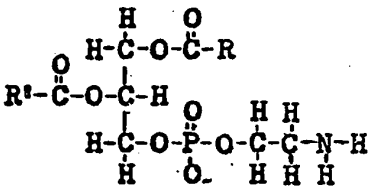
Phospholipids represent 5-10% of the dry weight of E. coli cells (13,20) and constitute more than 90% of the total lipids of these

microorganisms (21). The greater part of the phospholipids are localized in the cell membrane (22).

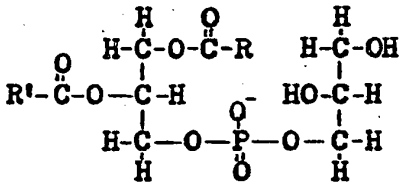
Phosphatidylethanolamine (PE) is the major phospholipid of E. coli, as well as of many other Gram negative organisms (23,24). The remaining phospholipids are principally phosphatidylglycerol (PG) and cardiolipin (CL) (12,24); the relative amounts of these are dependent on a number of conditions which will be discussed later. The presence of small amounts of phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol phosphate (PG-P), and several unidentified components have also been reported (12,24). Kaneyama, et al. (25) were unable to detect any PE plasmalogen in E. coli K-12. Benns and Proulx (26) have recently reported the formation of bis-phosphatidic acid or a close analogue in E. coli 015 under in vitro conditions. Further analyses of this component indicates that it consists of a mixture of mono- and diacyl-phosphatidylglycerol (27).

The structures of the three principle phospholipids of E. coli are given in Diagram 1. Those of PE and PG are well established; that of CL has been questioned. After its discovery by Pangburn in heart tissue(28), CL was the subject of debate for several years. Finally, the structure proposed by MacFarlane (29), that is, diphosphatidylglycerol, was shown to be correct by Lecocq and Ballon (30) and DeHaas and Van Deenen (31). Recentaly a report has again raised the question of the structure of CL obtained from rat tissues. Courtade, et al.(32) have proposed that the material designated as CL is really a mixture of compounds which have structures similar to that given, but containing fatty acids

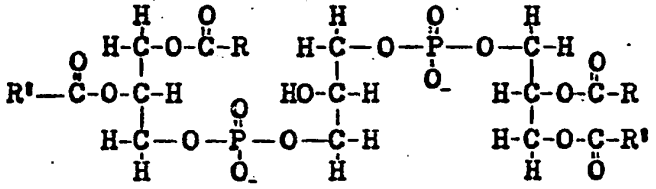
Diagram 1: Structure of the principle phospholipids of Escherichia coli.



PHOSPHATIDYLETHANOLAMINE



PHOSPHATIDYLGLYCEROL



CARDIOLIPIN

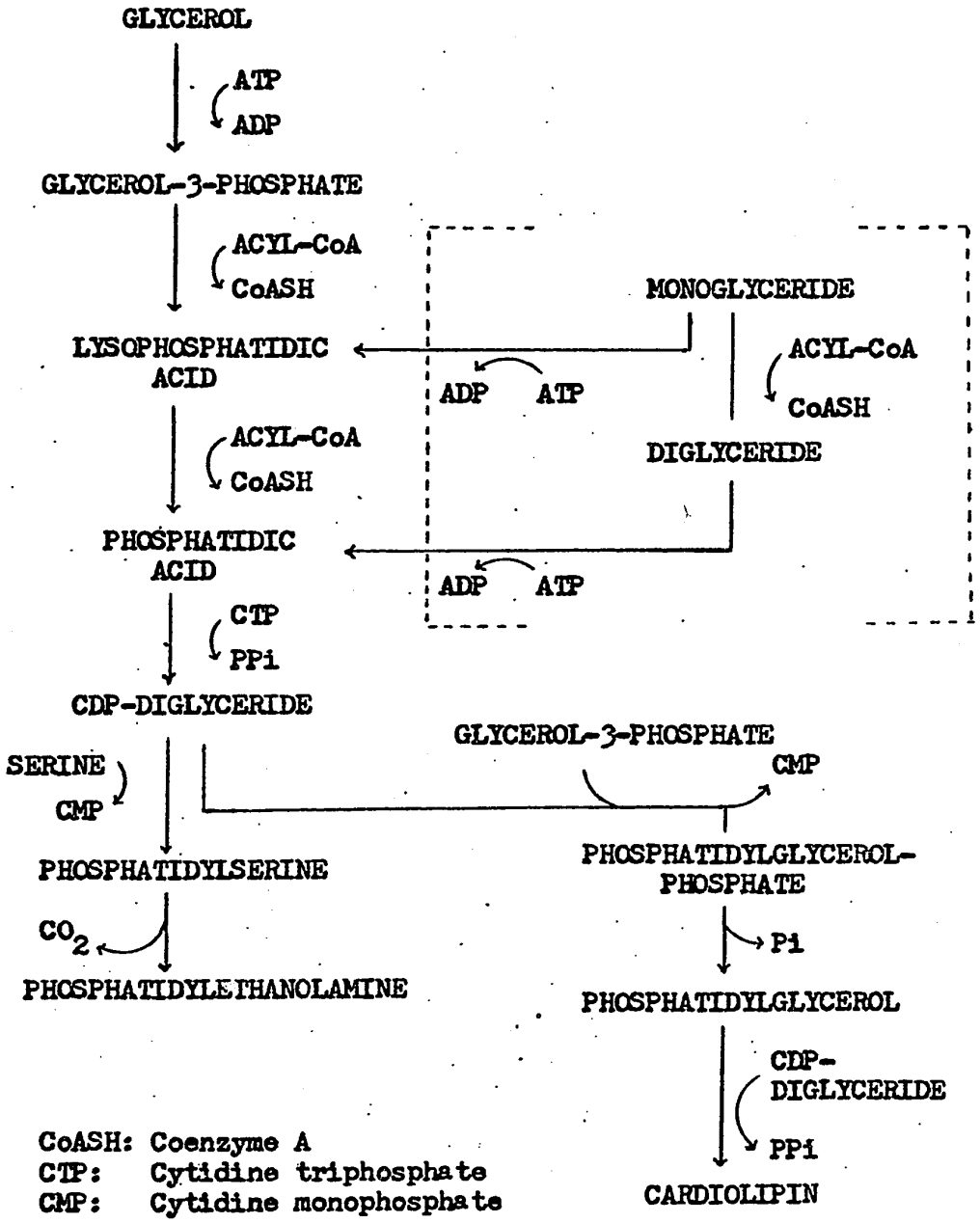
esterified to the hydroxyl group usually designated as free or to one of the phosphate moieties. Also, vitamin A might serve to conjugate one of the phosphate moieties in certain analogues. Using the methods of Courtade, et al., Nielson (33) was unable to confirm these findings. In E. coli, the structure of CL has been derived from chromatographic analyses of the deacylated product (13).

Synthesis of phospholipids in Escherichia coli

The general pathways of phospholipid synthesis in E. coli have been defined and are outlined in Diagram 2. The elegant work of Kennedy and co-workers (34-38) has shown the central role of cytidine diphosphate diglyceride (CDP-DG) in the synthesis of PE, PG and CL. The enzyme responsible for the formation of CDP-DG, i.e. cytidine triphosphate-phosphatidic acid cytidyl transferase, has been identified in the particulate fraction of E. coli by Carter (39) and the stepwise acylation of L-α- glycerol phosphate and lysophosphatidic acid (LPA) by acyl Coenzyme A (40-42), and to a lesser extent by acyl acyl-carrier protein (ACP) (42), has been described. Sinensky (43) has recently presented evidence that the acylation of L-α-glycerol phosphate and of LPA are catalyzed by two separate enzymes.

An alternate mechanism for the synthesis of CL has recently been proposed. Hirschberg and Kennedy (44) have shown that while CDP-DG does stimulate the synthesis of CL by a cell-free preparation of E. coli ML 308 as originally shown by Stanacev, et al. (36), the phosphorus moiety of this compound is not incorporated into the

Diagram 2: Pathways of phospholipid synthesis in Escherichia coli.



CoASH: Coenzyme A
CTP: Cytidine triphosphate
CMP: Cytidine monophosphate

cardiolipin. Comparison of exogenous doubly labeled PG with the labeling of CL synthesized led to the conclusion that CL is synthesized directly from two molecules of PG with the release of one glycerol moiety. Hostetler, et al.(45) have confirmed these findings, but have also presented evidence which suggests that the CDP-DG pathway is operative in E. coli at high CDP-DG concentration.

E. coli is known to contain a mono- and diglyceride kinase (indicated in brackets in Diagram 2) (20,40,41). However, glycerol and acyl CoA, in the absence of ATP, cannot adequately substitute for L-α-glycerol phosphate in the synthesis of phospholipids in vitro and E. coli has no metabolically-active diglyceride pool (20). Therefore, the physiological significance of the enzyme(s) is not known.

Most of the enzymes of phospholipid synthesis have been shown to be associated with the particulate fraction of the cells (36-38, 41,46,47), the only exception being serine:CDP-DG phosphatidyl transferase which is found in the soluble fraction (48). Another common feature of L-α-glycerol phosphate-acyl CoA acyl transferase (41), LPA:acyl CoA acyl transferase (41), CTP:PA cytidyl transferase (39), phosphatidylglycerolphosphate phosphatase (38) and cardiolipin synthetase (49) is a requirement for magnesium ions.

In general, the pathways described for the synthesis of PE and PG have been shown to be present in other bacteria (50) and in mammalian tissues (47,51-53). Synthesis of PE in mammalian tissues is also accomplished via reaction (1), which is analogous to the

synthesis of phosphatidylcholine (PC) in these tissues.

$$\text{1,2-diglyceride} + \text{CDP-ethanolamine} \longrightarrow \text{PE} + \text{CMP} \qquad (1)$$

Until recently the formation of CL had been studied only in bacteria. Davidson and Stanacev (54,55) have now shown that mitochondria of guinea pig liver can catalyze the formation of CL as well as of PG and PG-P. Cardiolipin was formed only in a system generating endogenous CDP-DG, whereas PG and PG-P can incorporate exogenously supplied CDP-DG. The authors suggest that the pathway of CL synthesis in mitochondria is as given in Diagram 2 and that either the CL synthetase has a high specificity for endogenously formed CDP-DG (they did not state the source or composition of the exogenous CDP-DG) and/or the biosynthesis of polyglycerophosphatides in mitochondria is hightly compartmentalized.

Hostetler, et al. (49) have since reported that mitochondria of rat liver can synthesize CL from exogenous CDP-DG, but at a rate much slower than the synthesis of PG from these precursors.

In view of the report of Hirschberg and Kennedy (44) regarding a possible second route of CL synthesis in E. coli, Hostetler, et al. (45) have reported further studies on the mechanism of CL synthesis in mitochondria. They could detect no release of labeled glycerol during the synthesis of CL from phosphatidyl (^{14}C)glycerol in vitro. Also the CL formed from (2-^{3}H)-phosphatidyl (1-^{14}C)-glycerol in the presence of unlabeled CDP-DG had a ^{3}H/^{14}C ratio nearly identical to that of the original PG. These findings confirm the presence of a

CDP-DG pathway of CL synthesis in mitochondria *in vitro*.

Turnover of phospholipids of *Escherichia coli*

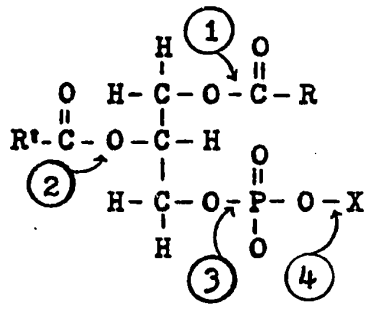
Very little was known of the turnover of *E. coli* phospholipids until Kanfer and Kennedy (12) described the metabolism of PE and PG in *E. coli* B. They reported that during exponential growth the phosphorus portion of PE is quite stable whereas that of PG showed considerable turnover. At the same time that Kanemasa, *et al.* (13) reported the presence of CL in *E. coli* cells, they also showed the turnover of this polyglycerophosphatide to be similar to that of PG.

Theoretically the degradation of phospholipids could follow several pathways. The sites of possible ester hydrolyses and the designation of the enzymes catalyzing individual reactions are given in Diagram 3.

Phospholipases A are ubiquitous, being found in nervous tissue (56), porcine pancreas (57), mosquito larvae (58), snake venoms (58-60) and *E. coli* (61,62).

Whole cell homogenates of *E. coli* studied by Fung and Proulx (62) were found to contain phospholipase A_1 predominantly, although lower levels of phospholipase A_2 were also detected at alkaline pH (63). Phospholipase A_1 in these preparations displayed two pH optima, one at pH 5 and the other at pH 8.4, as does the phospholipase A_1 activity of *E. coli* W spheroplasts (64). Scandella and Kornberg (65) have recently purified the alkaline phospholipase A_1 of *E. coli* and found this enzyme to have quite similar properties as those described

Diagram 3: Possible sites of hydrolysis of phospholipids and the enzymes catalyzing each reaction.



1 a) Phospholipase A_1 (Phosphoglyceride 1-acyl hydrolase)

b) Lysophospholipase (Monoacylglycerophosphoryl-X-acyl hydrolase)

c) Lipase

2 a) Phospholipase A_2 (Phosphoglyceride-2-acyl hydrolase)

b) Lysophospholipase (Monoacylglycerophosphoryl-X-acyl hydrolase)

c) Lipase

1 and 2 Phospholipase B

3 a) Phospholipase C (Phosphoglyceride phosphoryl-X hydrolase)

b) Phosphatidic acid phosphoryl hydrolase - in the case of phosphatidic acid

4 Phospholipase D (Phosphoglyceride -X hydrolase)

in crude preparations. It was devoid of A_2 activity and displayed a single pH preference of 8.4.

More recent evidence indicates, however, that there are at least two phospholipases A in E. coli. One, which is membrane-bound, is resistant to detergent, is activated by alcohol and has a broad specificity; the other, present in the cytosol fraction, is detergent sensitive, is inactivated by methanol and, according to Doi, et al. (66), acts primarily on PG. Lyso-PG did not accumulate when PG was exposed to the cytosol enzyme and possibly a phospholipase B was involved.

On the other hand, Bernard (67) described a similar cytosol enzyme which is greatly stimulated by ethanol in the presence of low concentrations of Triton X-100. Under her conditions, the enzyme seems to act on PE as well as PG and lyso-derivatives are formed. The author claims that the cytosol enzyme is, in fact, a phospholipase A_2.

In any case, the predominantly active phospholipase A of E. coli (62), as well as that of mitochondria and lysosomes (68,69), is associated with the particulate fraction. In general, these enzymes require or are activated by calcium ions. The only reported exception is that of an enzyme found in mosquito larvae (58).

A preliminary report by Proulx and Van Deenen (70) indicated the presence of phospholipase C activity in E. coli NCTC 2276 as tested with cell homogenates and exogenous substrates. Although these findings were extended to E. coli B (local stock) by Okuyama and Nojima (61), other strains tested in this laboratory, such as E. coli 015, 0118,

B (ATCC 11303 and 23226) as well as four hemolytic strains, did not degrade exogenous phosphatides to diglycerides. Phospholipase C is, therefore, not a typical enzyme of E. coli. Very likely, diglyceride formation from (^{14}C)-acyl labeled phosphatides could arise by recycling of any fatty acid released by phospholipase A. With respect to this, acylation of monoglyceride (40), as well as degradation of phosphatidate to diglyceride (61,70) has been described.

Phospholipase D has been reported to be present in Haemophilus parainfluenzae (71,72) and is specific for cardiolipin. This finding, however, has not been extended to other microorganisms.

The product of phospholipase A hydrolysis, lysophosphoglycerides, may be further degraded. Lysophospholipase activity of E. coli was first reported by Proulx and Van Deenen (70) and later confirmed by Okayama and Nojima (61) and Proulx and Fung (63). The hydrolysis of ^{32}P-LPE and ^{32}P-LPC (70) and the release of labeled fatty acids from ^{14}C-LPE (61) is catalyzed by a soluble fraction of sonicated cells. The enzyme of E. coli 015 does not require divalent cations for activity and has a broad alkaline pH optimum (63). The microbial enzyme differs from those of mammalian tissues in that it is not susceptible to several known inhibitors of lysophospholipase activity, e.g. NaF, KCN, and p-chloromercuribenzoic acid (61) and can be inhibited only by high concentrations of anionic detergents (61,63).

Whether lysophospholipase of E. coli is specific for either the 1- or 2-acyl position has not been shown. The lysophospholipase of rat liver is relatively nonspecific, catalyzing the hydrolysis of

1-acyl and 2-acyl LPC and of synthetic analogues with no mono-hydroxyl group or with the phosphoryl moiety in the 1 or 2 position (73).

Lysophosphatides may be reacylated to diacyl-phosphoglycerides as well as being degraded. A lysophosphatide:acyl CoA acyl transferase has been identified in E. coli B by Proulx and Van Deenen (74). The particulate fraction of sonicated cells catalyzed the conversion of LPE and, to a lesser extent, of LPC to diacyl-phosphoglycerides. No detailed properties of this E. coli enzyme have been reported but recent studies by Scandella and Kornberg (65) have confirmed its presence.

The products of the combined action of phospholipase A and lysophospholipase on PC and PE (i.e. glycerophosphorylcholine and glycerophosphorylethanolamine, respectively) were identified by Proulx and Van Deenen (70). Okuyama and Nojima (61) have postulated that the major pathway of phospholipid metabolism in E. coli is a stepwise deacylation involving these two enzymes. Significant amounts of L-α-glycerol phosphate and inorganic phosphate have also been detected in systems hydrolyzing PE in vitro (70). Okuyama and Nojima (61) describe the presence of phosphodiesterases attacking deacylated phosphatide products, but these enzymes have not yet been specifically identified.

Fatty acids of Escherichia coli

The phospholipids of E. coli contain the following fatty acids: myristate (14:0), palmitate (16:0), hexadecenoate (16:1), methylene hexadecanoate (17:v1), octadecenoate (18:1) and methylene

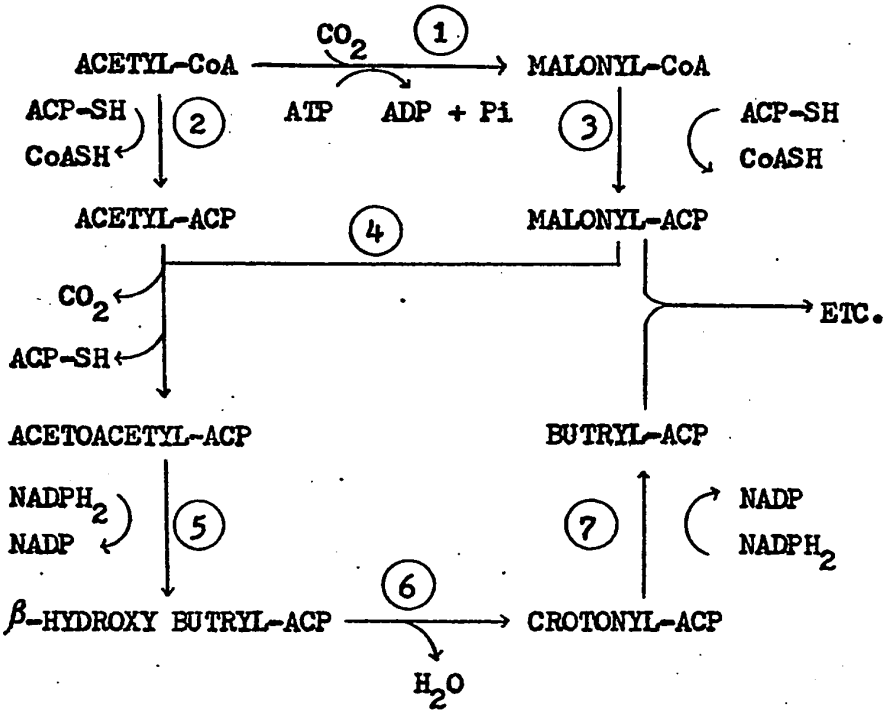
octadecanoate (19:v1) (25,79). Minor amounts of laurate (12:0), methylene tetradecanoate (15:v1) and β-hydroxy myristate have also been reported (75,76). It is now established that the octadecenoic acid is solely cis-vaccenate (18:1 11) (77) and that the hexadecenoic acid is palmitoleate (16:1 9) (77,78). The cyclopropane fatty acids are methylene derivatives of vaccenate and palmitoleate (78).

Synthesis of fatty acids by Escherichia coli

The pathway of fatty acid synthesis of E. coli is now well defined (79,80) as illustrated in Diagram 4. Two carbon units, via malonyl CoA with the loss of CO_2, are added to a growing chain. The final products in E. coli are predominately myristate and palmitate. The system is dependent on acyl-carrier protein (ACP) which can be synthesized in E. coli (80). Wakil and co-workers (see 80) have identified and characterized the enzymes responsible for the various reactions. Lynen (81), Majerus and Vagelos (79) and Wakil (82) have demonstrated the presence of the malonyl system of fatty acid synthesis in higher organisms. However, the enzymes of all systems thus far studied, except those of E. coli (80) and certain plants (83), are aggregate and are inactivated by dissociation.

The β-hydroxy-acyl ACP dehydrase (6 in Diagram 4) can catalyze the formation of both 2- and 3-monounsaturated acyl ACP's (84) of chain length of 10 carbons or less. The 3-unsaturated acyl ACP cannot be reduced to a saturated chain. Further elongation of the 3-decenoyl ACP produces palmitoleate and vaccenate. Synthesis of unsaturated

Diagram 4: Synthesis of fatty acids in Escherichia coli.



1 Acetyl Coenzyme A Carboxylase

2 Acetyl Coenzyme A-Acyl Carrier Protein Transacylase

3 Malonyl Coenzyme A-Acyl Carrier Protein Transacylase

4 β-Ketoacyl Acyl Carrier Protein Synthetase

5 β-Ketoacyl Acyl Carrier Protein Reductase

6 β-Hydroxylacyl Acyl Carrier Protein Dehydrase

7 Enol Acyl Carrier Protein Reductase

CoA: Coenzyme A

ACP: Acyl Carrier Protein

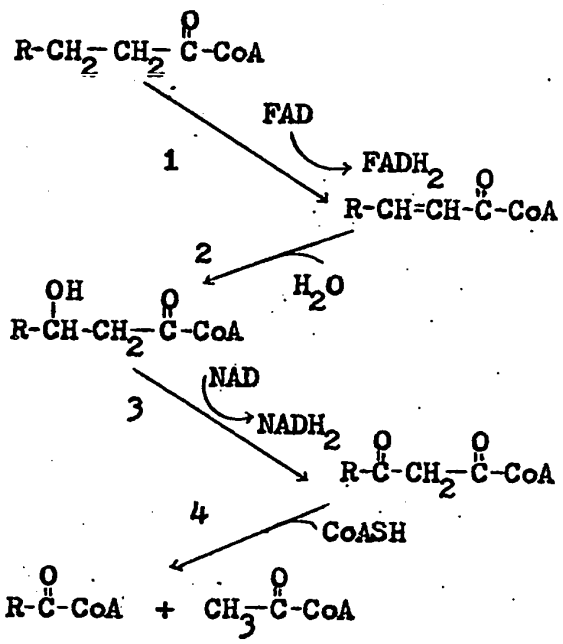
fatty acids of E. coli (85,86) and other Eubacterioles (21) is solely by this anaerobic mechanism, although higher organisms can also employ a monooxygenase for converting saturated fatty acids to unsaturated ones (87,88).

Cyclopropane fatty acids are formed by the transfer of a methyl group to the double bond of palmitoleate or to cis-vaccenate resulting in a type of substituted fatty acid (89). O'Leary (90) first suggested that S-adenosyl methionine was the methyl donor and Law, et al.(91) have shown that the presence of a number of other methyl donors in no way diminishes the incorporation of labeled methyl groups of methionine into cyclopropane fatty acids. The transfer of the methyl group is to an unsaturated fatty acid moiety of a phospholipid rather than to a free acid (92). The mechanism of addition to the unsaturated bond has been studied in greater detail by Law (93). The location of the cyclopropane fatty acid synthetaseof E. coli has not been determined but that of Pseudomonas fluorescens is found in the soluble fraction (94).

Oxidation of fatty acids by Escherichia coli

E. coli cells can convert a sizable fraction of many carbon sources into acetate (95). Wild type cells and various mutants are capable of growth in media containing fatty acids as the sole carbon source (96-98). The fatty acid supplied does not necessarily have to be one of those native to E. coli (97). The enzymes of β-oxidation, as given in Diagram 5, can be induced by fatty acids containing 14 or more carbons. The coordinate variation in the activities of the

Diagram 5: Beta-oxidation of fatty acids in Escherichia coli.



1 Acyl Dehydrogenase

2 Enoyl Hydrase

3 β-Hydroxylacyl Dehydrogenase

4 Thiolase

CoASH: Coenzyme A

enzymes suggest that they are under one gene control (97). The significance of the β-oxidation enzymes may not be great since bacteria in general can grow readily on glucose or other non-lipid fuels. They do not store neutral lipids as an energy source but E. coli can form glycogen granules (99).

The presence of a cyclopropane ring presents a block in the normal oxidative pathway. The ability to break the ring has been reported for the fungus, Fusarium oxysporum Schlectadehl (100), and the protozoa, Ochemonas danica (101), but not for E. coli. In any case, there is probably little catabolism of cyclopropane fatty acids in vivo since they have been shown to be stable for periods of up to 150 hours (10,91).

Effects of environmental factors on total lipids and phospholipids

In the normal life cycle of an E. coli culture grown at 37° C, total phospholipids decrease during the log phase then remain unchanged during the stationary phase (102). However, if the temperature of growth is 27° C, rather than 37° C, the total phospholipid content increases continuously throughout the log and stationary phases (102). Lowering the temperature of a culture growing exponentially at 37° C to 10° C, results in a 4.5-5 hour lag period. During this time total phospholipids increase about 20% (8), whereas other cellular components, e.g., protein, RNA and DNA, remain unchanged (19,20).

PE reaches a maximum concentration during the log phase of 37° C

cultures and then decreases during early stationary phase (24); PG follows the same general pattern. In contrast, CL increases during the stationary phase (24, 102). Whether or not there is a reciprocal relationship between the decrease in PG and the increase in CL is debatable as the reports are in conflict (24, 102). Haest, et al. (3) found no apparent differences in the distribution of the various phospholipid classes of E. coli grown to late log phase at 20°, 30°, or 40° C.

As mentioned previously, PG decreases and CL increases during the stationary growth phase. The same change in the proportions of these phospholipids is seen when exponentially growing cells are transferred to a medium containing no carbon source (9) or when filamentous growth (that is, growth without cell division) is induced by the presence of penicillin or by ultraviolet light (103). However, when cell growth and division is temporarily halted by subjecting cells to low temperatures, there is relatively less synthesis of CL than of either PE or PG and the proportion of CL of the total phospholipid does not increase (8).

The environmental temperature appears to influence the metabolism of phospholipids also. As mentioned previously, PG and CL turnover during normal exponential growth at 37° C, whereas PE is stable (12,13). Okuyama (8) has reported that the phosphate moieties of all major phospholipids of E. coli B, including PE, show considerable turnover during a 5 hour cold-induced lag phase. The acyl moieties of the phospholipids, however, did not give this response. The author

postulated that there was, in fact, a turnover of the total phospholipid molecule, but that the acyl moieties, as acyl ester groups or fatty acids, were being efficiently reutilized.

Effects of environmental factors on individual fatty acids

Under most conditions, palmitate represents the largest percentage of the total fatty acids of E. coli phospholipids. Palmitoleate and cis-vaccenate and/or their methylene derivatives are the next most abundant. Whether the fatty acid distribution of the individual phospholipids are the same is not yet clear. Kanemasa (13) has reported that the percent of each fatty acid in PE, PG and CL of 37° C stationary cultures are much the same. On the other hand, DeSiervo (102) found that in 27° C stationary cultures, the percent of cis-vaccenate was much greater and those of palmitate and palmitoleate were much less in PG than in either PE or CL.

The fatty acid composition as well as the amounts of the phospholipids themselves change throughout the growth cycle. As a culture approaches the stationary phase, the percent of monoenoic fatty acid decreases while that of the cyclopropane fatty acids increases (10,89). The methylation of monoenoic fatty acids does not proceed at the same rate within all phospholipids, but begins sooner and proceeds more slowly in PE than in either PG or CL. The net result is that the cyclopropane fatty acid composition of all phospholipids is the same at stationary phase.

The conversion of palmitoleate to methylene palmitate appears

to preceed that of vaccenate to methylene stearate (89). Methylene hexadecanoate represented 9% of the total fatty acid present before any methylene octadecanoate could be detected. After the 17:ν1 acid had reached a level of 30% of the total fatty acids, any further methylation appeared to be as the expense of 18:1.

In Pseudomonas fluorescens, the amount of cyclopropane fatty acid is inversely related to the degree of aeration of the culture due to an induction of cyclopropane fatty acid synthetase at low oxygen tensions (94,104). In E. coli, cyclopropane fatty acid accumulation at early stationary and during stationary phase could be reduced somewhat by increasing aeration. However, there was little effect on the percent of cyclopropane fatty acids present during exponential growth (104).

The concentration of cyclopropane fatty acids in E. coli is dependent on many factors, including the temperature of incubation. Knivett and Cullen (89) found that the percent of cyclopropane fatty acids of the total fatty acids was progressively less in E. coli grown at 37°, 30°, and 23° C. The percentage of cyclopropane fatty acids may also be decreased by an alkaline medium, the presence of citrate (85) and by filamentous growth induced by penicillin or ultraviolet light (76).

The effect of temperature on the fatty acid composition of E. coli is a well known phenomenon. Reducing the temperature at which cells are grown (3,89) or transferring exponentially growing cells to a lower temperature (8,19,74) leads to an increase in the percent

of unsaturated fatty acids and to a decrease in the percent of cyclopropane fatty acids. The adaptation of cells transferred to a colder medium is not complete. For example, the fatty acid composition of cells transferred from 37° to 10° C resembled that of cells grown at 25° C, rather than that of cells grown at 10° C.

Alterations in fatty acid composition due to changes in environmental temperatures is dependent on a source of carbon. Shaw and Ingraham (19) found no change in the fatty acid composition of E. coli kept for 4.5 hours at 10° C if glucose was withheld from the medium. These cells were capable of resuming growth at 10° C after this lag period when glucose was supplied. Thus growth at low temperatures is not dependent on the fatty acid composition of the cells. Conversely, a rather large change in fatty acid composition had no measurable effect on growth rate of E. coli cultures (105).

Positional specificity of fatty acid distribution in phospholipids

It is a general finding in phospholipids and in neutral lipids that the acyl groups esterified in the α-position are predominately saturated and those esterified in the β-position are cis-unsaturated. The only reported exceptions are CL of various mammalian tissues (106-108) and PE of Clostridium butyricum (109).

The means by which this asymmetry is established and maintained has been much debated. Lands and Hart (110) found stearate and linoleate to be incorporated equally well into both the 1- and 2-positions of phospholipids synthesized from α-glycerol phosphate

by microsomes of rat and guinea pig liver. At that time they suggested that "the specificities of the acyl transferase reactions leading to diacyl glycerol phosphate are not adequate to provide the pattern of fatty acids that is known to occur in glycerolipids in nature".

This view was supported by other reports of studies with rat brain (111) and liver (112), but was later questioned. It was subsequently found that the fatty acid distribution of PA of rat liver is nonrandom, which would indicate specific orientations of fatty acids in the de novo synthesis of phospholipids (113). However, the fatty acid distribution of PE and PC were different from that of PA suggesting that deacylation and reacylation have a role in determining the final composition and positional distribution of fatty acids in individual phospholipids. Recently, Lands has endorsed these findings and concurs with the concept of original nonrandom fatty acid distribution with further nonrandomization being established by a deacylation-reacylation mechanism (Pers. Comm. to Dr. P. Proulx).

In vitro studies with a particulate enzyme preparation from an E. coli mutant have recently shown that the acylation of L-α-glycerol phosphate is responsive to the type of fatty acid available (114). Palmitic acid was found to be esterified exclusively in position 1 and unsaturated fatty acids in position 2. The enzyme(s) responsible for the acylation has an unusual thermolability. Therefore, the relevance of this finding to other strains of E. coli is not yet known.

The development of silver ion thin layer chromatography has

opened a new field in the study of the positional specificity of fatty acids of phospholipids (115). Combined with gas-liquid chromatography analysis of fatty acids, one may now determine the relative amounts of precisely defined phospholipids, e.g. 1-palmitoyl, 2-vaccenyl PE, instead of total fatty acid composition of this lipid class.

Fatty acid auxotrophs of E. coli have also proven especially useful in studying changes in phospholipid species (11,116). These mutants can incorporate various exogenously supplied fatty acids (some of which are foreign to the cells (116)) intact into phospholipids.

The positional specificity of the fatty acids in the phospholipids of E. coli is not exact and E. coli cells contain some 1,2-disaturated (excluding 1-saturated, 2-cyclopropane) and 1,2-diunsaturated species. In the predominant 1-saturated, 2-unsaturated species, the longer the unsaturated fatty acid incorporated into the 2-position, the larger the myristate/palmitate ratio in the 1-position (11). In a diunsaturated species, shorter monoenoic fatty acids are preferentially acylated in the 1-position. In like manner, shorter saturated fatty acids are preferred over longer ones in the 2-position of disaturated species.

Trans-monoenoic acids may be incorporated into either the 1- or 2-position (11). Since these acids have a straighter configuration than their cis counterparts, they share some structural features and physical properties in common with saturated fatty acids, while at the same time, they are more reactive than long chain saturated fatty acids in transacylations involving the 2-position (116). When trans-

vaccenate was supplied exogenously to a fatty acid auxotroph of E. coli, the phospholipid formed contained less myristate than if cis-vaccenate were supplied.

Cyclopropane fatty acids are normally located in the 2-position (109) in accord with the fact that they are synthesized from unsaturated fatty acids already attached to a phospholipid molecule. Clostridium butyricum contains unsaturated fatty acids in both the 1- and 2-positions. However, the 2-fatty acid is methylated almost exclusively, indicating a positional "methylating" specificity of cyclopropane fatty acid synthetase in this organism at least (109).

GENERAL METHODS

Preparation of Escherichia coli 015 cells

Escherichia coli 015 was obtained from the Department of Microbiology of the University of Ottawa and maintained on agar slants. Cultures were grown in either nutrient broth or a modification of Medium 56 (117) as given in table I. One liter cultures were grown in large culture flasks in a shaking Labline incubator at 37° C. Smaller cultures were grown in Erlenmeyer flasks in an Eberbach water bath-shaker at 37° C with moderate shaking.

The optical density of the culture was followed using a Coleman Junior Spectrophotometer at 620 mμ. The growth curve of E. coli 015 in minimal medium is shown in figure 1. This medium supports full growth of E. coli ML (117); E. coli 015 does not grow as well in this medium as in complex medium. In most cases, cells were harvested at early or mid-log phase, at a time when growth was proceeding exponentially at a specific growth rate* of 0.44 hour^{-1}.

Each culture was checked for purity by Gram staining. Also, cells were streaked onto a nutrient plate and at least eight individual colonies were transferred to a MacConkey plate. The growth of red colonies indicated the presence of Gram negative organisms. A streak from these colonies was placed on a citrate slant to differentiate between E. coli (no growth) and Aerobacter (growth with a change in color

*specific growth rate: $k = \frac{2.303\ (\log_{10} x_2 - \log_{10} x_1)}{t_2 - t_1}$

where x_1 and x_2 are the optical densities at t_1 and t_2, respectively.

Table I. Media used for culturing Escherichia coli 015.

COMPLEX		MINIMAL	
	g/l		g/l
Bactopeptone	15.0	KH_2PO_4	13.6
Yeast Extract	1.0	$(NH_4)_2SO_4$	2.0
NaCl	5.0	$MgSO_4$	0.098
		$CaCl_2 \cdot 2\ H_2O$	0.1325
		$FeSO_4$	0.005
		Sodium Acetate	1.0
*Glucose	20.0	*Glucose	2.0

*Autoclaved separately

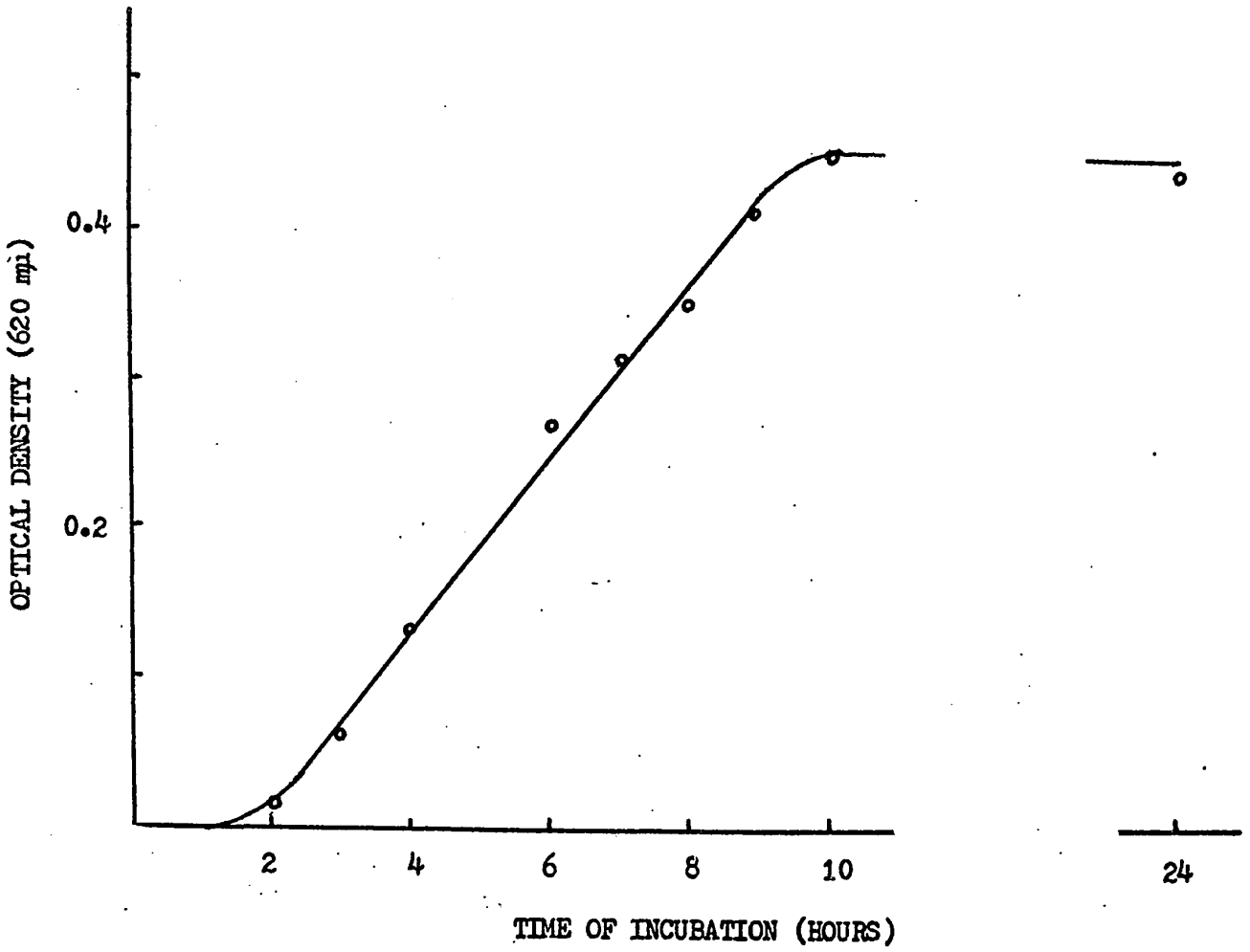


Figure 1: Growth of Escherichia coli 015 in Medium "56" at 37° C.

of the medium from green to blue). Periodically sample cultures were sent to the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa for typing and for checking for maintenance as smooth cultures by Miss Rhoda Laidly.

Cells were harvested by centrifuging at 10,000 rpm for 15 minutes in a Lourdes Model 1-A refrigerated centrifuge at 0° C.

Extraction of lipids

The standard procedure for extraction of total lipids from cells and of individual lipids from silica gel after thin layer chromatography was a modification of the procedure of Bligh and Dyer (118). A mixture of chloroform (redistilled), methanol and water (1:2.5:1, v/v/v) was added to the material to be extracted. The suspension was stirred for 30 minutes. One volume of each chloroform and water was added and the stirring continued for another 20 minutes. The two phases were separated by centrifuging at 3,000 rpm for 10 minutes and the lower chloroform phase was transferred to an evaporation flask with a Pasteur pipet. The remaining water and protein or silica gel layer were extracted again with one volume of chloroform for 30 minutes and the lower phase added to the first extract. The pooled extracts were evaporated to dryness at 37° C on a Buchi Rotovapor R Flash evaporator and the lipids were stored under nitrogen at -20° C.

Separation of lipids and water-soluble materials

Lipids were routinely separated on TLC plates (5x20 or 20x20 cm)

coated with 0.75 mm of silica gel made with 45 g of silica gel G and 90 ml of 5% ammonium sulfate (26) or with 0.5 mm of silica gel G. The former type of TLC plate will be referred to as "ammonia plates". The Rf values of the various neutral lipids and phospholipids in the solvent systems normally employed for separations are given in table II. Other types of plates and solvent systems will be described with the experiments in which they were used.

Two systems were used for separation and identification of water-soluble materials: 1) n-propanol-ammonia-water (60:30:10, v/v/v) on 0.5 mm cellulose plates activated at 110° C for two hours, and 2) phenol-water (5:1, v/v) on 3 mm Whatman filter paper (descending). The Rf values of standards are given in table III.

Both plates and papers on which radioactive materials were separated were scanned using a Nuclear Chicago Actigraph III ratio-chromatograph scanner.

Mild Alkaline Hydrolysis

Lipids were deacylated according to the procedure of Dawson, et al. (119) as modified by Ferrani and Benson (120). The material to be hydrolyzed was incubated with 5 ml of 1% toluene in methanol and 5 ml of 0.2 N KOH in methanol for 15 minutes at 37° C. The reaction was stopped by placing the incubation tube in ice and adding 5.0 ml of ice cold water. Excess KOH was removed by adding a 50% suspension of Dowex (50W x 80, 100-200 mesh) until the pink color of a drop of added phenolphthalein disappeared. The tubes were centrifuged at 3,000 rpm

Table II: Rf values of neutral lipids and phospholipids in various thin layer chromatography systems.

	Silica Gel G plates[1]		"Ammonia" plates[1]
	A[2]	B[2]	A[2]
Triglycerides	1.00	0.86	1.00
Fatty Acids	0.73	0.63	1.00
Diglycerides	1.00	0.29[3] 0.21	1.00
Monoglycerides	1.00	0.04	1.00
Phosphatidyl-ethanolamine	0.46	0.00	0.66
Phosphatidylglycerol	0.46	0.00	0.75
Cardiolipin	0.75	0.00	0.96
Phosphatidic Acid	0.70	0.00	0.96
Phosphatidylcholine	0.25	0.00	0.37
Lysophosphatidyl-ethanolamine	0.20	0.00	--
Lysophosphatidyl-glycerol	0.15	0.00	--

[1] see text for description of thin layer plates.

[2] A: chloroform-methanol-water (65:25:4, v/v), B: petroleum ether (30-60°C) -ethyl ether-formic acid (75:25:1.5, v/v).

[3] 1,3- and 1,2-diglycerides, respectively.

Table III. Rf values of water-soluble materials in systems 1 and 2 as indicated in the text.

	1	2
Inorganic phosphate	0.00	0.00
α-Glycerol phosphate	0.06	0.19
*Glycerophosphorylglycero-phosphorylglycerol (GPGPG)	0.45	0.22
O-Phosphorylethanolamine	0.42	0.32
*Glycerophosphorylglycerol (GPG)	0.70	0.53
*Glycerophosphorylethanolamine (GPE)	0.70	0.66

*Deacylated CL, PG and PE, respectively, isolated from E. coli lipids.

for 10 minutes. The supernatant containing the mixed water- and chloroform-soluble materials was removed to an evaporation flask with a Pasteur pipet. The Dowex resin was washed once with 2 ml of methanol and 2 ml of water. The supernatant and the wash solution were pooled and evaporated to dryness on a rotary evaporator at 50° C. Chloroform, isobutanol and water were mixed in a ratio of 100:50:75 (v/v/v) and allowed to separate into two phases. One ml of the upper phase and 2 ml of the lower phase were added to the evaporation flask and the contents mixed thoroughly and transferred to a centrifuge tube. The flask was washed with the same volumes of the upper and lower phases and the mixture added to the centrifuge tube. The contents were centrifuged and the upper phase containing the water-soluble materials were removed; the solvent was evaporated on a rotary evaporator at 50° C.

When fatty acids were to be analyzed, the hydrolysis was stopped by adding 5.0 ml of cold 0.2 N HCl, followed by 2.5 ml of methanol and 5.0 ml of chloroform. The fatty acids were extracted by the Bligh and Dyer procedure described previously. Recovery of fatty acids by this procedure was better than 95%.

Counting of radioactive materials

A Nuclear Chicago Mark IV scintillation counter was employed in some of the earlier work. The scintillation fluid contained 5% PPO and 0.3% dimethyl POPOP in toluene. Four percent Cabo-Sil was added to the fluid in order to count ^{14}C labeled materials on silica gel without prior elution. Quenching corrections were made using chloroform and

a chanels ratio method.

Later a Beckman LS-133 scintillation counter was used. The scintillation fluid contained 5% PPO in toluene, to which 100 ml of methanol and 3.5 ml of glacial acetic acid/liter were added (121). This mixture was adequate for accurate counting of ^{14}C labeled materials not previously eluted from silica gel. Tritium labeled materials were eluted by the modified procedure of Bligh and Dyer prior to counting. An external standard ratio was used for quench corrections.

Water-soluble materials were counted in the methanol-acetic acid scintillation fluid in the presence of 2.0 ml of Triton X-100 per 0.1 ml of water.

Materials

Bactopeptone and yeast extract were obtained from Difco; silica gel G from CanLab, Montreal; PPO and POPOP from Fraser Medical Supplies, Vancouver; Cabo-Sil from Packard Instrument, Co., Downers Grove, Ill., and amino-naphthosulfonic acid (ANS) from Fisher Scientific Co., Montreal.

The radio-isotopically labeled materials used were: ^{32}P-phosphoric acid, 1-^{3}H-acetate (sodium salt) and ^{3}H-methyl methionine from New England Nuclear; 1-^{14}C-palmitic acid (sodium salt), Nuclear Chicago and 1-^{14}C-acetic acid (sodium salt) from Amersham/Searle.

The commercially prepared enzymes were phospholipase D from cabbage (Calbiochem), phospholipase A_2 from Crotalus adamantus venom (Sigma Chemical Company) and porcine pancreatic lipase (Worthington Biochemical Company).

EXPERIMENTAL RESULTS

I. IN VIVO METABOLISM OF PHOSPHOLIPIDS OF ESCHERICHIA COLI 015 AT 37° C.

Introduction

At least two of the major phospholipids of E. coli are actively metabolized during exponential growth at 37° C. Kanfer and Kennedy (12) found that half of the radioactivity of prelabeled ^{32}P-PG was lost in 40-50 minutes. Kanemasa et al. (13) confirmed the turnover of PG and found that CL was similarily metabolized during a three hour growth period. PE, on the other hand, appears to be quite stable. In cells prelabeled with ^{32}P then transferred to nonradioactive medium, the label in PE increased for 30-60 minutes, then remained constant for up to three hours (12,13).

Several enzymes which participate in the hydrolysis of phospholipids have been identified in E. coli. Okuyama and Nojima (61) and Fung and Proulx (62) reported the presence of phospholipase A in E. coli B and E. coli 015 and 0118, respectively. In these studies PE, which is native to E. coli, and PC, which is not, served as substrates.

Doi, et al.(66) have presented evidence that certain strains of E. coli contain two phospholipases, differing in their susceptibility to inactivation by detergents, heat and organic solvents as well as localization and substrate specificity. Both PE and PG are hydrolyzed by the particulate enzyme, whereas PG and, to a lesser extent, CL are degraded by the soluble fraction.

Lysophospholipids which would be formed by phospholipase A

hydrolysis of phospholipids may be degraded by a soluble fraction of E. coli cells. Lysophospholipase activity was first reported by Proulx and Van Deenen (74) and later confirmed by Okayama and Nojima (61) and Proulx and Fung (63). On the other hand, the particulate fraction of E. coli cells can catalyze the reacylation of lyso-PE and, to a lesser extent, of lyso-PC to diacylphosphoglycerides (74).

The following experiments deal with the in vivo metabolism of phospholipids, both the phosphorus and fatty acyl moieties, of E. coli 015 at 37° C. The relative metabolism of the 1- and 2-acyl groups is discussed in relation to the enzymes present in this strain of E. coli.

Methods

E. coli 015 cells were grown to early log phase at 37° C in 300 ml of minimal medium 56 which contained 0.3 g sodium acetate, 0.6 g glucose and 100 μc of 1-^{14}C-acetate. The cells were harvested and resuspended in minimal medium containing the same amounts of sodium acetate and glucose as the growth medium, but from which the phosphate was omitted. The cell suspension was divided into two equal aliquots and returned to the 37° C water bath. After 5 minutes of incubation, 1 mc of ^{32}P was added to one of the flasks and the incubation continued for another 10 minutes.

The cells in each flask were harvested separately and resuspended in 150 ml of minimal medium in which the acetate concentration was increased to 0.075 M and the phosphate concentration was 0.1 M (The original growth medium was 0.0122 M acetate and 0.029 M phosphate) Aliquots of 25 ml were incubated at 37° C for 0, 30, 90 and 180 minutes. One series of flasks, containing the ^{14}C-labeled cells only, was used to assess the turnover of whole phospholipids as well as the turnover of individual acyl groups. The other series, doubly labeled, was used to assess turnover of ^{32}P-moieties. Simultaneous counting of the ^{14}C and ^{32}P-labels in this series gave an additional value for the turnover of carbon units and served to check whether identical conditions prevailed in both series as far as lipid turnover was concerned.

Cells were harvested and the lipids separated, extracted and counted as described in General Methods.

Labeling of the acyl groups by ^{14}C acetate was determined by

subjecting aliqouts of the phospholipids to mild alkaline hydrolysis (General Methods). Less than 4% of the total ^{14}C label of the phospholipid was found in the water soluble portion (table IV). Therefore, in subsequent experiments the total ^{14}C label was assumed to represent mainly the labeling of the acyl groups.

Labeling of the acyl group in position 2 was assessed with the use of phospholipase A_2 of Crotalus adamantus venom. The enzyme preparation contained a lysophospholipase which could not be entirely inactivated by heating at 60° C at pH 5.0 for 10 minutes. In order to minimize lysophospholipid hydrolysis, the ethereal system of Hildebrand and Law (109), in which lysophospholipids precipitate as they are formed, was used. A mixture containing the substrate in 2.0 ml of ethyl ether, 30 µl of 0.15 N NH_4OH and 30 µl of Tris buffer (0.1 N, pH 7.2) containing 0.01 M $CaCl_2$ and 3-5 mg of snake venom/ml was incubated with shaking at room temperature for 6-14 hours, i.e. until no trace of diacyl phospholipids remained. The reaction was stopped by adding 2.0 ml of 98% ethanol. The solvents were removed by evaporation.

The lipids were not extracted from the small quantity of protein present but were dissolved in chloroform-methanol-acetic acid (1:4:0.15, v/v/v) and spotted directly on silica gel G plates. Lysophospholipids and fatty acids were separated by developing the plates first in chloroform-methanol-water (12:6:1, v/v/v) for three-quarters of the height of the plate, drying the plate at room temperature, then developing it in petroleum ether-ethyl ether-formic acid (50:50:1.5, v/v/v) the full length of the plate.

Table IV: ^{14}C-Labeling of the glycerol and ethanolamine moieties[1] of phospholipids of Escherichia coli 015 grown in the presence of ^{14}C-acetate and chased.

Time of Incubation	PE	PG	CL
minutes	percent of the total phospholipid dpm		
0	0.6	1.4	1.1
30	0.9	3.7	2.4
90	0.9	2.4	2.5
180	0.9	2.2	2.9

[1]As determined by mild alkaline hydrolysis and recovery of the water-soluble materials.

^{14}C-labeling of the 1-acyl group was calculated as total ^{14}C counts minus the ^{14}C-counts of the fatty acids obtained by hydrolysis of the lipid with phospholipase A_2. The validity of this method was indicated by the results which always showed an equal distribution of label in both acyl ester positions of phospholipids isolated from E. coli cultures prior to a chase.

Isotopically labeled phospholipids for use as substrates in in vitro enzyme assays were prepared biosynthetically using liver of young rats. Incorporation of ^{32}P into phospholipids was as follows: 0.3 g of finely cut liver in 0.2 ml of Krebs-Ringer solution (0.1 M, pH 7.4) was incubated with 1-2 mg of CTP and 2.5 mc of ^{32}P at 37° C for 4 hours with 95% O_2-5% CO_2 aeration. The incubation mixture for ^{14}C incorporation contained 50 μc of 1-^{14}C-palmitate (sodium salt), 3.0 ml of Tris buffer (0.02 M, pH 7.4) containing 0.125 M KCl, 75 mg ATP and 2.5 mg CoA, and 3.0 ml of 20% liver homogenate in the same Tris buffer. Incubation was at 37° C for 45-60 minutes. The labeled lipids were extracted and separated as described in General Methods. The bands were stained with Ponceau red (0.05% and 0.2% uranyl nitrate in 0.01 N HCl). The phospholipids were immediately removed from the plates, extracted and stored under N_2 at -20° C.

1-^{14}C-palmitoyl PG was prepared by transphosphatidylation of 1-^{14}C-palmitoyl PC using phospholipase D from cabbage according to the method of Yang, et al.(122). The labeled substrate was incubated with 0.5 ml of ethyl ether, 0.2 ml of 10% glycerol, 0.1 ml of 0.4 M $CaCl_2$ and 0.3 ml of phospholipase D (3.5 units/ml in 0.4 M acetate buffer,

pH 5.6) for 30 minutes at room temperature. After the system was inactivated with 0.1 ml of 1 N HCl, the lipids were extracted with ethyl ether-95% ethanol (4:1, v/v) and separated on silica gel G plates in chloroform-methanol-water (65:25:4, v/v/v). As PG does not stain with Ponceau red, the bands were located by scanning. Labeled PG was eluted from the silica gel and stored under N_2 at -20° C.

^{32}P-PG and -CL were isolated from E. coli cells which were grown in nutrient broth containing 1 mc of ^{32}P for 6 hours. The extracted lipids were separated on "ammonia plates" as described in General Methods. The purity of the CL band was checked by rechromatographing an aliquot on 0.5 mm silica gel G plates developed in chloroform-methanol-ammonia-water, (70:30:4:2, v/v/v/v), a system in which PA separates from CL. Only one radioactive band corresponding to a CL standard was found.

A further check of the purity of both PG and CL was made by subjecting a fraction of each to mild alkaline hydrolysis and chromatographing the water soluble products on cellulose plates and on paper as given in General Methods. Only single components corresponding to GPG and GPGPG for PG and CL, respectively, were found.

The crude enzyme for assaying phospholipase A activity was prepared from E. coli 015 cells cultured in nutrient broth for 7 hours and harvested as described in General Methods. Cells from a 1 liter culture were suspended in 50 ml of Tris buffer (0.1 M, pH 8.4). Protein concentrations were 20-30 mg/ml. The suspension was sonicated intermittently for 30 minutes at 125 W using a Biosonik II ultra-

sonicator to disrupt the cells.

One ml of the enzyme preparation was mixed with 10 ml of Tris buffer (0.1 M, pH 8.4) containing 0.1 mM $CaCl_2$ and 100 mg of sodium deoxycholate. Two ml of this preparation were added to the substrate dissolved in 0.1 ml of ethyl ether. The mixture was incubated at 37° C and the reaction stopped at various times by the addition of 2.0 ml of chloroform and 5.0 ml of methanol.

The lipids were extracted and separated on silica gel G or "ammonia" plates. The bands were detected by scanning and by I_2 vapor visualization. In some instances the lipids were eluted from the silica gel and subjected to mild alkaline hydrolysis. The water-soluble products were separated on cellulose plates or on paper.

Lysophospholipase and lysophospholipid acylating enzyme activity were tested simultaneously by a modification of the method described by Proulx and Van Deenen (74). E. coli cells from a 7 hour culture were suspended in 0.1 M phosphate buffer (pH 6.0) and sonicated for 5 minutes. The pH was readjusted to 6.0. One ml of the total sonicate and 1.0 ml of phosphate buffer (0.1 M, pH 6.0) containing 3 mg CoA and 75 mg ATP/5 ml were incubated with ^{32}P-lyso-PE (prepared with snake venom phospholipase A_2 as described previously in this section) at 37° C for 3 hours. The reaction was stopped by adding 14 ml of methanol-chloroform (2.5:1, v/v) and the lipids were extracted and separated as previously described.

Results

The turnover of the ^{32}P- and of the ^{14}C- components of total lipids of individual phospholipids of E. coli 015 during exponential growth at 37° C are shown in figures 2 and 3. As less than 4% of the total label was found in the water-soluble products after mild alkaline hydrolysis of the phospholipids, the terms ^{14}C-labeled material and acyl moieties will be used interchangeably in the discussion.

Total lipids remained fairly constant throughout the time of incubation (figure 2). PE increased about 20% in 90 minutes then remained constant. The response of PE was essentially the same as originally found by Kanfer and Kennedy (12). They postulated that the initial increase in labeling of PE during growth of labeled cells in nonradioactive medium was due to synthesis of PE from an intracellular pool of labeled precursors.

Both ^{32}P- and ^{14}C-labeled PG and CL decreased during the three hour incubation in non-radioactive medium (figure 3). The turnover of the phosphorus moiety of these polyglycerophosphatides in E. coli 015 is much the same as has been shown for other strains of E. coli (12,13). The turnover of the acyl moieties of the phosphatides of E. coli at 37° C has not been previously reported.

The pattern of turnover of the two labels indicates a parallel turnover of the acyl and phosphate moieties of PG and CL. Nevertheless, there could still be a selective turnover of one of the two acyl groups. In order to check this possibility, aliquots of PE, PG and CL were subjected to mild alkaline hydrolysis and hydrolysis with snake venom

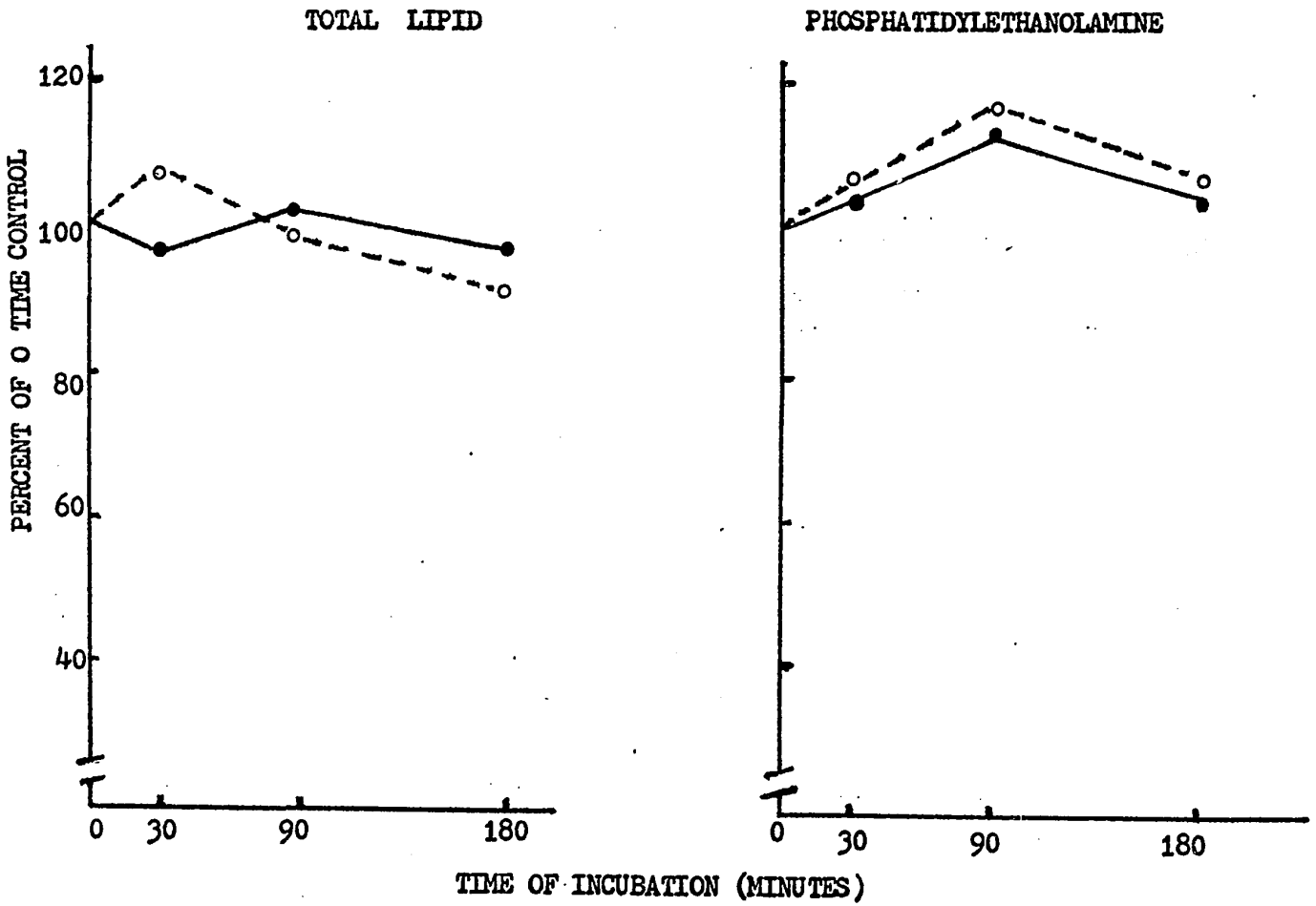


Figure 2: Turnover of ^{32}P (o---o) and ^{14}C (●—●) moieties of the total lipids and of phosphatidylethanolamine of prelabeled Escherichia coli 015 cells during a three hour incubation in nonradioactive medium at 37° C.

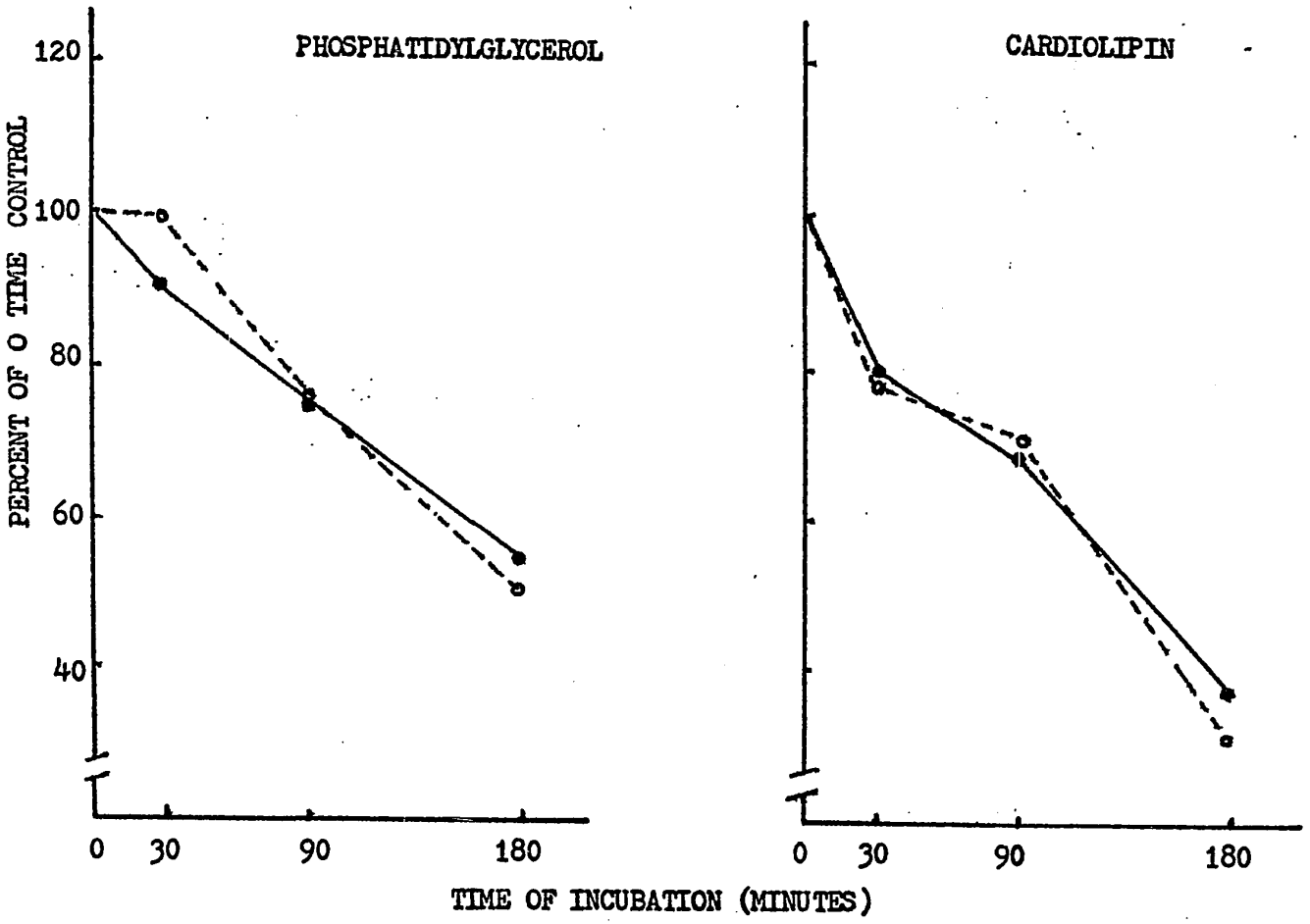


Figure 3: Turnover of ^{32}P (o--o) and ^{14}C(•—•) moieties of phosphatidylglycerol and cardiolipin of prelabeled Escherichia coli 015 during a three hour incubation in nonradioactive medium at 37° C.

phospholipase A_2 and the total fatty acids liberated in each case were isolated and counted. The results shown in figure 4 indicate that the percent of the ^{14}C-label in the 1 and in the 2 positions of each phospholipid did not change greatly with time. Thus the two acyl groups appear to turnover at the same rate.

No significant amounts of labeled neutral lipids could be detected during the incubation period (table V). The total water-soluble materials recovered during the incubation varied greatly. The components readily identified by paper and cellulose thin layer chromatography were ^{14}C-α-glycerol phosphate and ^{32}P-inorganic phosphate.

On one occasion, an attempt was made to account for all counts in the culture. The total ^{32}P counts increased in the medium at the expense of water-soluble material in the cell. Less than 2% of the radioactivity in the medium was lipid-soluble. It was apparent that the turnover of the water extractable material of cells pulse labeled with ^{32}P did not correspond precicely with that of the lipid. Rather, there was a considerable loss of ^{32}P to the medium, indicating a large degree of exchange with cold inorganic phosphate during the chase.

In cells labeled with ^{14}C-acetate and then allowed to equilibrate for 15 minutes with cold acetate in the medium, a different turnover pattern was observed. Changes in the water-soluble counts of the cells were small and were the inverse of those of the lipid fraction. There was no apparent loss of label to the medium which showed a constant radioactivity throughout the chase. It would appear from these results that the products of PG and CL turnover are reincorporated into PE, which

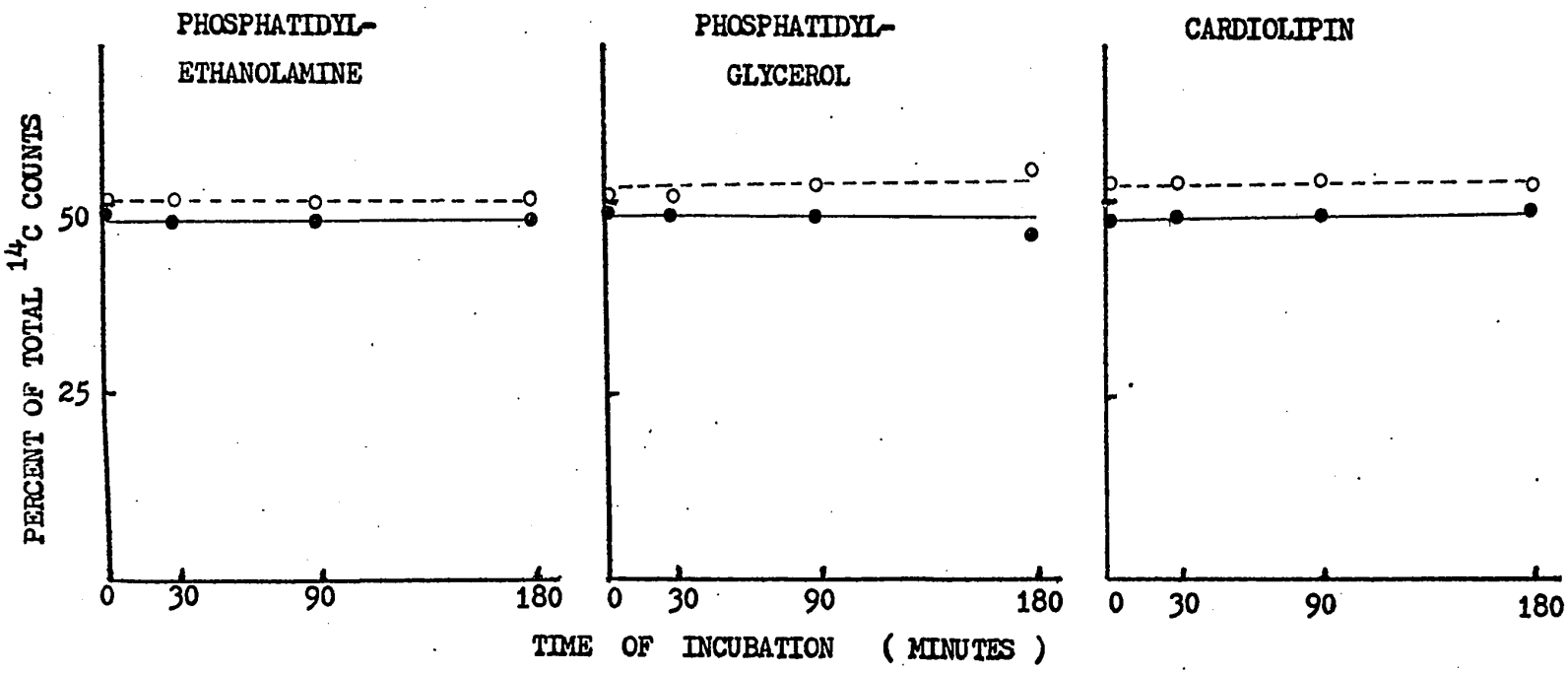


Figure 4: Distribution of the ^{14}C-label between the 1-acyl (o---o) and the 2-acyl (•—•) groups of the phospholipids of Escherichia coli 015 cells during a three hour incubation in nonradioactive medium.

Table V: ^{14}C-labeled total neutral lipids and fatty acids of *Escherichia coli* 015 labeled with ^{14}C-acetate and incubated at 37° C for three hours in high acetate non-radioactive medium.

Time of Incubation	Total Neutral Lipids	Fatty Acids
minutes	% of total lipids	
0	1.7	0.22
30	1.1	0.23
90	1.4	0.43
180	0.7	0.23

showed a net increase during the chase.

Figure 5 illustrates the results of incubating 1-(1-^{14}C-palmitoyl)-PG with sonicated E. coli cells. Approximately 50% of the PG was hydrolyzed in 120 minutes with the release of ^{14}C-fatty acids. Very little lyso-PG was found.

A further experiment using ^{32}P-labeled substrates (figure 6) confirms the presence of phospholipase A activity as opposed to a non-specific lipase activity. ^{32}P-lyso-PG was recovered when ^{32}P-PG was incubated with E. coli sonicate. The large proportion of counts recovered in the water-soluble fraction indicates that the lysophospholipase had not been completely inactivated in this incubation.

^{32}P-CL was also rapidly hydrolyzed by a sonicated cell preparation (figure 6) and both labeled chloroform- and water-soluble products were recovered. The chloroform-soluble material was chromatographed on silica gel G plates in chloroform-methanol-water (65:25:4, v/v/v) and the plates were scanned. The five bands found (figure 7) are probably lyso derivatives and are under further investigation in our laboratory.

The incubation of ^{32}P-lyso-PE with sonicated E. coli cells resulted in the formation of labeled PE and water-soluble products (table VI). The water-soluble material was identified as GPE by paper chromatography in phenol-water (5:1, v/v) (Rf 0.63). The formation of PE was most likely due to reacylation of lyso-PE since PE was the only labeled diacyl-phosphoglyceride formed. Analogous experiments performed by Mr. Guy Nantel with E. coli 015 and other strains showed that ^{14}C-lyso-PC was also converted to the labeled diacyl analogue.

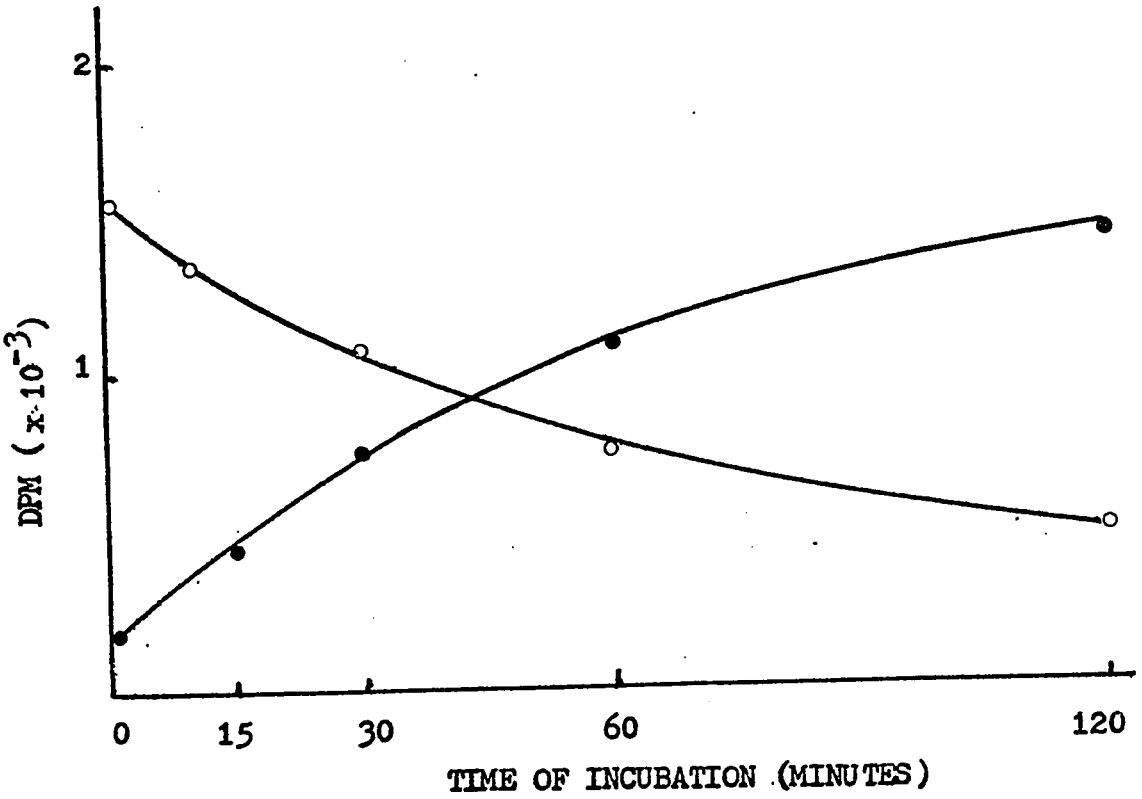


Figure 5: Hydrolysis of 1-(1-^{14}C-palmitoyl-phosphatidyl-glycerol by sonicated Escherichia coli 015 cells. (o) phosphatidylglycerol, (•) free fatty acids.

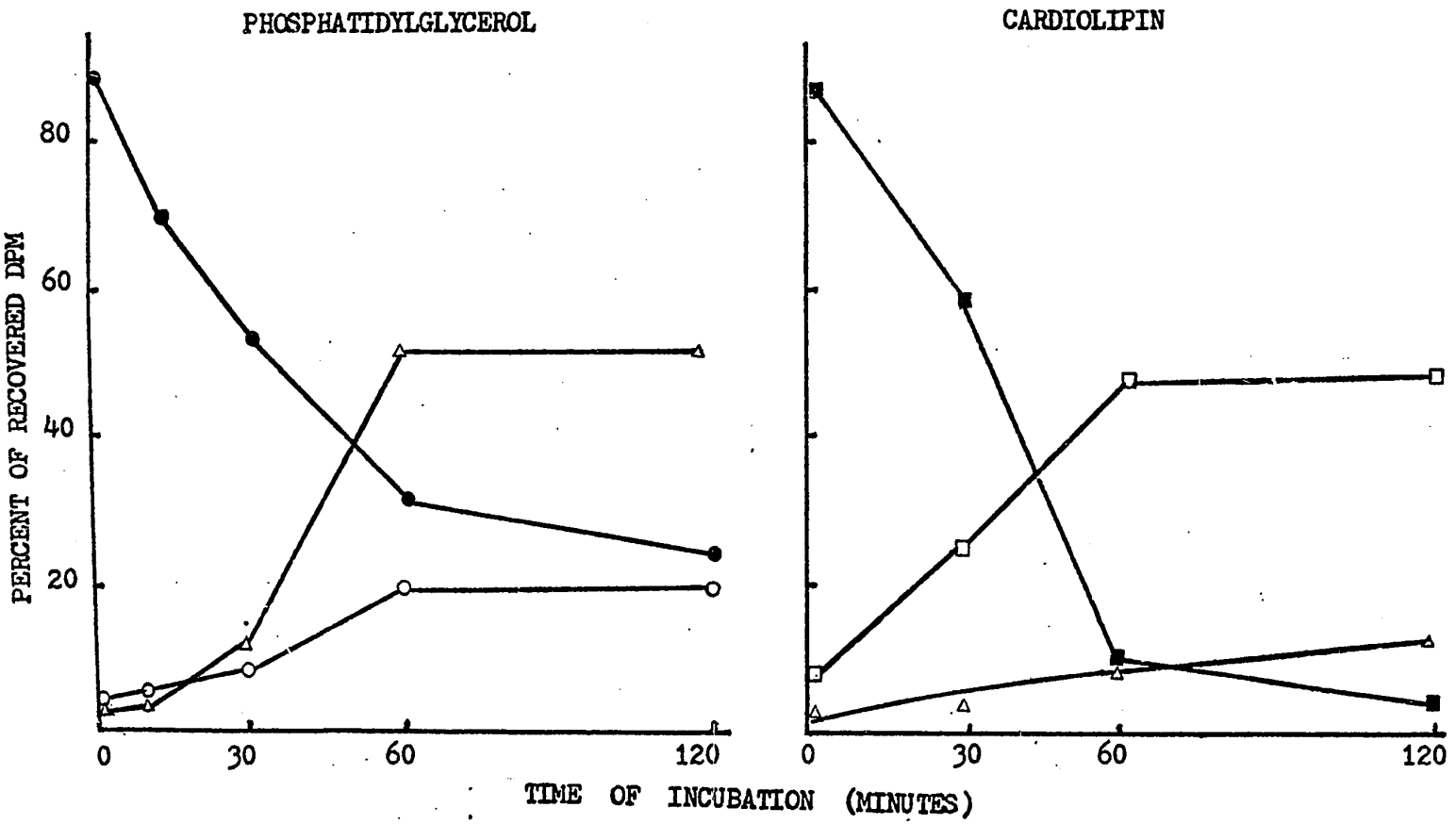


Figure 6: Hydrolysis of ^{32}P-phosphatidylglycerol and ^{32}P-cardiolipin by sonicated Escherichia coli 015 cells. (●) phosphatidylglycerol, (○) lysophosphatidylglycerol, (■) cardiolipin, (△) water-soluble products, and (□) total chloroform-soluble products of cardiolipin hydrolysis.

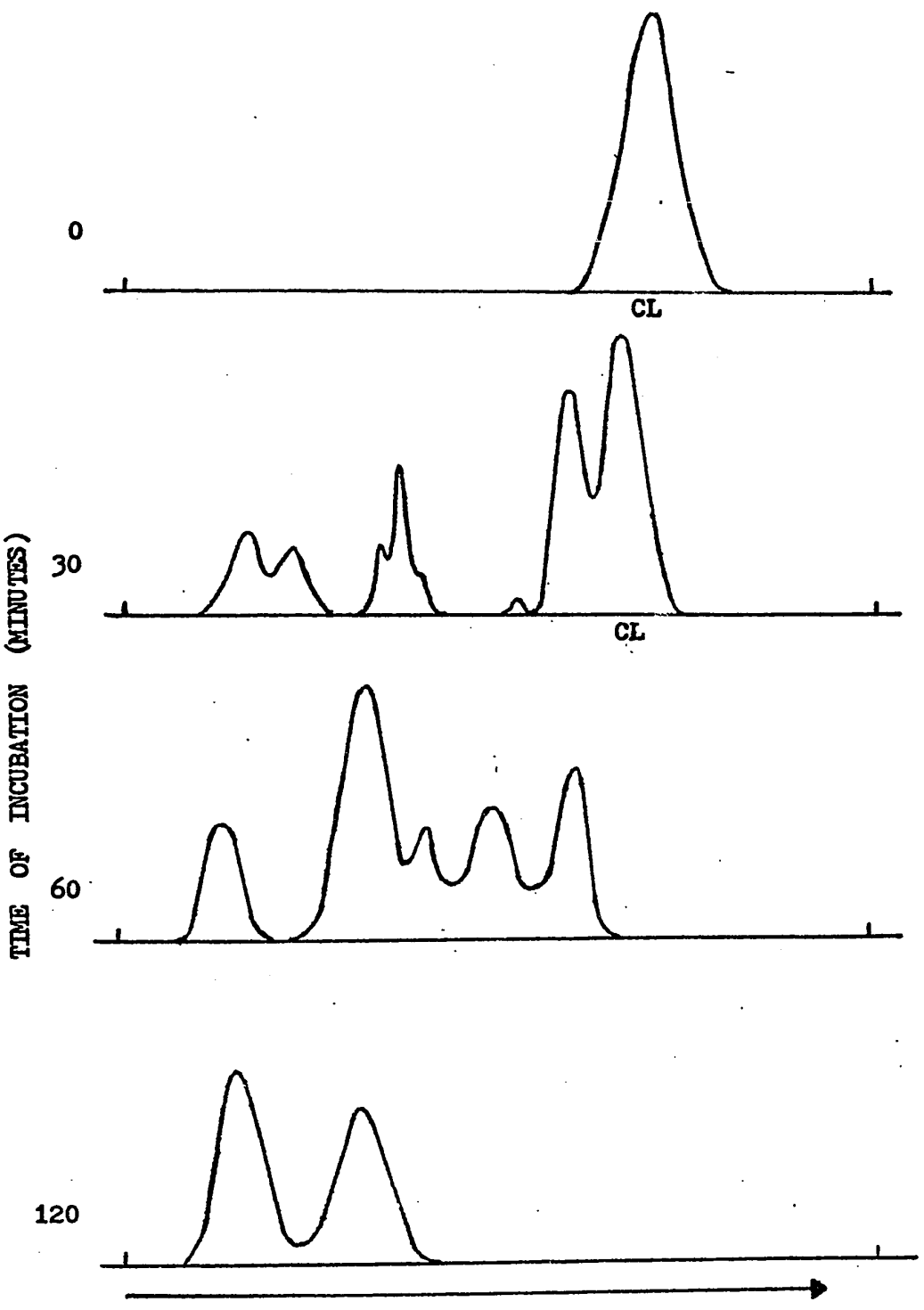


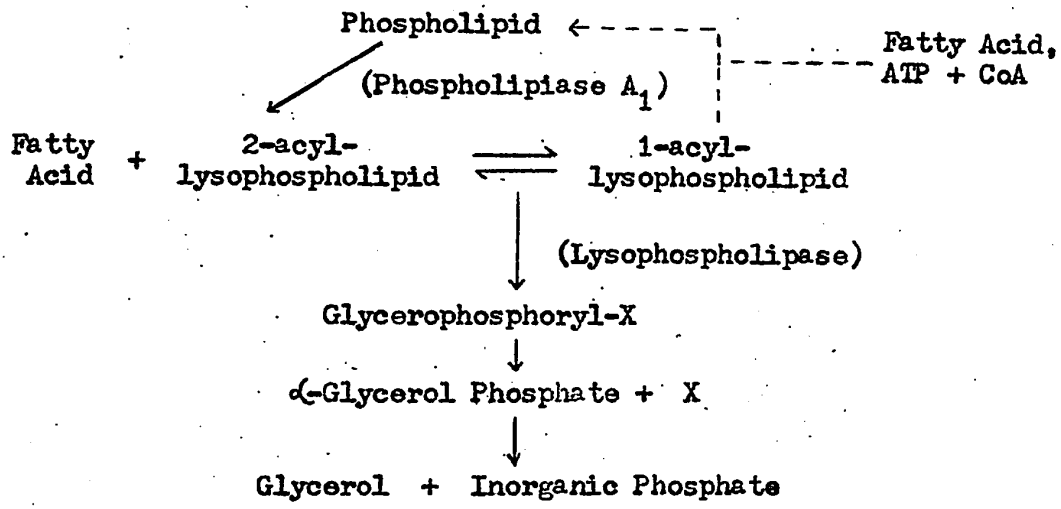
Figure 7: Chloroform-soluble products formed by the treatment of ^{32}P-cardiolipin with sonicated Escherichia coli 015 cells, separated on 0.5 mm silica gel G plates in chloroform-methanol-water (65:25:4, v/v/v).

Table VI: ^{32}P-labeled products resulting from the incubation of ^{32}P-lysophosphatidylethanolamine with sonicated Escherichia coli 015 cells.

	LPE	PE	PG	CL	Water-Solubles
	percent of recovered dpm				
Control	99				1
Sample 1	38	16	0.5	0.1	45
2	38	16	0.6	0.3	45

Dicussion

The in vitro experiments reveal the presence of active phospholipase A and lysophospholipase in E. coli 015, which could participate in the metabolism of phosphoglycerides as outlined in the following scheme:



Evidence from this and other studies (63) indicates that the acyl moiety in position 1 of the phospholipid is the first to be removed. Phospholipase A_2-like activity is not detectable in high amounts and could be accounted for by the combined action of several enzymes in the crude homogenate. A detailed study by Bernard (67) has shown, however, the presence of a distinct cytosol phospholipase A_2 in E. coli which is inhibited by high detergent concentrations but activated by ethanol. Whether or not lysophospholipase has positional specificity is unimportant since acyl migration between the 1- and 2-positions of lysophospholipids in spontaneous.

E. coli 015 contains a lysophospholipid acylating enzyme. This

would constitute a route for partial recycling of lysophospholipids as proposed by Lands (110) and indicated in the scheme by the dashed lines. However, results with intact cells indicate that the turnover of the acyl and phosphorus moieties of PG and of CL are parallel since ^{32}P and ^{14}C counts of doubly labeled lipids decrease at the same rate. Also we found that the ratio of labeling of acyl moieties in the 1- and 2-positions of all the phosphoglycerides studied remained constant over the three hour chase period.

The conversion of lyso-PE to water-soluble product(s) *in vitro* is about three times that of its reacylation to PE. The data of Proulx and Van Deenen (74) also showed that hydrolysis of lyso-PE was greater than its reacylation. It has been found in the microsomes of house fly larvae that the apparent Km of lysophospholipid transacylase was 20 times greater than that of the lysophospholipase (123). It was concluded that at low concentrations of lyso-compounds found *in vivo*, deacylation would be the preferred route. The lysophospholipid acylating enzyme could possibly function as a lysophosphatidic acid acylating enzyme in the *de novo* synthesis of phospholipids. However, no studies of a purified enzyme have yet determined its specificity.

The turnover of PG and of CL does not necessarily imply that phospholipase A degradation is involved. Turnover of PG could be accounted for entirely on the basis of its conversion to CL, although *in vitro* experiments do show that PG is a suitable substrate for phospholipase A. The ultimate catabolic drain would involve the breakdown of CL of which so little is known at present. There is

mounting evidence that phospholipase A_1 hydrolysis of endogenous phospholipids is triggered by adverse conditions, but that the enzyme is otherwise inactive (64,65).

II. *IN VIVO* METABOLISM OF PHOSPHOLIPIDS OF *ESCHERICHIA COLI* 015 DURING A COLD-INDUCED LAG PERIOD.

Introduction

The metabolism of bacterial lipids is known to be responsive to the environmental temperature (21). The fact that PE is not actively catabolized at 37° C is well confirmed (12,13). However, Okuyama (8) found that during the lag phase induced by transferring *E. coli* cells from 37° to 10° C, the ^{32}P-label of PE, as well as that of PG and CL, decreased over a five hour period.

In the previous section, we have shown that the phosphorus and fatty acyl moieties of PG and CL turnover at the same rate at 37° C. At 10° C, however, the ^{14}C-labeling of phospholipids showed no decline (8); it was suggested that the breakdown of phospholipids is complete but that an efficient reutilization of the fatty acids accounted for their apparent stability.

The environmental temperature is also known to affect the fatty acid composition of the lipids of bacteria (49,124), poikilotherms (125) and plants (126). In general, cells of microorganisms grown at low temperatures contain relatively more unsaturated fatty acids (21). Lowering the temperature of exponentially growing cells also results in an increase in the percent of unsaturated fatty acids (8,19). There are two possible ways in which these changes might occur: 1) existing phospholipids are altered either by desaturation of the fatty acid constituents

in situ or by a deacylation-reacylation cycle involving selectively, newly synthesized unsaturated fatty acids or 2) newly synthesized phospholipids are richer in unsaturated fatty acids than are existing ones. In the latter case, control could be exercised at the point of fatty acid synthesis or of acylation of α-glycerol phosphate and lysophosphatidic acid.

The synthesis of unsaturated fatty acids in *E. coli* does not require molecular oxygen (21,85,86); the desaturation of fatty acids has not been demonstrated in *E. coli* and most likely does not occur (21). Studies reported in the previous section of the present work indicated that a deacylation-reacylation cycle cannot account for the turnover of acyl groups in phospholipids. Results, however, were from cells grown at 37° C and may not necessarily represent the situation at lower temperatures. Temperature dependence has been demonstrated in certain strains of *Bacillus* which were found to desaturate palmitic acid to palmitoleic at 20° C but not at 30° C (126). Poikilotherms also demonstrate this particular temperature-dependent characteristic (125).

In the following set of experiments, the *in vivo* metabolism of the phospholipids of *E. coli* 015 during a cold-induced lag phase was studied. We also investigated mechanisms by which the degree of unsaturation of fatty acids of phospholipids increases during this time using a procedure which allowed a differentiation between those phospholipids synthesized prior to and those synthesized during the three hour incubation.

Methods

E. coli 015 cells were grown, labeled and harvested as described in the Experimental Section I. Aliquots of 25 ml each were incubated at 10° for 0, 30, 90, and 180 minutes. The temperature of the cold water bath was maintained at 10° ± 2° C by circulating precooled ethylene glycol through copper tubing in the bottom of the bath. Lipids were extracted, separated and counted as described in General Methods.

For phosphorus determination, bands of lipids on silica gel were transferred directly from TLC plates to micro-Kjeldahl flasks. One ml of 70% $HClO_4$ was added to each flask and these were heated 30 minutes on a micro-Kjeldahl digestion apparatus. After the flasks had cooled for 15 minutes, 6.0 ml of water was added, the contents were thoroughly shaken, transferred to centrifuge tubes and centrifuged for 15 minutes to remove the silica gel. Five ml of the supernatant was taken for the determination of inorganic phosphate by a modification of the Fiske and Subbarow procedure (128) as described below.

With each set of assays, several 1.0 ml aliquots of $HClO_4$ were placed in hydrolysis flasks and heated in the same manner as were the samples. After the flasks had cooled, 6.0 ml of distilled water was added, the contents were mixed and 5.0 ml aliquots were placed in tubes. One drop of phenolphthalein was added to three of the blank tubes, and 12 N NaOH was added dropwise from a 1.0 ml pipet until a pink color developed. A volume of 12 N NaOH equal to the average of the volumes used for the three blank tubes was added to other blank and sample tubes. Four of the blank tubes were used to prepare standards containing 5,

10, 15 and 20 µg of inorganic phosphate. One-half ml of 5% ammonium molybdate in 10 N H_2SO_4 was thoroughly mixed with a blank, standards and samples and 0.4 ml of 2.5% aminonaphthosulfonic acid in 15% $NaHSO_3$-20% Na_2SO_3 (195:1, v/v) was added to each tube. The contents were mixed and allowed to stand exactly 10 minutes. The color was read at 600 mµ using a Coleman Junior Spectrophotometer.

In a second series of experiments, E. coli were grown in 350 ml of minimal medium containing 250 µc of 2-^{3}H-acetate. The cells were harvested and resuspended in fresh medium containing 6.1 g/l of cold acetate and 125 µc of 1-^{14}C-acetate. Twenty-five ml aliquots were incubated at either 10° or 37° C for up to three hours. The phospholipids were extracted, separated and recovered as previously described.

Aliquots of PG and PE were treated with phospholipase C prepared from Bacillus cerius grown for 18 hours at 37° C in a medium containing (in g/l): bactopeptone, 10; yeast extract, 10; NaCl, 5; and NaH_2PO_4, 0.4. (The B. cerius culture was obtained from the Department of Microbiology of the University of Ottawa.) The procedure, according to Chu (129), was as follows: 10 g of $(NH_4)_2SO_4$ was added to 100 ml of the culture medium remaining after the cells had been harvested and the mixture was allowed to stand at -20° C overnight. The precipitate was collected by centrifugation, dissolved in 2.0 ml of Tris buffer (0.1 M, pH 7.4) and stored at -20° C. The crude enzyme preparation was used without further treatment.

The phospholipase C hydrolysis mixture contained 5.0 ml of ethyl ether, 3.0 ml of Tris buffer (0.1 M, pH 7.0), 0.4 ml of 0.05 M $CaCl_2$

and 0.6 ml of phospholipase C preparation. For the hydrolysis of PE approximately 1 mg of egg lecithin was also included. Quantities of phospholipids used were such that hydrolysis at room temperature was complete in two hours. The diglycerides formed were extracted with two 5.0 ml volumes of ethyl ether.

Diglycerides were separated into disaturated (including monosaturated-monocyclopropane), monosaturated-monounsaturated and diunsaturated species on silver nitrate plates as described by Van Golde and Van Deenen (130). The plates (about 0.5 mm thick) were prepared with 40 g of silica gel G and 80 ml of 6.75% silver nitrate with a custom-made plexiglass spreader designed by A. Lysionok of the Department of Biochemistry, University of Ottawa.

The plates were activated overnight at 110° C; the solvent system was chloroform-ethanol (98:2, v/v) (130). The plates were sprayed with 0.05% Rhodamine 6G and the diglycerides were visualized under ultraviolet light, or sprayed with 20% ammonium sulfate and charred at 110° C. A typical separation is shown in figure 8. The diglyceride bands were removed from the plates, which had been sprayed with Rhodamine 6G, eluted and counted. The residual pink color does not affect the counting efficiency in the system used.

In some instances, aliquots of the diglycerides were further hydrolyzed with porcine pancreatic lipase using the method of Van Golde and Van Deenen (131) which employs 9 mg of pancreatic lipase, 1 ml of 1 M Tris buffer (pH 8.0), 0.1 ml of 22% $CaCl_2$ and 0.25 ml of 1% sodium deoxycholate. The mixture was warmed 1 minute at 40° C, then shaken

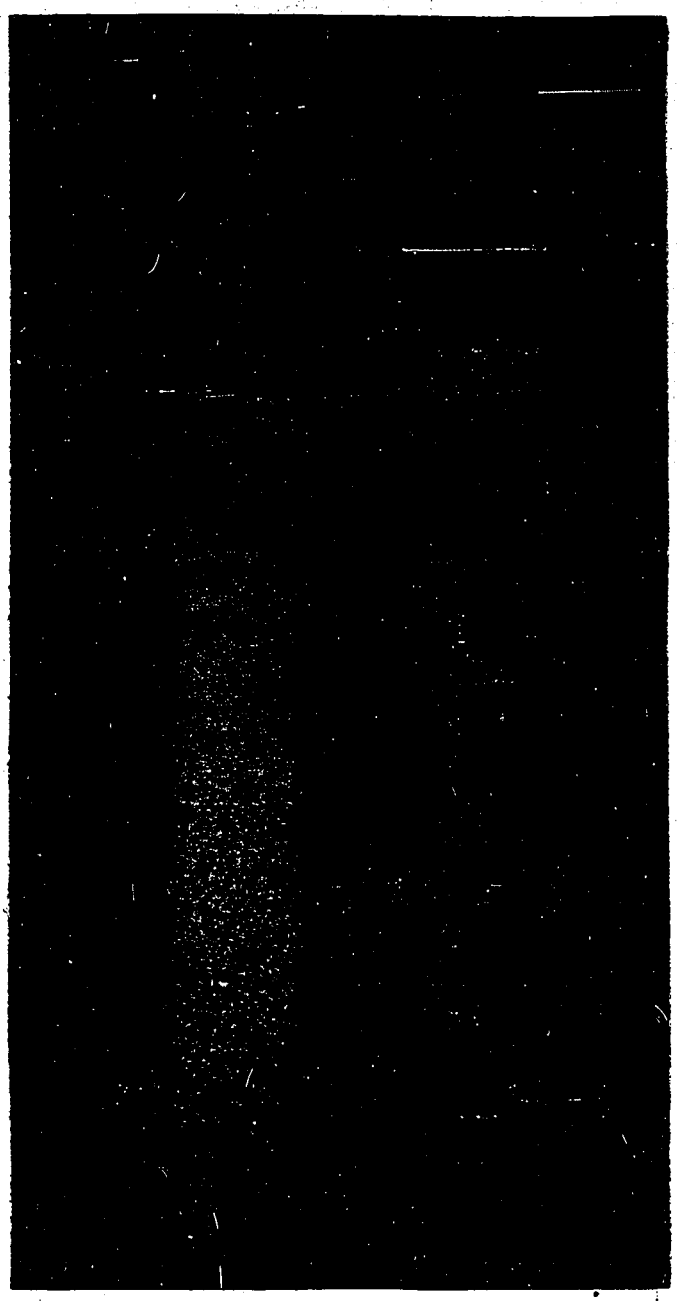


Figure 8: Representative separation of diglycerides (prepared by phospholipase C hydrolysis of phospholipids of Escherichia coli 015) on silver nitrate plates developed in chloroform- ethanol (98:2, v/v) See text for details. Bands were revealed by charring.

vigorously for 5 minutes at that temperature. One-half ml of 6 M HCl was added and the lipids were extracted by the method of Bligh and Dyer (119). The fatty acids and monoglycerides formed were separated on silica gel G plates in petroleum ether (30-60° C)-ethyl ether-formic acid (55:45:1.5, v/v/v), eluted and counted.

Methyl esters of the fatty acids of total lipids were prepared as described by Kates (132). Lipid aliquots were dried under nitrogen in 50 ml Erlenmeyer flasks with sealed on 5 ml side tubes and refluxed for one hour with 4.5 ml of 2.5% methanolic HCl. After the flasks were cooled, 0.5 ml of distilled water was added. The lipids were extracted three times with petroleum ether (40-60° C) and dried under nitrogen.

The methyl esters were dissolved in a small portion of chloroform for analysis using a Fractoevap Model GT, Carlo Erba (division of Aparachi Scientific)gas chromatograph. The gasses and flow rates were: nitrogen (carrier gas)-0.6 kg/cm^2; hydrogen (for flame detector)-9.7 kg/cm^2; and air-1.5 kg/cm^2. The column temperature was 190° C; those of the injection port and of the detector were 270° and 235° C, respectively. The column was 10% butanediol succinate on Chromosorb W AW-DMCS (60-80 mesh).

Results

Results of studies of the turnover of phospholipids are illustrated in figures 9 and 10. Neither the ^{14}C nor ^{32}P label of the total phospholipids nor of PE changed greatly over the three hour incubation period. Neither the phosphorus nor acyl moieties of PG or CL (figure 10) showed the pronounced turnover that was seen at 37° C (see figure 3).

In no case was the percent of neutral lipids (table VII) as great as that reported to Okuyama (8) in E. coli B cells during a cold-induced lag phase. Neither did we have any indication that neutral lipids were converted to phospholipids during this time as he has reported. Rather, there was a slight increase in the neutral lipids, specifically the fatty acid fraction, while the percent of all other fractions remained at a stable and very low level.

Figure 11 shows that cells kept stationary at 10° C readily incorporate ^{32}P and ^{14}C-acetate into all phospholipid fractions, although at a rate slower than at 37° C (figure 12), indicating continued de novo synthesis of lipids at low environmental temperatures. This conclusion is further supported by the fact that total lipid phosphorus of the cells increased during this time (figure 13).

As shown in figure 14, when E. coli cells were grown in a medium containing 2-^{3}H-acetate at 37° C, the level of incorporation of the label into each phosphatide corresponds approximately to the relative amount of each. Because of the low turnover of PG and CL, as

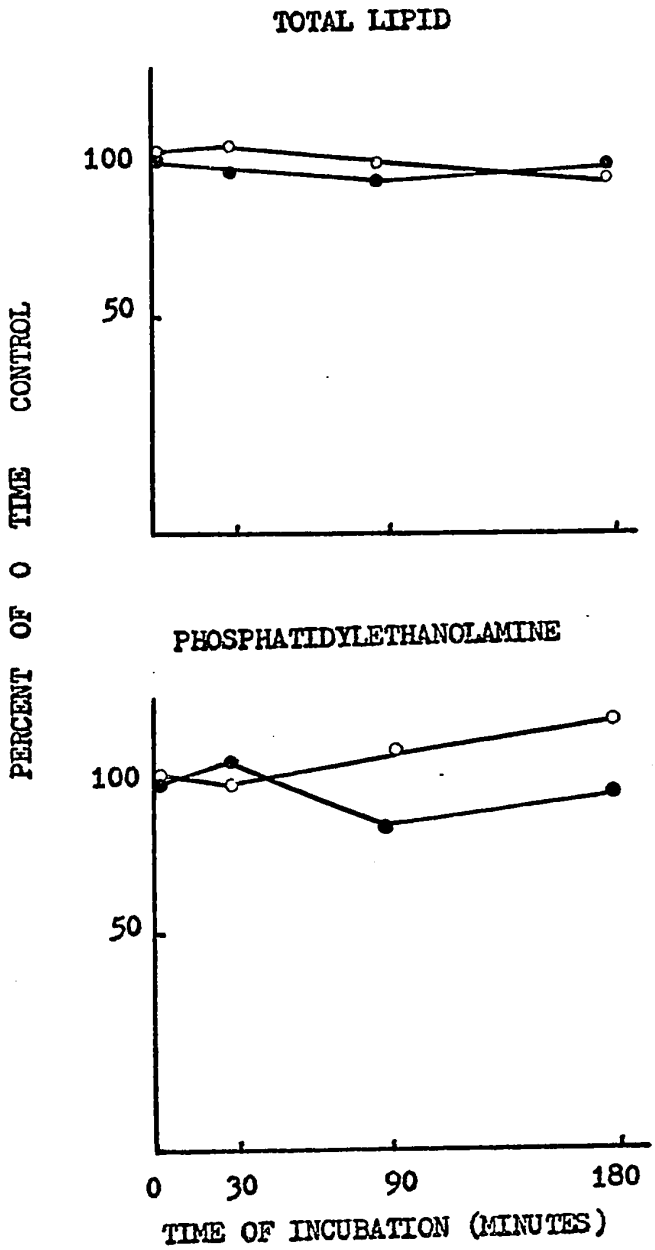


Figure 9: Turnover of ^{32}P (o—o) and of ^{14}C (•—•) moieties of the total lipids and of phosphatidylethanolamine of prelabeled Escherichia coli 015 cells during a three hour incubation in nonradioactive medium at 10° C.

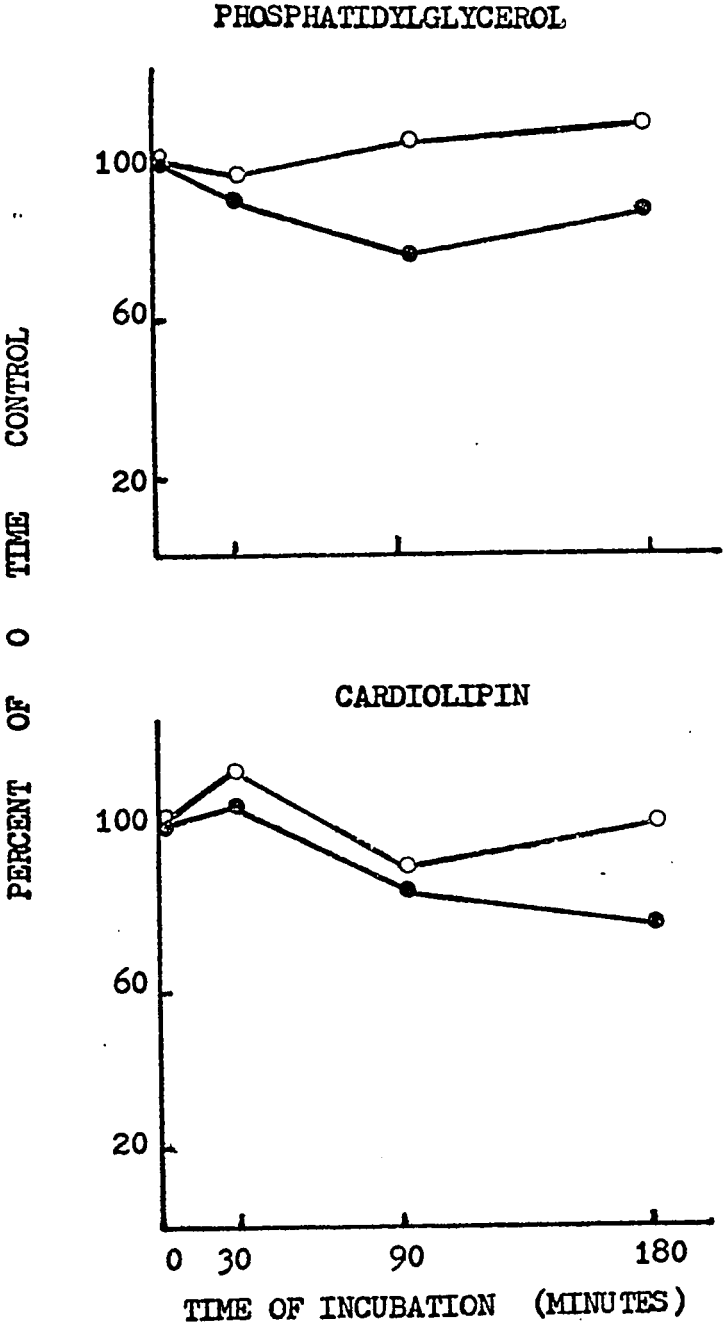


Figure 10: Turnover of ^{32}P (o—o) and of ^{14}C (●—●) moieties of phosphatidylglycerol and cardiolipin of prelabeled Escherichia coli 015 cells during a three hour incubation in nonradioactive medium at 10° C.

Table VII: ^{14}C labeled neutral lipids of Escherichia coli 015 cells which were prelabeled with 1-^{14}C-acetate and incubated for three hours in a high acetate nonradioactive medium at 10° C.

Time of Incubation	TNL[1]	TG	FA	DG	MG
minutes	percent of total dpm				
0	0.67	0.15	0.39	0.06	0.07
30	0.71	0.11	0.50	0.04	0.06
90	0.96	0.09	0.75	0.05	0.07
180	1.11	0.16	0.87	0.04	0.04

[1] TNL: total neutral lipid; TG: triglycerides; FA: fatty acids; DG: diglycerides; MG: monoglycerides.

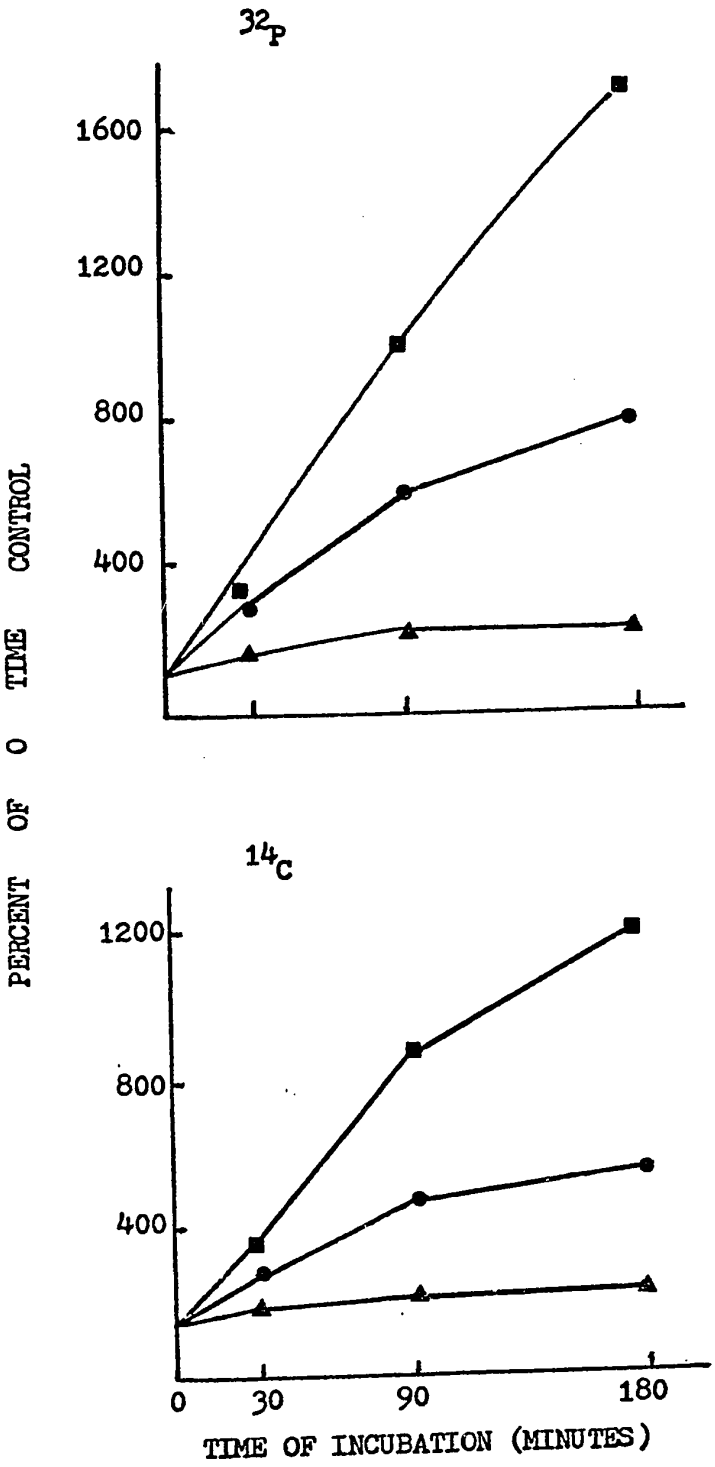


Figure 11: Incorporation of ^{32}P and 1-^{14}C-acetate into phospholipids of Escherichia coli 015 at 10° C. (■)phosphatidylethanolamine, (●) phosphatidylglycerol and (▲) cardiolipin

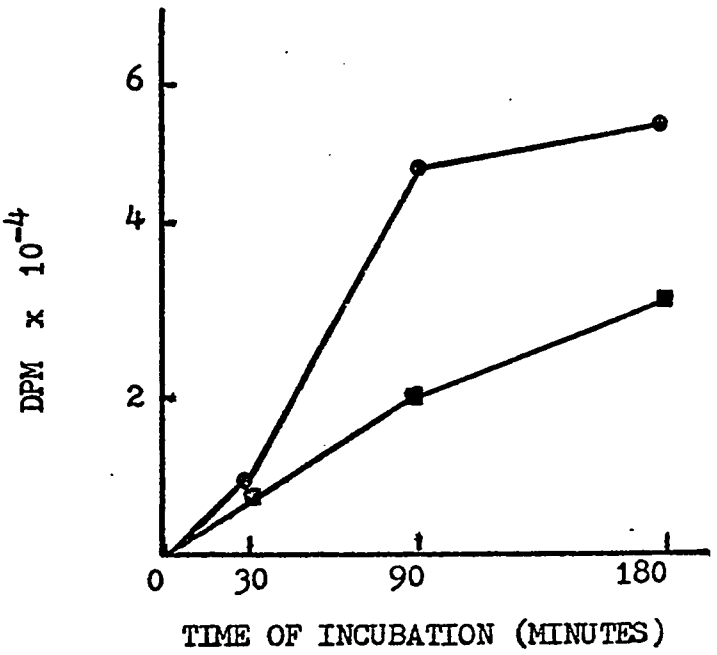


Figure 12: Incorporation of 1-^{14}C-acetate into the total phospholipids of Escherichia coli 015 at 10° (■—■) and 37° C (●—●).

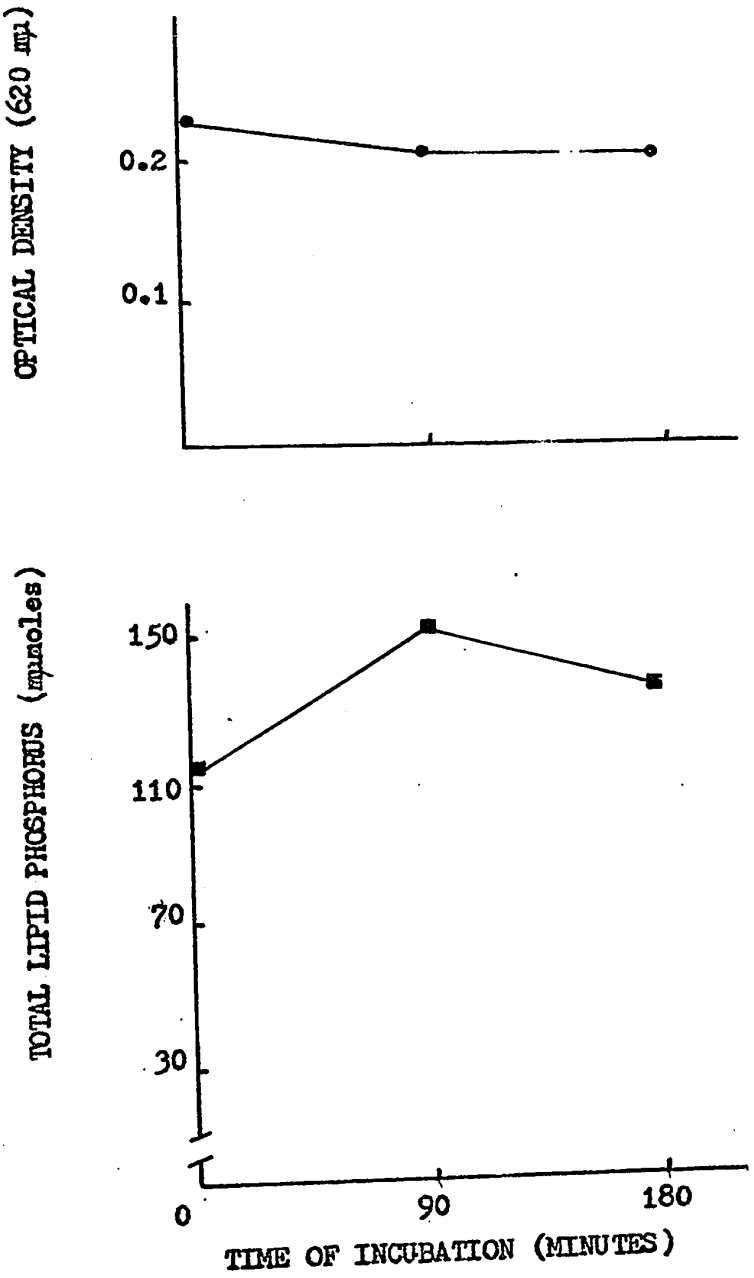


Figure 13: Optical density and total lipid phosphorus of Escherichia coli 015 cells during a a three hour incubation at 10° C.

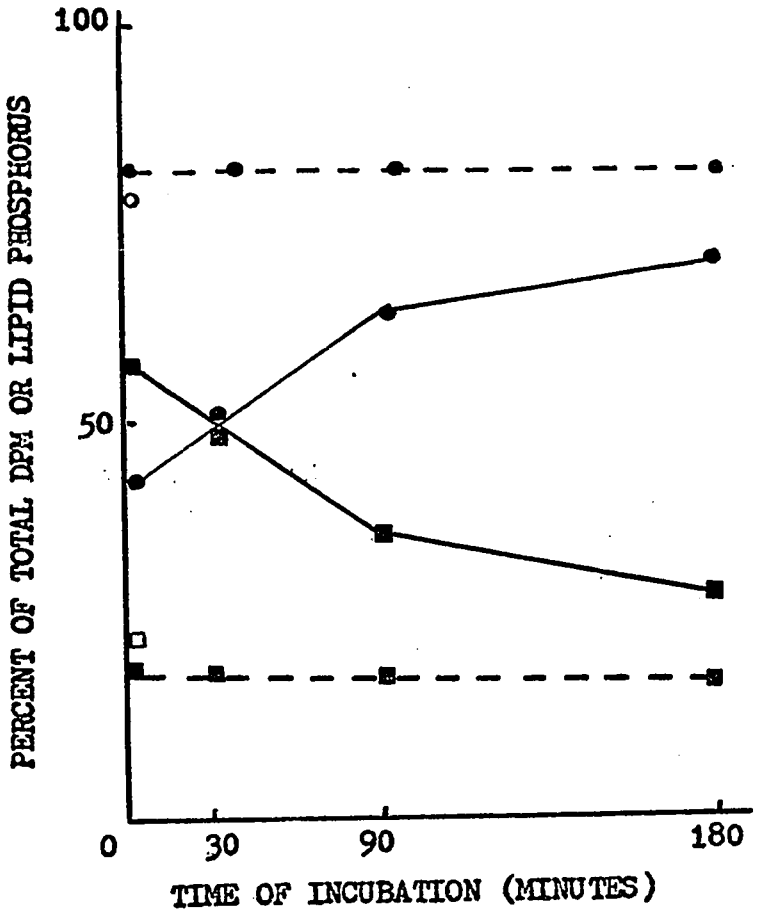


Figure 14: Turnover of ^{3}H-prelabeled phosphatidylethanolamine and the combined phosphatidylglycerol-cardiolipin fraction and the incorporation of ^{14}C-acetate into these phosphoglycerides of Escherichia coli 015 cells during a three hour incubation at 10° C. (●-●) ^{3}H-phosphatidylethanolamine, (●—●) ^{14}C-phosphatidylethanolamine, (■—■) ^{14}C-polyglycerophosphatides and (■-■) ^{3}H-polyglycerophosphatides. ○ and □ indicate the percent of phosphatidylethanolamine and polyglycerophosphatides, respectively, as determined by phosphorus analysis.

well as that of PE, at 10° C, the proportion of the ^{3}H-labeling is maintained during the three hour lag period. However, the rate of incorporation of 1-^{14}C-acetate into the polyglycerophosphatides at the beginning of the 10° C incubation is about the same as the incorporation into PE. Only by 180 minutes does the distribution of the ^{14}C-labeling approach that of the ^{3}H-labeling.

As it is evident that phosphoglyceride catabolism is greatly diminished, while de novo synthesis is still functioning at 10° C, it was decided to investigate the possibility that the phospholipids synthesized during the cold-induced lag phase were qualitatively different from those formed prior to exposure of the cells to the low temperatures. This was accomplished by incubating cells prelabeled with 2-^{3}H-acetate in a medium containing 1-^{14}C-acetate at 10° C. Diglycerides were prepared from PE and PG isolated at various times during the lag phase and the previously formed (^{3}H) and newly formed (^{14}C) diglyceride species were compared. These values were compared to those of a similar study at 37° C. The results are illustrated in figures 15-18.

At 37° C, ^{3}H-labeled monounsaturated (SU) and diunsaturated (UU) species of PE (figure 15) decreased with time. The ^{3}H disaturated (SS) species (which also contained monosaturated-monocyclopropane species) increased after a short lag period. This result could be explained on the basis of a conversion of unsaturated fatty acids to cyclopropane fatty acids. A similar picture was obtained for the PG fraction (figure 16). However, loss of unsaturated species (SU and UU) was not completely accounted for by the gain in SS species. In this fraction

PHOSPHATIDYLETHANOLAMINE

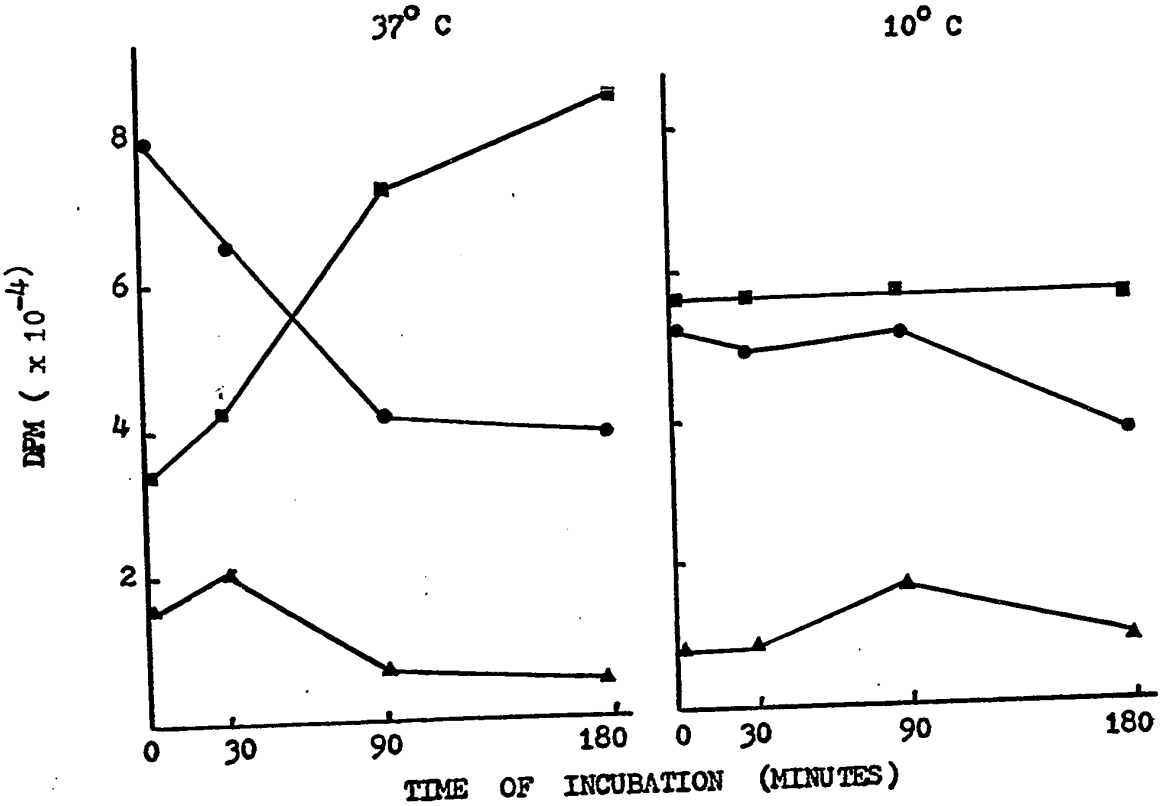


Figure 15: Turnover of ^{3}H in the disaturated (■), monosaturated-monounsaturated (●) and the diunsaturated (▲) species of phosphatidylethanolamine of Escherichia coli 015 during a three hour incubation at 37° and 10° C.

PHOSPHATIDYLGLYCEROL

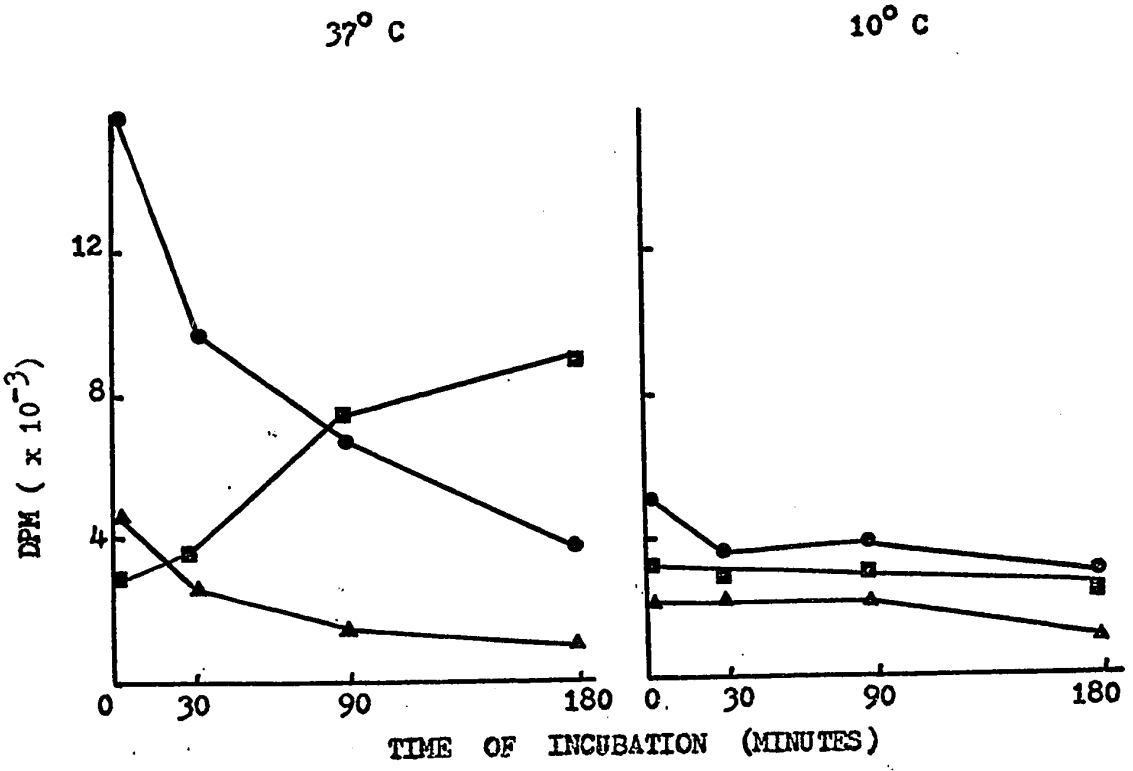


Figure 16: Turnover of ^{3}H in the disaturated (■), monosaturated-monounsaturated (●) and the diunsaturated (▲) species of phosphatidylglycerol of Escherichia coli 015 during a three hour incubation at 37° C or 10° C.

PHOSPHATIDYLETHANOLAMINE

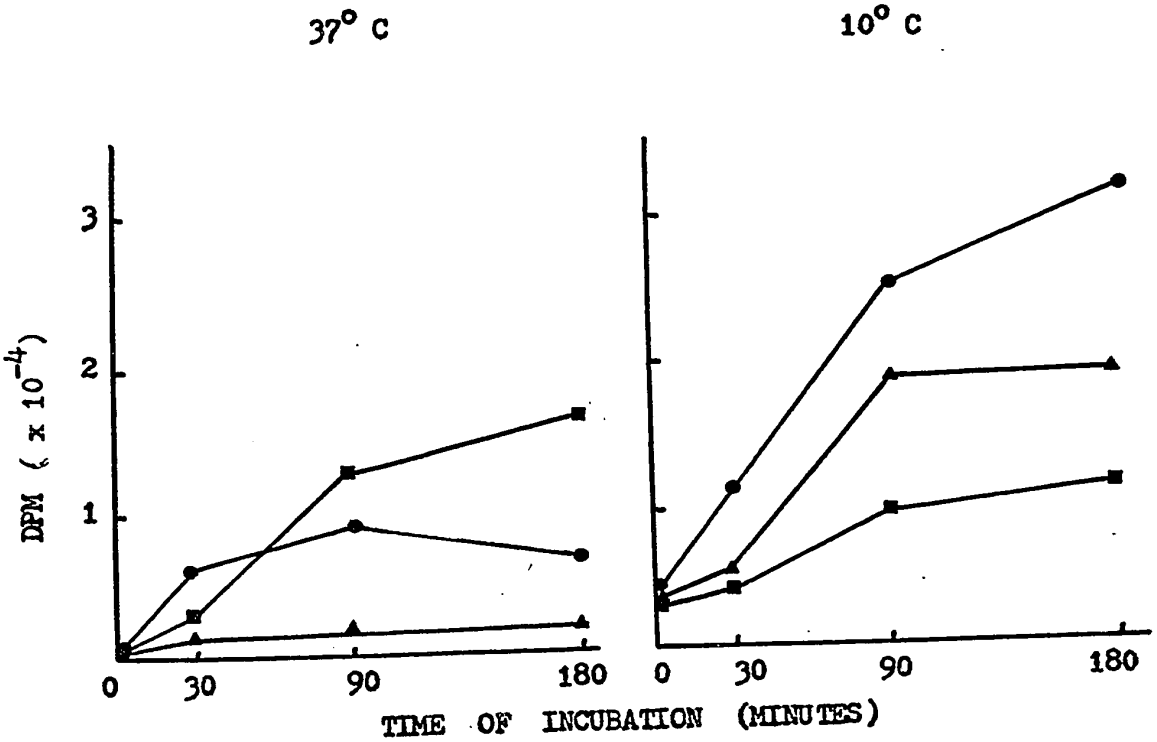


Figure 17: Incorporation of ^{14}C-acetate into the disaturated (■), monosaturated-monounsaturated (●) and diunsaturated (▲) species of phosphatidylethanolamine of Escherichia coli 015 during a three hour incubation at 37° and 10° C.

PHOSPHATIDYLGLYCEROL

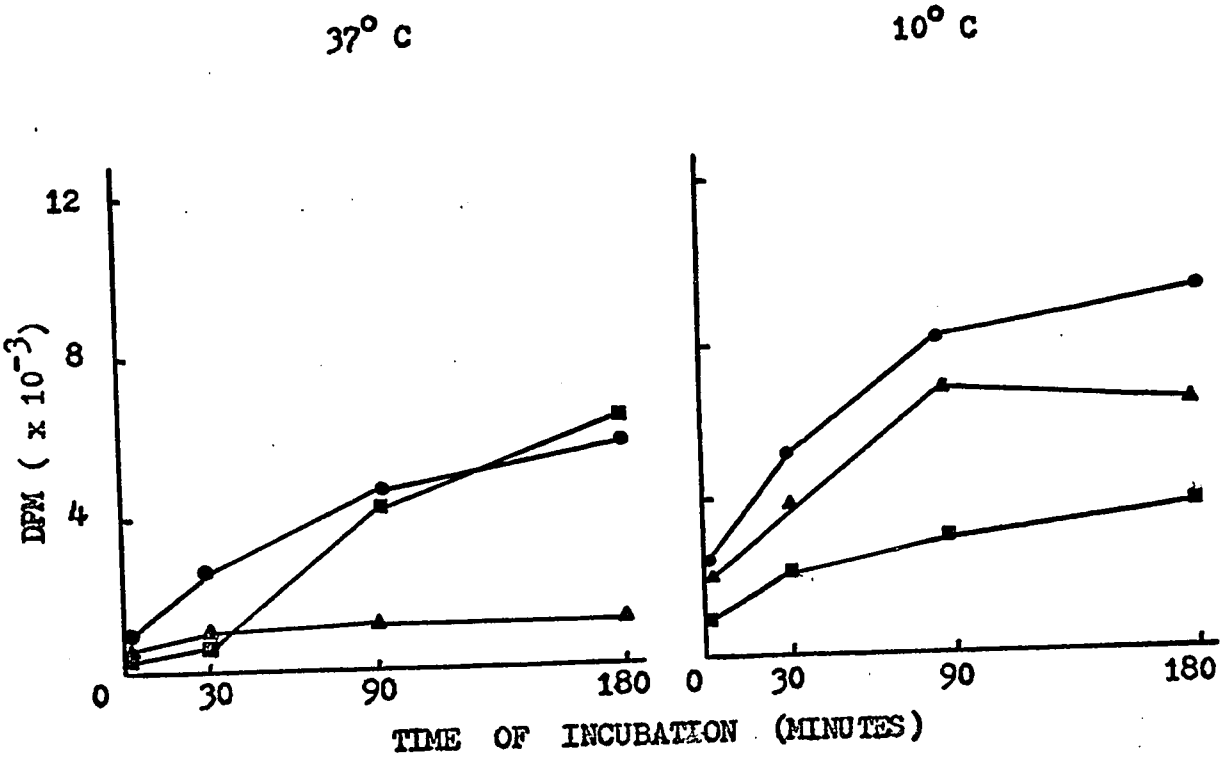


Figure 18: Incorporation of ^{14}C-acetate into the di-saturated (■), monosaturated-monounsaturated (●) and diunsaturated (▲) species of phosphatidylglycerol of Escherichia coli 015 during a three hour incubation at 37° and 10° C.

there was obviously a turnover of SU and UU species which is not related to the conversion of unsaturated fatty acids to their corresponding cyclopropane analogues.

In contrast, at 10° C, the ^{3}H-SS species are fairly constant and ^{3}H-labeled UU and SU components showed only a slight decrease (figures 15 and 16). This is in accord with our previous results indicating very little catabolism of phospholipids under these conditions.

The incorporation of ^{14}C-acetate into the various species of PE and PG was found to be temperature dependent (figures 17 and 18). At 10° C, the ^{14}C-UU fractions represented about 45% of the total phosphoglyceride as compared to 10% at 37° C. The formation of ^{14}C-SS species of PE and of PG was greatly reduced at the lower temperature. The SU species represented about 45% of the total diglycerides at both temperatures.

The difference in the relative amounts of the species of PE and of PG at 10° and 37° C could be explained on the basis of greater synthesis of the unsaturated species at the lower temperature. There might also be a decreased formation of cyclopropane fatty acids containing species from the unsaturated species. To check the latter possibility, the total fatty acid pattern of phospholipids of cells at 10° C was compared with those of cells at 37° C in minimal medium and also in buffered saline in order to eliminate growth as a factor. As can be seen in table VIII, the percentage of methylene hexadecanoic acid of cells at 37° C was 5-6 times that of cells incubated at 10° C. Some methylene octadecanoic acid was also detected in the phospholipids of cells

Table VIII: Fatty acid composition of the phospholipids of Escherichia coli 015 cells incubated for three hours either at 10° or 37° C in minimal medium or at 37° C in buffered saline.

Fatty Acids	Minimal Medium		Buffered Saline
	10° C	37° C	37° C
	percent of the total		
14:0	2.2	5.1	4.4
16:0	40.4	47.1	47.4
16:1	24.0	4.4	10.5
17:▽1	4.1	24.0	21.9
18:0 (?)	3.0	-	-
18:1	26.4	10.3	15.8
?	-	3.2	-
19:▽1	-	5.8	-

growing at 37° C in minimal medium. Thus, the "conservation" of monounsaturated fatty acids due to the low environmental temperature could contribute to the overall increase in unsaturation found under these conditions.

We have previously established that the turnover of PG and CL at 37° C was not selective for either the 1- or 2-acyl groups and that neither position of PE shows any turnover. Although there appears to be little, if any, turnover of phospholipids at 10° C, we sought to verify the absence of a specific turnover of one of the acyl groups at this lower temperature. To do so, PE and PG were isolated from cells grown in medium containing ^{3}H-acetate at 37° C, harvested, then incubated in a medium containing ^{14}C-acetate at 10° C. Diglyceride fractions of PE and PG were prepared, separated and converted to monoglycerides and fatty acids as previously described. If a ^{14}C-labeled fatty acid were replacing a ^{3}H-fatty acid preferentially in one position, the $^{3}H/^{14}C$ ratio of the fatty acids in that position should decrease more rapidly than the ratio of the fatty acids of the other position. As can be seen in figures 19 and 20, the $^{3}H/^{14}C$ ratio of the monoglyceride (i.e., the 2-acyl group) and the fatty acid (i.e., the 1-acyl group) were essentially the same. Therefore, there does not appear to be a selective turnover of one of the acyl groups at 10° C.

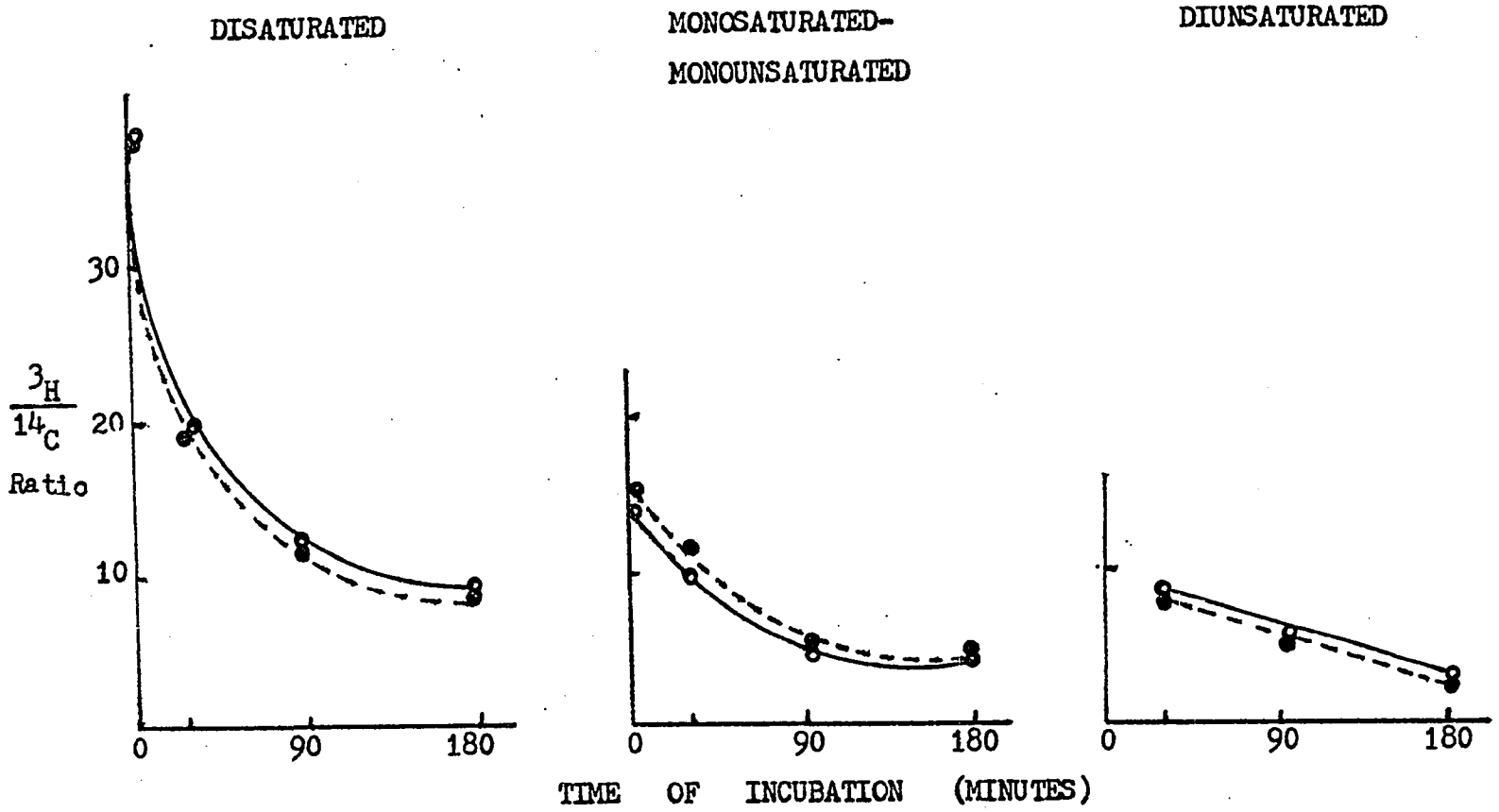


Figure 19: $^3H/^{14}C$ ratio of the fatty acid groups of the 1- (o—o) and the 2- (●---●) positions of species of phosphatidylethanolamine of Escherichia coli 015 which were prelabeled with 2-^{3}H-acetate and subsequently incubated with 1-^{14}C-acetate at 10° C.

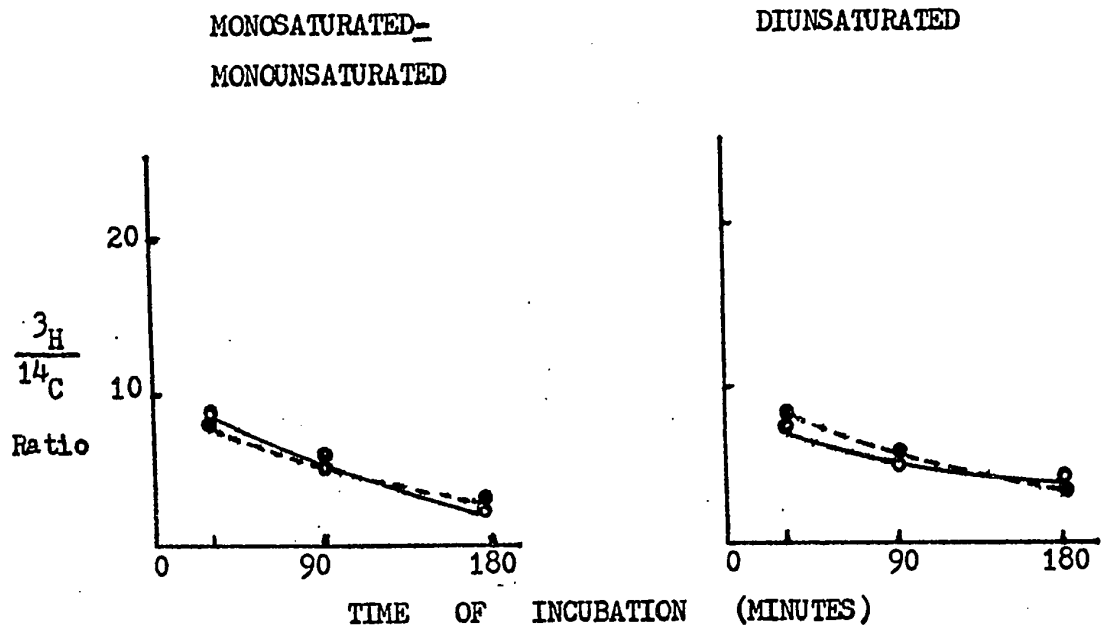


Figure 20: $^3H/^{14}C$ ratio of the fatty acid groups of the 1- (o——o) and the 2- (o--o) positions of species of phosphatidylglycerol of Escherichia coli 015 which were prelabeled with 2-^{3}H-acetate and subsequently incubated with 1-^{14}C-acetate at 10° C. No values are given for the disaturated species as ^{14}C incorporation into this fraction was not sufficient for accurate measurement.

Discussion

All phospholipids of E. coli 015 appear to be relatively stable during a three hour cold-induced lag period. Neither the phosphorus nor acyl moieties of PG or CL showed the pronounced turnover that was seen in cells incubated at 37° C. This contrasts with the report of Okuyama (8), who found that all three major phospholipids of E. coli B turnover during cold incubation. The level of neutral lipids of E. coli 015 was also much lower than in the organism used by Okuyama indicating that the discrepancies between our results may be due to the difference in the strains of E. coli used.

Synthesis, as well as catabolism, of phospholipids was diminished at the lower temperature. However, there was still significant incorporation of ^{32}P and ^{14}C-acetate into all fractions. Initially, ^{14}C-acetate incorporation into PE and into the polyglycerophosphatides proceeded at the same rate; only by 180 minutes does the ratio of ^{14}C-labeled PE and PG-CL approach the usual ratio of these phosphatides found in growing cells.

Complex mechanisms controlling the over-all levels of phosphatide content of the cell would necessarily be linked to the rate of membrane synthesis and consequently to cell growth and division. There would also have to be a form of regulation which affects the relative amounts of each phosphatide produced in order to maintain the PE/PG+CL ratio at about 3. It will be recalled that the synthesis of phosphatides in E. coli involves a branched pathway, the bifurcation occurring after CDP-DG formation. At 37° C there is a continual draining of poly-

glycerophospholipids due to their turnover whereas PE does not turnover appreciably. Thus, at this temperature, the higher proportion of PE could be largely explained on this basis and the idea of regulation at the branch point need not be invoked although it cannot be precluded. At 10° C, however, turnover of polyglycerophosphatides is appreciably decreased. Initially ^{14}C-labeled polyglycerophosphatide formation proceeded at a rate equal to that of PE but then fell such that, after three hours, the proportions of both labeled fractions reached their usual distribution values. This is highly suggestive of a mechanism of control operating on synthesis at the point of bifurcation under certain conditions. Further studies are being made in our laboratory to evaluate this possibility.

There was a greater percentage of unsaturated fatty acids and a lesser percentage of saturated and cyclopropane fatty acids in 10° C cells than in either growing or starving stationary cells at 37° C. Thus the difference in fatty acid composition of the phospholipids is very likely attributable to an effect of temperature rather than of cessation of growth.

The difference in the fatty acid pattern of cells incubated at 10° and at 37° C could be the result of changes in pre-existing phospholipids or of control of newly synthesized ones. An alteration in pre-existing phospholipids which would contribute to the increase in the degree of unsaturation could be accomplished either by a desaturation of existing fatty acids <u>in situ</u> or by a turnover of fatty acyl moieties on the phospholipid molecule.

The presence of a significantly active desaturase system in E. coli is unlikely since unsaturated fatty acid synthesis in this microorganism is anaerobic (21,81), whereas desaturations generally involve mixed function oxidases which require molecular oxygen. Also, if such a system were operative, the level of ^{3}H (i.e., pre-existing) disaturated species of phospholipids would be expected to decrease and that of the ^{3}H-monounsaturated-monosaturated and -diunsaturated species to increase. It was found, however, that the unsaturated species decreased rather than increased and that pre-existing saturated species remained unchanged during the three hour incubation at 10° C. The finding of Okuyama (8) that the absolute amount of cis-vaccenate in phospholipids increased during a cold-induced lag phase without a concomittant change in the saturated fatty acids is also inconsistant with the presence of a desaturase system.

A second possible mechanism by which pre-existing phospholipids might be altered would be by a deacylation-reacylation cycle as proposed by Lands (110). Such a mechanism was not found to operate in E. coli cells grown at 37° C when PG and CL were rapidly metabolized. At 10° C the fall in $^{3}H/^{14}C$ ratio was essentially the same for each of the 1- and 2-acyl groups of PE and PG indicating that there was no selective turnover of one or the other of the fatty acyl moieties. Thus the incorporation of ^{14}C-acetate represents a net synthesis of phospholipids, which is further supported by the finding that total lipid phosphorus increased 16% over the three hour incubation period; Okuyama (8) reported a 20% increase.

Our results indicate that the newly synthesized phospholipids are mainly unsaturated species. Accordingly, PE and PG synthesized during this time contain a greater percentage of diunsaturated species and a smaller proportion of disaturated species than do PE and PG synthesized prior to the cold incubation or by cells incubated at 37° C.

There is the further possibility that during cold exposure the high degree of unsaturation is maintained by the diminished conversion of monounsaturated fatty acids to their corresponding cyclopropane analogues. Data from this and other studies show that cyclopropane fatty acid synthesis in E. coli (10,89) and in Serratia marcescens (133) is diminished at low temperatures. However, the major cyclopropane fatty acid of E. coli is methylene-hexadecanoate formed from palmitoleate, whereas cis-vaccenate is the fatty acid component which is the most greatly increased during a cold-induced lag phase (8) or by growing cells at a low temperature (3). This would indicate that while control of cyclopropane fatty acid synthesis may help to maintain the level of palmitoleate, it cannot account entirely for the increased levels of cis-vaccenate.

The degree of unsaturation of the fatty acids of phospholipids may be the result of a control of de novo synthesis of either the fatty acids or of the phospholipids themselves. The control site in the latter case could be the incorporation of fatty acids into phospholipids. Starting with exogenous fatty acids, the incorporation process is known to involve first the formation of acyl-coenzyme A derivatives and second, the transacylation of acyl CoA to L-α-glycerol phosphate.

Sinensky has studied the effect of temperature on these two enzymes of E. coli (43). The rate of formation of palmitoyl- and oleoyl-CoA in vitro was the same whether the cells were grown at 25°, 34°, or 42° C. However, the palmitate-to-oleate ratio of incorporation into lyso-PA was directly dependent on the temperature at which the cells were grown. It was also shown that the second acylation (i.e., lyso-PA:acyl CoA transacylation) is also temperature dependent, relatively more oleate than palmitate being incorporated into PA at lower temperatures. Thus control of the acylation steps of phospholipid synthesis is indicated as a factor in the regulation of the species of phospholipids produced in response to changes in environmental temperature.

If the mechanism proposed by Sinensky (43) operates with respect to endogenous fatty acid utilization, one might expect that inappropriate fatty acids would accumulate. At either 37° or 10° C, there was no accumulation of labeled fatty acids. Possibly β-oxidation of undesirable fatty acids occurred. However, it may be recalled that this oxidative pathway is inducible, but otherwise not highly functional in E. coli (96). Since the cultures were prepared in minimal medium which contained no fatty acids, β-oxidation would likely not account for the lack of labeled fatty acid accumulation.

Control might be exerted directly on fatty acid synthesis. It has been shown that the type of fatty acids synthesized in vitro by an unsaturated fatty acid auxotroph of E. coli is dependent on the type of fatty acids supplied to the cells during growth (11,134). This

led the authors to conclude that fatty acid synthesis is regulated in such a manner as to supply those fatty acids necessary for minimizing variations in the physical properties of the phospholipids.

A similar control of fatty acid synthesis has not been directly demonstrated under the conditions of the present study. However, the lack of accumulation of labeled fatty acids indicates that some regulation ensuring production of only those fatty acids suitable for the acylation of L-α-glycerol phosphate and lyso-PA must exist. Therefore, we propose that *in vivo* control of the species of phospholipids produced at low environmental temperatures is a result of both selective fatty acid synthesis and selective acylation of L-α-glycerol phosphate and lyso-PA during *de novo* synthesis of phospholipids.

III. IN VIVO METABOLISM OF PHOSPHOLIPIDS OF ESCHERICHIA COLI 015 DURING A CARBON STARVATION-INDUCED STATIONARY PHASE.

Introduction

In the previous sections it was shown that phospholipid metabolism of E. coli 015 during a cold-induced lag phase is different from that of exponentially growing cells. It is also different from that of cells which have been grown at 37° C and have reached a stationary phase due to depletion of the energy source. In this latter case, total phospholipids and PE of the cells remain constant. However, the absolute amount of PG decreases, while that of CL increases (24,102). The same change in the polyglycerophosphatides is seen during stationary phases induced by the addition of colocine to the medium (135) and by oleate starvation of a fatty acid auxotroph (136). Rampini et al. (9) also demonstrated this phenomenon in E. coli cells harvested during exponential growth and incubated in buffered saline solution containing no carbon source. In all instances it was suggested that PG is converted to CL.

PG is known to be an intermediate in CL synthesis in E. coli (25) according to the following equation:

$$PG + CDP\text{-}DG \longrightarrow CL + CMP \qquad (1)$$

A recent communication of Davidson and Stanacev (51) showed that while no exogenous CDP-DG was incorporated into CL by guinea pig mitochondria in vitro, the synthesis of this phosphatide was dependent

on CTP. On the other hand, Hostetler, et al. (45) found that CDP-DG was incorporated into the CL of rat liver mitochondria in vitro.

However, the CL found in E. coli cells incubated in buffered saline may not be formed in the same manner. No energy source was available and the synthesis of CTP for CDP-DG production would be unlikely. Rampini et al. (9) suggested rather that the conversion of PG to CL was according to the following equation:

$$2\ PG \longrightarrow CL + \text{glycerol} \qquad (2)$$

This reaction was first proposed by Brundish et al.(137), who found that GPG, i.e. the deacylated product of PG, was converted to GPGPG, i.e. the deacylated product of CL, prepared from Pneumococcus lipids. Stanacev and Stuhne-Sekalec (138) have demonstrated the in vitro formation of CL from purified PG in the presence of phospholipase D from cabbage and suggested a similar transphosphatidylation mechanism.

Until recently phospholipase D was believed to be confined to higher plants. Ono and White (71,72) have now demonstrated the presence of this enzyme in Haemophilus parainfluenzae which specifically degrades CL to PG and PA. The authors have postulated that the enzyme might also function in the formation of CL from two molecules of PG. This mechanism is responsible for the synthesis of CL in Staphylococcus aureus (139).

DeSiervo and Salton (140) have described a membrane bound enzyme of Micrococcus lysodeikticus which catalyzes the conversion of PG to

CL. The enzyme has been tentatively designated as "cardiolipin synthetase". Synthesis of CL proceeds in the absence of any detectable CDP-DG. The independent turnover of the glycerol portion of PG in E. coli under certain conditions (142) would indicate the possibility of phospholipase D-like activity in at least some strains of this microorganism.

In the following experiments, the metabolism of ^{32}P- and ^{14}C-labeled phospholipids of E. coli 015 during a stationary phase induced by carbon starvation was studied.

Since the completion of these experiments, Hirschberg and Kennedy (44) have reported that cell-free preparations of E. coli ML 308 catalyze the conversion of PG to CL according to equation (2). Hostetler, et al. (45) have confirmed this finding in E. coli K12, but also presented evidence which suggests that the CDP-DG pathway (equation 1) is operative in E. coli at high concentrations of CDP-DG.

Methods

E. coli 015 cells were cultured to early log phase in 300 ml of nutrient broth containing 0.25 mc ^{32}P or 50 µc 1-^{14}C-acetate. The harvested cells were resuspended in an equal volume of 0.02 M potassium phosphate buffer (pH 7.4) containing 0.1 M NaCl. The optical density of the cells in this buffered saline remained essentially the same for a further three hour incubation period (figure 21).

In a second set of experiments the cells were grown in nonradioactive medium to early log phase, harvested and then incubated for three hours in 300 ml of either buffered saline or minimal medium, each of which contained 100 µc of ^{3}H-methyl methionine.

Cells were harvested and lipids were extracted, separated and counted as given in General Methods.

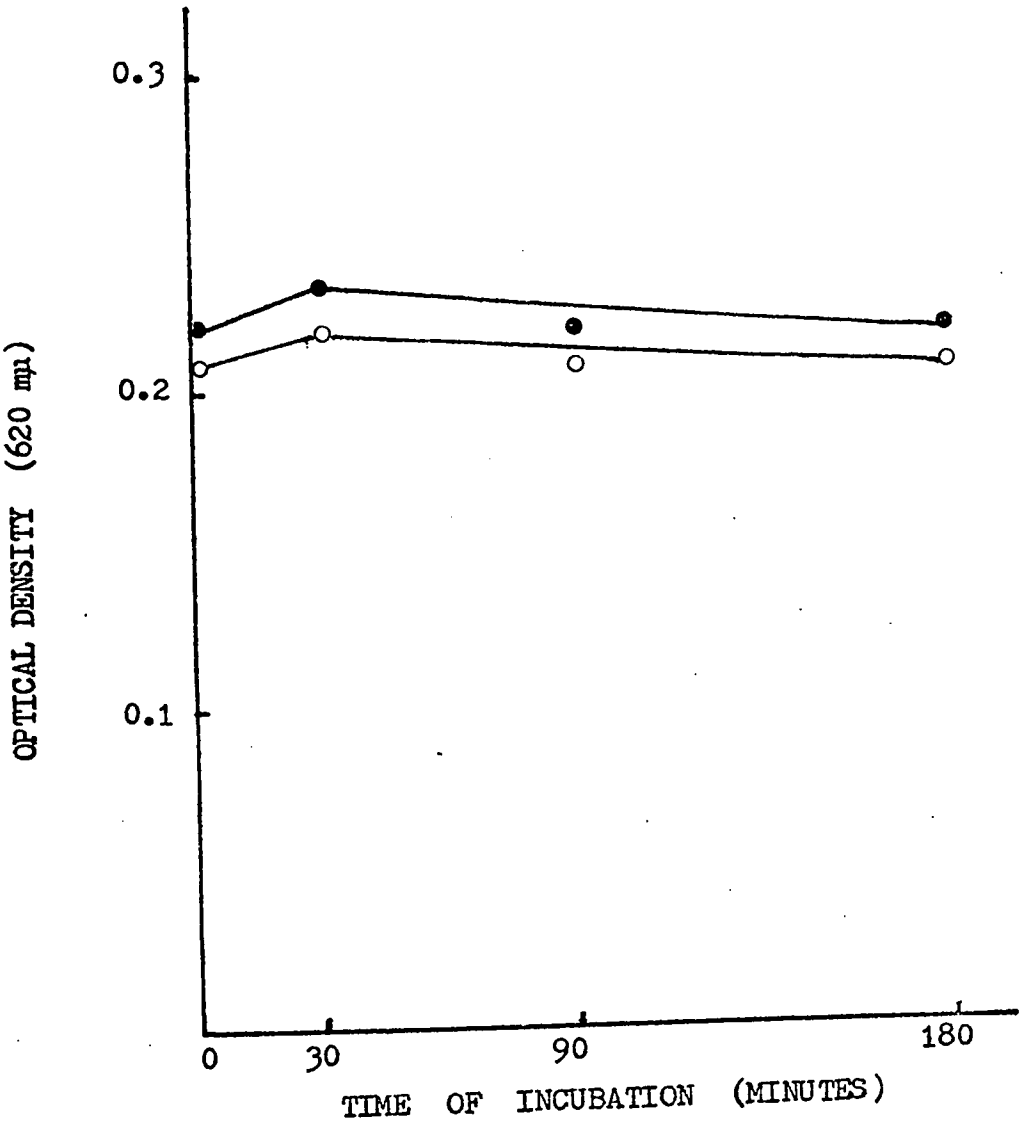


Figure 21: Optical densities of cultures of Escherichia coli 015 transferred during exponential growth in nutrient broth to buffered saline at 37° C. (●) ^{14}C studies and (○) ^{32}P studies.

Results

Figure 22 shows the turnover of the ^{14}C-label of total lipids and of individual phospholipids of E. coli cells incubated in buffered saline at 37° C. There was only a small turnover of the label in the total lipids, PE or in the total polyglycerophosphatide fraction. On the other hand, PG decreased slowly at first, then rapidly after 90 minutes. ^{14}C-CL remained constant for 90 minutes then shown a pronounced increase.

Essentially the same results were obtained with ^{32}P-labeled cells (figure 23) except that during the first 90 minutes there was a greater decrease in the total polyglycerophosphatides. Following this period, PG continued to decrease at a rate corresponding to the increase in CL. The slight difference in results obtained with the two labels could be due to the fact that ^{32}P-labeled cells were chased, whereas ^{14}C-labeled cells were not.

The "CL" present at the end of the three hour incubation was isolated and an aliquot was rechromatographed on silica gel G plates in chloroform-methanol-ammonia-water (70:30:4:2, v/v/v/v). Only one band corresponding to CL was found (Rf. values: CL = 0.50, PA = 0.20). Another aliquot was subjected to mild alkaline hydrolysis. The water-soluble products were chromatographed on 3 mm Whatman paper with phenol-water as the solvent as previously described and a single band corresponding to GPGPG was found (Rf. 0.24).

In a further experiment cells were prelabeled with 1-^{14}C-acetate then incubated in buffered saline solution containing 200 μc ^{32}P. As shown in figure 24, the usual increase in ^{14}C-labeled CL was found,

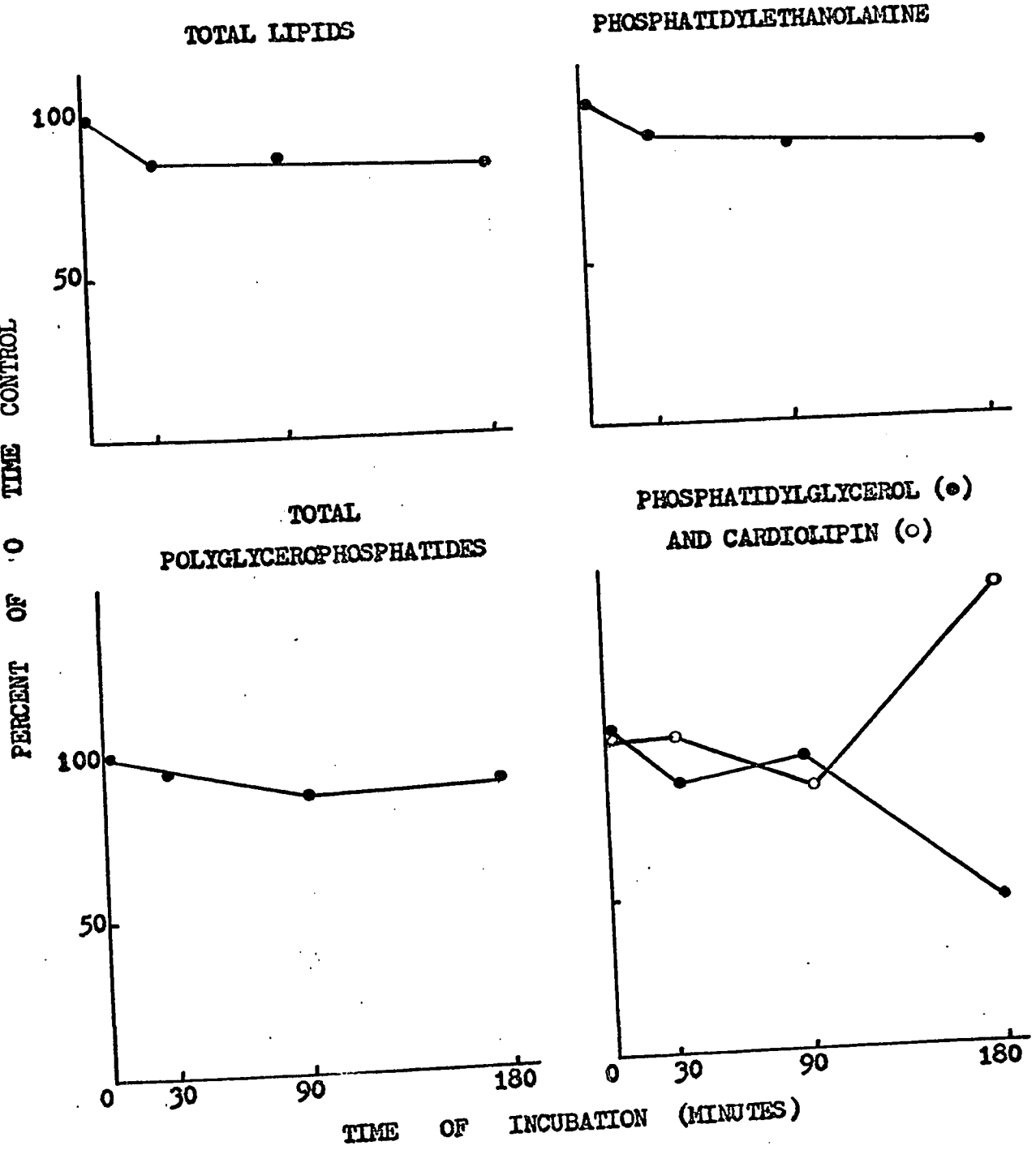


Figure 22: Turnover of ^{14}C in the total lipids and phospholipids of Escherichia coli 015 cells harvested during exponential growth and subsequently incubated in buffered saline for three hours.

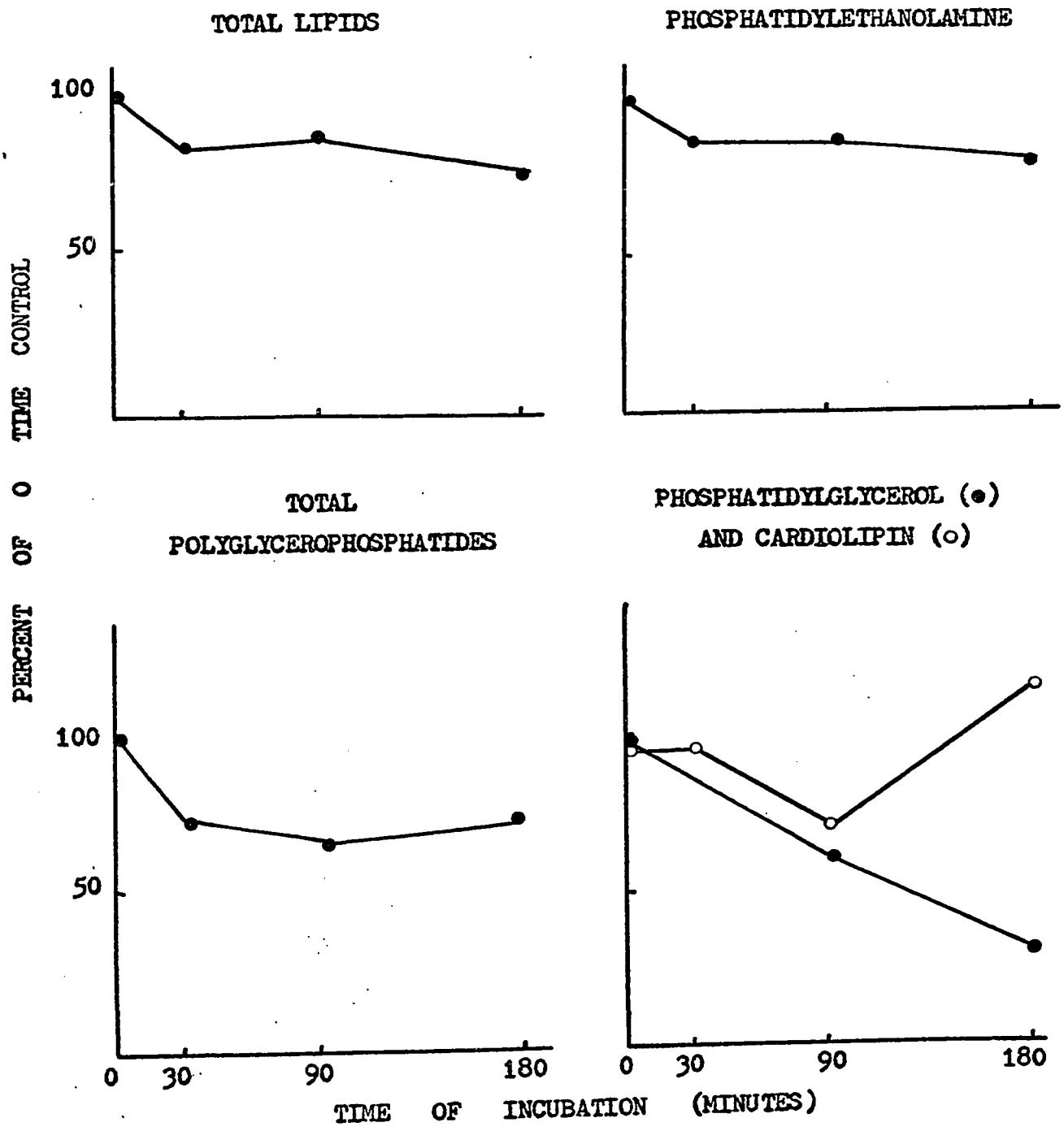


Figure 23: Turnover of ^{32}P in the total lipids and phospholipids of Escherichia coli 015 cells harvested during exponential growth and subsequently incubated in buffered saline for three hours.

while essentially no ^{32}P was incorporated into the total phospholipids.

The incorporation of the methyl group of ^{3}H-methyl methionine into total phospholipids in either buffered saline or minimal medium is illustrated in figure 25. In buffered saline, the incorporation is complete in 30 minutes, whereas in minimal medium the incorporation of the labeled methyl group continued over the three hour period.

While the total incorporation of ^{3}H-label into the lipids does not proceed beyond 30 minutes in buffered saline, the proportions of the individual phosphatides do not remain constant during the remainder of the incubation. As can be seen in figure 26, PG decreases and CL increases after 90 minutes in a manner similar to that found with the ^{32}P- and ^{14}C-labeled cells.

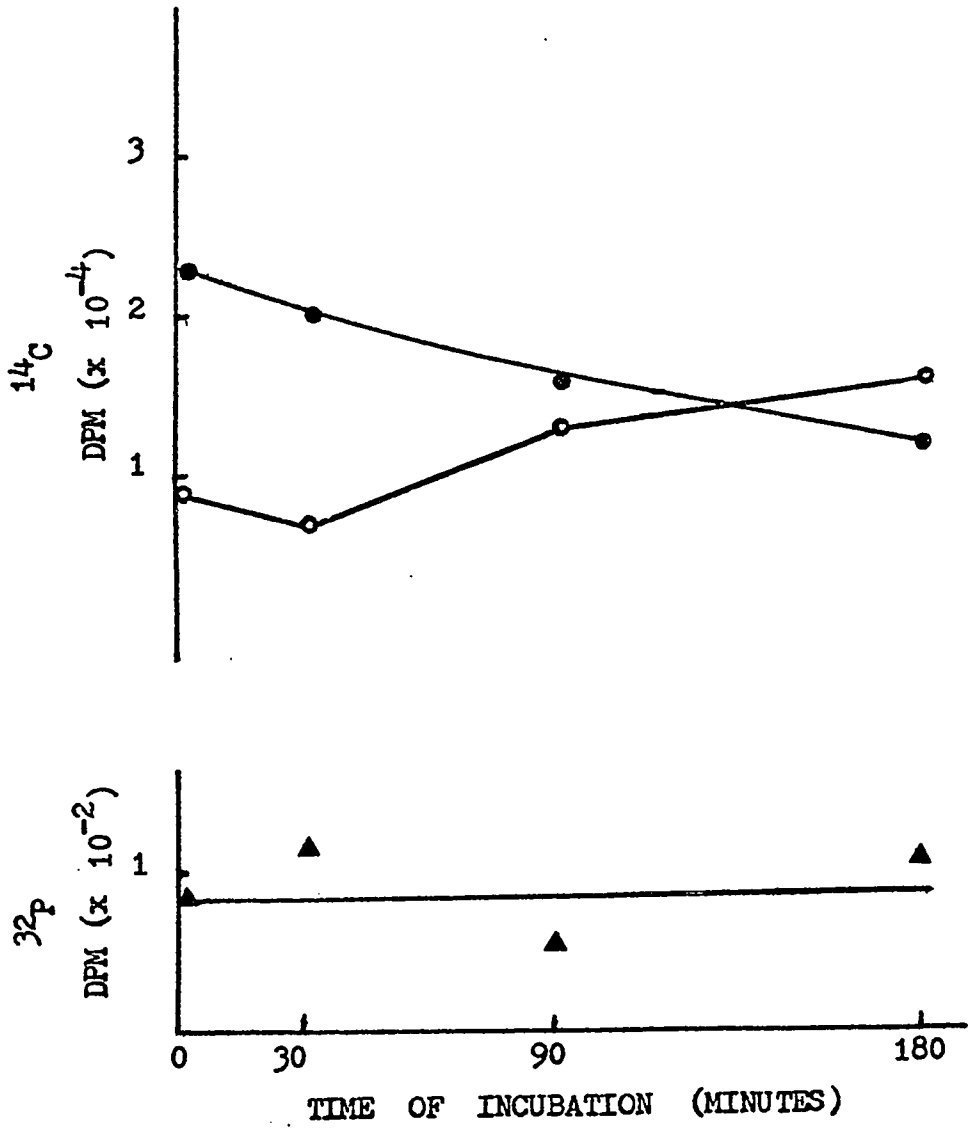


Figure 24: Turnover of ^{14}C in phosphatidylglycerol (●) and in cardiolipin (⊖) and the incorporation of ^{32}P (▲) into the total lipids of Escherichia coli 015 incubated in buffered saline.

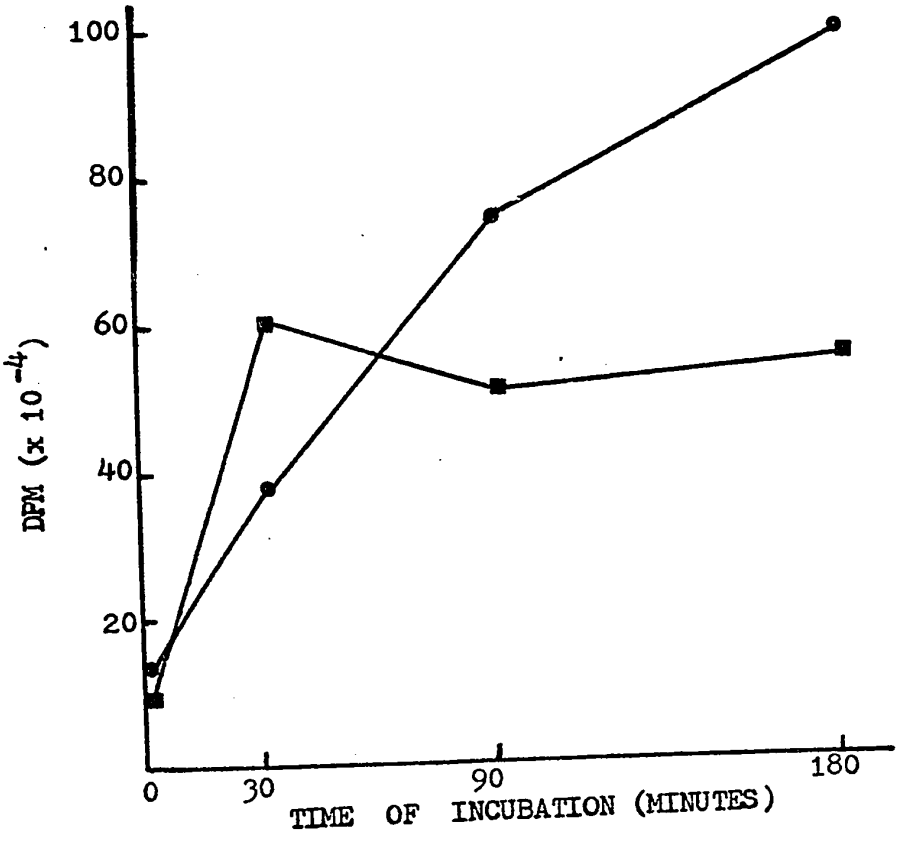


Figure 25: Incorporation of the methyl group of ^{3}H-methyl methionine into total lipids of Escherichia coli 015 harvested during exponential growth and subsequently incubated in buffered saline (■) or minimal medium (●).

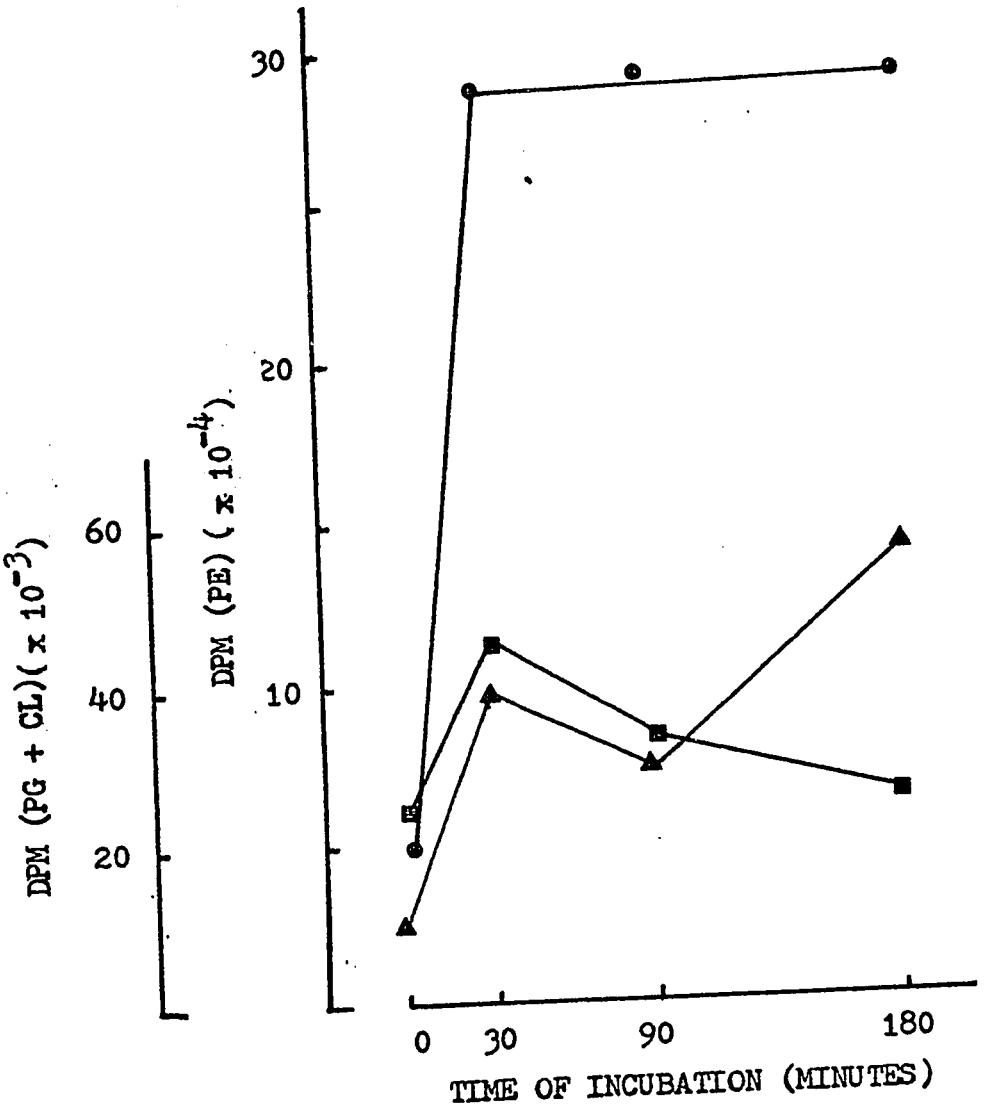


Figure 26: ^{3}H labeling of individual phospholipids of Escherichia coli 015 grown in medium containing ^{3}H-methyl methionine and subsequently incubated in buffered saline. (●) phosphatidylethanolamine, (■) phosphatidylglycerol and (▲) cardiolipin

Discussion

The results obtained by incubating prelabeled ^{14}C- or ^{32}P-PG and -CL in buffered saline could be interpreted as: 1) PG is being converted to a mixture of compounds, possibly acylated PG or PA; these two compounds do not clearly separate from CL in the original TLC system used, 2) PG is completely degraded and the inorganic phosphate and fatty acyl moieties are reused for de novo synthesis of CL, or 3) PG is converted directly to CL.

Acyl-PG appears to be only a minor lipid component of E. coli although it is readily formed under specific in vitro conditions (27). PA is also a trace lipid (24). That neither of these contributed substantially to the labeled "CL" fraction was verified by further chromatographic analysis of the intact lipid and of its water-soluble component. Only one compound, CL, accounted for this fraction.

The second possibility, i.e., the de novo synthesis of CL from inorganic phosphorus produced in the degradation of PG was also eliminated. That no de novo phospholipid synthesis occurs during the incubation in buffered saline was shown by the lack of ^{32}P incorporation during this time.

The results favor the third possibility, that PG is directly converted to CL. The conversion could possibily make use of endogenous CDP-DG. However, we have failed to detect this precursor and there are no reports of its being a significant component of E. coli lipids. Undoubtedly the CDP-DG pool is very small as would be expected from its very transient function. Also in a separate series of experiments,

it was found that the transfer of the methyl group of methionine via S-adenosyl methionine to form cyclopropane fatty acids of total phospholipids did not proceed beyond 30 minutes in cultures of buffered saline, whereas in cells in minimal medium, the incorporation continued for at least three hours (figure 25). This would indicate that the residual energy supply of the buffered saline culture was depleted in 30 minutes. Therefore, it likely did not contribute to the increase in ^{3}H-CL which was noted only after 90 minutes of incubation (figure 26).

At any rate, if endogenous CDP-DG or a system generating this precursor contributed significantly to the synthesis of CL, a parallel increase should have occurred in PE which turns over at a rate even slower than that of CL. This was not found. Therefore, it is not likely that CL is synthesized by the currently accepted CDP-DG pathway under these energy-free conditions. The only likely possibility remaining is that CL is formed from two molecules of PG as suggested by Rampini (9).

A report of a cell-free preparation of E. coli capable of synthesizing CL from two molecules of PG has recently appeared in the wake of reports of this mechanism in several other microorganisms (71,72,139,140). Hirschberg and Kennedy (43) found that, while CDP-DG stimulated the synthesis of CL from PG, the phosphorus moiety of this precursor was not incorporated into the CL formed. Further evidence was supplied by the finding that the $^{3}H/^{32}P$ ratio of the CL formed was exactly half of that of PG doubly labeled with ^{32}P and ^{3}H in the free glycerol. The labeled glycerol eliminated was recovered and unambiguously identified.

One could surmise that the operation of this alternate pathway may be provoked during the stationary phase of a normal growth cycle, when a depleted energy supply in the medium is accompanied by a conversion of PG to CL. This would be in accord with the findings of Hostetler, *et al.*(45) that both pathways of CL synthesis are functional in *E. coli in vitro*, although the CDP-DG pathway can be demonstrated only at high concentrations of CDP-DG.

Further work with cell-free preparations of *E. coli* will be needed to elucidate the steps involved in this alternate pathway of CL synthesis.

GENERAL DISCUSSION

We have investigated the *in vivo* metabolism of phospholipids of *Escherichia coli* 015 under normal growth conditions and in two different circumstances, low environmental temperature and carbon starvation, in which growth is greatly diminished. In the latter two instances, phospholipids of the cells are different from those of cells growning exponentially. During a cold-induced lag phase, there is little turnover of the phospholipids, but some *de novo* synthesis does occur. The newly formed PE and PG are richer in unsaturated species than are those phosphoglycerides synthesized prior to the lag phase. The change, therfore, is in the fatty acid composition of the phosphatides. In contrast, during a carbon starvation-induced stationary phase, there is a change in the relative amounts of the types of phosphatides, *i.e.*, the amount of PG decreases while that of CL increases with time.

The increase in the degree of unsaturation of phospholipids of cells exposed to low environmental temperatures is well documented (21). Also, it is known that the percent of unsaturated fatty acids of the total fatty acids is progressively greater in thermophilic, mesophilic and psychrophilic strains of *Clostridia* (124). Our results suggest that *de novo* synthesis of unsaturated species of phosphatides would account for this phenomenon. Control of the species of phosphoglycerides formed is most likely a combination of selective acylation of L-α-glycerol phosphate and lysophosphatidic acid (which Sinensky (43) has shown to be temperature sensitive) and regulation of the fatty acids synthesized

either by temperature sensitive enzymes of the fatty acid synthetase complex or by an effective feedback control mechanism. In a fatty acid auxotroph of E. coli (11) and in Lactobacillus planarium (131,132), fatty acid biosynthesis is subject to control by exogenous fatty acids.

A second factor operates to maintain the levels of unsaturated fatty acids in cells exposed to low environmental temperatures. The synthesis of cyclopropane fatty acids from palmitoleate and vaccenate was markedly decreased. This type of adaptation to low environmental temperatures has been reported for mesophilic Serratia marcescens (133). The same study revealed that a psychrophilic strain of this organism had apparently lost the ability to synthesize cyclopropane fatty acids. One could reason that the exposure of cells to low environmental temperatures exerts no direct control on the enzyme, but that the culture fails to attain the proper conditions for its activation. In vitro studies would be required to show whether cyclopropane fatty acid synthesis is in fact temperature sensitive under well controlled conditions.

Although it is generally observed that cyclopropane fatty acids accumulate in E. coli cells only as the culture approaches the stationary phase in the normal growth cycle at 37° C (10,69), the triggering of cyclopropane fatty acid synthesis is not clearly understood as yet. The physiological significance of cyclopropane fatty acid synthesis is also not yet clear. The original hypothesis was that the conversion of monounsaturated fatty acids to their cyclopropane derivatives was to prevent oxidation of the double bonds and thus limit fatty acid expenditure. The fact that fewer cyclopropane fatty acids

are formed in aerated than in partially anaerobic cultures of E. coli and Pseudomonas fluorescens (94,104) would argue against this explanation. There would seem to be no advantage of either having or not having cyclopropane fatty acids to cells at low environmental temperatures, as they afford the same physical properties to the membrane as do their monounsaturated counterparts. One could speculate that the methylation of monounsaturated fatty acids as the cells approach the stationary phase of normal growth cycle renders the phospholipids less susceptible to degradation. Phospholipase A of snake venom (Crotalus adamantus) and of porcine pancreas hydrolyze phospholipids containing unsaturated fatty acids more readily than those containing saturated fatty acids (141). No such studies have been made with the phospholipase A of E. coli or with synthetic cyclopropane fatty acid-containing species of phospholipids.

We found no evidence that a phospholipase A-lysophospholipid: acyl CoA acyl transferase cycle (i.e., the Land's cycle) was operative in E. coli, under the conditions studied, although the necessary enzymes have been shown to be present in this microorganism. The Land's cycle has been studied predominately in mammalian tissue and is thought to act most specifically for the incorporation of polyunsaturated fatty acids into phospholipids (113). As E. coli and most other microorganisms contain no polyunsaturated fatty acids (21), this cycle may not be widely utilized by them.

Phospholipase A_1 and lysophospholipase may function in the complete hydrolysis of phospholipids of E. coli although our evidence does not

definitely show this to be the case in intact cells. However, if this is so, it raises an interesting question regarding the mechanism of control of these enzymes. Why does PE, which is a suitable substrate *in vitro*, show no turnover during normal growth or during periods of reduced growth due to cold exposure, lack of carbon (as shown in these studies) or by insufficient available nitrogen (142). As results in this and other laboratories have shown, endogenous PE is readily degraded by phospholipase A when cells are damaged. Is the role of phospholipase A, therefore, only autolytic? If so, then what pathways would account for the turnover of polyglycerophosphatides in intact cells?

The purpose of lysophospholipid acylating enzyme is even more obscure as it does not appear to reacylate intermediate products in the normal turnover of PG or CL. Perhaps, this enzyme might also be involved in the incorporation of exogenous fatty acids and would serve to reacylate any lysophospholipids formed in "damaged" cells in which phospholipase A_1 might be active. The latter possibility might be investigated by determining the incorporation of exogenous fatty acids into diacylphospholipids under various conditions as compared to that of endogenous fatty acids (*i.e.*, those synthesized *de novo* from acetate).

The second condition studied in relation to normally growing *E. coli* was that of cells in a carbon starvation-induced stationary phase. The PE was found to be stable; PG and CL decreased for 60-90 minutes. PG continued to decrease for another 90 minutes, but during this time, there was a net increase in CL with no *de novo* synthesis of phospholipids. The synthesis of CL appears to be at the expense of PG but not to be

an energy requiring process involving a CDP-DG precursor.

A mechanism for the synthesis of CL from two molecules of PG has been demonstrated in E. coli (66) as well as in other microorganisms (67,138-140). This raises the question of the role of CDP-DG which stimulates this reaction in E. coli while not appearing to be directly involved in the formation of CL from PG.

Even more intriguing, perhaps, is the mechanism of control of CL synthesis. During exponential growth CL is synthesized continuously from PG by a mechanism which cannot be definitely stated at this time. As the culture approaches the stationary phase the synthesis of all phospholipids diminishes and appears to stop for a period. Then PG decreases and CL increases (102). One would wonder what is responsible for this biphasic synthesis of CL and whether or not two separate mechanisms are involved.

The phenomenon is equally interesting in cells incubated in buffered saline. Both PG and CL decrease for a period; then CL increases rather abruptly. Why does CL not continuously increase from the onset of the incubation as the PG decreases. What are the degradation products of PG during the time when it does not appear to be converted to CL and does their accumulation trigger the synthesis of CL? Investigations along this line might be very illuminating.

As the majority of the phospholipids of E. coli are located in the membrane of the cells (22), our results are directly relevant to the composition of the membrane. The removal of lipids from a membrane does not alter the electron-microscopic appearance of the membrane (143).

Nevertheless, the phospholipids are considered essential components of the fine structure of membranes. They are involved in the control of the permeability of the membrane and in the activity of certain membrane-bound enzymes (144-151). The latter effect may be mediated either by maintaining a proper hydrophobic environment or by a specific interaction of the lipids with an enzyme protein to change its conformation.

As we have seen, the lipid composition of a membrane may be altered in two ways: the relative amounts of individual phospholipids may be changed and the fatty acid composition of the individual phospholipids may vary. There is evidence to indicate that both factors must be considered in studying the relationship of lipids to the function of membranes. For example, the addition of CL to a monolayer of PC and PA increases the passage of both anions and cations through the layer (144), neutral amino acids pass more freely through membranes formed of unsaturated rather than saturated PC (145), and the uptake of glycerol by Mycoplasma laidlawii cells increases as the ratio of 18:2 to 16:0 increases (146). The list of enzymes and transport functions related directly to a particular lipid is growing rapidly, including β-galactoside and β-glucoside transport (147) and lactose transport (148) in E. coli. D(-)β-hydroxybutyric apodehydrogenase of beef heart mitochondria has an absolute requirement for unsaturated PC (149); the synthesis of the lipid A portion of lipopolysaccharide of Staphlococcus requires PE, specifically, 1,2,-diunsaturated or 1-unsaturated-2-cyclopropane PE (150). The ATPase activity and Ca^{++} transport of sarcoplasmic reticulum of muscle requires PC, lyso-PC or PA (151).

One can assume that in situations consistent with life, an organism will make those metabolic adaptations most favorable to its continued survival and growth. We must ask then what advantages were gained by the adaptations we have noted in cells during cold exposure and during periods of carbon starvation.

In the first instance, cold exposure, the increase in the degree of unsaturation has long been equated with the necessity of maintaining the fluidity of the membrane. This is based on the reasoning that membranes contract as the temperature is lowered (152), most likely due to the decreased mobility of the acyl chains of the phospholipids. The introduction of unsaturated fatty acids in E. coli (8,19,21) or branched or short chain fatty acids in other organisms (153-155) would be necessary to counteract this effect. The purpose would be to form a membrane consistent with the required permeability characteristics and with the activity of the necessary enzymes as previously mentioned.

It is interesting in this regard, that Shaw and Ingraham (19) found that changes in the fatty acid composition of cells during a cold-induced lag phase were not a requirement for their continued growth. If glucose were withheld during the period of cold exposure, there was no change in the relative amounts of saturated and unsaturated fatty acids of the cells. The addition of glucose after 5 hours resulted in immediate cell growth at this low temperature with fatty acids as found in the original 37° C culture. These same authors found that the lag phase usually noted upon the transfer of E. coli from 37° to 10° C was eliminated if the initial culturing at 37° C was anaerobic (20). The growth rate of

anaerobically grown cells was slowed but not halted upon transfer to 10° C under either anaerobic or aerobic conditions. It is unfortunate that they did not report the lipid composition of the cells under these conditions. However, the authors postulate that the lag phase induced by low environmental temperatures is for the removal of previously formed waste products and not merely a period of lipid adaptation.

Changes in the relative porportions of the individual phospholipids would influence the surface of the lipid portion of the membrane rather than its internal characteristics. For example, an increase in the PE to PG ratio would result in a net decrease in the negative charge of the polar head groups and could influence the binding of proteins and/or the transport of permeants across the membrane.

The significance of a change in the PG to CL ratio noted during carbon starvation is not yet known. One might postulate that such a change favors the maintenance of the membrane rather than a change in function. Doi, et al. (66) found that both the particulate and soluble fractions of E. coli were less effective in the phospholipase A hydrolysis of CL than in that of the other phosphatides. Thus the conversion of PG to CL might have a sparing effect on polyglycerophosphatides as conditions unfavorable for growth and survival prevailed.

On the other hand, our own results show that CL is readily degraded by whole E. coli sonicates and the work of Scandella and Kornberg (65) demonstrated the suitability of CL as a substrate for purified E. coli phospholipase A_1. In normally growing cells, CL turns over at a moderate rate yet there is no clear indication from our work that phospholipase A

is involved. When cells are starved or filamentous growth is induced (103), the CL/PG ratio increases substantially as though the conversion of PG to CL is greatly increased and the breakdown of CL is inhibited. Clearly more work is required to understand this phenomenon and to elucidate the catabolism of CL *in vivo* since there is yet no satisfactory mechanism accounting for its turnover.

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