POLYGLYCEROPHOSPHATIDE METABOLISM IN *ESCHERICHIA COLI*

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LIST OF ABBREVIATIONS

PE	-	Phosphatidylethanolamine
PG	-	Phosphatidylglycerol
CL	-	Cardiolipin
PS	-	Phosphatidylserine
PA	-	Phosphatidic Acid
PC	-	Phosphatidylcholine
DG	-	Diglyceride
CDP-DG	-	Cytidinediphosphate Diglyceride
CTP	-	Cytidine Triphosphate
CDP	-	Cytidine Diphosphate
CMP	-	Cytidine Monophosphate
UDP	-	Uridine Diphosphate
UMP	-	Uridine Monophosphate
ATP	-	Adenosine Triphosphate
CoA	-	Coenzyme A
ACP	-	Acyl Carrier Protein
NAD	-	Nicotinamide Adenine Dinucleotide
NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
FAD	-	Flavin Adenine Dinucleotide
DHAP	-	Dihydroxyacetone Phosphate
PP_i	-	Inorganic Pyrophosphate
P_i	-	Inorganic Orthophosphate

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GENERAL INTRODUCTION

General Structural Features of the Bacterial Cell Surface

Bacteria in general possess at their surface, a cytomembrane and a cell wall. In gram-positive organisms these two entities are easily distinguished by electron microscopy and biochemical techniques. The cell wall material of Bacilli for example, can be easily removed by muralytic agents and the plasmalemma isolated in relatively pure form after osmotic shock (1).

The cell surface of Gram-negative bacteria such as E. coli appears to be more complex. Electron microscopy reveals in this case several distinct layers (2,3), including an inner cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane, and a second membranous structure the "outer membrane" at the outer surface of the cell. The surface assemply is generally designated as the cell envelope. Attempts to separate the different layers in pure form have met with only moderate success. The methods are complicated and have not been readily adaptable to different strains of the same species. The ML strains of E. coli used by Kaback for example, are more amenable to cytoplasmic membrane isolation than the W strains which possess more "complete" cell wall structures (4).

The Cytoplasmic Membrane

Definite information regarding the structure and precise composition of bacterial membranes is limited to organisms in which adherence of cell wall material to the membrane does not pose a serious technical problem. Among the organisms most commonly studied are the Bacilli and pleuropneumonia-like organisms. In the case of *Mycoplasma laidlawii* at least, it seems that the architecture of the membrane is quite similar to that of animal plasmalemma i.e., the basic structural entities are protein and lipids arranged as bilayers (5,6). At this stage, one can only speculate that general knowledge on the structure of cytomembranes also applies to *E. coli* plasmalemma, although preliminary X-ray diffraction data indicate that the bulk of the lipid in both the inner and outer membranes is arranged as a bilayer (6).

Structural Models of the Plasmalemma

The surface of all cells is lined with a semipermeable membrane composed mainly of protein and lipid. Up until 1960 this barrier, the plasmalemma, was generally believed to be composed of a lipid bilayer "sandwiched" between two layers of protein. This simple concept proposed by Davson and Danielli (7) and Stein (8) and still dogmatized by Robertson (9) has been challenged

by several investigators (cf review article by E.D. Korn (10)) from 1960 onwards. The sea of diversity that resulted was not successful in submerging the original Davson-Danielli model although it has eroded some of the rigid proposals of Robertson. Accordingly, the newest evidence although supporting the bilayer concept, tolerates more liberal acceptance of the model, inasmuch as one need no longer ascertain that all the lipid is covered by protein in extended conformation (11). In some membranes at least, the structure may involve a mosaic of lipid bilayers and protein globules, each entity projecting polar groups at the surface (12,13)

The Fluidity of Biomembranes

Recent evidence has shown that in pure hydrated lipid bilayers there is flexibility of acyl chains in the plane of the bilayer (14) and that fluidity increases in the chains in proportion to the distance from the carboxyl group (14). Flip-flop perturbations are also detected as seldom and slow processes (15). The thermotropic mesomorphism of lipid in the membrane is largely controlled by either the cholesterol content and, or the degree of unsaturation of the acyl chains (16,17). In membranes which contain it, cholesterol acts as a regulator of fluidity. It liquifies straight saturated chains by

mechanical disruption of hydrophobic bonds and it diminishes the fluidity of cis-unsaturated chains either by some condensing effect as in the case of mono 9-cis unsaturated chains (16) or by sterically hindering the motion of mono-unsaturated chains with which it hydrophobically combines. The net result is that a liquid crystalline state is maintained (17).

Whether or not cholesterol is present in the membrane, unsaturation in the chains would naturally be a dominant factor influencing fluidity of the lipid core. The degree of unsaturation is alterable by diet (18) and in poikilothermic organisms including E. coli as well as in surface cells, by temperature (16,18,19). Cold increases the degree of unsaturation and the opposite is true.

Whether, and to what extent motion of lipids is restricted by the presence of protein is not completely known but it is now established that at physiological temperature, the acyl chains in many isolated biomembranes studied are in a liquid crystalline state (14,16,20,21). It is known from differential thermal analyses and spectrometric studies performed on bacterial and mammalian membranes (6,21) that the acyl chains can be reversibly melted without effecting gross conformational changes in the protein. Denaturation of the protein at higher temperature does not

affect the melts of the acyl chains. It is probable therefore that the structure of the membrane does not depend on extensive hydrophobic interactions between protein and lipid and that the gross physicochemical properties of one structural counterpart is not affected by the presence of the other. This evidence is complementary to the model of Singer and Nicolson who see the biomembrane as a liquid mosaic of lipid bilayers and protein globules (13). Both entities are mobile in a plane parallel to but, usually not, in a plane perpendicular to the surface of the membrane. The concept of mobility of protein in the lateral plane is based on the fact that fluorescein-labelled antibodies will react with cell surface antigens in a completely randomized manner. There are apparently no organized protein patterns at the surface of the cell (13). Also, the work of Frye and Ediden (22) has shown that when one cell which is labelled at its surface with fluorescein-labelled antibody fuses with an untagged cell, the resultant membrane is, at first, asymmetrical with respect to antibody distribution. With time, the label spreads uniformly over the whole fused cell surface. On the other hand the antibodies will react at only one surface of the membrane depending on the distribution of antigen.

This indicates considerable asymmetry of protein arrangements in the perpendicular plane.

The model of Singer and Nicholson seems quite reasonable from the evidence at hand although it could possibly be argued that interaction of surface proteins with tagged antibodies perturbs any organized protein at the surface of the cell and might actually cause randomization.

Since the protein mobility is postulated to occur in the parallel plane only, the model does take into account the well-documented asymmetry of protein distribution (23) but it assumes that the nature of this asymmetry is unimportant to the maintenance of surface phenomena such as active transport, and facilitated diffusion. It is doubtful that the model could apply without modification, to the inner mitochondrial membrane where coupled oxidative phosphorylation may well depend on a vectorial arrangement and lateral organization of the electron carriers and coupling factors (24,25).

The Function of Phospholipids in Biomembranes

Phosphoglycerides are found mainly in biomembranes where they serve a structural role. The amphipathic properties of these lipids compel them to form bilayers in aqueous media (11,26) and this seems now to be the universal structural basis for formation of all membranes.

Probably any amphipathic substance which is not wedge-shaped (27) would have been equally suitable to Nature. What seems to influence the structural properties of the biomembrane is not so much the nature of the polar head-groups of phosphoglycerides but the fluidity of their acyl chains. The force-area curves of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine with identical fatty acid compositions are much the same (28,29) although this generalization does not include phosphatidylglycerol and cardiolipin. Many of the passive or static properties of the biomembrane, that is, electrical properties and permeability to small uncharged molecules are attributable to the bilayer arrangement of lipids (26,30). The presence of protein, may however influence these parameters to some extent.

That the acyl chains be in liquid crystalline state seems essential for the normal functioning of membranes in general. The influence of fluidity on permeability has been extensively studied with liposomes (26). The penetration of glycerol, for example, is dependent on the degree of unsaturation in the acyl chains of liposomal lipids. The mechanical properties of the membrane are also influenced in a similar manner. Cells with membranes which have lipid components of excessive or too little fluidity are expected to function

in an aberrant manner. This is observed with *E. coli* unsaturated fatty acid auxotrophs which are supplemented with abnormal fatty acids. Such cells divide abnormally and lyse when shifted in temperature (31).

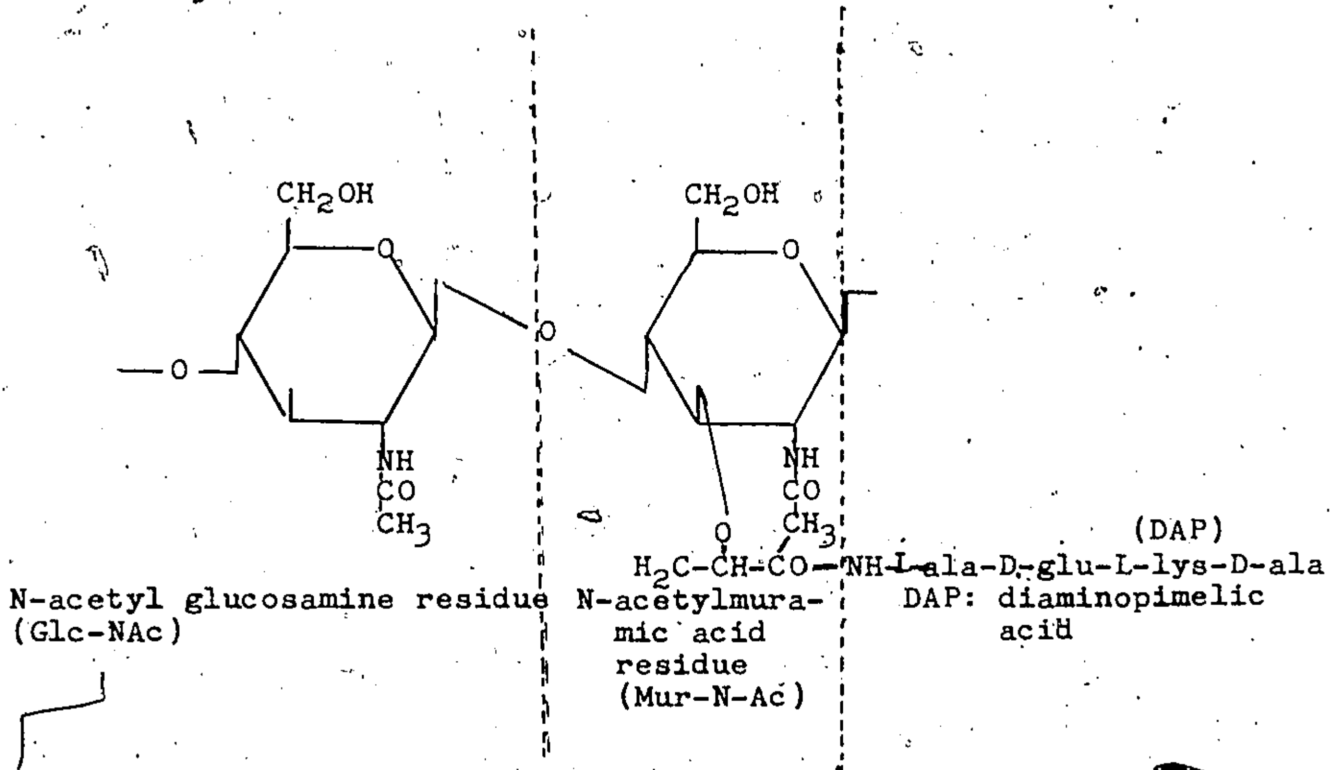
Phospholipids may also have functions not directly related to their influence on membrane rigidity and permeability. Specific phospholipids are associated with various enzyme and transport activities, for example, phosphatidylglycerol with the vectorial phosphorylation of α-methylglucosides in isolated membrane preparations of *E. coli* (32,33) and cardiolipin with cytochrome oxidase and cytochrome C of mitochondria (34,35). Even in such instances the fatty acid composition may be significant. Hydrogenation of cardiolipin has been found to reduce its capacity to bind cytochrome C *in vitro*.

The Peptidoglycan Layer

The cell wall material of Gram-positive and Gram-negative bacteria has one structural feature in common, i.e., both types of organisms contain peptidoglycan polymers which are chemically very similar. The basic repeating unit is a muropeptide, the structure of which is illustrated in Diagram I.

Diagram I

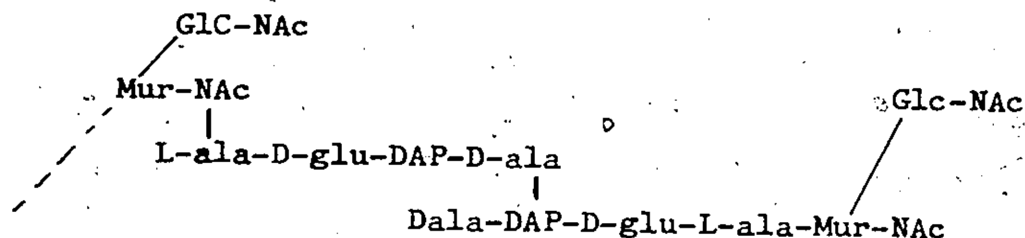
Structure of the Muropeptide Unit



The structure of the peptidoglycan may differ according to the nature of the crosslinkages. In Staphylococcus aureus, the muropeptide units are crossed-linked by a pentaglycyl unit conjugating the terminal D-alanine residue of one repeating unit and the lysine residue of another. In E. coli cross-linking dispenses with the pentaglycyl unit and occurs directly between the terminal D-alanine residue and the diaminopimelic residue of two vicinal repeating units as illustrated in Diagram 2.

Diagram 2

Structure of E. coli Peptidoglycan



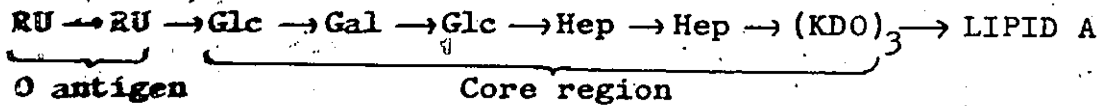
For a more complete description of structures and biosynthesis of peptidoglycans the reader is referred to excellent reviews articles by Osborn (36) and by Lennarz and Scher (37).

The Outer Membrane

The outer membrane of Gram-negative bacteria seems to have a complex architecture composed of lipopolysaccharide;polysaccharides and phosphoglycerides (36) combined with protein and, or arranged as bilayers (7). The lipopolysaccharide is characterized by three distinct regions. The outermost, contains the O-antigen which is a basis for the serological typing of Gram-negative organisms. It is conjugated with a core region which is in turn linked to the innermost component, lipid A (36) as illustrated in Diagram 3.

Diagram 3

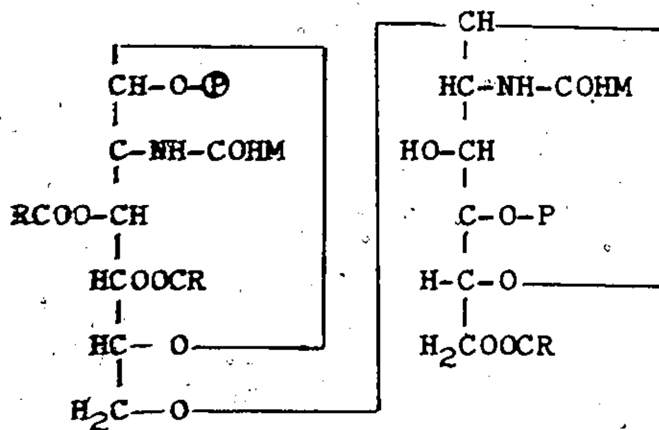
A) Structure of Lipopolysaccharide



RU: repeating oligosaccharide units variable in composition depending on the strain

Glc: glucose, Gal: galactose, Hep: heptose, KDO: keto-deoxyoctonate

B) Structure of Lipid A



HM: hydroxymyristate

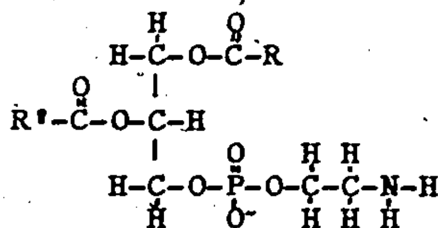
R: fatty acid residue

The structure for lipid A of Salmonella minnesota shown in Diagram 3 is tentative (38), that of E. coli is not completely elucidated and appears to consist basically of a polymer of N-acylated D-glucosamine units some of which are phosphorylated (39).

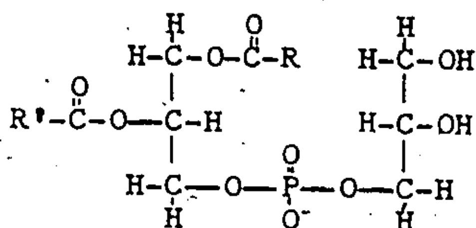
The Phospholipids of E. coli

The major phosphoglycerides of E. coli have been identified as phosphatidylethanolamine (PE), corresponding to 75% of the total lipids of growing cells, phosphatidyl glycerol (PG) and cardiolipin (CL) accounting for most of the remainder. Minor lipids such as phosphatidylserine (PS), lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), phosphatidylglycerophosphate (PGP), CDP-diglyceride (31) and phosphopolyisoprenoids (36) have also been reported to occur although their identification in some cases is not yet unequivocal (31) The structures of the major phospholipids of E. coli are illustrated in Diagram 4.

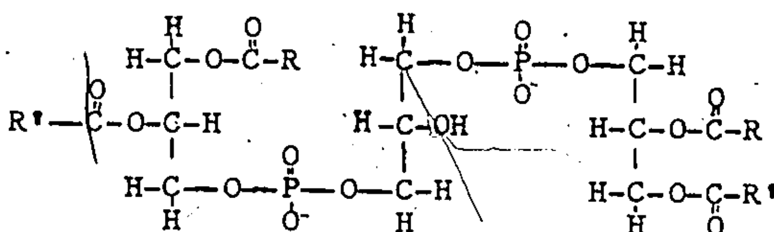
Diagram 4: Structure of the principle phospholipids of Escherichia coli



PHOSPHATIDYLETHANOLAMINE



PHOSPHATIDYLGLYCEROL



CARDIOLIPIN

The Characterization of Major E. coli Phospholipids

Phosphatidylethanolamine has been identified by cochromatography with authentic PE in many chromatographic systems, by determination of the molar ratios of constituent groups, by the production of glycerophosphorylethanolamine upon mild alkaline hydrolysis, by degradation with specific phospholipases to the expected products and by infrared spectroscopy (31).

Phosphatidylglycerol has been characterized by cochromatography with authentic PG the determination of the molar ratios of constituent groups, the presence of

vicinal hydroxyl groups in the intact lipid and the formation of glycerophosphorylglycerol by mild alkaline hydrolysis (31).

Cardiolipin has been identified by cochromatography with beef heart cardiolipin, the finding of glycerol, phosphate, and acyl esters in a 3:2:4 ratio, the formation of di(glycerophosphoryl)-glycerol upon mild alkaline hydrolysis and by degradation with specific phospholipases (31).

Some Properties of E. coli Phosphoglycerides

(1) Phosphatidylethanolamine

Phosphatidylethanolamine, the most abundant phospholipid of *E. coli*, is a slightly acidic, amphipathic substance and can form bilayers when mixed with other lipids. It forms typical liposomes with the lipopolysaccharide of *E. coli* and this interaction involves a stoichiometry of 5-10 molecules of phosphatidylethanolamine for 1 molecule of lipopolysaccharide as well as hydrophobic bonding since a high salt concentration does not dissociate the complex (40).

The main function of PE is likely structural but a role for its participation in the biosynthesis of lipopolysaccharide of *E. coli* has been described by Rothfield (40). PE was found to be essential and to act

catalytically in the transfer of sugar residues from a nucleoside sugar precursor to the non-reducing end of the growing polysaccharide chain forming the core of the lipopolysaccharide. Phosphatidylcholine (PC) was inactive in this capacity. Rothfield surmised that changes in the charge on the polar headgroup are not responsible for the effect and the difference is probably due to steric factors and the state of hydration of polar groups of choline.

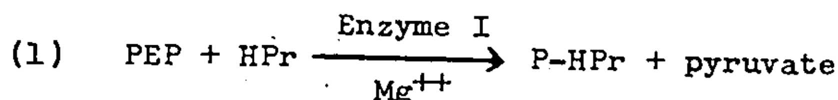
(2) Phosphatidylglycerol

Phosphatidylglycerol is usually the second most abundant phosphoglyceride in E. coli representing at most, 15 percent of the total phospholipid. It, too, is an acidic phospholipid more so than PE.

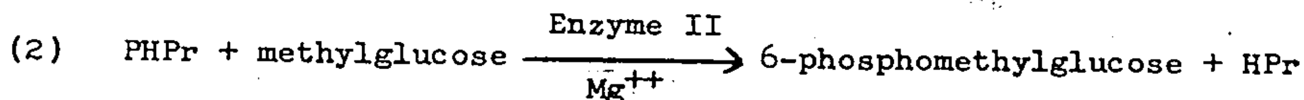
In Staphylococcus aureus and other Gram-positive bacteria PG is found partly as an aminoacyl derivative. Haest et al (41) showed that when PG/lysyl-PG ratio is increased in the staphylococcus membrane, the uptake of positive ions is also increased because of the greater negative charge in the lipid polar headgroups. They showed also that the penetration of non-electrolytes in lysyl-PG monolayers is greater than in PG monolayers, a result which can be explained on the basis that lysyl-PG has polar groups which are more bulky. Thus more loosely packed monolayers are formed. From this evidence one

could propose that the degree of aminoacylation of PG in the membrane influences the permeability of Gram-positive cells to electrolytes and non-electrolytes. In <u>E. coli</u> aminoacyl esters of PG have been systematically sought (31) but not found.

In many bacteria including <u>E. coli</u> the transport of monosaccharides and their analogues is mediated by phosphoenol pyruvate (PEP) phosphotransferase. The biochemical and genetic evidence for this was reviewed recently by Kaback (32). The reaction proceeds in two steps as follows:



$$(1)\quad \text{PEP} + \text{HPr} \xrightarrow[\text{Mg}^{++}]{\text{Enzyme I}} \text{P-HPr} + \text{pyruvate}$$



$$(2)\quad \text{PHPr} + \text{methylglucose} \xrightarrow[\text{Mg}^{++}]{\text{Enzyme II}} \text{6-phosphomethylglucose} + \text{HPr}$$

HPr is a low molecular weight heat-resistant protein. In some species, but not in <u>E. coli</u>, reaction 2 requires as well, a third component, Factor III. Kundig and Roseman were able to purify partially enzyme II from <u>E. coli</u> membranes and showed that it had a strict requirement for PG (c.f. ref. 32). The implication of PG in the transport of sugars was confirmed in the laboratory of Kaback (33) who found that specific destruction

of this lipid by treatment of isolated membrane preparations with phospholipase D resulted in loss of activity to transport and phosphorylate α-methylglucose.

(3) Cardiolipin

The third major phospholipid described for E. coli is cardiolipin which accounts for at least five to ten percent of the total phospholipid. Like other lipids, its role is likely structural. However from the unique structural features of this lipid i.e., its strong acidity and its two apolar diglyceride moieties one might expect it to have distinctive physicochemical properties. Shah and Shulman (42) in fact showed that in contrast to other phospholipids, the surface-pressure area curves of CL contract by some 10-13 percent in the presence of divalent metal ions. As they suggested, this contraction would involve a reduction in the size of the phosphodiester parts of the molecule by appropriate rotation of bonds. Since divalent cations bound to CL, can be replaced by Na ions, one might expect that changes in ionic environment would cause conformational changes in CL which in turn would affect membrane structure.

Other than a structural role, no definite function has yet been assigned to CL in E. coli. It is remarkable however that the levels of this lipid increase with factors

detrimental to the cell such as aging, starvation, the presence of colicins or various inhibitors of oxidative or substrate level phosphorylation, other factors which interfere with either protein synthesis or cell wall synthesis and yet other agents such as organic solvents which directly attack the membrane (31).

(4) Other Phosphoglycerides

It is very likely that the main function of minor phospholipids such as phosphatidic acid, phosphatidylglycerophosphate, phosphatidylserine and CDP-diglyceride is to serve as intermediates in the biosynthesis of the major phosphatides.

The Phosphoisoprenoids

The occurrence and function of phosphoisoprenoids has been recently reviewed by Lennarz and Scher (37). Their role in glycosylation reactions of bacteria and yeast is now well established.

Polyisoprenoid alcohol phosphates are a minor class of lipids present in the membranes of various bacteria. They act as carriers of substituted saccharides in glycosylation pathways leading to formation of complex polysaccharide structures. Usually the polysaccharide material that is formed serves as part of a coat external to the cytoplasmic membrane.

In the synthesis of peptidoglycans, undecaprenylphosphate reacts with UDP-N-acetyl muramyl pentapeptide to yield the undecaprenyl pyrophosphate derivative of the glycopeptide and UMP. Since the polyisoprenoid unit is buried within the lipid core of the membrane, this reaction serves to anchor the glycopeptide moiety to the membrane. Glycosylation with UDP-N-acetyl glucosamine then follows and the peptidyl disaccharide that is formed remains attached to the lipid carrier until it is transferred to a suitable acceptor, i.e., the growing peptidoglycan unit. Very likely this acceptor is itself attached to the membrane via a polyisoprenoid unit so that one can picture the formation of peptidoglycan as a process remaining bound to the cytomembrane until completion. Otherwise, the diffusion of intermediates into the growth medium would prevent an organized synthesis of wall material.

In a somewhat similar manner, phosphopolyisoprenoids are involved as saccharide carriers in the synthesis of O antigen of lipopolysaccharides (36,37). They are also involved in the formation of yeast mannan and capsular polysaccharide of certain bacteria (37) and possibly in the synthesis of teichoic acids characteristic of Gram-positive bacteria (37).

Fatty Acid Composition of *E. coli* Lipids

The fatty acid composition of *E. coli* lipids includes palmitic acid as the predominant saturated constituent, palmitoleic and cis-vaccenic as the major unsaturated analogues as well as C_{17} and C_{19} cyclopropane fatty acids in lower amounts (31,43). The cyclopropane fatty acid content increases with age. Myristate, laurate, and β -hydroxy myristate have also been found (31).

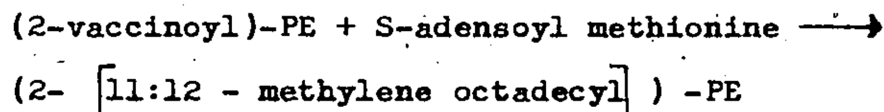
Synthesis of Fatty Acids by *E. coli*

The synthesis of fatty acids in *E. coli* has been reviewed by several authors (44,45,46,47) and can be summarized as indicated in Scheme I. Basically the scheme involves the addition of C_2 units to acetyl acyl carrier protein (ACP) until β -hydroxydecanoyl ACP is formed. At this point branching in the pathway occurs to yield either saturated or unsaturated fatty acids. The branching depends on the presence of β -hydroxydecanoyl ACP dehydrase activities, which can form 3-cis-decanoyl ACP the precursor of palmitoleic and cis-vaccenic acids and one which can form 2-trans-decanoyl ACP the precursor of palmitic acid. It is noteworthy that the synthesis of unsaturated fatty acids in *E. coli* is completely anaerobic and involves synthesis *de novo*. By contrast, animal and plant cells as well as certain microorganisms form their unsaturated fatty acids from saturated analogues and make use of desaturase systems requiring oxygen (48,49).

The first enzyme in the sequence, acetyl CoA carboxylase is a multienzyme complex. One subunit, biotin carboxyl carrier protein contains covalently-bound biotin which is carboxylated by biotin carboxylase, another subunit, in the presence of ATP and HCO_3^-. The carboxyl group is transferred from carboxy-biotin to acetyl CoA to form malonyl CoA by a transcarboxylase, a third subunit (50).

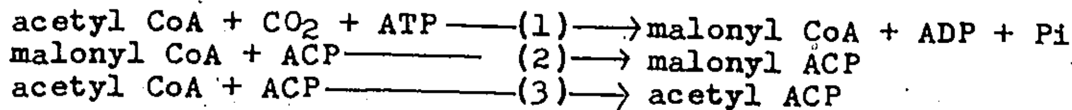
Whereas, in mammalian and yeast cells the fatty acid biosynthetic enzymes exist as an undissociated multienzyme complex of the cytosol (47,51), in E. coli and certain plants these same enzymes are present as soluble, easily dissociated complex (47). Even in E. coli however, the biosynthetic system is structured since there is now evidence to show that ACP is located close on the inner surface of the cell membrane (31).

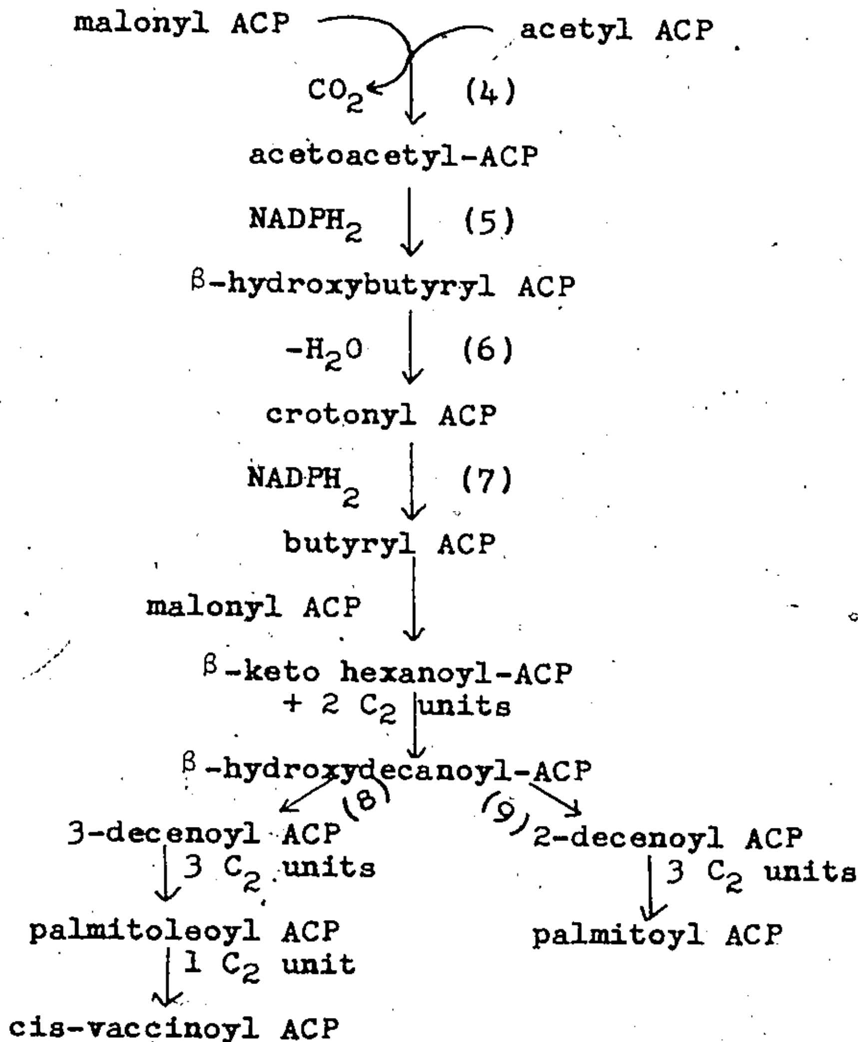
Cyclopropane fatty acids are formed by the transfer of a methyl group to the double bond of palmitoleate or to cis-vaccinate, resulting in a type of substituted fatty acid (52,53). The transfer of the methyl group is to an unsaturated fatty acid moiety of a phospholipid rather than to a free acid (54,55,56) according to the following reaction



(2-vaccinoyl)-PE + S-adensoyl methionine ⟶
(2- [11:12 - methylene octadecyl]) -PE

Scheme I



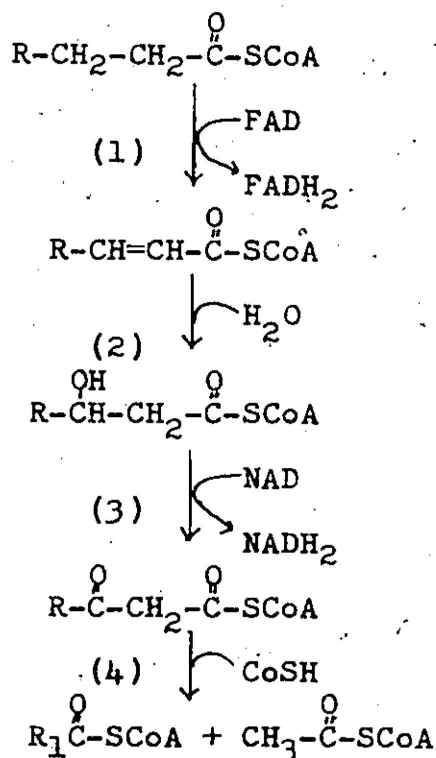


(1) Acetyl CoA carboxylase
(2) Acetyl CoA acyl carrier protein transacylase
(3) Malonyl CoA acyl carrier protein transacylase
(4) β-keto acyl carrier protein synthetase
(5) β-keto acyl carrier protein reductase
(6) β-hydroxy acyl carrier protein dehydrase
(7) Enol acyl carrier protein reductase
(8) (β,γ) hydroxydecanoyl acyl carrier protein dehydrase activity
(9) (α,β) hydroxydecanoyl acyl carrier protein dehydrase activity

Oxidation of Fatty Acids in *E. coli*

Although fatty acids are not the best sources of carbon for *E. coli* growth, β-oxidation enzymes (see Scheme II) can be induced in this organism by the presence of exogenous fatty acids (57,58). Only in mutants are these enzymes constitutive (57). The role of these enzymes may be other than to meet the energy demands of the cell. This idea is also generally supported by the work of Silbert et al (59).

Scheme II

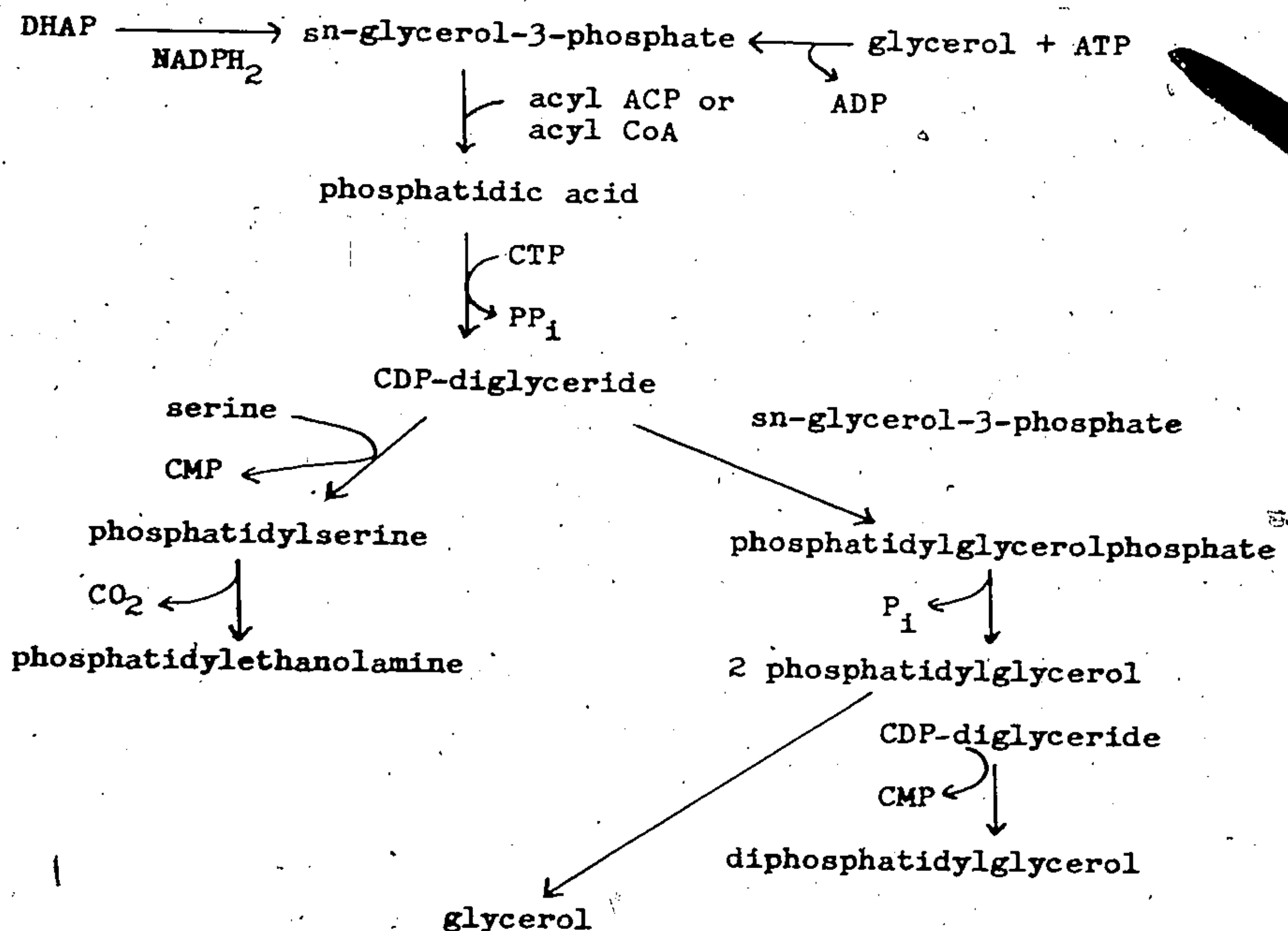


(1) Acyl dehydrogenase
(2) Enoyl hydrase
(3) β-hydroxy acyl dehydrogenase
(4) Thiolase

The Biosynthesis of Phosphoglycerides

The synthesis of the three major phospholipids of *E. coli* has been described in several articles by Kennedy and co-workers (60-64). These and other contributions to this field have been reviewed recently by Cronan and Vagelos (31) and can be summarized according to Scheme III.

Scheme III



Most of the enzymes involved have been found in the cell envelope (31,60-64) and tentatively so in the inner cytoplasmic membrane (65,66). The phosphorylation of glycerol and the formation of PS from CDP-diglyceride and serine however appear to involve soluble enzymes (41).

(I) Glycerol-kinase and DHAP Reductase

Glycerol-kinase is a cytosol enzyme which specifically phosphorylates glycerol to form sn-glycero-3-phosphate (67). *In vitro* glycerol alone cannot serve as precursor of phosphatides unless ATP is added (68). In the absence of glycerol, the formation of sn-glycero-3-phosphate can be achieved by a pyridine-nucleotide linked sn-glycero-3-phosphate dehydrogenase which reduces dihydroxyacetone phosphate (DHAP) obtained from glycolysis to produce sn-glycero-3-phosphate required for lipid synthesis. Using a mutant lacking this particular dehydrogenase Hsu and Fox (69) showed that glycerol starvation produced a block in lipid synthesis. The enzyme prefers reduced NADP although NADH can also serve as cofactor. The product of the reaction, sn-glycero-3-phosphate inhibits the enzyme thereby regulating its own synthesis (70).

(2) The Biosynthesis of Phosphatidic Acid

Ray et al (71) reported evidence that the acylation of sn-glycero-3-phosphate involves two activities, one that catalyzes the positionally-specific acylation of sn-glycero-3-phosphate by either saturated or unsaturated fatty acyl CoA to form monoacyl glycero-3-phosphate, and a second activity which acylates 1-acyl-glycero-3-phosphate to form phosphatidic acid.

It is now known that either acyl CoA or acyl ACP can serve as acyl donors. When palmitoyl ACP is used to acylate glycerophosphate either monoglyceride or lyso PA is formed but not phosphatidic acid. The appearance of monoglyceride results from the action of phosphatidate phosphohydrolase on lyso PA (72). Phosphatidate formation occurs when CoA esters are used with sn-glycerophosphate acceptor or when unsaturated acyl ACP or acyl CoA is the donor and 1-acyl-sn-glycero-3-phosphate is the acceptor. It appears that the acyltransferase (72) responsible for acylation at position 2 is quite specific for unsaturated acyl groups especially when acyl carrier protein is involved.

Phosphatidate can also be formed in E. coli by diglyceride kinase which can phosphorylate diglyceride and monoglyceride (68,73). This enzyme together with phosphatidate hydrolase could operate as a cycle as was suggested for animal tissues (72), but there is no direct evidence in support of this.

In fact, Chang and Kennedy (61) were unable to find a metabolically active pool of diglyceride in viable cells and moreover, glyceride synthesis under these conditions was very limited. They concluded that sn-glycero-3-phosphate is an obligate intermediate in the formation of phosphoglycerides. To date the function of DG kinase and PA hydrolase in E. coli remains obscure.

Another pathway for PA formation involves direct acylation of DHAP to be followed by reduction of the keto-lipid and further acylation. This pathway has been demonstrated in mammalian mitochondria (74,75). The possible occurrence of this metabolic sequence in E. coli is part of this investigation.

Synthesis of CDP-Diglyceride

CDP-diglyceride is formed by the action of CTP: phosphatidate cytidyl transferase. This is a particulate enzyme studied in crude form by Carter (76). The enzyme requires Mg^{++} and shows optimal activity at pH 6.5: above pH 7.0 and below pH 5.8 the rate of reaction drops rapidly. Magnesium ions play an interesting role in vitro as it has two separate inhibiting effects at high concentrations, i.e., both phosphatidic acid and the enzyme are precipitated by this cation.

Synthesis of Phosphatidylserine

Phosphatidylserine formation is catalysed by an L-serine-CMP: phosphatidyltransferase. Raetz and Kennedy (77) located this enzyme on the ribosomes of E. coli. They suggested that its presence there rather than in membrane, might be a means by which the cell coordinates synthesis of protein and membrane lipids. On the other hand Vagelos and Cronan (31) found a considerable portion of this enzyme in the washed envelope fraction provided the cells were gently lysed.

Synthesis of Phosphatidylethanolamine

Synthesis of phosphatidylethanolamine is catalysed by phosphatidylserine decarboxylase. This enzyme has been extracted from the membrane and purified to homogeneity (78). It requires no divalent cations but since the activity is inhibited by hydroxylamine and 4-bromo-3-hydroxylbenzyloxyamine, the enzyme may require a pyridoxal cofactor.

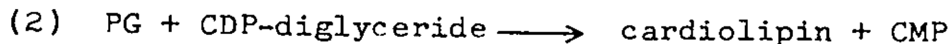
Synthesis of Phosphatidylglycerol

The synthesis of phosphatidylglycerophosphate (PGP) from CDP-diglyceride and sn-glycero-3-phosphate is catalysed by sn-glycero-3-phosphate: CMP phosphatidyl transferase (63). The enzyme is particulate, requires Mg^{++}

or Mn^{++}, detergent, and a pH of 8.0 for optimal activity. The K_m values for CDP-dipalmitin and sn-glycero-3-phosphate are 2.5×10^{-5}M and 2.5×10^{-4}M respectively. The product of this reaction is then hydrolysed by a specific phosphohydrolase to yield PG and inorganic phosphate (64). PGP phosphatase which is much more active than the preceding enzyme requires Mg^{++} and is stimulated by Triton X-100. It does not hydrolyze sn-glycero-3-phosphate or phosphatidic acid.

Cardiolipin Synthesis

Stanacev et al (62) were the first to describe the conversion of PG to cardiolipin in *E. coli*. CDP-diglyceride had a stimulatory effect on this reaction and on this basis it was believed to act as a substrate according to reaction 2.



(2) PG + CDP-diglyceride $\longrightarrow$ cardiolipin + CMP

Recent evidence by Hirschberg and Kennedy (79) and by Hostetler et al (80) showed that the mechanism of this reaction involves the condensation of two molecules of PG with the elimination of glycerol. CDP-diglyceride stimulates but does not participate as a donor in this reaction. This mechanism has been supported by the earlier work of Lusk (81) and Rampini (82).

Turnover of Phosphoglycerides in *E. coli*

It is well-established from the work of Kanfer and Kennedy (83) and other workers (84-86) that normally growing cells display turnover of polyglycerophosphatides but not of PE. In isotopically-labelled cells exposed to cold, however, the turnover of PE has been noticed along with an increased turnover of polyglycerophosphatides and neutral lipids. In such cells, neutral lipid acyl groups were reported to be converted to phosphatides such that there was no actual loss of phospholipid during turnover. Rather than a loss, cold exposure increased total phospholipid content which was likely due to an augmented synthesis of unsaturated species (85).

Bright-Gaertner and Proulx (86) confirmed the results of Kennedy et al for normally growing cells. In cold-exposed cells however there was no turnover of PE and diminished turnover of PG and CL. The main effect of cold was to increase the total lipid phosphorus and the proportions of newly synthesized unsaturated phosphatide species. This change was accompanied by a diminished conversion of unsaturated to cyclopropane fatty acid-containing species. At either 37°C or 10°C the turnover of acyl groups in the 1 and 2 positions of PE and PG was parallel and so was the turnover of ^{32}P and ^{14}C labelled phosphatides studied in the same culture. There appeared to be no partial turnover

pathways in operation when cells were chased at different temperatures under the conditions specified by these authors.

The turnover of phosphatidylglycerol can be explained by its conversion to cardiolipin, however, there is yet no definite proof that this is the sole mechanism involved. The turnover of cardiolipin is also ill-explained. Part of the present thesis is involved with further elucidation of PG and CL metabolism.

Enzymes Involved with Breakdown of *E. coli* Lipids

Investigations with cell homogenates by Proulx and Fung indicated the presence of phospholipase A_1 and lysophospholipase as the predominant lypolytic enzymes of *E. coli* (87,88). Other activities reported such as phospholipase C (89,90,91) and phospholipase A_2 (88,91) have been detected in different strains by independent workers but have not been studied in detail. A survey in this laboratory indicated the absence of phospholipase C in all of several strains studied. Phospholipase A_2 activity on the other hand may represent the combined action of phospholipase A and residual lysophopholipase activity, not inhibited by detergents although some authors claim otherwise (91,95-97).

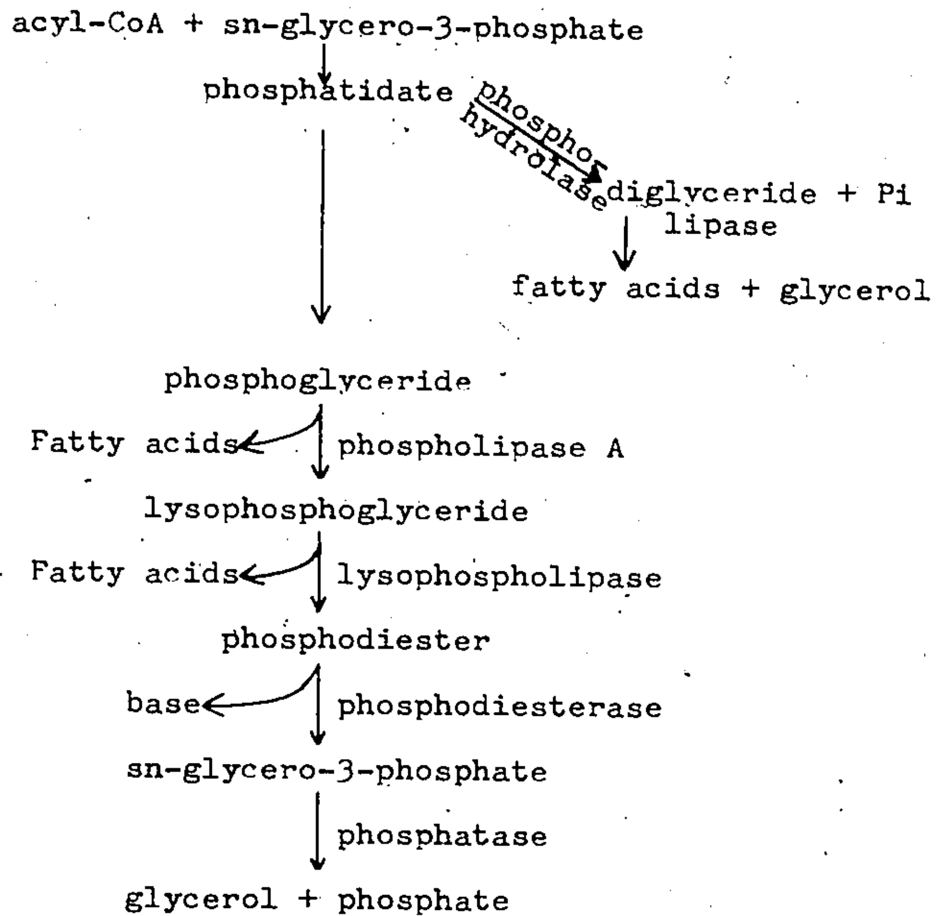
Present indications are that there exist in *E. coli* several types of phospholipase A. There is a detergent-resistent phospholipase A (92) which is membrane bound and appears to be identical to the alkaline phospholipase A_1 detected by Proulx and Fung (87,88) and purified by Scandella and Kornberg (93). There likely exists an acid phospholipase A_1 detectable in whole homogenates (88) and in spheroplasts (94) which is detergent sensitive: either this, or acid pH favors the conversion of a zymogen to the well-characterized alkaline phospholipase A. There is perhaps also an alcohol-activated phospholipase A which is tightly bound to the membrane and releases either free fatty acids or forms esters with the alcohol used (92). This enzyme may be identical with the purified phospholipase A_1 described by Scandella and Kornberg although there is yet no definite evidence indicating this. There appears to be another alcohol-activated phospholipase A which is either loosely bound to the membrane or is easily releasable by sonication, and is not markedly activated by detergents. It attacks the 2-acyl ester bond of phosphoglycerides (90,94-96). Finally there is a cytosol phospholipase A which is inhibited by alcohol or detergents and hydrolyzes readily only PG (92). Its positional specificity is unknown. The other phospholipases A have broad

substrate specificity and act on all _E. coli_ lipids as well as PC (87-93).

Van den Bosch and Vagelos (72) have also reported the presence in _E. coli_ of a phospholipase hydrolysing phosphatidate or its lyso analogue to glycerides and inorganic phosphate. The function of this enzyme is obscure since in _E. coli_ phospholipid synthesis does not occur by addition of diglyceride to a cytidine nucleotide-activated base. This enzyme may form a cycle with diglyceride phosphokinase also present in this organism, the sum reaction being ATPase activity. Such apparently futile pathways are generally well regulated and operate in only one direction for a given set of physiological conditions (viz. the phosphofructokinase, fructosediphosphatase reactions of glycolysis). Alternatively, the phosphatidate phosphatase may operate in sequence with lipase (98) to liberate fatty acids and glycerol and thus serve in some way to shut off phospholipid synthesis.

The overall catabolic sequence elucidated by _in vitro_ studies is summarized in Scheme IV.

Scheme IV



Appropriate phosphoesterase activities have been detected in *E. coli* which complete the degradation by lipolytic enzymes (90).

The Function of Lipolytic Enzymes in *E. coli*

Despite the presence of the enzymes described in Scheme IV, it is doubtful they are all operative in normally growing cells. In studies on turnover of *E. coli* phosphoglycerides, lysophosphoglycerides are never detected as breakdown intermediates. One could argue that either lysophospholipase activity or reacylation of such intermediates would prevent their detection. This is possible, however, when *E. coli* cells are damaged lysophosphoglycerides accumulate in large proportions and in this case at least their removal appears to be quite slow (98). Although reacylation of lysophosphatides has been shown to occur in *E. coli* homogenates (99), this pathway is not generally operative *in vivo* since various experiments by Bright-Gaertner and Proulx have shown that there is no sparing of acyl groups at either position 1 or 2 of phosphoglycerides during their turnover. It would seem that phospholipase A is an enzyme involved with autolysis rather than a functional turnover of phospholipid. It may be added that phospholipase purified by Scandella and Kornberg (93) was found to contain

lysophospholipase activity and results in this laboratory indicate that it possesses lipase activity as well. The possibility that this enzyme is a non-specific esterase has to be further explored.

There appears to be a distinct cytosol lysophospholipase in E. coli (unpublished data Nantel and Proulx) but a similar enzyme purified from mammalian tissues has the properties of a non-specific esterase (Van den Bosch personal communication to Dr. Proulx). Again the function of this lipolytic enzyme in E. coli has to be clarified.

Regulation of Fatty Acid and Phospholipid Biosynthesis

Temperature Effects

The degree of unsaturation in E. coli lipids is well known to depend on temperature of growth. Cold exposure results in the greater synthesis of unsaturated phosphatide species, a diminished formation of saturated and cyclopropane fatty acid species. There is evidence indicating that conversion of unsaturated acyl chains to cyclopropane acyl chains is diminished by cold shock but this would not constitute the sole mechanism for increasing unsaturated fatty acids levels (86). Temperature-sensitive control could be expected at the level of fatty acid synthesis itself and at the level of phosphatide formation. Starting with exogenous fatty acids, the incorporation process is known to involve

first the formation of acyl CoA derivatives and second, the transacylation of acyl CoA to sn-glycero-3-phosphate. Sinensky has studied the effect of temperature on these two enzymes of E. coli (100). The rate of formation of palmitoyl and oleoyl-CoA in vitro was the same whether the cells were grown at 25°C, 34°C or 42°C. However, the palmitate 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 phosphatides produced in response to changes in environmental temperature.

With respect to control acting 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 (101). This led the authors to conclude that fatty acid synthesis is regulated in such a manner as to supply the fatty acids necessary for minimizing variations in the physical properties of the phospholipids.

Effect of Changes in Energy Metabolism

The transition from logarithmic to stationary phase in E. coli cultures is accompanied by an increased conversion of PG to cardiolipin (102,103). This conversion also occurs during the following conditions; addition of colicin K (104) dinitrophenol (104) penicillin (102) or cyanide (105) and infection with bacteriophage (105). These and other conditions (31), although very diverse, have one property in common, they all cause a decrease in cellular phosphorylating ability. Lusk and Kennedy (81) showed a similar increase of PG conversion to cardiolipin when cells were exposed to high concentrations of Na^+. This distorted metabolism was normalized however by the addition of Mg^{++}.

Effects of Medium Composition

The amount of lipid in E. coli has been shown to be independent of the growth medium used (106). Many carbon sources have been altered, but no significant differences were seen in the lipid or fatty acid composition (106). Starvation or limitation of phosphate or of carbon source does not decrease the amount of phospholipid and produces no marked changes in the fatty acid composition of the phospholipids. From these observations Cronan and Vagelos (31) suggest that phospholipid and fatty acid

biosyntheses in normally growing *E. coli* are not regulated at the level of small molecule precursors. These same authors however point out the inducibility of acyl CoA synthetase, and of the β-oxidation enzymes and underline conditions in the medium which interfere with the energy metabolism or result in a shift of cation levels, all of which affect phosphatide levels. On this basis, it would seem that further work with whole cells homogenates and purified enzymes might be warranted in order to preclude or ascertain simple regulatory mechanisms.

Effects of Simple Metabolites *in vitro*

It is very likely that the rate of phospholipid synthesis is controlled in some way by mechanisms which are related to membrane synthesis. This does not preclude however the possibility of simple metabolite regulation. The control of phosphatidate biosynthesis in cell free systems has not been studied in great detail to date. Kito and Pizer (107) found that phosphatidate itself was ineffective in inhibiting the acylation of sn-glycero-3-phosphate. However the effectiveness of this type of feedback might not be easily noticeable unless lipids are added in a suitably micellized form. Their studies did nevertheless show that the incorporation of palmitate into PA was markedly inhibited by low ATP concentrations.

It can be emphasized that the biosynthetic route for E. coli lipids is a branched pathway. As will be stated later, there are many examples indicating that simple metabolite regulation occurs in pathways at a branch point as well as prior to such a site. Furthermore, in E. coli where relative levels of polyglycerophosphatides and PE are quite predictable for a given condition of growth, simple and rapid control mechanisms acting on phosphoglyceride metabolism are likely to operate. Part of the present thesis is involved with the detection of such mechanisms.

AIMS OF RESEARCH

A. Preliminary evidence indicated that ATP and Mg^{++} stimulated the incorporation of sn-glycero-3-phosphate into phosphoglycerides, particularly phosphatidylglycerol. The first aim of this thesis was to define the effects of ATP and Mg^{++} on phosphoglyceride metabolism under optimal cofactor and substrate concentrations.

B. To investigate the occurence of the acyldihydroxyacetone phosphate pathway and the diglyceride kinase pathway in *E. coli* and to see if these pathways are functional in the synthesis of more complex lipids.

C. To study the catabolism of polyglycerophosphatides *in vitro* and to account for their turnover which occurs in growing cells.

GENERAL METHODS

Preparation of E. coli Cells:

The culture of E. coli used for most studies was supplied by Dr. M. Beaulieu of the Department of Bacteriology at the University of Ottawa. Typing of the strain as E. coli 015 was performed by Miss Rhoda Laidley and Mr. D. Peters at the Laboratory Centre for Disease Control, Health and Welfare Canada. E. coli B (ATCC 11303) was obtained from the American Type Culture Collection.

The stock culture was maintained on nutrient agar slants. Litre batches of broth in shallow flasks were inoculated with a needle from freshly grown slants of nutrient culture. The broth medium contained per litre, 15 grams of bactopeptone, 1 gram of yeast extract, 20 grams of glucose and 5 grams of sodium chloride, and this was adjusted to pH 7.3 with 0.1 N sodium hydroxide. After 7 hours of growth on a rotary shaker at 37°C the pH of the medium was 4.6 and the optical density of the culture was about 0.26 at 630 nm. The growth curve is illustrated in Fig. 1. The purity of the cell culture was checked by routine methods, on MacConkey and citrate agar and staining by Gram's method. The cells were harvested by centrifugation at 5,000 g for ten minutes in a Lourdes refrigerated centrifuge at 5°C. The sediment was washed in 0.01 M phosphate

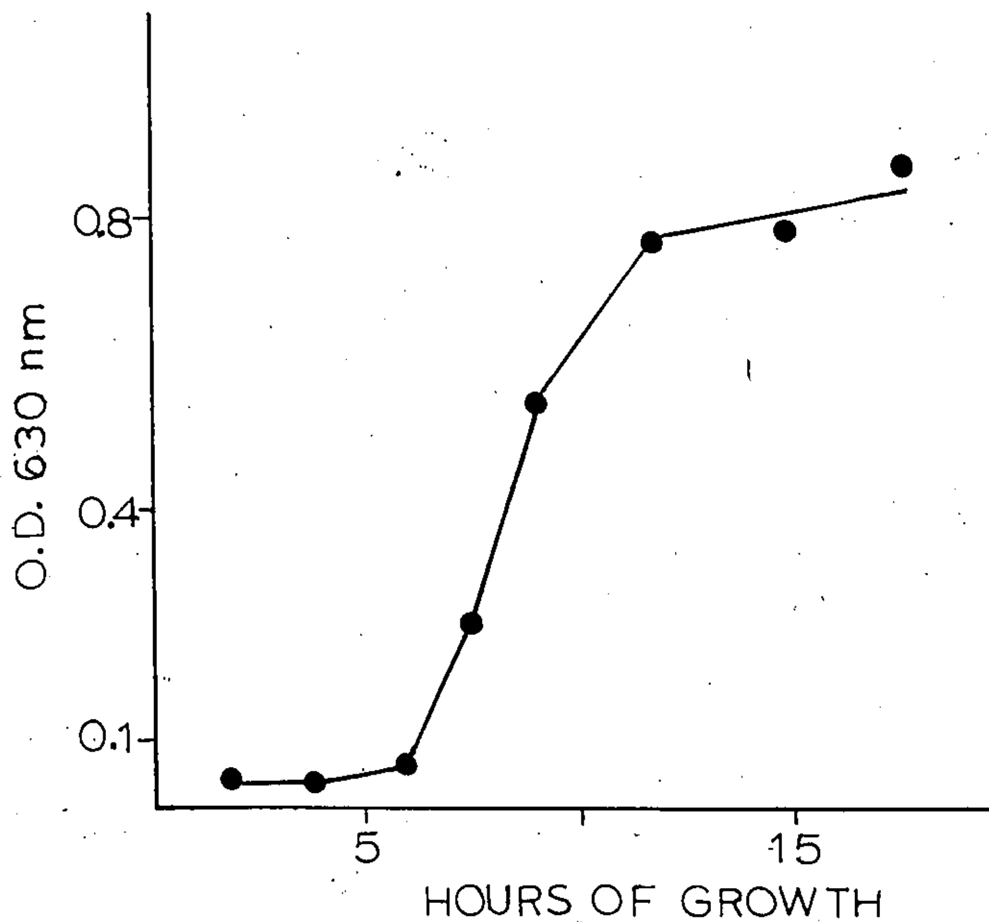


Figure 1. Growth curve of E. coli O15.

buffer pH 7.3 and resuspended in 25 ml. volume of buffer.

Preparation of Cell Homogenates:

The suspended cells were disrupted by intermittent sonication in a salt-ice mixture for 15 minutes using a Biosonik II Ultrasonicator (Bronwill Scientific Co.) at 125 w. Unbroken cells were removed by centrifuging 10 min. at 3,000 g.

The protein concentration in the sonicated cell suspension was assayed by the method of Lowry et al (108). The homogenate prepared as described contained 20 - 25 mg. protein per ml.

Extraction of Lipids

Labelled lipids were extracted by adapting the method of Bligh and Dyer (109) to our purposes. The incubation mixture (either (A) 0.8 or (B) 1 volume) was mixed with 2.5 volumes of methanol and 1 volume of chloroform. This monophasic mixture was stirred for 30 minutes at room temperature and 1 volume each of chloroform and water were added. The resulting biphasic system was stirred 20 min. and separated by centrifugation. The lower chloroform layer was then transferred to an evaporating flask with a Pasteur pipet. The top aqueous phase was re-extracted with one volume of chloroform and stirred 20 minutes. The mixture was centrifuged and the lower chloroform extract was pooled

with the first and evaporated to dryness. The final proportions of extraction solvents used in case A gave a point in the chloroform-methanol-water phase diagram just slightly above the maximum chloroform tie-line estimated at 0°C by Bligh and Dyer. The extraction of lipids was quantitative in both case A and B.

Some incubation mixtures contained ether. This solvent was removed by evaporation at reduced pressure before extraction of lipids.

Separation of Lipids

Neutral Lipids

Neutral lipids were separated in silica gel G plates with a mixture of petroleum ether (B.P. 60°-90°)-ether-formic acid, 55:45:1.5 by volume (system A) or 75:25:1.5 by volume system B. The adsorbent was applied as a slurry of 25 gms. in 50 ml. of water, to glass plates 20 x 20 or 20 x 5 cm, and after drying at room temperature were activated at 110°C for 1 hour. System B was especially useful for the separation of monoglyceride from phospholipids remaining at the origin.

Phospholipids

Several chromatography systems were used for separating and identifying phospholipids.

System C: A slurry of 25 g silica gel H in 50 ml. 1 percent sodium bicarbonate, was spread as a layer 0.75 mm thick on glass plates (110). These were activated 1 hour at 110°C just before use. Chromatograms were developed with chloroform-methanol - 1 N NH_4OH 80:36:2 by volume.

System D: A slurry of 25 gms. silica gel G in 50 ml. of water was spread as a layer 0.25 mm thick. After activation for 1 hr. at 110° and application of sample, the chromatograms were developed in chloroform: methanol:water, 65:25:4 by volume.

System E: In a method described by Randerath (111) for the separation of acidic detergents, the adsorbent consisted of silica gel G impregnated with ammonium sulfate. We modified this method for the isolation of phosphatidylglycerol. A slurry of 25 gms. of silica gel H in 75 ml. of water was spread as a layer .25 mm thick. After drying in air, the plates were activated at 110°C for 2 hrs. and used immediately after cooling. The chromatograms were developed with chloroform:methanol:water 65:25:4 v/v/v.

System F: This system was similar to system D except that the developing solvent consisted of a mixture of chloroform:methanol:ammonia:water, 70:30:4:2 by volume.

System G: This system was similar to system D except that a mixture of tetrahydrofuran:methylal:methanol:4N aqueous ammonia, 10:5:5:1 by volume was used as developing solvent (112).

System H: Phospholipids were separated on silicic acid impregnated paper prepared as described by Marinetti (113) using Whatman 3 mm filter paper. The solvent mixture used was 2,6-dimethyl-4-heptanone:acetic acid:sodium chloride (.85%), 40:20:3 by volume.

System I: This system was similar to system D except that the developing solvent consisted of a mixture of chloroform:methanol:acetic acid, 65:25:8 by volume (114).

R_f values of various lipids using these systems are given in Table Ia.

Localization and Isolation of Components

A. General Procedures:

Lipid components were routinely revealed by exposure of the chromatograms to iodine vapors however such iodine-stained lipids were not usually used as substrates. Iodine was removed by aeration prior to further staining or to counting.

Table Ia

R_f Values of Lipid for Various Chromatographic Systems

	Chromatographic System								
	A	B	C	D	E	F	G	H	I
PG			.75	.37	.69	.35	.53	.60	.60-.65
PE			.57	.52	.51	.51	.03	.50	
CL			.80	.74	.81	.35-.40	.50	.81	.83-.86
PA						.05-0.15	0.09	.93	.83-.86
FA	.75	.50							
TG	.91	.75							
MG	.09	.03							
DG	.55	.19							

Labelled components were located by scanning with an Actigraph III Detector (Nuclear Chicago). When the material was to be retrieved, localization of nitrogen-containing lipids was accomplished by spraying chromatograms with a solution of Ponceau red. Ponceau red also stains other types of lipids but less effectively. The staining solution was prepared as follows: two grams of uranyl nitrate and 0.05 gms. of Ponceau red were dissolved in 1 litre of 0.01 N hydrochloric acid. The stained components were scraped off, suspended in water and extracted by the method of Bligh and Dyer (109). The Ponceau red dye dissociates from the lipid and partitions completely into the aqueous phase whereas the silica gel forms a layer between the two phases. Lipids were recovered by evaporating the chloroform phase and stored under nitrogen at -20°.

B. Amino Groups:

0.5% ninhydrin solution in acetone was used as a spray to detect phospholipids that contain free amino groups such as PE. After spraying the chromatoplate was heated 5-10 minutes at 110°C.

C. Vicinal Hydroxyl Groups:

To detect components which contained vicinal hydroxyl groups, the chromatoplate was sprayed with a 2 percent aqueous solution of sodium metaperiodate and allowed to react 5 to 10 minutes as described by Baddiley (115).

After the reaction with sodium metaperiodate was completed the chromatoplate was placed in a tank containing sulfur dioxide, then sprayed with Schiff's reagent and re-exposed to sulfur dioxide. Phosphatidylglycerol gave a typical violet colour. The Schiff reagent spray was prepared by dissolving 1 gm. of pararosaniline in 50 ml. of water. The solution was decolorized with sulphur dioxide and activated charcoal and diluted to 1000 ml. with water.

D. Phosphate Groups:

Lipids containing phosphate group were detected on chromatoplates by spraying with Hanes-Isherwood reagent (116), prepared as follows: Three gms. of ammonium molybdate were dissolved in 25 ml. of distilled water. To this was added 30 ml. of 1 N hydrochloric acid and 15 ml. of 60 percent perchloric acid. The reaction was allowed to develop at 105°C for 20 minutes at which time phosphate containing compounds appeared as blue spots.

Mild Alkaline Hydrolysis of Lipids

Intact phospholipid was hydrolyzed by the method of Dawson (117) as modified by Benson and Ferrari (118).

A chloroform extract of the sample to be hydrolyzed was reduced to 0.5 ml. and to this was added 5.0 ml. of a 0.2 N methanolic potassium hydroxide solution. The sample was incubated for 15 minutes at 37°C in a water bath to

effect hydrolysis, after which time it was immediately set in ice and 5 ml. of ice cold water were added. Two drops of 1% phenolphthalein were added and the hydrolysate was titrated with a suspension of 30 percent Dowex 50W - X8 H^+ form. The suspension was centrifuged, the resin washed with 3 ml. of water and 3 ml. of methanol and the original supernatant plus washings were evaporated to dryness at 50°C.

Extraction solvents were prepared as follows: to 100 ml. of chloroform in a separatory funnel 50 ml. of isobutanol and 75 ml. of water were added and shaken. One ml. of the upper phase and 2 mls. of the lower phase were added to the dried hydrolysate and mixed vigorously. The biphasic solution was transferred to a centrifuge tube and the flask was rinsed with the same proportion of both solvents. After centrifugation the upper layer was removed and evaporated to dryness. The residue was dissolved in water for counting or for paper chromatographic analysis.

Identification of Water-Soluble Products by Paper Chromatography

Water-soluble products were separated and identified by descending paper chromatography using Whatman No. 3 mm and the following solvent systems:

1. phenol/water, 5/2, w/w.
2. propanol/ammonia/water, 6/3/1, v/v/v.
3. butanol/acetic acid/water, 5/4/1, v/v/v.
4. 1M ammonium acetate pH 7.5/ethanol, 35/65, v/v.

R_f values of various compounds using these systems are expressed in Table Ib.

The developed chromatograms were scanned with a Nuclear Chicago Actigraph III scanner or stained with appropriate reagents described for thin layer chromatography. Water-soluble products containing phosphate were revealed by staining with the Hanes-Isherwood reagent modified by Garcia (119), as follows: 8.0 ml. of a 12.5% w/v solution of ammonium molybdate, 3.0 ml. of 11 N HCl, 12 ml. of 12 N perchloric acid and 86 ml. of acetone were admixed. After the chromatogram had been sprayed with this reagent, it was exposed to ultra-violet light for 15 minutes.

Liquid Scintillation Counting Procedures

Method A: To measure the radioactivity present in a lipid solution, an aliquot was transferred to 15 mls. of toluene containing 0.5% 2.5-diphenyloxazole and 0.03% 2-phenylenebis (5-phenyloxazole) w/v. Counting was usually performed with a Nuclear Chicago "Mark I" spectrometer. In later work, a Beckman LS 133 spectrometer was used in which

Table Ib

R_f Values of Products of Phospholipid Degradation for Various Paper Chromatographic Systems.

	Paper Chromatographic System			
	1	2	3	4
GPG	.46	.50	.15	.63
GPE	.63	.60	.24	.22
GPGPG	.15	.55	.05	.58
GP	.29	.18	.23	.29
Glycerol	.77	.74	.60	.77
PGP		.03		.04

case the secondary fluor was omitted. This method was used mainly for ^{3}H-labelled samples which required elution prior to counting. For elution, agitation of the silica gel in chloroform methanol 1:1 was effective although Bligh and Dyer (109) extraction of the lipid from the gel was the method usually chosen.

<u>Method B</u>: To measure the radioactivity in an aqueous sample, an aliquot not exceeding 100 ul was transferred to 15 mls. of scintillation solution described for method A admixed with 1 ml. of Triton X-100.

<u>Method C</u>: A thixotropic gel, Cab-o-sil, was used to suspend ^{14}C- or ^{32}P-labelled materials without prior elution from the adsorbent. Cab-o-sil (4 g/100 ml.) was added to the "cocktail" described for method A.

<u>Method D</u>: ^{14}C labelled components separated by thin layer chromatography were also counted directly without elution or the use of thixotropic gel (120). For this purpose, the scintillation mixture of method A was modified to contain 10 percent methanol and 0.35 percent acetic acid by volume.

For each counting procedure, quench curves were prepared with chloroform using a channel's ratio method. When simultaneous counting of ^{3}H and ^{14}C was

necessary, correction for contribution of ^{14}C counts in the ^{3}H channel were made using appropriate quench curves prepared as described in the Nuclear Chicago Mark I manual. Values obtained were periodically checked with internal standards by the sequential addition and counting of ^{3}H toluene and ^{14}C-toluene or appropriate water-soluble standards to a vial containing the pre-counted sample.

Reproducibility of Results

For most of these experiments at least two or more experiments were done. Occasionally the results of one experiment were reported to corroborate other evidence.

PART A

Factors Influencing Incorporation of sn-Glycero-3-Phosphate into *E. coli* Phosphoglycerides.

Introduction

The enzymes for the synthesis of phosphoglycerides in *E. coli* are known to require activated fatty acids in the form of acyl ACP or acyl CoA as well as sn-glycero-3-phosphate, CTP, Mg^{++} and serine. These requirements have been amply shown by the study of the individual steps of the synthetic sequence; however little is known about the effect of substrate, cofactor or product concentrations on the overall rate of lipid biosynthesis or on the relative yield of each phosphatide. The few studies that have been made with cell-free preparations have all been under conditions which produce only labelled phosphatidic acid, or its lyso analogue (100). No such studies were made under conditions allowing incorporation of sn-glycero-3-phosphate into both phosphatidylethanolamine (PE) and polyglycerophosphatides.

There is very good evidence that PE and polyglycerophosphatide syntheses share common precursors until CDP-diglyceride is formed, thereafter branching in the synthetic sequence occurs such that *in vivo*, at least, the net production of PE, well exceeds that of polyglycerophosphatides. Again *in vivo* there is a catabolic drain of polyglycerophosphatides

whereas PE does not breakdown. This in itself could explain the net syntheses of lipids favoring PE. An intriguing fact is that E. coli is equipped with a phospholipase A1 of broad specificity and its action on PE, PG, and cardiolipin can be easily demonstrated in vitro. In viable cells, however, neither PE nor polyglycerophosphatides appear to be degraded by this enzyme (86) albeit its activity becomes quite noticeable once the cells are damaged (98). Thus although the rate of breakdown of polyglycerophosphatides might be an important factor regulating the levels of PG and CL, their ultimate mechanism of breakdown remains as obscure as the manner by which their turnover is regulated.

Control may also occur prior to the branch point. This could be pictured as regulation by simple metabolites very likely subject to more complex phenomena regulating formation of either the membrane or apoprotein with which lipids combine. A negative feed-back mechanism operating at such a site would decrease lipid synthesis without affecting the PE/polyglycerophosphatide ratio. A positive modulation on the other hand could increase the levels of all phosphatides but there could also result an increase in the PE/polyglycerophosphatide ratio since increased phosphatidate formation would make less sn-glycero-3-phosphate available for phosphatidyglycerol synthesis. Kito and Pizer (107) were among the few to study metabolite

control on a step prior to the branch point and their results indicated that ATP inhibited the formation of phosphatidic acid.

Possibly this ATP effect is allosteric or it could involve enzymatically-catalyzed chemical modification of the sn-glycero-3-phosphate acylating enzyme. Chemical modification of enzymes, is not a common regulatory mechanism in bacteria although adenylylation of glutamine synthetase has been reported to occur in E. coli thereby converting an "a" form into a "b", less active form (121). On the other hand inhibitory effects of ATP often involve chelation of Mg^{++} ions necessary for a particular enzyme. This possibility in the case of ATP effects on phosphatidic acid formation was not precluded by Kito and Pizer (107).

With the experiments that follow, we have initiated a study aiming at the detection of possible regulatory mechanisms concerned with the synthesis of E. coli phosphoglycerides. For this purpose using cell-free preparations, we examined mainly the effect of various metabolites and Mg^{++} on the incorporation of labelled sn-glycero-3-phosphate into phosphoglycerides.

Materials

sn-Glycero-3-phosphate-U-^{14}C (Sp. act. 10-20 uC/umole) was purchased from International Chemical and

Nuclear Corporation or from New England Nuclear Corporation. The purity of this substrate was checked by paper chromatography and in most instances found to be completely radiochemically pure. Certain batches, however, contained cyclic glycerophosphate in relatively large amounts and were not used. L-serine-U-^{14}C (Sp. act. 118 uC/umole) was obtained from New England Nuclear and found to be radiochemically pure. Beef heart cardiolipin and phosphatidic acid (sodium salt) were obtained in pure form from General Biochemicals Inc. ATP, CTP, CoA and palmitoyl CoA were purchased from Sigma Chemicals or Nutritional Biochemicals Corporation. Triton X-100 (Rohm and Haas) and Cutscum were obtained from Fisher Scientific Co.

Phosphatidylglycerol and phosphatidylethanolamine were obtained by Bligh and Dyer (109) extraction of E. coli 015 cells and preparative thin layer chromatography of the lipids with the sequential use of systems A, D and E. They were characterized by staining for vicinal hydroxyl groups and amino groups respectively and by identification of the water-soluble products obtained after mild alkaline hydrolysis.

Methods

Phosphopholipase C Preparation

This was done according to the method of Chu (122). Bacillus cereus was grown 18 hours in a shallow flask on a rotary shaker in one litre of broth containing 10 grams of bactopeptone, 10 grams of yeast extract, 5 grams of sodium chloride and 0.4 grams of sodium monobasic phosphate. The medium was harvested by centrifugation and to 100 ml., 60 grams of ammonium sulphate was added. The solution was allowed to stand at 0°C. The precipitate containing the enzyme was collected by centrifuging 1 h at 18,000 g. and dissolved in 0.02 M Tris buffer, pH 7.2, to give a protein concentration of 5-6 mgm/ml. The enzyme was stored at -20°C.

Hydrolysis of Phosphoglycerides with Phospholipase C

This was done according to the method of Plackett (123). A lipid sample, 5 mgm or less, was dissolved in 5 ml. of ether twice-washed with water. 0.6 ml. of crude enzyme and 3.4 ml. of 0.02 M Tris buffer, pH 7.2, containing 0.5 mM $CaCl_2$ were added. The mixture was incubated at room temperature for 3-4 hours on a horizontal shaker. The lipid was extracted by Bligh and Dyer (109) extraction after removal of ether. Water-soluble products were identified

by paper chromatographic and staining methods described previously. Lipids were separated and identified by chromatography in system A.

Studies Involving Incorporation of Labelled Precursors

The conditions for labelling lipids with different precursors were varied depending on the type of study and are therefore described individually in the text that follows. The preparation of cell-free homogenates of *E. coli*, the extraction of lipids and the analytical procedures relating to the experiments that follow are described in General Methods. Of vital importance to these studies was the identification of the lipid products obtained after an incubation; however, in routine work, it was impractical or impossible to rigorously identify these products. Identification was usually made on the basis of separation patterns obtained with a particular thin layer chromatography system. In preliminary work these patterns were well defined by cochromatography with reference lipids or by identification of mild alkaline products derived from the components. In cases when an unusual component appeared, more detailed analyses were made as described in the text.

RESULTS

(1) The Effect of pH on the Incorporation of sn-Glycero-3-phosphate-U-^{14}C into Total Phospholipids

Results summarized in Figure 2 indicate that optimum incorporation of labelled precursor into total phospholipids occurred between pH 6.8 and 7.4. The effect of different buffers and ionic strength at a given pH was not extensively studied. In subsequent studies, a 0.07 M phosphate buffer, pH 7.3, was used.

(2) The Effect of Protein Concentration on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into Lipid Classes

Results summarized in Figure 3 reveal a linear incorporation of sn-glycero-3-phosphate-U-^{14}C into total lipids with protein concentrations up to 11 mg/ml at 22°C and up to 8 mg/ml at 37°C when incubation conditions were similar except for temperature. As would be expected the incorporation was greater at 37°C than at 22°C although at 37°C incorporation decreased at protein concentrations above 8 mg/ml. In most subsequent studies the protein concentration, the incubation time and the amount of substrate were varied such that saturation conditions prevailed as calculated from the curves in Figure 3 or as verified by varying the enzyme

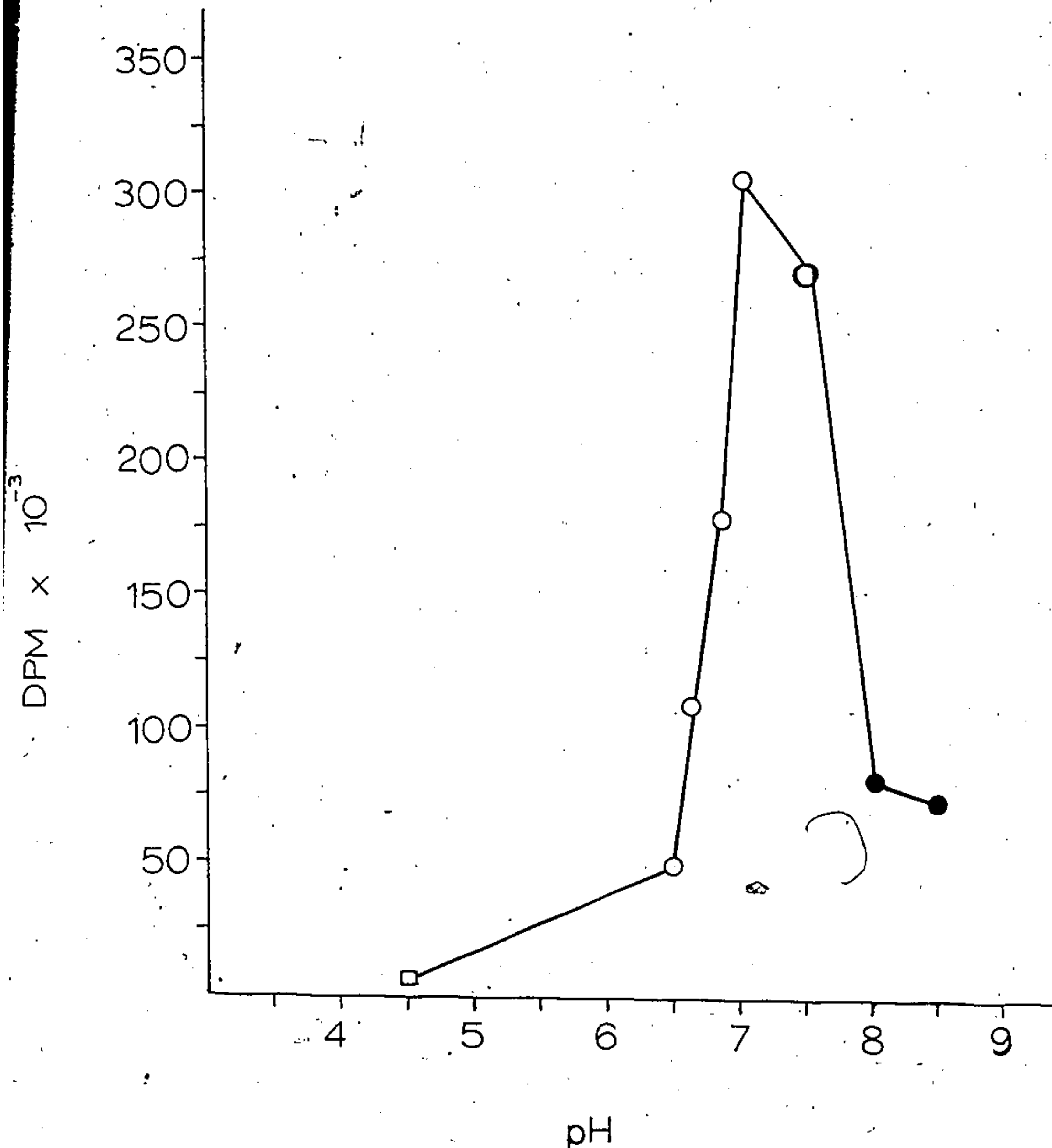


Figure 2. Effect of pH on the incorporation of sn-glycero-3-Phosphate-U-^{14}C into total lipids of E. coli 015. Incorporation medium contained in a volume of 4 ml; CoA, 0.1 mM; CTP, 0.4 mM; ATP, 3.7 mM; $MgCl_2$, 10 mM; stearic acid, 0.5 uM; E. coli sonicate, 10 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C, 1 uCi (Sp. Act. 10 uCi/umole); acetate buffer; (pH 4.5) 0.2 M,□□ ; phosphate buffer (pH 6.5-7.5), 0.07 M,○○ ; tris (pH 8.0-8.5) 0.02 M,●● ; Incubation was at 25°C for 120 minutes. Lipids were extracted by the method of Bligh and Dyer (109) and counted by method A.

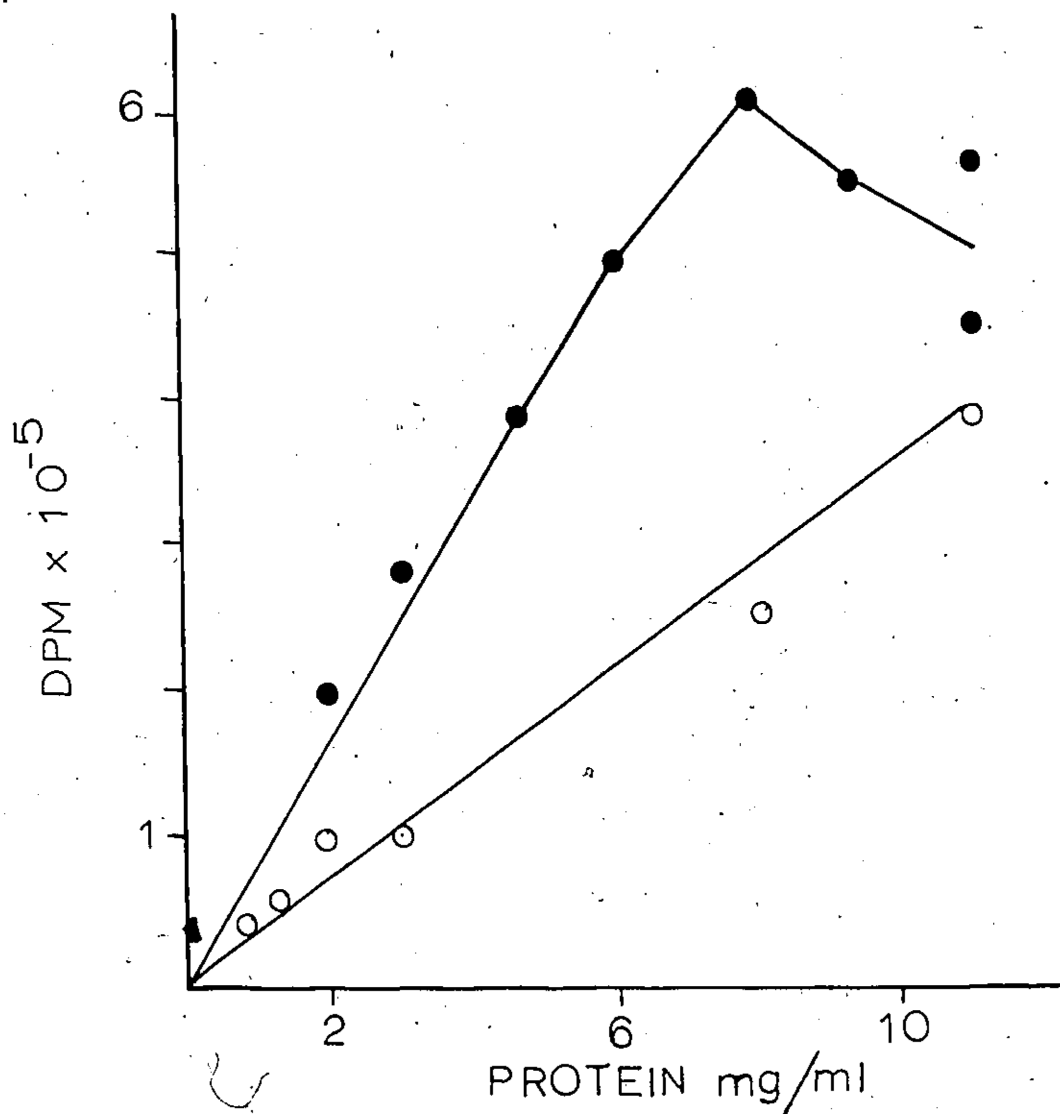


Figure 3. Effect of protein concentration on the incorporation of sn-glycero-3-phosphate-U-^{14}C into total lipids of E. coli 015. Incubation conditions were as stated for Table IIb except that the protein concentration was varied. ●–● 37°C, ○–○ 22°C.

concentration under a new set of conditions. Saturation kinetics were defined in terms of total lipid counts. Since the distribution of label within the lipid fractions was almost entirely in the glycerol moieties (Table IIa and IX) it follows that only two steps were involved in the incorporation of labelled sn-glycero-3-phosphate. The results of Figure 3 establish that saturation kinetics prevailed for the first step (acylation of the precursor) but the second enzyme involved (CMP: sn-glycero-3-phosphate phosphatidyl transferase) may not have been saturated under the conditions specified.

(3) The Effect of Temperature on the Distribution of Label within the Lipid Classes

Results in Table IIb reveal that an increase in temperature from 22°C to 37°C, increases the labelling in all phosphoglycerides. Phosphatidylglycerol is the most highly labelled lipid at both temperatures. However, at 37°C the ^{14}CPG/ ^{14}CPE ratio falls somewhat. Subsequent experiments (Table VIII) showed a less marked stimulation at 37°C when higher concentrations of protein (11 mg/ml) were used. Nevertheless distribution of label in these experiments was still much higher in the polyglycerophosphatide fraction than in PE.

Table IIa

Distribution of ^{14}C Isotope within the Phosphoglyceride Moieties of E. coli 015 Labelled at 37°C

Intact Phosphatide	Percent of total DPM Incorporated	Distribution of Label after Mild Alkaline Hydrolysis	
		Fatty Acid	Water Soluble product
PE	31	3	95
PG	54	8	92
CL	15	-	-

The 4 ml. incubation mixture contained phosphate buffer 0.07 M, pH 7.3, $MgCl_2$ 0.01 M, CTP 0.4 mM, ATP 3.7 mM, CoA 0.1 mM, sonicated cells, 9-13 mg. protein per ml.; sn-glycero-3-phosphate-U-^{14}C, 2.0 uCi (Sp. Act. 16 mCi/mMole) palmitic acid 0.1 mM, and oleic acid, 0.2 mM. The mixture was incubated 1 h at 37°C.

Table IIb

The Effect of Temperature on Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into the Phosphoglyceride Classes

	DPM Incorporated	
Phosphoglyceride	25°C	37°C
PE	61,300	251,200
PG	135,800	310,500
CL	25,300	50,100

The incubation mixture contained in 5.0 ml., 0.07 M phosphate buffer pH 7.3 as solvent and diluent, 0.1 mM palmitic acid, 0.2 mM oleic acid (added as sonicated suspensions in buffer), 0.4 mM CTP, 0.1 mM CoA, 2.8 mM ATP, 10 mM $MgCl_2$, E. coli homogenate (8 mg. protein/ml.) and 1 uCi of sn-glycero-3-phosphate (Sp. Act. 10 uCi/umole). After incubation for 1 h at 25°C or 37°C, the reaction was stopped with methanol addition. Lipids were extracted by a method of Bligh and Dyer (109) and suitable aliquots of lipid extract were subjected to chromatography in system E.

(4) Time Course of sn-Glycero-3-Phosphate Incorporation into Lipid Classes

Results summarized in Figure 4 indicate that the incorporation of labelled substrate was essentially linear in all fractions during at least one hour for those conditions stated. Throughout the experiment the labelling in PG exceeded that in PE although after 1 hour the rate of PG synthesis fell. A likely explanation for this decreased synthesis of PG is that an enzyme required for this synthesis is not stable or that there is a turn-over of PG. The conversion of PG to Cl would account for a portion of this reduction of label in PG.

From the data expressed in Table TI and Figures 3 and 4, it was apparent that protein concentration, temperature and time of incubation could be varied over a moderate range without significantly changing the labelling pattern. This simplified the devising of proper incubation systems for the study of cofactor and substrate effects on the synthesis of phosphoglycerides.

It was noticed in subsequent experiments however, that when incubations times were short and protein concentrations low, in a same experiment, the major lipid synthesized was PA (cf. Table VI). This labelled intermediate tended to separate together with Cl in the chromatographic systems

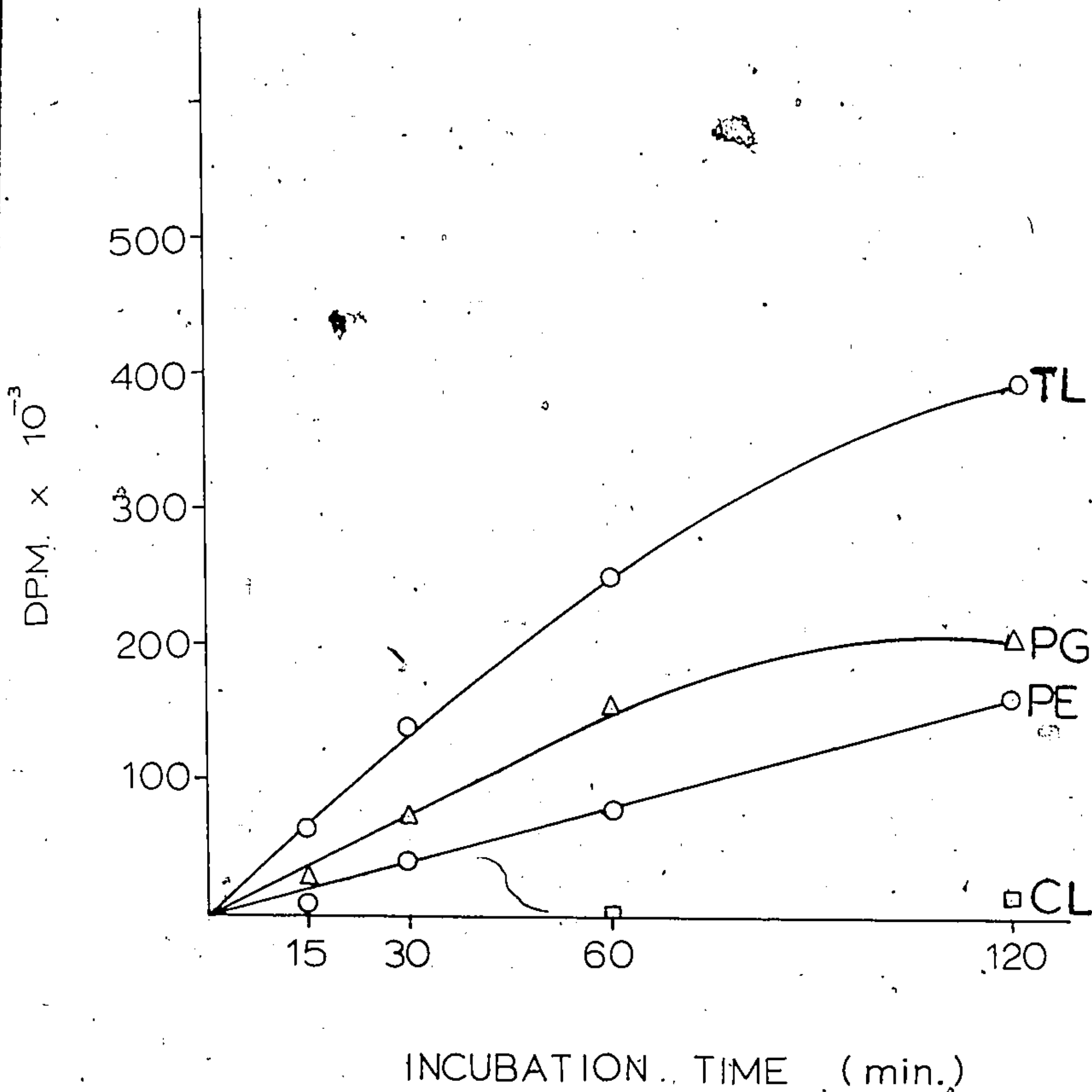


Figure 4. Time course of incorporation of sn-glycero-3-phosphate-U-^{14}C. The incorporation medium contained in a volume of 4. ml, phosphate buffer (pH 7.3), 0.07 M, $MgCl_2$, 10 mM; palmitic acid, 0.1 mM, oleic acid, 0.2 mM; CTP, 0.4 mM, CoA, 0.1 mM, ATP 3.7 mM; E. coli sonicate, 10 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C, 1 uC (Sp. Act. 20 uCi/umole). Incubation was at 25 C for 15, 30, 60 and 120 minutes. Lipids were extracted by the method of Bligh and Dyer (109) separated by thin layer chromatography in system E and counted by method D.

used (systems C, D, and E) for most of our studies. It is possible therefore that some of the CL counts given in Figure 3 are in fact partly due to the presence of PA particularly when small protein concentrations or short incubation times were used. At higher protein concentrations, or with longer incubation times the designated CL fraction yielded mainly GPGPG upon mild alkaline hydrolysis but occasionally trace amounts of glycerophosphate and GPG were found.

(5) The Effect of Serine on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into *E. coli* Lipids.

Since the synthesis of PE and polyglycerophosphatides depends on a common precursor, CDP-diglyceride, one would expect that the [serine]/[sn-glycero-3-phosphate] ratio could greatly influence the relative amounts of each type of phosphatide formed. Results to date have indicated a greater incorporation of sn-glycero-3-phosphate-U-^{14}C into PG in the absence of added serine. Possibly this preferred incorporation into polyglycerophosphatide was due to a lack of this amino acid.

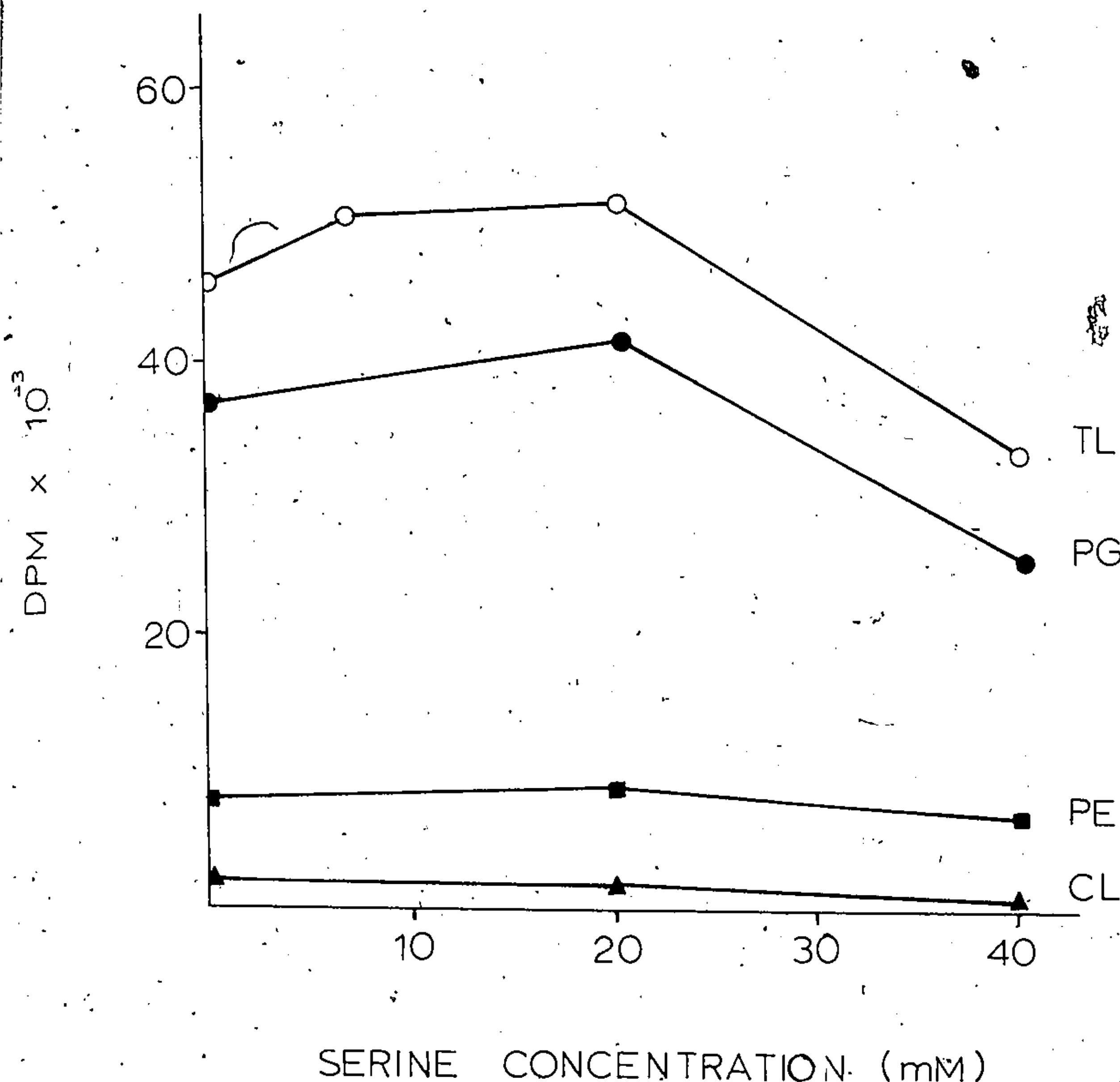


Figure 5. Effect of L-serine on the incorporation of sn-glycero-3-phosphate-U-^{14}C into phosphoglycerides of <u>E. coli</u> 015. The incubation mixture contained in 2 ml., 0.07 M phosphate buffer, pH 7.3 as solvent and diluent, 0.2 mM CoA, 10 mM $MgCl_2$, 0.1 mM palmitic acid sonicated in buffer 0.2 uCi of sn-glycero-3-phosphate-U-^{14}C (Sp. Act. 16 uCi/umole) 0.4 mM CTP and <u>E. coli</u> homogenate (1.2 mg. protein/ml). Serine was added at varied concentrations. After incubation for 1 h at 25°C, the mixture was inactivated with 2.5 volumes of methanol and lipids were extracted by the method of Bligh and Dyer (109). One aliquot of total lipid extract was counted directly and another was subjected to chromatography using system I. Lipid components counted by method D.

Results summarized in Figure 5 indicate that serine addition had no effect on the labelling of PE. At a rather high concentration of 40 mM, this amino acid inhibited the formation of PG which increased the $^{14}C_{PE}/^{14}C_{PG}$ ratio but this effect is not likely physiological. The lack of stimulation of PE synthesis by serine addition would indicate that the CDP-diglyceride concentration is rate-limiting and that endogenous serine levels are sufficient to permit maximal PE synthesis under the conditions prevailing.

(6) The Effect of Glycerophosphate on the Incorporation of Serine-U-^{14}C into *E. coli* Lipids

If at the branch point, the endogenous CDP-diglyceride concentration were non-saturating for both phosphatidyl transferases, addition of sn-glycero-3-phosphate could stimulate formation of polyglycerophosphatides at the expense of PE synthesis and addition of serine should produce the opposite result, provided the endogenous concentrations of these substrates were not already saturating.

Results summarized in Figure 6 illustrate the incorporation of ^{14}C-serine into total lipids, PE and PS. Since the previous experiment indicated that the endogenous pool of serine was saturating, the fairly linear increase

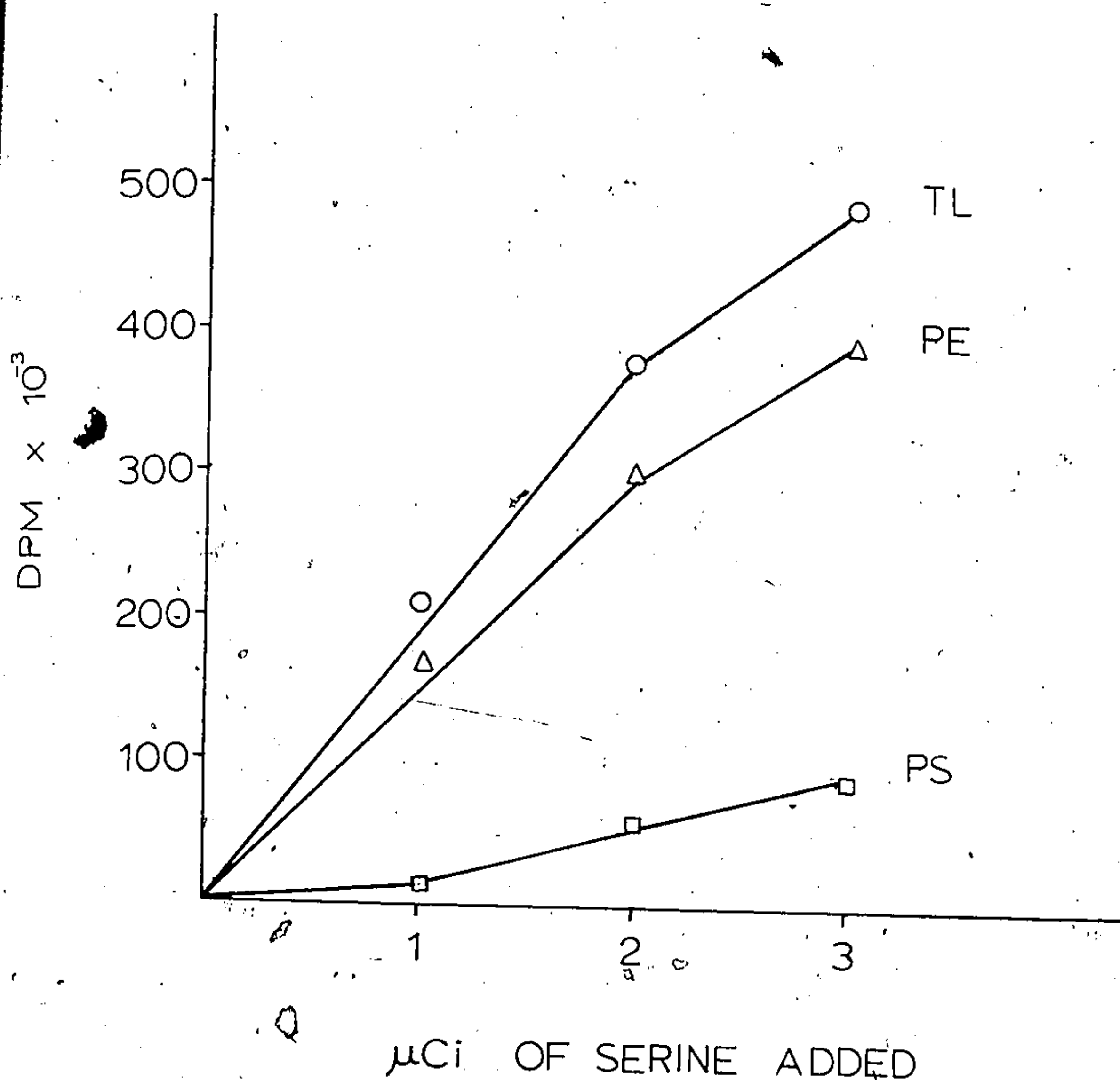


Figure 6. Incorporation of serine-U-14C into lipids of E. coli 015. Conditions were as stated for Figure 5 except that the labelled precursor in this case was serine-U-14C (Sp. Act. 118 uCi/umole). O total lipids, △ phosphatidylethanolamine, □ phosphatidylserine. Phosphatidyl-serine-U-14C was identified by cochromatography with reference compound purchased from General Biochemicals, Ohio.

in incorporation obtained with increase in labelled serine would simply reflect a resultant increase in specific activity of the substrate. Results summarized in Figure 7 indicate a maximal, 22%, inhibition of ^{14}C-serine incorporation when 5 mM rac-glycero-3-phosphate was added. The inhibition was most notable in the PE fraction. The results are most easily explained on the basis that the endogenous levels of glycerophosphate were insufficient to saturate the glycerophosphate: CDP-diglyceride phosphatidyl transferase. Glycerophosphate addition increased the activity of this enzyme and made less CDP-diglyceride available for PE formation. It can be noted from the results presented thus far that the incorporation of sn-glycero-3-phosphate is greater in PG than it is in PE. This was not due to lack of serine in the medium. Addition of serine to the incubation mixture did not increase the synthesis of labelled PE and it would appear that under our conditions, the endogenous concentration of this amino acid was saturating.

(7) The Combined Effect of Mg^{++} Coenzyme A, ATP and CTP on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into *E. coli*

From the known synthesis pathways of *E. coli* it can be deduced that substrate and cofactor requirements for

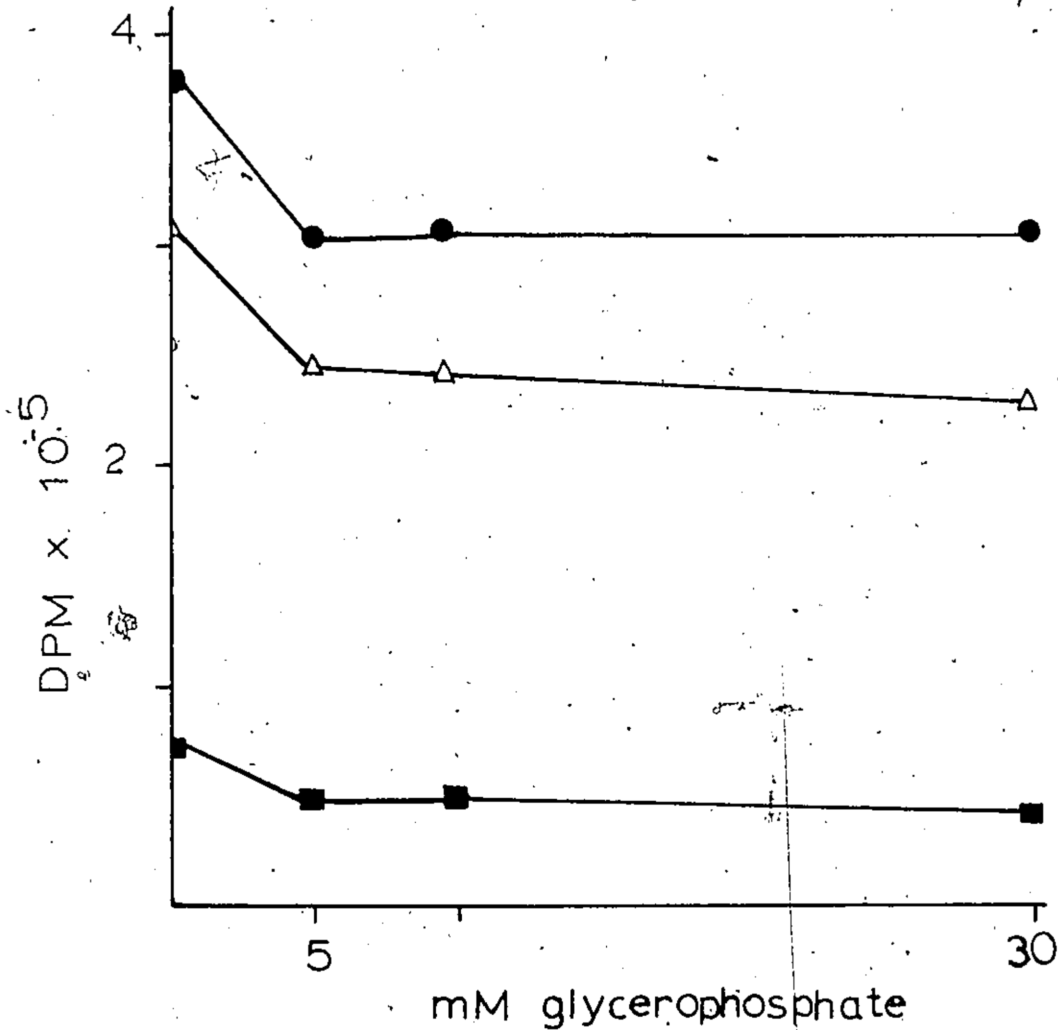


Figure 7. The effect of rac-glycero-3-phosphate on the incorporation of serine-U-^{14}C into lipids of E. coli 015. Conditions were similar to those specified for Figure 6 except that the medium contained a fixed amount of labelled serine (2 uCi) and various concentrations of rac-glycero-3-phosphate. ● total lipids, Δ phosphatidylethanolamine, ■ phosphatidylserine.

glycerophosphate incorporation are as follows; fatty acids, ATP, CoA, Mg^{++}, CTP and serine. The availability of these various substrates for the incorporation of sn-glycero-3-phosphate-U-^{14}C could affect not only the synthesis of total lipids but also the labelling pattern in the major lipid classes, as well as the distribution of label within a particular lipid. Our results have indicated that serine addition was without significant effect. A detailed study of the other cofactor requirements might possibly disclose any regulation by metabolites or cofactors acting at one or more steps of the pathway. This in turn might lead to an explanation for the preferred incorporation of labelled glycerophosphate into PG. In our preliminary experiments we studied under several conditions, the effects of CTP, ATP and CoA combined, and of Mg^{++}. At first the concentrations of these various additives were arbitrarily chosen from conditions described in the literature. Later, the cofactor requirements were studied systematically.

Results summarized in Table III indicate that whether the cofactors ATP, CTP and CoA were present or not, Mg^{++} addition caused a shift of labelling in favour of phosphatidylglycerol. The addition of ATP, CTP and CoA together, greatly accentuated the Mg^{++} effect. The rise in PG labelling due to Mg^{++} was usually accompanied by a fall

Table III

The Effect of Various Cofactors on the Labelling of <u>E. coli</u> Phosphoglycerides by sn-Glycero-3-Phosphate-U-^{14}C at 37°C

	DPM Incorporated into each Fraction			
	PE	PG	CL	Other Lipids
A Complete	151,500	207,800	13,500	5,300
B - (ATP, CTP, CoA, Mg^{++})	141,300	134,200	4,100	5,300
C - (ATP, CTP, CoA)	93,500	150,600	3,300	3,400
D - Mg^{++}	169,600	102,100	3,900	4,500

Incorporation medium contained in a volume of 4 ml: phosphate buffer (pH 7.3), 0.07 M; palmitic acid, 0.1 mM; oleic acid, 0.2 mM; <u>E. coli</u> sonicate, 5 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C 0.2 uCi/ml (Sp. Act 10 mC/mM). To incubation A was added CoA, 40 uM; ATP, 1.4 mM, CTP, 0.2 mM, $MgCl_2$, 10 mM. To incubation C was added Mg, 10 mM; To incubation D was added CoA, 40 uM; ATP, 1.4 mM; CTP, 0.2 mM. Incubations were at 37°C for 120 minutes. Lipids were extracted by the method of Bligh and Dyer (109), separated in system C and counted by method C.

in PE synthesis (compare A and D and B and C, Table III), but later studies showed that this was not always a marked effect.

(8) The Effect of Cytidine Triphosphate and Coenzyme A on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C

Results shown in Figure 8 indicate no marked effect of CTP on the incorporation of labelled precursor into the polyglycerophosphatide fractions. This nucleotide did cause however, a slight increase in the labelling of PE. Since CTP is required for the formation of CDP-diglyceride, an intermediate in the *de novo* synthesis of *E. coli* lipids, it was concluded that the levels of endogenous CTP or metabolically related substances were sufficient to allow almost maximal incorporation under our conditions. Other results, not shown, also indicated that the incubation medium did not require CoA supplement since addition of this cofactor at 0.5 mM concentration produced a maximal stimulation of less than 5% in total lipid counts.

From this evidence it was tentatively concluded that the combined effect of added ATP, CTP and CoA (Table III) was due to ATP itself.

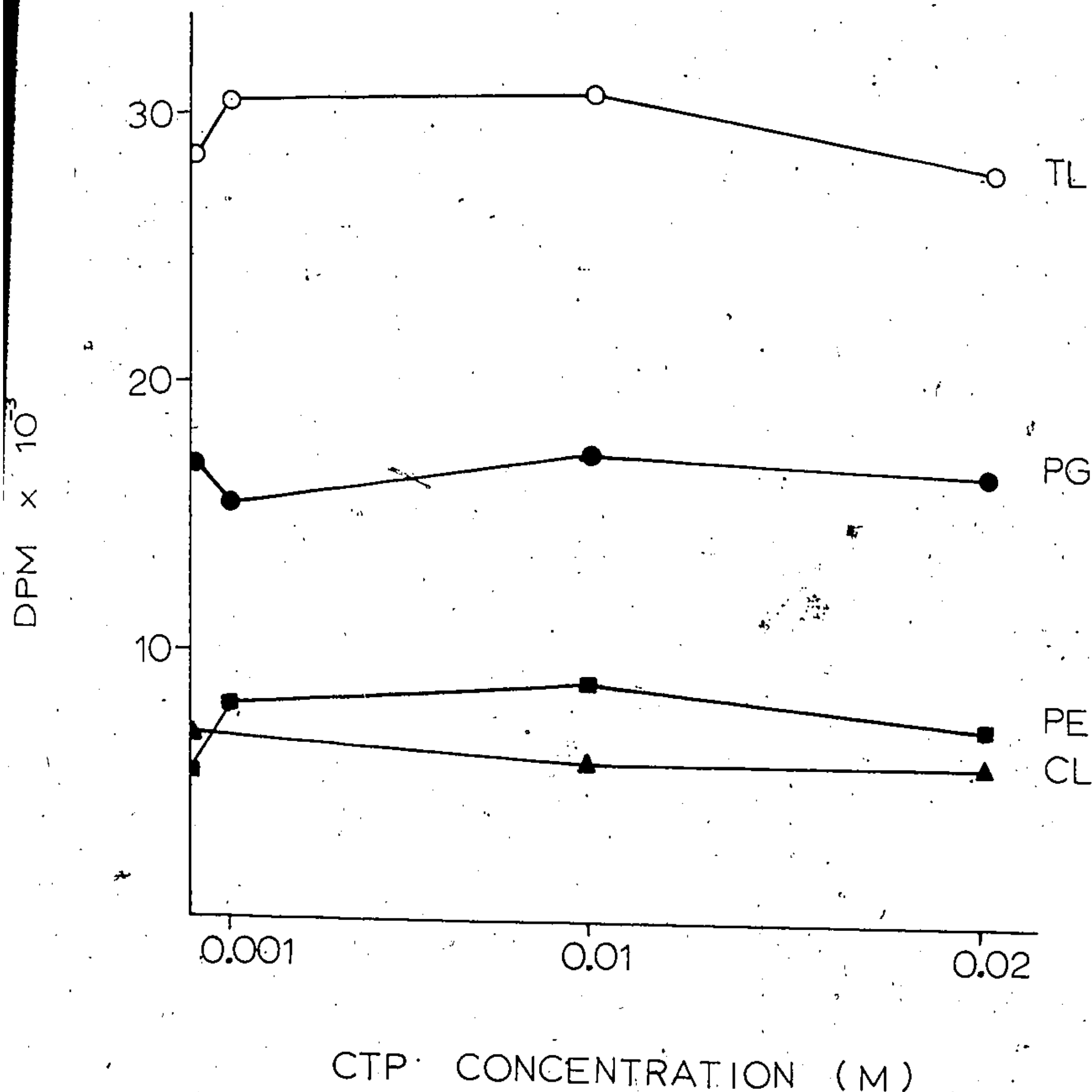


Figure 8. Effect of CTP on the incorporation of sn-glycero-3-phosphate-U-^{14}C into lipids of E. coli 015. The incubation mixture contained in 2 ml., 0.07 M phosphate buffer, pH 7.3, as solvent and diluent, 0.2 mM CoA, 10 mM $MgCl_2$, 0.1 mM palmitic acid, 0.2 uCi of sn-glycero-3-phosphate-U-^{14}C (Sp. Act 10 uCi/umole), E. coli homogenate (1.2 mg protein/ml) and varied concentrations of CTP. After incubations of 1 h at 25°C the mixture was inactivated with 2.5 volumes of methanol and lipids were extracted by the method of Bligh and Dyer (109). One aliquot of total lipid extract was counted directly and another was subjected to chromatography in system I. Lipids were then counted by method D.

(9) The Effect of Fatty Acid on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into *E. coli* lipids

As can be appreciated from Table IV the addition of palmitic acid to the incubation mixture resulted in a greater incorporation of labelled substrate into all lipid fractions provided ATP was also added. This result is explanable on the basis that ATP and fatty acid are involved in the synthesis of acyl CoA, a suitable acyl donor for the synthesis of PA. In the absence of added fatty acid however ATP still caused a marked increase in the incorporation of labelled substrate but this occurred only in the PG fraction. It would seem from this result that ATP stimulates the incorporation of labelled sn-glycero-3-phosphate in a manner that is not solely involved with the acylation step.

(10) The Effect of ATP and Mg^{++} on the Synthesis of Major *E. coli* Lipids

Figure 9 reveals that Mg^{++} alone, at 25°C, had a moderate stimulatory effect on the synthesis of *E. coli* lipids at concentrations of 10 mM or higher, whereas ATP alone (Figure 10) caused a marked inhibition of incorporation at concentrations above 10 mM.

When the incubation mixture was supplemented with 10 mM Mg^{++} at 25°C the addition of ATP up to a concentration

Table IV

The Effect of Fatty Acid on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into E. coli Lipids

Conditions	DPM Recovered in each Fraction		
	PE	PG	CL
No ATP, no fatty acid	6,200	8,800	900
No ATP	6,500	8,500	1,000
No fatty acid	5,900	28,900	1,100
Complete	9,400	32,100	1,200

The complete system contained in 2 ml., 0.07 M phosphate buffer pH 7.3 as solvent and diluent, 0.2 mM CoA, 2.8 mM ATP, 10 mM $MgCl_2$, 0.1 mM palmitic acid sonicated in buffer, 0.2 uCi of sn-glycero-3-phosphate-U-^{14}C (Sp. Act. 16 uCi/umole) 0.4 mM CTP and E. coli homogenate (1.2 mg. protein/ml.). Incubations were for 1 hour at room temperature (25°C).

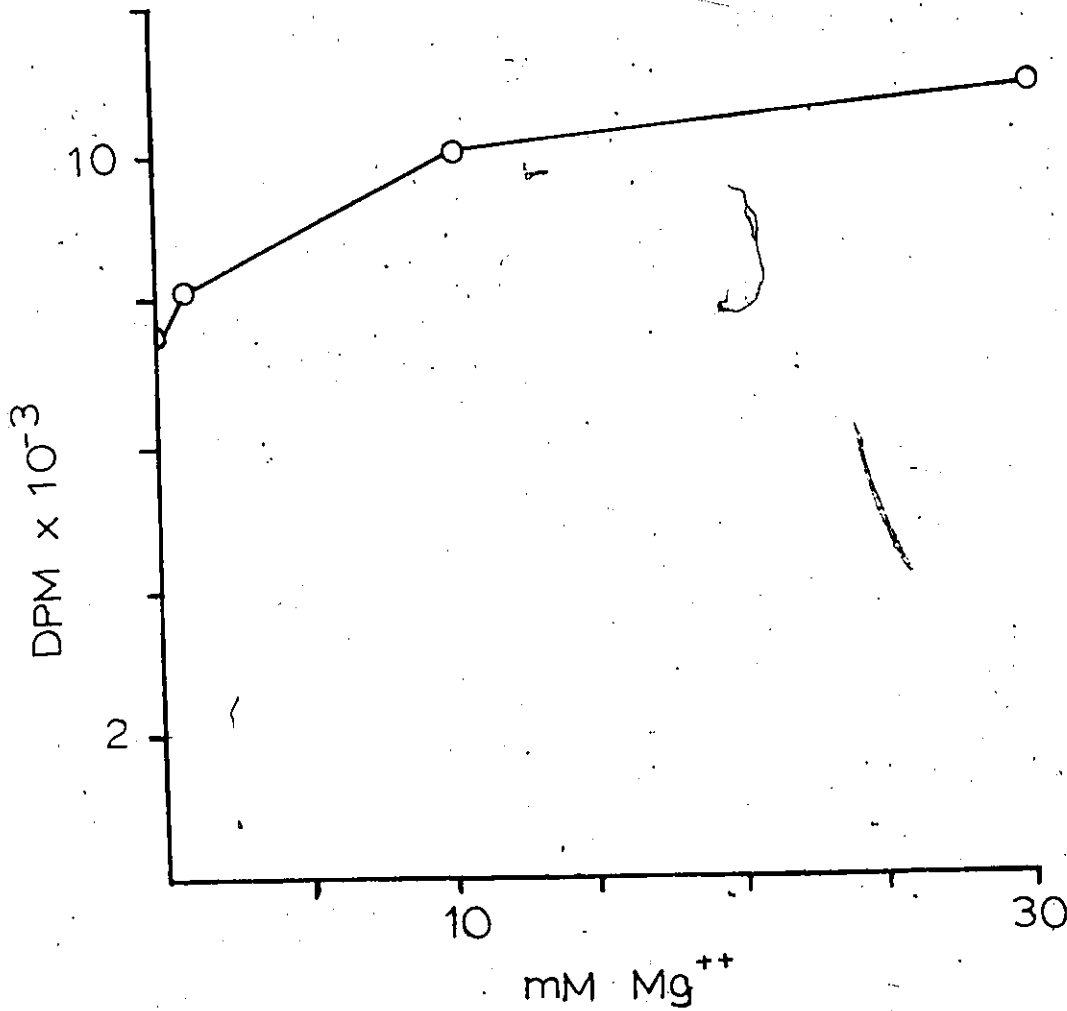


Figure 9. Effect of Mg^{++} on the incorporation of sn-glycero-3-phosphate-U-^{14}C into the total lipids of E. coli O15. Incubation conditions were as stated for Table IV except that no ATP was added and Mg^{++} concentration was varied.

of 10 mM stimulated incorporation (cf. Figure 11). Total lipid incorporation as well as that of each lipid was affected in a similar manner although the most marked stimulation was noted in the PG fraction At higher concentrations of this nucleotide stimulation by Mg^{++} was abolished and there always resulted a small but consistant inhibition of incorporation into all lipid fractions. Results summarized in Figure 12 illustrate the stimulatory role of Mg^{++} in the presence of 10 mM ATP. In general it was noted that maximal labelling occurred when the added Mg^{++} concentration was 10 mM provided the $[ATP] / [Mg^{++}]$ ratio did not exceed 1. Again, although Mg^{++} and ATP stimulated incorporation into all lipids, the most marked effect was noted in the PG fraction.

At 37°C similar effects of Mg^{++} and ATP were noted. Inhibition by high concentrations of ATP (Table V) could be gradually reversed by increasing the Mg^{++} concentration. The data expressed in Figures 10, 11, and 12 and Table V support the conclusion that the inhibition by ATP is due either to a chelation of Mg^{++} by excess nucleotide or to some negative allosteric effect on one or several biosynthetic enzymes, which is reversed by the addition of Mg^{++}. At any rate the inhibition was not considerable.

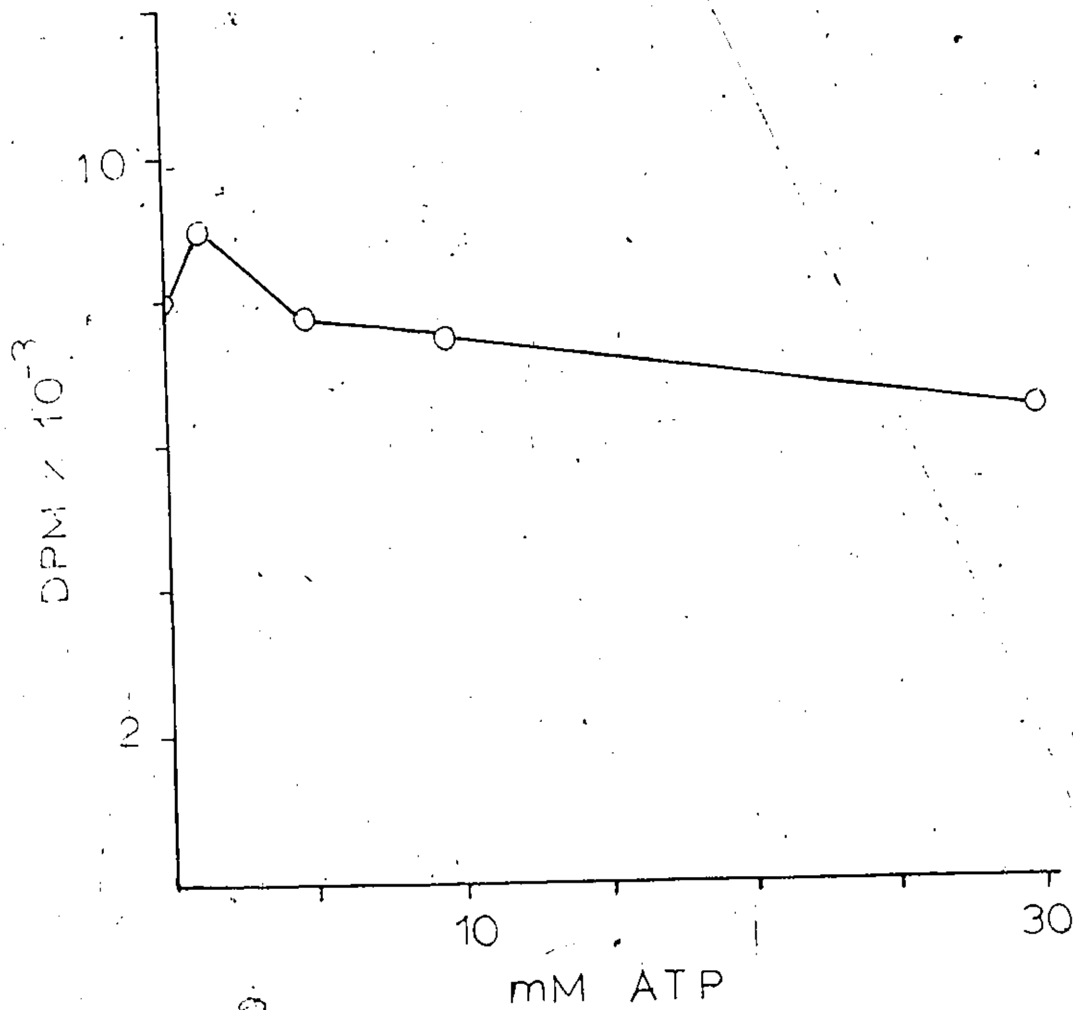


Figure 10. Effect of ATP on the incorporation of sn-glycero-3-phosphate-U-^{14}C into total lipids of E. coli O15. Incubation conditions were as stated for Table IV except that Mg^{++} was omitted and ATP concentration was varied.

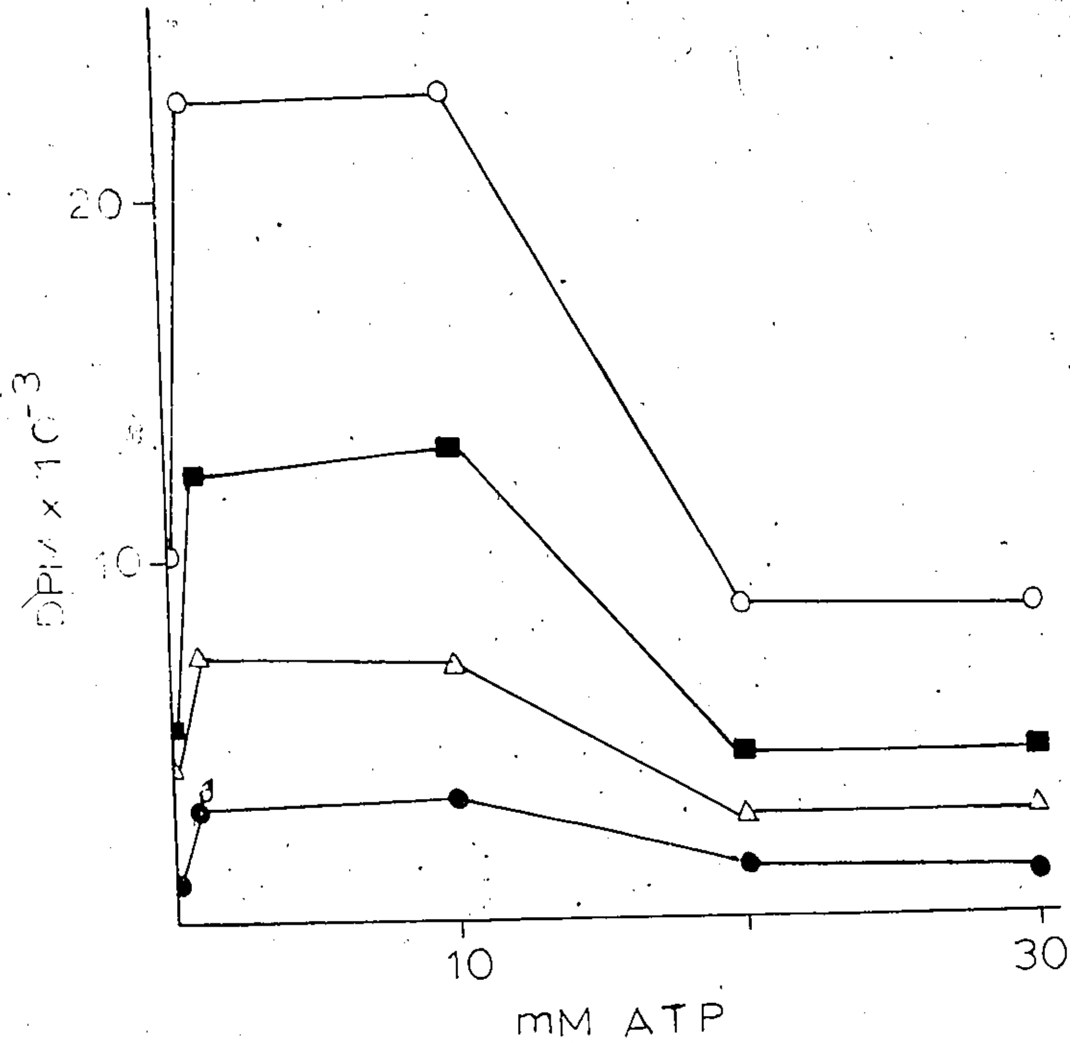


Figure 11. The effect of ATP in the presence of Mg^{++} on the incorporation of sn-glycero-3-phosphate-U-^{14}C into lipids of E. coli O15. Conditions were as stated for Table IV except that the ATP concentration was varied. ○ total lipids, ■ phosphatidylglycerol, △ phosphatidylethanolamine, ● cardiolipin.

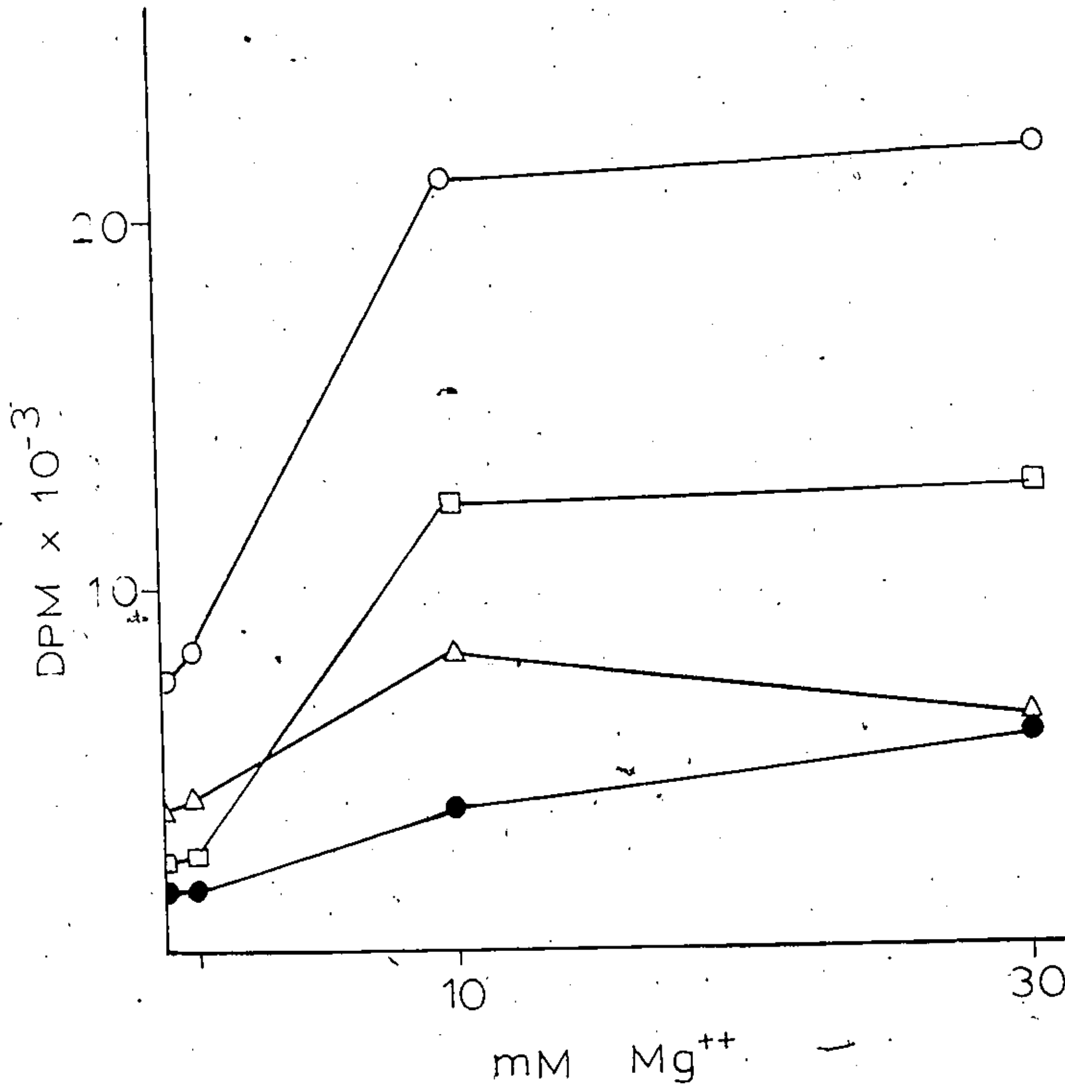


Figure 12. The effect of Mg^{++} in the presence of ATP on the incorporation of sn-glycero-3-phosphate-U-^{14}C into lipids of E. coli O15. Conditions were as stated for Table IV except that the Mg^{++} concentration was varied. ○ total lipids, □ phosphatidylglycerol, △ phosphatidylethanolamine, ● cardiolipin.

Table V

The Reversal of ATP Inhibition by Mg^{++}

Lipid Fraction	DPM Recovered in Each Fraction					
	No Mg^{++} ATP		10 mM Mg^{++} ATP		20 mM Mg^{++} ATP	
	2.8 mM	28 mM	2.8 mM	28 mM	2.8 mM	28 mM
PE	10,100	3,500	22,700	6,400	23,500	14,400
PG	5,400	3,900	124,000	6,700	131,900	63,800
CL	1,200	700	10,200	1,200	15,400	5,000

Incorporation medium contained in a volume of 5 ml; phosphate buffer (pH 7.3), 0.07 M; palmitic acid, 0.1 mM; oleic acid, 0.2 mM; CTP, 0.4 mM; CoA, 0.1 mM; ATP, 2.8 mM or 28 mM; *E. coli* sonicate, 3 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C, 1 uCi/5 ml (Sp. Act. 20 uC/umole). $MgCl_2$ was added at concentrations of 10 and 20 mM. Incubation was at 37°C for 60 minutes. Total lipid was extracted by the method of Bligh and Dyer (109) separated in systems B, D, E and counted by method D.

(ii) The Effect of ATP on the Synthesis of Phosphatidic Acid

Kito and Pizer (107) reported that low concentrations of ATP had a marked inhibitory effect on the synthesis of PA and concluded that this nucleotide had a regulatory role relating to phospholipid synthesis in *E. coli*. The inhibition was noted when a particulate fraction was the source of enzyme, palmitoyl CoA was added at optimal concentrations and incubations were carried out for short periods at 10°C. Our results with whole homogenates seemed to be compatible with those of Kito and Pizer insofar as an inhibitory effect of ATP on major phosphatide synthesis was noted. In our experiments, however, inhibition occurred only at high [ATP] / $[Mg^{++}]$ ratios and was reversed by increasing the Mg^{++} concentration. The [ATP] / $[Mg^{++}]$ ratio that was used by Kito and Pizer did not cause inhibition under our conditions. To characterize further the nature and locate the site of ATP intervention we adopted the conditions of Kito and Pizer which are those first described by Goldfine et al (124). However, our experiments were performed at 25°C instead of 10°C. The minimal protein concentrations and short incubation times used by these authors resulted in the formation of phosphatidic acid as the sole biosynthetic product when labelled sn-glycero-3-phosphate was the precursor.

As can be seen from the results in Table VI only one labelled product was obtained when low concentrations of particulate fraction (0.3 mg protein/ml) were used. The product was cochromatographed with phosphatidic acid in chromatographic system F (R_f .05). Mild alkaline hydrolysis of the product yielded glycerophosphate in systems 1 (R_f 0.28) and 2 (R_f 0.19). ATP under these conditions did cause a 12% inhibition in the acylation of the precursor. This result is in contrast with the 60% inhibition obtained by Kito and Pizer under similar conditions except for temperature and strain of E. coli used. With higher concentrations of particulate fraction (1.7 mg/ml) labelled sn-glycero-3-phosphate was incorporated mainly into PA but some significant labelling of PG and PE also occurred. The addition of ATP to this system increased the labelling in all phosphatides but the most significant stimulation on a percentage basis occurred in the PG fraction. When whole sonicate was used (2.5 mg total protein/ml containing 0.3 mgm of particulate protein/ml) a significantly lower incorporation of labelled precursor occurred if these results are compared to those obtained with an equivalent amount of particulate fraction. This fall is best explained by a dilution of the label with cytosol sn-glycero-3-phosphate or related metabolites. Again under these conditions, PA

Table VI

The Effect of ATP on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into Phosphoglycerides by *E. coli* Preparations

Protein Concentration mg/ml	ATP Concentration mM	DPM Incorporated into Each Lipid		
		PA	PG	PE
Particulate Fraction				
0.3	0	51,900	0	0
0.3	2.0	44,800	0	0
1.7	0	130,000	20,000	4,600
1.7	2.0	137,700	25,200	5,000
Whole Homogenate				
2.5	0	25,100	7,100	1,700
2.5	2.0	17,500	12,200	1,500

Incorporation medium contained in a volume of 5.0 ml: phosphate buffer (pH 7.0), 0.07 M; $MgCl_2$, 0.01 M; palmitoyl CoA, 43 uM; cysteine, 0.3 mM; rac-glycero-3-phosphate, 0.3 mM; sn-glycero-3-phosphate-U-^{14}C 1 uCi (Sp. Act. 20 uCi/umole): *E. coli* whole homogenate or *E. coli* particulate fraction. Both fractions were incubated with and without added 10 umoles of ATP. The incubations were at 25°C for 20 minutes. The particulate fraction was prepared by centrifuging the cell-free extract at 30,000 g for 30 minutes.

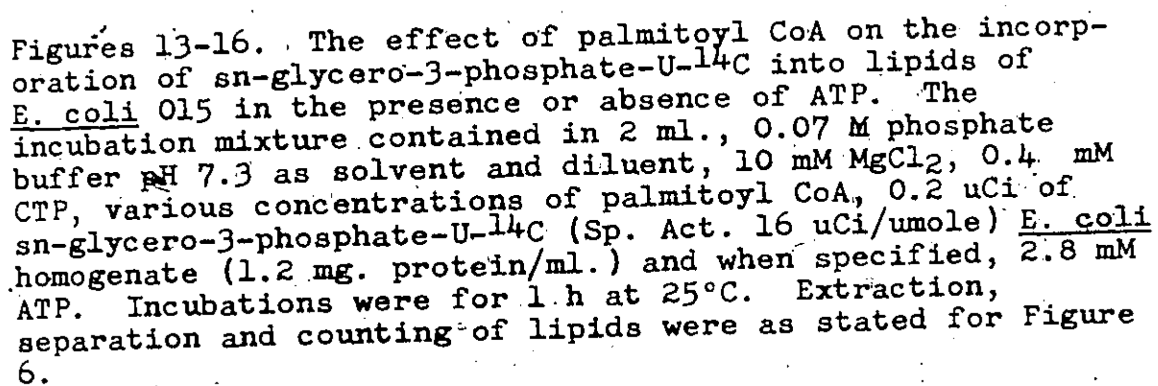
was the main phosphatide labelled, but PG and PE were also formed from the isotope added. ATP addition caused a marked diminution of the label in PA. However this fall can be explained by a sizeable conversion of PA to PG. At most, about 10-25% inhibition of PA synthesis occurred if this conversion is taken into account. The higher 25% value would apply only if the distribution of label in PG favors the unacylated glycerol as was later found (cf. Table IX).

In summary, it can be said that ATP causes only a small, probably insignificant inhibition of PA synthesis under conditions similar to those described by Kito and Pizer (107). We have no clear explanation for our contrasting results although strain differences and/or temperature effects may be involved. The reason that Kito and Pizer gave for using such a low incubation temperature is that the PA synthesizing enzyme(s) were unstable at higher temperatures. Again our results indicate otherwise. At higher temperatures phospholipid synthesis was greater in our case and the enzymes involved seemed quite stable. Increasing the particulate enzyme concentration to a level higher than that used by Kito and Pizer or adding cytosol to the particulate fraction causes a conversion of PA to other phosphatides. The main effect of ATP under these conditions is to increase the labelling of PG. This stimulation can be noticed with particulate fraction alone

but is more marked when cytosol is also present. These results which as a whole contrast with those of Kito and Pizer do not weaken the conclusion that ATP has a stimulating effect on phosphoglyceride synthesis provided the Mg^{++} concentration is equal to or higher than that of the nucleotide.

(12) The Effect of Mg^{++} and ATP in the Presence of Optimal Palmitoyl CoA Concentrations

Results in Table IV indicated that only part of the ATP effect was due to its participation in the formation of acyl CoA. Results in Table VI showed that ATP stimulated the synthesis of PG even in the presence of added palmitoyl CoA and under these conditions, did not significantly affect PA synthesis. Consequently, ATP had to be involved at a step beyond the de novo formation of PA. This conclusion was further verified. Results summarized in Figure 13 again indicate a stimulating effect of ATP + Mg^{++} on total lipid labelling even in the presence of optimal palmitoyl CoA concentrations. On the other hand, the stimulatory effect of ATP on PE and cardiolipin formation is completely abolished by the addition of palmitoyl CoA (cf. Figures 14 and 16). This ATP effect, additive to that of palmitoyl CoA, noted in Figure 13 is explained by the stimulation of PG formation in the presence of this



Figures 13-16. The effect of palmitoyl CoA on the incorporation of sn-glycero-3-phosphate-U-^{14}C into lipids of E. coli O15 in the presence or absence of ATP. The incubation mixture contained in 2 ml., 0.07 M phosphate buffer pH 7.3 as solvent and diluent, 10 mM $MgCl_2$, 0.4 mM CTP, various concentrations of palmitoyl CoA, 0.2 uCi of sn-glycero-3-phosphate-U-^{14}C (Sp. Act. 16 uCi/umole) E. coli homogenate (1.2 mg. protein/ml.) and when specified, 2.8 mM ATP. Incubations were for 1 h at 25°C. Extraction, separation and counting of lipids were as stated for Figure 6.

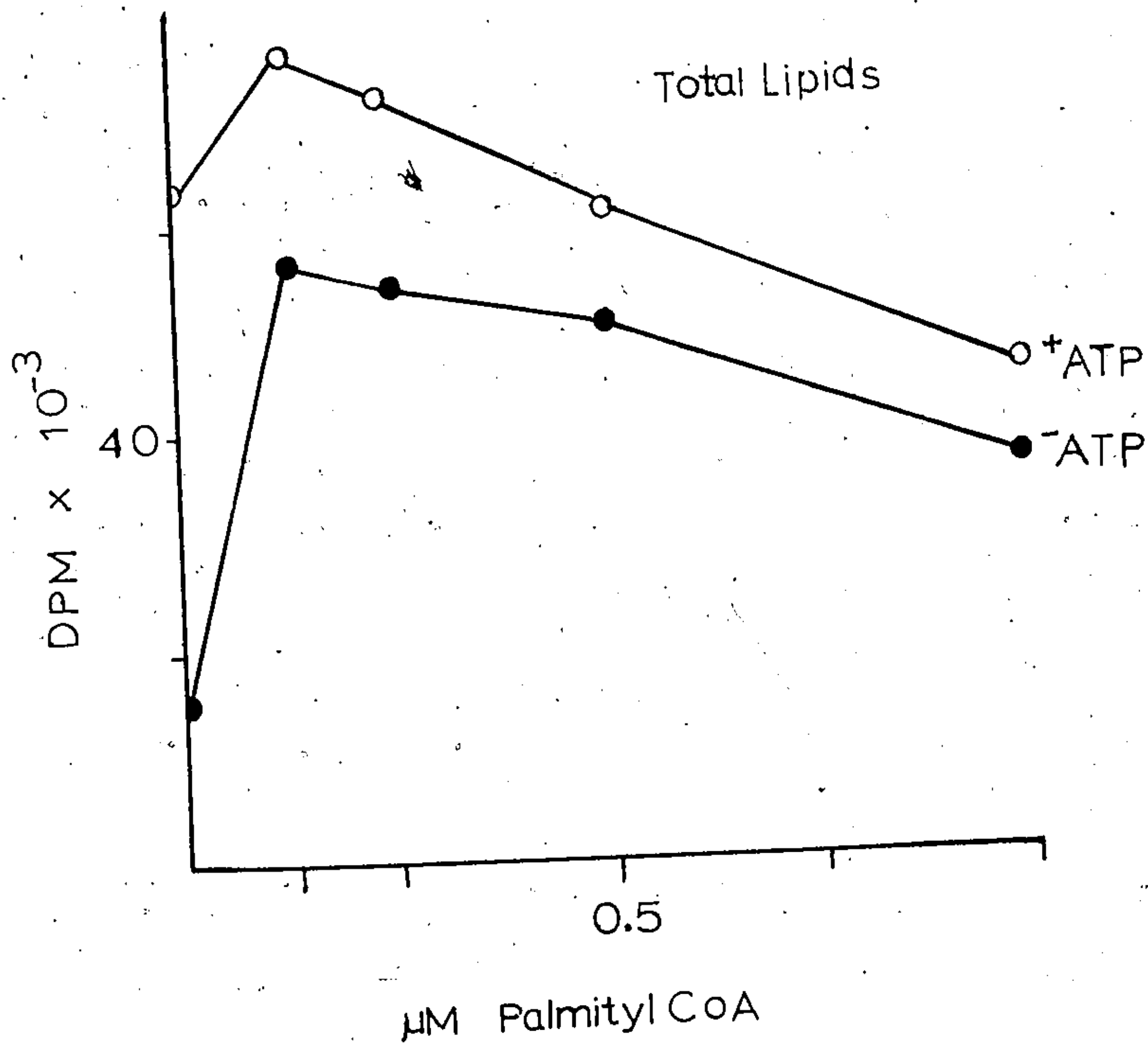


Figure 13

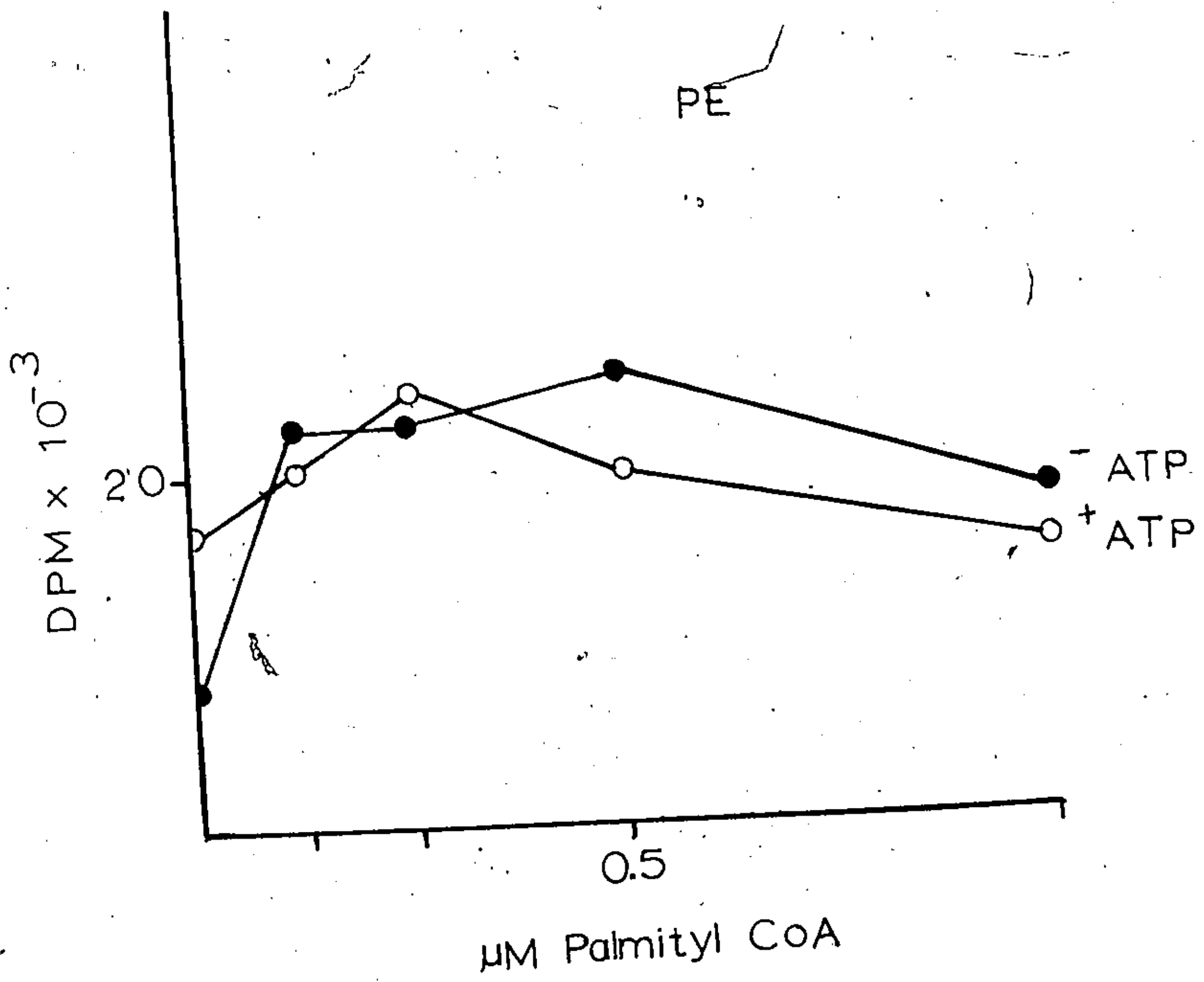


Figure 14

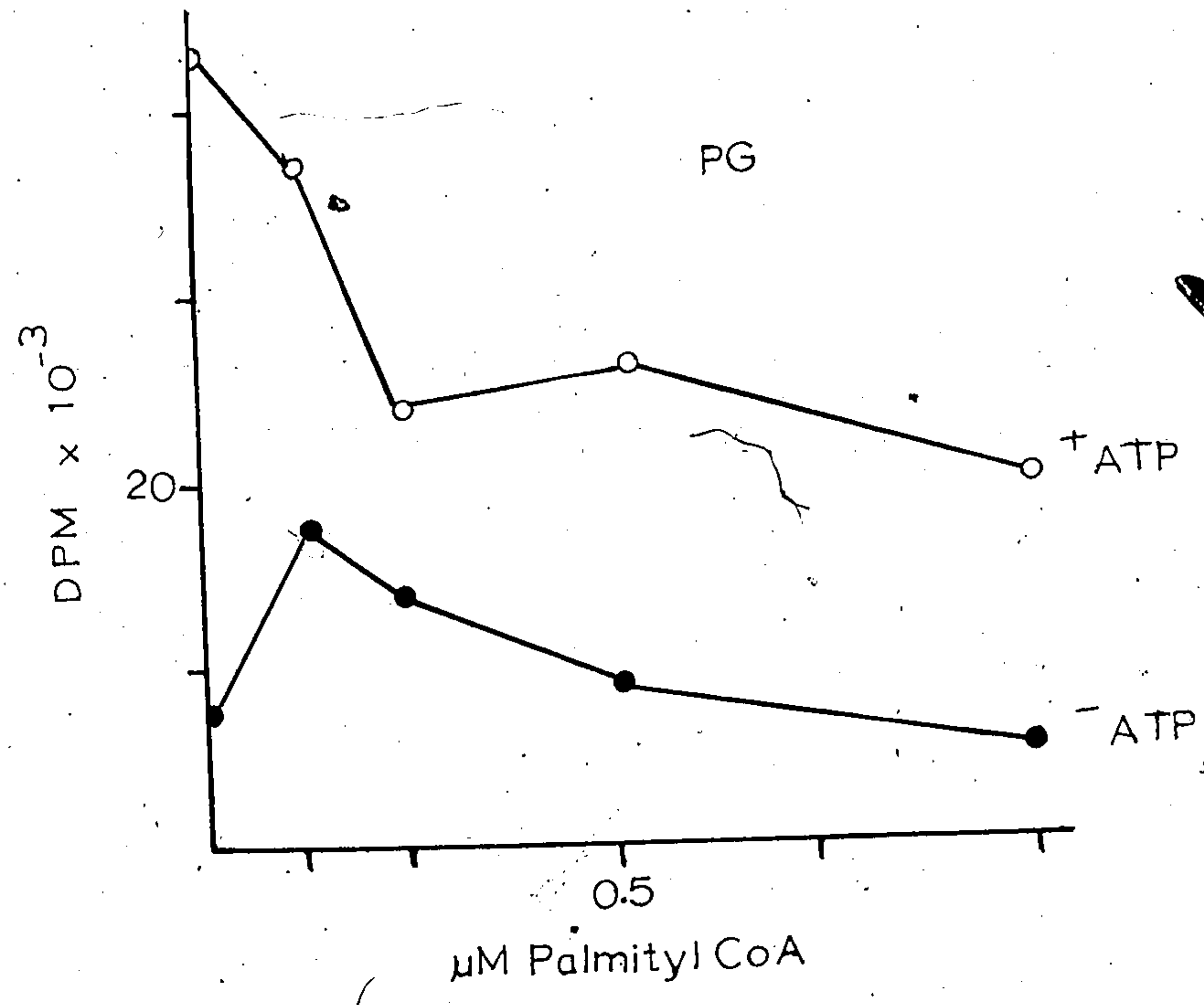


Figure 15

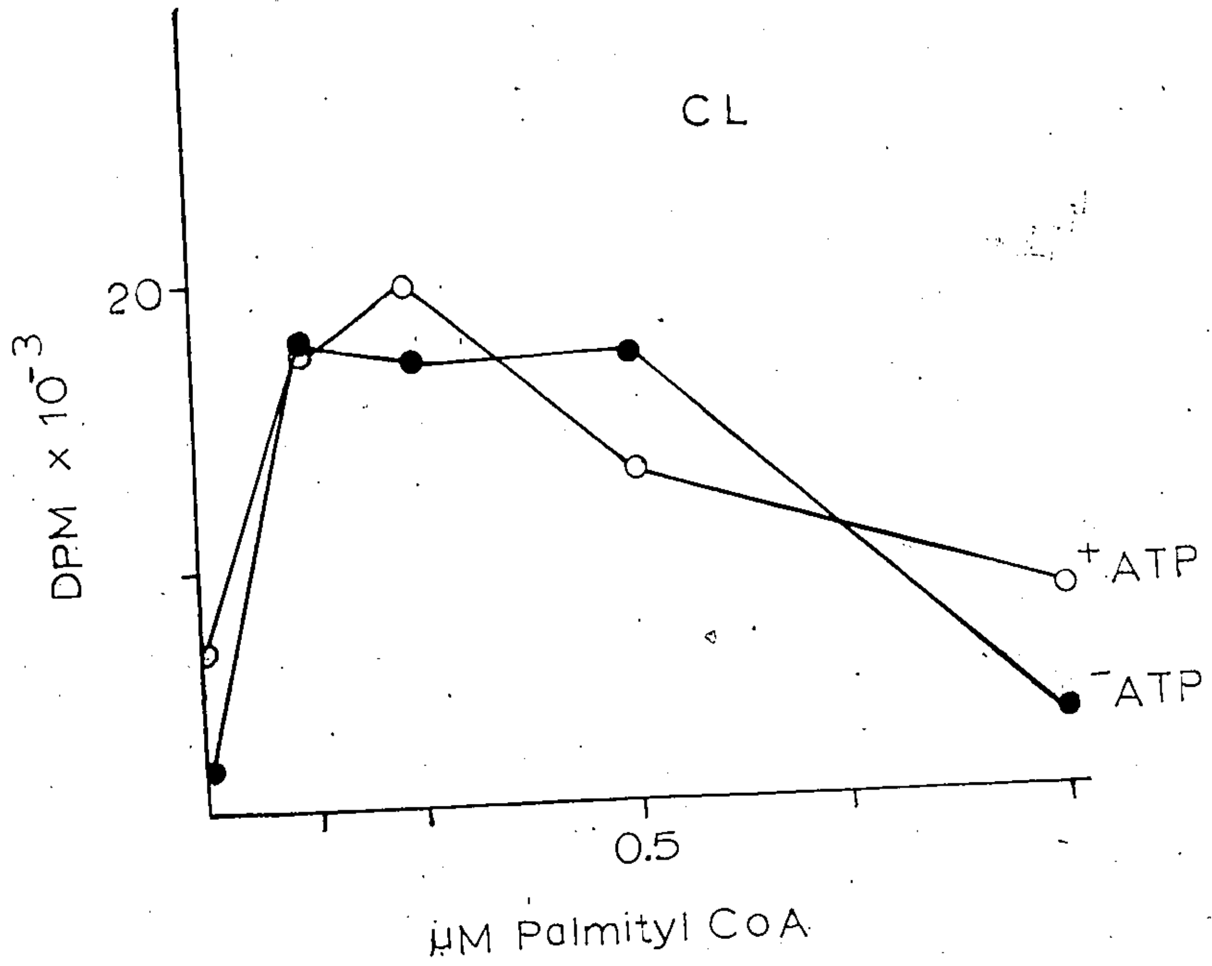


Figure 16

nucleotide and Mg^{++}. As can be seen from Figure 15, ATP + Mg^{++} greatly stimulated PG formation even in the presence of optimal palmitoyl CoA concentrations.

(ii) The Effects of ATP and Mg^{++} on the Distribution of Label Among Lipid Classes and Within Phosphatidylglycerol

In normally growing E. coli cells PE is the major phosphatide and represents approximately 75% of the total lipid phosphorous. If a culture is grown from a small inoculum in medium containing glycerol-1,3-^{14}C, labelling of lipids proceeds in a manner which reflects roughly the pool size of each lipid. This is illustrated in Table VII.

In most of our experiments with cell homogenates, it was noted that labelled glycerophosphate incorporated in a manner quite different than would be expected from the pool size of each lipid. When the medium was supplemented with ATP and Mg^{++}, the incorporation into PG well exceeded that into PE Although 1 mole of PG formed de novo incorporates twice as much glycerophosphate as 1 mole of PE, this alone would not account for the high proportion of labelling in PG by cell homogenates If it did per se the labelling pattern obtained with intact cells and homogenates should be quite similar. These conclusions prompted a more detailed investigation of those factors influencing the distribution of label within phosphatide classes.

Table VII*

Incorporation of Glycerol-1,3-^{14}C into Phosphoglycerides of Growing *E. coli* Cells

Lipid Fraction	DPM Incorporated	Distribution of Label as Per cent of Total Lipid Counts
PE	225,400	59.8
PG	88,600	23.5
CL	54,200	14.4
Other Lipids	7,600	2.3

E. coli 015 cells were grown 6 h at 37°C in a medium containing per 200 ml, 3 g Bactopeptone, 0.2 g yeast extract, 1.0 g sodium chloride; 50 uCi of glycerol-1,3-^{14}C and 4 g glucose autoclaved separately. Lipids were extracted by the method of Bligh and Dyer (109) and separated in system I. Components were counted by method D.

*Data obtained by Dr. K.S. Cho.

Results summarized in Table VIII corroborate those described earlier in Table II and in Figure 12. Addition of Mg^{++} in the presence of ATP causes a shift of incorporation favoring the polyglycerophosphate fraction. The effects of Mg^{++} are essentially the same at 25°C and 37°C. Temperature effects summarized in Table VIII are less marked than those illustrated in Table II. This is explained by the higher protein concentration used in the present experiment. As was noted earlier (Table III) with Mg^{++} and ATP addition there is an accompanying decrease in the labelling of PE which is intensified at higher temperature.

Aliquots of PG obtained from the proceeding experiment were degraded by phospholipase C from Bacillus cereus. Results shown in Table IX reveal that even in the absence of added Mg^{++} (but in the presence of ATP) labelled precursor incorporates mainly in the unacylated glycerol moiety of PG. Addition of Mg^{++} accentuated the uneven distribution by increasing the activity in the unacylated glycerol moiety. In these experiments the absolute counts in the diglyceride moiety remain constant, however in previous studies (cf. Tables III and VIII) a decreased formation of labelled diacylglycerol was evident with the decrease in labelled PE formed in the presence of Mg^{++}.

Table VIII

The Effect of Mg^{++} and Temperature on the Labelling of E. coli Phosphoglycerides by sn-Glycero-3-Phosphate-U-^{14}C

Lipid	DPM Incorporated in Each Fraction			
	25°C		37°C	
	No Mg^{++}	$+Mg^{++}$	No Mg^{++}	$+Mg^{++}$
PE	124,800	120,700	192,100	131,600
PG	132,800	240,900	127,300	231,900
CL	8,400	24,300	29,300	67,300

The incorporation medium contained in a volume of 4 ml; phosphate buffer (pH 7.3), 0.07 M; palmitic acid, 0.1 mM; oleic acid, 0.2 mM; CTP, 0.4 mM; CoA, 0.1 mM; ATP, 3.7 mM; E. coli sonicate, 9-13 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C, 1 uCi (Sp. Act. 16 uCi/umole). Incubation was at 25°C or 37°C for 1 h with or without the addition of 40 umoles of $MgCl_2$. Lipids were extracted by the method of Bligh and Dyer (109), separated in system E .

Table IX

The Effect of Mg^{++} and Temperature on the Distribution of ^{14}C Label Within PG Formed from sn-Glycero-3-Phosphate-U-^{14}C

Conditions for PG Synthesis			Radioactivity Recovered in the Products Obtained after Phospholipase C Hydrolysis of PG	
°C	$[Mg^{++}]$ mM	PG Formed DPM	Diglyceride DPM	Glycerophosphate DPM
25	0	132,800	39,800	92,900
25	10	240,900	43,300	192,700
37	0	123,300	41,900	80,100
37	10	231,900	30,100	176,200

PG obtained as described for Table VIII was hydrolyzed by phospholipase C of Bacillus cereus. After removal of the ether the lipids were extracted by the method of Bligh and Dyer (109). The radioactivity in aqueous and chloroform phases was counted by methods A, B and D. The lipids were separated in system B. Glycerophosphate was identified in system 1.

Discussion

The foregoing experiments were aimed at establishing optimal conditions for synthesis of phosphoglycerides and revealing possible metabolite control of this process in E. coli cell-free preparations. For this purpose, the influence of substrate and cofactor availability on incorporation of labelled sn-glycero-3-phosphate into lipid classes was studied. Of the substrates added few had marked effects. Palmitoyl CoA at certain low concentrations stimulated incorporation as would be expected and fatty acid in the presence of CoA, ATP and Mg^{++} mimicked the effect of palmitoyl CoA. Serine, CoA and CTP each had little or no effect at the concentrations tried, a result indicating their ample reserve as endogenous precursors. For similar reasons addition of unlabelled rac-glycero-3-phosphate did not appreciably affect the incorporation of serine-U-^{14}C into PE.

Among the cofactors tried, ATP and Mg^{++} were the most effective. ATP had very interesting effects in that at high concentrations it inhibited and at low concentrations it stimulated phosphoglyceride synthesis. Initially we tried explaining the inhibitory effect in the light of the results of Kito and Pizer (107) who showed that ATP inhibited

PA synthesis under certain conditions. We observed however that ATP inhibition was reversed by increasing the Mg^{++} concentration. This prompted the conclusion that ATP inhibited by chelating Mg^{++}, a cation required for most of the biosynthetic reactions. Under conditions similar to those of Kito and Pizer no significant inhibition of PA synthesis by ATP could be demonstrated with our E. coli strain. On this basis a possible negative effector function of ATP on PA synthesis was dismissed.

More interesting yet was the stimulatory effect of ATP which occurred in the presence of sufficient Mg^{++}. The stimulatory effect was not completely abolished by palmitoyl CoA addition and therefore implicated a step other than just the formation of acyl CoA derivatives. With sn-glycero-3-phosphate-U-^{14}C as precursor the stimulation occurred only in the polyglycerophosphatide fraction which was accompanied by a decreased labelling of PE. A preliminary conclusion was that ATP exerted a modulator effect at the branch-point favoring the formation of PG. This idea however was not substantiated by further experimentation. Analyses of the distribution of the label within PG with phospholipase C revealed that the unacylated glycerol moiety contained more isotope than did the acylated glycerol moiety. ATP plus

Mg^{++} addition accentuated this uneven distribution. It was obvious from these results that PG was synthesized partly by a de novo process (since there was some labelling in the diglyceride moiety) and partly from a process making use of an endogenous diacylglycerol precursor. The effect of ATP plus Mg^{++} was to increase the production of this unlabelled precursor, or to increase its incorporation into both PG and PE.

As endogenous diacylglycerol precursors, diglyceride and PA were considered eminent possibilities even though these lipids are present in only trace amounts (61). The fact is, their small pools could be constantly generated under our incubation conditions by a pathway hitherto unknown or of unexplained function.

One mechanism for the formation of endogenous diacylglycerol precursor would involve the acyldihydroxyacetone phosphate pathway) implicating a de novo process starting from a simple unlabelled precursor. The stimulatory role of ATP plus Mg^{++} could then be explained by a secondary reaction such as rephosphorylation of diglyceride overcoming any phosphatidate phosphohydrolase activity. This would then imply the action of diglyceride kinase.

One other possibility would involve a phospholipase D - like activity generating unlabelled PA. There is however not much precedent for involving this enzyme in bacterial lipid metabolism. White and collaborators (125) did find it in *Hemophilus parainfluenzae* and in *E. coli*, and recent evidence does indicate that cardiolipin synthesis proceeds via a transphosphatidylation reaction involving two molecules of PG as substrate. If a phospholipase D activity could be demonstrated in *E. coli* with one or more phosphoglyceride substrates the effects of ATP plus Mg^{++} would still require explanation.

The remainder of this thesis consists of experiments designed to show from what the unlabelled diglyceride originates, how it is formed, and how ATP plus Mg^{++} intervene.

PART B

Studies on Alternative Pathways for the Synthesis of Phosphatidic Acid

Introduction

In the foregoing section, results indicated that when sn-glycero-3-phosphate-U-^{14}C incorporates into PG, the unacylated glycerol moiety becomes more highly labelled than the acylated glycerol moiety. These findings prompted a search for pathways yielding an acylated glycerol or phosphatidyl moiety from an unlabelled precursor. A likely possibility was that the product of such a pathway, PA or diglyceride, would dilute any phosphatidate formed from labelled sn-glycero-3-phosphate.

Since in our studies, cell homogenates were used, various unlabelled glycolytic intermediates in the incubation mixture could serve to form dihydroxyacetone phosphate (DHAP), a suitable substrate for the synthesis of PA in mammalian tissues (75). Although the occurrence of a pathway involving the acylation of DHAP had not been shown in E. coli, there was no definite evidence that acylation of sn-glycero-3-phosphate occurred directly or that this pathway was the only manner by which phosphatidic acid synthesis resulted. In the investigation that follows, this point is further clarified.

One other pathway for the formation of unlabelled PA could involve diglyceride kinase, an enzyme which was found by independent workers (61,73) to occur in certain *E. coli* strains. The function of this enzyme remains uncertain since diglyceride represents a small pool of lipids which appears to be metabolically stable under conditions studied by Kennedy et al (61). In our attempts to further elucidate the function of diglyceride kinase and to explain the uneven labelling in PG, we have investigated the occurrence of this enzyme in *E. coli* 015, and under conditions which have been used to detect its activity we tested the effect of diglyceride on the incorporation of labelled sn-glycero-3-phosphate into *E. coli* lipids.

Materials and Methods

Preparation of Dihydroxyacetone Phosphate

A small amount, 0.3 mg of dihydroxyacetone phosphate dimethylketal monocyclohexylamine salt purchased from Sigma Chemicals was stirred with 0.4 gm of Dowex 50 (H^+) for 4 h at 37°C. The suspension was filtered and the filtrate was adjusted to pH 6.5, and then evaporated to dryness. This method was described by the supplier.

Incorporation of Doubly-labelled Glycerol in Intact Cells

Sixty ml of an *E. coli* 015 culture grown to the late log phase in nutrient broth containing 2% glucose were centrifuged 10 minutes at 5000 g. The sedimented cells were resuspended in 10 ml of the same medium containing a mixture of glycerol-2-^3H (0.151 mCi) and glycerol-1,3-^{14}C (0.039 mCi) and incubated 20 minutes at 37°C. After harvesting, the cells were extracted by the method of Bligh and Dyer (109). The lipids obtained and labelled substrate precursor used were counted using methods A and B respectively.

Incorporation of Various Precursors in *E. coli* Homogenates

Cell-free extracts of *E. coli* were prepared as

described in the General Methods. A standard 4 ml incubation mixture contained $MgCl_2$, 0.01 M; CTP, 0.4 mM; ATP, 3.7 mM; CoA, 0.1 mM; palmitic acid, 0.1 mM; and oleic acid 0.2 mM; whole <u>E. coli</u> homogenate (10 mg protein/ml) and 0.07 M phosphate buffer, pH 7.3 as solvent and diluent. Fatty acids were added as a sonicated dispersion. Labelled precursors and other additives were as specified in the text. Incubations were carried out for 1 h at 25°C with vigorous agitation.

<u>Various Analytical Procedures</u>

Lipids were extracted as described in the General Methods and separated by chromatography in system E. PE and PG were always the main products obtained and the identity of these components was checked by mild alkaline hydrolysis followed by paper chromatography of the water soluble products using systems 1 and 4.

Lipid components were scraped into scintillation vials and these as well as water-soluble products were counted as described in the General Methods.

Phospholipase C prepared was used to analyze the distribution of label in phosphatidylglycerol as previously described on page 59. Diglycerides obtained in this manner were separated by chromatography in system B and counted in the usual manner.

Preparation of Diglyceride

^{14}C-labelled diglyceride was obtained from the total lipid prepared by incubating E. coli 015 homogenates with sn-glycero-3-phosphate-U-^{14}C. Total lipids extracted by the method of Bligh and Dyer (109) were hydrolyzed with phospholipase C of B. cereus by the method of Plackett (123). After hydrolysis, total lipid was extracted by method of Bligh and Dyer (109) and the lipids were separated by thin layer chromatography in system A The diglyceride component was identified by cochromatography with authentic dipalmitin. The labelled diglyceride was removed from the adsorbent by extraction using the method of Bligh and Dyer (109) Dipalmitin obtained from Mann Research Laboratories was found to be chromatographically pure.

Assay of Diglyceride Kinase

Cell-free extracts of E. coli 015 were prepared as described in the General Methods with 0.01 M phosphate buffer pH 7.0, containing 0.01 M cysteine and centrifuged at 30,000 g for 30 minutes to obtain a particulate fraction. The sediment was washed and resuspended in buffer and heated 10 minutes in a boiling water bath as indicated by Pieringer and Kunnes (68). ^{14}C-diglyceride prepared as described in the Methods for this section was sonicated in 1% cutscum (v/v). The incubation medium was essentially that used by these authors.

Results

1. *In vivo* Incorporation of Doubly-labelled Glycerol into *E. coli* Phosphatides

Results summarized in Table X indicate that intact cells incorporate doubly-labelled glycerol into total lipids without a change in the ^3H-^{14}C ratio. A conversion of labelled glycerol to dihydroxyacetone phosphate prior to lipid formation would have resulted in a loss of ^3H relative to ^{14}C. Consequently, conversion of glycerol to lipids must have involved a direct acylation of any sn-glycero-3-phosphate formed.

If these results do not preclude the possibility of a functional acyldihydroxyacetone phosphate pathway in intact cells, this would imply that the sn-glycero-3-phosphate and DHAP pools are physically separate or that the rate of acylation of sn-glycero-3-phosphate is much larger than the rate of oxidation.

2. Incorporation by *E. coli* Homogenates of Various Precursors into *E. coli* Lipids

To test further the possible presence of an acyl DHAP pathway in *E. coli*, studies were pursued with cell homogenates. If under our *in vitro* conditions this pathway did function appreciably, addition of unlabelled DHAP should lower the specific activity of the diacylglycerol precursors to a larger extent than sn-glycero-3-phosphate itself. As a consequence, the uneven distribution of ^{14}C

Table X

Incorporation of Glycerol-1,3-^{14}C and Glycerol-2-^{3}H into Lipids of <u>E. coli</u> in Intact <u>E. coli</u> cells

Fraction	^{3}H/^{14}C
Glycerol 1,3-^{14}C plus Glycerol-2-^{3}H	3.6
Total Lipids	3.7

Standard conditions were used as stated in the text

label in the glycerol moieties of PG should become more pronounced. This did not occur (Table XI). Instead a decrease of about 15 - 20% in the radioactivity of PE and PG resulted. These results indicated that a direct reduction rather than an acylation of added DHAP had occurred.

If again the acyldihydroxyacetone phosphate pathway functioned in E. coli, and no other factor intervened in the uneven labelling of the glycerol moieties of PG (Tables IX and XI) a substitution of precursor by glucose 3,4-^{14}C should alter this distribution. In this case the labelling in the diglyceride moiety should be greater or at least equal to that in the unacylated moiety of phosphatidylglycerol. There was only a very low incorporation (Table XII), of the ^{14}C-label into total lipids as compared to the other precursor used. This result could be explained on basis of a greater dilution of label when glucose is the precursor used or a limited conversion of labelled DHAP to sn-glycero-3-phosphate in the absence of added reducing cofactor. Nevertheless, there was a considerably higher incorporation of ^{14}C-label in the unacylated than in the acylated glycerol moiety of PG (Table XII) when this lipid was degraded with phospholipase C.

This uneven distribution of label as well as that noted when sn-glycero-3-phosphate was the precursor had to be due to a diluent pool of phosphatidyl moieties which was not being formed by acylation of dihydroxyacetone phosphate.

Table XI

The Effect of Dihydroxyacetone Phosphate on the Incorporation of Labelled sn-Glycero-3-Phosphate into *E. coli* Phosphoglycerides

Fraction	Without DHAP Addition	With DHAP Addition
	DPM Recovered	
PG	362,500	312,000
PE	160,100	128,000
	Percent of PG Counts Recovered	
Unacylated Moiety of PG	74	72

The conditions were standard as stated for Table IIa except that 1.4 mM dihydroxyacetonephosphate and 6.7 mM sodium fluoride were also added (75). The mixture was incubated 1 h at 25°C.

Table XII

Distribution of Label in Phosphatidylglycerol

Produced from Glucose 3,4-^{14}C

Phosphatidylglycerol Moiety	DPM Recovered
Diglyceride	1600
Glycerophosphate	3600

The conditions were standard except that 8 uCi glucose -3,4-^{14}C (Spec. Act. 13.8 uCi mM) was used as precursor. The mixture was incubated 1 h at 22°C. The counts given are those of an aliquot representating 25% of the total counts in PG.

The results as a whole do largely extend those experimental conditions under which DHAP is not a suitable acyl acceptor in E. coli (72).

3. Characterization of Diglyceride Kinase

The data summarized in Table XIII indicate a 38% conversion of labelled diglyceride to phosphatidic acid under the experimental conditions stated.

Lipids were first analyzed in system A which separated diglyceride from phosphoglyceride. The phosphoglyceride fraction was eluted by Bligh and Dyer (109) extraction and rechromatographed in system D. Only 1 component was detected. This component was eluted by Bligh and Dyer extraction (109) an aliquot was counted and the remainder, subjected to mild alkaline hydrolysis.

Results summarized in Table XIV reveal the identity of the phosphoglyceride product as phosphatidic acid.

4. The Effect of Unlabelled Dipalmitin on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into Phosphoglycerides

Since a diglyceride kinase was detectable in our strain of E. coli, we tested the effect of unlabelled diglyceride on incorporation of sn-glycero-3-phosphate-U-^{14}C

Table XIII

Formation of Phosphatidic Acid from
^{14}C-Diglyceride by E. coli 015

Condition	DPM Recovered		
	Diglyceride	Phosphatidate	Other Lipids
Heat Inactivated Enzyme	26,800	0	0
Active Enzyme	16,900	10,500	0

The 2 ml. incubation mixture contained 4.8×10^4 DPM of ^{14}C-diglyceride added as sonicate in 1% cutscum (v/v); ATP, 2.4 mM; $MgCl_2$, 100 mM, cutscum 1% (v/v); phosphate buffer (pH 7.0), 0.07 M containing 10 mM cysteine; E. coli particulate protein, 2 mgm. Incubations were for 2 hours at 37°C. Lipids were extracted by the method of Bligh and Dyer (109).

Table XIV

Identity of Phosphoglyceride Product obtained after Incubation of ^{14}C-Diglyceride with <u>E. coli</u> 015

	R_f Value		
	System A	System F	System 1
Intact Lipid	0	.1	-
Phosphatidic Acid Disodium Salt	0	.1	-
Mild Alkaline Hydrolysis Product	-	-	.25
Glycerophosphate	-	-	.25

into lipid. In this case, whole sonicates were used without heat treatment and cutscum was replaced by Triton-X 100. Under similar conditions Chang and Kennedy (61) were able to demonstrate diglyceride kinase activity in their E. coli strain. If diglyceride kinase were active under conditions which reveal the stimulatory effect of ATP plus Mg^{++}, addition of cold diglyceride should decrease the incorporation of any labelled diacylglycerol precursors into PE and PG; however, incorporation into the unacylated glycerol moiety of PG should be stimulated. The net predictable result should be a decrease in labelled PE and an increase in labelled PG.

The data expressed in Table XV show no effect of added dipalmitin on the incorporation of sn-glycero-3-phosphate-U-^{14}C. It should be noted however that diglyceride kinase is stereospecific for sn-1,2 diglycerides whereas the dipalmitin used in our case contained a mixture of 1,2 - 2,3 and 1,3 isomers. No more than approximately 20% of the dipalmitin had the correct conformation. Increasing this dipalmitin to a concentration of 14 mM was also without effect. The results would indicate that if diglyceride kinase was at all active under our conditions, it formed a pool of PA which was unsuitable for incorporation into more complex lipids and consequently did not affect the pattern of phosphoglyceride labelling.

Table XV

The Effect of Dipalmitin on the Incorporation of sn-Glycero-3-Phosphate-U-^{14}C into Phosphoglycerides

Condition	DPM Incorporated into Each Fraction	
	PE	PG
No added dipalmitin	6,400	10,100
1.4 mM dipalmitin	7,600	10,800

The incorporation mixture contained in 2 ml; phosphate buffer (pH 7.3), 0.07 M; CoA, 40 uM; CTP, 0.2 mM; ATP, 1.4 mM; $MgCl_2$, 10 mM; palmitic acid, 0.1 mM; oleic acid, 0.2 mM; E. coli sonicate, 3 mg protein/ml; sn-glycero-3-phosphate-U-^{14}C, 0.2 uC; (Sp. Act. 10 uCi/umole). Triton-X-100 was added to a final concentration of 150 mg/ml. Incubation was at 37°C for 30 minutes in the presence of added dipalmitin (1.4 mM) or in its absence. Lipids were extracted by method of Bligh and Dyer (109) separated in system C and counted by method C.

Discussion

From the results in Part A, it could be deduced that under our conditions not all the diglyceride moiety of phosphoglycerides was synthesized via the acylation of sn-glycero-3-phosphate. This was supported by the fact that the unacylated glycerol moiety of PG was more highly labelled than the acylated moiety. Addition of ATP and Mg^{++} accentuated this effect. To explain these results we put forward several postulates (cf. discussion of Part A).

One of these possibilities was that part of the diglyceride moiety was synthesized from a small molecule precursor other than sn-glycero-3-phosphate and we visualized the possible operation of the acyldihydroxyacetonephosphate pathway. Our attempts to demonstrate the occurrence of this pathway in E. coli were unsuccessful. Perhaps under different conditions its functioning may be eventually demonstrated, although other workers have indicated that DHAP is an unsuitable acyl acceptor in E. coli (72).

Another possibility was that the diglyceride kinase pathway might function to form an unlabelled pool of phosphatidic acid. The peculiar effects of ATP plus Mg^{++} of decreasing the extent of labelling in the diglyceride moieties of phosphoglycerides could be conveniently explained on this basis. Although we could demonstrate the presence of this pathway in E. coli 015, unlabelled exogenous diglyceride did not produce the expected decrease in labelling of diglyceride

moieties by sn-glycero-3-phosphate-U-^{14}C. Possibly the diglyceride was not added in a suitably "micellized" form although under similar incubation conditions we were able to show formation of PA from ^{14}C-diglyceride derived from E. coli. The idea that in broken cells diglyceride kinase forms a pool of PA unsuitable for incorporation into more complex lipids has to be considered as an explanation. It can be added here that the conditions used to detect diglyceride kinase in E. coli have not been shown to be suitable for conversion of PA to other phosphatides. Perhaps in intact cells under suitable conditions diglyceride kinase may serve some role in scavenging exogenous partially acylated glycerides for the formation of phospholipids.

It would appear from the results of this section that neither the operation of the acyldihydroxyacetonephosphate pathway nor the diglyceride kinase pathway could account for the uneven incorporation of sn-glycero-3-phosphate in phosphatidylglycerol under our conditions. Even if the diglyceride kinase pathway were implicated, one would still have to demonstrate a mechanism for formation of unlabelled diacyl glycerol precursor. This is the object of our studies described in section C.

PART C

Studies on the Metabolism of Polyglycerophosphatides in *E. coli*.

Introduction

Results presented and described in Section B indicated that endogenous diacylglycerol precursor was not formed via the acydihydroxyacetone phosphate pathway under the experimental conditions chosen. Also, despite the presence of diglyceride kinase in *E. coli*, addition of unlabelled diglyceride in the presence of ATP plus Mg^{++} did not affect the labelling of phosphatides by sn-glycero-3-phosphate-U-^{14}C as might have been expected if an active PA pool had formed. It was tentatively concluded that under our conditions diglyceride kinase was probably not implicated in the incorporation of unlabelled diacylglycerol moieties into phosphoglycerides of *E. coli*. It is conceded, however, that this enzyme could still be involved in forming an active PA pool under more appropriate conditions not easily definable at this time. These negative results, and the fact that diglyceride and PA represent very small endogenous pools in *E. coli*, led to the consideration of another possibility, namely, that unlabelled diglyceride moieties were being generated during incubation from endogenous

phosphoglycerides. Such a mechanism if shown to occur could explain the uneven distribution of label in the glycerol moieties of PG provided that its response to ATP plus Mg^{++} could also be demonstrated.

Previous work indicated that whereas PE is a relatively stable lipid in intact E. coli (83) PG and CL display moderate turnover rates. However the mechanism(s) of breakdown and interconversion of polyglycerophosphatides remains largely unexplained. For these reasons we chose to investigate possible mechanisms of hydrolysis of PG and CL with cell homogenates. These studies led to the discovery of two hitherto undescribed pathways implicating polyglycerophosphatide metabolism in E. coli and point to a possibly important regulatory mechanism involving Mg^{++} and ATP.

Materials

PG and PE, for use as chromatographic standards, were obtained as described in Part B. PG, labelled approximately 70-75% in the unacylated glycerol moiety was obtained by incubating E. coli cell-free preparations with sn-glycero-3-phosphate-U-^{14}C as described for Table VIII.

(Tetrapalmitoyl) bis PA was purchased from Serdary Research Chemicals. This compound separated as a single phosphate containing, iodine-stainable, component

in systems F and G. Its infra-red spectrum (cf. Figure 17B) revealed absorption at 3200 and 1600 cm^{-1} possibly signifying some hydroxyl group content, likely due to water of hydration.

Labelled acylated PG was prepared by dissolving 10 mg of ^{14}C labelled PG in 0.5 ml trifluoracetic acid containing excess palmitoylchloride (100 mg). The reaction was carried out at 50°C with stirring for 1 hour. The trifluoracetic acid was removed under reduced pressure in a rotary evaporator. One ml of water was added and lipids were extracted by the method of Bligh and Dyer (109). Acyl-PG was then purified by sequential chromatography in systems B, D and F. The infra-red spectrum of this compound (cf. Figure 17A) indicated that it was devoid of free hydroxyl groups.

Beef heart CL and sodium phosphatidate were purchased from General Biochemicals Inc. ^{32}P-labelled CL was obtained from 1 litre of E. coli B, (ATCC 11303) cells cultured to the stationary phase in nutrient broth containing 5 mCi of ^{32}P-orthophosphate (New England Nuclear), isolated and then transferred to a starving medium (5 percent saline) for 3 hours. This latter step was reported to increase the levels of CL in E. coli (82). The labelled CL was isolated by Bligh and Dyer extraction (109) of the cells and successive preparative thin layer chromatography using systems D, F and I. The isolated material yielded GPGPG as

the sole labelled product of mild alkaline hydrolysis, and was identified by paper chromatography with system 1.

Phospholipase D was purchased from Calbiochem, and phospholipase C was prepared as described in the methods, Part B.

Methods

Enzyme Preparation for Studies on Phosphatidylglycerol Metabolism

E. coli 015 cultures were prepared as previously described in the General Methods. The cells were suspended in 25 ml of 0.07 M phosphate buffer, pH 7.4, containing 0.01 M cysteine hydrochloride and sonicated for 15 one minute periods in ice. Whole cells were removed by centrifugation at 5,000 g for 10 minutes and the supernatant obtained was centrifuged at 30,000 g for 30 minutes. The particulate fraction, washed once and resuspended in cysteine-phosphate buffer, gave a protein concentration of 12 mg/ml as determined by the method of Lowry et al (108).

Enzyme Preparation for Studies on Cardiolipin Metabolism

E. coli 015 cell-free homogenates were prepared as described in the General Methods. The suspension was diluted with 0.07 M phosphate buffer, pH 7.3, to give a

protein concentration of 7 mg/ml. E. coli B (ATCC 11303) was purchased from General Biochemicals Inc. as a frozen sediment of cells obtained from a culture in the late log phase. It was treated as described for E. coli 015 and the suspension was diluted in buffer to give a protein concentration of 36 mg/ml.

The extraction, chromatographic analyses, and staining of intact lipids, as well as mild alkaline hydrolysis of lipids and chromatographic analyses of the products obtained were performed as described in General Methods.

Acetic Acid Hydrolysis of Lipid and Water Soluble Samples

The products were hydrolyzed in a boiling water bath for twenty minutes with 90 percent acetic acid according to the method of Coulon-Morelec et al (126).

Hydrolysis with Phospholipase D

Phospholipids were hydrolyzed with Phospholipase D according to the method of Yang et al (127). Phospholipase D, prepared from cabbage, was obtained from Calbiochem Inc. 7.0 mg (containing 35 units) was dissolved in 0.4 M $CaCl_2$ and 0.2 ml phospholipase D preparation and was incubated at 25°C for 30 minutes. The reaction was stopped by the addition of 0.1 ml of 1 N hydrochloric acid. The lipids were extracted

by the method of Bligh and Dyer (109) after removal of the ether by evaporation at 40°C. Lipids were separated in systems B and F.

Ester Determination

The number of ester groups contained in aliquots of the unidentified lipid, PG (from E. coli) and commercial Cl, was determined using the method of Antonis (128). The dried lipid (containing 0.5 to 4 ueq ester) was dissolved in isopropyl ether (3.0 ml) and alkaline 2 M hydroxylamine solution (1.0 ml) was added, stoppered shaken and allowed to react at room temperature. After 30 minutes reaction time 6.0 ml ferric perchlorate solution (1.2 mgm ferric perchlorate) was added, stoppered, shaken, and allowed to react 30 minutes in the dark before reading the absorbance at 515 nm using a Coleman Junior Spectrophotometer. A standard curve was prepared from a working standard of tributyrin (99% pure) containing 10.25 mgm/25 ml of chloroform.

Infra-Red Analysis

Phospholipid, dissolved in chloroform, was layered between two sodium chloride discs for analysis with a Unicam SP 200 infra-red spectrophotometer.

Results

Metabolism of Phosphatidylglycerol

1. Formation of an Uncharacterized Lipid

Previous results in this laboratory had shown that when PG is incubated with cell-free homogenates in the presence of anionic detergents and Ca^{++} (86) it is attacked by phospholipase A to yield the characteristic lyso derivative. Purified phospholipase A of E. coli was also shown to attack readily this lipid. Other workers showed that in the presence of Mg^{++} and nonionic detergent, E. coli preparations will transform PG to CL via a transphosphatidylation reaction (80). Dr. Cho of this laboratory was able to confirm the functioning of the latter pathway using conditions specified by Hirschberg and Kennedy (79). It is apparent from these results that the metabolism of PG in broken cell preparations is highly dependent on conditions such as type of cation added and the charge on the substrate micelles increased by detergent addition. In the present study we aimed initially at conditions which might allow hydrolysis of PG by phospholipase D activity and chose therefore incubation conditions which are known to favour this reaction. Accordingly, the test was made in the presence of ether, Ca^{++}, and acetate buffer pH 5.6. Under

these conditions no evidence for the presence of phospholipase D activity was obtained. When conditions were modified slightly and the test was carried out in the presence of phosphate-cysteine buffer, pH 7.4, 10 mM $CaCl_2$ and ether, PG was readily converted to a major, less polar, compound having the same mobility as CL in chromatographic system D. Two other minor products were also detected. The major labelled component was not stainable by Periodate-Schiff or ninhydrin reagent indicating the absence of vicinal hydroxyl and free amino groups.

The compound was eluted by Bligh and Dyer extraction (109) and subjected to mild alkaline hydrolysis. Part of the water soluble product was analyzed directly by paper chromatography and identified as GPG. The remaining portion was hydrolyzed in 90 percent acetic acid and then analyzed by paper chromatography. The product in this case was glycerophosphate and glycerol (cf. Table XVIa). It was fairly evident from these results that the major incubation product was other than CL and consisted of a PG derivative containing no extra polar entities such as hydroxyl or aminoacyl groups. The unknown substance, designated Lipid X, was likely semi lyso bis PA (acyl PG) or bis PA.

2. Studies on the Identity of Lipid X

(a) Chromatographic Analyses

Lipid X, isolated by preparative chromatography

Table XVIa

Chromatographic Analysis of the Deacylation Product of Lipid-X

Substance	R_f Value Obtained in Various Systems			
	1	2	3	4
Diglycerophosphorylglycerol (GPGPG)	0.15	-	.05	0.58
Glycerophosphorylglycerophosphate (GPGP)*	-	-	-	0.20
Glycerophosphate (GP)	0.28	0.18	.23	0.26
Glycerol (G)	0.77	0.72	-	0.77
Glycerol-1,3-diphosphate (GDP)	-	-	-	0.05
Glycerophosphorylglycerol (GPG)	0.44	0.57	.15	0.67
Deacylated Lipid-X	0.41	0.56	.18	0.64
Deacylated Lipid-X further Hydrolysed with 90% acetic acid (126)	-	-	-	0.26 0.77

* R_f value obtained by Chang and Kennedy (63).

with systems B, D and F was subjected to further analysis using several other chromatographic systems (cf. Table XVIb). In systems D and F it cochromatographed with commercial tetrapalmitoyl bis PA and labelled acylated PG and could be easily distinguished from PA, PE and CL. System A revealed that Lipid-X was free of neutral lipid contaminants. Earlier work (84) had shown that PG sometimes separated erratically into two components depending on salt forms or the presence of PE in an extract. When Lipid-X was mixed with PE plus PG and re-chromatographed in system D it separated as a distinct component of unchanged R_f value. On this basis and because the unknown substance did not stain with periodate-Schiff reagent, Lipid-X could not have been a form of PG which separated erratically. Indications at this point were that Lipid-X was in fact bis PA or a close analogue. Chromatography in system G revealed however that Lipid-X ran more slowly than bis-PA or acylated PG and had an R_f value reported for semilyso bis PA (112). Its identity as bis PA could not be precluded on this evidence alone since for a tetra-acylated compound the fatty acid composition might be expected to affect its mobility. Unfortunately we had at our disposal no authentic bis-PA and semilyso bis-PA standards of different fatty acid composition.

Table XVIb

R_f Values of Lipid-X and Related Phospholipids for Various Chromatographic Systems

	Chromatographic System			
Phospholipid	D	F	G	H
Lipid-X	.68-.74	.72-.84	.75-.94	.69-.79
CL	.74	.35-.40	0.40-.50	.81
PG	.37	.35	.53	.60
Acylated PG[1]	.70-.74	.81-.88	.77-.81	-
Bis-PA[2]	.74	.77	.97	.70
PA	-	.05-.15	0.09	.93
PE	.52	.51	.03	0.50

[1] acylated PG= phosphatidylglycerol acylated as described in Methods Part C.

[2] bis-PA= tetrapalmitoyl bis phosphatidic acid obtained from Serdary Co.

(b) Acetic Acid Hydrolysis of Lipid-X

Phosphatides containing in their polar moieties an hydroxyl group vicinal to the phosphate ester, such as PG for example, hydrolyze in 90 percent acetic acid to yield diglyceride and a phosphomonoester. This method used by Coulon-Morelec et al (126) in their identification of CL, could in theory serve to distinguish between such compounds as bis-PA or 2'acyl-PG which would not hydrolyze and 3'-acyl-PG which would breakdown to diglyceride and lyso-PA. Success with this method however would be highly dependent on whether cyclic phosphate formation or acyl migration could be prevented. The procedure designed by Coulon-Morelec et al has not been applied to a large variety of compounds but with a limited number of lipids tried by them, cyclization could be avoided if 90 percent acetic acid rather than glacial acetic acid were used.

When Lipid-X was subjected to hydrolysis using the procedure of Coulon-Morelec et al little or no water-soluble products were obtained and about 20 percent of the label was found in diglyceride (separated in system A). The remainder of labelled lipid had the same R_f value as intact Lipid-X in system F. Since about 25-30 percent of the label in the glycerolphosphorylglycerol backbone structure of

Lipid-X belonged to the sn-glycero-3-phosphate moiety, it follows that acetic acid hydrolysis had proceeded to near completion. Yet labelled lyso PA could not be identified as a product. It is likely therefore that hydrolysis resulted in cyclic lyso PA formation. The latter substance could not be identified for lack of authentic reference material.

(c) Treatment of Lipid-X with Phospholipase C and D

Phospholipases C and D are often used for the elucidation of phosphatide structures. In theory hydrolysis of Lipid-X with these enzymes and identification of the products should allow a choice between the two possible structures, namely bis-PA and semi lyso bis-PA (acyl-PG). Unfortunately Lipid-X was found to resist prolonged (3-4 h) treatment with phospholipase C or D as did our acylated PG preparation. No structural information could be derived by use of these enzymes.

(d) Infra-Red Spectrometric Analysis of Lipid-X

The infra-red spectrum of Lipid-X (cf. Figure 17 D) is quite similar to that of commercial bis-PA (Figure 17 B) and CL (Figure 17 E) especially in the region (3200 cm^{-1}) indicative of free hydroxyl groups. This absorption band was

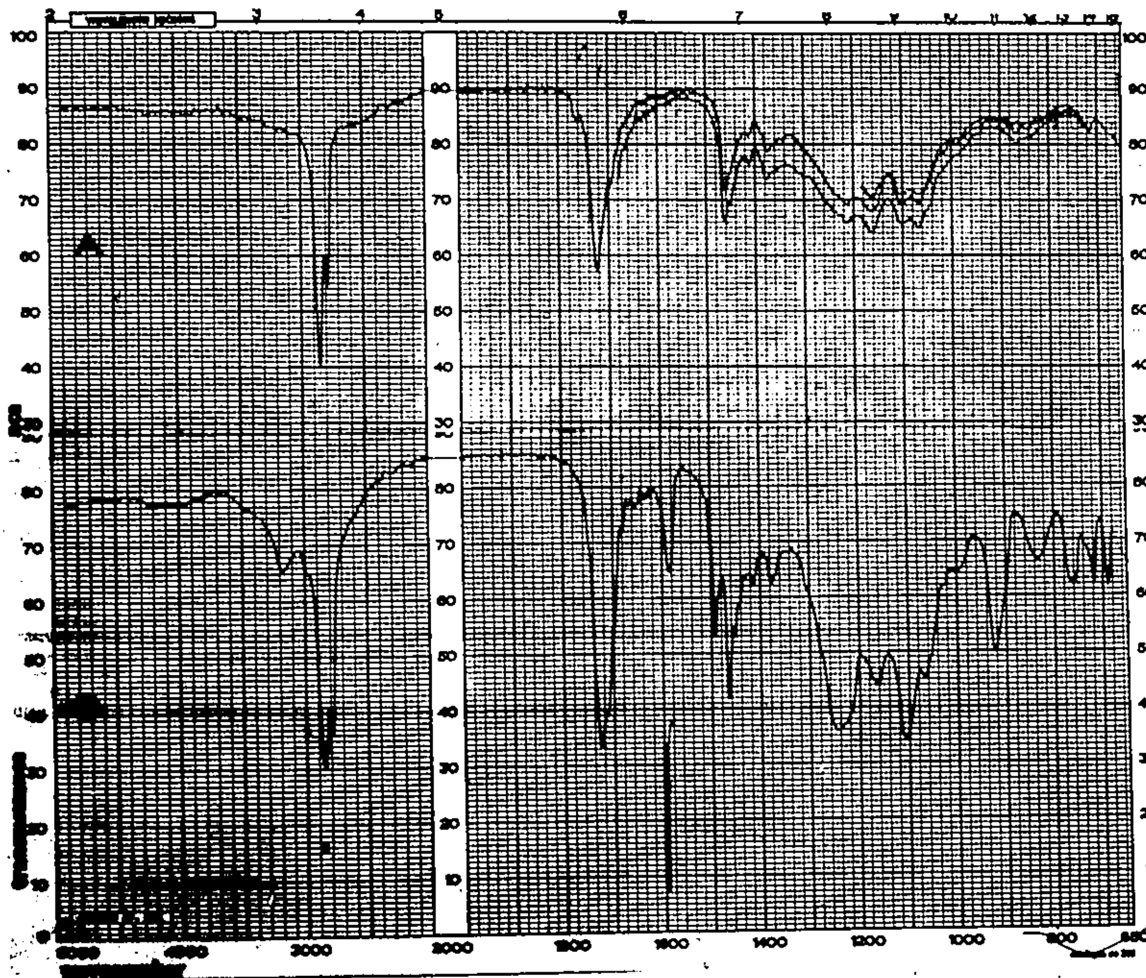


Figure 17., A - Infra-red spectrum of acylated ^{14}C-phosphatidylglycerol.

B - Infra-red spectrum of commercial tetrapalmitoyl bis-phosphatidic acid.

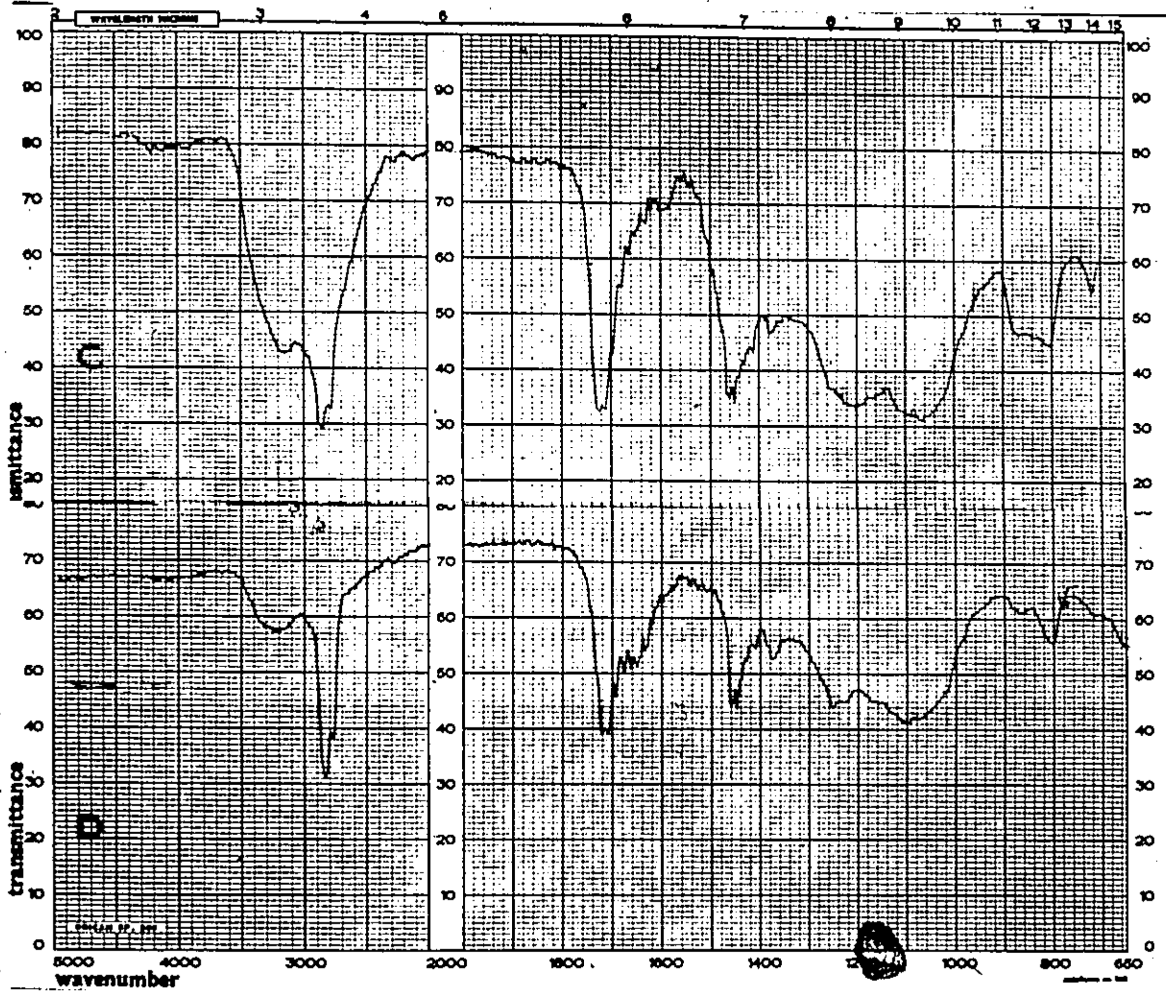


Figure 17. C - Infra-red spectrum of phosphatidylglycerol.

D - Infra-red spectrum of Lipid-X.

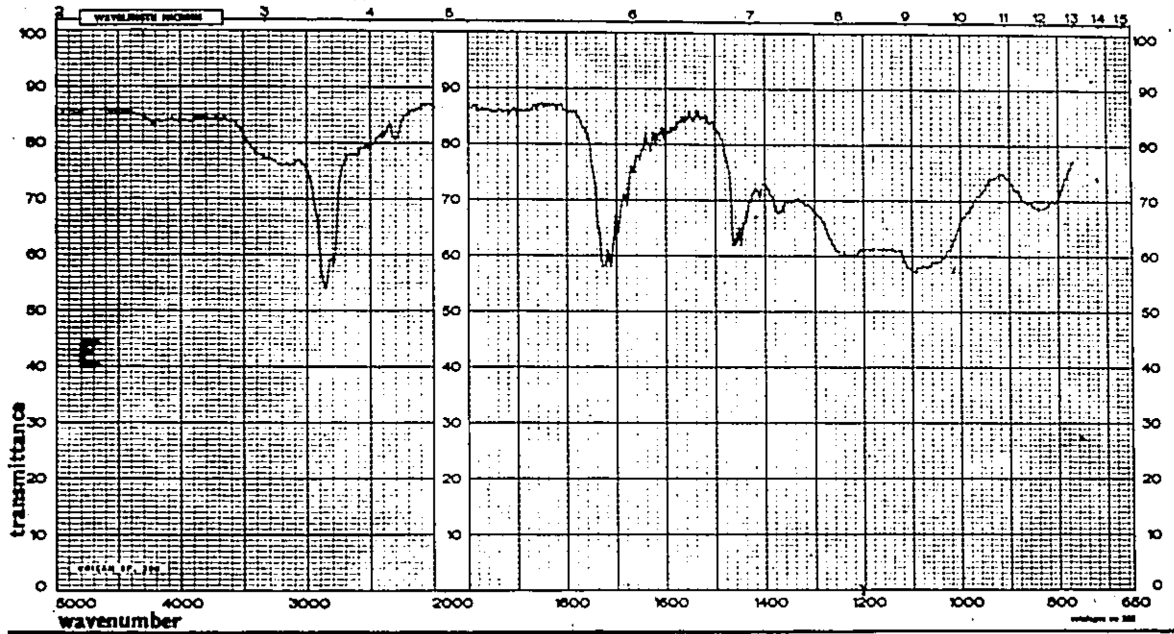


Figure 17. E - Infra-red spectrum of beef heart cardiolipin.

much stronger for PG (Figure C) than for the other phos-pholipids analyzed. The acylated PG prepared as described showed no absorption in this region.

The water absorption band at 1600 cm^{-1} was strong in the commercial preparation of bis-PA but was lacking in the spectra of Lipid-X, PG, acylated PG and CL.

From these scans the presence of free hydroxyl groups in Lipid-X indicates that it is a semi-lyso bis-PA rather than a fully acylated bis-PA.

(e) Ester and Phosphorous Content of Lipid-X

Results shown in Table XVII reveal for Lipid-X an ester/phosphorous ratio of 3.4 a value slightly higher than expected for acyl-PG. PG and CL also analyzed as control samples gave the expected ratios of approximately 2.1.

Conclusion

On the basis of the results presented it appears that *E. coli* under specific conditions can form an acylated derivative of PG, likely acyl-PG. Definite identification of Lipid-X was curtailed by the difficulty of obtaining sufficient amounts of chemically pure material. Radiochemical purity of the compound was however established and a tentative identification was made mainly on the basis of chromatographic analyses of the intact lipid and its deacylated derivative.

Table XVII

Phosphorous and Ester Contents of Isolated Phospholipids

Phospholipid	Micro equivilents of Phosphorous per mg	Micromoles of ester per mg	Ester/ Phosphorous
Lipid-X	0.5	1.7	3.4
PG	1.3	2.5	1.9
CL	1.25	2.7	2.2

Since these findings although interesting did not offer any solutions to the questions posed in Sections A and B further identification of Lipid-X and studies on its mechanism of formation were not pursued. Since no energy supply is needed for the reaction it can be surmised that the formation of acylated PG likely involves a transacylation reaction between PG and a suitable acyl donor, possibly PG itself or another phospholipid.

Cardiolipin Metabolism

One major difficulty in attempting to demonstrate a mechanism of breakdown for CL had been the isolation of sufficient labelled substrate from E. coli or other sources to carry out detailed experiments. Attempts to obtain highly labelled material by tritiation of ox-heart CL failed because of radiochemical and chemical decomposition. Using a method based on a report by Rampini et al (82) that CL levels greatly increased when E. coli cells were starved we were able to isolate sufficient ^{32}P-labelled CL (6.4×10^6 DPM) to allow its extensive purification and the pursuit of detailed experiments.

One other factor which curtailed advancement with the problem of CL breakdown was our experimental approach itself. We reasoned that addition of anabolic cofactors

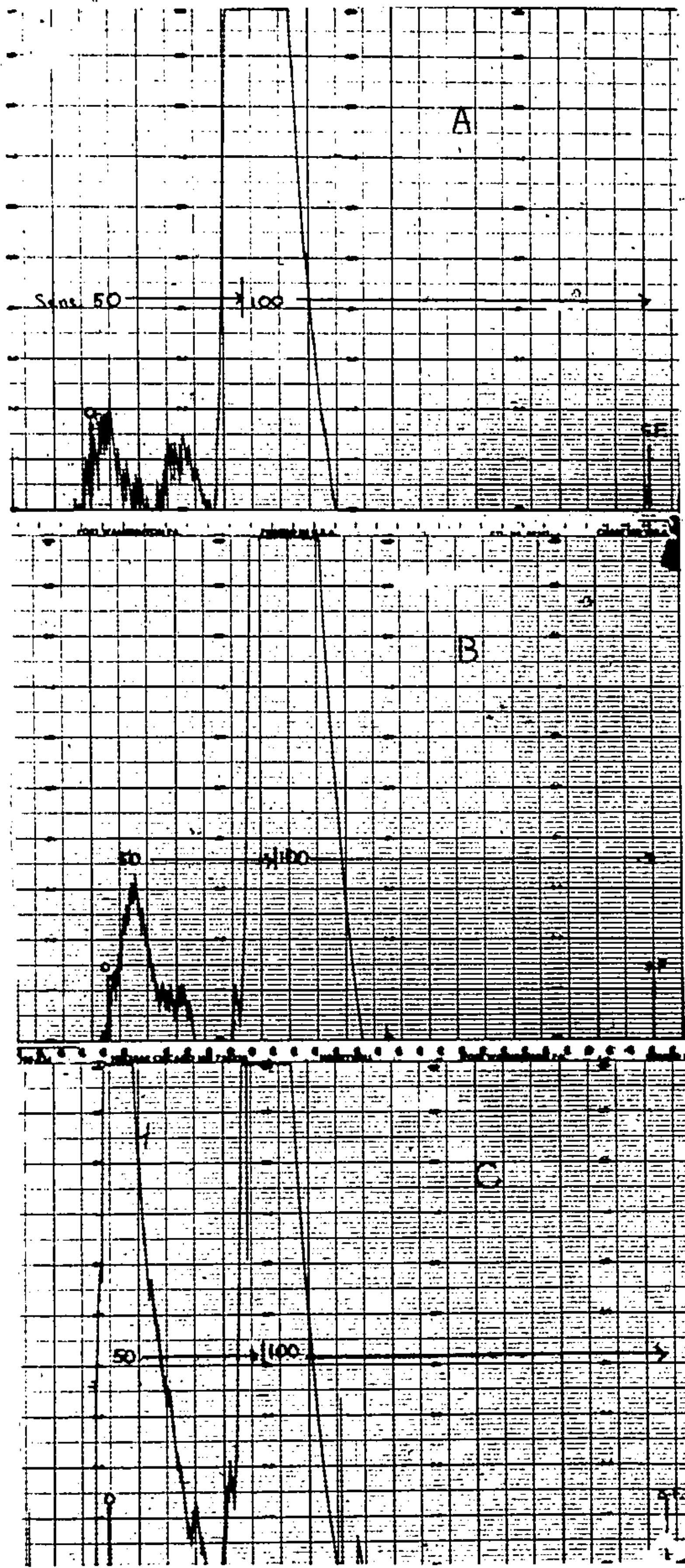
such as ATP would not be required for a reactinn that in fact involves hydrolysis. This reasoning was reinforced when White and his collaborators (129) reported hydrolysis of CL to PA and PG by Hemophilus parainfluenzae under rather simple conditions i.e., in the presence of nonionic detergent and Mg^{++}. Adopting their conditions, however, we were unable to definitely demonstrate phospholipase D activity in E. coli preparations. It was then decided to use reaction mixtures similar to those which allowed uneven labelling of the glycerol moieties of PG by sn-glycero-3-phosphate-U-^{14}C. Accordingly conditions described for Table VIII were chosen and after incubation, lipids were extracted by the method of Bligh and Dyer (109).

When aliquots of lipid extract were analyzed by thin layer chromatography in system F, the following results were obtained (Figure 18): little or no hydrolysis occurred with heat-inactivated enzyme (A) but extensive breakdown resulted when both ATP and Mg^{++} were added to fresh enzyme preparation (C). In the presence of Mg^{++} only, hydrolysis was much less extensive (B). Essentially the same results were obtained with E. coli 015 and B (ATCC 11303). Component 1, R_f 0.05-0.08 cochromatographed with authentic PA and component 3 (R_f .35-.40) cochromatographed with PG and CL.

When aliquots of lipid extract were subjected to thin layer chromatography in system I, the following

Figure 18. The incubation mixture contained in 2 ml, 7 mg of E. coli 015 sonicated cells, ^{32}P-cardiolipin (20,000-40,000 DPM), 10 mM Mg^{++} 0.07 M phosphate buffer pH 7.3 and either no ATP (B) or 2.5 mM ATP (A and C). Incubations were for 90 minutes at 37°C. Lipids were separated in system F. Figures are tracings of radioactivity on chromatoplates scanned with Actigraph III.

Figure 18



results were obtained (Figure 19): heat-inactivated enzyme was devoid of activity (results not shown) whereas with the fresh enzyme in the presence of Mn^{++} only (A) there was little activity detectable by the scanning technique. In the presence of Mn^{++} plus ATP hydrolysis was much more extensive (B). Results obtained with Mg^{++} with and without added ATP also indicated the necessity of nucleotide addition for marked hydrolysis. Again E. coli 015 and B showed identical results. Component 2, (R_f 0.60-0.65) cochromatographed with PG and stained with periodate-Schiff reagent. Component 3 (R_f 0.83-0.86) cochromatographed with PA and CL. When component 2 was eluted by Bligh and Dyer extraction (109) and subjected to chromatography in system F, it cochromatographed with PG and moved much higher than PA. The component yielded a single spot with either iodine or periodate-Schiff staining. Component 1, Figure 19 B remaining at the origin was not identified.

When aliquots of lipid extract were analyzed in neutral system D, the following results were obtained (Figure 20): heat-inactivated enzyme caused no hydrolysis (A); with fresh enzyme and Mg^{++} (B) or Mn^{++} (C) alone hydrolysis occurred only to a small extent. In the presence of ATP plus Mg^{++} (D) or Mn^{++} (E) hydrolysis was extensive. Component 1 (R_f 0-0.05) could not be readily identified in this system. When PA as a calcium salt was applied in this system most of the material remained at the origin as a circular spot, however a portion of the sample streaked up to an R_f value of 0.65. Component 2 (R_f

Figure 19. The incubation mixture contained in 2 ml, 32 mg of _E. coli_ B, ^{32}P-cardiolipin (20,000-40,000 DPM), 10 mM Mn^{++}, 0.07 M phosphate buffer pH 7.3 and either no ATP (A) or 2.5 mM ATP (B). Incubations were for 90 minutes at 37°C. Lipids were separated in system I. Figures are tracings of radioactivity on chromatoplates scanned with Actigraph III.

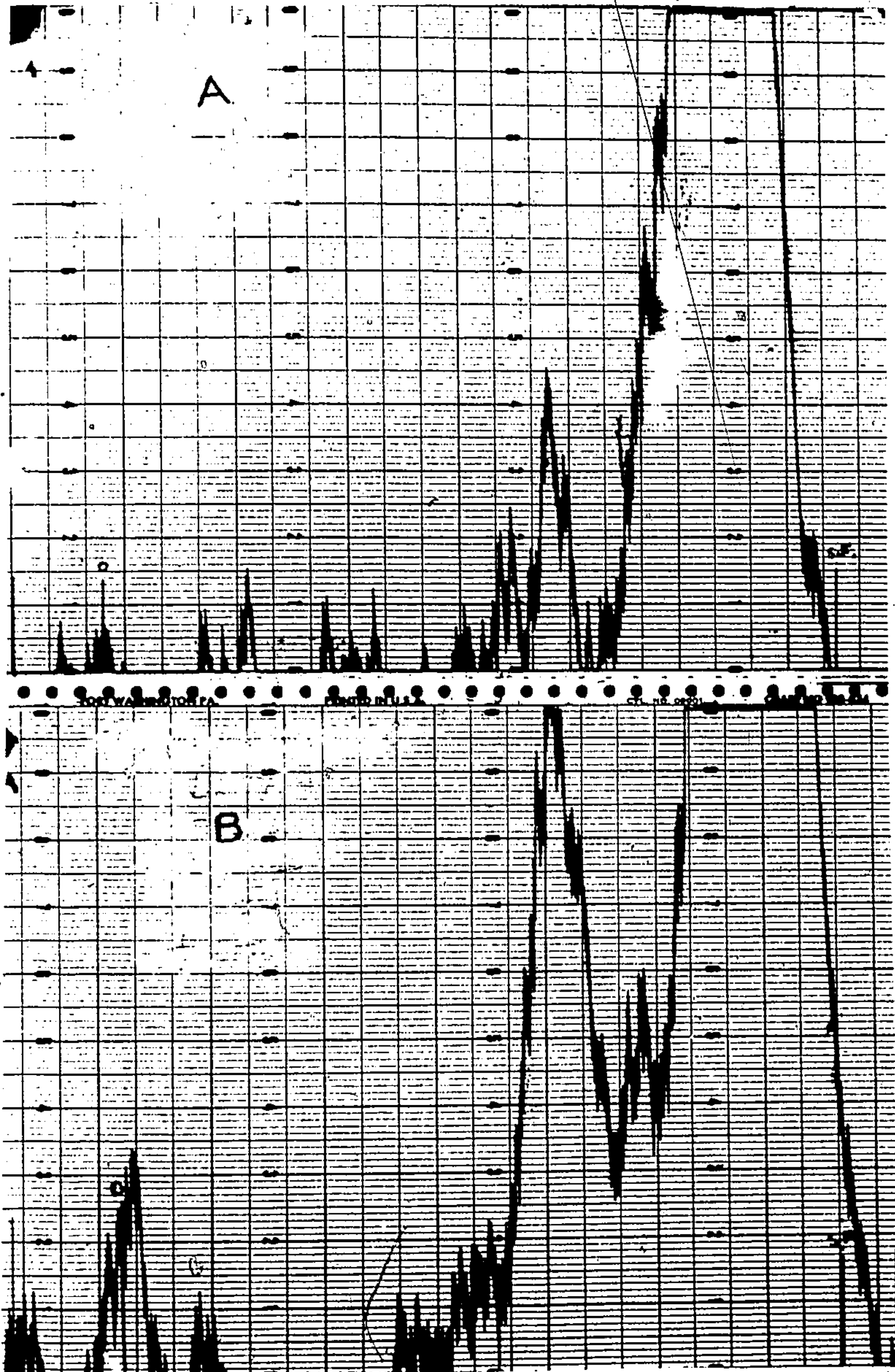


Figure 19

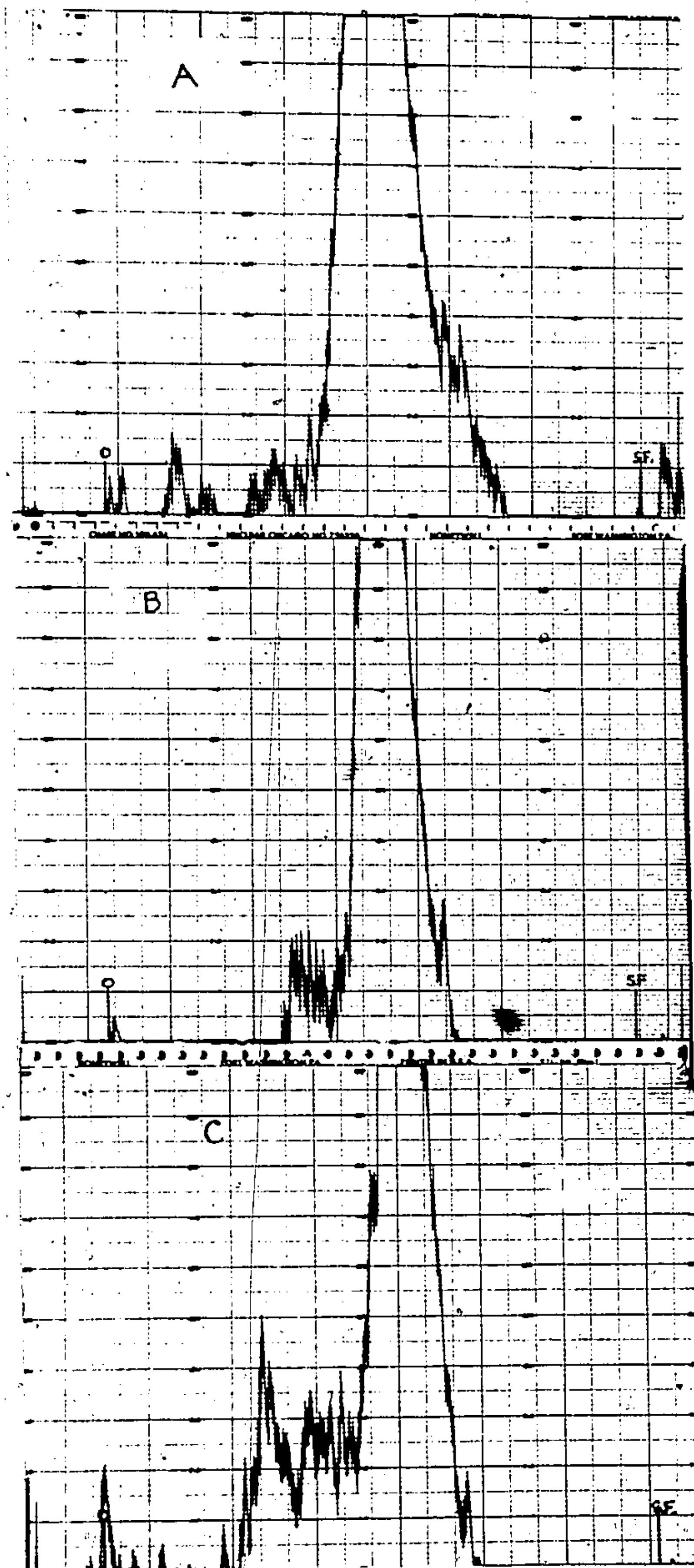


Figure 20

Figure 20. The incubation conditions were as stated for Figure 19. (A) inactivated enzyme in the presence of ATP; and Mg^{++}; (B) Mg^{++} only; (C) Mn^{++} only; (D) Mg^{++} + ATP; (E) Mn^{++} + ATP. Lipids were separated in system D. Figures are tracings of radioactivity on chromatoplates scanned with Actigraph III.

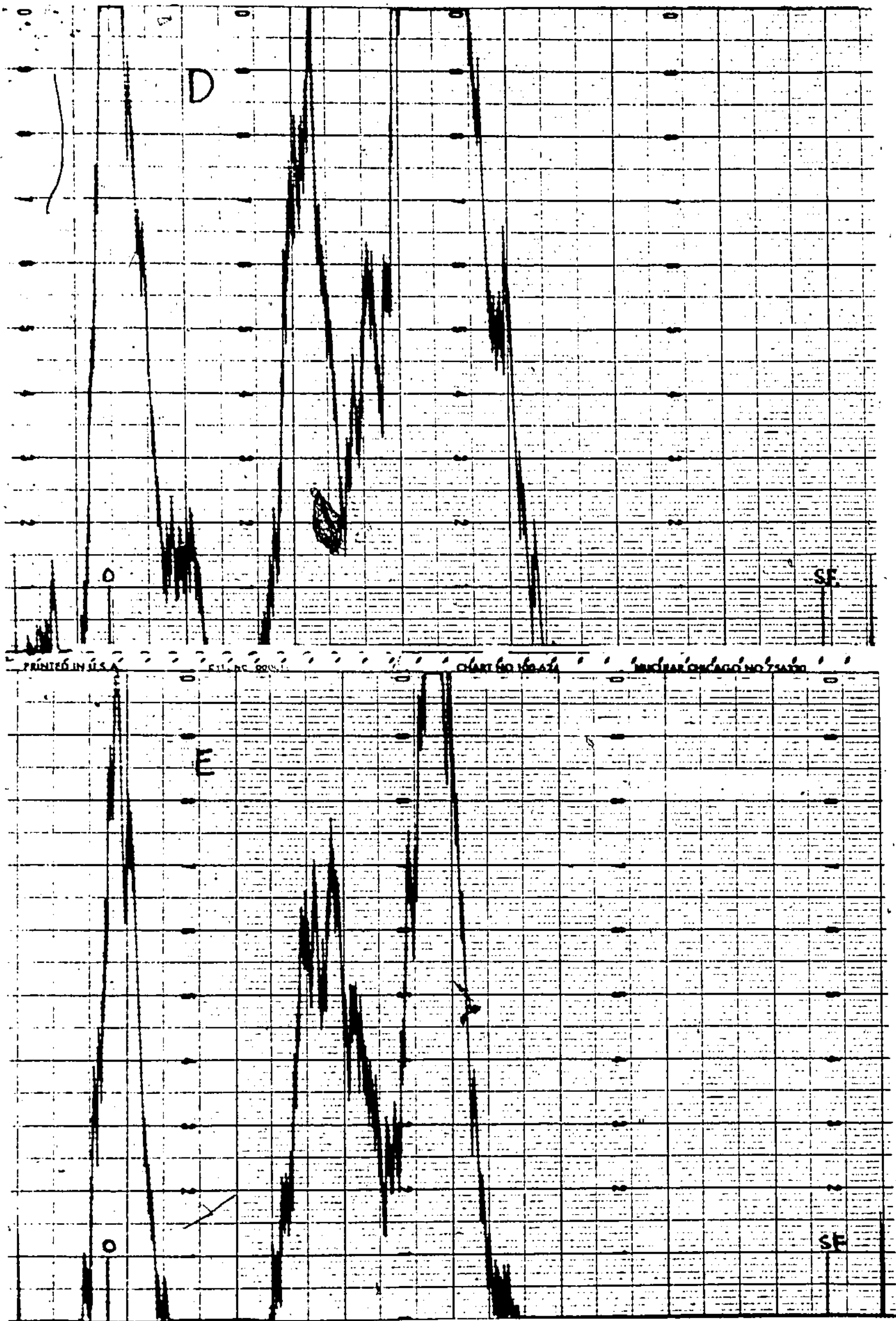


Figure 20

.25-.30) cochromatographed with PG and stained with periodate-Schiff reagent, whereas component 3 (R_f .5) cochromatographed with CL. When component 1 was eluted and rechromatographed on silica gel H with chloroform,-90 percent acetic acid - methanol 30:2:4 v/v/v, it cochromatographed with PA (Figure 21 A) and moved much higher than the reported value for CDP-diglyceride (130). When an aliquot of total lipid extract, obtained from an E. coli B preparation incubated with Mn^{++} plus ATP, was chromatographed in this last system (Figure 21 B) only one hydrolysis product was revealed (component 2 R_f 0.65) which cochromatographed with PG and stained with periodate-Schiff reagent. Component 3 (R_f 0.88) cochromatographed with CL and PA.

When aliquots of lipid extract were subjected to mild alkaline hydrolysis and then analyzed by paper chromatography with phenol-water as solvent the following results were obtained (Figure 22): with heat-inactivated enzyme (A) no hydrolysis occurred and only GPGPG could be identified whereas with active enzyme in the presence of Mg^{++} only (B) or Mn^{++} only (C) hydrolysis occurred to a limited extent. In the presence of ATP plus Mg^{++} (D) or Mn^{++} (E) hydrolysis was extensive and two additional water soluble products were identified, namely GPG and glycerophosphate (GP).

Quantitative results expressed in Table XVIII indicate that under optimal conditions (ATP plus Mg^{++} or

Figure 21A. The material remaining at the origin (component 1) in system D and derived from conditions stated for Figure 20 D and E was eluted by acid Bligh and Dyer extraction (109) and pooled. It cochromatographed with phosphatidic acid in the system described by Marshall and Kates (130). Figures are tracings of radioactivity on chromatoplates scanned with Actigr

Figure 21B. Total lipid extract obtained under conditions stated for Figure 20 D was chromatographed in the system described by Marshall and Kates (130). Figures are tracings of radioactivity on chromatoplates scanned with Actigraph III.

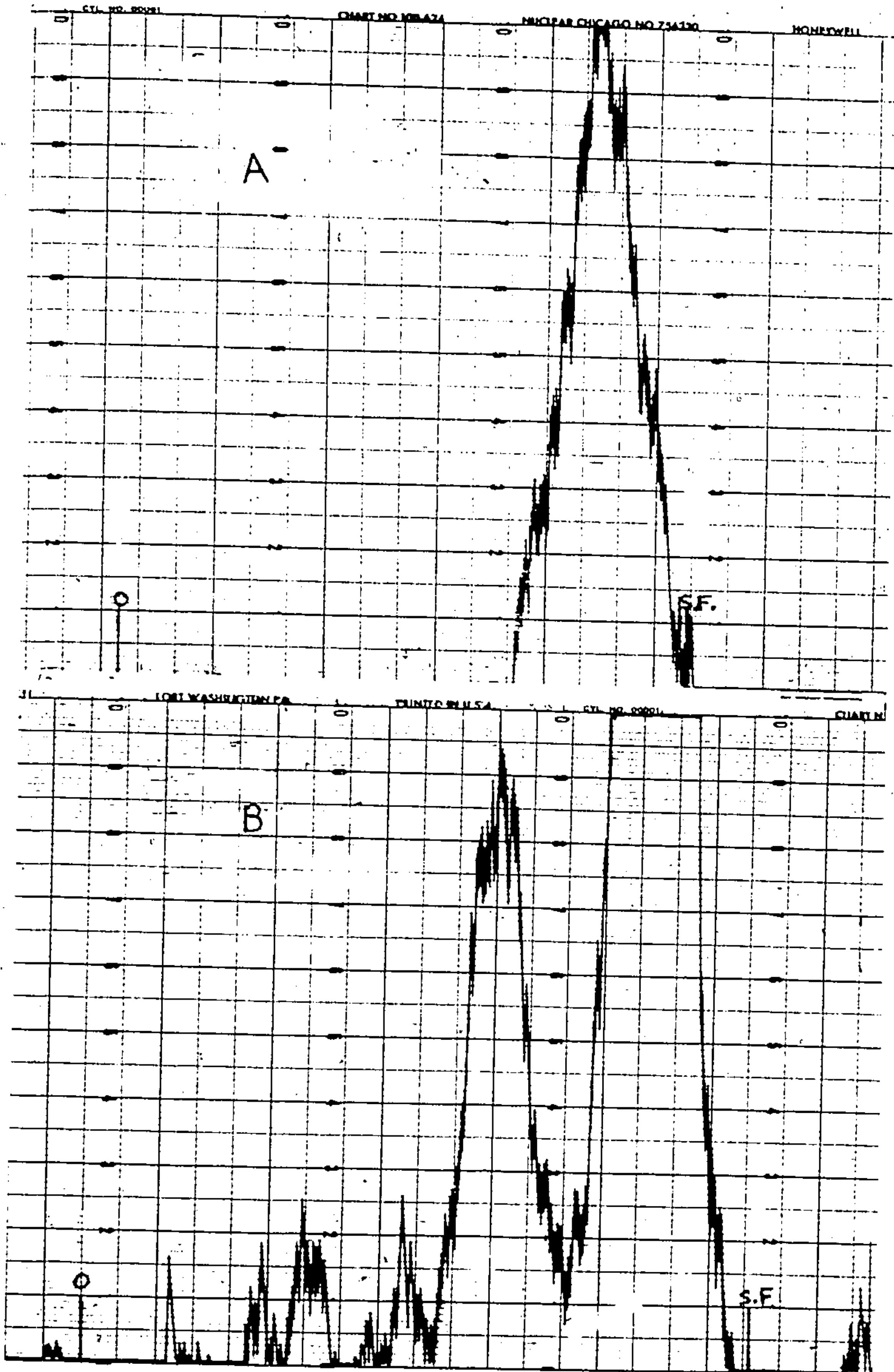


Figure 21

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Figure 22. Mild alkaline hydrolyses products of total lipid extracts obtained after incubations of E. coli B sonicate with ^{32}P-cardiolipin. Conditions were similar to those described in Figure 18 (A) enzyme in the presence of ATP + Mg^{++}; (B) Mg^{++} only; (C) Mn^{++} only; (D) Mg^{++} + ATP; (E) Mn^{++} + ATP. Water soluble products were separated by paper chromatography with phenol water (5:2 w/w) as solvent. Figures are tracings of radioactivity on chromatoplates scanned with Actigraph III.

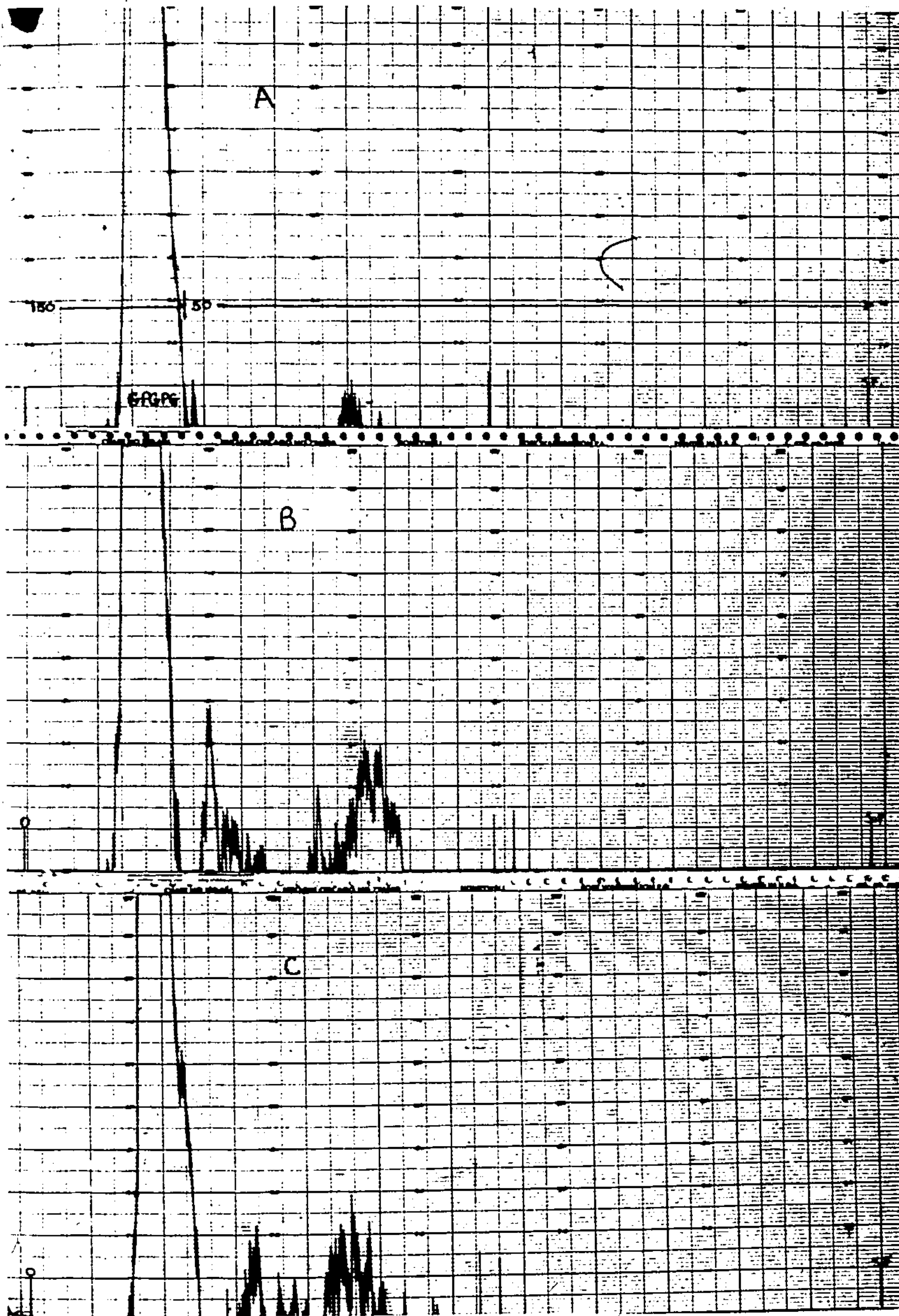


Figure 22

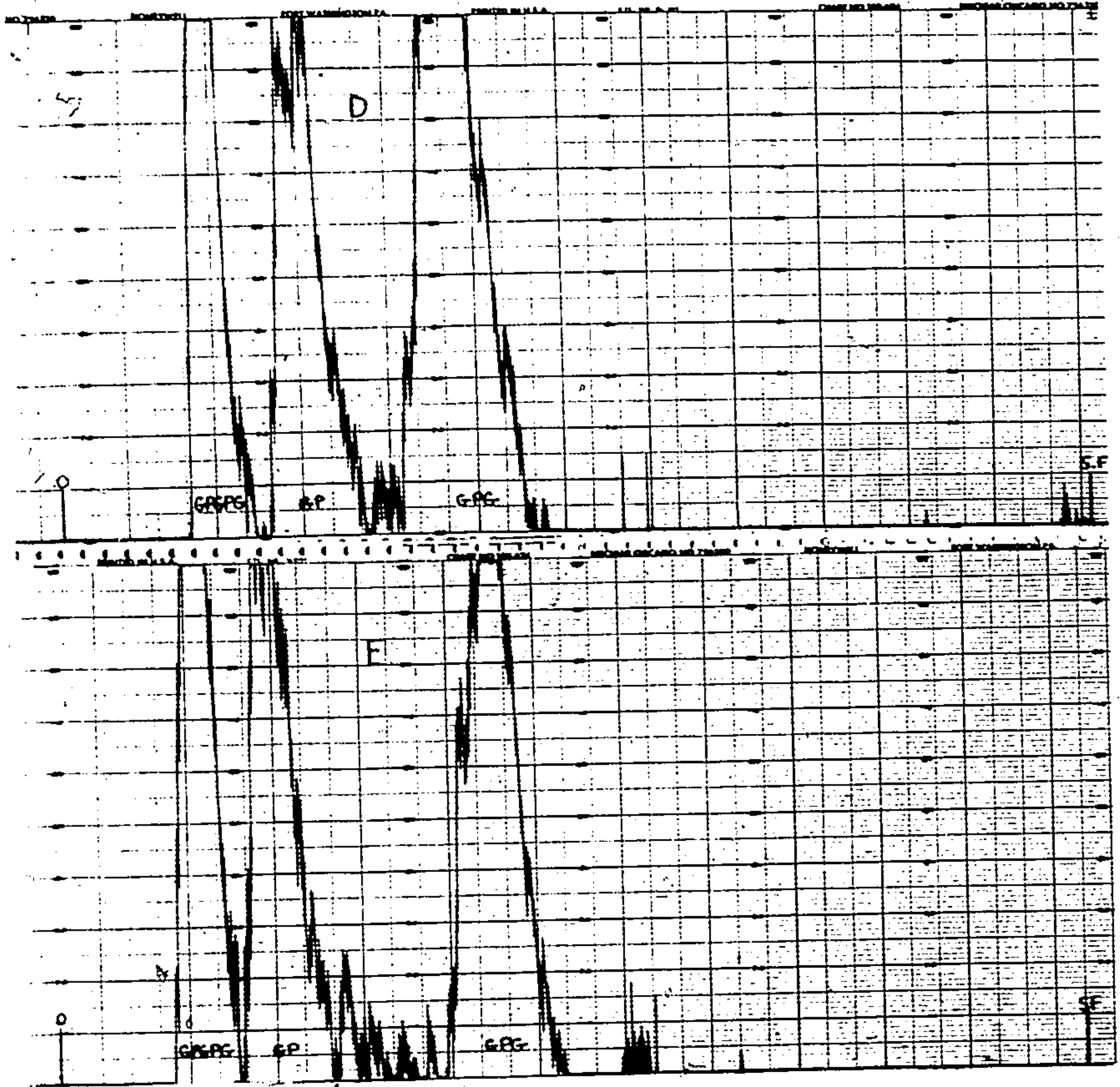


Figure 22

Table XVIII

Hydrolysis of ^{32}P-Cardiolipin by E. coli B Homogenates

Conditions	DPM Recovered in Each Deacylated Fraction		
	CL	PG	PA
Heated Preparation + ATP + Mg^{++}	10,600	50	70
Fresh Preparation + Mg^{++}	6,900	400	300
Fresh Preparation + Mn^{++}	6,900	500	400
Fresh Preparation + ATP	6,900	900	1,400
Fresh Preparation + ATP + Mg^{++}	4,900	2,300	1,800
Fresh Preparation + ATP + Mn^{++}	5,100	2,100	1,800

Incubation conditions were as stated for Figure 21. After extraction lipids were deacylated, separated by paper chromatography in phenol-water system 1 and areas of the chromatogram corresponding to each phosphodiester were cut into small strips and counted by method D.

Mn^{++}) about 45-50 percent hydrolysis occurred in 90 minutes at 37°C. Considerable breakdown also occurred in the presence of ATP alone indicating the presence of endogenous cations. In the absence of ATP hydrolysis fell to less than 10 percent. Recoveries of total counts varied between 80-90 percent and were in the lower range when ATP was absent. Possibly a parallel hydrolysis by the combined action of phospholipase A and lysophospholipase on CL took place resulting in a complete degradation to phosphodiesters and this would account for some loss of activity. Alternatively degradation of PA to diglyceride and inorganic phosphate may have also occurred and would account not only for loss of activity but for the fact that recoveries of PA were usually lower than those of PG.

The water soluble products of the reaction were not counted.

Preliminary results not shown indicated that Ca^{++} could not replace Mg^{++} or Mn^{++} and that 2.8 mM CTP in the presence of added Mg^{++} or Mn^{++} was ineffective in stimulating hydrolysis of CL.

Conclusion

From the data presented in this section, it can be concluded that CL is hydrolyzed by *E. coli* preparations

via phospholipase D activity. The lipid substrate specificity of the enzyme has not been determined. To date results in this laboratory have indicated that PE and PG are attacked by phospholipase A rather than this enzyme, however, the effect of ATP on the hydrolysis of these substrates has not been assessed. It is apparent however that <u>E. coli</u> phospholipase D has a requirement for both divalent cation and ATP. The precise involvement of these cofactors in the hydrolysis reaction requires further investigation.

GENERAL DISCUSSION

The biosynthesis of the various phospholipid classes found in E. coli, begins with the common precursor sn-glycero-3-phosphate and follows a single pathway up to the production of CDP-diglyceride. What regulates the incorporation of at least 75 percent of this CDP-diglyceride into PE and the remainder into PG and CL has not been studied extensively. It is known that CL can be formed from PG and in the absence of an energy source. Also CL levels are increased in the stationary phase of growth or under conditions where available energy is at a minimum (31).

A survey of factors that affect the incorporation of sn-glycero-3-phosphate into the three major phospholipids was done with a cell-free homogenates. It was found that under our conditions labelled sn-glycero-3-phosphate incorporated mainly into PG components. The fact that PG is derived from two glycerophosphate units does not account for the high percentage incorporation into this lipid. This preferential incorporation was not due to lack of available L-serine as the endogenous level of this precursor was found to be adequate for maximal incorporation of sn-glycero-3-phosphate into PE. As expected, ATP and Mg^{++} increased the acylation of sn-glycero-3-phosphate with the resultant increased incorporation of

this precursor into all the lipid classes. In the presence of optimal concentrations of palmitoyl CoA, the addition of ATP to the incubation mixture was shown to stimulate incorporation of labelled sn-glycero-3-phosphate into PG but in such a system PE and CL were not affected. This ATP effect on PG in the presence of added palmitoyl CoA was quite unexpected and not readily explicable on the basis of the known pathways.

The possibility that ATP and Mg^{++} were stimulatory by positive modulation on the CDP-diglyceride: sn-glycero-3-phosphate phosphatidyl transferase was dismissed when it was found that Mg^{++} and ATP increased the incorporation of sn-glycero-3-phosphate only into the free glycerol moiety of PG. If the transferase reaction had been stimulated the level of incorporation into the diglyceride moiety should have increased also but it was found that incorporation into the diglyceride moiety was in fact often decreased. This indicated that the precursor diglyceride formed from sn-glycero-3-phosphate was being diluted with diglyceride moieties derived from an endogenous source and that the effect of ATP was to increase the availability of this unlabelled precursor.

The possibility that ATP and Mg^{++} were inactivating the CDP-diglyceride: L-serine phosphatidyl transferase is unlikely if one considers the small decrease in labelling of PE compared to the large effect on the incorporation into free

glycerol moiety of PG. It also follows from this that any production of endogenous diglyceride moieties did not have any marked inhibitory effects on the synthesis of labelled diglyceride moieties. Although there would result from this production a dilution of the labelled diglyceride precursor, the transferase enzymes were probably not saturated with respect to liponucleotide. Consequently the decrease in labelling of diglyceride moieties of PE and PG was not very great or did not consistantly occur.

Our results are in agreement with those of Thomas et al (131) who concurrently found that ATP stimulated the incorporation of serine and sn-glycero-3-phosphate into the phospholipids of E. coli by increasing the endogenous pool of diglyceride. They explained this effect of ATP as an increased diglyceride kinase activity but provided no definite proof that this enzyme was actually involved. In our experience the addition of cold diglyceride, in the presence of added ATP, showed no effect on the incorporation of sn-glycero-3-phosphate into PE and PG although it was established that diglyceride kinase is present in our strain of E. coli. There exists the possibility that the diglyceride added was not in a proper micellized form for its incorporation into phospholipid. Also a mixture of rac 1,2 dipalmitin and 1,3 dipalmitin was used whereas it is known that diglyceride kinase is stereospecific for the sn-1,2 isomer.

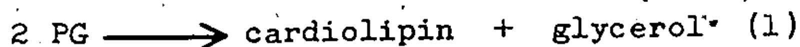
Nevertheless there is no evidence that the diglyceride kinase reaction and its stimulation by ATP and Mg^{++} provided the endogenous diglyceride for the synthesis of phospholipid, in their case as in ours. The fact is only very low levels of endogenous diglyceride are found in E. coli and on this basis alone diglyceride kinase involvement would have to be dismissed unless a pathway for generating this lipid was clearly shown to operate under our conditions used for the incorporation of sn-glycero-3-phosphate. The indications were that there was indeed an unlabelled diglyceride precursor of some sort and this generated a search for its identity.

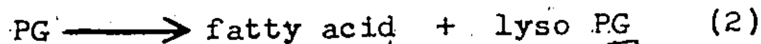
The acylation of dihydroxyacetonephosphate prior to its reduction to lysophosphatidic acid has been shown to occur in mammalian systems and could supply, if operative under our conditions, the unlabelled diglyceride moiety for synthesis of both PE and PG. We tried several means of demonstrating this pathway and found no evidence for its existence in E. coli.

Unlike PE which is a stable pool *in vivo*, both CL and PG display moderate turnover involving mechanisms not fully elucidated (83). Therefore these lipids were chosen as possible suppliers of diglyceride precursor. Phospholipase C activity on PG could perform such a function but the conditions

necessary to demonstrate the presence of this enzyme i.e. ether and calcium would make this activity unlikely under our usual conditions for incorporation of sn-glycero-3-phosphate. Nevertheless we examined this possibility using the particulate fraction only. We found quite unexpectedly that in the presence of ether and Ca^{++} PG did not degrade to diglyceride but was transformed into an acylated PG which separated like CL in the usual chromatographic systems used. This acylated PG has not been described previously in *E. coli* and represents a new pathway for the conversion of PG.

Since our discovery and identification in *E. coli* of a reaction forming acylated PG further work has been done in this laboratory to study the conditions which affect the production of this compound. Dr. K.S. Cho found that calcium and ether are absolute requisites for demonstrating this activity. If Mg^{++} and non-ionic detergent are present instead of Ca^{++} and ether PG is slowly converted to CL as was reported by others (79 , 80). In the presence of Ca^{++} and Triton-X-100, PG is converted to lysophosphatidylglycerol via the action of phospholipase-A. The fate of PG as it turns over *in vivo* could be depicted by the following reactions which have been elucidated *in vitro*.


2 PG ⟶ cardiolipin + glycerol (1)


PG ⟶ fatty acid + lyso PG (2)

PG + PX ⟶ acyl PG + lyso-PX (3)

The first reaction involving a transphosphatidylation is reasonably well established now. It is interesting that CDP-diglyceride stimulates this reaction but according to Hirschberg and Kennedy (79) this liponucleotide does not serve as substrate. Very likely the stimulatory effect of CDP-diglyceride is due to its detergent properties. Accordingly in our experiments we noted that whenever a detergent was added to an incubation mixture the amount of CL formed was always appreciably increased. This was certainly the case when palmitoyl CoA (c.f. Figure 15) or other detergents were present. However that reaction 1 occurs in viable cells is only weakly supported by the fact that whenever low energy conditions prevail the CL content increases (31). This would not be the case if CL synthesis required CDP-diglyceride as a substrate. There is in fact no direct evidence indicating the occurence of reaction 1 _in vivo_.

Again the evidence in favour of reaction 2 is derived mainly from experiments using cell-free preparations. Since there is a lysophosphoglyceride: acyl CoA acyl transferase in _E. coli_ one could picture the occurence of a cycle implicating this enzyme and phospholipase A in the partial turnover of phosphoglyceride acyl groups. There has been one claim to this effect (132) although results by Bright-Gaertner and Proulx (86) and by Wakil et al (133) have failed to indicate such a partial turnover mechanism in intact cells.

Present indications from our laboratory as well as from other groups (31) are that phospholipase A is active only in cells which are damaged or subjected to adverse conditions (93). Its location in coliforms on the exterior membrane of the cell envelope (134) make this enzyme quite suitable for a scavenger role.

As for reaction 3 the evidence in favour of a simple transacylation is still fragmentary. Recent results by Dr. Cho indicate it is indeed acyl PG that is the product of this reaction. Confirming our own results he found that the formation of acyl PG requires no ATP and free fatty acids are unsuitable as substrates. The reaction could be pictured as either involving two molecules of PG or 1 molecule of PG plus another low energy (acyl donor) ester as substrates. At this point we would like to suggest the possibility that the synthesis of acyl PG is catalyzed by phospholipase A_1. The work of Doi et al (92) and Bernard et al (91) indicates that phospholipase A of E. coli can catalyze ester hydrolysis as well as transacylation much as the lipoprotein lipase of post-heparin serum.

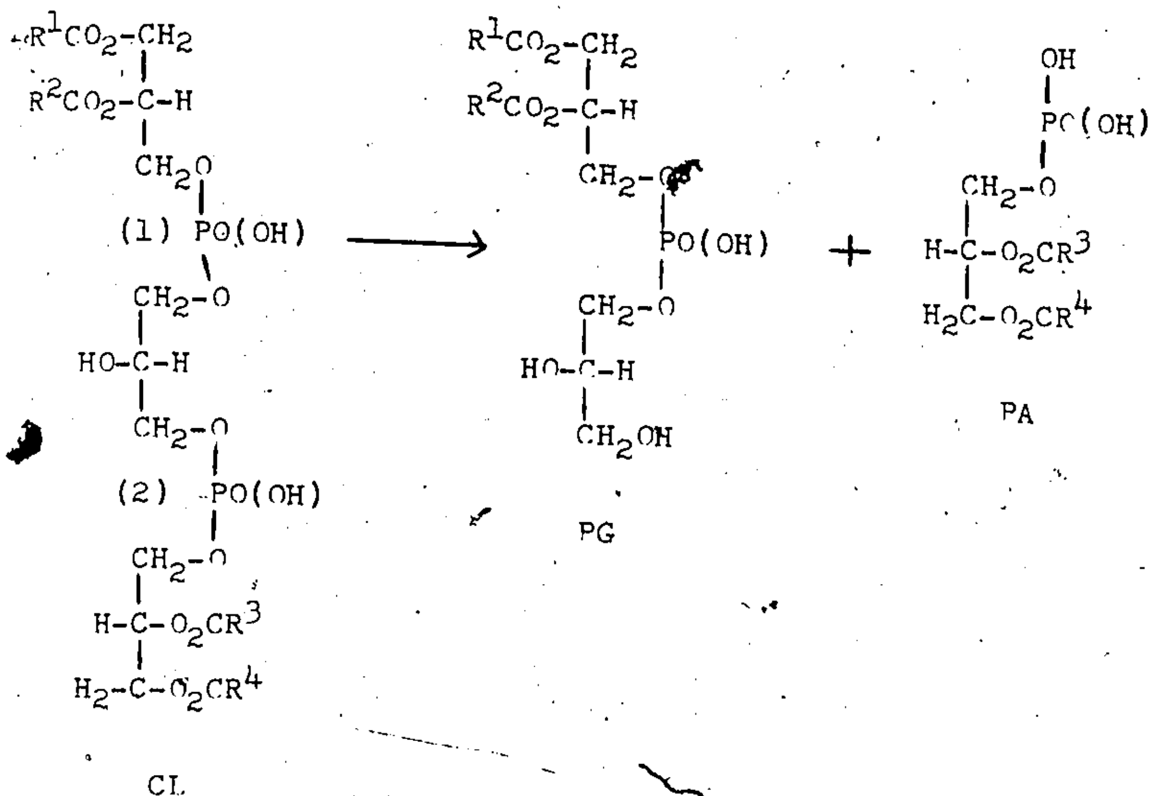
The role of acylated PG derivatives found in bacteria (135,136) and mammalian tissue (112) remains obscure. Do these substrates arise simply as by-products of phospholipase A activity or are they formed to satisfy a specific role of the membrane? Olsen and Ballou (135) pursuing the work of

Ames (114) identified sn-3-phosphatidyl-sn-1'-(3'-acyl) glycerol in Salmonella typhimurium, found this substance to turn over at the moderate rate characteristic of PG. This turnover involved complete synthesis and breakdown. Consequently the compound is not a transient anabolic precursor to other substances and does not serve in any special way for the temporary storage of acyl groups. At any rate, acyl PG occurs in Salmonella typhimurium as a very minor component and hence the importance of this substance in Enterobacteriaceae will likely remain unknown for some time.

Since the uneven incorporation of sn-glycero-3-phosphate into PG was favoured when ATP and Mg^{++} were present in the medium similar conditions were chosen to study the hydrolysis of CL by E. coli preparations. It was found that ATP plus Mg^{++} or Mn^{++} stimulated the conversion of CL to PA and this represents a second pathway for E. coli hitherto undescribed in the literature.

The conversion of CL to PA and PG can be represented by the following scheme:

Scheme V



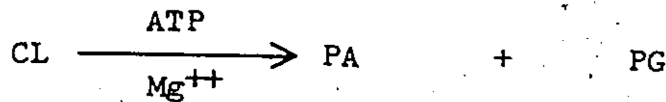
In the case of E. coli it is not yet known whether hydrolysis proceeds to form sn-3-phosphatidyl-sn-1'-glycerol i.e. whether the attack is at position 1 or 2. Astrachan found that the cardiolipin - specific phospholipase D of Hemophilus parainfluenzae splits the molecule at position 2 to yield sn-3-phosphatidic acid and sn-3-phosphatidyl-sn-1'-glycerol (137). Also regarding the E. coli enzyme its substrate specificity has not yet been systematically studied although the variety of conditions used in this laboratory for promoting hydrolysis of PG and PE have not permitted the detection of phospholipase D with these substrates.

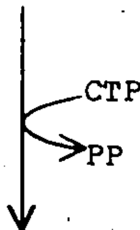
Involving the phospholipase D activity of E. coli we can now explain the enigmas arising in Part A of this thesis. The PA formed from endogenous CL can be converted to CDP-diglyceride which would dilute any labelled diacylglycerol precursor formed from sn-glycero-3-phosphate-U-^{14}C. The conseqence would be as was indeed found, the formation of a PG pool which is more intensely labelled in the free glycerol moiety than in the diglyceride moiety. Also the incorporation into PG would exceed that into PE.

As can be seen from Scheme VI the cycling of PG and CL in the presence of ATP and Mg^{++} would result in an enrichment of the label in the unacylated glycerol moiety of PG. Any PA formed from endogenous CL would remain unlabelled as it cycles through the polyglycerophosphatides and conseqently there would be no stimulation in the labelling of PE by these cofactors.

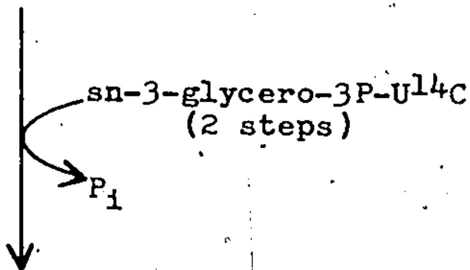
It is interesting to note that the sum of the reactions of the PG-CL cycle is a phospholipase D attack on PG. On this basis different turnover rates for the glycerol moieties of PG should be demonstratable in intact cells under appropriate chase conditions. This has been definitely shown with Hemophilus parainfluenzae (138) cells but not yet with E. coli.

Scheme VI

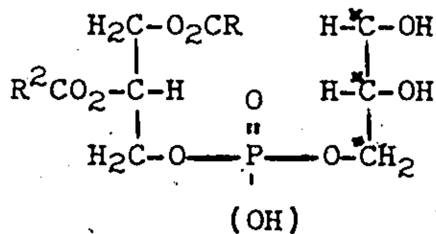




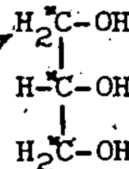
CDP-DG

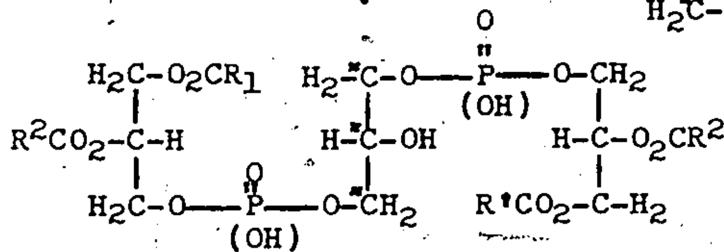


PA



ATP + Mg++





The stimulation of CL hydrolysis by ATP plus Mg^{++} could be explained on the basis of an allosteric effect or by an enzymatically catalyzed chemical modification of phospholipase D involving a phosphorylation or adenylylation. The first possibility could be explored by kinetic studies in the presence and absence of ATP. Allosteric enzymes usually display sigmoidal kinetics which are characteristically modified by a modulator substance. The second possibility would require the difficult task of isolating or proving definitely the existence of active and inactive forms of the enzyme.

The work of White et al indicated that the conversion of CL to PA and PG catalyzed by _H. parainfluenzae_ extracts (129) occurred without ATP addition. It must be added however that White et al did not test the effect of ATP on this conversion. Also if one pictures possible active and inactive states of the enzyme, ATP addition _in vitro_ might not necessarily produce very intense effects. All would depend on the growth stage at which the bacteria are isolated and the energy supply at that stage. Enzymes prepared from cells depleted in ATP would likely contain less activated enzyme and in such a case ATP might produce a greater stimulation. That the _H. parainfluenzae_ enzyme may require ATP is supported by the work of White et al (139)

who described two pools of CL with different turnover rates. The turnover of the most active pool was inhibited by uncouplers of oxidative phosphorylation. As with *E. coli*, factors which cause a decrease in the energy supply of *H. parainfluenzae* cells also cause an increase in CL content.

The stimulation of CL hydrolysis by ATP may be of great significance with respect to control of energy metabolism. As was pointed out for the first time by Macfarlane, CL is mainly associated with membranes carrying out respiration and oxidative phosphorylation. In mitochondria it is present in the inner membrane only (25) and in *Salmonella typhimurium* it is located mainly in the cytoplasmic membrane of the cell envelope (134) where respiratory processes occur. The precise function of this lipid is not completely known but one could picture its importance as imparting on the membrane a correct structural conformation such that the arrangement of electron carriers and phosphorylation intermediates is optimal for ATP formation. Interesting work by Racker et al (140) revealed that reconstitution of the phosphorylation process in lipid-depleted mitochondrial vesicles could be achieved by addition of PE and/or lecithin. The restoring activity of these two phospholipids was greatly enhanced by concurrent addition of CL. Addition of CL, however, was not very effective in the absence of other phosphatides.

Besides favoring the phosphorylating process CL may be essential for respiration. Phospholipids have been considered as the site of binding of cytochrome C and Quinn and Dawson (34) suggest that if indeed cytochrome C is associated with phospholipid in mitochondria, then CL would fulfill the characteristics of the binding most adequately. In short there is evidence that the presence of adequate levels of CL in respiratory membranes serve to favour ATP production and this is ascribed to CL maintaining organization of the electron carrier system. Our discovery that in *E. coli* ATP stimulates the breakdown of CL may provide a mechanism for the regulation of ATP production. One can picture conformational changes in the membrane as a likely result of CL hydrolysis, and this disruption of the electron carrier system could interrupt respiration to produce a fall in ATP. In the extreme event when phosphorylation activity is negligible (as in the stationary phase of the growth curve) CL levels are increased by phospholipase D transphosphatidylation activity, and with this increase in CL, the cell can be pictured as maximizing its effects to restore ATP levels. To hypothesize further, we put forward the idea that phospholipase D undergoes change in the presence of ATP to alter its activity from one of transphosphatidylation to that of hydrolysis with the ultimate effect of decreasing the production of ATP due to

disruption of the electron carrier oxidative phosphorylation system. Hirschberg and Kennedy's work (79) supports the reverse effect that occurs in the absence of ATP. Such a working hypothesis for which we offer only preliminary evidence naturally invites much further experimentation.

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CLAIMS TO ORIGINALITY

1) A detailed study was made of the cofactor and substrate requirements for the synthesis of phosphoglycerides in *E. coli* extracts and of their effects on the levels of each phosphatide formed.

2) The incorporation of sn-glycero-3-phosphate-U-^{14}C under defined *in vitro* conditions was found to favour greatly the unacylated glycerol moiety of phosphatidylglycerol, relative to acylated glycerol moieties in both phosphatidylethanolamine and phosphatidylglycerol. This effect could not be explained by the known pathways for *de novo* synthesis of phosphoglyceride in *E. coli*.

3) The acyldihydroxyacetone phosphate pathway was found not to operate in *E. coli* under conditions promoting formation of phosphoglycerides and the enhanced labelling of the unacylated glycerol moiety of phosphatidylglycerol by various labelled precursors.

4) The observation in claim #2 led to the hypothesis that formation of phosphoglyceride could proceed by a partial synthesis pathway making use of an endogenous pool of phosphatidyl moieties. Addition of ATP plus Mg^{++} increased the availability and/or rate of utilization of this endogenous precursor. A search for the metabolic route leading to the formation of an

endogenous diacylglycerol moieties led to the discovery of two new pathways implicating polyglycerophosphatides in E. coli.

5) The first of these pathways concerns the formation of an acylated phosphatidylglycerol derivative partially characterized as semilysobisphosphatidic acid. The formation of this substance requires Ca^{++} but no energy supply and thus very likely involves a transacylation implicating phosphatidylglycerol as acyl acceptor.

6) The second of these pathways involves the hydrolysis of cardiolipin to phosphatidic acid and phosphatidylglycerol via phospholipase D-like activity. This activity requires added Mg^{++} and is markedly stimulated by ATP.

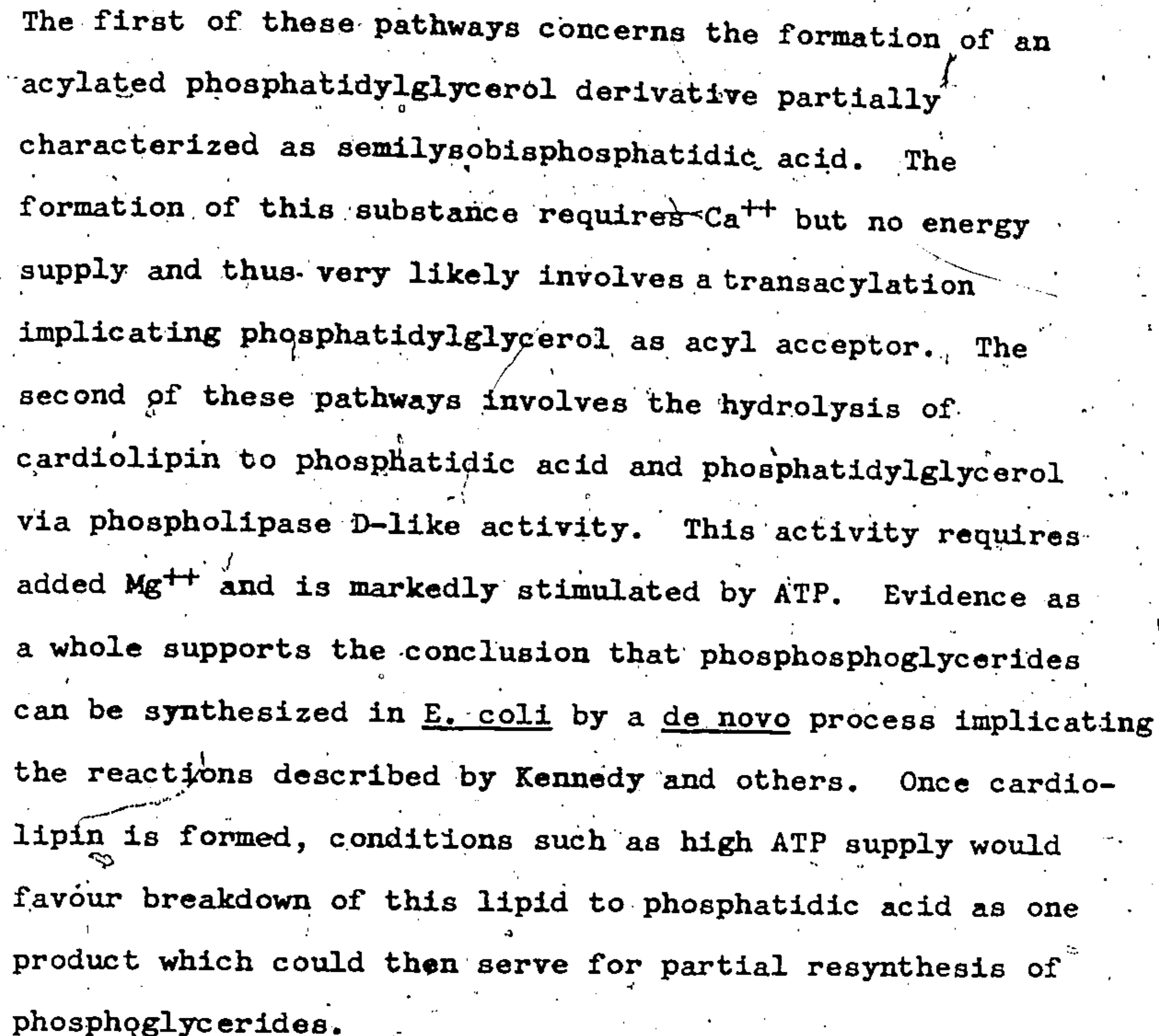
7) Evidence as a whole supports the conclusion that phosphosphoglycerides can be synthesized in E. coli by a de novo process implicating the reactions described by Kennedy and others. Once cardiolipin is formed, conditions such as high ATP supply would favour breakdown of this lipid to phosphatidic acid as one product which could then serve for partial resynthesis of phosphoglycerides.

8) From the evidence obtained in the literature and from our own results it is proposed for the first time, that E. coli is perhaps typical of many organisms by being equipped with a phosphatidylglycerol-cardiolipin cycle which is under ATP control. Formation of ATP is dependent on the

particular architecture which cardiolipin imparts to the respiratory membrane. Hydrolysis of cardiolipin in the presence of excess ATP would disrupt the fine organization of the membrane required for oxidative phosphorylation and as a consequence would limit ATP production.

ABSTRACT

A detailed study was made of the cofactor and substrate requirements for the synthesis of phosphoglycerides in _E. coli_ extracts and of their effects on the levels of each phosphatide formed. The incorporation of sn-glycero-3-phosphate-U-^{14}C under defined _in vitro_ conditions were found to favour greatly the unacylated glycerol moiety of phosphatidylglycerol, relative to acylated glycerol moieties in both phosphatidylethanolamine and phosphatidylglycerol. This effect could not be explained by the known pathways for _de novo_ synthesis of phosphoglyceride in _E. coli_. The acyldihydroxyacetone phosphate pathway was found not to operate in _E. coli_ under conditions promoting formation of phosphoglycerides and the enhanced labelling of the unacylated glycerol moiety of phosphatidylglycerol by various labelled precursors. Evidence is given that formation of phosphoglyceride could proceed by a partial synthesis pathway making use of an endogenous pool of diglyceride moieties. Addition of ATP plus Mg^{++} increased the availability and/or rate of utilization of this endogenous precursor. A search for the metabolic route leading to the formation of an endogenous diacylglycerol moiety led to the discovery of two new pathways implicating polyglycerophosphatides in _E. coli_.



The first of these pathways concerns the formation of an acylated phosphatidylglycerol derivative partially characterized as semilysobisphosphatidic acid. The formation of this substance requires Ca^{++} but no energy supply and thus very likely involves a transacylation implicating phosphatidylglycerol as acyl acceptor. The second of these pathways involves the hydrolysis of cardiolipin to phosphatidic acid and phosphatidylglycerol via phospholipase D-like activity. This activity requires added Mg^{++} and is markedly stimulated by ATP. Evidence as a whole supports the conclusion that phosphophoglycerides can be synthesized in E. coli by a de novo process implicating the reactions described by Kennedy and others. Once cardiolipin is formed, conditions such as high ATP supply would favour breakdown of this lipid to phosphatidic acid as one product which could then serve for partial resynthesis of phosphoglycerides.