Catabolism of Cardiolipin In *Escherichia coli* And Other Gram-Negative Bacteria

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A Thesis

Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Department of Biochemistry
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April, 1976

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List of Abbreviations

PA	-	Phosphatidic Acid
PG	-	Phosphatidylglycerol
PE	-	Phosphatidylethanolamine
CL	-	Cardiolipin
PS	-	Phosphatidylserine
PC	-	Phosphatidylcholine
GP	-	Glycerophosphate
GPG	-	Glycerophosphorylglycerol
GPE	-	Glycerophosphorylethanolamine
GPGPG	-	Glycero - (diphosphoryl) - glycerol
PGP	-	Phosphatidylglycerophosphate
MAG	-	Mild Alkaline Hydrolysis
NAD	-	Nicotinamide Adenine Dinucleotide
ACP	-	Acyl Carrier Protein
CoA	-	Coenzyme A
RNA	-	Ribonucleic Acid
AMP	-	Adenosine Monophosphate
ADP	-	Adenosine Diphosphate
ATP	-	Adenosine Triphosphate
CMP	-	Cytidine Monophosphate
CDP	-	Cytidine Diphosphate
dCDP	-	Deoxycytidine Diphosphate
CTP	-	Cytidine Triphosphate
GMP	-	Guanosine Monophosphate
GDP	-	Guanosine Diphosphate
GTP	-	Guanosine Triphosphate
TTP	-	Thymidine Triphosphate
UMP	-	Uridine Monophosphate
UDP	-	Uridine Diphosphate
UTP	-	Uridine Triphosphate
PEP	-	Phosphoenolypyruvate
SDS	-	Sodium Dodecyl Sulfate
EDTA	-	Ethylenediaminetetraacetic Acid
DTT	-	Dithiothreitol
TCS	-	3,3', 4, 5' - Tetrachlorosalicylanilide
M-CCCP	-	Carbonylcyanide m - Chlorophenylhydrazone

Acknowledgments

This research was funded by the Medical Research Council of Canada.

My appreciation is extended to Dr. P. Proulx whose direction and aid was invaluable.

Special thanks are extended to Michele Robillard for typing this thesis.

For Pat, because I love her.

INTRODUCTION

Phospholipids have long been conceded a structural role in membranes. Indeed, the membrane owes its basic, liquid-crystalline, protein-accommodating, lipid bilayer structure to the amphipathic character of these lipids and to their variable degree of unsaturation.[1] To this fundamental architectural function, which is being more clearly understood each year in terms of detailed molecular motions and asymmetric associations[2,3], one must add several other allied structural functions. These depend at least in part on the polar head-groups of the lipids and relate to particular enzyme or transport activities which they complement. A case in point is the phosphatidylserine - requiring $Na^+ - K^+$ adenosine triphosphatase of erythrocyte membranes.[3]

Cardiolipin (CL), like other phosphoglycerides, probably plays a structural role in biological membranes. However, one might deduce a more specific use for this lipid from the fact that it is located almost exclusively in membranes carrying out respiration and oxidative phosphorylation.[4-6] Recent solubilization and reconstitution studies on the inner mitochondrial membrane have in fact shown that cardiolipin is intimately associated with components of both the electron transport[7,8] and oxidative phosphorylation[9] processes. Optimal reconstitution of membranes displaying

phosphorylating activity requires added cardiolipin.[9] There is good reason therefore to suspect that CL plays an important structural and activating role in respiratory membranes although it is not yet possible to explain the nature of its participation in detail.

The present thesis focuses on the metabolism of lipids in E. *coli* and other Gram-negative bacteria. More specifically, it concerns the elucidation of a pathway implicated in the turnover of the metabolically-related polyglycerophosphatides, phosphatidyl glycerol (PG) and CL. This study was prompted by the fact that comparatively little was known about the enzymes governing polyglycerophosphatide metabolism in E *coli*. In animal cells, this metabolism occurs in mitochondria and is fairly well understood because of the recent studies of Stanacev et al[10,11] and other workers.[12,13] Also, in the light of what was stated previously about the possible function of CL in membranes, one cannot help but assign particular significance to the fact that the turnover of coliform polyglycerophosphatides is closely related to the energy state of the cell. Indeed, when the energy supply or metabolism is defective in E. *coli* and other Gram-negative bacteria such as when the culture reaches the stationary phase or when the cells respond to respiratory or phosphorylation inhibitors[14], CL levels increase at the expense of PG. The situation is reversed when the energy

supply is abundant.[14] This interesting phenomenon could well be related to an important regulatory mechanism coupling respiratory and phosphorylating activities to structural changes in the membrane resulting from polyglycerophosphatide interconversions. It therefore became important to know what these interconversions implied first of all, in terms of the reactions and types of enzymes involved.

The review of the literature to follow, presents material which relates directly to the particular interests of this thesis, namely, the metabolism and function of lipids in *E. coli* with particular emphasis on some of the degradative enzymes involved. Since the present investigation did lead to the characterization of a particular type of phospholipase D attacking CL specifically, detailed attention has been given to this lipolytic enzyme and the different properties it displays depending on its source. For the sake of clarity and simplicity, an exhaustive review of the literature dealing more generally with bacterial lipid research is not given. For a broader evaluation of this field, the reader may wish to refer to several complementary reviews.[14-16]

A. Phosphoglyceride Species of *E. coli*

The cell envelope of *E. coli* contains the entire lipid complement of the cell.[17] The lipids are composed

mainly of phosphoglycerides which are located in both the outer and inner membranes of the cell envelope. The phosphoglyceride compositions of the cell wall-outer membrane and cytoplasmic membrane fractions are similar although the phospholipid content per milligram of cytoplasmic membrane protein is almost twice that of the outer layers.[18] The same results have been described for *Salmonella typhimurium* by Osborn et al.[19]

(i) Phosphatidic Acid

Phosphatidic acid (PA) is the precursor of all the various phosphoglycerides of *E. coli*, but is present only in trace amounts in the cell.[14] The main function of this minor phosphoglyceride is to serve as an intermediate in phospholipid biosynthesis. That this lipid is of the sn-3-configuration has been determined by the reaction of its deacylated derivative with sn-glycero-3-phosphate:NAD oxidoreductase.[20]

(ii) Phosphatidylethanolamine

Between 70 and 80% of the *E. coli* phospholipid consists of phosphatidylethanolamine (PE). This major phosphoglyceride is metabolically stable[14] and its function in the cell envelope is thought to be mainly structural although it

is required for other purposes as well. For instance, PE acts as a cofactor in the synthesis of the core region of lipopolysaccharide.[21] PE has also been shown recently to be involved in the reconstitution of E. *coli* membrane particles and in the reconstitution of nitrate reductase activity in these particles.[22]

(iii) Phosphatidylglycerol

As the second major phosphoglyceride of E. *coli*, PG, an acidic lipid, comprises from 5 - 15% of the total phospholipid. Unlike the stable PE pool, the PG pool is metabolically active and the levels of this lipid in E *coli* vary in accordance with the levels of CL^{14} as discussed in a later section. The main role of PG in the cell envelope would appear to be structural but it is also a requirement for the functioning of the phosphoenol pyruvate phosphotransferase system responsible in part for the uptake of hexose sugars.[23-25] Two enzymes comprise this system - Enzyme I and Enzyme II. Enzyme II confers sugar specificity with respect to the transferring of phosphate from phosphoenol pyruvate to various carbohydrates and exhibits a strict requirement for PG.[23] Accordingly, destruction of the PG in isolated bacterial membrane preparations by added phospholipase D has been

reported to inhibit the vectorial phosphorylation of α-methylglucoside.[26] There is, however, some disagreement with these results. Long and Dittmer could not substantiate the specific hydrolysis of PG in vesicles by phospholipase D and suggested that some other component of the crude phospholipase D preparation may inhibit transport in the membrane preparation.[27]

(iv) Cardiolipin

The remaining 5 - 15% of the *E. coli* phosphoglyceride consists of CL, the stereochemistry of which is such that it contains two sn-3-phosphatidyl moieties per molecule.[28] This strongly acidic phospholipid is thought to serve mainly a structural function in the cell envelope.

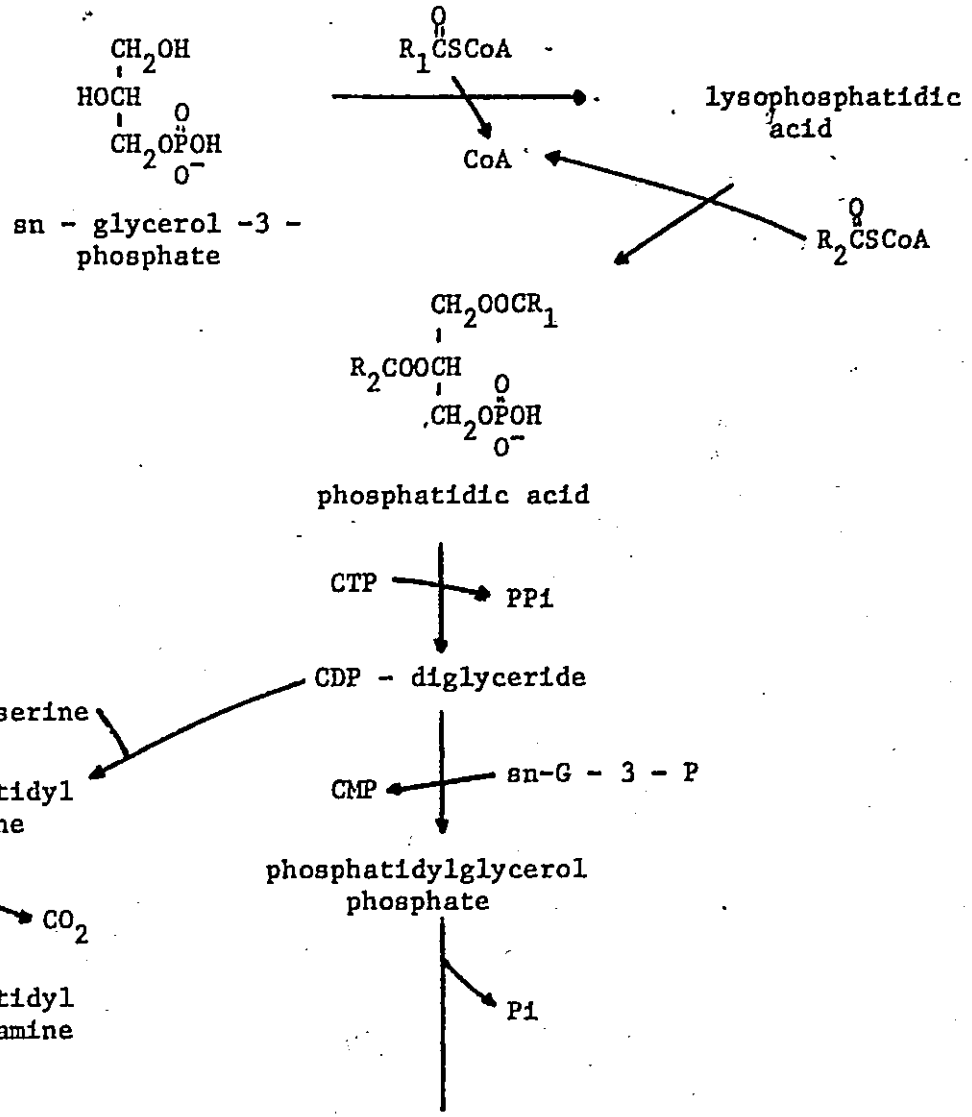
(v) Other Phosphoglycerides

The cytosine-containing liponucleotide fraction of *E. coli* has recently been isolated[29] and was shown to contain almost equal amounts of CDP - and dCDP - diglycerides. The total amount of cytosine liponucleotide in *E. coli* cells was low, consisting of about 0.04% of the total lipids. In this respect, *E. coli* cells differ from some mammalian tissues such as beef liver[30] and rat pineal gland[31] in which only CDP-diglyceride was detected.

Phosphatidylserine (PS) is also present in *E. coli* in minute amounts as an intermediate in the synthesis of PE.[32-34] The presence of phosphatidylglycerol phosphate (PGP) in *E. coli* has been reported by some workers[33,35] but others[34] including investigators from this laboratory did not detect this lipid. Another minor phosphoglyceride is acyl phosphatidylglycerol[36,37] which accumulates when PG is incubated with particulate fractions of *E. coli* under energy-free conditions. This lipid has also been characterized in extracts of *E. coli* as a minor component.[38]

B. Biosynthesis of Phosphoglycerides in *E. coli*

Phospholipid biosynthesis in *E. coli*, as it is now understood, follows the pathways shown in Figure 1. Much of the basic work in this field has been performed by Kennedy and coworkers using cell-free systems. Under the most common analytical conditions, nearly all of the biosynthetic enzymes are found in the particulate fraction of broken cells. Some enzymes, such as CDP-diglyceride: L-serine phosphatidyltransferase and the enzymes of fatty acid biosynthesis are recovered in the soluble fraction of the cell but are most probably in close association with the inner membrane in vivo.[14,39,40] All the other enzymes involved in the biosynthesis of the phosphoglycerides are localized in the inner cytoplasmic membrane.[41,42] It is approp-


CH2OH
HOCH
CH2OPOH
O⁻
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R1CSCoA
CoA
lysophosphatidic acid
R2CSCoA
CH2OOCR1
R2COOCH
CH2OPOH
O⁻
phosphatidic acid
CTP
PPi
CDP - diglyceride
serine
phosphatidyl serine
CO2
phosphatidyl ethanolamine
CMP
sn-G - 3 - P
phosphatidylglycerol phosphate
Pi

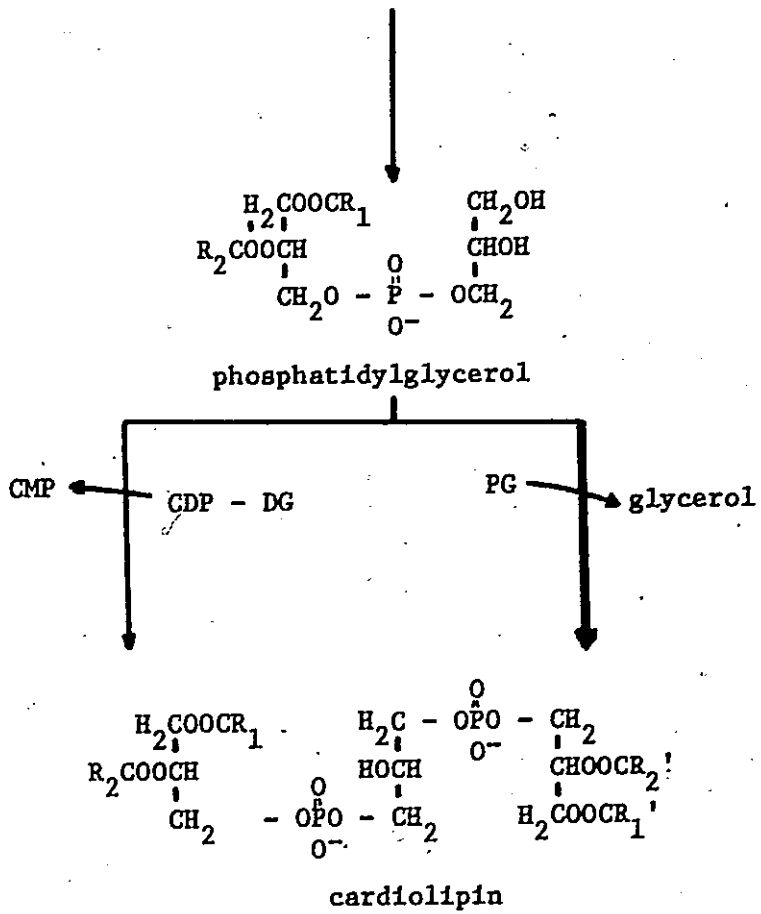


FIGURE 1. Biosynthesis of the *E. coli* phosphoglycerides.

riate to underline the similar lipid composition of inner and outer membranes[18] and the completely asymmetric distribution of the biosynthetic enzymes. This calls for a translocation of phospholipid from inner to outer membrane.[43]

(i) Synthesis of Phosphatidic Acid

Sn-glycero-3-phosphate (GP) is the form in which the glycerol moiety is incorporated into the phosphoglycerides. This compound is therefore the key precursor of the E. *coli* phospholipids. The stepwise acylation of GP by glycerophosphate acyltransferase and monoacyl glycerophosphate acyltransferase produces PA.[44] The selective acylation of GP is thought to be the step at which the asymmetric distribution of fatty acids in the phospholipid molecule is achieved[15] as most naturally-occurring phosphoglycerides are saturated at the 1-acyl position and unsaturated at the 2-acyl position.[45]

The acyl donor in the synthesis of PA can be either the thioester of acyl carrier protein (ACP) or CoA as both function in cell extracts. However, there is a greater specificity towards the acylation of 1-acyl lyso PA with unsaturated acyl groups when ACP thioesters are substrates.[46] Also, with acyl CoA synthetase being inducible,[47] it is likely that this enzyme is involved mainly with the incorporation of exogenous fatty acids.

In *E. coli*, diglyceride and monoglyceride can be phosphorylated by the action of diglyceride kinase to form PA.[48-50] This, however, would imply the presence of a metabolically active pool of diglyceride in *E. coli* which has yet to be found.[20] Therefore, in *E. coli*, the de novo synthesis of PA would appear to involve only the pathway using sn-glycero-3-phosphate as the intermediate.

(ii) Synthesis of CDP-Diglyceride

CDP-diglyceride plays a central role in the biosynthesis of membrane lipids in *E. coli* by acting as a donor of phosphatidyl residues in the biosynthesis of PS and PGP[51-53] and of CL.[54] The enzyme system which catalyzes the synthesis of CDP-diglyceride from CTP and PA is located in the particulate fraction of broken *E. coli* cells.[55,56] Actually, there are two analogous forms of activated diglyceride, CDP- and dCDP-diglyceride, both of which turn over rapidly in vivo.[29] Raetz and Kennedy[29] have shown that dCDP-diglyceride is a better substrate in the synthesis of PGP and that CDP-diglyceride is a better substrate in the synthesis of PS. Therefore, it may be possible that the specificity for dCDP or CDP may be at the basis of a regulatory mechanism for the synthesis of phospholipids. Also, Raetz and Kennedy suggest that because the levels of cytosine liponucleotide relative to PA are very low, the conversion of PA to CDP-or dCDP-diglyceride may be the rate-limiting step in phosphoglyceride synthesis.

(iii) Synthesis of Phosphatidylserine

CDP-diglyceride: L-serine phosphatidyltransferase (PS synthetase) of *E. coli* differs from other enzymes of phospholipid synthesis in that it may be found in the soluble fraction of broken cells[52] tightly bound to ribosomes.[57] If the cells are gently lysed, however, a good part of the activity is recovered in the washed particulate fraction.[14] Accordingly, Ishinaga and Kito[40] conclude from their studies that this enzyme is loosely associated with the membrane in vivo but is probably removed from the membrane as a result of harsh fractionation procedures.

Since the PS synthetase is associated with the membrane, it is possible that the envelope phosphoglycerides interact with this enzyme. Indeed, Ishinaga et al have found that CL activates while PG strongly inhibits the activity of this enzyme.[58] This may be the basis for another regulatory mechanism affecting PS and PE syntheses. Therefore, it is possible that PS and PE syntheses are controlled by the products of the other phosphoglyceride pathway, namely PG and CL.

PS synthetase has been purified over 100-fold and its properties have been studied by Raetz and Kennedy.[59] They suggest that there is a phosphatidyl-enzyme intermediate involved in the synthesis of PS since the formation of PS from CDP-digylceride and L-serine appears to be reversible in vitro.[59]

(iv) Synthesis of Phosphatidylethanolamine

Phosphatidylserine decarboxylase is the membrane-bound enzyme that catalyzes the synthesis of PE from PS. The activity of this enzyme is much greater than that of phosphatidylserine synthetase[52] which explains why PS does not accumulate in vivo. This decarboxylase from E. *coli* has been purified to near homogeneity by Kennedy and coworkers.[60,61] The purified enzyme is inhibited by reagents that attack carbonyl groups which suggest that either a pyridoxal phosphate moiety or a keto-acid may be involved in the decarboxylation reaction.[61]

(v) Synthesis of Phosphatidylglycerol

The two enzymes that catalyze the conversion of CDP-diglyceride to PG have been partially purified from the particulate fraction of broken E. *coli* cells.[53,62] Phosphatidylglycerol phosphate is thought to be an intermediate in this reaction although it has yet to be found in E. *coli*. Sn-glycerol-3-phosphate: CMP phosphatidyltransferase has a strict requirement for added Mg^{++} or Mn^{++} and is entirely dependent upon added CDP-diglyceride for activity.[3] This enzyme is relatively inactive when compared to PGP phosphatase which would explain why PGP does not accumulate in vivo. PGP phosphatase also has an absolute requirement for added Mg^{++} and has a high affinity for its substrate PGP.[62]

(vi) Synthesis of Cardiolipin

Earlier work on the synthesis of CL in *E. coli* suggested that the phospholipid was formed according to equation (1) since unlabelled CDP-dipalmitin stimulated the synthesis of CL from PG.[54] However, subsequent studies have shown that CDP-diglyceride does stimulate CL synthesis, but most probably through a detergent effect or as an allosteric effector since it does not act as a phosphatidyl donor.[63]

(1) PG + CDP-diglyceride ⟶ CL + CMP

(2) 2PG ⟶ CL + Glycerol

The work of Rampini et al[64] with intact cells indicated that *E. coli* could synthesize CL under conditions of energy depletion. On this basis they proposed a pathway for CL synthesis involving transphosphatidylation with two moles of PG. Lusk and Kennedy[65] have also found that after pulse labelling cells with tritiated glycerol, the metabolism of PG involved the cleaving of the labelled glycerol from PG. Stanacev and Stuhne-Sekalec[66] were the first to show with a cell-free system that CL can be formed from PG in a reaction not dependent on CDP-diglyceride. It is now evident that the synthesis of CL in *E. coli* occurs via equation (2) since Hirschberg and Kennedy[63] found that an equimolar release of glycerol resulted during the formation of CL from PG.

These results have been corroborated by Hostetler et al[13] and by Tunaitis and Cronan.[67] The CDP-diglyceride pathway may still operate in vivo when high concentrations of CDP-diglyceride are present as Hostetler et al have suggested[13] although this has not been confirmed.

C. Turnover of Phosphoglycerides in *E. coli* and the Effect of Culture Conditions on this Turnover

Work performed by Kanfer and Kennedy[34] and corroborated by other researchers[68-71] has shown that while PE levels in normally growing *E. coli* cells (i.e., exponentially growing at 37°) remain stable, the other phospholipids undergo rapid turnover. The minor phospholipids such as PA, CDP-diglyceride, PGP and PS are metabolized rapidly since they are intermediates in the biosynthesis of the major phosphoglycerides.

One explanation for the turnover of PG is its conversion to CL.[63] However, Kanfer and Kennedy[34] found that there was a net loss of label from the total lipid fraction that accompanied turnover of labelled PG. Therefore, the conversion of PG to CL may not be the only metabolic fate of PG. Kennedy et al have since reported that PG contributes to the synthesis of oligosaccharides in *E. coli*.[72] They found that label lost from the PG and CL fractions in exponentially growing cells appeared in a group of oligo-

saccharides composed of glycerol and phosphoric acid in equimolar amounts and of glucose as the only hexose sugar. Another metabolic fate of PG may be the formation of acyl PG,[36] although more work is required to elucidate and assess the significance of this pathway.

Preliminary work by Benns and Proulx[73,74] has shown that the turnover of CL is via its hydrolysis by the action of a phospholipase D. They found through studies involving the incorporation of U - (^{14}C) - sn - glycero - 3 - phosphate into PG that the label was distributed unequally between the acylated and nonacylated glycerols of PG. In order to obtain this unequal label distribution, there must have been an enlargement of a pool of endogenous, non-labelled phosphatidyl precursors. Since CL caused a slight stimulation of the incorporation of sn-glycero-3-phosphate into the unacylated glycerol moiety of PG, this indicated that phospholipase D action on CL was supplying an alternative pool of PA.[74] Further studies on the hydrolysis of CL by phospholipase D from *E. coli* form the basis of this thesis.

Changes occur in the turnover of these major phospholipids if the culture conditions are altered in any of several ways. If *E. coli* cultures are allowed to grow to the stationary phase, there results an increase in the CL level[33,75,76] and a corresponding decrease in the PG

level.[33,34,75] Along with these two changes comes an increase in cyclopropane fatty acids which is accompanied by a decrease in unsaturated fatty acids.[76-78] PE levels do not change.

The conversion of PG to CL observed during growth is also seen in cells which have become unable to phosphorylate ADP because of various factors. For instance, growth of cells in the presence of penicillin,[75] cyanide,[79] colicin K,[80] dinitrophenol,[80] phenethyl alcohol,[81,82] formaldehyde, toluene, chloroform[80] or sodium[65] results in this increased conversion of PG to CL. *E. coli* infected with bacteriophage gives the same results[79,83] as does growing cells in a medium lacking an energy source.[64] Treatment of *E. coli* with levorphanol, a morphine analogue, results in an increase in CL levels and a decrease in both PG and PE levels.[84]

Alterations also occur in the phospholipid metabolism of *E. coli* when cells are grown at temperatures other than 37°. Bright-Gaertner and Proulx[70] found that there is a lower turnover rate of PG and CL when cells are shocked at 10°. PE did not turnover at all. Other effects of cold included an increase in the total lipid phosphorus, a higher degree of fatty acid unsaturation and a lower content of cyclopropane fatty acids.[70] These results confirm the

earlier work of DeSiervo[35] who found that when cells were grown at 27°, there was also an increase in the total lipid phosphorus and a decrease in the turnover of CL. The phospholipid composition of *E. coli* undergoes different changes when grown at higher temperatures. Bell et al[85] found that the relative rates of PG and CL synthesis (especially that of PG) increases when temperature-sensitive mutants are grown at 40°. This increase was believed to be due to the increase in the activity of glycerol-3-phosphate: CMP phosphatidyl transferase at that temperature.[85]

It has been amply shown that PE levels remain constant under normal conditions. In a few cases, an unusual accumulation or turnover of this lipid has been shown. For instance, Barbu et al[82] noted an apparent conversion of PG and CL to PE in cells that were treated with phenylethanol. The same observations were made by Bell et al[85] while studying the phospholipid metabolism of a temperature-sensitive DNA initiation mutant of *E. coli* at the restrictive temperature of 40°. Golden and Powell[86] also found a marked turnover of PE in amino acid-starved *E. coli* cells with a genetic lesion in RNA control. Other workers[87] have found that there is an increase in PE synthesis when filamentous *E. coli* cells were induced to produce septa. These results may indicate that PE synthesis is related to cell growth and division and that pools of this normally stable lipid change only at

these times.[83]

D. Catabolism of Phosphoglycerides in E. coli

Several types of phospholipase A_1 have been located in E. coli. Scandella and Kornberg,[88] purified a membrane-bound, alkaline phospholipase A_1 that appears to be the same enzyme first observed by Proulx and Fung[89,90] and Okuyama and Nojima.[91] An acid phospholipase A_1 has been detected in whole cell homogenates[90] and in E. coli spheroplasts.[92] Doi et al[93] have designated two kinds of phospholipase A in E. coli, a detergent-resistant and a detergent-sensitive type. Further work on the positional specificity of the detergent-resistant phospholipase A showed that this enzyme had both A_1 and A_2 activities,[94] the A_1 activity being similar to that purified by Scandella and Kornberg.[88] More evidence is now needed to confirm the existence of a distinct, highly specific phospholipase A_1 in E. coli. Doi and Nojima were not able to show that the A_1 and A_2 were separate enzymes.[94] Albright et al also found both A_1 and A_2 activities in the cell wall and could neither establish nor preclude that there were two different enzymes involved.[95] Bernard et al[96-98] have, however, reported that both the high-speed supernatant and particulate fractions of broken E. coli cells contained only phospholipase A_2 activity. In the light of most of the present evidence, their conclusions seem incorrect.

The purified phospholipase A_1 of *E. coli* also contains lysophospholipase activity[94] and might better be designated phospholipase B. However, a separate lysophospholipase has recently been purified to near-homogeneity by Doi and Nojima.[99] This enzyme hydrolyzes 1-acyl-GPE, 2-acyl-GPE, 1-acyl-GPG, and 1-acylglycerol but does not attack diacyl phospholipids. Albright et al[95] have detected three types of lysophospholipase activities in *E. coli*. There is a lysophospholipase A_1 activity in the cell wall that seems to be associated with the phospholipase A_1. There is also a lysophospholipase A_1 in the membrane and cytosol and a lysophospholipase A_2 in the inner membrane.[95] A PG-specific phospholipase A present in the cytosol fraction has also been described.[93,95]

E. coli also possesses lipase activity.[100] The enzyme (s) involved hydrolyzes triglycerides, diglycerides, monoglycerides and simple fatty acyl esters at an alkaline pH and requires Ca^{++} together with detergents for activity. In crude homogenates, the level of lipase activity towards trioleoylglycerol is similar to the level of phospholipase A_1 activity.[88] Doi and Nojima have confirmed these results and gave genetic evidence indicating that a single enzyme is associated with triglyceride and phospholipid hydrolysis.[101] It seems from the foregoing that *E. coli* is well equipped with lipolytic activities capable of completely hydrolyzing

phospholipids and triglycerides to water-soluble products (c.f. Figure 2). A phosphodiesterase that catalyzes the hydrolysis of glycerophosphorylethanolamine to sn-glycerol-3-phosphate and ethanolamine has also been found in *E. coli*.[95] Whether several distinct lipolytic enzymes exist or whether relatively few such enzymes account for all the lipid hydrolyses noted in cell extracts remains to be clarified.

All of these studies have shown the presence of lipolytic activities in cell extracts. Audet et al[102] have recently revealed phospholipase A_1 activity in growing *E. coli* cells. However, levels of this activity were significant only in one *E. coli* strain examined in which the structure of the cell envelope was less stable than in normal cells. This fact, plus other evidence[88,92] suggests that it is adverse conditions that triggers phospholipase A_1 activity in vivo.[102] In normal cells phospholipase A_1 does not function in the turnover of endogenous lipids but may serve for the degradation of exogenous lipids.

E. Phospholipase D

Phospholipase D is a phosphatidohydrolase that, until five years ago, was thought to exist only in plants. It has since been detected in bacteria and in mammalian cells.

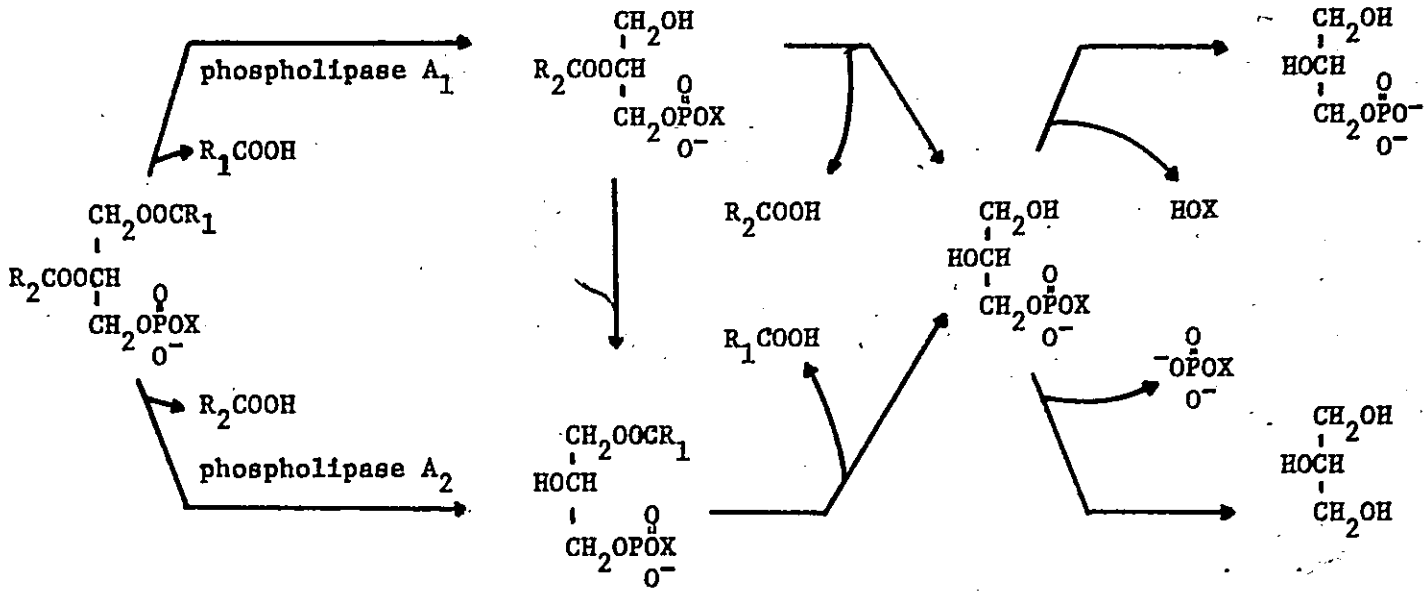


FIGURE 2

Possible reactions involved in the catabolism of phosphatidylethanolamine. X represents the ethanolamine moiety. (95)

(i) In Plants

Phospholipase D has been purified extensively from cabbage leaves.[103-106] This enzyme catalyzes the hydrolysis of the terminal phosphodiester bond of phosphatidylcholine (PC) as well as of lyso PE,[107] PE, PS and PG and has been given the systematic name, phosphatidylcholine phosphatidohydrolase (E.C. 3.1.4.4.). The product of the hydrolysis of the diacyl phosphoglycerides by phospholipase D is PA. Lyso PC is hydrolyzed to cyclic lyso PA and choline.[107] Enzyme activity requires added calcium ions and ether. Ether is required either to solubilize the lipid substrate or to change the hydrophilic nature of the enzyme. The phospholipase D that has been purified is a soluble enzyme[106] although phospholipase D activity has been found associated with plastids in an insoluble form.[108,109]

Purified phospholipase D from cabbage exhibits transphosphatidylase activity as well as phosphatidohydrolase activity.[105,110] This activity is involved in the transfer of the phosphatidyl group of PC to either methanol, ethanol, 2-propanol, glycerol, ethanolamine, choline or serine. When glycerol is the acceptor, both isomers of PG are formed while in nature only the sn-1-configuration of the unacylated glycerol is found, so it is possible that this transphosphatidylase activity does not occur in vivo. Since enzyme activity is inhibited by p-chloromercuribenzoate,

a phosphothioester linkage may be involved in a phosphatidyl-enzyme complex.[105] The transphosphatidylase activity of commercial phospholipase D from cabbage has also been shown to catalyze the formation of CL in a reaction for which PG acts as the donor and acceptor of the phosphatidyl moiety.[66]

There is some evidence that different enzymes or different sites on a single enzyme may be responsible for the base-exchange and phosphatidohydrolase activities. Calcium has been found to stimulate the phosphatidohydrolase activity while inhibiting the transphosphatidylase activity.[111] Recently, Saito et al[112] have shown that the two activities in commercial phospholipase D from cabbage do show different characteristics. For example, the transphosphatidylase activity has a pH optimum of 9.0, requires calcium ions (optimum concentration being 4mM) and is inhibited by hemicholinium-3, a non-competitive inhibitor of ethanolamine and choline incorporation into phospholipids. The phosphatidohydrolase activity has a pH optimum of 5.6, requires calcium ions for activity (optimum concentration being 28mM) and is not inhibited by hemicholinium-3.[112]

Phospholipase D from peanut seeds has been purified to a high specific activity.[113] The enzyme requires

calcium ions and ether for activity and has a pH optimum of 5.6 although it is unstable at acid pH. Work on the amino acid composition has shown that the N-terminal group is glycine. Molecular weight determinations ranged from 22,000 to 200,000 depending on the method of measurement used.[113] Peanut seed phospholipase D hydrolyzes PC[113] and will attack CL if the diethyl ether is replaced by deoxycholate.[114] Besides being present in cabbage and peanuts, phospholipase D has been detected in a wide variety of plants.[109,115]

(ii) In Mammalian Cells

It is only recently that the phospholipase D activity of mammalian cells was reported. Saito and Kanfer were the first to discover this phosphatidohydrolase activity in solubilized rat brain preparations from particulate fractions.[116] Further work[117] confirmed that PC is hydrolyzed to form PA by these preparations. The phosphatidohydrolase activity has a pH optimum of 6.0. Calcium ions stimulated the hydrolysis but were not essential and could be replaced by magnesium ions. Diethyl ether strongly inhibited the reaction as did p-chloromercuriophenyl sulfonate but dithiothreitol could overcome this inhibition which suggests the involvement of a sulfhydryl group in the enzyme-substrate complex. The same preparations that show

phosphatidohydrolase activity also have transphosphatidylase activity displaying a pH optimum of 7.2.[117]

Taki et al[118] have also reported the presence of phospholipase D activity in rat brain homogenates. Incubation of labelled homogenates with 10mM Ca^{++} at pH 7.6 resulted in the formation of labelled PA. The activity was very low, however.

Rat brain microsomes contain a lysophospholipase D that hydrolyzes alkyl- and alk-1-enyl phospholipids.[119] Whereas magnesium ions are required for activity, calcium ions are not and inhibit at higher concentrations. This activity, therefore, does not appear to be related to the Ca^{++} - stimulated phospholipase D of rat brain.

(iii) In Bacteria

Prior to the studies described in this thesis, phospholipase D activity had been detected in only one type of bacteria. It was Ono and White[120] who first discovered an active phospholipase D in *Haemophilus parainfluenzae* that specifically hydrolyzes CL to PG and PA in the presence of added Mg^{++}. This enzyme does not attack other phospholipids such as PE, PG, PS, PC or methylated phosphatidylethanolamines. It differs from

plant phospholipase D in that it has a pH optimum between 7.5 and 8.0 and is inactive in the presence of added organic solvents or calcium ions. The detergents SDS, Tween 20 and hexadecylpyridinium chloride are strongly inhibitory while the detergents Triton, deoxycholate and Sarcosyl had little effect on the enzyme activity.[120,121] Most of the *H. parainfluenzae* phospholipase D activity is found in the membrane fraction although some activity remains in the supernatant after ultrasonic disruption of the cell. This enzyme has also been found to function in the normal turnover of the polyglycerophosphatides of this organism.[121] Work by Astrachan[122] on the mode of action of this cardiolipin - specific phospholipase D revealed that it is specifically the bond between the phosphate and the central glycerol at the sn-3 position of CL that cleaves. (Figure 3).

Two other types of bacteria have now been shown to produce phospholipase D in the culture medium. One is *Streptomyces hachijoensis*[123] and the other, *Corynebacterium ovis*.[124,125] The latter bacterium produces a toxin which, in sheep erythrocytes, hydrolyzes sphingomyelins and lyso PC releasing free choline. This phospholipase D activity has been partially purified and has been found to be active at pH 8.0 in the absence of both Ca^{++} and diethylether.[125] The phospholipase D that *Streptomyces hachijoensis* produces

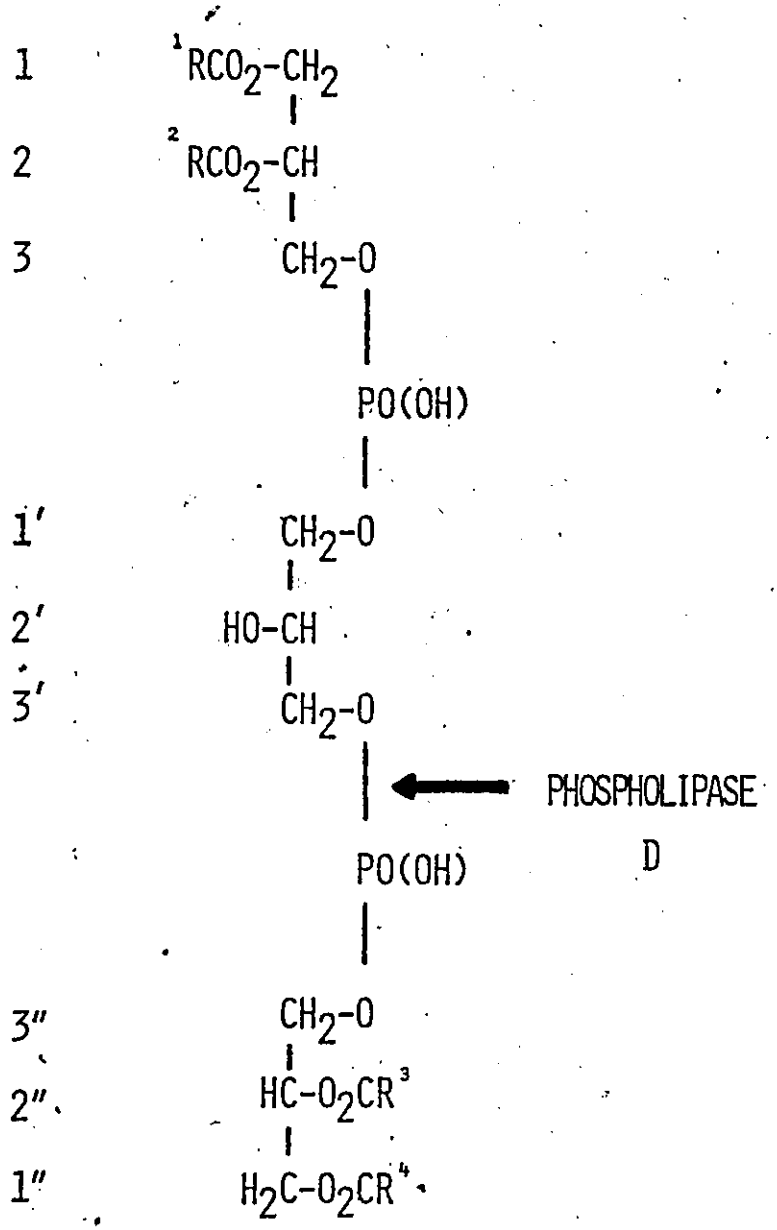


Figure 3. Bond Specificity of Cardiolipin- Specific Phospholipase D from *Haemophilus parainfluenza.* (119)

has also been purified. The enzyme has a molecular weight of 16,000, an optimal pH of 7.5, and is stimulated by ethyl ether, Triton X-100 and Ca^{++} but is inhibited by sodium dodecyl sulfate. This phosphatidohydrolase has a broad substrate specificity in that it hydrolyzes PE, PC, CL, PS, lyso PC and sphingomyelin liberating the corresponding bases.[123]

The *Haemophilus parainfluenzae* phospholipase D differs from these two bacterial enzymes in that it is found within the cell rather than outside. It also has a requirement for Mg^{++} and an apparently strict substrate specificity. This strict specificity for CL is a distinguishing feature and makes the systematic name phosphatidylcholine phosphatidohydrolase obviously inappropriate. A new name may have to be suggested taking into account this specificity for CL to distinguish it from the plant and other phospholipases D.[122] Since the enzyme has not been purified and its substrate specificity has not been rigorously determined with synthetic analogues, it is difficult at present to justify the use of a systematic name such as sn-1,3-diphosphatidylglycerol-sn-3-phosphatidohydrolase. Consequently, a trivial designation such as cardiolipin - specific or cardiolipin-hydrolyzing phospholipase D will be used throughout the rest of this thesis.

AIMS OF RESEARCH

1. To further characterize the cardiolipin-hydrolyzing phospholipase D of *E. coli* extracts in terms of its mode of action, substrate specificity, cofactor and pH requirements as well as its subcellular localization.

2. To investigate in particular an ATP activation of this enzyme.

3. To determine the effect of culture age on the levels and on certain of the properties of this enzyme.

4. To survey a number of organisms for the presence of this enzyme.

MATERIALS AND METHODS

A. Materials

The following bacteria were obtained from the American Type Culture Collection: *Escherichia coli* B (ATCC 11303), *Salmonella typhimurium* (ATCC 13311), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 12606), *Bacillus cereus* (ATCC 14579) and *Bacillus subtilis* (ATCC 6051). *Saccharomyces cerevisiae* (ATCC 2338) cells were obtained from the same source.

E. coli B cells grown commercially in a high peptone medium to the late stage were purchased as a frozen sediment of cells from either General Biochemicals Inc. or Grain Processing Corp.

Dithiothreitol (DTT), Tris (hydroxymethyl) aminomethane (Tris), nucleotides and phosphoenolpyruvate were purchased from Grand Island Biological Co. and/or from Sigma Chemical Co. Phospholipids such as beef heart cardiolipin, phosphatidic acid (sodium salt) and phosphatidylethanolamine were obtained from General Biochemicals Inc. Fine chemicals as well as common chemicals and solvents from various sources were of the purest grade possible. Chloroform was redistilled prior to use.

PSC solubilizer, (8-^{14}C) - adenosine triphosphate and γ - (^{32}P) - adenosine triphosphate were procured from Amersham/Searle Co. whereas all other radiochemicals were obtained from New England Nuclear Corp. Culture reagents such as bactopeptone and yeast extract were purchased from Difco Co. Silica gel G was a product of Macherey Nagel and Co.

B. Methods

(i) Growth of *E. coli* Cells

Stock cultures of *E. coli* B were maintained on agar slants. One liter cultures were grown in large low form erlenmyer flasks at 37° with constant shaking. The nutrient broth contained per liter of distilled water; 15 grams of bactopeptone, 1 gram of yeast extract, 20 grams of glucose and 5 grams of sodium chloride. Cells were normally grown to the late log stage (7 hours) as was determined from a growth curve. The purity of the culture was checked by the Gram stain and by plating on MacConkey and citrate agars. Cells were harvested by centrifugation at 4,000 x g for 10 min. in a Sorvall automatic refrigerated centrifuge (model RC2-B) at 4°.

(ii) Preparation of Cell-Free Homogenates of *E. coli* B Cells

Fresh *E. coli* B cells prepared in this way were

suspended in 9.0 ml. of 0.1 M potassium phosphate buffer pH 7.3. The cell suspension was then sonicated for four, one minute periods on ice using a Biosonik II Ultrasonicator (Bronwill Scientific Co.) at a setting of 60 watts. Between sonications, the suspension was allowed to cool so that the temperature never exceeded 8°. Centrifugation at 3,000 x g for 10 min. at 4° removed unbroken cells. Supernatant fractions from the cell-free sonicates were obtained by centrifugations at 17,000 x g (in a Sorvall centrifuge), 100,000 x g and/or 200,000 x g (in a Beckman L-2-65B Ultracentrifuge) as designated for each experiment.

Alternatively, *E. coli* B cells that were grown commercially were used. Cell-free homogenates of these cells were obtained as described for freshly prepared cells but in this case there was usually a greater loss of material to the 3,000 x g debris, possibly because of membrane aggregation.

The protein concentrations of these and all other enzyme preparations were determined by the method of Lowry et al.[126]

(iii) Preparation of Cell-Free Extracts of Various Organisms

The various bacteria, *Salmonella typhimurium*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*,

Bacillus cereus and *Bacillus subtilis* were grown for 6h. in 1 liter cultures of the medium described previously for the growth of *E. coli*. The bacterial cells were harvested by centrifugation, suspended in 0.1 M potassium phosphate buffer pH 7.0 and sonicated for 4, one minute periods on ice.

Saccharomyces cereviseae cells were grown for 23h. at 28° in 1 liter of medium containing 10 g yeast extract, 10g bactopeptone and 20g dextrose.[127] The yeast cells were then harvested as above, resuspended in 9.0 ml., 0.1M phosphate buffer pH 7.0 and were homogenized with a Braun homogenizer using a bead size of 0.45 - 0.55 mm and a bead to cell volume ratio of 3 to 2. Cells were shaken for two, 2.5 min. periods while being cooled with dry ice.

Unbroken yeast and bacterial cells were removed by centrifugation at 7,000 x g and 3,000 x g respectively for 10 min. at 4° and the cell-free extracts were diluted with buffer to give final protein concentrations of 4.0 mg/ml.

Crude rat liver mitochondria were prepared by the method of McMurray and Dawson[128] but in the absence of 0.1mM ethylenediaminetetraacetic acid (EDTA).

(iv) Extraction of Lipids

Lipids were extracted from various sources (i.e., cells, enzyme assays) by the method of Bligh and Dyer.[129] To 0.8 volume of the aqueous suspension of biological material was added 2 volumes of methanol and 1 volume of distilled chloroform. After mixing this monophasic mixture at room temperature, 1 volume of each of chloroform and water were added. This biphasic system was then centrifuged and the lower chloroform layer was transferred to an evaporating flask using a Pasteur pipette. The top aqueous phase was washed once with 2 volumes of chloroform, the chloroform layers were pooled and the lipid extract was evaporated to dryness.

With large extraction volumes involving more than 8 mg. of tissue protein, the monophasic systems were mixed for 30 min. periods and the biphasic systems for 5 min. periods. With smaller extraction volumes (eg. 4mg protein in a volume of 2.5 ml.) mixing was done on a vortex mixer for 20 sec. periods.

(v) Separation of the Phosphoglycerides

Silica gel plates, 0.5mm thick, were made using a slurry of 55g. of silica gel G and 110ml. of water.

Phosphoglycerides were separated on these plates using one of the following three systems (or a sequential combination of the three);

System 1 - chloroform: methanol: 7N ammonium hydroxide (60:35:5 by volume)

System 2 - chloroform: methanol: glacial acetic acid (65:25:8 by volume)

System 3 - chloroform: methanol: water (65:25:4 by volume)

The Rf values of the various phospholipids in each of these systems are given in Table 1.[130] The silica gel plates were usually activated at 110° for 60 min. before use. However, in the case of system 3 better separation between the CL and the PE was obtained when the plates were activated for only 15 min. at 110°. For routine analyses of the phospholipase D assay, phospholipids were separated on silica gel G plates with system 3. The scan shown in Figure 4 illustrates the separation of PA, PG, PE and CL in this system.

(vi) Mild Alkaline Hydrolysis of the Phosphoglycerides and the Separation of the Water-Soluble Products

In order to further identify the phospholipids, they were degraded to their water-soluble products by the mild-alkaline hydrolysis method of Marshall and Kates.[131]

The dried lipid sample was dissolved in 0.5 ml. of methanol: chloroform (3:2 by volume) which was then

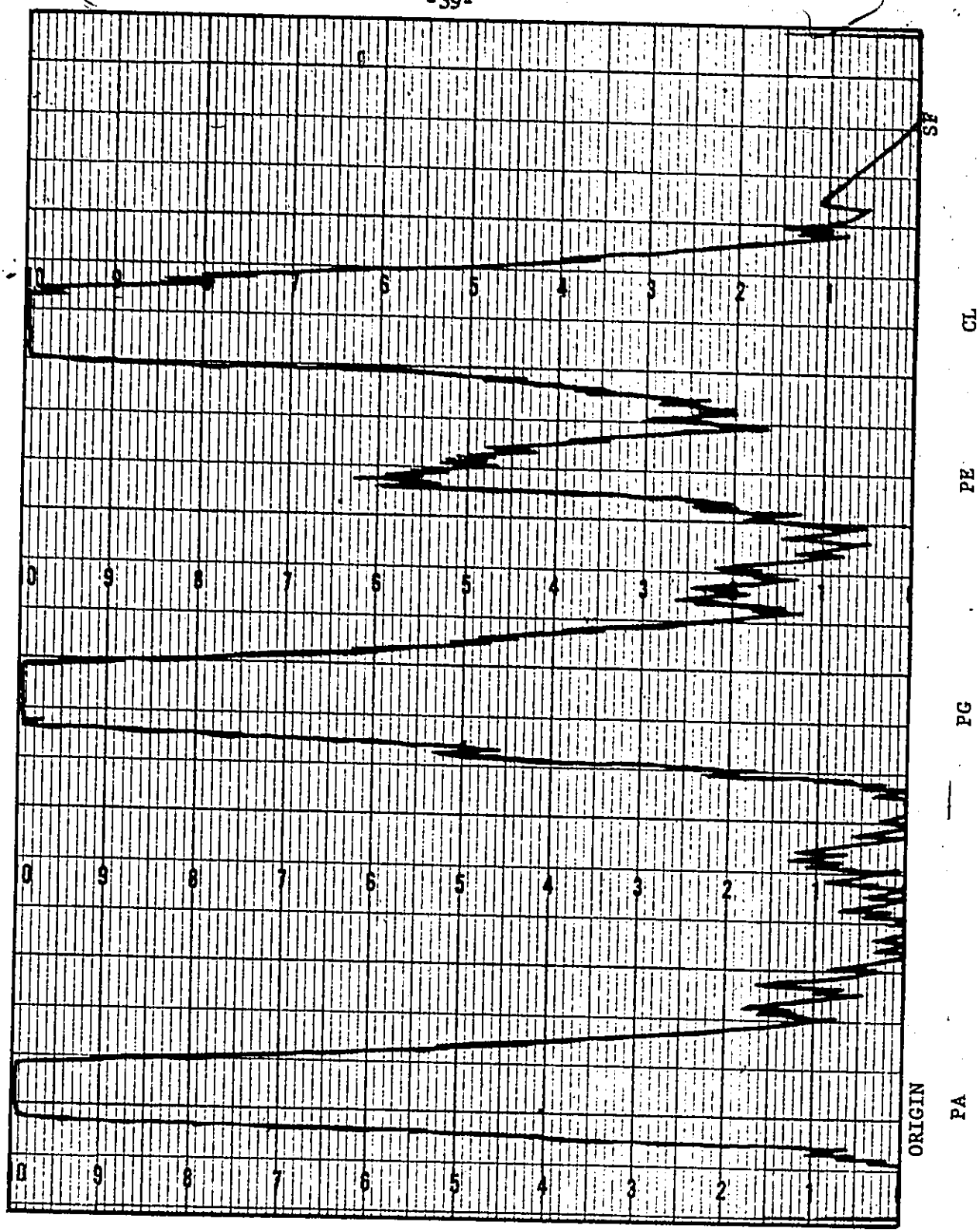
TABLE 1

Rf VALVES OF PHOSPHOLIPIDS AND THEIR DEACYLATION PRODUCTS IN VARIOUS CHROMATOGRAPHIC SYSTEMS

	SYSTEM				
	1	2	3	4	5
PA	0.08	0.58	0.00		
PG	0.52	0.56	0.32		
PE	0.38	0.35	0.40		
CL	0.55	0.95	0.68		
GP				0.29	0.27
GPG				0.46	0.64
GPE				0.63	0.22
GPGPG				0.15	0.50

FIGURE 4

Separation of phosphoglycerides with system 3. The figure is a tracing of radioactivity on a chromatoplate scanned with an Actigraph III (Nuclear Chicago Corp.) gas flow monitor.


AS
CL
PE
PG
PA
ORIGIN

incubated at room temperature for 15 min. with 0.5 ml. of 0.2N methanolic NaOH. The water-soluble products were extracted by the addition of 1ml. of methanol:chloroform (1:4 by volume) and 0.9 ml. water with mixing (ie. a Bligh and Dyer extraction). This biphasic system was centrifuged for 1min. at 600 x g and the upper aqueous phase was extracted with a Pasteur pipette. The upper phase was neutralized with 0.3-0.5 ml. of Dowex -50 in the presence of 1% phenolphthalein. The neutralized upper phase was made slightly alkaline with 1.5 M methanolic NH_4OH and was then evaporated to dryness. This water-soluble fraction was then dissolved in methanol: water (10:9 by volume) to spot.

The water-soluble products were separated by ascending chromatography on Whatman #1 paper in either or both of 2 systems:

System 4 - 90% liquefied phenol: water (20:5 by volume giving a final phenol: water proportion of 5:2 by weight)

System 5 - 1M ammonium acetate pH 4.5:95% ethanol (35:65 by volume) (ref. 50)

The Rf values of the water-soluble hydrolysis products of the phosphoglycerides are given in Table 1.

(vii) Detection and Identification of the Phosphoglycerides

The lipid components separated on silica gel plates

were revealed routinely by briefly exposing the plates to iodine vapours. The iodine was then removed by aeration.

Nitrogen-containing phospholipids were identified by spraying the plates with a solution of Ponceau red, which was made by dissolving 2g uranylnitrate and 0.05g Ponceau red in one l. of 0.01N HCL. The Ponceau red could be removed from the lipid components by Bligh and Dyer extraction since the dye remains in the aqueous phase.

Phospholipids containing free amino groups (eg. PE) were also detected by spraying with 0.5% ninhydrin in acetone:butanol (1:1) and then heating the plates at 110° for 5 - 10 minutes.

Phosphate-containing lipids were identified by spraying with Hanes-Isherwood reagent[132] and then developing the plates at 110° for 20 minutes.

Phosphoglycerides containing vicinal hydroxyl groups (eg. PG) were detected by spraying the plates with periodate - Schiff reagents as indicated by Baddiley et al.[133] This consisted of first spraying the plate with 2% sodium metaperiodate. After 10 min. the plate was transferred to a tank of sulfur dioxide, then sprayed with Schiff's reagent and was then re-exposed to sulfur dioxide. Only one

E. coli lipid gave a positive mauve color when treated in this manner and this component corresponded to PG.

To detect labelled compounds, chromatoplates and paper strips were scanned with an Actigraph III gas flow-detector (Nuclear Chicago). Alternatively, labelled compounds were identified by autoradiography on Kodak (no screen) Blue Brand x-ray film. Films were developed using Kodak developer and fixer.

(viii) Preparation of ^{32}P-Labelled Cardiolipin

A 1 liter culture of *E. coli* was incubated to the stationary phase (16h.) at 37° with the addition of 5m Ci of $H_3{}^{32}PO_4$. The cells were grown to the stationary phase in order to increase the level of cardiolipin.[75,76] Alternatively, cardiolipin levels could be raised by transferring cells at the late log stage to a saline medium for 2h. before final harvesting.[134]

Lipids were extracted from the harvested cells by the method of Bligh and Dyer with three washings of the aqueous phase. The medium was then neutralized, sterilized and fresh glucose was added. The medium was then inoculated with a 5ml. broth culture of *E. coli* B and another lipid fraction was obtained as stated above which was pooled with the first

The total lipid extract was separated by TLC in system 1. This system separated any labelled orthophosphate, minor lipids such as PA or acyl PG and most of the PE from the labelled PG and CL which ran close together. These latter two phospholipids were extracted as one fraction which was then run in system 2. The CL brand that was extracted from system 2 often contained an unidentified lipid that was determined to be an artifact produced during chromatography. This compound ran at the following Rf values in the systems:

System 1 - Rf ≈ 0.77,
System 2 - Rf ≈ 0.96 and
System 3 - Rf ≈ 0.70

The mild alkaline hydrolysis product of the compound ran at an Rf of 0.77 in system 4 and corresponded to no known lipid. Mild alkaline hydrolysis of whole lipid extract did not produce this fast running component. The CL was therefore purified by re-chromatography in system 1. The (^{32}P)-labelled CL was usually pure at this stage. The (^{32}P)-labelled PE and PG fractions extracted from systems 1 and 2 respectively were purified by chromatography in system 3. The purities of these three (^{32}P)-labelled phosphoglycerides were checked by subsequent mild-alkaline hydrolysis and paper chromatography of the water-soluble products in either system 4 or 5.

Lipid phosphorus was determined by the method of Bartlett.[135]

(ix) Liquid Scintillation Counting

Liquid scintillation counting was performed in disposable polyethylene vials with a Beckman LS 133 spectrometer. Material from TLC plates was counted without prior elution in the following scintillation fluid: 5g of 2,5 - diphenyloxazole in one l. toluene mixed with 100 ml. methanol and 3.5 ml. glacial acetic acid.[136] Material from paper chromatograms was counted by cutting appropriate areas into sufficiently large sections and standing these sections in the vial. Counting of both (^{32}P)- and (^{14}C) - isotopes was according to the channel's ratio method.

(x) Dialysis

Seamless cellulose dialysis tubing (average diameter dry, inflated = 0.7.9") was prepared for use by the method described by McPhie.[137] 5 ml. aliquots of *E. coli* preparations (40mg. protein /ml.) were dialyzed against three changes of 3 l. of 0.1M phosphate buffer pH 7.0 at 4° for 18 hours.

(xi) Ammonium Sulfate Fractionation

Ammonium sulfate fractions of *E. coli* B cell extracts were obtained by the sequential addition of the required amount of ammonium sulfate to 5ml. aliquots of cell extract (approximately 30mg. protein/ml.) to obtain the following percent saturated solutions: 35%, 50%, 75% and 100%. The amounts of ammonium sulfate necessary to obtain these fractions were calculated from the nomograph of di Jeso.[138] Protein precipitations were at 0° for 15 min. and the pH of the solution was maintained at 7.0. Precipitates were obtained by centrifugation at 10,000 x g for 10 min. at 4°, were washed three times with an ammonium sulfate solution of the same concentration and were then dissolved in 2.0 ml. of 0.1M phosphate buffer pH 7.0.

(xii) Calcium Phosphate Gel Fractionation

Calcium phosphate gel prepared from calcium oxide and phosphoric acid by the method of Swingle and Tiselius[139] was donated by Dr. D. Williamson. Calcium phosphate gel pH 7.0 (4.0 ml.) was added to a 1 ml. aliquot of *E. coli* B cell extract (approximately 30mg protein/ml) prepared in 1mM phosphate buffer pH 7.0. The suspension was stirred occasionally on ice for 30 min. and was then centrifuged at 5,000 x g for 10 min. at 4°. The 1mM phosphate buffer protein fraction

was decanted and kept. To the calcium phosphate pellet was added 1ml. of 1M phosphate buffer pH 7.0 and a 1M phosphate buffer protein fraction and a calcium phosphate gel pellet were obtained as described above.

RESULTS AND DISCUSSION

A. Identification of Phospholipase D Activity in E. coli Extracts

In the initial stages of this study conditions for studying cardiolipin hydrolysis were arbitrarily chosen although preliminary work had indicated a requirement for ATP.[73,74] As the results in Figures 5 and 6 show, CL was not hydrolyzed by the cell-free sonicate of commercial *E. coli* B when EDTA was added to the assay. When Mg^{++} alone was included, a slight hydrolysis of CL was observed. However, significant hydrolysis was observed only in the presence of both Mg^{++} and ATP. One of the hydrolysis products co-chromatographed with authentic PA in systems 1, 2 and 3. The other radioactive spot reacted positively with periodate - Schiff stain and co-chromatographed with pure *E. coli* PG in the same systems. The labelled CL ran together with beef heart CL but occasionally contained a slight amount (less than 1-2%) of (^{32}P)-labelled PE.

The identity of the enzyme hydrolysis products of CL were further verified by analysis of their deacylation

FIGURE 5

Autoradiograms of lipid products obtained by incubating (^{32}P)-labelled CL with homogenates of commercial E. coli B cells in the presence (A) EDTA + ATP and (B) Mg^{++} + ATP. Development was first in the horizontal direction with system 1 and then in the vertical direction with system 2. The solvent fronts were the extremities of the autoradiograms. The basic assay contained in 2ml., 0.1M potassium phosphate buffer pH 7.3 as solvent and diluent, E. coli cell-free sonicate (3.9-4.0 mg protein) and 9 nmoles of (^{32}P)-cardiolipin (59,000 DPM) dispersed in buffer by sonication. When specified, 2.8 mM ATP, 10mM $MgCl_2$ and 30mM EDTA were added. Incubations were for 1h. at 37° and reactions were stopped by lipid extraction.

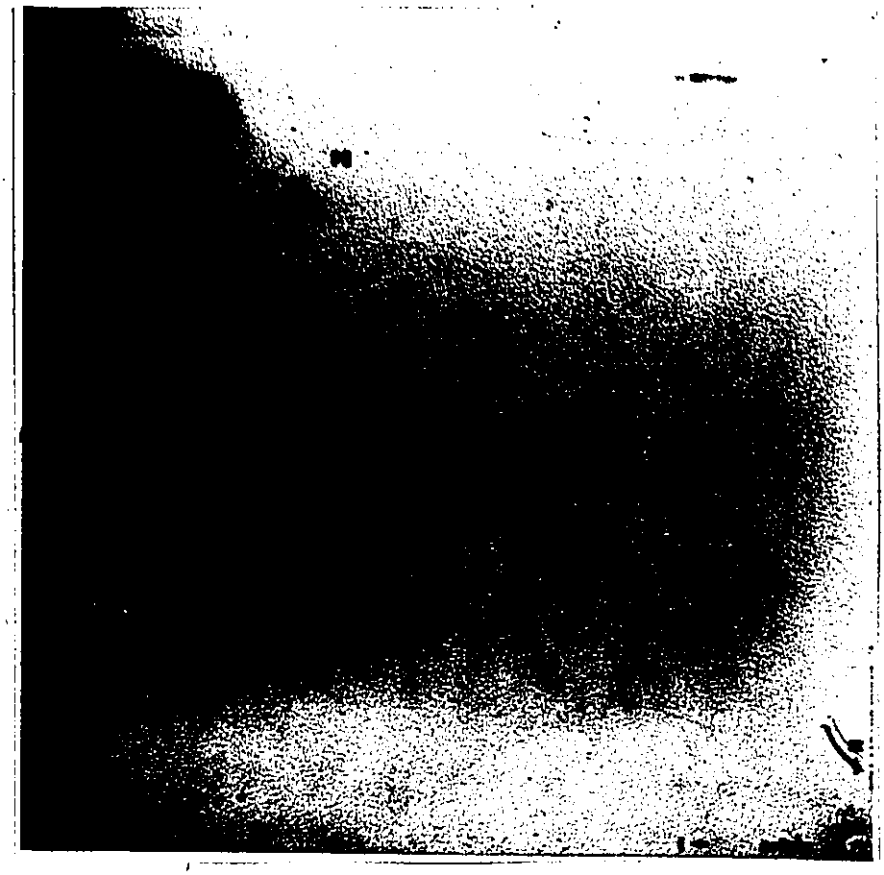
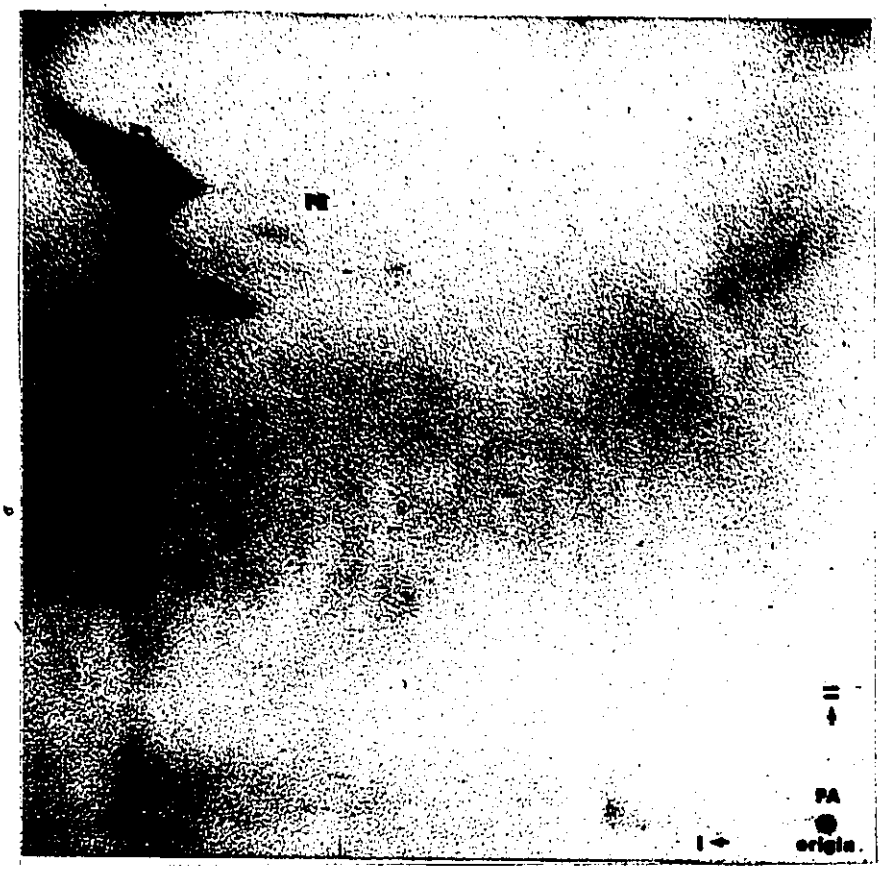


FIGURE 5 (A)

FIGURE 5 (B)

FIGURE 6

Autoradiogram of lipid products obtained by incubating (^{32}P)-cardiolipin with homogenates of commercial E. coli B cells in the presence of Mg^{++} and ATP. Development was first in the horizontal direction with system 1, and then in the vertical direction with system 3. The solvent fronts were the extremities of the autoradiogram. The assay conditions were the same as those described for Fig.5.


PE
PA
origin

products (Fig. 7). Mild alkaline hydrolysis of the total lipids extracted from incubation mixtures containing ATP and Mg^{++} yielded di-(glycerophosphoryl)-glycerol, glycerophosphate and glycerophosphorylglycerol as the labelled products. The identity of these compounds was ascertained by co-chromatography with the deacylated products of pure *E. coli* CL, PA and PG respectively. Occasionally a slight contamination of the labelled CL by labelled PE resulted in the detection of trace amounts of glycerophosphorylethanolamine (GPE). Except for this occasional PE contaminant, the only labelled deacylation product observed from the total lipid extracted from incubation mixtures containing EDTA was di-(glycerophosphoryl)-glycerol.

The ATP-stimulation of CL hydrolysis by extracts of commercially obtained *E. coli* B could not be readily duplicated with extracts of freshly grown *E. coli* B cells. As the results in Table 2 show, the addition of magnesium alone produced maximum hydrolysis of CL when fresh cells were used. No hydrolysis occurred with ATP alone and the addition of ATP plus Mg^{++} showed the same effect as Mg^{++} alone. However, if the extract from fresh *E. coli* B cells was frozen and thawed several times, little hydrolysis occurred in the presence of added Mg^{++} alone. Enzyme activity in this case was dependent on both added Mg^{++} and ATP. This ATP effect was, however, more marked if commercial *E. coli* B cells were used.

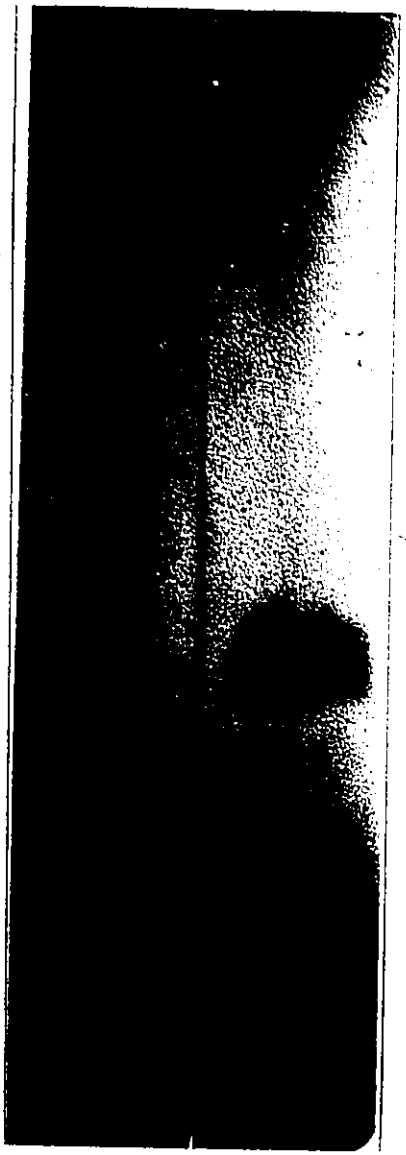


FIGURE 7. Autoradiogram of deacylated lipid products obtained by incubating ^{32}P-labelled CL with *E.coli* homogenates in the presence of (A) EDTA and (B) Mg^{++} + ATP. The assay conditions were the same as those described for Fig. 5.

TABLE 2

Effect of Mg^{++} and ATP on the Hydrolysis of Cardiolipin by *E. coli* Extracts

Conditions	A % DPM recovered as product		B % DPM recovered as product			C % DPM recovered as product		
	PA	PG	PA	PG	PE	PA	PG	PE
ATP + EDTA	2	2	2	2	1	0	1	1
Mg^{++}	14	17	7	9	2	1	3	1
ATP	3	3	1	2	1	0	1	2
ATP + Mg^{++}	14	18	20	23	1	30	26	1

A *E. coli* B homogenate used without freezing and thawing

B *E. coli* B homogenate used after freezing and thawing several times

C *E. coli* B homogenate of frozen cells obtained commercially and thawed once

The assay conditions were the same as those described for Figure 5.

All counts not recovered as PA or PG were found in the substrate remaining.

These results establish unequivocally, for the first time, the presence of a phospholipase D in *E. coli*. This enzyme hydrolyzes CL, the products being PA and PG. Similar activity has been detected in cell-free sonicates of *Haemophilus parainfluenzae* under slightly different conditions.[120,121] In both cases, however, added Mg^{++} is a requirement for enzyme activity and both enzymes are active at a similar pH (7.3 in the case of *E. coli* and 7.5 in the case of *H. parainfluenzae*). ATP did not constitute a cofactor requirement for *H. parainfluenzae* enzyme although in this case the effect of nucleotides was not tried. The enzyme from *H. parainfluenzae* was similar to the enzyme found in freshly prepared *E. coli* B extracts. However, as the results summarized in Table 2 show, ATP stimulation of phospholipase D in *E. coli* sonicates was demonstrable in fresh preparations that had been frozen and thawed or in preparations from commercial cells that were obtained in a frozen state. This treatment likely decreased endogenous levels of ATP or else reversibly inactivated an enzyme requiring ATP in vivo.

B. Studies on the Properties of Crude E. *coli* Phospholipase D

(i) Optimal Conditions for the Hydrolysis of Cardiolipin by Phospholipase D

As Figure 8 shows, the hydrolysis of CL by the cell-free sonicate of commercial E. *coli* B cells was linear up to 15 min. of incubation. The rate of the reaction was also linear with protein concentrations up to 400 ug of protein (Fig. 9) Results in Figure 10 indicate that CL hydrolysis occurred only within a narrow pH range. The curve displayed an optimum at pH 7.0 while no activity whatsoever occurred below pH 6.2 or above pH 8.0. The enzyme activity at either pH 7.3 or 7.6 did not change if Tris-HCl buffer was substituted for phosphate buffer.

As the Lineweaver-Burke plot illustrated in Figure 11 shows, the Km for ATP is 54uM. The optimal concentration of ATP was found to be about 0.3mM but no inhibition occurred with higher concentrations of up to 6.0mM under the assay conditions used (Figure 12). Maximal hydrolysis of CL occured at concentrations of Mg^{++} that were 8mM or higher while no enzyme activity was observed in the absence of Mg^{++}. The Km for Mg^{++} was 5.0mM as determined from the Lineweaver-Burke plot in Figure 13. Under optimal cofactor conditions, the rate of hydrolysis of CL increased with

FIGURE 8

Time course of the hydrolysis of cardiolipin by *E. coli* phospholipase D. The incubation mixture contained in 2ml. final volume, 0.1M potassium phosphate buffer pH 7.0 as solvent and diluent, 4nmoles of (^{32}P)-cardiolipin (20,000 DPM) sonicated in buffer, 2.8 mM ATP, 10mM Mg^{++} and *E. coli* preparations* (0.4 mg protein). In the control, 30mM EDTA was substituted for the Mg^{++}. Incubations were at 37° and the reactions were stopped by lipid extraction. In this study and for all other assays except when indicated phospholipids were separated with system 4. The radioactive bands were then scraped into vials and counted.

* cell-free extract of commercial cells

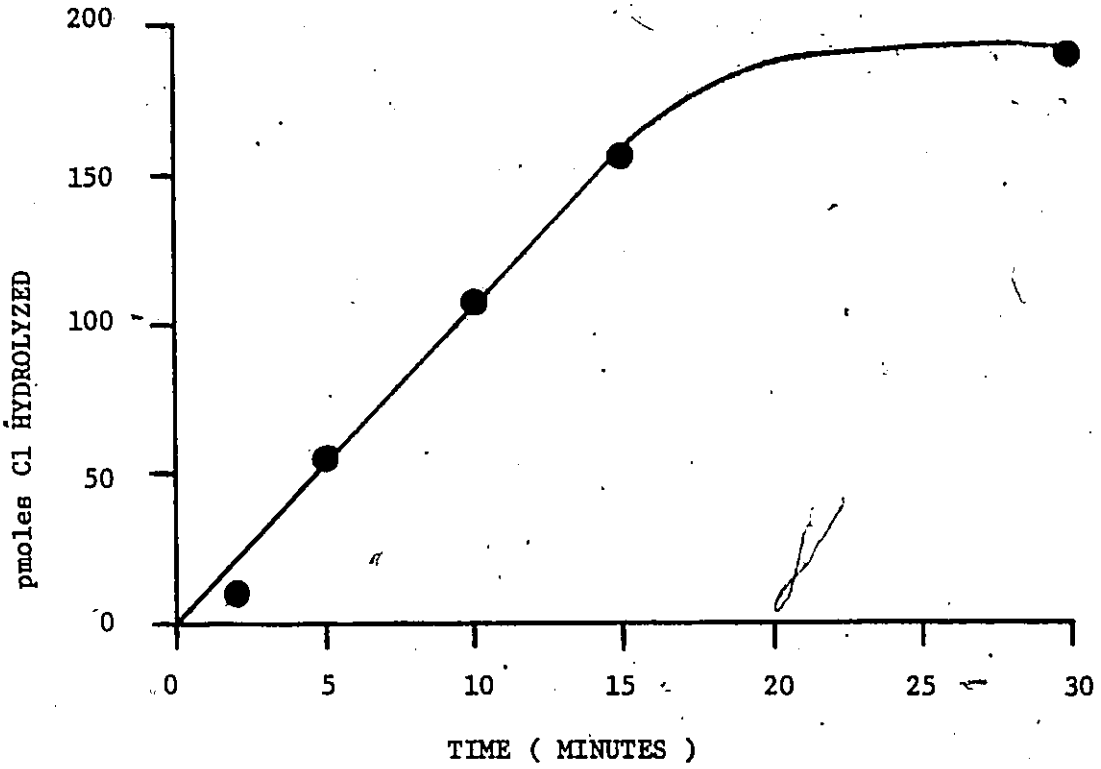

200
150
100
50
0
pmoles Cl HYDROLYZED
0
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 9

The effect of protein concentration on the hydrolysis of cardiolipin. The enzyme assay was the same as that described for Fig. 8. Incubations were for 15 min. at 37°.

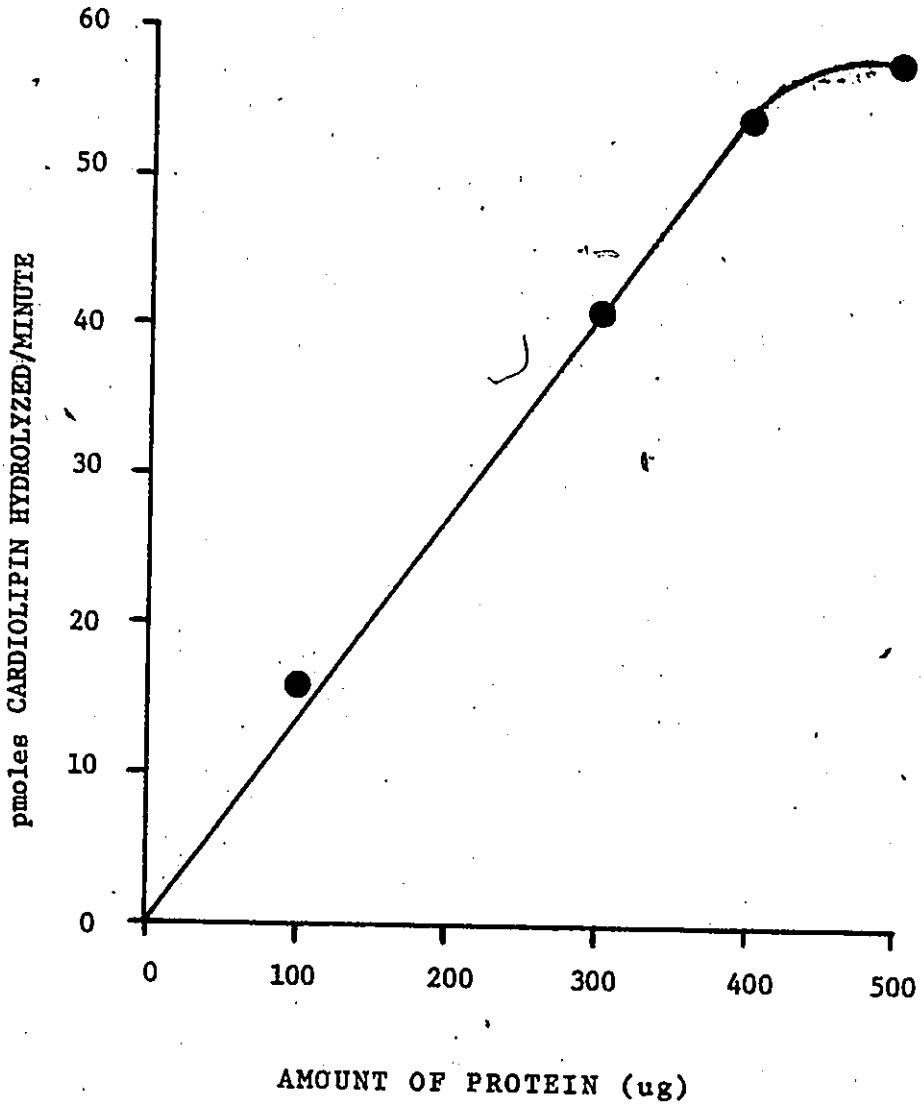
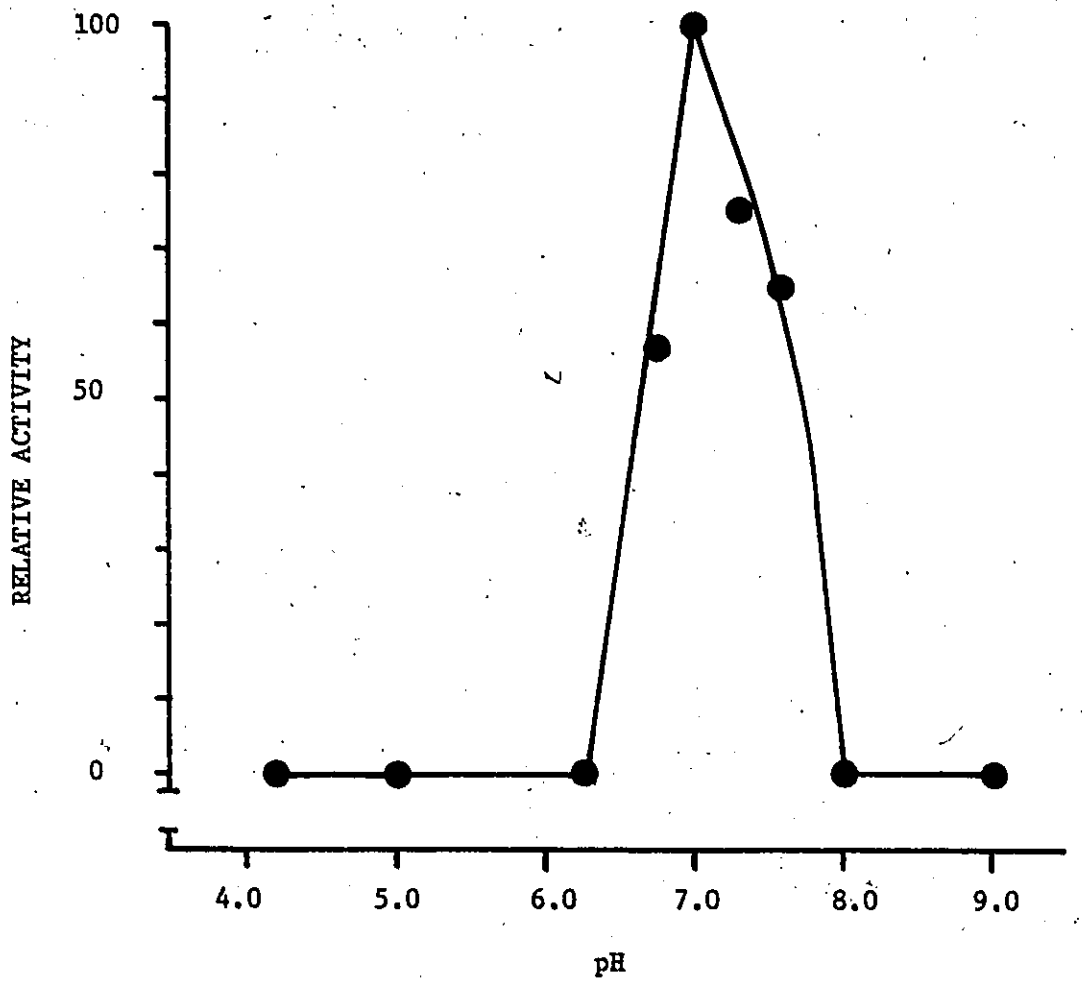

60
50
40
30
20
10
0
pmoles CARDIOLIPIN HYDROLYZED/MINUTE
0
100
200
300
400
500
AMOUNT OF PROTEIN (ug)

FIGURE 10

The effect of pH on the hydrolysis of cardiolipin. The incubation mixture contained in 2ml. final volume, 4 nmoles (^{32}P)-cardiolipin (20,000 DPM) sonicated in appropriate buffer, 2.8 mM ATP, 10mM Mg^{++} and E. coli cell-free sonicate. In the pH range 6.2-7.6, 0.1M phosphate buffer was used as solvent and diluent. Below this range the buffer was 0.1M acetate and above this range the pH was maintained with 0.1M Tris-HCl. The latter buffer was also used at pH 7.3 and 7.6 in place of the phosphate buffer. Incubations were for 15 min. at 37°.


100
50
0
RELATIVE ACTIVITY
4.0
5.0
6.0
7.0
8.0
9.0
pH

FIGURES 11 and 12

Effect of ATP concentration on the rate of hydrolysis of CL by *E.coli* phospholipase D. The assay conditions were the same as those described for Figure 9 except that increasing concentrations of ATP were used.

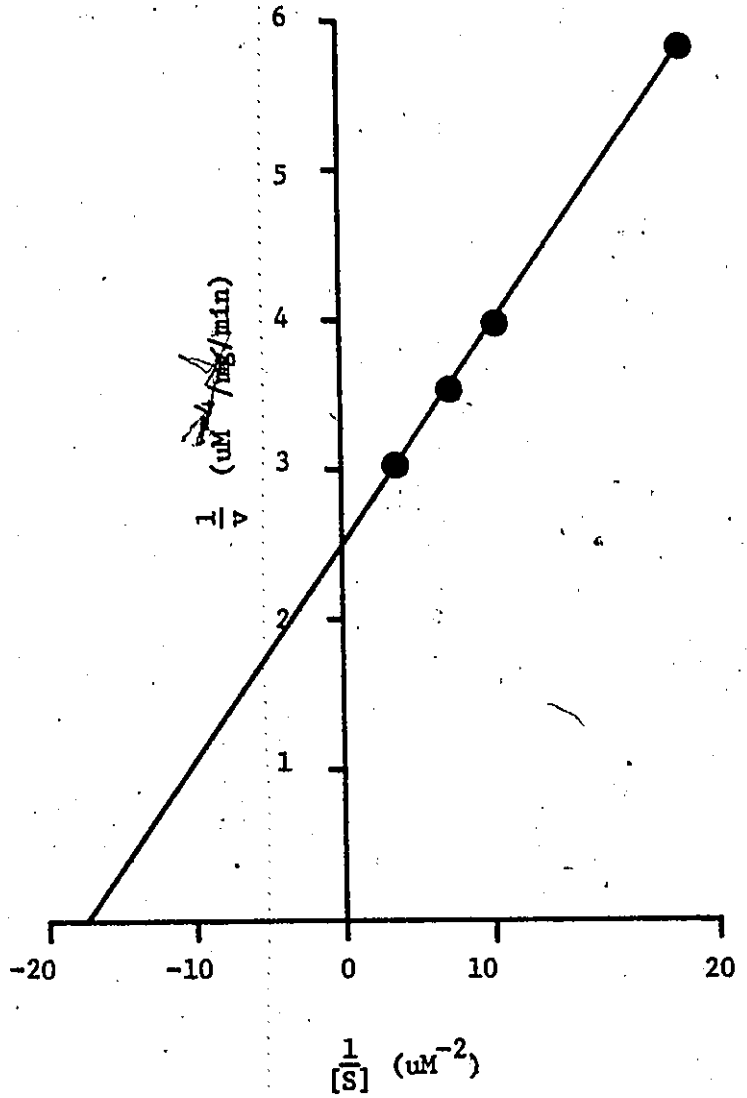


FIGURE 11

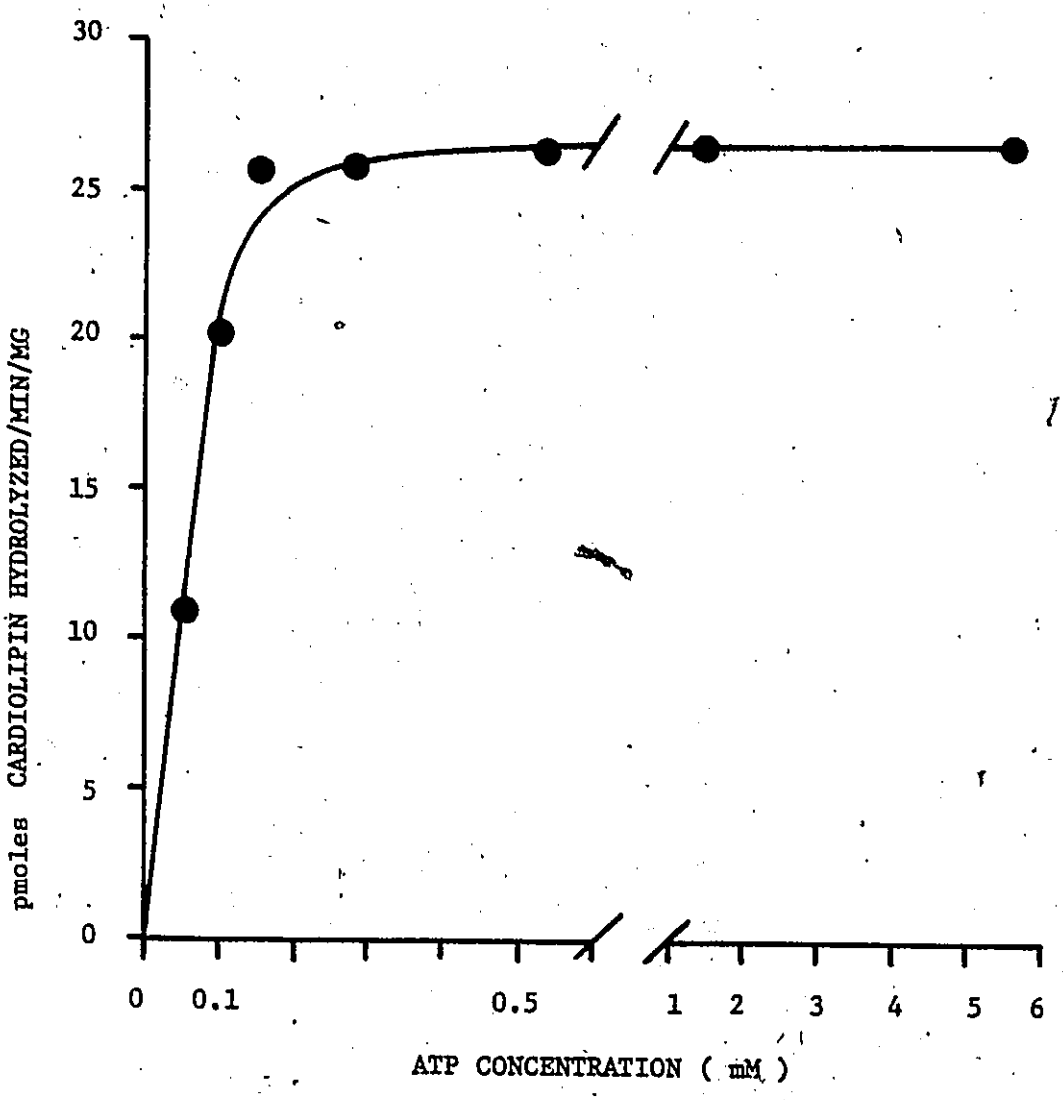

30
25
20
15
10
5
0
pmoles CARDIOLIPIN HYDROLYZED/MIN/MG
0
0.1
0.5
1
2
3
4
5
6
ATP CONCENTRATION (mM)

FIGURE 12

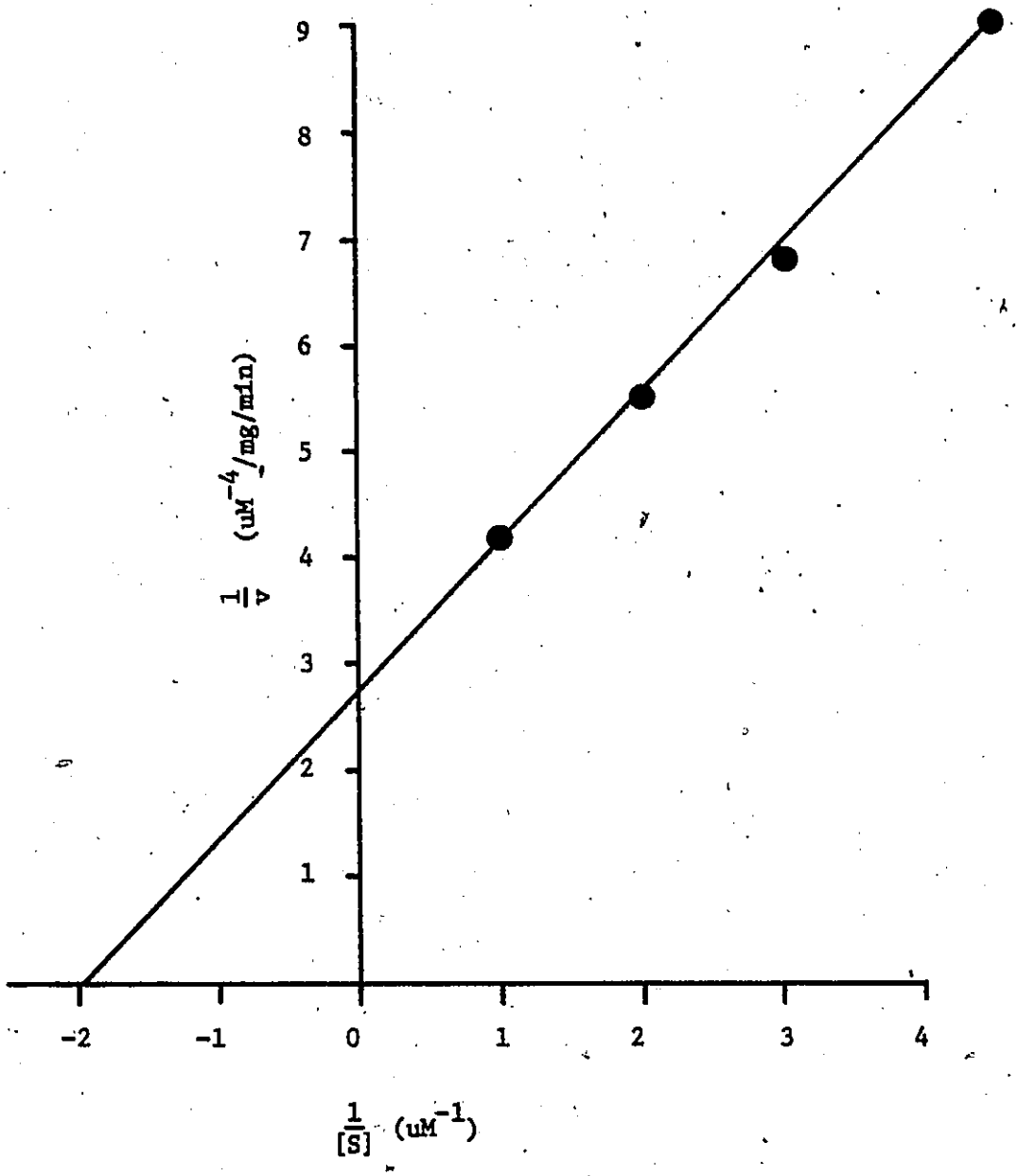


FIGURE 13. Effect of Mg^{++} concentration on the rate of hydrolysis of cardiolipin by *E. coli* phospholipase D. The assay conditions were described in Fig. 9.

substrate concentration. However the kinetic parameters could not be accurately determined in this case because of an apparent inhibition of the enzyme at higher substrate concentrations (Figure 14). An extrapolated Vmax value of 64pM CL hydrolyzed per min. per mg protein was obtained. Experimentally the Vmax value was obtained at 5.8mM substrate. The unusual kinetics may be due in part to insolubility of the substrate which at higher concentrations probably aggregates into larger micellar structures, a phenomenon which would decrease its accessibility to the enzyme and could prevent enzyme complexing with remaining monomeric substrate. Unfortunately, as will be illustrated shortly, dispersion of the substrate with detergents did not improve the assay conditions since the enzyme was sensitive to surfactants.

(ii) Effect of Nucleotides

When various nucleotides were tested with dialyzed preparations, ATP and ADP stimulated the reaction most effectively (Table 3). GTP, CTP and UTP were less stimulatory. CL was not hydrolyzed when ATP was substituted by TTP, any of the mononucleotides or dinucleotides other than ADP or by cyclic AMP or phosphoenolpyruvate. These results indicate a marked preference by the enzyme for ATP

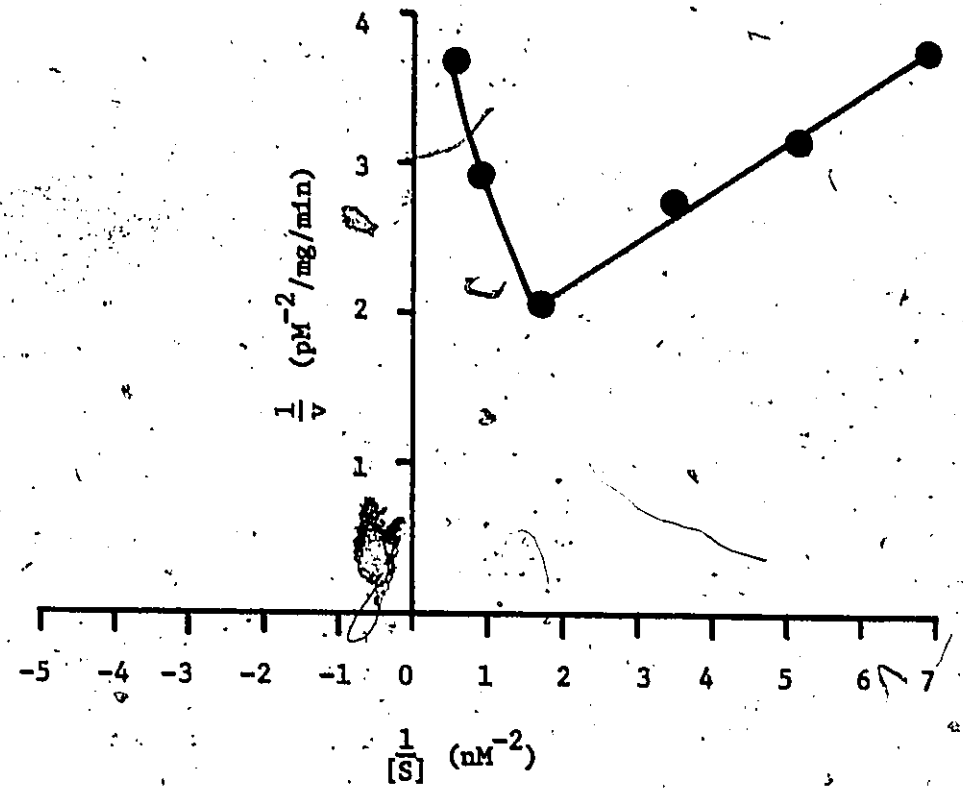


FIGURE 14. Effect of cardiolipin concentration on the rate of hydrolysis by *E. coli* phospholipase D. The assay conditions were the same as those described for Figure 9.

TABLE 3

EFFECT OF NUCLEOTIDES ON PHOSPHOLIPASE D ACTIVITY IN DIALYZED ENZYME PREPARATIONS

Nucleotide	Cardiolipin Hydrolysis pm/min/mg
ATP	60
GTP	35
CTP	17
UTP	13
TTP	0
ADP	59
GDP	0
CDP	8
UDP	0
AMP	0
GMP	0
CMP	0
UMP	0
CYCLIC AMP	0
PEP	0

The assay conditions were the same as those described for Figure 9 except that a dialyzed *E. coli* cell-free extract (0.4 mg protein) was used and the above nucleotides (2.8mM) were substituted for ATP. The cell-free extract was freshly prepared.

as nucleotide cofactor as well as the likely involvement of adenylate kinase in the E. *coli* extract which would explain the stimulation by ADP. Adenylate kinase activity was also deduced from the fact that in non-dialyzed preparations, nucleotide specificity was not apparent and all the trinucleotides except TTP could stimulate phospholipase D activity equally well (Table 4).

(iii) Effect of Cations and Detergents

As shown earlier, phospholipase D - mediated hydrolysis of CL is stimulated by increasing amounts of Mg^{++} up to 10mM (Table 5). In the presence of 1mM Mg^{++}, Mn^{++} further stimulated the enzyme although not to the same extent as an equivalent amount of Mg^{++}. At a concentration of 3mM, Co^{++} stimulated phospholipase D activity but at a higher concentration there was a lack of stimulation above that seen with 1mM Mg^{++}. Of the other cations tested, Ba^{++}, Ca^{++} and Fe^{+++} showed no additive effect. Zn^{++} inhibited enzyme activity by about 50% while Cu^{++} and EDTA caused complete inhibition.

The three detergents tested, sodium dodecyl sulfate, sodium deoxycholate and Triton X-100 were all strongly inhibitory (Table 5).

TABLE 4

EFFECT OF NUCLEOTIDES ON PHOSPHOLIPASE D ACTIVITY WITH NON-DIALIZED ENZYME PREPARATIONS

Nucleotide	Cardiolipin Hydrolysis pm/min/mg
ATP	60
GTP	58
CTP	58
UTP	55
ADP	51
CDP	19

The assay conditions were the same as those described for Figure 9 except that different nucleotides (2.8mM) were substituted for ATP.

TABLE 5

EFFECT OF CATIONS AND DETERGENTS ON E. *coli* PHOSPHOLIPASE D

Additive	Relative Activity
none (no Mg^{++})	0
Mg^{++}, 1 mM	100
3 mM	170
10 mM	220
Mn^{++}, 3 mM	140
10 mM	150
Co^{++}, 3 mM	150
10 mM	105
Cu^{++}, 1 mM	0
Zn^{++}, 1 mM	27
Ba^{++}, 1 mM	43
Fe^{+++}, 1 mM	45
Ca^{++}, 1 mM	43
EDTA, 30 mM	0
Sodium Deoxycholate, 10 mM	9
Sodium Dodecyl Sulfate, 20 mM	6
Triton X 100, 0.07% w/v	8

The incubation mixture contained in 2 ml final volume, 0.1 M phosphate buffer, pH 7.3, 4 nMoles of ^{32}P-labelled cardiolipin sonicated in buffer, 2.8 mM ATP, 1 mM Mg^{++} and *E. coli* cell-free sonicate (0.4 mg protein). The basic assay was complemented with the above additives of described concentrations. The relative enzyme activity of the basic assay was 60. The cell-free extract was not dialyzed.

(iv) Substrate Specificity of the Phospholipase D of *E. coli*

The phospholipase D in cell extracts of commercially obtained *E. coli* B cells did not hydrolyze either PE or PG under conditions allowing CL hydrolysis (Table 6). Neither were these phosphoglycerides attacked by varying these conditions. For example, no PA was produced from either PE or PG at pH 7.0 in the presence of Mg^{++} alone, ATP alone or without either cofactor. Varying the pH from 4.3 to 8.0 also had no effect. When Mg^{++} was replaced by Ca^{++} over this same pH range, no phospholipase D hydrolysis of PE was observed. All of the results in this study were duplicated with preparations from freshly grown cells.

(v) Subcellular Distribution of the Phospholipase D in *E. coli* Cells

In the case of freshly grown *E. coli* B cells, the phospholipase D was found in the supernatant fraction after centrifugation at 100,000 x g and 200,000 x g (Table 7). No activity whatsoever was observed in the 100,000 x g debris and only slight activity was detected in the 200,000 x g debris. However, the enzyme activity decreased with successive centrifugation steps so that the 200,000 x g supernatant contained only a third of the activity originally present in the 3,000 x g supernatant. This decrease in enzyme activity could possibly be due to the loss of some

TABLE 6

Substrate Specificity of the Phospholipase D in *E. coli*

Conditions	% Initial Counts						
	PE	PA	PG	PA	CL	PA	PG
without Mg^{++} or ATP	98	2	99	1	98	1	1
Mg^{++}	97	3	99	1	70	13	17
ATP	97	3	97	1	97	1	2
Mg^{++} + ATP	98	2	98	2	68	14	18
pH 4.3	98	2	98	2	98	1	1
5.0	97	3	98	2	98	1	1
6.2	98	2	97	3	98	1	1
7.3	98	2	98	2	75	11	14
8.0	92	2	98	2	98	1	1

The assay conditions were the same as those described for Figure 9 except for the buffers which were varied to obtain different pH. These buffers are those stated for Figure 10. Where indicated 20,000 DPM of the substrates were used upon sonication in the appropriate buffer: (^{32}P)-Cl (Sp. Act. 2.2 Ci/mole), (^{32}P)-PG (Sp. Act 0.86 Ci/mole) and (^{32}P)-PE (Sp. Act. 4.65 Ci/mole).

TABLE 7

Subcellular Distribution of Phospholipase D Activity In *E. coli* B Cells

E. coli B	Subcellular fraction	Total Activity mM/min
Freshly Grown	3,000 x g supernatant	25
	17,000 x g debris	0
	100,000 x g debris	0
	100,000 x g supernatant	13
	200,000 x g debris	1
	200,000 x g supernatant	8
Commercially Obtained	3,000 x g supernatant	20
	17,000 x g debris	1
	100,000 x g debris	1
	100,000 x g supernatant	17
	200,000 x g debris	1
	200,000 x g supernatant	11

The assay conditions were the same as those described for Figure 9. All subcellular fractions were assayed for activity using protein concentrations of 400 ug.

required cofactor or protein to the debris or to autolysis. At any rate, this component would have to be non-dialyzable since results illustrated in a later section show that no loss of activity is observed as a consequence of dialysis. Further work on the reconstitution of the subcellular fractions will have to be done to clarify this point.

A similar distribution of phospholipase D activity was seen with commercial *E. coli* B cells. Trace activity was observed in each of the debris fractions but the major part of the activity remained in the supernatant fractions. Again, centrifugation decreased the phospholipase D activity as only one half of the activity in the 3,000 x g supernatant remained after centrifugation at 200,000 x g. It seems that although freshly grown cells and commercially obtained cells had a different requirement for ATP, the distribution of the activity was identical in both these sources of enzyme. This means that if ATP activation involves an enzymatic chemical modification of phospholipase D, the enzymes promoting this modification are distributed in the cell in a manner similar to phospholipase D (i.e., they are also soluble).

(vi) Fractionation of Cardiolipin - Hydrolyzing Phospholipase D Activity from *E. coli* Extracts

In preliminary experiments, ammonium sulfate fract-

ionation was tested as a means of purifying the phospholipase D activity of E. *coli* . The only ammonium sulfate fraction to contain significant phospholipase D activity was the 36-50% saturated fraction (Table 8). This fraction comprised only 40% of the original protein but unfortunately the specific activity of the phospholipase D was only about one half of the corresponding value for the enzyme in the 17,000 x g supernatant of commercial E. *coli* B cells.

Similar results were obtained if the protein in this 17,000 x g supernatant was fractionated from calcium phosphate gel by sequential elution with increasing concentrations of phosphate buffer (Table 9). All of the phospholipase D activity was found in the fraction eluted with 1 M buffer. This fraction contained only 30% of the original protein but the specific activity was again about one third of the original activity. This loss of activity was prevented if the fractionation was performed in the presence of dithiothreitol. In this case the specific activity of the phospholipase D in the 1 M buffer fraction was double that of the original value. Glycerol was also effective in protecting the enzyme activity although not to the same extent as DTT.

The results of these studies indicate that E. *coli* phospholipase D most closely resembles the phosphatidohydrolase

TABLE 8

Ammonium Sulfate Fractionation of Phospholipase D Activity From E. coli

Ammonium Sulfate Fraction (% Saturation)	Protein Concentration mg	Specific Activity pM Hydrolyzed/min/mg
0-35	22	6
36-50	56	35
51-75	24	6
76-100	36	0

The assay conditions described for Figure 9 were used. The starting material was 140 mg. protein of the 17,000 x g supernatant of commercial E. coli B cells.

The specific activity of the phospholipase D in this preparation was 70 pM CL hydrolyzed/min/mg protein.

TABLE 9

Calcium Phosphate Gel Fractionation of Phospholipase D Activity

Protein Fraction	Protein Concentration	Enzyme Activity pM CL hydrolyzed/min/m
17,000 x g supernatant of commercial *E. coli* B cells	30	70
1 mM buffer fraction	6.8	0
1 mM buffer fraction + DTT	6.8	0
1 mM buffer fraction + glycerol	6.8	0
1 M buffer fraction	9.4	37
1 M buffer fraction + DTT	9.4	135
1 M buffer fraction + glycerol	9.4	80
calcium phosphate gel after elution with 1 M buffer	13	0

The assay conditions described for Figure 9 were used. Where indicated, dithiothreitol or glycerol was added to the calcium phosphate gel and to the buffers to a final concentration of 0.5 mM and 20% respectively.

found in *H. parainfluenzae*.[120,121] Both enzymes are dependent on high Mg^{++} concentrations, the Km for Mg^{++} for the *H. parainfluenzae* phospholipase D being 1.3mM and the corresponding value for the *E. coli* enzyme being 5mM. Since the calculated soluble Mg^{++} concentration in *E. coli* is 4mM,[140] the enzyme could function to near maximum capacity in vivo. Indeed, high Mg^{++} concentrations are required by other *E. coli* enzymes such as DNA polymerase[141] or the amino acid-activating enzymes.[142] The divalent cation requirement of both phospholipases D could be satisfied to some extent by Mn^{++} and Co^{++} (stimulation of phospholipase D by Co^{++} was also observed for the enzyme from *Streptomyces hachijoensis*).[123] The fact that Ca^{++} has no effect on the bacterial enzymes clearly distinguishes these from the plant phospholipase D. Also, the pH optimum of 5.6 for the plant enzyme differs from that for both the *E. coli* enzyme which is 7.0 and the *H. parainfluenzae* enzyme which is 7.5.

The *E. coli* phospholipase D exhibits the same strict specificity for CL as does the enzyme from *H. parainfluenzae*. Neither PG nor PE was hydrolyzed either under the conditions for CL hydrolysis or under altered conditions. Metals such as Cu^{++} and Zn^{++} markedly inhibited both enzymes as did EDTA and sodium dodecyl sulfate. However, Triton X-100

and sodium deoxycholate both had little effect on the *H. parainfluenzae* enzyme and could be used to solubilize substrate. This fact may account for the apparently greater activity of the *H. parainfluenzae* phospholipase D. This latter enzyme has a specific activity of 20nM CL hydrolyzed/min/mg protein while the *E. coli* enzyme displays substrate inhibition and thus maximal values are more than 100 fold smaller than those for the *H. parainfluenzae* enzyme. Because of the required differences in assay procedures and the substrate inhibition however, the activities of both these enzymes could not be accurately compared.

In their studies on the cardiolipin-specific phospholipase D of *H. parainfluenzae*, Ono and White[121] did not describe any nucleotide dependence of the enzyme. However, they did find from in vivo experiments that two inhibitors of oxidative phosphorylation, 3,3', 4,5-tetrachlorosalicylanilide (TCS) and carbonyl cyanide m-chlorophenyl hydrazone (m-CCCP) blocked the hydrolysis of CL.[143] Therefore, phospholipase D activity in that bacterium appears to be linked to energy metabolism. In *E. coli*, a definite requirement for ATP has been shown under certain conditions and this requirement can be satisfied only by ADP and ATP, the ADP acting through

the probable mediation of adenylate kinase.

The cardiolipin-hydrolyzing phospholipase D from *E. coli* appears to be a soluble enzyme being found in the 200,000 x g supernatant fraction. In this respect it differs from the *H. parainfluenzae* enzyme which is located in the 25,000 x g debris fraction.[118]

The *E. coli* enzyme is recovered in the 36-50% ammonium sulfate fraction and also in the protein fraction eluted from calcium phosphate gel with 1M phosphate buffer. These fractionation procedures, as well as subcellular separations, cause a decrease in the specific activity of the phospholipase D. However, retention of activity after calcium phosphate gel fractionation is achieved by the addition of glycerol as was found for the purification of peanut phospholipase D.[110] The addition of DTT during this fractionation procedure also affords a further protection of the enzyme implying the involvement of sulfhydryl groups in the stability of the phospholipase D. The stabilizing effect of DTT on phospholipase D activity has been observed for the cabbage enzyme.[105]

C. Further Investigation of the ATP-Requirement of the Cardiolipin-Hydrolyzing Phospholipase D of *E. coli*

As previous results did show, the phospholipase D

from *E. coli* can be shown to have an ATP-requirement by either freezing and thawing fresh cells several times or by using commercially grown cells that are obtained in a frozen state. By further defining the methods by which an ATP effect could be shown in fresh cells, it was hoped that the mechanism of ATP intervention could be elucidated.

The results in Table 10 show that dialysis of fresh cells by itself did not enhance the nucleotide dependence of the phospholipase D. Most of the endogenous nucleotides were removed by dialysis. As a control, 200,000 DPM of (^{32}P)-γ-ATP were dialyzed under the same conditions as the cell extract and 92% of the label was lost to the dialyzing buffer. Such dialyzed preparations could develop a nucleotide requirement when subjected to a 15min. preincubation in the presence of Mg^{++} prior to the addition of substrate. Preincubation of undialyzed preparations in the presence of Mg^{++} for longer periods (i.e.,1h.) at 37° or their storage for periods over a week at 4° produced similar results. Preincubation without Mg^{++} did not produce an ATP effect. There was considerable loss of activity (approx. 70%) however, if preincubations at 37° were prolonged for an hour or more but shorter preincubations were effective only with dialyzed preparations. The loss of activity is probably due to general proteolysis

TABLE 10

Study of the ATP-Requirement of the Phospholipase D in Freshly Prepared *E. coli* Cells

Treatment of Enzyme	Assay Conditions	
	Mg^{++}	Mg^{++} + ATP
	(% Hydrolysis)	
Without Dialysis or Preincubation	36	43
Dialysis	36	44
Dialysis + Preincubation for 15 min. with Mg^{++}	5	36
Preincubation for 60 min. Without Mg^{++}	13	15
Preincubation for 60 min. With Mg^{++}	3	14

The assay was the same as that described for Figure 9. Where indicated, before the assay, the 17,000 x g supernatant of an *E. coli* B cell-free extract was subjected to either dialysis and/or preincubation for either 15 min. or 60 min. or both. When preincubation was performed with added 10mM Mg^{++}, Mg^{++} was not added for the assay. The cell-free extract was freshly prepared.

attacking phospholipase D and other proteins. The phospholipase D activity of dialyzed preparations was not stable and most of the activity was lost within a week even at 0° storage.

Another attempt to elucidate the mechanism of ATP stimulation consisted of trying different conditions which would abolish the ATP effect in commercial cells. To do this, the 200,000 x g supernatant of sonicated commercial *E. coli* B cells was preincubated under various conditions and was then subjected to ammonium sulfate fractionation. The 36-50% saturated fraction was retained and was assayed for cardiolipin-hydrolyzing phospholipase D activity with and without added ATP.

As the results in Table 11 show, the phospholipase D activity in the 36-50% saturated ammonium sulfate fraction required ATP for activity as was the case for the high-speed supernatant fraction of the commercial cells. However, if the 200,000 x g supernatant fraction was preincubated for 15 min. at 37° with either ATP alone or Mg^{++} plus ATP before the addition of substrate, the corresponding 36-50% ammonium sulfate fraction did not exhibit an appreciable ATP effect. Preincubation of the high-speed supernatant fraction with Mg^{++} alone completely eliminated phospholipase D activity.

TABLE 11

Conditions affecting the ATP-Requirement of the Cardiolipin-Hydrolyzing Phospholipase D from Commerical *E. coli* B

Conditions	Enzyme Activity pM CL hydrolyzed/min./mg. protein	
	Mg^{++}	Mg^{++} + ATP
200,000 x g supernatant of commercial *E. coli* B cells (A)	5	55
36-50% saturated ammonium sulfate fraction of (A)	3	38
36-50% saturated ammonium sulfate fraction of (A) pre-incubated with ATP	21	29
36-50% saturated ammonium sulfate fraction of (A) pre-incubated with Mg^{++}	0	0
36-50% saturated ammonium sulfate fraction of (A) pre-incubated with Mg^{++} + ATP	22	28

Cardiolipin-hydrolyzing phospholipase D activity was assayed under the standard conditions. When indicated 20 mg. protein of the 200,000 x g supernatant of sonicated commercial *E. coli* B cells was preincubated for 15 min. at 37° with 9mM ATP and/or 34mM Mg^{++}. The 36-50% saturated ammonium sulfate fraction was then obtained and 400 ug protein was assayed for activity in the presence of either Mg^{++} or Mg^{++} + ATP.

To further investigate the role of ATP in the phospholipase D-catalyzed hydrolysis of CL, labelling of the 200,000 x g supernatant fraction with (^{14}C)- or (^{32}P)-γ-labelled ATP was attempted (Table 12). However, in either case, relatively little label, was incorporated in the 36-50% ammonium sulfate fraction after incubation with the corresponding labelled ATP. Since this incorporation was not appreciable and because of the expense of labelled ATP, this line of investigation was concluded.

There was no labelling by ATP of either CL or its hydrolysis products under the assay conditions used for phospholipase D. The label from 5-30uCi of (^{32}P)-γ-ATP was not found in any of these phospholipids after incubation with cold ATP, Mg^{++} and a cell-free extract under the usual assay concentrations.

The results of Table 10 show that the CL-hydrolyzing phospholipase D in cell-free extracts of freshly prepared *E. coli* B cells can, under certain conditions be made to exhibit a requirement for ATP. Removal of endogenous ATP by dialysis alone had no effect on enzyme activity. However, a nucleotide requirement became evident when dialysis was coupled with a preincubation period of 15 min. at 37°. These results suggest the presence of active and inactive forms of phospholipase D.

TABLE 12

Attempt to Label a Phospholipase D-Containing Protein Fraction with Labelled ATP

Protein Concentration (mg)	Cofactor	DPM in 36-50% saturated ammonium sulfate fraction
10	25uCi(^{32}P)-ATP	926
	1uCi(^{14}C)-ATP	1343

Aliquots of the 200,000 x g supernatant of sonicated commercial E. *coli* B cells were preincubated for 15 min. at 37° with either 25uCi of γ-(^{32}P)-ATP or 1uCi of (8-^{14}C)-ATP. The 36-50% saturated ammonium sulfate fraction was obtained and was counted in PCS solubilizer.

The fact that dialyzed enzyme was not stimulated by added ATP is noteworthy. One can suggest several explanations on this basis. One possibility is that ATP does not act as an allosteric effector or if it does, it binds very tightly to the enzyme. So long as the enzyme is saturated with endogenous ATP in this way, an exogenous ATP requirement would not be demonstrable. An alternative explanation is that ATP directly serves in the chemical modification of the enzyme (adenylation or phosphorylation) or is in some way indirectly involved in a process producing a relatively long lasting effect on the enzyme conformation. The preincubation studies seem to indicate that there is a Mg^{++} - dependent, probably enzyme catalyzed inactivation of phospholipase D which can be partially reversed by ATP addition. The non-reversible inactivation is probably due to autolytic proteolysis.

The results in Table 11 unfortunately do not eliminate any of the above mentioned possibilities. When extracts of commercial cells were preincubated with either ATP or ATP plus Mg^{++}, the phospholipase D found in the subsequent 36-50% saturated ammonium sulfate precipitate did not require ATP for activity. Preincubation may have resulted in a tight binding of ATP not removable by ammonium sulfate precipitation or it may have favored a process

similar to adenylation or phosphorylation.

The ammonium sulfate precipitate of non-preincubated supernatant did respond to ATP addition. This result indicates that either the effect of ATP is allosteric or there is co-precipitation with phospholipase D, of an enzyme system using ATP as substrate which serves to activate the phospholipase D. As can be appreciated, the pilot experiments which were designed to help reveal the mechanism of ATP action were not completely successful. The results are compatible with a tight binding of ATP to the enzyme or a chemical modification of the enzyme resulting from the appropriation of an ATP group. In either case it is clear that the enzyme exists in an active and inactive form and that the enzyme is in some way ATP regulated.

D. Breakdown of Cardiolipin by Transphosphatidylation in *E. coli* Extracts

Evidence summarized in Table 13 reveals that CL breakdown occurring in dialyzed cell-free homogenates or in the 100,000 x g supernatants of *E. coli* cells greatly favored PG production over that of PA when glycerol was added to the medium. The ratio of PG to PA increased with increasing concentrations of glycerol.

TABLE 13

Effect of glycerol on cardiolipin-specific phospholipase D of *E. coli* cell-free preparations

Conditions	Counts Recovered as products	Ratio of product counts $\frac{\text{phosphatidyl glycerol}}{\text{phosphatidic acid}}$
(A)		
Mg^{++}, ATP	1070	1.4
Mg^{++}, ATP, 1% glycerol	1100	1.6
Mg^{++}, ATP, 4% glycerol	1230	2.2
Mg^{++}, ATP, 10% glycerol	1690	2.7
(B)		
Mg^{++}, ATP	5840	1.5
Mg^{++}, ATP, 4% glycerol	5500	2.9
(C)		
Mg^{++}, ATP, 4% glycerol	0	0

(A) The incubation mixture contained in 2ml, 2.8 mM ATP, 10mM Mg^{++}, dialyzed cell-free *E. coli* B sonicate (2.8mg protein) 0.1M phosphate buffer pH 7.3, ^{32}P-labelled cardiolipin (6700 DPM) and glycerol as specified. Incubations were for 30 min. at 37°.

(B) The conditions were similar to those in (A) except that the 100,000g x 1 h supernatant of *E. coli* B (3.4 mg protein) and 9700 DPM ^{32}P-labelled cardiolipin were used.

(C) The conditions were similar to those in (A) except that the 17,000 x g debris of *E. coli* B (4mg protein) were used.

Glycerol was the only acceptor to function in this transphosphatidylation reaction (Table 14). Normal CL hydrolysis to PG and PA via phospholipase D activity occured when either serine and CTP or α-glycerophosphate were added to the assay. In the presence of added ethanolamine, CL breakdown was inhibited completely. No label appeared in the PE fraction when either serine or ethanolamine was added.

To further show that CL breakdown can also occur via transphosphatidylation with glycerol, dialyzed, cell-free *E. coli* B sonicates were incubated with labelled glycerol under various conditions (Table 15). Subsequent analysis of the endogenous phospholipids showed that when Mg^{++} alone was added some label appeared in the PG fraction. The amount of label found in this phospholipid increased when Mg^{++} plus ATP were added to the incubation. No radioactivity was detected in either the PA, PE or CL fractions.

These results reveal that, in *E. coli* extracts, breakdown of CL can proceed via a transphosphatidylation causing an apparent reversal of the cardiolipin synthase reaction:

$$\text{cardiolipin} + \text{glycerol} \xrightarrow{Mg^{++},\ ATP} 2\ \text{phosphatidylglycerol}$$

The transphosphatidylase activity appears to be specific

TABLE 14

Effect of Various Acceptors on the Transphosphatidylase Activity of E. coli Extracts

Conditions	Counts recovered as products	Ratio of Product Counts PG/PA
complete	1070	1.4
glycerol	1290	2.3
ethanolamine	0	0
serine + CTP	910	1.1
DL-α-glycerophosphate	950	1.3

The incubation mixture was identical to that used in Table 13 (A). When specified, either 5% glycerol, 4% ethanolamine, 5.0mM serine, 1.5mM CTP or 3mM DL-α-glycerophosphate was added.

TABLE 15

Incorporation of (^{14}C)-Glycerol into Phospholipids Under the Conditions for the Assay of Phospholipase D Activity

Conditions	Counts Recovered			
	PA	PG	PE	CL
Mg^{++}	93	815	81	77
Mg^{++} + ATP	70	2179	153	70

The basic assay contained in 2.0ml. of 0.1M phosphate buffer pH 7.3, dialyzed, cell-free *E. coli* B, sonicate (4 mg. protein) and 55,000 DPM (1,3-^{14}C)-glycerol.
To this assay, the following components were added where indicated; 10mM Mg^{++}, and/or 2.8mM ATP. Incubations were for 30 min. at 37°.

for glycerol as neither serine, ethanolamine nor α-glycerophosphate could act as acceptors. Mg^{++} and either ATP or CTP were also required for activity.

Although the conditions used in these experiments also favour the conversion of glycerol to sn-3-glycerophosphate, the enzymes which mediate the synthesis of PG from this compound are particulate enzymes.[14] Therefore, by using the 100,000 x g supernatant fraction, glycerol could not have been incorporated into PG except through trans-phosphatidylation. Also, phosphatidylglycerophosphate could not be detected as an intermediate which further indicates direct involvement of glycerol.

It is unlikely that the transphosphatidylase activity is associated with cardiolipin synthase since the latter enzyme is a particulate enzyme[63] and in these experiments no transphosphatidylation was observed using the 17,000 x g debris. Rather, this activity was present in the 100,000 x g supernatant and is therefore more probably associated with the phospholipase D activity located in the same fraction. It is not uncommon that transphosphatidylase and phospholipase D activities are associated with the same enzyme. Purified phospholipase D from cabbage has been found to cause the phosphatidylations

of ethanol, ethanolamine and glycerol by PC as well as to catalyze the exchange between choline and PC.[105]

E. Effect of Cell Age on Cardiolipin-Hydrolyzing Phospholipase D Activity in E. coli Cells

In E. coli cells, the amount of phospholipase D activity and the effect of added ATP on this activity varied with cell growth. Cells were harvested at various growth stages according to the growth curve shown in Figure 15 and the cell-free extracts of these cells were assayed for cardiolipin-hydrolyzing phospholipase D activity. As the results in Table 16 show, no enzyme activity was observed in the early log phase. Phospholipase D activity appeared in the mid log stage and required added ATP for maximal activity. In the late log stage, phospholipase D activity reached its maximum and required only Mg^{++} for activity. This activity decreased in the stationary stage but still was active with added Mg^{++} alone. If cells grown to the late log stage, where maximum phospholipase D activity was observed, were incubated in saline solution for 3h. at 37°, the total phospholipase D activity decreased by 65% and the enzyme at this stage required ATP for maximal activity.

These results indicate that the CL-hydrolyzing phospholipase D probably plays little or no role in the E. coli

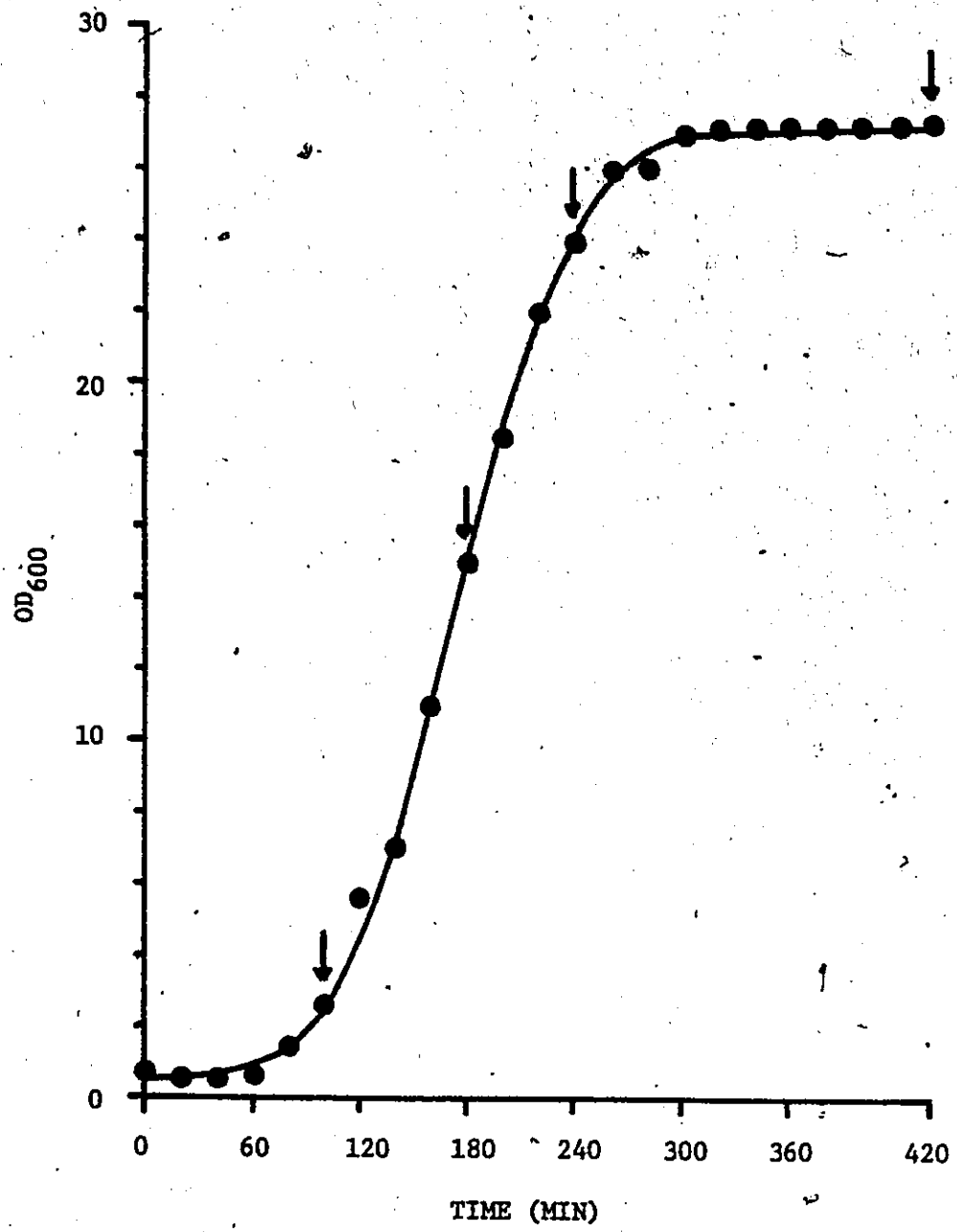


Figure 15 Growth curve for E. *coli* B

TABLE 16

Effect of Cell Growth on Cardiolipin-hydrolyzing Phospholipase D from *E. coli*

growth stage	Culture Time (min)	Enzyme Activity pM CL hydrolyzed/min/mg protein	
		Mg^{++}	Mg^{++} + ATP
early log	100	0	0
mid log	180	3	17
late log	240	63	70
stationary	420	50	58
saline medium*		11	25

E. coli B Cells grown to the required stage in 150ml. of medium were harvested and cell-free extracts were obtained as previously described. These extracts were then assayed for cardiolipin-hydrolyzing phospholipase D activity with the assay conditions described for Figure 9.

* Cells from the late log phase were resuspended in 0.1M saline pH 7.0 (150ml), were incubated for 3h. at 37° prior to assay.

cell in the early or mid log stage of cell growth. The enzyme exhibits maximum activity in the late log stage which may explain the low concentration of cardiolipin at that stage when compared to PG.[14] In the stationary phase, the CL concentration is known to increase at the expense of PG[33,34,75,76] which may be partly due to the decrease in phospholipase D activity at that stage. This conversion of PG to CL is also seen when cells are transferred to a saline medium in the case of both *E. coli*[64] and *Staphylococcus aureus*.[134] Tunaitis and Cronan[67] have studied *E. coli* cells under conditions which promote CL synthesis and found that the increase in CL was not due to an increase in CL-synthetase activity. They suggested that the lack of an energy requirement for this enzyme and the presence of large amounts of cellular PG accounted for this increase in CL synthesis. However, results shown here indicate that CL-hydrolyzing phospholipase D activity decreases greatly under energy-starved conditions. Also, the enzyme at this stage requires ATP for maximal activity and since the medium is energy-free, this enzyme probably exhibits very little activity in vivo under those conditions. Therefore, the relative increase in CL under starved conditions may be due to a decrease in CL catabolism rather than an increase in its synthesis or perhaps a combination of both.

F. Occurrence of Cardiolipin-Hydrolysing Phospholipase D Activity in Bacteria

Under the standard conditions for the assay of the cardiolipin-specific phospholipase D of *E. coli*, CL was hydrolyzed by cell-free extracts of three other Gram-negative bacteria- *Salmonella typhimurium*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Table 17). As would be expected from the results with freshly grown *E. coli* cells, ATP did not stimulate breakdown in these extracts but they all had a similar requirement for Mg^{++}. No activity was detected with added ATP, Ca^{++} or EDTA alone.

Cardiolipin hydrolysis was not observed in any of the Gram-positive bacteria tested (i.e., *Staphylococcus aureus*, *Bacillus cereus or Bacillus subtilis*) or in *Saccharomyces cerevisiae* or in rat liver mitochondria under the standard assay conditions. Neither could activity be detected in these organisms using increased protein aliquots of up to 4mg or incubation times of up to 30 min. Phospholipase D activity was not observed in any of the organisms tested in the presence of added Ca^{++}, ATP or EDTA alone. (Table 18)

The identity of the hydrolysis products obtained by incubating cell-free homogenates of *E. coli*, *Salmonella*

TABLE 17

Hydrolysis of Cardiolipin by Extracts of Various Organisms

Organism	pM Cardiolipin Hydrolyzed				
	Mg^{++}	ATP	Mg^{++} + ATP	Ca^{++}	EDTA
Salmonella typhimurium	60	0	65	0	0
Proteus vulgaris	63	0	64	0	0
Pseudomonas aeruginosa	36	0	38	0	0
Escherichia coli	70	0	73	0	0
Bacillus cereus	0	0	0	0	0
Bacillus subtilis	0	0	0	0	0
Staphylococcus aureus	0	0	0	0	0
Saccharomyces cereviseae	0	0	0	0	0
Rat liver mitochondria	0	0	0	0	0

The standard incubation mixture contained in 2ml., 0.1M phosphate buffer pH 7.0, 2-4 nmoles of (^{32}P)-cardiolipin (Sp. Act. 2.2 Ci/mole) sonicated in buffer and cell-free extracts of the various organisms (0.4 mg protein). When indicated 2.8 mM ATP, 10mM Mg^{++}, 10mM Ca^{++} and 30mM EDTA were added. Incubations were for 15 min. at 37°. The cell-free extracts were freshly prepared and non-dialyzed.

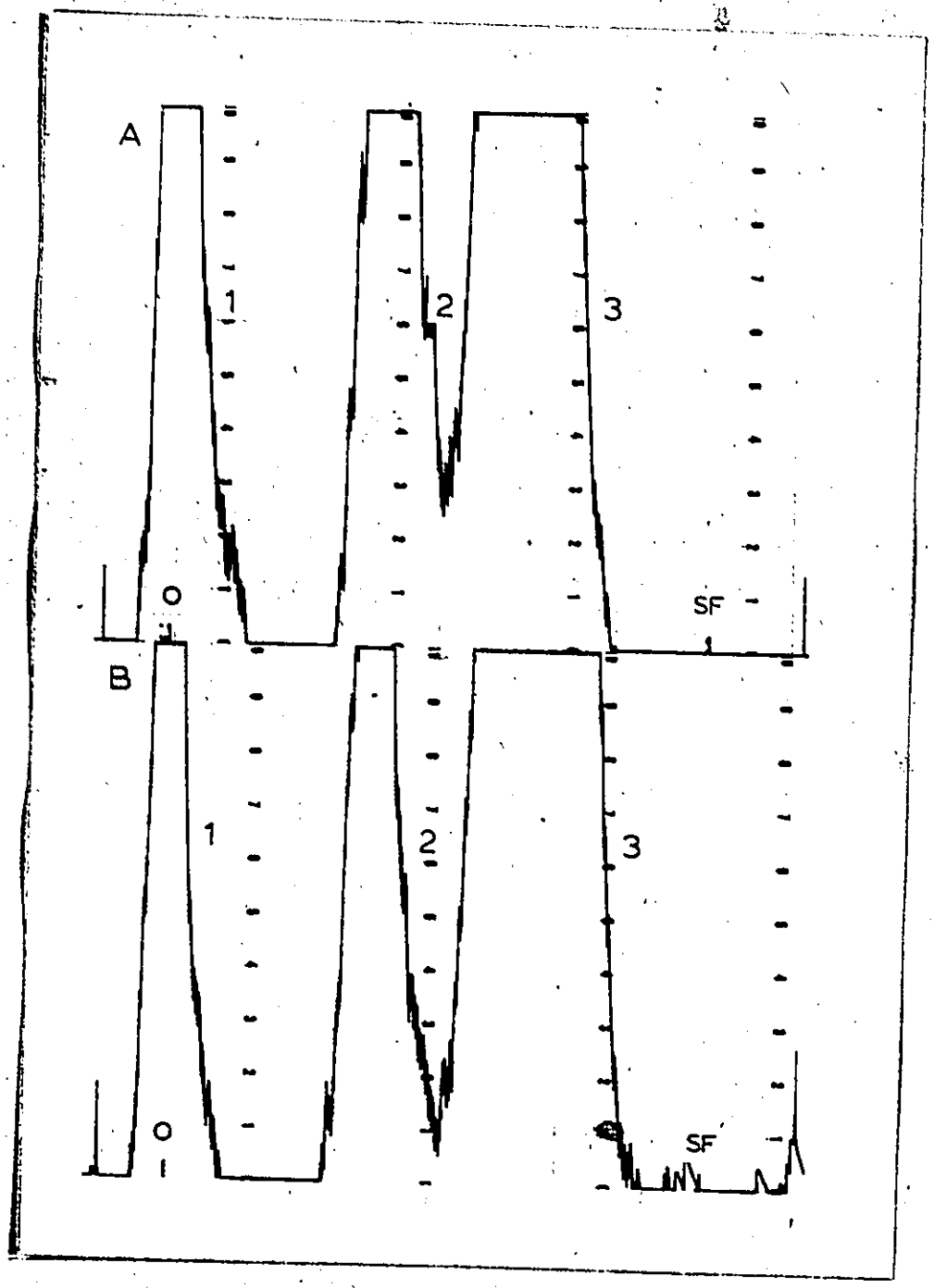
typhimurium, *Proteus vulgaris* and *Pseudomonas aeruginosa* with (^{32}P)-labelled CL are revealed in Figure 16. Components 1, 2, and 3 were identified respectively as PA, PG and CL by cochromatography with reference lipids in system 3. Also, after elution and deacylation of these components from the assay with the S. *typhimurium* extract by mild alkaline hydrolysis, components 1, 2 and 3 were identified as corresponding to glycerophosphate, glycerophosphatidylglycerol and di-(glycerophosphoryl)-glycerol. (Figure 17)

Results illustrated in Figure 18 corroborate those just presented. When whole lipid extracts of active incubation mixtures were subjected to mild alkaline hydrolysis, the products obtained were the same for each Gram-negative bacteria and were identical to GP, GPG and GPGPG in system 6.

For all four bacteria containing cardiolipin-hydrolyzing phospholipase D there was a similar pH requirement (Table 18). Activity was detected only at pH 7.0 with little or no activity seen at pH 6.2 or lower or at pH 8.0. Phospholipase D activity could not be detected in cell-free extracts of *Staphylococcus aureus* or *Saccharomyces cerevisiae* in the pH range tested (Table 18). In the case of *Staphylococcus aureus* hydrolysis of either PG or CL by phospholipase D activity could not be detected at pH 4.4 in the presence of either Mg^{++} alone, Mg^{++} plus ATP or Ca^{++} alone although

Figure 16

Scans of radioactivity of the hydrolysis products obtained by incubating (^{32}P)-labelled cardiolipin with cell-free preparations of (A) *Escherichia coli*, (B) *Salmonella typhimurium*, (C) *Proteus vulgaris* and (D) *Pseudomonas aeruginosa*. The incubation mixture contained in 2ml., 0.1M phosphate buffer pH 7.0, 2-4 nmoles of (^{32}P)-cardiolipin (Sp. Act. 2.2 Ci/mole) sonicated in buffer 10mM Mg^{++}, 2.8 mM ATP and cell-free extracts of the various organisms (0.4 mg protein). Incubations were for 15 min. at 37°.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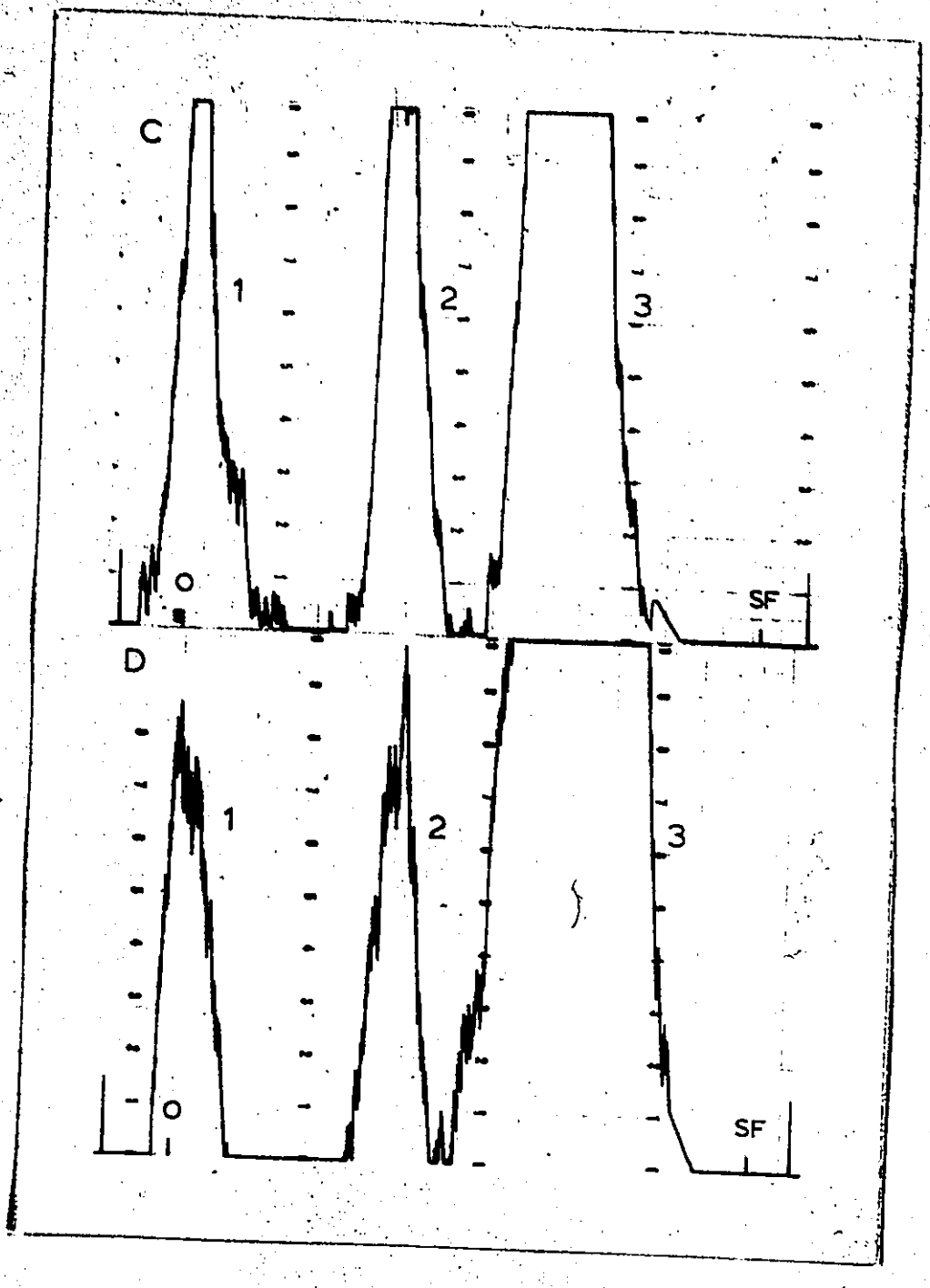
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Figure 17 Autoradiogram of deacylation products obtained by mild alkaline hydrolysis of component 1(B), component 2 (C) and component 3 (A) illustrated in Figure 16B. The products were separated on Whatman No. 1 paper with ethanol - 1M ammonium acetate pH 7.5 (65:35 v/v) (System 5) as solvent by the ascending technique.

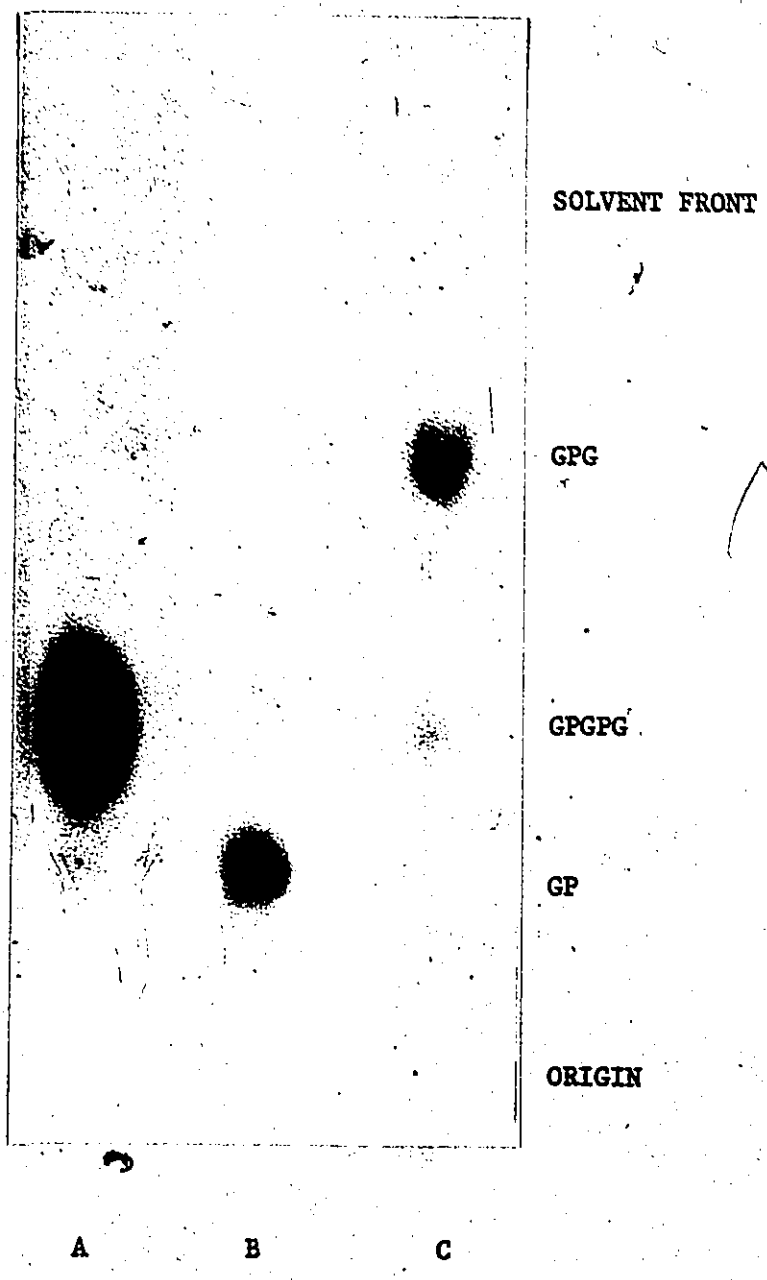
SOLVENT FRONT
GPG
GPGPG
GP
ORIGIN
A
B
C

Figure 18 Autoradiogram of deacylation products obtained by mild alkaline hydrolysis of lipid extracts of homogenates of (B) *Escherichia coli*, (C) *Salmonella typhimurium*, (D) *Proteus vulgaris* and (E) *Pseudomonas aeruginosa* incubated with (^{32}P)-labelled cardiolipin under the conditions described for Figure 15. Separation of the water-soluble products was with system 5. In (A) the incubation mixture contained *E. coli* homogenate and 30 mM EDTA.

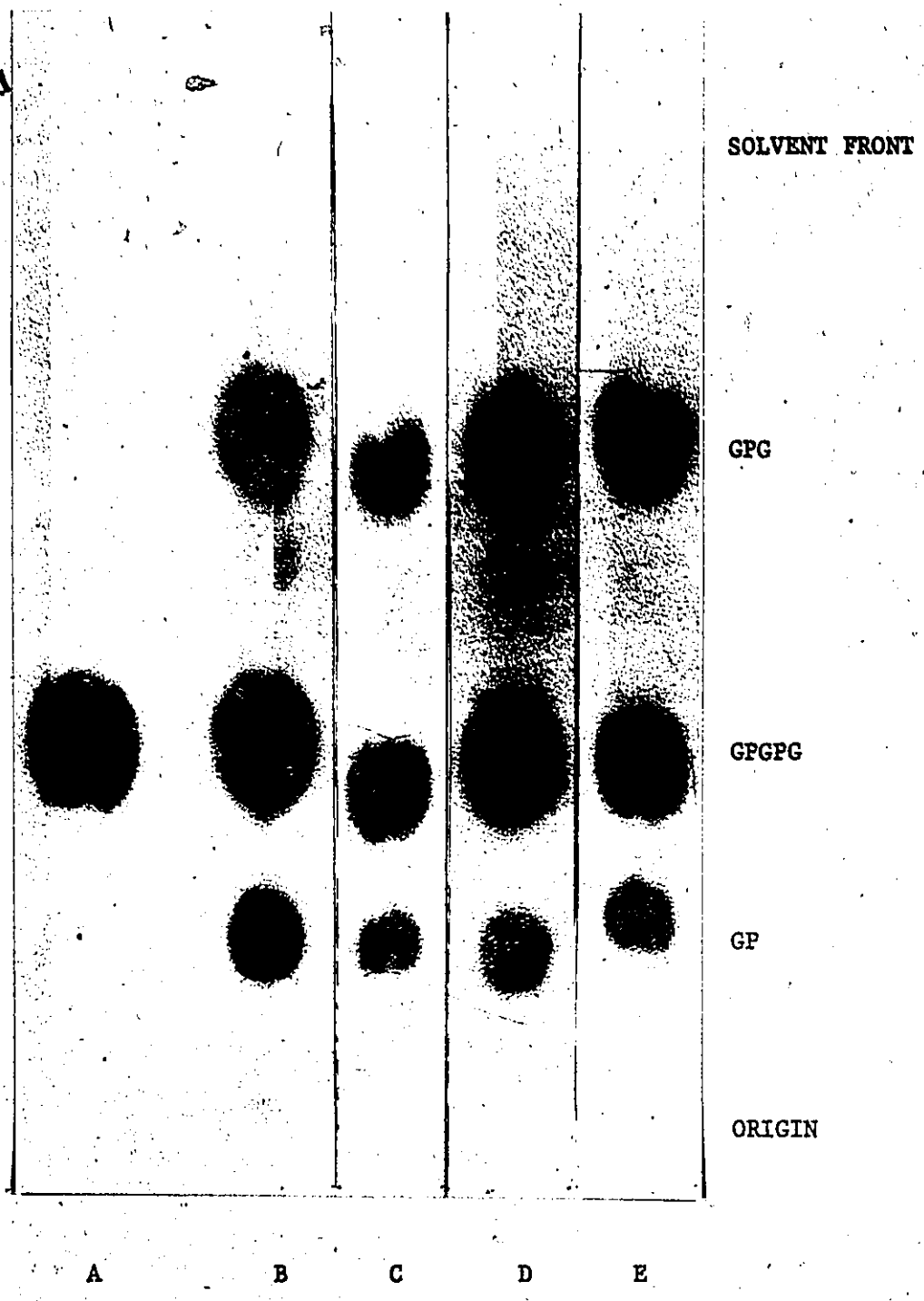
SOLVENT FRONT
GPG
GPGPG
GP
ORIGIN
A
B
C
D
E

TABLE 18

Effect of pH on the Hydrolysis of Cardiolipin by Extracts of Various Organisms

Organism	pM Cardiolipin Hydrolyzed pH				
	4.3	5.0	6.2	7.0	8.0
Salmonella typhimurium	0	0	14	75	7
Proteus vulgaris	0	0	1	65	4
Pseudomonas aeruginosa	0	0	2	42	1
Escherichia coli	0	0	0	75	0
Staphylococcus aureus	0	0	0	0	0
Saccharomyces cereviseae	0	0	0	0	0

The assay conditions were the same as those described for Figure 15. In the pH range 6.2 - 7.0, 0.1M phosphate buffer was used. Below this range the buffer was 0.1M acetate and above this range the pH was maintained with 0.1M Tris-HCL.

at this pH synthesis of CL by transphosphatidylation with PG has been reported to occur.[63,66]

With extracts from *Proteus vulgaris* and *Pseudomonas aeruginosa*, neither PG nor PE were hydrolyzed under conditions allowing the breakdown of CL to PA and PG (Table 19). Nor could hydrolysis of these two phospholipid substrates be observed by increasing the incubation time to 30 min. or the protein concentration of these extracts to 4 mg.

It appears from these results that there is a typical phospholipase D found in Gram-negative bacteria that hydrolyzes CL to PA and PG. Of all the organisms tested for this activity, only the five Gram-negative bacteria shown in Table 20 exhibit this enzyme activity.

This phospholipase D activity was not detected in the Gram-positive bacteria or in yeast cells or in crude rat liver mitochondria under a variety of conditions. In the case of *Staphylococcus aureus* these negative results were suprising since this bacterium forms CL by transphosphatidylation with PG and, much like *Haemophilus parainfluenzae*, responds to inhibitors of oxidative phosphorylation or proton gradient formation by accumulating CL.[144]

TABLE 19

Substrate Specificity of the Phospholipase D from *Proteus vulgaris* and *Pseudomonas aeruginosa*

Bacteria	pM Substrate Hydrolysed		
	PE	PG	CL
Proteus vulgaris	0	0	64
Pseudomonas aeruginosa	0	0	38

The assay conditions were the same as those described for Table 6.

TABLE 20

The Occurrence of Cardiolipin-Hydrolyzing Phospholipase D in Various Organisms

Organism	Phospholipase D activity
Escherichia coli	+
Proteus vulgaris	+
Salmonella typhimurium	+
Pseudomonas aeruginosa	+
*Haemophilus parainfluenzae**	+
Staphylococcus aureus	-
Bacillus cereus	-
Bacillus subtilis	-
Saccharomyces cerevisiae	-
Rat liver mitochondria	-

* results of Ono and White[117,118]

The phospholipase D found in *Salmonella typhimurium*, *Proteus vulgaris* and *Pseudomonas aeruginosa* cell-free extracts show characteristics similar to the *E. coli* enzyme in that they exhibit a strict requirement for Mg^{++} and are active only at about neutral pH, the pH optimum of the *E. coli* enzyme being 7.0. Also, none of these phosphatidohydrolases tested acts on either PE or PG. ATP does not stimulate CL breakdown by any of these freshly prepared cell-free extracts of Gram-negative bacteria. However, it was previously shown that an ATP-requirement for the *E. coli* phospholipase D was displayed only by cell extracts that had been frozen and thawed. It is quite likely that the phospholipases D of these Gram-negative bacteria, like those of *E. coli* and *H. parainfluenzae* are involved in the turnover of CL which occurs in response to the energy state of the cell.

CONCLUDING REMARKS

The present research has shown conclusively that E. *coli* cell extracts contain a phospholipase D activity that specifically hydrolyzes cardiolipin according to the following equation:

cardiolipin $\xrightarrow{Mg^{++},\ ATP}$ phosphatidic acid + phosphatidylglycerol

Recently, work performed by Audet *et al*[145] has indicated that a functional phospholipase D acting on CL exists in intact E. *coli* cells. This enzyme activity coupled with the synthesis of CL via the transphosphatidylation with two moles of PG[63] has been tentatively designated as the polyglycerophosphatide cycle.[145] (Figure 19)

Since the combined actions of these two enzyme activities are equivalent to a phospholipase D attack on PG, the activity of this cycle in intact cells should result in a sparing of the phosphatidyl moiety of PG and a faster rate of turnover of the non-acylated glycerol moiety. Audet *et al*[145] found that the non-acylated glycerol moiety of PG does indeed turn over initially at twice the initial rate of the acylated glycerol moiety in growing cultures of E. *coli* B and E. *coli* K19.

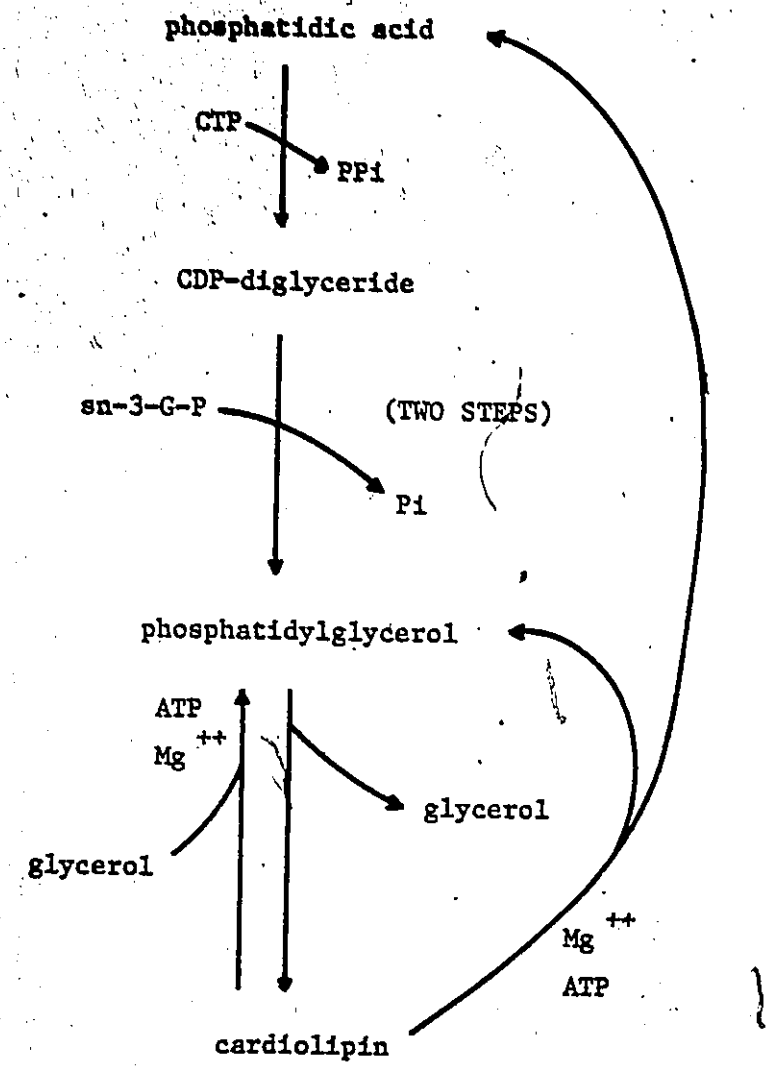

Figure 19. Polyglycerophosphatide Cycle in *E. coli*

The involvement of CL-specific phospholipase D in the polyglycerophosphatide metabolism of intact *E.coli* B cells was further tested by culturing cells in a complex medium with (^{32}P)-orthophosphate and then transferring the cells to an energy-free medium. Under these conditions, levels of CL increase at the expense of PG.[64] When these starved cells were returned to the original energy-rich medium, PG levels increased while CL levels decreased. However, no PA accumulation was observed.

The possibility that the PA formed from phospholipase D action on CL was completely incorporated into PG was examined by incubating labelled CL with cell-free *E. coli* B extracts in the absence of detergent. Under these conditions, the CL-specific phospholipase D was active and degraded the CL to approximately equal amounts of PA and PG. However, when Triton X-100, which inhibits cardiolipin-hydrolyzing phospholipase D, was added to a mixture of ATP, Mg^{++}, CTP, L-serine, α -glycerophosphate, labelled CL and cell-free extracts, CL was not broken down. Rather, PA was converted to PG. These results show that PA produced from CL breakdown can be re-incorporated into the polyglycerophosphatides.

An alternative explanation for the lack of PA accumulation observed by Audet et al in intact *E.coli* cells

is that CL is not hydrolyzed in vivo by the phospholipase D but instead CL is broken down via a transphosphatidylation with glycerol. Results given in this thesis have shown that this transphosphatidylase activity is found in broken *E. coli* cells, very likely associated with phospholipase D activity and is specific for glycerol as the acceptor.

Ballesta *et al*[146] have also reported unequal rates of turnover in the glycerol moieties of *E. coli* PG. Their results are compatible with a functional polyglycero-phosphatide cycle although they proposed a mechanism involving an exchange between the non-acylated glycerol of PG and free glycerol.

Rampini[147] has recently confirmed the results of Audet *et al* concerning the functioning of the cardiolipin-specific phospholipase D in intact *E.coli* cells. He found that if cells were transferred from an energy-rich medium to a saline medium and back to an energy-rich medium, the CL that had accumulated in the saline medium was hydrolyzed to PG mainly. PA did not accumulate. They found, however, that synthesis of PE and PG from (^{32}P)-orthophosphate occured readily under these conditions and concluded that any PA formed from CL must be rechannelled towards the syntheses of PE and PG. The author offered no proof to that effect however. Audet *et al*[145] did not observe an incorporation of PA into PE under in vitro conditions.

At any rate, evidence seems to indicate the involvement of a functional polyglycerophosphatide cycle in intact E. *coli* cells. Since the phospholipase D exhibits an ATP-requirement, this cycle would be under energy control and would explain the responsiveness of PG and CL levels to the energy supply in the cell.[14,64]

Some control over this cycle may be exerted by the phospholipids themselves. De Siervo[149] found that in membrane preparations of *Micrococcus Lysodeikticus* , cardiolipin synthetase was inhibited by its end products CL and glycerol. PE and PA were also inhibitory, PA being the strongest inhibitor. In the case of CL-specific phospholipase D of E. *coli* the inhibitory effects of phosphoglycerides were not studied. However ethanolamine was found to be inhibitory. A higher level of control may be exerted over this cycle. Levels of CL-synthetase in E. *coli* remain constant under varying conditions even though PG and CL levels may change.[67] But studies here have shown that levels of cardiolipin-specific phospholipase D change with cell age. It is possible that control over the levels of this enzyme may also influence the cycle.

The CL-hydrolyzing phospholipase D is found not only in E. *coli* . Its presence has been reported in

Haemophilus parainfluenzae[120,121] and research presented here has proved its occurrence in *Salmonella typhimurium*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Cardiolipin synthetase activity has not yet been confirmed in the three latter bacteria so it is not certain whether a polyglycerophosphatide cycle functions in these organisms. However in *Salmonella typhimurium*[130] turnover studies have shown that label lost from PG is incorporated into CL. Also, in *H. parainfluenzae*[121] CL accumulated and PG disappeared in the membrane fraction when inhibitors of phospholipase D activity were added to the growth medium.

In the case of the non-fermentative marine bacteria *Pseudomonas aeruginosa*, only a slight amount of CL synthetase activity has been observed by De Siervo and Reynolds.[148] These workers found that in accordance with this low activity, this bacterium also contained little or no CL. This may explain why we found less cardiolipin-specific phospholipase D activity in *Pseudomonas aeruginosa* than in some of the other bacteria examined.

The Gram-positive bacteria tested showed no CL-specific phospholipase D activity. This is surprising since *Staphylococcus aureus* is known to contain CL synthetase[144] and the unacylated glycerol moiety of PG turns over

more rapidly than the diacylated glycerol moiety[150] as is the case in *E. coli*. Cardiolipin synthetase is also found in *Micrococcus lysodeikticus*.[151] A glycerol-requiring mutant of *Bacillus subtilis* which was shown in the present study to contain no CL-hydrolyzing phospholipase D has been seen to accumulate CL and lose PG in pulse-chase experiments under conditions of glycerol deprivation.[152]

There are two possibilities which may account for the failure to detect CL-specific phospholipase D activity in Gram-positive bacteria. The enzyme may be active in these bacteria but under conditions that differ from the Gram-negative enzyme although variations in the assay conditions were tested in this study. The other possibility is that CL is not degraded by phospholipase D action in these bacteria. Lillich and White[152] did find, however, that in their glycerol-requiring mutant of *Bacillis subtilis*, CL counts were rapidly lost to PG when glycerol was added to glycerol-deprived cells. This would indicate that CL is being degraded by a transphosphatidylation reaction involving glycerol as acceptor. The enzyme involved must be quite different from the cardiolipin-specific phospholipase D activity detected so far only in Gram-negative bacteria. In rat liver cells, CL degradation appears to involve the lysosomes in which acid hydrolases degrade CL to yield water-soluble products and free fatty acids.[153]

The studies presented here have shown that the CL-specific phospholipase D of *E. coli* is regulated by ATP. However, the means of this regulation has not been clearly defined. It appears that the enzyme exists in an active and inactive form and that the activation involves either an allosteric effect by ATP or an enzymatically catalyzed chemical modification of the phospholipase D. Alternatively the ATP could be indirectly acting on some modifier. In order to distinguish conclusively between these possibilities, the phospholipase D will have to be purified. For example, with purified enzyme, isolation of a covalent complex of the ATP-enzyme using both (^{14}C)- and γ-(^{32}P)-labelled ATP would ascertain chemical modification as the mechanism of activation.

ATP stimulation of CL hydrolysis within the cell membrane may be significant in the control of energy metabolism in *E. coli* and other Gram-negative bacteria. As was discussed in the introduction of this thesis, CL is closely associated in respiratory membranes with components of both electron transport[7,8] and oxidative phosphorylation[9] and is a requirement for the optimal reconstitution of membranes capable of phosphorylating ADP.[9] One may hypothesize that CL is involved in maintaining the organization of the electron transport system for optimal ATP formation.

White[154] has found that changes occur in the lipid content, distribution and metabolism when the composition of the bacterial electron system is altered. He suggests that post-synthetic modifications of the phospholipids, including changes in the polar head groups may be the mechanism of the involvement of lipids in membrane changes.

Some properties of CL lend themselves to control of structure and function in membranes. De Siervo and Reynolds[151] have suggested that, in marine Gram-negative bacteria at least, CL is involved in the stabilization of cell membranes as is Mg^{++}. The increased stability may result from the particular structure of CL which may permit the bridging of independent lipoprotein structures. Also, molecular packing of cardiolipin is known to be affected by Mg^{++}, undergoing a 10 - 13% contraction in the presence of this divalent metal ion.[155] These unique properties of CL may impart a particular organization within the membrane which could be altered by CL-specific phospholipase D. Such hydrolysis could also change the fluidity of the membrane which is dependent, in part, on the nature of the phospholipid head group.[156,157] If the hydrolysis of CL does affect the organization and thus the function-

ing of the electron transport system, then the fact that ATP stimulates this hydrolysis may provide a mechanism for the regulation of ATP production.

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ABSTRACT

Cell-free extracts of *Escherichia coli* were found to catalyze the enzymatic hydrolysis of cardiolipin (CL), the products being phosphatidylglycerol and phosphatidic acid. This phosphatidohydrolase activity differs from the plant phospholipase D in its strict specificity for CL and has been designated cardiolipin-specific phospholipase D.

This enzyme exhibits a strict requirement for Mg^{++} and is active within a limited pH range, the optimum being 7.0. The activity, which is located in the high-speed supernatant fraction of broken cells, is inhibited by detergents and EDTA.

The cardiolipin-specific phospholipase D exists in an active and an inactive form. ATP is required to activate the enzyme. However it is not known whether the effect of ATP is allosteric or if the ATP is used as a substrate for an enzyme which activates the phospholipase D.

The form of the cardiolipin-specific phospholipase D and the activity of the enzyme vary with cell age and the availability of an energy source in the cell medium. It is suggested that the relative increase in CL levels observed both in the stationary growth stage and under starved conditions may be due to a decrease in CL catabolism.

In *E. coli* cell-free extracts, CL can also be broken down via a [illegible] phosphatidylation with glycerol. This enzyme activity is specific for glycerol as the acceptor and is probably associated with the CL-specific phospholipase D activity.

The cardiolipin-specific phospholipase D was detected in all the Gram-negative bacteria tested and exhibited similar characteristics. Its activity was not observed in Gram-positive bacteria, yeast or rat liver mitochondria.

The cardiolipin-specific phospholipase D activity coupled with cardiolipin synthetase, in *E. coli* at least, has been tentatively designated as the polyglycerophosphatide cycle. Since the phospholipase D exhibits an ATP-requirement, this cycle would be under energy control and would explain the responsiveness of PG and CL levels to the energy supply in the cell. The hypothesis is put forward that the ATP-stimulated hydrolysis of CL could provide a mechanism for the regulation of ATP production.