Isolation and characterization of cardiolipin synthetase from *Bacillus stearothermophilus*

Peter E. Coderre

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THE ISOLATION AND CHARACTERIZATION OF CARDIOLIPIN SYNTHETASE FROM BACILLUS STEAROTHERMOPHILUS

by

Peter E. Coderre

B.S., University of Vermont, 1979

Presented in partial fulfillment of the requirements for the degree of

Master of Science

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Approved by

Chairman, Board of Examiners

Dean, Graduate School

Date
Cardiolipin synthetase activity of Bacillus stearothermophilus has been characterized in two membrane preparations: 1.) a washed membrane preparation in which about 30% of the total membrane protein was removed and 2.) a Triton X-100-extracted membrane preparation in which 66% of the total membrane protein had been removed. Enzymatic activity was recovered in the particulate fractions of all fractionation steps; no enzymatic activity was recovered in any of the supernatant fluids after centrifugation at 150,000 x g for 60 minutes. Cardiolipin synthetase activity was assayed by the conversion of endogenous [32P] labeled phosphatidylglycerol to [32P] labeled cardiolipin in washed membranes prepared from cells grown in a tryptase yeast extract medium containing H3[32P]O4. Cardiolipin synthetase activity of the washed membrane preparations showed an optimal pH and temperature of 5.5 and 55°C respectively. No stimulation by divalent cations or inhibition by EDTA was observed. Enzymatic activity was reduced by 80% at concentrations of Triton X-100 equal to or above 0.15% in the enzyme assay mixture. A rapid assay procedure was developed in which cardiolipin synthetase activity was monitored by the release of [2-3H] glycerol. By using a mixture of purified phosphatidylglycerol labeled with [32P] and [2-3H] glycerol, it was shown that one molecule of glycerol was released for every molecule of cardiolipin formed. Attempts to solubilize cardiolipin synthetase with detergents such as octyl glucoside, sodium cholate, zwittergent 3-14, and Triton X-100 were unsuccessful. However, sequential extractions of washed membranes with 0.05% and 0.10% Triton X-100 yielded an enzyme preparation that contained 34% of the original total membrane protein and showed a 1.22 fold increase in specific activity. The characteristics of the Triton X-100-extracted membrane preparation were different than the washed membrane preparation. Enzymatic activity from the detergent extracted membranes had an optimal pH of 6.0, a temperature optimum of 60°C, and was stimulated by divalent cations. This enzyme preparation had a Km = 6.67 x 10^-4 M. The enzyme was inhibited by the addition of cardiolipin and stimulated in the presence of total phospholipid from Bacillus stearothermophilus. Inability to solubilize the enzyme with any of the detergents previously mentioned and enzyme stimulation in the presence of total phospholipid suggested a requirement for specific phospholipids for enzymatic activity. Disruption of the protein-lipid interactions may account for the inability to purify cardiolipin synthetase to date.
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<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>14</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>21</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>45</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>58</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>61</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cardiolipin synthetase activity in whole cells and membrane fractions</td>
<td>22</td>
</tr>
<tr>
<td>2.</td>
<td>Effects of divalent cations on cardiolipin synthetase activity of washed membranes</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>Treatment of washed membranes with detergents</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Preparation of Triton X-100-extracted membranes</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of cation and nucleotides on cardiolipin synthetase activity of Triton X-100-extracted membranes</td>
<td>40</td>
</tr>
<tr>
<td>6.</td>
<td>Effects of phospholipids on cardiolipin synthetase activity from Triton X-100-extracted membranes</td>
<td>41</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Enzymatic synthesis of phospholipids in <em>Escherichia coli</em></td>
<td>13</td>
</tr>
<tr>
<td>2.</td>
<td>The effect of pH on cardiolipin synthetase activity in washed membranes</td>
<td>23</td>
</tr>
<tr>
<td>3.</td>
<td>The effect of temperature on the conversion of endogenous PG to CL in washed membranes</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>The effect of Triton X-100 on cardiolipin synthetase in washed membranes</td>
<td>27</td>
</tr>
<tr>
<td>5.</td>
<td>Comparison of $[2-^3H^-]$glycerol release and $[^{32}P^-]$PG conversion by washed membranes</td>
<td>29</td>
</tr>
<tr>
<td>6.</td>
<td>Comparison of nmoles $[2-^3H^-]$glycerol release and nmoles $[^{32}P^-]$CL formation by washed membranes</td>
<td>30</td>
</tr>
<tr>
<td>7.</td>
<td>Effects of substrate concentration on $[2-^3H^-]$glycerol release in Triton X-100-extracted membranes</td>
<td>35</td>
</tr>
<tr>
<td>8.</td>
<td>Time course for $[2-^3H^-]$glycerol release in Triton X-100-extracted membranes</td>
<td>36</td>
</tr>
<tr>
<td>10.</td>
<td>Optimal pH for $[2-^3H^-]$glycerol release by Triton X-100-extracted membranes</td>
<td>38</td>
</tr>
<tr>
<td>11.</td>
<td>Kinetics of cardiolipin synthetase of Triton X-100-extracted membranes</td>
<td>44</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine 5' diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5' triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BBL</td>
<td>Baltimore Biological Laboratories</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytosine 5' triphosphate</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamintetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5' triphosphate</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>l</td>
<td>Liter</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>min.</td>
<td>Minutes</td>
</tr>
<tr>
<td>n mole</td>
<td>Nanomole</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Inorganic phosphorus</td>
</tr>
</tbody>
</table>

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rpm ........................................ Revolutions per minute
S ........................................ Substrate concentration
Tris ....................................... Tris (hydroxymethyl) aminomethane
TYE ..................................... Trypticase yeast extract
μCi ....................................... Microcuries
μg ......................................... Micrograms
μl ......................................... Microliters
UTP ....................................... Uridine 5' triphosphate
v ........................................... Velocity
Chapter I.

Introduction

One of the most significant contributions to molecular biology in the last 15 years has been the Fluid Mosaic model of membrane structure proposed by Singer and Nicolson in 1972. The model proposes that all membranes are a dynamic, yet thermodynamically stable fluid bilayer composed of lipids and proteins (93). According to the Trauble and Overath version (98), 80% of this lipid constitutes the bilayer while the remaining 20% is closely associated with integral membrane proteins. Membrane protein can be divided into two categories: peripheral and integral.

Peripheral (also called extrinsic) proteins are easily dissociated from the membrane by treatments with chelating agents or by increasing the ionic strength of the immediate environment. These proteins are usually devoid of lipid and are soluble in aqueous buffers. The various characteristics of peripheral proteins infer that they are attached to the membrane by weak noncovalent bonds (perhaps electrostatic interactions). More often than not, they are attached to the membrane via association with integral membrane proteins (93).

Integral (also called intrinsic) proteins require harsh treatment to be dissociated from the membrane. This is achieved by the use of detergents or surfactants, protein denaturants, or organic solvents. Lipid is often tenaciously bound to the protein; this lipid contributes to the protein's stability. This is evident by the insolubility of these proteins in aqueous buffers upon removal of the lipid (78). Integral proteins comprise 70% of the total membrane protein of *Escherichia coli* and are assumed to be important for maintenance of the structural inte-
integrity of the membrane (93).

The other major component of membranes is the lipid matrix. In bacteria, this matrix consists of several different types of phospholipids. Each individual phospholipid molecule is amphipathic in nature, consisting of two distinct parts, the polar head group and the hydrophobic fatty acid chains. Individual phospholipid molecules can differ from one another both in the structures of their fatty acids and in the structures of their polar head groups.

The importance of the fatty acid composition (the hydrophobic portion of the phospholipid molecule) in determining the physical properties of the membrane is well established (see ref. 22 for a review). The relative concentrations of straight chain saturated and unsaturated fatty acids and branched chain fatty acids change in response to changes in the growth temperature. The changes in fatty acid composition are correlated with changes in the phase transition temperature of the membrane. It therefore appears that the major function of the non-polar (i.e. fatty acid) component of the phospholipid molecule is to regulate membrane fluidity.

At present, it is unclear why membranes contain several different polar head groups in phospholipids. As pointed out by Cronan (22), if the primary function of the phospholipid component is to form a bilayer with appropriate physical properties, it is not clear why several different types of phospholipid (i.e. in terms of the polar groups) would be formed and why these are maintained at relatively constant concentrations under steady-state growth conditions. Likewise, the structural role offers no explanation for the turnover of a portion of the phospholipid pool.
Through the study of phospholipid synthesis, insight may be gained towards the understanding of the function and assembly of the polar head groups. Most of the work done on phospholipid synthesis has been with *Escherichia coli*. With minor modifications, the scheme constructed by Kennedy can be applied to most bacteria including *Bacillus stearothermophilus* (see figure 1).

The precursors for phospholipid synthesis include the fatty acids, sn-glycero-3-phosphate, L-serine, and cytidine-5'-triphosphate (CTP). Water soluble enzymes are involved in the synthesis of fatty acids (9,10). A double bond is formed at the C₁₀ position of the fatty acid by a particular 6-oxo-hydroxydecanol acyl carrier protein hydrase (9,23,10). This enzyme is positioned at a metabolic point of divergence in which either palmitic acid (16:0) or palmitoleic acid and cis-vaccenic acid (18:1) are formed. Another major precursor is sn-glycero-3-phosphate which is formed from dihydroxyacetone phosphate. This reaction is catalyzed by a specific dehydrogenase which uses the reduced form of either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) (59).

All the enzymes of phospholipid synthesis occur on the inner membrane (8,107) with the exception of phosphatidylserine synthetase which is associated with the ribosomes (29,7). In the initial step, sn-glycero-3-phosphate is acylated in the first position with a saturated fatty acid by an acyl transferase which preferentially utilizes palmitoyl coenzyme A, but if presented with sufficient substrate, it can utilize unsaturated fatty acids (67,66,101). Acylation of the second position occurs by a different acyl transferase (7) which preferentially utilizes unsaturated fatty acyl coenzyme A (67,68) to form phosphatidic acid.
acid. The sequential acylation of sn-glycero-3-phosphate is believed to provide a logical mechanism for regulation of fatty acid composition (92).

The first acyl transferase has been partially purified using an extraction procedure with Triton X-100. The enzyme is not active in the presence of non-ionic detergents such as Triton X-100 (18,47). But, activity can be recovered if the detergent is diluted with Escherichia coli phospholipids (94). Sucrose gradient analysis indicated that a large amount of lipid was bound to this enzyme (94). The second acyltransferase has not been purified to date. Once both of these enzymes are available in a homogenous form, their properties may provide valuable insight into the metabolic control of phospholipid synthesis since these enzymes are located at a metabolic branch point.

The product of these sequential acylations, phosphatidic acid, reacts with CTP or deoxyCTP to form CDP-diglyceride, a liponucleotide derivative (17). The enzyme responsible is CDP-diglyceride synthetase, which is present in all bacteria (8,17,63,70,107). It is located in the membrane (8,107). It has been extracted with digitonin and can be further purified with a diethylaminoethly-cellulose column in the presence of digitonin(73). The enzyme from Escherichia coli does not require lipids for optimal activity. In contrast to the other enzymes of phospholipid metabolism, CDP-diglyceride synthetase requires phosphatidic acid esterified with unsaturated fatty acids and the enzyme does not utilize dipalmitoyl phosphatidic acid (8). Divalent cations and potassium ions are required for maximal activity (8). Both phosphatidic acid and liponucleotides are metabolized rapidly with the former present in a 10-fold excess over the latter (76).
At this point, CDP-diglyceride can be converted to one of two different phosphodiester products. The phosphatidyl moiety can be donated to either the hydroxyl group at position 1 of sn-glycero-3-phosphate (to form phosphatidylglycerophosphate) (18,52) or to L-serine to form phosphatidylserine (49). Regulation of the phosphatidylserine to phosphatidylglycerophosphate ratio is believed to occur at this branch site.

The first alternative of CDP-diglyceride metabolism involves the formation of phosphatidylserine from CDP-diglyceride and L-serine by phosphatidylserine synthetase (49). This enzyme is the only enzyme of the phospholipid pathway not located within the membrane. It is found tightly bound to ribosomes with 10-30% located in the cytosol (75). It is found to be associated with both subunits (74) and can be extracted by aqueous polymer partitioning in the presence of 5 M NaCl with subsequent absorption (54,76), and substrate specific elution with CDP-diglyceride from phosphocellulose (54). Low concentrations of Triton X-100 enhance the rate of reaction, but concentrations greater than 0.2% are inhibitory (49,55,76). Interaction of mixed micelles of Triton X-100 and CDP-diglyceride with the enzyme has been proposed (55). It is unknown whether this represents an effect on the enzyme structure or an interaction with the enzyme. Since a biological substitute has yet to be found for Triton X-100, this is a difficult hypothesis to support.

Phosphatidylserine is present in low concentrations in Escherichia coli and it is rapidly decarboxylated to a more stable phospholipid, phosphatidylethanolamine (49,74). The enzyme responsible for this, phosphatidylserine decarboxylase, has been extracted from membranes with Triton X-100 and purified to homogeneity by a combination of ion-exchange
chromatography, gel filtration, and density gradient centrifugation (28). To prevent aggregation of the enzyme, Triton X-100 must be included in all steps of the purification procedure (28). The enzyme is inhibited by hydroxylamine indicating an essential ketone or aldehyde group at the active site (28,71,87); specifically, pyruvate was found to be a bound cofactor (87), suggesting post-translational modification of the enzyme. The presence of pyruvate as a cofactor is not unusual and has been found to be a cofactor for a variety of decarboxylases (79). The enzyme does not require divalent cation and requires Triton X-100 in the homogeneous state (28). The enzyme does not fuse with phosphatidylserine liposomes or intact membranes of erythrocytes unless detergent is present (105). Alteration of buoyant density in sucrose density gradients of enzyme with bound Triton X-100 compared to soluble proteins of similar size suggests that Triton X-100 has a direct effect on the enzyme rather than affecting the substrate. The enzyme is inhibited by high concentrations of Triton X-100 (28) possibly due to dilution of the substrate in mixed micelles (105,106).

The second alternative of CDP-diglyceride metabolism is the formation of phosphatidylglycerophosphate by phosphatidylglycerophosphate synthetase. This enzyme attaches CDP-diglyceride or dCDP-diglyceride to the hydroxyl group at position 1 of sn-glycero-3-phosphate (18,40,49,74). Similar to the synthesis of phosphatidylserine, the enzymatic reaction releases CMP (18,41). To date, all procaryotes and eucaryotes examined have been found to possess phosphatidylglycerophosphate synthetase (18,19,31,50,54,70). The enzyme has been extracted from Escherichia coli membranes and purified to homogeneity (18,41). It appeared that the enzyme bound a large amount of detergent (41) and is stimulated by
Phosphatidylglycerophosphate is a short lived intermediate in the phospholipid biosynthetic pathway. It is rapidly dephosphorylated by phosphatidylglycerophosphate phosphatase to yield phosphatidylglycerol (PG) (19). Very little is known about this enzyme, probably due to the rapid turnover of the substrate. Like most phospholipid biosynthetic enzymes, it requires Mg$^{++}$ and Triton X-100 for activity (19). However, the phosphatase has not been localized within the membrane nor has it been purified to homogeneity (19).

Recently, Nishijima et al. (63) have discovered another possible route for phosphatidylglycerophosphate. They constructed two mutants of which the first (pgs A1B1) had a partially inactivated phosphatidylglycerophosphate synthetase. It synthesized two-thirds of the normal level of phosphatidylglycerol but was not temperature sensitive. The second lesion, pgs Gl, caused temperature sensitive growth and normal phosphatidylglycerol synthesis in strains with the pgs A1B1 lesion. These two lesions were found to be separate yet they interacted with one another. Together these lesions caused cessation of growth, decreased phosphatidylglycerol synthesis, and accumulation of a 'lipid Y' at non-permissive temperatures. The pgs B1 mutant by itself induced an accumulation of a 'lipid X', which consisted of a diphosphorylated, glucosamine-containing disaccharide. The structure was derivatized with two amide-linked and two ester-linked 3-deoxyoctulosonic acids (KDO) (65). It is believed that 'lipid X' is a precursor of the lipid A of lipopolysaccharide (LPS). 'Lipid Y' has been shown to be structurally similar to 'lipid X' except that 'lipid Y' has one mole extra of palmitic acid. It is suggested (65) that 'lipid Y' may be synthesized from...
'lipid X' via an acylation reaction, which is stimulated by a decreased synthesis of phosphatidylglycerol.

The subsequent metabolism of phosphatidylglycerol (PG) remains the most evasive yet most intriguing event in the phospholipid biosynthetic pathway. Some of the confusion is well illustrated by the early work of Stanacev et al. (95) who suggested that both phosphatidylglycerol and CDP-diglyceride were needed to form cardiolipin.

\[ \text{(1) phosphatidylglycerol} + \text{CDP-diglyceride} \]
\[ \rightarrow \text{cardiolipin} + \text{CMP} \]

Evidence for this reaction came from studies which showed that radioactive phosphatidylglycerol was converted to cardiolipin and that CDP-diglyceride stimulated the reaction three-fold (42,95). However, further studies were quite contradictory to the above mentioned scheme (27,42,77,95). Rampini et al. (77) showed that when CDP-diglyceride formation was blocked by inhibition of energy generation, the synthesis of cardiolipin was not terminated. They observed that as levels of cardiolipin dropped, levels of phosphatidylglycerol increased. Thus, the following scheme was proposed:

\[ \text{(2) 2 phosphatidylglycerol} \rightarrow \text{cardiolipin} + \text{glycerol} \]

This scheme is supported by the work of Lusk and Kennedy (61) who demonstrated with radiolabeling studies that labeled free glycerol was released upon formation of cardiolipin.

While the major phospholipid of *Escherichia coli*, phosphatidylethanolamine (PE), remains in relatively stable concentrations (3,4,11,15,48,56,90), phosphatidylglycerol is actively metabolized to cardiolipin by cardiolipin synthetase in a variety of organisms (13,15,26,31,37,42). The function of cardiolipin has yet to be determined. Several lines of
circumstantial evidence suggest a role in oxidative energy metabolism. In eucaryotes, the synthesis of cardiolipin occurs in the matrix of mitochondria. Awasthi et al. (2) found that cardiolipin is found primarily on the inner membrane. It is tenaciously bound by cytochrome oxidase which requires cardiolipin for catalytic activity (34,80). In *Bacillus stearothermophilus*, cardiolipin is the predominant phospholipid in a lipid-depleted membrane fragment which contains the terminal electron transport system and ATPase complex (16). Santiago et al. (83) discovered a correlation between cardiolipin degradation and loss of ATPase activity. Work with various phospholipases have yielded a wealth of information. Phospholipase C from *Clostridium welchii* hydrolyzes phosphatidylcholine and phosphatidylethanolamine but not cardiolipin. When this is used on mitochondria, phosphorylative activity is retained (12). Treatment with phospholipase C from *Bacillus cereus* (which hydrolyzes phosphatidylcholine, phosphatidylethanolamine, and cardiolipin) results in a significant reduction in oxidative phosphorylation (12). Card et al. (16) and others (35) have shown that cardiolipin turnover ceases and cardiolipin accumulates in the membrane upon inhibition of oxidative energy metabolism by inhibitors or oxygen deprivation. These results suggest that cardiolipin and/or cardiolipin turnover require energy or are linked to an energy requiring system.

Another possible product of phosphatidylglycerol and/or cardiolipin metabolism was recognized by Van Golde et al. in 1973 (102). Although Kanfer and Kennedy (48) first recognized the turnover of phosphatidylglycerol and cardiolipin, it was not until ten years later that a possible alternate product was discovered. Van Golde et al. (102) found a unique class of water soluble oligosaccharides (MDO). MDO consists of glycero-
phosphate moieties linked as phosphodiesters to the 6 position of glucose (51, 102). The sugar groups consist of 8-10 molecules of glucose per molecule which may be derived from UDP-glucose as is evident by a requirement of UDP-glucose for MDO biosynthesis (51, 89, 102). The sn-glycero-1-phosphate moiety is derived from the polar head groups of cardiolipin and/or phosphatidylglycerol, more likely from the latter (102). The average molecular weight of MDO is about 2,000 (95). This low molecular weight compound is found in the periplasmic space and accounts for approximately 1% of the dry weight of the cell (51, 89, 90, 102).

MDO synthesis accounts for about 75% of the polyphosphatide turnover. If phospholipases and lysophospholipases are responsible for some lipid metabolism, it represents a minimum of the lipid turnover. Several different lines of evidence do indeed suggest that MDO is derived from phosphatidylglycerol and/or cardiolipin and account for the turnover of these polyphosphatides. Pulse-chase experiments with \( \text{sn-}[2-{^{3}}\text{H}]\)-glycero-3-[\(^{32}\text{P}\)]-phosphate demonstrate that radioactivity in the MDO increased while that of phosphatidylglycerol and cardiolipin decrease. Simultaneously, the ratio of \( ^{3}\text{H} \) to \( ^{32}\text{P} \) remains constant (102). It would be feasible for the MDO to accept the glycerophosphate head group of phosphatidylglycerol or cardiolipin (51), resulting in the production of a molecule of \( \text{sn-1}, 2\)-diglyceride (73). MDO synthesis can be blocked in a mutant in which cell growth is not inhibited (89, 90). The function of MDO is unknown. It may be that this molecule is involved in a transport system, but this is mere conjecture (73). Since it is not required for growth and it is localized in the periplasmic space, it is unlikely that it is involved in the regulation of fatty acid synthesis (9).
Gram positive organisms do not contain MDO, but do contain a similar structure to MDO designated as lipoteichoic acid (LTA)—a membrane bound, water soluble glycerophosphate polymer (53). There are two types of teichoic acids found in Gram positive bacteria: a cell wall teichoic acid and a membrane-associated lipoteichoic acid. There are many different types of cell wall teichoic acids but most Gram positive bacteria contain a single LTA composed of polyglycerophosphate moieties (53). LTA's almost always possess 1,3-phosphodiester linked chains of 25-30 glycerophosphate moieties. These are variously substituted with glycosyl or D-alanyl ester groups and terminal fatty acids. A number of studies have been performed on LTA's biological activities (53) and antigenic properties (108) as well as their possible location in mesosomes (13, 14) yet their function has yet to be established.

It has been suggested that lipoteichoic acid plays some role in the cell division cycle by inhibiting cell autolysins or that it binds and sequesters divalent cations (53). It is rather intriguing that cardiolipin also binds divalent cations and is a more potent inhibitor of Streptococcus faecalis autolysins than LTA (20).

Pulse chase experiments with $[^2\text{H}]$-glycerol have provided the strongest evidence for a link between cardiolipin/phosphatidylglycerol turnover and lipoteichoic acid synthesis. Glaser and Lindsey (36) with Staphylococcus aureus and Emdur and Chiu (30) with Streptococcus sanguis showed that during a 45 minute chase period, 60% of the $[^2\text{H}]$-glycerol lost from phosphatidylglycerol was accountable in the radioactivity of the LTA. Card et al. (16) found that 75% of the $[^3\text{H}]$-glycerol was found in the phenol-water extract by using the hot 40%-phenol water procedure (108).
It appears that phosphatidylglycerol/cardiolipin turnover is linked to the synthesis of LTA. Both molecules are hypothesized to have similar functions yet in different parts of the cell. If phosphatidylglycerol/cardiolipin turnover does indeed correlate with LTA synthesis, characterization of the involved enzymes and optimal conditions for synthesis of each molecule may provide insight into the functions of these molecules.

Statement of thesis

The goal of this work is the characterization of cardiolipin synthetase from *Bacillus stearothermophilus*. The study consisted of the following aspects:


2. Cell fractionation and analysis of cardiolipin synthetase activity in different cell fractions.

3. Analysis of cardiolipin synthetase activity in membranes which were stripped of peripheral proteins.

4. An attempt to solubilize and subsequently purify cardiolipin synthetase.
Figure 1. Enzymatic synthesis of phospholipids in *Escherichia coli* (73).
Chapter II.

Materials and Methods

Reagents

Bovine serum albumin, lysozyme, dithiothreitol, octylglucoside, cholic acid, and Triton X-100 were purchased from Sigma; DNAase was purchased from Worthington Biochemical.

Organism

The organism used in these studies was Bacillus stearothermophilus (NCA 2184) which was kindly supplied by Dr. George L. Card, Department of Microbiology, University of Montana, Missoula, Montana.

Growth of Bacillus stearothermophilus

Unless otherwise noted, B. stearothermophilus was grown in medium containing 2% Trypticase peptone (BBL) and 1% yeast extract (Difco) (TYE medium). Cultures were grown in a New Brunswick fermentor (model MF 114) with vigorous aeration (air flow of 6 liters/min). Smaller cultures were grown in two liter baffled flasks (Bellco) with volumes not in excess of 500 ml/flask on a New Brunswick gyratory shaker (model 625). The temperature was maintained at 60°C ± 2°C unless specified elsewhere. Cultures were inoculated to an O.D. 600 of 0.025 and harvested at an O.D. 600 of 0.400 (Coleman Jr. II). For fermentor cultures, cells were harvested on crushed ice and collected by centrifugation (Sharples model T-1). The cells were then washed with ice cold 50 mM Tris-HCl (pH 7.8) containing 0.15 M NaCl and 10 mM MgCl₂ (wash buffer). For smaller cultures, cells were harvested in 250 ml plastic centrifuge
bottles containing crushed ice. Cells were collected by centrifugation on an International centrifuge (model V) at three-fourths speed for 20 min. Cells were then washed in the wash buffer.

**Lipid Extraction:**

A modified Bligh and Dyer (82) procedure, adapted for different sizes of samples, was used for all lipid extractions. For the preparations of carrier lipid and unlabeled PG, cells (1 to 5 g wet weight) were suspended in 50 ml methanol and 10 ml of 0.3% NaCl and refluxed over boiling water for 5 min. After cooling, 25 ml of chloroform and 10 ml of 0.3% NaCl were added. The suspension was stirred at room temperature for 60 min, then centrifuged and the supernatant fluid transferred to a separatory funnel. The pellet was re-extracted two additional times as described above. The supernatant fluids were combined and sufficient chloroform and 0.3% NaCl were added to give a final chloroform-methanol-0.3% NaCl ratio of 2:2:1.8. After vigorous shaking and separation of the two phases, the lower (chloroform) phase was transferred to a round bottom flask and evaporated to dryness. The lipid was resuspended in a small volume of chloroform-methanol (2:1), analyzed for phosphorus (1), and the relative phospholipid composition determined by chromatography as described below. Lipid preparations were stored at -15°C.

Small samples (less than one mg protein) were extracted in 12 ml conical centrifuge tubes. Chloroform, methanol, and 0.3% NaCl were mixed to give the first phase Bligh and Dyer ratios (1:2:0.8). After carrier lipid was added, the mixture was incubated at 60°C for 15 min.
then at 4°C for 60 min. Chloroform and 0.3% NaCl were then added to form the two phase system of Bligh and Dyer proportions (2:2:1.8). After centrifugation in an International clinical centrifuge (model CL) at full speed for 10 minutes, the bottom chloroform layer was removed. The upper water:methanol phase was subsequently washed with two 1.5 ml volumes of chloroform. Chloroform extracts were pooled and dried on a Buchler Evapomix (model 2281) at 30-35°C. Solvent was dried under a stream of nitrogen. The sides of the tubes were rinsed with benzene and then dried under a stream of nitrogen. Each lipid sample was dissolved in 100 µl chloroform:methanol (2:1). Aliquots were analyzed for total radioactivity by scintillation spectrometry and for lipid composition by chromatographic techniques.

**Lipid Analysis**

Lipids were separated by chromatography on silica gel loaded papers (Whatman SG-81) or thin layer plates (Whatman LK-6). Papers were developed in one dimension with chloroform:methanol:diisobutylketone:acetic acid:water (23:10:45:25:4) (solvent A) for approximately two hours (16). Thin layer plates were developed in chloroform: methanol:acetic acid (65:25:8) (solvent B) for approximately 100 minutes (82). Chromatograms were visualized either with a molybdate spray (103) for lipid phosphorus or by autoradiography with Kodak X-Omat R Film (X-RI). In the case of autoradiography, X-ray film was developed and aligned with the chromatogram. Radioactive phospholipid spots were cut from papers or scraped from thin layer
plates with a single edged razor blade, placed in individual scintillation vials, and mixed with Beckman Ready Solv EP liquid scintillation cocktail. Samples were counted on a Nuclear-Chicago Unilux III liquid scintillation counter.

**Preparation of labeled phosphatidylglycerol**

Phosphatidylglycerol was labeled with either $\text{H}^{32}\text{PO}_4$ or $\left[2^{-3}\text{H}\right]$-glycerol. For $\left[32\text{P}\right]$ labeling, cells were grown for at least four cell doublings in a defined medium containing 200 μCi of $\text{H}^{32}\text{PO}_4$. Cells were labeled with $\left[2^{-3}\text{H}\right]$-glycerol by pulse labeling for 4 minutes with $\left[2^{-3}\text{H}\right]$-glycerol at a cell density of about $2 \times 10^9$ cells/ml (OD 0.4 at 600 nm). Immediately after the pulse period, growth was stopped by pouring the cell suspension over crushed ice. PG isolated from cells labeled in this way contained between 75% and 80% of the $\left[2^{-3}\text{H}\right]$- in the non-acylated glycerol.

The phospholipids were spotted on silica gel preparative thin layer plates (Whatman PLK 5) and developed with chloroform:methanol:acetic acid (65:25:8). Chromatograms were visualized via autoradiography. Silica gel containing the radio-labeled PG was scraped from the plates with a razor blade. The PG was further purified by lipid extraction and column chromatography. The PG was loaded onto a Bio Rad silicic acid column (Bio-Sil HA minus 325 mesh) equilibrated with chloroform. The column was developed with (a) two volumes of chloroform, (b) two volumes of chloroform:methanol (10:1), and (c) four volumes of chloroform:methanol (2:1). The PG was eluted with the chloroform:
methanol (2:1) volumes which were pooled and taken to dryness. The PG was dissolved in chloroform:methanol (2:1) and stored at -15°C. Phospholipid content was quantified by a phosphorus determination (1).

For the enzyme assays the labeled PG was adjusted to a concentration of 1 nmole/µl and 500 cpm/µl. The dried PG was emulsified in 200 µl of either 0.5% Triton X-100 or 25-50 mM octyl glucoside in distilled water. The preparation was then sonified to clarity (about 15-20 minutes) with a Sonifier cell disrupter microtip (model W-350) at 50% duty cycle at an output control of '1' (about 100 watts).

Enzyme Assay

a. $[^{2-3}\text{H}]$-glycerol release

Release of $[^{2-3}\text{H}]$-glycerol into the water soluble phase was determined as follows. The assay mixture contained 100 µg membrane protein, 10 nmoles total of emulsified $[^{2-3}\text{H}]$-PG (40,000 cpm) and $[^{32}\text{P}]$-PG (8,000 cpm), 400 µg of BSA, 10 mM MgCl$_2$, 100 mM sodium acetate buffer (pH 5.5) in a total volume of 0.2 ml. The reaction was terminated after 10 minutes by addition of 0.2 ml of 20% ice cold trichloroacetic acid. The reaction mixture was centrifuged in a clinical centrifuge at full speed for 10 minutes, and a 200 µl aliquot of the supernatant fluid was analyzed for $[^{3}\text{H}]$.

b. $[^{32}\text{P}]$-cardiolipin formation

The method of Burritt and Henderson (13) was performed with some modifications. The assay mixture contained 100 µg membrane
protein, 20 nmoles \([^{32}\text{P}]\) or \([^{3}\text{H}]\)-PG in 0.5% Triton X-100, and 100 mM sodium acetate buffer (pH 5.5) in a total volume of 0.5 ml. The assay was performed at 60°C for 20 minutes unless noted otherwise. Samples were then placed on ice. The reaction was terminated with the addition of 0.2 ml of 0.3% NaCl and 3.0 ml of chloroform:methanol:1N-HCl (1:2:.03). After carrier lipid was added, the mixture was incubated at 60°C for 15 minutes, then at 4°C for 60 minutes. The phospholipids were extracted, chromatographed, and radioactivity was determined in each phospholipid.

Collection of crude membranes

Membranes were prepared by the method of Card et al. (16). Cells were incubated in 0.2 M NaCl-50 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$ containing 150 µg of lysozyme/ml. After 30 minutes of incubation at 25°C, protoplasts were collected by centrifugation at 30,000 x g for 10 minutes. Protoplasts were subsequently lysed in 50 mM Tris-HCl (pH 7.8). DNAase (10 µg/ml) was added to reduce viscosity and membrane fragments were collected by centrifugation at 30,000 x g for 20 minutes. Membrane fragments were washed once in buffer containing 0.2 M NaCl, 50 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$ then washed 4-5 times in 50 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$. Membranes not used immediately were stored in 5% glycerol, 50 mM Tris-HCl (pH 7.8), 5 mM MgCl$_2$, 1 mM dithiothreitol at -20°C. These membranes are termed washed membranes in the following discussion.

Enzyme Solubilization

Solubilization was performed according to the method of Racker.
et al. (72) for protein reconstitution. Aliquots of washed membranes containing 8 mg protein were emulsified in 4 ml of either Triton X-100, sodium cholate, octyl glucoside, or zwittergent (see Results). The mixtures were incubated for 30-40 minutes at 4°C then centrifuged for one hour at 50,000 rpm. The supernatant fluid and pellet were separated for protein determination (60). The pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.0). Aliquots containing 100 µg protein were assayed for cardiolipin formation or [2-\(^3\)H]-glycerol release.

**Butanol Solubilization**

Butanol solubilization was according to Huesgen and Gerisch (43) with some modification. Five ml of washed membranes (12.8 mg) in 50 mM sodium acetate buffer (pH 5.5) were mixed with 3.75 ml of butanol and allowed to stand at 4°C for one hour. The mixture was centrifuged at 5,000 rpm. This gave two phases and a pellet at the bottom of the water soluble phase. The pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.0). The pellet and the butanol phase were assayed for cardiolipin formation. The buffer soluble material was centrifuged at 150,000 x g for one hour, then the supernatant and pellet were assayed for cardiolipin formation.
Chapter III.

Results

I. Cellular distribution of cardiolipin synthetase activity

Various cell fractions were assayed for cardiolipin synthetase activity. Table 1 shows that the enzyme is tightly bound to the membrane. There was no loss of enzyme activity when protoplasts were formed and subsequently lysed. Washed membrane, which was stripped of between 20 and 30% of the total protein of crude membrane, retained full activity. In fact; it appeared to have slightly higher enzyme activity, possibly due to the loss of inhibitory peripheral protein upon washing. No CL-synthetase activity could be detected in any of the supernatant fractions from the centrifugation steps listed in Table 1.

II. Cardiolipin synthetase activity of washed membrane preparations

1. Optimum pH and temperature

The washed membrane preparations were used to determine the optimum conditions for CL-synthetase activity. The results shown in Figures 1, 2, and 3 were obtained with washed membrane preparations isolated from cells grown for at least four cell doublings in TYE medium containing $\text{H}_3\text{PO}_4$ (2 μCi/ml). The optimum pH for the conversion of endogenous PG to CL was 5.5 with a sharp drop in activity below pH 5.0 (figure 2). No marked differences in activity were noted when phosphate or acetate buffers were used to adjust the pH. The optimum temperature for the conversion of endogenous PG to CL was between 55° and 60°C (Figure 3). The rate of CL synthesis decreased
Table 1. Cardiolipin synthetase activity in whole cells and membrane preparations.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Phospholipid\textsuperscript{b}</th>
<th>Percentage Composition before 20 min. incubation at 60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cells</td>
<td>PG</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>30.4</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>PG</td>
<td>ND\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>ND</td>
</tr>
<tr>
<td>Membranes</td>
<td>PG</td>
<td>50.8</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>30.3</td>
</tr>
<tr>
<td>Washed Membranes</td>
<td>PG</td>
<td>52.3</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>30.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Preparations (1000 µg protein/ml) were suspended in 100mM sodium acetate buffer (pH 5.5) and assayed before and after incubation at 60°C for 20 minutes. Whole cells labeled with $^{32}$P (2 µCi/ml culture) were fractionated as described in the text. Samples of each fraction containing 100 µg of protein were suspended in 100 mM sodium acetate buffer (pH 5.5). Lipids were extracted and assayed before and after incubation at 60°C for 20 minutes as described in Materials and Methods.

\textsuperscript{b} PG = phosphatidylglycerol, PE = phosphatidylethanolamine, CL = cardiolipin

\textsuperscript{c} ND = not done

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Figure 2. The effect of pH on CL-synthetase activity in washed membranes. Total phospholipid content was 350 nmoles/mg protein. Enzyme assay contained 100 μg membrane protein from cells pulsed with $[^{32}P]$, (200 μCi/m mole PO₄) and 100 mM buffer in a total volume of 400 μl. After 20 minutes at 60°C, the reaction was stopped by the addition of the lipid extraction mixture as described in Materials and Methods. The extracted lipid was separated by thin layer chromatography. Sodium acetate buffer was used from pH 4.0 to 5.5 and phosphate buffer from pH 6.0 to 8.0.
Figure 3. The effect of temperature on the conversion of endogenous PG to CL in washed membranes. The assay mixture contained 100 µg of washed membranes in 400 µl of 100 mM Na-acetate buffer (pH 5.5). The initial CL concentration was 12.6% of the total phospholipid. The reaction was stopped and the lipids were extracted and quantified as described in Figure 2.
rapidly with decreases in temperature below 55°C.

2. Effect of divalent cation on cardiolipin synthetase activity

Table 2 shows the effects of Mg$^{++}$, Ca$^{++}$, and EDTA on the conversion of endogenous phosphatidylglycerol to cardiolipin. The lack of substantial decrease in cardiolipin formation in the presence of 1 mM EDTA suggested that divalent cation was not required for enzyme activity. In fact, enzyme activity appeared to be enhanced by the presence of EDTA.

3. The effects of Triton X-100 on cardiolipin synthetase activity

The effect of Triton X-100 on the conversion of exogenous $^{32}$P-labeled PG to $^{32}$P-labeled CL is shown in figure 4. Concentrations greater than 0.1% were inhibitory and enzyme activity decreased by almost 80% at concentrations of Triton X-100 greater than 0.15%. The inhibition was not irreversible, however, because when concentrations of 0.2% Triton X-100 or less were diluted to a final concentration of 0.05% Triton X-100 in the assay mixture, activity was the same as mixtures without Triton X-100.

The effect of Triton X-100 on CL-synthetase activity was important for two reasons. First, this detergent has been successfully used to solubilize a number of integral membrane proteins, including some enzymes involved in phospholipid biosynthesis. Second, the physical state in which the exogenous PG was added to the reaction mixture has a marked effect on the rate of CL synthesis and the total amount of CL formed by the membrane preparations. The proper concentration of Triton X-100 in the exogenous PG preparation was
Table 2. Effect of divalent cations on cardiolipin synthetase activity of washed membranes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phospholipid</th>
<th>Percent of total phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control\textsuperscript{b}</td>
<td>PG</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>30.4</td>
</tr>
<tr>
<td>None</td>
<td>PG</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>47.9</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>26.2</td>
</tr>
<tr>
<td>10 mM MgCl\textsubscript{2}</td>
<td>PG</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>31.5</td>
</tr>
<tr>
<td>10 mM CaCl\textsubscript{2}</td>
<td>PG</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>33.2</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>32.3</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>PG</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>47.8</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>27.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Washed membranes from \textsuperscript{[32P]} labeled cells (2 \textmu Ci/ml culture medium) were suspended in 100 mM sodium acetate buffer (pH 5.5). After 20 minutes at 60\textdegree C, the lipid was extracted and analyzed as described in the Materials and Methods section.

\textsuperscript{b} Composition of the washed membrane preparations prior to the incubation period.
Figure 4. Effects of Triton X-100 on cardiolipin synthetase in washed membranes. The assay mixture: 100 μg washed membrane, 20 nmols of $^{32}$P$_{PO_4}$ (200 μCi $^{32}$P/mole PO$_4$), 100 mM Na-acetate buffer (pH 5.5), and Triton X-100 at the concentrations shown. After 20 minutes of 60°C, the reaction was stopped and the lipid analysed as described in Figure 2.
critical to ensure sufficient dispersion of the PG into micelles capable of fusing with CL-synthetase in the membrane preparations. Consistent results were obtained by using PG preparations of 1 nmole/μl dispersed in 0.5% Triton X-100.

4. Assay of cardiolipin synthetase by the release of \( \left[ 2-^3\text{H} \right] \)-glycerol from labeled PG.

The time and materials required for the assay of CL-synthetase by \( [32\text{P}] \)-CL formation has restricted the progress which might be made in isolating the enzyme. Therefore, a second assay was developed in which the free glycerol produced by the CL-synthetase reaction was determined rather than the CL formed.

PG was labeled by briefly pulsing cells with \( \left[ 2-^3\text{H} \right] \)-glycerol (4μCi/ml of culture) at cell densities above 2 x 10^9 cells/ml. The PG preparation used in the experiment shown in figure 5 contained 500 cpm/nmole PG. After treatment with phospholipase D, 25% of the label was recovered as phosphatidic acid and 75% as free glycerol. Figure 5 shows that the rate of \( \left[ ^3\text{H} \right] \)-glycerol release parallels the quantity of \( [32\text{P}] \)-labeled phosphorus recovered in the CL from the TCA precipitate. Data from this curve were used to calculate the nmoles glycerol released as follows:

\[
\frac{\text{cpm} \left[ 3\text{H} \right]}{\text{nmole PG}} \times .75 = \frac{\text{cpm} \left[ 3\text{H} \right] \text{in nonacylated glycerol}}{\text{nmole PG}}
\]

\[
\text{cpm} \left[ ^3\text{H} \right] \text{released/sample} \times \frac{\text{cpm} \left[ 3\text{H} \right] \text{in nonacylated glycerol}}{\text{nmole PG}} = \text{nmoles } \left[ ^3\text{H} \right] \text{-glycerol released}
\]

Note that in figure 6 the values for the nmoles \( \left[ ^3\text{H} \right] \)-glycerol released were equivalent to the values for nmoles \( [32\text{P}] \)-CL formed. Therefore, one mole of glycerol was released for every nmole CL formed.
Figure 5. Comparison of $[2-^3H]$-glycerol release and $[^{32}P]$-PG converted by washed membrane. The assay mixture contained various concentrations of PG with a specific activity of 470 cpm $[^3H]$/n mole PG and 350 cpm $[^{32}P]$/n mole PG. Seventy-five percent of the $[^3H]$ label was in the non-acylated glycerol of PG. After 10 minutes of 60°C, the reaction was stopped by placing the tubes in an ice bath and adding 200 μl of ice cold 20% trichloroacetic acid (TCA). After 10 minutes on ice, the tubes were centrifuged and the TCA soluble portion carefully removed. The TCA precipitate was then extracted and analyzed as described in Figure 2.

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Figure 6. Comparison of nmoles $\left[2-{ }^{3} \mathrm{H}\right]$ glycerol release and nmoles $\left[{ }^{32} \mathrm{P}\right]$-CL formation by washed membranes. Conditions for the enzyme assay are described in the legend for Figure 5. Calculation of nmoles $\left[3{ }^{3} \mathrm{H}\right]$ glycerol released are described in the text. Calculation of nmoles $\left[{ }^{32} \mathrm{P}\right]$-CL was by dividing the nmoles PO$_4$ (Figure 5) by 2.
III. Detergent solubilization of cardiolipin synthetase.

A variety of detergents were used in attempting to solubilize cardiolipin synthetase from washed membranes (Table 3). In each experiment, washed membranes were treated with detergent and then centrifuged at high speed. If the enzyme remained in the supernatant, it was considered solubilized. Detergent concentrations were usually at or above the critical micelle concentration (CMC) except for 3-14 zwittergent which was used at one-quarter its CMC. This concentration of zwittergent 3-14 was less inhibitory and solubilized as much membrane-bound protein as 0.012% zwittergent 3-14 (its CMC).

No enzymatic activity was recovered in the supernatant fractions. Enzymatic activity always remained in the pellet. The most successful detergent in solubilizing membrane protein was 0.1% Triton X-100. It solubilized 66% of the total membrane protein and provided for an increase in enzyme specific activity in the pellet fraction. Other detergents such as octyl glucoside alone and in combination with sodium cholate removed 79% and 55% of the total membrane protein, but showed a marked decrease in CL-synthetase activity in the particulate fraction.

IV. Partial purification of cardiolipin synthetase by extraction with Triton X-100.

Cardiolipin synthetase was partially purified by extraction of washed membranes with Triton X-100 (Table 4). The enzyme was not solubilized as seen by the lack of enzymatic activity in any of the supernatant fractions. Enzyme activity was recovered only in the pelleted membrane preparations. Washed membranes treated with
Table 3. Treatment of washed membranes with detergents.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of washed membrane protein recovered in pellet fraction</th>
<th>nmol of CL formed/mg protein (pellet fraction)</th>
<th>fraction of washed membrane-specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.00</td>
<td>42.6</td>
<td>1.00</td>
</tr>
<tr>
<td>40 mM octyl glucoside pellet</td>
<td>0.21</td>
<td>30.6</td>
<td>0.72</td>
</tr>
<tr>
<td>14 mM sodium cholate</td>
<td>0.70</td>
<td>30.8</td>
<td>0.72</td>
</tr>
<tr>
<td>0.003% 3-14 zwittergent</td>
<td>0.86</td>
<td>37.9</td>
<td>0.89</td>
</tr>
<tr>
<td>12 mM sodium cholate + 27 mM octyl glucoside</td>
<td>0.45</td>
<td>45.2</td>
<td>1.06</td>
</tr>
<tr>
<td>0.1% Triton X-100 butanol</td>
<td>0.34</td>
<td>68.7</td>
<td>1.61</td>
</tr>
<tr>
<td>1. 50K pellet of buffer soluble protein\textsuperscript{b}</td>
<td>0.02</td>
<td>18.5</td>
<td>0.43</td>
</tr>
<tr>
<td>2. butanol soluble protein</td>
<td>0.52</td>
<td>2.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Washed membrane was suspended at a concentration of 2mg/ml in the reagents listed. After 10 minutes at 60°C, the suspension was centrifuged at 150,000 x g for 60 minutes. No CL-synthetase activity was found in any of the supernatant fluid fractions.

\textsuperscript{b} After solubilization of 12.8 mg washed membranes in 50 mM sodium acetate buffer (pH 5.5) with 3.75 ml of butanol, the mixture was incubated for 1 hour at 4°C and then centrifuged at 5,000 rpm. The buffer soluble material was centrifuged at 150,000 x g for 1 hour then the supernatant and pellet were assayed for enzymatic activity.

\textsuperscript{c} CL-synthetase was assayed for cardiolipin formation as previously described in the Materials and Methods.
Table 4. Preparation of Triton X-100-extracted membranes.a

<table>
<thead>
<tr>
<th>fraction</th>
<th>volume (ml)</th>
<th>protein (mg)</th>
<th>protein yield</th>
<th>total units</th>
<th>specific activity</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>washed membranes</td>
<td>8</td>
<td>27.94</td>
<td>1.00</td>
<td>83.6</td>
<td>2.99</td>
<td>1.00</td>
</tr>
<tr>
<td>0.05% Triton X-100 supernatant</td>
<td>8</td>
<td>10.26</td>
<td>0.37</td>
<td>2.1</td>
<td>0.20</td>
<td>0.03</td>
</tr>
<tr>
<td>0.05% Triton X-100 pellet</td>
<td>8</td>
<td>17.68</td>
<td>0.63</td>
<td>24.3</td>
<td>1.37</td>
<td>0.29</td>
</tr>
<tr>
<td>0.10% Triton X-100 supernatant</td>
<td>8</td>
<td>4.03</td>
<td>0.14</td>
<td>0.5</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>0.10% Triton X-100 pellet</td>
<td>2</td>
<td>9.44</td>
<td>0.34</td>
<td>34.5</td>
<td>3.65</td>
<td>0.41</td>
</tr>
</tbody>
</table>

a. Each fraction was assayed as described in figure 7 except that 10 nmoles of PG were added.

b. Unit of activity is defined as the amount of enzyme which produces 1 nmole of CL per minute at 60°C.
0.05% Triton X-100 had 37% of the total membrane protein removed while retaining 28% of the CL-synthetase activity. When the pellet of this preparation was again extracted with Triton X-100, another 29% of the total membrane protein was removed, while 41% of the original enzymatic activity was recovered. This resulted in an increase of specific activity from 2.99 units/mg protein for washed membranes to 3.65 units/mg protein for the 0.1% Triton X-100 extracted pellet. This preparation was used for further characterization of cardiolipin synthetase.

1. Effect of substrate concentration.

In assay mixtures containing 100 μg protein, the release of [2-3H]-glycerol was linear up to 50 nmoles of PG added (Figure 7). The enzyme appeared to be saturated at concentrations of PG above 50 nmoles. In contrast, the washed membrane preparations showed a linear relationship up to 100 nmoles PG added (Figure 5). The release of [2-3H]-glycerol during a 20 minute incubation period is shown in Figure 8. The reaction was linear for 10 minutes. About 70% of the total glycerol released in the 20 minute period was released in the first 10 minutes.

2. Optimum pH and temperature for the Triton extracted membrane.

The rate of glycerol release was monitored at temperatures between 35° and 80°C (Figure 9). The optimal temperature for [3H]-glycerol formation was 60°C. Significant decreases in glycerol formation occurred below 45°C and above 70°C. Figure 10 shows the effects of pH on [3H]-glycerol formation. The optimal pH for [3H]-glycerol formation was at pH 6.0 with significant decreases below pH 4.5 and above pH 7.0. It should be noted that both the shape of the curves...
Figure 7. Effects of substrate concentration on [2-^3H-]glycerol release in Triton X-100-extracted membranes. The assay mixture contained 100 µg of Triton X-100-extracted membranes, various amounts of PG (40,000 cpm [^3H] and 8,000 cpm[^32P] / nmole PG), 100 mM Na-acetate buffer (pH 5.5) in a total volume of 200 µl. The reaction was stopped after 10 minutes with the addition of 20% TCA. The release of [2-^3H-]glycerol into the TCA soluble phase was determined as described in Materials and Methods.
Figure 8. Time course for \([2-^{3}H\text{-}]\)glycerol release in Triton X-100-extracted membranes. CL-synthetase activity was assayed as described in Figure 7.
Figure 9. The effect of temperature on $[^2\text{H}]$glycerol release in Triton X-100-extracted membranes. CL-synthetase activity was assayed as described in Figure 7.
Figure 10. Optimum pH for \([2-\text{H}^-]\) glycerol release by Triton X-100-extracted membranes. CL-synthetase activity was assayed as described in Figure 7 except that the concentration of buffer was 25 mM. Sodium acetate buffer was used from pH 4.0 to 5.5 and phosphate buffer from pH 6.0 to 8.0. The reaction was stopped after 10 minutes at each pH and glycerol released determined as described in Figure 7.
and optimum points for the Triton extracted preparations were different than those obtained with the washed membrane preparation (Figures 2 and 3).

3. Effect of divalent cations and nucleotides on the Triton extracted membrane.

Table 5 shows the effects of a variety of divalent cations and nucleotide triphosphates (NTP's) on CL-synthetase activity in Triton extracted membranes. Divalent cations had a stimulating effect on enzyme activity (50%), whereas EDTA inhibited enzymatic activity by about 23%. All of the NTP's had inhibitory effects on the enzyme, particularly ATP. The addition of PO$_4$ or ADP had little effect on $^3$H-glycerol release. Again it should be noted that the washed membrane preparations (Table 2) showed a different response to divalent cations and EDTA.


Table 6 shows the effect of various phospholipids on $^3$H-glycerol release. The addition of phosphatidylethanolamine at lower concentrations (50 μM) had a somewhat inhibitory effect, but higher concentrations of phosphatidylethanolamine seemed to have little effect on enzyme activity. Substantial decreases in $^3$H-glycerol release were seen with the addition of cardiolipin. In the presence of 500 μM cardiolipin, the amount of $^3$H-glycerol released was 45% of that observed for the control preparation. The addition of total phospholipid appeared to slightly increase enzyme activity. The total phospholipid extract consisted of 52.3% PG, 14.2% CL, and 30.3% PE.
Table 5. Effect of cations and nucleotides on cardiolipin synthetase activity of Triton X-100-extracted membrane.a

<table>
<thead>
<tr>
<th>Addition</th>
<th>cpm [3H] glycerol released (x 10^3)</th>
<th>Fraction of control (no addition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.28</td>
<td>1.00</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>6.07</td>
<td>1.42</td>
</tr>
<tr>
<td>10 mM MnCl₂</td>
<td>6.15</td>
<td>1.44</td>
</tr>
<tr>
<td>10 mM CaCl₂</td>
<td>6.63</td>
<td>1.55</td>
</tr>
<tr>
<td>10 mM EDTA-Na₂</td>
<td>3.29</td>
<td>0.77</td>
</tr>
<tr>
<td>10 mM P₁</td>
<td>4.32</td>
<td>1.01</td>
</tr>
<tr>
<td>10 mM ADP</td>
<td>4.35</td>
<td>1.02</td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>2.74</td>
<td>0.64</td>
</tr>
<tr>
<td>10 mM CTP</td>
<td>3.79</td>
<td>0.89</td>
</tr>
<tr>
<td>10 mM GTP</td>
<td>3.86</td>
<td>0.90</td>
</tr>
<tr>
<td>10 mM UTP</td>
<td>3.81</td>
<td>0.89</td>
</tr>
</tbody>
</table>

a. CL-synthetase activity was assayed as described in figure 7.
Table 6. Effects of phospholipids on cardiolipin synthetase activity from Triton X-100-extracted membranes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Addition\textsuperscript{b}</th>
<th>cpm $[^{3}H]$-glycerol released ($\times 10^3$)</th>
<th>Fraction of control (no added phospholipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.06</td>
<td>1.00</td>
</tr>
<tr>
<td>50 $\mu$M PE</td>
<td>3.25</td>
<td>0.80</td>
</tr>
<tr>
<td>250 $\mu$M PE</td>
<td>3.86</td>
<td>0.95</td>
</tr>
<tr>
<td>500 $\mu$M PE</td>
<td>3.98</td>
<td>0.98</td>
</tr>
<tr>
<td>50 $\mu$M CL</td>
<td>3.37</td>
<td>0.83</td>
</tr>
<tr>
<td>250 $\mu$M CL</td>
<td>2.43</td>
<td>0.60</td>
</tr>
<tr>
<td>500 $\mu$M CL</td>
<td>1.81</td>
<td>0.45</td>
</tr>
<tr>
<td>50 $\mu$M TPL\textsuperscript{c}</td>
<td>4.23</td>
<td>1.15</td>
</tr>
<tr>
<td>250 $\mu$M TPL</td>
<td>4.21</td>
<td>3.63</td>
</tr>
<tr>
<td>500 $\mu$M TPL</td>
<td>4.56</td>
<td>6.74</td>
</tr>
</tbody>
</table>

a. Cardiolipin synthetase activity assayed as described in figure 7.
b. Phospholipid concentration expressed as lipid phosphorus.
c. TPL represents total phospholipid extracted from Bacillus stearothermophilus. This consisted of 52.3% PG, 30.3% PE, and 14.2% CL.
The specific activity of the PG was therefore reduced from 4,000 cpm $\left[^{3}\text{H}\right]/\text{n mole PG}$ to 670 cpm $\left[^{3}\text{H}\right]/\text{n mole PG}$. Based on these calculations, it appeared that the addition of the mixed phospholipid preparation resulted in a 4-12% increase in CL-synthetase activity.

5. The kinetics of the cardiolipin synthetase reaction.

The $K_m$ and $V_{\text{max}}$ for the Triton X-100-treated membrane preparation were determined by plotting the reciprocal of the velocity versus the reciprocal of the substrate concentration (Lineweaver-Burk plot). It was determined that the $K_m = 6.67 \times 10^{-4} \text{M}$ and the $V_{\text{max}} = 2.78 \times 10^{-4} \text{M}^{-1} \times \text{min}$. These values must be considered with some reservation because of possible errors in estimation of the specific activity of CL in reaction mixtures. The slope of the substrate concentration for Triton X-100-extracted membranes (Figure 7) is not equal to the slope of the same curve for washed membranes (Figure 5). It is also important to consider that saturation occurred at 50 nmoles PG added for the Triton X-100-extracted membranes whereas the enzyme present in the washed membrane preparation was not saturated at 50 nmoles PG added.
Figure 11. Kinetics of cardiolipin synthetase activity of Triton X-100-extracted membranes. It was calculated that 1600 cpm of $[^3H]$-glycerol released was equivalent to 1 nmole CL formed.
$K_m = 6.67 \times 10^{-4} \text{ M}$

$\frac{1}{S} (\times 10^4 \text{ M}^{-1})$

(1 x 10^{-4} \text{ moles x min}^{-1})
In this study, CL-synthetase activity in *Bacillus stearothermophilus* was located within the cell membrane, characterized in washed membranes, and characterized in a partially purified Triton X-100-extracted membrane preparation. Attempts to solubilize the enzyme with a variety of detergents were unsuccessful.

Enzymatic activity was located within the membrane. Whole cells, protoplasts, crude membranes, and washed membranes all displayed CL-synthetase activity. Washed membranes showed enhanced activity compared to other cell fractions, possibly due to loss of peripheral proteins which may have interfered with enzyme-substrate binding. Consequently, washed membranes were used to determine various characteristics of cardiolipin synthesis.

The pH optimum for cardiolipin formation was pH 5.5; the activity decreased rapidly below pH 5. The temperature range for enzymatic activity was broad with an optimum at 55°C. It appeared that CL-synthetase was stable over a broad range of temperatures. Enzymatic activity was not stimulated by divalent cation but activity was somewhat stimulated by the presence of EDTA. This suggested that EDTA had chelated an inhibitory metal or perhaps a divalent cation-dependent inhibitor. When Triton X-100 was added to the assay mixture, cardiolipin formation was significantly inhibited especially at detergent concentrations of 0.1% or greater. At a concentration of 0.15% Triton X-100, the critical micelle concentration, (CMC), for Triton X-100, there was a decrease of 80% in the rate of CL synthesis. Washed membranes treated
with concentrations of Triton X-100 up to 0.2% continued to demonstrate enzymatic activity provided that the detergent concentration was diluted to 0.05% prior to the assay (data not shown). It appeared that Triton X-100 at concentrations less than 0.2%, exerted an inhibitory effect by binding to the enzyme or removing essential boundary lipid from the enzyme rather than denaturing the enzyme. Enzymatic activity was irreversibly lost at concentrations of Triton X-100 greater than 0.2%.

Analysis of the products of CL-synthetase was by one of two methods: lipid extraction and separation of \(^{32}\text{P}\)-labeled phospholipids or by a 10% trichloroacetic acid (TCA) precipitation in which \(^{3}\text{H}\)-glycerol remained in the supernatant. Each of these methods measured different products; \(^{32}\text{P}\)-cardiolipin in the case of the former and \(^{3}\text{H}\)-glycerol release in the latter case. Each of these methods had particular advantages and disadvantages which must be considered upon analysis of the data.

The initial method of monitoring enzymatic activity involved lipid extraction and chromatography to separate the phospholipids. The corresponding spots for PG and CL on the chromatogram were analyzed for radioactivity. The strongest asset of this method was the direct measurement of CL. Thin layer plates were sprayed with a molybdate spray for lipid phosphorus to detect phospholipids, thus avoiding the extended periods of time required for autoradiography. However, there are some drawbacks to this method.

One of the greatest disadvantages with this method was the amount of time necessary to complete the procedure (10 hours). Another disadvantage was the numerous opportunities for error because of the number of different steps involved in the analysis. As a result, data obtained
by this method has a greater chance of being inaccurate.

The other method of monitoring enzymatic activity was to detect $[^{3}\text{H}]-\text{glycerol}$ release in the supernatant fluid after precipitation with 10% trichloroacetic acid (TCA). The major advantages with this method were the speed with which the assay could be performed and the consistency of data obtained. There were fewer steps and therefore, fewer opportunities for error.

The disadvantages with the 10% TCA precipitation procedure involved the use of detergents. When protein samples containing detergent were analyzed, the results were misleading. The detergent formed micelles with the substrate such that some of the substrate was not precipitated by the 10% TCA. As a result, the $[^{3}\text{H}]-\text{PG}$ remained in the supernatant fluid and was mistakenly observed as radioactivity from glycerol release. Release of $[^{3}\text{H}]-\text{PG}$ into the supernatant fluid was monitored by the inclusion of $[^{32}\text{P}]-\text{PG}$ in the initial exogenous $[^{3}\text{H}]-\text{PG}$ preparation. If $[^{32}\text{P}]-\text{PG}$ was released into the supernatant fluid to be assayed, $[^{32}\text{P}]$ as well as $[^{3}\text{H}]$ radioactivity was monitored and the radioactivity of the $[^{3}\text{H}]$ was adjusted accordingly.

Another disadvantage with this assay was that in the presence of detergents in the assay mixture, $[^{3}\text{H}]$ was quenched more than in the absence of detergents from the assay mixture. In the presence of Triton X-100, 50% of the $[^{3}\text{H}]$ was quenched whereas 33% of the $[^{3}\text{H}]$ was quenched in the absence of Triton X-100 in the assay mixture. These values were adjusted by multiplying cpm values by 2 (for samples from assay mixtures containing detergent) or by 1.5 (for samples from assay mixtures not containing detergent). Quenching in the presence of Triton X-100 was confusing because the scintillation cocktail contained Triton X-100.
Perhaps the detergent concentration was sufficiently elevated to create quenching.

The data from figure 5 indicated that $[^3H]$-glycerol release correlated with nmoles phosphorus recovered from CL after lipid extraction of the 10% TCA precipitate. Assuming that one $[^{32}P]$ labeled molecule of CL was formed from two $[^{32}P]$ molecules of PG, it was calculated from the data in figure 5 that equimolar amounts of CL and glycerol were formed in the reaction. These data lent credibility to the 10% TCA precipitation method. The amount of glycerol released was calculated from calculations showing that 75% of the $[^3H]$ label was present in the non-acylated glycerol of PG (see Results for calculations).

The data from figures 5 and 6 supported the view that the CL synthetase was not discriminating between endogenous, nonlabeled and exogenous, labeled PG. Errors in calculating the amount of CL formed could have occurred if there was not a uniform mixing of the unlabeled endogenous PG with the labeled exogenous PG and if the glycerol was preferentially released from either the endogenous or exogenous PG. If there was no mixing of endogenous and exogenous PG, the amount of CL formed calculated from the amount of $[^3H]$ glycerol released would not have been equivalent to the amount of CL formed calculated from $[^{32}P]$ recovered in the CL isolated from the chromatograms. Therefore it appeared that the exogenous PG vesicles fused with the membrane, then the labeled and unlabeled PG mixed freely to form equimolar amounts of CL and glycerol. Perhaps the initial fusion process was dependent upon divalent cations or lipid cofactors to ensure proper membrane-exogenous PG vesicle fusion.
One of the goals of this study was to solubilize CL-synthetase. Although this was not accomplished, the preparation with the highest specific activity was prepared by a two step washing procedure with Triton X-100. While many other detergents removed lipid from membrane bilayers, Triton X-100 was very efficient at removing protein as noted by the removal of 66% of the total membrane protein using the two step extraction procedure. At the same time, 41% of the enzyme was retained in the membrane with an increase in specific activity from 2.99 units/mg protein for washed membranes to 3.65 units/mg protein for the Triton X-100-extracted membranes. Although the increase in specific activity was not overwhelming, a large quantity of protein was removed. Either much of the enzyme was removed or Triton X-100 had inhibitory effects on the CL-synthetase. Inhibition of the enzyme by Triton X-100 may have been due to the loss of essential boundary lipid (see p. 53 for a discussion of boundary lipid).

The characteristics of the Triton X-100-extracted membranes were quite different compared to the enzyme in the washed membranes. First, the pH optimum for the Triton X-100-extracted membranes was 6.0 as opposed to pH 5.5 for washed membranes. The pH curve for the detergent-extracted membranes was narrower than the pH curve for washed membranes suggesting that the enzyme in the detergent-extracted membranes was more sensitive to pH. Tanford and Reynolds (97) suggested that detergent micelles interact more readily at more alkaline pH. Perhaps the higher pH allowed for greater interaction between detergent micelles and phospholipid.

The other major difference between the washed membrane preparation and the detergent-extracted membrane preparation was the effect of the cation. Surprisingly, the detergent-extracted membrane preparation was
stimulated by the divalent cations Ca\(^{++}\), Mg\(^{++}\), and Mn\(^{++}\) and inhibited by EDTA. It was also curious to note that nucleotide triphosphates such as ATP, CTP, GTP, and UTP were also inhibitory to enzymatic activity. This was initially interpreted as a link between energy generation and cardiolipin formation. However, further evaluation led to the interpretation that these nucleotide triphosphates (NTP's) may have been acting as chelators of divalent cations. For instance, O'Sullivan and Perrin have found that Mg-ATP has a very high stability constant (69).

All three divalent cations were as effective in stimulating CL-synthetase from detergent-extracted membranes. This suggested that divalent cation served to aid in phospholipid vesicle fusion rather than a divalent cation requirement. This suggestion is supported by observations that mono and divalent cations aid in phospholipid vesicle fusion (97).

The substrate concentration curve for detergent-extracted membranes was linear from 0-50 nmoles PG added after which the rate of \(^{3}\text{H}\) -glycerol release plateaued. This curve contrasts greatly with the substrate concentration curve for washed membranes which remained linear through 100 nmoles PG added. Enzyme saturation may have occurred in the case of detergent extracted membrane (see Figure 7) or perhaps boundary lipid requirements for stabilization of the enzyme were not fulfilled by the higher concentrations of PG.

The optimal temperature for \(^{3}\text{H}\) -glycerol release was 60°C, which is near the optimal growth temperature (62°C). The rate of glycerol release at temperatures greater than 70°C rapidly decreased possibly due to irreversible denaturation of the enzyme.
When various phospholipids were added to the Triton X-100-extracted membranes, certain phospholipids stimulated enzyme activity while others inhibited enzyme activity. As expected, the addition of CL substantially decreased $[^3H]_2$-glycerol release. This was probably due to product inhibition. The reason for the decrease in enzymatic in the presence of PE was not known, but perhaps the replacement of regulatory phospholipid with PE inhibited cardiolipin synthetase. In the presence of *Bacillus stearothermophilus* total phospholipid, the rate of $[^3H]_2$-glycerol release increased significantly (Table 6). Decreases in specific activity were accounted for by the dilution of the $[^3H]_2$-PG. Perhaps if the lipid and detergent were allowed to interact for an extended period of time, an even greater increase in enzymatic activity would have occurred.

There have been conflicting views on the recognition site on the phospholipid molecules for lipid dependent proteins. However, it has been generally accepted that the polar head group has been responsible for specific recognition of the phospholipid by membrane protein (21, 33, 81, 99). Cardiolipin synthetase appeared to be no exception (Table 6). Enzymatic activity from Triton X-100-extracted membranes were 1) not stimulated by the addition of PE, 2) inhibited by the addition of CL, and 3) stimulated by the addition of total phospholipid.

It was intriguing to note that more CL was formed in the presence of total phospholipid than when equivalent amounts of purified PG were presented to the CL-synthetase from detergent-extracted membranes. It was calculated that the CL formed had a specific activity of 1600 cpm $[^3H]_2$/nmole CL formed. When 60 nmols of PG were added to the detergent-extracted membranes, 15.6 nmols of CL were formed. When 100 nmols of
total phospholipid were added to the detergent-extracted membranes, 22.8 nmoles of CL were formed. This total phospholipid preparation contained 50 nmoles of unlabeled PG, as well as 10 nmoles of \([^{3}H]\)-PG. This represented a 46.2% increase in the CL formed. It was calculated that the specific activity of the \([^{3}H]\)-PG dropped from 1600 cpm/nmoles CL formed to 667 cpm/nmoles CL formed. Perhaps it was the concentrations of particular phospholipids in the total phospholipid mixture that regulated the CL-synthetase. Perhaps these phospholipids directed the enzyme to the substrate or stabilized the enzyme. Whatever the function of the phospholipid, it was apparent that certain phospholipids in particular concentrations were required for proper enzymatic activity.

Activation of CL-synthetase from detergent extracted membranes by total phospholipid could also explain the effects of substrate concentration on washed membranes and Triton X-100-extracted membranes. Perhaps the abundance of various phospholipids in the washed membranes stabilize the cardiolipin synthetase such that enzyme and substrate were allowed contact more readily whereas the lack of phospholipid in detergent extracted membranes did not allow proper contact between substrate and enzyme.

It was also important to be aware that some Triton X-100 remained bound to the membrane even after centrifugation and therefore may have affected the behavior of cardiolipin synthetase. It has been shown that specificities of several membrane proteins for phospholipids differed in enzymatic assays performed in the presence and absence of detergent (86). A detergent may relieve lipid dispersion problems or latency of an enzyme (86). However, the Triton X-100 appeared to have replaced essential boundary phospholipid.

When a time course was performed on the Triton X-100-extracted mem-
branes, 70% of the $[^3\text{H}]$-glycerol released in 20 minutes was released within the first 10 minutes. The velocity was linear for the first 10 minutes thus allowing for the calculation of a $K_m$ from the saturation kinetics data (Figure 8). The $K_m$ obtained for this preparation was $6.67 \times 10^{-4}\text{M}$ which was similar to the $K_m$ of $2.21 \times 10^{-4}\text{M}$ obtained by Short and White (91) for *Staphylococcus aureus* membranes.

Attempts to solubilize cardiolipin synthetase with various detergents were unsuccessful. A number of detergents were used at their CMC or above since it has been shown that amphipathic proteins bind detergent near their CMC (98). Detergent activation of CL-synthetase was not observed. It may be that the CL-synthetase was solubilized but was denatured simultaneously due to removal of essential boundary lipid. There is strong evidence that solubilization alone is not sufficient for activation of at least one lipid depleted enzyme. In the case of $C_55$ isoprenoid alcohol kinase, phospholipids must be added in the presence of Triton X-100 for full activation to occur (84,85).

It is apparent that integral membrane proteins have distinct "boundary lipids" (26,45,46,47,56,58,64,96,97) in the membrane that are immobile relative to the bulk lipid phase. These boundary lipids often have an intimate relationship with the protein such that excess lipid or detergent used in solubilization procedures tend to cause disaggregation of the proteins. Preferential lipid-protein associations could minimize mismatching of boundary lipid around the surfaces of membrane proteins. Heterogenous lipid compositions may also be required to satisfy the different packing requirements of membrane embedded proteins.

Cardiolipin synthetase appears to be a membrane-bound enzyme that
requires phospholipids for enzymatic activity. Lipid involvement with membrane proteins was first demonstrated in studies in which re-binding phospholipids to solvent-extracted mitochondria led to reactivation of respiratory activity (33). Later it was shown that phospholipids were required for each component of the respiratory chain (for both intact mitochondria and purified systems) (10,100). Cardiolipin, lecithin, and total mitochondrial phospholipid could restore activity but the mode of reconstitution was critical.

Several other enzymes require specific phospholipids for enzymatic activity. Mitochondrial D(-)-hydroxybutyric acid dehydrogenase has an absolute specificity for lecithin (97); other phospholipids do not restore activity. The isoprenoid alcohol phosphokinase from Staphylococcus aureus which phosphorylates a C55 isoprenoid alcohol intermediate in peptidoglycan synthesis, has an absolute requirement for PG or CL (39) in the presence of desoxycholate. Activity is not recovered in preparations lacking one or the other.

Enzymes of lipid metabolism also have phospholipid requirements (57,58). Results of attempts to solubilize CL-synthetase indicated a strong affinity (Table 6) and possible requirement for phospholipid. It was unknown whether lipid was an absolute requirement for enzymatic activity, a regulator of enzymatic activity, or both. If lipid was required for enzymatic activity, there should be loss of enzymatic activity upon delipidation, which would be restored upon reconstitution with lipids. If the lipid acted as a regulator, the lipid may have been required for enzymatic catalysis of non-polar substrates.

CL-synthetase may require lipids to form an appropriate binding site for PG. This would have been unusual because the physical state
of phospholipid affects the enzymatic reactions when phospholipids act as substrates and activators (25). The activity of lipid requiring enzymes (26) have been shown to be influenced by 1) the substrate mass, in which the enzyme is dependent upon the interface with the phospholipid available for enzymatic attack, 2) the extent of molecular packing around the enzyme, 3) the addition of solvent, which may dilute polar head groups and 4) the electrostatic field which affects adsorption and penetration of the enzyme.

Green and Tzagoloff (38) have offered a model for the role of lipid. They suggested that membranes are composed of a bidimensional association of lipoprotein subunits along a plane in which phospholipids contribute a hydrophilic surface. This polar surface prevents polymerization of proteins into 3-dimensional aggregates. Such aggregates would not allow contact of the functional reactive groups of the enzyme and the substrate. Soluble enzymes would spontaneously form micelles in water due to the amphipathic nature of the detergent with which the enzyme is dispersed.

By acting as regulators, lipids could create a finer level of organization in the membrane. They may direct precise contact between particular proteins in the membrane to assure that multistep catalysis occurs in a concerted manner (14). Phospholipids may act as enzyme regulators by insuring proper conformation of the protein. The conformation of the protein is optimal when exterior apolar amino acid residues are in contact with the lipid.

Perhaps this finer level of organization has been disrupted in Triton X-100-extracted membranes containing cardiolipin synthetase. Perhaps the enzyme has an absolute requirement for the lipid bilayer.
in order to function properly. This was once thought to be the case for the Ca\textsuperscript{++} ATPase in which cholate-mediated lipid substitution techniques were employed (62). However, Banerjee et al. (5) were able to partially purify the Ca\textsuperscript{++} ATPase by extraction with octyl glucoside at high salt concentrations.

Octyl glucoside appears to be one of the most promising tools for protein solubilization. Baron and Thompson (6) found that this detergent could solubilize more bacterial membrane proteins than Triton X-100 or Brij 36-T while not affecting enzymatic activity. The detergent was more easily dialyzed than Triton X-100. The results of the present study have shown that octyl glucoside alone or with sodium cholate, extracted large amounts of membrane protein (Table 3).

Earlier attempts (13,19) as well as the present study have failed to solubilize CL-synthetase. Solubilization of the enzyme has been complicated by the failure to discover a detergent that solubilizes the enzyme in an active form. The small increase in specific activity and significant decrease in total units observed in detergent-extracted membranes (Table 1) suggests that CL-synthetase may have been solubilized by Triton X-100 but not in an active form.

Disruption of important lipid-protein interactions by detergent may require lipid reconstitution of the detergent solubilized CL-synthetase. Among the methods of lipid reconstitution available are: removal of detergent by dialysis, detergent dilution, sonification, and sonification with freeze-thaw (72). All of these methods involve the replacement of detergent with phospholipids such that enzymatic activity is regained. Attempts to reconstitute CL-synthetase by any
of the above mentioned methods would be the next logical step towards the purification of CI-synthetase.
Chapter V.

Summary

Cardiolipin synthetase from *Bacillus stearothermophilus* has been characterized in two membrane preparations: 1.) a washed membrane preparation in which about 30% of the total membrane protein was removed and 2.) a Triton X-100-extracted membrane preparation in which 66% of the total membrane protein has been removed.

Cardiolipin synthetase activity in washed membranes was assayed by the conversion of $^{32}$P--PG to $^{32}$P--CL. The characteristics of CL-synthetase from washed membranes were as follows:

1.) The optimal pH and temperature were 5.5 and 55°C respectively. CL-synthetase activity below pH 5.0 decreased rapidly. Enzyme activity below 55°C also decreased rapidly. Both curves were broad indicating that CL-synthetase was stable over a wide range of temperatures and pH.

2.) CL-synthetase from washed membranes was not stimulated by divalent cations nor was it inhibited by EDTA.

3.) CL-synthetase was inhibited in the presence of concentrations of Triton X-100 of 0.1% or greater. At concentrations of 0.15% or greater, enzymatic activity decreased by 80%.

A rapid assay system was developed which monitored the release of $[2-^3$H--]glycerol into the supernatant of a 10% TCA precipitation. It was found that the amount of $[2-^3$H--]glycerol released was equivalent to the amount of CL formed.

Attempts to solubilize CL-synthetase with detergents such as octyl
glucoside, sodium cholate, zwittergent 3-14, and Triton X-100 were unsuccessful. However, sequential extractions of washed membranes with 0.05% and 0.10% Triton X-100 yielded an enzyme preparation that contained 34% of the original membrane protein and showed a 1.22 fold increase in specific activity. The characteristics of the detergent extracted membrane preparation were different than the washed membrane preparation.

1.) The optimal pH and temperature were 6.0 and 60°C respectively. The peaks and the curves for these characteristics were different than those of the washed membrane preparation. The data suggest that the Triton X-100-extracted membrane preparation was more susceptible to pH and temperature.

2.) Enzymatic activity was stimulated in the presence of Ca$$^{++}$$, Mn$$^{++}$$, and Mg$$^{++}$$ whereas the enzyme was inhibited by EDTA. Enzymatic activity decreased in the presence of ATP, CTP, GTP, and UTP. These data suggest a divalent cation requirement by the enzyme.

3.) Enzymatic activity was linear until 50 nmoles of PG were added at which point $[^3H]glycerol release plateaued. The $K_m$ was 6.67 x 10^{-4} M. When a time course was performed, 70% of the $[^3H]glycerol released over a 20 minute incubation period was released within the first 10 minutes of incubation.

4.) The addition of various phospholipids to the enzyme assay mixture had different effects on enzymatic activity. In the presence of added PE, enzyme activity was lowered at low concentrations of PE but enzymatic activity increased with higher concentrations of PE. CL-synthetase was strongly inhibited in the presence of added CL suggesting product inhibition. With the addition of total phospholipid from
Bacillus stearothermophilus, enzyme activity was strongly enhanced suggesting a lipid requirement.

5.) The inability to solubilize CL-synthetase and stimulation of the enzyme in the presence of total phospholipid suggest a specific lipid requirement. Disruption of the protein-lipid interactions in the membrane may account for the failure to solubilize CL-synthetase to date.
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