1975

# Effects of membrane association and membrane phospholipid composition on ATPase activity in Bacillus stearothermophilus

Paul Edwin Orndorff
*The University of Montana*

# EFFECTS OF MEMBRANE ASSOCIATION AND MEMBRANE PHOSPHOLIPID COMPOSITION ON ATPASE ACTIVITY IN BACILLUS STEAROTHERMOPHILUS

By

Paul E. Orndorff

B.A., Knox College, 1972

Presented in partial fulfillment of the requirements
for the degree of

Master of Science

UNIVERSITY OF MONTANA

1975

Approved by:

George L. Card
Chairman, Board of Examiners

John M. Stewart
Dean, Graduate School

Dec 12, 1975
Date





ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

## ABSTRACT

Orndorff, Paul E., M.S., December, 1975, Microbiology

Effects of Membrane Association and Membrane Phospholipid Composition on ATPase Activity in Bacillus stearothermophilus ( 79 pp.)

Director, Dr. G. L. Card

Membrane phospholipid composition in whole cells of Bacillus stearothermophilus was altered by a 20-min period of anaerobiosis. During this period there was a decrease in phosphatidylglycerol and corresponding increase in cardiolipin (CL). Membrane-associated ATPase was solubilized by dialysis of the membrane fractions against 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The optimum conditions for ATPase activity in all fractions was at pH 8.4 in the presence of 1.0 mM $MgCl_2$. The temperature optimum for the membrane-bound fractions (high CL and control) was at 64°C, whereas soluble ATPase exhibited maximum activity at 59°C. Enzyme activity remained stable in the control membrane fraction, whereas the soluble enzyme lost 100% activity after 30-min incubation at 80°C; and the activity of the high CL membrane fraction was reduced 50%. The high CL membrane fraction retained three times more ATPase than the control membrane fraction when the fractions were washed successively with (i) 10 mM Tris-HCl (pH 7.5), 5.0 mM $MgCl_2$, (ii) 2 M LiCl, 0.25 M Tris-HCl (pH 7.5) and (iii) dialysed against 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Membrane CL concentration did not influence the reassociation of solubilized ATPase to ATPase-depleted membranes. A $K_m$ of 1.8 mM was determined for both the soluble and membrane fractions at 60°C. The $K_m$ remained constant in the soluble fraction, whereas the $K_m$ decreased to 1.1 mM in the control membrane fraction and decreased to 0.32 mM in the high CL membrane fraction at 45°C. The determination of the activation energy of membrane and soluble fractions revealed that the soluble enzyme underwent a change in activation energy at 56°C. The temperature at which there occurred a change in activation energy was displaced slightly from 56°C in the membrane-bound enzyme preparations. High CL membrane preparations showed a lower activation energy over a temperature range of 45°-56°C than did the control membrane preparations. These findings suggest that membrane association and membrane phospholipid composition affect both the physical and kinetic properties of ATPase in Bacillus stearothermophilus.

## ACKNOWLEDGMENTS

I wish to express my sincere thanks and appreciation to Dr. G. L. Card for his encouragement and guidance in the preparation of this thesis and in all aspects of my graduate work.

In addition, I would like to thank Kalene De Vore for her help and extraordinary patience in typing this thesis.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

## LIST OF ABBREVIATIONS

| Abbreviation | Explanation |
|---|---|
| ATPase | Adenosine Triphosphatase |
| BBL | Baltimore Biological Laboratories |
| Cal | Calories |
| CL | Cardiolipin |
| Co-Q | Coenzyme Q |
| DCCD | N, N-dicyclohexycarbodiimide |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxyribonuclease |
| E | Activation Energy |
| EDTA | Ethylenediaminetertraacetate |
| $\Delta H$ | Enthalpy |
| $K^{o}$ | Degrees Kelvin |
| $K_{m}$ | Michaelis Constant |
| NADH | Nicotinamide adenine dinucleotide |
| nm | Nanometers |
| O.D. | Optical Density |
| PG | Phosphatidylglycerol |
| PE | Phosphatidylethanolamine |
| Pi | Inorganic Phosphorous |
| R | Gas Constant (1.987 cal/mole) |
| RNA | Ribonucleic Acid |
| Tris | Tris (hydroxymethyl) Aminomethane |
| V | Velocity |

# CHAPTER I

## INTRODUCTION

During the past 20 years the biological membrane has passed from a sketchy and even dubious entity to a highly weighed, fractioned, analyzed and photographed cellular structure. The advances made in understanding the structure and function of membranes have come largely from novel and innovative methodology accompanied by liberal but enlightened theorizing. Yet, today both membrane structure and this structure's integral role in cellular metabolism remain an elusive and intriguing problem for the cellular physiologist.

Although the problems involved in the study of the structure and function of the membrane have been considerable, workers in this field have not gone entirely without reward. Through the years, membrane theory has progressed to the point where a general concept of the structure of biological membranes is now widely accepted. This model, albeit not as orthodox as once considered, now assumes that biomembranes (1) are fluid structures, at least in the plane parallel to the cell surface, (2) that their essential skeletons consist of a layer of lipids, the polar head groups of which extend into fluid phase, and (3) that proteins are somehow intercalated into these lipids by virtue of hydrophobic associations. It is, however, the proteins and their associated polysaccharides that seem to account for the specificity and individuality of membranes. Indeed, many of the immunologic, cohesive, and

transport properties of membrane surfaces are apparently subserved by discrete proteins, some of which have been isolated (16, 21, 28, 55, 60).

Although the proteins associated with the biological membrane seem to confer the membrane's individuality, the phospholipids which make up the bulk of membrane structure have been shown to influence the behavior of membrane proteins (55). The degree to which the membrane phospholipids affect associated proteins seems to depend on the nature of the association of the protein with the membrane (51, 52, 55).

Proteins associated with membranes fall into two classifications with regard to location: (1) Those proteins that are loosely associated with the membrane and can be solubilized by methods that do not require disruption of membrane structure are termed extrinsic or peripheral proteins. Extrinsic enzymes are typically involved in coupling cytoplasmic reactions with those more integrally associated with the membrane, e.g., succinate dehydrogenase, ATPase (55). (2) Proteins that are located in the lipid matrix of the membrane are termed integral or intrinsic proteins. These proteins usually require the use of lipid solvents to extract them from the membrane. Intrinsic enzymes frequently show loss of activity after solubilization. The most well characterized examples of intrinsic enzymes are the cytochrome oxido-reductases in mitochondria and the transferases involved in cell wall synthesis in bacteria (55).

The first indication that lipids could be activly involved in the metabolic processes relegated to the membrane came in 1961 when Lester and Fleischer (34) demonstrated that phospholipid and Coenzyme Q were necessary for electron transport activities. This discovery soon led to a series of experiments that expended the idea of lipid involvment to both extrinsic and intrinsic enzymes in mitochondria, microsomes and bacteria. In their initial studies, Lester and Fleischer (34) demonstrated that extraction of mitochondrial membranes with acetone containing 20% water removed Coenzyme Q. Extraction with acetone containing 10% water removed Coenzyme Q and 90% of the membrane phospholipid. Extraction with 80% or 90% acetone resulted in the loss of electron transport activity. Up to 50% of the activity could be restored only if both Co-Q and phospholipid were added back to the extracted mitochondrial membranes.

Subsequent experiments by Casu and Fleischer (15) in 1967 demonstrated that phospholipid was required for each of the complexes of the electron transfer chain in mitochondria. In these experiments the membrane phospholipids were first degraded by phospholipase A. This treatment resulted in the loss of electron transfer activities of succinate Co-Q reductase, Co-Q-cytochrome c reductase, cytochrome c oxidase, NADH-oxidase and $Mg^{2+}$ stimulated ATPase. In all cases enzyme activities reappeared when phospholipids were added back. The use of phospholipase A allowed the controlled degradation of membrane phospholipids. By this method

it was shown that only a portion of the phospholipid of the membrane was required for the activity of any one enzyme. In all cases the lipid most active in restoring activity was cardiolipin, which was approximately as active as the total mitochondrial phospholipid extract (55). Comparable concentrations of phosphatidylcholine showed negligible activity despite the fact phosphatidylcholine is the major phospholipid component of native mitochondria.

Lipids are required for full activity of a number of microsomal enzymes. Glucose-6-phosphatase is a microsomal enzyme that appears to require phospholipid for activity (18). Treatment of intact microsomes with phospholipase C caused a marked loss of glucose-6-phosphatase activity under conditions leading to the hydrolysis of approximately two-thirds of the total microsomal phospholipid. Activity was completely restored by the addition of a variety of phospholipids, but lysophosphatidylcholine was effective in restoring activity at much lower concentrations than were other phospholipids tested. Treatment with phospholipase C also inhibits both the ATPase activity and $Ca^{2+}$ transport of intact microsomes (40). Both functions can be fully restored by the addition of micellar dispersions of phosphatidylcholine, lysophosphatidylcholine, and phosphatidic acid.

A similar requirement for phospholipids has been reported for a $Na^{1+}$ - $K^{1+}$ activated ATPase activity from a membrane fraction of cerebral cortex (61) and kidney cortex (20). The enzyme activities in the two membranes were activated by different phospholipids. The brain enzyme displays full activity with

phosphatidylcholine whereas lysophosphatidylcholine and phosphatidic acid are less active, and the acidic phospholipid cardiolipin in inactive. On the other hand, activation of the kidney enzyme is best accomplished by the negatively charged phosphatidylserine.

The cellular envelopes of bacteria contain a diverse collection of enzymes. Bacterial membrane-associated enzymes are called upon to perform many tasks relegated to specific cells or organelles of higher organisms. Thus, bacteria, which offered a simplified system for molecular geneticists, pose special problems for the membrane physiologist. Nevertheless, several specific enzymes have been studied successfully with regard to lipid involvement in their activity.

The most well characterized bacterial membrane-associated enzymes are several of the transferase enzymes involved in the biosynthesis of bacterial lipopolysaccharides and in the transfer of phosphate from phosphoenolpyruvate (PEP) to sugars. Also membrane-associated bacterial ATPase has been the object of frequent studies.

Studies of purified glycosyltransferase involved in the synthesis of bacterial lipopolysaccharide has been shown to require lipid for activity (55). When lipids are extracted from the cell envelope fraction by treatment with organic solvents, the residual cell envelope still contains the galactose-deficient or glucose-deficient lipopolysaccharide, but it has lost its ability to act as acceptor in the transferase reaction. The glycosyl acceptor activity of the lipid-depleted cell envelope

can be completely restored by adding back the lipid extract. In this case the lipid appears to activate the substrate in the reaction rather than interacting directly with the enzyme (55).

The cell envelopes of many bacteria contain an enzyme system catalyzing the transfer of phosphate PEP to sugars. In Escherichia coli and Salmonella typhimurium at least four proteins are required for the PEP-dependent phosphorylating activity. Two of the protein components (IIA and IIB) appear to be located in the membrane, whereas the other two (HPr and enzyme I) are recovered in the soluble fraction of cell extracts. Roseman (53) demonstrated that lipid was required for activity of the membrane-bound components of the system, and subsequent studies revealed that the active system can be reconstituted in vitro from the protein components plus phospholipid. Phosphatidylglycerol was the active component in the system; other lipids showed much lower activity (53).

Bacterial ATPase has not as yet been studied as intensively with regard to phospholipid involvement in its activity as has mitochondrial ATPase. This has in part been due to the difficulties associated with working with the diverse bacterial systems of energy transduction in which ATPase appears to play a critical role (13, 29). In mitochondrial systems ATPase has been conclusively shown to be a critical link in the coupling of oxidation to phosphorylation and has become the object of intensive investigation to determine the factors which influence its activity. Although there have been a number of studies of phospholipid

involvement in ATPase activity in mitochondria, the manner in which membrane lipids influence ATPase activity is at present far from certain. Nevertheless, a working hypothesis regarding lipid involvement in enzyme activity has emerged and has served in guiding the experiments on bacterial ATPase in this study.

Membrane-bound enzymes can be viewed as one structural element in an organized array of other molecules. Since the behavior of many membrane enzymes is markedly affected by their insertion into the membrane structure, neighboring molecules (e.g., phospholipids and proteins) may play a major role in regulating the catalytic activity of these enzymes.

Membrane lipids are known to be in a dynamic state with significant rates of turnover of whole molecules and parts of individual phospholipid molecules (14, 64). Changes in membrane phospholipids may, therefore, serve a regular function in vivo. Indeed, in all of the studies discussed here, there was a specific requirement for certain phospholipids to restore full activity to lipid-depleted enzymes, and the phospholipids required were frequently only a minor class of the total membrane phospholipid. Although the notion of lipid composition as a control mechanism in enzyme activity is at present without basis, requirements for specific phospholipids in enzyme activity indicate that at least some form of interaction is necessary to supply the enzyme with its required lipid environment. A short list of possible functions of lipids in the activity of membrane-associated enzymes is presented below to show the variety of interactions, all of which

could influence ATPase in this study (55). (1) the lipid could activate the substrate. This mode of action appears to be active in the glycosyltransferase system mentioned previously. (2) The lipid could directly activate the enzyme, perhaps by inducing a conformational change in the enzyme. This possibility is implied in any study relating to enzyme activation when supplied with its appropriate lipid constituents. However, this possibility is difficult to prove conclusively in biological systems where the mechanism and sequence of the reaction catalyzed by the enzyme is not known. Although, under more controlled conditions (63), it has been shown that nonpolar compounds can induce reversible changes in protein structure. (3) The lipid could act as an organizer in multi-enzyme systems, directing the sequential arrangement of enzyme proteins within the membrane structure in systems such as the electron transport chain. Direct evidence of this mechanism is at present lacking. It is not possible at this time to relegate phospholipid involvement in ATPase activity to any of the above model systems in spite of the fact that the existence and location of membrane-associated ATPase itself have been known for quite some time.

Membrane-associated ATPase, a peripheral (or extrinsic) membrane-associated enzyme, is ubiquitous in nature and has been studied in both eucaryotic and procaryotic cells. In eucaryotic cells the ATPases most studied have been the mitochondrial $Ca^{2+}$-$Mg^{2+}$activated ATPase and the $Na^{1+}$-$K^{1+}$ activated ATPase of erythrocytes and sarcoplasmic reticulum. Bacterial ATPase

has been studied in many species, all of which possess the $Ca^{2+}$-$Mg^{2+}$ activated ATPase (22).

Membrane-associated ATPase has been shown to catalyze the hydrolysis of ATP to ADP and Pi according to the following reaction: ATP + $H_2O \rightarrow$ ADP + Pi (22). It has long been suspected that ATPase was involved in the reverse reaction to form ATP in the coupling of oxidation to phosphorylation through the electron transport system (7, 24, 27, 43, 47). The discovery by Racker (21) that the small mushroom-like particles (elementary particles) projecting from the inner mitochondrial membrane contained ATPase activity tended to strengthen the proposed model of ATPase as a link between the intrinsic electron transport chain and the matrix of the mitochondria. Other indirect evidence from ATPase-deficient mutants of E. coli tended to support the fundamental role of ATPase: the ATPase-deficient mutants could not couple oxidation to phosphorylation through the electron transport system (13, 58, 66). More recently, mitochondrial and sarcoplasmic ATPases have been shown to catalyze the formation of ATP from ADP and Pi in vitro (31, 49), and it now seems certain that ATPase plays a central role in the coupling of oxidation to phosphorylation.

Because this report deals with a bacterial ATPase which has properties more closely related to those of the mitochondrial ATPase, the following presentation of work with eucaryotic systems is directed towards the properties of mitochondrial ATPase. A discussion of mitochondrial ATPase is necessary because of the similarities of the two enzymes and the fact that work on

phospholipid involvement in ATPase activity in mitochondria is at a much more advanced state.

In 1966 Racker and his co-workers (9, 30) first demonstrated that ATPase in mitochondrial preparations would be stimulated by the presence of added phospholipids. Removal of lipids by cholate extraction of submitochondrial particles from rat liver resulted in a marked decrease in ATPase activity; the activity was partially restored by the addition of different phospholipids. Acidic phospholipids (e.g., phosphatidylglycerol and cardiolipin) were more active in ATPase stimulation, although no specific phospholipid could be shown to be required for activity. They further found that membrane-bound and soluble ATPase exhibited different sensitivity to the uncoupling agent, oligomycin; the soluble enzyme was resistant, whereas the membrane-bound form was sensitive to this agent (21, 48). From these observations, it appears that ATPase might be influenced by membrane lipids at two levels. (1) Cholate extraction greatly reduced the enzyme's activity which was restored by adding back acidic phospholipids. This would imply a critical lipid environment in the vicinity of the enzyme to insure its proper function. (2) In the studies involving the uncoupling agent, oligomycin, ATPase that was solubilized by methods not involving lipid solvents was still fully active and insensitive to the uncoupling agent, whereas the membrane-associated ATPase was sensitive. This suggests that the membrane exerts an allosteric effect on membrane-associated ATPase in some way altering its sensitivity to oligomycin. Thus, it appears

that the membrane exerts an effect on ATPase merely by virtue of its association as well as seeming to provide an integral unit (i.e., the unit removed by cholate extraction) necessary for the enzyme's activity.

Subsequent studies by Burstein *et al.* (12) suggested that the acidic phospholipid cardiolipin might play a role in oxidative phosphorylation. These investigators demonstrated that virtually all of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can be cleaved by treating submitochondrial particles with phospholipase C. The resultant particles, which have lost two-thirds of their phospholipids, catalyzed oxidative phosphorylation with an efficiency similar to untreated control particles. However, subsequent exposure of the digested particles to a phospholipase (from *Bacillus cereus*), which cleaves cardiolipin (CL) as well as PC and PE, resulted in a very marked decrease in phosphorylating capacity.

In 1971 Santiago *et al.* (56), using a system of selective degradation of phospholipids, demonstrated the gradual loss of ATPase activity concomitant with CL degradation in beef heart mitochondria. Although the requirement for cardiolipin in ATPase activity did not imply that ATPase was the ciritical cardiolipin-sensitive link in the work of Burstein *et al.* (12), it did demonstrate a component of the phosphorylating system to be specifically sensitive to cardiolipin degradation.

Work to reconstitute an *in vitro* controlled membrane system in order to examine phospholipid action on ATPase activity met

with little success (67) until Racker and Stoeckenius (49) in 1974 succeeded in creating a model system for the study of oxidative phosphorylation. Briefly, the system is composed of crude phospholipid (soybean) vesicles to which have been added a bacterial pigment "bacteriorodopsin" from Halobacterium halobium. This pigment is oriented so that, when exposed to light, protons are transmitted to the inside of the vesicles. The addition to purified bovine heart mitochondrial ATPase, coupling factors, ADP and Pi complete the system. With this system it was observed that exposure to light induces a rise in pH in the medium containing the vesicles concomitant with ATP formation. The phospholipid composition of the vesicles was also found to influence the reaction. No activity was observed with vesicles prepared with PE and PG as the only phospholipid constituents. However, if CL was added to the PE-PG vesicles to produce vesicles containing 10% CL, the activity of the enzyme was restored to 70% of its original activity in the crude phospholipid preparations. This work not only suggested the central role for ATPase in oxidative phosphorylation but also confirmed that phospholipid composition has a profound effect on this enzyme.

Progress on work attempting to show phospholipid involvement in bacterial ATPase has not advanced to the state it has with mitochondrial ATPase. Work dealing with bacterial ATPase activity has largely been confined to describing the properties of soluble and purified $Ca^{2+}$-$Mg^{2+}$ activated ATPase. Abrams and his co-workers in 1965 (1) first succeeded in solubilizing and purifying a

membrane-associated ATPase from Streptococcus fecalis. It was noted that solubilization of the enzyme could be accomplished simply by eluting the $Mg^{2+}$ ions (1). Also their studies revealed a hydrophobic component of ATPase, separated by agarose gel filtration, that was necessary (along with $Mg^{2+}$ ions) for reassociation to the membrane but not necessary for activity (2, 5, 6). Further studies by Abrams and his co-workers (5) on the reassociation of ATPase to the membrane indicated that the membrane could accommodate only a limited number of ATPase molecules. This led them to suggest that there were specific binding sites on the membrane for the attachment of ATPase with $Mg^{2+}$ acting as a bridge between the protein and the membrane. Their evidence, however, did not exclude the possibility that the cation could induce a change in the conformation of the membrane or the enzyme to facilitate binding. Also their reassociation experiment did not show conclusively that there were specific sites on the membrane for ATPase attachment.

Kepes et al. (51, 52) have reported that ATPase from E. coli can also be solubilized by decreasing the $Mg^{2+}$ ion concentration and that reassociation was facilitated with $Mg^{2+}$. They also found that the solubilized enzyme exhibited a shift of 1.5 pH units for optimum activity, became resistant to the uncoupling agent, N, N-dicyclohexylcarbodiimide (DCCD) and that there was a twofold increase in the $K_m$. Farias et al. (46, 59) found that membrane fluidity had an allosteric effect on $Ca^{2+}$ activated ATPase from E. coli. Bragg et al. (8) were able to isolate a

hydrophobic region of $Ca^{2+}$ activated ATPase from E. coli that was necessary for reassociation to the membrane but not necessary for activity.

ATPase from Bacillus subtilus (54), Bacillus megaterum (44, 45), Micrococcus lysodeiktycus (22, 33), and Bacillus stearothermophilus (23, 25, 26, 38, 39, 41, 42) has been described in detail regarding its requirements for activity, purification and properties of the purified enzyme, but contain virtually no information concerning possible effects of the membrane on the enzyme. The ATPase from the thermophilic bacterium, B. stearothermophilus, has been purified to a state considered homogeneous based on ultra centrifugation and electrophoresis analysis (25). The pH optimum for the purified and membrane-bound enzyme was found to be between 8.0 and 8.5. Divalent cations such as $Mg^{2+}$ and $Ca^{2+}$ activate the enzyme. In contrast to the enzyme from E. coli, sulfhdryl reagents and DCCD had no effect on either the membrane-bound or soluble enzyme (25). Hachimori et al. (25) noted an abrupt lowering of the activation energy of the purified enzyme at 55°C. They suggested that this resulted from a conformational change in the protein resulting in greater activity at higher temperatures. Other studies with mesophilic organisms (29, 46, 50, 59, 62) have shown that ATPase undergoes a lowering of activation energy corresponding to a temperature at which there is a change in phase of the membrane lipids and is not due to the proteins. Whether the lowering of activation energy of ATPase of B. stearothermophilus is a property of a possible lipid

component of the purified enzyme or due solely to some change in the protein has not been determined.

With the knowledge of the basic properties of ATPase in B. stearothermophilus, there are several avenues open to the investigation of possible effects the membrane might have on the enzyme. (1) Since B. stearothermophilus is a thermophile, the membrane might contribute to the thermostability of the enzyme. Soluble ATPase and pyrophosphatase from B. stearothermophilus have been reported to be stable at 65°C (38, 42). However, 65°C is approximately the optimum growth temperature of the organism, and higher temperatures have not been tested. (2) The membrane could exert allosteric effects on ATPase resulting in modifications of the enzyme's behavior in the membrane-bound state. The studies with E. coli revealed a shift in pH optimum and a change in the $K_m$ of the enzyme. (3) In the studies with mitochondrial ATPase the phospholipid cardiolipin was observed to play a significant but, as yet, an undefined role in the ATPase activity. This effect has not as yet been demonstrated in bacterial systems. Cardiolipin could influence the basic nature of the association of the enzyme with the membrane and in addition influence the kinetic and thermodynamic properties of the enzyme.

Bacillus stearothermophilus is an ideal model for studying the effects of membrane phospholipid composition on ATPase activity. The major phospholipids of the membrane are phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (CL). Rapid conversion of PG to CL can be achieved by subjecting cells to

anaerobiosis or by suspending the cells in a non-growth buffer medium. The conversion of PG to CL appears to be a non-energy-requiring reaction involving the direct conversion of two molecules of PG to one of CL (14). During a 20-minute period of anaerobiosis the ratio of PG:CL changed from 2.5 to 0.5. Membranes isolated from these cells provide a useful system for studying the effects of alteration of two of the major phospholipid components of the membrane on ATPase activity.

This study was divided into four parts. The first part was concerned with the isolation and characterization of soluble and membrane-bound ATPase, including the determination of the optimum conditions for enzyme activity (i.e., pH, temperature, divalent cation requirements). Second, the thermostabilitie of membrane-bound and soluble ATPase were examined. Third, the effects of phospholipid composition on the release and reassociation of the enzyme to the membrane were examined. The fourth part consisted of determining whether membrane phospholipid had any effect on the kinetic parameters ($K_m$ and $V_{max}$) of the enzyme and on the activation energy of the reaction.

# CHAPTER II

# MATERIALS AND METHODS

## Organism

Bacillus stearothermophilus (NCA-2184) was used exclusively to obtain the data presented in this manuscript. The organism was kindly supplied from stock cultures maintained by Dr. G. L. Card, University of Montana, Missoula, Montana.

## Growth of Bacillus stearothermophilus

Unless otherwise indicated, B. stearothermophilus was grown in medium containing 20 g Tryticase (BBL) and 10 g Yeast Extract (Difco) per liter of distilled water. Cultures were routinely grown in a fermentor (New Brunswick model MF114) with vigorous stirring (motor speed 400-500 rev/min) and vigorous aeration (air flow 10-12 liters/min). For smaller cultures (less than 2 liters) the cells were grown in 2-liter baffled flasks (Bellco) with volumes not exceeding 500 ml/flask on a gyratory incubator shaker (New Brunswick model 625). The growth temperature was routinely maintained at 60°C ± 2°C. For some experiments the growth temperature was varied; these instances are noted in the text. Cells were routinely harvested at an optical density (O.D) of between 0.4 and 0.5 (600 nm, Coleman Junior II). When grown in the fermentor, the culture was poured into 4-6 liter Erlenmeyer flasks filled with crushed ice, collected by centrifugation (Sharples model T-1), and then washed in ice-cold 10 mM Tris-HCl

(pH 7.5). When grown on the gyratory shaker incubator, the cells were harvested by pouring the culture into 250 ml plastic centrifuge bottles filled with crushed ice. The cells were then collected by centrifugation (International model V) and washed twice in Tris-HCl as above.

## Treatment of Cells to Achieve the Phosphatidylglycerol (PG) to Cardiolipin (CL) Conversion

When the O.D. of the culture reached 0.4-0.5 nm, a portion of the culture was poured into Erlenmeyer flasks of just sufficient volume to hold the sample. The flasks were then tightly stoppered and incubated at 62°C ± 2°C in a waterbath for 20 min. Within the first minute of this treatment, the oxygen concentration dropped to 0% as measured by an oxygen monitor (YSI model 53). At this time there was an abrupt decrease in growth rate and corresponding decrease in phospholipid synthesis (14). The relative concentration of PG decreased rapidly with a corresponding increase in CL concentration. The net effect of this treatment was that the molar ratio of PG to CL decreased from about 2.5 (characteristic of aerated cultures at this temperature) to less than 0.5 after the anaerobic treatment (14). When cells were grown in the fermentor, anaerobic conditions were obtained by flushing the vessel with nitrogen. The cells that underwent the anaerobic treatment are referred to as high cardiolipin cells. The cells harvested directly on ice are termed control cells.

## Fractionation of Cells and Preparation of Crude Enzyme

Cells were always fractionated within 12 h of collection.

All manipulations of the preparations, unless specified otherwise, were performed in an ice bath or in a cold room at 4°C. The cells were suspended in distilled water, then lysozyme was added to a concentration of 125 ug/ml. This mixture was incubated at room temperature or at 37°C until lysis was observed (usually about 20 min). Lysis could be observed by a clearing of the suspension. When observed microscopically, no whole cells were observed after the suspension had cleared. The viscosity of the lysed cell suspension was reduced by sonication or by DNase treatment. Cell lysates were sonified continuously for between 20-30 seconds at 40% duty-cycle, output control at 4°C (Bronson model W-350). The preparation was kept in an ice bath during sonication. The DNase treatment consisted of adding to the lysate DNase and $MgCl_2$ to a final concentration of 1 ug/ml DNase in 5 mM $MgCl_2$. Within 5 min the DNase-treated preparation exhibited the free-flowing characteristics of the sonified preparation. After either of these treatments, the lysate was centrifuged (Sorvall RC2B) at 30,000 x g for 30 min. The pellet from this centrifugation consisted primarily of the membrane fraction; the supernatant contained the cytoplasmic components. When sonication was employed, the supernatant contained some membrane fragments. This was determined by centrifugation at 150,000 x g for 2 h, after which a visible rust-colored membrane pellet was observed. Supernatants from DNase-treated preparations, when subjected to the same centrifugation step, yielded no observable pellet. Whether

sonication or DNase treatment was used, the supernatant fraction from the 30,000 x g centrifugation was regarded as "crude soluble enzyme", and the membrane fraction was regarded as "crude membrane-bound enzyme". Both crude soluble and crude membrane-bound enzymes exhibited no loss of activity for up to 3 weeks when stored at 4°C in 10 mM Tris-HCl (pH 7.5). Assays of these fractions, however, were normally run within 5 days after fractionation.

## Solubilization of Membrane-Bound ATPase

The procedure to elute ATPase from the membrane (Fig. 1) essentially follows that of Hachimori et al. (25). Cells were collected and fractionated as described in the preceding section. The pellet (membrane) fraction from the 30,000 x g centrifugation step was resuspended in 5 mM $MgCl_2$ in 10 mM Tris-HCl (pH 7.5) and allowed to stand overnight at 4°C. This preparation was then centrifuged for 30 min at 30,000 x g. The pellet from this step was then washed twice with 2 M LiCl in .25 M Tris-HCl (pH 7.5). The resultant pellet was then resuspended in 10 mM Tris-HCl (pH 7.5) and dialysed against distilled water containing 0.1 mM EDTA for 12 h at 4°C. The contents of the dialysis tube were then transferred to 5/8" by 3" polyallomer tubes (Beckman), and the pH was adjusted to 9 with 1 N NaOH. Following this step, the tubes were allowed to stand at 4°C for 30-45 min. The EDTA-alkaline treatment caused the ATPase to dissociate from the membrane. The tubes were then capped and centrifuged (Beckman L2-65B) at 150,000 x g for 2 h. The supernatant fraction was termed

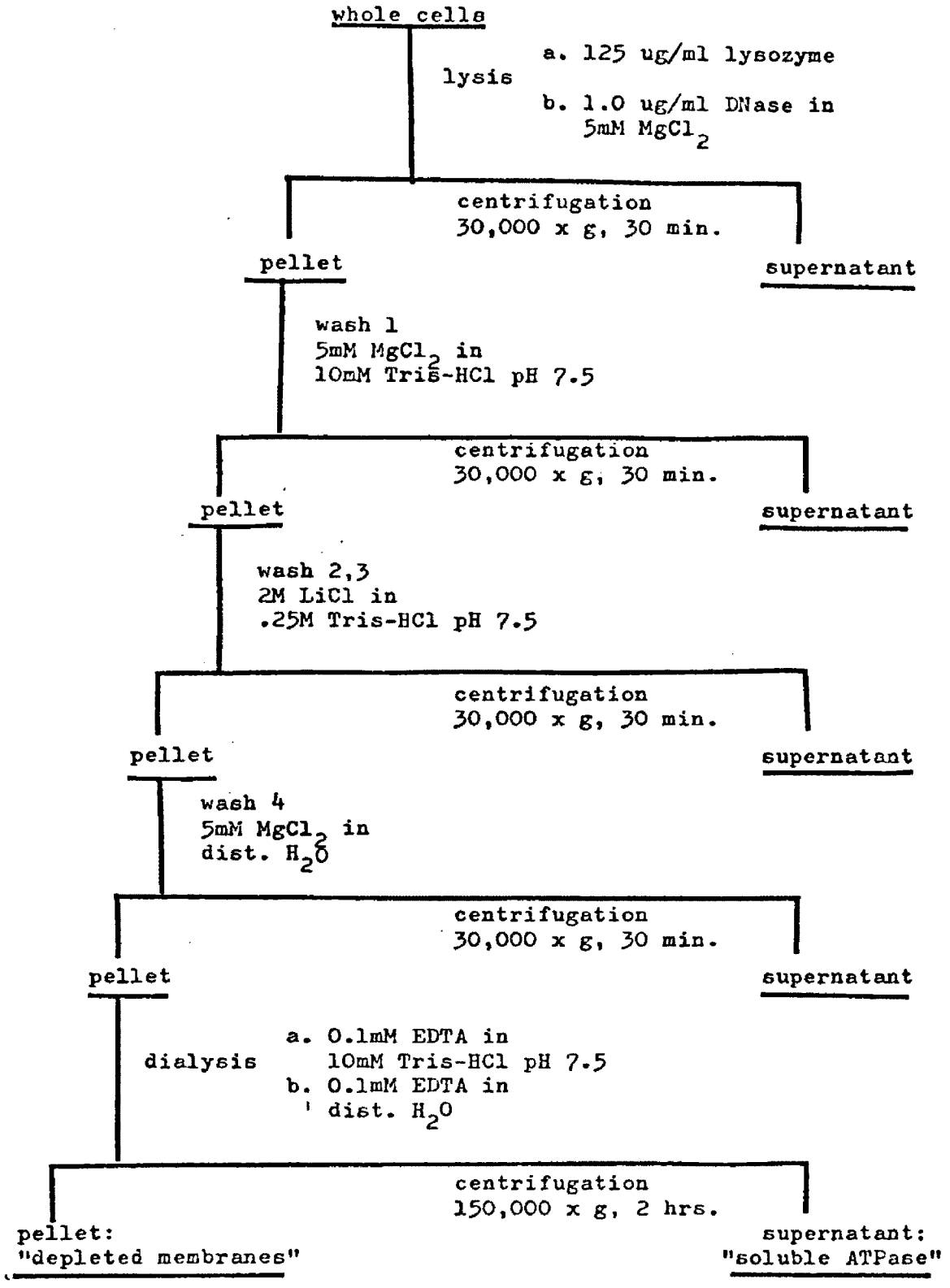



**Figure 1. Flow Sheet for the Preparation of "Depleted Membranes" and "Soluble ATPase".**

"soluble ATPase". The pellet fraction was termed the "depleted membrane fraction". It retained between 20 and 40% of the original ATPase activity found in the crude membrane preparation. In some instances both LiCl washes were omitted. LiCl removes proteins electrostatically bound to the membrane and has no effect on the release of ATPase. The washes were omitted so that the depleted membrane fraction would have a more representative population of proteins in studies where membrane integrity was needed. In experiments where concentrated ATPase was needed, soluble ATPase was concentrated by ammonium sulfate precipitation. Ammonium sulfate (powder) was added to plastic centrifuge tubes containing soluble ATPase in 10 mM Tris-HCl (pH 7.5) to 60% saturation at 4°C. The tubes were allowed to stand for 1 h at 4°C and then were centrifuged at 30,000 x g for 30 min. The pellet of precipitated protein was resuspended in a small volume of 10 mM Tris-HCl (pH 7.5) and dialysed against 10 mM Tris-HCl (pH 7.5) for 24 h at 4°C with two changes of buffer.

## Reassociation of Solubilized ATPase with the Depleted Membrane Fraction

Reassociation of solubilized ATPase with the depleted membrane fraction was attempted using membranes depleted by the method described above. Because membrane integrity was thought to be important in this experiment, the LiCl washes in the above procedure were omitted. Solubilized ATPase was concentrated as described above to yield 9.4 units of activity/ml in 10 mM Tris-HCl buffer (pH 7.5). Incubation of the depleted membranes with

the concentrated soluble ATPase was performed in 15 ml conical centrifuge tubes. The mixture contained 0.1 ml soluble ATPase (0.05 mg protein), 0.1 ml depleted membranes (0.08 mg protein) and 0.1 ml of 0.3 M $MgCl_2$ in 10 mM Tris-HCl (pH 7.5). To facilitate reassociation, the mixture of ATPase and depleted membranes was first brought to 60°C in a waterbath; the $MgCl_2$ was then added. This mixture was allowed to stand for 1 h at 22°C and then 4 h at 4°C. The mixture was then centrifuged at 10,000 x g (International model V) for 6 h. The supernatant was drawn off and assayed for ATPase activity. The pellet was resuspended in 0.5 ml 10 mM Tris-HCl (pH 7.5) and was then assayed for ATPase activity. Controls consisted of non-depleted membranes and soluble ATPase alone. The soluble ATPase above produced practically no pellet; however, values of ATPase activity found in this pellet were subtracted from values obtained when testing for reassociation.

## Conditions and Calculations for Determining the Activation Energy of Membrane-Associated and Solubilized ATPase

The velocity of enzyme-catalyzed reactions increases with increasing temperatures. The relationship between the velocity of the reaction and temperature can be expressed as the activation energy (E). This expression can be derived from the empirical Arrhenius equation relating the velocity of the reaction to temperature (65).

$$d\,\frac{\ln k}{dT} = \frac{E}{RT^2} \qquad (1)$$

where k is the reaction velocity constant, R the gas constant, T the absolute temperature and E a constant called the activation energy. Integration of equation (1) gives

$$\ln \frac{k_2}{k_1} = \frac{E}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \qquad (2)$$

from which the value for E can be obtained from the slope of the straight line when log k is plotted against $\frac{1}{T}$o. If the concentration of enzyme and substrate is held constant, the term $\ln \frac{k_2}{k_1}$ can be simplified since

$$\text{Rate} = k\,[E]\,[S] \text{ then}$$

$$\frac{\text{Rate at } T_2}{\text{Rate at } T_1} = \frac{k_{T_2}\,[E]\,[S]}{k_{T_1}\,[E]\,[S]}$$

This simplifies equation (2) to the form

$$\ln \frac{\text{activity at } T_2}{\text{activity at } T_1} = \frac{E}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \qquad (3)$$

From equation (3) one finds

$$E = 2.303\ R \cdot \text{slope}$$

or

$$E = 4.576 \times \text{slope where } R = 1.987 \text{ cal/mole.}$$

It should be noted that the term E is not the activation energy $\Delta H^*$ of the activated complex formed during an enzyme reaction but can be related to this quantity by $E = \Delta H^* + RT$ (17). The graphic relationship of E to an enzyme-catalyzed reaction is shown in Fig. 2. It should also be noted that an activation energy is a

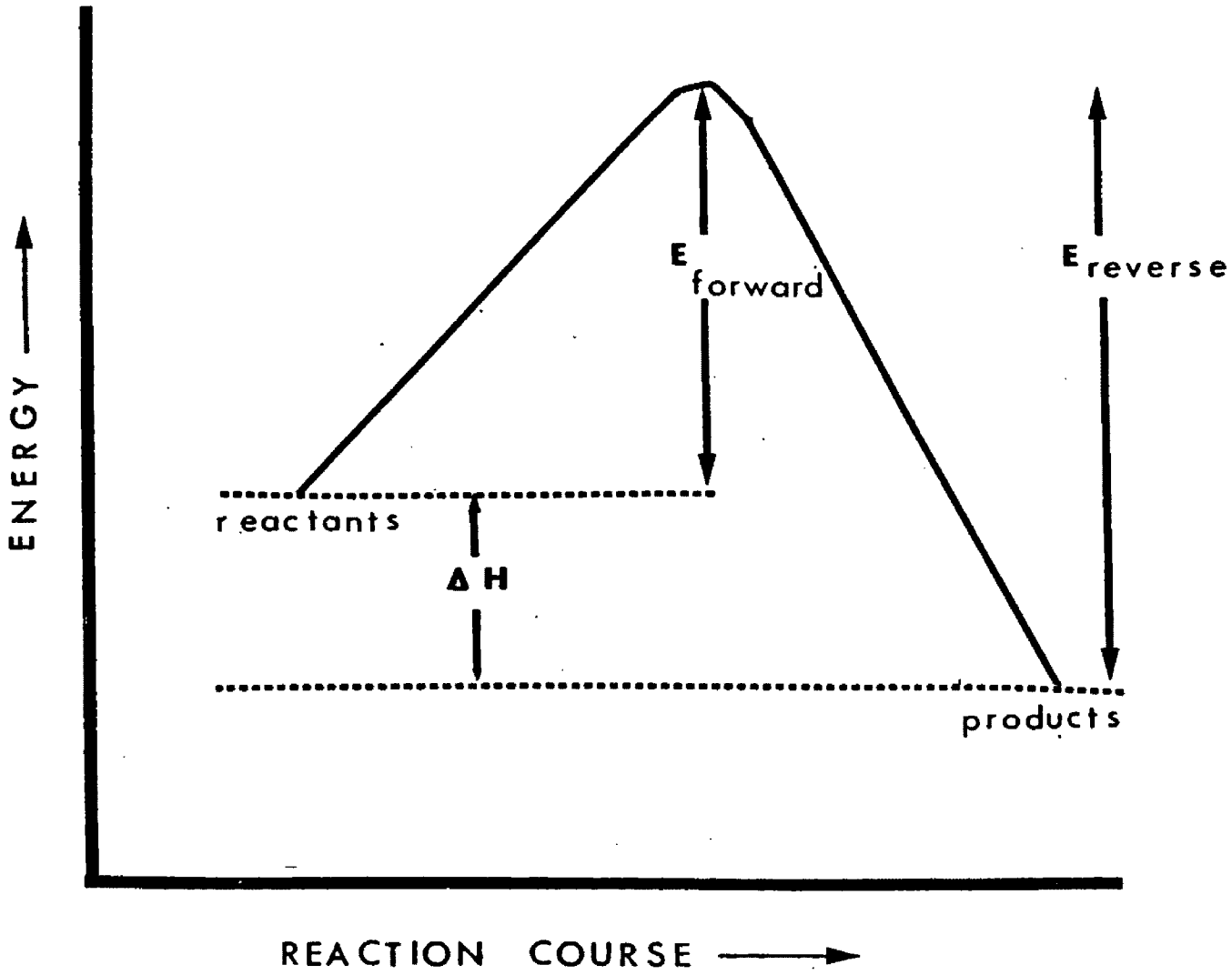



Figure 2. Energy Diagram for an Exothermic Reaction (65)

property of a reaction. However, with enzyme-catalyzed reactions, it has been the convention (50, 17) to refer to the "activation energy of the enzyme"; this convention is followed in this manuscript.

To determine the activation energy of membrane-bound and soluble ATPase, depleted membrane fractions and soluble ATPase were assayed over a temperature range from 40°C - 64°C using a Haake waterbath. This waterbath made it possible to assay activity at 2°C ± 0.2°C intervals necessary to obtain accurate results. The values obtained were plotted as described above, and the activation energy was then calculated.

## Assay of ATPase Activity

The reaction mixture for assaying ATPase activity, unless otherwise noted, consisted of: 50 mM Tris-HCl (pH 8.4, at 60°C), 2.5 mM ATP and 1.0 mM $MgCl_2$. To this mixture between .025 and 1.0 mg sample protein was added. The volume of the reaction mixture was varied throughout the studies ranging from 2.5 to 1 ml. The reaction volume did not affect the activity of the ATPase. To stop the reaction, an equal volume of ice-cold 5% $HClO_4$ was added. Precipitated protein was removed by centrifugation (Sorvall desktop). Between 0.5 and 1.0 ml of the clear supernatant was assayed for inorganic phosphorous (Pi) by the method of Fisk and Subbarow (19). This method consisted of adding to the sample in the following order: 0.4 ml 2.5 M $H_2SO_4$, 0.5 ml 2.5% ammonium molybdate, and 0.5 ml Fisk-Subbarow reagent

(composition shown in Table 1). Distilled water was then added to give a total volume of 10 ml. The optical density was then measured at 700 nm (Coleman Junior II) after exactly 10 min. Standard curves were prepared periodically throughout the study.

A typical reaction was initiated by adding the preheated reaction mixture to a premeasured amount of enzyme preparation. The enzyme preparation was kept in an ice bath and transferred to a waterbath in which the reaction mixture was preheating. The reaction mixture was added immediately following the transfer of enzyme to the waterbath. Usually 0.2 - 1.0 mg of protein was added from the membrane preparation, and 0.025 - 0.05 mg protein was added from the soluble preparation. To insure rapid and simultaneous initiation, the reaction mixture was added to the enzyme with an automatic pipet (Cornwall). Reactions were done in duplicate. Control preparations to detect non-enzymatic hydrolysis of ATP were prepared by adding 5% $HClO_4$ to the enzyme before adding the reaction mixture. This mixture was used to zero the spectrophotometer.

## Dry Weight Determination

The dry weights were determined with aliquots of cells that had been resuspended in distilled water and dried to constant weight at 90°C.

## Determination of Protein

Protein concentration was determined by the method of Lowry *et al.* (36) using bovine serum albumin as a standard.

Table 1. Composition of Fisk-Subarrow Reagent

| Stock Reagent, mix: | | |
|---|---|---|
| 1, 2, 4 | Amino-naphtholsulfonic acid | 2.5 g |
| | Sodium bisolfate | 124.5 g |
| | Sodium sulfite | 5.0 g |

| Reagent, dissolve: |
|---|
| 1.2 g stock powder in 20 ml distilled water |

## Reagents

Unless otherwise noted, chemicals used in this study were all reagent grade. DNase type I, lysozyme (egg white), ATP (disodium salt) and bovine serum albumin were purchased from the Sigma Company and were rated grade I.

# CHAPTER III

# RESULTS

## Enzyme Characterization

### Localization of the Enzyme; Effects of DNase vs. Sonication in Preparing Crude Fractions

*Bacillus stearothermophilus* was grown and harvested as described in Materials and Methods. Crude membrane and crude soluble fractions were assayed for ATPase activity. As shown in Table 2, the highest specific activity resided in the crude membrane fraction in both high CL and control preparations when DNase was employed. When sonication was used, the specific activity was more evenly divided between the soluble and membrane fractions. The distribution of total ATPase activity was also significantly affected by sonication and DNase treatment. As opposed to sonication, DNase treatment resulted in more of the total ATPase activity in the membrane fractions. The effect of a 300,000 x g centrifugation of a sonified preparation is shown in Fig. 3. Only about 20% of the total ATPase activity was recovered in the membrane fractions after centrifugation at 30,000 x g, whereas about 50% was recovered in the pellet after centrifugation at 300,000 x g for 2 h.

### Determination of Optimum Conditions for Enzyme Activity

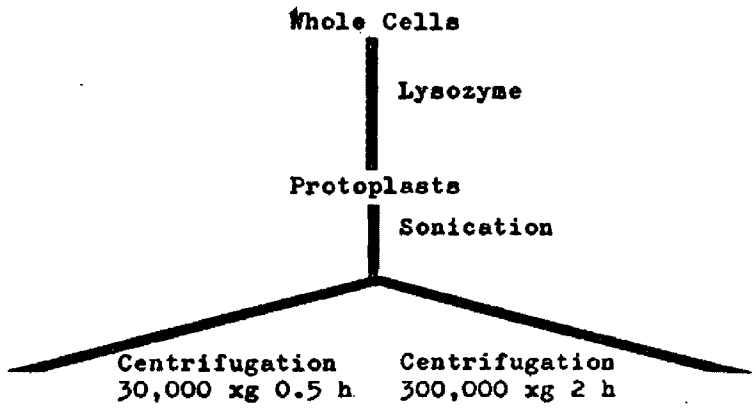
pH Optimum. High CL and control crude membrane preparations were assayed over a pH range from 5.5 to 9.2. All buffers were

Table 2. The Effects of Sonication vs. DNase on the Distribution of ATPase Activity[a] in Crude Soluble and Crude Membrane Fractions from High CL and Control Cells.

| | Sonication | | DNase | |
|---|---|---|---|---|
| | Units[b] / mg Protein | % Total Units | Units / mg Protein | % Total Units |
| Control Membrane Fraction | .5354 | 20.0 | .7193 | 67.0 |
| High CL Membrane Fraction | .4290 | 16.2 | .8064 | 87.0 |
| Control Soluble Fraction | .4516 | 80.0 | .1612 | 33.0 |
| High CL Soluble Fraction | .4700 | 83.8 | .2075 | 13.0 |

a. The reaction mixture for determining ATPase activity consisted of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$ and 2.5 mM ATP at 55°C.

b. A unit of activity is that amount of enzyme that liberates one u mole of Pi per minute under the reaction conditions described above.




| Pellet | % Activity | Super-natant | % Activity |
|---|---|---|---|
| Control | 22 | Control | 78 |
| High CL | 20 | High CL | 80 |

| Pellet | % Activity | Super-natant | % Activity |
|---|---|---|---|
| Control | 49 | Control | 51 |
| High Cl | 47 | High CL | 53 |

Figure 3. Preparation and Centrifugation of High CL and Control Cell Lysates; The Effects of Sonication on the Distribution of ATPase Activity. Percent activity was determined from the activities of the supernatant and pellet (membrane) fraction. The enzyme assay was performed in 50 mM borate-borax buffer, 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

adjusted to the proper pH at 60°C. Both the high CL and control crude membrane fractions exhibited a pH optimum between 8.2 and 8.9 (Fig. 4). In all subsequent experiments, the pH of the reaction mixture was kept at 8.4 with either borate-borax or Tris-HCl buffer.

Determination of Optimum Divalent Cation Concentration. High CL and control crude membrane fractions were employed in testing $Ca^{2+}$ and $Mg^{2+}$ ions to determine the optimum divalent cation concentration for ATPase activity. The results, shown in Fig. 5, indicated that optimum ATPase activity in both high CL and control preparations was obtained with between 1.0 and 1.5 mM $MgCl_2$ ($Mg^{2+}$/ATP = 0.4-0.6). In subsequent studies the enzyme assay was performed using 1.0 mM $MgCl_2$.

Determination of Optimum Temperature for Activity. Both crude soluble and crude membrane-bound enzymes were assayed at temperatures ranging from 40°C to 75°C. The temperature optimum for both high CL and control crude membrane fractions was 64°C (Fig. 6). The optimum temperature for the crude soluble enzyme was between 56°C and 59°C. Subsequent ATPase assays were, unless otherwise noted, performed at 60°C.

Thermal Stability of the Crude Membrane-Bound and Soluble Enzyme; The Effects of CL Concentration and $Mg^{2+}$ Ions

The possibility that the membrane could confer increased thermostability to membrane-associated ATPase was examined by incubating both crude membrane-bound and crude soluble enzyme

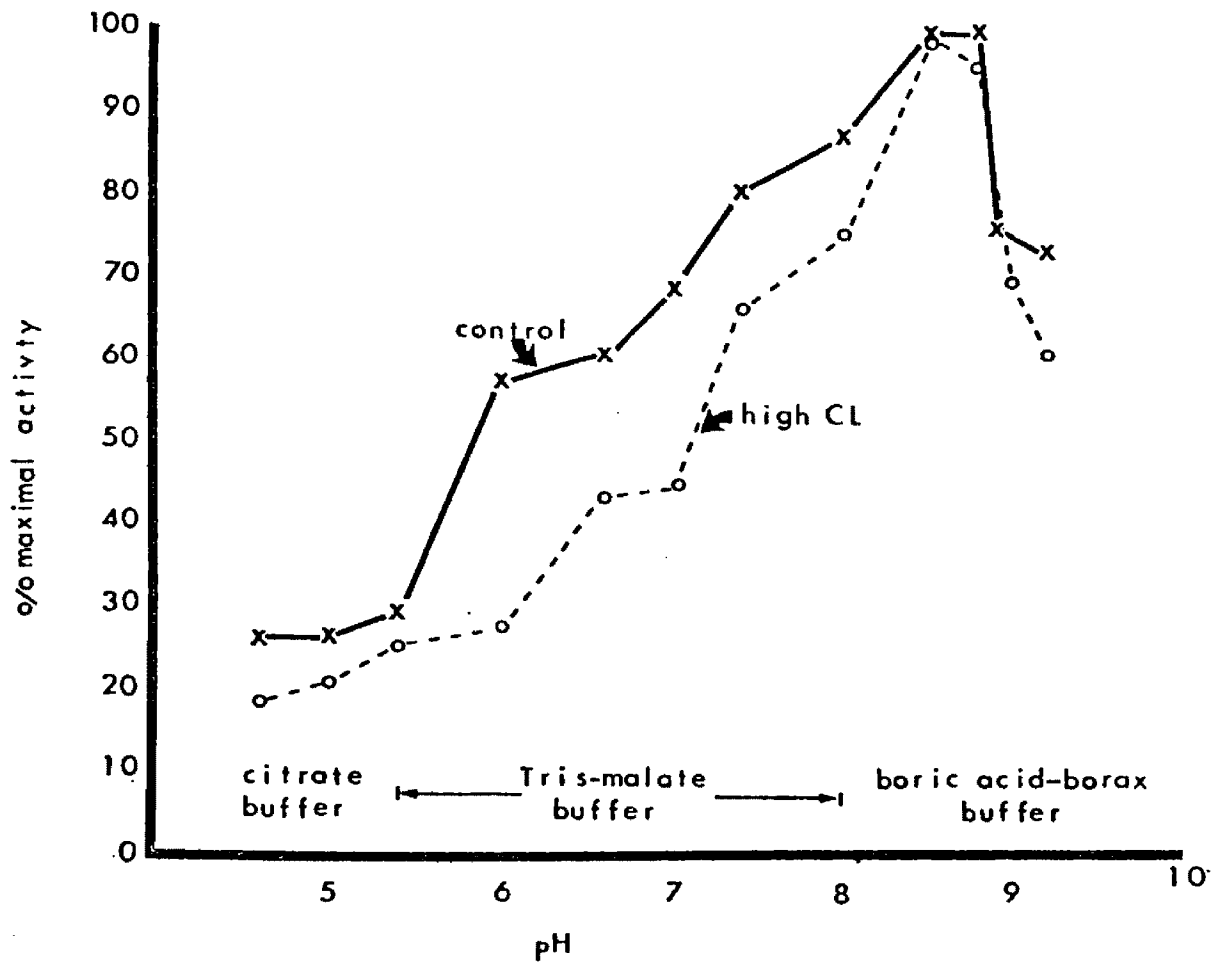



Figure 4. Determination of Optimum pH for ATPase Activity in High CL and Control Crude Membrane Fractions. The reaction mixture for determining ATPase activity consisted of 50 mM buffer, 1.2 mM $CaCl_2$ and 2.5 mM ATP at 55°C.

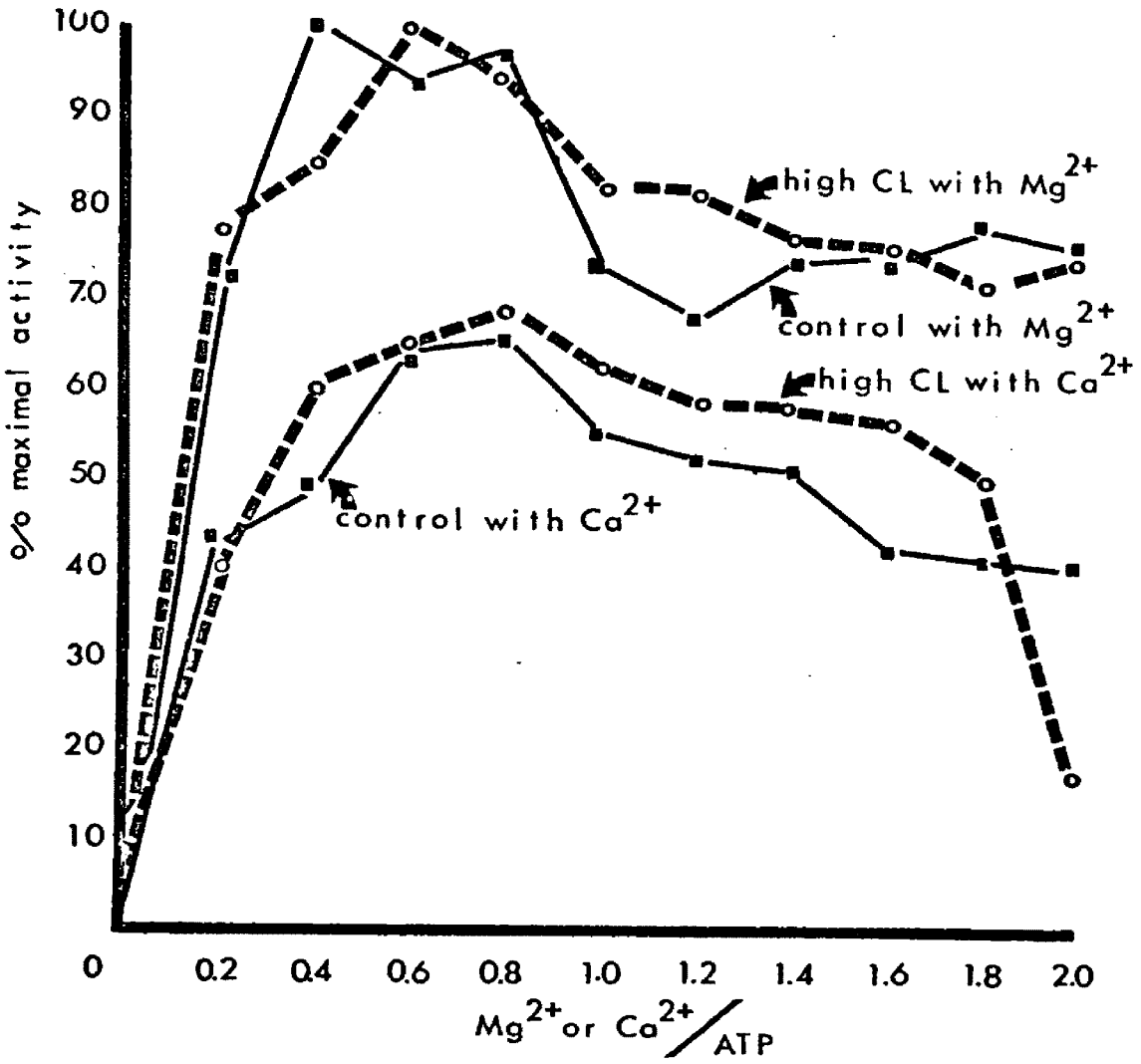



Figure 5. Optimum Divalent Cation Concentration for ATPase Activity in High CL and Control Crude Membrane Fractions. The enzyme assay was performed in 50 mM borate-borax buffer (pH 8.4), 2.5 mM ATP at 55°C. Cation concentrations are shown as a ratio in which ATP concentration remained constant throughout.

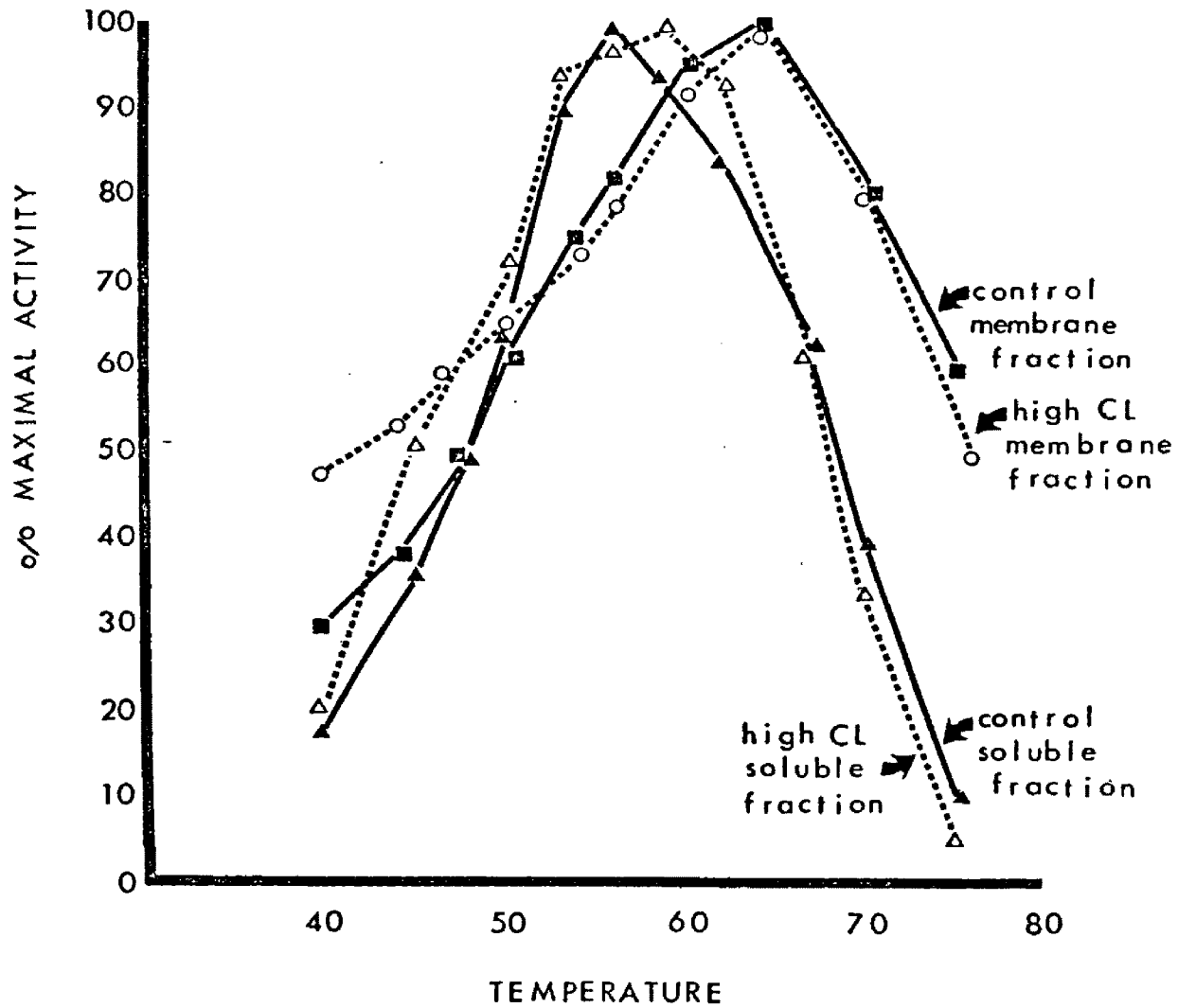



Figure 6. Optimum Temperature for ATPase Activity in Crude Soluble and Crude Membrane Fractions. The enzyme assay was performed in 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP.

preparations at 80°C, 75°C and 70°C in the presence or absence of 0.1 M $MgCl_2$ in 50 mM borate-borax buffer (pH 8.4) and then assayed at 60°C under optimum conditions. At 80°C (Figs. 7, 8) the effects of the membrane and $MgCl_2$ in stabilizing ATPase activity were clearly evident. The control crude membrane preparations remained quite stable (Fig. 7) whereas the activity of the soluble preparation dropped to zero (Fig. 8).

Prior incubation at 75°C (Figs. 9, 10) yielded somewhat different results. Again the crude membrane-bound preparations remained stable. However, the high CL membrane preparation, which showed an initial rise and then a rapid drop in activity at 80°C, now showed a rise and no subsequent drop (Fig. 9). The activity of the crude soluble enzyme showed a gradual decrease in activity (Fig. 10). Also, $MgCl_2$, which was necessary for the stability of the membrane fractions at 80°C, was no longer required for their stability at 75°C.

Prior incubation of the samples at 70°C (Figs. 11, 12) resulted in the gradual loss of activity in the crude membrane preparation in the presence of $Mg^{2+}$ (Fig.11), whereas the soluble preparation remained stable (Fig. 12).

The loss of activity at 70°C in the crude membrane preparations and not in the soluble preparations suggested that perhaps some metabolic activity not evidenced at 80°C or 75°C might be inhibiting ATPase activity in the crude membrane preparations. This possibility was examined by incubating crude soluble and crude membrane preparations at 65°C in the presence of $MgCl_2$ at

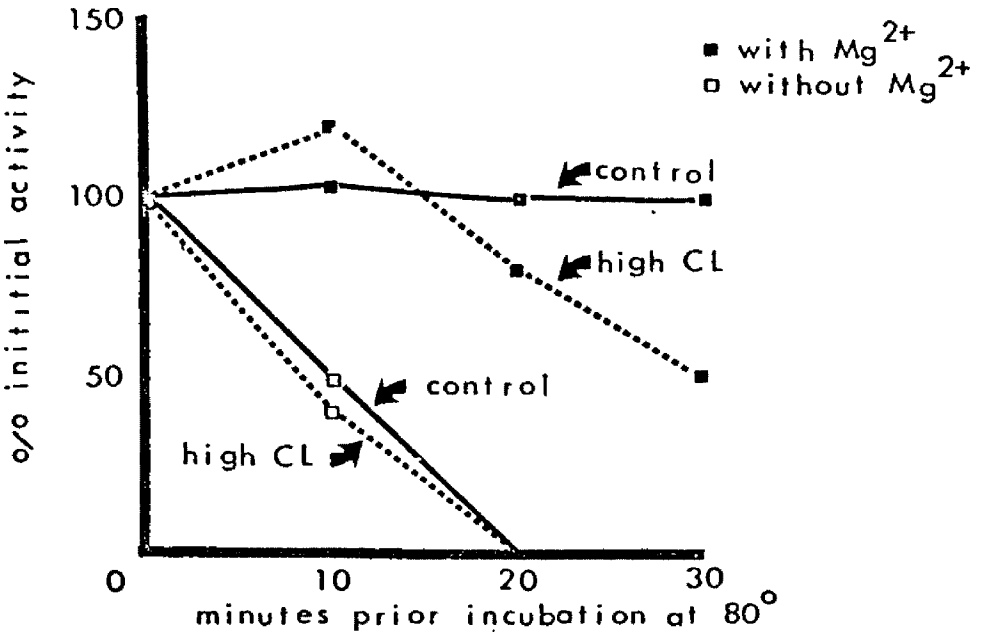



Figure 7. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Membrane Fractions at 80°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

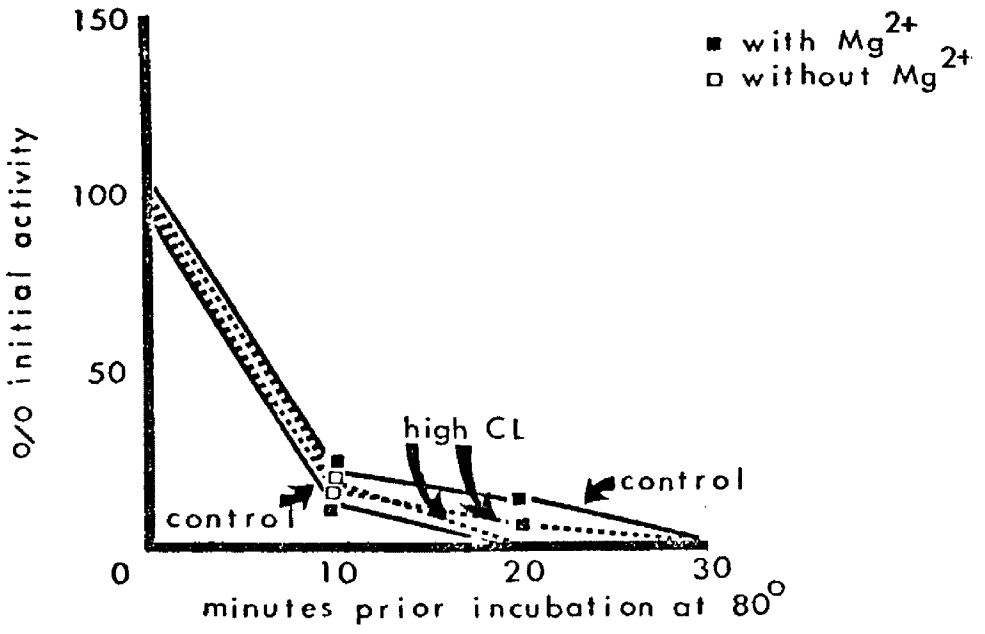



Figure 8. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Soluble Fractions at 80°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

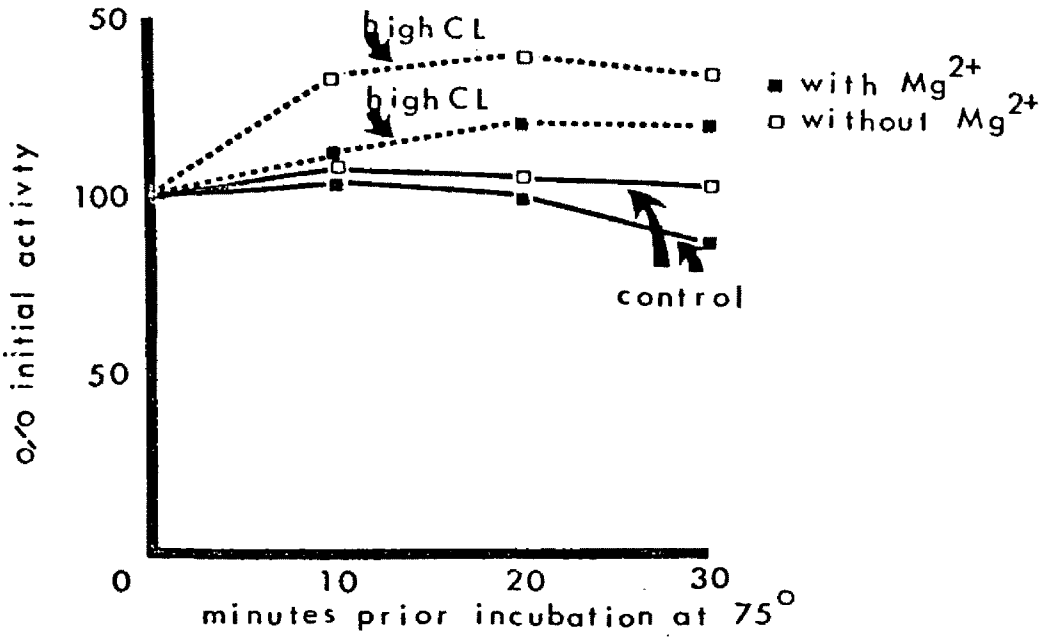



Figure 9. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Membrane Fractions at 75°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

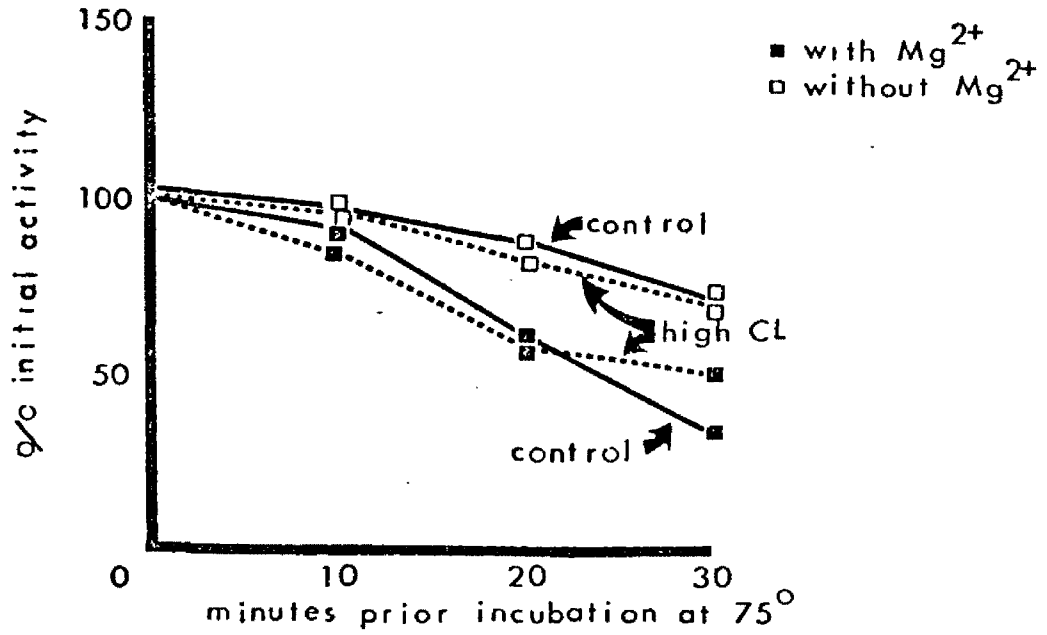



Figure 10. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Soluble Fractions at 75°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

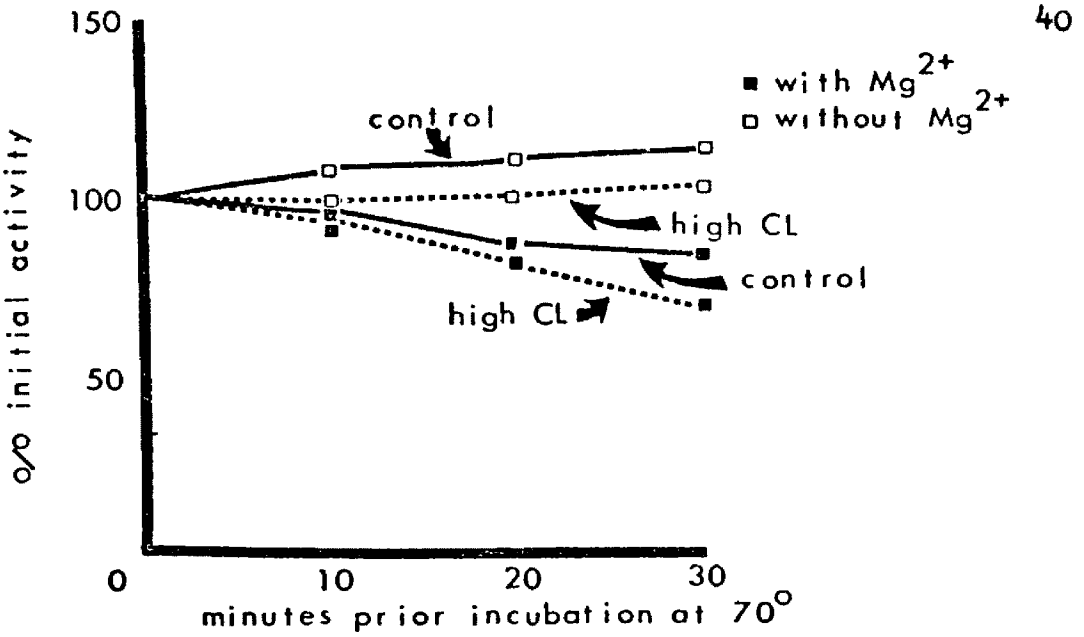



Figure 11. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Membrane Fractions at 70°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

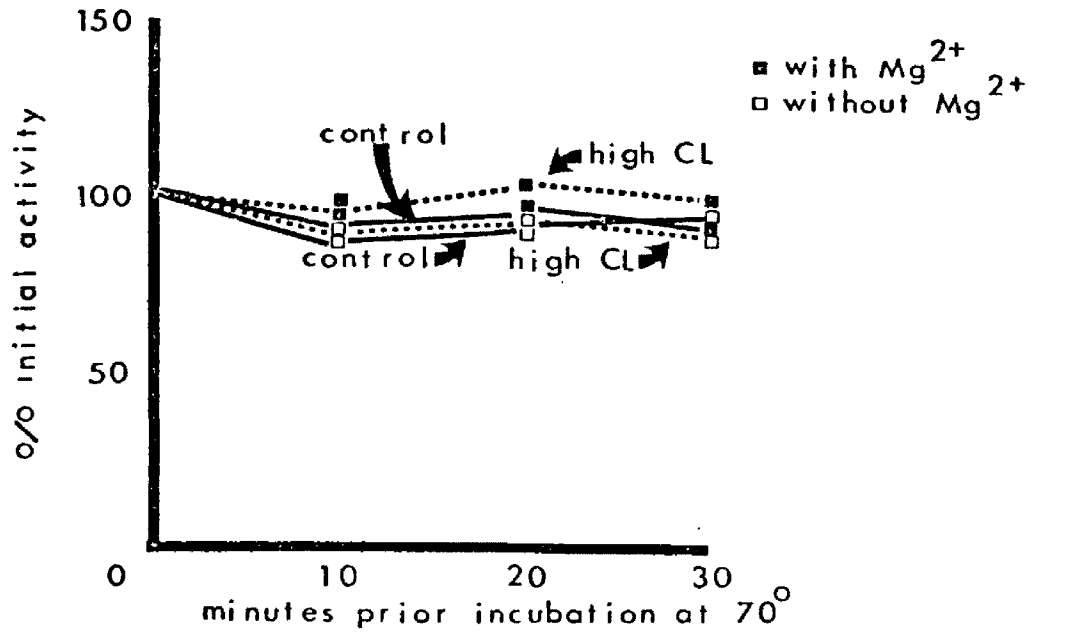



Figure 12. The Effects of $Mg^{2+}$ on ATPase Activity During Prior Incubation of Control and High CL Crude Soluble Fractions at 70°C. The prior incubation mixtures designated as "with $Mg^{2+}$" contained 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Those mixtures designated "without $MgCl_2$" consisted of 50 mM borate-borax buffer (pH 8.4). Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

a lower pH. The results of prior incubation of samples at pH 5.7 and pH 8.4 are shown in Figs. 13, 14. At pH 5.7 both the control and high CL crude membrane preparations remained stable, whereas at pH 8.4 the control membrane preparation fell off rapidly (Fig. 13). The soluble fraction remained stable at both pH 5.7 and pH 8.4 (Fig. 14).

## Solubilization and Reassociation of ATPase from High CL and Control Membrane Preparations

### Solubilization of Membrane-Associated ATPase

The results of the thermostability studies at 80°C suggested that the high CL membrane preparation might be releasing ATPase during the incubation, thus making the enzyme thermolabile, resulting in the rise and subsequent fall in activity shown in Fig. 7. This possibility was examined in experiments designed to show if CL concentrations could affect (1) ATPase binding characteristics and (2) ATPase binding capacity of the membrane.

The procedure used to solubilize ATPase from the membrane is described in Materials and Methods. An abbreviated procedure is shown in Fig. 15. The supernatant fractions ($S_1$ - $S_6$) and the final pellet fraction ($P_1$) from the high CL and control membrane preparations were assayed for ATPase activity. The results, shown in Table 3, indicated that both membrane preparations released approximately the same amount of protein; however, the high CL preparation selectively retained ATPase activity.

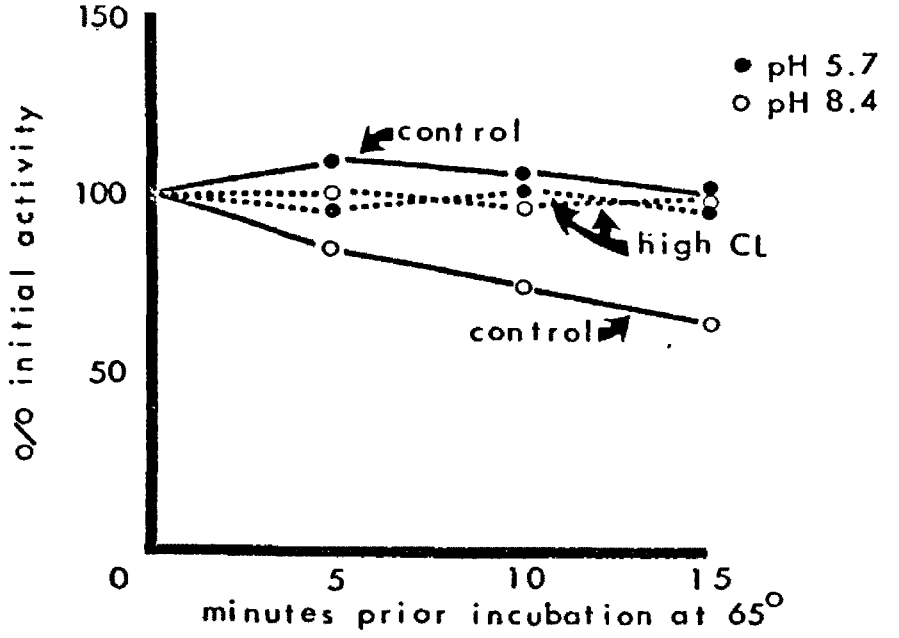



Figure 13. The Effect of pH on ATPase Activity During Prior Incubation of Control and High CL Crude Membrane Preparations at 65°C. The prior incubation mixtures at pH 5.7 consisted of 50 mM acetate buffer (pH 5.7) and 0.1 M $MgCl_2$. At pH 8.4 the prior incubation mixture consisted of 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

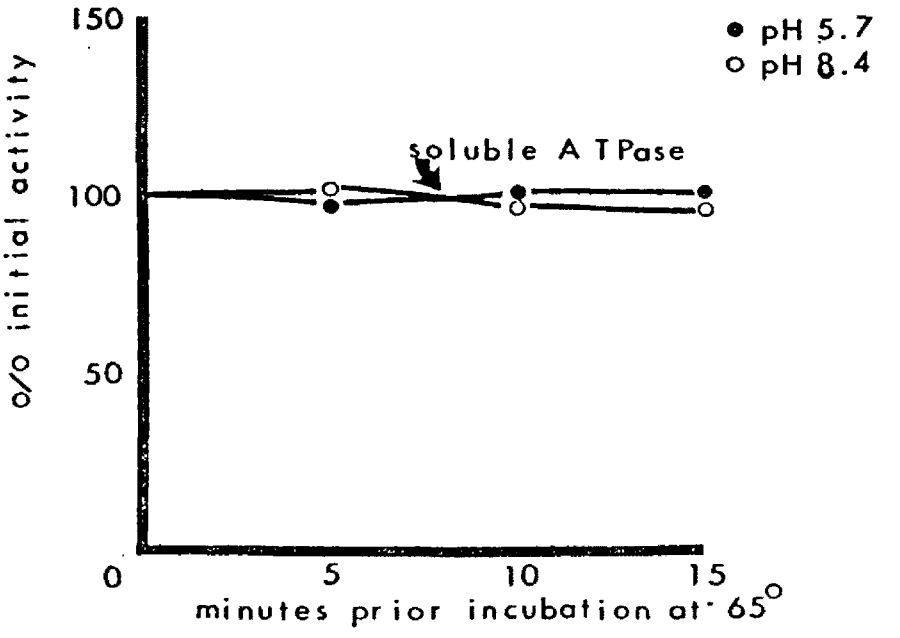



Figure 14. The Effect of pH on ATPase Activity During Prior Incubation of Soluble ATPase at 65°C. The prior incubation mixtures at pH 5.7 consisted of 50 mM acetate buffer (pH 5.7) and 0.1 M $MgCl_2$. At pH 8.4 the prior incubation mixture consisted of 50 mM borate-borax buffer (pH 8.4) and 0.1 M $MgCl_2$. Enzyme activity was determined in a reaction mixture consisting of 50 mM borate-borax buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C. Soluble ATPase (prepared as shown in Figure 27 from both high CL and control membrane preparations was used to obtain the data shown in Figure 14.

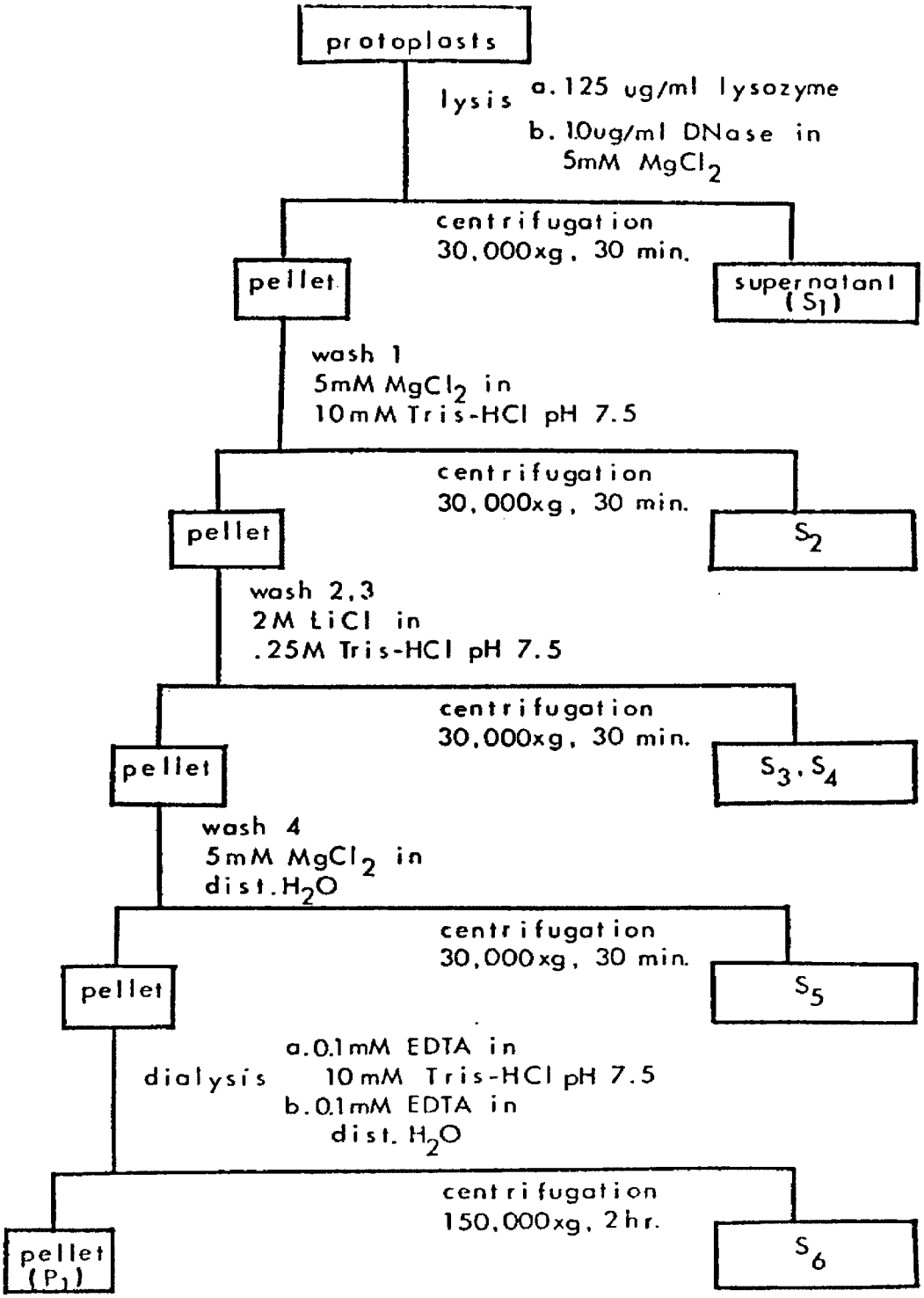



**Figure 15. Flow Sheet of the Preparation of Fractions $S_1$-$S_6$ and $P_1$.**

Table 3. ATPase Activities of Fractions $S_1$-$S_6$ and $P_1$.

| fraction | o/o total protein | | o/o total units[a] | |
|---|---|---|---|---|
| | control | high CL | control | high CL |
| $S_1$ | 75.5 | 75.7 | 23.1 | 17.1 |
| $S_2$ | 9.8 | 7.4 | 4.1 | 1.2 |
| $S_3$ | 3.6 | 3.3 | 2.9 | 0.0 |
| $S_4$ | 1.6 | 1.4 | 5.9 | 2.0 |
| $S_5$ | 0.1 | 0.02 | 1.6 | 0.6 |
| $S_6$ | 1.0 | 1.3 | 53.9 | 55.7 |
| $P_1$ | 8.2 | 10.7 | 8.2 | 23.3 |

a. ATPase activity was determined in a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$ and 2.5 mM ATP at 60°C.

## Reassociation of Solubilized ATPase with the ATPase-Depleted Membrane Fractions

Conditions for reassociation of solubilized ATPase to the depleted membrane preparations are discussed in Materials and Methods. The results (Table 4) indicated that the solubilized ATPase was bound to just under the original capacity of the non-depleted membrane. Neither the control nor the high CL preparations exhibited levels of ATPase activity above those found in the non-depleted control preparations.

## Kinetic Properties of Membrane-Bound and Soluble ATPase at 60°C and 45°C

Soluble and membrane-bound enzyme preparations for these experiments represent soluble ATPase and the corresponding depleted membrane fraction. The two LiCl washes (see Materials and Methods) were omitted in preparing these fractions so that the membrane fractions would have a more representative population of proteins.

The $K_m$ and $V_{max}$ of the control and high CL soluble and membrane-bound preparations at 60°C and 45°C were determined by graphing the reciprocal of velocity (where v = moles Pi liberated/min/mg protein) versus the reciprocal of the substrate concentration in millimoles (Lineweaver-Burk plot). From the results at 60°C (Fig. 16) a $K_m$ of 1.8 mM was calculated for all the fractions. At 45°C (Fig. 17) the results indicated that the $K_m$ for the soluble fractions remained at 1.8 mM, whereas the control membrane-bound had a $K_m$ value of 1.1, and the $K_m$ of the high CL membrane fraction was 0.35 mM. The relative changes in the $K_m$ and $V_{max}$

Table 4. Reassociation of Soluble ATPase with Depleted Membranes from High CL and Control Cells.

| | | ATPase bound to membranes | |
|---|---|---|---|
| soluble ATPase[a] added units/ml | | undepleted membranes units/ml | depleted[b] membranes units/ml |
| none | control | 8.514 | 4.450 |
| | high CL | 6.772 | 5.224 |
| 9.40 | control | 8.901 | 8.127 |
| | high CL | 6.966 | 6.579 |

a. Soluble ATPase was prepared from the membrane fractions of high CL and control cells as shown in Figure 2 except that the two LiCl washes were omitted. The soluble ATPase was precipitated with $(NH_4)_2 SO_4$ as described in Materials and Methods.

b. Depleted membrane fractions from control and high CL cells were prepared as shown in Figure 2 except that the two LiCl washes were omitted.

c. Units of activity were determined in a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at 60°C.

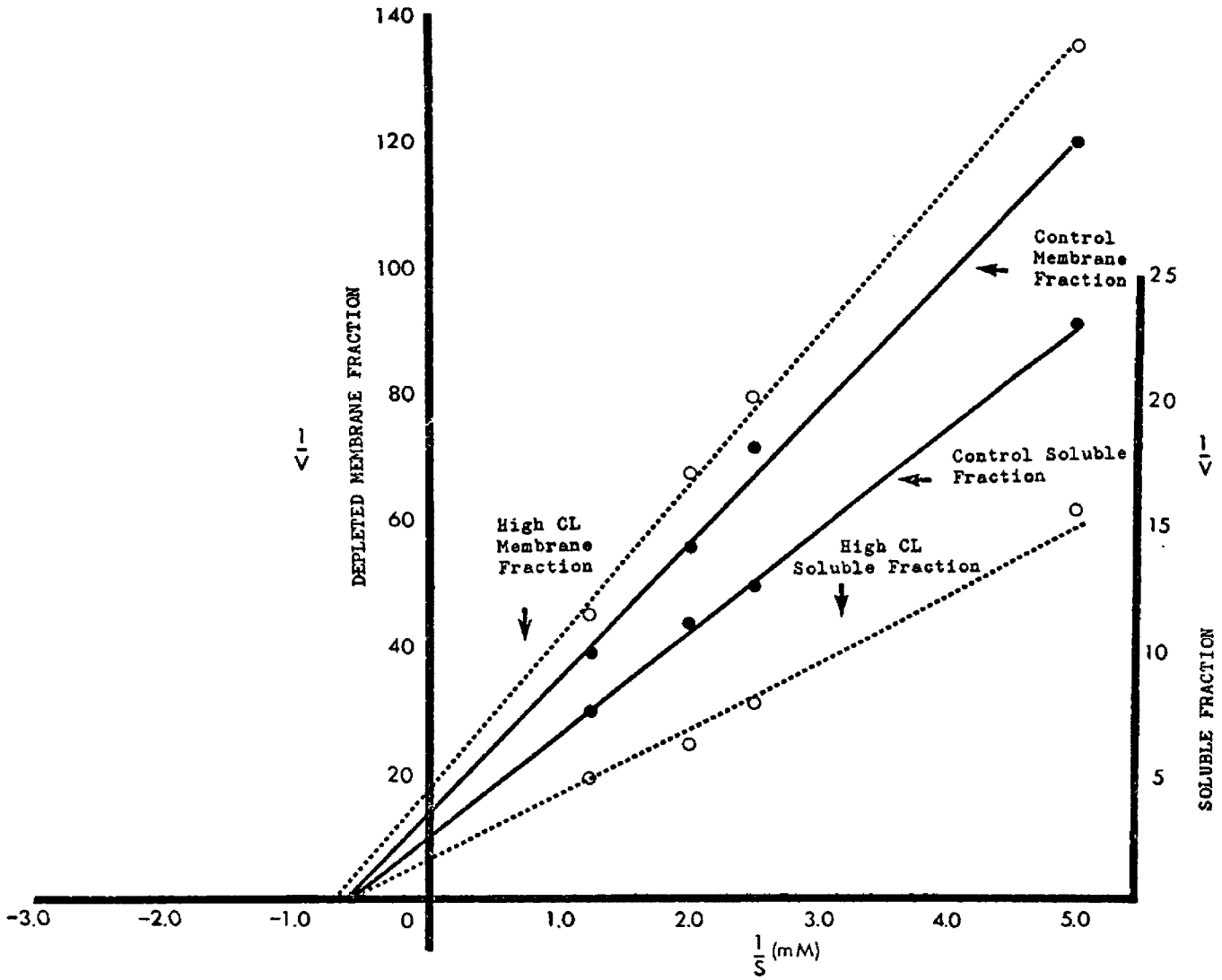



Figure 16. Kinetics of ATPase Activity in Soluble ATPase and Depleted Membrane Fractions of High CL and Control Cells at 60°C. V=u moles Pi liberated/min/mg protein under the following reaction conditions: 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$ at 60°C (substrate concentrations in millimoles are shown in the Figure).

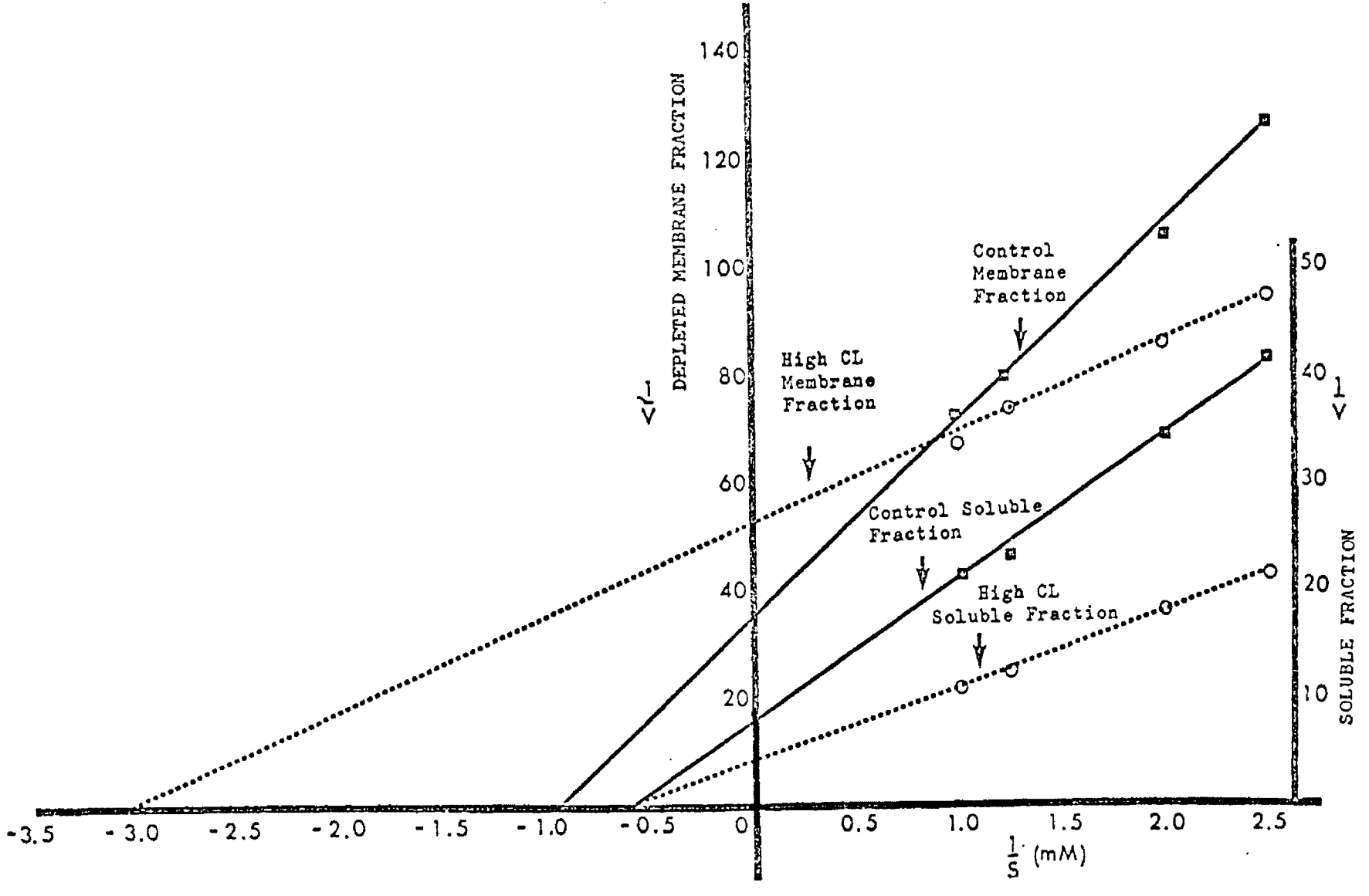



Figure 17. Kinetics of ATPase Activity in Soluble ATPase and Depleted Membrane Fractions of High CL and Control Cells at 45°C. V=u moles Pi liberated/min/mg protein under the following reaction conditions: 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$ at 45°C (substrate concentrations in millimoles are shown in the Figure).

at 60°C and 45°C are shown in Table 5. The values indicated that a uniform change (lowering) of $V_{max}$ occurred in all the fractions. The $K_m$ for the soluble fractions did not change. The $K_m$ for the membrane fractions, however, showed a threefold change in $K_m$ between 60°C and 45°C.

## The Effects of Temperature on the Kinetics of Membrane-Bound and Soluble ATPase

Membrane-associated enzymes often show a change in activation energy, i.e., discontinuity in Arrhenius plot (described in Materials and Methods). Raison (50) has suggested that in many cases, the change in activation energy is associated in some way with the physical properties of the membrane. The temperature at which a change in activation energy occurs has been shown in some cases (50) to occur at the phase transition temperature of the membrane lipids. Phase transition temperatures have not been determined for B. stearothermophilus; however, if the temperature at which there was a change in activation energy was influenced by the membrane, then we would expect to see a modification of any change, either in position (i.e., temperature) or in magnitude, in the activation energy as the membrane lipid composition was altered.

By growing B. stearothermophilus at 45°C, 50°C, 55°C and 60°C, characteristic concentrations of branched-chained fatty acids (68) as well as characteristic concentrations of CL are present in the membranes. The percent CL present in cells grown at the various temperatures is as follows: 27% at 60°C, 18% at

Table 5. Values of $K_m$ and $V_{max}$ from Figures 16 and 17, and the Comparison of the Differences in $K_m$ and $V_{max}$ Between the High CL and Control Preparations at 60° and 45°C.

| Fractions | $K_m$ (mM) | | $V_{max}$ | | $\frac{\text{High CL } 60°/45°}{\text{Control } 60°/45°}$ | |
|---|---|---|---|---|---|---|
| | 60° | 45° | 60° | 45° | $V_{max}$ | $K_m$ |
| Control Depleted Membrane Fraction | 1.8 | 1.1 | 0.0833 | 0.0290 | 3.40 | 1.14 |
| High CL Depleted Membrane Fraction | 1.8 | 0.32 | 0.0688 | 0.0190 | | |
| Control Soluble Fraction | 1.8 | 1.8 | 0.4000 | 0.1660 | 1.00 | 0.93 |
| High Cl Soluble Fraction | 1.8 | 1.8 | 0.666 | 0.2857 | | |

55°C , 14% at 50°C and 9% at 45°C (Card et al. in press). The membrane and soluble fractions from these cells could then be used to determine if CL concentration and/or branched-chained fatty acid concentration could influence the activation energy of ATPase.

Depleted membrane and soluble fractions were prepared from cultures of B. stearothermophilus grown at 45°C, 50°C, 55°C and 60°C. These fractions were assayed over a temperature range from 40°C - 64°C. The activities were plotted as described in Materials and Methods. Fig. 18 shows the plots of the depleted membrane and soluble fractions. The plots have been separated in order that the slopes and breaks (corresponding to the activation energy and a change in activation energy respectively) may be seen more clearly; they do not represent a comparison in activity of the fractions. As shown in Fig. 18 the change in activation energy associated with the depleted membrane fractions exhibited a gradual shift towards higher temperatures (indicated by the arrows) with higher growth temperature. Also, the activation energy tended to increase at post-transition temperatures (i.e., 64°C ≃56°C) with decreasing growth temperatures as indicated by the increasing slopes of the lines over the post-transition range (see Table 6). The soluble enzyme fraction exhibited, with one exception, a change in activation energy at 56°C, and the activation energies of the soluble preparation remained constant at post-transition temperatures (see Table 6).

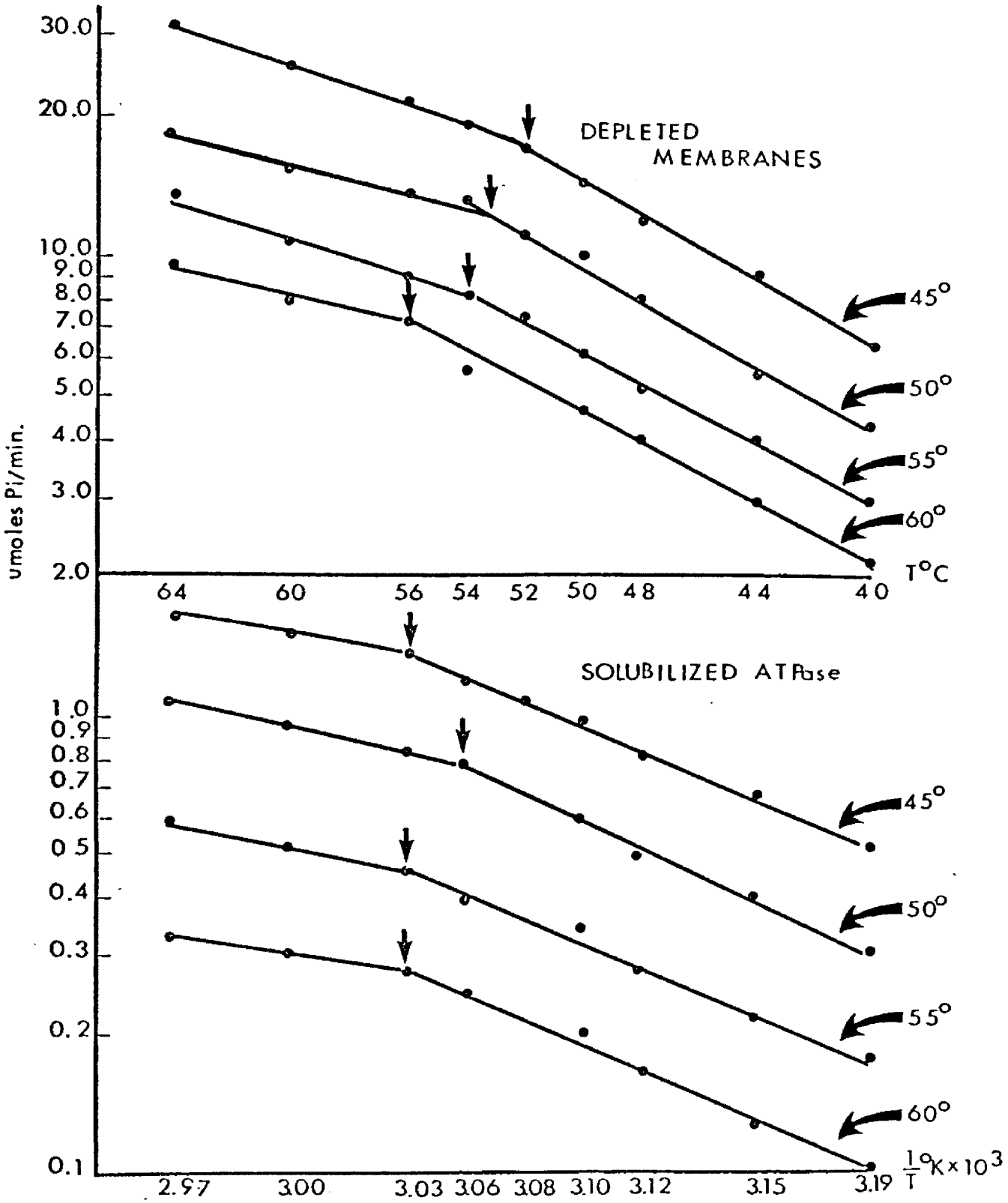



Figure 18. Arrhenius Plots of Soluble and Depleted Membrane Fractions from Cells Grown at 45, 50, 55 and 60°C. Enzyme activity was determined in a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at the temperatures shown.

The effects of high CL were next tested to determine if CL concentration alone would influence the activation energy. Cells for this experiment were grown at 60°C. Control and high CL depleted membrane fractions were used to obtain the data shown in Fig. 19. It is evident that in the high CL membrane preparations there was a reduction in the magnitude of the change in activation energy over the control preparations. The high CL membrane preparations showed a lower activation energy over the entire temperature range tested. Values of E in Kcal/mole for Figs. 18, 19 (Table 6) indicated that the high CL membrane influenced the activation energy at pre-transition temperatures. At post-transition temperatures the activation energy was the same as that for the control depleted membrane fractions and the soluble enzyme from cells grown at 60°C.

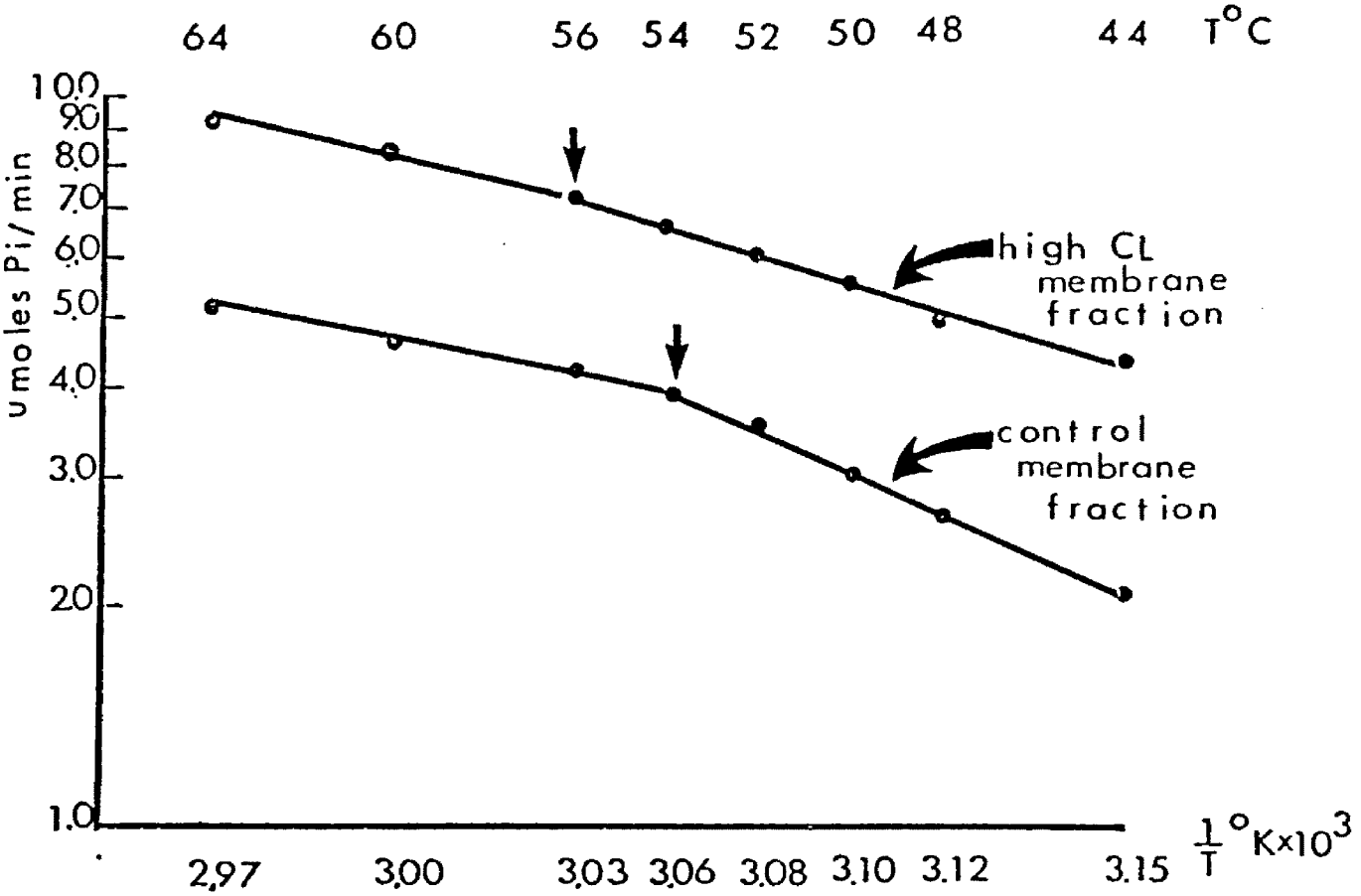



Figure 19. Arrhenius Plots of High CL and Control Depleted Membrane Fractions from Cells Grown at 60°C. Enzyme activity was determined in a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 8.4), 1.0 mM $MgCl_2$, 2.5 mM ATP at the temperatures shown.

Table 6. Activation Energies for the Fractions Shown in Figures 18 and 19.

| Fraction | Growth Temperature | Activation Energy[a] ($\frac{K\ cal}{mole}$) Post-Transition (64°-≃56°C) | Pre-Transition (56°-44°C) |
|---|---|---|---|
| Depleted Membrane | 45 | 13.0 | 15.0 |
| | 50 | 10.4 | 17.0 |
| | 55 | 11.0 | 15.0 |
| | 60 | 6.5 | 15.0 |
| Soluble | 45 | 6.5 | 15.0 |
| | 50 | 6.5 | 15.0 |
| | 55 | 6.5 | 15.0 |
| | 60 | 6.5 | 15.0 |
| Control Depleted Membrane | 60 | 6.5 | 15.0 |
| High CL Depleted Membrane | 60 | 6.5 | 9.0 |

a. Activation energies were calculated as described in Materials and Methods.

# CHAPTER IV

## DISCUSSION

In this study the activity of a membrane-associated ATPase from Bacillus stearothermophilus was shown to be influenced by changes in the phospholipid composition of the membrane. Under a number of experimental conditions, both the binding characteristics and the kinetic and thermodynamic properties of the enzyme were influenced by the alternation of membrane phospholipid.

The first part of the study, to locate and establish optimum conditions for activity, revealed that both the high CL and control membrane preparations exhibited maximal activity under the same assay conditions (Figs. 4, 5, 6). The treatment of the cell lysates to reduce viscosity prior to centrifugation of lysed suspension had a significant effect on the distribution of the enzyme in the crude soluble and crude membrane fractions. The membrane phospholipid composition also had an effect on this distribution (Table 2). DNase treatment produced an overall higher specific activity in the membrane fractions of both high CL and control cells compared to that seen when sonication was employed (Table 2). The specific activity of the high CL membrane preparations was higher than the control preparation when DNase was used, whereas the specific activity of the control membrane preparation was higher than the high CL membrane when sonication was employed. The amount of ATPase recovered in the membrane (30,000 x g pellet) fraction could be influenced by two factors: (1) the

release of individual ATPase molecules (perhaps as small lipoprotein units) or (2) the release of small membrane fragments (vesicles) which would not sediment at 30,000 x g. If the difference in the amounts of ATPase recovered in the 30,000 x g pellet fractions shown in Table 2 resulted from the first possibility, then the treatment might be influencing the binding of ATPase itself. On the other hand, if the ATPase is released with membrane vesicles, then the treatment might be influencing the general structure of the membrane rather than the specific binding of ATPase. From the results shown in Fig. 3, it appeared that most of the ATPase activity remaining in the supernatant fluid was present as free enzyme or at least particles so small that they remained in the supernatant after centrifugation at 300,000 x g for 2 h.

Both the high CL and control membrane preparations exhibited a higher temperature maximum than the crude soluble preparations (Fig. 6). It was also noted (Fig. 6) that the curves for the membrane-associated enzyme exhibited a broad temperature range, whereas the soluble enzyme showed a rather sharp optimum temperature for activity. This suggested that the enzyme associated with the membrane was more thermostable and required higher temperatures for maximal activity.

There have been many reports attempting to relate the thermostability and, indeed, the thermophilic nature of obligate thermophiles to some physiological characteristic of the organism. Two general hypotheses have been considered. One is that the

components of the thermophile are not necessarily thermostable but are rapidly replaced as they are denatured (10, 11). This hypothesis was supported by the studies of Allen (3, 4), who found that thermophiles lost viability as rapidly as mesophiles when heated at 65°C in a non-growth medium. The addition of nutrient to the medium allowed growth of the thermophile but did not prevent death of the mesophile. The other hypothesis is that molecular components of thermophiles are innately more thermostable than those from mesophiles. This stability could be an inherent property of the molecule or could be due to some protective factor(s) or both (32). The inherent thermostability of macromolecules from thermophiles has been demonstrated for a number of enzymes (37) and ribosomes (57) from B. stearothermophilus. On the other hand, Ljunger (35) has reported that if calcium and glucose were added to a suspension of B. stearothermophilus in Tris-HCl buffer, cell viability, when incubated at 65°C, was dramatically prolonged. He suggested that calcium was required for the stabilization of cellular proteins against heat denaturation.

ATPase from B. stearothermophilus has been shown to be thermostable at 65°C both in the soluble and membrane-bound states (38, 39). In the study presented here, much higher incubation temperatures were employed because our concern in this study was not so much with the nature of thermophily but rather with the question of whether the membrane has any influence on the structural properties of the enzyme molecule.

The thermostability studies, the second part of this study, demonstrated that the crude membrane-bound enzyme was more thermostable at 80°C and at 75°C than were the crude soluble fractions (Figs. 7, 8, 9, 10) and that $Mg^{2+}$ increased the stability of the membrane-bound enzyme (Fig. 7). This protective effect of $Mg^{2+}$ appeared to result from the interaction of $Mg^{2+}$ with the membrane or the membrane-enzyme complex rather than the enzyme alone because $Mg^{2+}$ had no protective effect on the soluble enzyme at any of the temperatures tested (Figs. 8, 10, 12). In fact, at 75°C (Fig. 10) the preparation containing $Mg^{2+}$ was less stable than the preparation without added $Mg^{2+}$. The crude membrane preparations showed a characteristic increase in activity on continued incubation at temperatures below 80°C (Figs. 9, 11). This increase in activity was more pronounced in preparations without added $Mg^{2+}$ (Fig. 9).

These results might be interpreted by considering the probable effects of $Mg^{2+}$ and CL, both of which have been shown to cause a contracting of the membrane (Dr. Card personal communication) which resulted in stabilizing the vesicles and at the same time reducing the availability of the enzyme to the substrate. This same interpretation can be used to explain the rise in activity during prior incubation of the membrane fractions at 75°C and 70°C in the absence of $Mg^{2+}$ (Figs. 9, 11). In these cases the rise in activity was more pronounced in the high CL preparations since they would have been in a more compact state than the control membrane preparations and show increasing activity

as the vesicles "unfolded" during prior incubation.

The thermostability of the control and high CL membrane preparations at 70°C (Fig. 11) was decreased in the presence of $MG^{2+}$ whereas the soluble preparations remained stable (Fig. 12). The decrease in activity was not, therefore, a result of enzyme denaturation. This apparent inconsistency was thought to be due to some form of metabolic activity occurring in the membrane fraction. Changing the pH from 8.4 to 5.7 and lowering the temperature to 65°C in the prior incubation mixture seemed to increase the stability of the membrane-bound enzyme. As shown in Figs. 13 and 14, the control (i.e., low CL) membrane preparation lost activity at pH 8.4 but not at pH 5.7. The high CL membrane fraction and soluble ATPase from both high CL and control membrane preparations showed no loss of activity at either pH. The optimum pH for CL synthesis is 5.8, and essentially no CL is synthesized above pH 7.5 (Dr. Card personal communication). Although it is evident that the high CL preparation was stable at both pH 5.7 and 8.4, caution must be exercised in relating the stability of the control preparations at pH 5.7 to an increase in CL. In a complex system such as this, the response to pH might result from some metabolic change which had nothing whatever to do with the change in phospholipid composition. It is evident, however, that the loss of activity observed was due to a membrane alteration because the soluble enzyme (Fig. 14) was not affected by pH at this temperature. Although it seemed apparent that the change in pH of the prior incubation mixture stabilized the activity of

the membrane-associated ATPase at 65°C, it was not determined if this could be the cause of the loss of activity observed at 70°C (Fig. 11). It was concluded that the factors influencing the thermostability of the membrane-bound enzyme at temperatures within physiologic range of the organism (e.g., 70-65°C) might be much more complex than at higher temperatures (e.g., 75-80°C) since any number of factors could be inactive at the higher temperatures and come into play at the lower temperatures.

The third part of this study was concerned with the binding characteristics of ATPase to control and high CL membrane preparations. One possible explanation for the loss of activity of the high CL preparation was that the release of ATPase could be more easily facilitated from the high CL membrane and thus account for the rapid loss in activity of the high CL membrane fraction when incubated at 80°C (Fig. 7). This did not prove to be the case. As shown in Table 3, the high CL membranes selectively retained ATPase while giving up approximately the same amounts of the protein as the control preparation. The evidence that ATPase was selectively held by the high CL membrane brought to mind a hypothesis originally proposed by Abrams (2). In his successful attempts to reassociate solubilized ATPase with ATPase-depleted membranes from Streptococcus fecalis, he postulated that the enzyme bound specifically to cardiolipin which was the major phospholipid constituent of the organism. The system employed for increasing the CL concentration in this study seemed an ideal way of testing Abrams' hypothesis. Since the CL alteration

occurs in whole cells, and no further synthesis of ATPase occurs subsequent to this alteration, it was necessary to show that high CL membranes depleted in ATPase could take up more ATPase during reassociation than was originally present prior to depletion. The results of the attempts to reassociate ATPase with the depleted membrane fractions are shown in Table 4. The results indicate that an increase in the CL concentration of the membrane did not result in a greater amount of activity than was originally present in the membrane prior to depletion. This suggested that there are probably a finite number of binding sites on the membrane; and that whereas CL seems to influence the strength of the bond, it does not dictate the binding sites or binding capacity of the membrane. This would seem valid since ATPase is postulated to be associated with electron transport. It would be reasonable to assume that sites for ATPase attachment would be dictated by the components involved in electron transport.

Although it was apparent from the thermostability studies that association with the membrane influenced the structural (physical) properties of the enzyme, the important question as far as the physiology of the cell is concerned is whether the membrane associations have any influence on the enzymatic properties of the ATPase. Difference in the $K_m$ of soluble and membrane-associated ATPase have been reported in *E. coli* (51, 55). In this organism a twofold increase in the $K_m$ of the solubilized enzyme over the membrane-bound enzyme was noted. In *B. stearothermophilus* the $K_m$ of the solubilized enzyme was determined by

Militzer to be 3.2 mM at 60°C (39, 41). Hachimori et al. (25, 26), using purified ATPase from B. stearothermophilus, found the $K_m$ to be 8.4 mM at 58°C. They also found that both the $K_m$ and $V_{max}$ of the enzyme showed a nonlinear relationship with increasing temperature. When determining $K_m$ and $V_{max}$ at temperatures ranging from 30°C to 65°C, they found an abrupt decrease in $K_m$ and increase in $V_{max}$ at 55°C, suggesting to them a conformational change in the enzyme at this temperature, causing an increased rate of catalysis above 55°C. The activities at 60°C of soluble and depleted membrane fractions from both high CL and control cells are shown in Fig. 16. The $K_m$ values for all the fractions were 1.8 mM, and values of $V_{max}$ were approximately additive. This suggested that the differences in activity observed in the soluble and membrane-bound preparations in previous experiments was probably due to the amount of enzyme present and not due to changes in the kinetic parameters of the enzyme. If the kinetic parameters (e.g., $K_{+2}$) of the soluble and membrane-bound enzyme are identical, then differences in activity should reflect differences in enzyme concentration.

The same assay performed at 45°C yielded very different results. In Fig. 17 the $K_m$ of the soluble and control membrane fractions remained about the same (1.8 mM). The $V_{max}$ dropped as would be expected at the lower temperatures. The fact that the $K_m$ remained unchanged in unusual considering that the lowered temperature would seem to affect the rate constants forming the E-S complex as well as affecting the rate constants for the

breakdown of the complex. However, the most interesting feature of the experiment was the drastic threefold reduction in $K_m$ in the high CL membrane preparations (Table 5). This observation indicated that membrane phospholipid could influence the catalytic properties of the enzyme by influencing the rate of formation of E-S complex. However, this influence of membrane phospholipids was manifest only at a sub-optimum temperature for catalysis (45°C).

The results of the kinetic study indicated that a potentially fruitful approach to tie in all the observations discussed above would be to determine the effect of CL on another thermodynamic parameter, the activation energy (E). Changes in the activation energy, or rather discontinuities in Arrhenius plots, are currently one of the most widely used measurements of the influence of membrane composition on membrane-associated enzymes (46, 50, 62).

Although most enzyme-catalyzed reactions show a constant activation energy over the physiological temperature range, a number of reactions have been reported where the plot of the logarithm of the velocity against the reciprocal of the absolute temperature (Arrhenius plot) shows two straight lines of different slope, i.e., a discontinuity or change in E with temperatures (50). In both procaryotic and eucaryotic organisms, enzymes associated with membranes are most frequently observed to show a change in activation energy. Raison (50) has suggested that, in most cases, the change is associated in some way with changes in the physical properties of the membrane. Evidence for membrane involvement in the activation energy of the membrane-associated

enzyme follows a basic pattern. Membrane-associated enzymes often show a discontinuity in an Arrhenius plot; the same preparation treated with detergents or the solubilized enzyme alone does not exhibit a discontinuity in an Arrhenius plot. Furthermore, a correlation can be made between the temperature at which there is a phase change of membrane lipids. This phase change can be observed by several different techniques and has been interpreted to represent a transition of the lipid bilayer from an ordered to a non-ordered state (going from temperatures below to temperatures above the transition temperature) (50). The temperature at which the phase transition occurs has been shown to be related to the fatty acid composition of the membrane lipids (50). The relative amounts of the different types of complex lipids may also influence the transition temperature, but this has not been demonstrated. Many membrane-associated enzymes show a change in activation energy which coincides with the lipid phase transition (50). Others have shown a change in activation energy at a different temperature than the lipid transition but still appear to be influenced by the lipid composition (50). Some purified enzymes (membrane free) also show changes in activation energy which are assumed to represent conformational changes in the protein molecule (17, 25, 26).

Bacillus stearothermophilus contains predominantly branched-chained fatty acids when grown at 45°C and predominantly straight-chained fatty acids when grown at 60°C (68). The lipid phase transition temperatures have not been determined for

B. stearothermophilus. However, if the change in activation energy of the ATPase was influenced by the membrane, then we would expect to see a change in the activation energy as the lipid composition of the membrane was changed. Although the differences in Fig. 18 must be regarded somewhat skeptically, it appeared that a difference of 4°C in the transition temperature had occurred in the membrane preparations from cells grown at the different temperatures. The transition temperature for the soluble enzyme from all except the "50°C cells" was 56°C (Fig. 18). This aspect requires further studies, but the tentative conclusion is that the membrane influences the temperature at which a change in activation energy occurs. The change in the activation energy of the free enzymes (Fig. 18) would appear to represent a conformational change in the enzyme molecule; however, even the free enzyme may possibly be a lipoprotein complex (26). Another aspect of the results shown in Fig. 18 was that the membrane-associated enzyme showed a trend towards increasing activation energies at post-transition temperatures (i.e., an increase in the slope of the plots at temperatures above 56°C - 54°C). The magnitude of this increase in activation energy appeared to be dependent upon growth temperature. As the growth temperature was lowered, the enzyme showed an increase in post-transitional activation energy (Table 6). Since membrane CL concentration decreases with decreasing growth temperature (Card et al. in press), it seemed that there might be some relationship between the decreasing CL and the increasing activation energy at post-transition temperature.

If CL influenced the activation energy, then the high CL membranes should show a lowering of the activation energy at post-transition temperature.

The influence of the high CL membrane on membrane-associated ATPase is shown in Fig. 19. The results suggested that CL concentration profoundly affected the activation energy at lower temperatures. However, the activation energy at post-transition temperatures was the same as the soluble enzyme and the control membrane preparation from cells grown at 60°C (Table 6). This observation did not confirm the postulated relationship of CL concentration in regulating activation energy at post-transition temperatures. It did, however, confirm that CL affects the activation energy at lower temperatures, resulting in a lower activation energy over a much broader temperature range.

These concluding experiments, which attempted to relate phospholipid composition to an alteration in the kinetic parameters of the enzyme, produced the most definitive information on how phospholipid composition affects ATPase activity. Although the kinetic data is in many ways incomplete, a number of conclusions can be drawn from these experiments. The first and foremost observation was that the temperature of growth only slightly influenced the temperature at which there was a change in activation energy of the membrane-associated enzyme. The solubilized enzyme exhibited a change in activation energy independent of growth temperature (Fig. 18). This suggested that the membrane fatty acid composition could influence the temperature at which

there occurs a change in activation energy. However, fatty acid composition appeared to have affected the temperature at which some change in the protein occurred rather than inducing the change by virtue of a phase change in membrane lipids as described by Raison (50), since the soluble enzyme underwent a change in activation energy at approximately the same temperature. Second, the membrane CL concentration appeared to have lowered the activation energy of membrane-associated ATPase over a temperature range of 40°C - 54°C (Table 6). Third, CL concentration did not appear to affect the activation energy at post-transition temperatures when growth temperature was not a factor (Table 6). The trend towards increasing activation energy at post-transition temperatures with decreasing growth temperature (Table 6) was tentatively thought to be due to the branched-chained fatty acids that appeared to influence the temperature at which the change in activation energy occurred (Fig. 18). Fourth, the membrane CL concentration appeared to have affected the $K_m$ of the enzyme at 45°C (Fig. 17).

In the above kinetic studies it was shown conclusively that phospholipid can play a direct role in catalysis. Although no model can be proposed on how the membrane phospholipid affects the catalytic process, these initial studies have shown that perhaps with further kinetic studies, a thermodynamic "profile" of the reaction could be constructed to determine which specific thermodynamic properties of the reaction are affected by the phospholipid.

The data gathered in this study have shown several ways by which phospholipid composition affects membrane-associated ATPase in B. stearothermophilus. In the first part of the study, ATPase was localized in the membrane fraction, and optimum conditions for activity were found to be at pH 8.4 in borate-borax buffer with 1 mM $MgCl_2$ at 60°C. Different temperature optima were noted for the crude soluble and crude membrane-bound ATPase. The second part of the study demonstrated that the control membrane preparation, in the presence of $Mg^{2+}$, stabilized ATPase activity at 80°C, whereas the crude soluble ATPase preparations lost 100% of their activity at 80°C. Subsequent experiments at 75°C, 70°C and 65°C showed that the membrane was instrumental in stabilizing the activity of ATPase at 75°C. At 70°C and 65°C the activity of the soluble fractions remained stable at both pH 8.4 and pH 5.7. At 70°C and 65°C the activity of the soluble fractions remained stable at both pH 8.4 and pH 5.7. The third part of the study demonstrated that the binding characteristics of ATPase were modified by the high CL membrane fraction. The high CL membrane fraction was shown to retain three times more activity when subjected to elution procedures. The CL concentration was not, however, found to increase the binding capacity of the membrane, as evidenced by attempts to reassociate ATPase to depleted high CL and control membrane preparations. In the fourth part of the study, it was found that at 60°C the $K_m$ for soluble and membrane-bound (both control and high CL) was 1.8 mM. At 45°C the $K_m$ for the high CL membrane fraction showed a threefold

decrease whereas the $K_m$ for the other fractions remained constant. The determination of the activation energy for ATPase associated with membranes each possessing characteristic branched-chained fatty acid constituents, showed that the activation energy was only slightly affected by this parameter. However, a pronounced change in activation energy was observed to occur at $\simeq 56^{\circ}C$ in both control membrane-bound and soluble fractions, and this was tentatively attributed to a conformational change in the protein. When the activation energy for a high CL membrane fraction was determined, very little change in activation energy was observed.

# CHAPTER V

## SUMMARY

The possible influence of membrane association and membrane phospholipid composition on an ATPase from Bacillus stearothermophilus was examined. Under a number of experimental conditions, it was found that ATPase activity in membrane fractions with a PG/CL ratio of 0.5 differed significantly with those possessing a PG/CL ratio of 2.0.

(1) The optimum pH for ATPase activity was between 8.3 and 8.9 in both high CL and control membrane fractions. The optimum divalent concentration for ATPase in high CL and control membrane fractions was observed with 1.0 mM $MgCl_2$ ($Mg^{2+}$/ATP = .4).

(2) The optimum temperature for activity for ATPase in high CL and control membrane fractions was at $\simeq 64^{\circ}C$. Crude soluble ATPase exhibited maximum activity at $59^{\circ}C$.

(3) The thermostability of ATPase activity in the high CL membrane fraction was decreased 50% after 30 min incubation at $80^{\circ}C$, whereas the control membrane preparations remained stable.

(4) ATPase activity in high CL membrane fractions was stable during prior incubation at $65^{\circ}C$ and pH 8.4, whereas the control membrane preparation lost 40% of its activity at the end of 15 min incubation.

(5) Membrane-bound ATPase was released by successive treatments with .01 M Tris-HCl (pH 7.5), 2 M LiCl, and .1 mM EDTA

followed by dialysis. Membranes with low concentration of cardiolipin released 92% of the total ATPase activity, whereas high cardiolipin fractions released 77%.

(6) Attempts to reassociate soluble ATPase with high CL and control ATPase-depleted membranes revealed that both high CL and control membranes took up ATPase to just under their original capacity.

(7) At 45°C the $K_m$ of ATPase associated with membranes high in CL exhibited a threefold decrease compared to that of control membrane preparations. At 60°C both high CL and control membrane preparations had a $K_m$ of 1.8 mM.

(8) The activation energy of ATPase associated with membranes high in CL was 9 Kcal/mole, whereas the activation energy for ATPase associated with control membranes was 15 Kcal/mole over a temperature range of 40°C - 54°C. The activation energy from 54°C to 64°C was 6.5 Kcal/mole in both high CL and control membrane fractions.

(9) ATPase solubilized from high CL membranes exhibited the same behavior as soluble ATPase from control cells, showing a 100% loss of activity after 30 min incubation at 80°C. The activity of soluble ATPase was stable after 15 min incubation at 65°C at both pH 5.7 and 8.4. The $K_m$ of soluble ATPase was 1.8 mM at 60°C and 45°C.

(10) Soluble ATPase underwent a change in activation energy at $\simeq$56°C. This suggested that there was a conformational change in the protein at the transition temperature of 56°C. The

activation energy of soluble ATPase was 15 Kcal/mole over a temperature range of 40°C - 56°C and was 6.5 Kcal/mole from 56°C - 64°C.

## LITERATURE CITED


1. Abrams, A. 1965. Soluble ATPase released from membranes of Streptococcus fecalis. J. Biol. Chem., 240:3575-3681.

2. Abrams, A. and C. Baron. 1967. The isolation and subunit structure of streptococcal membrane adenosine triphosphatase. Biochemistry, 6:225-229.

3. Allen, M. B. 1960. The dynamic nature of thermophily. J. Gen Physiol., 33:205-214.

4. Allen, M. B. 1953. Thermophilic aerobic spore forming bacteria. Bact. Rev., 17:125-173.

5. Baron, C. and A. Abrams. 1968. Reversible attachment of adenosine triphosphatase to streptococcal membranes and the effect of $Mg^{++}$ ions. Biochemistry, 7:501-506.

6. Baron, C. and A Abrams. 1971. Isolation of bacterial membrane protein, NECT is essential for the attachment of ATPase. J. Biol. Chem., 246:1542-1544.

7. Bertina, R. M., J. A. Steenstra and E. C. Slater. 1974. The mechanism of inhibition by oligomycin of oxidative phosphorylation in mitochondria. Biochim. Biophys. Acta., 368:279-297.

8. Bragg, P. O., D. L. Davies and C. How. 1973. Effect of removal or modification of subunit polypeptides on the coupling factor and hydrolytic activities of the $Ca^{++}$and $Mg^{++}$activated adenosine triphosphatase of Escherichia coli. Arch. Biochim. Biophys., 159:664-670.

9. Bulos, B. and E. Racker. 1968. Partial resolution of enzymes catalyzing oxidative phosphorylation. J. Biol. Chem., 243:3901-3913.

10. Bubella, B. and E. S. Holdsworth. 1966. Protein synthesis in Bacillus stearothermophilus. Biochim. Biophys. Acta., 123:366-375.

11. Bubella, B. and E. S. Holdsworth. 1966. Protein synthesis and turnover in a thermophile. Biochim. Biophys. Acta., 125:376-389.

12. Burstein, C., R. G. Morris and E. Racker. 1970. Effects of phospholipase C on the two sides of the inner mitochondrial membrane. Fed. Proc. Fed. Amer. Soc. Exp. Biol., 29:605.

13. Butlin, J. D., G. B. Cox and F. Gibson. 1971. Oxidative phosphorylation in Escherichia coli K12. Mutations affecting magnesium ion or calcium ion-stimulated adenosine triphosphatase. Biochem. J., 124:75-81.

14. Card, George L. 1973. Metabolism of phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin of Bacillus stearothermophilus. J. Bacteriol., 114:1125-1137.

15. Casu, A., B. Fleischer and S. Fleischer. 1966. The role of phospholipids in $Mg^{++}$ dependent ATPase activity. Fed. Proc. Fed. Amer. Soc. Exp. Biol., 25:413.

16. Coleman, R. 1972. Membrane-bound enzymes and membrane ultrastructure. Biochim. Biophys. Acta., 300:1-30.

17. Dixon. Malcolm and Edwin C. Webb. 1964. Enzymes. Academic Press, Inc., New York. 2nd Edition, pp. 150-165.

18. Duttera, S. M., W. L. Byrne and M. C. Ganoza. 1968. Studies on the phospholipid requirement of glucose-6-phosphatase. J. Biol. Chem., 243:2216-2228.

19. Fisk, C. H. and Y. Subbarow. 1925. The colormetric determination of phosphorous. J. Biol. Chem., 66:375-400.

20. Fenster, L. J. and J. H. Copenhaver, Jr. 1967. Phosphatidylserine requirement of $(Na^{+}-K^{+})$-activated adenosine triphosphatase from rat kidney and brain. Biochim. Biophys. Acta., 137:406-408.

21. Fesenden-Raden, J. M. and E. Racker. 1971. Structure and Function of Biological Membranes. (L. I. Rothfield, ed.) Ch. 9. Structure and functional organization of mitochondrial membranes. Academic Press, Inc., New York and London.

22. Fukui, Yoshio. 1972. Common peptides in Micrococcus lysodeiktycus membrane proteins. Biochim. Biophys. Acta., 288: 65-72.

23. Georgi, C. E., W. E. Militzer and S. T. Decher. 1955. The organelle nature of a particle isolated from Bacillus stearothermophilus. J. Bacteriol., 70:716-720.

24. Greville, G. C. 1969. Current Topics of Bioenergetics. Vol. III (D. R. Sandi, ed.) A scrutiny of Mitchell's chemiosmotic hypothesis of respiratory chain and photosynthetic phosphorylation. Academic Press, Inc., New York and London.

25. Hachimori, Akari, Namiko Muramatsu and Nosh Yoshiaki. 1970. Studies on an ATPase of thermophilic bacteria and purification and properties. Biochim. Biophys. Acta., 206:426-437.

26. Hachimori, Akari and Nosh Yoshiaki. 1973. Conformational change with temperature of ATPase from Bacillus stearothermophilus. Biochim. Biophys. Acta., 315:481-484.

27. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bac. Rev., 36:172-230.

28. Heppel, Leon A. 1971. Structure and Function of Biological Membranes. (L. I. Rothfield, ed.) Ch. 5. The concept of periplasmic enzymes. Academic Press, Inc., New York and London.

29. Hsung, Jean-Cheru, Leaf Huang, Daniel J. Joy and Alfred Haug. 1975. Lipid and temperature dependence of membrane bound ATPase activity of Acholeplasma laidlawii. Can. J. Biochem., 52:975-979.

30. Kagawa, Y. and E. Racker. 1966. Partial resolution of enzyme catalyzing oxidative phosphorylation. J. Biol. Chem., 241:2467-2475.

31. Knowles, Aileen and E. Racker. 1975. Formation of adenosine triphosphate from Pi and adenosine dephosphate by purified $Ca^{++}$ adenosine triphosphatase. J. Biol. Chem., 250:1949-1951.

32. Koffler, H. 1957. Protoplasmic differences between mesophiles and thermophiles. Bact. Rev., 21:227-240.

33. Lastras, Mercedes and Emilio Munozik. 1974. Membrane adenosine triphosphatase of Micrococcus lysodeiktycus: effects of millimolar $Mg^{++}$ in modulating the properties of membrane bound enzymes. J. Bacteriol, 119:593-601.

34. Lester, R. L. and Sidney Fleischer. 1961. The respiratory activity of acetone extracted beef-heart mitochondria, role of coenzyme Q and other lipids. Biochim. Biophys. Acta., 47:358-377.

35. Ljunger, C. 1970. On the nature of the heat resistance of thermophilic bacteria. Physiol. Plant., 25:351-364.

36. Lowry, H. O., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Measurement of protein with the folin phenol reagent. J. Biol. Chem., 193:265-275.

37. Manning, G. B., L. L. Campbell and R. J. Foster. 1961. Thermal stable alpha-amylase of Bacillus stearothermophilus. II. Physical properties and molecular weight. J. Biol. Chem., 236:2958-2961.

38. March, C. and W. E. Militzer. 1956. Thermal enzymes VII. Further data on adenosine triphosphatase. Arch. Biochim. Biophys., 60:433-438.

39. March, C. and W. E. Militzer. 1956. Thermal enzymes VIII. Properties of heat stable inorganic pyrophosphates. Arch. Biochim. Biophys., 60:449-451.

40. Martonosi, A., J. Donley and R. A. Halpin. 1968. The role of phospholipids in the adenosine triphosphatase activity and $Ca^{++}$ transport III. J. Biol. Chem., 243:61-70.

41. Militzer, W. E. and C. Tuttle. 1952. Thermal enzymes IV. Partial separation of adenosine triphosphatase. Arch. Biochim. Biophys., 31:422-428.

42. Militzer, W. E., C. Tuttle and C. E. Georgi. 1961. Thermal enzymes III. A pyrase from a thermophilic bacteria. Arch. Biochim. Biophys., 31:416-423.

43. Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature (London), 191:144-148.

44. Mirsky, Rhonda and Virginia Barlow. 1972. Alternative purification of the membrane-bound ATPase from Bacillus megaterium $K_m$ and some properties. Biochim. Biophys. Acta., 274:556-562.

45. Mirsky, R. and V. Barlow. 1972. Molecular weight, amino acid composition and other properties of membrane-bound ATPase from Bacillus megaterium. Biochim. Biophys. Acta., 291:480-488.

46. Moreno, Hortensia, Faustino Sineriz and Ricardo N. Farias. 1974. Heterotropic "macroeffector" for cooperative behavior of $Ca^{++}$-adenosine triphosphatase of Escherichia coli. J. Biol. Chem., 249:7701-7706.

47. Pullman, E. E., H. S. Penefesty, A. Datta and E. Racker. 1960. Partial resolution of enzymes catalyzing oxidative phosphorylation. J. Biol. Chem., 235:3322-3330.

48. Racker, E. 1972. Membrane Research. (C. Fred Fox, ed.) Membrane ATPase. Academic Press, Inc., New York and London.

49. Racker, E. and W. Stoeckenius. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. J. Biol. Chem., 249:662-663.

50. Raison, John K. 1973. Membrane Structure and Mechanisms of Biological Energy Transduction. (J. Avery, ed.) The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. Plum Press, London.

51. Roisin, Marie-Paul and Adam Kepes. 1973. The membrane ATPase of Escherichia coli: II release into solution, allotopic properties and reconstitution of membrane bound ATPase. Biochim. Biophys. Acta., 305:249-259.

52. Roisin, Marie-Paul and Adam Kepes. 1972. The membrane ATPase of Escherichia coli: I ion dependence and ATP-ADP exchange reaction. Biochim. Biophys. Acta., 275:333-346.

53. Roseman, S. 1969. Transport of carbohydrates by a bacterial phosphotransferase system. J. Cell Physiol., 54:138-184.

54. Rosenthal, S. S. and A. Matheson. 1973. ATPase isolated in membranes of Bacillus subtilus. Biochim. Biophys. Acta., 318:252-261.

55. Rothfield, L. and D. Romeo. Structure and Function of Biological Membranes. (L. I. Rothfield, ed.) Ch. 6. Enzyme reactions in biological membranes. Academic Press, Inc., New York and London.

56. Santiago, Estahen, Natilia Lopez-Moratalla and Josiah Segovia. 1973. Correlation between losses of mitochondrial ATPase activity and cardiolipin degradation. Biochim. Biophys. Res. Comm., 53:439-444.

57. Saunders, G. F. and L. L. Campbell. 1966. Ribonucleic acid and ribosomes of Bacillus stearothermophilus. J. Bacteriol., 91:332-339.

58. Schaira, Hausll and Dorothee Gruber. 1973. Mutants of Escherichia coli K12 defective in oxidative phosphorylation. Eur. J. Biochem., 37:282-286.

59. Sineriz, Faustino, Bloj Barnaby, Ricardo N. Farias and Raul T. Trucco. 1973. Regulation of membrane fluidity and the allosteric behavior of the ($Ca^{++}$)-adenosine triphosphatase from Escherichia coli. J. Bacteriol., 115:723-726.

60. Singer, S. J. 1971. Structure and Function of Biological Membranes. (L. I. Rothfield, ed.) Ch. 4. The molecular organization of biological membranes. Academic Press, Inc., New York and London.

61. Tanaka, R. and K. P. Strickland. 1965. Role of phospholipid in the activation of $Na^+$, $K^+$-activated adenosine triphosphatase of beef brain. Arch. Biochim. Biophys., 111:583-592.

62. Taniguchi, Kazanga and Shoichi Iida. 1972. The effect of phospholipids on the apparent activation energy of ($Na^+$-$K^+$) ATPase. Biochim. Biophys. Acta., 274:536-541.

63. Wettlaufer, D. B. and R. Lovrien. 1964. Induction of reversible structural changes in proteins by nonpolar substances. J. Biol. Chem., 239:596-603.

64. White, D. C. and A. N. Tucker. 1969. Phospholipid metabolism during bacterial growth. J. Lipid Res., 10:220-230.

65. William, Virginia R. and Hulen B. Williams. 1973. Basic Physical Chemistry for the Life Sciences. W. H. Freeman and Company, San Francisco, p. 316.

66. Yamomoto, T. H., M. Mevel-Nio and R. C. Valentine. 1973. Essential role of membrane ATPase or coupling factor for anaerobic growth and anaerobic active transport in Escherichia coli. Biochim. Biophys. Acta., 314:267-275.

67. Yamashita, S. and E. Racker. 1969. Resolution and reconstitution of the mitochondrial electron transport system. J. Biol. Chem., 244:1220-1227.

68. Yao, M., H. W. Walker and D. A. Lillard. 1970. Fatty acids from vegetative cells and spores of Bacillus stearothermophilus. J. Bacteriol., 102:877-878.