Fatty acid composition of protein-associated lipid in Bacillus stearothermophilus

Robert L. Ingram

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FATTY ACID COMPOSITION OF PROTEIN-
ASSOCIATED LIPID IN BACILLUS STEAROTHERMOPHILUS

by

Robert L. Ingram
B.S., Kent State University, 1975

Presented in partial fulfillment of the requirements
for the degree of

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Chairman, Board of Examiners

Dean, Graduate School

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Fatty Acid Composition of Protein-Associated Lipid in Bacillus stearothermophilus

Director, Dr. George L. Card

According to accepted models of membrane structure about 20% of the membrane lipid is associated with integral membrane protein. The nature of the lipid-protein interactions and the relationship between bulk lipid (bilayer-lipid) and protein associated lipid are unknown. The present study was undertaken to compare the fatty acid composition of protein associated lipid with the fatty acid composition of bulk lipid of Bacillus stearothermophilus. A lipid depleted membrane fragment (LDM-fragment) containing the terminal electron transport system and the ATPase complex was isolated by extraction of washed membranes with sodium cholate and gel filtration on Sepharose 6B columns. The non-covalently associated lipid was extracted from the LDM-fragment with chloroform/methanol/water (1:2:0.8 v/v). Covalently bound fatty acids were then released from the LDM-fragment residue by transesterification with 0.4 N H2SO4 in methanol or with 5% NaOH in 50% aqueous methanol. Investigations as to whether the covalently bound lipid was attached to the LDM-fragment through ester and/or amide linkages were also performed. Fatty acid analysis of proteolipid associated with the LDM-fragment was also performed. Fatty acid methyl esters were prepared from non-covalently bound lipid (the extract of the LDM-fragment) and the bulk lipid (cholate extracted lipid) by the same transesterification procedures. The fatty acid composition of each preparation was analyzed by gas-liquid chromatography. The fatty acid composition of the bulk lipid was similar to the fatty acid composition of the protein associated lipid. These lipids contained mostly branched chain fatty acids with a predominance of anteiso fatty acids. No covalently bound LDM-fragment fatty acids, no amide or ester linked LDM-fragment fatty acids, and no proteolipid associated fatty acids were detected.
DEDICATED TO MY PARENTS
ACKNOWLEDGEMENTS

The author acknowledges with sincere appreciation Dr. George L. Card for his guidance and patience which made this study possible.

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>23</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>38</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>75</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>82</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>85</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>95</td>
</tr>
</tbody>
</table>

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# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Calculation of ug FA/in² using quantitative FAME mixture H103.</td>
<td>39</td>
</tr>
<tr>
<td>2. Calculation of ug FA/in² using quantitative FAME mixture H103.</td>
<td>40</td>
</tr>
<tr>
<td>3. Calculation of the mean ug FA/in².</td>
<td>44</td>
</tr>
<tr>
<td>4. Fatty acid analysis of 80.0 ml chloroform plus 40.0 ml methanol.</td>
<td>45</td>
</tr>
<tr>
<td>5. Fatty acid analysis of 80.0 ml of chloroform.</td>
<td>46</td>
</tr>
<tr>
<td>6. Fatty acid analysis of 40.0 ml of methanol.</td>
<td>47</td>
</tr>
<tr>
<td>7. Fatty acid analysis of 80.0 ml of redistilled chloroform.</td>
<td>48</td>
</tr>
<tr>
<td>8. Fatty acid analysis of 40.0 ml of redistilled methanol.</td>
<td>49</td>
</tr>
<tr>
<td>9. Efficiency of acid transesterification versus base transesterification using cholate extracted bulk lipid.</td>
<td>50</td>
</tr>
<tr>
<td>10. Fatty acid composition of cholate extracted bulk lipid (experiment two).</td>
<td>56</td>
</tr>
<tr>
<td>11. Fatty acid composition of cholate extracted bulk lipid (experiment three).</td>
<td>57</td>
</tr>
<tr>
<td>12. Comparison of the cholate extracted bulk lipid with the Bligh and Dyer extracted LDM-fragment lipid (experiments two &amp; three).</td>
<td>65</td>
</tr>
<tr>
<td>13. Summary of ueq FA/mg LDM-fragment protein of the Bligh and Dyer extracted LDM-fragment lipid (experiments two and three).</td>
<td>66</td>
</tr>
<tr>
<td>14. Fatty acid analysis of total lipids from whole membranes.</td>
<td>73</td>
</tr>
<tr>
<td>15. Fatty acid analysis of 90% CL – 10% PE from whole membranes.</td>
<td>74</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Danielli-Davson model of the structure of cell membranes.</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>The Singer-Nicolson fluid mosaic model of the structure of cell membranes.</td>
<td>11</td>
</tr>
<tr>
<td>3.</td>
<td>Flow sheet for the preparation of LDM-fragments and cholate extracted bulk lipid.</td>
<td>25</td>
</tr>
<tr>
<td>4.</td>
<td>Flow sheet for the preparation of non-covalently associated and covalently bound LDM-fragment lipid.</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Flow sheet for the preparation of proteolipid associated with the LDM-fragments.</td>
<td>36</td>
</tr>
<tr>
<td>6.</td>
<td>Peak area calculation by triangulation.</td>
<td>42</td>
</tr>
<tr>
<td>7.</td>
<td>Gas liquid chromatograph of BC-Mix-L.</td>
<td>53</td>
</tr>
<tr>
<td>8.</td>
<td>Gas liquid chromatograph of a co-injection of BC-Mix-L plus FAME prepared from the cholate extracted bulk lipid.</td>
<td>55</td>
</tr>
<tr>
<td>9.</td>
<td>Gas liquid chromatograph of the FAME prepared from 80.0 ml of chloroform plus 40.0 ml of methanol.</td>
<td>59</td>
</tr>
<tr>
<td>10.</td>
<td>Gas liquid chromatograph of the FAME prepared from the fifth Bligh and Dyer extracted LDM-fragment lipid.</td>
<td>61</td>
</tr>
<tr>
<td>11.</td>
<td>Gas liquid chromatograph of the FAME prepared from the ninth Bligh and Dyer extracted LDM-fragment lipid.</td>
<td>63</td>
</tr>
<tr>
<td>12.</td>
<td>Gas liquid chromatograph of the FAME prepared from a mixture of 42 ml of chloroform, 22 ml of methanol, and 71 ml of chloroform-methanol (2:1).</td>
<td>70</td>
</tr>
<tr>
<td>13.</td>
<td>Gas liquid chromatograph of the FAME prepared from the proteolipid associated with the LDM-fragments.</td>
<td>72</td>
</tr>
</tbody>
</table>

vii

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ABBREVIATIONS

A  area in square inches
a-17:0  anteiso C-17 or 14-Methylhexadecanoate
ATP  adenosine triphosphate
ATPase  adenosine triphosphatase
ATT  attenuation
B  width in inches of triangle at 1/2 H
B and D  Bligh and Dyer
BL  cholate extracted bulk lipid
CL  cardiolipin
°C  degrees centigrade
DNase  deoxyribonuclease
EBFA  ester bound fatty acids
FA  fatty acid
FAME s  fatty acid methyl esters
g  gram
GLC  gas liquid chromatography
>  greater than
H  triangle height in inches
hr(s)  hour(s)
in  inches
i-14:0  iso C-14 or 12-methyltridecanoate
LDM-fragments  lipid depleted membrane fragments
< less than
LTA lipoteichoic acid
mg milligram
ml milliliter
mm millimeter
mM millimolar
MEM minimal essential medium
min minutes
M molar
n-18:0 stearate
n-18:1 oleate
nm nanometer
N normality
% percent
PE phosphatidylethanolamine
PW phenol water treatment
PG phosphatidyl glycerol
Pi inorganic phosphate
PO₄ inorganic phosphate
psig pounds per square inch gauge
SD standard deviation
µeq microequivalent
µg microgram
V volume
W weight
CHAPTER I
INTRODUCTION

In recent years it has become evident that the membrane plays a crucial role in almost all cellular activity. The first belief that cells may be enclosed by a thin membrane came over 100 years ago through ion permeability studies. Confirmation of such a structure was only recently substantiated with the advent of the electron microscope and through cell fractionation and membrane isolation. Today the enclosure of every living cell by a membrane portraying structural asymmetry is well established.

Although the past few years have witnessed advances in our appreciation of the structure of cell membranes and the molecular mechanisms underlying their different functions and properties, our understanding of the molecular organization of membranes is still rudimentary. Experience teaches that in order to achieve a satisfactory understanding of how any biological system functions, the detailed molecular composition and structure of that system must be known. The principal objective in dealing with membrane structure is to relate the chemical composition of individual membrane constituents to their intrinsic physical properties and to examine the nature of the interactions between these components in the assembled membrane.

The first indication that lipids might be an important constituent of biological membranes came in the last years of the nineteenth century.
from the work of Overton (25). Overton postulated in 1902 that the plasma membrane was composed of a thin layer of lipid. Gorter and Grendel (39) in 1926, using extracted lipids from washed erythrocytes spread as a monomolecular film on a Langmuir trough at low pressure, found that the film covered an area which was almost twice the total surface area calculated for the intact red cells used in the experiment. They concluded that the individual lipid molecules were presumably packed together in the monomolecular film in the Langmuir trough with the polar, hydrophilic ends all submerged in the water and the nonpolar, hydrophobic chains projecting upward into the air since in this configuration one obtains a minimum energy configuration for a thin film composed of amphipathic molecules. They further concluded that the cell membrane consisted of a bimolecular layer of lipids with the molecules standing on end packed with the hydrophobic ends opposed at the center and the hydrophilic ends facing inward and outward. Later it was demonstrated (108) that the erythrocyte surface area was approximately 50% greater than that calculated by Gorter and Grendel, and the reason that Gorter and Grendel's calculations turned out to be valid was because the acetone extraction procedure used failed to extract about one-third of the lipid present in the red blood cell ghosts. Gorter and Grendel also based their measurements on light microscopy and the assumption that erythrocytes are discs when in fact they are biconcave. Therefore, these errors tended to offset each other.
The presence of protein in membranes was first postulated based on the observation that interfacial tension at a water-oil interface is about 10 to 15 dynes/cm whereas surface tension of cells is 0.1 to 0.2 dynes/cm (25,26). Danielli and Harvey (20) noted in 1934 that interfacial tension between oil and water could be lowered to about 0.6 dynes/cm by adding some marine egg contents (protein) to the aqueous phase. In 1935, Danielli and Davson (26, Fig. 1), noting that the lowered surface tension of intact cells could also be explained by the aforementioned phenomena, proposed the first complete membrane model. Danielli, then at Princeton, and Davson envisioned the presence of a compact globular protein layer attached to the polar head groups on either side of Groter and Grendel's bimolecular lipid leaflet. Protein thus served to lower the surface tension of the interface. In order to account for the high penetration rate of water through the membrane, as noted in ion permeability studies, Danielli and Davson later modified this hypothesis to include polar pores of small dimensions extending through the lipid bilayer.

Although many membrane biologists challenged the validity of the Danielli-Davson bilayer model, evidence from x-ray diffraction and electron microscopy studies indicated that this model was correct. From x-ray diffraction studies of myelin sheath nerve axons Schmitt (112) proposed in 1941 that the sheath was composed of multiple lipid layers wrapped concentrically around the axon with successive lipid layers separated by layers of protein. The electron microscope studies of
Fig. 1. The Danielli-Davson model (top) and the Robertson model (bottom) of the structure of cell membranes (26).
Fernandez-Moran and Sjostrand (26) in the early 1950's using osmium-fixed myelin provided direct confirmation for this concentric laminar structure of myelin.

Robertson, using potassium permanganate fixation of myelin tissue, showed that each of the single dense lines seen upon osmium fixation could be resolved into two distinct dense lines, each approximately 25 Å thick separated by a light zone of about the same thickness (26). Although osmium was shown to bind preferentially with the olefinic bonds in lipid fatty acids forming an osmic acid diester of the glycerol derivative (45), Robertson (112) took the view that osmium binds to protein. Robertson based this view on the studies of Fleischer et al. (32) and later reinforced by Fleischer and co-workers (33). These experiments demonstrated that even after mitochondrial membrane lipid extraction using acetone followed by osmium fixation, the trilaminar appearance in electron micrographs was still evident. Thus Robertson interpreted the light interperiod zones in electron micrographs of osmium fixed membranes as representing the nonpolar hydrocarbon chains and the darker, denser lines as layers of protein. Robertson also noted that the differences in staining intensity of the "inside" and "outside" dark lines might represent differences in the protein layers of the inside and outside surfaces of the membrane and/or these differences might be caused by the presence of a polysaccharide in the exterior coat. These observations led Robertson (82) in 1959 to propose the "unit membrane" concept of membrane structure (Fig. 1). According to this model, membranes
consisted of a lipid bilayer leaflet 40 to 65 Å thick, asymmetrically coated on the interior by a thin protein layer in the B configuration and on the exterior by either a protein, a mucoprotein, or a mucopolysaccharide. Protein-protein and protein-lipid interactions were considered to be primarily electrostatic (27).

By the early 1960's the unit membrane model had been extended and strengthened by Robertson and others (112) into a far reaching statement of membrane structure that included all cellular membranes.

However, by the mid 1960's dissenting voices were heard, and by the end of the 1960's serious objections were being raised as to the simplicity and inclusiveness of this model. One of the main objections raised concerned the fact that the model was based primarily on the results of electron microscopy and x-ray diffraction studies of myelin. Myelin, functioning mainly as an electrical insulator, contains none of the complex enzyme systems associated with other membranes, such as bacterial, inner mitochondrial, or chloroplast lamellae membranes (112). Other difficulties arose from the dimensions proposed by Robertson as common to all membranes. Investigations by Sjostrand (92) and others (113) noted significant differences in total membrane thickness as revealed by high-resolution electron microscopy. These studies showed that membrane thickness varied from a minimum of 50 Å in smooth endoplasmic reticulum to 95 Å for the plasma membrane. In 1962 Luzzati and Husson (60) raised further doubt about the universality of the unit membrane model when they demonstrated using x-ray scattering diagrams that various lipid-water mixtures
reveal several kinds of structures depending upon concentration and temperature. They proposed three stable structures of lipid-water mixtures - micellelar, rectangular or a complex hexagonal arrangement. Another problem arose when Maddy (63) using infrared absorption, Wallach (105) using optical rotary dispersion, and Glaser (37) using circular dichroism noted that most membrane protein is largely randomly coiled with a significant percentage (20-50%) in the alpha helical form and not in the extended beta configuration.

With the exact nature of membrane structure still unknown, Benson (27) in 1966 proposed a somewhat different view of membrane structure. Benson viewed the membrane as having proteins extending through the entire thickness of the membrane, thus making up the basic framework of the membrane structure, with lipid molecules secondarily inserted into the basically hydrophobic protein network. Consequently, lipid-protein interactions were viewed as primarily hydrophobic whereas the unit membrane model of Robertson viewed these interactions as electrostatic. Benson's membrane model received strong support since many isolated membrane proteins were shown to be very insoluble in aqueous solutions unless detergents were present, implying that the proteins have a hydrophobic exterior unable to interact with the surrounding water. Thus, if membrane proteins exist in an ionic environment, as stated in the Robertson membrane model, one would expect the surface of the protein to be ionic.
Green and Perdue (42) proposed in 1966 that biological membranes are composed of repeating arrays of individual lipoprotein subunits. Green and Perdue's subunit model or protein crystal model was consistent with the observations made by many electron microscopists of repeating subunits in many membrane preparations, particularly in mitochondrial (92) and chloroplast (38) membranes. Branton (6,7), using freeze fracture electron microscopy, added further support to the protein crystal model when he noted particulate subunits embedded between the interior and exterior surfaces of chloroplast and of mitochondrial membranes. Observations in other laboratories (1) also noted this appearance in freeze fracture electron micrographs of vacular, nuclear and bacterial membranes.

As 1970 approached, membrane biologists were still seeking a unified model of membrane structure. Such a model (Fig. 2) was described in 1972 by Singer and Nicolson (91). According to this model, termed the fluid mosaic model, the matrix of cell membranes is a lipid bilayer to which proteins are either absorbed (i.e. extrinsic or peripheral protein) by predominantly polar forces, or are interpolated into the bilayer in direct contact with the hydrophobic region of the membrane (integral or intrinsic proteins). This structure, which exists in a fluid rather than a crystalline state, permits only short-range orderings, such as those required for the formation of integrated functional units like the multi-component electron transport chain of the inner mitochondrial membrane or the specific lipid-protein interactions necessary for the specific activity of many membrane bound enzymes and antigens (79).
Fig. 2 The Singer-Nicolson fluid mosaic model of the structure of cell membranes (53).
Evidence that the fluid mosaic model represented a truly unified model for membranes came from various experiments. The idea that the major portion of the phospholipids in the membrane are arranged in a bilayer form with integral proteins intercalated was provided by calorimetric and x-ray diffraction studies (28,56,91,95,105,111). The concept that the main components of biological membranes, e.g., proteins and lipids, are held in place only by non-covalent interactions was supported by the fact that the components of the cell membrane can be dispersed by solvents, detergents, or denaturing agents that do not involve the breaking of covalent bonds (25). Plasma membrane fluidity with rapid lateral lipid movement was supported by studies on lipid bilayers using spin label techniques (25), differential calorimetry (65,95) and x-ray diffraction studies (28,111). Integral membrane protein translational displacements within the bilayer was strongly verified by Frye and Edidin (36) based on cell fusion studies. Mouse L cells and human transformed cells labeled with the corresponding fluorescent labeled antibodies were induced to fuse using Sendai viruses. After initial fusion each cell surface could be recognized by their differing labels whereas after 40 minutes considerable inter-mixing of the antigens was noted. Inter-mixing was not prevented by inhibitors of protein synthesis, but at temperatures below 20°C low fluidity was observed and the intermixing of antigens was retarded. Taylor et al. (99) noted that in cells treated with a labeled antibody, the antigens are first randomly distributed but after sometime become clustered and agglomerate at one pole of the cell.
where pinocytosis of the antigen-antibody complexes takes place. This process, termed capping, is also inhibited by temperatures that crystallize the lipid bilayer.

Having somewhat resolved the mystery of membrane structure, many membrane biologists began focusing their attention upon the critical issue; the biogenesis of biological membranes.

One of the central thoughts of the fluid mosaic model is the existence of two types of proteins; extrinsic (peripheral) and intrinsic (integral) (83,91). Peripheral proteins, constituting 20-30% of membrane proteins, require only mild treatments, such as an increase in the ionic strength of the medium or the addition of a chelating agent, to disassociate them from the membrane. Separation of integral proteins requires much more drastic treatments, such as detergents, bile acids, protein denaturants, or organic solvents. Integral proteins, when freed of lipids, tend to aggregate in neutral aqueous buffers whereas peripheral proteins are relatively soluble in neutral aqueous buffers. Therefore, two possible types of lipid-proteins interactions are possible. Peripheral proteins tend to be held to the membrane by mainly electrostatic interactions whereas integral proteins tend to associate with the lipid bilayer through hydrophobic interactions.

One concept so far not stressed in the fluid mosaic model is the idea that part of an integral membrane proteins protrudes from the membrane. Thus proteins intrinsic to the membrane continuum are oriented at a lipid-aqueous interphase. The exact proportion of the total surface of
an integral protein exposed to either the water or hydrocarbon layers may vary for different proteins, the main point being that any protein capable of this kind of orientation at a water-lipid interface must contain a polar surface exposed to the aqueous phase and a nonpolar surface exposed to the hydrocarbon phase. Thus, in membranes one observes amphipathic proteins as well as amphipathic phospholipids. This concept, termed the bimodal principle, was first suggested by Vanderkooi and Green (104) as an intrinsic feature of the protein crystal model.

Realizing the existence of globular amphipathic membrane proteins extending into or through the lipid regions of the membrane, membrane biologists began questioning the nature of the lipid that existed between the fluid bilayer and the membrane proteins. Jost et al. (50-53), using a cytochrome oxidase model system, and employing the spin label 16-doxylstearic acid and later the phospholipid spin label 16-doxyl-phosphatidyl choline, were among the first to study lipid-protein interactions. Using electron spin resonance spectroscopy with the above mentioned spin labels, they were able to show the existence of a highly immobilized lipid layer between the hydrophobic cytochrome oxidase protein and the adjacent fluid bilayer region. This single layer of phospholipid surrounding the protein, termed boundary layer lipid, was shown to be in equilibrium with the adjacent bilayer region. Furthermore, this region consisted of a relatively constant amount of phospholipid associated with the hydrophobic portion of the protein (0.2 mg phospholipid/1.0 mg protein). Van and Griffith (102), using the extrinsic...
protein cytochrome C, showed that electron spin resonance spectroscopy measurements of boundary lipid in intact membranes were not interfered with by the presence of extrinsic proteins. The presence of a highly immobile boundary lipid layer (annulus or captive lipid) has been supported by many investigators (5,8,46,77,96,101,106).

During the past several years several studies have appeared that criticize the validity of highly immobile boundary lipid (16,19,22). Davoust et al. (22), using electron spin resonance spectroscopy on rhodopsin-egg lecithin vesicles under physiological conditions (37°C and high lipid to protein ratios), showed that the rhodopsin boundary lipid layer was associated with low microviscosity. Chapman et al. (16,19), using electron spin resonance spectroscopy on gramicidin A-egg lecithin vesicles, obtained similar results. This decrease in microviscosity at physiological temperatures of boundary lipid was also supported by Letellieret's et al. (57) observations that hydrophobic proteins will migrate preferentially into domains enriched with lipids containing predominately unsaturated fatty acids which tend to increase fluidity. Another observation possibly implying high mobility of boundary lipid is the observation that intrinsic membrane proteins tend to increase transbilayer movements of lipids — a process that requires high lipid mobility (110).

Whether boundary layer lipid exists in equilibrium with the bilayer lipid and is in a highly immobilized state or is in a more fluid state at physiological conditions is currently under debate. Further studies using spin label techniques, combined with lipid and fatty acid analysis of boundary layer lipid might help settle this debate.
Since protein-associated lipid constitutes 30-35% of the membrane lipid (9), investigators began to question whether this lipid served other functions besides preserving membrane integrity. Membrane-bound enzymes can be viewed as one structural element in an organized array of molecules that perform multi-step catalysis in a concerted way. Since many membrane enzymes are known to be 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 enzymes.

The involvement of lipids in the function of membrane enzymes was first demonstrated when it was shown that rebinding phospholipids to solvent extracted mitochondria led to reactivation of respiratory activity (9). In bacteria, requirements for specific phospholipids have been reported for phosphoenolpyruvate - phosphotransferase system, glycosyltransferase enzymes, isoprenoid alcohol phosphokinase, diglyceride kinase, ATPase enzyme, D-lactate oxidase, NADH oxidase, succinate oxidase, cytochrome oxidase, and pyruvate oxidase (29,50,55,66,76,86,87,98). It is interesting to note that electron transport enzymes are prominent among the phospholipid requiring enzymes.

Although it is generally accepted that many (if not all) membrane enzymes require phospholipids for their activity, little is known of the exact mechanism of the lipid-protein interactions. It is not known whether electrostatic interactions (through the head groups of the phospholipid) or whether hydrophobic interactions (through the fatty acyl side...
chains) are required for enzyme activity. Several studies have pointed to the importance of the fatty acid chains of phospholipids as regulators of enzyme activity. Swanljung and co-workers (98) noted an eighteen fold increase in mitochondrial ATPase by lysolecithin. More interesting was their observation that the amount of phospholipid required to give half maximal activation was inversely related to the number of fatty acid chains in the lipid. White and Frerman (109) noted an increase in unsaturated fatty acids with the aeration (stimulates the development of a functional electron transport system) of anaerobically (no membrane bound electron transport system present) growing *Staphylococcus aureus* cells. Consistent with this observation is Hsung's et al. (48) observation that high membrane fluidity (present when one has a high ratio of unsaturated to saturated fatty acids) is accompanied by high ATPase enzymatic activities in *Acholeplasma laidlawii*. In a few cases (73,74, 100) free fatty acids have been shown to regulate enzyme activity, but little is known of the mechanism of interaction with the enzymes.

Evidence has been presented for covalent fatty acid-protein interactions. Yamamoto and co-workers (114) have shown that the membrane enzyme penicillinase from *Bacillus licheniformis* 749/C contains phosphatidylserine that is covalently bound to the enzyme through the phosphate moiety. MacLennan (62) has shown that the Ca\(^{2+}\) transport ATPase enzyme of sarcoplasmic reticulum contains two fatty acid molecules covalently bounded per protein molecule. The outer membrane of *Escherichia coli* has been shown to contain a lipoprotein of 58 amino acid residues with an
N-terminal glycerclycysteine (44,110). Two fatty acids are bound to the N-terminus by ester linkages and one by an amide bond. Sone et al. (93), using the H\(^+\) translocating ATPase complex from the thermophilic bacterium PS 3, have shown that the central role in H\(^+\) conduction through the membrane is played by a DCCD-binding proteolipid. Proteolipids have also been isolated from sarcoplasmic Ca\(^{2+}\) ATPase (62,80), mitochondrial membranes (13), and myelin (34).

With the recent advances in the understanding of the structure of biological membranes, much interest has now been focused on the mode(s) of membrane growth. Receiving much attention is the mechanism(s) of insertion of integral protein into the lipid bilayer. Two theories have been proposed: an adaptation of the signal hypothesis (3,4,110) to membrane assembly (84) and a membrane trigger hypothesis (49,110).

According to the signal hypothesis, a protein destined to span the bilayer has a N-terminal signal sequence of 15-30 residues that causes it (and the ribosome on which synthesis is occurring) to bind to a specific membrane transport channel. The force of polypeptide chain elongation drives the peptide through this specific pore. On the opposite face of the bilayer, the emerging leader sequence is removed by a specific protease. When a hydrophilic sequence enters the protein pore, it is thought to signal the release of the peptide from this pore into the bilayer. Although leader (signal) peptides and membrane bound ribosomes have been identified (110), no direct evidence for the proposed protein pore exists.
The membrane triggered model minimizes the role of catalysis in integral protein assembly and emphasizes the ability of a membrane lipid bilayer to trigger the folding of a polypeptide into a conformation that spans the bilayer or is at least integrally associated with it (110). This model accounts for observations of integral membrane protein insertion after synthesis, as noted by an increasing number of investigators (110). In this model the leader peptide allows the growing chain to fold in a manner compatible with the aqueous environment. Membrane recognition is proposed to involve more than the leader peptide portion of the new protein, and the membrane element recognized may be either protein, lipid, or a particular physical property of the lipid phase. Upon binding to the appropriate membrane, the protein interacts with lipid components to fold into a conformation that exposes hydrophobic residues to the bilayer's fatty acyl chains. This interaction, which may begin before synthesis of the peptide chain is complete, ends with the N-terminal leader oligopeptide being removed proteolytically, rendering the process irreversible.

Although further studies are required to clarify the mechanism(s) of integral protein assembly, at the present most evidence tends to support the validity of the membrane trigger hypothesis. This hypothesis explains the following observations: (1) Isolated, water-soluble proteins can enter bilayers as integral proteins. Ito and Wickner (49) noted that procoat synthesis in M13 infected cells occurs on soluble polysomes. Procoat then enters the cell membrane and is converted to coat protein.
Other examples of membrane proteins being inserted into membranes post-translationally include: the post-translational processing and entry into chloroplasts of the small subunit of ribulose bisphosphate carboxylase (47), mitochondrial proteins that are made in the cytosol and enter the organelle after their synthesis is complete (e.g. cytochrome oxidase, 78), and the insertion of the soluble proteins melittin (43), staphylococcal alpha toxin (107), streptolysin S (35), and complement (110) into biological membranes. (2) Integral membrane proteins are capable of more than one functional conformation, and transformation between these states is triggered by specific lipids or cofactors. Protein II* from the \textit{E. coli} outer membrane (89) and \textit{E. coli} pyruvate oxidase (88) are the most often cited examples.

If the membrane triggered hypothesis turns out to be the universal mechanism for integral protein assembly, one immediately notes the importance of lipid in this assembly process. Fatty acid analysis of this lipid would add greatly to the understanding of the mechanism(s) of integral protein synthesis through lipid-protein interactions.

\textbf{Statement of Thesis}

As has been discussed, lipid-protein interactions are encountered in many areas of membrane biology. The identity of lipid interacting with protein has been investigated in many studies, but few investigators have looked at the fatty acid composition of this lipid. The present study was therefore undertaken to analyze and compare the fatty acid composition of bulk layer lipid with that of lipid interacting, both
covalently and non-covalently, with protein. Studies on the type of bonding (ester and/or amide) present in the covalently bound lipid were also carried out. Investigation for the presence of the chloroform-methanol soluble proteolipid described by many workers was also performed.

The present study employed lipid depleted membrane fragments (LDM-fragments) isolated from membranes of *B. stearothermophilus* as the lipid-protein system for investigating covalently and non-covalently protein bound lipid. These LDM-fragments contained associated lipid, a portion of the electron transport system (succinate dehydrogenase, cytochromes, and cytochrome oxidase), and the membrane bound ATPase. Bulk lipid was extracted from washed membranes of *B. stearothermophilus* grown in a minimal essential medium by the use of sodium cholate. LDM-fragments (with their associated lipid; presumably boundary layer lipid) were prepared by the centrifugation of the cholate extract followed by Sepharose 6B column chromatography of the resulting pellet. Non-covalently associated lipid was removed from the LDM-fragments by exhaustive Bligh and Dyer extractions. After exhaustive solvent extractions and phenol water treatment of the LDM-fragments to remove lipoteichoic acid like molecules, fatty acid methyl esters (FAMEs) were made directly from the resulting residue to remove any covalently bound lipid. To detect the presence of ester and/or amide linked lipid, LDM-fragments (after exhaustive Bligh and Dyer - phenol water treatment) were subjected to alkaline hydroxylamine extraction. The resulting supernantant contained ester
bound lipid and the pellet amide linked lipid. FAMEs were prepared from all samples by acid and/or base transesterification and analyzed by gas liquid chromatography (GLC).
CHAPTER II
MATERIALS AND METHODS

Organism

*Bacillus stearothermophilus* (NCA-2184) was used throughout this study. The organism was obtained 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 a modification of the minimal essential medium described by Rowf et al. (85, Appendix I). All cultures were grown at 60° ± 2° C in a fermentor (New Brunswick model MF 114) with stirring (400 rev/min) and vigorous aeration (6-8 liters/min). Fifty milliliters of a starter culture were grown in a 250 ml baffled flask on a gyratory incubator shaker (New Brunswick model G-25) at 60° ± 2° C. When an optical density of 0.43-0.45 (600nm, Coleman Junior II) was attained, the cultures were collected on ice, centrifuged (Sharples model T-1 or International model V) and washed twice with ice-cold 50 mM Tris-HCl buffer (pH 7.8), 0.15 M NaCl, 10 mM MgCl₂.

Preparation of Washed Membranes

Washed cells (Figure 3) were resuspended in 27.0 ml of 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.8), 10 mM MgCl₂ and three ml of a 1.5 mg/ml
Fig. 3. Flow sheet for the preparation of LDM-fragments and FAMEs from cholate-extracted bulk lipid.
Cholate extract

B and D extraction

Cholate Extracted

Bulk Lipid

Phosphorous determined

Base Transesterification

FAMEs

GLC

Washed Cells

Dry Weight

Lysozyme

Protoplasts

Washed Membranes

Protein determined

Suspended at 2 mg protein/ml with 1.5% Sodium Cholate, 0.25 M Na₂SO₄, 0.5 mM Tris HCl pH 7.8

Incubate 4°C, 1 hour

Centrifuge 140,000 x g 2 hrs.

Cholate Pellet

Protein determined

ATPase determined

Sepharose 6B Column

Pool Samples with ATPase Activity

Centrifuge 150,000 x 3 hrs

Supernatant

LDM-Fragments

Acid Transesterification

FAMEs

GLC

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lysozyme suspension (Sigma, Grade 1) were added. The mixture was incu-
bated at 25° C for 30 minutes then centifuged at 30,000 x g for 15
minutes (Sorvall RC2B). The protoplasts were resuspended in 0.05 M
Tris-HCl (pH 7.8) and DNase (Sigma, Type 1) was added to a final con-
centration of 10 ug/ml to reduce viscosity. After 5 minutes of in-
cubation at 25° C, the mixture was centifuged at 30,000 x g for 20 minutes.
The pellet was resuspended in 0.05 M Tris-HCl (pH 7.8), 0.1 M NaCl,
10 mM MgCl₂ and centrifuged 20 minutes at 30,000 x g followed by
washing with 0.05 M Tris-HCl (pH 7.8), 10 mM MgCl₂ until protein was no
longer detected in the supernatant fluid (usually 5 times). If not
used immediately, the pellet (designated as washed membranes) was stored
at 4° C as a pellet under 0.05 M Tris-HCl (pH 7-8), 10 mM MgCl₂.

Preparation of the Cholate Extracted Bulk Lipid and the LDM-Fragments

Washed membranes (Figure 3) were suspended at a concentration of
2 mg protein/ml in 1.5% sodium cholate, 0.25 M Na₂SO₄, 0.5 mM Tris-
HCl (pH 7.8) for 1 hour at 4° C. The mixture was then centifuged at
140,000 x g for 2 hours (Beckman L2-65B). The 140,000 x g pellet
fraction was labelled as the cholate-pellet and the supernatant fraction
as the cholate-extract. Lipid was extracted from the cholate-extract
by the Bligh and Dyer extraction procedure (2). Lipid phosphorous was
determined by a modification of the Fiske-Subba Row procedure (10,
Appendix III). Fatty acid methyl esters (FAMEs) were prepared from this
lipid by acid transesterification (81) and base transesterification
(68) and analyzed by gas liquid chromatography. The cholate pellet was

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resuspended in a small volume (2-3 ml) of 0.5% sodium cholate, 0.4 M Na₂SO₄, 50.0 mM Tris-HCl (pH 7.8) and protein concentration and ATPase activity were determined. The suspension was layered onto a Sepharose 6B column (2.7 x 32 cm) equilibrated with 0.5% sodium cholate, 0.4 M Na₂SO₄, 50.0 mM Tris-HCl (pH 7.8). Column fractions with ATPase activity were pooled and centrifuged at 150,000 x g for 3 hours (Beckman L2-65B). The pellet was termed the LDM-fragment preparation. If the LDM-fragments were not used immediately they were stored as a pellet at 4°C under a layer of 50.0 mM Tris-HCl (pH 7.8) containing 10 mM MgCl₂.

Determination of Protein

Protein concentration was determined by the method of Lowry et al. (59) using bovine serum albumin as a standard (Appendix II). All determinations were done in duplicate.

Phospholipid Extraction (Bligh and Dyer, 2)

The following procedure was used for large volume extractions. The sample was dried and suspended in 10.0 ml of 0.3% NaCl in a 250.0 ml round bottom flask. Fifty ml of methanol were added and the mixture refluxed over boiling water for 5 minutes. After cooling, 10.0 ml of 0.3% NaCl and 25.0 ml of chloroform were added and the mixture was stirred at room temperature for 1-2 hours. The mixture was then transferred to a 500-ml separatory funnel. After adding 25.0 ml of chloroform and 25.0 ml of 0.3% NaCl, the suspension was shaken vigorously. The

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addition of chloroform and NaCl resulted in the production of a two-phase system. The lower phase was collected, taken by dryness and re-suspended in a small volume (1 to 5 ml) of chloroform-methanol (2:1). A phosphorus determination on an aliquot of the chloroform-methanol solution was always performed at this point. For small sample extractions the volumes were adjusted so that the one phase system was chloroform-methanol-water (1:2:0.8), and the two phase system was chloroform-methanol-water (2:2:1.8).

Preparation of Fatty Acid Methyl Esters (FAMEs) by Acid Transesterification

Acid transesterification was performed by the procedure of Read and McElhaney (81). Lipid was placed in a 250 ml round bottom flask and dried under a vacuum. Ten ml of 0.4 N H₂SO₄ in methanol were added and the mixture refluxed at 70°C for 2 hours. After cooling, 20.0 ml of distilled water were added. The mixture was transferred to a 125.0 ml separatory funnel and extracted twice with 10.0 ml portions of n-hexane. FAMEs were dried under a vacuum, resuspended in a small volume (0.1-0.2 ml) of n-hexane and analyzed by gas liquid chromatography. FAMEs were stored in n-hexane under nitrogen at 0°C.

Preparation of Fatty Acid Methyl Esters by Base Transesterification

Base transesterification was performed by the procedure of Moss et al. (68). Lipid was placed in a screw cap tube and solvent removed by a vacuum (Buchler Evapo-Mix). Five ml of 5% NaOH in 5% aqueous methanol were added and the mixture heated at 100°C for 15 minutes.
The saponified material was cooled and acidified to pH 2 with concentrated HCl. A 4.0 ml portion of boron trifluoride methanol (Applied Science Laboratories, State College, Pa.) was added and the mixture heated for 5 minutes at 100°C. The contents of the tube were transferred to 10.0 ml of a saturated sodium chloride solution and the methyl esters extracted twice with 2.0 ml of 1:4 chloroform-hexane. The combined extracts were dried under a vacuum, resuspended in 0.1-0.2 ml of n-hexane and analyzed by gas liquid chromatography. FAMEs were stored in n-hexane under nitrogen at 0°C.

**Fatty Acid Analysis Using Gas Liquid Chromatography (GLC)**

Unless otherwise indicated the following conditions were employed in all GLC runs. Samples were analyzed using a Becker Model 409 gas chromatograph equipped with a flame ionization detector. The metal coiled column (1.0 meter x 1/8 inch outer diameter) was packed with 10% SE 30 coated on 80/100 mesh Chromosorb W-HP (Applied Science Laboratories, State College, Pa.). The amount of sample injected, the attenuation, and the range varied with the sample. Sensitivity was $2.5 \times 10^{-12}$ amperes. Samples (0.1 - 1.0 µl) were injected using a 1.0 µl syringe (Hamilton). After sample injection, the column oven was temperature-programmed from 100°C to 180°C at a rate of 2.5°C/min with a 10 minute isothermal preperiod or operated at 180°C isothermally. The injection port temperature was 180°C and the detector temperature was 200°C. The carrier gas (N$_2$) was maintained at an inlet pressure of 18 psig. Recorder (Honeywell Electronik 194) speed was maintained at 1 inch/10 minutes.
FAMEs were identified by peak enhancement with known standards (Appendix VI).

**Assay of Adenosine Triphosphatase (ATPase)**

Adenosine triphosphatase activity was determined by the isobutanol-benzene extraction procedure of Penniall (75, Appendix IV). All determinations were done in duplicate.

**Preparation of the Sepharose 6B Column**

A piece of glass tubing (2.7 x 32 cm) was washed thoroughly with Ajax cleaner. Sepharose 6B gel beads were washed 3-4 times with column buffer (0.5% sodium cholate, 0.4 M Na₂SO₄, 50 mM Tris-HCl, pH 7.8) under a vacuum to remove trapped air. After washing, the beads were made into a slurry and poured into the column. A closed system was created by placing rubber plugs in both ends of the column. An 18 gauge needle tip was inserted through each rubber plug and tubing connected to these tips. Column buffer was passed through the column to insure proper column packing. A 3 cm layer of column buffer was maintained on top of the beads to prevent disruption of the beads when column buffer entered from the top. Care was taken to insure that the distance from the column buffer inlet point to the column buffer outlet point did not exceed that of the bead height, thus preventing column collapse due to excessive head pressure. After the column was completely packed (column packing was followed by the presence of an interfacial band), 50.0 ml of column buffer was passed through the column until the flow rate was 5-10 ml/hour. To layer a sample onto the column, the top of the 3 cm
layer of column buffer was reduced to the top of the beads and the sample applied to the column by gently allowing the solution to run down the side of the column. When the sample had completely entered the beads, the 3 cm layer of column buffer was replaced and the system closed by replacing the top plug. Fractions (2.5 ml) were collected in a Gilson fraction collector.

**Extraction of Lipid Non-Covalently Associated with the LDM-fragments**

After determining protein, phosphorous and ATPase activity, LDM-fragments (Figure 4) were subjected to exhaustive chloroform - methanol (2) extractions (5-9 times).

**Extraction of Lipoteichoic Acid-Like Molecules Associated with the LDM-fragments**

After protein determination, the residue left after exhaustive Bligh and Dyer extractions of the LDM-fragments (Figure 4) was divided in half and one part subjected directly to acid transesterification. The other half was subjected to 40% phenol-water (P/W) treatment to remove lipoteichoic acid (LTA) - like molecules. After suspending the residue in 1.2 ml of 80% phenol in a 15 ml conical tube, an equal volume of distilled water was added. The solution was mixed well and incubated at 4°C for 70 minutes. The mixture was then centrifuged (Sorvall RC2B) at 30,000 x g until the upper water phase was clear (about 30 minutes). The upper phase (LTA) was removed and placed in a 250 ml round bottom flask. After water (1.2 ml) was added to repeat the extraction, the combined water extracts were evaporated under a vacuum at 70°C and subjected to acid transesterification.
Fig. 4. Flow sheet for the preparation of FAMEs from non-covalently associated and covalently bound LDM-fragment lipid.
LDM Fragments
- Protein, ATPase, Phosphorous determined
  Exhaustive B and D

Supernatant
- Phosphorous determined
  Acid Transesterify
  FAME
  GLC

Residue
- Protein determined
  Acid Transesterification
  Phenol
  Water
  Treatment
  FAME s
  GLC

Residue
- Supernant (LTA)
  Acid Transesterification

Acid Transesterification
- FAME s
- GLC

Acid Transesterification
- Alkaline
  Hydroxyamine
  Treatment

FAME s
- GLC

Supernant (EBFA)
- Acid Transesterification
- Residue

Acid Transesterification
- FAME s
- GLC

FAME s
- GLC
Extraction of Ester and Amide Linked LDM-fragment Lipid

After phenol water treatment, all traces of phenol were removed from the LDM-fragments (Figure 4) by washing the residue remaining after PW treatment twice with 1.2 ml of methanol. The residue was divided equally into 2 parts. One part was subjected to acid transesterification and the other part to alkaline hydroxylamine treatment (70). Briefly the hydroxylamine procedure was as follows. The residue was suspended in 1 ml of hydroxylamine reagent (Appendix V) in a screw cap tube and placed in a 65°C water bath for 2 minutes. After cooling, the mixture was acidified to pH 2 with acetic acid and extracted twice with n-hexane. The extracted upper phase represented ester linked lipid. Fatty acid methyl esters were prepared from this extract by acid transesterification. The residue left after hydroxylamine treatment was transferred to a 250-ml round bottom flask, dried, and subjected to acid transesterification. These fatty acid methyl esters represented amide linked fatty acids.

Extraction of Proteolipid Associated with the LDM-fragments

Proteolipids were extracted with chloroform-methanol (2:1) by the method of Fillingame (31). The LDM-fragments, prepared as described in Figure 3, were placed in a 250-ml round bottom flask at a protein concentration of 30 mg/ml (Figure 5). Twenty five volumes of chloroform-methanol (2:1) were added. After stirring overnight at 4°C, the solution was placed in conical tubes and centrifuged (International Clinical Model CL) for 30 minutes. The supernatant fluid was poured into a
Fig. 5. Flow sheet for the preparation of FAMEs from proteolipid associated with the LDM-fragments.
LDM Fragment (at 30-60 mg Protein/ml)
Protein determined
25 volumes CM (2:1) added
Stir overnight, 4°C
Centrifuge 30 min., clinical

Supernatant
Add 0.2 volume 0.05 N NaCl
Collect Lower Phase
Phosphorous on Lower Phase determined
Precipitate Lower phase with 4 volumes diethylether at -20°C; 24 hours
Centrifuge 1000 x g 2 hrs. at -20°C

Precipitate ("proteolipid")
Protein determined
Phosphorous determined
Acid Transesterification

FAME s
GLC
500-ml separatory funnel, and the residue in the conical tubes was washed once more with chloroform-methanol (2:1). The supernatant was again collected by centrifugation and added to a 500-ml separatory funnel. Two-tenth volumes of 0.05 N NaCl were added to the separatory funnel, and the phases were allowed to separate. The upper phase was removed with a capillary pipette, and the interface was washed carefully three times with small volumes (1-3 ml) of upper phase solvent without disturbing the lower phase. Together with the remaining rinsing fluid, the lower phase was made into one phase by adding methanol. One volume of chloroform plus adequate volumes of methanol to maintain clarity of the solution were added. The mixture was dried and resuspended in 1 ml chloroform-methanol (2:1) in a 12 ml conical tube. Four ml of diethylether were added slowly with stirring at 0°C. After 24 hours at -20°C, the proteolipid precipitate was removed by centrifugation at 1000 x g for 2 hours at -20°C (Sorvall RC2B). The proteolipid was resuspended in 1 ml of chloroform-methanol (2:1) and precipitated twice more with diethylether. The resulting precipitate was resuspended in 1.4 ml of chloroform-methanol (2:1). After determining protein and phosphorus content, the sample was placed in a 250 ml round bottom flask and subjected to acid transesterification.
CHAPTER III

RESULTS

Calculation of Microgram Fatty Acid/Inch²

The major portion of the present study was based upon the fatty acid analysis of various lipids using gas liquid chromatography (GLC). Unknowns were identified either by comparison of retention times of unknown fatty acids (FA) with known fatty acids or by co-injection of known fatty acids with unknown fatty acids. Unknown fatty acids were quantified by calculating GLC peak areas, since in GLC the area under a curve is directly proportional to the concentration of the substance producing that curve. In order to calculate the ratio of concentration/area, standard fatty acid mixtures of known concentration were employed. Microgram FA/in² was calculated using fatty acid methyl ester mixture H103 under two different sets of conditions (Tables 1 and 2). Peak area was calculated using the triangulation method (Figure 6). Microgram FA/in² was calculated as follows:

\[
\frac{100 \text{ mg FA mixture}}{0.5 \text{ ml n-hexane}} \times 0.1 \text{ ml removed} = 20 \text{ mg FA mixture} \\
\frac{20 \text{ mg FA mixture}}{0.2 \text{ ml n-hexane}} \times 0.0005 \text{ ml injected} \times \frac{1000 \text{ ug}}{\text{mg}} = 50 \text{ ug FA} \\
\frac{50 \text{ ug FA mixture}}{\text{injection}} \times \% \text{ of FA in the injection} \div \text{area of FA peak in inches}^2 = \text{ug FA/in}^2
\]
Table 1
Calculation of μgFA/in² Using Quantitative FAME Mixture H103

<table>
<thead>
<tr>
<th>FA</th>
<th>Area (in²)</th>
<th>Weight % of FA</th>
<th>μgFA/in²</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-15:0</td>
<td>0.48</td>
<td>14.6</td>
<td>15.2</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.59</td>
<td>17.9</td>
<td>15.2</td>
</tr>
<tr>
<td>n-17:0</td>
<td>0.60</td>
<td>18.2</td>
<td>15.2</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.76</td>
<td>23.3</td>
<td>15.2</td>
</tr>
<tr>
<td>n-19:0</td>
<td>0.86</td>
<td>26.1</td>
<td>15.2</td>
</tr>
<tr>
<td>Total</td>
<td>3.29</td>
<td>100.1</td>
<td></td>
</tr>
</tbody>
</table>

H103, which contains 100 mg of a FA mixture, was dried and resuspended in 0.5 ml n-hexane; 0.1 ml was removed from this mixture, dried, and resuspended in 0.2 ml n-hexane; 0.5 μl was removed and analyzed by GLC at ATT = 32, range = 10. Programming was 100-180°C at 2.5°C/min with a 10 minute isothermal preperiod. FAME = fatty acid methyl ester, FA = fatty acid, GLC = gas liquid chromatography, ATT = attenuation, in² = square inches.
Table 2

Calculation of µgFA/in² Using Quantitative FAME Mixture H103

<table>
<thead>
<tr>
<th>FA</th>
<th>Area (in²)</th>
<th>Weight % of FA</th>
<th>µgFA/in²</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-15:0</td>
<td>0.46</td>
<td>15.1</td>
<td>32.8</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.54</td>
<td>17.7</td>
<td>32.8</td>
</tr>
<tr>
<td>n-17:0</td>
<td>0.63</td>
<td>20.7</td>
<td>32.8</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.66</td>
<td>21.6</td>
<td>32.8</td>
</tr>
<tr>
<td>n-19:0</td>
<td>0.76</td>
<td>24.9</td>
<td>32.8</td>
</tr>
<tr>
<td>Total</td>
<td>3.05</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

aH103, which contains 100 mg of a FA mixture, was dried and resuspended in 0.5 ml n-hexane; 0.1 ml was removed, dried, and resuspended in 0.2 ml h-hexane; 1.0 µl was removed and analyzed by GLC at ATT = 64, range = 10, isothermal 180°C. FAME = fatty acid methyl ester, FA = fatty acid, GLC = gas liquid chromatography, ATT = attenuation.
Fig. 6. Peak area calculation by triangulation

Symbols:

B  Width (in inches) of the triangle at one half the triangle height.

H  Height of the triangle created by drawing tangents to the curve.

A  Area = B x H (e.g. B = 1.32 inches, H = 1.41 inches, A = 1.86 square inches).
The results summarized in Tables 1 and 2 demonstrated that at a specified range and attenuation the same value for ug FA/in\(^2\) was obtained whether the gas liquid chromatograph was being operated isothermally or by temperature programming. Table 3 gives ug FA/in\(^2\) at range 10 and various attenuations. After peak identification and peak area calculation, quantification was performed using Table 3.

**Calculation of Solvent Fatty Acid Contamination**

Before analysis of an unknown fatty acid mixture, it was necessary to determine if there existed any fatty acids in the solvents employed in the various experiments. The results shown in Table 4 demonstrated that there was considerable fatty acid contamination in 80.0 ml of chloroform plus 40.0 ml of methanol. The composition of chloroform and methanol are shown in Tables 5 and 6 respectively. Tables 7 and 8 show the composition of redistilled chloroform and redistilled methanol respectively. Acetone (employed in cleaning the syringe before each injection), sodium cholate, and n-hexane were free of fatty acids. These results were used to correct for solvent fatty acid contamination.

**Efficiency of Acid and Base Transesterification**

Cholate extracted bulk lipid was prepared as described in Materials and Methods, and FAMEs were prepared by acid and base transesterification. Table 9 gives the fatty acid composition of this lipid. It was concluded that acid and base transesterification transesterified all fatty acids with no discretion (comparison of the weight % of fatty acids) but acid
Table 3

Calculation of the Mean μg FA/in²
d

<table>
<thead>
<tr>
<th>ATT²</th>
<th>μg FA/in²</th>
<th>μg FA/in²</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>30.4</td>
<td>32.8⁵</td>
<td>31.6</td>
</tr>
<tr>
<td>32</td>
<td>15.2ᵇ</td>
<td>16.4</td>
<td>15.8</td>
</tr>
<tr>
<td>16</td>
<td>7.6</td>
<td>8.2</td>
<td>7.9</td>
</tr>
<tr>
<td>8</td>
<td>3.8</td>
<td>4.1</td>
<td>3.95</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>2.05</td>
<td>1.98</td>
</tr>
<tr>
<td>2</td>
<td>0.95</td>
<td>1.02</td>
<td>0.99</td>
</tr>
</tbody>
</table>

ᵃRange = 10
ᵇNote Table 1
⁵Note Table 2

This table was constructed using data from Tables 1 and 2 and the assumption that doubling the attenuation resulted in a one-half reduction in the peak area (note Tables 1 and 2). FA = fatty acid, ATT = attenuation.
Table 4

Fatty Acid (FA) Analysis of a Mixture of 80 ml Chloroform and 40 ml Methanol\textsuperscript{a,c}

<table>
<thead>
<tr>
<th>FA</th>
<th>Area (in\textsuperscript{2})</th>
<th>Weight % of FA</th>
<th>(\text{meq FA of sample transesterified} \times 10^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-12:0</td>
<td>0.09</td>
<td>3.5</td>
<td>8.3</td>
</tr>
<tr>
<td>n-13:0</td>
<td>0.02</td>
<td>0.8</td>
<td>2.2</td>
</tr>
<tr>
<td>n-14:0</td>
<td>0.08</td>
<td>3.1</td>
<td>6.1</td>
</tr>
<tr>
<td>n-15:0</td>
<td>0.04</td>
<td>1.6</td>
<td>2.7</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.30</td>
<td>11.7</td>
<td>22.0</td>
</tr>
<tr>
<td>n-17:0</td>
<td>0.05</td>
<td>2.0</td>
<td>3.6</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.71</td>
<td>27.6</td>
<td>47.3</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.44</td>
<td>17.1</td>
<td>29.2</td>
</tr>
<tr>
<td>&gt;n-19:0</td>
<td>0.84</td>
<td>33.7</td>
<td>166.3\textsuperscript{b}</td>
</tr>
<tr>
<td>&lt;n-20:0</td>
<td></td>
<td></td>
<td>121.4\textsuperscript{c}</td>
</tr>
<tr>
<td>Total</td>
<td>2.57</td>
<td>101.1</td>
<td>121.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} A mixture of 80 ml of chloroform plus 40 ml of methanol were acid transesterified. FAMEs were suspended in 0.1 ml n-hexane and 1.0 \(\mu\)l analyzed by GLC at ATT 4 range 10, 180\textdegree C isothermal.

\textsuperscript{b} \(\mu\)g FA/sample transesterified \(\times 10^{-2}\).

\textsuperscript{c} Total \(\text{meq FA/sample transesterified excluding >n-19:0 <n-20:0} \times 10^{-2}\). FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.
Table 5

Fatty Acid Analysis of Eighty Milliliters of Chloroform<sup>a</sup>

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Area (in²)</th>
<th>Weight % FA</th>
<th>μeq FA/ml Chloroform X 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-12:0</td>
<td>0.04</td>
<td>3.7</td>
<td>1.0</td>
</tr>
<tr>
<td>n-13:0</td>
<td>0.12</td>
<td>11.1</td>
<td>2.7</td>
</tr>
<tr>
<td>n-14:0</td>
<td>0.05</td>
<td>4.6</td>
<td>1.1</td>
</tr>
<tr>
<td>n-15:0</td>
<td>0.02</td>
<td>1.9</td>
<td>0.4</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.10</td>
<td>9.3</td>
<td>1.8</td>
</tr>
<tr>
<td>n-17:0</td>
<td>0.03</td>
<td>2.8</td>
<td>0.4</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.31</td>
<td>28.7</td>
<td>5.1</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.19</td>
<td>17.6</td>
<td>3.2</td>
</tr>
<tr>
<td>&gt;n-19:0&lt;n-20:0</td>
<td>0.22</td>
<td>20.4</td>
<td>1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>1.08</td>
<td>100.1</td>
<td>15.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 80 ml of chloroform were acid transesterified. FAMEs were suspended in 0.2 ml n-hexane and 1.0 μl analyzed by GLC at ATT 4, range 10, 180°C isothermal. FA = fatty acid, FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.

<sup>b</sup> μg FA/ml chloroform X 10⁻³.

<sup>c</sup>Total μeq FA/ml chloroform excluding >n-19:0<n-20:0 X 10⁻³.
Table 6

Fatty Acid Analysis of Forty Milliliters of Methanol

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Area (in²)</th>
<th>Weight %</th>
<th>μeq FA/ml Methanol x 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-14:0</td>
<td>0.01</td>
<td>2.9</td>
<td>0.3</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.05</td>
<td>14.7</td>
<td>1.9</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.05</td>
<td>14.7</td>
<td>1.7</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.03</td>
<td>8.8</td>
<td>0.9</td>
</tr>
<tr>
<td>&gt;n-19:0</td>
<td>0.20</td>
<td>58.8</td>
<td>2.0^{b}</td>
</tr>
<tr>
<td>&lt;n-20:0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.34</td>
<td>99.9</td>
<td>4.8^{c}</td>
</tr>
</tbody>
</table>

^{a} 40 ml of methanol were acid transesterified. FAMEs were suspended in 0.2 ml n-hexane and 1.0 μl analyzed by GLC at ATT 4, range 10, 180°C isothermal. FA = fatty acid, FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.

^{b} μgFA/ml methanol x 10^{-3}.

^{c} Total μeq FA/ml methanol excluding >n-19:0 <n-20:0 x 10^{-3}.
Table 7

Fatty Acid Analysis of Eighty Milliliters of Redistilled Chloroform

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Area (in²)</th>
<th>Weight % of FA</th>
<th>µeq FA ml Redistilled Chloroform x 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-12:0</td>
<td>0.02</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>n-13:0</td>
<td>0.01</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>n-14:0</td>
<td>0.04</td>
<td>4.7</td>
<td>2.1</td>
</tr>
<tr>
<td>n-15:0</td>
<td>0.02</td>
<td>2.3</td>
<td>0.8</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.10</td>
<td>11.6</td>
<td>4.8</td>
</tr>
<tr>
<td>n-17:0</td>
<td>0.05</td>
<td>5.8</td>
<td>2.0</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.35</td>
<td>40.7</td>
<td>14.6</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.27</td>
<td>31.4</td>
<td>11.3</td>
</tr>
<tr>
<td>Total</td>
<td>0.86</td>
<td>100.0</td>
<td>37.5</td>
</tr>
</tbody>
</table>

ₐ₈₀ ml of redistilled chloroform were acid transesterified. FAMEs were suspended in 0.1 ml n-hexane and 1.0 µl analyzed by GLC at ATT 2 range 10, 180°C isothermal. FA = fatty acid, FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Area (in²)</th>
<th>Weight % of FA</th>
<th>$\text{meq FA} \times 10^{-4}$ per ml Redistilled Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-14:0</td>
<td>0.02</td>
<td>15.4</td>
<td>1.9</td>
</tr>
<tr>
<td>n-15:0</td>
<td>0.01</td>
<td>7.7</td>
<td>0.5</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.06</td>
<td>46.2</td>
<td>5.5</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.02</td>
<td>15.4</td>
<td>2.1</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.02</td>
<td>15.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Total</td>
<td>0.13</td>
<td>100.1</td>
<td>11.3</td>
</tr>
</tbody>
</table>

$^a$ 40 ml of redistilled methanol were acid transesterified. FAMEs were suspended in 0.1 ml n-hexane and 1.0 µl analyzed by GLC at ATT 2, range 10, 180°C isothermal. FA = fatty acid, FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.
Table 9

Efficiency of Acid Transesterification Versus Base Transesterification Using Cholate Extracted Bulk Lipid

<table>
<thead>
<tr>
<th>FA</th>
<th>Weight % of FA</th>
<th>μeq FA/μeq PO₄</th>
<th>Weight % of FA</th>
<th>μeq FA/μeq PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-14:0</td>
<td>3.8</td>
<td>0.1</td>
<td>5.9</td>
<td>0.1</td>
</tr>
<tr>
<td>a-15:0</td>
<td>30.8</td>
<td>0.8</td>
<td>31.9</td>
<td>0.3</td>
</tr>
<tr>
<td>i-16:0</td>
<td>30.1</td>
<td>0.7</td>
<td>25.2</td>
<td>0.2</td>
</tr>
<tr>
<td>n-16:0</td>
<td>7.9</td>
<td>0.2</td>
<td>4.2</td>
<td>0.0</td>
</tr>
<tr>
<td>a-17:0</td>
<td>27.4</td>
<td>0.6</td>
<td>32.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>2.4</td>
<td>100.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Equal amounts of cholate extracted BL from one batch of cells were acid transesterified and base transesterified. BL = bulk lipid, FA = fatty acid.*
transesterification was more efficient (comparison of the ueq FA/μeq PO₄). This efficiency was based upon the fact that bulk lipid from membranes of *B. stearothermophilus* consisted of phospholipids, and these phospholipids contained 2.0 μeq FA/μeq PO₄ (11). Therefore, acid transesterification was employed in the preparation of all FAMES.

**Fatty Acid Composition of Cholate Extracted Bulk Lipid**

Two batches of cells were prepared and bulk lipid extracted as described in Materials and Methods. Using the principle of peak enhancement with known standards, Figures 7 and 8 show that the cholate extracted bulk lipid consisted of the following fatty acids: i-14:0, a-15:0, i-16:0, n-16:0, and a-17:0. Tables 10 and 11 give the fatty acid compositions of the cholate extracted bulk lipid from these two batches of cells after correction for solvent fatty acid contamination.

**Efficiency of the Bligh and Dyer Extraction Procedure**

Lipid depleted membrane fragments were prepared and non-covalently associated lipid removed by nine successive Bligh and Dyer extractions. Figure 9 represents a gas liquid chromatograph of FAMES prepared from 80 ml of chloroform plus 40 ml of methanol (solvent volumes used in each extraction). Figure 10 is a gas liquid chromatograph of the FAMES obtained from the fifth Bligh and Dyer extract of the LDM-fragments. Figure 11 gives the gas liquid chromatograph of the FAMES prepared from the ninth Bligh and Dyer extract of the LDM-fragments (same fragments as in Figure 10). A comparison of Figure 9 with Figure 11 shows all the
Fig. 7. Chromatograph of the standard FAME mixture BC-Mix-L. BC-Mix-L was suspended in 0.2 ml n-hexane and 0.8 µl analyzed at ATT 32, range 10. Programming was 100-180 at 2.5°C/min with a 10 minute isothermal preperiod. FAME = fatty acid methyl ester, ATT = attenuation.
Fig. 8. Chromatograph of a co-injection of FAME mixture BC-Mix-L plus FAMEs prepared from the cholate extracted bulk lipid. Both the FAME mixture BC-Mix-L and the cholate extracted FAMEs were suspended in 0.2 ml n-hexane. 0.5 μl of BC-Mix-L plus 0.5 μl of the FAMEs from the cholate extracted lipid were analyzed by co-injection at ATT 16, range 10. Programming was 100-180°C at 2.5°C/min with a 10 minute isothermal preperiod. FAME = fatty acid methyl ester, ATT = attenuation.
Table 10

Fatty Acid Composition of Cholate Extracted Bulk Lipid$^{a,f}$

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Area (in$^2$)</th>
<th>Weight$^c$ % of FA</th>
<th>(\mu\text{eq FA}^d) Sample Transesterified</th>
<th>(\mu\text{eq FA}^e) (\mu\text{eq P}_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-14:0</td>
<td>0.22</td>
<td>2.4</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>n-14:0</td>
<td>0.07</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>a-15:0</td>
<td>2.64</td>
<td>28.3</td>
<td>4.1</td>
<td>0.7</td>
</tr>
<tr>
<td>i-16:0</td>
<td>2.47</td>
<td>26.4</td>
<td>3.6</td>
<td>0.6</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.94</td>
<td>8.4</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>a-17:0</td>
<td>3.23</td>
<td>34.6</td>
<td>4.5</td>
<td>0.7</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.27</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.14</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>&gt;n-19:0&lt;n-20:0</td>
<td>0.12</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>9.34$^b$</td>
<td>100.1</td>
<td>13.7</td>
<td>2.3</td>
</tr>
</tbody>
</table>

$^a$FAMEs were prepared from the cholate extracted bulk lipid by acid transesterification.
$^b$FAMEs were suspended in 0.2 ml n-hexane, and 1.0 \(\mu\text{l}\) analyzed by GLC at ATT 4, range 10. Programming was 100-180°C at 2.5°C/min with a 10 min isothermal preperiod.
$^c$Corrected for solvent contamination.
$^d$Percentages corrected for solvent contamination.
$^e$Corrected for solvent contamination. 80 ml chloroform plus 50 ml of methanol were used in the preparation of the FAMEs.
$^f$The acid transesterified cholate bulk lipid contained 6.3 \(\mu\text{eq P}_4\).
$^f$This lipid was extracted from 603 mg of cells (dry weight). FA = fatty acid, FAMEs = fatty acid methyl esters, GLC = gas liquid chromatography, ATT = attenuation.
Table 11

Fatty Acid Composition of Cholate Extracted Bulk Lipid\(^a,^f\)

<table>
<thead>
<tr>
<th>FA</th>
<th>Area (in(^2))</th>
<th>Weight %(^c) of FA</th>
<th>µeq FA(^d) Sample Transesterified</th>
<th>µeq FA(^e) µeq PO(_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-12:0</td>
<td>0.03</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>n-13:0</td>
<td>0.01</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>i-14:0</td>
<td>0.05</td>
<td>2.8</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>n-14:0</td>
<td>0.05</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>a-15:0</td>
<td>0.38</td>
<td>21.0</td>
<td>5.8</td>
<td>0.5</td>
</tr>
<tr>
<td>i-16:0</td>
<td>0.59</td>
<td>32.6</td>
<td>8.6</td>
<td>0.8</td>
</tr>
<tr>
<td>n-16:0</td>
<td>0.24</td>
<td>6.1</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>a-17:0</td>
<td>0.68</td>
<td>37.6</td>
<td>9.5</td>
<td>0.8</td>
</tr>
<tr>
<td>n-18:0</td>
<td>0.24</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>n-18:1</td>
<td>0.14</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>&gt;n-19:0&lt;n-20:0</td>
<td>0.12</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>1.81(^b)</td>
<td>100.1</td>
<td>26.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\(^a\)FAMEs were prepared from the cholate extracted BL by acid transesterification. FAMEs were suspended in 1.0 ml n-hexane and 1.0 µl analyzed by GLC at ATT 8, range 10. Programming was 100-180°C at 2.5°C/min with a 10 min isothermal preperiod.

\(^b\)Percentages corrected for solvent contamination.

\(^c\)Corrected for solvent contamination.

\(^d\)Corrected for solvent contamination. 600 ml of chloroform plus 410 ml of methanol were used in the preparation of the FAMEs.

\(^e\)The acid transesterified cholate extracted bulk lipid contained 11.4 µeq PO\(_4\).

\(^f\)This lipid was extracted from 658 mg of cells (dry weight). FA = fatty acids, FAMEs = fatty acid methyl esters, BL = bulk lipid, GLC = gas liquid chromatography, ATT = attenuation.
Fig. 9. Chromatograph of the FAMEs prepared from 80 ml of chloroform plus 40 ml of methanol. FAMEs were suspended in 0.2 ml n-hexane and 1.0 µl analyzed at ATT 4, range 10,180°C isothermal. FAMEs = fatty acid methyl esters, ATT = attenuation.
Fig. 10. Gas liquid chromatograph of the fatty acid methyl esters prepared from the fifth Bligh and Dyer extracted LDM-fragment lipid. Fatty acid methyl esters were suspended in 0.2 ml n-hexane and 1.0 μl analyzed at attenuation 4, range 10, 180°C isothermal.

Symbol:

A Due to realignment of paper on the recorder.
Fig. 11. Chromatograph of the FAMEs prepared from the ninth Bligh and Dyer extracted LDM-fragment lipid. FAMEs were suspended in 0.2 ml n-hexane and 1.0 µl analyzed at ATT 4, range 10, 180°C isothermal. FAMEs = fatty acid methyl esters, ATT = attenuation.
fatty acids present were due to solvent contamination. Therefore, it was concluded that after nine Bligh and Dyer extractions no more lipid was being removed from the LDM-fragments. Nine Bligh and Dyer extractions were therefore designated as exhaustive extractions. Any lipid remaining was assumed to be bound to the fragments through covalent linkages.

**Fatty Acid Composition of Non-covalently Associated LDM-fragment Lipid**

Lipid depleted membrane fragments were prepared and non-covalently associated lipid removed by exhaustive Bligh and Dyer extractions. Table 12 gives the fatty acid composition of this lipid and a comparison with the corresponding cholate extracted bulk lipid. Results presented in Table 12 indicated that the fatty acid composition of cholate extracted bulk lipid was similar to that of non-covalently associated LDM-fragment lipid. Both lipid classes appeared to be phospholipid (note ueq FA/ueq PO₄ in Table 12). Table 13 represents a summary of ueq FA/mg LDM-fragment protein from the Bligh and Dyer extracted LDM-fragment lipid.

**Fatty Acid Analyses of the Phenol-Water Extract (LTA) from the LDM-fragments After Exhaustive Bligh and Dyer Extractions of the LDM-fragments**

After nine Bligh and Dyer extractions, LDM-fragments were subjected to phenol-water treatment as described in Materials and Methods to remove any lipoteichoic acid-like molecules. After deletion of solvent fatty acid contamination involved in sample preparation, no fatty acids could be detected in the PW extract from 44.5 mg of LDM-fragment protein.
Table 12

Comparison of the Cholate Extracted Bulk Lipid with the Bligh and Dyer Extracted LDM-fragment Lipid\(^{a,b}\)

<table>
<thead>
<tr>
<th>Experiment #2(^b)</th>
<th>Bligh and Dyer Extracted LDM-fragment Lipid</th>
<th>Experiment #3(^b)</th>
<th>Bligh and Dyer Extracted LDM-fragment Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate Extracted BL(^c)</td>
<td>Weight</td>
<td>% of FA</td>
<td>(\mu)eq FA</td>
</tr>
<tr>
<td>FA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i-14:0</td>
<td>2.4</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>a-15:0</td>
<td>28.3</td>
<td>0.7</td>
<td>29.1</td>
</tr>
<tr>
<td>i-16:0</td>
<td>26.4</td>
<td>0.6</td>
<td>22.3</td>
</tr>
<tr>
<td>n-16:0</td>
<td>8.4</td>
<td>0.2</td>
<td>6.3</td>
</tr>
<tr>
<td>a-17:0</td>
<td>34.6</td>
<td>0.7</td>
<td>40.3</td>
</tr>
<tr>
<td>Total</td>
<td>100.1</td>
<td>2.3</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\(^a\)Two different cell cultures were grown and their respective lipids isolated and compared.
\(^b\)FAMEs prepared by acid transesterification. BL = bulk lipid, FA = fatty acid.
\(^c\)Note Table 10.
\(^d\)Note Table 11.
Table 13

Summary of µeq FA/mg LDM-fragment Protein of the Bligh and Dyer Extracted LDM-fragment Lipid

<table>
<thead>
<tr>
<th>FA</th>
<th>µeq FA/mg LDM-fragment Protein</th>
<th>µeq FA/mg LDM-fragment Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-15:0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>i-16:0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>a-17:0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

aTwo different cell cultures were grown and the lipid isolated and compared. FA = fatty acid.
Fatty Acid Analysis of Ester and/or Amide Linked LDM-fragment Lipid

After nine Bligh and Dyer extractions and phenol-water treatment of the LDM-fragments, any lipid remaining was assumed to be covalently bound to the LDM-fragments. Removal of this lipid and conversion to FAMEs was accomplished by direct transesterification of the residue remaining after phenol-water treatment of the LDM-fragments. After correction for solvent fatty acid contamination involved in sample preparation, there were no detectable fatty acids released by acid transesterification.

Fatty Acid Analysis of Ester Linked LDM-fragment Lipid

To confirm the absence of lipid bound to the LDM-fragments (after nine Bligh and Dyer extractions and phenol-water treatment) through ester linkages, the alkaline hydroxylamine extract (EBFA, note Materials and Methods) was subjected to acid transesterification. No fatty acids were detected in a sample of 21.3 mg LDM-fragment protein after nine Bligh and Dyer extractions and phenol-water treatment.

Proteolipid Fatty Acid Analysis

Lipid depleted membrane fragments and their associated proteolipid were prepared as described in Materials and Methods (Figure 5). In the present study, 0.085 mg of proteolipid protein were isolated from 4.18 mg of LDM-fragment protein. The proteolipid did not contain detectable phosphorous, thus eliminating the possibility of any phospholipids being
associated with the proteolipid. Figure 12 represents a gas liquid chromatograph of FAMEs prepared from the amount of solvents used in the preparation of the proteolipid. Figure 13 denotes a gas liquid chromatograph of the FAMEs prepared from the proteolipid. After a comparison of Figure 12 with Figure 13 it was concluded that there were no detectable fatty acids associated with the proteolipid.

Fatty Acid Analyses of Total Lipid and Cardiolipin-Phosphatidylethanolamine From Whole Membranes

A preparation of total lipid (9% cardiolipin, 31% phosphatidylglycerol and 60% phosphatidylethanolamine) from membranes of *B. stearothermophilus* and a preparation of 90% cardiolipin - 10% phosphatidylethanolamine from whole membranes of *B. stearothermophilus* were kindly donated by Dr. G. L. Card, University of Montana. Fatty acid analyses of these two preparations demonstrated they had similar fatty acid compositions (Tables 14 and 15).
Fig. 12. Gas liquid chromatograph of the FAMEs prepared from the mixture; 42 ml chloroform, 22 ml methanol, 71 ml chloroform-methanol (2:1). FAMEs were suspended in 0.1 ml n-hexane and 1.0 µl analyzed at ATT 2, range 10, 180°C isothermal. FAMEs = fatty acid methyl esters, ATT = attenuation.
**Fig. 13.** Gas liquid chromatograph of the FAMEs prepared from proteolipid associated with the LDM-fragments. FAMEs were suspended in 0.3 ml n-hexane and 1.0 μl analyzed at ATT 2, range 10, 180°C isothermal. FAMEs = fatty acid methyl esters, ATT = attenuation.
Table 14
Fatty Acid Analysis of Total Lipids from Whole Membranes

<table>
<thead>
<tr>
<th>FA</th>
<th>GLC Run #1a,b</th>
<th>GLC Run #2a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area (in²)</td>
<td>Weight % of FA</td>
</tr>
<tr>
<td>i-14:0</td>
<td>0.49</td>
<td>2.2</td>
</tr>
<tr>
<td>a-15:0</td>
<td>9.40</td>
<td>41.8</td>
</tr>
<tr>
<td>i-16:0</td>
<td>1.80</td>
<td>8.0</td>
</tr>
<tr>
<td>a-17:0</td>
<td>10.8</td>
<td>48.0</td>
</tr>
<tr>
<td>Total</td>
<td>22.49</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*a* Calculations from two different GLC runs on the total lipid preparation.

*b* The weight % of n-16:0 could not be determined since the amount of solvent employed in sample preparation was not known. GLC = gas liquid chromatography, FA = fatty acid.
Table 15

Fatty Acid Analysis of Cardiolipin-Phosphatidylethanolamine Derived from Whole Membranes

<table>
<thead>
<tr>
<th>FA</th>
<th>GLC Run #1a,b</th>
<th>GLC Run #2a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area (in²)</td>
<td>Weight % of FA</td>
</tr>
<tr>
<td>i-14:0</td>
<td>0.23</td>
<td>2.6</td>
</tr>
<tr>
<td>a-15:0</td>
<td>3.85</td>
<td>43.8</td>
</tr>
<tr>
<td>i-16:0</td>
<td>0.76</td>
<td>8.6</td>
</tr>
<tr>
<td>a-17:0</td>
<td>3.96</td>
<td>45.0</td>
</tr>
<tr>
<td>Total</td>
<td>8.80</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\(^{a}\)Calculations from two different GLC runs on the cardiolipin-phosphatidylethanolamine preparation.

\(^{b}\)The weight % of n-16:0 could not be determined since the amount of solvent employed in sample preparation was not known. GLC = gas liquid chromatography, FA = fatty acid.
CHAPTER IV
DISCUSSION

According to accepted models of membrane structure about 20% of the membrane lipid is associated with integral membrane protein. The nature of lipid-protein interactions and the relationship between bulk lipid (bilayer-lipid) and protein-associated lipid are unknown. The purpose of the present investigation was to compare the fatty acid composition of protein-associated lipid with the fatty acid composition of bilayer lipid from Bacillus stearothermophilus NCA 2184.

The preponderance of branched-chain fatty acids has been shown to be a characteristic feature of the genus Bacillus (54). Results of the present study indicated the bulk membrane lipid from B. stearothermophilus NCA 2184 grown at 60° ± 2°C in a minimal essential medium contained the following fatty acids: 2.4% iso-C14, 28.3% anteiso-C15, 26.4% iso-C16, 8.4% C16 and 34.6% anteiso-C17. Although the analyses of membranes from different laboratories show a good measure of agreement in the nature of the principle fatty acids, there are both quantitative and qualitative differences in composition. These differences have been attributed to the marked effect that cultural conditions (such as composition of the growth medium and the growth temperature) have on lipid composition. These differences have also been attributed to differences between bacterial strains. Daron (21) noted that lipids from B. stearothermophilus FJW grown at
60°C in 1% glucose medium supplemented with 0.1% tryptone contained 62.5% palmitic acid. On the other hand, when this organism was grown at 60°C in 1% acetate medium supplemented with 0.1% tryptone, the lipid consisted of over 50% branched-chain fatty acids. Studies by Yao et al. (115) reported that Bacillus stearothermophilus ATCC 7953 grown in 1% trypticase (BBL)-0.2% yeast extract (Difco) contained predominately branched-chain fatty acids when grown at 45°C and mostly straight-chain fatty acids when grown at 55°C. From the work of Cho et al. (17) and Shen et al. (90) it was shown that B. stearothermophilus (FJW and NCA 2184) grown in 1% trypticase (BBL) - 0.2% yeast extract (Difco) contained predominately branched-chain fatty acids when grown at 55°C. Of these branched-chain fatty acids, 68.9% were iso-fatty acids (90). In contrast to the work of Shen et al. (90), results of the present study (Table 12) indicated bulk membrane lipid from B. stearothermophilus grown at 60 + 2°C in a minimal essential medium consisted of predominately anteiso-fatty acids.

Since approximately one-fifth of membrane lipid is associated with membrane proteins (enzymes), membrane researchers have questioned the possible function of this lipid. The involvement of lipid in the function of membrane enzymes was first demonstrated when it was shown that rebinding phospholipids to acetone extracted mitochondria led to reactivation of respiratory activity (9). In bacteria, requirements for specific phospholipids have been reported for ATPase, succinate oxidase, cytochrome oxidase and pyruvate oxidase (29,76). In a few cases (73,74,100), free fatty acids have been shown to influence enzyme activity.
Although many membrane enzymes are influenced by lipids, the exact mechanism (or nature) of lipid-enzyme interactions has received little attention. Recently, evidence has been presented for covalent fatty acid-protein interactions. Yamamoto and co-workers (114) have shown that the membrane enzyme penicillinase from *Bacillus licheniformis* 749/C contained phosphatidylserine covalently bound to the enzyme. MacLennan (62) has shown that the Ca\(^{2+}\) transport ATPase enzyme of sarcoplasmic reticulum contained two covalently bonded fatty acid molecules per protein molecule.

In this study, the possible presence of covalent lipid-protein interactions was investigated using a lipid depleted membrane fragment (LDM-fragment) isolated from membranes of *B. stearothermophilus* by detergent extraction and gel filtration chromatography. This fragment consisted of cytochromes, cytochrome oxidase, succinate dehydrogenase and the ATPase complex. No covalently bound fatty acids were detected after treating the LDM-fragment (21.3 mg protein) under conditions which would break ester or amide bonds (sensitivity limit 1.7 x 10\(^{-3}\) \(\mu\)eq FA).

The membrane ATPase complex (designated F\(_0\).F\(_1\)) is comprised of two components: an integral membrane component (F\(_0\)) and a peripheral component (F\(_1\)). The catalytic and peripheral moiety of the ATPase complex (F\(_1\)-ATPase) is composed of five subunits (\(\alpha, \beta, \gamma, \delta\) and E) and has a molecular weight of 3.8 X 10\(^5\) (from the thermophilic bacterium PS3,93). The membrane integral part of the ATPase (F\(_0\)) has been defined as a factor(s) that renders F\(_1\)-ATPase sensitive to energy transfer inhibitors, such as oligomycin and NN'-Dicyclohexylcarbodiimide (DCCD,93). Little
else is known about the construction of $F_0$ except that the smallest subunit of $F_0$ from the thermophilic bacterium PS3, which binds DCCD, is a proteolipid (93). Proteolipids have also been identified with the ATP energy transducing system of *E. coli* (31), the sarcoplasmic Ca$^{2+}$ ATPase (63,80) and the mitochondrial ATPase (13). The exact definition of proteolipid has been the subject of some controversy. Folch and Lees defined proteolipids as lipid-protein complexes that were soluble in organic solvents but insoluble in water (cited in ref. 13). Fillingame defined proteolipids as proteins that were soluble in chloroform-methanol (31). From Folch and Lees's definition, proteolipids may represent proteins covalently conjugated with lipids. To investigate the possible presence of lipid-protein conjugates that might have been extracted upon chloroform-methanol treatment of the LDM-fragment, fatty acid analysis of proteolipid associated with the LDM-fragment was performed. No fatty acids could be recovered after treating the chloroform-methanol soluble protein (85 micrograms) of the LDM-fragment under conditions which would break ester or amide linkages. Assuming the proteolipid had a molecular weight of 8000 daltons (93) and the limit of detection for the present assay ($1.7 \times 10^{-3} \mu$eq FA), one microequivalent of fatty acid per ten micromoles proteolipid could have been detected.

Inasmuch as lipid is required for the function of the enzymes present within the LDM-fragment from *B. stearothermophilus* NCA 2184 (except for succinate dehydrogenase, personal communication from Dr. George L. Card) and no lipid covalently bound to the LDM-fragment was noted, it appears
that non-covalent lipid-protein interactions must be responsible for the influence that lipids have on the function of these enzymes.

Card et al. (12) demonstrated that lipid associated with a membrane LDM-fragment isolated from *B. stearothermophilus* consisted of 75-90% cardiolipin. Results presented in the present study indicated the fatty acids associated with the LDM-fragment could be accounted for as phospholipid since the molar ratio of fatty acids to phosphorus was 2.1. The expected fatty acid/phosphorus ratio for the three major phospholipids of *B. stearothermophilus* - cardiolipin, phosphatidylglycerol and phosphatidylethanolamine - would be 2.0.

Although it appears that the enzymes present within the LDM-fragment interact with phospholipids non-covalently, there remains much controversy as to which part of the phospholipids molecule (polar or non-polar) is involved in the lipid-protein interactions. At neutral pH the majority of membrane phospholipids are either zwitterionic or bear a net negative charge (18). The head groups of the phospholipids are thus available for electrostatic bonding. In support of the importance of the polar portion of phospholipids in lipid-protein interactions, De Pont and co-workers (24) noted that phosphatidylserine, or negatively charged phospholipids in general, were essential for rabbit kidney outer medulla (Na\(^+\) + K\(^+\)) - ATPase activity. Further support for the importance of the polar head group in lipid-protein interaction was given by Esfahani et al. (29) when they noted a specificity of the polar group of phospholipids (especially cardiolipin) in the segment of the electron transport chain of *E. coli*.
from NADH dehydrogenase through coenzyme Q. In contrast, Swanljung et al. (98) stated that the hydrocarbon chains of fatty acids were the important part of negatively charged phospholipids in the activation of beef heart mitochondrial ATPase since the amount of phospholipid required to give half-maximal activation was dependent upon (or was inversely related to) the number of fatty acid chains in the lipid (e.g. the ATPase is activated up to 18-fold by lysolecithin and to a smaller extent by cardiolipin and phosphatidylethanolamine). It therefore appears that the specificity of lipid-protein interactions can reside in either the polar or non-polar portion of the phospholipid.

It has been shown by Card et al (12) that phospholipid non-covalently associated with a LDM-fragment was enriched in cardiolipin (78 ± 4% of the total phospholipids). In contrast, cholate extracted bulk lipid contained little cardiolipin (9% of the total phospholipids). Results (Tables 14 and 15) of the present study indicated that the total lipid (isolated from whole membranes) and a preparation containing 90% cardiolipin - 10% phosphatidylethanolamine had similar fatty acid compositions (major fatty acids were anteiso-C_{15}, iso-C_{16} and anteiso-C_{17}). It's clear from data shown in Table 12 that the fatty acid composition of lipid associated with the LDM-fragment was essentially the same as the cholate extracted bulk lipid. Together, the above results suggest that the specificity of cardiolipin toward the LDM-fragment resides in the polar headgroup and not in the non-polar segment.
In conclusion, results of the present study indicated that lipids and proteins present within the LDM-fragment interact non-covalently through the polar headgroup of phospholipid. These phospholipids, like the bulk layer phospholipids reported in this study and in other Bacilli, contained predominantly branched-chain fatty acids.
CHAPTER V
SUMMARY

The fatty acid composition of bulk (bilayer) membrane lipid and membrane protein-associated lipid from Bacillus stearothermophilus was investigated. A lipid-protein complex was isolated from membranes of B. stearothermophilus using sodium cholate and purified by column chromatography with Sepharose 6B. The lipid-protein complex (designated as the lipid depleted membrane fragment) contained cytochromes, succinate dehydrogenase, cytochrome oxidase and the adenosine triphosphatase complex (12). Fatty acid analyses of lipid covalently bound (through amide and/or ester linkages) and non-covalently associated with proteins in the lipid depleted membrane fragment (LDM-fragment) were performed. The fatty acid composition of proteolipid associated with the LDM-fragment was also investigated.

(1) The major fatty acids of cholate extracted bulk lipid from B. stearothermophilus grown in a minimal essential medium at 60 ± 2°C were anteiso-C_{15}, iso-C_{16} and anteiso-C_{17}.

(2) The fatty acid composition of the lipid non-covalently associated with the LDM-fragment was essentially the same as the cholate extracted bulk lipid. The lipid non-covalently associated with the LDM-fragment contained 0.3 microequivalents of fatty acids per milligram of LDM-fragment protein.
(3) The molar ratio of fatty acids to phosphorous of the lipid non-covalently associated with the LDM-fragment was 2.1. This suggested that the lipid-noncovalently associated with the LDM-fragment was phospholipid which would have an expected ratio of 2.0. All of the neutral lipid and free fatty acids were apparently extracted by cholate.

(4) A phenol water extract of the LDM-fragment after nine successive Bligh and Dyer extractions contained no detectable fatty acids. This suggests that no lipoteichoic acid like molecules (these are polyglycerolphosphates with fatty acids esterified to the terminal groups) were associated with the LDM-fragment.

(5) After exhaustive extractions of the LDM-fragment with chloroform-methanol to remove non-covalently bound lipid, no amide or ester linked fatty acids were detected.

(6) The chloroform-methanol soluble proteolipid (0.085 milligrams of protein) extracted from the LDM-fragment contained no detectable fatty acids.

(7) Total lipid (isolated from whole membranes of B. stearothermophilus) and a preparation containing 90% cardiolipin - 10% phosphatidylethanolamine (isolated from the aforementioned total lipid) had similar fatty acid compositions (major fatty acids were anteiso-C_{15}, iso-C_{16} and anteiso-C_{17}, Tables 14 and 15).
Data from this study and results obtained by Card et al (12) that LDM-fragments from *B. stearothermophilus* were enriched in cardiolipin led to the conclusion that the specificity of phospholipids associated with the LDM-fragment resides in the phospholipid polar head group and not in the phospholipid non-polar segment.


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# APPENDIX I

## Thermophilic Defined Minimal Essential Medium For B. stearothermophilus

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Amount (ml) of Stock Solution Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate-HCl (1%)</td>
<td>60.0</td>
</tr>
<tr>
<td>L-Glutamine (1%)</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Histidine-HCl(\cdot)(\text{H}_2\text{O}) (1%)</td>
<td>6.3</td>
</tr>
<tr>
<td>L-Isoleucine (1%)</td>
<td>15.0</td>
</tr>
<tr>
<td>L-Methionine (1%)</td>
<td>7.8</td>
</tr>
<tr>
<td>L-Valine (1%)</td>
<td>18.9</td>
</tr>
<tr>
<td>Biotin (10 mg/100 ml)</td>
<td>15.0</td>
</tr>
<tr>
<td>Thiamine-HCl (10 mg/100 ml)</td>
<td>15.0</td>
</tr>
<tr>
<td>Nicotinic Acid (10 mg/100 ml)</td>
<td>15.0</td>
</tr>
<tr>
<td>Anhydrous CaCl(_2) (5%)</td>
<td>0.15</td>
</tr>
<tr>
<td>FeCl(_3)(\cdot)6(\text{H}_2\text{O}) (0.5%)</td>
<td>0.15</td>
</tr>
<tr>
<td>ZnSO(_4)(\cdot)7(\text{H}_2\text{O}) (5%)</td>
<td>0.15</td>
</tr>
<tr>
<td>MnCl(_2) (10 mM)</td>
<td>0.15</td>
</tr>
<tr>
<td>Glucose (20%)</td>
<td>15.0</td>
</tr>
<tr>
<td>Mineral Salts (10 g NH(_4)Cl, 10 g NaCl, 4 g MgSO(_4) per liter)</td>
<td>150.0</td>
</tr>
<tr>
<td>KH(_2)PO(_4) (1M)</td>
<td>1.5</td>
</tr>
<tr>
<td>Tris (hydroxymethyl) aminomethane-Hydrochloride pH 7.8 (1M)</td>
<td>150.0</td>
</tr>
</tbody>
</table>

*a* To 1022.4 ml of sterile distilled water add the designated amounts of stock solutions in the order listed to prevent precipitation. Final pH is 7.8.

*b* Prepare and autoclave each stock solution separately.
APPENDIX II

Protein Determination by the Method of Lowry (59)

Reagents

1. 1N NaOH
2. 1N Folin Reagent
3. 1% CuSO₄
   2% Na-K Tartrate
   2% Na₂CO₃
4. Reagent C
   50 ml Na₂CO₃ + 0.5 ml Na-K Tartrate + 0.5 ml CuSO₄
   Make reagent C fresh each day

Procedure

1. Add 0.2 ml of sample (diluted if necessary) to a 13 x 100 mm test tube.
2. Prepare a blank using 0.2 ml of the compound that the sample is suspended in.
3. Add 0.25 ml of 1 N NaOH to each tube and place in a boiling water bath for 10 min.
4. Cool and add 2.5 ml of Reagent C. Allow to stand at room temperature for 10 min.
5. Add 0.25 ml of Folin reagent. Mix each tube immediately after adding Folin reagent.
6. Allow all tubes to stand at room temperature for 30 minutes then read the absorbance at 750 nm.
7. Calculate protein concentration by using the slope of a pre-determined standard curve. \( \text{ug/ml} = \text{Absorbance} \times \text{dilution factor} \)
x slope of standard curve. A standard curve must be generated each time CuSO$_4$, Na-K Tartrate or Na$_2$CO$_3$ is prepared

APPENDIX III

**Phosphorous Determination (10)**

**Reagents**

1. **Fiske-Subba Row Reagent**
   
   **stock reagent:** Mix well 2.5 g of 1, 2, 4-amino-naphthol sulfonic acid, 142.5 g sodium bisalifite, and 5.0 g sodium sulfite.
   
   **reagent:** Dissolve 1.2 g stock power in 20.0 ml distilled water (stable 3-4 days or until a white precipitate forms)

2. Ammonium molybdate - 2.5%

3. 70% perchloric acid

4. 1N H$_2$SO$_4$

**Procedure**

1. Add sample (0.1 - 1.0 ml) to a 19 x 150 mm clean test tube.

2. As a blank add the same amount of solvent to another 19 x 150 mm clean test tube.

3. Remove solvent with N$_2$, under vacuum, or by placing at 100°C.

4. Add 1.0 ml of 70% perchloric acid and digest for 3-4 hours at 170°C.
5. Cool then add 5.2 ml distilled H₂O, 0.5 ml 2.5% ammonium molybdate, and 0.5 ml of Fiske-Subba Row reagent, in that order, mixing after each addition.

6. Place tubes in boiling water bath and heat at 90-95°C for 7.5 minutes. Use a tube containing water and a thermometer to time the heating.

7. Cool then read at 820 nm.

8. If necessary, samples (plus the blank) can be diluted up to 1/20 (usually 1/5 or 1/10 dilutions will do) with 1N H₂SO₄. Read A₈₂₀ between 0.05 - 0.50.

9. ugPi/ml = Absorbance x 6.0 x dilution factor.

APPENDIX IV

ATPase Assay Procedure (75)

Reagents

1. 0.05 M silicotungstic acid
2. 0.28 M H₂SO₄
3. 1.0% ammonium molybdate
4. isobutanol - benzene (1:1 v/v)
5. 3.4% H₂SO₄ in absolute ethanol
6. 0.15% SnCl₂ in 1.5 M H₂SO₄
7. 10 mM ATP, 2 mM MgCl₂ (Store at 4°C).
8. 0.1 M Tricine NaOH (store at 4°C)
Procedure

A. Assay Mixture

1. Add 0.2 ml of sample (diluted if necessary) to a 13 X 100 mm test tube.
2. As a blank add 0.2 ml of solvent to a 13 X 100 mm test tube.
3. Add 0.3 ml of a mixture of reagents 7 and 8 (equal volumes) to each tube.
4. Mix immediately and incubate at 60°C for 20 minutes

B. Pi determination

1. Stop the reaction with 0.5 ml of ice cold 0.05 M silicotungstic acid (Reagent 1)
2. Add 3.0 ml of reagent 4 (isobutanol-benzene) and mix well
3. Add 1.0 ml of a mixture of reagents 2 and 3 (equal volumes) mix for approximately 20 seconds
4. Centrifuge (clinical) for 20 minutes to separate phases
5. Remove 1.0 ml of the upper phase and dilute to 5.0 ml with reagent 5 (3.4% H₂SO₄ in absolute ethanol)
6. Add 0.5 ml of reagent 6 (SnCl₂) and mix immediately
7. Read absorbance at 730 nm. Color stable for at least one hour
8. Units of ATPase activity = absorbance x dilution factor.

One unit of ATPase activity is defined as the amount liberating 1 ug of Pi/min.
APPENDIX V

Preparation of Alkaline Hydroxylamine Reagent

Reagent A

4% ethanolic hydroxylamine: 2g hydroxylamine dissolved in 2.5 ml \( \text{H}_2\text{O} \) then diluted to 50 ml with absolute ethanol

Reagent B

8% ethanolic \( \text{NaOH} \): 4g \( \text{NaOH} \) dissolved in 2.5 ml \( \text{H}_2\text{O} \) then diluted to 50 ml with absolute ethanol

Alkaline Hydroxylamine Reagent (make fresh daily)

1. Mix equal volumes of reagent A and reagent B in a 12 ml conical tube
2. Mix well then centrifuge (20 minutes International Clinical Model CL) and decant the supernatant (alkaline hydroxylamine reagent)

APPENDIX VI

Known Mixtures of FAME's

BC Mix-L (Applied Science State College, Pa.)

- \( i = 14:0 \)
- \( n - 14:0 \)
- \( a - 15:0 \)
- \( n - 15:0 \)
- \( i - 16:0 \)
- \( n - 16:0 \)
- \( a - 17:0 \)
H103 (Applied Science, State College, Pa.)
  n - 15:0
  n - 16:0
  n - 17:0
  n - 18:0
  n - 19:0

Mix KD (Applied Science, State College, Pa.)
  n - 14:0
  n - 16:0
  n - 16:1
  n - 18:0
  n - 18:1

K101 (Applied Science, State College, Pa.)
  n - 8:0
  n - 10:0
  n - 12:0
  n - 14:0
  n - 16:0
  n - 18:0
  n - 20:0
ME-Mix-24 (Alltech Associates, Arlington Heights, Ill.)
  n - 13:0
  n - 14:0
  n - 15:0
  n - 16:0
  n - 17:0

ME-Mix-26 (Alltech Associates, Arlington Heights, Ill.)
  n - 13:0
  n - 15:0
  n - 17:0
  n - 19:0
  n - 21:0

K103 (Applied Science, State College, Pa.)
  n - 12:0
  n - 14:0
  n - 14:1
  n - 16:0
  n - 16:1

K102 (Applied Science State College, Pa)
  n - 14:0
  n - 16:0
  n - 16:1
  n - 18:0
  n - 18:1