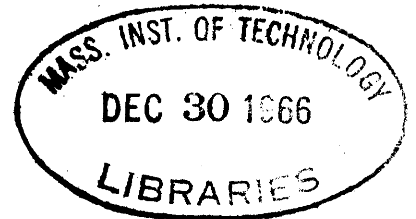

MASS. INST. OF TECHNOLOGY
DEC 30 1966
LIBRARIES

A STUDY OF LIPID COMPOSITION OF BACILLUS MEGATHERIUM

by

Cesar Maximo Vidalon M.

Ing. Agro., Universidad Agraria
Lima, Peru
(1963)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF
SCIENCE

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
July, 1966

Signature of Author ....
Cesar M. Vidalon M.
Department of Nutrition and Food Science
July 19, 1966

Certified by ....
Thesis Supervisor

Accepted by ....
Chai[illegible]e on Thesis

A Study of Lipid Composition Of

Bacillus Megatherium

by

Cesar Maximo Vidalon M.

Submitted to the Department of Nutrition and Food Science on July 19, 1966 in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The lipid composition of Bacillus megatherium was studied by extracting the lipids with chloroform-methanol (2:1) and purified by the Folch method. Lipids were fractionated by DEAE-cellulose column chromatography. Lipid classes were determined by thin layer chromatography. Diglycerides, hydroxy-fatty acids and free fatty acids were qualitatively determined as the major neutral lipids. Poly-B-hydroxybutyrate was indirectly calculated as 72% of the total chloroform-methanol lipid extract. Phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine and an unknown designed as #1 were the major phosphlipid compounds. Fatty acids were determined by gas liquid chromatography. The major portion of the normal saturated fatty acids was 16:0 (10.8%). The major compound of the total fatty acids was tentatively identified as a 15:branched chain acid. Fatty acids with 16 or fewer carbon atoms constituted 91.5% of the total fatty acids.

Thesis Supervisor: Phillip Issenberg

Title: Assistant Professor of Food Science

ACKNOWLEDGEMENTS

The author would like to express deep appreciation to his thesis advisor, Professor Phillip Issenberg, for his kindness, encouragement and patience.

The author is also grateful to his collegues Marshall Myers and Judy Goldstein for their assistance. Also, sincerest gratitude is expressed to Mr. Evangelos Paneras, who was particularly generous with his assistance.

TABLE OF CONTENTS

		Page
	Abstract....................................	2
	Acknowledgements............................	3
I.	Introduction................................	9
II.	Literature Survey...........................	11
	A. 1. General Consideration..................	11
	2. Extraction and Purification of Lipids.......	12
	3. Separation and Identification of Lipids....	13
	B. Bacterial Lipids..........................	14
	1. General..................................	14
	2. Lipid Composition in Various Bacteria......	19
	a. Enterobacteriaceae. Gram-negative......	19
	b. Lactobacillaceae. Gram-positive......	20
	c. Bacillaceae. Gram-positive............	20
	C. Bacillus megatherium......................	22
	1. Lipids of Whole Cells.....................	22
	2. Lipid Classes of the Cytoplasmic Membrane...	23
	3. Fatty Acid Composition of the Cytoplasmic	23
	Membrane................................	24
	4. Lipids of the Cell Walls..................	24
III.	Experimental Procedure.....................	27
	A. Growth of Bacteria........................	27
	B. Solvents..................................	27
	C. Extraction and Purification of Lipids.........	28
	1. Extraction by Solvents....................	28

page

2. Acid Hydrolysis........................ 29

3. Folch Washing.......................... 29

4. Dry Matter Determination............... 30

D. Thin Layer Chromatography (TLC)............ 30

1. Developing Solvents.................... 30

2. Preparation of TLC Plates.............. 31

3. Application of Samples................. 31

4. Development of Chromatograms........... 32

5. Detection and Identification of Spots.. 33

a. General Reagent..................... 33

b. Specific Reagents for Phospholipids. 33

c. Reagents for Sterols and Carbohydrates.............................. 35

6. TLC Prints............................. 35

E. DEAE Column Chromatography................. 36

1. Eluting Solvent System for DEAE Columns. 36

2. Preparation of DEAE for Column Chromatography........................ 37

3. Preparation of Columns................. 37

a. Preparation of 20-cm Columns........ 38

b. Preparation and Use of Microcolumns. 39

c. 20-cm Column Elution Procedure...... 39

F. Esterification Methods..................... 42

1. BF_3-Method............................ 42

2. Microesterification Methd 43

Page

a. Reagents.................................. 43

b. Apparatus.................................. 43

c. Procedure.................................. 43

G. Gas Liquid Chromatography (GLC).............. 45

1. Instrument.................................. 45

2. Conditions of Operation.................... 45

3. Injection of Samples.................... 45

IV. Results.................................. 48

A. Total Lipid.................................. 48

B. Chromatographic Separations.................. 48

1. Microcolumns.................................. 48

2. Final Chromatographic Separations.......... 50

C. Identification of Lipid Classes.............. 50

1. Identification of Polar Lipids.............. 50

2. Identification of Neutral Lipids.......... 53

D. Analysis of Fatty Acids Methyl Esters........ 54

1. Esterification.................................. 54

2. Gas Liquid Chromatography.................. 55

V. Discussion.................................. 78

A. Column and TLC Chromatography Separations..... 78

1. Lipid Classes Composition.................. 78

B. Fatty Acid Analysis.................................. 80

1. Esterification.................................. 80

2. Fatty Acid Composition.................. 81

VI. Conclusions.................................. 83

VII. Suggestions for Future Research.............. 85

Bibliography.................................. 87

LIST OF FIGURES AND TABLES

Figure Number — Page

1. Run #5. Thin Layer Chromatography (TLC) from DEAE-column chromatography. Progress of Elution.............................. 71
2. Run #5. TLC from DEAE-column Chromatography Before and After Folch Wash............... 72
3. TLC During Chemical Tests on the Plates... 73
4. TLC of Fractions I - VI Esterified by the Micromethod................................ 74
5. GLC. Plot Detector Response vs. Concentration 75
6. GLC. Plot Log. Retention Time vs. C-Number at 133°C.................................. 76
7. GLC. Plot Log. Retention Time vs. C-Number at 170°C.................................. 77

Table Number

1. Content and Overall Composition of Lipids in Various Bacteria.......................... 15
2. Phosphatide Composition in Various Bacteria 16
3. Major Fatty Acids of Various Bacteria..... 17
4. Summary of Data on Lipid Composition of Bacteria.................................. 18
5. Lipid Composition of Cytoplasmic Membrane of B. megatherium.......................... 25
6. Fatty Acid Composition of Cytoplasmic Membrane of B. megatherium.......................... 26
7. Identification of Samples.................. 47
8. Run #4. Volumes of Solvent Used for DEAE-cellulose column chromatography................. 59
9. Run #5. Lipid fractions from DEAE-Column Chromatography........................... 60
10. Runs #6 & #7. Elution scheme for DEAE-column Chromatography........................... 61
11. Runs #6 & #7. Weight of Fractions from DEAE Cellulose................................ 62

Page

12. Color Reactions and R_f Values of Phospholipids........................ 63

13. Color Reactions and R_f Values of Neutral Lipids...................... 64

14. BF_3-Method. Percentage Conversion to Methyl Esters........................ 65

15. Microesterification Method. Percentage Conversion to Methyl Esters......... 66

16. Percentage Composition of Fatty Acids in Total Lipid Before and After Folch Wash, and in Acid Extracted Lipid......... 67

17. Percentage Composition of Fatty Acids of Fractions I - VI.................... 68

18. Amount of Fatty Acids per mg of Total Lipid.................................. 69

I. INTRODUCTION

In the last ten years the world's population has grown by about a fifth, representing an average annual rate of increase of 2%, which is much faster than ever before in history. In a number of developing countries the annual increase now exceeds 3%. Because of the much more rapid population growth, the increase in per capita production is lower especially in developing countries, FAO (1965).

This social phenomenon reveals that agriculture is not as attractive or perhaps as competitive as manufacturing industries. A food shortage is likely in the future. A logical way to prevent it will require the use of new food sources and processing means which satisfy some basic requirements, such as:

1. Raw material ready available and at low cost.
2. An efficient means to transform the raw material into food under a variety of conditions.

The first point could be satisfied by using petroleum or nitrogen from the air or their derivatives. The second point would be solved by the use of microorganisms, which could eliminate the uncertainty of the climatalogical conditions in agriculture, and their high adaptability to different environments, make them a versatile food source for human beings.

Technological development creates a new aspect in food supply. The technology of space requires provision of food for man under a different environment. On his long voyages through the space man probably will have to produce his own food.

Many Bacteria have a wide range of adaptability to different

conditions. Bacillus megatherium is an apparently non-toxic species encountered all over the world. This bacterium is found on the soil, in marine air and in the upper air over land (Gregory, 1961).

Apart from adaptability of this organism the problem of nutritional suitability should be considered. In Gram-negative bacteria, toxins forming complexes with lipids and carborhydrates have been reported. Although toxins were not reported in Gram-positive bacteria this possibility should be examined.

Knowledge of the nature of the fatty acids is important. Some unsaturated fatty acids would oxidize rapidly during processing and storage. Absence of unsaturated fatty acids will increase storage stability but the nutritional value will decrease. In dehydrated cells the oxidation rate of unsaturated fatty acids will be enhanced.

The main purpose in the utilization of B. megatherium is as a source of protein. The lipids could be supplemented from other sources, but since always some kind of lipid will be present, a study of its composition is necessary for reasons mentioned above.

This thesis describes a preliminary study of the lipid classes present in B. megatherium and the fatty acid composition of each of these classes.

II LITERATURE SURVEY

A. 1. General Considerations.

The non-specific analyses of a few years ago, when interest was directed to the percentage of total lipids, and later to the determination of total fatty acids (O'Leary, 1962), provide little information about the distribution of the fatty acids among specific lipid classes (Huston, 1964). An explanation for this lag in knowledge can be found in the words of Porter (1950): "Pure fats are practically never found in bacteria, instead they occur together as complex mixture which are extremely difficult to separate and purify". With the developement of new techniques, especially gas chromatography, studies of lipids from all sources are becoming more specific and exact.

Bacterial lipids, in general, differ substantially from those of higher life forms in such respects as the absence of sterols, phospholipids low in nitrogen and high in carbohydrates, and presence of large proportions of free fatty acids, and the presence of certain fatty acids not ordinarily found in other life forms (Huston, 1964).

Many authors have observed quantitative and qualitative variations in bacterial lipids depending on factors such as: composition of the culture medium (Lemoigne, 1944; Porter, 1950; Woodbine, 1959; Sthephenson, 1966); the gaseous environment (Macrae, 1959); age of the culture (Lemoigne, 1944; Asselineau, 1960); temperature (Shaw, 1965;

Marr, 1962). Even different batches of the same strain will give different results, and the necessity for using a reproducible medium in order to get consistent results is emphasized by O'Leary (1962). Another difficulty in the analysis of bacterial lipids is the enzymatic alterations of the native lipids during extraction (Asselineau, 1960).

2. Extraction and Purification of Lipids.

Many factors affect lipid extractability. Lovern (1955) points out the following: (a) much of the lipid may be present in protein or carbohydrate complexes which are usually insoluble in fat solvents, (b) some lipids are only slightly soluble in fat solvents, (c) some fat solvents are also good solvents for certain non-lipid constituents of tissue. A further complication is that wet tissue cannot be efficiently extracted. There is no difficulty extracting triglycerides: they do not form complexes in the tissue and are soluble in practically all fat solvents. Phospholipids, sphingolipids and sterols are usually present in tissue partly in "bound" form.

The extraction method will depend on the kind of information required. If the purpose is to study specific lipids, vigorous conditions may damage or destroy some of the more labile constituents. If conditions are too mild the recovery of the constituents will not be complete (O'Leary, 1962). Many solvents, singly or as mixtures, have been used. A review is presented by Entenman (1957).

Non-lipid contaminants are always present in the extract and they have a two-fold origin: (a) acetone and alcohol, especially with wet tissue, are quite effective extractants for many of the non-lipid constituents of tissue, e.g. urea, amino acids, various nitrogenous bases, sugars, etc; (b) substances normally insoluble in fat solvents are readily soluble in the presence of phospholipids (Lovern, 1955). These contaminants are mainly water soluble substances and methods for their removal are based on this fact. Various methods have been used for purification, but the procedure developed by Folch (1956) appears to be the most widely accepted.

3. Separation and Identification of Lipids.

A review of separation methods is presented by Fontell (1961). In recent years adsorption chromatography on columns has proved to be a satisfactory method for separating lipid classes and single compounds. Adsorbents used include: silicic acid (Hanahan, 1957; Hirsch, 1958; Lea, 1955); combination of silicic acid column chromatography and paper chromatography (Vorbeck, 1965; Rouser, 1963); or combination with thin layer chromatography (TLC)(Smith, 1965; Rouser, 1963); diethyl amino ethyl cellulose (DEAE) column chromatography and TLC were used by Rouser (1963, 1964). Thin layer chromatography alone has been used by many workers, (Mangold, 1960; Skipski, 1962, 1964; Barret, 1962; Morris, 1963; Lepage, 1963; Blank, 1964; Nichols,

1964; Pelick, 1965; Freeman, 1966).

Gas liquid chromatography, introduced by James and Martin (1952), is at present widely used for the analysis of fatty acids, usually as methyl esters.

B. Bacterial Lipids

1. General

The early work on bacterial lipids was concerned only with determination of the percentage of total lipid of various organisms. Because the values reported can vary considerably with strains used and culture conditions these figures will give only general ideas of cell compositions.

Values of "free lipids" and "bound lipids" of whole cells for a number of bacteria are given by Asselineau (1960) and Kates (1964), the latter also gives values for neutral lipids and phospholipids. Tables I - IV give a general view of the total lipid content, phosphatides and major fatty acids in species of three bacterial families, most of the data were extracted from Kates (1964).

The description of bacterial lipids which follows will emphasize: (1) those compounds not ordinarily present in other forms of life and, (2) those compounds ordinarily present in other forms of life and absent or present only in trace levels in bacterial lipids.

TABLE I Content and Overall Composition of Lipids in Various Bacteria (6)

	Eubactecteriales				
	Gram-positive				Gram-negative
	Bacillaceae			Lacto bacillac.	Entero-bacter.
	B. meg.	B. cereus	Cl. perfr.	L. casei	E. coli
Lipid content, % cell dry wt.			1		
Free Lipids	19.1, 2.0	2.0	1.6	3.6	9.2
Bond Lipids	2.0, 2.0	-	-	1.1	-
Lipid composit, % of free lipids					
Phosphatide	90	50	45	70	86
Glycolipids	0	-	10	tr	-
Neutral lipids			30		4
Glycerides	≠	≠	↑	tr	↑
F. Fatty Acids	≠	-	↑	-	↑
Unsaponif.	-	5(alcohol)	↑	3.6	↑
Others	(B.hydroxy but.)	tr(Plasmalogen.)	-	-	-
References	(1)	(2)	(3)	(4)	(5)

1. Lemoigne, 1944; Weibull, 1957
2. Kates, 1962
3. Macfarlane, 1962
4. Ikawa, 1963
5. Kaneshiro and Marr, 1962

6. - = Not determined
≠ = Component present but amount unknown
tr = trace

TABLE 2 Phosphatide Composition in Various Bacteria
(% of Total Phosphatides)

	Eubacteriales				
	Gram-positive				Gram-negative
	Bacillaceae			Lacto-bacillac.	Entero-bacter.
	B. megatherium "M"	B. Cereus	Cl. perfringes	L. casei	E. coli
Phosph. Acid.	+	- , +	↑	-	-
Diphosph. glycerol	+?	12, +	12	-	-
Phosph. glycerol(PG)	+?	28, 35	↓	-	+
O-amino acid of PG	+?	+?, 8	88	+	+ ?
Phosph. inositol	0	- , +?	-	0	-
Phosph. serine	tr	0 , -	-	0	+
Phosph. ethanolamine	-	39, 46	-	0	> 90
Phosph.-N-methylethanolam.	-	- , -	-	-	-
Phosph.-N-dimethylethanolam.	-	- , -	-	-	-
Phosph. choline	0	+?, 0	-	0	0
	(1)	(2)	(3)	(4)	(5)

1. Weibull, 1957
2. Kates, et al, 1962; Houtsmuller and van Deenen, 1963
3. Macfarlane, 1962
4. Ikawa, 1963; 5) Law, 1961; Kaneshiro and Marr, 1962; Kanfer and Kennedy, 1963.

TABLE 3

TABLE 3 Major Fatty Acids of Various Bacteria (% of Total Fatty Acids)

		Eubacteriales				
		Gram-positive				Gram-negative
		Bacillaceae			Lacto-bacillaceae	Entero-bact.
	Fatty Acids	B. megatherium (6)	B. cereus	Cl. perfringes	L. Casei	E. coli
Normal sat.	12:0		-	-	-	2.86
	14:0		2.0	24.0	0.7	2.0
	16:		9.0	/	9.0	36.0
	18:0		tr	/	1.0	
	18:0		-	30.0	-	
Normal unsat.	16:1		5.0	-	10.0	21.9
	18:1		0	-	26.	20.02
Cyclopropane	17: cyc		0	-	0	8.8
	19: cyc		0	-	49.	4.9
Branched (1)	13: br		12	-	-	
	15: br		40	-	-	
	17: br		17	-	-	
Hydroxy acids	14: OH		-	-	-	5.9
References			(2)	(3)	(4)	(5)

1. Includes iso and anteiso isomers
2. Kates *et al*, 1962
3. Macfarlane, 1962 a,b
4. Thorne and Kodicek, 1962
5. Gavin, 1965
6. See Table 6

TABLE 4

Summary of Data on Lipid Composition of Bacteria According To Their Taxonomic Classification

	Eubacteriales			
	Gram-positive			Gram-negative
	Bacillaceae		Lacto-bacillac.	Entero-bacteriac.
	Bacilli	Clostridia		
M. Fatty A.	13: br	16:0	16:0	16:0
	15: br	16:1	16:1	16:1
	17: br	18:1	18:1	18:1
		17: cyc.		17: cyc.
		19: cyc.	19: cyc.	19: cyc.
M. Phosph.(1)	PE	PE		PE
	PG		PG	PG
	poly GP			poly GP
	PG-AA		PG-AA	PG-AA
		PS		PS
		Me-PE		
		Plasmalogen		

Abbreviations: br = branched; cyc. = cyclopropane; PG = Ph. Glycerol; poly-PG = Poly-glycerol phosphatide; PG-AA = O-amino acid ester of PG (lipamino acid); PS = Ph. serine; MePE = phosphatidyl -N-methyletanolamine; PE = Phosphatidyl ethanolamine; M. Fatty A. = Major Fatty Acids; M. Phosph. = Major Phosphatides.

2. Lipid Composition in Various Bacteria

a. Enterobacteriaceae. Gram-negative.

Escherichia coli has been studied in detail with regard to lipid composition by Kaneshiro and Marr (1962), Gavin (1965), Shaw (1965) and Law (1961).

Among the phospholipids, lecithin is absent and phosphatidyl ethanolamine constitutes most of the phospholipid. Although palmitic acid constitutes 36% of the fatty acids, fatty acids with 17 and 19-carbon atoms, containing the cyclopropane ring have been found. The C_{17} acid has been identified by Kaneshiro and Marr (1961) as cis-9, 10-methylene hexadecanoic acid and C_{19} has been indentified by Hoffman et al (1954, 1955) as cis-11,12-methylene octadecanoic acid (lactobacillic acid). No branched chains have been reported in E. coli. Among the unstaurated fatty acids 16:1 was identified as palmitoleic acid and the 18:1 was a mixture of 70% cis-11,12-octadecenoic acid (cis-vaccenic acid), and 30% oleic acid, (Kaneshiro and Marr, 1961). 3-Hydroxytetradecanoic acid (β-hydroxymyristic acid) has been reported by Gavin (1965) and Shaw (1965). Cells of E. coli also contain 1.4% of bound fatty acids, which includes all of the C_{10} and C_{14}-hydroxy acids found in the cell, together with a spectrum of fatty acids typical of the lipopolysacharide endotoxin,

(Law, 1961; Kates, 1964).

Branched chains have not been reported in Gram-negative bacteria. If present, they are probably in very low concentration. The same is the case for saturated fatty acids with more than 18-carbon atoms.

b. Lactobacillaceae. Gram-positive

Ikawa (1963) has reported that *L. casei*, *L. plantarum*, *Streptococcus faecalis*, *Pediococcus cerevisiae* and *Leuconostop mesenteroides* did not contain any detectable amount of inositol, choline, ethanolamine or serine, most of the nitrogen was accounted for by the presence of bound L-lysine. Phosphatidyl glycerol and O-amino acid esters of phosphotidyl glycerol have been reported as the main phospholipids (Kates, 1964). Thorne and Kodicek (1962) reported C_{19}-cycolpropane, 18:1, 16:1 and 16:0 as the major fatty acids, and only small amounts of 14:0, 15:br (iso), 16:br (iso) and 17:1 in *L. casei*, *L. plantarum* and *L. acidophilus*; 17:cycolpropane was not detected.

c. Bacillaceae. Gram-positive

Clostridium. An important new class of phosphatide, the O-amino acid esters of phosphatidyl glycerol (lipoamino acids) was reported by Macfarlane (1962) in *Clostridium perfringens*. This species contains 1.6% extractable lipids of which 30% is neutral lipid, the remainder being phosphatides and glycolipid. The phosphatides con-

tains about 12% of phosphatidic acid and 88% of several amino acid esters of phosphatidyl glycerol, chiefly the esters of alanine, lysine and ornithine. The glycolipid fraction contains mainly mannose. The total fatty acids were indentified as even carbon number normal saturated acids from C_{10} to C_{20}, predominantly C_{14}(24%) and C_{20} (30%).

In Cl. butyricum, Goldfine (1962) reported 25% of the total phospholipid was plasmalogen, another important component was an unidentified fraction containing N-methyl ethanolamine as the predominant base.

Bacillus. Kates et al (1962) investigated the lipids of Bacillus cereus. This bacterium contains about 2% of extractable lipids, of which about 50% is phospholipid: the reamined consisting of diglycerides, and unsaponifiable material. The phospholipid consists chiefly of phosphatidyl ethanolamine (36%), phosphatidyl clycerol (30%) and poly-glycerol phosphatide (10%); the minor components determined by color reaction were lecithin and and lysocompounds. However, Houstmuller and van Deenen (1963 ,) did not find lecithin nor lysocompounds but confirmed the presence of phosphatidyl ethanolanime and phosphatidyl glycerol. They reported the presence of diphosphatidyl glycerol (cardiolipin), phosphatidic acid and O-ornithine

ester of phosphatidyl glycerol (8%).

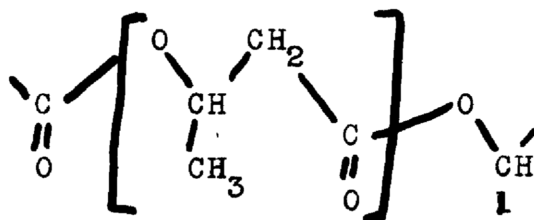
Bacillus polymyxa was also reported to contain mainly phosphatidyl glycerol, phosphatidyl ethanolamine, and small amounts of phosphatidic acid and lysophosphatides (Matches, et al 1964).

C. Bacillus megatherium

1. Lipids of whole cells.

In B. megatherium, Weibull (1957) reported 2% free lipids in whole cells and Lemoigne (1944) found 19.1% free lipids in whole cells and 2% as bound lipids. He also reported the presence of poly-β-hydroxybutyrate in amounts of 8 to 26% on dry cell basis for B. megatherium and 15-19% for B. cereus. The presence of phosphatidyl glycerol is reported by Haverkate , et al in Kates (1964). The fatty acid composition of the membrane (which would be very similar to that of the whole cell) is qualitatively very much the same as that of B. cereus, differing mainly in the proportions of branched-C_{15}(iso and anteiso). (Kates, 1964).

Lemoigne (1944) working with B. Megatherium and B. cereus found that all of the poly-β-hydroxybutyrate is present in the lipid inclusions. The structural formula of this compound is;



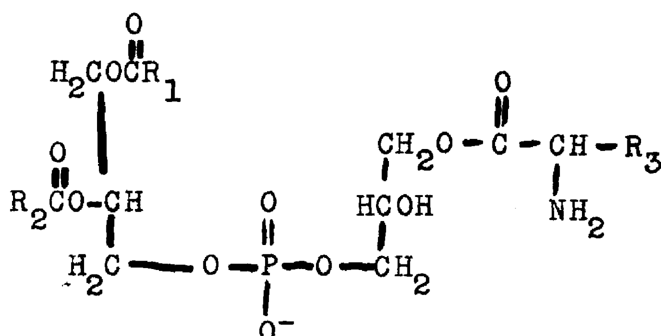
the most highly polymerized fractions $(C_4H_6O_2)_n$(m.p.179°),

contain about 110 residues (Asselineau, 1960). Williamson, (1958) reported that the intracellular lipid inclusions in *B. cereus* grown under a variety of cultural conditions contains 89% of poly-β-hydroxybutyrate and 11% of ether-soluble lipid. The role of this compound is not well elucidated, Macrae (1958) concluded that this compound is a storage material, and he did not find enough evidence to establish its role as a reserve of carbon and energy sources.

Poly-β-hydroxybutyrate was reported in a variety of other bacteria such as *Azotobacter agilis*, *Rhizobium sp*, *Chromobacterium sp*, *Pseudomonas solanarum* and *P. antimycetica* by Forsyth *et al* (1958); in *Micrococcus halodenitrificans* by Kates *et al* (1961).

2. Lipid classes of the cytoplasmic membrane.

In Table 5 is presented a summary of data reported by Weibull (1957, 1958) and Yudkin (1962). Furthermore, high concentrations of lipoamino acids were reported by Hunter and Godsall (1961) in protoplasts of *B. megatherium*. Macfarlane (1962b) discovered that the lipoamino acids are actually O-amino acid esters of phosphatidyl glycerol, the structure of which is,



Where: R_1 and R_2 = fatty acid residues
R_3 = amino acid residue.

She further found that high proportions of lipoamino acids could be isolated if precautions were taken to reduce to a minimum the action of hydrolytic enzymes during the isolation procedure. These anzymes hydrolyze the amino acid ester linkage, resulting in the formation of phosphatidyl glycerol and free amino acids. The activity of the enzyme was found to vary in different species such as B. cereus, B. megatherium, P. stutzeri and S. marcescens by Houtsmuller and van Deenen (1963), and in lactic acid bacteria by Ikawa (1963). It was suggested also that lipoamino acids accumulate in the stationary phase of growth (Kates, 1964). Hunter and Goodsall (1961) reported that a variety of amino acids were incorporated into lipoamino acids of B. megatherium protoplasts. Phenylalanine and arginine appeared to be incorporated to a greater extent than other aminoacids.

3. Fatty Acid Composition of Cytoplasmic Membrane.

The fatty acid composition of the cytoplasmic membrane of B. megatherium is presented in Table 6. Inasmuch as the lipids of gram-positive bacteria are largley associated with the cytolplasmic membrane, one would expect the fatty acid composition of the latter to resemble closely that of the whole cells (Kates, 1964).

4. Lipids of Cell Walls.

Studies on lipid composition of Gram-positive and Gram-negative bacteria lead to the conclusion that cell walls of Gram-negative species contain large amounts of lipids (up to 26%) whereas Gram-positive bacteria have little or no cell wall lipids. For B. megatherium zero total lipid in the cell wall was reported by Kates (1964).

TABLE 5

Lipid Composition of Cytoplasmic Membrane of Bacillus megatherium

	Strain M		Strain KM	
Overall composition				
Protein, % of membrane dry wt.	63-69		75	Weibull(1958)
Lipids, " " " "	16-21		23	Yudkin(1962)
P, % of total lipids	3.6	(1)	3.4	(2)
Total neutral lipid %	-	(1)	56	(2)
Total phosphatides %	-	(1)	44	(2)
phosphatidic acid	+	(1)	-	(2)
diphosph. glycerol	+ } 90	(1)	-	(2)
phosph. glycerol	+	(1)	-	(2)
phosph. inositol	0	(1)	-	(2)
phosph. ethanolamine	0	(1)	97	(2)
phosph. serine	0	(1)	-	(2)
lipoamino acids	-	(1)	+	(2)

(1) Weibull (1957)
(2) Yudkin (1962)

TABLE 6

Fatty Acids Composition of Cytoplasmic Membrane of B. megatherium, Strain KM

Fatty Acids	Total Membrane lips.(1)	Neutral lipids(2)	Phosphatides(2)	Acetone Sol. Lipoamino A.	Lipoarg. Complex(3)
12:0	3.5(4)	tr	tr	2.1	tr
13:br	tr	tr	tr	tr	tr
13:0	tr	tr	tr	tr	tr
14:br	3	4	13	11	tr
14:0	3	3	6	3	tr
15:br(iso)	26 }				
15:br(ant)	29 }	25	35	37	4
15:0	3	3	4	2	1
16:br(iso)	1 }				
16:br(ant)	2 }	9	8	13	5
16:0	7	15	22	10	24
16:1	3	14	8	12	6
17:br	3	6	5	7	7
17:0	-	1	0.5	tr	6
17:1	1	-	-	-	-
18:0	3	4	2	tr	15
18:1	9	11	4	3	24
18:2	-	5	tr	tr	tr
19:br(ant)	-	-	-	-	-
19:cyc.	-	-	-	-	-
21:0	2	-	-	-	-

1. Thorne and Kodicek, 1962
2. Data for lipid fractions of total cells, Hunter and James, 1963.
3. Data for lipoamino acids of protoplasts, Hunter and James, 1963
4. Includes fatty acids with less than 12-carbon atoms.

III. EXPERIMENTAL PROCEDURE

A. Growth of bacteria.

Bacillus megatherium was cultured in a pilot plant fermentator using a synthetic medium. The conditions and medium are described by Tannenbaum *et al* (1966).

The culture was harvested by centrifugation and the concentrated bacterial suspension transferred to one pint polyethylene screw-capped bottles and stored at -26°C. After six months of storage the concentrated suspension was diluted 2:1 (v/v) with water and the cells were disintergrated at a pressure of 8,000 psi using a modified laboratory Manton-Gaulin homogenizer (Everett, Mass.). The percentage of disintegration was estimated to be 70% by observing under the microscope.

B. Solvents

The following reagent grade solvents were used: Methanol, absolute, redistilled; chloroform, redistilled, and 1% of methanol was added as a preservative and stored at 5°C; acetone, redistilled; benzene, redistilled; petroleum ether, redistilled; fraction collected 40-50°C (all Fisher certified Reagent); isopropyl ether (Eastman Organic Chemicals), redistilled; glacial acetic acid (Dupont); diethyl ether anlydrous (MallinKrodt Chemical Works), redistilled as follows: 30g of $SO_4Fe \cdot 7H_2O$ was added to 1.5 liter of ether and stirred for one hour using a magnetic stirrer, following by distillation.

C. Extraction and purification of lipids.

1. Extraction by solvents

The lipids were extracted using chloroform-methanol. The residue was acidified with HCl and extracted. Four batches of wet disintegrated cells making a total of 100.08 g were homogenized for five minutes in a Waring Blendor using 20 ml of deoxygenated chloroform-methanol 2:1 (v/v), for each gram of wet sample. The solvents were deoxygenated by bubling prepurified nitrogen through them for several minutes. The homogenized material was filtered using a coarse sintered glass filter. A nitrogen atmosphere was maintained during the filtration and subsequent operations. The residue was extracted twice with half the volume of solvent used for the first extraction and then filtered as above. The filtrates were evaporated on a rotary vacuum evaporator (Buchler Instruments Inc., New York, N.Y.).

The tissue water was removed azeotropically, with three additions of 25 ml portions of absolute ethanol and evaporated. Nearly all of the non-lipid organic matter which will not re-suspend in organic solvents after drying remains along the walls.of the evaporating flask (Moore, 1966). This insoluble residue was discarded. The dried lipids were re-suspended in chloroform-methanol (2:1), filtered through a medium porosity sintered glass filter, transferred to a tared 100 ml round bottom flask, and evaporated to dryness on the rotary

evaporator. The flask containing the lipid was dried by placing it in a vacuum desiccator over KOH. The pressure was reduced with a water aspirator and the desiccator was filled with nitrogen. The sample was dried overnight and weighed.

2. Acid hydrolysis.

The residue after solvent extraction was refluxed for two hours with 6N HCl, and extracted three times with diethyl ether in a separatory funnel. The ether extract was transferred to a tared flask, evaporated to dryness on the rotary evaporator, further dried over KOH, and weighed.

3. Folch Washing (Folch J., et al, 1957)

a. The dry lipid was transferred to a 40 ml graduated centrifuge tube and dissolved with chloroform-methanol (2:1), diluted to 20 ml and 5 ml of o.7% NaCl solution was added.

b. The solution was stirred with a glass rod, then centrifuged at 1800 RPM for 30 min. at approximately $0^{o}C$ in an International Portable Refrigerated Centrifuge, Model PR-2.

c. After centrifugation, two phases are observed. The lower chloroform layer was removed with a Pasteur pipet and transferred to a tared 200 ml round bottom flask.

d. The upper phase containing most of the water soluble impurities was washed twice; first with 15 ml

and second with 10 ml of lower phase which contains chloroform-methanol-water in the ratio of 86:14:1 ($^{v}/_{v}$). Each wash was followed by centrifugation as described in step (b).

e. The two lower layers of step (d) were added to the lower layer of step (c) and evaporated to dryness on a rotary vacuum evaporator. After drying, the lipid color was pale yellow and the washing was repeated as in step (c).

f. The lipid was dried overnight in a vacuum desiccator over KOH. The pressure was reduced with a water aspirator and the desiccator was filled with nitrogen.

g. The lipid was weighed, dissolved in chloroform in a graduated flask, made to 10 ml volume, and stored at -26°C.

4..Dry matter determination.

Samples were dried under a vacuum of 30 in. of Hg for 24 hours at 70°C. Four samples of bacterial suspension were used.

D. Thin layer Chromatography (TLC)

1. Development Solvents for TLC

a. Polar solvent. Chloroform-methanol-glacial acetic acid-water were mixed in the ratio of 85:15:10:4 ($^{v}/_{v}$) (Nichols,B.W.,1964).

b. Non-polar solvent. Petroleum ether-diethyl ether glacial acetic acid were mixed in the ratio of

90:10:1 (v/v), (Nichols, B.W., 1964).

c. Isopropyl ether-glacial acetic acid were mixed in the ratio of 96:4 (v/v), (Skipski, V.P., et al, 1965).

2. Preparation of TLC plates.

Silica gel H (E. Merck; Brinkmann Instruments, Inc.) was used throughout. Glass plates, 20x20 cm were thoroughly cleaned with a sulfo-chromic acid cleaning solution. Two different thickness layers were used: 250μ during the preliminary work and 500μ during the final work. For the 250μ layers 25g of silica gel was slurried with 72ml of water, for 500μ layers, 50 g of silica gel with 117ml of water, for five 20x20 cm glass plates. The plates were made by using an Automatic Plate Leveller and an interchangeable spreader (Quickfit and Quartz Ltd., England). The plates were dried at room temperature, activated for one hour at 110°C, and cooled for 30 minuted before spotting. Plates to be developed in the non-polar solvent were pre-washed for two hours in the same solvent, dried and then activated. Otherwise, a wide, dark band appeared on the chromatogram in the area of hydrocarbons and cholesterol esters after H_2SO_4-dichromate spray. Distortion due to edge effects was prevented by making vertical lines with a scriber (Quickfit) near the edges prior to development in solvents.

3. Application of samples.

The samples were applied with 10 and 50 ul Hamilton

syringes, 2.5 to 3.0 cm from the bottom edges of the plates. The amount of standard compounds applied ranged from 6 to 12 ug; whereas, the unknowns (lipid fractions from DEAE column chromatography) were applied in greater quantities.

4. <u>Development of chromatograms.</u>

Chromatographic chambers with capacity for five plates (Quickfit) and two plates (Brinkmann) at a time were used. The chambers were lined with Whatman filter paper #1 and allowed to reach saturation with the solvents before use.

Plates used to test the presence of phospholipids were developed in the polar solvent (approximately 1.5 hours), and plates to be tested for neutral lipids were developed in non-polar solvents (approximately 45 min.). During the last part of this experiment a two-step solvent system in one single direction was used for neutral lipids. In this case the mixture isopropyl ether-glacial acetic acid 96:4 ($^v/_v$), (more polar solvent) was used first and allowed to move approximately 7-8 cm from the bottom of the plate (approximately 15 min.). The plate was dried at room temperature for 30-40 minutes and then developed in the second solvent system, petroleum ether-diethyl ether-glacial acetic acid, 90:10:1 ($^v/_v$) (less-polar) and the solvent was allowed to move until approximately 0.5 cm from the top edge (approximately 45 minutes).

5. Detection and identification of spots.

A metalloglass sprayer (Metalloglass Inc., Boston)was used. The reagents used were as follows:

a. Sulfuric acid was used for general detection. Prepared by dissolving 1.2gr of $K_2Cr_2O_7$ in 200 ml of 55% reagent grade H_2SO_4, (Rouser, G., et al, 1964).

b. Specific reagents for phospholipis. These chemical tests were made on the plates. b_1 to b_5 in (Skimdore and Entenman, 1962).

b_1. Ninhydrin (Nin) to detect amino phosphatides, Dry plates were sprayed with a solution of 0.3 g ninhydrin in 5 ml lutidine and 95 ml n-butanol saturated with water. As the plates were dried at room temperature, red-violet spots appeared on a white background.

b_2. Molybdic acid (Mo) to detect phosphatides. Dry plates were sprayed with a solution of 5 ml 60% w/v perchloric acid, $HClO_4$ (Baker), 10 ml N HCl, and 25 ml 4% w/v ammonium molybdate $(NH_4)_6Mo_7O_{24}$ (Baker). Blue spots appeared on a white background as the plates were dried at room temperature.

b_3. Ferric Chloride-sulfosalicylic acid (Fe) to detect phosphate groups. Dry plates wer sprayed with a solution of 7.0 g sulfosalicylic acid, 0.1 g $FeCl_3 \cdot 6H_2O$, and 25 ml water diluted to 100 ml with 95% ethanol. White fluorescent spots appeared on a

purple background as the plates were dried at room temperature.

b_4. Ammoniacal silver nitrate (Ag) to detect glycerol and inositol. Dry plates were sprayed with a mixture of equal volumes of 0.1 N $AgNO_3$ and 7 N ammonium hydroxide. The plates were then heated at 110° until dark brown spots appeared on a white background.

b_5. Dragendorf reagent (Bi) to detect choline. Dry plates were sprayed with a mixture of 4 ml solution I, 1 ml solution II, and 20 ml distilled water. Solution I contained 1.7 g $Bi(NO_3)_3 \cdot 5H_2O$ diluted to 100 ml with 20% v/v acetic acid. Solution II contained 40 g KI in 100 ml water. As the plates were dried at room temperature, free choline produced a purple spot and choline-containing compounds produced orange spots.

b_6. Dipicrylamine to detect choline. Dry plates were sprayed with a solution of 0.2 g dipicrylamine in 50 ml acetone and 50 ml twice-distilled water. Choline and its derivatives appear as red spots on a yellow background, (Stahl, 1965).

b_7. Chargaff's reagent to detect choline and choline-containing substances. Solution I, 1g phosphomolibdic acid is dissolved in 100 ml of a mixture consisting of equal volumes of ethanol and chloroform. Solution II, 1g stannous chloride is dissolved in 100 ml 3N HCl. Prepare freshly before use. Spray

with I, dry for 3 minutes, spray with II, dry for 10 minutes, (Stahl, 1965).

c. Bial reagent to detect glycolipids, 40.7 ml concentrated H_2SO_4, 0.1 orcinol, 1 ml 1% ferric chloride solution, diluted to 50 ml with water.

The plates are kept in an atmosphere of HCl at 80°C for 90 minutes, and are then sprayed with Bial reagent. The color is developed by replacing the plates in the HCl atmosphere at 80°C until violet spots appear on a white background (Randerath, 1964).

d. Anisaldehyde-sulfuric acid to detect steroids, terpenes, sugars, etc.

Freshly prepared solution of 5 ml anisaldehyde in 50 ml glacial acetic acid, with addition of 1 ml of H_2SO_4(d 1.84). Heat to 100-110°C for 5 to 10 minutes. The pink background is brightened by treatment with water vapor (from a steam bath). Phenols, terpenes, sugars and steroids will stain violet, blue, red, grey or green (Stahl, 1965).

6. TLC Prints

Thin layers chromatograms were recorded using 200SS Ozalid paper (General Aniline and Film Corp., New York) and a 30 W Glow-Box (Instruments for Research and Industry, Cheltenham, Pa.). The TLC plate was placed over the glow-box with the coated face up and a sheet of ozalid paper over it. After exposure for a few minutes, the paper was put in a glass jar containing an open

beaker of NH_4OH. After one minute all the spots turned blue.

E. DEAE Column Chromatography.

1. Eluting solvent system.

During the preliminary work with microcolumns the following systems were used:

Run #1. Solvent Pre-1, Benzene-acetone, 9:1; ether benzene, 8:2; chloroform-methanol, 7:3; ethylacetate-ether, 1:1; ethyl acetate-methanol, 1:1 and containing 0.1% of NH_4OH, (all v/v).

Run #2. Solvent Pre-2. Chrloroform-methanol, 95:5; ether-benzene, 9:1; chloroform-methanol, 7:3; etheyl acetate-ether, 1:1; ethyl acetate-methanol, 1:1 and containing 0.1% of NH_4OH, (all v/v).

Run #3. Solvent Pre-3. System given by Rouser et al (1964). Chloroform-methanol, 9:1; chloroform-methanol, 7:3; methanol; glacial acetic acid-chloroform, 6:1; glacial acetic acid; methanol (for washing of the acid); chloroform-methanol, 4:1 and containing 20 ml of 28% aqueous ammonia per liter, and made 0.01 M respect to ammonium acetate, (all v/v).

During preliminary work with 20-cm columns:

Run #4. Solvent Pre-4. Chloroform-acetone, 95:5; chloroform-methanol, 9:1; chloroform-methanol, 7:3; methanol; glacial acetic acid-chloroform, 6:1; glacial acetic acid; methanol (for washing of the acid); chloroform-methanol, 4:1 and containing 20 ml of 28%

aqueous ammonia per liter and made 0.01 M respect to ammonium acetate, (all v/v).

Run #5. Solvent 5. Chloroform-acetone, 95:5; chloroform-methanol, 9:1; chloroform-methanol, 7:3; methanol; glacial acetic acid-chloroform, 6:1; methanol (for washing of the acid); chloroform-methanol, 4:1 and containing 20 ml per liter of 28% aqueous ammonia and made to 0.01 M respect to ammonium acetate, (all v/v).

Runs #6 and #7. Solvent 5 was used.

2. Preparation of DEAE for column chromatography.

Selectacel diethyl amino ethyl cellulose (DEAE) type 20, capacity 0.83 meq per gram (Carl Schleicher and Schuell Co., Keene, N.H.) was used.

Washing of DEAE. The procedure described here is that given by Rouser et al (1963). One hundred grams of DEAE was placed in a 1.5 liter capacity buchner funnel over which has been placed several layers of filter paper. The DEAE was washed with 1 N aqueous HCl, water, 1 N aqueous KOH, and water, this sequence of washes constitutes one cycle. After three wash cycles the bed was washed with methanol. The bed was then air dried on the filter under mild suction from a water aspirator, transferred to a vacuum desiccator and thoroughly dried over KOH.

3. Preparation of Columns.

Two different sizes of columns were used, the

characteristics of which are as follows:

a. Preparation of 20.cm columns.

Essentially the columns were prepared by the procedure outlined by Rouser et al (1963). Glass columns 40 cm x 2.5 cm I.D. (Kontes Glass Company, Vineland, N.J.) equipped with a reservoir for solvent, nitrogen inlet, coarse sintered glass disc and teflon stopcock were used. A 15 g portion of dried DEAE was placed in a beaker and allowed to stand overnight in glacial acetic acid. The ion exchange cellulose was pressed gently with a pestle in a mortar until it takes on a uniform appearance; this procedure ensures through wetting of the ion exchanger with acetic acid and a uniformly packed column.

Small portions of a very dilute slurry of DEAE in glacial acetic acid were passed into the chromatographic tube. After each addition the DEAE bed was pressed lightly with a large diameter glass rod. At the end, the DEAE bed heighth was about 25 cm. Two bed volumes of glacial acetic acid were passed through the column and the acid was removed with three volumes of methanol. Removal of acetic acid was tested with pH-paper. Methanol was removed with chloroform, and chloroform replaced by the solvent mixture to be used as the first eluting solvent. Two bed volumes of the first eluting solvent were passed before appliaction of the sample. At this stage the bed height was about 20 cm.

b. Microcolumns

These columns were used only during the preliminary work. A small plug of glass wool was placed at the bottom of a 7 mm I. D. Pasteur pipet which was packed with a dilute slurry of DEAE to a height of 7 cm. Less than one gram of dried DEAE is needed for each column. The columns, during packing, were pressed in order to have a flow of 0.5 ml/min with the first eluting solvent. During operation the columns were placed inside a plastic box under a nitrogen atmosphere.

Ten mg samples were applied in 2 ml of the first solvent; 40 ml of each solvent was applied and fractions of 10 ml collected; which were evaporated under a stream of nitrogen to a final volume of approximately 0.2 ml. Fifty microliter samples of these fractions were applied to duplicate TLC plates to determine the progress of fractionation.

Three solvent systems, Pre-1, Pre-2 and Pre-3 were tested. Mixtures of standards containing neutral lipids and phospholipids were applied to these columns and eluted with solvent sustems Pre-2 and Pre-3. The order of elution of the different compounds was established by TLC analysis of eluted fractions.

C. 20-cm Column Elution Procedure

The total lipid sample, approximately 250 mg, was dissolved in 5 ml of the first eluting solvent and applied to the

surface of the DEAE column bed. It was allowed to drain into the column and immediately additional solvent was added carefully from the solvent reservoir. A fraction collector (Rinco Instruments Company, Greenville, Illinois) was used, and fractions of approximately 14 ml were collected. Pressure was applied by connecting the solvent reservoir to a prepurified nitrogen tank which permitted control of flow rate at approximately 3 ml/min. A low flow of nitrogen was also connected to the column tip on which a plastic hood was fixed in order to provide a nitrogen atmosphere during the elution. The progress of the fractionation in each run was followed by TLC on each even numbered tube. When it was necessary to change to a new solvent, the new solvent was added after the previous solvent had reached the surface of the DEAE bed. TLC plates were developed in polar and non-polar solvents.

During the preliminary work samples were separated on two columns. The purpose of the first was to determine the volume of each solvent required to elute a given fraction. In this run even numbered fractions were evaporated to a final colume of 0.2 ml from which 50 ul was applied to TLC plates; the odd numbered fractions were discarded. The second run was designed to obtain groups of fractions by matching the TLC results with the tube fractions. In this run 5 ml of each even numbered tube was evaporated to a final volume of approximately 0.1 ml from which 50 ul was applied to TLC plates. The size of sample to be applied to

TLC and the necessity for having fast results required a change in layer thickness from 250μ to 500μ . The odd numbered fractions and the rest of the even numbered fractions were stored over ice until the TLC results were obtained. In this way eight different fractions were formed, see Table 9, evaporated under vacuum in the rotary evaporator, dried overnight over KOH, and weighed. Fraction #VIII from chloroform-methanol 4:1 was observed to contain ammonia salts and was further extracted using petroleum ether. The TLC plates were spotted alternately with standards, sprayed with ninhydrin, and then with H_2SO_4-dicromate.

During the final stages of the work the total dry lipid was further purified by the method outlined by Folch et al (1957), and two more 20-cm columns were run (Runs #6 and #7) in order to obtain an adequate amount of lipid fractions for further analysis. TLC plates were spotted with 50 ul directly from each even numbered tube fraction. Unfortunately, this procedure did not permit observation of spots, probably because the samples were too dilute. The final fractions, designed I to VI, were formed on the basis of previous results. The purification of fraction VI obtained from chloroform-methanol, 4:1, and all the operations until the weighing of the fractions were as described above.

The dried fractions were diluted with chloroform to known volumes, samples were applied to duplicate TLC plates,

and developed in polar and non-polar solvents. Chemical tests were made on individual plates.

F. Esterification.

Two methods were tested; BF_3-method and a micromethod using methanol-HCl.

1. BF_3-Method.

This method, described by Metcalfe (1966), is based on a rapid mild saponification followed by esterification with BF_3. Metcalfe (1966) worked with samples of about 150 mg; in the present experiment a slight modification was made to adapt this method to samples of about 2 mg. Samples of 50, 25, 10, 5 and 2 mg of triolein, tripalmitin and total bacterial lipid, before and after Folch wash, were used.

The samples were placed in 25-ml volumetric flasks. Four ml of 0.5 N methanolic sodium hydroxide was added and the mixture was heated on a steam bath for 5 min. at about $60 \pm 5^{\circ}C$. Five ml of BF_3-methanol 14% (w/v) (Applied Science Laboratories) was added to the flask and the mixture boiled for 2 minutes. Eight ml of saturated NaCl solution and 8 ml of petroleum ether were added, shaken for one minute, and let stand for 10 minutes. The petroleum ether layer was transferred, with a Pasteur pipet, to a 50-ml Ferlemeyer flask. Two more extractions with petroleum ether were made. After addition of approximately 2 g of anhydrous sodium sulfate, each flask was swirled slowly and let stand for one hour.

The petroleum layer was carefully transferred to a tared glass tube with glass stopper. The Erlemeyer flask was washed twice with 2 ml portions of petroleum ether, which was then evaporated on a 60°C water bath. Samples were dried overnight in a desiccator over KOH and weighed. In one hour six samples can be esterified simultaneoulsy and the equipment used is inexpensive.

Table 7 gives the identification of the samples. Samples #50, 25 and 20 were esterified by this method and analyzed by gas liquid chromatography.

2. Microesterification method.

The method described by Staffel *et al* (1959) was used.

a. Reagents.

5% HCl in methanol; sodium sulfate-sodium bicarbonate mixture, reagent frade, anhydrous, 4 to 1 mixture by weight; petroleum ether, redistilled, 40° - 50°C; benzene dried over sodium and distilled.

b. Apparatus.

Microinteresterification assembly, 19/38, consisting of round-bottomed test tubes, Liebig condensers, cold fingers, and six-place manihold, with nitrogen inlet, (Metro Industries, N.Y.); McLeod gage; vacuum pump; cold trap; water bath.

c. Procedure.

(1) Aliquots of lipid fractions designated as TL and I to VI, containing 5 mg of lipids were placed

in the microsublimation tubes and evaporated to dryness with a stream of nitrogen. Six samples were used in each run.

(2) Four ml of 5% HCl in methanol and 0.5 ml of dry benzene were added.

(3) A condenser with a $CaCl_2$ trap was connected and the mixture refluxed in a water bath at $86^{\circ} \pm 2^{\circ}C$ for two hours. The system was removed from the bath and cooled to room temperature.

(4) Two volumes of water were added, and the methyl esters were extracted three times with 3 ml portions of petroleum ether by shaking the stoppered tubes. With a Pasteur pipet, the petroleum layer was transferred to a stoppered 50-ml Erlemeyer flask containing about 2 g of the Na_2SO_4-Na_2HCO_3 mixture. The Erlemeyer flasks were washed twice with the solvent and allowed to stand for one hour.

(5) The petroleum ether layer was transferred to a second microsublimation tube. The contents of the flask was washed once with 1 ml solvent, and evaporated to dryness in the rotary evaporator connected to a water aspirator.

(6) Microsublimation. After the microsublimation tube was fitted to the cold finger, a vacuum of about $25 \pm 5\mu$ of Hg was produced. The system was then placed in a water bath at $60^{\circ} \pm 2^{\circ}C$ for one hour.

(7) After cooling, the sublimed esters were rinsed with hexane into a graduated flask and diluted to 0.5 ml.

Esterification of 5 mg samples of total lipid (TL) and fraction I-VI was performed by this method. The esterification of TL samples and Fraction I were repeated with 15 mg samples. Only 2.5 mg of fraction III collected from two columns was used.

G. Gas Liquid Chromatography (GLC) of Fatty Acids Methyl Esters

1. Instrument.

A flame ionization chromatograph (Aerograph HiFi, Model 600C), equipped with a Hydrogen Generator (Aerograph Model 650) and a Linear Temperature Programmer (Aerograph), was used.

2. Conditions of Operation.

A 5' x 1/8" O.D. coiled stainless steel column packed with 10% diethylene glycol succinate (DEGS) on Anakron ABS (100-110 mash) was used. The flow of nitrogen carrier gas, was 21 ml/min.; injector temperature was maintained at 270°C. Separations were performed at column temeperatures of 133° ± 1°C and 170° ± 1°C. The lower temperatur was used for compounds up to 16-carbon atoms, and the higher temperature for compounds with more than 16-carbon atoms.

3. Injection of Samples.

Samples of 1 ul were injected with a 1 ul Hamilton syringe #7101. The following samples were injected:

#50, 25, 20, TL and fractions I to VI. The samples #50 and 25 were used in order to determine whether there was a variation in fatty acid composition due to the Folch washings. Sample #20 was analyzed to determine the fatty acid composition of bound lipids. Samples of fractions I to VI showed the relative composition of each fraction.

A standard curve was determined at 133°C using the following standard mixture, (Applied Science Laboratora-tories, Inc.): $C_{10:0}$, 4.55 mg; $C_{12:0}$, 6.05 mg; $C_{14:0}$, 11.65 mg; $C_{16:0}$, 24.6 mg. The following dilutions (mg/ml), with respect to $C_{16:0}$, were made: 9.84, 4.92, 2.46, 1.23 and 0.615. The areas (cm^2) of each peak were found by using the formula $(\frac{wxl}{2})$ Range x Attenuation, where w = peak width and h = peak height. Using the peak areas of $C_{16:0}$ as a reference, a plot of detector response (area) versus size of sample injected was made as shown in figure (13).

Absolute areas for $C_{16:0}$ were determined at 133° and 170°C and a conversion factor determined to compensate for the effect of temperature on detector response.

The areas of the unknown peaks were determined by the formula given above. Areas were converted to weight/volume (ug/ul) by using the relation:

$$\frac{\text{Unknown peak area, cm}^2}{\text{The area of 1 ug } C_{16:0} \text{ std, cm}^2}$$

The weight of each unknown peak (ug) was calculated by using $(\frac{ug}{ul})$ (500 ul). The percentage fatty acids of the unknown in

TABLE 7

Identification of Samples Used For Esterification and Gas Liquid Chromatography

Sample Code	Kind of Sample (1)	Esterification Sample Size, (mg)	Esterification Method(2)	Remarks
50	DTL	50.3	BF_3	Before Folch wash
25	DTL	25.0	BF_3	After Folch wash
20	AEL	20.0	BF_3	-----
TL	DTL	15.0	Micro M.	After Folch wash
Neutral lipid(3)				
I	DEAE-Fr.	15.0	Micro M.	After Folch wash
II	" "	5.0	" "	" "
Phospholipids (3)				
III	" "	2.5	" "	" "
IV	" "	5.0	" "	" "
V	" "	5.0	" "	" "
VI	" "	5.0	" "	" "

(3) I-VI Eluted from DEAE column chromatography with the following solvents: I, C/A, 95.:5; II, C/M, 9:1; III, C/M, 7:3; IV, C/M, 7:3 and M; V, AcH/C, 6:1; VI, C/M, 4:1.

(1) DTL - Dry Total Lipid
AEL - Acid Extracted Lipid

(2) Micro M. - Microesterification Method.

each fraction and in the total lipids were calculated (and were expressed as percentage in total fatty acids). These results are presented in Tables 16 and 17.

All the unknown peaks were expressed alsonon the basis of 1 mg total lipid by using the formula:

$$\frac{\text{Weight unknown, mg}}{\text{Weight Sample for esterification, mg}}$$

These results are presented in Table 18.

III RESULTS

A. Total Lipids

Total solids as determined by drying in a vacuum of 30 in of Hg, and 70°C for 24 hours was 8.86%. The lipids as percentage of dry matter are: total lipid before Folch wash, 29.5%, (2.6115 g); lipid extracted after hydrolysis, 0.26% (0.0232 g); total lipid after Folch wash, 16.46%. The last result shows that about half of the total material extracted by chloroform-methanol 2:1 was non-lipid.

B. Chromatographic Separation

1. Microcolumns

During preliminary chromatographic studies, after elution with solvent system Pre-1, a small amount of insoluble material was observed on the surface of the DEAE column. Solubility tests showed that only mixture of chloroform-menthanol completely dissolved this material.

It was slightly soluble in mixture of chloroform-acetone and chloroform-ether. TLC analysis of fractions eluted from DEAE-columns with solvent systems Pre-2 and Pre-3 permitted establishing order of elution of standards. Poor separation of fractions from total lipid samples, lead to the decision to discontinue use of microcolumns.

The results of run #4 with a 20-cm DEAE cellulose column established the volume of each solvent required. These volumes, shown in Table 8 were used in all future column chromatography. It was also determined from this run that no solutes were eluted by acetic acid. Acetic acid was not used in subsequent analyses.

The lipid fractions (8 in total) prepared during run #5 using solvent system #5 are described in Table 9. Figure 1 shows the progress of elution as determined by TLC. The advantage of combining these two methods is illustrated. The thin layer chromotograms, developed in polar and non-polar solvents, of the DEAE column fractions are presented in figure 2. TL chromatograms of fractions obtained after Folch wash are shown in Figure 2. The ninhydrin reagent showed some yellow spots on Fractions IV, V and VI which indicates the presence of some non-lipid contaminants eluted with the last portion of solvent C/M, 7:3 and with methanol. Fraction VII shows many spots not clearly separated.

Comparing the TLC plates of run 5 (before washing), Figure 2 with runs 6 and 7, (after washing) on the same

figure, shows no difference for the last fraction eluted with, C/M, 4:1. Fractions eluted with AcH/C, 6:1 were different: only a few spots appear after washing. There were also differences for fractions eluted with C/M. 7:3.

2. Final Chromatographic Separations.

The elution schemes of runs 6 and 7 using solvent system #5 and lipid samples after Folch washing are presented in Table 10. The TL chromatograms obtained by spotting 50 ul of the tube fraction on the plates did not give satisfactory results because of low concentration. Fractions were combined on the basis of previous studies and results are presented in Table 11. Neutral lipids represents 81.2% and phospholipids 14.7% of the total lipid. The reproducibility of the column chromatography method, with the exception of fraction V, is satisfactory as seen in Table 11. Recovery from the column was 95.8%. The TLC of the fractions developed in polar and non-polar solvent are shown in Figure 2. TL chromatograms developed in non-polar solvent showed absence of neutral lipid in fractions IV, V and VI. Some material does not migrate in fractions I-III. Plates developed with polar solvents showed that only fractions III-VI contain phospholipids.

C. Identification of Lipid Classes

1. Idenitification of Polar Lipid.

The disposition of the fractions and standards used for chemical tests is presented in Figure 3. Tentative identifications of the unknowns are presented in Tables

12 and 13. A total of eleven spots were detected using polar solvents and seven using non-polar solvents.

Ninhydrin was the most sensitive reagent used for specific chemical tests. Ferric chloride-sulfosalicylic acid does not react clearly with the minor compounds. Molybdic acid and Chargaff's reagents did not give clear results.

Only two compounds, 9 and 11 in fraction VI, do not contain NH_2 groups (Ni-negative), and they are also eluted with a solvent mixture for acidic compounds (C/M, 4:1). Lecithin, lysolecithin and sphingomyelin are not present since 1 to 8 all are Ni-positive. On the basis of chemical tests on cerebroside and sulfatide standards it was concluded that these compounds are not present. These results suggest that the phospholipids compounds observed (1 to 8) should contain either ethanolamine or serine or both.

Compound #1 exhibited a positive reaction for compounds containing choline, (Drag-positive), but it is also Ni-positive which is not typical for these compounds. From the results of these experiments, it is not possible to give a tentative identification for this compound.

Compounds #2 (Fraction IV) and 6 (Fraction V), probably are the same. They yield the same reactions and have the same R_f value as oxidized phosphatidyl ethanolamine. Phosphatidyl ehtanolamine has been reported to be present in B. megatherium strain M by Weibull (1957) and

in strain NRRLB939 by Mizushima (1966). This compound was reported also in B. Cereus by Houtsmuller and van Deenen (1963) as one of the major phospholipid compounds in B. megatherium. Phosphatidyl ethanolamine is easily oxidized (Hanaham, 1960), and with one year old samples used in this experiment, the probability of oxidation is high.

Compound #3, (Fraction IV), two compounds are suspected to be present in this spot. The lower spot will be the same as compound #7 (Fraction V), both have the same R_f value as phosphatidyl serine.

Compounds #4 (Fraction IV), 8 (Fraction V) and 10 (Fraction VI), are present only in very small amounts. The reagents used were not sensitive enough to give tentative identifications of these compounds.

Acidic lipids. Compound #9 (Fraction VI), contains phosphorus but no free amino groups and has an R_f value near that of phosphatidyl ethanolamine. Nichols (1964) working with plant phospholipids under conditions similar to this experiment (silica gel H, plates activation, development solvent), found that phosphatidyl glycerol has an R_f value slightly less than phosphatidyl ethanolamine. This compound has been reported in B. megatherium by Weibull (1957), Haverkate, et al (1962) and Mizushima (1966). Kates (1964) lists this compound as one of the major phospholipids in bacilli.

Compound #11 (Fraction VI), is another acidic lipid.

There are two compounds besides cerebroside with R_f values similar to that of compound #11. Diphosphatidyl glycerol (cardiolipin) is one of them. Rouser et al (1964) has used cerebroside as a standard to determine diphosphatidyl glycerol in brain lipids. The other compound is phosphatidic acid as reported by Nichols (1964) in plant phospholipids working under conditions similar to those used in this experiment. A small amount of diphosphatidyl glycerold was reported by Weibull (1957) in B. megatherium strain M. Mizushima (1966) reported this compound in an amount of 27% of membrane lipid in B. megatherium strain NRRLB939. Both compounds probably will give the same reactions with the chemical tests used in this experiment.

2. Idenitification of Neutral Lipids.

The two-step development system allowed the separation of the following classes of lipid compounds: Hydrocarbons, cholesterol esters, methyl esters of fatty acids, triglycerides, fatty acids, diglycerides and hydroxy acids. The tentative identification of each of these compounds is given in Table 13 and shown graphically in Figure 3. The anisaldehyde reagent gave a well defined reaction and showed the absence of free cholesterol.

The hydroxy acids and cholesterol have approximately the same R_f value under the condition used in this experiment. The color reaction obtained using the anisaldehyde reagent indicated the absence of cholesterol. The compounds observed were tentatively identified as hydroxy-acids.

Comparing fractions III (after Folch wash) versus III_{bF} (before Folch wash) in Figure 2, it is possible to observe that Folch washing removed the diglycerides. Some esterification appears to have occurred during the washing procedure.

D. Analysis of Fatty Acid Methyl Esters.

1. Esterification.

In Table 14 the percentage conversion obtained using the BF_3-method and microesterification method are presented. The BF_3-method yielded, on the average, 90% conversion for all sample sized with triolein. Efficiency decreased with decreasing amount of tripalmitin. With total lipid, the efficiency in general is very low, but is increased after Folch washing. A 6.4% conversion was obtained after washing for the same size of samples. Triolein and tripalmitin efficiency were calculated by weighing with an analytical balance. Values given for total lipid were calculated from GLC.

Conversion using the microesterification method shows, in general, that the efficiency is lower with fractions containing neutral lipids, especially fraction I. The esterification was repeated using 15 mg samples for total lipid (TL) and fraction I. An increased conversion is observed in the direction of increasing polarity of the solvents used to elute this fraction. Conversion is greatest, 44%, with the acidic lipids of fraction VI.

The low yield of methyl esters (microesterification method), and the different values obtained for each

fraction was investigated qualitatively using TLC. The results, as seen in Figure 4, shows that phospholipids were no longer present. Thin layer chromatograms developed in non-polar solvent using the two-step development system showed (from top down) presence of carbohydrates and absence of chelesterol esters. Spots corresponding to esters for fraction I-VI (equal columes being applied) reveal qualitatively the relative proportions of esters obtained from each fraction. Spots very near the triglyceride position are observed for fraction I, V and VI. Free fatty acids are also present in fractions II, V and VI. Other spots, probably diglycerides and hydroxy-acids, are present in all fractions, mainly in fractions V and VI. The spots at the left corresponds to sample #20 (extracted after acid hydrolysis). Tests made with total lipid before and after esterification gave results similar to that described for frations I to VI.

2. Gas-liquid Chromatography

a. Standard Curve.

A plot of detector response vs. concentration with methyl palmitate as reference is shown in Figure 5. Linearity was obtained up to 5 ug. The value 525 cm^2 per ug sample was used to obtain the concentration of all the unknown peaks as tabulated in Tables 16 and 17.

A plot of log retention time of standards (at 133° and 170^oC) versus carbon numbers is shown in

Figures 6 and 7.

The absolute areas for $C_{16:0}$ at 133° and 170°C (average from 6 injections of 1.23 ug samples) were 697 ± 2cm^2 and 689.8 ± 2cm^2, respectively, from which a conversion factor of 1.01 was calculated. All the peak areas, from #13 up, caluclated at 170°C, were multiplied by this factor before conversion to concentration.

b. Fatty Acid Composition

A comparison will be made first, of fatty acid composition before and after Folch washing. In table 16 are presented the fatty acid composition of samples #50, 25 and TL. Although there is a difference in the method of esterification, see Table 7, the changes observed are consistent. After Folch washing there is an increase of 30% for compounds #7 and 50% for #8 and #10. Few compounds, for example #2 and #3, show a decrease to trace level. Important changes are the removal of about 75% and 90% of compounds identified as $C_{14:0}$ and $C_{18:1}$, respectively; there is a decrease also of about 50% for $C_{18:0}$. Compound #9, present before Folch washing and in smaller proportion after, when esterified using the BF_3 method, was never observed using the microesterification method. Another outstanding change is the presence of 7.7% of the compound identified as $C_{20:0}$ after washing which was observed only in trace quantity

before washing, the use of the microesterification method yielded only 0.16% (see Table 17) of this compound.

Sample #20 (extracted after acid hydrolysis) shows the presence of almost all of the compounds with the exception of the lower carbon number fatty acids. Compounds #10, 11 (16:0) and #12 are present in bigger amounts than in samples #50, 25 or TL.

The fatty acid composition of samples TL (total lipid) and fraction I-VI, all esterified by the same method is presented in Table 17. In general, there is consistency in the composition of each chromatographic peak as obtained from TL with that obtained from fractions I-VI. The only major difference is with respect to peak #22 which consituted 3.28% of Fraction I-VI, but only 0.87% when using sample TL.

The composition of peak #6 is included in peak #7 because resolution of these compounds was not achieved. It was observed that most of the fatty acids, 93.9%, came from the phospholipid fractions III-VI, and mostly, 72.2%, from fraction VI.

Although fatty acid compounds with more than 16 carbons were observed, 91.5% are compounds with 12-16 carbon atoms per chain. These compounds were determined at $133^{o} \pm 1^{o}C$. The only major compound with more than 16 carbons is peak #22, 3.16% in fraction V. $C_{20:0}$, (7.73%) was observed using the BF_3 method.

The saturated compounds constitute only 16.0% of the total esterified fatty acids, with palmitic acid, 10.8%, the major compound. The identified unsaturated compounds, 18:1 and 18:2, are present in amounts of 1.97% and 0.27% respectively.

Six compounds constitute 91.3% of the total fatty acids, they are:

Peak #	%	
4	14.8	
7	43.2	Incls. Peak #6
10	13.7	
11	10.8	
12	5.5	
22	3.3	

Of these compounds only peak #11 was identified as palmitic acid. Identification of the others was not possible from the reference dataavailable. Probably the most interesting to elucidate is peak #7.

TABLE 8

Run #4. Volumes of Solvents Used for DEAE-Cellulose Column Chromatography

Solvents (1)	Volumes, ml
C/A 95:5	350
C/M 9:1	400
C/M 7:3	500
M	350
AcH/C 6:1	300
M	300 (for washing AcH)
C/M 4:1	750

TABLE 9

Run #5. Lipid fractions from 20-cm DEAE column Chromat.

Sample applied: 250 mg dissolved in 5 ml of C/A, 95:5

Lipid Fractions	Weight of Fractions, mg	Solvent (1)	Tube Nos.
I	108.8	C/A 95:5	1-32
II	13.9	C/M 9:1	33-63
III	3.2	C/M 7:3	64-72
IV	3.9	C/M 7:3	73-87
V	21.4	C/M 7:3	88-105
VI	45.3	M	106-143
VII	29.2	AcH/C 6:1	144-167
VIII	2.9	C/M 4:1	168-227
	1.3	Menthanol washing	
TOTAL..............	229.9		
PERCENTAGE RECOVERY	91.9		

Abbreviations: C = chloroform; A = Acetone; M = Methanol;

AcH = Acetic acid.

TABLE 10

Runs #6 and #7. Elution scheme for 20-cm DEAE column and thin layer chromatography monitoring

Sample Size: 250 mg Folch washed lipid and applied in 2 ml C/A, 95:5

SOLVENTS	VOLUME SOLVENT APPLIED, ML	TUBE NOS. COLLECTED	TLC PLATE NO.	TUBE NOS. APPLIED TO TLC
C/A, 95:5	350	1-31	1	1,2-16
			2	18-30
C/M, 9:1	400	32-65	2	32, 34
			3	36-52
			4	54-64
C/M. 7:3	500	66-108	4	66-70
			5	72-88
			6	90-106
			7	108
M	350	109-138	7	110-124
			8	126-138
AcH/C, 6:1	300	139-171	8	140, 142
			9	150-166
M	300			(washing)
C/M. 4:1	750	172-end	10	172-end

TABLE 11 Runs #6 and #7. Weight of Fractions Obtained with Each Solvent

Lipid frac.	Wgt. of fractions, mg Run #6	Run #7	Average	% of total lipid	Eluted with Solvent	Tube No.
Neutral lipids;						
I	193.5	185.0	189.2	75.7	C/A,95:5	1-39
II	lost	13.8	13.8	5.5	C/M, 9:1	40-72
Phosphalipids;						
III	1.5	1.7	1.6	0.7	C/M, 7:3	73-94
IV	16.0	17.8	16.9	6.8	C/M, 7:3 M	95-116 118-149
V	2.5	14.1	8.3	3.3	AcH/C,6:1	150-171
VI	9.2	9.9	9.6	3.9	C/M, 4:1	172-end
Total......		242.3	239.4			
Percentage Recovery...		96.5	95.8			

(1) It was observed that after the change of solvent, eight tubes were filled before the new solvent started to elute. In this Table, the eight additional tubes were included in the previous solvent fraction.

TABLE 12

Color Reactions and R_f Values of Phosphalipids (1).

Fraction	Spot	R_f	Ni	Fe	Drag	Ag	Bial	H_2SO_4
All	A		-	-	-	-	-	+
III	1	0.30	+	+	+	-	-	+
IV	2	0.11	+	(2)	-	-	-	+ OPE
	3	0.18	+	+	-	-	-	+ PS
	4	0.28	+	(2)	-	-	-	+
V	5	0.06	+	(2)	-	-	-	+
	6	0.10	+	+	-	-	-	+ OPE
	7	0.16	+	(2)	-	-	-	+ PS
	8	0.27	+	(2)	-	-	-	+
VI	9	0.22	-	+	-	-	-	+ PG
	10	0.30	+	(2)	-	-	-	+
	11	0.65	-	+	-	-	-	+ PG or PA
Standards								
Cer.		0.55	-	-	-	-	+	+
PE		0.36	+	+	-	-	-	+
PC		0.16	-	+/	+	-	-	+
PS		0.12	+	+	-	-	-	+
Sulf.		0.10	-	-	-	-	+	+
Sphing.		0.07	-	+	+	-	-	+
OPE		0.11	+	+	-	-	-	+
Lysol		0.05	-	+	+	-	-	+

Abbreviations: For reagents are given in experimental procedure
For Standards: Cer = Cerebroside; PE = Phosphatidyl ethanolamine; PC = phosphatidyl choline; PS = phosphatidyl serine; sulf. = sulfatide; sphing = sphingomyelin; OPE = oxidized PE; PA = phosphatidic acid.
(2) Not clear color reaction.

TABLE 13

Color Reactions and R_f Values for Neutral Lipids

Spot #	R_f	H_2SO_4	Anisadehyde	Compound
Ch	0.15	+	pink	Cholesterol
1	0.15	+	gray	Hydroxy acids (2)
2	0.24	+	(1)	Diglycerides
3	0.31	+	(1)	Free Fatty Acids
4	0.42	+	(1)	Triglycerides
5	0.57	+	dark blue	Fatty Acid Esters
6	0.70	+	violet	Cholesterol esters
7	0.80	+	yellow	Carbohydrates

(1) Different tones of gray

(2) Tentatively assigned

TABLE 14

TABLE 14 Percentage Conversion to Methyl Esters for Total Lipids and Fractions from DEAE-Column Chromatography

$$\text{Conversion, \%} = \left(\frac{\text{weight esters}}{\text{weight sample}}\right) 100$$

BF_3-Method. Saponification-Esterification, Metcalfe (1966)

	Triolein	Tripalmitin	total lipid Before F wash		total lipid after F wash		Acid hydrolysis extracted
Conversion, %	90	84-30	1.24	tr	6.4	(x)	5.56
Sample Size,mg	50-2	50-2	50-25	25	25	7-2	20

(x) Qualitative test made using TLC gave positive results.

TABLE 15

Microinteresterification Method, Staffel et al (1959)

Percentage Conversion to Methyl Esters

Sample Code	Wt. Lipid Sample, mg	Weight esters, mg	Conversion, %
TL	15.0	0.707	4.71
I	15.0	0.206	1.37
II	5.0	0.120	2.40
III	2.5	0.092	3.68
IV	5	0.051	1.02
V	5.0	0.419	8.38
VI	5.0	2.201	44.02

TABLE 16

Percentage Composition of Fatty Acids in *B. megatherium* lipid, before and after Folch wash and in lipid residue extracted after acid hydrolysis.

Standard	rel ret.	Peak #	Rel Ret.	50	25	20	TL
10:0	0.09	1	0.09	tr	⊖	⊖	tr
12:0	0.20	2	0.20	0.72	tr	0.45	tr
		3	0.33	0.80	tr	⊖	tr
		4	0.36	15.60	18.35	3.25	17.15
14:0	0.45	5	0.44	13.65	2.07	0.45	1.01
		6	0.54	(x)	(x)	(x)	(x)
		7	0.57	34.00	39.50	24.45	44.50
15:0 (1)		8	0.67	0.72	2.35	2.88	1.51
		9	0.74	4.16	2.07	⊖	⊖
		10	0.81	10.30	11.90	24.35	15.75
16:0	1.00	11	0.97	7.85	9.30	17.75	9.50
		12	1.29	3.85	4.62	16.95	6.02
		13	1.64	1.28	0.48	2.34	0.87
18:0	1.89	14	1.91	2.25	0.85	2.35	1.30
18:1	2.21	15	2.22	4.50	0.48	0.63	0.65
		16	2.44	⊖	⊖	tr	⊖
19:0 (1)		17	2.60	⊖	⊖	tr	⊖
18:2	2.85	18	2.87	0.32	0.28	0.36	tr
20:0	3.61	19	3.49	t	7.73	2.35	⊖
		20	4.10	⊖	⊖	0.45	⊖
		21	4.62	⊖	⊖	0.45	tr
		22	5.90	o	o	0.36	0.87
22:0 (1)		23	6.65	o	o	0.18	0.87
				100.00	100.00	100.00	100.00

Abbreviations for Tables 16 and 17: tr = traces
(1) = determined from plot log. ret. time vs. C-number
(x) = included in unknown #7

TABLE 17 Percentage Composition of Fatty Acids of Fractions I-VI

Standard	Rel. Ret.	Peak #	Rel. Ret.	TL	I	II	III	IV	V	VI	
10:0	0.09	1	0.09	t	t	o	o	o	o	0.16	0.16
12:0	0.20	2	0.20	t	t	t	o	o	t	t	t
		3	0.33	t	0.01	t	t	o	t	t	0.01
		4	0.36	17.15	0.26	0.20	0.46	0.13	1.15	12.00	14.80
14:0	0.45	5	0.44	1.01	0.03	0.10	0.10	t	0.24	0.75	1.22
		6	0.54	(x)	(x)	0.10	(x)	(x)	(x)	(x)	0.10
		7	0.57	44.50	0.88	0.62	1.50	0.40	3.80	36.00	43.20
15:0 (1)		8	0.67	1.51	0.04	0.28	0.29	0.23	0.50	0.65	1.99
		9	0.75	o	o	o	o	o	o	o	o
		10	0.81	15.75	0.32	0.40	0.75	0.28	1.55	10.40	13.70
16:0	1.00	11	0.97	9.50	0.26	0.82	1.20	0.44	1.60	6.50	10.82
		12	1.29	6.02	0.20	0.15	0.22	0.13	0.55	4.25	5.50
		13	1.64	0.87	0.02	0.34	t	o	0.05	0.48	0.89
18:0	1.89	14	1.91	1.30	0.05	0.52	0.23	0.08	0.35	0.45	1.68
18:1	2.21	15	2.22	0.65	0.04	0.32	1.40	o	o	0.21	1.97
		16	2.44	o	o	o	o	o	o	o	o
19:0 (1)		17	2.60	o	o	o	o	o	o	o	o
18:2	2.85	18	2.87	t	t	0.06	o	0.05	0.06	0.10	0.27
20:0	3.61	19	3.49	o	o	o	o	o	0.16	o	0.16
		20	4.10	o	o	o	o	o	o	o	o
		21	4.62	t	o	o	o	o	o	0.25	0.25
		22	5.90	0.87	0.12	-	o	o	3.16	o	3.28
22:0 (1)		23	6.65	0.87	o	o	o	o	o	t	
				100.00	2.23	3.91	6.15	1.74	13.77	72.20	100.00

TABLE 18

Amount of Each Known and Unknown Fatty Acids per mg of Total Lipid (1)

Fatty Acid	Peak #	Fatty Acid Ratio, x 10^3 (5)	Major Portion in Fraction
10:0	1	0.17 (2)	VI
12:0	2	-	-
	3	0.45	I
	4	7.92	VI, V
14:0	5	0.47	VI, V
	6	-	} VI, V
	7	20.70	
15:0	8	0.70	VI, V
	9	-	-
	10	7.30	VI, V
16:0	11	4.40	VI, V
	12	2.80	VI
	13	0.40	II, VI
18:0	14	0.60	II, VI
18:1	15	0.30	III
	16	-	-
19:0	17	-	-
18:2	18	0.28	VI
20:0	19	3.30 (3)	Sample #25, V
	20	-	-
	21	0.27	VI
	22	6.40 (4)	V
22:0	23	0.40	

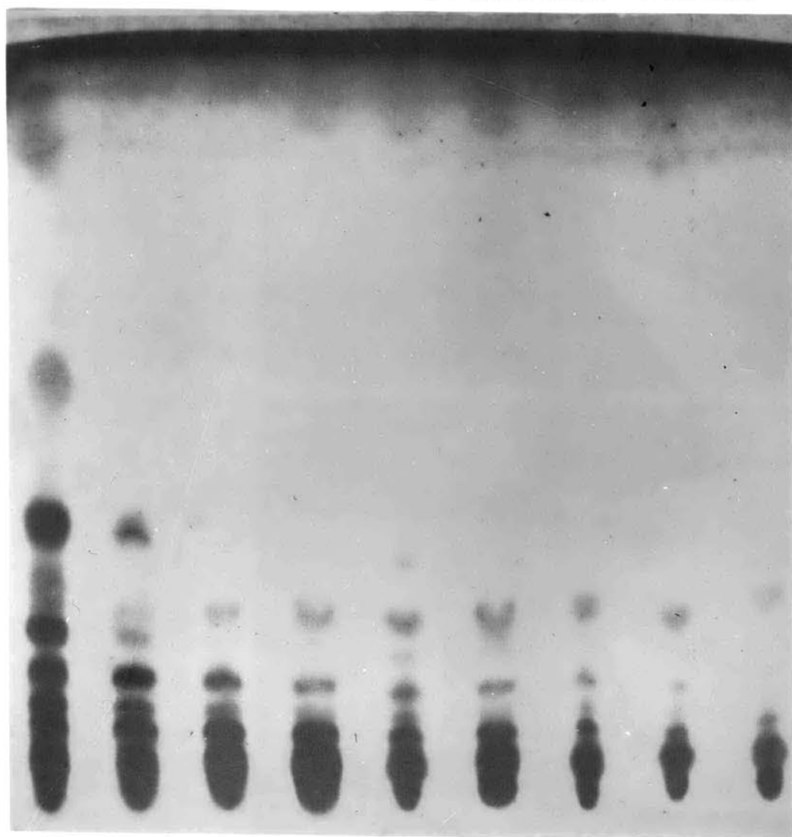
1. Calculated from TL esterification
2. Calculated from Fraction VI
3. Calculated from Sample #25
4. Calculated from Fraction V
5. $\frac{\text{Fatty acid, (mg)}}{\text{One mg total lipid}}$

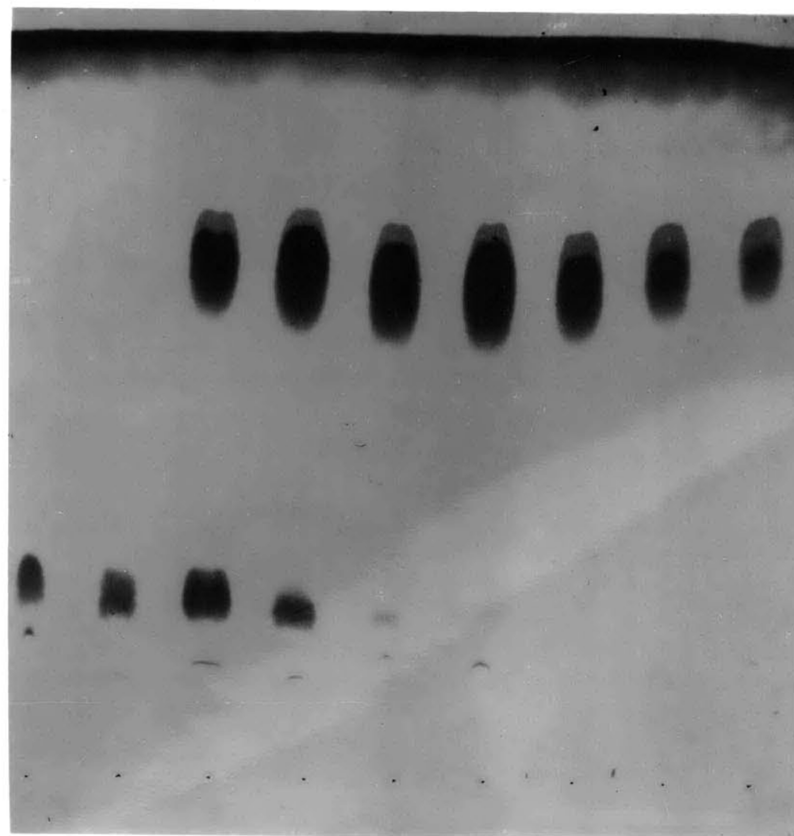
TABLE 19 Abbreviations Used For Figures I-IV

Ch. E	=	Cholesterol ester
F.A.E.	=	Fatty Acid Ester
Trig	=	Triglyceride
Dig	=	Diglyceride
Monog	=	Monoglyceride
Ch	=	Cholesterol
L.P.Ch.	=	Lysophosphatidyl Choline
Sphin	=	Sphingomyelin

Other abbreviations are the same as used before.

FIG 1 RUN 5. THIN LAYER CHROMATOGRAPHY OF FRACTIONS FROM DEAE-COLUMN CHROMATOGRAPHY._ PROGRESS OF ELUTION
DEVELOPMENT SOLVENT C/M/AcH/W 85:15:10:4





144 146 148 150 152 154 156 158 160

TUBE NUMBER →

← ELUTED WHIT AcH/C 6:1 →

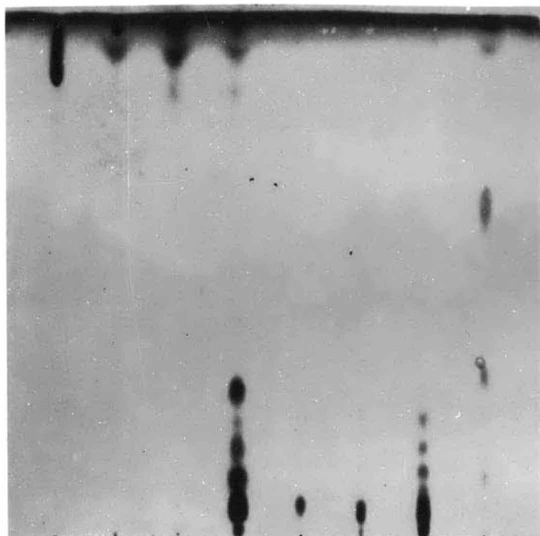
180 182 184 186 188 190 192 194 196

← ELUTED WHIT C/M 4:1 →

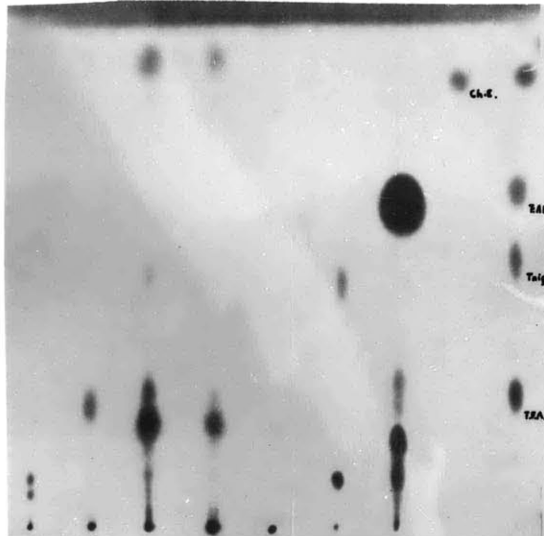
FIG 2. THIN LAYER CHROMATOGRAPHY OF FRACTIONS FROM DEAE-CELLULOSE COLUMN CHROMATOGRAPHY BEFORE FOLCH WASH (RUN 5, FIG a and b) AND AFTER FOLCH WASH (RUN 6 AND 7, FIG c and d)

a. DEVELOP. SOLVENT : C/M/AcH/W 85:15:10:1

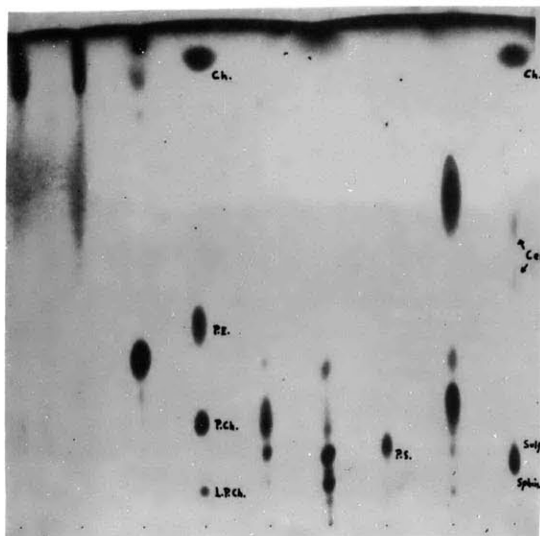
b. PET. ETHER/ETHER/AcH, 90:10:1



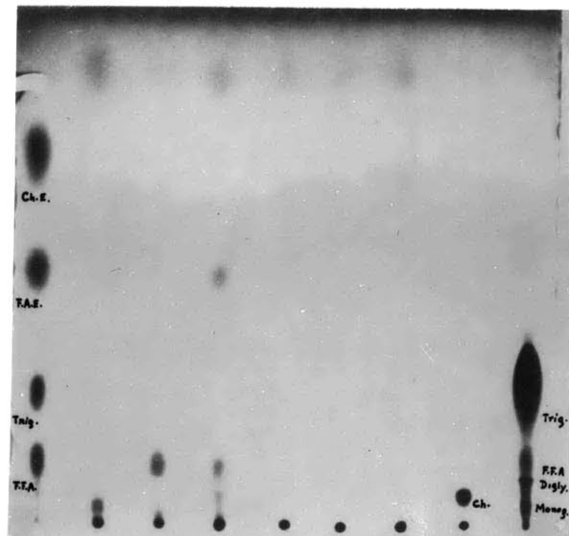
FRACTION No	I	II	III	IV	V	VI	VII	VIII
SAMPLE, μg.	108	140	96	97	43	45	146	29



	I	II	III	IV	V	Tripalm.	M.oleate	STD.	STD.
	108	140	96	97	43	12	-	6	48



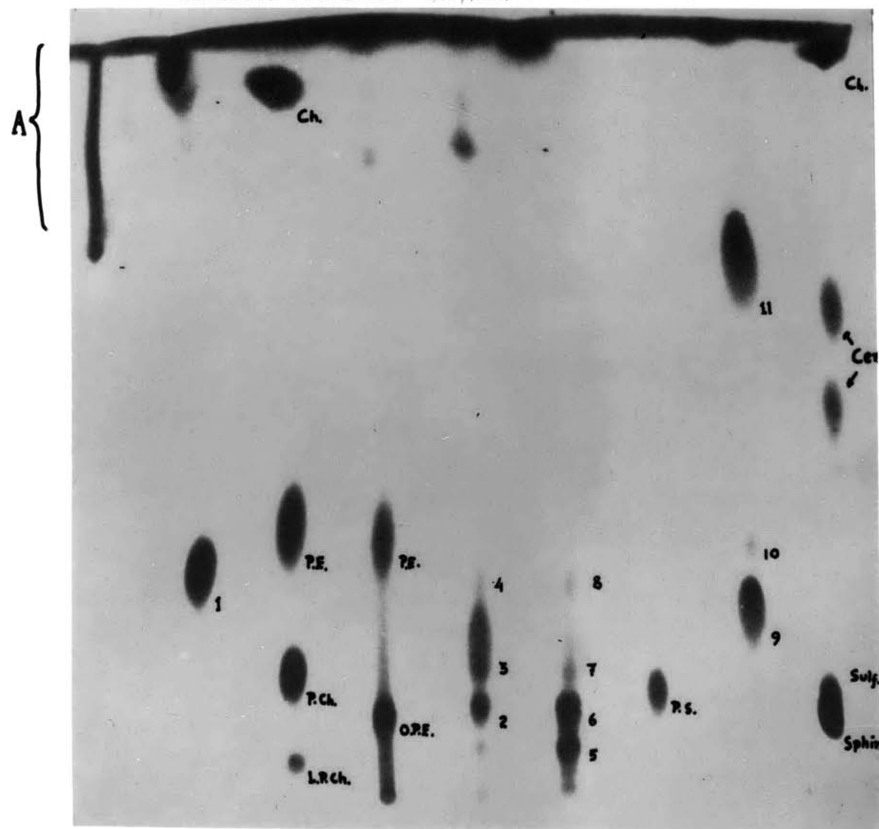
FRACTION No	I	II	III	STD.	IV	V	STD.	VI	STD.
SAMPLE μg.	235	138	64	24	70	50	6	46	24



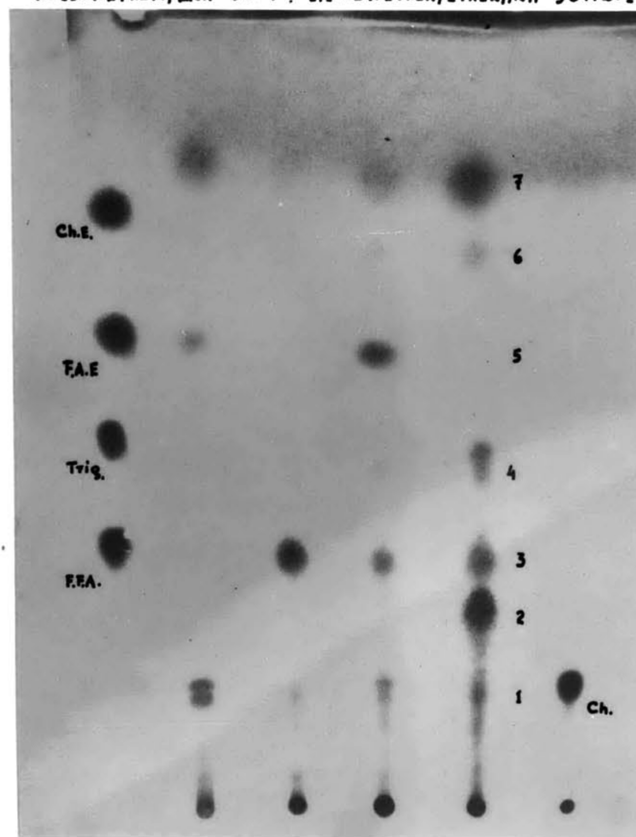
STD.	I	II	III	IV	V	VI	STD.	STD.
40	380	180	83	70	50	46	24	-

FIG 3. THIN LAYER CROMATOGRAPHY DURING CHEMICAL ANALYSIS ON THE PLATES

DEVELOP. SOLVENT: C/M/AcH/W 85:15:10:4



1.- ISOP. ETHER/AcH 96:4 . 2.- PET. ETHER/ETHER/AcH 90:10:1



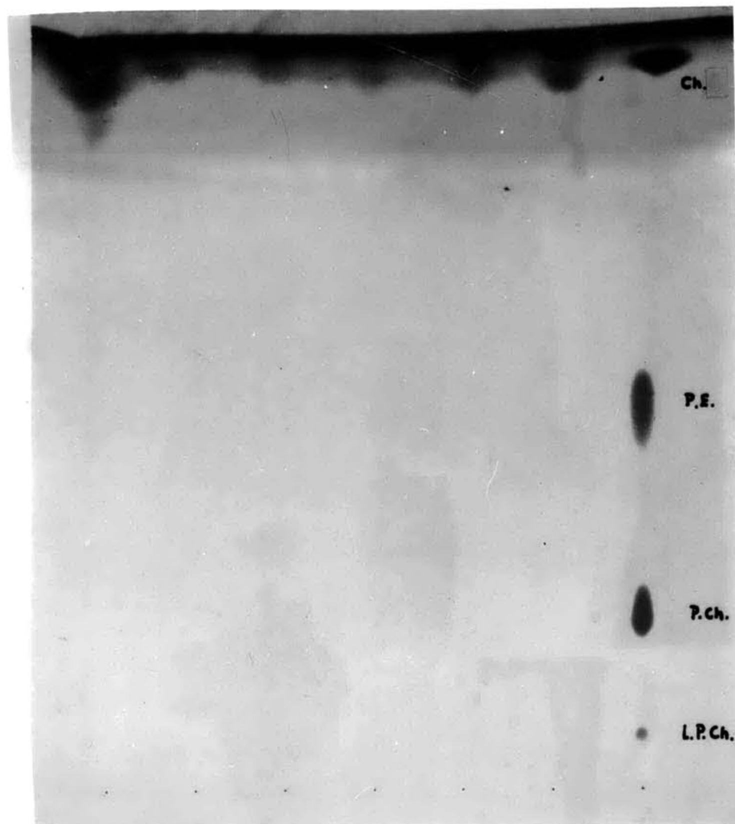
FRACTIONS:	II	III	STD.	STD.	IV	V	STD.	VI	STD.
SIZE SAMPLE, μg	207	128	80	16	280	125	20	110	80

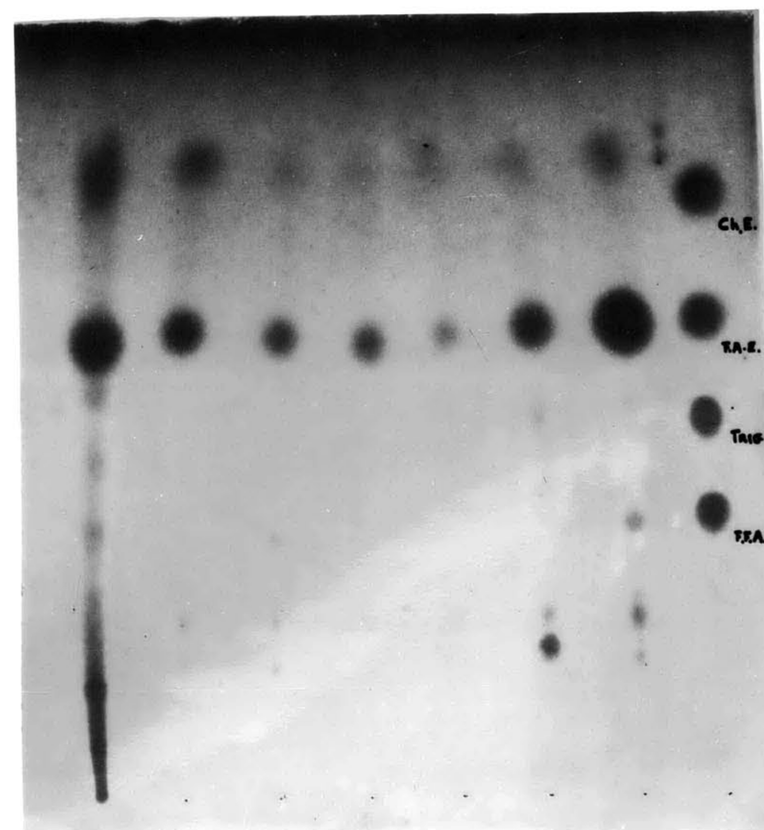
STD.	I	II	III	III$_{bF}$	STD.
64	440	207	96	96	24

FIG 4. THIN LAYER CROMATOGRAPHY OF SAMPLES ESTERIFIED BY THE MICROESTERIFICATION METHOD.

DEVELOPMENT SOLVENT: C/M/AcH/W

1. ISOPROPYL ETHER/AcH 96:4 ; 2. PET.ETHER/ETHER/AcH, 90:10:1





FRACTIONS: 20 II III IV V VI STD.

20 I II III IV V VI STD.

FRACTIONS I-VI: EQUAL SAMPLES WERE ESTERIFIED, DILUTED TO EQUAL VOLUMES AND 15 μl APPLIED TO TLC PLATES.

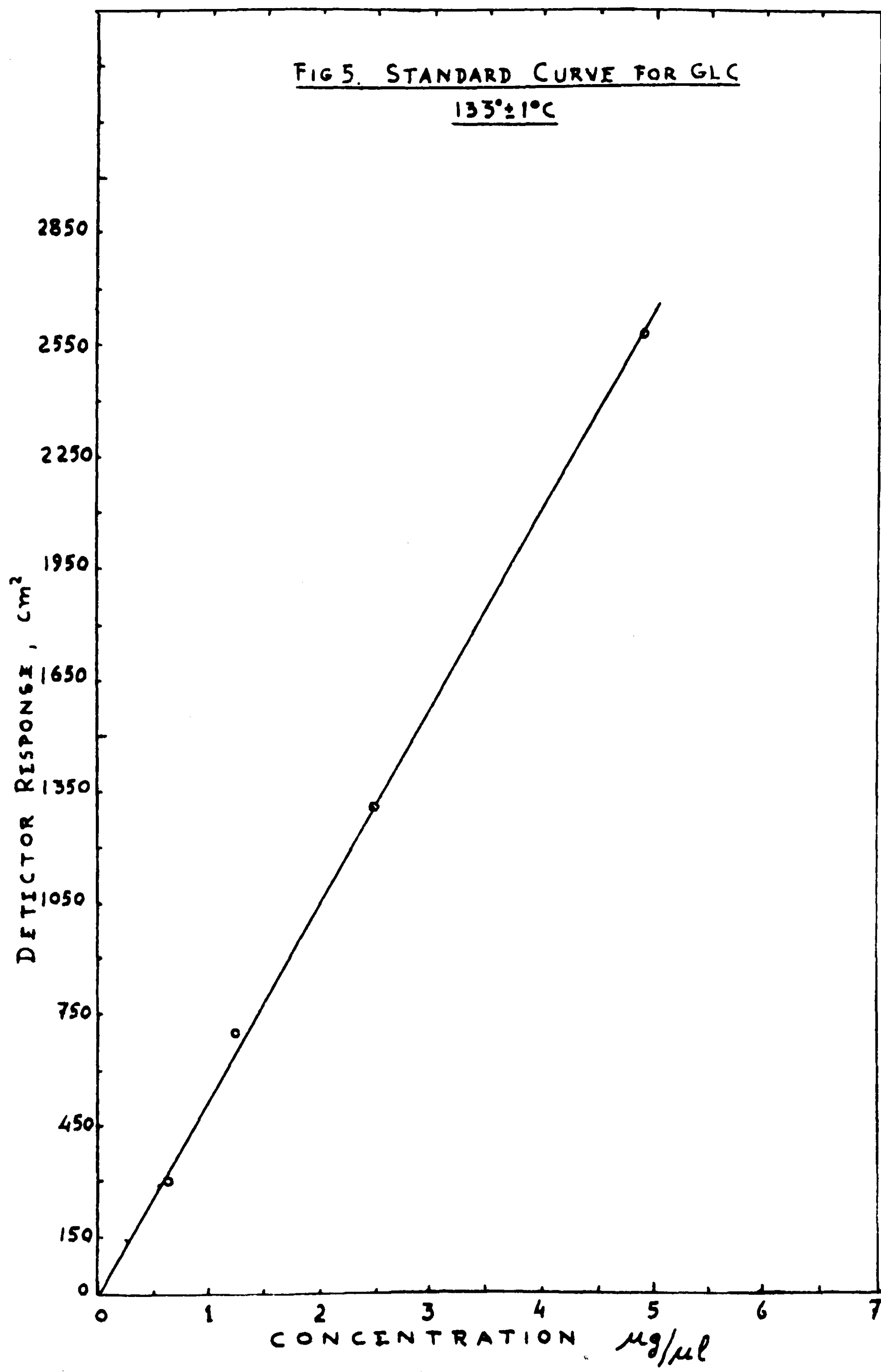


FIG 5. STANDARD CURVE FOR GLC
133°±1°C
DETECTOR RESPONSE, cm^2
2850
2550
2250
1950
1650
1350
1050
750
450
150
0
0
1
2
3
4
5
6
7
CONCENTRATION $\mu g/\mu l$

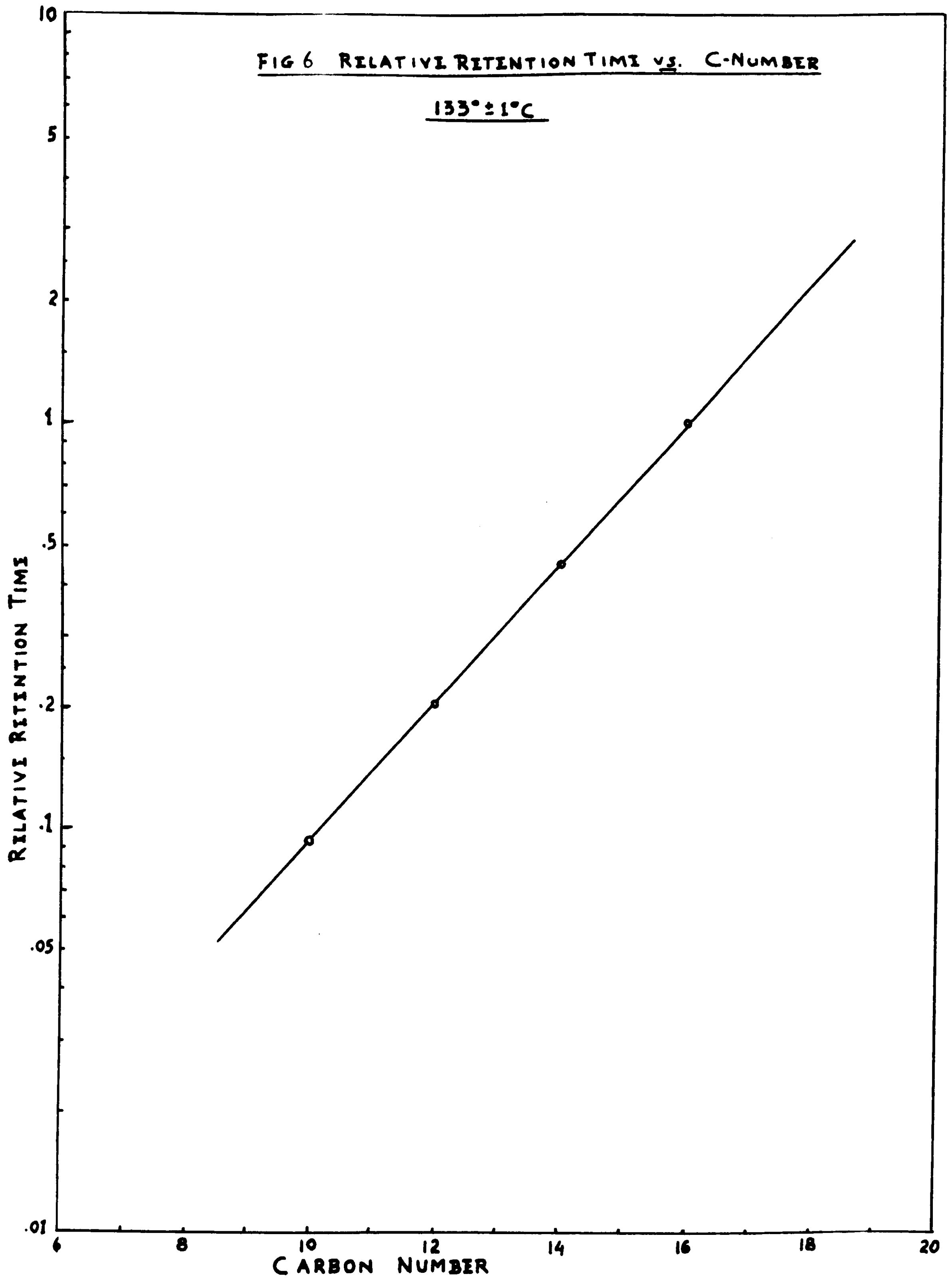


FIG 6 RELATIVE RETENTION TIME vs. C-NUMBER
133° ± 1°C
RELATIVE RETENTION TIME
10
5
2
1
.5
.2
.1
.05
.01
6
8
10
12
14
16
18
20
CARBON NUMBER

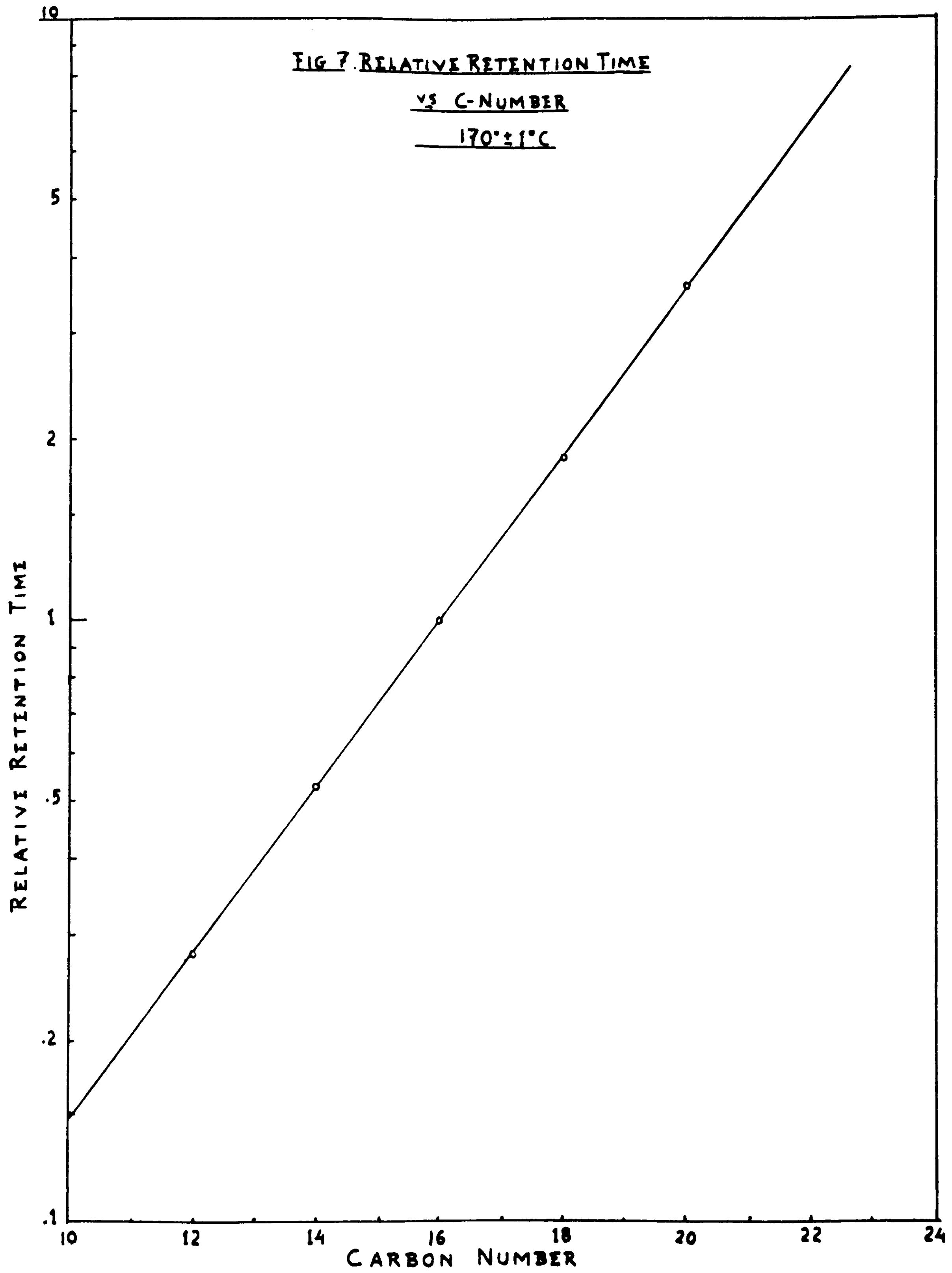


FIG 7. RELATIVE RETENTION TIME
VS C-NUMBER
170° ± 1°C
RELATIVE RETENTION TIME
CARBON NUMBER
10
5
2
1
.5
.2
.1
10
12
14
16
18
20
22
24

V. Discussion

A. Column Chromatography Separation

1. Lipid Classes Composition.

Column chromatography using DEAE-cellulose gave a value of 81.2% (203mg) for neutral lipids and 14.7% (36.4 mg) for phospholipids. The percentage of neutral lipids in this experiment is high as compared with those reported by Mizushima (1966) in cytoplasmic membrane of B. *Megatherium*. He reported 66.7% of phospholipids and 33% of neutral lipids without considering poly-β-hydroxybutyrate (PβHB). Williamson (1958) reported that lipid inclusions under a variety of cultural conditions contain 89% of PβHB. Kates (1964) states that the composition of the cytolplasmic membrane should be very similar to that of the whole cell.

In this experiment, evidence for the presence of PβHB in fractions I and II would be accounted from the following results:

a. Fractions I (440 ug sample) and II (207 ug sample) when developed in non-polar solvent showed a large amount of non-migrating material, and only hydroxy-fatty acids and free fatty acids were observed as the major neutral compounds. The same fractions when developed in polar solvents shows no presence of phospholipid but the previously non-migrating material forms a large streak near the solvent front.

b. During esterification of fractions I-VI, the percentage of conversion, with the exception of fraction IV, is lowest for fraction I (1.37%) and there is an increase toward fraction VI (44.02%), see Table 15. The presence of a compound like PβHB would be one of the reasons for the low yield for fractions I and II. Fraction IV was eluted with the last portion of the solvent C/M, 7:3, and methanol. The last solvent was used with the purpose to remove all the water soluble non-lipid contaminants which probably remained after the Folch wash, and this would be the reason for its low yield of methyl esters.

Disregarding fraction III (1.6 mg) which contains neutral lipids and phospholipid, and using the results of this experiment together with those reported in the literature the following values can be obtained:

a. Poly-β-hydroxybutyrate. Eightynine per cent of 203 mg (fraction I $\neq$ II) gives a value of 180.7 mg of PβHB, which is equivalent to 72.2% of total lipid extracted by C/M, 2:1.

b. The amount of neutral lipid without considering PβHB is 22.0 mg, so that the new per cent composition without considering PβHB would be : Neutral lipids 37.7% (22.0 mg) and phospholipids 58.4% (36.4 mg). These new values are very close to those reported by Mizushima (1966).

Among the neutral lipids, diglycerides was observed as the major neutral lipid before Folch Wash.

These results show that the major neutral lipid compounds are diglycerides, free fatty acids and hydroxy-fatty acids. Mizushima (1966) reported diglycerides (17%) (1,3-and 1,2-diglycerides) and free fatty acids (6.6%) as the major neutral lipid compounds. The absence of cholesterol observed in this experiment was reported also by Mizushima (1966), Yudkin (1962) and Weibull (1957).

The phospholipids tentatively identified in this experiment as phosphatidyl ethanolamine, phosphatidyl glycerol, diphosphatidyl glycerol were reported as the major phospholipid compounds by Kates (1964), Mizushima (1966). The compound called polyglycerol phosphate by Kates (1964) is the same as diphosphatidyl glycerol (Ansell, 1964). Lipoamino acids have been reported by Hunder and Goodsall (1961) as a major lipid class which by enzymatic hydrolysis will give phosphatidyl glycerol and free amino acids. Although lipoamino acids were not positively identified in this experiment, there are some unidentified compounds which most of them were Ninhydrin-positive. Phosphatidyl serine was found in the present experiment and Weibull (1957) also reported small amounts of this compound.

B. Fatty Acid Composition

1. Esterification

Under the conditions of this experiment, the micro-esterification method (Stoffel, 1959) did not yield the methyl esters in a pure state. This result can be observed on Figure 4 on which carbohydrates, free fatty

acids, and other compounds, probably mono and diglycerides, are present. Formation of diglycerides from diphosphatidyl glycerol during acid hydrolysis was reported by Ansell (1964). The reason of the contamination observed may be due to a fault in the technique, however, Cho (1966) working with lipids of bacterial membrane, and using the same method outlined by Stoffel, had to purify the crude methyl esters using Florisil-column chromatography.

The esterification using BF_3 gave better results after Folch wash. Thorne and Kodicek (1962) have used alkaline and acid hydrolysis for esterification because acid hydrolysis destroy lactobacillic acid, and alkaline hydrolysis gave 12% lower yield of methyl esters. Lactobacillic acid (19:cyclopropane) has not been reported in B. megatherium, but Table 16 shows that compound #19 (identified as 20:0) of sample #25 constitutes 7.73% of the total fatty acids when the esterification is carried out under alkaline conditions. This compound (20:0) is present only in trace level (Table 17) when using acid hydrolysis.

2. Fatty Acid Composition

This experiment showed that 16:0 (10.8%) is the major saturated fatty acid. However, the other compounds which constitute the major portion of the fatty acids were not identified. For B. megatherium the major fatty acids are 15:branched iso and anteiso, which constitute 55% of the total (Thorne and Kodicek, 1962, Mizushima, 1966).

Probably compound #7 (43.2%) in this experiment corresponds to 15: br. Thorne and Kodicek (1962) using polyethylene glycol columns at 180°C and 18:0 as reference gave a relative retrention time of 0.31 for 15:br anti iso; in this experiment working at 170°C and taken 18:0 as reference the relative retention time of compound #7 is 0.35. Other branched chain acids reported in B. megatherium are 14: br (3.0%), 16:br iso and anti iso (3.4%), 17:br anti iso (2.6%) and 18:br iso (0.7%), (Thorne and Kodicek, 1962).

The most part of the fatty acids reported on B. megatherium contains 16 or less than 16-carbon atoms, which agrees with the value of 91.5% obtained in this experiment, Table 7.

In general, the composition of fatty acids with fewer than 20- carbon atoms reported in this experiment are in agreement with those given in the literature. Fatty acids with more than 20-carbon atoms were found in small amounts in the present experiment, mainly in sample #20 (extracted after acid hydrolysis). In fraction V compount #22 constitutes 3.16% of the total fatty acids, however, its identity was not possible to determine from the plot log retention time vs carbon number. Thorne and Kodicek (1962) reported 2% of a saturated fatty acid with 21-carbon atoms.

VI. Conclusions

From the results of this experiment the following conclusions can be stated:

1. Almost complete extraction of the lipids, together with non-lipid contaminants was accomplished with the mixture chloroform-methanol 2:1. After removal of water soluble contaminants, the total lipid consitutes 16.5% dry basis.
2. Only a small lipid portion, 0.26% dry basis, is extracted after acid hydrolysis.
3. DEAE-cellulose column chromatography accomplished the separation of lipid fractions.
4. Poly-β-hydroxybutyrate (PβHB) which is soluble in chloroform or chloroform-methanol mixtures was suspected to be present in fractions I and II. An indirect calculation of PβHB gave a value of 72.2% of total lipid extracted by C/M (2:1).
5. The amount of neutral lipid was computed as 37.7% and phospholipid 58.4% of total PβHB-free lipid.
6. The two-step development system for thin layer chromatography gave good results for the characterization of neutral lipids:

   Diglyceride, hydroxy-fatty acids and free fatty acids were the major meutral lipid compounds. Triglycerides, cholesterol ester and carbohydrate are also present. Cholesterol was not detected.
7. The polar solvent for TLC used for characterization

of phospholipids permitted the separation of these compounds. However, the oval-shape of the spots prevented complete separation specially in the case of fractions IV and V. The following phospholipids were tentatively identified:

Phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, and an unidentified compound, #1, as the major compounds. Probably phosphatidic acid is also present, but not separated from diphosphatidyl glycerol. Phosphatidyl serine was detected. Several minor ninhydrin-positive compounds were not identified.

8. Gas liquid chromatography permitted the separation of the fatty acids as their methyl esters.

   A temperature of 133°C was used to identify fatty acids up to 16-carbon atoms. These fatty acids constitute 91.5% of the total. A temperature of 170°C was used for identification of compounds with more than 16-carbon atoms.

   Palmitic acid (10.8%) is the most abundant saturated fatty acid. Peak #7 (44%) was tentatively identified as 15:branched chain acid. Unidentified major compounds with less than 16-carbon atoms are peak #4 (14.8%), #10 (13.7%) and #12 (5.5%); with more than 20-carbon atoms peak #22 (3.3%).

9. The Folch method of purification removed almost quantitatively the diglycerides and myristic acid (14:0).

10. Only very small amounts of the unsaturated fatty acids such as linoleic and oleic acid, considered as essential fatty

acids for human nutrition were observed. Although the total lipid content of B. megatherium can be increased or decreased with culture conditions, the nutritional implications of the branched chain fatty acids which are the major compounds should be investigated.

VII. Suggestions for Future Work

1. The analyses described here should be repeated using freshly harvested cells to determine the extent of autoxidation which occurred.
2. Poly-β-hydroxybutyrate should be removed before Folch washing by precipitating the polymer with diethyl ether, cooling and separation by centrifugation. Poly-β-hydroxybutyric acid can be estimated by the method of Law (1961).
3. DEAE-cellulose column chromatography should be monitored again with thin layer chromatography because the elimination of PβHB probably will simplify the elution of the neutral lipids.
4. The removal of diglycerides and myristic acid by Folch wash should be taken in consideration.
5. Fraction V should be subfractionated in order to improve the separation.
6. Individual phospholipids (major compounds) should be isolated from TLC plates and them deacylated to confirm their identity.
7. Alkaline and acid esterification would be desirable to

study the fatty acid composition.

8. A complete study of bacterial lipid stability should be initiated. Rates of oxygen absorption in dehydrated cells should be investigated.

BIBLIOGRAPHY

1. Ansell, G. B. and J. N. Hawthorne, Phospholipids, Chemistry, Metabolism, and Function., Elsevier Pub. Co. pp 11-39, 1964.

2. Asselineau, J. and E. Lederer. Chemistry and Metabolism of Bacterial Lipids, in Lipide Metabolism. K. Bloch (ed) John Wiley & Sons, Inc. U. S. A. pp 337-406, 1960.

3. Barrett, C. B., M. S. J. Dallas and F. B. Padley. The Separation of Glycerides by Thin Layer Chromatography on Silica Impregnated with Silver Nitrate, Chem. and Ind. 1050, 1962.

4. Blank, M. L., J. A. Schmit, and O. S. Privett. Quantitative Analysis of Lipids by Thin Layer Chromatography, J. A. O. C. S. 41, 371, 1964.

5. Cho, K. Y. and M. R. J. Salton. Fatty Acid Composition of Bacterial Membrane and Wall Lipids, Biochem. et Biophys. Acta. 116, 73, 1966.

6. Entenman C., General Procedures for Separating Lipid Components of Tissue, in Methods in Enzymology Vol. 3, S. P. Colowick and N. O. Kaplan (Eds). Acad. Press, pp 299-317, 1957.

7. Folch, J., et al. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, J. Biol. Chem. 226, 497, 1957.

8. Fontell, K. et al. Some New Methods for Separation and Analysis of Fatty Acids and Other Lipids, J. Lipid Res. 1, 391, 1960.

9. Forsyth, W. G. C. et al. Occurrence of Poly. G. Hydroxybutric Acid in Acrobic Gram-negative Bacteria, Nature 182, 800, 1958.

10. Freeman, C. P. and D. West. Complete Separation of Lipid Classes on a Single Thin Layer Plate, J. Lipid Res. 7, 324, 1966.

11. Gavin, J. J. and W. W. Umbreit, Effect of Biotin on Fatty Acid Distribution in Escherichia coli, J. of Bacteriol - 89, 437, 1965.

12. Goldfine, H. and K. Bloch. On The Origin of Unsaturated Fatty Acids and Clostridia. J. Biolog. Chem. 236, 2596, 1961.

13. Gregory, P. H. The Microbiology of the Atmosphere Plant Science Monographs. N. Polunin (Ed). Leonard Hill (Books) Limited, London. pp 109-147, 1961.

14. Hanahan, D. J. Lipide Chemistry. John Wiley & Sons, Inc., pp 42-105, 1960.

15. Haverkate, F. et al, The Enzymic Hydrolysis and Structure of Phosphatidyl Glycerol, Biochem et Biophys. Acta, 63, 547m 1962.

16. Hirsch, J. and E. H. Ahrens, Jr. The Separation of Complex Lipide Mixtures by the Use of Silicic Acid Chromatography; J. of Biol. Chem. 233, 311, 1958.

17. Hoffman, K. and Ch. Panos. The Biotin-like Activity of Lactobacillic Acid and Related Compounds, J. of Biol Chem. 210, 687, 1954.

18. Hoffman, K. and F. Tausing. On The Identity of Phytomonic and Lactobacillic Acids. A Reinvestigation of the Fatty Acid Spectrum of Agrobacterium (Phytomonas) Tumefaciens. J. of Biol. Chem. 213, 425, 1955.

19. Houtsmuller, U. M. T. and L. L. M. Van Deenen. Identification of a Bacterial Phospholipid as an O-ornithine Ester of Phosphatidyl Glycerol. Biochem. et Biophys. Acta 70, 211, 1963.

20. Hunter, G. D. and A. T. James. Lipoamino Acids from Bacillus megatherium, Nature 198, 789, 1963.

21. Hunter, G. D. and R. A. Goodsall. Lipoamino Acid Complexes from Bacillus megatherium and Their Possible Role in Protein Synthesis, Biochem. J. 78, 564, 1961.

22. Huston, Ch. K. and P. W. Albro. Lipids of Sarcine, lutea. I. Fatty Acid Composition of the Extractable Lipids, J. of Bacteriol 88, 425, 1964.

23. Ikawa, M. Nature of the Lipids of Some Lactic Acid Bacteria, J. of Microb. 85, 772, 1963.

24. James, A. T. and A. J. P. Martin Gas-Liquid Partition Chromatography: The Separation and Microesterimation of Volatile Fatty Acids from Formic Acid to Dodecanoic Acid. Biochem. J. 50, 679, 1952.

25. Kates, M. et al, The Lipid Composition of Micrococcus Halodenitrificans as Influences by Salt Concetnration. Can. J. of Microbiol. 7, 427, 1961.

26. Kates, M. et al, The Lipid Composition of Baccillus cereus as Influenced by the Presence of Alcohols in the Culture Medium. Can. J. of Biochem. and Physiol. 40, 83, 1962.

27. Kates, M. Bacterial Lipids, in Advances in Lipid Research Vol. 2. R. Paoletti and D. Kritchevsky (Eds) Acad. Press pp 17-84, 1964.

28. Kaneshiro, T. and A. G. Marr. Cis-9, 10-Methylene Hexadecanoic Acid from the Phospholipids of Escherichia coli. The J. of Biol. Chem. 236, 2615, 1961.

29. Kaneshiro, T. and A. G. Marr. Phospholipids of Azotobacter Agilis, Agrobacterium tumefaciens, and Escherichia coli, J. Lipid Research 3, 184, 1962.

30. Law, J. H. and R. A. Slepecky. Assay of Poly-β-hydroxybutiric Acid. J. Bacteriol. 82, 33, 1961.

31. Lea, C. H., D. N. Rhoses and R. D. Stoll. Phospholipids. 3. On the Chromatographic Separation of Glycerophospholipids, Biochem. M. 60, 353, 1955.

32. Lemoigne, M., G. Delaforte and M. Croson. A L'etude Botanique et Biocimique des Bacteries due Genre Bacillus. Valeur du test des Lipides β-Hydroxybutyriques pour la caracterisation des Especies, Ann. Inst. Pasteur, 70, 224, 1944.

33. Lepage M. The Separation and Identification of Plant Phospholipids and Glycolipids by Two-dimensional Thin Layer Chromatography, J. of Chromatog. 13, 99, 1964.

34. Lovern, J. A. Lipids of Biochemical Signifigance. Methuen & Co., Ltd., London. pp 43-81, 1957.

35. Macfarlane, M. G. Characterization of Lipoamino-acids as O-aminoacids of Phospatidyl Glycerol, Nature 196, 136, 1962.

36. Macrae, R. M. and J. F. Wilkinson. Poly-β-hydroxybutyrate Metabolism in Washed Suspensions of Bacillus cereus and Bacillus megatherium, J. Gen. Microbiol. 19, 210, 1958.

37. Mangold, H. K. and D. C. Malins. Fractionation of Fats, Oils, and Waxes on Thin Layers of Silicic Acid. A. O. Ch. J. 37, 383, 1960.

38. Matches, J. R. et al. Phospholipids in Vegative Cells and Spores of Bacillus polymyxa, J. Bacteriol 87, 16, 1964.

39. Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. Rapid Preparation of Fatty Acid Esters from Lipids for Gas Chromotographic Analysis, Anal. Chem. 38, 514, 1966.

40. Mizushima, S. et al. Chemical Composition of the Protoplast Membrane of Bacillus megatherium, J. of Biochem. 59, 374, 1966.

41. Moore, T. Personal Communication, M.I.T. Dept. Food Science and Nutrition, 1966.

42. Morris, L. J. Separation of Isomeric Long-chain Pòlyhydroxy Acids by Thin Layer Chromatography, J. of Chromatog. 12, 321, 1963.

43. Nichols, B. W., Separation of Plant Phospholipid and Glycolipids, in New New Biochemical Separation. A.T. James and L. J. Morris (Eds). D. Van Nostrand Co., Ltd. London pp 321-337, 1964.

44. O'Leary, W. M. The Fatty Acids of Bacteria, Bacteriol. Rev. 26, 421, 1962.

45. Pelick, N. et al Some Practical Aspects of Thin Layer Chromatography of Lipids J. A. O. C. S. 42, 393, 1965.

46. Porter, J. R. Bacterial Chemistry and Physiology. J. Wiley & Sons, Inc., 5th Edit. p 406, 1950.

47. Randerath K. Thin Layer Chromatography. Verlag Chemie. Germany pp 126-151, 1964.

48. Rouser, G. et al Lipid Composition of Beef Brain, Beef Liver, and the Sea Anemone: Two Approaches to Quantative Fractionation of Complex Lipid Mixtures. J. A. O. C. S., 40, 425, 1963.

49. Rouser, G. C. Galli and E. Lieber. Analytical Fractionation of Comples Lipid Mixtures: DEAE Cellulose Column Chromatography Combined with Quantitative Thin Layer Chromatography, J. A. O. C. S. 41, 836, 1964.

50. Shaw, M. K. and J. L. Ingraham. Fatty Acid Composition of Escherichia coli as a Possible Controlling Factor of the Minimal Growth Temperature. J. of Bacteriol. 90, 141, 1965.

51. Skidmore, W. D. and C. Eentenman. Two-dimensional Thin Layer Chromatography of Rat Liver Phosphatides. J. Lipid Research 3, 471, 1962.

52. Skipski, V. P., et al. Separation of Lipid Classes by Thin Layer Chromatography, Biochem. et Biophys. Acta 106, 386, 1965.

53. Skipski, V. P. Separation of Phosphatidyl Ethanolamine, Phosphatidyl Serine, and Other phospholipids by Thin Layer Chromatography, J. Lipid Research 3, 467, 1962.

54. Smith, F. P. and C. V. Henrikson. Glucose-containing Phospholipids in Mycoplasma Laidlarvii, Strain B, J. Lipid Research 6, 106, 1965.

55. Sthephenson M. Bacterial Metabolism. The MIT Press, Camb. Mass. 1966.

56. Stahl, E. Thin Layer Chromatography, A Laboratory Handbook Springer-Verlag. Berlin pp 485-502, 1965.

57. Stoffel, W., F. Chu and E. H. Ahrens Jr. Analysis of Long-chain Fatty Acids by Gas-liquid Chromatography. Micro-method for Preparation of Methyl Esters, Anal. Chem. 31, 307, 1959.

58. Tannenbaum, S. R., Matches, R. I. and G. R. Capco. Processing of Bacteria for the Production of Protein Concentrates. Advances in Chemistry Series, A. C. S. 1966 (in press).

59. Thorne, K. J. I. and E. Kodicek. The Metabolism of Acetate and Mevalonic Acid by Lactobacilli, IV. An Analysis of the Fatty Acids by Gas-liquid Chromatography, Biochem. Biophys. Acta 59, 306, 1963.

60. Vorbeck, M. L. and G. V. Marinetti. Separation of Glycosyl diglycerides from Phosphatides Using Silicic Acid Column Chromatography. J. Lipid Research 6, 3, 1965.

61. Weibull, C. The Lipids of a Lysozyme Sensitive Bacillus Species (Bacillus "M"). Acta Chem. Scand. 11, 881, 1957.

62. Weibull, C. and L. Bergstrom. The Chemical Nature of the Cytoplasmic Membrane and Cell Walls of Bacillus megatherium, strain M. Biochem. Biophys. Acta 30, 340, 1958.

63. Williamson, D. H. and J. F. Wilkinson. The Isolation and Estimation of the Poly-β-hydroxybutyrate Inclusions of Bacillus species, J. Gen. Microbiol. 19, 198, 1958.

64. Woodbine, M. Microbial Fat: Microorganisms as Potential Fat Producers, in Progress in Industrial Microbiology. . Vol I D. J. D. Jockenhull (Ed). Hergwood and Co. London, pp 179-245, 1959.

65. Yudkin, M. D. Chemical Studies of the Protoplast Membrane of Bacillus megatherium K. M. Biochem J. 82, 40:, 1962.

66. United Nations. Food and Agriculture Organization, The State of Food and Agriculture, 1965. Rome, 1965.