AN ABSTRACT OF THE THESIS OF

Marian Lorraine McCollough for the M.S. in Microbiology
(Name) (Degree) (Major)

Date thesis is presented September 1, 1966

Title STUDIES ON THE FATTY ACID AND LIPID COMPOSITION OF CLOSTRIDIUM BOTULINUM

Abstract approved Redacted for Privacy
(Major professor)

Routine studies of bacterial fatty acid and lipid composition, made possible by recently developed techniques, have brought about an increasing interest in the physiological significance of these compounds. The purpose of this study was to characterize the fatty acids of the vegetative cells of Clostridium botulinum 33A, as the first step in an investigation of the possible relationship between the fatty acid content and radiation resistance of this organism. Lipids were extracted from lyophilized cells. Free fatty acids were separated by column chromatography. Methyl esters of the free fatty acids and the fatty acids from the other lipids were characterized by gas-liquid chromatography. The lipid extract was separated into classes using thin-layer chromatography.

The results show that this organism possesses a complete fatty acid spectrum ranging from C_{10} to C_{20} with the exception of

C_{19}. The great majority of the fatty acids present are straight-chain saturates or straight-chain monounsaturates. The lipids include large amounts of free fatty acids and phospholipids.

STUDIES ON THE FATTY ACID AND LIPID COMPOSITION OF CLOSTRIDIUM BOTULINUM

by

MARIAN LORRAINE MCCOLLOUGH

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1967

APPROVED:

Redacted for Privacy

Professor of Microbiology

In Charge of Major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented September 1, 1966

Typed by Joanne Wenstrom

ACKNOWLEDGMENTS

I wish to thank my major professor, Dr. Arthur W. Anderson, for giving me the opportunity to conduct this study and for his guidance and encouragement during the course of the investigation. I have learned a great deal from him.

Thanks are due to Dr. Ian Tinsley, Mr. Robert Lowry, and Mr. James Saddler of the Department of Agricultural Chemistry for the use of their gas chromatograph. Special thanks go to Mr. Lowry for permitting me to use his laboratory facilities and materials, for his many helpful suggestions, and for his generous and patient assistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

STUDIES ON THE FATTY ACID AND LIPID COMPOSITION OF *CLOSTRIDIUM BOTULINUM*

INTRODUCTION

Before the development of techniques such as thin-layer chromatography, gas-liquid chromatography, infrared spectroscopy, and mass spectrometry relatively little was known about bacterial lipids and fatty acids. Investigation was hampered by the inconvenient physical characteristics of lipids and by the lack of a means for accurately analyzing very small samples.

The routine application of these techniques has greatly facilitated work in this area and information about the composition and function of bacterial lipids and their component fatty acids has steadily accumulated. Certain differences such as the absence of sterols and the presence of unusual fatty acids have been found between the lipids of these and other organisms.

The physiological significance of lipids is being investigated and a thorough knowledge of their component fatty acids is an essential prerequisite for complete understanding. The purpose of this study was to determine the fatty acid composition of the vegetative cells of *Clostridium botulinum* 33A, a highly radiation resistant anaerobic sporeformer, as the first step in an investigation of the possible relationship between the fatty acid composition of this organism and its resistance to radiation.

REVIEW OF LITERATURE

Bacterial Lipids

The lipid content of bacterial cells varies from species to species but generally ranges from 1-10% of the dry cell weight. The lipids known to occur in bacteria may be divided into eight classes: (1) free fatty acids, (2) fatty acid polymers, (3) glycerides, (4) phospholipids, (5) glycolipids, (6) peptidolipids, (7) waxes, and (8) bound lipids.

Free Fatty Acids

The proportion of bacterial lipid that occurs as free fatty acid varies widely in the bacteria that have been studied. It is often considerably greater than that found in other organisms. Huston and Albro (40) found 2.1% free fatty acid in Sarcina lutea while Asselineau (7) reported that Corynebacterium diphtheriae contains 32% free fatty acid. Twenty percent or more free fatty acid has been found in a number of bacteria including Lactobacillus acidophilus, Bacillus megaterium, Agrobacterium tumefaciens, Hemophilus pertussis, and Mycobacterium leprae (19, 53, 74, 89). Some reports of very high free fatty acid content may be the result of lipid hydrolysis. In 1939 Akasi (1) concluded that almost all the lipid of

Salmonella typhimurium is free fatty acid but recently MacFarlane (62) found glycerides and a considerable amount of phospholipid in this organism Normal saturated and unsaturated acids, branched chain acids and hydroxy acids have been identified in the free fatty acid fraction of bacteria (9, 19).

Fatty Acid Polymers

A polymer of β-hydroxybutyric acid is present in a wide variety of bacteria. Azotobacter chroococcum and Bacillus cereus contain inclusions that are predominantly poly β-hydroxybutyric acid (55, 92). This polymer has also been identified in species of Chromobacterium, Rhizobium, Pseudomonas (23), Hydrogenomonas, Spirillum, Ferrobacillus, Rhodospirillum (59), and Micrococcus (87).

Glycerides

Mono-, di-, and triglycerides have been observed in bacteria although they are less common here than in plant and animal cells. Glycerides account for 12% of the lipid of Agrobacterium tumefaciens (89) and 35% of the lipid of Lactobacillus acidophilus (19). Glycerides containing saturated C_{16} and unsaturated C_{18} fatty acids occur in several species of Salmonella (9).

Monoglycerides. Monoglycerides have been reported in Sarcina lutea (40) and several workers have investigated the monoglycerides of Mycobacterium tuberculosis. An α-glyceryl ester of a C_{16} or C_{18} fatty acid has been identified in the acetone soluble fat of this organism (72) and an α-monoglyceride of mycolic acid is present in the methanol insoluble "purified wax" fraction (71, 13).

Diglycerides. The most thoroughly characterized bacterial lipids are those of Mycobacterium tuberculosis. A 1, 3-diglyceride containing palmitic and stearic acids has been found (72). Two diglycerides with more complex fatty acids are known to occur in this bacteria. One contains hexacosanoic (C_{26}) acid and the other has one palmitic or similar acid and one mycolic acid residue per molecule (13). The latter was discovered in the BCG strain. Sarcina lutea possesses diglycerides (40) and a diglyceride with a branched C_{15} acid as its major component is present in Micrococcus lysodeikticus (61).

Triglycerides. The presence of triglycerides has been detected in Sarcina lutea (40) and Mycobacterium tuberculosis. Some mycobacterial triglycerides are known to contain mycocerosic acid (10) and normal C_{22}-C_{26} fatty acids (71) but such long chain acids are not always present. Bloch et al. (13) identified a triglyceride

in M. tuberculosis which has palmitic or similar acids as its principal components.

Phospholipids

The phospholipids constitute a major fraction of the lipids of many bacteria. A number of bases have been identified in bacterial lipids. The most common ones are ethanolamine, glycerol, and inositol (74, 50). Phosphatidyl ethanolamine is the predominant phospholipid of Escherichia coli (54), Micrococcus halodenitrificans (51), Salmonella typhimurium (62), and Proteus P 18 (70). Glycerol phosphatides comprise over 25% of the phospholipid of Clostridium botulinum (11), Micrococcus lysodeikticus (61), and Sarcina lutea (41). Choline has been identified only in the genus Agrobacterium (25, 48, 28) although a wide variety of bacteria have been examined for it (28). Some bacterial phospholipids contain amino acids. Phosphatidyl serine has been found in several bacteria (41, 49, 11) and the existence of amino acid esters of phosphatidyl glycerol has been established (60, 50). A phospho-glycolipid containing inositol and mannose occurs in Mycobacterium tuberculosis (90, 72). Part of the phospholipid of some anaerobic bacteria exists in the plasmalogen form (11, 26, 3, 91) but thus far plasmalogens have not been found in aerobic bacteria.

Glycolipids

The glycolipids of bacteria are composed of a fatty acid or fatty alcohol and a sugar. They do not contain sphingosine. Trehalose-6,6' dimycolate is present in Mycobacterium tuberculosis (71). Trehalose-fatty acid esters have also been reported in Corynebacterium diphtheriae (2) as have galactose esters (9). A glycolipid of rhamnose and β-hydroxydecanoic acid present in Pseudomonas aeruginosa has been described (12, 44). MacFarlane (60, 62) reports a glucose containing glycolipid in Staphylococcus aureus and a mannose glycolipid in Clostridium perfringens.

Peptidolipids

There is little information on bacterial peptidolipids. These substances are fat soluble combinations of peptides and lipids which should be distinguished from the water soluble lipopeptides (9). A peptidolipid of Mycobacterium avium was reported by Smith et al. (86) and has been described by Asselineau and Lederer (9). Nocardia asteroides possesses a peptidolipid composed of 70% peptide and 30% lipid (30).

Waxes

True waxes are esters of long chain fatty acids and long chain

fatty alcohols. Their occurrence in bacteria is limited mainly to the genera Mycobacterium and Corynebacterium. Phthiocerol-dimycoceranate has been identified in M. tuberculosis (71) and esters of complex fatty acids with 2-eicosanol and 2-octadecanol have been reported in M. avium and M. phlei (9). Palmitates and stearates of octadecanol and docosanol (5) as well as fatty acid esters of corinnic alcohol (9) are present in C. diphtheriae.

Bound Lipids

"Bound lipids" are those which can be extracted with organic solvents only after hydrolysis of "defatted" cells. The fatty acid composition of bacterial bound lipids has been studied but the nature of the lipid-carbohydrate or lipid-protein complexes has not been determined. Bound lipids comprise about 20% of the total lipid of Azotobacter agilis (47) and Corynebacterium diphtheriae (7) while 80% of the lipid of Lactobacillus arabinosus (35) and L. casei (36) is bound.

Bacterial Fatty Acids

The fatty acids of bacteria can be divided into five groups: (1) straight-chain saturated acids, (2) straight-chain unsaturated acids, (3) branched-chain acids, (4) cyclopropane acids, and (5) hydroxy acids. Some of the more complex fatty acids possess the characteristics of two groups.

Straight-chain Saturated Acids

The normal saturated acids with an even number of carbon atoms are common constituents of bacterial lipids. The short chain ones (C_1-C_{10}) have been found in all bacteria examined for them. Those occurring most frequently in large amounts are the C_{12}-C_{18} (lauric, myristic, palmitic, stearic) acids (74). Palmitic acid is the major fatty acid of many bacteria including Lactobacillus acidophilus (19), Corynebacterium diphtheriae (24), Clostridium butyricum (27), Escherichia coli (46, 67), Streptococcus lactis (63), Aerobacter aerogenes (75), and Mycobacterium tuberculosis (18). It comprises from 30-49% of their fatty acids. Normal saturated acids with 20 or more carbons are relatively rare. Arachidic (C_{20}) and behenic (C_{22}) acids have been identified in Pseudomonas aeruginosa (79) and behenic, lignoceric (C_{24}), and cerotic (C_{26}) acids have been reported in Corynebacterium diphtheriae (4, 6). Normal saturates with an odd number of carbons have been detected in a few bacteria. Several Nocardia species contain C_{15}-C_{19} acids (14), C_{13} and C_{15} acids are present in Clostridium butyricum (27), and C_{15}-C_{21} acids occur in Pseudomonas aeruginosa (79).

Straight-chain Unsaturated Acids

The great majority of unsaturated fatty acids in bacteria are

monoenoic. Of these the most important are the hexadecenoic (16:1) and octadecenoic (18:1) acids. The predominant 16:1 acid is palmitoleic (9-10 hexadecenoic) but 7-8, 10-11, and 11-12 hexadecenoic acids are also known to occur in bacteria (14, 38, 83). Large amounts of hexadecenoic acid are present in Corynebacterium diphtheriae, Micrococcus lysodeikticus (24), M. halodenitrificans (51), and Escherichia coli (67). The two 18:1 acids that are frequently found in bacterial lipids are oleic (9-10 octadecenoic) acid and cis-vaccenic (11-12 octadecenoic) acid. Oleic acid has been identified as the only octadecenoic acid in Mycobacterium phlei (57) and Nocardia sp. (14). Large quantities of cis-vaccenic acid are present in Lactobacillus arabinosus (35), L. casei (36), and Agrobacterium tumefaciens (37). Group C Streptococcus (38), Escherichia coli (46), and Clostridium butyricum (83) contain a mixture of oleic and cis-vaccenic acids. Fifty-two percent of the fatty acids of Micrococcus halodenitrificans consists of octadecenoic acid (51). Tetradecenoic (14:1) acid has been detected in several bacteria (4, 51, 79, 41). An unsaturated hydroxy acid of 31-32 carbons has been reported in Corynebacterium diphtheriae (77). Small amounts of some short chain (73, 29), and odd carbon chain length (41, 79, 14) unsaturates occur in a few bacteria.

Branched-chain Acids

Branched chain acids account for a majority of the fatty acids of several gram positive bacteria. Bacillus subtilis (45), Sarcina lutea (41), and Micrococcus lysodeikticus (24) contain 60% branched acids. Ninety-three percent of the fatty acids of Bacillus megaterium are branched (24). The C_{15} and C_{17} branched acids predominate. The iso form of these acids has been identified in Bacillus natto (81, 82) but the anteiso form is abundant in Bacillus subtilis (45), Micrococcus lysodeikticus (56), and Staphylococcus aureus (62). Ten-methyl stearic acid (C_{19}) has been characterized in Mycobacterium tuberculosis (18) and M. phlei (57). Another branched C_{19} acid is present in Pasteurella pestis (8). Pseudomonas aeruginosa possesses all the C_9-C_{19} odd carbon branched acids (43). Hausmann and Craig (32) found 6-methyl octanoic and iso-octanoic acids in polymixin B, the antibiotic produced by Bacillus polymyxa. Several complex branched-chain and branched, hydroxy fatty acids are present in Corynebacterium diphtheriae and Mycobacterium tuberculosis. The review by Asselineau and Lederer (9) contains a discussion of them.

Cyclopropane Acids

A 19 carbon cyclopropane fatty acid, discovered in Lactobacillus arabinosus (34), was named lactobacillic acid. It has

subsequently been identified in L. casei (36), L. delbruckii (39), L. plantarum (33), Agrobacterium tumefaciens (37), and Escherichia coli (46) and comprises from 7 to 30% of the fatty acids of these organisms. The occurrence of the cyclopropane ring at the 11-12 position in this acid was established by Marco and Hofmann (65). Similar acids have been reported in Salmonella typhimurium (62), Aerobacter aerogenes (75), Clostridium butyricum (27), and Serratia marcescens (94). Analogous C_{17} cyclopropane fatty acids comprise 20-30% of the fatty acids of Salmonella typhimurium (62), Escherichia coli (46), Aerobacter aerogenes (75), and Serratia marcescens (94). It has been determined that the cyclopropane ring of the C_{17} acid in E. coli occurs at the 9-10 position (46). Small amounts of C_{13} and C_{15} cyclopropane acids have been reported in Clostridium butyricum (27).

Hydroxy Acids

Most of the hydroxy acids of bacteria are β-hydroxy acids. A polymer of β-hydroxybutyric acid is present in many bacteria and has been discussed in the section on bacterial lipids. Mevalonic acid (3, 5-dihydroxy-3-methylpentanoic acid) has been isolated from cultures of lactobacilli (85, 88). The complete series of acids from β-hydroxyoctanoic through β-hydroxymyristic has been reported in Pseudomonas aeruginosa (12, 79). β-hydroxymyristic acid has

also been identified in Escherichia coli (42, 67), and Proteus P 18 (70). β-hydroxydecanoic acid occurs in a number of bacteria including E. coli (54), Serratia marcescens (16, 17), and Azotobacter agilis (47). Dihydroxystearic acid has been found in Lactobacillus acidophilus (19).

METHODS AND MATERIALS

Source Of Organism

The organism used throughout this study was Clostridium botulinum strain 33A, obtained from the Quartermaster Food and Development Command for the Armed Forces, Natick, Mass.

Culture Conditions

Cells were grown in 1.5 liter quantities of trypticase-peptone-glucose (TPG) broth made using: 75g, trypticase (BBL); 7.5g, bacto-peptone (Difco); 15g, glucose (anhydrous, reagent); 1500 ml distilled water. The presence of a fermentable sugar inhibits sporulation. The pH before autoclaving was 6.8. The inoculum was 5 ml of a 48 hour TPG broth culture which had been inoculated from a TPG-cooked meat broth vegetative cell stock culture. The use of successive vegetative cell inocula decreases sporulation (84). The flasks were inoculated soon after autoclaving to eliminate the need for incubation in an anaerobic atmosphere.

The inoculated flasks were incubated at 30° C for 72 hours. This incubation time was found to yield the maximum cell mass with a minimum of autolysis and sporulation. The cultures were checked for sporulation by gram stain and for purity by gram stain and growth

on blood agar plates incubated aerobically and anaerobically at 30°C. The cells were harvested by centrifugation at 7000 x g for 20 minutes in a Sorvall centrifuge and washed three times with distilled water. The washed cells were resuspended in a minimum amount of water and lyophilized.

Reagents

All solvents and other chemicals used were reagent grade with the exception of the hexane. "High Purity" hexane was glass distilled twice before being used. Absolute methanol and anhydrous ether were used. The ether was glass distilled before use.

Extraction of Lipids for Fatty Acid Studies

Lipids were extracted from the lyophilized cells by the following procedures, based on the method of Huston and Albro (40).

Extraction of Free Lipids

One to two grams lyophilized cells were shaken with 100 ml acetone for one hour. The extract was filtered through Whatman No. 1 filter paper and the cell residue was shaken with 100 ml chloroform-methanol (2:1 v/v) for two hours. This extract was filtered off and the cell residue was again shaken with 100 ml chloroform-methanol (2:1 v/v) for two hours. After filtration, the

cell residue was shaken with 100 ml chloroform-methanol (1:1 v/v) for one hour. This extract was filtered and the four extracts were combined and evaporated to dryness under a stream of nitrogen in a 50°C water bath. The lipid residue was taken up in 9 ml chloroform-methanol (2 :1 v/v) and washed free of non-lipid contaminants by the method of Folch, Lees, and Sloane Stanley (22) using sodium chloride in the wash solution and upper phase. Traces of water were removed with excess anhydrous sodium sulfate and the washed extract was evaporated to dryness under nitrogen. The "free" lipid residue was taken up in ether.

Extraction of Bound Lipids

The cell residue remaining after the final solvent extraction was subjected to a 2 hour reflux with 2 N aqueous potassium hydroxide in a nitrogen atmosphere. The mixture was acidified with 6 N hydrochloric acid and the bound lipids were extracted four times with 200 ml of chloroform (4 x 50 ml). The chloroform extract was evaporated to dryness in a 50°C water bath under a stream of nitrogen and the residue was taken up in 9 ml chloroform-methanol (2 :1 v/v). This bound lipid extract was treated as described above for the free lipid extract and finally taken up in ether.

Extraction of Lipids for Lipid Class Studies

The extraction method used was based on that of Folch, Lees, and Sloane Stanley (22). The lyophilized cells were diluted 20 fold (w/v) with chloroform-methanol (2:1 v/v) and the mixture was shaken for two hours at room temperature in a nitrogen atmosphere. The extract was filtered off and this procedure was repeated. The two extracts were combined and evaporated to dryness under a stream of nitrogen on a 50° C water bath. The residue was taken up in 9 ml chloroform-methanol (2:1 v/v) and washed (22). The washed extract was evaporated to dryness in a stream of nitrogen. The residue was weighed and taken up in chloroform (10 mg/ml). The lipid sample was kept in the refrigerator under nitrogen in a screw cap tube with an aluminum foil cap liner.

Separation of Free Fatty Acids

The free fatty acids were separated from the free lipid extract on an isopropanol-potassium hydroxide treated silicic acid column as described by McCarthy and Duthie (68). A modification of their method (58) permitted the recovery of the phospholipids. The methanol fraction containing the phospholipids and potassium formate was evaporated to dryness under nitrogen and the residue was taken up in chloroform. Dilute hydrochloric acid was added to

dissolve the potassium formate. The chloroform phase was separated from the aqueous phase and evaporated to dryness in a stream of nitrogen. The phospholipid residue was taken up in ether. The eluting solvents were evaporated in a stream of nitrogen from the glyceride and free fatty acid fractions and the residues were taken up in ether.

Preparation of Methyl Esters of Fatty Acids

The glyceride, phospholipid, and bound lipid fractions were combined to form a "total" lipid fraction. Fatty acid methyl esters were prepared by refluxing this total lipid fraction with 100 ml methanol-benzene-conc. sulfuric acid (17:2:1 v/v/v) for one hour in a nitrogen atmosphere. Water was added to stop the reaction and the mixture was neutralized with aqueous sodium hydroxide. The fatty acid methyl esters were extracted with 200 ml double distilled hexane (4 x 50 ml). The extract was evaporated to dryness in a stream of nitrogen, weighed, and taken up in hexane (10mg/ml). Methyl esters of the free fatty acids were prepared in the same manner.

Purification of Fatty Acid Methyl Esters

The "total" fatty acid methyl esters and the free fatty acid methyl esters were purified by thin-layer chromatography. Standard

glass plates (20 x 20 cm) were spread with a 250 μ layer of ether and chloroform washed silicAR TLC 7GF and activated by heating at 110°C for one hour.[1] The sample was applied as a band 1.5 cm from the bottom edge of the plate. A standard fatty acid methyl ester mixture was spotted near one end of the band. The plates were developed by the ascending method in hexane-ether (95:5 v/v) for 20 minutes in a nitrogen atmosphere. 2', 7' dichlorofluorescein (0.2% in 96% ethanol) was used for visualization and the fatty acid methyl ester band of the sample was determined by comparing its position with that of the standard. This section of the thin layer was removed from the plate by vacuum aspiration and the purified fatty acid methyl esters were eluted with chloroform, evaporated to dryness in a stream of nitrogen, and taken up in hexane (10 mg/ml). These purified preparations were kept in the refrigerator under nitrogen in screw cap tubes with aluminum foil cap liners.

Hydrogenation of Fatty Acid Methyl Esters

A portion of the "total" and free fatty acid methyl ester samples was hydrogenated using the microhydrogenation method of Farquhar et al. (21). Hexane was used as the solvent instead of ethanol to prevent transesterification (76). The hydrogenation flask

[1] Mallinkrodt Chemical Works, St. Louis, Mo.

was flushed with nitrogen before the catalyst was added. The reaction conditions were maintained for 30 minutes to insure complete hydrogenation of all unsaturated fatty acid esters.

Gas-Liquid Chromatographic Analysis of Fatty Acid Methyl Esters

Coating and Packing of Column

In choosing the inert support for a column, both the theoretical advantages of small and large particle size and the actual results of the investigator must be considered. A very fine support gives maximum column efficiency while a coarse support is easier to pack uniformly and requires a smaller pressure differential to produce the desired carrier gas flow rate (52). Chromosorb P 60-80 mesh, acid washed was chosen as the inert support for the column used in this investigation.[2]

Ethylene glycol succinate (EGS) was used as the stationary phase. The ratio of stationary phase to inert support was 15 : 85. When a low ratio of stationary phase to support is used, the film of stationary liquid is thinner and column efficiency is increased since a shorter time is required for transfer of methyl esters between liquid and gas phases (21). Three g, EGS was dissolved in 42.5 ml chloroform and 17g, chromosorb P was added. This mixture was allowed to sit overnight. The chloroform was then

[2] F and M Scientific Co., Avondale, Pa.

evaporated on a steam bath with occasional stirring. The coated support was packed into a 6' x 1/8" O. D. length of aluminum tubing which had one end plugged with glass wool. An electric vibratory tool was used to insure a tight, uniform packing. The column was flushed with nitrogen and conditioned at 190°C with no gas flow for two days.

Chromatographic Instrument

The instrument used for the analysis was a modified Beckman GC-2 gas chromatograph equipped with a hydrogen flame ionization detector. This type of detector is highly sensitive and is not affected by water or carbon dioxide. The following operating conditions were used throughout this study:

Column: 6' x 1/8" O. D. 15% EGS on Chromosorb P 60-80 mesh

Column temperature: 193° C

Carrier gas: Helium

Flow rate: 40 ml/min

Sample size: 5μl

Column Characteristics

The efficiency of a chromatographic column is measured in terms of its number of theoretical plates. A theoretical plate

consists of the establishment of equilibrium between the liquid and gas phases and the subsequent separation of the phases. At each equilibration, the components of the sample being analyzed are partitioned between the two phases. The number of theoretical plates of a column (n) may be calculated for any component of a sample using the formula $n = 16(tr/W)^2$ where tr = the retention time of the component measured from the point of sample injection to the midpoint of the component's elution curve and W = the base width of the component. The number of theoretical plates of the column used for this analysis, measured at the methyl stearate curve, was 1547. Since the gas and liquid phases in a column are continually in motion relative to each other, the establishment of complete equilibrium at a given point is impossible. For this reason the height equivalent to a theoretical plate (HETP) is also measured. HETP = length of column (cm) ÷ n. For this column HETP = 0.12cm.

The knowledge of a column's ability to separate components having similar retention times, such as a saturated fatty acid methyl ester and its monounsaturated homologue, is also important in analytical work. Component resolution = $\frac{2\Delta y}{ya+yb}$ where Δy = the distance between the peak maxima of the two components and ya and yb = the base widths of components a and b. A solution to the equation of 1 or greater indicates that the components are completely separated. The components usually selected for this

determination are methyl stearate and methyl oleate. The resolving power of a column decreases with use. At the time the column was used for this analysis, its resolution of methyl stearate and methyl oleate was 0.86.

Identification and Quantitation of Fatty Acid Methyl Esters

All unknown and known samples were chromatographed in 5μl quantities. A mixture of known fatty acid methyl esters was chromatographed at intervals between unknown samples.[3] A combination of methods was used to tentatively identify the components of the samples. The relative retention times of the unknown component peaks were compared with those of the known esters. The chromatograms of samples before and after hydrogenation were compared. The carbon number of each component was determined (93).

A quantitative analysis of the samples was made using Carroll's method (15) in which peak area is calculated as the product of retention time and peak height. This method of calculation is possible because of the linear relationship between peak width and retention time. Although it is not as widely used as triangulation, this method has been found to give more reproducible results (15, 20).

[3] Sigma Chemical Co., St. Louis, Mo.

Thin-Layer Chromatography of Lipids

The lipid extract was separated into lipid classes using thin-layer chromatography as described by Mangold (64). Standard glass plates were spread with a 250μ layer of silica gel G (Stahl) and activated by heating at 110°C for two hours.[4] Plates were developed in hexane-ether-acetic acid (90:10:1 v/v/v) by the ascending method in unlined jars that had been flushed with nitrogen. Lipids of known composition were chromatographed side-by-side with the unknown sample. These included monostearin and oleic acid given by Dr. E. A. Day, Department of Food Technology, Wesson oil which consists mainly of triglycerides, and a standard mixture of fatty acid methyl esters. Several indicators were used to visualize the chromatograms. Rhodamine B(0.5% in ethanol) was used as a general lipid indicator (64). Ninhydrin (0.5% in butanol) was used to test for aminophosphatides (80) and 2,4-dinitrophenylhydrazine (0.4% in 2N hydrochloric acid) was used to test for plasmalogens (78, 66). The presence of all organic material was detected by spraying the plate with 50% sulfuric acid followed by charring (69).

[4] Brinkmann Instruments Inc., Westbury, N. Y.

RESULTS AND DISCUSSION

Gas-Liquid Chromatography of Fatty Acids

The composition of the fatty acids of Clostridium botulinum 33A was determined using gas-liquid chromatography. Twenty-two acids were detected in the free fatty acid fraction. Of these, sixteen were tentatively identified (Figure 1, Table II). Of the twenty-one acids found in the "total" fatty acid fraction, seventeen were tentatively identified (Figure 2, Table III). Where possible, identification was based on a comparison of the relative retention times of the unknowns with those of authentic samples and on the reaction of the unknowns to hydrogenation, which converts an unsaturated acid to its saturated homologue. The standards used included the complete series of n-saturated fatty acid methyl esters from C_8 to C_{22} and the following n-unsaturates: 16:1, 18:1, 18:2, 18:3, 20:1. The relative retention times determined for these compounds are listed in Table I. The tentative identification of the compounds for which standards were not available was based on their carbon numbers and their reaction to hydrogenation.

The overall fatty acid pattern of this organism includes straight chain saturated and unsaturated acids and cyclopropane acids. This pattern is similar to those found in other clostridia

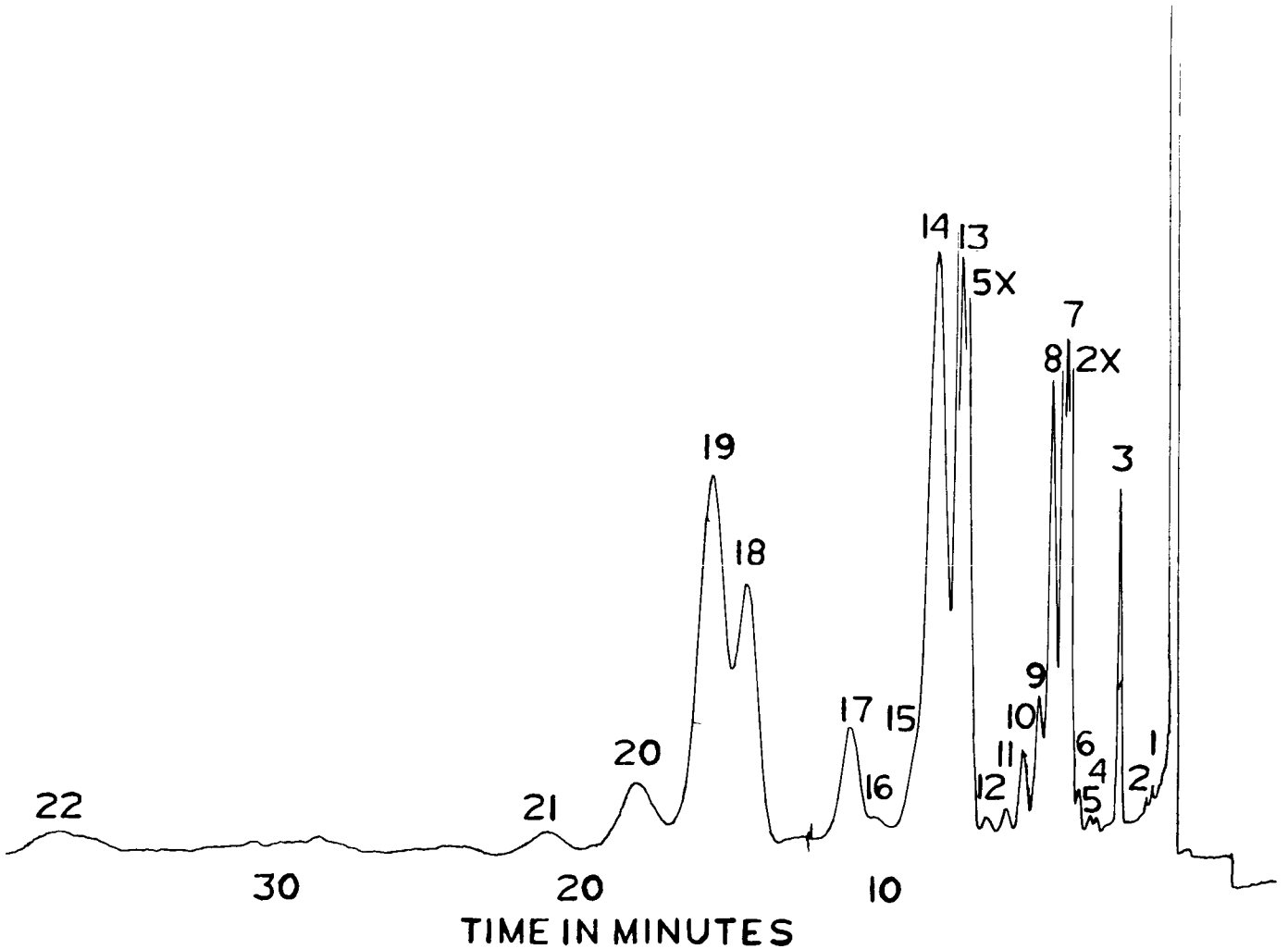

Figure 1. Chromatogram of methyl esters of free fatty acids of Cl. botulinum 33A

Table I. Relative Retention times of standard fatty acid methyl esters

Compound[1]	Rel. Ret. Time Tr. / Tr.	Carbon number
8:0	0.043	8.0
9:0	0.064	9.0
10:0	0.087	10.0
11:0	0.114	11.0
12.0	0.150	12.0
13:0	0.201	13.0
14:0	0.275	14.0
15:0	0.376	15.0
16:0	0.525	16.0
16:1	0.579	16.3
17:0	0.716	17.0
18:0	1.000	18.0
18:1	1.069	18.3
18:2	1.246	18.7
19:0	1.390	19.0
18:3	1.448	19.2
20:0	1.924	20.0
20:1	2.045	20.2
21:0	2.683	21.0
22:0	3.726	22.0

[1]Number of carbon atoms in acid: number of double bonds

Table II. Free fatty acid composition of Clostridium botulinum 33A grown at 30°C in TPG medium

Peak no.	Rel. Ret. Time Tr. / Tr.	Compound[1]	Carbon no.	% Composition
1	0.063	un[2]	9.6	trace[4]
2	0.073	10:0	10.0	trace
3	0.140	12:0	12.0	1.8
4	0.191	13:0	13.0	0.1
5	0.209	13:0cy[3]	13.3	0.1
6	0.234	un	13.6	0.4
7	0.266	14:0	14.0	27.4
8	0.300	14:1	14.4	5.4
9	0.332	un	14.7	1.8
10	0.368	15:0	15.0	1.3
11	0.407	15:1	15.3	0.5
12	0.456	un	15.7	0.4
13	0.512	16:0	16.0	24.1
14	0.569	16:1	16.3	14.7
15	0.609	un	16.5	1.0
16	0.715	17:0	17.0	0.6
17	0.767	17:1	17.3	1.4
	0.767	17:0cy	17.3	2.1
18	1.000	18:0	18.0	3.3
19	1.081	18:1	18.3	8.0
20	1.250	18:2	18.7	1.8
21	1.451	un	19.1	0.6
22	2.574	20:2	20.8	1.2

[1]Number of carbon atoms in acid: number of double bonds

[2]un = unidentified

[3]cy = cyclopropane

[4]trace = less than 0.1%

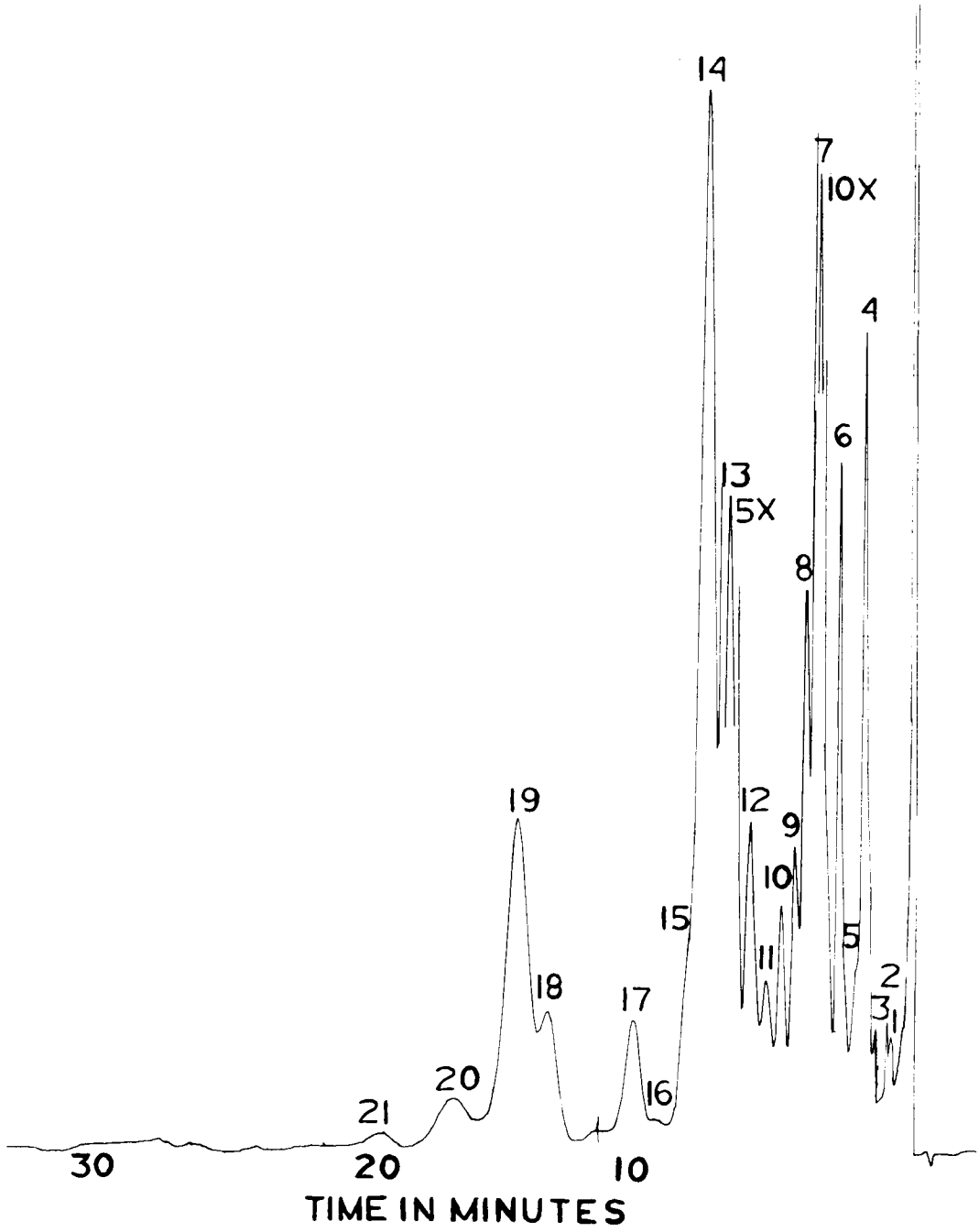

Figure 2. Chromatogram of methyl esters of total fatty acids Cl. botulinum 33A

Table III. Total fatty acid composition of Clostridium botulinum 33A grown at 30°C in TPG medium

Peak no.	Rel. Ret. Time Tr. / Tr.	Compound[1]	Carbon no.	% Composition
1	0.650	un[2]	9.6	trace[4]
2	0.750	10:0	10.0	trace
3	0.114	11:0cy[3]	11.3	0.1
4	0.141	12:0	12.0	1.8
5	0.161	12:1	12.4	0.4
6	0.210	13:0cy	13.3	2.2
7	0.268	14:0	14.0	42.4
8	0.303	14:1	14.4	2.6
9	0.333	un	14.7	1.4
10	0.370	15:0	15.0	1.3
11	0.411	15:0cy	15.3	0.8
12	0.456	un	15.7	2.2
13	0.514	16:0	16.0	26.7
14	0.574	16:1	16.3	9.8
15	0.614	un	16.5	2.0
16	0.715	17:0	17.0	0.1
17	0.769	17:1	17.3	0.4
	0.769	17:0cy	17.3	1.0
18	1.000	18:0	18.0	1.1
19	1.082	18.1	18.3	2.9
20	1.253	18:2	18.7	0.5
21	1.462	18:3	19.2	0.2

[1]Number of carbon atoms in acid: number of double bonds

[2]un = unidentified

[3]cy = cyclopropane

[4]trace = less than 0.1%

(60, 27). The normal saturates predominate in both the free and "total" fatty acid fractions, accounting for 58.6% of the former and 73.4% of the latter. Normal unsaturates comprise 33.0% of the free fatty acids and 16.8% of the "total" fatty acids. The complete spectrum of fatty acids from C_{10} to C_{20}, with the exception of C_{19} is present.

The free fatty acids constitute an important fraction of the lipids of this organism. Care was taken to avoid artificially increasing the amount of free fatty acids. During extraction and treatment of the lipid sample physical, chemical, or enzymatic degredation of the lipids may occur. To prevent this, lyophilized cells (9) were extracted at room temperature in a nitrogen atmosphere (66). Because ether is known to activate lipolytic enzymes (31), it was not used for lipid extraction. Substantial amounts of free fatty acids occur in other bacteria (9, 53).

Over 60% of the fatty acids present are straight chain saturates. Large amounts of such acids are quite common in bacteria (74). The predominant acid here is myristic (14:0). Although myristic acid is a frequently reported constituent of bacterial fatty acids (14, 24, 38, 46, 79, 63, 56), it is usually not present in great quantity. However, MacFarlane (60) found 24% of the fatty acids of Clostridium perfringens to be myristic acid. The special significance, if any, of the large amount of myristate in these bacteria

is not known. The other major saturated acid present is palmitic acid (16:0). This is an almost universal constituent of bacterial fatty acids, often accounting for 40% or more of the total acids present (19, 27, 46, 75). Small amounts of normal saturates with an odd number of carbons (C_{13}-C_{17}) are present. Such acids have recently been reported as minor components of several bacteria including Pseudomonas aeruginosa (79), Clostridium butyricum (27), Nocardia sp. (14), and Sarcina lutea (41).

The group of normal unsaturated acids is the second most abundant and consists mainly of monoenoic acids. The position of the double bonds in these acids has not been determined. The major acid of this group is 16:1, a common constituent of the fatty acids of many bacteria (24, 67, 41, 38, 83). Palmitoleic acid, with the double bond in the 9-10 position, occurs most frequently but 7-8, 10-11, and 11-12 hexadecenoates have also been reported (14, 38, 83). An octadecenoic acid is present. Early investigators tended to identify octadecenoic acid as oleic acid without actually establishing that the double bond was in the 9-10 position (74). Reinvestigation has shown in some cases (37) that 11-12 octadecenoic (cis-vaccenic) acid is present instead. Both oleic and cis-vaccenic acids have been found in a number of bacteria (46, 36, 14, 83). The other monounsaturates present here are much less common but several of them have been found in Pseudomonas aeruginosa (79)

and Sarcina lutea (41). Higher unsaturates such as 18:2, 18:3 and 20:2 have been reported occasionally (38, 40, 58).

Cyclopropane acids are common constituents of gram negative bacteria, lactobacilli, and clostridia (50). Four such acids have been tentatively identified in Clostridium botulinum 33A on the basis of their relative retention times and insensitivity to mild hydrogenation as compared with unsaturated acids. The 17 and 19 carbon cyclopropane acids occur most frequently (39, 8, 94) but the 13 and 15 carbon cyclopropanes have also been reported (27).

Thin-Layer Chromatography of Lipids

The lipid sample of Clostridium botulinum 33A was resolved into its component classes using thin-layer chromatography (Figure 3). Not all of the classes could be identified but there was evidence for the presence of hydrocarbons, which travel with the solvent front, triglycerides, free fatty acids, and phospholipids, which do not migrate from the origin. Huston and Albro (40) found that the lipids of Sarcina lutea are composed of hydrocarbons, free fatty acids, mono-, di-, and triglycerides, and a mixture of phospholipids. On the basis of the time it took them to turn brown when charring (69), it appears that the free fatty acids and phospholipids contain unsaturated fatty acids. The phospholipid gave a positive reaction with ninhydrin and 2,4-dinitrophenylhydrazine indicating

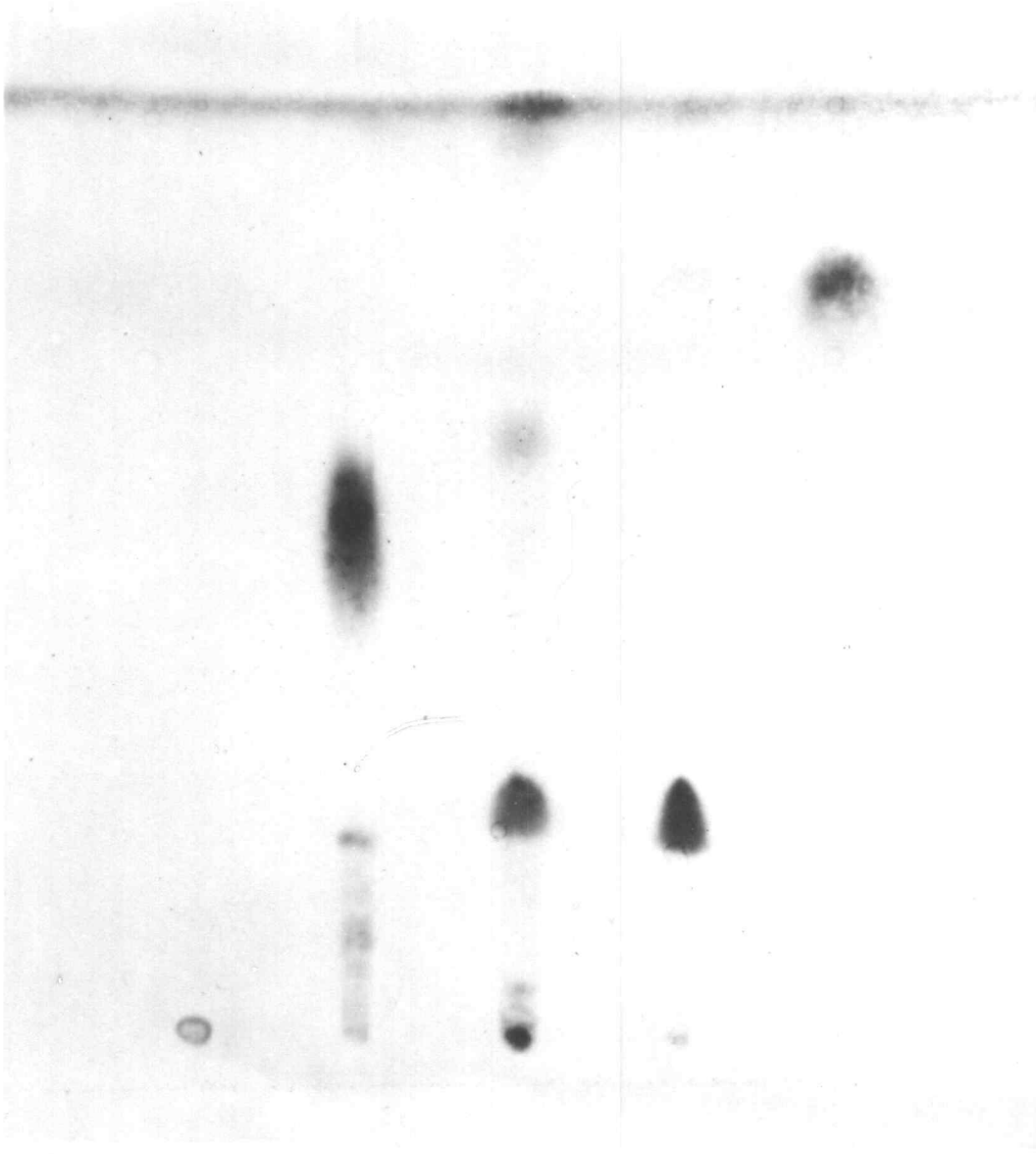

Figure 3. Thin-layer chromatogram of lipid classes on Silica Gel. Solvent: hexane-ether-acetic acid 90:10:1 v/v/v. Development time: 25 min. (1) monostearin (2) wesson oil (3) lipids of Cl. botulinum 33A (4) oleic acid (5) fatty acid methyl esters.

respectively that aminophosphatides and plasmalogens are present. The most commonly occuring and often the most abundant amino-phosphatide of bacterial lipids is phosphatidyl ethanolamine. It has been found in a wide variety of bacteria (50). Phosphatidyl serine also occurs in bacterial lipids. MacFarlane (60) discovered o-amino acid esters of phosphatidyl glycerol in Clostridium perfringens and Staphylococcus aureus. Fairly recently it was discovered that the lipids of some bacteria contain plasmalogens. Thus far they have been found only in the anaerobic bacteria. Part of the phospholipids of Clostridium butyricum (26, 11) and some species of rumen bacteria (3, 91) exist in the plasmalogen form.

SUMMARY

A study was made of the fatty acid and lipid composition of Clostridium botulinum 33A vegetative cells. The methyl esters of the fatty acids were characterized using gas-liquid chromatography and the lipids were resolved into classes by thin-layer chromatography.

The component lipids of this organism include hydrocarbons, triglycerides, free fatty acids, and phospholipids. The complete fatty acid spectrum from C_{10} through C_{20}, with the exception of C_{19}, is present. The major fatty acid group is the normal saturated acids and myristic acid is the single most abundant fatty acid. Small amounts of cyclopropane acids are present.

BIBLIOGRAPHY

1. Akasi, S. Fat substance obtained from the typhoid bacillus of mice. Journal of Biochemistry (Tokyo) 29: 13-20. 1939. (Abstracted in Chemical Abstracts 33: 4285-5. 1939)

2. Alimova, E. K. The distribution of lipids between the cell membrane and other component parts of the cell in diphtheria microbes. Biochemistry (USSR) 23: 193-198. 1958.

3. Allison, M. J. et al. Metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. II. Biosynthesis of higher branched-chain fatty acids and aldehydes. Journal of Bacteriology 83: 1084-1093. 1962.

4. Asano, M. and H. Takahashi. Bacterial components of Corynebacterium diphtheriae. I. Studies of fats. Journal of the Pharmaceutical Society of Japan 65: 17-19. 1945. (Abstracted in Chemical Abstracts 45: 3906f. 1951)

5. _____. Bacterial components of Corynebacterium diphtheriae. II. Phosphatides and wax. Journal of the Pharmaceutical Society of Japan 65: 81. 1945. (Abstracted in Chemical Abstracts 45: 4303c. 1951)

6. _____. Bacterial components of Corynebacterium diphtheriae. III. Firmly bound lipides. Journal of the Pharmaceutical Society of Japan 68: 186-188. 1948. (Abstracted in Chemical Abstracts 47: 8825a. 1953)

7. Asselineau, J. Sur la composition des lipides de Corynebacterium diphtheriae. Biochimica et Biophysica Acta 54: 359-360. 1961.

8. _____. Sur quelques applications de la chromatographie en phase gazeuse a l'étude d'acides gras bactériens. Annales de l'Institut Pasteur 100: 109-119. 1961.

9. Asselineau, J. and E. Lederer. Chemistry and metabolism of bacterial lipides. In: Lipide metabolism, ed. by Konrad Bloch. New York, Wiley, 1960. p. 337-406.

10. Asselineau, J. and J. Moron. Sur la composition des graisses acétonosolubles de souches humaines de bacille tuberculeux. Bulletin de la Société de Chimie Biologique 40: 899-911. 1958.

11. Baumann, N. A., P. O. Hagen and H. Goldfine. Phospholipids of Clostridium butyricum. Studies on plasmalogen composition and biosynthesis. Journal of Biological Chemistry 240: 1559-1567. 1965.

12. Bergström, S., H. Theorell and H. Davide. Pyolipic acid, a metabolic product of Pseudomonas pyocyanea, active against Mycobacterium tuberculosis. Archives of Biochemistry 10: 165-166. 1946.

13. Bloch, H. et al. Constituents of a "toxic-lipid" obtained from Mycobacterium tuberculosis. Biochimica et Biophysica Acta 23: 312-321. 1957.

14. Bordet, C. and G. Michel. Étude des acides gras isolés de plusieurs espèces de Nocardia. Biochimica et Biophysica Acta 70: 613-626. 1963.

15. Carroll, K. K. Quantitative estimation of peak areas in gas-liquid chromatography. Nature 191: 377-378. 1961.

16. Cartwright, N. J. Serratamic acid, a derivative of L-serine produced by organisms of the Serratia group. Biochemical Journal 60: 238-242. 1955.

17. _____. The structure of serratamic acid. Biochemical Journal 67: 663-669. 1957.

18. Cason, James et al. Certain characteristics of the fatty acids from the lipides of the tubercle bacillus. Journal of Biological Chemistry 205: 435-447. 1953.

19. Crowder, J. A. and R. J. Anderson. A contribution to the chemistry of Lactobacillus acidophilus II. Composition of the neutral fat. Journal of Biological Chemistry 104: 399-406. 1934.

20. Dijkstra, A. Quantitative estimation of peak areas in gas-liquid chromatography. Nature 192: 965-966. 1961.

21. Farquhar, John W. et al. The analysis of fatty acid mixtures by gas-liquid chromatography: Construction and operation of an ionization chamber instrument. Nutrition Reviews 17 (Pt. 2, Supplement): 1-30. 1959.

22. Folch, J., M. Lees and G. H. Sloane Stanley. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry 226: 497-509. 1957.

23. Forsyth, W. G. C., A. C. Hayward and J. B. Roberts. Occurrence of poly- β-hydroxybutyric acid in aerobic gram negative bacteria. Nature 182: 800-801. 1958.

24. Fulco, Armand, Ronald Levy and Konrad Bloch. The biosynthesis of Δ^9 - and Δ^5 -monounsaturated fatty acids by bacteria. Journal of Biological Chemistry 239: 998-1003. 1964.

25. Geiger, W. B. and R. J. Anderson. The chemistry of Phytomonas tumefaciens. The composition of the phosphatide. Journal of Biological Chemistry 129: 519-529. 1939.

26. Goldfine, H. Phospholipids of Clostridium butyricum; Plasmalogens. (Abstract) Federation Proceedings 22: 415. 1963.

27. Goldfine, H. and K. Bloch. On the origin of unsaturated fatty acids in Clostridia. Journal of Biological Chemistry 236: 2596-2601. 1961.

28. Goldfine, H. and M. E. Ellis. N-methyl groups in bacterial lipids. Journal of Bacteriology 87: 8-15. 1964.

29. Gubarev, E. M., G. D. Bolgova and E. K. Alimova. A study of free and bound lipid fractions of the Brucella type suis 44 by the method of chromatography. Biochemistry (USSR) 24: 185-189. 1959.

30. Guinand, M., G. Michel and E. Lederer. Sur les lipides de Nocardia astéroides; isolement de lipopeptides. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 246: 848-851. 1958.

31. Hanahan, D. J. The enzymatic degradation of phosphatidyl choline in diethyl ether. Journal of Biological Chemistry 195: 199-206. 1952.

32. Hausmann, Werner and Lyman C. Craig. Polymixin B_1. Fractionation, molecular weight determination, amino acid, and fatty acid composition. Journal of the American Chemical Society 76: 4892-4896. 1954.

33. Henderson, T. O., J. J. McCeill and S. B. Tove. Folic acid involvement in cyclopropane fatty acid synthesis in lactobacilli. Journal of Bacteriology 90: 1283-1287. 1965.

34. Hofmann, K. and R. Lucas. The chemical nature of a unique fatty acid. Journal of the American Chemical Society 72: 4328-4329. 1950.

35. Hofmann, K., R. Lucas and S. M. Sax. The chemical nature of the fatty acids of Lactobacillus arabinosus. Journal of Biological Chemistry 195: 473-485. 1952.

36. Hofmann, K. and S. M. Sax. The chemical nature of the fatty acids of Lactobacillus casei. Journal of Biological Chemistry 205: 55-63. 1953.

37. Hofmann, K. and F. Tausig. On the identify of phytomonic and lactobacillic acids. A reinvestigation of the fatty acid spectrum of Agrobacterium (Phytomonas) tumefaciens. Journal of Biological Chemistry 213: 425-432. 1955.

38. _____. The chemical nature of the fatty acids of a group C Streptococcus species. Journal of Biological Chemistry 213: 415-423. 1955.

39. Hofmann, K. et al. The estimation of the fatty acid composition of bacterial lipides. Journal of Biological Chemistry 217: 49-60. 1955.

40. Huston, C. K. and P. W. Albro. Lipids of Sarcina lutea. I. Fatty acid composition of the extractable lipids. Journal of Bacteriology 88: 425-432. 1964.

41. Huston, C. K., P. W. Albro and G. B. Grindey. Lipids of Sarcina lutea. III. Composition of the complex lipids. Journal of Bacteriology 89: 768-775. 1965.

42. Ikawa, M. et al. An agent from E. coli causing hemorrhage and regression of an experimental mouse tumor. III. The component fatty acids of the phospholipide moiety. Journal of the American Chemical Society 75: 1035-1038. 1953.

43. James, A. T. and A. J. P. Martin. Gas-liquid chromatography. The separation and identification of the methyl esters of saturated and unsaturated acids from formic acid to n-octadecanoic acid. Biochemical Journal 63: 144-152. 1956.

44. Jarvis, F. G. and M. J. Johnson. A glycolipide produced by Pseudomonas aeruginosa. Journal of the American Chemical Society 71: 4124-4126. 1949.

45. Kaneda, Toshi. Biosynthesis of branched chain fatty acids. I. Isolation and identification of fatty acids from Bacillus subtilis (ATCC 7059). Journal of Biological Chemistry 238: 1222-1228. 1963.

46. Kaneshiro, Tsuneo and Allen G. Marr. Cis-9, 10-methylene hexadecanoic acid from the phospholipids of Escherichia coli. Journal of Biological Chemistry 236: 2615-2619. 1961.

47. _____. Hydroxy fatty acids of Azotobacter agilis. Biochimica et Biophysica Acta 70: 271-277. 1963.

48. _____. Phospholipids of Azotobacter agilis, Agrobacterium tumefaciens, and Escherichia coli. Journal of Lipid Research 3: 184-189. 1962.

49. Kanfer, J. and E. P. Kennedy. Metabolism and function of bacterial lipids. I. Metabolism of phospholipids in Escherichia coli B. Journal of Biological Chemistry 238: 2919-2922. 1963.

50. Kates, M. Bacterial lipids. Advances in Lipid Research 2: 17-90. 1964.

51. Kates, M., S. N. Sehgal and N. E. Gibbons. The lipid composition of Micrococcus halodenitrificans as influenced by salt concentration. Canadian Journal of Microbiology 7: 427-435. 1961.

52. Keulemans, A. I. M. Gas chromatography. 2d ed. New York, Reinhold, 1959. 234p.

53. Knaysi, G. Chemistry of the bacterial cell. In: Bacterial physiology, ed. by C. H. Werkman and P. W. Wilson. New York, Academic Press, 1951. p. 1-27.

54. Law, John H. Lipids of Escherichia coli. (Abstract) Bacteriological Proceedings 1961, p. 129.

55. Lemoigne, M. and H. Girard. Reserves lipidiques β-hydroxybutyriques chez Azotobacter chroococcum. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 217: 557-558. 1943.

56. Lennarz, W. J. The role of isoleucine in the biosynthesis of branched-chain fatty acids by Micrococcus lysodeikticus. Biochemical and Biophysical Research Communications 6: 112- 116. 1961.

57. Lennarz, W. J., G. Scheuerbrandt and K. Bloch. The biosynthesis of oleic and 10-methylstearic acids in Mycobacterium phlei. Journal of Biological Chemistry 237: 664-671. 1962.

58. Lewis, Norman F. Studies on the lipid and fatty acid composition of Micrococcus radiodurans. Master's thesis. Corvallis, Oregon State University, 1965. 53 numb. leaves.

59. Lundgren, D. G. et al. Characterization of poly- β-hydroxybutyrate extracted from different bacteria. Journal of Bacteriology 89: 245-251. 1965.

60. MacFarlane, M. G. Characterization of lipoamino acids as o-amino-acid esters of phosphatidyl glycerol. Nature 196: 136-138. 1962.

61. _____. Composition of lipid from protoplast membranes and whole cells of Micrococcus lysodeikticus. Biochemical Journal 79: 4P-5P. 1961.

62. _____. Lipid components of Staphylococcus aureus and Salmonella typhimurium. Biochemical Journal 82: 40P-41P. 1962.

63. Macleod, P. et al. Quantity and fatty acid composition of lipid extracted from cells of Streptococcus lactis. Journal of Bacteriology 83: 806-810. 1962.

64. Mangold, Helmut K. Thin-layer chromatography of lipids. Journal of the American Oil Chemists' Society 38: 708-727. 1961.

65. Marco, G. J. and K. Hofmann. Structural studies on lactobacillic acid and other longchain fatty acids containing the cyclopropane ring. (Abstract) Federation Proceedings 15: 308. 1956.

66. Marinetti, G. V. Chromatographic separation, identification, and analysis of phosphatides. Journal of Lipid Research 3: 1-20. 1962.

67. Marr, Allen G. and John L. Ingraham. Effect of temperature on the composition of fatty acids in Escherichia coli. Journal of Bacteriology 84: 1260-1267. 1962.

68. McCarthy, R. D. and A. H. Duthie. A rapid quantitative method for the separation of free fatty acids from other lipids. Journal of Lipid Research 3: 117-119. 1962.

69. Morris, L. J., R. T. Holman and K. Fontell. Naturally occurring epoxy acids: I. detection and evaluation of epoxy fatty acids by paper, thin-layer and gas-liquid chromatography. Journal of Lipid Research 2: 68-76. 1961.

70. Nesbitt, J. A. and W. J. Lennarz. Composition of lipids and lipopolysaccharide from the bacillary and L forms of Proteus P18. Journal of Bacteriology 89: 1020-1025. 1965.

71. Noll, Hans. The chemistry of some native constituents of the purified wax of Mycobacterium tuberculosis. Journal of Biological Chemistry 224: 149-164. 1957.

72. Noll, Hans and Eugene Jackim. The chemistry of the native constituents of the acetone-soluble fat of Mycobacterium tuberculosis (Brevannes). I. Glycerides and phospholipids. Journal of Biological Chemistry 232: 903-917. 1958.

73. Norris, A. T. and K. Bloch. On the mechanism of the enzymatic synthesis of unsaturated fatty acids in Escherichia coli. Journal of Biological Chemistry 238: PC 3133-3134. 1963.

74. O'Leary, W. M. The fatty acids of bacteria. Bacteriological Reviews 26: 421-447. 1962.

75. _____. S-adenosylmethionine in the biosynthesis of bacterial fatty acids. Journal of Bacteriology 84: 967-972. 1962.

76. Poukka, R., L. Vasenius and O. Turpeinen. Catalytic hydrogenation of fatty acid methyl esters for gas-liquid chromatography. Journal of Lipid Research 3: 128-129. 1962.

77. Pustovalov, V. L. Higher fatty acids of the diphtheria bacillus. Biochemistry (USSR) 21: 33-42. 1956.

78. Reitsema, R. H. Characterization of essential oils by chromatography. Analytical Chemistry 26: 960-963. 1954.

79. Romero, E. and R. R. Brenner. Fatty acids synthesized from hexadecane by Pseudomonas aeruginosa. Journal of Bacteriology 91: 183-188. 1966.

80. Rouser, G., J. O'Brien and D. Heller. The separation of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography. Journal of the American Oil Chemists' Society 38: 14-19. 1961.

81. Saito, K. Chromatographic studies on bacterial fatty acids. Journal of Biochemistry (Tokyo) 47: 699-709. 1960.

82. _____. Studies on bacterial fatty acids; the structure of subtilopentadecanoic and subtiloheptadecanoic acids. Journal of Biochemistry (Tokyo) 47: 710-719. 1960.

83. Scheuerbrandt, G. et al. A novel mechanism for the biosynthesis of unsaturated fatty acids. Journal of Biological Chemistry 236: PC70. 1961.

84. Schmidt, C. F. Some factors affecting growth and sporulation of Clostridium botulinum strain 62A. (Abstract) Bacteriological Proceedings 1960, p. 44.

85. Skeggs, Helen R. et al. Discovery of a new acetate replacing factor. Journal of Bacteriology 72: 519-524. 1956.

86. Smith, D. W. et al. The characterization of mycobacterial strains by the composition of their lipide extracts. Annals of the New York Academy of Sciences 69: 145-157. 1957.

87. Smithies, W. R., N. E. Gibbons and S. T. Bayley. The chemical composition of the cell and cell wall of some halophilic bacteria. Canadian Journal of Microbiology 1: 605-613. 1955.

88. Tamura G. and K. Folkers. Identity of mevalonic and hiochic acids. Journal of Organic Chemistry 23: 772. 1958.

89. Velick, S. F. and R. J. Anderson. The chemistry of Phytomonas tumefaciens. II. Composition of the acetone-soluble fat. Journal of Biological Chemistry 152: 523-531. 1944.

90. Vilkas E. and E. Lederer. Isolement d'un phosphatidyl-inositodimannoside a partir d'un phosphatide de Mycobactéries. Bulletin de la Société de Chimie Biologique 38: 111. 1956.

91. Wegner, G. H. and E. M. Foster. Incorporation of isobutyrate and valerate into cellular plasmalogens by Bacteroides succinogenes. Journal of Bacteriology 85: 53-61. 1963.

92. Williamson, D. H. and J. F. Wilkinson. The isolation and estimation of the poly-β-hydroxybutyrate inclusions of Bacillus species. Journal of General Microbiology 19: 198-209. 1958.

93. Woodford, F. P. and C. M. VanGent. Gas-liquid chromtography of fatty acid methyl esters: the "carbon number" as a parameter for composition of columns. Journal of Lipid Research 1: 188-190. 1960.

94. Zalkin, Howard and John H. Law. Enzymatic synthesis of the cyclopropane ring. (Abstract) Federation Proceedings 21: 287. 1962.