


AN ABSTRACT OF THE THESIS OF

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(Name of Student) (Degree)

in Microbiology presented on October 27, 1967  
(Major) (Date)

Title: The Lipids of Spore Types B, E, and F and Vegetative  
Cell Types E and F of Clostridium Botulinum

Abstract approved:   
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The primary purpose of this investigation was to characterize the lipids of the spores and vegetative cells of Cl. botulinum. A second purpose was to explore the possibility that lipids might serve as a means of differentiating the chiefly proteolytic Cl. botulinum type B from the nonproteolytic Cl. botulinum types E and F.

The total lipid extracted accounted for 3.7%, 3.3%, 2.0%, 2.7%, and 3.0% of the dry weight of Cl. botulinum vegetative cell types 61E and F; and spore types 61E, F, and 115B, respectively.

The fatty acids were analyzed in the form of their methyl esters by gas-liquid chromatography. Infrared spectroscopy, mercuric acetate fractionation, and silver nitrate-thin layer chromatography served as complementary means of analysis. The total fatty acids included straight chain saturated, unsaturated, and cyclopropane acids. Palmitic and myristic were the predominant acids

in both the spores and vegetative cells of types 61E, F, and 115B. Together, they made up over 50% of the total fatty acids.

Unsaturated acids were the second major group. These were primarily 7,8-tetradecenoic, 9,10-hexadecenoic, 7,8-hexadecenoic, 11,12-octadecenoic, and 9,10-octadecenoic acids. Types E and F possessed an 18-carbon diunsaturate, which was not found in the vegetative cells or spores of type 115B. However, insufficient quantities prevented its further characterization. The vegetative cells and spores also contained C<sub>15</sub>, C<sub>17</sub>, C<sub>19</sub> cyclopropane fatty acids as adjudged by their infrared spectra and gas-liquid chromatographic behavior.

The phospholipids accounted for approximately 60% of the total lipids in the vegetative cells and 40% of that in the spores. The primary phospholipid was phosphatidylethanolamine. Qualitative tests for plasmalogens, glycolipids, and phosphatidic acid were positive for both spores and vegetative cells.

THE LIPIDS OF SPORE TYPES B, E, AND F AND VEGETATIVE  
CELL TYPES E AND F OF CLOSTRIDIUM BOTULINUM

by

Charles Edward Kimble

A THESIS

submitted to

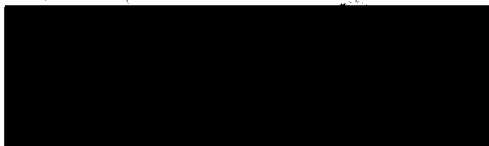
Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Master of Science

June 1968

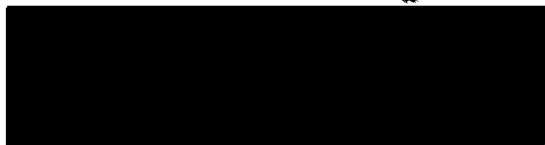
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Date thesis is presented October 27, 1967

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## ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. A. W. Anderson for his able guidance during the course of this investigation and the preparation of the thesis.

Funds to support this study were made available by the U. S. Atomic Energy Commission Grant AT (45-1)-1930. The author would like to express his gratitude to this agency for making this study possible.

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# THE LIPIDS OF SPORE TYPES B, E, AND F AND VEGETATIVE CELL TYPES E AND F OF CLOSTRIDIUM BOTULINUM

## INTRODUCTION

The expansion of knowledge regarding the chemistry, metabolism, and function of carbohydrates, proteins, enzymes and co-enzymes, and nucleic acids has been such that texts only a few years old now seem sketchy and uninformative. However, lipids were not included in this surge of study until very recently. In the past few years the development of new and reliable methods for the isolation and analysis of lipids has led to a marked increase in bacterial lipid research.

In general, bacterial lipids differ substantially from those of higher life forms. They lack sterols, possess phospholipids low in nitrogen and high in carbohydrate; they contain a relatively large proportion of free fatty acids and possess certain fatty acids not ordinarily found in other life forms. These differences have led to speculation on the probable relationship of bacterial lipids to pathogenicity and to heat and radiation resistance. However, before the function of bacterial lipids can be effectively explored, their qualitative nature must be elucidated.

The primary purpose of this study is to elucidate the chemical nature of the lipids of both the spores and vegetative cells of Clostridium

botulinum. A second purpose is to explore the possibility of lipids acting as a means of differentiating the chiefly proteolytic Cl. botulinum type B from the nonproteolytic Cl. botulinum types E and F.

## LITERATURE REVIEW

Research in the field of bacterial lipids has increased markedly during the past five years. However, only the lipids of a few bacterial species have been intensively examined. In proportion to the number of species of bacteria actually catalogued, those which have had their lipids examined, even summarily, are very few indeed.

The tubercle bacillus is illustrative of a bacterial species whose lipids have been subject to a great deal of research. This is because it was discovered early that lipids enhance the pathogenicity of this bacillus (4, 53). The lipids of certain *Pseudomonas* strains have been investigated because of their involvement in antibiotic factors (4).

Reviews on the chemistry and biochemistry of bacterial lipids have been published by Anderson (1), by Asselineau and Lederer (2, 5), by O'Leary (75), by Kates (44), and Lennarz (54).

One of the difficulties in studying bacterial lipids is that the lipid content of bacterial cells can be markedly affected, both quantitatively and qualitatively, by various aspects of the culture procedure; including the composition of the medium, the physical conditions under which the culture is inoculated, the age of the culture when the cells are harvested, and even by the particular strain of organisms grown. A number of authors have discussed these aspects of

bacterial lipids in some detail (33, 44, 75, 78, 89).

The alterations in bacterial lipid content due to changes in the medium are many and varied. Some generalizations can be made, however. The primary one is that high nitrogen content decreases cellular lipid content and high carbohydrate concentration in the medium increases cellular lipid content. Even the nature of the carbon source affects cellular lipids. Replacement of glucose with glycerol, for example, greatly decreases the amount of lipid (44).

In a study of the fatty acid content of Escherichia coli (67) it was found that nitrogen-limited cells had higher concentrations of saturated acids and lower concentrations of unsaturated acids than cells of the same strains that were glucose limited.

Temperature is another important aspect of the environment that affects microbial lipid composition. Bacteria increase their content of unsaturated fatty acids at low temperatures, and decrease the relative amounts of these acids at higher temperatures. O'Leary suggests that this represents an effort to keep lipids liquid and therefore metabolizable at the lower temperatures (74).

Many workers have reported the increasing degree of saturation of bacterial fatty acids as a culture ages. Such changes have been seen in Escherichia coli (18), Lactobacillus arabinosus (18), and Serratia marcescens (45, 50). This is one of the evidences often adduced to support the concept that at least some of the fatty

materials in these organisms are not food storage substances since they accumulate as exogenous food is depleted.

### Fatty Acids of Bacteria

Fatty acids are the basic components of more complex lipids, and as such their analysis is of considerable import. As free fatty acids, fatty acids constitute a small proportion of the lipids of both Gram-negative and Gram-positive eubacteria. For example, in Azotobacter agilis, Agrobacterium tumefaciens, and Escherichia coli, the free fatty acid content is less than 10% of the total lipid (41), while in Sarcina lutea it is 2.1% (38).

Four general classes of fatty acid have been found in eubacteria: saturated and unsaturated fatty acids, and branched chain and cyclopropane fatty acids.

#### Saturated Fatty Acids

The saturated fatty acids are similar to those encountered in other forms of life and are the simplest of the bacterial lipids. In most microorganisms palmitic acid is the predominant fatty acid; stearic, myristic, and lauric are also common. Acids with chains of fewer than 12 carbon atoms are more important as metabolic intermediates than as constituents of cellular lipid complexes (74).

## Unsaturated Fatty Acids

Unsaturated fatty acids usually constitute a sizable portion of the total fatty acid content of microorganisms. There are two separate pathways for the synthesis of monounsaturated fatty acids (9, 12).

Many aerobic bacteria are able to desaturate stearic and palmitic acids (55). This is an oxygen-dependent conversion and is similar to that found in higher forms of life; however, it has been shown that the desaturation of palmitic and stearic acids by M. phlei requires  $\text{Fe}^{++}$  and a flavin derivative in addition to TPNH and  $\text{O}_2$  (25). Fulco, Levy, and Bloch (26) have observed that B. megaterium KM desaturates palmitic and stearic acids to cis-5-hexadecenoic and cis-5-octadecenoic acids, respectively. These reactions are both oxygen dependent.

Facultatively aerobic and anaerobic bacteria are unable to desaturate stearic or palmitic acids either in the absence or presence of oxygen. Sheuerbrandt et al. (86) have observed that monounsaturated acids are synthesized by Clostridium butyricum from shorter-chain precursors. More recently Norris, Matsumura, and Bloch (71) have isolated from E. coli B an enzyme that acts specifically on  $\beta$ -hydroxydecanoate thioesters and gives mixtures of  $\alpha$ ,  $\beta$ - and  $\beta$ ,  $\gamma$ -decenoates.

Polyunsaturated fatty acids do not occur in significant amounts in bacterial lipids, whether anaerobic or not. Scheuerbrandt et al.

(86) suggest that "the appearance of these compounds was a still later evolutionary event." This is in contrast to other micro-organisms, plants, and animals.

#### Branched-chain Fatty Acids

Branched-chain acids have been increasingly reported as constituents of bacterial lipids. They constitute an appreciable portion of the fatty acid content of many species, notably the Bacillaceae (34, 75) and various Gram-positive cocci. MacFarlane has reported that 15- and 17- carbon branched-chain acids make up 80-90% of the total acids of Micrococcus lysodeikticus (61) and 70% of the total fatty acids of Staphylococcus aureus (63). Most of the branched acids that have been identified have proved to be iso- or anteiso-branched methyl compounds.

#### Cyclopropane Fatty Acids

The cyclopropane fatty acids are the most extraordinary fatty acids found in bacteria. They have been reviewed by Asselineau and Lederer (5), Hofmann (34), Kates (44), and O'Leary (75). The C<sub>17</sub> and C<sub>19</sub> cyclopropane acids are commonly found among bacteria (3, 34, 44, 75). They are present in the lipids of Gram-negative bacteria such as S. marcescens (7), E. coli (41), and Agrobacterium tumefaciens (37) and of the Gram-positive bacteria, such as streptococci and lactobacilli (34). It has been clearly established that



cyclopropane acids are formed by the addition of a (-CH<sub>2</sub>-) group derived from the methyl of S-adenosylmethionine across the double bond of an unsaturated fatty acid (58, 73), but the exact details of the addition are unclear and the nature of the intermediate compounds, if any, is unknown.

### Complex Bacterial Lipids

#### Glycerides

Although monoglycerides, diglycerides, and notably triglycerides are major components of plant and animal lipids, they constitute relatively minor fractions in the total cellular lipids of bacteria (74). In bacteria and other microorganisms lipids have different predominant forms and different principal functions. The major portion of their glycerol-fatty acid combination are incorporated into more complex forms such as glycolipids and phosphoglycerides.

#### Phospholipids

Phospholipids are the most diverse and perhaps most important category of lipids found in bacteria. They frequently comprise as much as 60-90% of the total lipids. The most common phospholipids may be classified into two structural groups, the nonnitrogenous and the nitrogenous derivatives of phosphatidic acid.

With respect to the former group K. Bloch (10, 11) then Macheboeuf and Faure (64) showed the presence of phosphatidic acids in the phospholipids of the tubercle bacillus. Cmelik (16) characterized phosphatidic acids in a strain of Salmonella typhi, Huston et al. (38) in a strain of Sarcina lutea and Saito and Akashi (85) in a strain of Alcaligenes faecalis.

It must be realized that many of these compounds may arise from the hydrolysis of more complex phospholipids in the course of the extraction procedures required for their isolation. Some enzymes may easily hydrolyze glycerophosphatides into phosphatidic acids in organic solvents, such as moist ether (51).

Usually, a sole nitrogen base is present in the phospholipids of a bacterial species. Ethanolamine alone is most frequently encountered: it has been identified in the phospholipids of Azotobacter vinelandii (68), Azotobacter agilis (42), Alcaligenes faecalis (85), Bacillus cereus (46), Clostridium butyricum (28), Pseudomonas denitrificans (85), Staphylococcus aureus (63), Salmonella typhi (16) and Vibrio cholerae (8). Choline alone has been detected in three cases: Neisseria gonorrhoeae (90), Sarcina lutea (38) and Lactobacillus acidophilus (19).

Phosphatidylserine has been found in the lipids of E. coli (43), of Serratia marcescens (45) and in the endotoxin of Pseudomonas aeruginosa, a serine-containing phospholipid has been detected (36).

Phosphatidylglycerols and diphosphatidyl glycerols have been detected in the phospholipids of Thiobacillus thiooxidans (40, Streptococcus faecalis (39) and Staphylococcus aureus (62).

Recently, plasmalogen-type compounds have been encountered in species of Clostridium and Bacteroides (29, 92). Plasmalogens are phosphoglycerides in which one fatty acid is replaced by a fatty aldehyde linked to the glycerol moiety through an ether rather than an ester linkage. Little is yet known regarding these substances which are otherwise rarely encountered in microorganisms.

### Glycolipids

The term glycolipid designates the lipid fractions which contain sugars and remain soluble in organic solvents, in contrast to lipopolysaccharides, also composed of lipids and sugars, but water-soluble.

In the mycobacteria several complicated glycolipids are known to occur. These are phosphatidylinositols linked to monosaccharides, disaccharides, or oligosaccharides (3, 49). Mannose is commonly found, and both dimannoside and pentamannoside derivatives of phosphatidylinositols have been found in strains of Mycobacterium. Recently Panos (17, 76) has reported a diglucosyl-diglyceride in the lipids of protoplasmic membranes from both cells and L forms of Streptococcus pyogenes. A similar structure has been detected in

the lipids of Staphylococcus aureus by Polonovski and his associates (77). A dimannosyldiglyceride has been found in Micrococcus lyso-deikticus and glucosyl-diglyceride and galactosyl-glucosyl-diglyceride have been shown in various strains of pneumococci (14).

### Peptidolipids

Peptidolipids are combinations of fatty acids and small peptides or individual amino acids. The simplest representative of this group of lipids is serratamic acid, which was isolated from a species of Serratia by Cartwright (15). It contains serine and 3-hydroxy-decanoic acid. Compounds containing peptides rather than single amino acids have been isolated from Nocardia asteroides, Bacillus megatherium, and Escherichia coli (3).

### Waxes

Waxes are esters of long-chain fatty acids and long-chain fatty alcohols. With the exceptions of mycobacteria and corynebacteria they are quite uncommon.

Human and bovine strains of M. tuberculosis contain diesters of mycocerosic acid and alcohols of the phthiocerol group (27). The number of diesters possible is high since phthiocerol is a mixture of at least five alcohols and mycocerosic acid of at least three acids.

C. diphtheriae has been reported to contain esters of octadecanol and docosanol (32) and of corinic alcohol  $C_{35}H_{70}$ , m. p.  $54^{\circ}C$ .

### Polymers of Fatty Acids

Lemoigne, Milhaud, and Croson (53) demonstrated sometime ago that Bacillus megaterium produce polymers of (-)-3-hydroxybutyric acid. Subsequent studies showed that many other species of bacteria contain this type of polymer often in high concentrations. Studies of Junggren and his associates (59) show that poly- $\beta$ -hydroxybutyrates from different species do vary in chain length. The authors point out, however, that the polymer is highly susceptible to hydrolysis.

Until recently, the  $\beta$ -hydroxybutyrate polymer was the only such structure known to occur in bacteria. However, Thorne and Kodicek have found a lipid polymer formed in lactobacilli from mevalonic acid (91).

## MATERIALS AND METHODS

### Microorganisms Used

Original cultures of Cl. botulinum types 61E and 115B were obtained from the Quartermaster Food and Container Institute for the Armed Forces, Natick, Massachusetts. The parent strain of Cl. botulinum type F used throughout this study was obtained from the stock culture collection maintained by the Department of Microbiology at Oregon State University.

### Culture Media and Growth Conditions

Vegetative cells were produced in 15-liter quantities using a trypticase-proteose-glucose medium (TPG medium). The medium consisted of 5.0% trypticase (Baltimore Biological Laboratory), 0.5% peptone (Difco), 1.0% glucose, and 0.01% sodium thioglycolate in distilled water. The pH was adjusted to 7.1 before sterilization. A 3.0% inoculum of a 24 hour culture was used when the cells were to be grown in large quantities. Vegetative cultures were incubated for 12, 24, 36, and 48 hour periods at 30°C. The cells were then harvested in a cooled, closed Sharples Super-Centrifuge (model T-1P) with a standard clarifier bowl. Cells were washed four times in 0.85% NaCl and lyophilized to facilitate extraction.

For the preparation of spores, stock spore suspensions in

sterile M/15 phosphate buffer pH 7.0 were heat-shocked at 65°C for 15 minutes (80°C for 10 minutes for the more heat resistant type 115B spores) to kill residual vegetative cells and to activate the spores to germination. Immediately after heat shock, a 1.0% inoculum was introduced into freshly steamed trypticase-thioglycollate broth (trypticase, 5%; Bacto-peptone, 1.5%; glucose, 0.01%; sodium thioglycollate, 0.01%; thiamine-HCl, 0.01%; pH 7.0). The thiamine-HCl was autoclaved separately and added after sterilization. The inoculated flasks were incubated for ten days to allow the formation of a maximum number of spores. After the ten day growth period the spores were harvested and washed three times with sterile 0.067 M phosphate buffer at pH 7.

#### Spore Cleaning Procedure

Liberation of spores from residual vegetative sporangia was accomplished by a modification of the procedure of Grecz and Anellis (31). The cleaning procedure required the use of lytic enzymes and sonic oscillation. The spores were re-suspended in 0.067 M sterile phosphate buffer solution at pH 7.0, and the suspension of crude spores was digested with trypsin (150 µg/ml) and lysozyme (200 µg/ml). Lysis of sporangia was induced by ultrasonic oscillation of the reacting mixture at 10 kc (Bronson Instruments Inc., Model LS75, Sonifier). The mixture was sonified for

four minute periods at 0, 0.25, 0.5, 1.0, 2.0, 3.5, and 7.0 hours. Incubation of the reacting mixture between the sonic treatments was at 45°C. The cleaning procedure was completed by repeated washing of the spores with distilled water. The degree of purification of spores was estimated microscopically from the reduction in vegetative sporangia and loss of stainability by crystal violet. Spore cleanliness was also checked by spore behavior in the aqueous-polymer system Y of Sacks and Alderton (84).

#### Estimation of Viable Spore Numbers

The spore suspensions were heat-shocked at 65°C for 15 minutes, except for type B spores. The latter were heat-shocked at 80°C for ten minutes. Serial decimal dilutions were made in neutral phosphate buffer; 1-ml samples of each dilution were inoculated in each of five replicate tubes of Wynne's broth (95). Growth in the medium was usually completed in 14 days. The most probable number of viable spores was calculated from the statistical tables of Fisher and Yates (23).

#### Toxin Assay

Cultures were periodically tested for toxin production. Toxin was assayed by duplicate intraperitoneal injections into white mice (16 to 20g). A positive reaction was indicated by death of the mice



within three days. The specific types of toxin were confirmed by toxin-antitoxin neutralization tests. Tenfold serial dilutions were made of the culture supernatant fluids in a gelatin-phosphate diluent. Gelatin protein appears to minimize inactivation of the toxin, reduce pyrogenic reactions, and potentiate the effect of botulinum toxin (93).

### Lipid Extraction

#### Reagents

All chemicals were of reagent grade. Solvents were redistilled immediately prior to use.

#### Vegetative Cells

The cells were dried to constant weight in vacuo. One gram of lyophilized vegetative cells was then suspended in 50 ml chloroform-methanol (2:1, v/v) and extracted three hours at room temperature by continuous agitation with a magnetic stirrer. This and all other extractions were carried out under nitrogen. The resulting suspension was filtered on a Buchner funnel. The filter cake was re-extracted twice more with chloroform-methanol as described above. Finally, the residue was extracted with 50 ml. ethanol:ether (3:1, v/v) for two hours. The extracts were pooled and evaporated under a stream of nitrogen at 45°C. The residue was taken up in chloroform-methanol, 2:1, and washed by the method of Folch, Lees, and

Sloan-Stanley (24) to remove nonlipid contaminants. Nonlipid contaminants that were not removed by the Folch washing procedure were separated on a DEAE cellulose column according to Rouser et al. (82).

The remaining cell residue was examined for nonextractable lipids by subjecting it to a three hour reflux with 10% KOH (w/v) in 50% aqueous methanol (v/v). Nonsaponifiable material was removed by extracting with petroleum ether (b. p. 40-60°C). The lipid was then extracted with anhydrous ethyl ether after acidification.

### Spores

To facilitate extraction spores were first disrupted. They were mixed with chloroform to form a paste and placed into a glass homogenization flask with five times their wet weight of glass beads (0.11 to 0.12mm). The spores were then homogenized in a Bronwell carbon dioxide cooled mechanical homogenizer (MSK) at 4000 cycles per minute. An eight minute treatment was sufficient to cause disruption of the spores. The disrupted spores were then extracted as previously described.

### Fatty Acid Analysis

#### Esterification

Fatty acids were examined in the form of their methyl esters.

The total lipids were esterified by a modification of the method of Metcalfe and Schmitz (69). Four milliliters of a 12% solution of  $\text{BF}_3$  (w/v) in methanol were added to 100 mg lipid in a test tube. The mixture was boiled on a steam bath three minutes. The boiled mixture was transferred from the test tube to a 60 ml separatory funnel with six volumes of water; 5 ml of n-pentane was added, mixed, and the layers allowed to separate. The hydrocarbon layer was washed with water until washings were neutral to litmus. Anhydrous sodium sulfate was used to remove excess water from the hydrocarbon layer. The sulfate was then removed by filtering through a Buchner funnel and washed twice with 4 ml portions of n-pentane. The solvent was removed in vacuo and the esters taken up in benzene for gas-liquid chromatography analysis (GLC).

The  $\text{BF}_3$  methanolysis is simple, rapid, and quantitative. Since boron fluoride alcoholates behave like strong acids (70), they promote methanolysis in a manner similar to HCl or  $\text{H}_2\text{SO}_4$  added to methanol with the added advantage conferred by the extreme electropolarity of the boron fluoride.

#### Gas Liquid Chromatography

Chromatograms were obtained with an F & M model 402 gas chromatograph with a flame ionization detector (F & M Scientific Corporation, Avondale, Pennsylvania). All samples for qualitative

study were analyzed on at least one polar and one nonpolar column. The following column packing and conditions were used: (a) silicone gum rubber (GE SE-30), 3.8% on 80/100 mesh Diatport S; the column was temperature-programmed from 160 to 250°C at 10°C per minute; (b) ethylene glycol succinate (EGS), 15% on 60/80 mesh Chromosorb P; the column was maintained isothermally at 170°C, 180°C, or 190°C; (c) Apiezon L (ApL), 10% on Chromosorb P 60/80 mesh; the column was maintained isothermally at 225°C; (d) neopentyl glycol succinate (NPGS), 10% on 60/80 mesh Chromosorb W; the column was maintained isothermally at 200°C; (e) Carbowax 20M, 10% on 60/80 mesh Chromosorb P; the column was maintained isothermally at 185°C; (f) FFAP (a polar liquid phase obtained from Varian Aerograph) 10% on 60/80 mesh Chromosorb P, the column was maintained isothermally at 180°C; (g) benzylamine adipate, 20% on 35/80 mesh Chromosorb W; the column was maintained isothermally at 125°C. Helium was used as the carrier gas in all cases.

Preparative GLC was carried out on columns (6'x3/8") containing 20% ethylene glycol succinate on Chromosorb P, 60/80 mesh. Temperatures and pressures were varied, depending on the fraction to be separated. The effluent stream was split; approximately 4% went to the detector, and the remainder to a teflon collection tube placed over the splitter exhaust.

The effluent splitter was of an annular design, located in the

center of the effluent stream. If the sample being analyzed contained components having a wide range of molecular weights, the annular split became a very important design feature when compared to the simple tee configuration. There is a slight tendency for larger molecules to take the path of least resistance with the latter. A T-shaped split results in disproportionate amounts of the heavier molecules continuing through the straight leg of the splitter. This is undesirable when analyzing quantitatively with simultaneous detection. However, the annular split does not require the stream to turn a corner at the splitting point, and this overcomes the problem of the component concentrations becoming nonrepresentative.

#### Separation of Saturated and Unsaturated Esters

Fatty acid methyl esters were reacted with mercuric acetate according to Mangold (65), and the adducts were separated by chromatography on silicic acid by a modification of the technique of Erwin and Bloch (21). After preparation of the adducts, the solvents and excess acetic acid were removed on a rotary evaporator. Five milligrams of the dried adducts were taken up in benzene and placed on a column of 600 mg silicic acid (100 mesh), which had been prepared from a slurry in benzene. The column was then eluted three times with benzene to a total of 27 ml. The combined benzene eluates contained the saturated fraction. The mercuric acetate adducts of

the unsaturated fatty acid esters were eluted by 12 ml of 5% acetic acid in methanol.

The solvents were evaporated and the free methyl esters regenerated by reaction of the adducts with 0.3 ml 12 N HCl in 5 ml of methanol for two hours at room temperature. Water was then added to the point of turbidity, and the regenerated methyl esters were extracted into freshly redistilled petroleum ether and analyzed by gas-liquid chromatography.

The monounsaturated and diunsaturated fatty acid methyl esters were separated by chromatography on silicic acid impregnated with  $\text{AgNO}_3$  (94). Thirty grams Silica Gel G (E. Merck, A.G., Darmstadt, Germany) was slurried with 60 ml of 12.5%  $\text{AgNO}_3$  (w/v) in 28-30%  $\text{NH}_4\text{OH}$ . Uniform 0.25 mm layers were then spread on 2 x 20 cm and 20 x 20 cm glass plates with a Desaga-Brinkman applicator. After the chromatoplates had air dried for 30 minutes, they were activated in an oven for 30 minutes at  $110^\circ\text{C}$ . Approximately  $50\mu\text{g}$  of the fatty acids methyl esters were applied 2 cm from the lower edge of the plate. Development of the chromatoplates was carried out in a chamber saturated with chloroform-ethanol 99:1 (v/v). The separations were visualized either by charring according to the procedure of Privett and Blank (79) or by spraying with a 0.2% solution of 2', 7'-dichlorofluorescein in ethanol.

### Structural Analysis

Gas-liquid chromatography was used to determine the chain length of the saturated fatty acid produced by catalytic hydrogenation (47). The number of double bonds in the molecule was then deducible from the retention time of the original fatty acid methyl ester, the chromatographic behavior of its mercuric acetate adduct, and its behavior on  $\text{AgNO}_3$ -thin layer chromatography.

The number of double bonds in the molecule was confirmed, and their positions were established by analysis of the products of permanganate-periodate oxidation performed by a modification (47) of the procedure of van Rudloff (83). Each unsaturated fatty acid was degraded to a dicarboxylic acid that was derived from the carbon atoms from the carboxyl group to the first double bond, and a monocarboxylic acid that was derived from the carbon atoms from the last double bond to the methyl terminal end. These fatty acids were identified by GLC of their methyl esters.

Infrared spectra of the various methyl ester samples and lipid fractions were made from thin films on KBr pellets or NaCl crystals. Analyses were performed with a Beckman IR5 recording spectrophotometer.

## Complex Lipids

### Column Chromatography

Lipid separations were obtained on a diethylaminoethyl (DEAE) cellulose column. An important feature of a DEAE column is the relatively large amount of lipid that can be applied to it. Rouser (81) suggests 350 mg for a 2.5 x 20 cm column containing 15 g of DEAE.

The column packing was prepared as follows: Fifteen grams of DEAE cellulose (Sigma) was placed in a beaker and allowed to stand overnight in redistilled glacial acetic acid. To insure thorough wetting of the DEAE cellulose with acetic acid and a uniformly packed column, the DEAE was pressed gently against the walls of a mortar with a pestle to break up the lumps.

A small plug of glass wool was placed at the bottom of the chromatography tube, and the DEAE slurry in glacial acetic acid added in four equal portions. After each addition the excess acid was forced out under 3 psig nitrogen pressure, and the DEAE bed pressed lightly with a glass rod. For best column performance, it was imperative that all air pockets and any nonuniformity be removed while packing the column. Three bed-volumes of glacial acetic acid were passed through the column and then washed out with methanol until the washings were neutral to litmus. Methanol



was washed out with chloroform.

The non-polar lipids were eluted with chloroform, zwitterion lipids with chloroform-methanol (9:1, v/v), basic lipids with chloroform-methanol (7:3, v/v), water-soluble nonlipid substances with methanol, and acidic lipids with chloroform-glacial acetic acid (3:1, v/v) containing 0.001 M potassium acetate (82).

### Thin Layer Chromatography

Both preparative and analytical separations were carried out on Silica gel G plates prepared according to Stahl (88). By applying the lipids dissolved in chloroform-methanol (2:1) to the origin as a series of contiguous spots, up to 20 mg of lipids could be separated on a plate (20 cm x 20 cm). Chromatography with appropriate reference compounds and reaction with indicator reagents were employed as criteria of identification.

Hexane-ethyl ether-acetic acid (90:10:1, v/v/v) was used for lipid class separation. Chloroform-methanol-water (67:23:4, v/v/v), chloroform-methanol - 14% aqueous ammonia (17:7:1, v/v/v), and diisobutyl ketone-acetic acid-water (8:5:1, v/v/v) (56) were used as solvent systems for complex lipid analysis. The spots were detected with 0.05% aqueous Rhodamine B; iodine vapors; Dragendorff's reagent for choline and quaternary ammonium compounds; ammoniacal silver nitrate for phosphatidic acid, phosphatidyl glycerol, and

inositides; Bial's reagent (30) for glycolipids;  $\alpha$ -cyclodextrin for monochain lipids; 0.2% ninhydrin (20) in butanol for aminophosphatides; fuchsin- $H_2SO_4$  (13) for plasmalogens; and phosphomolybdic acid for glycerides and unsaturated fatty acids.

### Analytical Methods

Phosphorous was determined by a modification of the procedure of Bartlett (6). Seventy percent perchloric acid was used to digest the sample rather than sulfuric acid. To a sample 0.9 ml of 70% perchloric acid was added. Digestion was carried out for 15 minutes on a gas flame. After the tubes had cooled, 7.0 ml of distilled water, 1.5 ml of 2.5% ammonium molybdate, and 0.2 ml of the aminonaphtholsulfonic acid reagent described by Bartlett were added. The tubes were placed in boiling water for seven minutes, removed, and allowed to cool for 20 minutes. The optical densities were determined at 700 m $\mu$  with a Bausch and Lomb Spectronic 20 equipped with a red filter and a red sensitive phototube.

Fatty aldehydes of plasmalogens were detected qualitatively by precipitation of hydrazones after reacting with a 2.7% alcoholic solution of acidic 2,4-dinitrophenyl hydrazine or were determined quantitatively by reaction with an acidic solution of p-nitrophenyl hydrazine (80).

## RESULTS AND DISCUSSION

The total lipid extracted accounted for 3.7%, 3.3%, 2.0%, 2.7%, and 3.0% of the dry weight of Cl. botulinum vegetative cell types 61E and F<sup>1</sup> and spore types 61E, F, and 115B, respectively. The fatty acid composition of the spores and vegetative cells is shown in Table I.

The fatty acids were analyzed in the form of their methyl esters by gas-liquid chromatography. The overall fatty acid pattern included straight chain saturated, unsaturated, and cyclopropane acids. Myristic and palmitic acids were the predominant acids in both the spores and vegetative cells of 61E, F, and 115B.

A typical chromatograph of the total fatty acid methyl esters of Cl. botulinum 61E is shown in Figure I. Peaks I, II, III, V, VII, IX, and XIII were identified as the methyl esters of caprylic (8:0),<sup>2</sup> capric (10:0), lauric (12:0), myristic (14:0), pentadecanoic (15:0), palmitic (16:0), and stearic (18:0) acids, respectively. These esters had the same retention times as known standards in six gas-liquid chromatographic systems. They migrated with the solvent front on

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<sup>1</sup> Percentages are based upon 36-hour vegetative cells cultured as described in the Materials and Methods.

<sup>2</sup> The first number used refers to the number of carbon atoms in the fatty acid chain; the second number, to the number of double bonds in the molecule.

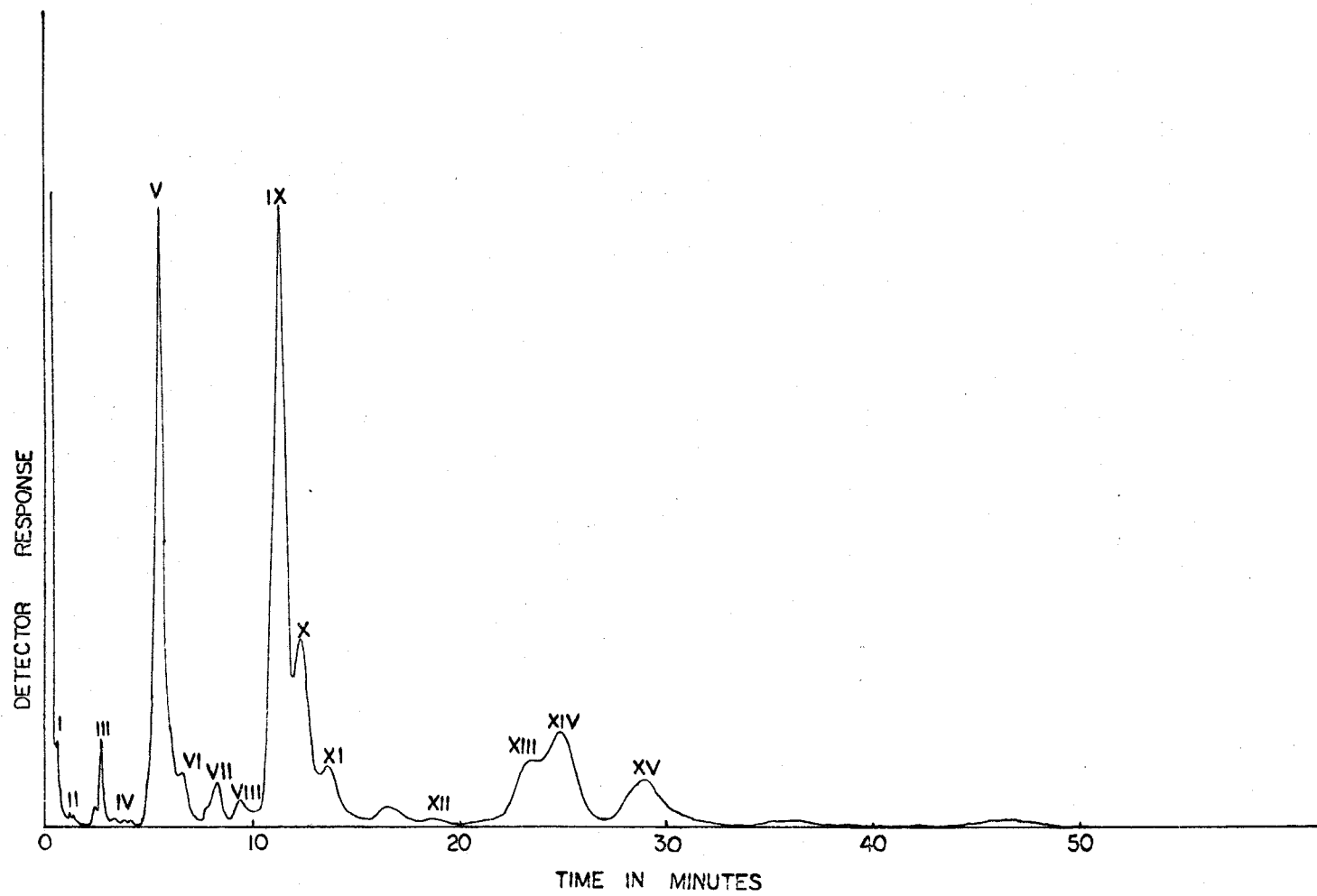
Table 1. Fatty Acid Composition of *C. botulinum*

Fatty Acid	Relative Fatty Acid Composition (% of Total)				
	61E Vegetative	F Vegetative	61E Spores	F Spores	115B Spores
caprylic	0.6	0.8	---	---	---
pelargonic	+	+ <sup>a</sup>	---	---	---
capric	0.3	0.5	+	0.2	---
undecanoic	+	+	---	---	---
lauric	2.8	2.0	0.9	1.5	1.0
Tridecanoic	0.2	---	+	0.2	+
methylene-dodecanoic	0.3	0.4	+	+	+
7,8-Tetradecenoic	3.2	4.2	1.1	1.6	1.2
methylene-tetradecanoic	1.5	1.5	1.0	0.9	0.6
Myristic	25.4	18.0	25.1	24.4	13.3
Pentadecanoic	3.2	1.5	1.0	0.8	1.0
Br <sup>b</sup> -pentadecanoic	---	---	0.4	0.7	---
palmitic	32.3	22.6	35.0	32.1	55.7
9,10-hexadecenoic	6.3	7.9	5.3	6.6	1.4
7,8-hexadecenoic	2.7	5.3	2.3	4.4	0.6
methylene-hexadecanoic	4.2	8.4	1.5	2.2	1.0
heptadecanoic	0.8	1.5	1.5	2.2	1.8
Br-heptadecanoic	3.0	9.5	2.3	2.2	3.1
stearic	3.3	3.6	4.0	4.3	9.2
11,12-octadecenoic	3.2	4.3	4.2	7.1	4.3
9,10-octadecenoic	1.4	2.8	2.0	3.3	1.8
octadecadienoic	+	+	---	---	---
methylene-octadecanoic	1.1	1.4	2.3	1.9	1.5
nonadecanoic	0.4	1.0	0.3	1.3	1.4
Arachidic	0.5	1.3	0.5	---	0.4
Heneicosanoic	0.4	1.7	---	---	---

<sup>a</sup> A + sign indicates that the component was detected but that it did not occur to an extent greater than 0.1%.

<sup>b</sup> Br equals Branched.

Figure 1. Gas liquid chromatogram of total fatty acid methyl esters from vegetative cells of C1. botulinum 61E. The column was 6 feet x 1/8 inch (O.D.) It was composed of 15% ethylene glycol-succinate polyester on Chromosorb P (60/80 mesh) operated at a column temperature of 180°; flow rate, 40 ml of He per minute. Peaks V and IX are shown at a 2X attenuation to keep them on scale.



$\text{AgNO}_3$ -TLC and did not form mercuric acetate adducts. Moreover, their chromatographic positions were unchanged by hydrogenation. Infrared spectrophotometry of these esters revealed no unusual bands.

Peak VI was identified as the methyl ester of 7,8-tetradecenoic acid. This peak disappeared completely when the sample was hydrogenated. The compound behaved as a monoenoic acid upon fractionation of the mercuric acetate adducts. Silver nitrate-thin layer chromatography established that it was a monoene. Oxidation with performic acid and periodate yielded only heptanoic and pimelic acids (Table II). This compound did not show an absorption band at  $965\text{ cm}^{-1}$ , which is characteristic of the out-of-plane hydrogen deformation of trans olefins. Thus, the acid was identified as cis-7,8-tetradecenoic acid.

Peak X was identified as the methyl ester of 9,10-hexadecenoic acid. However, there was a small quantity of 7,8-hexadecenoate methyl ester present. This peak disappeared after hydrogenation and the peak corresponding to methyl palmitate increased by an amount equivalent to the monoene. The monocarboxylic acids resulting from oxidative degradation were heptanoic and nonanoic (pelargonic) acids (Table II). The dicarboxylic acids were identified as azelaic and pimelic acids by GLC of their methyl esters. Therefore, the acids were assigned the structures 9,10-hexadecenoic and

Table 2. Fragments<sup>a</sup> isolated after oxidative degradation of unsaturated acids from Cl. botulinum 61E.

Peak No.	Monocarboxylic Acids		Dicarboxylic Acids		Parent Unsaturated Acid	Percentage of Total Peak Area
VI	heptanoic	C <sub>7</sub>	pimelic	C <sub>7</sub>	7, 8-tetradecenoic	100
XI	heptanoic	C <sub>7</sub>	Azelaic	C <sub>9</sub>	9, 10-hexadecenoic	69
	pelargonic	C <sub>9</sub>	pimelic	C <sub>7</sub>	7, 8-hexadecenoic	31
XVI	heptanoic	C <sub>7</sub>	undecanedioic	C <sub>11</sub>	11, 12-octadecenoic	73
	pelargonic	C <sub>9</sub>	Azelaic	C <sub>9</sub>	9, 10-octadecenoic	27

<sup>a</sup>The composition of each fraction was calculated from the results of a single oxidation. The percentage composition of the mixture of the isomers was based on the relative areas of the peaks corresponding to the recovered dicarboxylic acids.



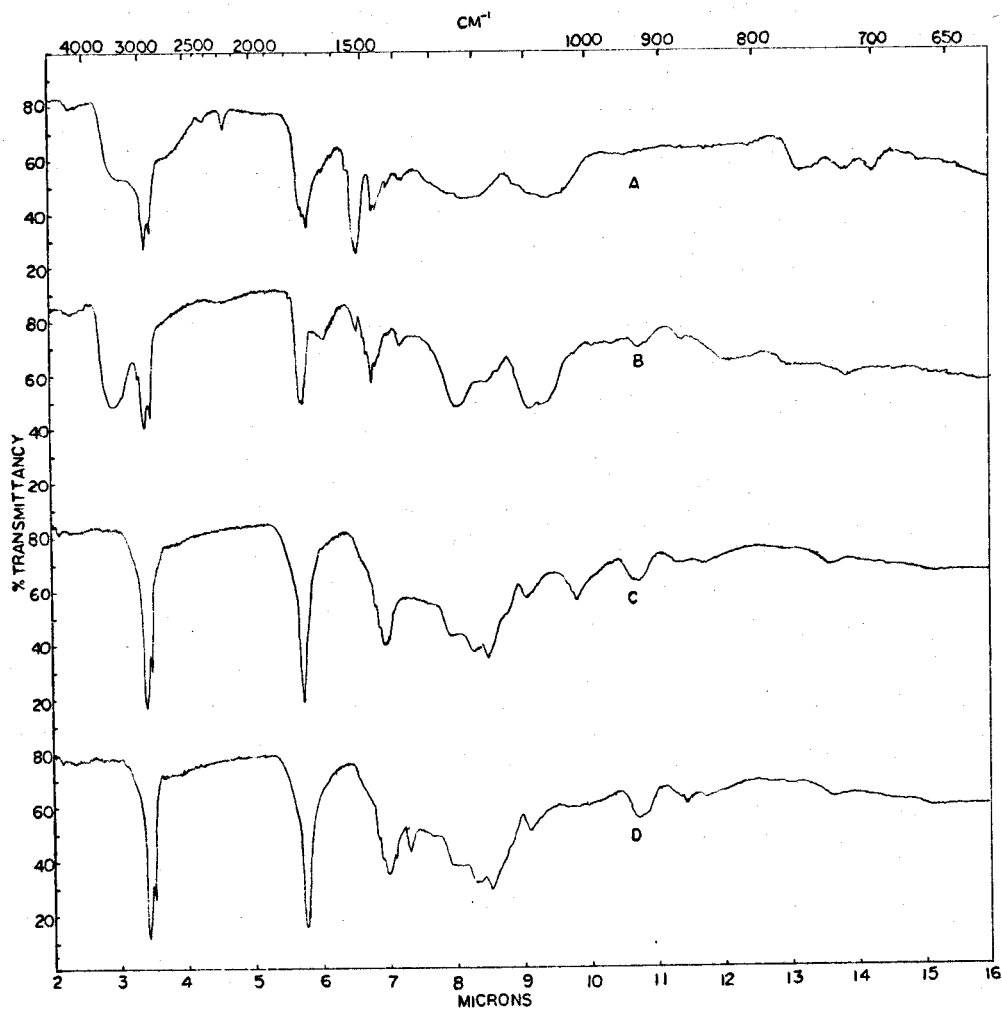
7, 8-hexadecenoic acids.

In a similar manner peak XIV was identified as primarily the methyl ester of 11, 12-octadecenoic acid with a lesser quantity of 9, 10-octadecenoate. The oxidative degradation products are shown in Table II and support the structural designations. This peak behaved as a monoene upon  $\text{AgNO}_3$ -TLC and mercuric acetate fractionation. It disappeared after hydrogenation and the methyl stearate peak increased by an amount equivalent to its monoene analogue.

Peaks VIII, XII, and XV were identified as the methyl esters of cyclopropane fatty acids. The fatty acids contained 15, 17, and 19 carbon atoms. They exhibited properties similar to monounsaturated acids on GLC, moving ahead of the corresponding saturated fatty acid on nonpolar columns and behind on polar columns. The peaks showed an absorption band at  $1020 \text{ cm}^{-1}$  characteristic of cyclopropane compounds (Figure 2C) and were unaffected by mild hydrogenation. Catalytic hydrogenation of the free acid in glacial acetic acid (22) opened the cyclopropane ring. The infrared band at  $1020 \text{ cm}^{-1}$  disappeared and a new band at  $1370 \text{ cm}^{-1}$ , characteristic of methyl-branched alkanes, appeared (Figure 2D).

The small quantity of compound IV prevented its structural analysis. However, a plot of the logarithm of the retention time against the number of atoms disclosed that components IV, VIII, XII, and XV could be joined by a straight line with the same slope as that

Figure 2. Infrared spectra of (A) total lipids from vegetative cells of Cl. botulinum 61E, spread evenly as a film over NaCl crystals; (B) phosphatidylethanolamine from 61E vegetative cells, (C) methylene hexadecanoic acid from Cl. botulinum F, and (D) hydrogenated methylene hexadecanoic acid. Samples B, C, and D were spread evenly as films over pellets of KBr.



for methyl esters of the normal saturated fatty acids. This peak was tentatively identified as a 13-carbon cyclopropane acid.

Peak XI was identified as a branched-chain 17-carbon fatty acid methyl ester. Branched-chain saturates were distinguished from monounsaturates emerging at the same point from polyester columns by noting their reversed order on ApL and by comparing their degree of separation from n-saturates on EGS at two different temperatures (41).

Both Cl. botulinum 61E and F possessed an 18-carbon diunsaturate as evidenced by its behavior on  $\text{AgNO}_3$ -TLC (Figure 3). This component eluted slightly ahead of linoleic (9,12-octadecadienoic) fatty acid ester on GLC. After hydrogenation the retention time was identical to that of methyl stearate. The fact that this component eluted slightly ahead of methyl linoleate on an EGS column suggested that its double bonds were closer to the carboxyl end. Theoretically each additional double bond present in the carbon beyond the 9,10-position increases the polarity (48). For instance, methyl 8,11-linoleate would be eluted before methyl 9,12-linoleate. This acid was not found in the vegetative cells of 115B. Lack of sufficient quantities prevented further identification of this diunsaturate.

Where quantities of fatty acid methyl esters were insufficient for structural analysis, identification was based on comparison of

relative retention times of unknown peaks with those of methyl ester standards. Silver nitrate-thin layer chromatography, mercuric acetate fractionation, and GLC after hydrogenation served as complementary means of analysis.

The presence of tetradecenoic, hexadecenoic, and octadecenoic acids are consistent with the concept of an elongation (i. e. non-oxidative) pathway (54) for the biosynthesis of unsaturated fatty acids in bacteria. A proposed mechanism for the formation of long-chain saturated and unsaturated fatty acids via  $\beta$ -hydroxy acid intermediate is shown in Figure 5. It would appear that the anaerobic elongation mode of forming unsaturated fatty acids is a major and distinctive metabolic activity in Cl. botulinum.

Hofmann and colleagues (35), who first isolated and established the structure of a cyclopropane acid, lactobacillic acid, as cis-11,12 methylene octadecanoic acid, proposed that it was synthesized by the addition of a  $C_1$  unit across the double bond of cis-11 octadecenoic acid; the  $C_{17}$  cyclopropane acid, cis-9,10-methylene hexadecanoic acid, would presumably be synthesized by analogous reactions starting with cis-9-hexadecenoic acid. The presence of  $C_{15}$ ,  $C_{17}$ , and  $C_{19}$  cyclopropane acids in Cl. botulinum with their possible  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  unsaturated precursors are consistent with this mode of synthesis (Figure 5).

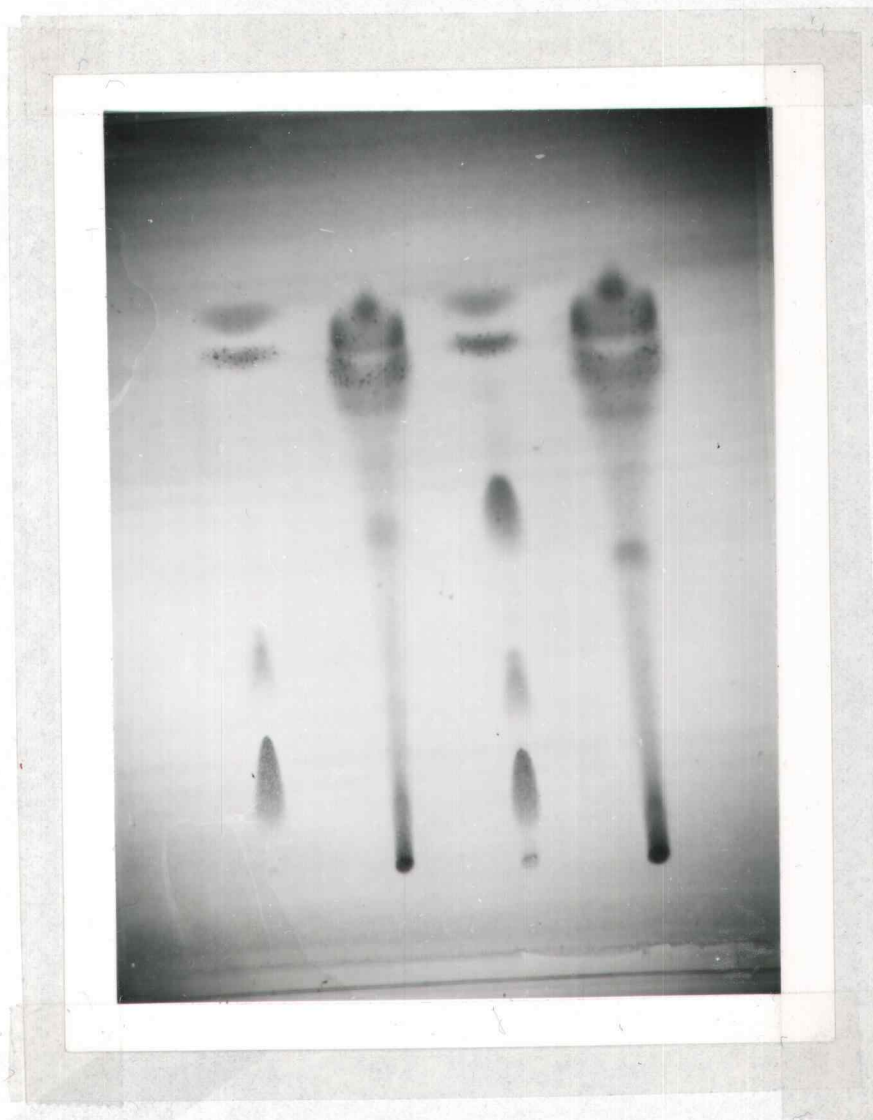


Figure 3. Silver nitrate-thin layer chromatography of methyl esters. (1) linolenic, linoleic, and saturated methyl ester standards, (2) total methyl esters from type F vegetative cells, (3) linolenic, linoleic, oleic, and saturated fatty acid methyl ester standards, and (4) total esters from type 61E vegetative cells.

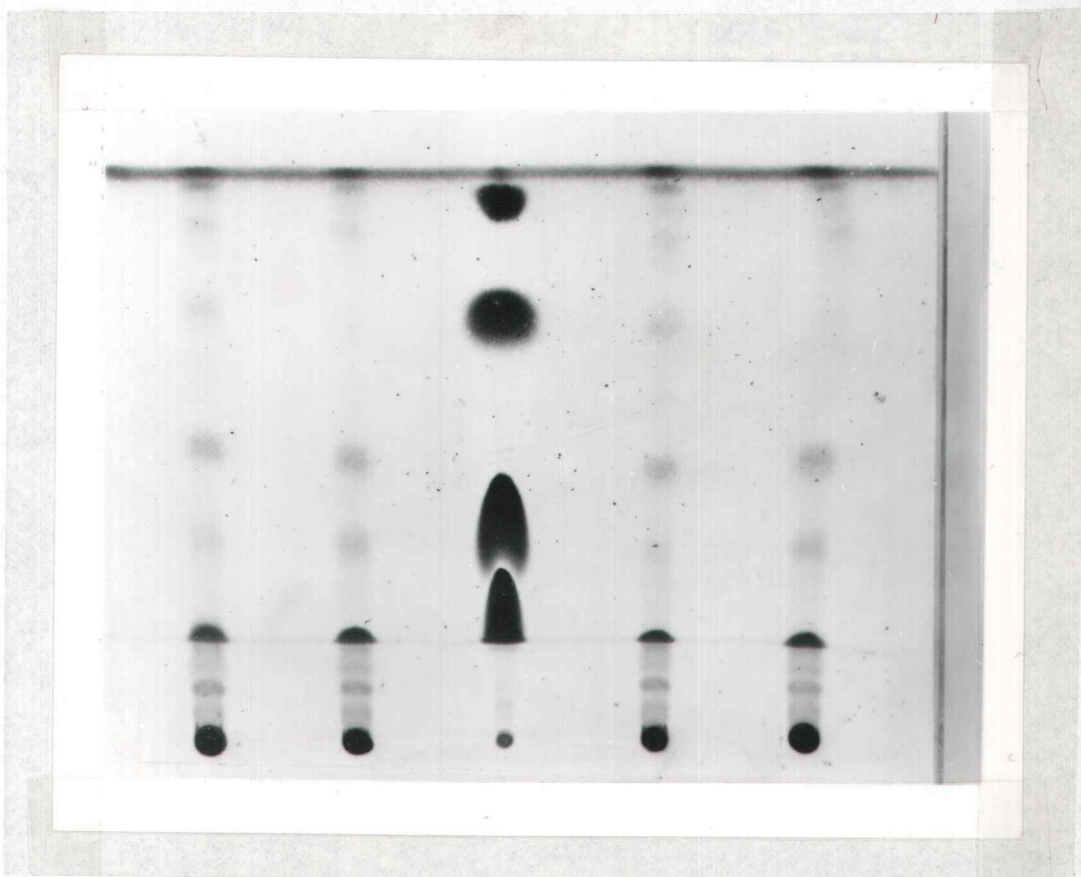


Figure 4. Thin layer chromatoplate of lipid classes on silica gel. Solvent: hexane-ethylether-acetic acid, 90:10:1 (v/v/v). (1) type F vegetative cells (2) type 61E vegetative cells (3) lipid mixture of oleic acid, triolein, methyl oleate, and cholesteryl oleate in ascending order (4) type 61E spores (5) type F spores. (Left to Right)

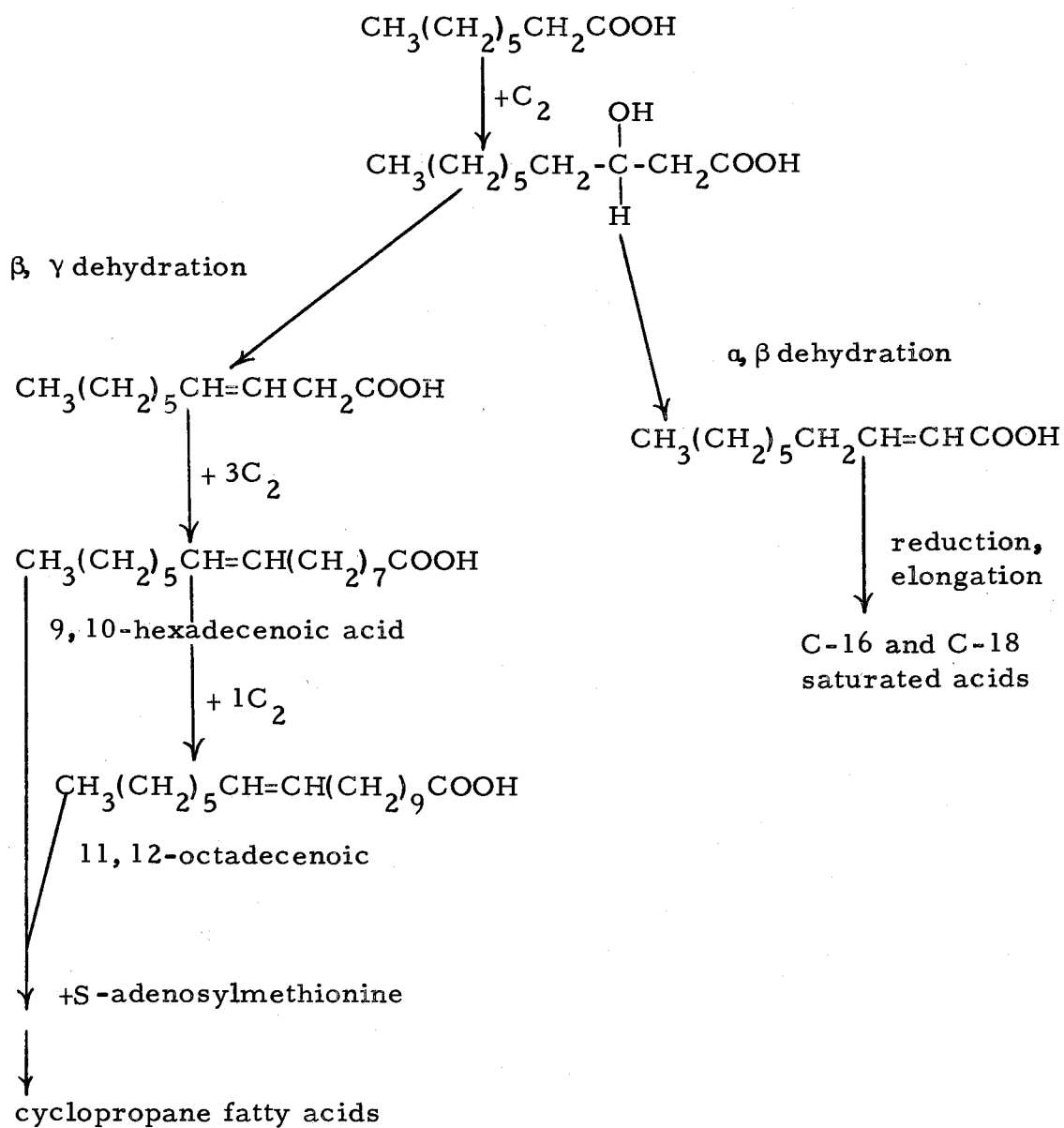


Figure 5. Proposed mechanism for the formation of long-chain saturated, unsaturated, and cyclopropane fatty acids via  $\beta$ -hydroxy acid intermediate.



The lipid classes of Cl. botulinum were fractionated on thin layer chromatography (Figure 4) and on DEAE cellulose column chromatography.

The nonpolar fraction from DEAE chromatography contained less than 1% phosphorous. A low absorption at  $1740\text{ cm}^{-1}$  indicated the absence of significant quantities of glycerides and other carboxylic esters. There was no phosphatidylcholine present in either the vegetative cells or spores of Cl. botulinum.

The phospholipid eluted with chloroform-methanol (7:3, v/v) was evaporated to dryness under reduced pressure and redissolved in a minimal volume of chloroform. The phospholipid was precipitated from this solution by the addition of six volumes of cold acetone. The precipitate was collected by centrifugation and dissolved in benzene. When examined on TLC it had the same  $R_f$  value as a pure phosphatidylethanolamine. An ester absorption at  $1740\text{ cm}^{-1}$ , P-O-C stretch at  $1030$  and  $1075\text{ cm}^{-1}$ , P-O stretch at  $1225\text{ cm}^{-1}$ , and absence of a band at  $968\text{ cm}^{-1}$  were noted and are characteristic of phosphatidylethanolamine (Figure 2B) (66).

The chloroform-glacial acetic acid eluate yielded the "free" fatty acids and phosphatidic acid. The latter may have been the product of hydrolysis, however. The phospholipid comprised approximately 60% of the lipid extracted from the vegetative cells and

4.0% of that extracted from the spore lipids.

Bial's reagent indicated the presence of glycolipids in both spores and vegetative cells. The presence of plasmalogens was shown in spores and vegetative cells by the fuchsin- $H_2SO_4$  test. Qualitatively, the phospholipids of Cl. botulinum appear to be similar to other species of Clostridia. In the phospholipid fraction of Cl. welchii type A, McFarlane (60) has characterized phosphatidic acids, phosphatidylglycerols, and esters of phosphatidylglycerols with amino acids (primarily lysine and ornithine). Goldfine (28) has fractionated the phospholipid fraction of Cl. butyricum by silicic acid column chromatography. He found that just after the phosphatidylethanolamine peak a fraction containing phosphatidyl-N-methylethanolamine emerged. Goldfine found that most of these phospholipids occur as plasmalogens. This is not the case in Cl. botulinum. Aliquots of the total lipids were subjected to methanolysis in order to obtain the methyl esters of the fatty acids and the aldehyde dimethylacetals. The mixture was saponified and the dimethylacetals extracted from the alkaline solution. Only the dimethylacetal of palmitaldehyde could be detected and it amounted to less than 1/100 the weight of the fatty acids from Cl. botulinum 61E and F vegetative cells.

## SUMMARY

The lipid composition of Cl. botulinum spores and vegetative cells was studied. The total lipid extracted accounted for 3.7%, 3.3%, 2.0%, 2.7%, and 3.0% of the dry weight of Cl. botulinum vegetative cell types 61E and F; and spore types 61E, F, and 115B, respectively.

The fatty acids were analyzed in the form of their methyl esters by gas-liquid chromatography. The total fatty acids included straight chain saturated, unsaturated, and cyclopropane acids. Myristic and palmitic acids were the predominant acids in both the spores and vegetative cells of types 61E, F, and 115B. Together, they made up over 50% of the total fatty acids.

Unsaturated acids were the second major group. These were primarily 7,8-tetradecenoic, 9,10-hexadecenoic, 7,8-hexadecenoic, 11,12-octadecenoic, and 9,10-octadecenoic acids. Types E and F possessed an 18 carbon diunsaturate, which was not found in the vegetative cells or spores of 115B. However, insufficient quantities prevented its further characterization. The vegetative cells and spores also contained C<sub>15</sub>, C<sub>17</sub>, and C<sub>19</sub> cyclopropane fatty acids. A possible mechanism for the formation of long-chain saturated and unsaturated fatty acids via  $\beta$ -hydroxy acid intermediates was proposed.

The phospholipids accounted for approximately 60% of the total lipids in the vegetative cells and 40% of that in the spores. The primary phospholipid was phosphatidylethanolamine. Qualitative tests for plasmalogens, glycolipids, and phosphatidic acid were positive for both spores and vegetative cells.

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