FATTY ACID COMPOSITION OF BACTERIAL LIPIDS AS A MEANS OF DIFFERENTIATING SPECIES.

BY

KARL ELMER KRUSHEL

A Thesis presented to

The Faculty of Graduate Studies and Research

of The University of Manitoba

in partial fulfillment of the requirements for

The Degree of Doctor of Philosophy.

1969

Ph. D. Biochemistry

Karl Elmer Krushel

FATTY ACID COMPOSITION OF BACTERIAL LIPIDS AS A MEANS OF DIFFERENTIATING SPECIES

ABSTRACT

The fatty acid composition of twenty-seven bacterial strains, including seventeen different species of Corynebacterium have been studied. Fatty acids were extracted directly from whole washed cells and examined as methyl esters by gas liquid chromatography. The most abundant acid in four corynebacteria species was a C_{15}-saturated branched-chain acid. The fatty acid profiles of these four plant pathogens strongly resemble those of certain species of Propionibacterium and on this basis it was felt that these four species could be reclassified. The most abundant fatty acids of the remaining corynebacteria were hexadecanoic and octadecenoic acid.

Visual and quantitative comparisons of the chromatograms for the presence and relative amounts of large major peaks allowed rapid differentiation of corynebacteria into two major groups. Differences within each group were not as great, but significant enough to distinguish most species from each other.

The experimental method was evaluated and discussed as regards medium, period of bacterial growth, harvesting method, extraction solvent and analytical technique.

ACKNOWLEDGEMENT

The author would like to acknowledge his gratefulness to Dr. A. D. Robinson for his patient supervision, constant interest and encouragement which he provided throughout this course of study.

The author would also like to express his appreciation to Dr. E. M. D. Cleveland for his invaluable assistance in providing the bacterial cultures as well as a continuous supply of information and advice.

The experimental work was carried out in the Pathology Department at Deer Lodge Hospital to whom the author would like to express his gratitude for providing equipment and " time-off " to carry out this project. The co-operation of Dr. W. L. Parker , Dr. P. T. Green , Miss Helen Lillie and the Special Chemistry staff was greatly appreciated.

"...The chymists are a strange class of mortals impelled by an almost insane impulse to seek their pleasure among smoke and vapour, soot and flame, poisons and poverty, yet among all these evils I seem to live so sweetly, that may I die if I would change places with the Persian King..."

Johann Joachim Becker,
"Acta Laboratorii Chymica
Novacencis, sen Physica
Subterranea" (1669)

TABLE OF CONTENTS

LIST OF CHROMATOGRAMS.

23	C. xerosis	7711	100
24	C. fascians	CF-1	101
25	C. "Q"	149	102
26	C. pseudodiphtheriticum	10700	103
27	C. pseudodiphtheriticum	10701	103
28	C. pseudotuberculosis	1033	104
29	C. pseudodiphtheriticum	6981	105
30	C. bovis	3224	106
31	C. enzymicum	8155	107
32	C. diphtheriae	U 61 A	108
33	C. diphtheriae	U 61 B	108
34	C. hoagii	7005	109
35	C. equi	1621	110
36	C. ulcerans	7906	111
37	C. diphtheriae	DL 210 B	112
38	C. xerosis	DL 7813	113
39	C. segmentosum	934	114
	GLC using LAC-728 column:		
40	Std. Mix D		115
41	Std. Mix BC-L		115
42	Std. Mix BC-1		115
43	Std. Mix L-205		115
44	Std. Mix 3		116
45	Std. Mix A		116
46	C. insidiosum	2A	117

LIST OF FIGURES.

INTRODUCTION

The mystery of bacterial evolution has resulted in the failure to produce a universally accepted natural classification of bacteria. An attempt at a phylogenetic classification was published by Bergey (12) in 1923, in order to stimulate efforts to perfect the classification of bacteria into orders, families, and genera as had been suggested earlier by a Committee of the Society of American Bacteriologists. However, this classification was never entirely satisfactory to authorities, as can be noted by comparing the sixth (1948) edition of Bergey's "Manual of Determinative Bacteriology" with the seventh (1957) edition (17). The sixth edition grouped bacteria into six orders containing 36 families while the latter has ten orders and 47 families. This revision was probably due, in part, to the fact that the characteristics of micro-organisms, upon which classification is based, are relatively few.

Early classifications were based on structural characteristics, particularly the shape of the cells. This had been found useful in drawing up natural classifications of higher plants and animals. However, as the number of known bacterial species increased, it was no longer adequate to know what the cells looked like, so it became necessary to consider what they did. Even this often failed to differentiate between certain species and so they were subgrouped according to their habitat or isolation source relationships and pathogenicity towards the host. This eventually led to the present mode of classification which is based on the following properties:

(a) Morphology:

Gross appearance of the culture,

Fine structure of the cell,

Staining properties

(b) Biochemical characteristics:

Optimal growth conditions

Fermentation and aerobic oxidation products

Reductions

Light absorption and emission

Exoenzyme production.

(c) Serology:

Specific antigen-antibody reactions.

(d) Toxigenic properties:

Exotoxin production

Endotoxin production.

Very often it was difficult to assign a certain organism to a definite category on this basis, since the cell shape might not be significantly different and the reactions, that it takes part in very limited. As a result, the search for better means of classification continued.

In 1956, Cummins and Harris (31) suggested that differences in composition of bacteria cell walls might be used to classify this form of life. In the same year, James and Martin (42) published a report describing the use of a new technique, gas-liquid chromatography, to determine fatty acids found in the culture media upon which bacteria had been grown. In 1961, Asselineau (7) suggested that GLC could be applied to the determination of bacterial fatty acids. Abel et al (1) in 1963, using gas-liquid chromatography, showed that different families of

one order have distinguishably different cellular fatty acid composition. Brown and Cosenza (20), in 1964, used lipids to distinguish between genera of bacteria, while Yamakawa and Ueta (86), in 1964, used carbohydrate, as well as GLC analysis of cellular fatty acids, to distinguish between species of Neisseria.

Research of bacterial lipids using gas-liquid chromatography, up to the present time has employed several approaches: some workers have analyzed chemical compounds extracted directly from cells (37, 68), others have separated out the membrane components and worked with these (79), some have confined themselves to the metabolic products of bacterial growth left in the media (63, 69, 19), workers have thermally decomposed the organisms and have studied the products of pyrolysis (77, 78, 36) while others have studied the bacteria by chromatographing the head-space vapors from milk (10).

Despite the amount of work done in this field, no one has attempted to differentiate all the species within a single genus. It is therefore our aim to study one genus in detail, with the hope that a simple and uniform technique might be developed for classification.

The genus Corynebacterium was selected for this study since, at the present time, classical bacteriological techniques have not been able to classify this genus adequately. This is probably due to the fact that, except for C. diphtheriæ, these organisms are quite inactive, hence hard to tell apart and relatively unimportant from a medical point of view, due to their low pathogenicity. In several instances, the differentiation has been based upon isolation source, rather than upon biochemical or morphological properties.

Thus, our study takes on the following objectives:

(a) to use the technique of gas-liquid chromatography to distinguish one species of Corynebacterium from another on the basis of fatty acid composition.

(b) to analyze the cellular fatty acid composition of one genus as completely as possible at this time.

(c) to shed some light on the controversy which enshrouds the classification of this genus.

This thesis is divided into four sections. The first section includes a review of basic microbiology as well as a literature survey. The concepts of microbiology presented here are elementary to a bacteriologist but provide basic information to a chemist without a background of microbiology. The secondsection lists the sources of the materials used and describes the method employed. The third section discusses the method as well as the experimental findings. The fourth and final section summarizes the work done and presents the conclusions.

HISTORICAL LITERATURE REVIEW

SOME BASIC CONCEPTS OF MICROBIOLOGY

The history of microbiology can be traced back to Antony van Leeuwenhoek (1632-1723) whose greatest claim to fame was not the invention of the microscope but rather the discovery of the microbial world (82). When scientists were able to see this "vast world of microscopic creatures" with their own eyes, the science of microbiology began to develop. Scientists soon wondered where these various forms originated. Some believed that microorganisms were formed spontaneously from the nonliving materials present in infusions, while others believed that the "seeds" or "germs" of these microscopic creatures were always present in the air, from which they could enter and grow under suitable growth conditions.

From numerous scientific reports it began to become apparent that spontaneous generation in plants and animals does not occur. However, it took the experiments of Pasteur and Tyndall, which showed that living matter does not arise de novo on our planet, to finally discredit the doctrine of spontaneous generation.

The second major scientific advance which accurred in the 19th century was the recognition of biological evolution. In 1859 Darwin published his book "The Origin of Species", and for the first time man saw life as a historical process which began with the evolution of our planet, and gradually developed more complex organisms to yield the various kinds we have today.

Meanwhile the quest for scientific clues leading to the primary origin of life on earth continued. Two scientific hypotheses to account for this existed. The first suggested that spores transported through space from some other

cosmic habitation infected the earth. This hypothesis has lost significance as a result of our increasing knowledge of outer space. The second hypothesis expressed the belief, that living matter arose from the mutation of non-living matter on the earth itself. This also seemed hard to accept since even the simplest microorganism is very complex in organization and chemical structure. Not until fairly recently have men been able to show that the latter proposal is a possibility. Haldane and Oparin (82) pointed out that before any microorganism existed on earth, any organic chemicals that might have been formed by chemical means would have possessed far greater stability than at present since there were no microorganisms to destroy them. They suggested that at an early time in the earth's history, a massive synthesis and accumulation of organic matter took place by photochemical reactions in the primitive atmosphere. This matter accumulated in the primitive oceans where it underwent subsequent chemical change to produce molecules of an increased complexity. Thus chemical evolution led to self reproduction which resulted in the formation of cellular organisms and eventually biological evolution. If one accepts this explanation and applies these ideas to microorganisms, it is to be expected that no clear cut distinction between many of the bacterial species would exist, but a great overlap in structural characteristics would result. It is not hard to see where the difficulty lies in attempts to classify bacteria by relating organisms to their evolutionary stage (phylogenetic classification) especially since until only recently, no supporting evidence has been obtained from fossilization.

Until about 1830, the living world could be readily divided into two different kinds of organisns: plants and animals. As explorations of the microbial world became more intensive, it became evident that many microbial groups were

difficult to fit into either of the two traditional groups. In the light of evolutionary ideas of today, this can be understood by thinking of the microbial groups as having branched off the pathway before the two other biological lines leading to plants and animals appeared.

To avoid the arbitrary assignment of transitional groups to one or the other kingdom, Haeckel, in 1866, proposed the establishment of a third kingdom, Protista. He distinguished members of this kingdom from true plants and animals, on the basis of their simple biological organization: they are unicellular, or if multicellular, do not show characteristically different regions of tissue. The finer details of their cellular structure could not be revealed by scientists until the electron microscope had been developed. This showed the existence of two different kinds of cells among organisms: a highly evolved type called the eucaryotic cell found in all plants, animals and in several groups of protists, and a simple type, the procaryotic cell. On this basis, the protists were divided into two groups (a) higher protists (with an eucaryotic cell structure) consisting of fungi, protozoa and most algae; (b) lower protists (with procaryotic cell structure) containing all bacteria and the blue-green algae.

The basic difference between the two types of cells lies in the fact that the eucaryotic cell nucleus is separated from the cytoplasm by a nuclear membrane and contains highly organized sub-units called chromosomes, while the procaryotic cell does not have its nucleus and cytoplasm clearly differentiated and is less highly organized. The sub-divisions of the Protista are shown in Fig. 1 on the next page.

The lower protists can readily be subdivided into blue-green algae

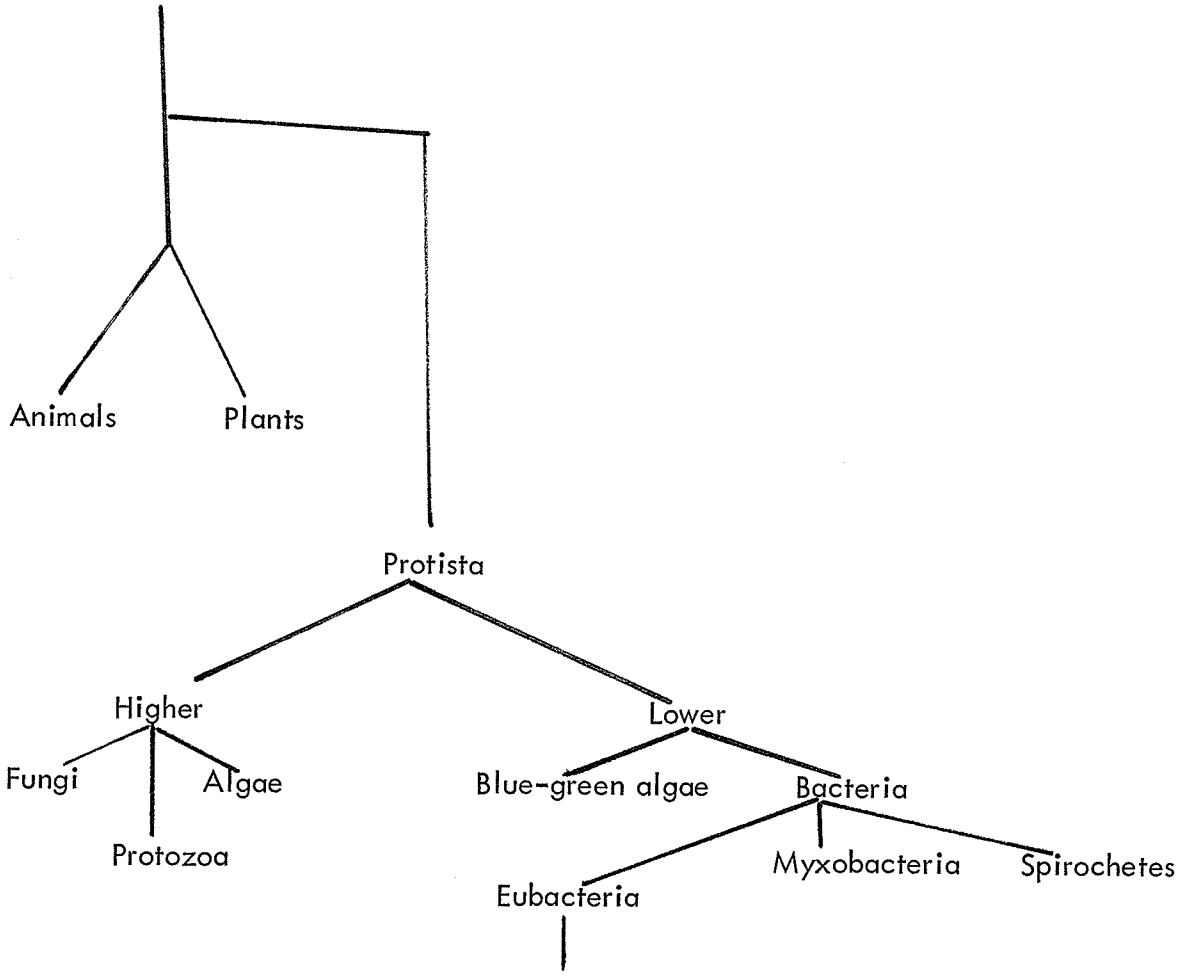


Fig. 1. Subdivisions of Protista.

and bacteria on the basis of certain distinctive characteristics common to the algae. The bacteria meanwhile can be further split into myxobacteria, spirochetes and eubacteria (or true bacteria) by considering their mechanism of movement and cell wall character. This brings us finally to the eubacteria which constitutes the largest and most diversified group of lower protists. Some of the basic structural characteristics in relation to chemical composition will be considered here, to provide some of the background upon which this research is based.

The presence of eubacteria was originally demonstrated by a differential staining procedure developed by Christian Gram in 1884 whereby bacteria are treated with crystal violet and iodine, followed by a washing in either acetone or alcohol. On this basis the "gram-positive" eubacteria turn violet and remain so after washing, while "Gram-negative" eubacteria become colorless after the solvent wash. While this was developed originally on an empirical basis, it became evident that the staining properties are correlated to some basic chemical difference of the cell. The difference was found to be in the cell wall: Gram-negative eubacteria have a high lipid content, while that of Gram-positive eubacteria is low (82). The cell wall, which is a protective structure designed to prevent the uptake of water in hypotonic solution to cause the cell to swell and burst, is mainly a macromolecular mesh composed of polysaccharides. In the lower protists, these polysaccharides are long chain polymers of simple sugars or amino sugars. Procaryotic organisms also have other chemical constituents in their cell walls such as lipids and proteins.

If one removes the cell wall enzymatically from Gram-positive bacteria, the existence of a cytoplasmic membrane may be demonstrated. Its

chemical composition is quite different from that of the cell wall, consisting mainly of protein and lipids with smaller amounts of RNA (82, 79). No membrane has been isolated from Gram-negative bacteria so far, due to difficulty in dissociating it from the cell wall.

The cytoplasm itself consists mainly of RNA found in the ribosomes. It may also contain reserve material such as poly-β-hydroxybutyric acid, polymerized inorganic metaphosphate or elemental sulphur.

The nucleus containing DNA has been discovered only recently, since nucleic acid staining resulted in the RNA of cytoplasm masking the DNA of the nucleus. Other parts of the bacterial cell are: (a) flagella -- long strands of fibrous protein always associated with "motility"; (b) capsules and slime layers -- organic polymers accumulated on the outer surface of some bacterial cell walls and usually composed of polysaccharides of varied composition; (c) endospores -- highly resistant bodies formed by some bacteria. The chemical structure is different from that of the original cell.

As can be seen from Fig. 1, page 9, eubacteria may be subdivided into many groups based upon a combination of shape, physiology and/or biochemical activities. This raises a difficult question in setting up taxonomic divisions, namely, which of these characteristics should be of primary importance. Should a group be established on the basis of similar physiological (or biochemical) activities while containing a variety of cell shapes, or should similar cell shapes be grouped containing a variety of physiological activities ?

This has caused a great confusion of

classifications to appear in the past. (25) but was resolved to an extent by Bergey's publication in 1923 of, "The Manual of Determinative Bacteriology" (12). The manual has gradually gained general acceptance in succeeding editions. Bergey attempted to establish a natural or phylogenetic classification in which organisms were grouped as much as possible according to the supposed degree of evolutionary relatedness. The order of precedence (in decreasing rank) as applied to the classification system is as follows:

Kingdom - Protista

Division - Lower protists

Class - Bacteria

Order - Eubacteria

Family - Corynebacteriaceæ

Genus - Corynebacterium

Species - (a) diphtheriæ

(b) xerosis

(c) pseudodiphtheriticum, etc.

A "species" has been defined as any primary subdivision of a genus and is considered by Cowan and Steel (30) as a basal unit. If a number of basal units containing similar characteristics are combined, we get the next higher unit, the genus; groups of genera combine to form a family, etc.

LITERATURE SURVEY

The family of bacteria we are mainly concerned with in this study are the Propionic acid and Corynebacteria (as Stanier (82) calls them) or Bergey's (17) Family XII, the Corynebacteriaceæ.

Stanier et al (82) give them this heading since propionic acid is produced in their metabolic products (as well as acetic acid, carbon dioxide and occasionally succinic acid). The corynebacteria can be distinguished from propionic acid bacteria by a physiological difference as may be shown by a comparison of their reactions (especially to oxygen) in Fig. 2, page 14 . They are club-shaped rods from which they derive their name: the Greek word (koryne) meaning a club and the Latin (Bacterion) a little staff (25). They are Gram-positive, rod-shaped bacteria that usually occur in palisade, V-form or cuneiform arrangements, as a result of their failure to separate completely during division. This peculiar mode of cell division is known as "snapping-fission".

The corynebacteria are a group of microorganisms characterised by their apparent lack of pathogenicity for man except for C. diphtherieæ which is notorious for its human pathogenicity. As a result, almost all the interest in the past has been concentrated on it while the remaining species of this genus have been left almost untouched. The disease diphtheria was recognized by Bretonneau (18) in 1826 but its cause was unknown. Two years later Trousseau (84) confirmed and extended Bretonneau's work. In 1883 Klebs (51) described the organism under the name of Microsporon diphthericum. However, he admitted having problems in obtaining pure cultures from diphtheritic membranes. Finally, however, in 1883 Löffler (58) showed that he could isolate the organisms in their characteristic arrangement from victims' organs, grow them on solidified blood serum and reproduce the disease experimentally in guinea pigs. The bacteria were given a specific rank by Trevisan between 1842 and 1890, but it was not until 1896 that Lehmann and Neumann (54) elevated the species of diphtheria and diphtheria-like organisms

	Propionic acid bacteria (Propionibacterium)	Coryneform bacteria (Corynebacterium)
Nature of energy-yielding metabolism	Propionic acid fermentation of sugars and usually of lactic acid	Respiration or sugar fermentation of lactic or propionic type
Relations to oxygen	Anaerobic	Aerobic; some facultatively anaerobic
Presence of cytochromes and catalase	+	+
Growth factors required (amino acids & vitamins)	few	few
Motility	absent	absent

Fig. 2. Distinguishing features of main groups of Gram-positive, non-spore forming rods.

to the generic rank and named them Corynebacterium. Since then, many organisms have turned up which fit this description in a general way and have been included in the genus Corynebacterium for lack of a better place. As a result of these inclusions the genus as a whole began to resemble the original less and less and began to develop characteristics of its own. The first edition of Bergey's Manual (12) split the genus into two groups of species: (a) the true diphtheria organism and (b) the "diphtheroid" group (non-toxigenic, parasitic, corynebacteria). To the latter category H. L. Jensen (44, 45) added a number of organisms found in soils of Australia and, worst of all (according to bacterial taxonomists), a plant pathogen. Soon other plant pathogens found their way into this genus until it was so broad and ill-defined that identification in many cases had to be based on isolation source. Five sources are now recognized: (1) human (2) domestic and laboratory animals (3) insects (4) plants and (5) soil and water.

In 1947 H. J. Conn (29) pointed out that with this tendency to include a greater and greater variety of organisms there would soon be no reason for excluding almost any Gram-positive, non-spore forming rod. He suggested that the genus be re-defined to exclude some of the microorganisms, especially the plant and soil organisms, whose characteristics did not agree very well with the rest. Clark (27) and Murray (15) voiced their agreement and argued that limitations for the genus should be established. With this in mind, Murray and Cleveland (25, 16, 26) set out to obtain as complete a collection of Corynebacteria species as possible and, with a standard method, record the activities of the organisms to define the limits of the genus. By morphology, carbohydrate fermentations and biochemical reactions

they showed that a classification not entirely dependent upon source information could be made. However, the reactions of the plant and soil organisms did not differ enough from the others to suggest that they be placed in another genus.

Meanwhile in other areas of research, new approaches were being investigated for possible use in bacterial taxonomy. Chemical compounds were beginning to be extracted from cells which were part of the cell structure, with the hope that these might provide a clue to the relationship between species. In 1955, Hofmann (47) used column chromatography to show variations in fatty acid content of Lactobacilli, while Cummins and Harris (31) a year later, using paper chromatography to determine amino acids, amino peptides and sugars, showed that cell wall composition could be used to distinguish between certain families of Gram-positive bacteria. Wolochow (85) felt that extraction and identification of structural elements could be used to differentiate between individual species based on the fact that these differences were governed by natural or evolutionary relationships. Almost a decade later Boone and Pine (14) refined the method and were able to separate certain species of the genus Actinomyces on this basis. Later, amino acid analysis alone was found useful in differentiating anaerobic corynebacteria from certain aerobic members of the genus (48). Other approaches have been developed recently which include polyacrylamide gel electrophoresis (76), DNA base composition of cells (38) and nucleic acid hybridization (9).

The most extensive and rewarding work has been concerned with lipid determination of bacterial cells or cell walls. The event mainly responsible for this was the introduction of the gas chromatograph by James and Martin in 1952

(41). Here, finally was an instrument that would permit development of methods that were relatively simple and yet so sensitive that only extremely small samples were required. Four years after its introduction, they were able to use GLC to determine the fatty acids found in culture media upon which Pseudomonas aeruginosa had been grown (42). Following this, the application of gas-liquid chromatography (GLC) to microbiology began to grow. Cason and Tavs (21) studied fatty acid composition of Mycobacterium tuberculosis, while Asselineau (7) suggested that GLC could be applied to determination of any bacterial fatty acids.

With gas chromatography a very promising new tool, a number of approaches to lipid analysis were undertaken by workers in the field. Whole bacterial cells were pyrolyzed and the products analysed by GLC (7, 77, 78, 37, 36), with conflicting reports as to the usefulness of this method. Using a modification of the Davison method (32), Reiner (77) was able to produce unique "pyrograms" for various strains of E. coli which he felt were reproducible. While Garner and Gennaro (37) agreed with Reiner and felt that this method could be applied to taxonomy of microorganisms, Fontanges et al (36) disagreed. Following a systematic study they agreed that although certain species could be identified by this means, nevertheless most of them showed a similar pyrogram or one very close to that of the culture media. Hence they felt that this technique could not be used for bacterial taxonomy.

Staining procedures, especially the Gram stain, indicated that bacterial cell walls had a considerable lipid content so several workers isolated bacterial membranes or "envelopes" and determined the fatty acid composition of these (24, 18, 13). However, none of these data were applied to classification.

In a follow-up to earlier work of James and Martin (42) lipid extracts of growth medium were checked by the following men, who used GLC to determine metabolic products. Bassett and Claydon (10) chromatographed the head space vapors of milk in an attempt to characterize the bacteria present. Lewis, Moss and Jones (56) identified the volatile fatty acids of Clostridium using the conditions for gas chromatography as described by Henis, Gould and Alexander (39). O'Brien (69) applied the technique of Miturka and Alexander (63) to metabolic products of glucose metabolism, while Bowden and Bassette (11) used volatile metabolic products to distinguish between E. coli and Aerobacter aerogenes.

Because of its simplicity and apparent potential in characterization of bacterial species, the most popular approach to lipid analysis has been the GLC analysis of cellular fatty acids. The whole cells are washed, hydrolyzed, fatty acids extracted, methylated and determined by gas chromatography. Using this method in 1963, Abel, deSchmertzing and Peterson (1) examined the fatty acid composition of a wide range of bacteria and attempted to correlate lipid composition of these bacteria with their taxonomic classification. They concluded that the idea had potential feasibility, as they were able to demonstrate differences at the family level.

The following year, 1964, Brown and Cosenza (20) in a similar study were able to distinguish between the genera Gaffkya and Micrococcus of the family Micrococcaceae. In the same year, Yamakawa and Ueta (86) went one step farther and, using GLC analysis of cellular fatty acids plus carbohydrate studies, showed that they could differentiate bacteria at the species level: they were able to distinguish Neisseria haemolysans from other Neisseria species.

Since that time, several workers have used GLC for analysis of fatty acid methyl esters of microorganisms and have established some taxonomic relationships by comparing their profiles (49, 68, 67, 57, 75, 66). For example, Kaneda (49) was able to distinguish Bacillus cereus from nine other Bacillus species on the basis of iso C_{15} content.

Moss and Lewis (68), in the first of a series of reports, stated that they were able to distinguish three species of Clostridium from each other and from other species. Later, however, in nine species of Neisseria they found a fairly homogeneous group of fatty acids, none of which were distinct for this genus. They next turned their attention to the "anaerobic diphtheroids" following a recommendation by the American Society for Microbiology that the anaerobic species of corynebacteria be placed in the genus Propionibacterium (ASM News, page 29-30, August 1966). They eventually showed that the seven species of Propionibacterium could be split into two groups based on their C_{15} saturated branched chain fatty acid content. Group I, whose predominant fatty acid was the ante iso C_{15} isomer, contained P. freudenreichii and P. shermanii while group II, which had iso C_{15} predominating, contained P. arabinosum, P. jensenii, P. pentosaceum, P. theonii and P. zeæ. Six anaerobic corynebacteria species were found to have patterns very much alike and somewhat similar to the patterns of the Group II Propionibacterium. However, they could easily be distinguished from Group I Propionibacterium species. An investigation of Listeria monocytogenes (75) revealed a fatty acid profile, also similar to that of Propionibacterium, but the species of Listeria were not consistent among themselves. They had either ante iso C_{15} or iso C_{15} as the major fatty acid present, although the former was present in amounts four times that of the latter. As a result

of this work they considered recommending, in agreement with Douglas and Gunter (33) and Moore and Cato (64), the change of C. acnes to P. acnes, and in any case designation of C. acnes as the only anaerobic species of the genus Corynebacterium.

The most recent advance in methodology along these lines was introduced by Farshtchi and Moss (35) in 1969. After the application of Trimethysilyl (TMS) derivatives for chromatographic identification of many organic compounds (81) it was successfully applied to biological material (83). Farshtchi and Moss (35) applied this technique to the TMS derivatives of whole cell hemolysates and found it to have good potential as a method for differentiation of bacteria.

The chemical nature of bacterial lipids has been summarized in a number of good reviews (72, 70, 50, 55, 8, 59). Some of these authors (8) have pointed out that bacterial lipids differ from those of higher forms of life in several respects: the presence of large proportions of free fatty acids, the frequent presence of unusual fatty acids not seen in other organisms, and the absence of sterols as well as classical lecithins and cephalins.

Early studies were confined mainly to lipid determinations as percentage of total weight, however they did show that in most bacterial species lipid contents range between one and ten percent of dry cell weight (70). Of the total lipid, free fatty acids frequently make up less than ten percent but of total fatty acids more than twenty percent often occurred as free fatty acids (70). In one organism, Salmonella typhimurium, the lipid fraction has been reported to consist almost entirely of free fatty acids (8).

In general, the chain length of various free fatty acid types ranges from C_{14} to C_{23} (55). Palmitic (C_{16}) acid is the most frequent and, usually, most

abundant of any saturated straight chain fatty acid found.* C_{19} to C_{28} saturated straight chain fatty acids occur only in a few species. The most common unsaturated fatty acids are cis, 9, 10 - hexadecenoic ($C_{16:1}$) and cis, 9, 10- and 11, 12-octadecenoic acids with the latter two the most frequently encountered. Unsaturated fatty acids longer than C_{18} have been reported in C. diphtheriæ (3, 3a, 2, 4, 74). The latter three unsaturated fatty acids have been shown to be precursors of the corresponding cyclopropane fatty acids, cis 9, 10-methylenehexadecanoic acid, cis 9, 10 - and 11, 12-methylene-octadecanoic acid. The most commonly encountered branched chain fatty acids are 12- or 13-methyltetradecanoic acids (anteiso C_{15} and iso C_{15}) and 15-methylhexadecanoic (iso C_{17}) acid (65, 49). Occasional reports of hydroxy acids can be found (70) but their significance is unknown. Some complex hydroxy acids peculiar to C. diphtheriæ were reported by Asselineau (5, 8) named corynemycolic acid ($C_{32}H_{64}O_3$), corynemycolenic acid ($C_{32}H_{62}O_3$) and corynine ($C_{52}H_{104}O_4$) a dihydroxy compound.

A clear-cut distinction between the fatty acids of Gram-positive and Gram-negative bacteria appears to exist (55). Practically all the Gram-positive organisms contain branched chain fatty acids; often they are its major component. On the other hand, little or no branched chain acids have been found in Gram-negative organisms, which typically contain saturated, unsaturated and cyclopropane fatty acids. Marr and Ingraham (61) pointed out that bacterial growth time should be kept constant in making comparisons like this since they found the cyclopropane methylene/ fatty acids, /hexadecanoic and methyleneoctadecanoic, to be formed only after the cessation of exponential growth.

Other lipid fractions found in bacteria have not been established to

* Nomenclature: See page 80.

the extent of the fatty acids. Mono-, di- and triglycerides have been reported in bacteria (8) but many species apparently contain none at all. In early studies, Chargaff (22, 23) showed that C. diphtheriæ contained about five percent free lipids, eighty percent of which was triglycerides; the remainder consisted of "waxes" and small amounts of phosphatides. Waxes appear to be uncommon to bacteria except for mycobacteria and corynebacteria (8) where they appear in very small amounts. Phospholipids (or phosphatides) are widespread in bacteria, frequently representing major portions of the total lipid content but they have not been investigated with modern analytical techniques (60). Glycolipids are well known among bacterial constituents (53). The carbohydrates isolated from glycolipids are: glucose, galactose, arabinose, mannose, rhamnose, trehalose, as well as amino sugars. Lipopolysaccharides are merely complex glycolipid polymers.

Most of the lipid analysis done in the genus Corynebacterium were concerned with one species, C. diphtheriæ, which has by now received a thorough "going-over" (8, 22, 23, 5, 3, 3a, 2, 4, 74). A recent study of C. diphtheriæ cells walls (62) found its components to be of three types: 27.7% glycosaminopeptides, 20.6% free lipids, and 40.6% polysaccharides of pentose and hexose. No interest has been shown in other members of this family until recently. We have already referred to the work of Moss and Lewis (67) on the anaerobic corynebacteria. Lipid studies on aerobic corynebacteria have been limited to carotenoids of C. fascians (73), lipids of C. ovis (52) and electron microscope studies of cell walls of C. sepedonicum (80).

EXPERIMENTAL

MATERIALS

Media:

Tryptic Soy Broth Lot #443709	Difco Laboratories, Inc.
Trypticase Soy Broth Lot. #803675	Baltimore Biological Laboratories
Mueller-Hinton Medium Lot #602633	Baltimore Biological Laboratories
Loeffler Medium Lot #706325	Baltimore Biological Laboratories
Trypticase Soy Agar Lot #810676	Baltimore Biological Laboratories
Blood Agar (25cc blood/500cc Trypti-case Soy Agar Lot. #810676)	Baltimore Biological Laboratories

Reagents:

Hexane (Redistilled) - Certified ACS Grade	Fisher Scientific Co.
Potassium Hydroxide - Reagent ACS Grade	Anachemia Chem. Ltd.
Methyl Alcohol (absolute) 'Baker Analyzed' Reagent	J.T. Baker Chem. Co.
Hydrochloric Acid - ASP Grade	Can. Indust. Ltd.
14% w/v Boron Trifluoride-Methanol Lot #784-25 Lot # 0048	Applied Science Laboratories
Ethyl Ether USP	E.R. Squibb & Sons, Ltd.

Chromatographic Materials

SE-30 Silicone Gum Rubber (Methyl)	Wilkens Instrument & Res. Inc.
Gas Chrom Q (100-120 mesh) Lot 749/28	Applied Science Lab. Inc., State College, Pa.

Chromatographic Materials cont'd

6% LAC-728 on Diatoport S (80-100 mesh)	Hewlett-Packard, Toronto, Ont.
Silica Gel G (Merck)	Kensington Scientific Corp. Oakland Calif.
Thin Layer Chromatography Spreader (Kensco)	Kensington Scientific Corp.

Reference Standards:

National Institute of Health Type Mixture Kits: Applied Science Laboratories.

Mixture C: C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}.

Mixture D: C_{14}, C_{16}, $C_{16:1}$, C_{18}, $C_{18:1}$

Mixture F: C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}.

BC Mix L:

iC_{14}	Methyl 12-Methyltridecanoate
nC_{14}	Methyl Myristate
aC_{15}	Methyl 12-methyltetradecanoate
nC_{15}	Methyl Pentadecanoate
iC_{16}	Methyl 14-methylpentadecanoate
nC_{16}	Methyl Palmitate
aC_{17}	Methyl 14-methylhexadecanoate

BC Mix 1:

iC_{18}	Methyl 16-Methylheptadecanoate
nC_{18}	Methyl Octadecanoate
aC_{19}	Methyl 16-Methyloctadecanoate
iC_{20}	Methyl 18-Methylnonadecanoate
nC_{20}	Methyl Eicosanoate
aC_{21}	Methyl 18-Methyleicosanoate

Mix H103:

C_{15}	Methyl Pentadecanoate
C_{16}	Methyl Palmitate
C_{17}	Methyl Heptadecanoate
C_{18}	Methyl Stearate (Octadecanoate)

Mix L205:

C_{18}	Methyl Stearate
$C_{18:1}$	Methyl Oleate
$C_{18:2}$	Methyl Linoleate
$C_{18:3}$	Methyl Linolineate

Cultures

All cultures used in this study were obtained from the collections of Dr. E.M.D. Cleveland (25). The nomenclature used to describe the various species shall be that outlined in Bergey's Manual (17), unless otherwise stated in the text. The origin of these cultures is detailed below:

Number	Genus and species	Origin
C-69	Nocardia corallina	Dr. O. Khairet, U. of Manitoba
DL7813	C. xerosis	Deer Lodge Hospital Isolate
DL210B	C. diphtheriæ	Deer Lodge Hospital Isolate
6981	C. pseudodiphtheriticum	ATCC
Mc97	L. monocytogenes	Dr. E.G.D. Murray, U. of McGill
3224	C. bovis	NCTC
1621	C. equi	NCTC
CF-1	C. fascians	Dr. W.H. Burckholder, Cornell University

Cultures cont'd

Number	Genus and species	Origin
CM-1	C. michiganense	Dr. W.H. Burckholder, Cornell University
2A	C. insidiosum	Dr. W.H. Burckholder, Cornell University
U61A	C. diphtheriæ	University of Manitoba Isolate
U61B	C. diphtheriæ	University of Manitoba Isolate
7005	C. hoagii	ATCC
373	C. xerosis	ATCC
7711	C. xerosis	ATCC
7562	C. creatinovorans (now Arthrobacter ureafaciens)	ATCC
7906	C. ulcerans	NCTC
149	C. "Q"	Dr. M.O. Pollock, Lister Inst.
1033	C. pseudotuberculosis	NCTC
8155	C. enzymicum	ATCC
6451	C. renale	NCTC
934	C. segmentosum	NCTC
CF-3	C. flaccumfaciens	Dr. W.H. Burckholder, Cornell U.
CP-2	C. pointsettiæ	Dr. W.H. Burckholder, Cornell U.
10700	C. pseudodiphtheriticum	ATCC
10701	C. pseudodiphtheriticum	ATCC
DL-1	E. coli	Deer Lodge Isolate

****

NCTC - National Culture Type Collection

ATCC - American Type Culture Collection

METHOD.

The corynebacteria used for this study were lyophilized cultures from the collection of Dr. E. M. D. Cleveland. These cultures were reactivated on slants of Loeffler's medium, and incubated at 37°C or 25°C, depending upon their demands, until growth was established. They were then checked by him as regards purity and cultural characteristics using standard microscopic, cultural, biochemical and toxigenic tests.

All organisms were subcultured into 10 ml. of low fat medium, Tryptic Soy Broth (Difco), and allowed to grow for 96 hours. The same lot of medium was used throughout this study. They were then subcultured into a second contained of TSB, and allowed to incubate for a further 96 hour period. This procedure eliminated any differences in fatty acid pattern which may have been caused by the medium of original isolation.

The bacteria were then harvested in total by centrifugation as a precipitate from the medium. The medium was aspirated off, and the bacteria washed three times with distilled water. This step removed any adherent medium which might contaminate the analysis. The washed bacteria were then subjected to chemical treatment. This involved saponification and acidification to free the fatty acids, followed by extraction of these into hexane. The fatty acids were then esterified and the solvent evaporated to dryness.

The fatty acid methyl esters were separated on two different columns using the technique of gas liquid chromatography. The resulting chromatogram was then examined. The distinctive peaks were located and the relative percent of the total of each calculated.

Details of the procedure are presented in the following section.

A. Growth Conditions

i) Medium:

To insure uniformity and growth in a low fat environment all organisms were subcultured into a tube (20 x 100mm) containing Tryptic Soy Broth (TSB, 10 ml) using a sterile wire loop. TSB (pH 7.3) contains 1.7% Tryptose (Difco), 0.3% Soytone (Difco), 0.25% dextrose (Difco), 0.5% NaCl and 0.35% K_2HPO_4. All media was autoclaved at 15 lbs psi (121°C) for 20 minutes immediately following preparation and stored at 4°C in a refrigerator until required.

a) Initial Growth:

The cultures were then allowed to grow for 96 hours at 37°C (or 25°C if growth was prohibited at the higher temperature). The choice of temperature was based upon previous knowledge of the growth requirements.

b) Secondary Growth:

The cultures were then subcultured once more into a flat-bottom glass jar (150 ml) containing TSB (10 ml) using a sterile Pasteur pipette and allowed to grow for a further 96 hours. In cases where growth following this was not of sufficient quantity for analysis, the procedure was repeated with a larger initial growth sample, and subcultured into an Erlenmeyer flask (250 ml) containing TSB (100 ml).

B. Harvesting:

At the end of precisely 96 hours incubation, the cultures were centrifuged and the medium aspirated off. The residue of bacterial bodies thus obtained was washed 3 times with distilled water and suspended in alcoholic KOH (20 ml, 15% (w/v) KOH in 50% (v/v) methanol.)

C. Chemical Preparation

The method used was that of Moss and Lewis (68) with some minor modifications. These are discussed in the following section, "Analysis of Experimental Method". The methanolic-KOH suspension was subjected to saponification at 70°C for 4 hours in a flask (100 ml) connected to a reflux condenser. After cooling, the non-saponifiable material was extracted with redistilled hexane (3 x 10ml). The residue (aqueous layer) was acidified to pH 2 with concentrated HCl and the free fatty acids extracted into redistilled hexane (3 x 10ml). The three extracts were combined into a centrifuge tube (50 ml) and evaporated to dryness under vacuum while shaking with a mechanical shaker. Final drying was facilitated by a stream of nitrogen at room temperature. The fatty acid residue was mixed with Boron Trifluoride-Methanol reagent (2 ml, 14% w/v) and heated for 5 minutes in an 80°C water bath to facilitate methylation. The flask was then cooled and distilled water (10 ml) added. The fatty acid methyl esters were extracted by shaking for 1 minute with hexane (3 x 10 ml). The hexane was evaporated to dryness as before, transferred to a microcentrifuge tube with two hexane rinses and evaporated under a nitrogen stream. The tubes were stoppered using an aluminum foil liner to prevent any contact with organic matter and stored at -10°C under nitrogen until ready for analysis. The fatty acid methyl ester residue was then redissolved in 10 - 15 microlitres of redistilled hexane.

D. Gas Liquid Chromatography

All gas chromatographic analyses were performed on dual column F and M Scientific Model 402 Gas Chromatograph (Hewlett-Packard, Toronto) equipped with dual hydrogen flame ionization detectors.

All samples were analyzed with both polar and non-polar liquid phase coated supports, in 6 foot U-shaped glass columns having 1/4" o.d. and 1/8" i.d. The non-polar phase was 1.5% methyl silicone rubber gum (SE-30) on Gas Chrom Q (100-120 mesh). This column was prepared as described by Haahti (46), details of which are given in the following section. This column was operated at a pressure of 40 psi (60 ml/min) using helium as carrier gas and was temperature programmed from 95°C to 275°C at 3° per minute with the sample injected when the temperature reached 100°C. Other parameters were: Flash-heater temperature 180°C, detector temperature 230°C, hydrogen 15 psi(35 ml/min), air 20 psi(300ml/min), recorder speed 15" per hour.

The polar column used was 6% LAC-728 on Diatoport S (80-100 mesh) prepared commercially and purchased from Hewlett-Packard, Toronto. (This column is a DEGS, diethyleneglycol succinate type, used commonly in fatty acid work. However, company specifications indicated LAC-728 to have better stability). The operating parameters were the same as stated above except for the following changes: the column was operated isothermally at 180°C, flash-heater temperature 220°C, detector temperature 260°C.

For routine analysis two or three microlitres of the methyl ester solution were injected directly into the carrier gas stream.

The peaks were identified by careful comparison of retention times related to those for highly purified standards of fatty acid methyl esters obtained from Applied Science Lab., State College. Pa. The peaks areas were calculated by multiplying peak height by peak width at 1/2 the peak height. The evaluation was shown to be as accurate as the methods of peak wt. and planimetry. After peak

areas had been determined, the percentage of the total represented by each peak was calculated. This was done to convert the quantitative amount of each fatty acid entity to a relative basis, to eliminate the problem of peak size difference introduced by analysing different amounts of bacteria. (See Fig. 3 and Fig. 4).

E. Miscellaneous

i. Preparation of 1.5% SE-30 on Gas Chrom Q

One volume of siliconized support (Gas Chrom Q) was suspended in 4 volumes of a 1.5% solution of stationary phase(SE-30) in toluene. Air was removed from the support with vacuum and the mixture shaken vigorously. The support was separated by means of a Buchner-type funnel using a coarse filter paper and the coating solution drawn off under reduced pressure, until the solvent flow or foaming was over. The support was spread on filter paper in a thin layer and dried in an oven at 110°C.

This coating procedure was reported (46) to be reproducible and convenient. In general, n% (w/v) coating solution gave approximately n% (w/w) coated porous support.

ii. GLC sample injection technique

The "solvent flush" injection technique was found to give good reproducibility for injection of small samples. The plunger of a clean, dry syringe was washed with solvent and wiped with tissue paper or towel. The plunger was then inserted into the syringe taking care not to touch the plunger body with the fingers. The syringe was repeatedly filled with solvent to wet the barrel and plunger. For two microlitres of flush, the needle was dipped into solvent and the plunger pulled back to a reading of 1.2 microlitres, keeping in mind that the needle volume is approxi-

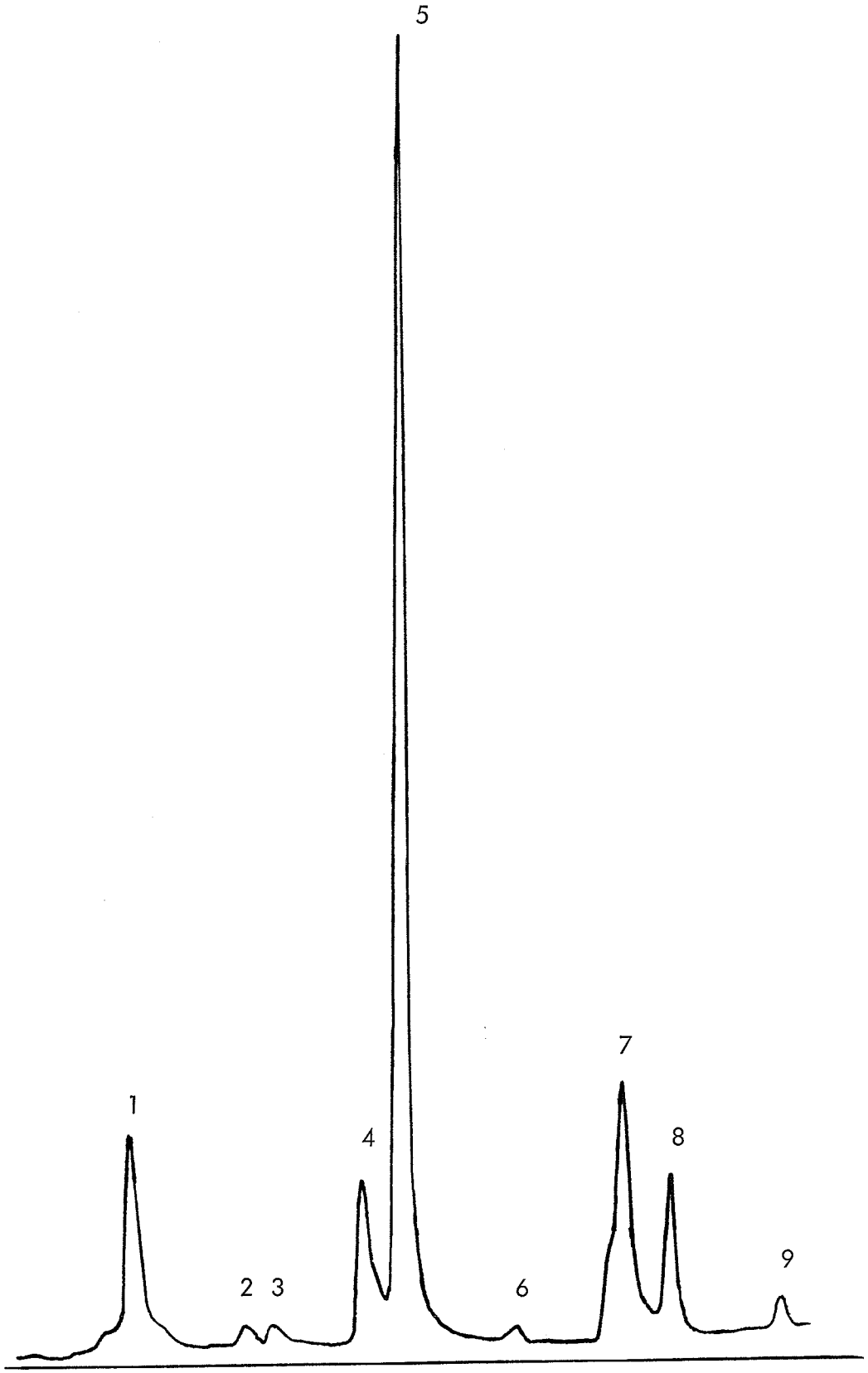


Fig. 3. An example of a chromatogram and arbitrary peak identification.

Peak	Ht. (mm)	W. (mm)	Area	Relative %
1	34	2.5	85.0	10.2
2	3	3.0	9.0	1.1
3	3	3.0	9.0	1.1
4	27	3.0	81.0	9.7
5	211	2.0	422.0	51.1
6	2	4.0	8.0	1.0
7a	13	2.5	32.5	3.9
7b	41	2.5	103.0	12.4
8	27	2.5	67.5	8.1
9	5	2.5	12.5	1.4
Total			824.5	100.0

Fig. 4a. Example of the relative peak calculation.

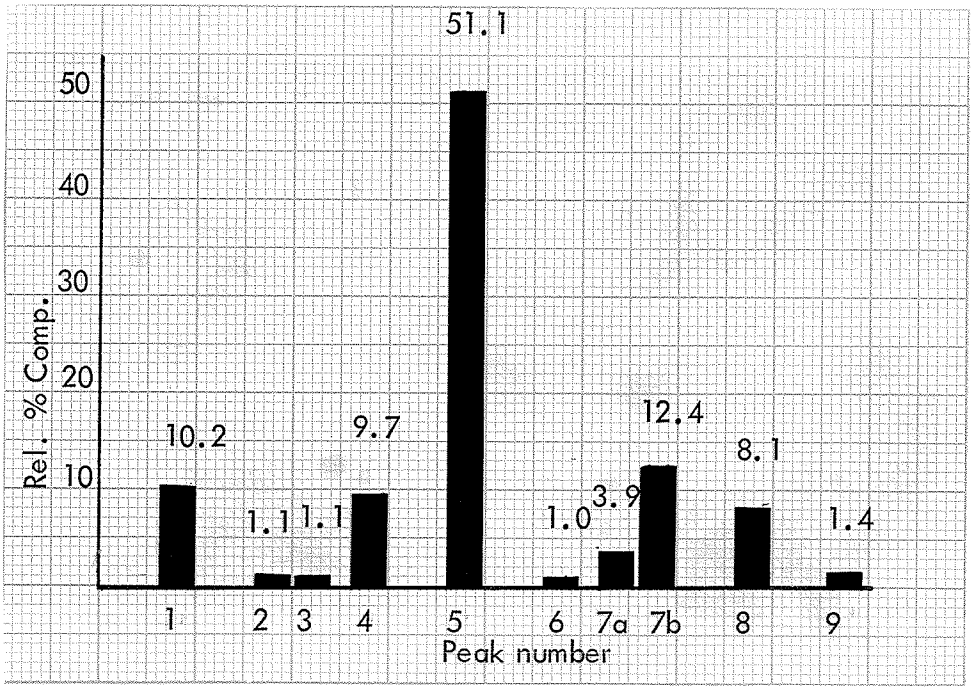
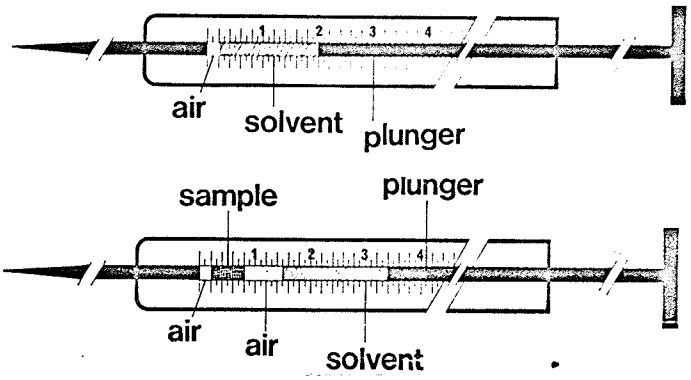


Fig. 4b. Example of the graphical representation of data.

mately 0.8 microlitres. The needle was then removed from the solvent and the plunger pulled back until an airspace was visible at the needle end of the syringe (see fig. A). The needle was now immersed in the sample and the desired amount drawn into the syringe. In fig. B , 0.6 microlitres sample size is shown. The needle was removed from the solution, the plunger pulled back until the sample size could be read and injected into the chromatograph.



A.

B.

iii. Preparation of microcentrifuge tubes

The fine end of a Pasteur pipette was cut off and sealed by heating, so that a tapered length of approximately 1 inch was obtained. The pipette was cut with a file, at a point 1" above the tapered portion, and this cut firepolished. In this way we obtained a centrifuge tube, 2" in length, which would allow the needle of a Hamilton syringe to go right to the bottom. Fatty acid samples could be dissolved in a volume of 10-15 microlitres and a sample of 2-3 microlitres easily removed.

iv. Thin layer chromatography

Standard plates 2" x 8" Pyrex glass were used as the support for T. L.C. The spreader gate was adjusted to give a 250μ film thickness. 25 grams of Silica Gel G was added to 50 ml water in a 250 ml Erlenmeyer flask, stoppered, and shaken immediately for 1 minute. The slurry was then poured into the spreader and

the plates coated. After allowing the plates to set, they were activated at 110°C for one hour, then stored in a dessicator cabinet over indicating Silica Gel until used. Plates were not activated again before use but were used only if not more than one week old.

Immediately before use, the sides of each plate were evened by running a fingernail along each side. The movement of the solvent front was stopped by scoring the plate with a sharp pencil at the point desired (usually 10 cm from sample application point). The sample was spotted with a Pasteur pipette drawn to a fine tip or ultimately with a Hamilton syringe.

The tank containing fresh solvent was lined with a paper towel and the solvent allowed to rise to the top indicating the atmosphere was saturated. The solvent used in this case was hexane:ether:glacialacetic acid in proportions of 85: 15:2 respectively. After the plate was developed it was removed from the tank, dried under an air current and sprayed with 50% sulphuric acid. The plate was then heated on a hot plate at 150°C until fuming ceased.

When this method was used for preparative work, the plate was either sprayed with distilled water to locate the spots or another plate run under identical conditions and spots developed. The fatty acid esters could be recovered by scraping the area desired into a centrifuge tube and eluting with hexane.

RESULTS AND DISCUSSION

ANALYSIS OF EXPERIMENTAL METHOD

A. General Discussion.

In choosing a medium for bacterial growth we were concerned with two things: providing nourishment for the bacteria, and avoiding fatty acid contamination by medium lipids. Obviously, the ideal medium would contain no fatty acid. However, since corynebacteria are very demanding and require a certain amount of fatty acids we were forced to use the lowest fatty acid containing medium which still supported growth. Various media were checked and the one selected was Tryptic Soy Broth.

It had been observed that the same bacteria grown on different media would exhibit slightly different fatty acid patterns. We were able to show that if a culture was plated onto Loeffler's medium and Blood agar medium, two slightly different patterns arose. If the two cultures were inoculated into TSB the fatty acid patterns became quite similar. If subcultured twice into TSB, the patterns became identical. Thus, two successive subcultures in TSB eliminated differences due to the medium of original isolation.

We observed that the fatty acid content of the bacteria varied somewhat with the length of time they were allowed to grow. We therefore determined a "growth curve" using spectroturbidimetric techniques and analyzed the fatty acid composition at various points on the curve. This demonstrated that the composition was quite consistent at points in the same region of the curve but different at points more widely separated on the curve. Since we had decided, for the sake of convenience, to harvest at the end of 96 hours incubation, we set about to determine how much variation in fatty acid pattern would occur from 72 to 124 hours.

Parallel cultures were estblished and harvested at 72, 92, 96, 100, 124 hours. It was seen that the fatty acid pattern changed only slightly over this period and that the three cultures analyzed on the fourth day were in close agreement. This showed that the time for harvesting bacteria was not absolutely critical but, if harvested at 96 hours, no problem due to time change should interfere.

A liquid medium rather than a solid medium was decided on. Broth cultures can be harvested by centrifugation and aspiration -- solid media cultures by surface washing. Therefore, Tryptic Soy Broth was selected because it provided an easier, more uniform, and more complete method of harvesting the bacteria.

The washing technique was next examined. A series of cultures was grown and each was washed a different number of times. It was shown that the pattern changed with successive washing. This was probably due to removal of adherent medium. It was seen that by the third wash the pattern remained stable, (no more medium being removed). Thus, three washes was deemed sufficient to remove medium contamination. As a further verification of the efficiency of the washing procedure, the water washes were run on a thin layer chromatography plate. This demonstrated that much material was removed in the first wash, a small amount removed in the second wash, and no detectable material in the third wash. Thus, once again, three washings were shown to be sufficient.

A trial was set up to determine the best solvent for extraction from the saponification mixture. It was seen that pure hexane was more efficient than the previously suggested hexane-ether mixture. A further trial demonstrated that the chemical preparation technique produced a chromatographic pattern entirely made up of fatty acid methyl esters and was therefore deemed valid.

Using various accepted methods for determining the relative peak amounts, we concluded that the quickest, most efficient and most accurate technique was that of measuring the peak height and multiplying by the peak width at one half peak height to determine the area.

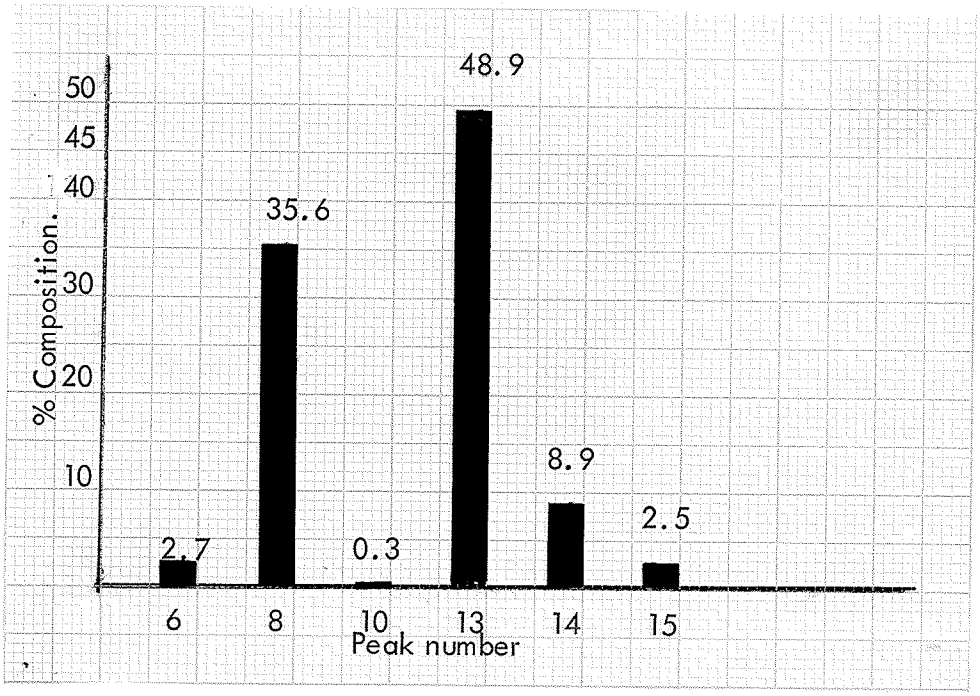
The reproducibility of the entire procedure was determined by analyzing the fatty acid pattern of 15 identical cultures. It was shown that on a qualitative basis the runs agreed with 100% accuracy. On a quantitative basis it was shown that any run using our technique would have a 99% probability that the peaks lie within 4.2% of the determined value. The practical implication of this result was that, to differentiate between the peaks of different bacteria, there must be a difference of 8.4%.

Details of the procedure outlined above will now be considered.

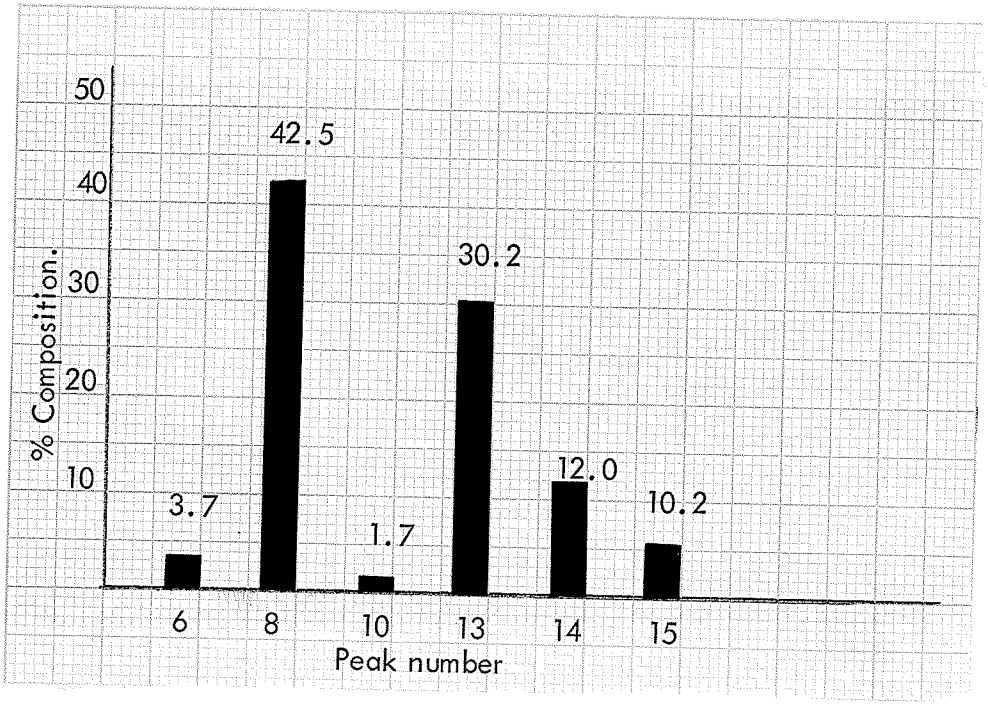
B. Growth Conditions.

i. Medium.

It was observed by Fontanges et al (36), and confirmed by ourselves that the same bacteria grown on different media would exhibit different fatty acid composition when analyzed (Fig.5, page 41). This was due to three possible factors: (1) that harvesting and washing techniques allowed a certain amount of the medium to be carried over into the saponification mixture; (2) that a certain amount of medium was adsorbed onto or included in the bacteria and was not removed during the washing process ; (3) that different nutrient material in different media would force the bacteria to use different raw materials, and thus possibly change the fatty acid content of the bacteria. For this reason an ideal medium would contain no fatty acids. But corynebacteria are generally very demanding in



a) Loeffler"s Medium.



b) Blood Agar Medium.

Fig 5 . Fatty acid composition of C. pseudodiphtheriticum grown on two different media.

their growth requirements and a fat-free medium was thought to be impossible. In fact, it was shown by Pollock and co-workers (71) that fatty acids, notably oleic, are essential for the growth of Corynebacterium "Q". With this knowledge available it was felt that the only alternative would be to use a medium which would support all species and yet demonstrate a very low fatty acid content. Thus, various media were analyzed for the fatty acid content and the chromatogram shown on page 89. In all cases this represents the total fatty acids obtained from 10 ml of media.

From these results it may be seen that either Tryptic Soy Broth or Trypticase Soy Broth could be used. These media are basically the same, but manufactured by two different companies. Tryptic Soy Broth (Difco) was chosen and the same lot used throughout the study to avoid any change that the medium might cause. This would eliminate the problems due to the three aforementioned causes for differential media growth, namely: (1) since the fatty acid content of the entire medium was insignificant when compared to the fatty acid content of the bacteria, it was inconceivable that carry-over of small amounts of medium could alter the fatty acid pattern; (2) at harvest, after centrifugation, the volume of bacterial bodies was usually about 0.5 ml. It is obvious that if the entire medium (10 ml) would not produce significant interference then 1/20th of this amount was of no concern. Furthermore, even if the bacteria had included enough medium to completely fill their bodies(which, of course, they cannot do) they could not include more than 0.5 ml, an insignificant amount. Thus the problem of medium adsorption and/or inclusion was justifiably ignorable. (3) If we assume that nutrient material in the medium could affect the fatty acid content of the bacteria, we must standardize the culture procedure to equalize the process. For this reason, the bacteria were subcultured twice in Tryptic Soy Broth.

By doing this the bacteria would fully adapt to this environment and would show no difference due to the media from whence they originated.

To study this, a C. pseudodiphtheriticum species was cultured on a Blood Agar Plate and on a Loeffler's slant. After 72 hours they were each subcultured into 10 ml of Tryptic Soy Broth. The bacteria from the Blood agar and the Loeffler's were then analyzed for fatty acid content. The results may be seen in Fig. 6. After the first subcultures had been incubated for 96 hours they were subcultured a second time into TSB and the remaining bacteria analyzed. It is seen that a slight difference still existed. The second subculture was allowed to incubate for 96 hours and the bacteria analyzed. The fatty acid patterns are now identical. Thus, by two successive cultures, in a standardized medium any differences due to the original isolation medium were nullified.

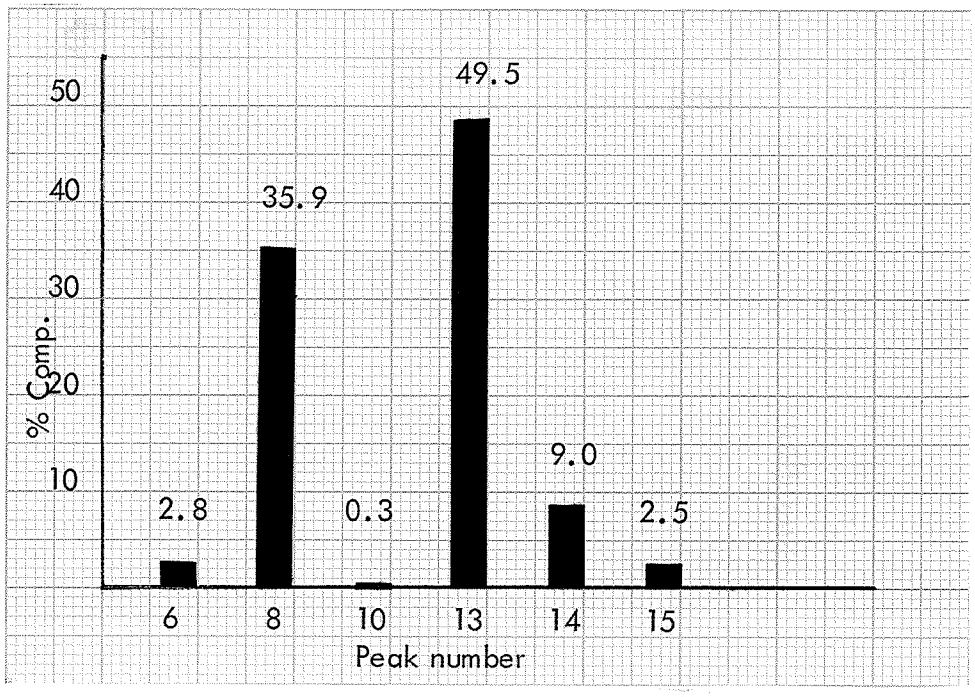
Thus, it could be shown that although growth on different media induced changes in the fatty acid patterns, these differences could be eliminated by subculture through two media.

ii. Length of Bacterial Growth.

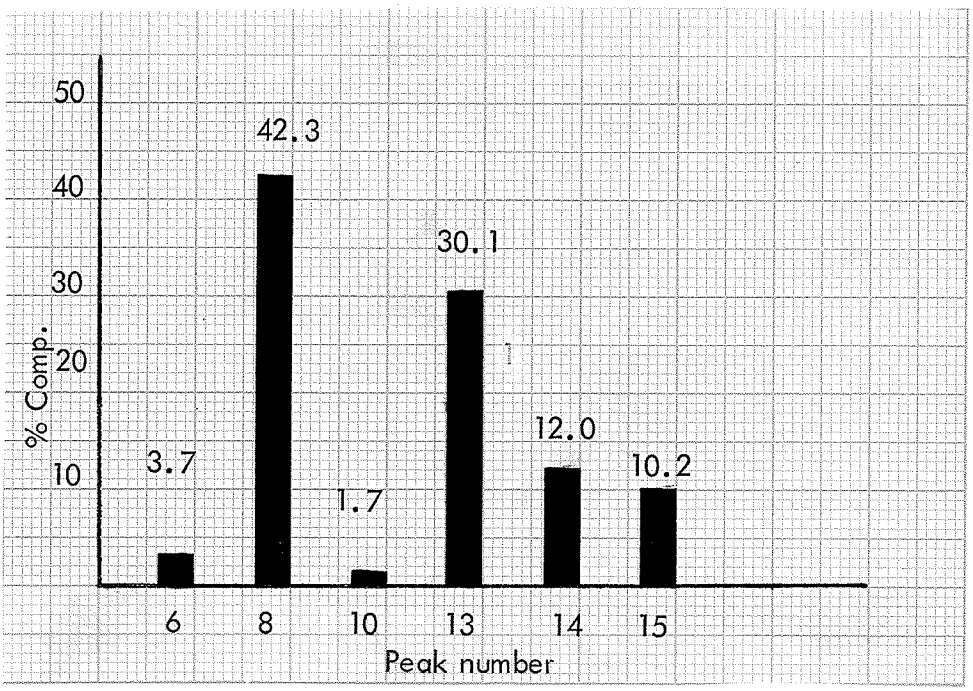
We observed in preliminary trials that the fatty acid content of the bacteria varied somewhat with the length of time they were allowed to grow. It was decided to observe the change in fatty acid composition at different stages of the bacterial growth curve. This was done by setting up two trials. In one the growth curve was plotted and fatty acid analysis made at three different points. In the second trial, five analyses were carried out at culture ages ranging from 3-5 days.

(a) Growth Curve

Thirty culture tubes were obtained and 10 ml of Tryptic Soy Broth

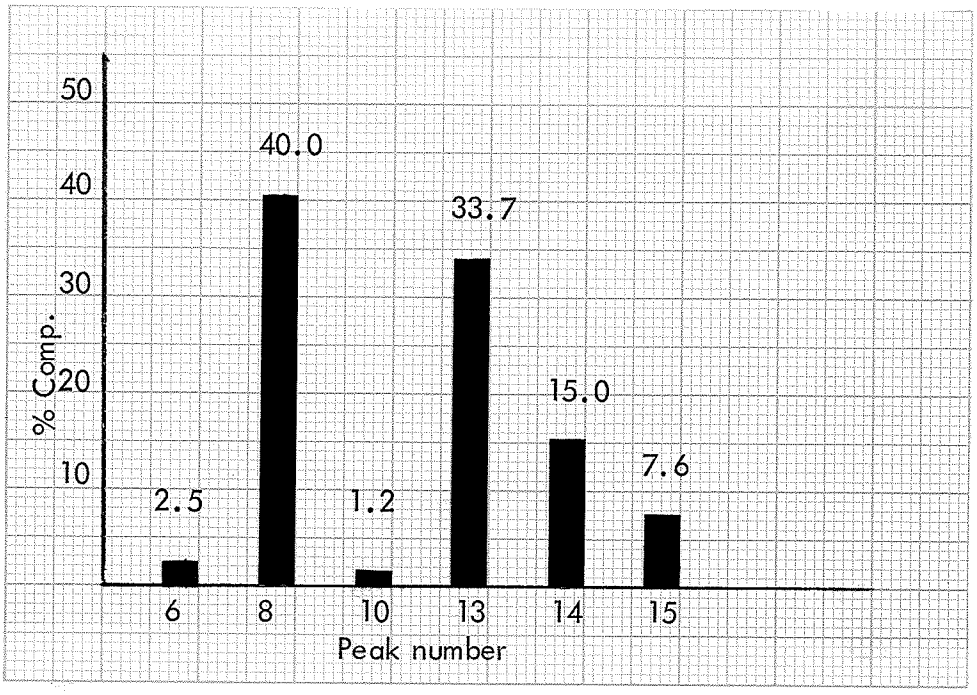


a. Growth on Loeffler's slant.

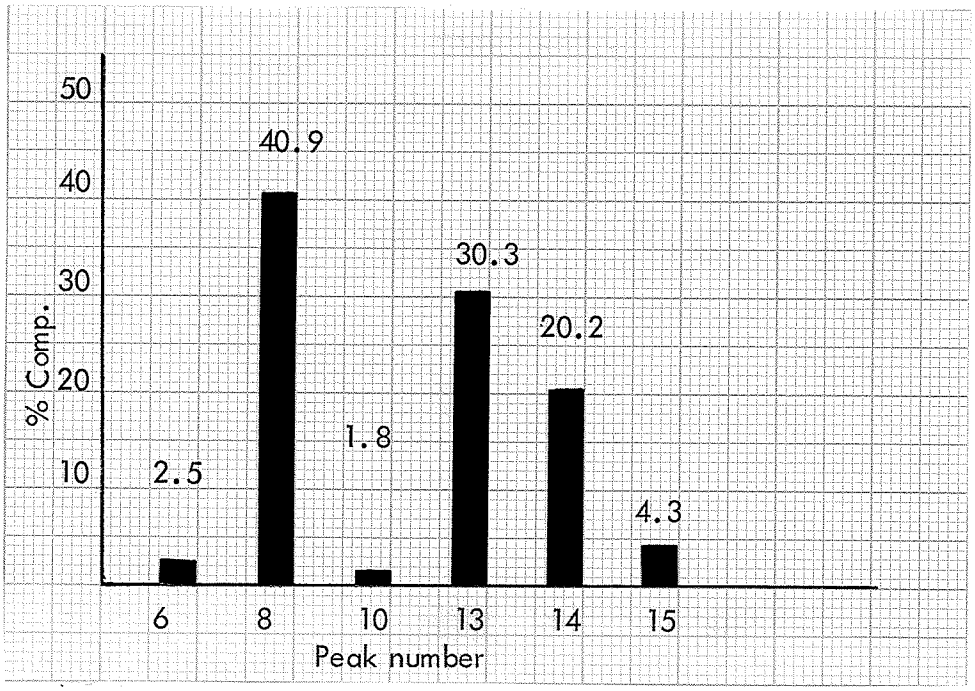


b. Growth on Blood Agar plate.

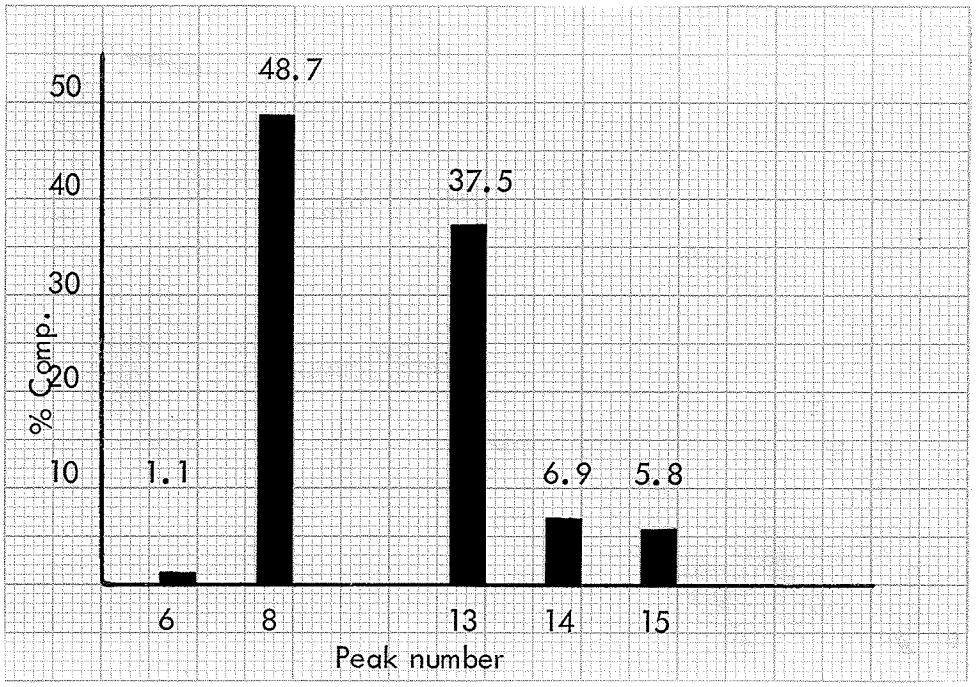
Fig. 6. Fatty acid composition of C. pseudodiphtheriticum after various subcultures from two different media.



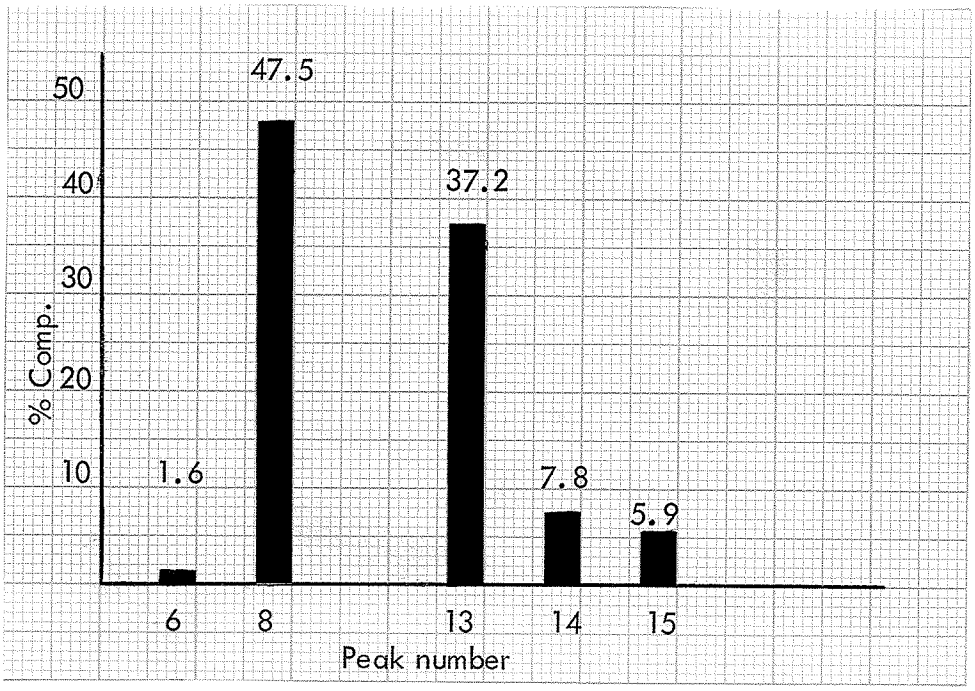
c. Growth on Loeffler's slant subcultured once into TSB.



d. Growth on Blood Agar plate subcultured once into TSB.



e. Growth on Loeffler's slant subcultured twice into TSB.



f. Growth on Blood Agar plate subcultured twice into TSB.

placed in each. The tubes were calibrated in a filter photometer at 580 mμ and each tube inoculated with two drops of a liquid medium containing a viable growth of C. michiganense. Two tubes were not inoculated and served as a medium blank. Cultures were incubated at 23°C which is slightly lower than the optimum growing temperature for this organism. This provided a slow growth which enabled us to determine the growth curve most accurately. At frequent intervals the tubes were placed in the spectrophotometer and the turbidity read in optical density units. For greater precision at least 10 tubes were analyzed. At various points along the curve, cultures were harvested and analyzed for their fatty acid content. Fig. 7 shows the growth curve and the points at which bacteria were harvested while Fig. 8 shows fatty acid patterns obtained. It is seen that some differences exist. Fig. 8b and 8c are quite similar, but Fig. 8a is different. From this it can be seen that the fatty acid pattern does not differ at points which are in the same region of the curve, but do differ at more remote points. The growth curve data are shown in Table I, page 84. Thus, it was necessary to determine precisely how much change could be expected near the 96 hour period we chose for growth.

(b) Detailed Analysis over 124 hour period.

Because it was obvious that such an extensive growth period was not expedient, it was decided to use conditions which would speed up the growth. Thus blood culture bottles providing a surface area of medium measuring 3 x 6 cms were used. This allowed a harvestable quantity to be produced in three days.

A four day growth period was decided upon to ensure growth of all species. This time period would also be of reasonable length for possible future use in a routine method for bacterial differentiation. A trial was set up to determine

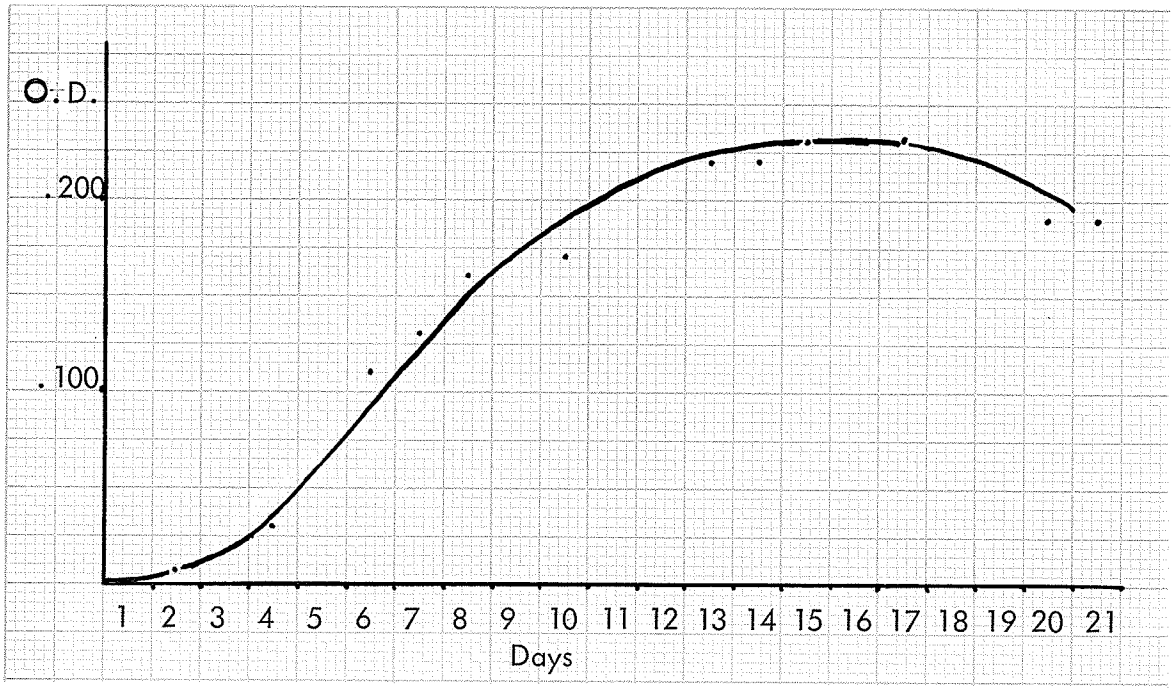


Fig. 7. Growth Curve.

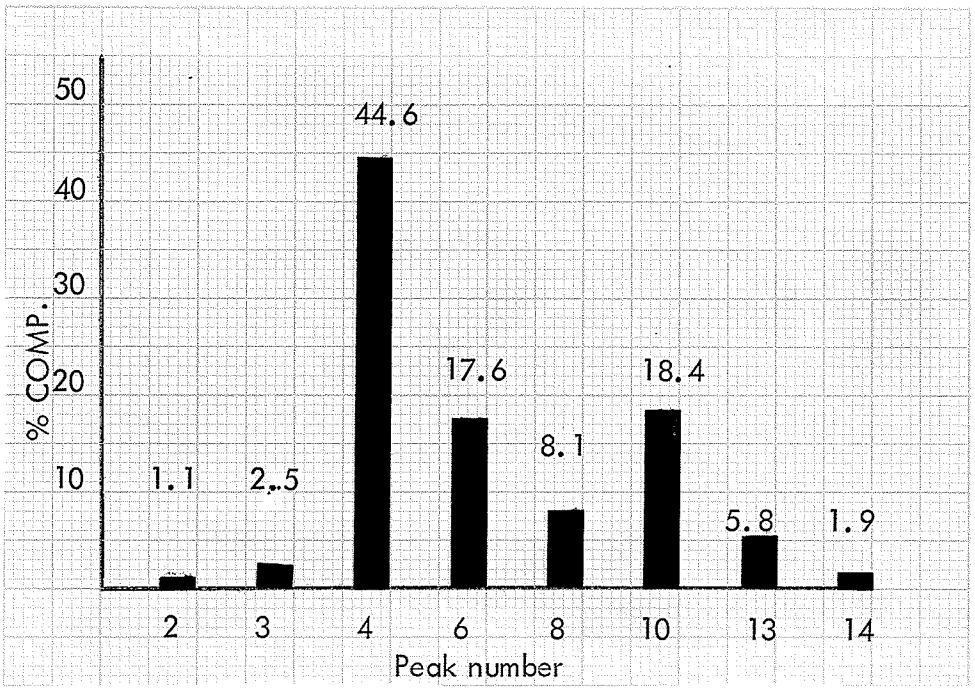


Fig. 8a. Fatty acid analysis after 7 days of growth.

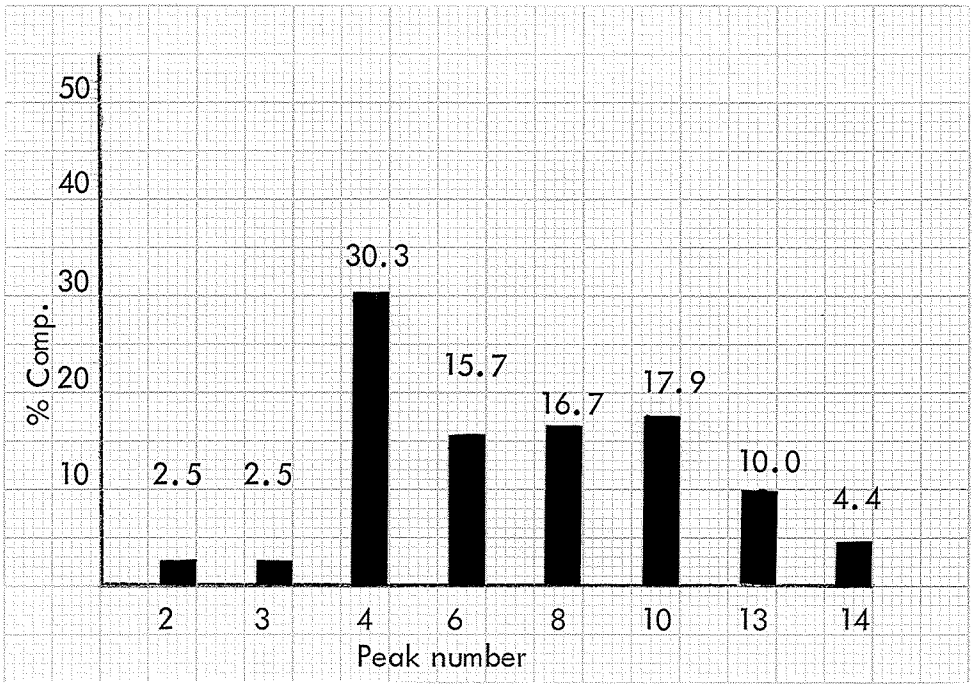


Fig. 8b. Fatty acid analysis after 14 days of growth.

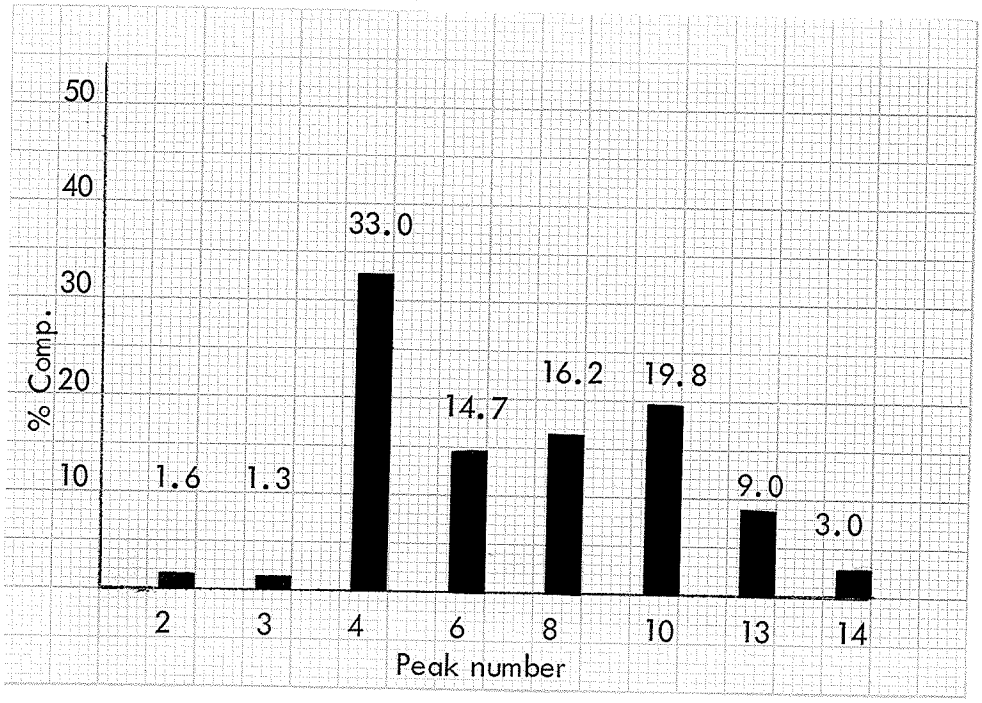


Fig. 8c. Fatty acid analysis after 17 days of growth.

how much variation in the bacterial fatty acid pattern could be expected in this four day period.

Thus, five cultures of C. fascians were set up. At the end of exactly three days (72 hours) one of the cultures was removed, harvested and analyzed. The remaining tubes were left for 20 further hours (92 hours) and another specimen removed. Four hours later (96 hours) a third specimen was removed and four hours later (100 hours) a fourth specimen was removed. The final specimen was removed after five days of growth (124 hours). The results are shown in Fig. 9, page 51.

It can be seen that there is very little change over this period of time. Thus, time is not absolutely critical. However, for precision, harvesting should be made at 96 hours.

C. Harvesting.

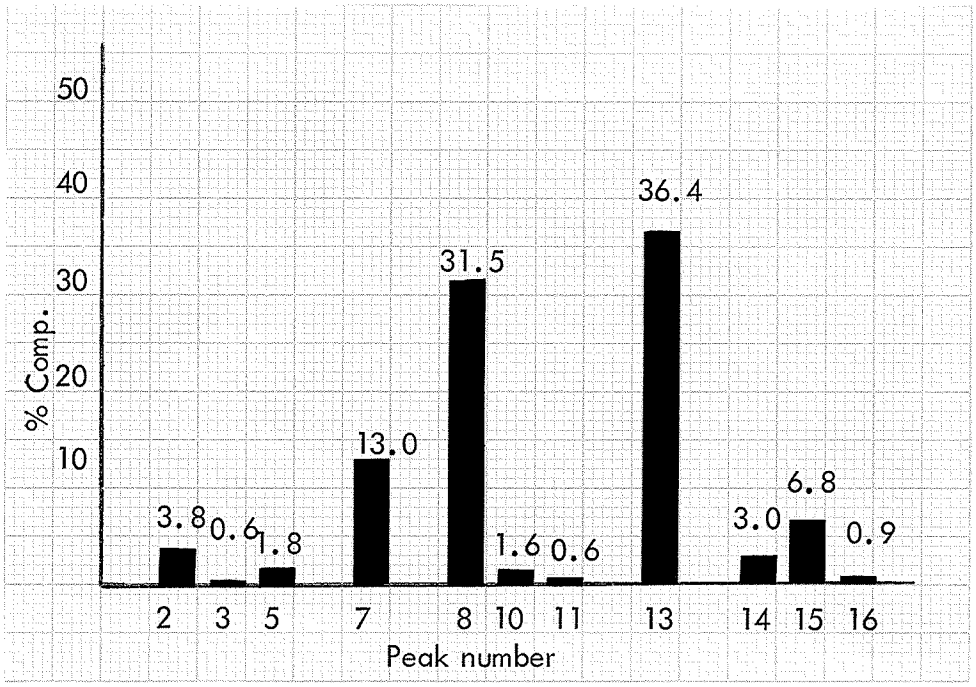
i. Choice of medium.

The choice of liquid medium rather than solid agar was based upon the ease of harvesting. With solid agar, a surface washing technique was needed which did not lend itself to uniformity. On the other hand, the culture grown in liquid medium was easily harvested by centrifugation and aspiration of the medium.

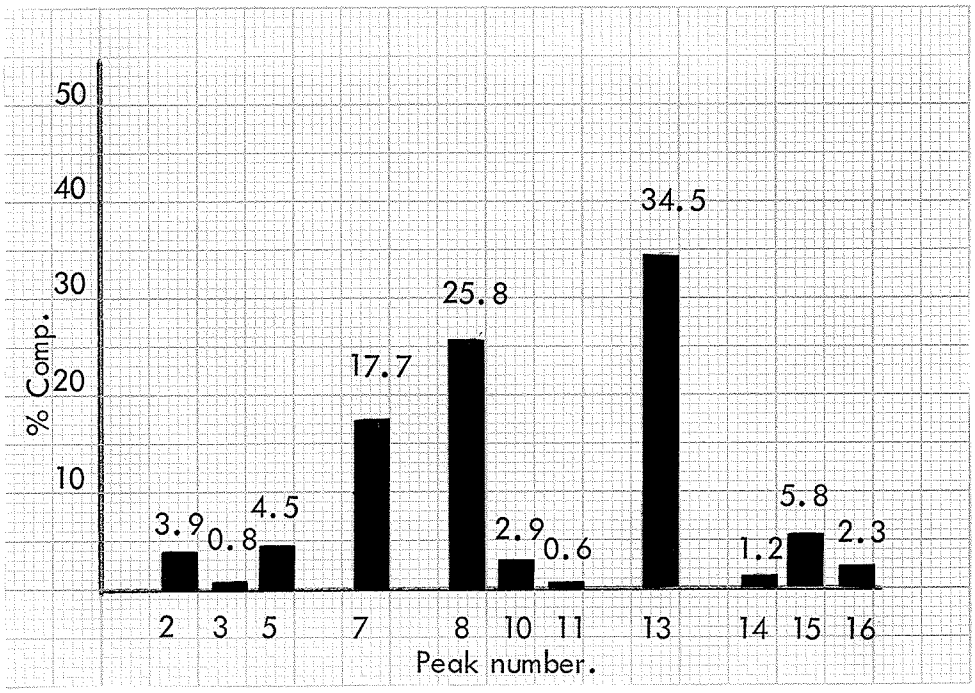
ii. Washing Technique.

Three washings with distilled water were performed. This number of washings was shown to be adequate by Fontanges et al (36) and confirmed by ourselves using the following technique:

A series of five specimens of one bacterial culture was grown, sub-cultured and harvested in identical fashion. The first specimen was analyzed with-

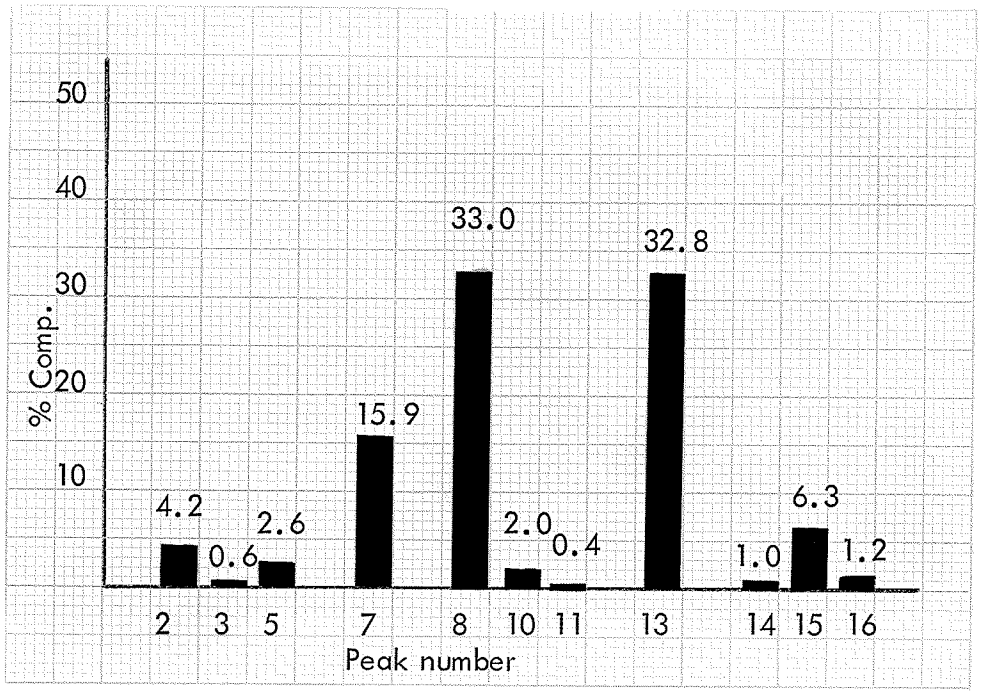


9 a. Period of growth: 72 hrs.

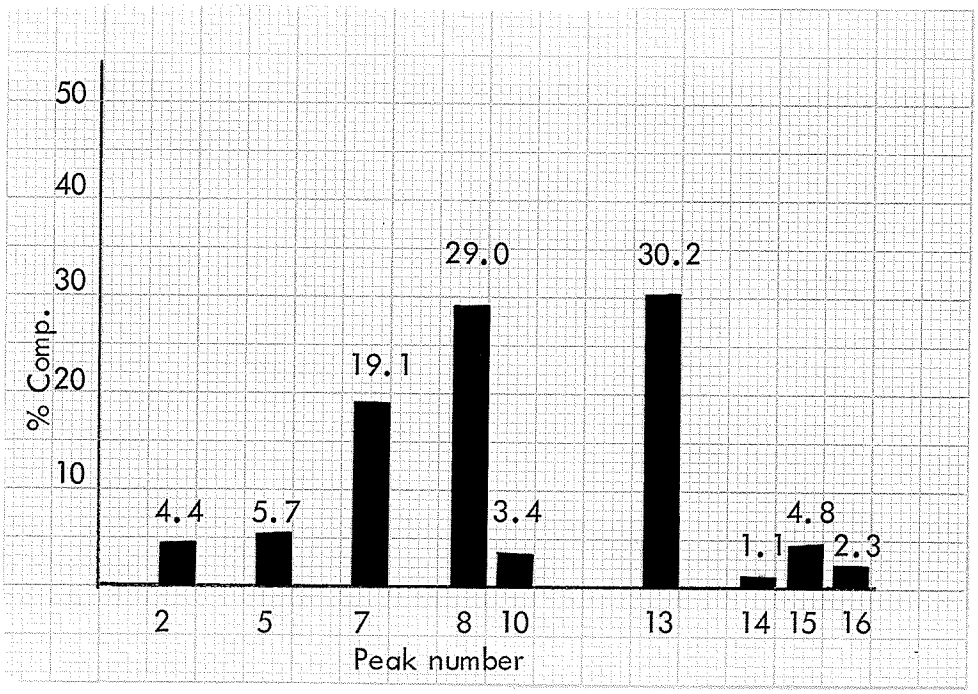


9 b. Period of growth: 92 hrs.

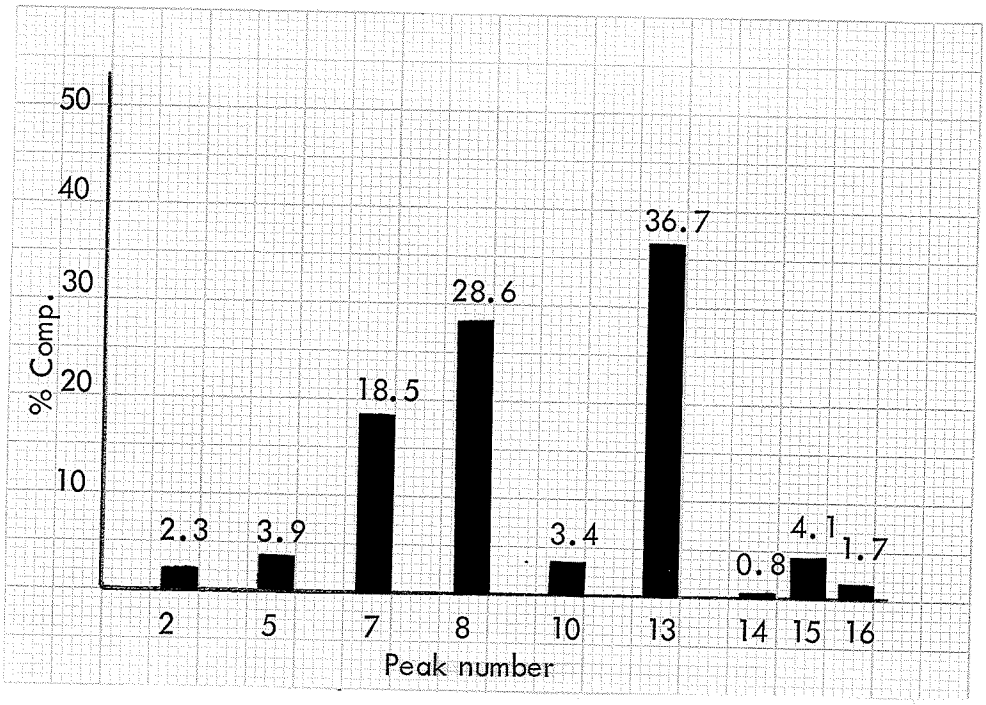
Fig. 9. Fatty acid analysis of bacteria harvested at different intervals of time.



9 c. Period of growth: 96 hrs.



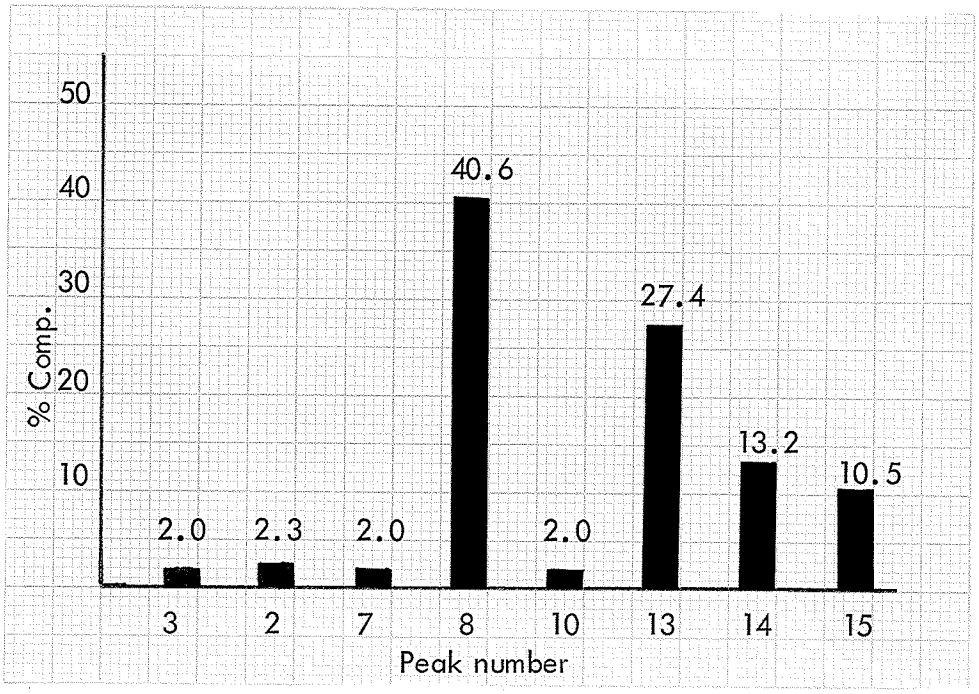
9d. Period of growth: 100 hrs.



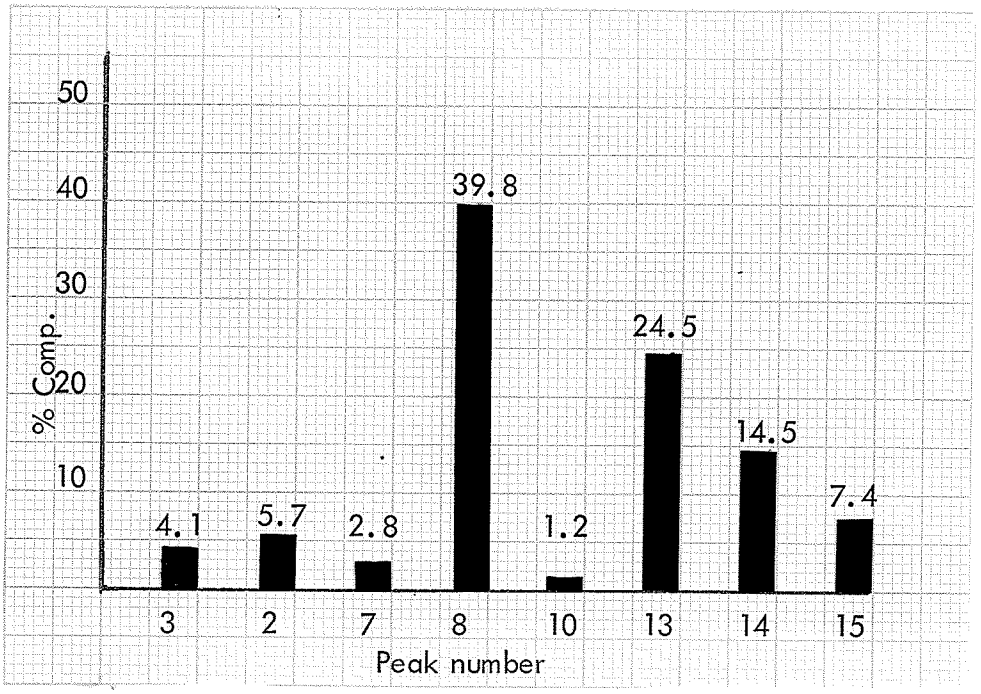
9e. Period of growth: 124 hrs.

out washing. The second was washed once, and analyzed. The third was washed twice and then analyzed. The fourth received three washings prior to analysis and the fifth received four. It was assumed that the washing process removed adherent material on the bacterial surfaces. Therefore, adequate washing would produce a stage in which the bacteria no longer had adherent material. Consequently any further washing would not change the fatty acid pattern. The data exhibited in Fig.10 show that with no washings a particular fatty acid pattern was obtained; following one wash a slightly different fatty acid pattern was seen. We concluded that a single washing alters the fatty acid pattern by removal of adsorbed material. Next, the bacteria were washed once more. Once again, a difference was evident, and once again material must have been washed away. Similar conclusions can be made with regard to three washings. However, when the bacteria were washed four times, essentially no difference in peaks was seen. Thus, the fourth wash did not remove any material. Fig. 10 shows that the peaks "level out" between three and four washings. Thus, it was demonstrated that three washings were adequate to remove the adsorbed material.

A further trial was carried out to determine the efficiency of the washing procedure. The washings of the bacterial culture were saved, extracted into hexane, concentrated and run on a thin layer chromatography plate (Fig. 11). Equal amounts were utilized in every case. It may be seen that the first wash contained a large amount of material, the second wash contained a small amount of material, and the third wash contains no observable amount of material. Thus, three washings were sufficient to remove contaminating matter.

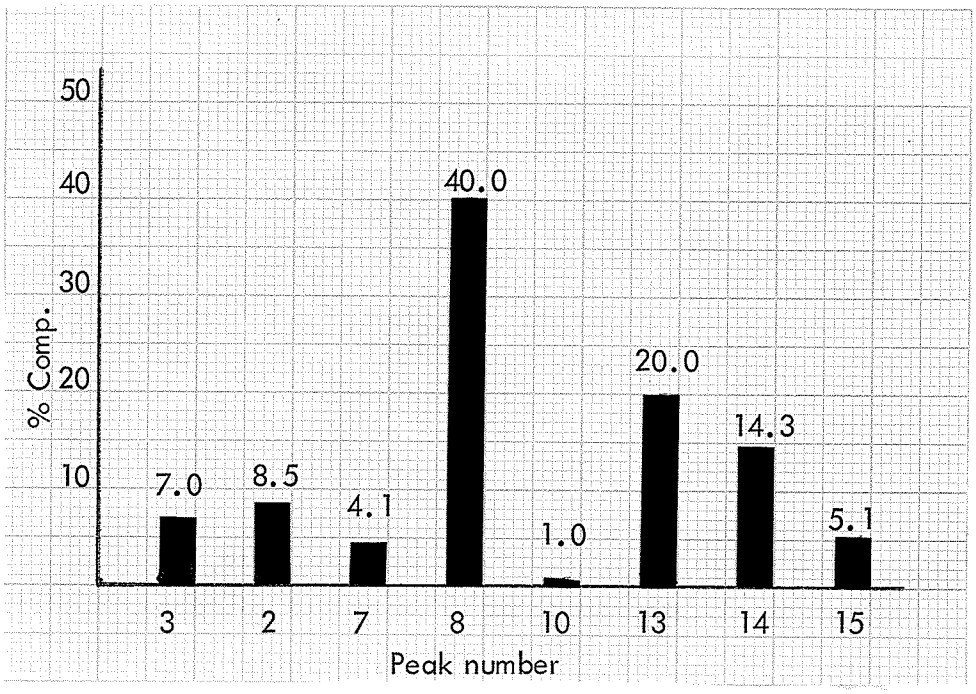


10a. No washes.

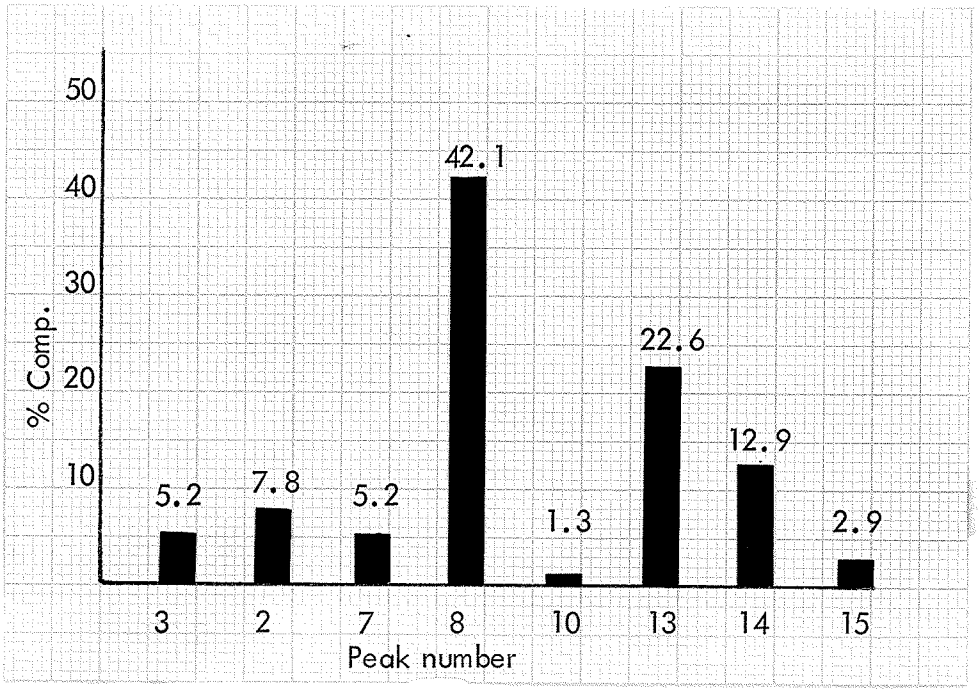


10b. One wash.

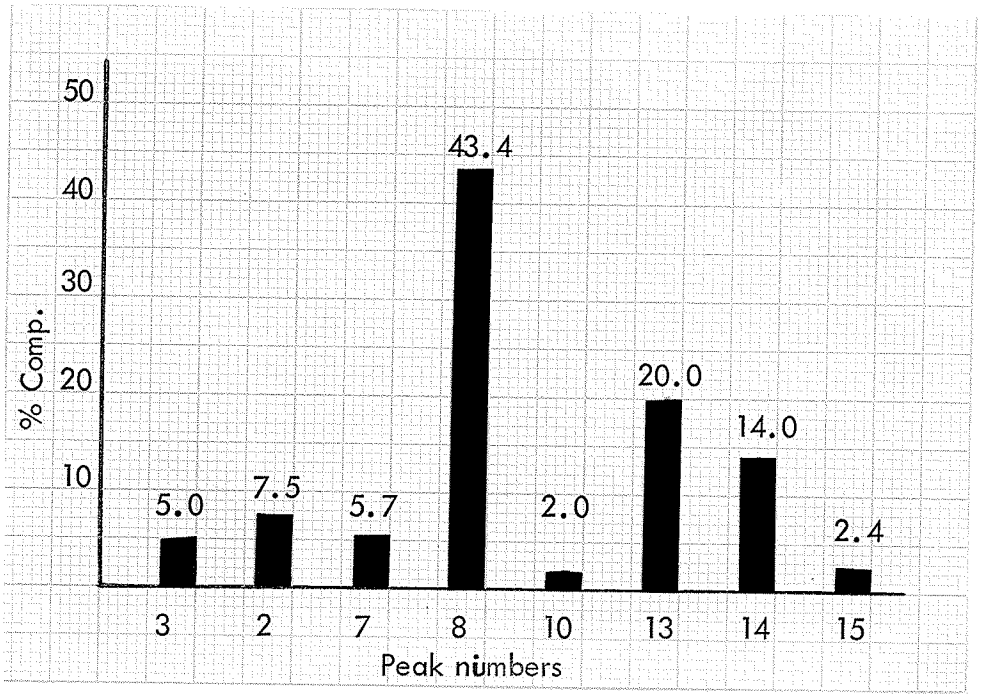
Fig. 10. Fatty acid composition of bacteria after various washes.



10c. Two washes.



10d. Three washes.



10e. Four washes.

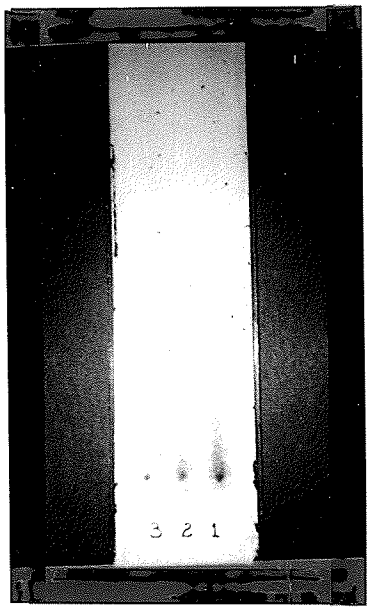


Fig. 11. Thin Layer Chromatogram of bacterial washings.

Plates: 0.25 mm. Silica Gel G on glass plates.

Solvent: Chloroform:Methanol: Water (65: 25: 4)

Color Dev.: 50% Sulphuric acid and heating.

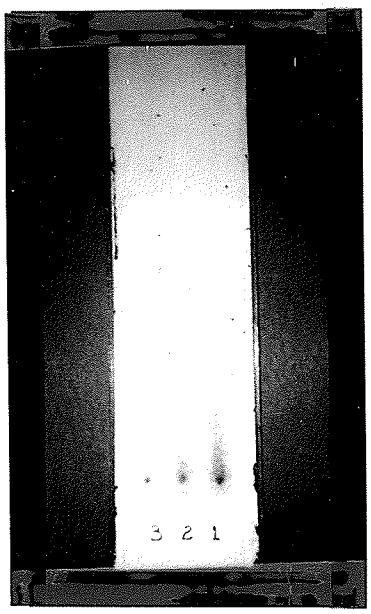


Fig. 11. Thin Layer Chromatogram of bacterial washings.

Plates: 0.25 mm. Silica Gel G on glass plates.

Solvent: Chloroform:Methanol: Water (65: 25: 4)

Color Dev.: 50% Sulphuric acid and heating.

D. Chemical Preparation Technique.

i. Solvent Extraction Efficiency.

Moss and Lewis (68) suggested the use of hexane-ether mixture as a solvent for extraction. By the following trial it was demonstrated that hexane alone is a better solvent, (see Fig. 12).

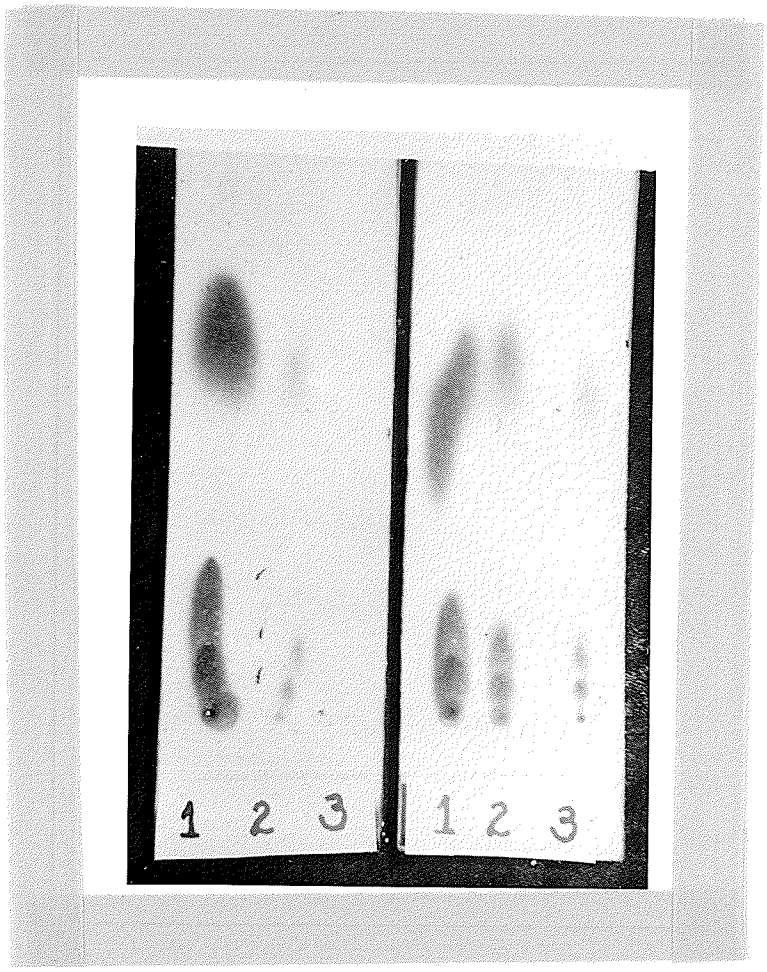
Two identical aliquots of a saponification mixture (from step 1 of the method) were washed in the normal fashion. One specimen was washed with pure hexane, the other with hexane-ether mixture. The washings were concentrated to dryness by evaporation under vacuum, then redissolved in 0.1 ml of pure hexane and run on a thin layer chromatography plate.

It is seen that by the third wash the hexane had removed the non-saponifiable material, where the mixture was still in the process of removing the material.

ii. Purityof the Final Product.

To determine the purity of the final product of the chemical preparation technique, an aliquot of the final product was run on a thin layer plate. It was seen that although the final product was almost entirely fatty acid methyl ester, there was a trace of other materials with smaller Rf values. To determine whether or not these trace compounds would affect the final chromatogram, a subsequent trial was undertaken.

Here, a part of the final product was introduced directly into the chromatography and a pattern obtained. Next, the final product was run on a TLC plate and the fatty acid methyl esters separated from the trace components and eluted into hexane. They were run through the chromatograph and patterns of these two runs compared. See Chromatograms No.24 and 8 . Table II page 85 shows that they



a. b.

Fig. 12. Thin Layer Chromatograms of a) hexane, b) hexane:ether (1:1) extractions.

Plates: 0.25 mm. Silica Gel G on glass plates.

Solvent: Hexane:ether:acetic acid (85:15:2)

Color Dev. : 50% Sulphuric acid and heating.

differ to a very slight degree (average peak difference 0.58%, σ of 0.8).

Thus, the trace components have no effect on the final chromatographic pattern. The chemical preparation technique is valid.

E. Peak Calculation Technique

The chromatographic run was analyzed by three techniques:

1. Tracing on to graph paper and calculating the area by counting the squares;

2. Weighing out the peaks which we had cut from the chart;

3. Using formula: Area = height x width (at 1/2 the peak height).

The Results:

Peak	Tracing Tech. (No. of squares each 1 sq. mm)		Weighing Tech. (mg)		Formula Tech. (Area in mm^2)	
1	20	4.7%	0.72	2.3%	18	4.2%
2	30	6.9%	1.11	3.5%	27	6.2%
3	15	3.4%	2.00	6.3%	15	3.5%
4	225	51.3%	14.90	47.3%	226	52.4%
5	3	0.7%	0.02	0.1%	2.5	0.6%
6	50	11.5%	5.41	17.2%	53	12.3%
7	10	2.3%	0.98	3.1%	7	1.6%
8	4	0.9%	0.45	1.4%	3	0.7%
9	80	18.3%	5.92	18.8%	80	18.5%

A calculation shows that tracing and formula techniques differ by an average of 0.5% per peak (S.D. 0.63), whereas tracing and weight differ by 2.3% (S.D. 3.7). Obviously the formula technique is the quickest and most accurate.

Weighing is not accurate due to the small size of the cut-out peaks.

F. Reproducibility.

To evaluate the reproducibility of the technical procedure we inoculated 15 media with a species of corynebacteria (DL 317B). After four days these were further subcultured into the same media, as outlined earlier. These were harvested, and run through the chemical preparation technique and then analyzed with the Gas Chromatograph. The results are tabulated on page 86 (see Table III.). The average value for each peak was then calculated, and the standard deviation from this average was also determined (see Fig. 13).

It is seen that on a qualitative basis the runs agree with 100% accuracy. On a quantitative basis it is determined that any run using our technique will have a 99% probability that the peaks will lie within 4.2% of the determined value. The practical implication of this result was that, to differentiate between two peaks of different bacteria on a quantitative basis, there must be a difference of 8.4% between two peaks.

G. Blanks.

(a) Redistilled hexane (90 ml) was evaporated under vacuum and reconstituted to 10 microlitres. Two µl. were injected into the chromatograph and the sample temperature programmed to 250°C. Chromatogram No.6, page 92, shows the resulting straight base line with no interferences.

(b) A reagent blank was determined using 10 ml distilled water. The water blank was treated exactly like a bacterial sample and carried through the whole procedure. The resulting chromatogram is shown on page 92, Chromatogram No. 7 .

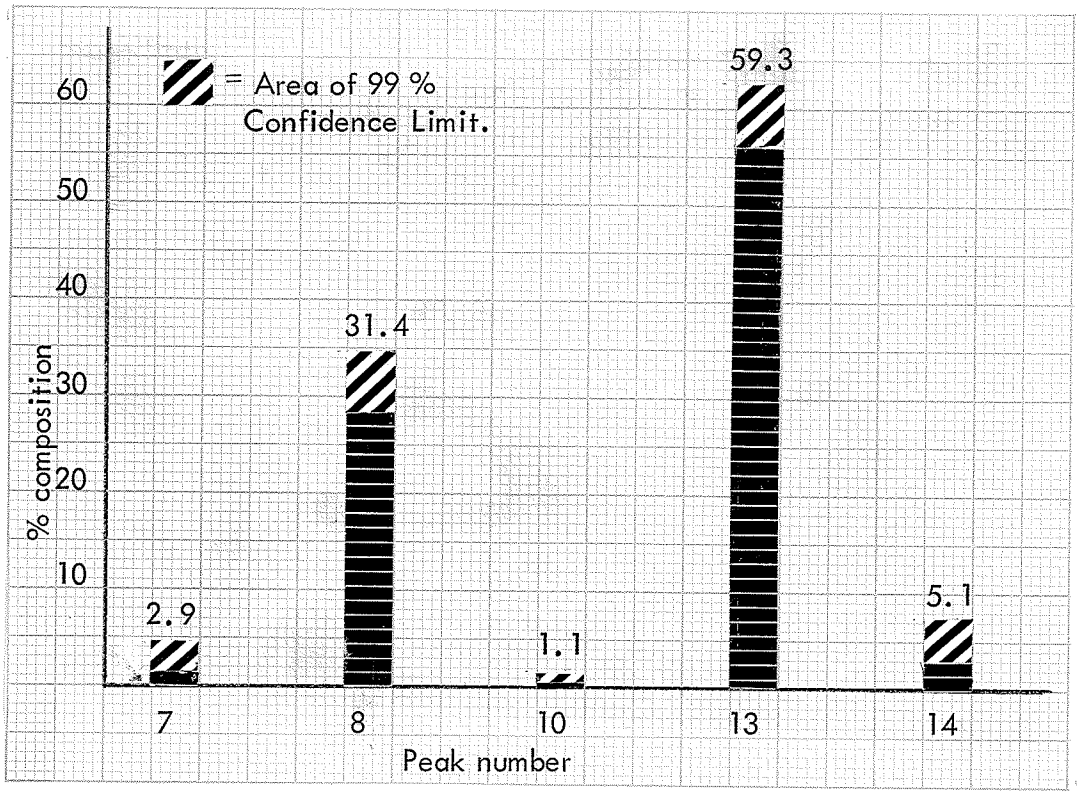


Fig. 13. Graphical representation of fatty acid composition showing the area of confidence limits.

Average Standard Deviation:	1.0	(from Table III)
	0.4	
	2.0	
	2.2	
	1.5	
Total	7.1	

Av. Std. Deviation = 7.1/5 = 1.4

99% Confidence Limits for the technique: 1.4 X 3 = ± 4.2%

Range of 99% Confidence Limits = 8.4%

H. Calibration and Identification.

It is well known that a linear relationship exists between the logarithm of the retention volume and the number of carbon atoms of a homologous series of fatty acids (40). In temperature programming, however, it is more convenient to determine and plot elution temperatures rather than retention volume. The same linear relationship is found when plotting the logarithm of relative elution temperature versus the carbon number (34). As with retention volume, each homologous series will have a different slope. The fatty acids described here were characterized by gas chromatographic elution data determined on two columns, SE-30 and LAC-728. The measured relative elution temperatures were compared with figures obtained under the same conditions with highly purified fatty acid methyl esters obtained from Applied Science Laboratories. They supplied the National Institute of Health kits of methyl ester standards. Fig. 14, shows a typical carbon number versus elution temperature plot and the following table gives an example of the method of calculation.

Compound	Abs. Elut. Temp °C	Rel. Elut. Temp.	Log(Rel. Elut. Temp)
C15:0	142	0.95	$\bar{1}$.978
C16:0	150	1.00	0.000
C17:0	158	1.05	0.021
C18:0	167	1.11	0.045
C19:0	175	1.17	0.068
C20:0	185	1.23	0.090
C22:0	202	1.34	0.127

With temperature programmed runs using the SE-30 column, the data reproduced very well from day to day. However, when a new column was prepared the data usually changed somewhat from that of the old column and so each new column was initially calibrated and then re-calibrated at intervals for the lifetime

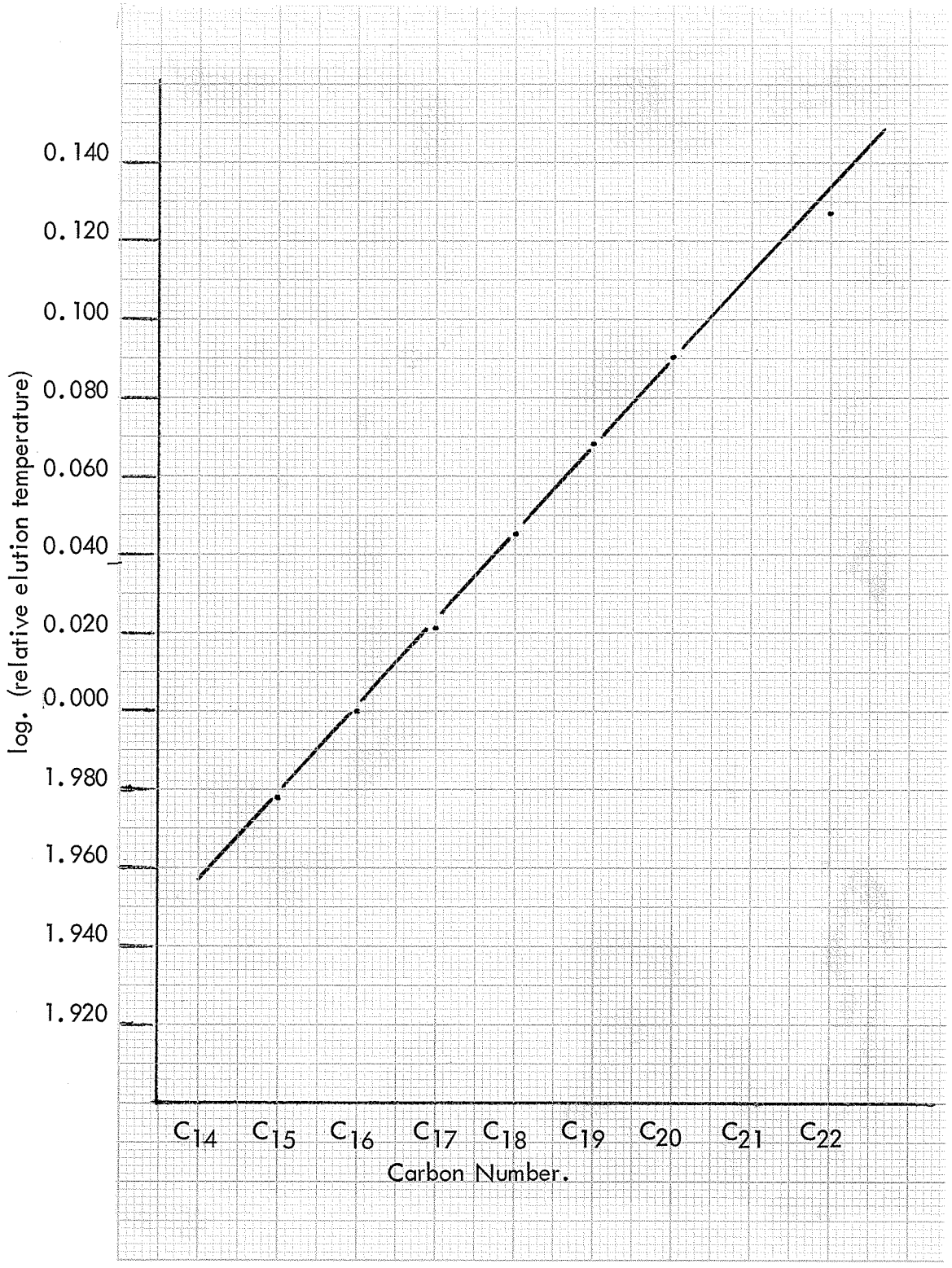


Fig. 14. Carbon number vs. elution temperature plot.

of the column. Calibration was carried out by running a mixture of fatty acid standards, e.g. std. Mix 2, Chromatogram #15, and each peak identified by comparing it with the chromatographic data of the individual components of the mixture (Std. Mix BC-L and BC-1). A clear plastic sheet was used to prepare a template, on which the elution temperature and peak locations of the standards were marked. This template could then be placed on a sample chromatogram, lined up with the corresponding elution temperature and the peaks rapidly identified.

The LAC-728 column was used isothermally since this coating decomposed at high temperatures. Since temperature was constant, retention time was used in calibration data rather than elution temperature. A plot of the logarithm of retention time versus carbon number again produced a linear relationship. Since the retention time varies significantly with temperature, a column recalibration run was carried out every day that the LAC-728 column was used, to nullify any influence due to temperature variations. The identification procedure used was the same as described above. A fatty acid standard mixture was run (each component of which had been earlier identified) and a plastic template made from that day's result. The injection point was recorded and then the location of each standard peak marked on the template. Sample peaks were then rapidly identified by placing the template on the sample chromatogram, lining up the point of injection and matching location of unknown peaks with the peaks of the previously run standard sample.

Two chromatograms are shown in which an internal standard was added to the sample. Oleic acid was added to Tryptic Soy Broth (Chromatogram #2).

Comparison of this chromatogram with the original for Tryptic Soy (Chromatogram # 1) shows peak 13 only to be greatly increased, confirming our earlier identification of peak 13 as octadecenoic acid. Chromatogram # 9 shows the results of the fatty acid analysis of Nocardia corallina with Mixture D added as internal standard. Comparison with the original (Chromatogram #70) shows that the relative sizes of five components in sample C69 are increased following the addition of Mixture D. The five elevated peaks correspond to peaks 2, 7, 8, 13 and 14, as identified in the original sample chromatogram. Mixture D is known to contain C_{14}, C_{16}, $C_{16:1}$, C_{18}, and $C_{18:1}$ (peaks No. 2, 7, 8, 13, and 14.) Since no new peaks appeared but merely an elevation of peaks 2, 7, 8, 13, and 14 occurred, the five peaks of the sample must be the same as the five peaks of the standard and thus identification of these peaks as shown is confirmed.

An attenuation change was made at the point marked by an asterisk(Chromatogram # 70c). For all peaks to be on the same scale , peak 2 should be reduced to 1/4 of the height shown.

Due to the reduction in size of the original chromatogram to fit the thesis page, the elevation of peaks 2, 7, and 8 is more difficult to see than in the original. However, peaks 13 and 14 are clearly elevated.

ANALYSIS OF RESULTS.

Abel, deSchmertzing and Peterson (1) were able to show that fatty acid analysis by GLC could differentiate between bacterial families. We substantiated this by analyzing the cellular fatty acids of Nocardia corallina, Escherichia coli and three different genera of the family Corynebacteriaceae. The chromatographic patterns are shown on page 135. These bacterial cultures represent two different Orders, three different families and five different genera. Nocardia corallina belongs to Class II, Order V, Family II; Escherichia coli is in Class II, Order IV, Family IV, while C. xerosis, L. monocytogenes and A. ureafaciens are in Class II, Order IV, Family XII (17). On the genus level, the three different genera of Corynebacteriaceae are Corynebacterium xerosis (Genus I), Listeria monocytogenes (Genus II) and Arthrobacter ureafaciens (Genus VI). The chromatogram of A. ureafaciens is shown on page 99 labelled as C. creatinovorans, which is its old name.

Obvious differences in fatty acid profiles can be seen if the patterns are compared. E. coli and N. corallina differ in a number of peaks-- 2, 4, 13, 15; this represents a difference between Orders. The absence of peaks 9 and 15 (unidentified fatty acids) in the three genera of Corynebacteriaceae, distinguish them from Nocardia or Escherichia; this represents a difference between families. Visual comparison of the fatty acid profiles of C. xerosis, L. monocytogenes and A. ureafaciens shows significant differences; a difference between genera.

The work reported in this thesis concerns the differentiation of various species of Genus I, Corynebacterium, of the family Corynebacteriaceae. The fatty acid patterns are shown on pages 97-114. Seventeen species (including a number of strains of some species) were successfully analyzed. C. sepedonicum failed to grow

in sufficient amounts for analysis. It probably required the presence of one or more fatty acids in the medium for growth. One of the organisms, Corynebacterium "Q" is an exceedingly rare organism which we were fortunate enough to obtain from Dr. Cleveland. He has one of the few cultures of this organism in the world, obtained a number of years ago from the original isolator, Dr. Pollock. While this organism is very demanding, requiring either oleic acid or a long chain unsaturated fatty acid as a growth factor (71), we did manage to grow it on our medium in sufficient quantity for analysis.

Two strains of each C. xerosis (ATCC #373 and 7711) and C. pseudodiphtheriticum (ATCC #10700 and 10701) were run. For the purposes of our work we define as "strain" an isolate whose characteristics resemble those of an original type species described in Bergey's manual (17). These strains were originally obtained from the American Type Culture Collection. In addition, we ran a number of strains isolated locally by Dr. Cleveland. The patterns of the local isolates agreed well with the others. We were also able to analyse an orange pigmented strain of C. pseudodiptheriticum obtained originally from the ATCC (No. 6981) but which is no longer listed with them. Our analysis showed this strain to have one more fatty acid peak than the other two ATCC strains of C. pseudodiphtheriticum. One other organism available to us was the plant pathogen Arthrobacter ureafaciens. This organism was originally obtained from the ATCC as C. creatinovorans by Dr. Cleveland. Since it was originally throught to be a member of the genus corynebacterium it was included in our study of corynebacteria. A search through the literature since completion of our experimental work failed to produce any information on C. creatinovorans until it was suddenly discovered that it has been renamed to Arthrobacter ureafasciens comb.

nov. (16). The reactions of this organism apparently fit more closely the genus Arthrobacter and it has thus been reclassified as such*. We will thus include it in our table of data as an example of a different genus of the family Corynebacteriaceæ. A local isolate of C. xerosis was used in our reproducibility study and was subcultured into fifteen individual tubes of media. The results have been presented earlier.

Three samples of C. diphtheriæ were grown, two were conventional types while one (DL 210 B) was an unusual, apparently atoxigenic variant. Its fatty acid profile will be discussed later.

The principal fatty acid composition of the bacterial cells examined in this study are shown in Table IV. The fatty acids detected fell in the range between C_{14} and C_{21} with saturated, unsaturated and branched chain acids represented. Three other components which were not identified at this time occurred rarely or in trace amounts. These could possibly be cyclopropane type fatty acids, however we were unable to obtain standards for these and so were unable to compare the retention times to provide tentative identification. Cyclopropane acids are known to occur in bacteria (70, 19) although Marr and Ingraham (61) reported that they found them to be formed only after cessation of exponential growth. The first one reported was C_{19} cyclopropane acid (lactobacillic acid) now known to be cis-11, 12-methylene-octadecanoic acid. Others reported are C_{17} cyclopropane acid (cis-9, 10-methylenehexadecanoic acid) and C_{15} cyclopropane acid. The unidentified acids fall roughly in the areas where the C_{15}, C_{17} and C_{19} cyclopropane acids might be expected to occur.

*Arthrobacter is another genus of the same family Corynebacteriacæ. The genera of this family are Corynebacterium, Listeria, Erysipelothrix, Microbacterium, Cellulomonas, and Arthrobacter.

Both even- and odd-numbered straight chain saturated fatty acids from C_{14} to C_{18} were represented. However, the odd-carbon number acids were found in much smaller amounts than the even-numbered acids. Only C_{16} and C_{18} unsaturated fatty acids were found, with mere traces of $C_{18:2}$. A number of species were found to have a high percentage of branched chain fatty acids, indeed, in some species these were the major components.

The four predominant fatty acids to occur were saturated acids, C_{15} branched chain ($C_{15:0br}$), C_{17} branched chain ($C_{17:0br}$), C_{16} straight chain ($C_{16:0}$, hexadecanoic) acid, and C_{18}-monoenoic ($C_{18:1}$, octadecenoic) acid. The large percentages of $C_{15:0br}$ and $C_{17:0br}$ acids were characteristic of the four plant pathogens tested , although differences were observed in relative amounts of these acids among species. These four species (C. insidiosum, C. pointsettiae, C. michiganse, C. flaccumfaciens) had the last-named two acids in much higher concentration than any of the other species tested. Hexadecanoic and octadecenoic acids were commonly found but in widely varying amounts. Hexadecanoic acid was found in every sample tested and ranged in concentration from 3.6% in C. insidiosum to 55.4% in C. equi. It was the major constituent in a number of species. Octadecenoic acid, on the other hand, was also found in all samples but in much smaller concentrations; it ranged from 1.3% in the plant pathogen C. insidiosum to 49.1% in the animal parasite C. renale. It, too, was the major fatty acid found in a number of species. The remaining fatty acids were, as a rule, found in low and variable concentration . Of the remaining fatty acids the one appearing most consistently, but in low concentration, was $C_{14:0}$ (myristic) acid. The three unidentified fatty acids, if present, were so in amounts less than 3.5% except for three species where they ranged from 5.8 to 9.3%.

A number of unusual, long chain fatty acids have been reported in C. diphtheria, in early work by Asselinau (6) and Alimova (3, 3a, 2). These lipids were obtained from the whole cells by solvent extractions and fractionated by a series of solvent separations. The lipids were characterized by classical biochemical techniques such as Iodine number, melting point, molecular weights by neutralization number, etc. In our study no evidence of these complex lipids was found even though two samples of C. diptheriæ were temperature programmed to 275°C and then kept isothermally at this temperature for 255 minutes. The possibility exists that these lipids did not extract in our method or may have broken down when the cells were subjected to alcoholic hydrolysis. It may be pointed out that no lipids of this type have been recorded in any of the numerous bacterial lipid analyses carried out using GLC. Since C. diphtheriæ was not of primary interest to us in this study, we did not follow this up any further, however, a gas chromatographic investigation of the lipids extracted by the above method on C. diphtheriæ could well be worthwhile in providing more up-to-date information.

Visual comparison of gas chromatography methyl ester profiles of fatty acids extracted from 17 species of corynebacteria permitted a rapid separation into distinct groups. One of these groups could be further subdivided into three sections so that, in all, a possibility of four groups exists. The first separation could be made on the basis of presence or absence of major peaks, while the second subdivision into three further sections, on the basis of relative sizes of major peaks.

If we attempt to group the chromatograms of the 17 corynebacteria species by visual comparison, two distinguishable "landmarks" can first be established. They are two pairs of fatty acid peaks found throughout, although in widely ranging quantities. The first pair appearing in the vicinity of 150°C corresponds to $C_{16:1}$

and C_{16}, while the second pair eluted at about 170°C corresponds to $C_{18:1}$ and C_{18}. A distinction between the first and second group can be made on the basis of Group 1 having neither one of these two pairs as its major peak while Group 2 has either one or more of these peaks as its largest fatty acid fraction. The major peaks of Group 1 are two odd-numbered branched-chain fatty acids ($C_{15:0\,br}$ and $C_{17:0\,br}$), while Group 2 has both of these acids in only minor amounts or completely absent. $C_{15:0\,br}$ is the predominant fatty acid in Group 1 ranging in concentration from 33.3% to 69.5%, with $C_{17:0\,br}$ next highest, its concentration ranging from 15.0% to 31.6%. Group 2, on the other hand, has these two acids in maximum concentrations of only 2.0% and 3.4% respectively. On the basis of cellular fatty acid content, a distinct difference between the corynebacteria of Group 1 and the rest of the genus can be seen. Interestingly enough, the Group 1 bacteria are all plant pathogens or parasites, the very ones that bacteriologists have for years suspected belonging to another bacterial group but have so far been unable to change, due to their close resemblance in morphology and biochemical reactions to other members of the genus! It would appear that, on the basis of cellular fatty acids, a way of differentiation has at last been found.

Group 2, which includes the majority of corynebacteria checked in this study, all contain at least one fatty acid of each "landmark" pair as its major component. This large group can be subdivided into three sections by a consideration of the hexadecanoic ($C_{16:0}$) acid and octadecenoic ($C_{18:1}$) acid content. The groupings are as follows:

Group I

C. insidiosum

C. pointsettiae

C. michiganense

C. flaccumfaciens

*A. ureafaciens**

Group 2

a. *C. enzymicum*

C. diphtheriae

C. hoagii

C. equi

C. ulcerans

b. *C. pseudotuberculosis*

C. bovis

C. pseudodiphtheriticum

C. "Q"

C. fascians

C. segmentosum

c. *C. xerosis*

C. renale

* Originally analyzed as *C. creatinovorans.*

Group 2a can be distinguished from the other two groups by the presence of a large hexadecanoic acid ($C_{16:0}$) peak, its major component. Its secondary peak was octadecenoic acid ($C_{18:1}$) with varying amounts of other smaller peaks. Group 2c, on the other hand, had hexadecanoic and octadecenoic acids in concentrations reversed to Group 2a. Octadecenoic acid ($C_{18:1}$) is its major peak while hexadecanoic acid ($C_{16:0}$) is secondary, with only trace amounts of any shorter chain fatty acid. Group 2b appears to be an intermediate; its members have large and roughly equal amounts of both hexadecanoic ($C_{16:0}$) and octadecenoic ($C_{18:1}$) acid. It would appear that, according to fatty acid analysis, a distinct separation of species does not exist in this group but rather a spectrum of species with C. pseudodiphtheriticum centred between C. diphtheriae and C. xerosis.

Two strains of each , C. diphtheriae (U 61A and U 61B), C. xerosis (7711 and 373), and C. pseudodiphtheriticum (10700 and 10701) were checked and the results, as shown in Table IV, page 87 agree very well with each other. The unusual isolate of C. diphtheriae (DL 210B) was not differentiated very well from the other two. A local isolate of C. xerosis (DL 7813), while varying somewhat in its minor components, could readily be identified as belonging

to C. xerosis. The strain of C. pseudodiphtheriticum which, for some reason, is no longer listed with ATCC (originally #6981) bore strong resemblances to the ATCC strains #10700 and 10701 except for one relatively large peak; an unidentified fatty acid which made up 9.3% of its total fatty acids.

The work presented here confirms or supplements a number of statements in the work of Murray and Cleveland as pointed out in Cleveland's thesis (25). He mentions that C. equi is "an unusual organism amongst animal and human pathogenic parasitic corynebacteria, yet easily recognized by its pink growth". However, C. hoagii which also forms a pink pigment, was indistinguishable from C. equi in their study. He was thus lead to believe that "the C. hoagii culture was mistakenly identified". Cellular fatty acid analysis of these two organisms show practically identical composition, confirming the earlier suspicions.

Another interesting point was the observation that all the plant pathogens tested except C. fascians "split esculin and arbutin or at least esculin". In our work all the plant pathogens except C. fascians could be separated from the rest of the corynebacteria into Group 1 on the basis of a high content of $C_{15:0br}$ and $C_{17:0br}$ fatty acids. Thus, in two independent investigations, C. fascians has been observed to be different than the other plant pathogens of the genus Corynebacterium.

The only organism which we failed to analyze successfully was C. sepedonicum, and perhaps the answer can be found in an observation made by Cleveland (25) when discussing his various species. "C. sepedonicum is an extremely slow growing organism, the culture examined taking from 5 days to a week to produce colonies only 0.2 mm in diameter and becoming deeply yellow pigmented." Our growth period of four days was probably not of sufficient length to produce enough material to work with.

It should perhaps be pointed out that, in this discussion, we mention

"odd-numbered branched chain" fatty acids a number of times in referring to $C_{15:0br}$ and $C_{17:0br}$ acids without making any reference to their possible configuration. However, in TableIV, we specify a configuration and call them aC_{15} and aC_{17} representing anteiso C_{15} and anteiso C_{17} as identified from retention data of our standards. Since we could not obtain iC_{15} and iC_{17} standards, we were unable to show the difference in retention time that exists between the "iso" and "anteiso" isomers. Moss (65) and Kaneda (49) have already shown that these isomers do separate on columns very similar to the ones we were using. Since we found only one peak in our samples, which corresponded exactly to the "anteiso" standard, we feel that our "odd-numbered branched chain" fatty acids are probably of "anteiso" configuration.

While we have been able to analyze only one culture of most corynebacteria species, some suggestions, based on our experimental results, can be made regarding taxonomy. Moss et al (66) have recently published a description of the cultural characteristics and fatty acid composition of species belonging to the genus Propionibacterium which relates to our work. They were able to separate the fatty acid patterns of seven Propionibacterium species into two groups. Group I contained anteiso C_{15} (aC_{15}) in greatest abundance, with $C_{17:0br}$ the next highest in concentration. Group II, on the other hand, had the isoC_{15} (iC_{15}) isomer as the most abundant acid, followed by a smaller amount of the aC_{15} isomer and only small amounts of $C_{17:0br}$. The four plant pathogens, that we analyzed and were able to separate from the rest of the corynebacteria, have the same characteristic peaks as Group I Propionibacterium of Moss and co-workers. Both had aC_{15} as their major component with $C_{17:0br}$ next in abundance. On this basis we feel that these four

organisms might well fit into the genus Propionibacterium. The anaerobic species of Corynebacterium have already been shown (67, 65), to be very similar to certain members of the genus Propionibacterium. Indeed, on the basis of biochemical and morphological characteristics one of the anaerobic corynebacteria, C. acnes, has been tentatively reclassified to Propionibacterium by some workers (33, 64). Moss et al (66) found all of the anaerobic corynebacteria they tested to fit into their Group II Propionibacterium. On the basis of our work and that of Moss et al (66) the genus Corynebacterium could possibly be split into three fractions. The four plant and soil organisms C. insidiosum, C. pointsettiae, C. michiganense and C. flaccumfaciens could be placed into Group I Propionibacterium along with P. freudenreichii, and P. shermanii. The anaerobic corynebacteria could fit into Group II alongside P. arabinosum, P. jensenii, P. pentosaceum, P. theonii, and P. zeae while the remaining aerobic corynebacteria (our Group 2) would remain in the genus Corynebacterium.

This would remove the four plant and soil organisms from the genus Corynebacterium, something that some bacterial taxonomists feel should have happened long ago except that no good reason for doing so could be found.

Another possibility exists, although more remote at this time. Earlier in this discussion we made mention of the fact that C. creatinovorans had been reclassified to Arthrobacter ureafaciens. The four members of our Group I had striking resemblances to the pattern of this organism. If we consider the fatty acid profile of A. ureafaciens (alias C. creatinovorans) to be representative of the genus Arthrobacter, we could well consider suggesting that the four members (Group I) could be candidates for admittance to the genus Arthrobacter. Alternately, A. ureafaciens

could be reclassified as Group I Propionibacterium . Since this was the only Arthrobacter culture we had, the fatty acid data on it were too limited to warrant any definite statement at this time regarding its reclassification.

TABLES

AND

CHROMATOGRAMS

KEY TO PEAK NUMBERING.

Number	Fatty acid	
1	iC_{14} ($C_{14:0br}$)	
2	$C_{14:0}$	Myristic acid
3	Unidentified	
4	aC_{15} ($C_{15:0br}$)	
5	$C_{15:0}$	
6	iC_{16} ($C_{16:0\ br}$)	
7	$C_{16:1}$	Hexadecenoic acid
8	$C_{16:0}$	Hexadecanoic acid
9	Unidentified	
10	aC_{17} ($C_{17:0br}$)	
11	$C_{17:0}$	
12	iC_{18} ($C_{18:0br}$)	
13	$C_{18:1}$	Octadecenoic acid
14	$C_{18:0}$	Octadecanoic acid
15	Unidentified	
16	aC_{19} ($C_{19:0br}$)	
17	$C_{19:0}$	
18	iC_{20} ($C_{20:0br}$)	
19	$C_{20:0}$	
20	aC_{21} ($C_{21:0br}$)	
21	$C_{18:2}$	Linoleic acid
22	$C_{18:3}$	Linolenic acid

Number	Fatty Acid
23	$C_{22:0}$
24	$C_{24:0}$
25	Unidentified

Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; br= branched chain; a = anteiso; i = iso

KEY to NAMING of SPECIES

Code Number	Genus and Species
C69	Nocardia corallina
DL 7813	C. xerosis
DL 210B	C. diphtheriae
6981	C. pseudodiphtheriticum
Mc 97	L. monocytogenes
3224	C. bovis
1621	C. equi
CF-1	C. fascians
CM-1	C. michiganense
2A	C. insidiosum
U61A	C. diphtheriae
U61B	C. diphtheriae
7005	C. hoagii
373	C. xerosis
7711	C. xerosis
7562	C. creatinovorans
7906	C. ulcerans
149	C. "Q"
1033	C. pseudotuberculosis
8155	C. enzymicum
6451	C. renale

Code Number	Genus and Species
934	C. segmentosum
CF-3	C. flaccumfaciens
CP-2	C. pointsettiae
10700	C. pseudodiphtheriticum
10701	C. pseudodiphtheriticum
DL-1	E. coli

DAY	2	4	6	7	8	10	13	14	15	17	20	21
	.001	.025	.110	.130	.150	.140	.230	.200	.210	.220	.190	.190
	.000	.028	.140	.170	.160	.170	.200	.240	.210	.230	.190	.190
	.009	.032	.110	.110	.150	.180	.230	.180	.270	.220	.200	.180
	.004	.037	.100	.130	.150	.150	.170	.210	.220	.260	.190	.200
	.012	.030	.110	.110	.140	.150	.200	.200	.210	.220	.180	.190
	.002	.023	.100	.100	.170	.200	.230	.210	.240	.210	.170	.180
	.005	.035	.100	.130	.160	.170	.240	.230	.230	.240	.190	.190
	.010	.028	.120	.150	.160	.130	.210	.220	.220	.210	.190	.200
	.017	.032	.100	.130	.180	.190	.200	.240	.230	.230	.180	.190
	.007	.030	.110	.110	.150	.170	.240	.220	.260	.260	.200	.180
Mean O.D.	.007	.030	.110	.130	.160	.170	.220	.220	.230	.230	.190	.190
Std. Deviation	.005	.004	.004	.020	.016	.021	.022	.020	.017	.019	.009	.007

TABLE I Optical Density readings during growth

F.A. PEAK	BEFORE TLC	AFTER TLC	Δ	Δ^2
2	2.3	2.0	0.3	0.09
5	3.9	4.3	0.4	0.16
7	18.5	17.2	1.3	1.69
8	28.7	29.5	0.8	0.64
10	3.4	3.5	0.1	0.01
11		0.7	0.7	0.49
13	36.8	35.2	1.6	2.56
14	0.8	1.0	0.2	0.04
15	4.1	4.3	0.2	0.04
16	1.7	2.0	0.3	0.09
				5.81

$$\sigma = \pm\sqrt{5.81/9} = \pm 0.8$$

Table II. Analysis of sample before and after Thin Layer Chromatography.

	Peak #7		Peak #8		Peak #10		Peak #13		Peak #14	
	%		%		%		%		%	
1	4.5	1.6	29.9	1.5	1.1	0.0	57.8	1.5	6.7	1.6
2	4.6	1.7	28.8	2.6	1.3	0.2	59.4	0.1	5.9	0.8
3	3.6	0.7	30.6	0.8	1.6	0.4	57.0	2.3	7.2	2.1
4	3.7	0.8	29.9	1.5	1.4	0.3	57.7	1.6	7.3	2.2
5	1.7	1.2	33.7	2.3	0.9	0.2	60.3	1.0	3.4	1.3
6	2.5	0.4	31.4	0.0	1.1	0.0	56.8	2.5	8.2	3.1
7	2.3	0.6	32.2	0.8	0.4	0.7	60.3	1.0	4.8	0.3
8	2.6	0.3	33.8	2.4	0.8	0.3	59.9	0.6	2.9	2.2
9	3.1	0.2	34.1	2.7	1.4	0.3	56.2	3.1	5.2	0.1
10	4.1	1.2	28.3	3.1	1.2	0.1	62.9	3.6	3.5	1.6
11	1.8	1.1	30.2	1.2	0.8	0.3	61.7	2.4	5.5	0.4
12	1.9	1.0	30.4	1.0	0.6	0.5	62.9	3.6	4.2	0.9
13	2.3	0.6	31.2	0.2	0.8	0.3	61.7	2.4	4.0	1.1
14	3.3	0.4	33.2	1.8	1.6	0.5	57.2	2.1	4.7	0.4
15	1.7	1.2	34.0	2.6	0.8	0.3	60.0	0.7	3.5	1.6
TOTAL	43.7		471.7		15.8		891.8		77.0	
Average	2.9		31.4		1.1		59.3		5.1	
Std. Dev.	1.0		2.0		0.4		2.2		1.5	

TABLE III REPRODUCIBILITY

Table IV.
Percentage composition of the principal fatty acids of representative strains of Corynebacterium species.

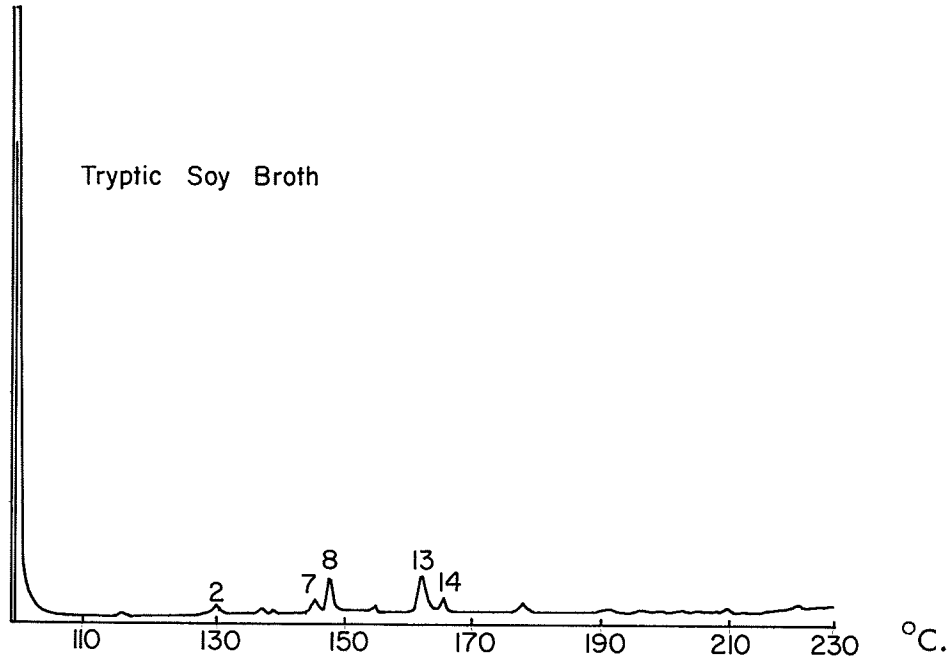
Culture #		C_{14}	Un	aC_{15}	C_{15}	iC_{16}	C_{16}	$C_{16:1}$	Un	aC_{17}	C_{17}	iC_{18}	C_{18}	$C_{18:1}$	Un	$C_{18:2}$	aC_{19}	C_{19}	
2A			1.9	69.5		8.7	3.6			15.0				1.3					
CP-2		2.0		45.5	0.3	4.6	10.0	1.8		29.8			2.2	3.8					
CM-1		1.6	1.3	33.3		12.4	16.3	2.4		19.9			3.0	9.1	0.7				
CF-3		3.1		40.4	1.7	2.7	12.1	0.7		31.6			2.3	2.5		2.9			
7562	0.8[2]	1.4		50.0	1.5	12.7	12.2	0.7		14.1			2.9	2.4		1.3			
6451		0.5		0. 3			18.6	1.2		0.5	1.3	0.7	27.8	49.1					
7711							28.1	3.6		1.3	2.6	0.6	14.3	48.8					0.7[3]
373							25.9	2.6		0.3	0.9	0.3	21.8	48.2					
CF-1		3.9			4.5		25.8	17.7		2.9	0.6		1.2	31.6	5.8	3.8	2.2		
149		3.1		1.9			31.7	4.7		2.9		0.8	8.2	34.8		7.6			2.5[3] 1.8[4]
10700		1.3					47.9	2.6					5.6	42.6					
10701		1.5					48.8	4.5					3.6	41.6					
1033		3.1		2.0		0.5	32.4	4.1		2.4		1.1	9.0	30.8	2.0	7.9			2.0[3] 2.7[4]

Table IV. (cont'd).

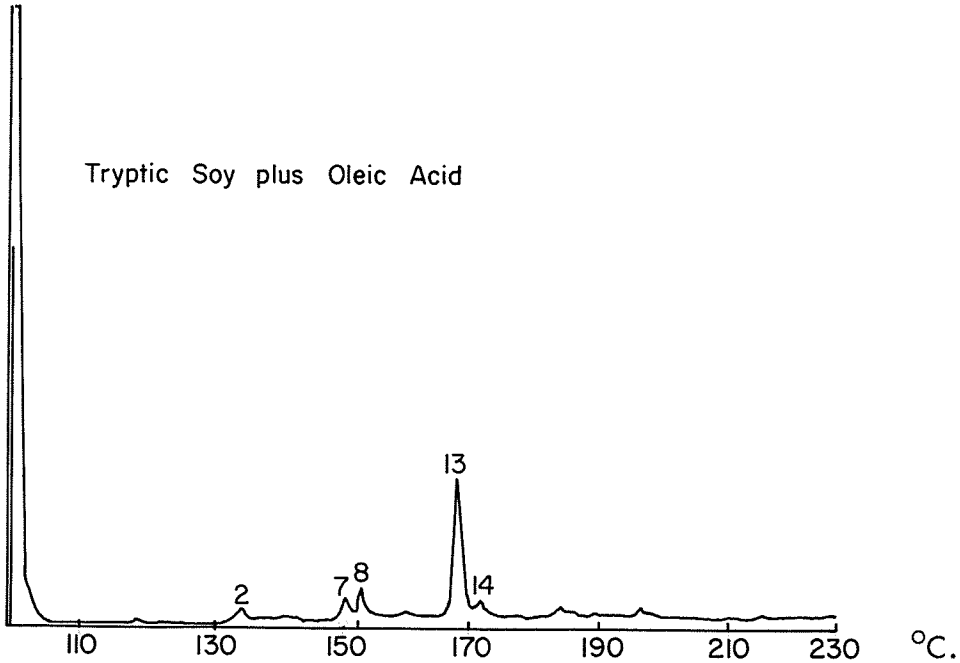
Culture #		C_{14}	Un	aC_{15}	C_{15}	iC_{16}	C_{16}	$C_{16:1}$	Un	aC_{17}	C_{17}	iC_{18}	C_{18}	$C_{18:1}$	Un	$C_{18:2}$	aC_{19}	C_{19}	
6981		0.4					43.4	1.3					5.1	40.5	9.3				
3224	1.1[2]	2.3		2.3			29.4	7.2		2.8		1.0	9.4	23.4	4.7	9.8		2.3	4.3[3]
8155		5.5		1.8	2.4	0.5	45.8	3.4	0.7	1.1	1.0		9.0	15.1			9.1		2.2[3]
U61A		5.3	0.7	1.1	1.4		53.6	8.1		0.7	0.9		3.8	12.3		12.1			2.4[5]
U61B		2.8		0.4	0.8		54.7	11.5		1.0	0.8	0.8	3.6	19.5		4.1			
7005		12.7			7.3		51.9	9.7		2.4	0.8			13.6	1.6				
1621		10.2			5.4		55.4	12.9		1.6				12.1	2.4				
7906		8.1					50.8	26.5					2.0	8.3		4.3			
DL210B	3.4[1]	6.8				1.1	42.5	7.4				1.7	13.0	18.1		6.0			
DL7813							27.1	2.9		2.0	3.4		29.3	33.2		2.1			
934	2.5[1]	3.6		2.6		1.0	26.4	4.9		1.5			9.0	21.4		20.9			3.3[3] 2.9[4]

Footnotes: 1= unidentified, 2= iC_{14} 3 = iC_{20} 4 = C_{20} 5 = aC_{21}

a. Numbers refer to percentages of total acids.
b. Code numbers of bacterial cultures refer to ATCC orNCTC Strain numbers. See page 82 for key to names of species.
c. Re fatty acid naming: Number to the left of the colon refers to number of carbon atoms; number to right refers tothe number of double bonds; br = branched chain; Un = Unidentified; a = anteiso; i = iso.

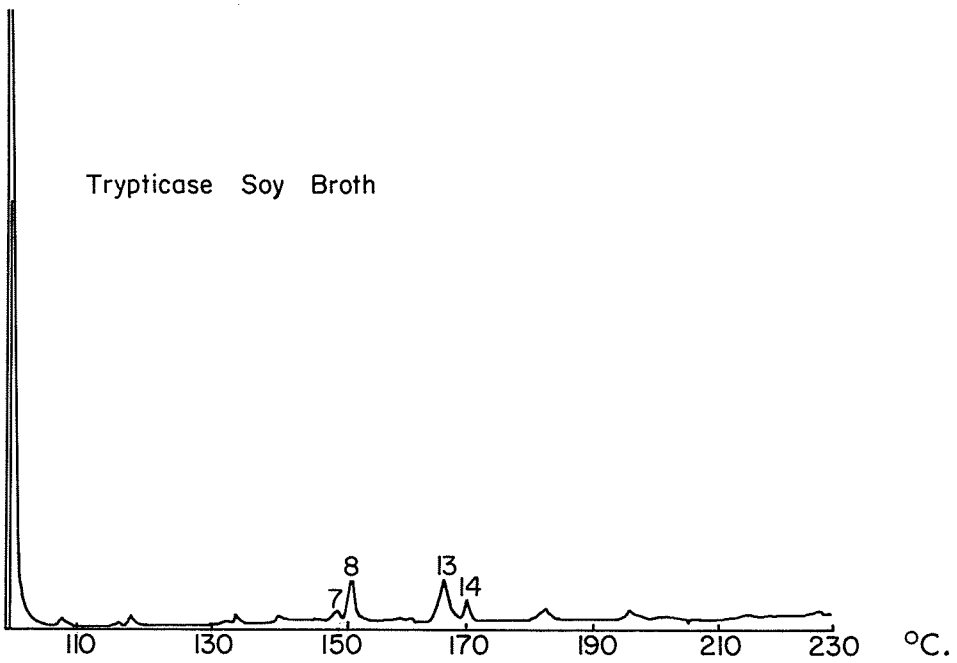


Chromatogram # 1.

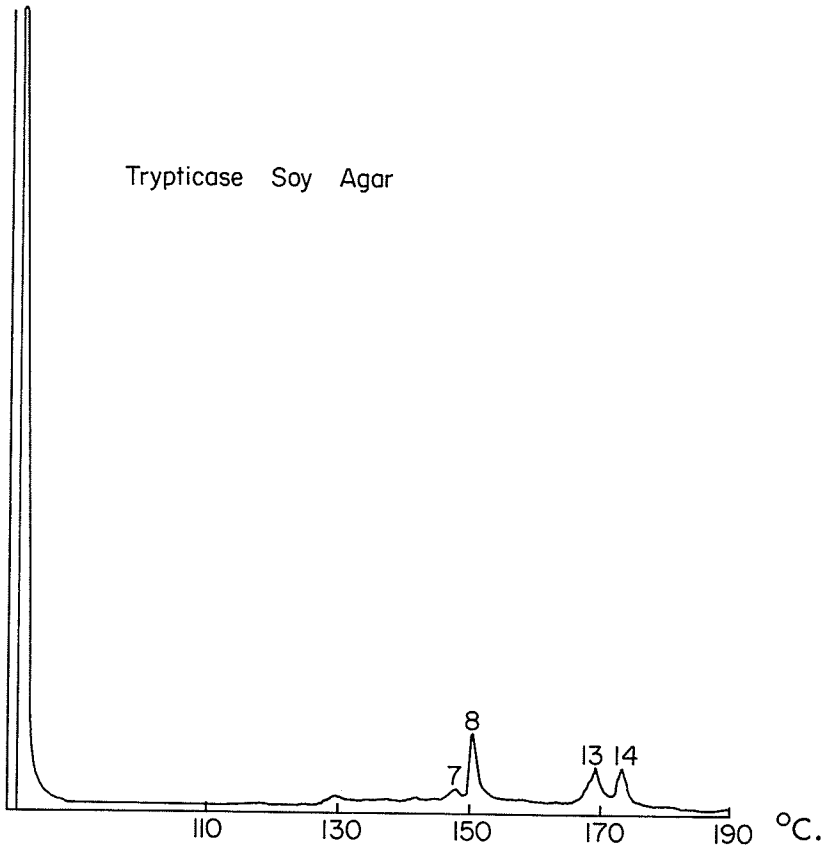


Chromatogram # 2.

Media fatty acids analyzed on SE-30.

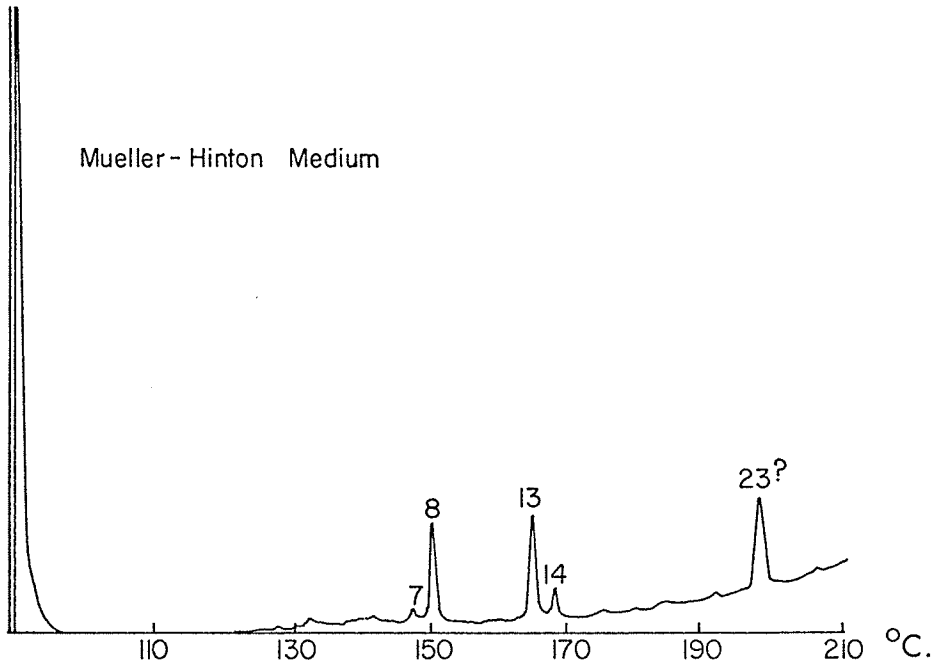


Chromatogram # 3.



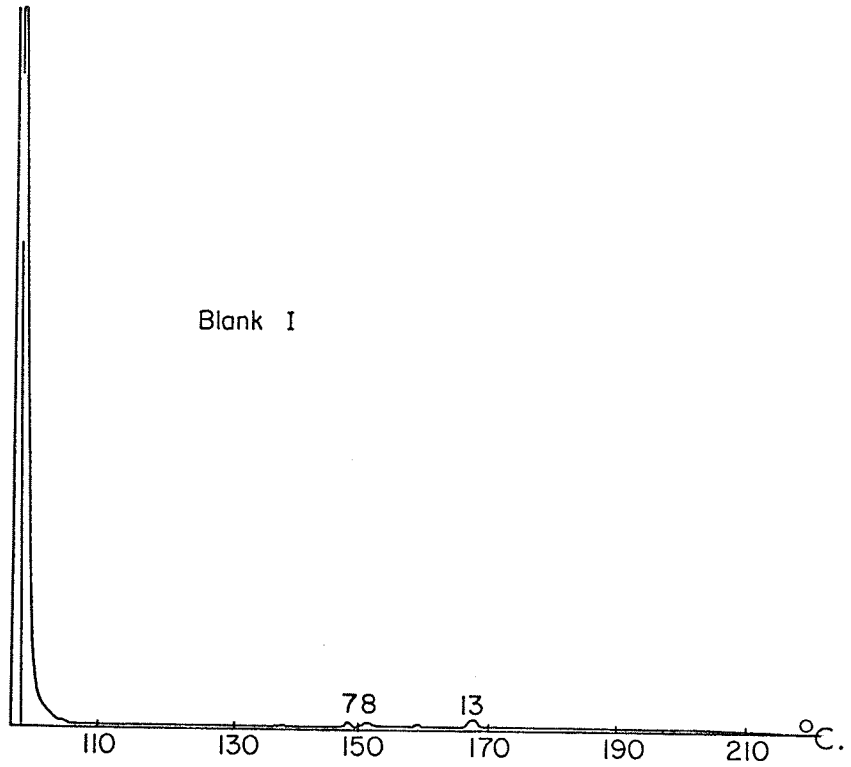
Chromatogram # 4.

Media fatty acids analyzed on SE-30.

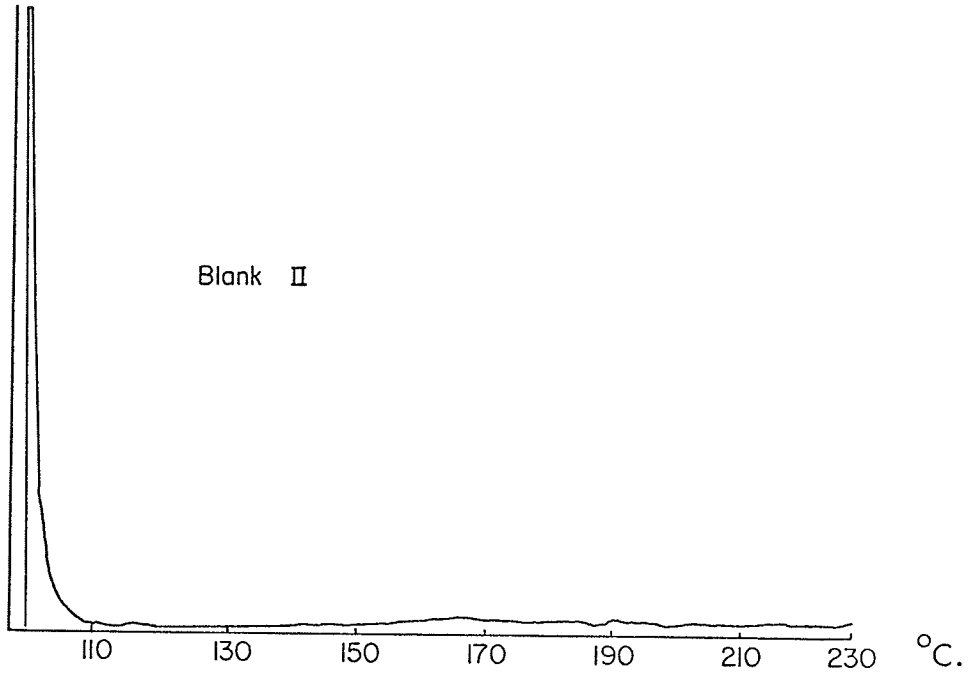


Chromatogram # 5.

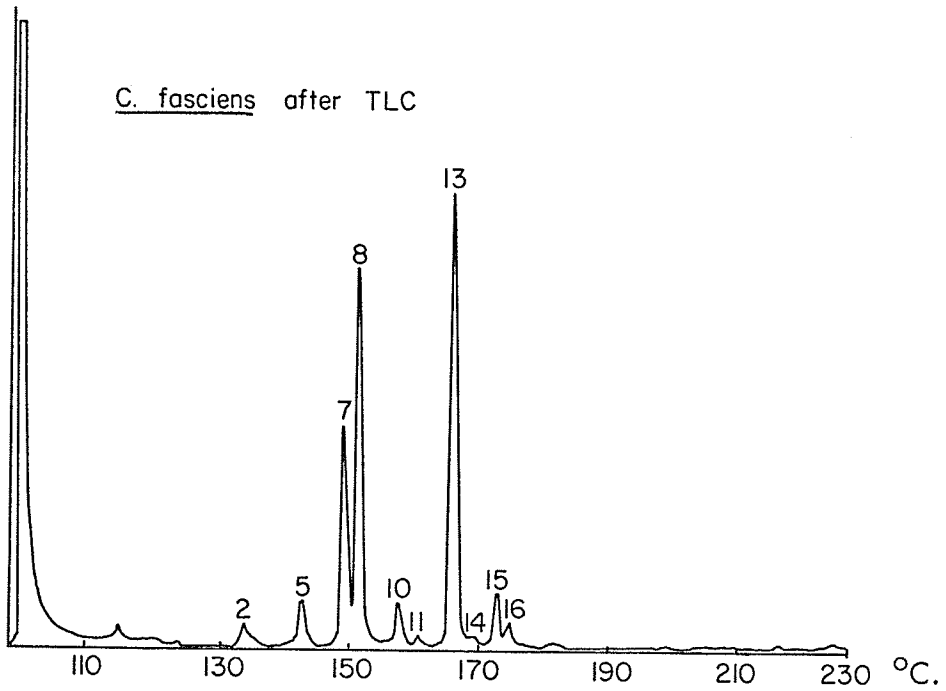
Media fatty acids analyzed on SE-30.



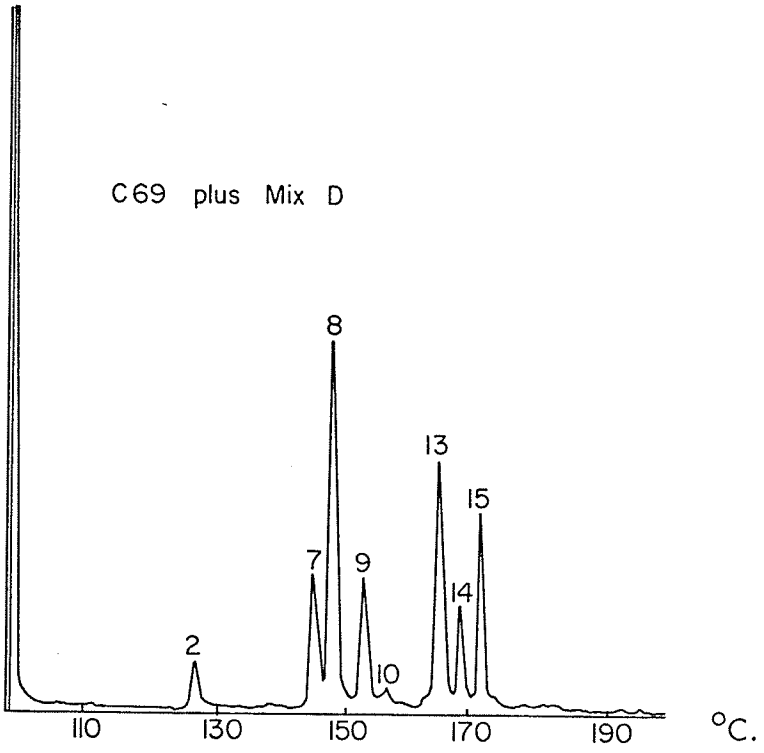
6. Hexane blank run on SE-30.



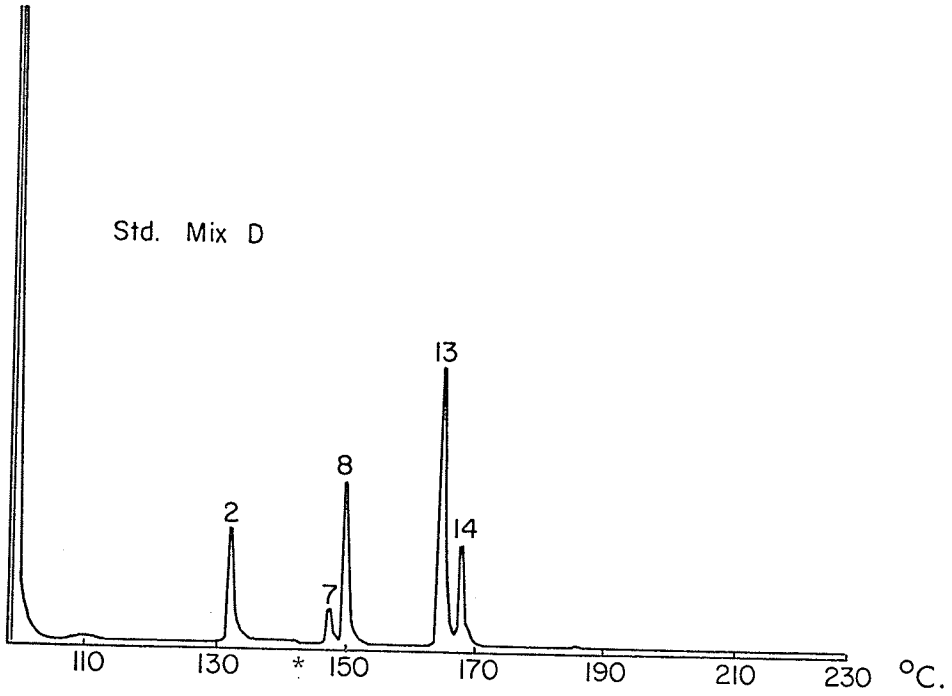
7. Reagent blank run on SE-30.



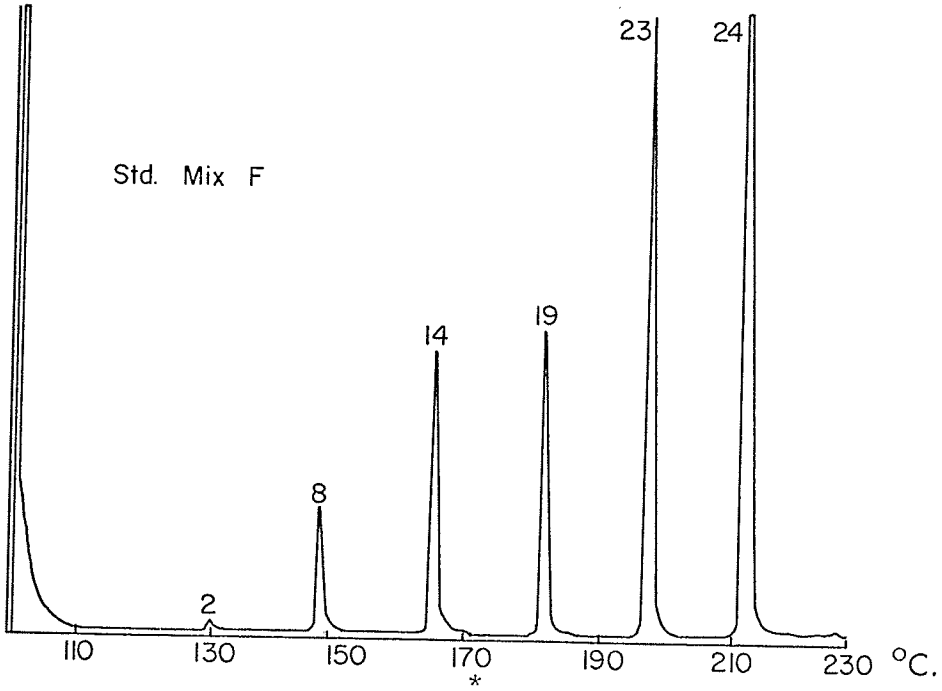
8. Fatty acids of C. fasciens after TLC purification. (SE-30)



9. Fatty acids of N. coralling with Std. Mix D added as internal standard. (SE-30)

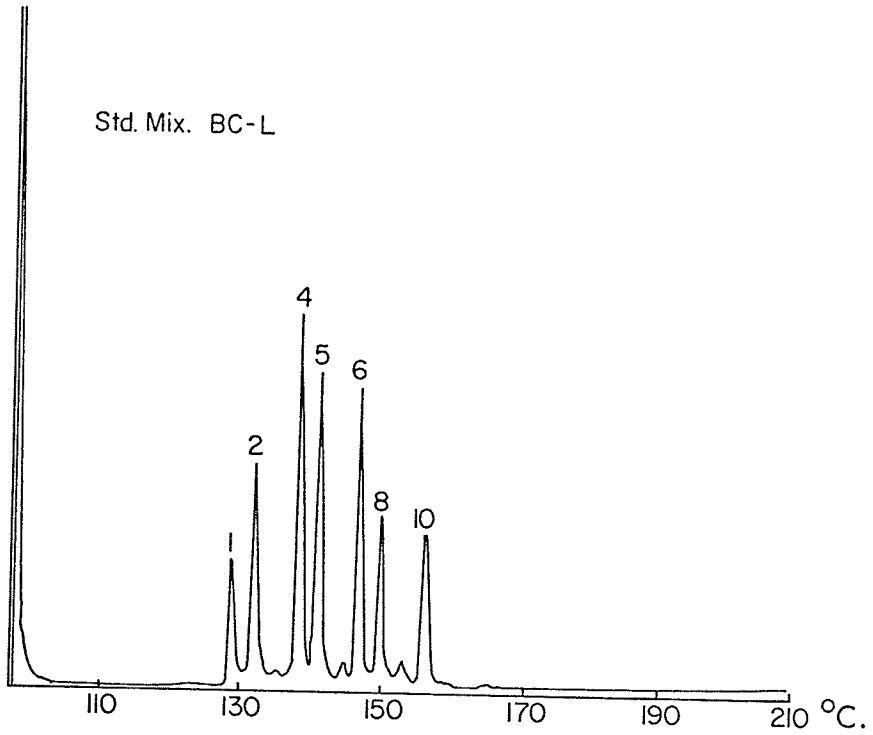


10.

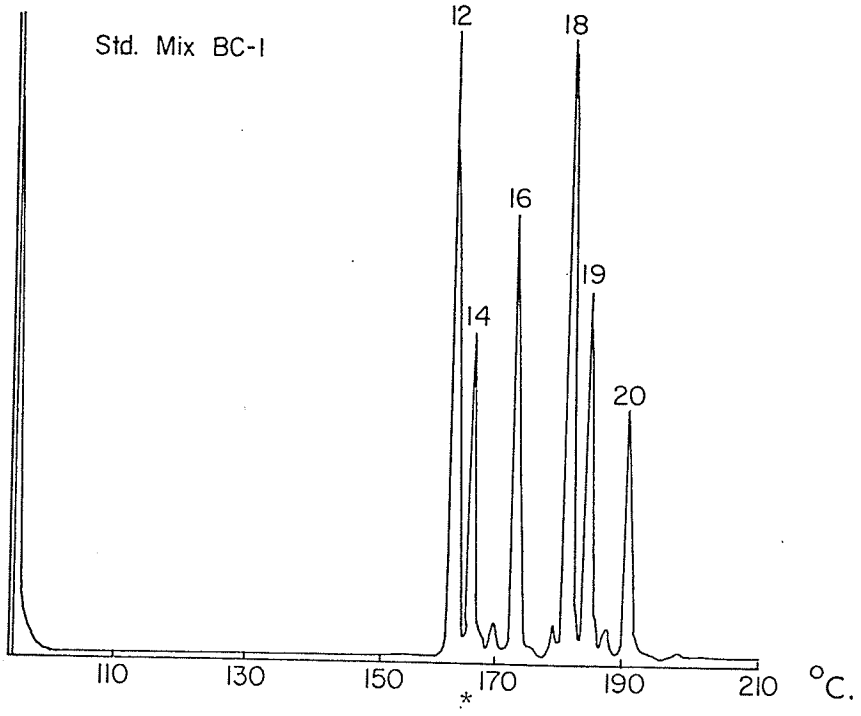


11.

Fatty acid standards run on SE-30.



12.



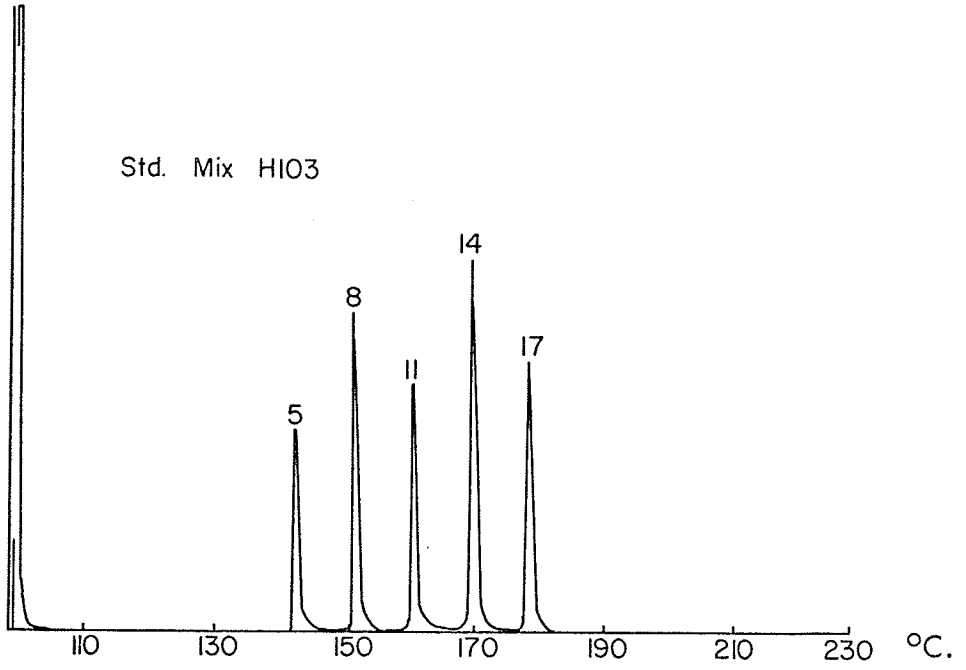
13.

Fatty acid standards run on SE-30.

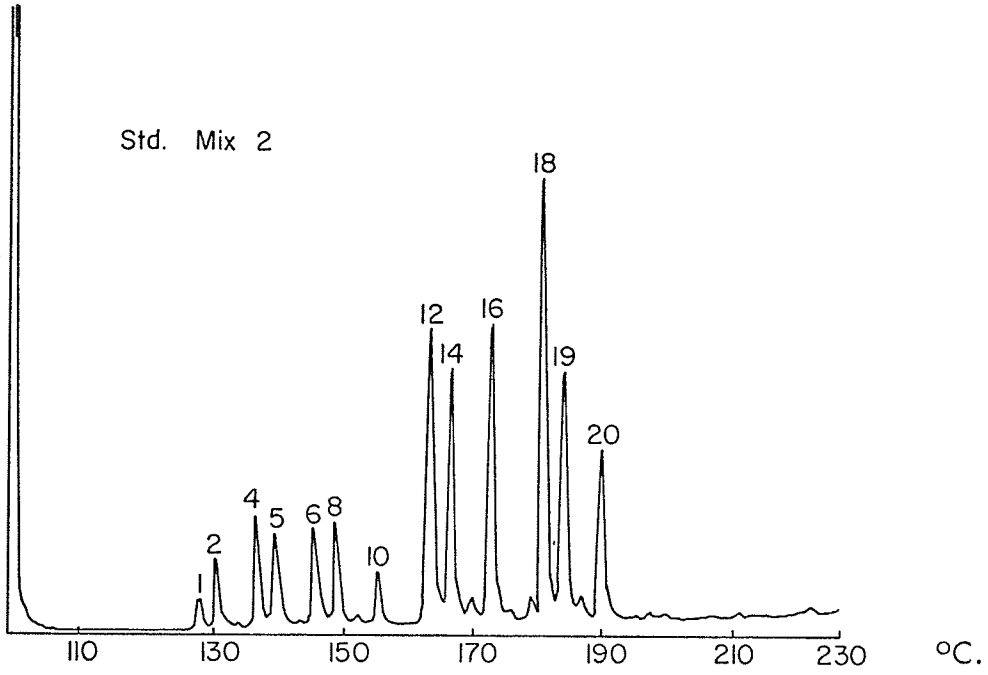
Chromatogram # 10. An attenuation change was made at the point marked with an asterisk(*). For all peaks to be on the same scale reduce peak 2 height by 1/2.

Chromatogram # 11. For all peaks to be on the same scale reduce the height of peaks 2, 8, and 14 by 1/2.

Chromatogram # 13. For all peaks to be on the same scale reduce the height of peak 12 by 1/2.

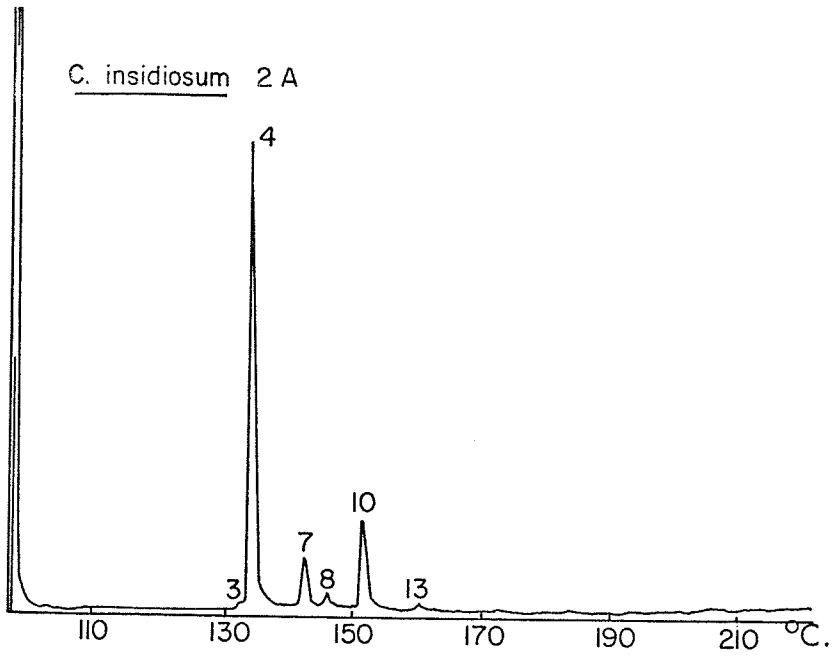


14.

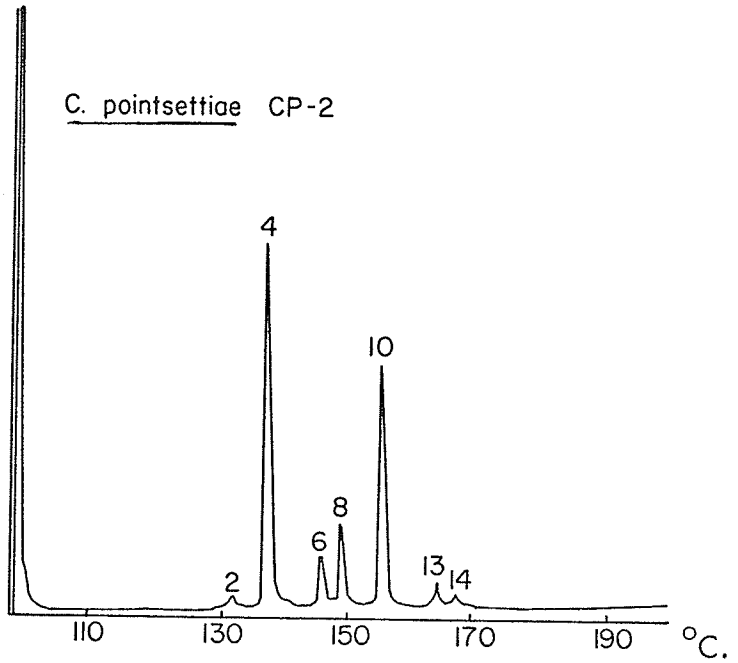


15.

Fatty acid standards run on SE-30.

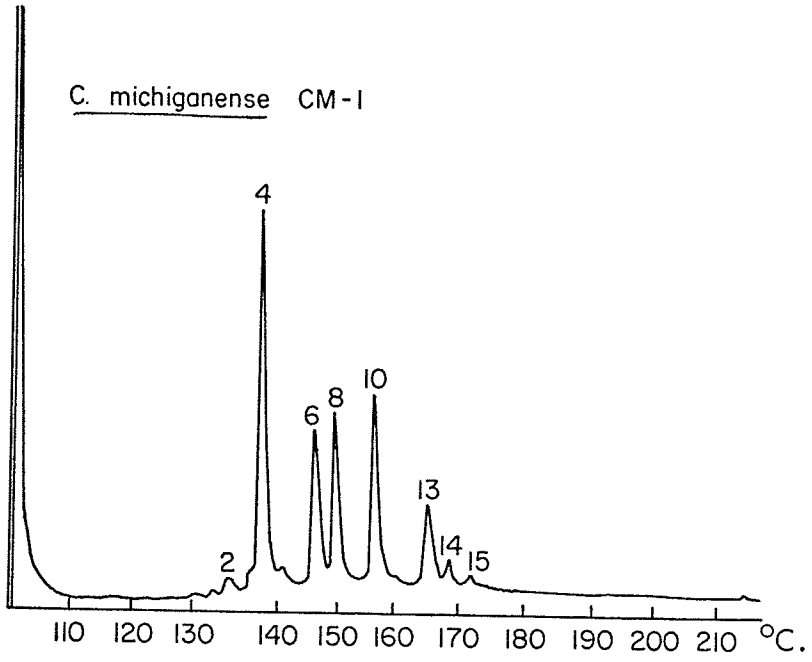


16.

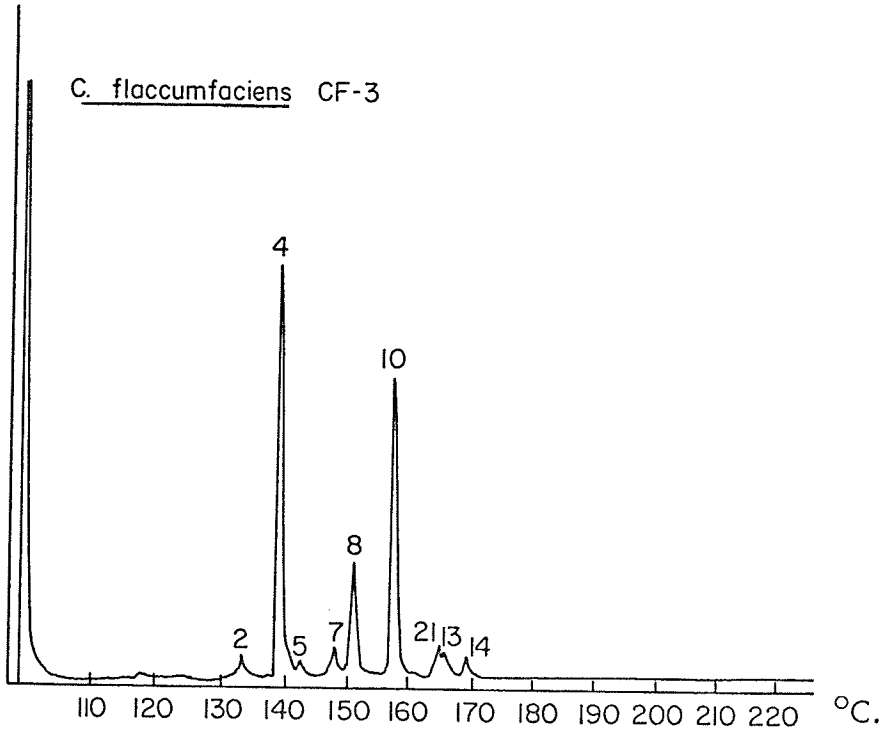


17.

Fatty acid analysis on SE-30 column.

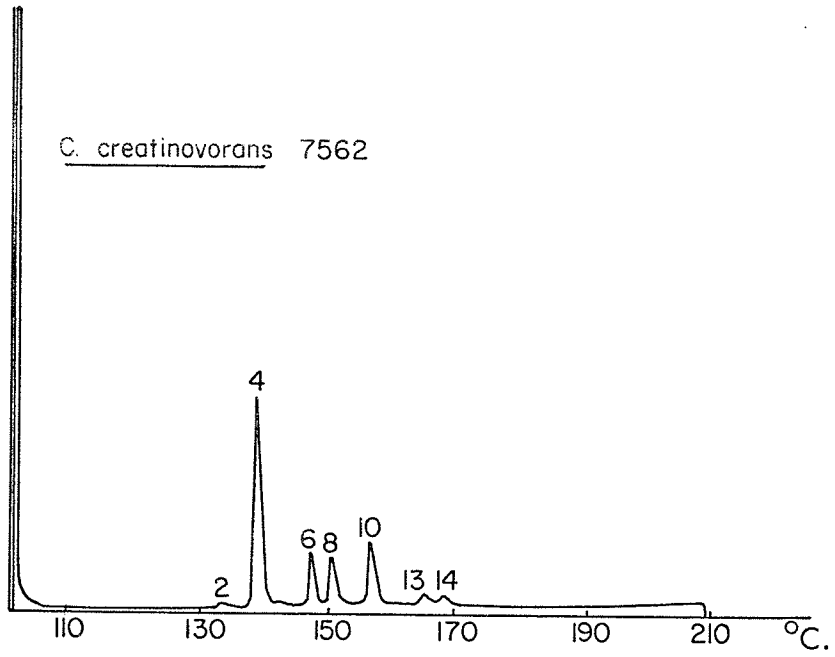


18.

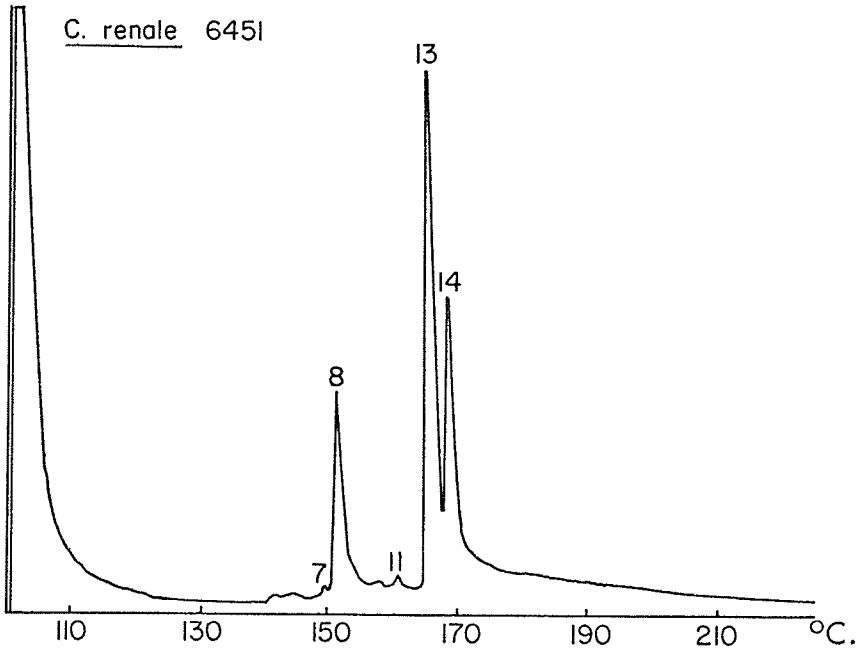


19.

Fatty acid analysis on SE-30.

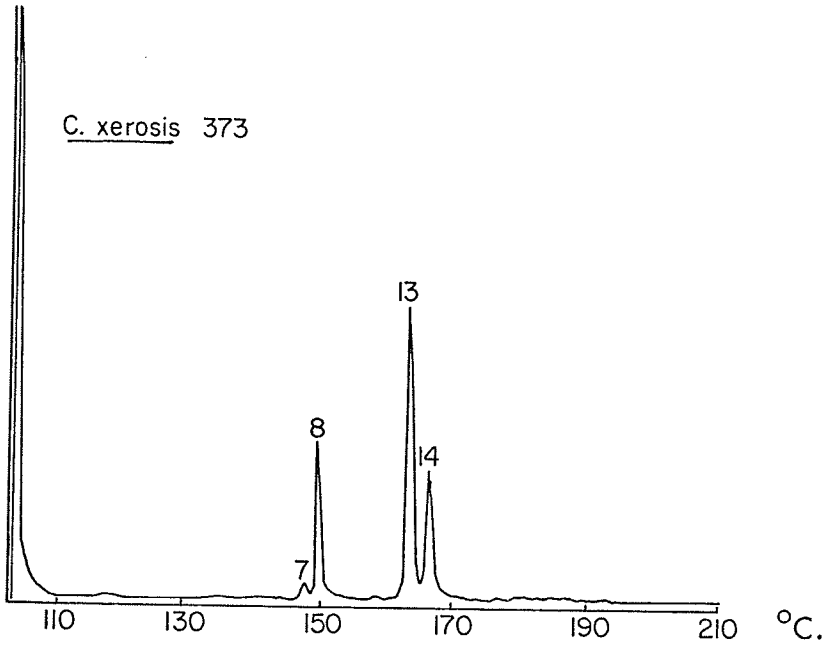


20.

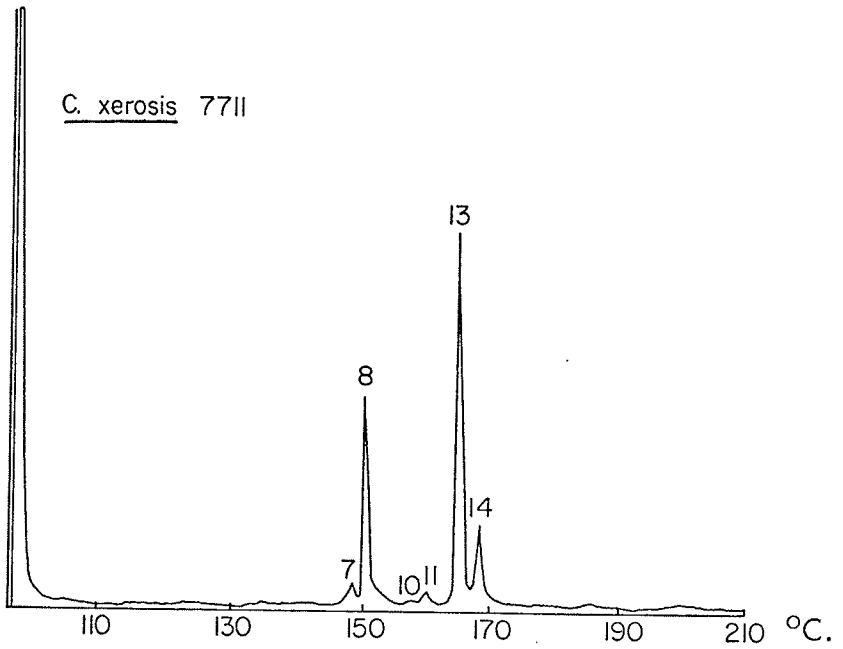


21.

Fatty acid analysis on SE-30.

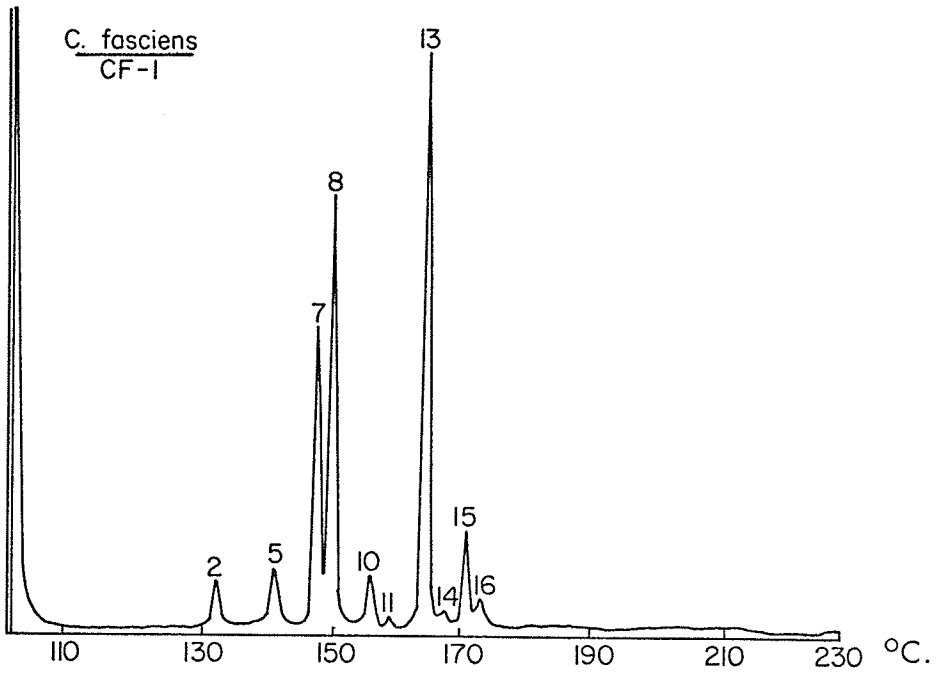


22.



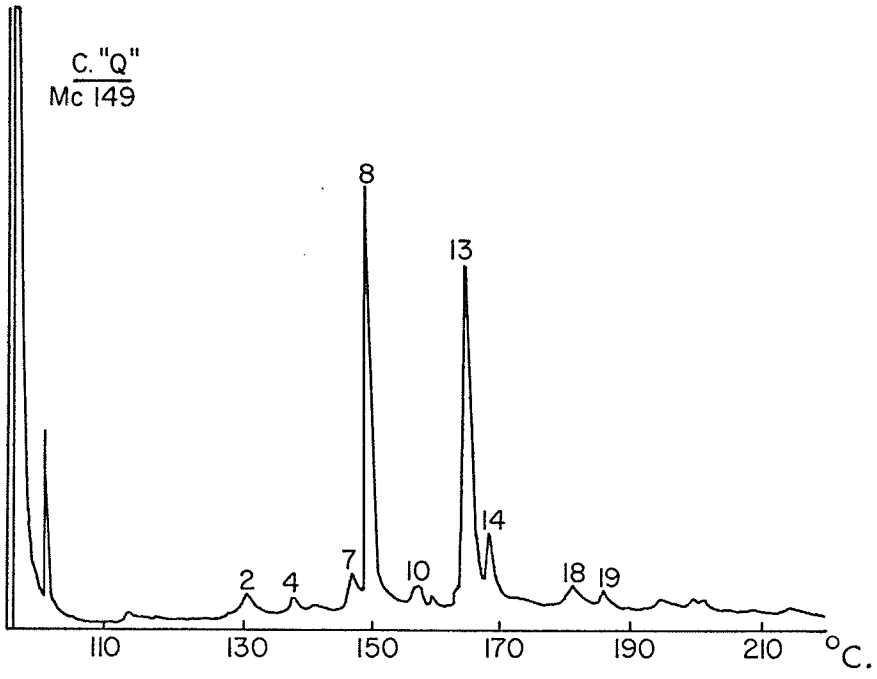
23.

Fatty acid analysis on SE-30.



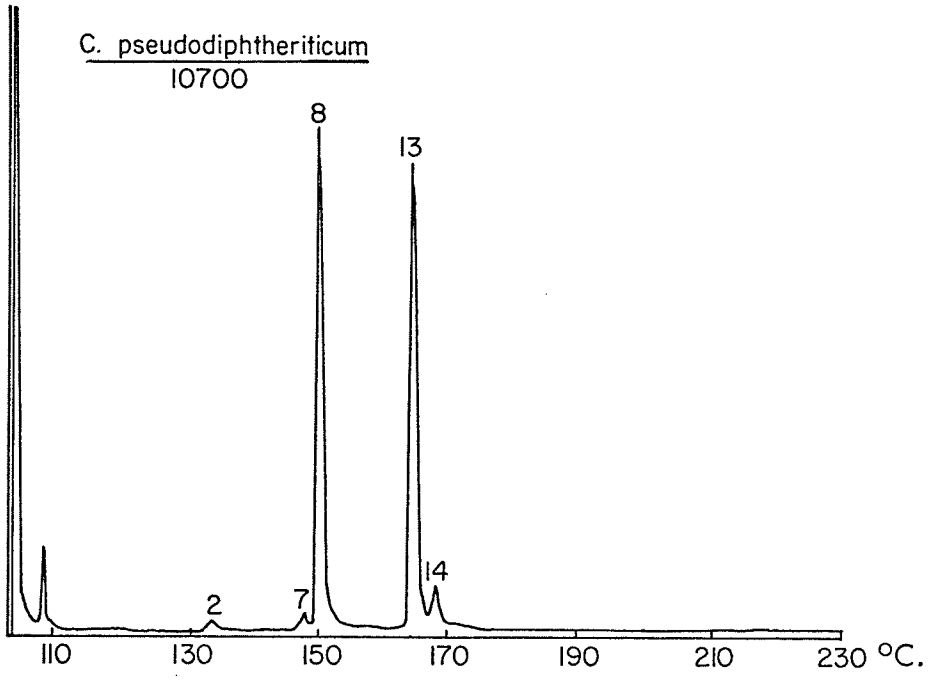
24.

Fatty acid analysis on SE-30.

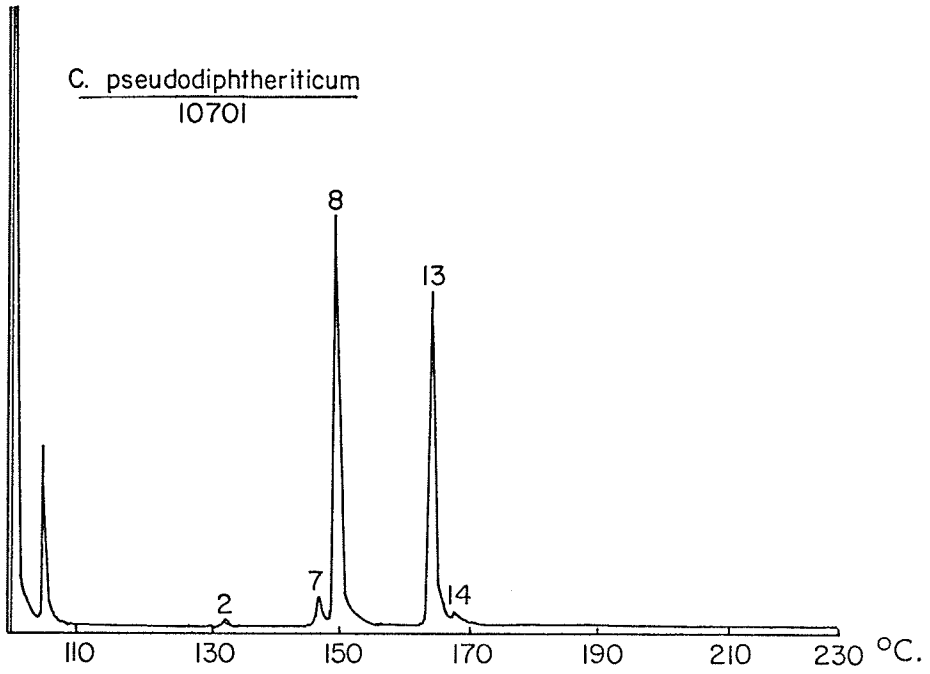


25.

Fatty acid analysis on SE-30.

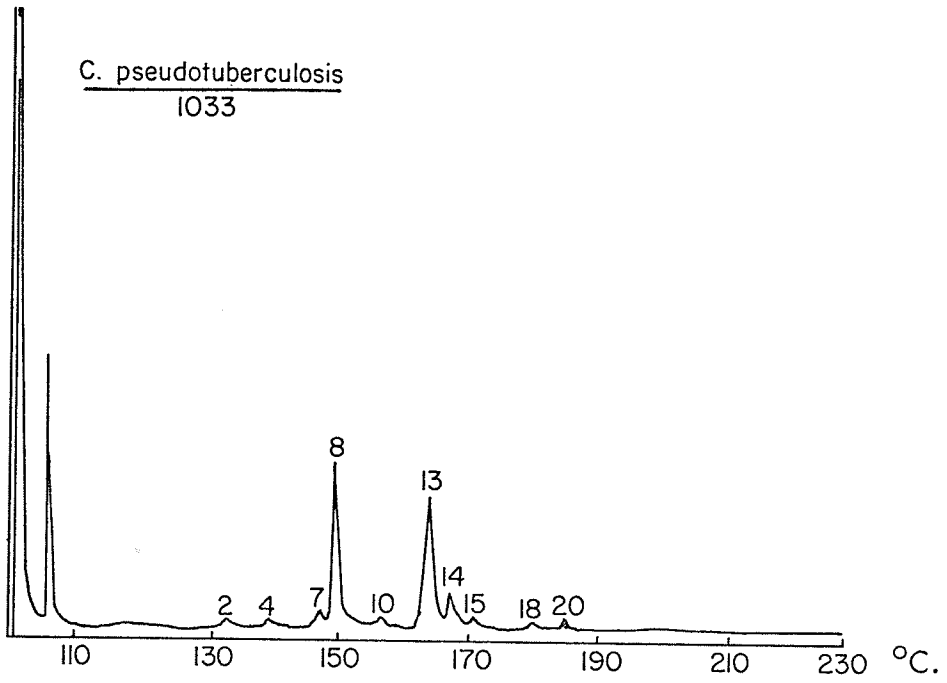


26.



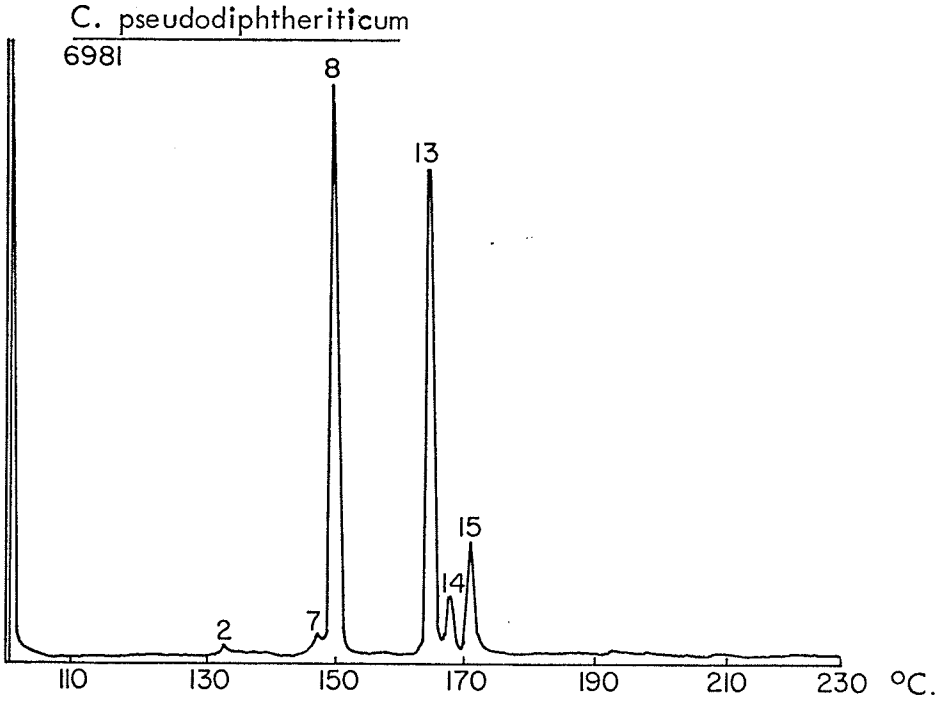
27.

Fatty acid analysis on SE-30.



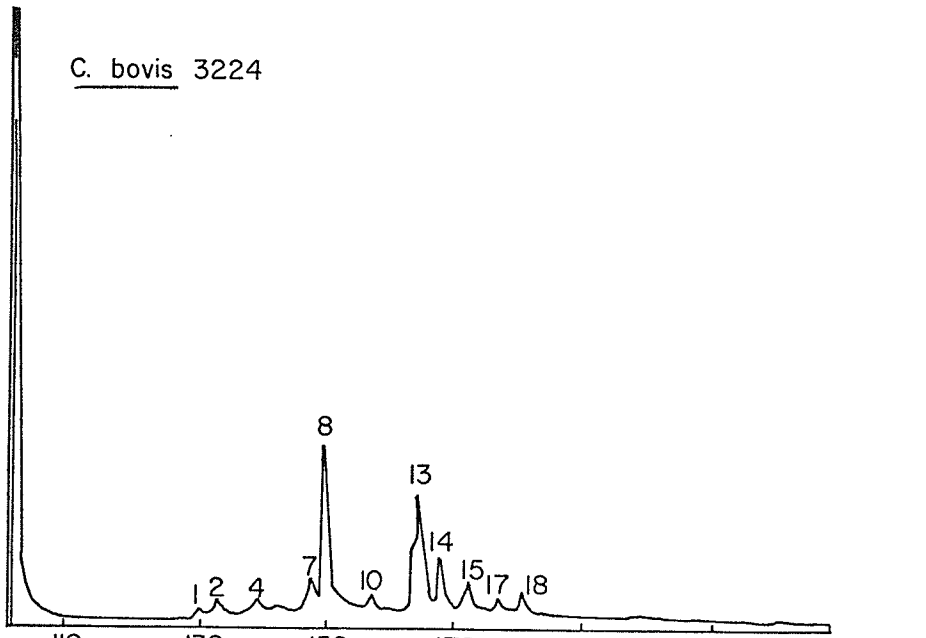
28.

Fatty acid analysis on SE-30.



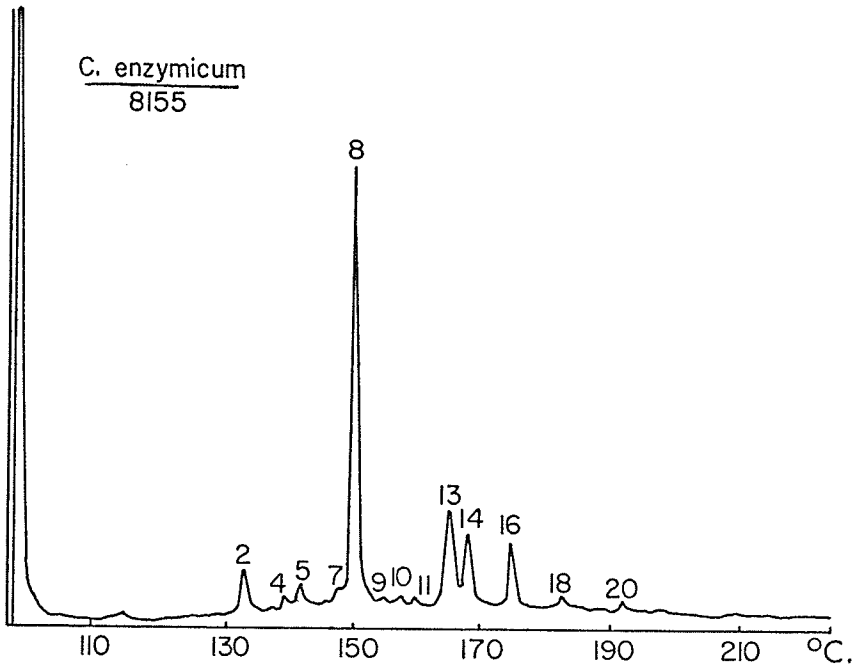
29.

Fatty acid analysis on SE-30.



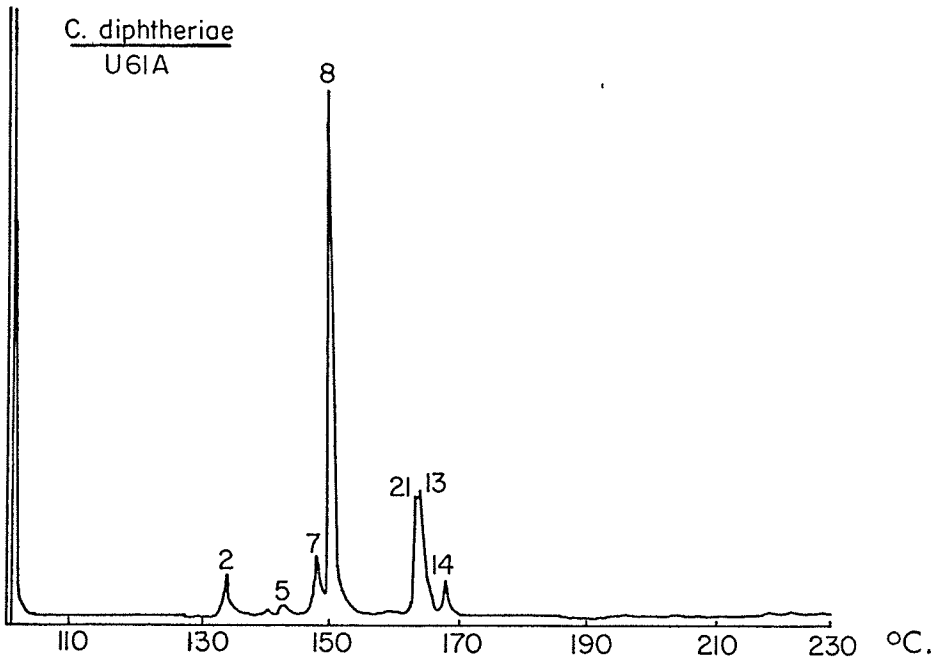
30.

Fatty acid analysis on SE-30.

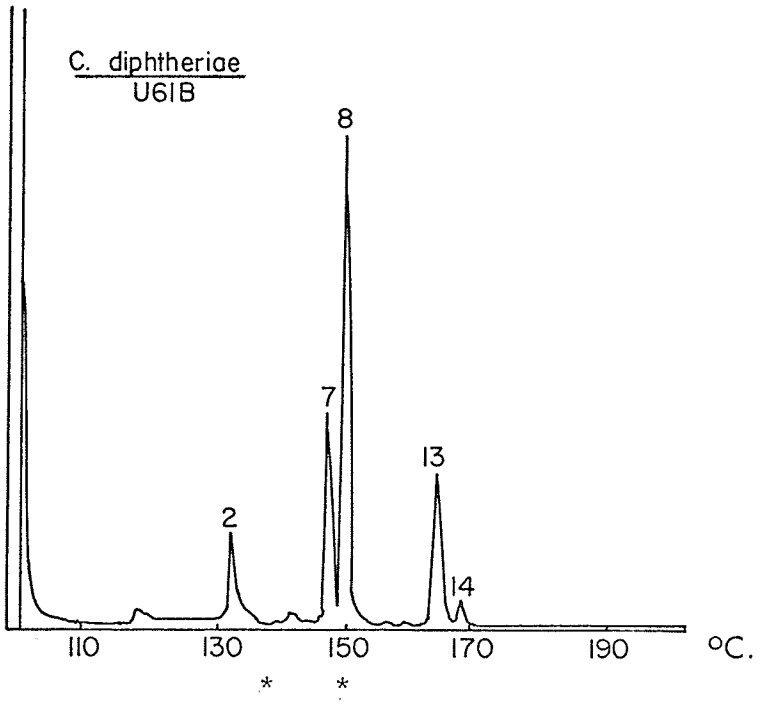


31.

Fatty acid analysis on SE-30.



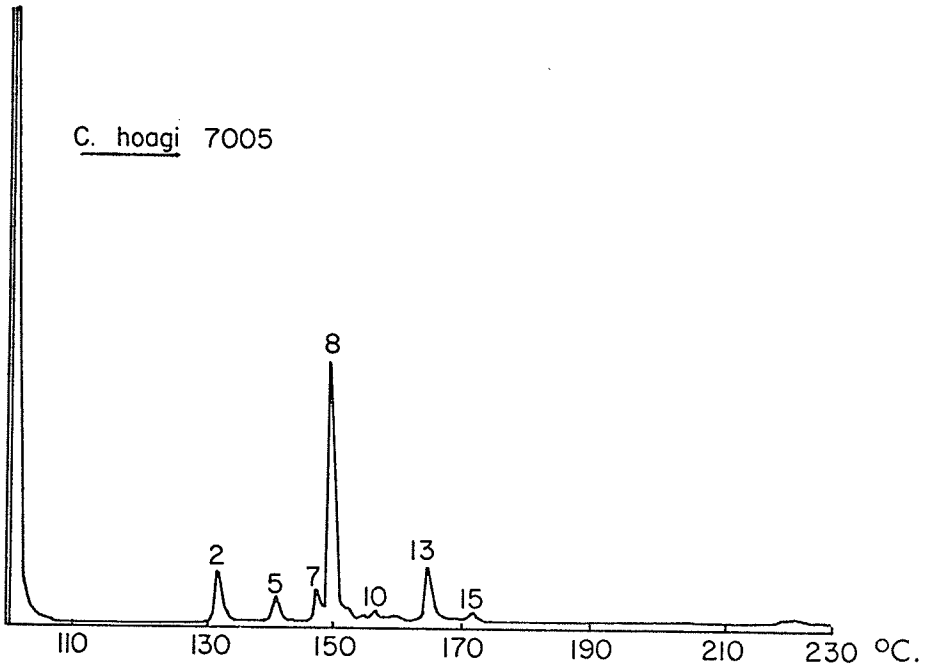
32.



33.

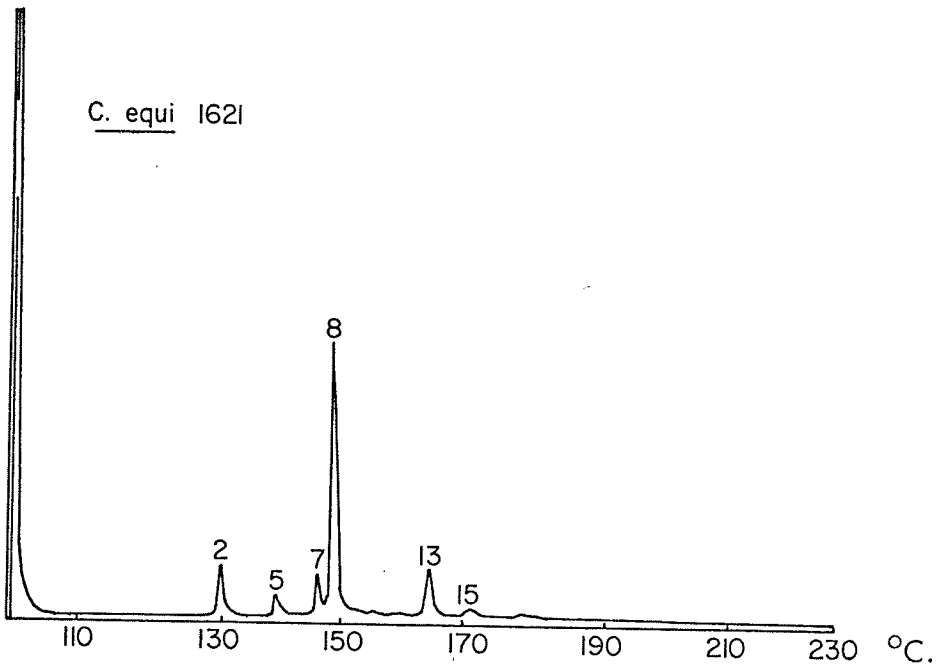
Fatty acid analysis on SE-30.

Chromatogram # 33. Two attenuation changes were made as marked by an asterisk (*). For all peaks to be on the same scale reduce peak 2 to 1/4 the height shown, peak 7 to 1/2 the height shown. The remaining peaks then appear in the proper heights.



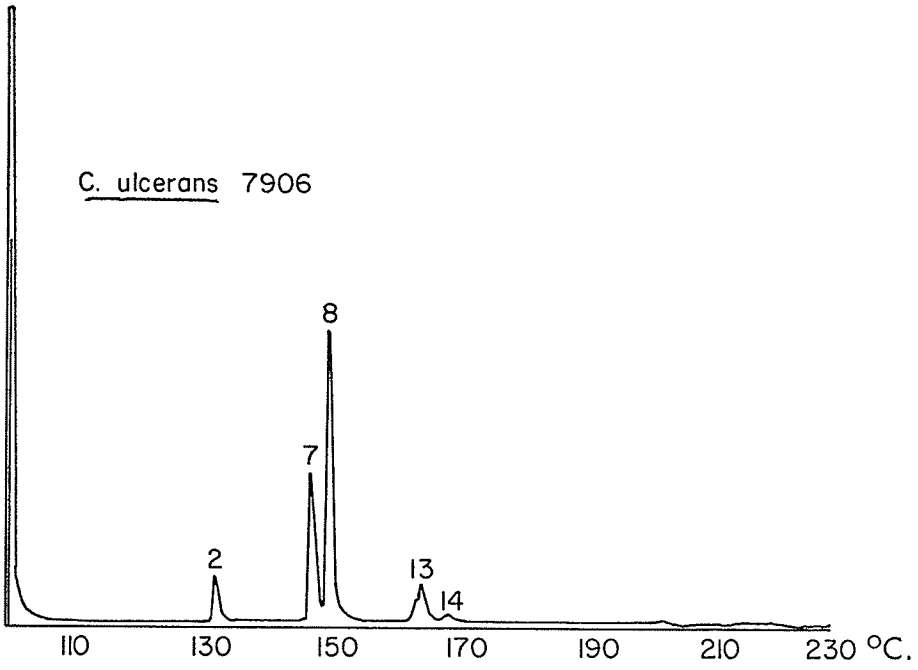
34.

Fatty acid analysis on SE-30.



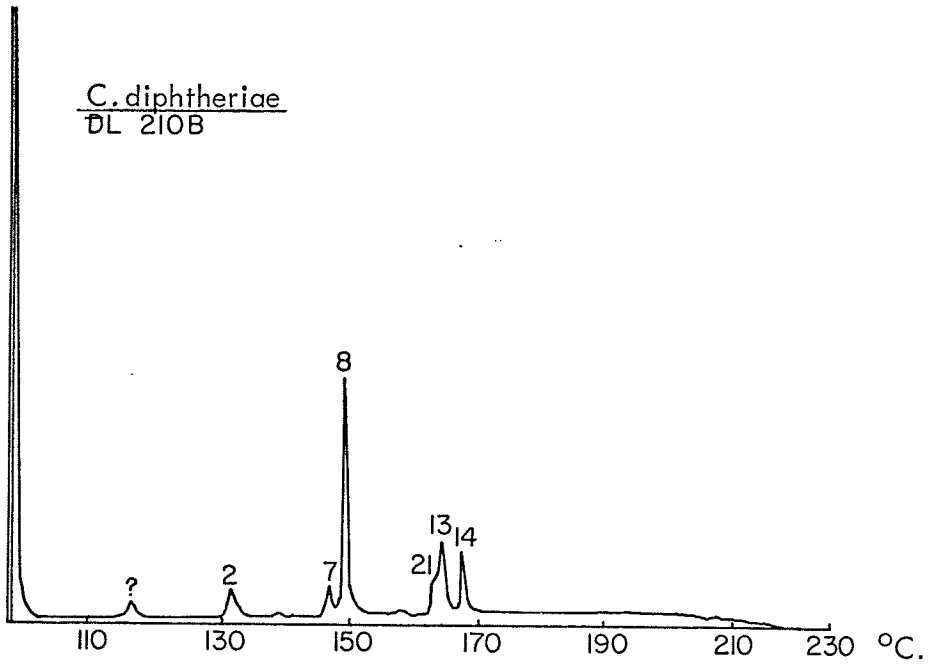
35.

Fatty acid analysis on SE-30.



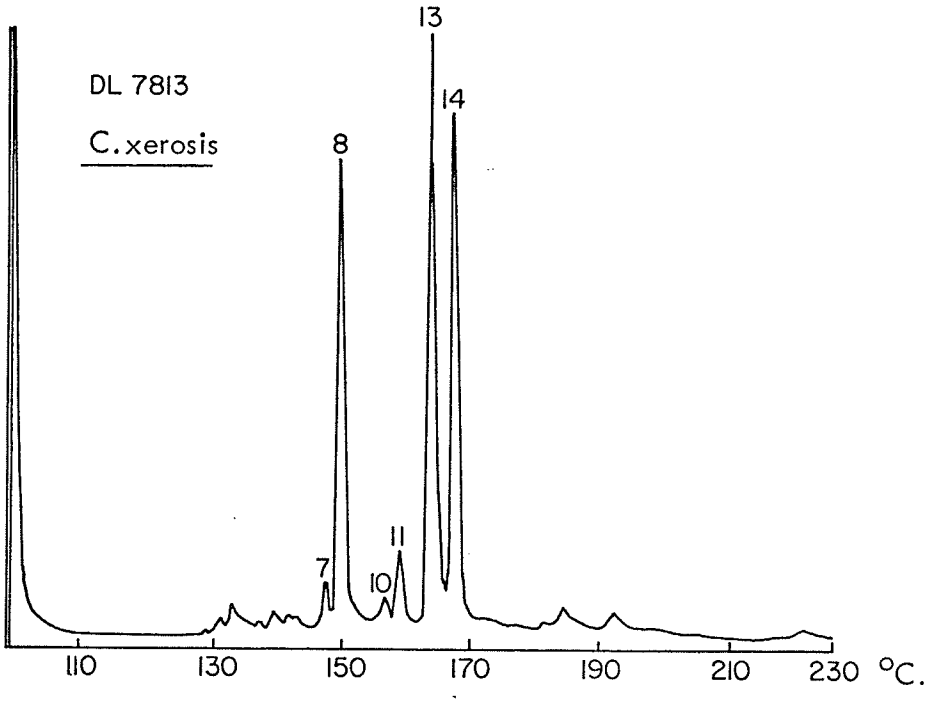
36.

Fatty acid analysis on SE-30.



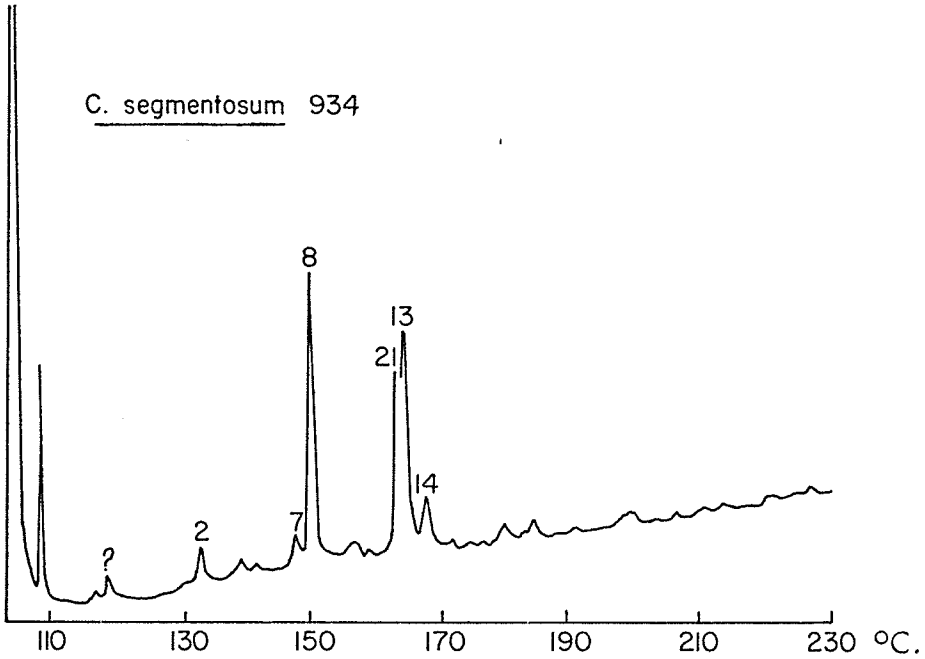
37.

Fatty acid analysis on SE-30.



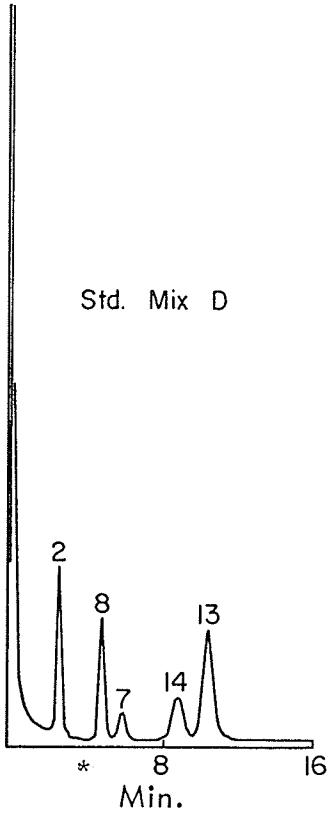
38.

Fatty acid analysis on SE-30.

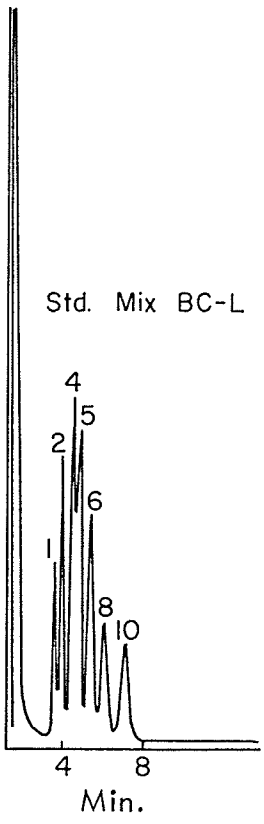


39.

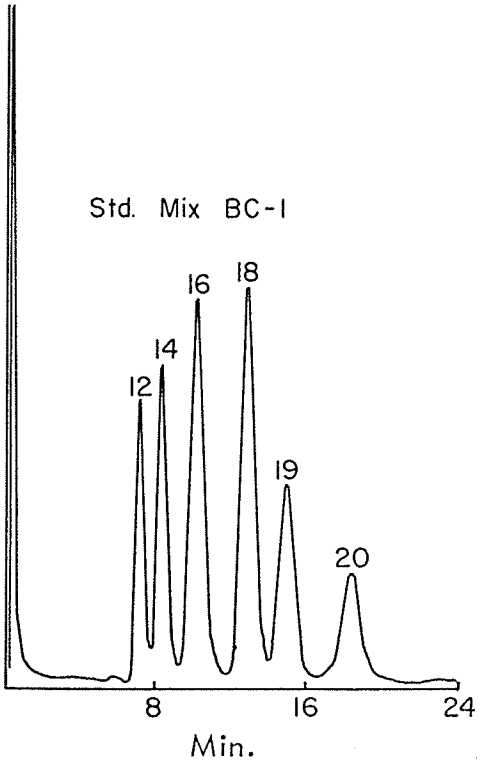
Fatty acid analysis on SE-30.



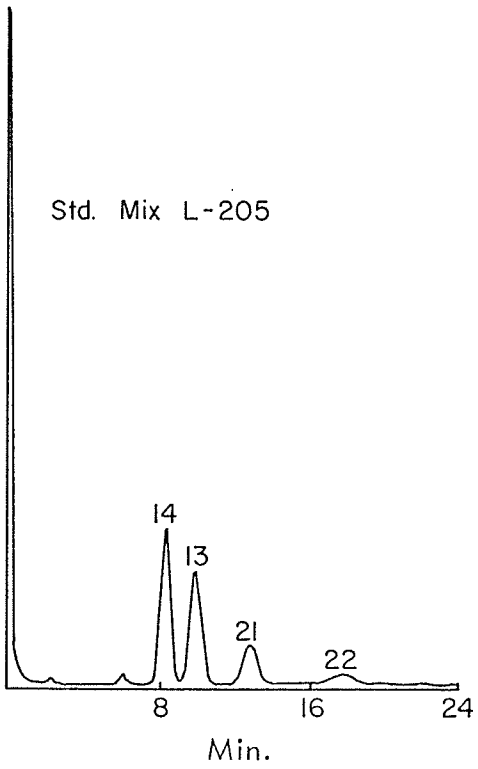
40.



41.

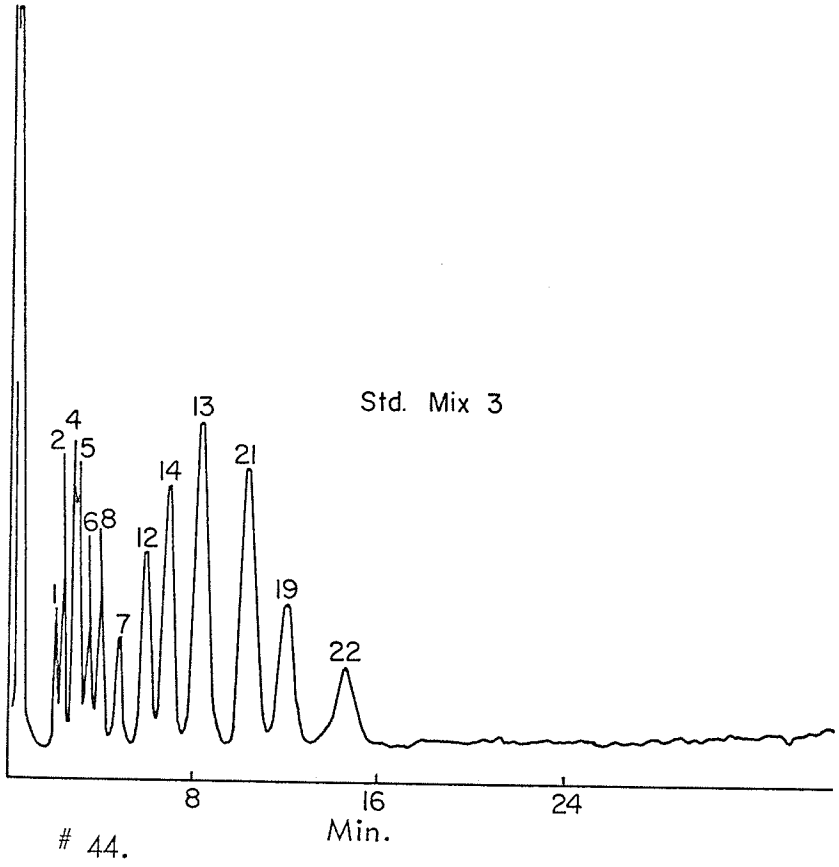


42.

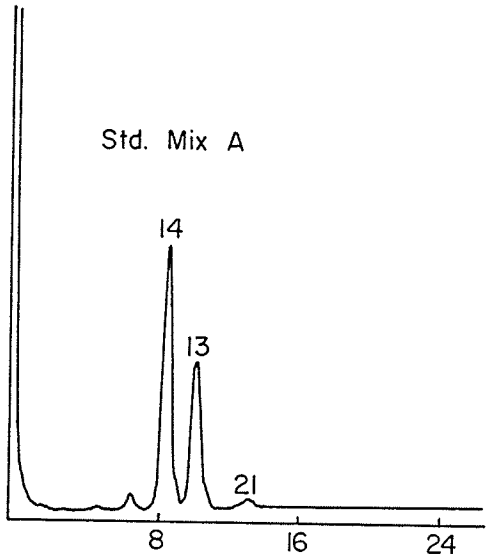


43.

Fatty acid standards run on LAC-728 column.



44.



45.

Fatty acid standards run on LAC-728 column.

Std. Mix 3 contains:

Std. Mix BC-L

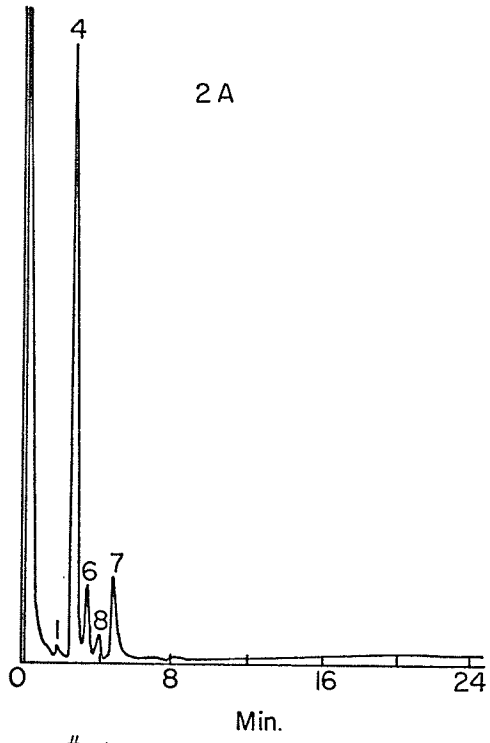
Std. Mix BC-1

Std. Mix L-205

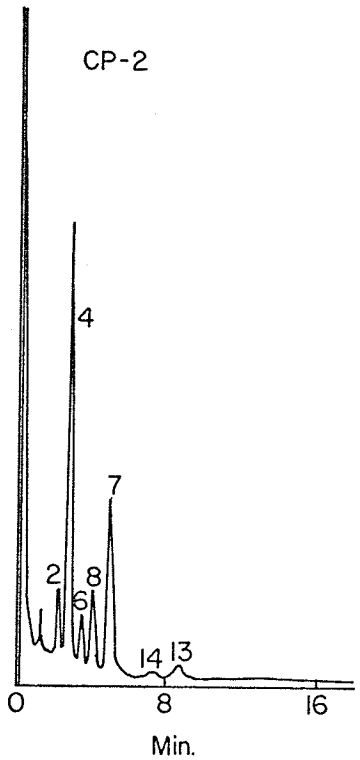
It may be noted that not all the individual peaks appear in Chromatogram # 44. This is due to a number of peaks not being separated on this type of column. The overlapping pairs are: peaks 7 and 10; peaks 13 and 16; peaks 18 and 21; peaks 20 and 22.

For convenience, each peak was marked with only one number.

Chromatogram # 40. Due to an attenuation change (*) reduce the height of peak 2 by 1/2.

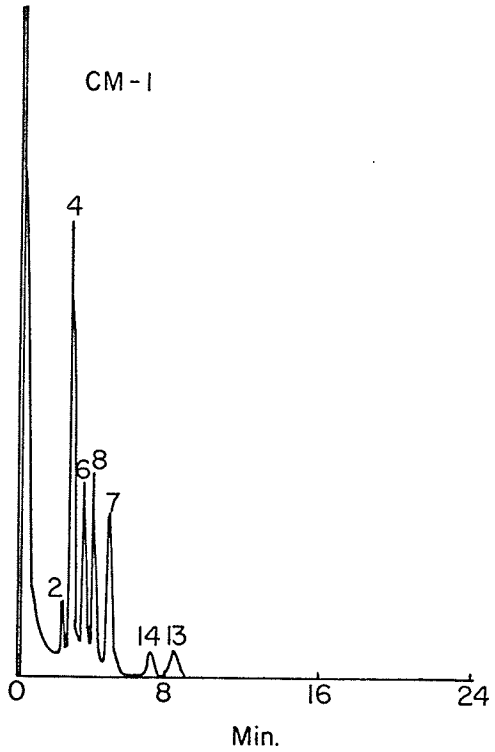


46.

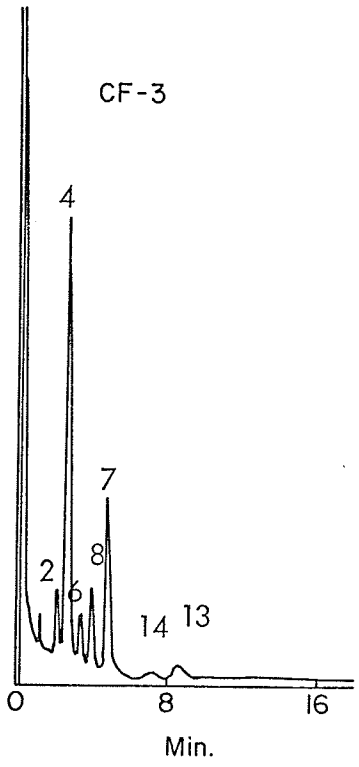


47.

Fatty acid analysis on LAC-728 column.

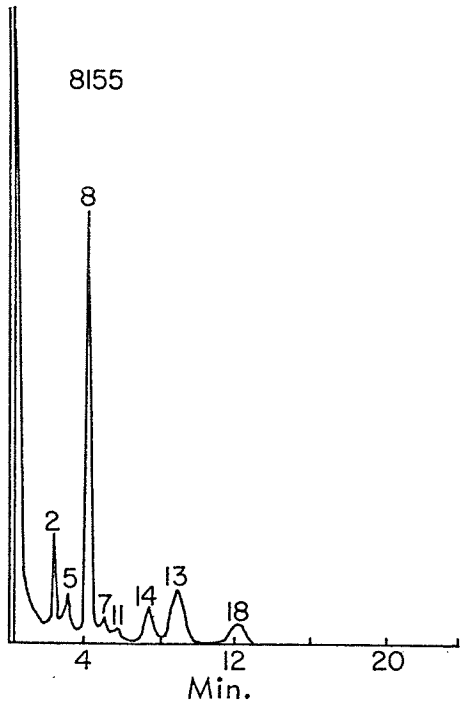


48.

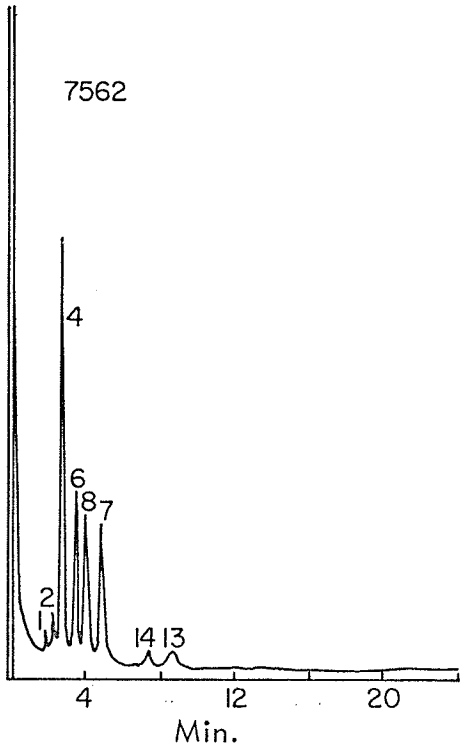


49.

Fatty acid analysis on LAC-728.

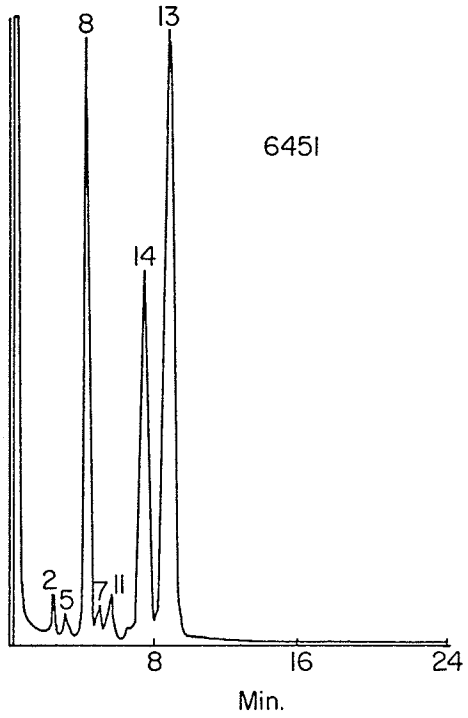


50.



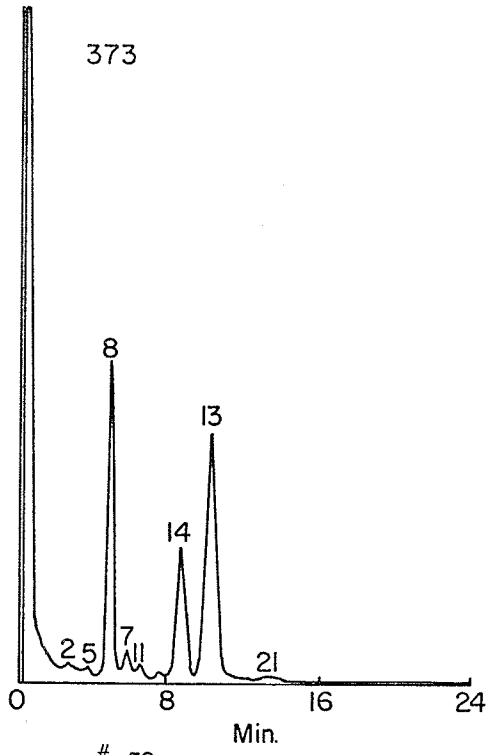
51.

Fatty acid analysis on LAC-728.

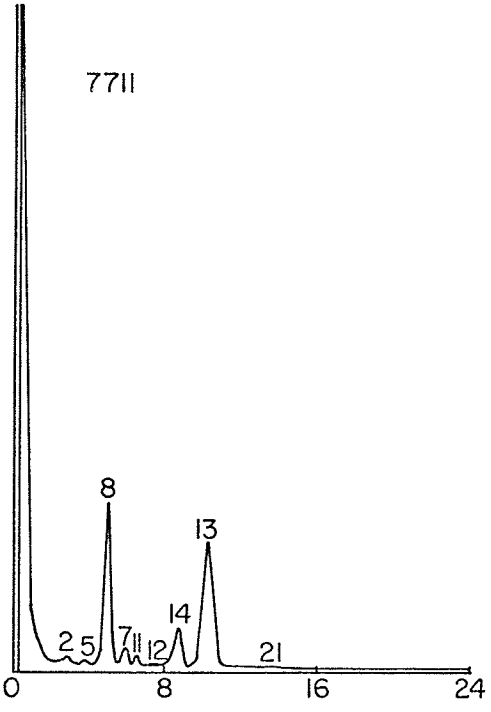


52.

Fatty acid analysis on LAC-728.

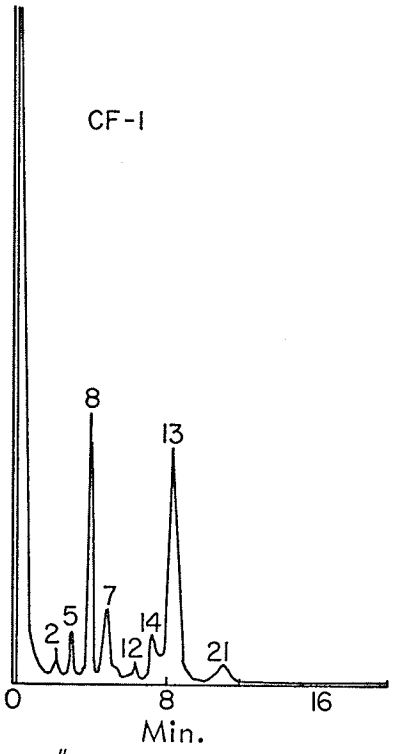


53.



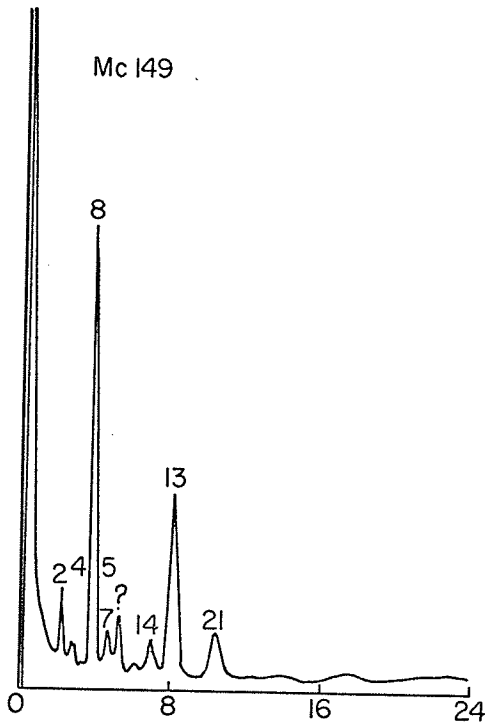
54.

Fatty acid analysis on LAC-728.



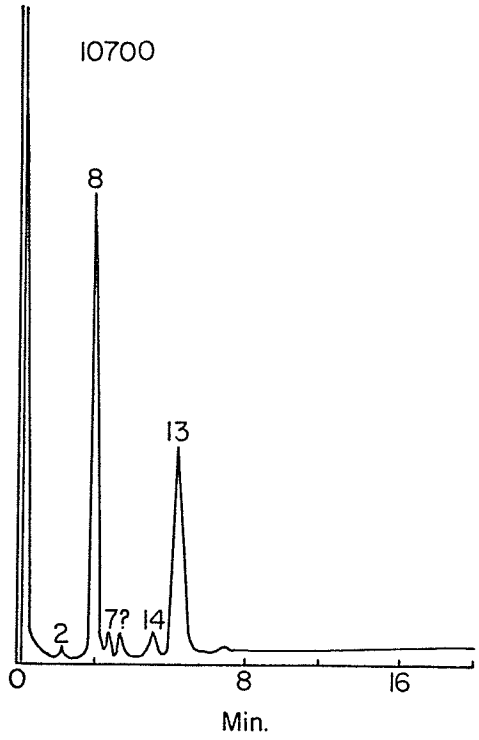
55.

Fatty acid analysis on LAC-728.

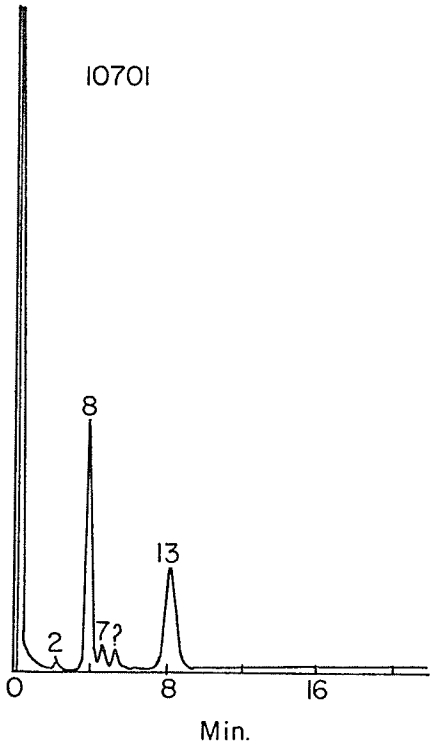


56.

Fatty acid analysis on LAC-728.

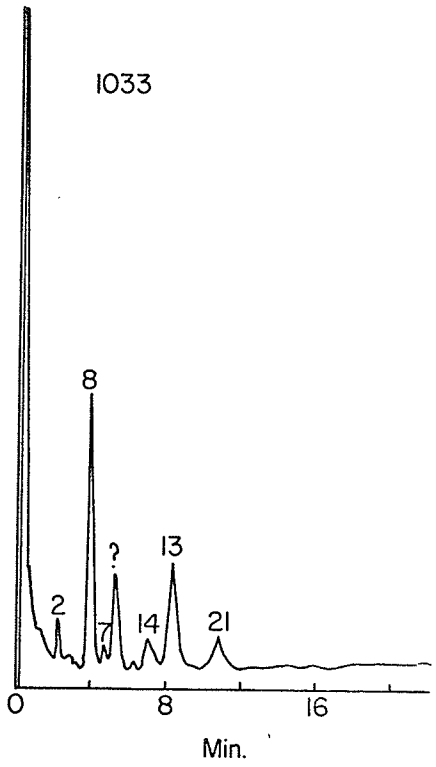


57.



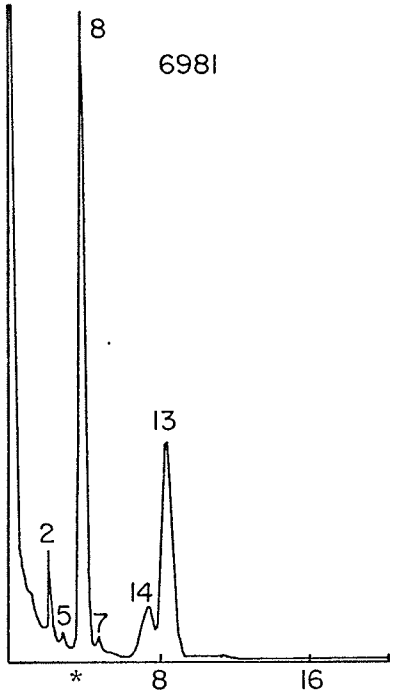
58.

Fatty acid analysis on LAC-728.



59.

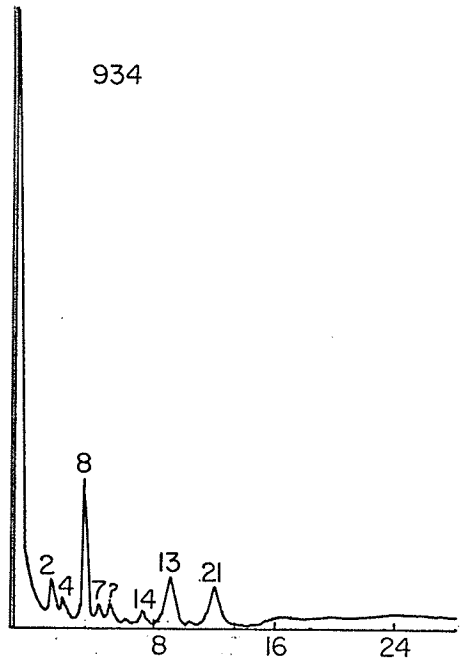
Fatty acid analysis on LAC-728.



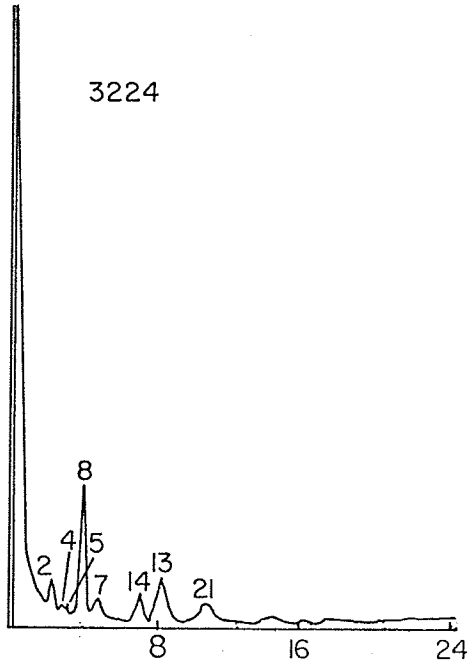
60.

Fatty acid analysis on LAC-728.

(*) reduce the height of peaks 2 and 5 by 1/4 to keep all peaks on the same scale.

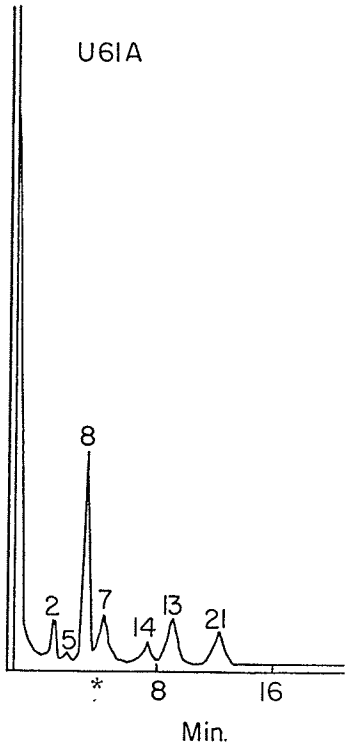


61.



62.

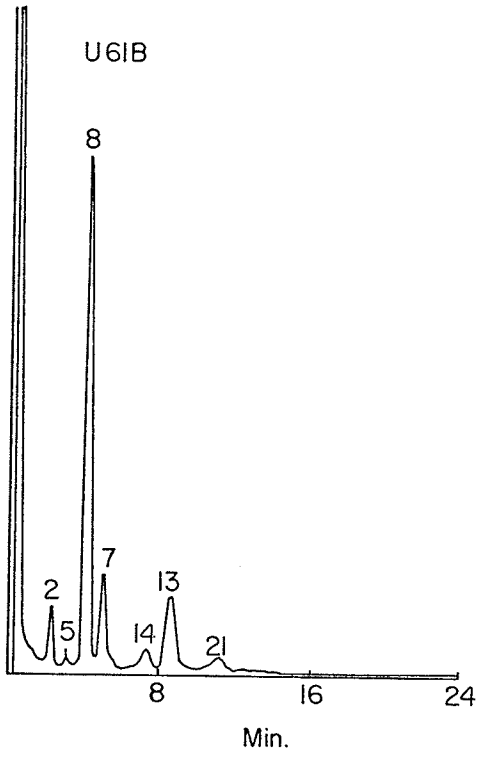
Fatty acid analysis on LAC-728.



#63.

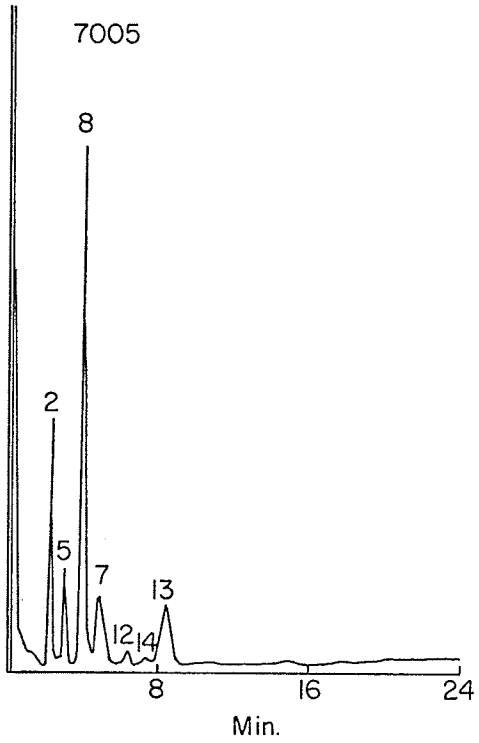
Fatty acid analysis on LAC-728.

(*) attenuation change. Multiply the peak height of # 2, 5, and 8 by 2.



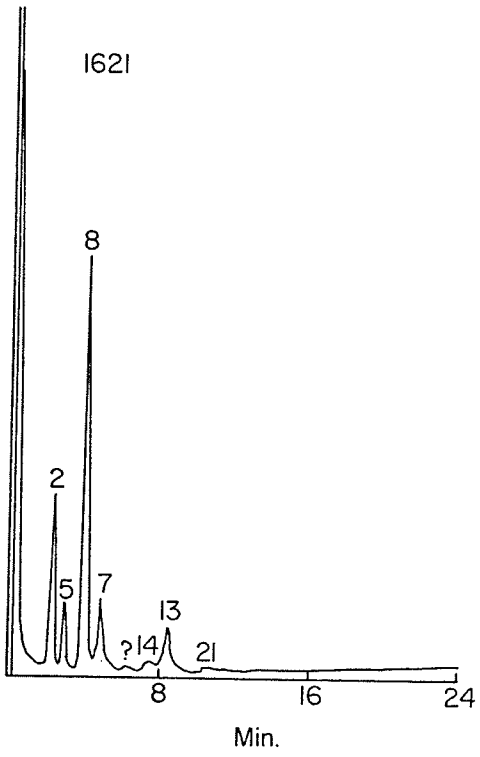
64.

Fatty acid analysis on LAC-728.



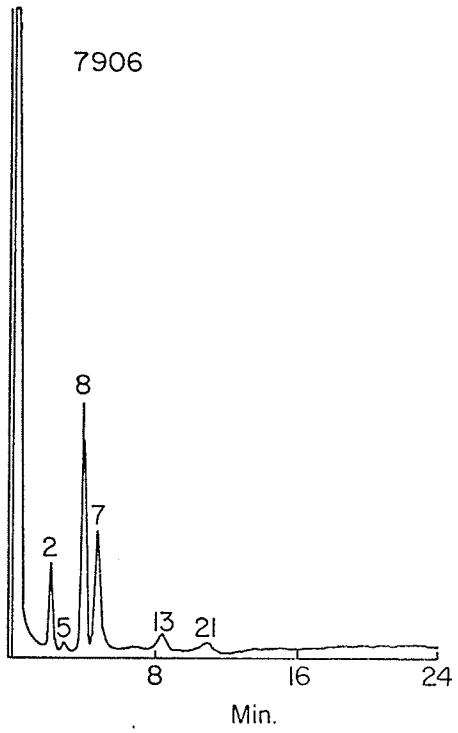
65.

Fatty acid analysis on LAC-728.



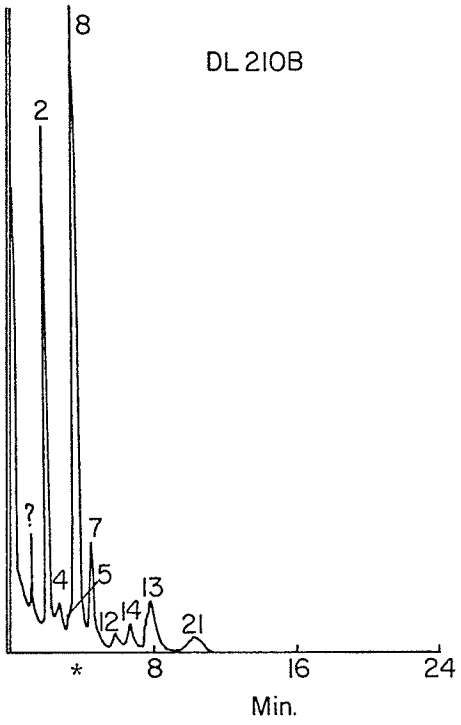
66.

Fatty acid analysis on LAC - 728.



67.

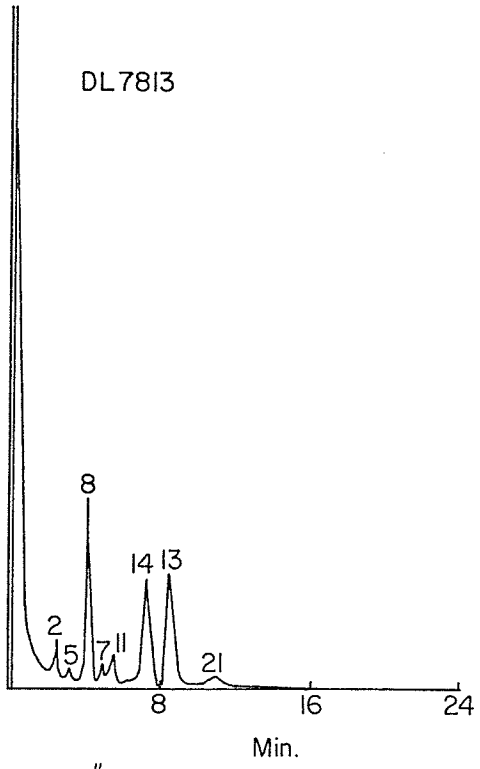
Fatty acid analysis on LAC-728.



68.

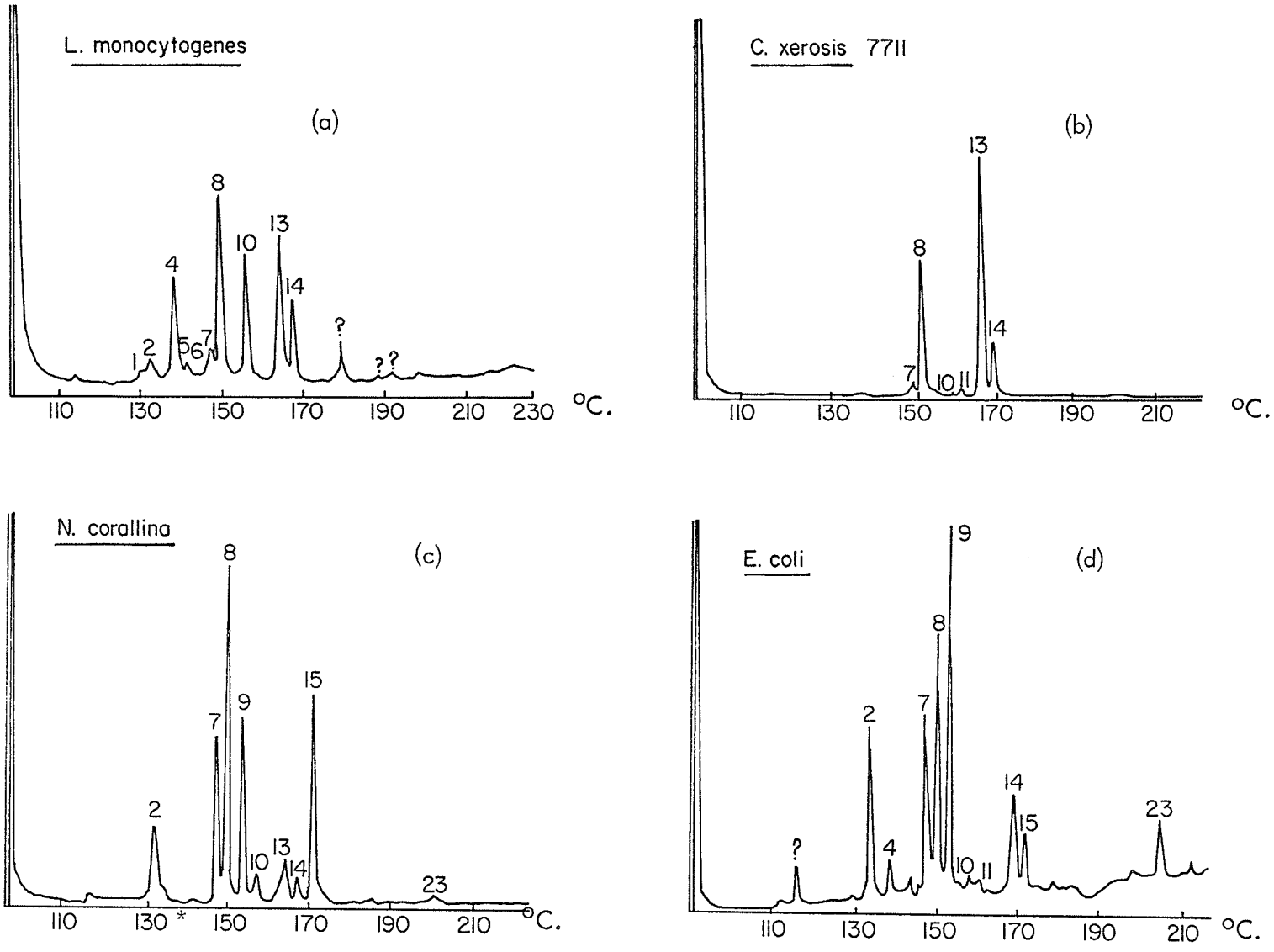
Fatty acid analysis on LAC-728.

(*) attenuation change. Reduce the peak heights of # 2, 4, and 5 by 1/2.



69.

Fatty acid analysis on LAC-728.



Chromatogram # 70. Fatty acid analysis of different families and genera of bacteria. (SE-30)

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS.

The nature of the fatty acids of twenty-seven bacterial strains including seventeen different corynebacteria have been studied by gas-liquid chromatography. In some cases a number of different strains of each *Corynebacterium* species were analyzed. Relative percentage composition of each fatty acid constituent was determined and tabulated. Fatty acids were found to range from C_{14} to C_{21} in carbon number, with saturated, unsaturated and branched chain acids represented. The most frequently encountered acids were C_{14} (myristic), C_{16} (hexadecanoic), $C_{16:1}$ (hexadecenoic), C_{18} (octadecanoic) and $C_{18:1}$ (octadecenoic). Two branched chain fatty acids appearing in a number of species were identified as $C_{15:0br}$ and $C_{17:0br}$. These two fatty acids were the major fractions in a number of plant pathogens.

The experimental method was thoroughly checked for validity before being used. The following conclusions were reached regarding our method:

i) The growth medium, Tryptic Soy Broth, contained minor traces of fatty acids but not in amounts to introduce contamination.

ii) Two successive subcultures in the same medium eliminated differences due to the medium of original isolation.

iii) The variation of fatty acid content with length of growth period, was found to be small within the same portion of the growth curve, but varied significantly at more widely separated points on the curve.

iv) Over the period of time which we selected for growth (96 hours) the fatty acid pattern did not vary significantly and therefore time was not a critical factor.

v) The choice of liquid medium rather than solid, was made on the basis of its providing an easier, more uniform, and more complete method of harvesting.

vi) Three washings of the live bacteria were sufficient to remove all adherent medium contaminant.

vii) Hexane was the most efficient extraction solvent.

viii) The purity of the final product was assured as established by TLC.

ix) The formula method of calculating the relative peak areas was the fastest, most efficient, most accurate technique available to us.

x) No interfering substances were introduced during the chemical manipulations, as shown by our reagent blank.

xi) Reproducibility was 100% qualitatively and allowed a 99% probability that a peak will lie within 4.2% of the determined value.

On the basis of fatty acid methyl ester analysis of seventeen species of corynebacteria we were able to conclude that:

i) The corynebacteria species tested could easily be divided into two main groups: Group 1 containing four plant pathogens, C. insidiosum, C. pointsettiae, C. michiganense and C. flaccumfaciens. The other species fell into Group 2.

ii) Group 2 could be subdivided into three sections on the basis of relative amounts of C_{16} (hexadecanoic) and $C_{18:1}$ (octadecenoic) acids.

Section (a)- (hexadecanoic acid the major peak)- contained C. enzymicum, C. diphtheriae, C. hoagii, C. equi and C. ulcerans.

Section (c)- (octadecenoic acid the major peak)- contained C. xerosis and C. renale.

Section (b)-(hexadecanoic and octadecenoic in roughly equal amounts)- contained C. bovis, C. pseudotuberculosis, C. pseudodiphtheriticum, C. "Q".

C. fascians, and C. segmentosum.

iii). Group 1 showed similarities in its fatty acid pattern to Group I Propionibacterium of Moss et al (66), thus it was felt that the four plant pathogens could possibly be reclassified as such.

iv). A.ureafaciens, a member of another genus showed the same fatty acid pattern as our group I members.

v). Corynebacteria of Group 2 could not be differentiated from one another as clearly but at best could be divided into three sections. Three species, however, each representing a different section of Group 2 could quite readily be distinguished from each other.

The main objectives of this study have been realized:

(a) the technique of gas liquid chromatography for analysis of bacterial cellular fatty acids has been successfully applied to the differentiation of species of corynebacteria.

(b) the cellular fatty acids of these species have been determined.

(c) as a result of being able to make a concrete suggestion as regards the classification of the plant pathogens, we have been able to shed some light on the controversy which enshrouds the classification of this genus.

BIBLIOGRAPHY

1. Abel, K. deSchmertzing, H. and Peterson, J. I. J. Bacteriol. 85:1039 (1963)

2. Alimova, G.K. Biochemistry (U.S.S.R.) 23:193 (1958)

3. Alimova, E.K. Biokhimiia 24:605 (1959)

3a Alimova, E.K. Biokhimiia 24:722 (1959)

4. Asano, M. and Takahashi, H. J. Pharm. Soc. Japan 65:17 (1945)
Chem. Abstract 45:3906 (1951)

5. Asselineau, J. 1957 Les lipides bacteriens, p. 90
In W. Ruhland (ed.) Handbuch der Pflanzenphysiologie, v. VII Springer-Verlag, Berlin

6. Asselineau, J. Biochem. Biophys. Acta 54:359 (1961)

7. Asselineau, J. Ann. inst. Pasteur 100:109 (1961)

8. Asselineau, J. and Lederer, E. 1960. Chemistry and metabolism of bacterial lipids, p. 337-406.
In K. Block (ed.) Lipide Metabolism.
John Wiley & Sons, Inc. New York.

9. Basden, E.H. II, Tourtellotte, M.E., Plastridge, W.N., and Tucker, J.S.
J. Bacteriol. 95: 439 (1968)

10. Bassette, R. and Claydon, T.J. J. Dairy Sci. 48:775 (1965)

11. Bawden, R.E. and Bassette, R. J. Dairy Sci. 49:624 (1966)

12. Bergey, D.H. 1923 Bergey's Manual of determinative bacteriology ed. I
The Williams and Wilkens Company, Baltimore, Md.

13. Bobo, R.A. and Eagon, R.G. Can. J. Microbiol. 14:503 (1968)

14. Boone, C.J. and Pine, L. Applied Microbiology 16(2):279 (1968)

15. Breed, R.S. Murray E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of determinative bacteriology, 6th ed.
The Williams and Wilkens Company, Baltimore, Md.

16. Breed, R.S., Cleveland, E.M.D., Clark, F.E., Couch, J.N., Morse, E.V., Nellis, L., Philip, C.B., Pittman, M., Seeliger, H., and Starr, M.P.
Bacteriol. Rev. 19:273 (1955)

17. Breed, R.S. Murray, E.G.D., Smith, N.R. 1957. Bergey's Manual of determinative bacteriology 7th ed. The Williams and Wilkens Company, Baltimore, Md.

18. Bretonneau, P. Traite de la diphtherite, Paris, 1826, cited by Nuttall, G.H.F. and Graham-Smith, E.S. The bacteriology of diphtheria, Cambridge University Press, 1913

19. Brian, B.L. and Gardner, E.W. Appl. Microbiol. 16(4): 549 (1968)

20. Brown, J.P. and Cosenza, B.J. Nature 204:802 (1964)

21. Cason, J. and Tavs, P. J. Biol. Chem. 234:1401 (1959)

22. Chargaff, E. Z Physiol. Chem. 201:191 (1931)

23. Chargaff, E. Z Physiol. Chem. 218:223 (1933)

24. Cho, K.Y. and Salton, M.R.J. Biochem. Biophys. Acta 116:73 (1966)

25. Cleveland, E.M.D. Ph.D. Thesis, McGill Univ. 1955

26. Cleveland, E.M.D. Man. Med. Rev. 43:86 (1963)

27. Clark, F.E. Int. Bull. of Bact. Nom. and Tax. 2:45 (1952)

28. Cohen, M. and Panos, C. Biochemistry 5 (7): 2385 (1966)

29. Conn, H.J. and Dimmick, I. J. Bacteriol. 54: 291 (1947)

30. Cowan, S.T. and Steel, K.J. 1965 Manual for the Identification of Medical Bacteria, Cambridge University Press

31. Cummins, C.S. and Harris, H. J. gen. Microbiol. 14:583 (1956)

32. Davison, W.H.T., Slaney, S., and Wragg, A L. Chem. and Indust. p.1356 (1954)

33. Douglas, H.C. and Gunter, S.E. J. Bacteriol. 52:15 (1946)

34. F & M Scientific Methods Bulletin No. 117 p.12 (1966) (Qualitative and Quantitative Lipid Analysis by Gas Chromatography)

35. Fatshtchi, D. and Moss, C.W. Appl. Microbiol. 17(2):262 (1969)

36. Fontanges, R., Blandenet, G. and Queignec, R. Ann. inst. Pasteur 112:10 (1967)

37. Garner, W. amd Gennaro, R.M. Chem. Eng. News 43:69 (1965)

38. Gasser, F., and Mondel, M. J. Bacteriol. 96:580 (1968)

39. Henis, Y., Gould, J.R. and Alexander, M. Appl. Microbiol, 14:573 (1966)

40. James, A.T. J. Chromatography 2:552 (1959)

41. James, A.T. and Martin, A.J.P. Biochem. J. 50:679 (1952)

42. James, A.T. and Martin, A.J.P. Biochem. J.63:144 (1956)

43. Jawetz, E. Melnick, J.L. and Adelberg, E.A. 1966 Review of Medical Microbiology, 7th Edition Lange Medical Publications, Los Altos, California

44. Jensen, H.L. Proc. Linnean Soc. N.S.W. 59: 19 (1934)

45. Jensen, H.L. Ann. Rev. Microbiol. 6:77 (1952)

46. Haahti, E. Scand. J. Clin. Invest., 59: supple 13, 1 (1962)

47. Hofmann, K., Hsiao, C.Y., Henis, D.B., and Panos, C. J. Biol. Chem. 217:49 (1955)

48. Iizuka, H. Iida, M. Unami, Y. and Hoshino, Y. Z.Allg. Mikrobiol. 8: 145 (1968) CA69:49933

49. Kaneda, T. J. Bacteriol. 93:894 (1967)

50. Kates, M. Adv. Lipid Res. 2:17 (1964)

51. Klebs, E. Verhandl. d. Kong, f. inn. Med. II:125 (1883) (cited in (25).)

52. Lacave, C. Asselineau, J. and Toubiana, R. Eur. J. Biochem. 2:37 (1967) Chem. Abstr. 67:70606j

53. Lederer, E. 1958 Glycolipids of bacteria, plants and lower animals, p. 119-146 In Colloquium der Gesellschaft fur physiol. Chemie 8. Springer-Verlag, Berlin

54. Lehmann, K.D. and Neumann, R.O. Atlas and Grundriss der Bakteriologie Munchen, III Aufl. 1904

55. Lennarz, W.J. Adv. Lipid Res. 4: 175 (1966)

56. Lewis, V.J. Moss, C.W. and Jones, W.L. Can. J. Microbiol. 13:1033 (1967)

57. Lewis, V.J. Weaver, R.E. and Hollis, D.G. J. Bact. 96(1):1 (1968)

58. Loffler, F. Mitte a.d. K. Gesundheitsamte II:451 (1884) Cited in (25).

59. Lovern, J.A. 1955 The phosphatides and glycolipids p.376 In W. Ruhland (ed.) Handbuch der Pflanzenphysiologie, v. VII Springer-Verlag, Berlin

60. Lovern, J.A. 1955. The chemistry of lipids of biochemical significance. John Wiley and Sons, Inc. New York

61. Marr, A.G. and Ingraham, J.L. J. Bact. 84(6):1260 (1962)

62. Matsubara, T. Nara Igaku Zasshi 16(4):358 (1965) Chem. Abstr. 65: 2658d (1966)

63. Mitruka, B.M. and Alexander, M. Appl. Microbiol. 16(4):636 (1968)

64. Moore, W.E.C. and Cato, E.J. J. Bacteriol. 85:870 (1963)

65. Moss, C.W. and Cherry, W.B. J. Bacteriol. 456:241 (1968)

66. Moss, C.W. Dowell, V.R. Jr., Farshtchi, D. Raines, L.J. and Cherry, V.B. J. Bacteriol. 97(2):561 (1969)

67. Moss C.W., Dowell, V.R. Jr., Lewis, V.J. and Schekter, M.A. J. Bacteriol. 94(5):1300 (1907)

68. Moss, C.W. and Lewis, V.J. Appl. Microbiol 15(2): 390 (1967)

69. O'Brien, R.T. Food Technol. 21:1130 (1967)

70. O'Leary, W.M. Bacteriol. Rev. 26:421 (1962)

71. Pollock, M.R. Howard, G.A. and Boughton, B.W. Biochem, J. 45:417 (1949)

72. Porter, J.R. (1946) The Chemical Composition of Microorganisms p.352 In "Bacterial Chemistry and Physiology" John Wiley and Sons, Inc. New York

73. Prebble, J. J. Gen. Microbiol. 52: 15 (1968)

74. Pustovalov, V.L. Biochemistry (U.S.S.R.) 21:33 (1956)

75. Raines, L.J., Moss, C.W., Farshtchi, D. and Pittman, B. J. Bacteriol. 96(6):2175 (1968)

76. Razin, S. and Rottem, S. J. Bacteriol. 94(6): 1807 (1967)

77. Reiner, E. Nature, 206: 1272 (1965)

78. Reiner, E. J. Gas Chromatog. 5:65 (1967)

79. Salton, M.R.J. and Freer, J.H. Biochem. Biophys. Acta 107:531 (1965)

80. Santaolalla, M. Microbiol. Espan. 20:87 (1967) Chem. Abstr. 69:57552k (1968)

81. Sharkey, A.G. Jr., Friedel, R.A. and Langer, S.H. Anal. Chem. 29:770 (1957)

82. Stanier, R.Y. Dondoroff, M. and Adelberg, E.A. 1963 The Microbial World 2nd Ed. Prentice-Hall Inc., Englewood Cliffs, N.J.

83. Swelley, C.C. Bentley, R. Makita, M. and Wells, W.W. J. Am. Chem. Soc. 85:2497 (1963)

84. Trousseau, Rapport sur l'epidermie de bologne, Clinique Medicale de l'hotel dieu de Paris, Vol I, 1828 (cited in (25).)

85. Wolochow, H. Armed Services Technical Information Agency Rept. No. 211170, p.2

86. Yamakawa, T. and Ueta, N. Japan J. Exptl, Med. 34:361 (1964)