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TECHNICAL MANUSCRIPT 123

THE LIPIDS OF SARCINA LUTEA.

I. FATTY ACID COMPOSITION OF THE EXTRACTABLE LIPIDS

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THE LIPIDS OF *SARCINA LUTEA*.
I. FATTY ACID COMPOSITION OF THE EXTRACTABLE LIPIDS

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ABSTRACT

The extractable lipids of Sarcina lutea were separated into several fractions by a combination of column and thin-layer chromatography. Qualitative and quantitative characterization of the fatty acid content of these lipid fractions was accomplished by means of gas-liquid chromatography and infrared analysis.

Of the total extract, the lipids consisted of 2.1 per cent free fatty acids, 51.0 per cent glycerides, and 22.7 per cent complex lipids; they had a fatty acid content with a complete spectrum of carbon numbers from C_8 to C_{22}. The fatty acids included a large component of branched-acids in addition to the normal straight-chain acids. The branched-acids, comprising 40 per cent of the fatty acids analyzed, constituted a homologous series of iso acids from C_{12} to C_{19}. Two 18-carbon unsaturates were found, cis-9-octadecenoate and cis-11-octadecenoate.

A relatively high percentage (20.5 per cent) of the extractable material from S. lutea was found to be hydrocarbon. This material was not further characterized.

I. INTRODUCTION

Bacterial lipids, in general, differ substantially from those of higher life forms in such respects as: (a) the absence of sterols, (b) phospholipids low in nitrogen and high in carbohydrate, (c) the presence of large proportions of free fatty acids, and (d) the presence of certain fatty acids not ordinarily found in other life forms. The presence of these unusual fatty acids and their possible relation to bacterial pathogenicity has stimulated much research.

A review of the literature on bacterial fatty acids was made by Porter[1] and more recently by Asselineau and Lederer[2] and by O'Leary.[3] Although investigators have reported the fatty acid composition of some bacterial lipid fractions,[4,5] little information is available on the distribution of fatty acids among specific bacterial lipid classes.

Sarcina lutea, a Gram-positive, aerobic (facultatively anaerobic), nonmotile, pigment-producing micrococcus, has been found in air, soil, and water all over the earth.[6] The only reported analysis of the lipids of Sarcina is that by Akashi and Saito,[7] who studied the fatty acid composition of the acetone-soluble and -insoluble fractions.

The communication reports on the distribution of fatty acids among the various general lipid classes present in the extractable lipids of Sarcina lutea. For the purposes of this study, only the C_8 to C_{22} fatty acids are discussed.

II. EXPERIMENTAL PROCEDURE

A. CULTURE CONDITIONS

Sarcina lutea (ATCC 533) was cultured at 25°C for 48 hours in continuously aerated trypticase soy broth (pH 7) that was found to contain less than 0.02 per cent lipid. The cells were harvested by centrifugation, washed free of medium with 0.1M aqueous potassium chloride (KCl) solution, and lyophilized to constant weight.

B. EXTRACTION

Ten grams of lyophilized cells were shaken at room temperature with 150-milliliter portions of solvent according to the extraction scheme outlined in Figure 1. Extracts 1 through 4 were combined, evaporated to

dryness *in vacuo*, and the residue taken up in chloroform-methanol, 2:1 (v/v). This solution was washed according to the method of Folch *et al*[8] to remove nonlipid contaminants. The chloroform portion was evaporated to dryness *in vacuo* and weighed.

The remaining cell residue was examined for nonextractable lipids by subjecting it to a two-hour reflux with 2N aqueous potassium hydroxide (KOH). The resulting material was acidified, and a chloroform extract of this was examined separately.

Shaking the cells first with acetone permitted a more rapid and complete extraction by the other solvent systems. All extractions were carried out at room temperature to prevent alterations in complex lipid structure,[9] and as rapidly as possible to avoid prolonged exposure to methanol.[10]

Spot tests with Rhodamine 6G indicated that no further lipid was extracted with chloroform-methanol, 2:1 (v/v), after two shakings. The final extraction with chloroform-methanol, 1:1 (v/v), did, however, yield a small additional quantity of lipid as well as appreciable amounts of nonlipid.

C. SILICIC ACID COLUMN CHROMATOGRAPHY

The extracted lipids were separated according to class and purified on three columns prepared as follows: Column I consisted of a 1.7 by 150 centimeter glass tube packed to a depth of 120 centimeters with a hexane slurry of activated Mallinckrodt silicic acid (30 grams, 100/120 mesh) and Hyflo Super-Cel (15 grams). The column was washed with 100-milliliter portions of acetone, ethyl ether, and n-hexane in that order. Columns II and III consisted of 0.8 by 30 centimeter glass tubes. Column II was packed with 15 grams of 160/200 mesh activated silicic acid and Column III with 15 grams of 100/140 mesh activated silicic acid. Both II and III were washed with 50-milliliter portions of acetone, ethyl ether, and n-hexane and, in addition, III received a final wash with 50 milliliters of chloroform.

The lipid extract was separated into general classes on Column I, using a flow rate of eight milliliters per minute. A crude hydrocarbon fraction was eluted with 150 milliliters of n-hexane-benzene, 49:1 (v/v). The simple lipids were eluted next with 300 milliliters of chloroform, but the complex lipids required 300 milliliters of chloroform-methanol, 1:1 (v/v), followed by 100 milliliters of methanol, for their complete removal from the column. Each fraction was evaporated to dryness *in vacuo*, and then weighed and redissolved in chloroform pending further treatment. Over-all recovery of lipid from this and succeeding columns averaged 99.5 per cent.

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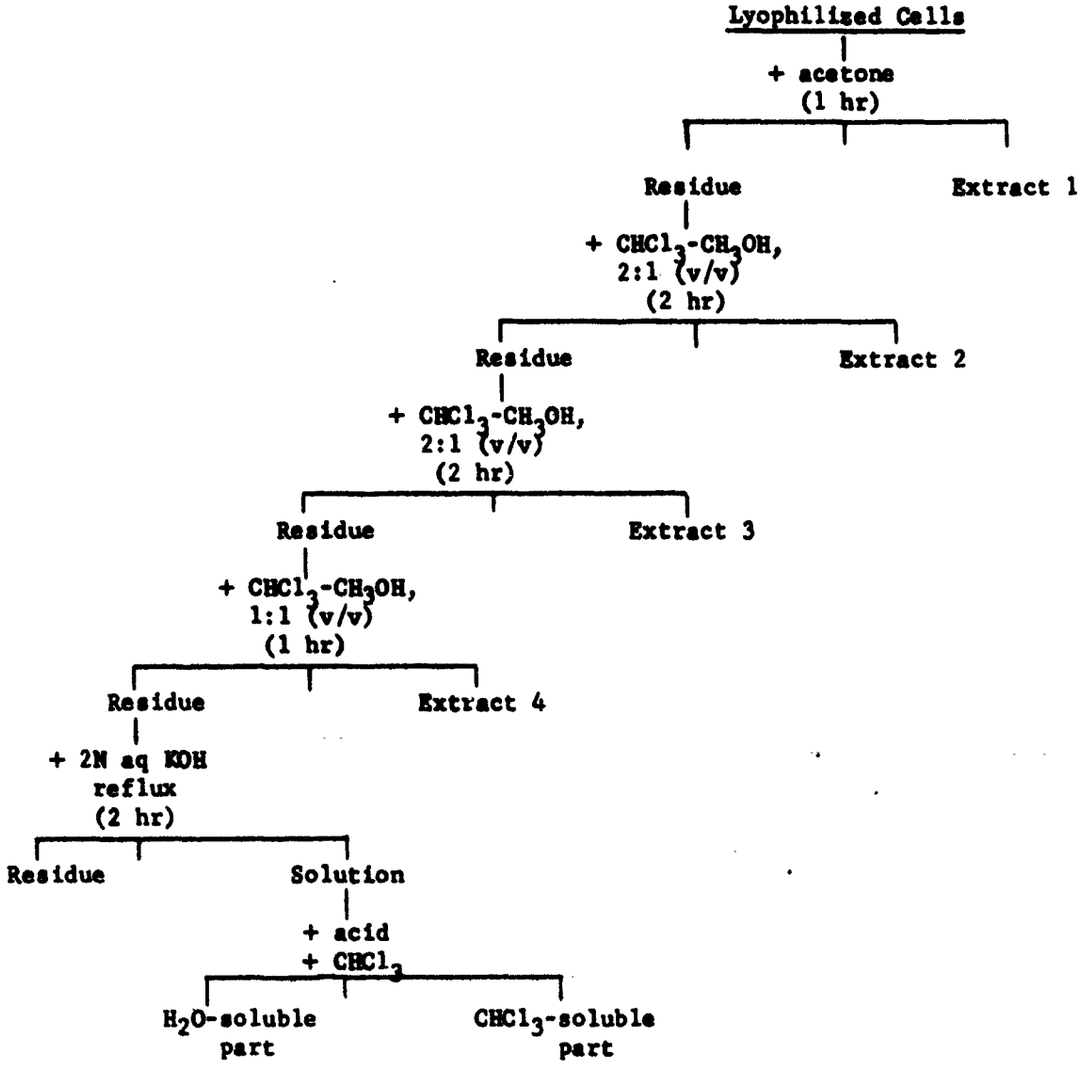

Figure 1. Sarcina lutea Extraction Scheme. Extracts 1 through 4 were combined, washed, and examined as the "total extract," The chloroform-soluble part of the base-hydrolyzed residue was examined as the "base hydrolyzate."

The crude hydrocarbon fraction was further resolved on Column II. A pure hydrocarbon fraction was eluted with 100 milliliters of n-hexane. Nonhydrocarbon carry-over was then eluted with 50 milliliters of ethyl ether and added to the simple lipid fraction.

Simple lipid impurities were separated from the complex lipids on Column III by eluting them with 125 milliliters of chloroform. This eluted material was added to the simple lipid fraction. The complex lipids were finally eluted with 200 milliliters of chloroform-methanol, 1:2 (v/v). The combined simple lipid fraction was further fractionated into a free fatty acid fraction and a glyceride fraction on a KOH-treated silicic acid column as described by McCarthy and Duthie.[11]

D. THIN-LAYER CHROMATOGRAPHY (TLC)

The various lipid fractions were monitored on thin layers (250 to 275 per microns) of silica gel G (Stahl) on 20 by 20 centimeter standard glass plates. All plates were spread with a Desaga-Brinkman nonadjustable applicator*, activated for two hours at 110°C, anddeveloped by the ascending method in unlined tanks.

All column cuts were examined for lipid classes by developing the plates in n-hexane-ethyl ether-glacial acetic acid, 90:10:1 (v/v/v).[12] The hydrocarbon fraction from Column II was developed in n-hexane-benzene, 90:10 (v/v). The complex lipids from Column III were resolved in a system of chloroform-methanol-4.3N ammonium hydroxide, 17:7:1 (v/v/v). The simple lipid fraction was resolved into glyceride types by development according to Brown and Johnston.[13]

Methyl ester preparations were examined for the presence of hydroxyl- or epoxy-esters by chromatographing them on silica gel G impregnated with silver nitrate.[14] The methyl esters were also resolved into saturated and unsaturated fractions by preparative TLC of the acetoxymercuri-methoxy derivatives.[15] Two fractions of these derivatives were eluted from the silica gel G and hydrolyzed to the original esters for further analysis.

Spots on the variously developed plates were made visible with (a) 0.001 per cent aqueous Rhodamine 6G, (b) saturated chromic-sulfuric acid with charring, (c) iodine vapors, (d) 0.4 per cent ninhydrin in water-saturated n-butanol, and (e) a molybdate reagent.[16]

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* Brinkman Instruments, Inc., Great Neck, N.Y.

E. GAS-LIQUID CHROMATOGRAPHY (GLC)

Fatty acid methyl esters were prepared for GLC by refluxing the lipid material for two hours in methanol containing 0.5 per cent by weight concentrated sulfuric acid and 5 per cent by weight 2,2-dimethoxypropane as a water scavenger. After stopping the reaction with water, the esters were extracted with gas-chromatographically pure n-hexane.

All chromatograms were obtained with an F & M Model 500 Gas Chromatograph with a Model 1609 Flame Ionization Detector.* Helium, at an outlet flow rate of 88 milliliters per minute, was used as the carrier gas, and all columns were of 1/4-inch O.D. coiled copper tubing. The following column packings and conditions were used:

(a) Silicone Gum Rubber (GE SE-30), 5 per cent on 100/120 mesh Gas Chrom P.** Column (two feet) temperature programmed from 150° to 288°C at 15°C per minute.

(b) Apieson L (ApL), 10 per cent on 50/60 mesh Anakrom ABS.*** Column (four feet) maintained isothermally at 210°C.

(c) Ethylene Glycol Succinate (EGS), 10 per cent on 120/140 mesh Gas Chrom P. Column (eight feet) maintained isothermally at 150° or 180°C.

(d) Ethylene Glycol Glutarate (EGGlu), 10 per cent on 70/80 mesh Anakrom ABS. Column (eight feet) maintained isothermally at 180°C.

(e) Cyclohexanedimethanol Succinate** (CDMS), 11 per cent on 80/100 mesh Chromosorb W. Column (eight feet) maintained isothermally at 185°C.

F. INFRARED ANALYSIS

Infrared spectra of the various lipid fractions and methyl ester samples were made from thin films on potassium bromide (KBr) pellets using a Perkin-Elmer Model 21 Recording Infrared Spectrophotometer.

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* F & M Scientific Company, Avondale, Pa.
** Applied Science Laboratories, Inc., State College, Pa.
*** Analytical Engineering Laboratories, Inc., Hamden, Conn.

III. RESULTS

A. TOTAL LIPID COMPOSITION

Lyophilized cells of S. lutea were found to contain, on the average, 1.30 per cent solvent-extractable lipid. An additional 0.24 per cent lipid material could be obtained by base hydrolysis of the cell residue.

The extractable material fractionated on the silicic acid columns was found to consist of 20.5 per cent hydrocarbon material, 59.1 per cent simple lipid (2.1 per cent free fatty acids and 51.0 per cent glycerides), and 22.7 per cent complex lipid.

Thin-layer chromatography indicated the presence of hydrocarbons, free fatty acids, mono-, di- and triglycerides, and a complex mixture of highly polar phosphorus-containing compounds. The complex lipids contained both ninhydrin-positive and -negative components. There was no evidence for the presence of fatty alcohols, sterols, sterol esters, waxes, or sphingomyelins.

B. FATTY ACIDS

Tables I, II, and III list the fatty acid composition as determined for the various lipids of S. lutea. Peak areas, determined with a planimeter, were obtained from chromatograms using the EGS column at 180°C. The area percentages are approximately equivalent to weight percentages of the range covered.[17] Although the EGS column did not resolve as many components as did the EGGlu column, the clean separation of these components that were resolved on EGS made quantitation less arbitrary.

The methyl ester peaks were identified on the basis of their relative retention times as compared with methyl stearate, their equivalent chain lengths from plots of carbon number versus retention time for n-saturated methyl ester standards, and their separation factors from n-saturates. In some cases, additional confirmation of identity was obtained from infrared spectra of particular components trapped from the column effluent ahead of the detector. Monounsaturates were distinguished from branched-chain saturates emerging at the same point from polyester columns by noting their reversed order on ApL and by comparing their degree of separation from n-saturates on EGS at two different temperatures.[18] Figure 2 illustrates the separation obtained on EGS at 180°C. Saturates in general were distinguished from unsaturates by comparison of the chromatograms of the two fractions obtained by TLC of the acetoxymercurimethoxy derivatives. The position of methyl branches and the location and configuration of double bonds could, in some cases, be tentatively determined from relative retention times on EGGlu. An example of the separation possible on EGGlu is shown in Figure 3. When possible, such identifications were verified by chromatographing known standards of the suspected ester constituents.

TABLE I. FATTY ACID COMPOSITION OF S. LUTEA LIPIDS

Fatty Acid Carbon Chain[a]	Relative Retention Time	Composition, per cent	
		Total Extract	Base Hydrolyzate
8:0-10:0	0.06+	5.2	36.5
A	0.14	2.2	5.4
12:0	0.19	8.1	7.2
B	0.21	2.6	0.4
13:0	0.24	1.8	3.0
13:1	0.27	---	0.2
br-14:0[c]	0.30	0.9	---
14:0	0.33	5.2	1.1
C	0.37	26.4	19.1
15:0	0.42	1.5	trace[b]
br-16:0	0.50	3.2	0.9
16:0	0.60	10.1	8.0
D	0.67	0.9	---
16:1	0.70	2.7	0.9
16:2	0.83	---	0.2
E	0.93	2.6	5.6
18:0	1:00	5.7	5.0
F	1.18	4.0	0.2
19:0	1.32	2.5	trace
18:2	1.50	---	0.2
20:0	1.74	1.9	0.2
21:1	2.29	2.6	---
22:0	3.00	0.9	0.2
20:4	3.40	3.4	0.1

a. Number of carbon atoms in acid : number of double bonds
b. trace = less than 0.1 per cent
c. br = branched

TABLE II. FATTY ACID COMPOSITION OF MAJOR LIPID CLASSES IN *S. LUTEA*

Fatty Acid Carbon Chain[a]	Composition, per cent	
	Simple Lipids[b]	Complex Lipids
8:0-10:0	8.6	1.6
A	5.4	trace[c]
12:0	9.4	3.5
B	2.8	2.1
13:0	5.2	1.4
13:1	---	---
br-14:0[d]	0.8	1.0
14:0	2.4	6.1
C	13:0	33.9
15:0	2.8	trace
br-16:0	2.6	3.7
16:0	8.8	12.3
D	0.4	1.3
16:1	0.7	3.1
16:2	---	---
E	2.8	1.0
18:0	5.4	9.1
F	2.6	8.5
19:0	4.4	1.6
18:2	---	---
20:0	1.2	2.1
21:1	2.6	2.7
22:0	9.0	2.7
20:4	4.0	---

a. Number of carbon atoms in acid : number of double bonds
b. Free fatty acids plus glycerides
c. trace = less than 0.1 per cent
d. br = branched

TABLE III. FATTY ACID COMPOSITION OF S. LUTEA SIMPLE LIPIDS

Fatty Acid Carbon Chain[a]	Composition, per cent	
	Free Fatty Acids	Glycerides
8:0-10:0	2.0	9.2
A	0.3	5.9
12:0	1.5	10.0
B	0.8	3.0
13:0	0.2	5.6
13:1	---	---
br-14:0[b]	0.1	0.9
14:0	14.9	2.3
C	2.2	14.0
15:0	3.4	2.9
br-16:0	1.2	2.8
16:0	32.5	8.6
D	26.0	trace[c]
16:1	3.7	trace
16:2	---	---
E	1.8	3.0
18:0	2.1	5.8
F	0.9	2.8
19:0	0.1	4.7
18:2	---	---
20:0	0.7	1.3
21:1	0.5	2.8
22:0	0.2	9.7
20:4	1.0	4.3

a. Number of carbon atoms in acid : number of double bonds
b. br = branched
c. trace = less than 0.1 per cent

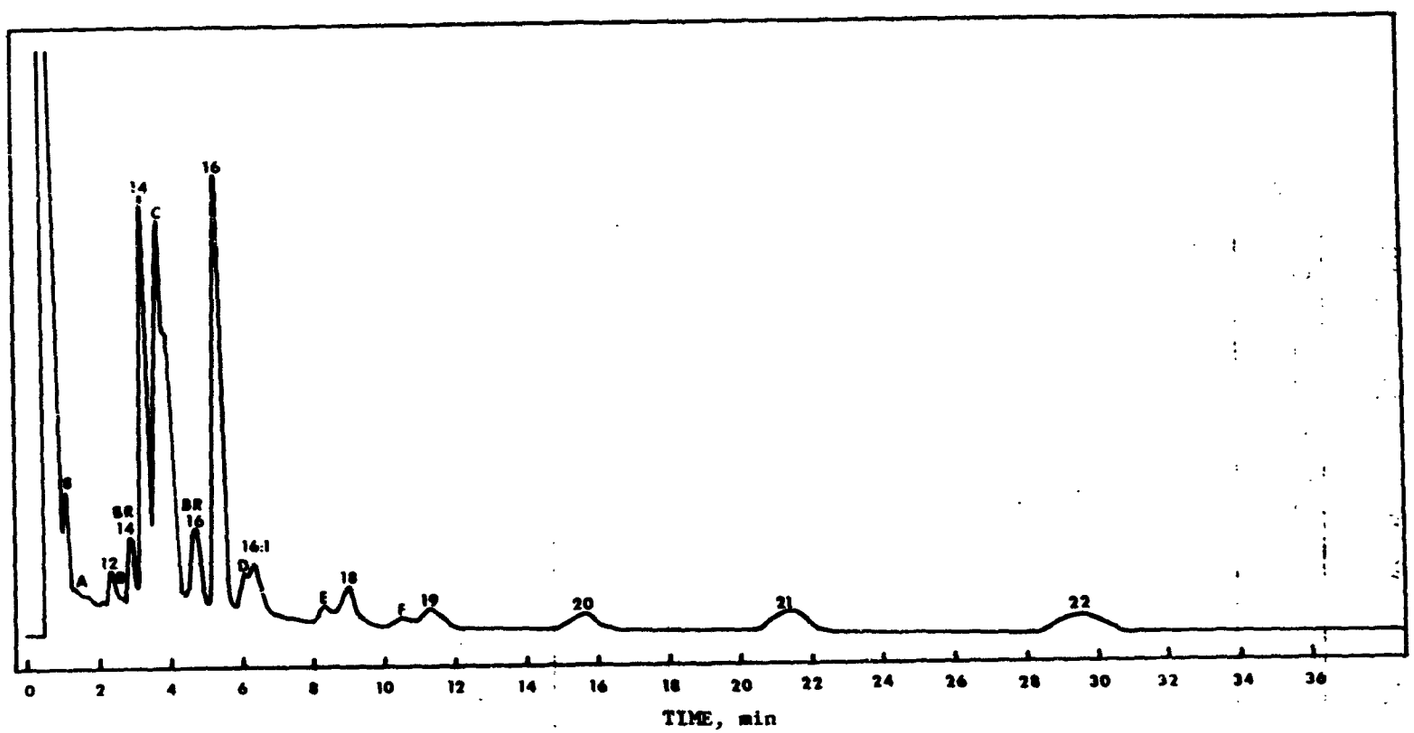


Figure 2. Gas-Liquid Chromatography on an Ethylene Glycol Succinate Column at 180°C of the Methyl Esters of the Fatty Acids from Sarcina lutea. Chromatography conditions are described in Section II. The initial off-scale band is from the solvent. Designations used are: A to F corraspond to the letters listed in Table I and described in Section III.; whole numbers refer to methyl esters of n-saturated acids of the same carbon number; 16:1, methyl ester of monounsaturated C_{16} acid; BR-14 and BR-16, methyl esters of branched C_{14} and C_{16} acids. Peak C is shown at a 4X attenuation to keep it on scale.

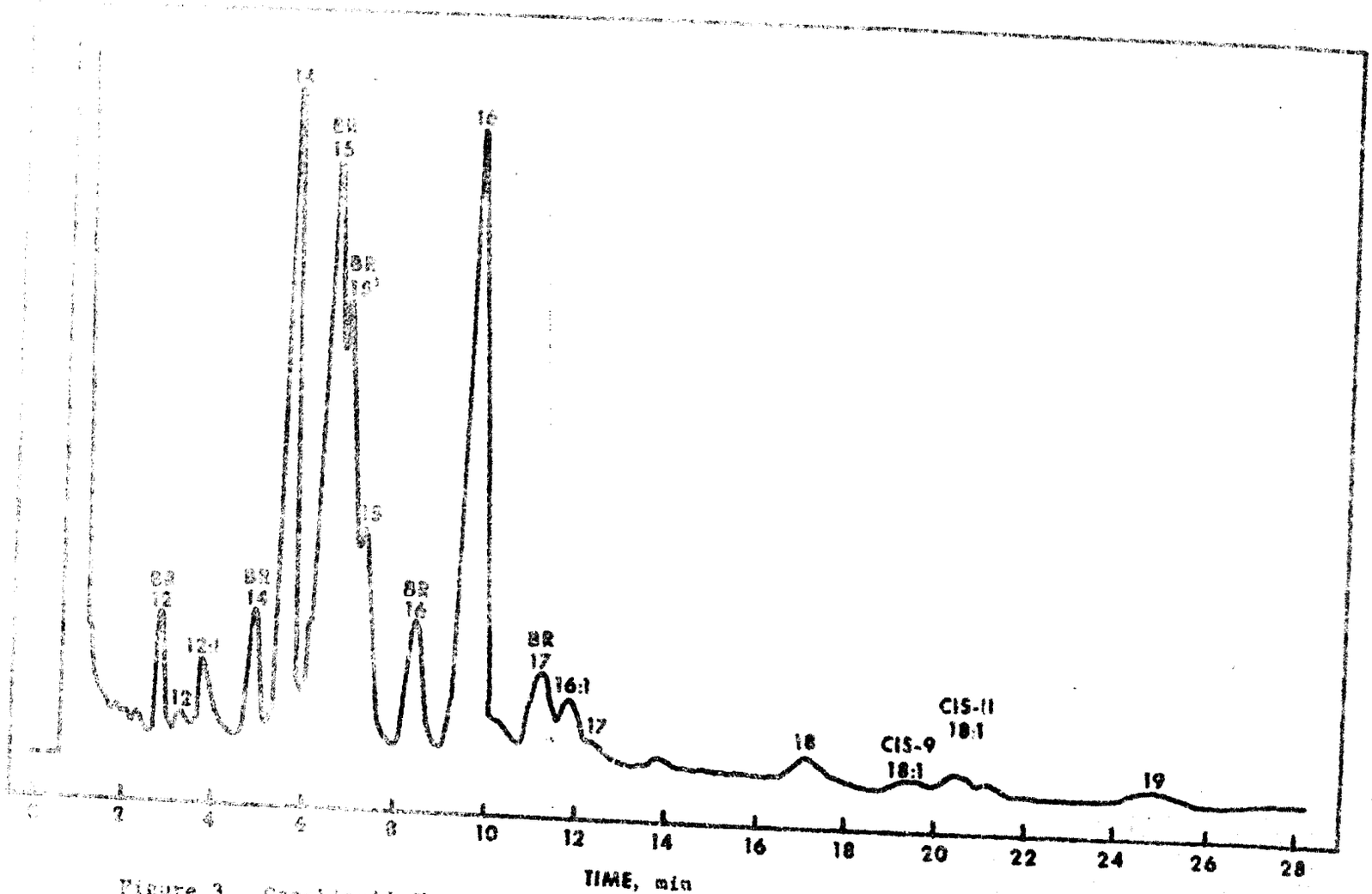
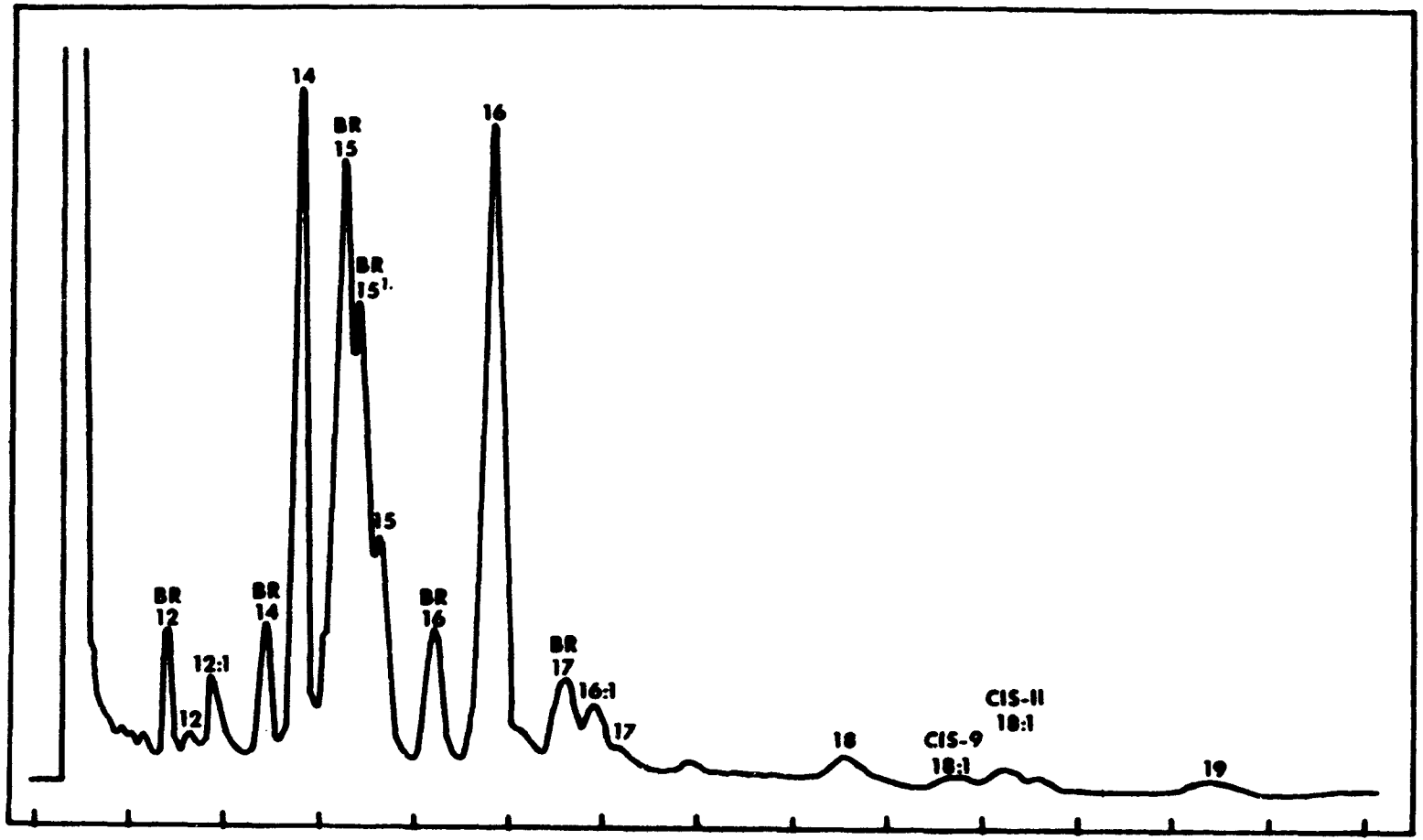
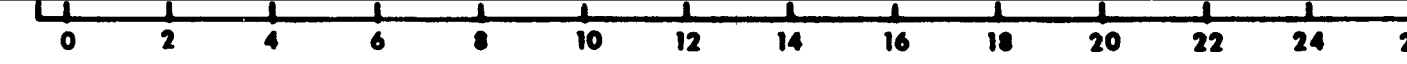


Figure 3. Gas-Liquid Chromatography on an Ethylene Glycol Glutarate Column of the Methyl Esters of the Fatty Acids from Sarcina lutea. Chromatography conditions are described in Section II. The initial off-scale band is from the solvent. Designations used are: Whole numbers refer to methyl esters of n-saturated acids of the same carbon number; 12:1 and 16:1, methyl esters of monounsaturated C_{12} and C_{16} acids; CIS-9-18:1 and CIS-11-18:1, methyl esters of cis-9-octadecenoic acid and cis-11-octadecenoic acid; BR-, methyl esters of iso-branched acids of the same carbon number; BR-15[1], methyl ester of anteiso-branched C_{15} acid. The peak containing BR-15, BR-15[1] and 15 is shown at a 4X attenuation to keep it on scale.


14
BR 15
BR 15¹.
16
15
BR 12
BR 14
BR 16
12:1
12
BR 17
16:1
17
18
CIS-9 18:1
CIS-11 18:1
19



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Chromatograms obtained with the SE-30 column indicated that the complete spectrum of carbon numbers from C_8 to C_{22} was present in the various lipid fractions. The CMDS column indicated that the bulk of the C_8 to C_{10} acids from the base hydrolyzate were C_8 acids.

Peaks A to F in the tables were found, with the exception of peak D, to be resolved into more than a single component on EGGlu. On the basis of the previously mentioned retention characteristics applied to the EGGlu column, tentative identifications of these peaks were:

"A" was composed of n-hendencanoate and 10-methylhendecanoate, with the latter as the major component.

"B" consisted of dodecanoate and 11-methyldodecanoate, with the branched-acid again the major component.

"C", containing by far the largest percentage of the esters in the total extract, was resolved into three components corresponding to tetradecenoate and two branched-acids. The branched-acids made up the majority of "C" and corresponded to 13-methyltetradecanoate and 12-methyltetradecanoate.[19] An infrared spectrum of this fraction indicated the presence of both an iso- and anteiso-configuration.[20]

"D" corresponded to 15-methylhexadecanoate and neoheptadecanoate.[21] An infrared spectrum indicated the presence of both the iso- and tertiary-configurations in this fraction.

"E" corresponded to a mixture of heptadecenoate, 16-methyl-heptadecanoate, and 9,10-methylenehexadecanoate.[4] An infrared spectrum of "E" indicated the presence of cis-configuration but no cyclopropane absorption.[22]

"F" was another triplet containing 9-methyloctadecanoate,[23] cis-9-octadecenoate (oleate), and cis-11-octadecenoate (cis-vaccenate). Had lactobacillate (cis-11,12-methylene octadecanoate) been present, it would have emerged with the branched member of this triplet. However, no cyclopropane absorption could be observed in the infrared spectrum of this fraction.

Evidence exists for the inclusion of two homologous series of branched-acids besides the iso- series, although the exact nature of these trace components has not been established. Moreover, the ApL column indicated that, in opposition to what has been found for other bacteria, n-hexadecanoic constitutes only a small percentage of the fatty acid content of S. lutea. The bulk of the material designated 16:0 in Table I eluted slightly before authentic hexadecanoate.

IV. DISCUSSION

In S. lutea, the largest class of lipids was found to be the simple lipids, consisting of free fatty acids and glycerides. As previously mentioned, the cell content of free fatty acids is usually higher in bacteria than in most other life forms. A number of bacteria have been reported to contain free fatty acids comprising more than 20 per cent of the fatty acid content.[2] Although mono-, di- and triglycerides have been reported in many bacteria,[1,24] their concentrations are usually lower than in plant and animal cells. In fact, many bacteria reportedly lack glycerides.[2] S. lutea, on the other hand, was found to contain lipids with only 2.1 per cent free fatty acids and 51.0 per cent glycerides. Recent studies[25] suggest that reports of high free fatty acid contents in lipid material may be due to lipolysis during extraction and chromatography on silicic acid. As ether is known to activate certain lipolytic enzymes,[26] the use of this solvent for extraction or column chromatography was avoided in this study. Moreover, ketones are known to inhibit the action of certain lipases.[27] Thus, the use of acetone as the initial extracting solvent may have prevented the lipolysis of S. lutea lipids.

The complex lipids were not characterized for this study other than by a determination of their gross fatty acid content. The fact that several ninhydrin-positive and -negative components were detected by TLC indicates that this fraction is justifiably termed "complex."

An interesting point is the relatively high percentage of hydrocarbon material (20.5 per cent) extracted with the lipids. Since S. lutea has a yellow pigment, it is not unreasonable to expect that hydrocarbons might be present in the extract. Gamma-carotene has been reported in several bacterial species,[28,29] although little quantitative data are available. Further comment will have to await a more complete characterization of this material.

The major group of fatty acids found in S. lutea was the branched-acids, comprising more than 40 per cent (ca) of the C_8 to C_{22} acids. With the exception of two, all of the branched-acids present in amounts greater than 0.1 per cent belonged to a homologous series of iso- acids. The complete series of iso- acids from C_{12} to C_{19} was observed. The two exceptions were 12-methyltetradecanoate and 9-methyloctadecanoate. Akashi and Saito[7] have reported a branched-acid (C_{15}) in the lipids of Sarcina, which they named sarcinic acid. Saito, in subsequent studies,[30,31] reported the isolation and identification of 13-methyltetradecanoic acid and 15-methylhexadecanoic acid from B. subtilis, but did not report whether the C_{15} acid from B. subtilis was identical with sarcinic acid. Asselineau[32] also reported finding two branched-acids in B. subtilis, one C_{15} and the other C_{16}. In the same paper there is mention of a C_{19} branched-acid that was also found in Pasteurella pestis. Kates, et al[33] reported that B. cereus contained a

homologous series of branched-acids from C_{12} to C_{18}, but did not report the position of the branch. They reported also that the major fatty acid of this organism was a branched C_{15} acid. Agre and Cason[34] reported a series of branched-acids from C_{15} to C_{19} in tubercle bacillus.

The relatively low unsaturated fatty acid content was concentrated mainly in the C_{16} and C_{18} portions of the chromatograms. The hexadecenoate corresponds to cis-9-hexadecenoic acid. Both this acid and cis-11-hexadecenoic acid have been found in bacteria.[35,36] Oleic acid has been reported in streptococci[35] and the tubercle bacillus;[37] cis-11-octadecenoate has been reported in several microorganisms.[38,39] Since cis-11-octadecenoate is a precurser for lactobacillic acid,[40] the low concentration of unsaturates may have resulted in there being too little lactobacillate produced for infrared analysis.

A more detailed quantitative study of the distribution of the fatty acids of S. lutea in relation to culture conditions is required before any significant biochemical conclusions can be drawn.

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