THE CHEMICAL NATURE OF THE FATTY ACIDS OF A GROUP C STREPTOCOCCUS SPECIES*

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The mechanism of stimulation of bacterial growth by lipides is as yet but little understood. Since it seemed to us that exact knowledge of the fatty acid composition of a number of microorganisms might provide a basis for further advances in this field, we have recently examined the fatty acid spectra of Lactobacillus arabinosus (1, 2) and of Lactobacillus casei (3). It was found that the fatty acid patterns of these organisms are practically identical; both lactobacilli produce palmitic, stearic, cis-vaccenic, and lactobacillic acids as the major lipide components.

The present investigation extends our fatty acid studies to a group C Streptococcus species.

EXPERIMENTAL

Cultivation and Extraction of Cells

The starting material for this study was a large quantity of cells of a group C Streptococcus species, strain H46A (ATCC 10706), grown on Christensen's medium (4). At the end of the incubation period, a quantity of Hyflo Super-Cel was added to the culture to facilitate collection of the cells and the mixture filtered; the moist filter cake (5340 gm.), composed of streptococcal cells and Hyflo Super-Cel (inactivated by the addition of n-hexylresorcinol), was then shipped to our laboratory. Immediately on arrival, this material was suspended in 14 liters of acetone and the suspension was kept at 5° for 48 hours. The acetone extract was then decanted from the insoluble residues which were reextracted with six additional 5 liter portions of acetone. The combined acetone extracts were evaporated

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1 The melting points were determined with short stem Anschütz thermometers and are uncorrected. Freshly distilled solvents were used, and operations were performed under nitrogen whenever necessary. The iodine numbers were determined by the Wijs method.
2 We wish to express our sincere appreciation to Dr. Brian Hutchings of the Lederle Laboratories Division of the American Cyanamid Company for supplying us with this material.
to a small volume in vacuo, and the resulting suspension was extracted with ether. The ether extract was washed with 5 per cent sodium bicarbonate, dried over sodium sulfate, and evaporated to give a yellow oil (12 gm.) composed of "free lipides" and n-hexylresorcinol. The material insoluble in acetone was collected and dried to constant weight in vacuo. The dry residue weighed 34.20 gm. and contained 430 gm. of combustible substance (streptococcal cells) as determined by ashing of a small aliquot. For the isolation of the "bound lipides" this residue was suspended in 12.5 liters of 2 N sulfuric acid and the mixture was autoclaved at 121° for 2 hours. The cooled hydrolysate was filtered through large Buchner funnels and the filter cakes were exhaustively extracted with ether. The combined ether extracts were washed with 5 per cent sodium bicarbonate, dried over sodium sulfate, and evaporated in vacuo to give 15.1 gm. of "bound lipides." The filtrate was discarded, as exhaustive extraction of a 300 ml. aliquot yielded only a negligible quantity (13 mg.) of ether-soluble material.

Isolation and Characterization of Fatty Acids from "Bound Lipides"

Preparation and Separation of Methyl Esters

Saponification of the "bound lipides" (15.1 gm.) (2) gave 13.6 gm. of tan-colored fatty acids (iodine number 36.6) and 0.39 gm. of a light brown, semisolid, non-saponifiable fraction which was not investigated further. A microspectrophotometric analysis (5) of the fatty acids demonstrated the presence of 2.8 per cent of dienoic acids, 0.2 per cent of trienoic acids, and 0.0 per cent of tetraenoic acids. A sample of the fatty acids (12.6 gm.) was esterified with diazomethane and the methyl esters (12.93 gm.) were fractionally distilled in a spinning band, semimicro, Piros Glover fractionating column (2). From the distillation curve (Fig. 1), the following composition was estimated: C₁₂ esters, 0.26 gm. (2.0 per cent); C₁₄ esters, 0.67 gm. (5.2 per cent); C₁₆ esters, 4.85 gm. (37.5 per cent); C₁₈ esters, 5.62 gm. (43.4 per cent); and still pot residues 1.54 gm. (13.9 per cent).

\( \text{C}_{12} \text{ Fatty Acid} \)

The C₁₂ methyl ester Fractions³ 1 and 2 (0.215 gm.) melted at 3.2-4.8° and gave no depression of melting point on admixture with methyl laurate. Saponification followed by recrystallization from acetone afforded lauric acid, m.p. 43.8-44.8°; neutral equivalent, calculated, 200; found, 204.

\( \text{C}_{14} \text{ Fatty Acid} \)

The C₁₄ methyl ester Fractions 5 and 6 (0.356 gm.) melted at 17.3-17.8° and gave no depression of melting point on admixture with methyl my-

³ The individual fractions collected during the course of the distillation were numbered consecutively and individual points on the distillation curve (Fig. 1), from left to right, correspond to fraction numbers.
ristate. Saponification and recrystallization from acetone afforded myristic acid, m.p. 54.2–54.8°; neutral equivalent, calculated, 228; found, 228.

\( C_{16} \) Fatty Acids

The \( C_{16} \) methyl ester Fractions 19 and 20 (1.192 gm.) melted at 29.0–30.0° and gave no depression of melting point on admixture with methyl palmitate. Saponification followed by recrystallization from acetone afforded palmitic acid, m.p. 63.0–63.5°; neutral equivalent, calculated, 256.4; found, 256. A sample of the acid was converted into the amide which melted at 105.0–106.1° and gave no depression on admixture with palmitamide. The \( C_{16} \) methyl ester Fractions 11 to 15 (1.855 gm.) were

![Distillation curve of the methyl esters derived from the “bound lipides”](http://www.jbc.org/)
cent ethanol at 5° and three recrystallizations from 95 per cent ethanol at
-20°, m.p. 77.2-79.6°. (Literature, melting point of the low melting di-
hydroxy derivative of palmitoleic acid is 85-86° (8).)

\[ \text{C}_{14}\text{H}_{22}\text{O}_4. \text{ Calculated, C 66.6, H 11.2; found, C 66.3, H 10.9} \]

The dihydroxyhexadecanoic acid fraction (200 mg.) was oxidized with
periodic acid (9) and the oxidation mixture was steam-distilled. The non-
volatile fragments (aldehydicarboxylic acids) were converted into the cor-
responding dibasic acids by oxidation with silver oxide in the presence of
dilute sodium hydroxide in the manner previously described (2). The
dibasic acids (75 mg.) melted at 83.6-90.8° and had a neutral equivalent of
98. Samples of these dibasic acids were subjected to chromatography on
phosphate-buffered silica gel columns according to the method of Klenk
and Bongard (10). Klenk’s Column B served as the stationary phase and
chloroform containing 5 per cent of n-butanol was employed for the elution
of undecadienoic acid; azelaic acid was eluted with chloroform containing
30 per cent of n-butanol. A typical run, with an 8.6 mg. sample of the
dibasic acids, is illustrated in Fig. 2. The azelaic acid fractions from two
chromatograms were combined for isolation of the pure acid, m.p. 104.8-
106.8°; there was no depression on admixture with an authentic sample.

\[ \text{C}_{18} \text{Fatty Acids} \]

The C_{18} methyl ester Fractions 36, 37, and 39 (0.937 gm.), m.p. 36.5-
38.0°, were saponified to give stearic acid, m.p. 68.9-69.8°; neutral equiv-
alent, calculated, 284; found, 283. The C_{18} methyl ester Fractions 28 to
32 (2.370 gm.) were saponified, and a 1.950 gm. sample of the resulting
acids was separated into stearic acid, m.p. 68.6-69.6° (0.630 gm.), and a
liquid acid fraction (1.294 gm.) by low temperature recrystallization es-
sentially in the manner previously described (3). Ten additional re-
crystallizations of the liquid acid fraction from acetone at -60° gave a ma-
terial (0.694 gm.) having the following properties: m.p. 10.5-12.0°, iodine
number, 89.7, neutral equivalent, 280; calculated, iodine number, 89.9;
neutral equivalent, 282. No depression of the melting point was observed
when this material was admixed with a sample of synthetic cis-vaccenic
acid; a 1:1 mixture with oleic acid melted at 2.0-3.2°. The oxirane deriv-
ative of the liquid acid (1) melted at 46.0-47.2° and did not depress the
melting point of a sample of the oxirane derivative of synthetic cis-vaccenic
acid, m.p. 46.0-47.6°. On admixture with the oxirane derivative of oleic
acid, m.p. 57.0-58.0°, a marked depression of the melting point was ob-
served.

Perfomic acid oxidation (7) of a 99 mg. sample of the liquid acid af-
forded a dihydroxyoctadecanoic acid melting at 89.6-91.2°. This ma-
material did not depress the melting point of an authentic sample of the low melting dihydroxy derivative of synthetic cis-vaccenic acid, but a marked depression resulted on admixture with the low melting dihydroxy derivative of oleic acid.

\[ \text{C}_{18}\text{H}_{36}\text{O}_4. \text{ Calculated, C } 68.3, \text{ H } 11.5; \text{ found, C } 68.1, \text{ H } 11.3 \]

A sample of the dihydroxy derivative (35 mg.) was oxidized with periodic acid followed by silver oxide, as previously described (2), and the resulting dibasic acids were dissolved in 10 ml. of chloroform. Aliquots of this solution were chromatographed on Klenk's Column B with chloroform containing 5 and 30 per cent, respectively, of \( n \)-butanol serving as eluting agents. A typical run, with a 2 ml. aliquot of the chloroform solution of the dibasic acids, is illustrated in Fig. 2.

**Examination of Still Pot Residues**

The dark brown, viscous, still pot residues (1.542 gm.) were distilled at 0.05 mm. to give 0.894 gm. of pale yellow distillate and 0.640 gm. of a resinous material which was not investigated further. The distillate was saponified, and the resulting acids were separated into a solid fraction.
(0.342 gm.) and a liquid fraction (0.466 gm.) by fractional recrystallization from acetone at −20°. Spectroscopic examination of the liquid fraction in the infra-red region failed to reveal the presence of lactobacillic acid.

**Isolation and Characterization of Fatty Acids from “Free Lipides”**

The substances extractable with acetone (12.0 gm.) consisting of *n*-hexylresorcinol and “free lipides” were saponified to give an alkali-soluble fraction (*n*-hexylresorcinol plus fatty acids) (9.0 gm.) and alkali-insoluble materials (0.36 gm.) which were not investigated further. The alkali-soluble portion (8.783 gm.) was exposed to an excess of diazomethane for 10 minutes, and the resulting material (8.955 gm.) was distilled. This material was composed of *n*-hexylresorcinol, 2.924 gm.; low boiling esters, 0.256 gm.; C₁₆ esters, 1.627 gm.; C₁₈ esters, 1.007 gm.; and still pot residues, 2.541 gm.

**C₁₆ Fatty Acids**

A sample of the C₁₆ esters (1.300 gm.) was saponified, and the resulting acids (1.235 gm.) were separated into palmitic acid, m.p. 62.2–63.4° (0.700 gm.), and a liquid fraction (0.535 gm.) by fractional recrystallization from acetone at −30°. The liquid material was further purified by four recrystallizations from acetone at −80° to give a fraction (0.297 gm.) melting at −10° to −8°; neutral equivalent, 253; iodine number, 100.4.

The dihydroxy derivative melted at 79.0–83.0° and did not depress the melting point of a sample of the corresponding dihydroxy derivative derived from the “bound lipides.”

C₁₆H₃₂O₄. Calculated, C 66.6, H 11.2; found, C 66.6, H 11.0

**C₁₈ Fatty Acids**

A sample of the C₁₈ methyl esters (1.179 gm.) was saponified and the resulting acids were separated into stearic acid, m.p. 67.8–69.2° (0.260 gm.), and a liquid fraction (0.858 gm.) by fractional recrystallization from acetone at −25°. The liquid material was further purified by six recrystallizations from acetone and two recrystallizations from methanol at −60° to give a fraction (0.413 gm.) melting at 9.0–11.0°; neutral equivalent, 281; iodine number, 89.6. Admixture with cis-vaccenic acid gave no lowering of the melting point, but a marked depression resulted on admixture with oleic acid.

The dihydroxy derivative melted at 90.8–92.4° and gave no depression when admixed with a sample of the corresponding dihydroxy derivative from the “bound lipides.”

* The presence of large amounts of *n*-hexylresorcinol in the substances extractable with acetone interfered with the isolation of the “free lipide” fatty acids and the quantitative evaluation of the complete fatty acid spectrum.
DISCUSSION

As far as we are able to ascertain, the present investigation represents the first systematic study of the fatty acid spectrum of a Streptococcus. The total lipide content of the organisms (grown under the conditions specified) was 4.5 per cent, based on the weight of dry cells. The major portion of these lipides was present in a "bound" form, and only a small proportion of acetone-extractable "free lipides" was isolated. The "bound lipides" contained 2.8 per cent of dienoic acids; the proportion of tri- and tetraenoic acids was negligible. The "bound lipide" methyl esters consisted of large proportions of C₁₆ and C₁₈ esters and of small proportions of C₁₂ and C₁₄ esters. Pure samples of lauric and myristic acids were isolated from the lowest boiling ester fractions. The C₁₆ esters contained both solid and liquid constituents. The solid acid was identified as palmitic acid; the neutral equivalent and iodine number of the highly purified liquid fraction were those of a monoethenoid hexadecanoic acid. Hydrogenation converted the material into palmitic acid, demonstrating the presence of a straight carbon chain. The dihydroxy derivative of the liquid fraction melted unsharply and considerably lower than the low melting isomer of 9,10-dihydroxyhexadecanoic acid. Its oxidative cleavage resulted in the formation of azelaic and undecanedioic acids in the ratio of 89:11 on a molar basis. We conclude that the liquid fraction contains large proportions of 9,10-hexadecenoic acid (palmitoleic acid) and smaller proportions of 11,12-hexadecenoic acid (palmitvacenic acid). We are unaware of any previous report indicating the presence of the latter material in a natural source.

In addition to stearic acid, the C₁₈ acids contained liquid constituents which exhibited the neutral equivalent and iodine number of a monoethenoid octadecanoic acid. The liquid material and its dihydroxy and oxirane derivatives failed to depress the melting point of cis-vaccenic acid and of its corresponding derivatives, respectively. A marked depression resulted with the corresponding compounds of the oleic acid series. These observations suggest the presence of cis-vaccenic acid contaminated by small quantities of a closely similar monoethenoid octadecanoic acid or acids. The x-ray analysis of the dihydroxy derivative also pointed to the presence of a mixture largely consisting of 11,12-dihydroxyoctadecanoic acid and a small proportion of a closely similar material, possibly 9,10-dihydroxyoctadecanoic acid. The presence of undecanediioic and azelaic acids in the ratio of 9:1 on a molar basis in the dibasic cleavage fragments of this dihydroxy derivative leads to the final characterization of the liquid fraction as a mixture of cis-vaccenic and oleic acids.

We wish to thank Dr. E. S. Lutton of The Procter and Gamble Company for the x-ray pattern and its evaluation.
Thus far, we have investigated the fatty acid patterns of *L. arabinosus* and *L. casei* (2, 3), and have shown that these organisms contain cis-vaccenic acid as the major monoethenoid octadecanoic acid component of their lipides. The characterization of the cis-vaccenic acid was based on rigorous physical and chemical criteria. The streptococcal lipides also contain large proportions of cis-vaccenic acid, with minor proportions of oleic acid. It is of interest to note that the double bond of the major monounsaturated C₁₈ component (cis-vaccenic acid) of the streptococcal lipides occupies the 11,12 position, whereas the double bond of the major monoethenoid hexadecanoic acid (palmitoleic acid) is located in the 9,10 position.

Of particular significance is the absence of lactobacillic acid from the streptococcal lipides. This fatty acid is present in significant proportion in the lipides of the previously mentioned organisms. In our isolations of this cyclopropane fatty acid, we had resorted to diazomethane for conversion of the fatty acids into their methyl esters. Diazomethane has the property of adding across certain double bonds. This suggested the remote possibility that lactobacillic acid may be an artifact produced by the addition of diazomethane to the double bond of cis-vaccenic acid, which occurs in these lipides. In the present investigation we have again employed the diazomethane method of esterification and have not been able to detect lactobacillic acid, despite the presence of cis-vaccenic acid. Lactobacillic acid is thus a true metabolite and not an artifact produced during the isolation.

**SUMMARY**

The fatty acid spectrum of a Group C Streptococcus species, strain H46A, has been qualitatively evaluated. The lipide content of the dry cells was 4.5 per cent. The major portion of these lipides was present in a "bound" form, and only a minor fraction was acetone-extractable ("free lipides"). The "bound lipides" were composed of small proportions of lauric and myristic acids and large proportions of C₁₆ and C₁₈ fatty acids. The C₁₆ fatty acid fraction contained palmitic, 9,10-hexadecenoic, and a small proportion of 11,12-hexadecenoic acids. In addition to stearic acid, the C₁₈ fraction consisted of a large proportion of cis-vaccenic acid and small proportions of oleic acid. A careful investigation of the highest boiling ester fractions derived from the "bound lipides" failed to reveal the presence of lactobacillic acid. The composition of the "free lipides" was similar to that of the "bound lipides."

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