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Decenoic, Dodecenoic, and Tetradecenoic Acids in the *Lactobacteriaceae**

William M. O'Leary

ABSTRACT: Gas-chromatographic analyses of the fatty acids of several species of the *Lactobacteriaceae* demonstrated the presence of C_{10}, C_{12}, and C_{14} monoenoic acids. Further chemical studies of such compounds recovered by preparative scale gas chromatography indicated that they were, respectively, *cis*-3-decenoic, *cis*-5-dodecenoic, and *cis*-7-tetradecenoic acids, members of the *cis*-11-octadecenoic acid series (i.e., $CH_3(CH_2)_3CH{=}CH(CH_2)_nCOOH$). There were also evidences of small amounts of Δ^3-dodecenoic and Δ^5-tetradecenoic acids in two species of streptococcus. This demonstration of the presence of shorter members of the *cis*-11-octadecenoic acid series in these organisms is particularly significant. Several years ago, it was postulated, even though it was not possible with the methodology then available to detect these compounds, that they should be intermediates in the lengthening mode of monoenoic fatty acid biosynthesis which occurs in these, and other, microorganisms. The evidence presented in this paper of the occurrence of these compounds supports the validity of earlier concepts.

As a result of studies of fatty acid requirements and metabolism in lactobacilli and other bacteria, it was suggested several years ago that at least one mechanism for the biosynthesis of long-chain monounsaturated fatty acids in microorganisms was the progressive lengthening of already unsaturated short chain fatty acids (O'Leary and Hofmann, 1957; Hofmann *et al.*, 1959). Further studies and expansions of this concept have been presented by Bloch and his co-workers (Bloch *et al.*, 1961; Scheuerbrandt and Bloch, 1962; Erwin and Bloch, 1964) who have shown that two major pathways for unsaturated fatty acid biosynthesis occur in microorganisms, aerobic dehydrogenation of long-chain saturated acids and anaerobic elongation of short-chain monoenoic acids. This subject has been extensively reviewed by O'Leary (1962a) and Kates (1964).

Investigations of the lengthening process have been done mostly with bacteria containing *cis*-11-octadecenoic acid as their sole or principal octadecenoic acid and palmitoleic acid as their hexadecenoic acid. These acids have a similar structure from the terminal methyl group through the double bond and differ from there to the carboxyl group only in the number of intervening methylene groups. It has been found that in these organisms members of a homologous series of such acids down to at least ten carbons in chain length satisfy exogenous fatty acid requirements and are converted ultimately into the longer chain intracellular acids (O'Leary, 1962a). However, the hypothesis that the lengthening pathway of fatty acid biosynthesis involves a series of monoenoic acids as intermediate steps has suffered from the failure actually to detect the shorter members of the series in bacterial cells. Aside from the report of Bloch *et al.* (1961) of the occurrence of tetradecenoic acid in certain clostridia and the report of a similar acid in corynebacteria by Asano and Takahashi in 1945, the presence in bacteria of unsaturated fatty acids shorter than hexadecenoic acid has been doubtful. This paper reports the occurrence of

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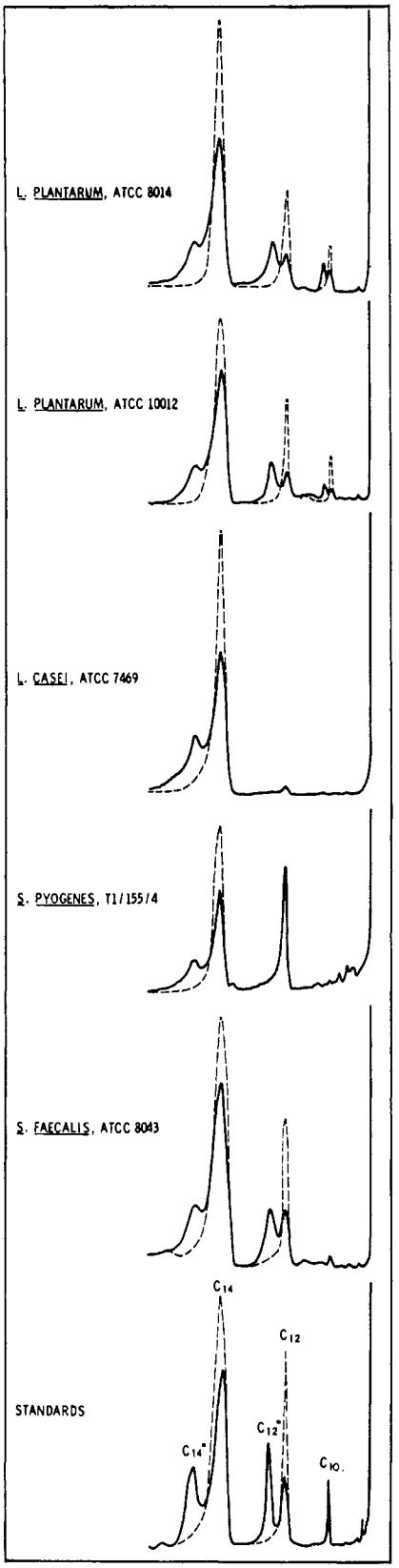

FIGURE 1: Gas chromatograms of the methyl esters of fatty acids up to C_{15} extracted from members of the *Lactobacteriaceae*. Solid lines, before hydrogenation; dotted lines, after hydrogenation. Stationary phase, diethylene glycol succinate; column temperature, 210°; sample size, 2 μl; gas, He; flow rate, *ca.* 100 ml/min.

C_{10}, C_{12}, and C_{14} unsaturated fatty acids in various species of the family *Lactobacteriaceae*.

Experimental

Microorganisms and Cultivation Methods. *Lactobacillus acidophilus* ATCC 9857, *Lactobacillus plantarum* ATCC 8014 and 10012, *Lactobacillus casei* ATCC 7469, *Streptococcus faecalis* ATCC 8034, and *Leuconostoc mesenteroides* ATCC 9135 were obtained from the American Type Culture Collection. *Streptococcus pyogenes* Group A, Type 1, strain T1/155/4, was obtained from Dr. Fred Gill of the Department of Medicine, Cornell University Medical College, New York. The methods used for the maintenance and large scale culture of lactobacilli have been described in detail elsewhere (Hofmann, *et al.*, 1957; O'Leary, 1959a,b). Streptococci were maintained on trypticase soy agar. For large-scale culture of streptococci both the medium of Mickelson (1964) and, later, that of Updyke and Nickle (1954) were employed, as we found little difference in the fatty acid content of cells grown on each medium and greater cell yields were obtained using the latter medium. *Leuconostoc mesenteroides* was grown on the medium described by Ikawa (1963).

Fatty Acid Standards. Chromatographic standards of most fatty acid methyl esters were obtained from Applied Science Laboratories, University Park, Pa., and the Hormel Institute, Austin, Minn. *n*-Heptanoic acid was obtained from Distillation Products Industries, Inc., Rochester, N.Y., and purified by fractional recrystallization from ethanol at −20°. *cis*-3-Decenoic, *cis*-5-dodecenoic, and *cis*-7-tetradecenoic acids were synthesized and purified by methods described by Hofmann *et al.* (1959).

Extraction of Fatty Acids. Initial studies involved the analyses of fatty acids in the materials extracted from whole cells by the method of Folch *et al.* (1957). Later work employed the direct alkaline hydrolysis-extraction method of Hofmann *et al.* (1957).

Chromatographic Methods. Analytical gas chromatography was performed using a Perkin-Elmer Model 154-D fractometer fitted with a 4 m × 6.4 mm column of diethylene glycol succinate for cellular fatty acids and a DC-200 silicone column of similar dimensions for analyses of unsaturated acid cleavate products. Preparative scale gas chromatography was performed with a Wilkens Autoprep Model A-700 fitted with 6.5 m- × 9.6-cm columns containing the same stationary phases. Operating conditions are shown in the various figures.

Chemical Methods. Methylation of all fatty acid preparations was accomplished by the use of boron trifluoride in methanol. Free fatty acids were recovered from methyl esters by the usual saponification-reacidification procedures. Unsaturated fatty acids were hydrogenated using Adams catalyst in glacial acetic acid.

To determine the location of double bonds in the unsaturated fatty acids recovered from bacterial cells, individual acids were converted to their dihydroxy derivatives using performic acid and cleaved to alde-

TABLE I: Occurrence of C_{10}, C_{12}, and C_{14} Monoenoic Acids in Various Bacteria.

Organism	Strain	Monoenoic Acids (as per cent of total fatty acids) C_{10}	C_{12}	C_{14}
Lactobacillus plantarum	ATCC 8014	0.3	0.9	1.3
Lactobacillus plantarum	ATCC 10012	0.4	1.2	1.5
Lactobacillus casei	ATCC 7469	<0.1[a]	<0.1	1.7
Lactobacillus acidophilus	ATCC 9857	<0.1	<0.1	<0.1
Streptococcus pyogenes	T1/155/4	<0.1	<0.1	1.0
Streptococcus faecalis	ATCC 8043	<0.1	0.2	1.4
Leuconostoc mesenteroides	ATCC 9135	<0.1	<0.1	<0.1

[a] Less than this amount, if present, could not be reliably detected by the analytical methods used.

hydes and aldehyde acids as described by Huber (1951). Aldehyde fractions were recovered by steam distillation under alkaline conditions and oxidized to aliphatic acids with potassium permanganate (Schneider, 1964). These cleavage products were then examined by gas chromatography as described above.

Infrared spectra were obtained using a Perkin-Elmer Model 237 Infracord in which samples were inserted as thin films between NaCl disks.

Results

The first indications of the presence of unsaturated acids of medium chain lengths in lactobacilli were obtained during gas chromatographic analyses of fatty acids in lipid complexes recovered from *Lactobacillus plantarum* ATCC 8014 by mild solvent extraction (Folch *et al.*, 1957) which had been intended as a first step in the determination of fatty acid distribution among lipid classes in that organism. Certain recorder peaks in these analyses indicated the presence of C_{10}, C_{12}, and C_{14} monoenoic acids. Hydrogenation of these samples eliminated the suspect peaks and correspondingly increased the analogous saturated acid peaks (Figure 1).

To determine whether these acids were limited to this strain or were more widely distributed, we studied other lactobacilli and members of other genera in the family *Lactobacteriaceae*. Chromatograms obtained in the course of these studies are also included in Figure 1. *Lactobacillus plantarum* ATCC 10012 contained unsaturated acids of the same chain lengths found in *L. plantarum* ATCC 8014. *L. casei* ATCC 7469 and *Streptococcus pyogenes* T1/155/4 contained tetradecenoic acid, but no dodecenoic or decenoic acids were detected. *S. faecalis* ATCC 8043 contained tetradecenoic acid and dodecenoic acid, but no decenoic acid could be found. None of these three monoenoic acids was detected in the mixed fatty acids extracted from either *L. acidophilus* ATCC 9857 or *Leuconostoc mesenteroides* ATCC 9135.

The percentages of total extracted cellular fatty acids represented by the three acids in each organism are shown in Table I.

After the analytical scale gas chromatography indicated the presence of these medium chain length monoenoic acids in certain organisms, sufficient amounts of each acid were collected by preparative gas chromatography to permit determinations of neutral equivalent, iodine number, and elemental composition. Table II gives the results obtained in the examination of acids isolated from *L. plantarum* ATCC 8014. Essentially identical results were obtained with acids from other organisms studied.

TABLE II: Analytical Data for Medium Chain Length Monoenoic Fatty Acids Isolated from *Lactobacillus plantarum*.

Analysis	Monoenolic Acid C_{10}	C_{12}	C_{14}
Neutral equivalent			
Calcd	170.2	198.3	226.3
Found	170.8	197.2	226.1
Iodine number			
Calcd	149.1	128.0	112.2
Found	148.9	129.0	112.7
Per cent carbon			
Calcd	70.5	72.7	74.3
Found	70.5	72.1	74.9
Per cent hydrogen			
Calcd	10.7	11.2	11.6
Found	10.6	11.3	12.1

The infrared absorption spectra of these isolated acids were consonant with what would be expected of *cis* monoenoic monocarboxylic aliphatic acids. Spectra of the methyl esters of tetradecenoic acids isolated from

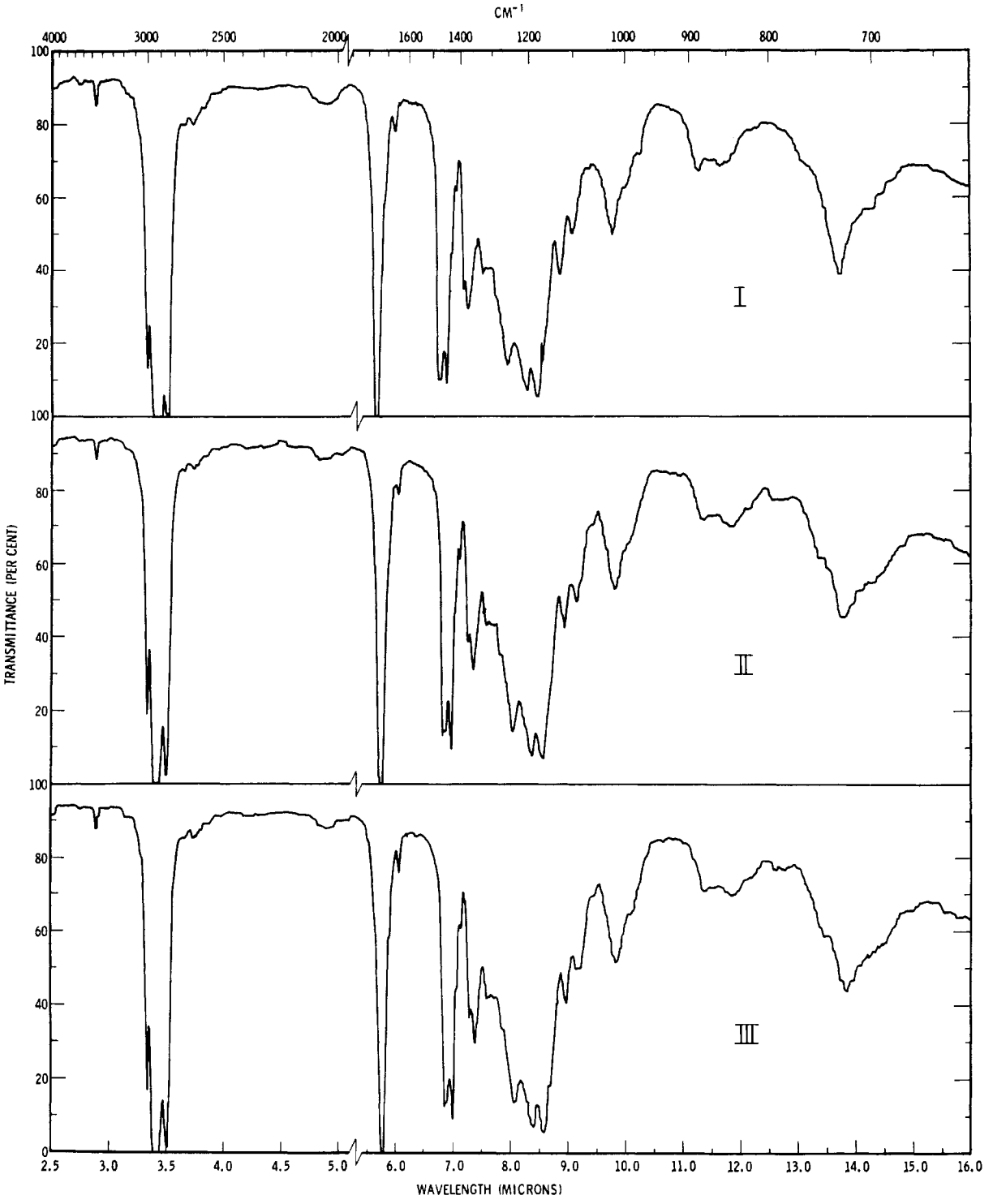

FIGURE 2: Infrared absorption spectra of C_{14} monoenoic methyl esters of fatty acids isolated from *L. plantarum* (I) and from *S. pyogenes* (II), and of an authentic sample of *cis*-7-tetradecenoic acid (III).

L. plantarum ATCC 8014 and *S. pyogenes* T1/155/4 are shown in Figure 2.

These data indicated that the compounds under investigation were indeed decenoic, dodecenoic, and tetradecenoic acids. The further question, then, was specifically which acids they were. For several of the organisms investigated here, it was already known that their longer chain unsaturated fatty acids were members of a homologous series having the configuration shown (Hofmann, 1963; Kates, 1964; O'Leary, 1962a):

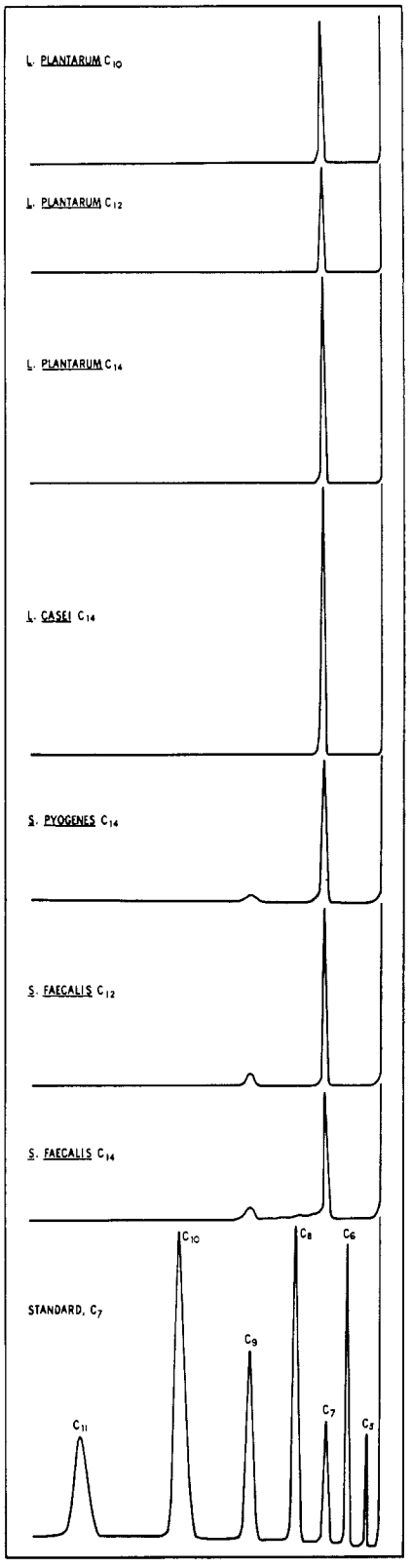

FIGURE 3: Gas chromatographic analyses of the methyl esters of fatty acids derived from the methyl ends of monoenoic bacterial fatty acids. Stationary phase, DC-200 silicone; column temperature, 180°; sample size, 2 μl; gas, He; flow rate, approximately 80 ml/min.

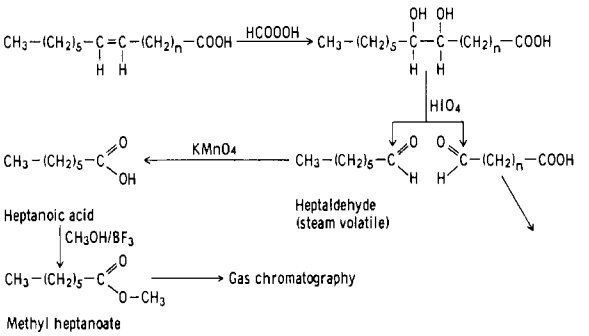

FIGURE 4: Cleavage of monoenoic fatty acids from bacteria and preparation of aliphatic acid from the methyl end of the original acid.

$$CH_3(CH_2)_5C(H)=C(H)(CH_2)_nCOOH$$

If the elongation concept of unsaturated fatty acid biosynthesis in these organisms is correct, then the shorter acids of this series should have a similar configuration in the methyl end of their molecules, and for decenoic, dodecenoic, and tetradecenoic acids *n* should be respectively 1, 3, and 5.

To determine the locations of the unsaturation, the isolated acids were cleaved at the sites of the double bonds and the methyl "ends" were converted to the monocarboxylic saturated aliphatic acids which were then methylated and analyzed by gas chromatography on DC-200 silicone stationary phase as described above. The results of these studies are shown in Figure 3. In each case, the methyl ester obtained was chromatographically identical with methyl *n*-heptanoate, and such a product could only have been obtained by the sequence of reactions illustrated in Figure 4, which is adapted from Hofmann (1953).

Gas co-chromatography of the individual methyl esters of decenoic, dodecenoic, and tetradecenoic acids isolated from bacteria when mixed with analogous methyl esters of *cis*-3-decenoic, *cis*-5-dodecenoic, or *cis*-7-tetradecenoic acids gave single peaks in each instance.

Discussion

The fatty acids of some of the organisms employed in this investigation have been repeatedly examined in the past (see, for example, Asselineau, 1962; Hofmann, 1963; Kates, 1964; O'Leary, 1962a) without detecting the presence of unsaturated acids with chains of less than 16 carbon atoms. It now appears that this was a procedural limitation in that it simply was not possible with the methods heretofore employed to detect such fatty acids because of the low concentrations in which they occur in bacteria and the difficulty of separating them from related compounds. Their detection at this time was actually a fortuitous consequence of experimenta-

tion directed toward determining the mode of distribution of fatty acids among lipid classes in eubacteria, an investigation which has yet to be concluded. In these studies, we were employing a gas chromatographic method that proved to be sufficiently sensitive and discriminating to demonstrate the presence of small amounts of 10, 12, and 14 carbon monoenoic acids. Thus we were able to detect compounds which had always been present but which previously had eluded detection. Understandably, this line of investigation was then pursued at the expense of our original studies.

The fact that *cis*-3-decenoic, *cis*-5-dodecenoic, and *cis*-7-tetradecenoic acids have now been found to occur in various members of the *Lactobacteriaceae* is, of course, important in itself in that it adds to our over-all knowledge of microbial lipids. In addition, these compounds are of particular interest in that they are just the ones that were predicted 8 years ago to be necessary intermediate stages in the biosynthesis of *cis*-11-octadecenoic acid in these organisms. While nutritional studies have shown that such acids when supplied exogenously could be converted to the longer chain monoethenoid acids found in relative profusion in such microorganisms, it has not previously been shown that the shorter acids do indeed naturally occur in the cells. Thus, the findings reported in this paper provide strong, if not final, evidence for the elongation hypothesis of unsaturated fatty acid biosynthesis in the lactobacteria and related organisms.

The data obtained in the present studies show that the occurrence of monoenoic acids of medium chain length is not limited to one strain, species, or genus, at least within the *Lactobacteriaceae*. However, on the basis of the relatively limited information now available, it does appear that the spectrum of unsaturated cellular acids becomes more limited as nutritional requirements become more fastidious: *L. plantarum* has more acids than *L. casei* which has more than *L. acidophilus*. Similarly, *S. faecalis* has more than *S. pyogenes*. Whether this is a limited or more widespread phenomenon is yet to be determined. The absence of any medium chain length unsaturated fatty acids in *Leuconostoc mesenteroides* is in agreement with the recent findings of Ikawa (1964) using a different strain of the same species. We have employed similar methods in studies of the lipids of various members of the *Enterobacteriaceae* (O'Leary, 1962b, 1965) and did not see any evidence of 10, 12, or 14 carbon monoenoic acids. A study of the fatty acids of a pleuropneumonia-like organism, however, did produce an unidentified fraction which might, in the light of the findings reported in this paper, be a tetradecenoic acid (O'Leary, 1962c). Reinvestigation of this organism would be most desirable.

In the cleavage products from the acids of the streptococci examined a small amount of what appeared to be nonanoic acid was detected, suggesting that some of the monoenoic acids in these organisms had the configuration of the oleic acid homologous series (i.e., $CH_3(CH_2)_7CH{=}CH(CH_2)_nCOOH$). This would agree well with the paper of Hofmann and Tausig (1955) in which they reported that a Group C *Streptococcus* contained hexedecenoic and octadecenoic acids of both the oleic and the *cis*-11-octadecenoic acid series.

The fact that not all members of the pertinent homologous series were found in every organism examined may reflect either species and generic dissimilarities, or, more likely, the possibility that the concentrations of undetected compounds are so low as to require even more exquisitely sensitive analytical methods for their detection. It has been suggested that turnover of the shortest intermediates, either free or in complexes, may be so rapid that too little exists at any one moment to be isolated by the hydrolysis and extraction methods so far employed (O'Leary, 1965). Isotope procedures, particularly biosynthetic labeling, may prove useful in the detection of very minute amounts of such acids that may occur in organisms in which these compounds have not yet been found by chemcal analytical techniques. High turnover rates may also account for the relatively low concentrations of these medium chain length intermediates (if such they are) in the biosynthesis of long chain monoenoic acids which occur in bacterial cells in much larger amounts.

It has been clearly established in recent years that the cyclopropane fatty acids occurring in many bacteria are biosynthesized by the addition of a 1-carbon fragment across the double bond of long chain monoenoic acids (O'Leary, 1959a; O'Leary, 1959b; Liu and Hofmann, 1962) and that the 1-carbon unit is derived from the methyl carbon of methionine by way of *S*-adenosylmethionine (O'Leary, 1959a; Liu and Hofmann, 1962; O'Leary, 1962b; O'Leary, 1965). It has now been found that several cyclopropane fatty acids occur in bacteria, including the C_{19}, C_{17}, C_{15}, and C_{13} acids (Kates, 1964), and it appears that these acids are generated only from the analogous monoethenoid acids and not by lengthening of shorter chain cyclopropane acids. The findings reported in this paper strongly support this biosynthetic scheme which has been postulated previously in various forms by numerous investigators (*vide supra*). The involvement of this mechanism with the metabolic functions of biotin has been discussed extensively elsewhere (O'Leary, 1957; Hofmann, *et al.*, 1959).

At this point, at least two major questions yet remain to be resolved. One is the nature of the precursor of decenoic acid. In the case of the *cis*-11-octadecenoic acid homologous series which obtains in the organisms investigated in this paper (i.e., $CH_3(CH_2)_5CH{=}CH(CH_2)_nCOOH$) it is possible to maintain the apparently essential configuration of the methyl end of the chain only down to decenoic (and, conceivably, to nonenoic) acid without moving the site of the double bond. Numerous hypotheses have been advanced regarding this problem (see, for example, Scheuerbrandt and Bloch, 1962; Erwin and Bloch, 1964; Kates, 1964). However, as yet, no experimental evidence has been obtained to support these hypotheses. A similar problem exists for the oleic acid homologous series (i.e., $CH_3(CH_2)_7CH{=}CH(CH_2)_nCOOH$). For this series, it would be possible to maintain the methyl end of the

chain without moving the site of the double bond only down to dodecenoic (and, conceivably, to undecenoic) acid. Again, no precursor has been demonstrated.

The other major problem is the fate of the end products of all this biosynthetic turmoil, namely the cyclopropane fatty acids. To date, no metabolic function, structural role, unique lipid complex, specific cytological location, in short, any purpose has been reported for these most uncommon compounds. This is especially peculiar in view of their extreme structural specificity and the relatively high concentrations in which they occur (O'Leary, 1962a, 1965). Consequently, present concepts are of necessity speculative and inconclusive. Some workers have suggested a structural function. Others have pointed out that in view of their structural specificity, rigid and relatively high concentration levels, and absolute requirements for growth in many organisms, a more kinetic role is indicated such as a transport or semistabile storage form of 1-carbon fragments in the form of the ring methylene. There are no data, and only future studies can resolve this matter. Meanwhile, the present investigations have strengthened the concepts of the other stages of this train of biosynthetic events.

The complete and conclusive elucidation of the biosynthetic pathway leading to unsaturated and cyclopropane fatty acids is, of course, of basic and important interest to the biochemist and molecular biologist. Beyond this, however, is the possibility of the application of such fundamental matters to such things as therapeutic measures. For example, the occurrence of uncommon (or, more exactly, non-mammalian) lipid metabolism pathways in microorganisms such as the lengthening mechanism, or the essential functions of cyclopropane compounds in certain microorganisms, may provide specific areas of attack for antimicrobial preparations. If enough biochemical mechanisms peculiar to bacteria can be elucidated, we may be able to abandon our presently largely empirical approach to antibacterial agents and begin to search for specific compounds which will bring about specific inhibitions.

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