Composition of the saturated and monounsaturated fatty acids of *Mycobacterium phlei*

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ABSTRACT Combined gas–liquid chromatography–mass spectrometry has been used to identify unusual fatty acids of *Mycobacterium phlei*. In addition to many normal saturated and monounsaturated fatty acids, series of iso, anteiso, 10-methyl, and (*n*–8)-methyl substituted fatty acids have been found and identified. These mid-chain branched acids may arise by methylation of monounsaturated acids followed (if necessary) by chain elongation.

SUPPLEMENTARY KEY WORDS GLC–mass spectrometry · intensity ratios · iso · anteiso · 10-methyl · (*n*–8)-methyl substituted fatty acids · biosynthesis

In their paper on the biosynthesis of oleic acid and 10-methyloctadecanoic acid in *Mycobacterium phlei*, Lennarz, Scheuerbrandt, Bloch, and Ryhage (1) suggested that small amounts of a branched-chain C_{17} fatty acid were present in the organism in addition to the studied substances and a number of normal and monoenoic fatty acids. Because of lack of material, definitive identification was impossible. However, it was inferred from gas-chromatographic and radioisotopic data that the substance was an analogue of 10-methyloctadecanoic acid. In this paper, which represents a detailed study of the fatty acids of *M. phlei*, we establish that 10-methylhexadecanoic acid is present in the lipids of the organism. Other branched-chain fatty acids, including members of the iso and anteiso series, have been encountered.

MATERIALS AND METHODS

GLC and GLC–MS methods were as described in the preceding paper (2).

Standard normal (C_{12}, C_{14}, C_{15}–C_{20}, C_{22}, and C_{24}), iso (C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, and C_{24}), anteiso (C_{15}, C_{17}, C_{19}, C_{21}, and C_{23}), and monoenoic (C_{16}, C_{18}, C_{20}, C_{22}, and C_{24}) fatty acid methyl esters were obtained from Applied Science Laboratories, Inc., State College, Pa., and Supelco, Inc., Bellefonte, Pa.

Preparation of M. phlei Fatty Acid Methyl Esters and Preliminary GLC

M. phlei, the gift of Professor M. Weber, was grown, harvested, and extracted as previously described (3). The nonpolar lipids were treated at room temperature with methanolic hydrochloric acid for 10 hr; the solution was evaporated to dryness and the product taken up in ethyl acetate. The temperature-programmed GLC trace obtained from this preparation is shown in Fig. 1.

GLC–MS of the Mixture

The methanolyzed lipid mixture was analyzed by combined GLC–MS. An electron energy of 17 ev was used. We detected saturated fatty acid methyl esters by scanning the spectra for the following characteristic fragmentation features:

(*a*) A parent molecular ion of value m/e 60 + 14*a* (*a* = 1, 2, 3, . . .);

(*b*) A base peak at m/e 74;

(*c*) Prominent losses from the parent molecular ion of 29, 31, and 43 units of mass;

(*d*) A sequence of fragment ions of m/e ratio 87 + 14*b* (*b* = 0, 1, 2 . . .).

We detected the monounsaturated fatty acid methyl esters most conveniently by scanning the spectra for a pair of ions of general formula, 68 + 14*c* and 26 +

Abbreviations: GLC, gas–liquid chromatography; GLC–MS, combined GLC–mass spectrometry.

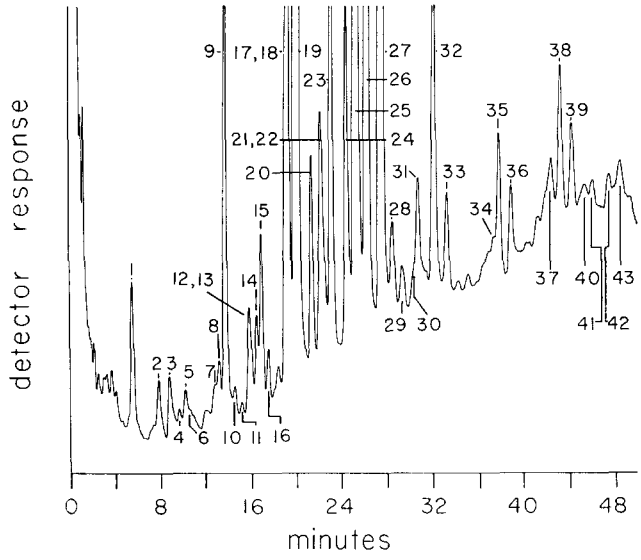


FIG. 1. GLC of methanolyzed total lipids of *Mycobacterium phlei* on 3% OV-1; temperature-programmed from 120 to 250°C at 3°C/min. All but 10 of the peaks were attributable to fatty acid methyl esters.

14*c* (*c* = 1, 2, 3 . . .). These correspond to the P–32 and P–74 fragment ions and have always been found, even in those cases where the parent ion is relatively weak.

RESULTS

Of the components of Fig. 1, the saturated and monounsaturated fatty acid methyl esters were easily picked out when each mass spectrum was scanned for the fragmentation characteristics described under Methods. We operated the mass spectrometer at 17 ev since at this voltage the parent ions are relatively more intense than they are at 70 ev, spectra are much less complicated by secondary fragmentation patterns, and ready differentiation between isomeric fatty acid derivatives is possible (2). Components 2, 6, 7, 9, 12, 13, 15, 17, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, and 43 were saturated, and components 8, 14, 18, 22, 25, 28, 34, 37, and 42 monounsaturated fatty acid methyl esters.

To determine which of the saturated fatty acid esters belonged to the normal, iso, and anteiso series, we gas-chromatographed the methanolyzed lipids from *M. phlei* together with a standard mixture of such materials. From the resultant chromatogram and the retention time–carbon number plot derived from it, components 2, 6, 9, 15, 19, 23, 26, 29, 32, 35, 38, and 43 were respectively identified as C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, C_{22}, C_{24}, and C_{26} normal fatty acid methyl esters. Measurement of the 17 ev mass spectral intensity ratios, (P–29)/(P–31)/(P–43), for the compounds confirmed these structure assignments (2).

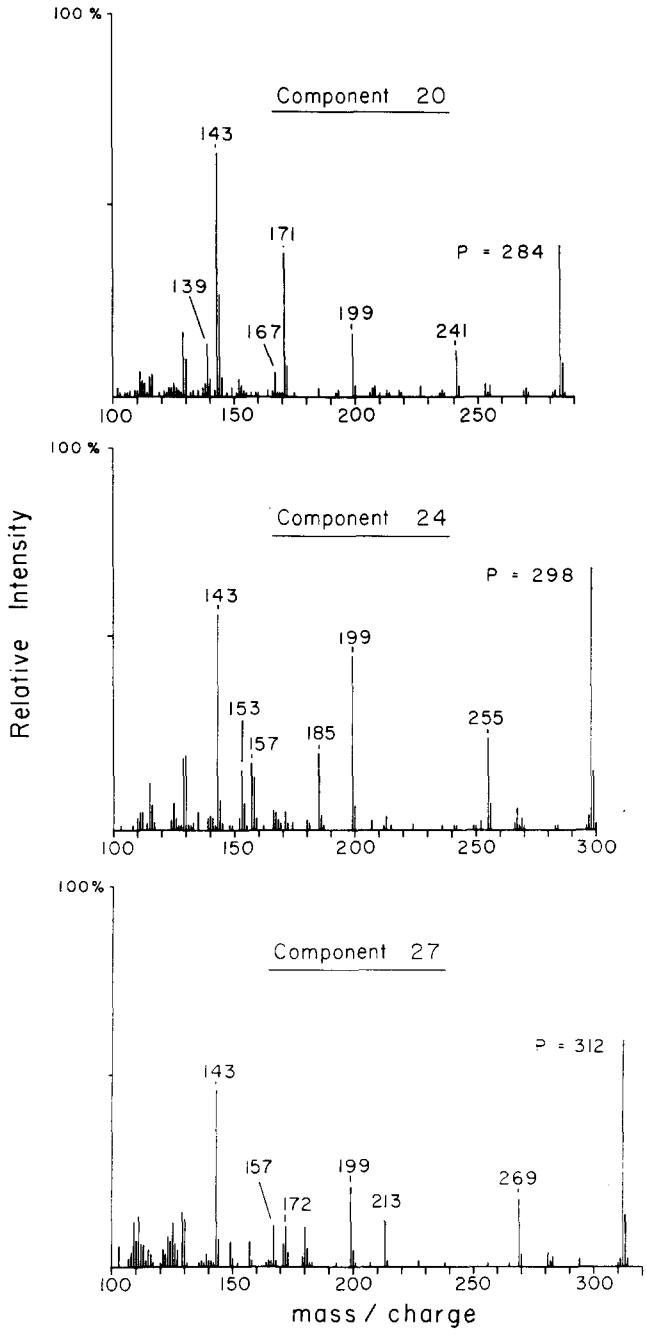


FIG. 2. Mass spectra (17 ev) of components 20, 24, and 27.

On the OV-1 (methyl silicone polymer) columns used, the iso and anteiso esters of the same carbon number are only just separated. Thus, although it can be said that components 7, 12, 13, 17, and 21 were the iso *or* anteiso methyl esters of carbon number C_{14}, C_{15}, C_{15}, C_{16}, and C_{17}, respectively, no reliable GLC discrimination between the two possibilities could be made. However, as has been shown previously (2), the intensity ratios (P–29)/(P–31)/$(P–43)_{17ev}$ can differentiate between the two isomeric structures. Iso esters have such a ratio in the

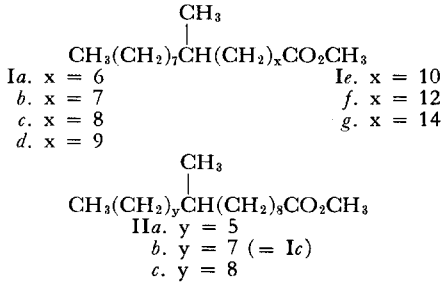
TABLE 1 SATURATED FATTY ACID METHYL ESTERS OBTAINED FROM *M. phlei*

Component	Parent Ion (m/e)	GLC Retention Time Corresponds to	(P–29)/ (P–31)/(P–43)$_{17ev}$	Allocated Structure: Methyl
2	214[a]	*n*-C_{12}	52/65/100	Dodecanoate
6	228[b]	*n*-C_{13}	35/54/100	Tridecanoate
7	242[c]	i/a-C_{14}	14/20/100	Isotetradecanoate
9	242	*n*-C_{14}	20/48/100	*Tetradecanoate*
12	256	i/a-C_{15}	21/36/100	Isopentadecanoate
13	256	i/a-C_{15}	35/30/100	Anteisopentadecanoate
15	256	*n*-C_{15}	24/58/100	*Pentadecanoate*
17	270[d]	i/a-C_{16}	20/35/100	Isohexadecanoate
19	270	*n*-C_{16}	24/55/100	*Hexadecanoate*
20	284	—	24/28/100	8- and 10-Methylhexadecanoate
21	284	i/a-C_{17}	43/22/100	Anteisoheptadecanoate
23	284[e]	*n*-C_{17}	26/51/100	*Heptadecanoate*
24	298	—	22/29/100	9-Methylheptadecanoate
26	298	*n*-C_{18}	21/54/100	*Octadecanoate*
27	312	—	16/25/100	*10-Methyloctadecanoate*
29	312[f]	*n*-C_{19}	—	Nonadecanoate
30	326	—	20/33/100	10- and 11-Methylnonadecanoate
32	326	*n*-C_{20}	23/49/100	Eicosanoate
33	340	—	28/24/100	12-Methyleicosanoate
35	354	*n*-C_{22}	27/49/100	Docosanoate
36	368	—	15/21/100	14-Methyldocosanoate
38	386[g]	*n*-C_{24}	23/55/100	*Tetracosanoate*
39	396	—	ca. 50/36/100	16-Methyltetracosanoate
43	410[h]	*n*-C_{26}	—	Hexacosanoate

Italicized structures same as reported by Lennarz et al. (1). i/a, iso or anteiso. A superscript letter denotes that the component was slightly contaminated by material with parent ion(s) having m/e at (*a*) 227, (*b*) 188 and 232, (*c*) 232, 234 and 240, (*d*) 268 (methyl hexadecenoate), (*e*) 256, (*f*) 326 and 333, (*g*) 357, and (*h*) 388.

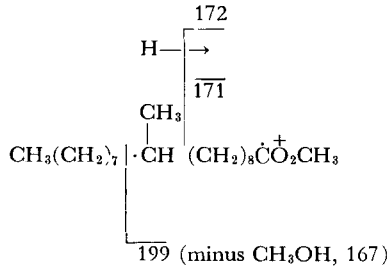
range (10–20)/(16–36)/100, while anteiso esters have values in the range (32–58)/(16–38)/100. By this criterion, components 7, 12, and 17 are the C_{14}, C_{15}, and C_{16} iso esters, while components 13 and 21 are the C_{15} and C_{17} anteiso esters (Table 1).

After the normal, iso, and anteiso fatty acid methyl esters had been identified, seven unidentified saturated esters remained: components 20, 24, 27, 30, 33, 36, and 39. From their GLC retention times and mass spectral characteristics, they appeared to be monomethyl substituted fatty acid esters of carbon number C_{17}, C_{18}, C_{19}, C_{20}, C_{21}, C_{23}, and C_{25}.



The structure of component 27 was deduced to be methyl 10-methyloctadecanoate (Ic) by comparing its 70 ev mass spectrum with that already published for this substance (4). Study of the 17 ev spectrum of this component (Fig. 2), indicated that the Ryhage–Stenhagen Rule for locating branched-chain methyl groups in monomethyl substituted fatty acid methyl esters (5) is valid at this lower ionization voltage. This valuable rule can be formally stated as follows: "A methyl branch in a monomethyl substituted fatty acid methyl ester will be located at carbon number *n*, if there are relatively intense ions in its mass spectrum at m/e 59 + 14*n*, 32 + 14*n* and/or 31 + 14*n*, and 27 + 14*n*, and a relatively weak ion at m/e 45 + 14*n*, where *n* = 2, 3, 4, . . . "

The basis for this rule is shown below for the case of methyl 10-methyloctadecanoate (Ic), *n* = 10.



The easiest way to use the Rule in structure determination is to search the mass spectrum for an intense ion at m/e 59 + 14*n* which has sizeable ions 27, 28, and 32 units of mass below it, and which has little or no intensity 14 masses units below it. When such a situation is found the methyl group is located at carbon *n*. Thus, examination of the spectrum of component 24 (Fig. 2) indicated that this C_{18} branched-chain methyl ester

was methyl 9-methylheptadecanoate (I*b*), because there were intense ions at m/e 185, 158, 157, and 153 and only weak intensity at m/e 171.

By similar methods it was deduced that:

component 33 was methyl 12-methyleicosanoate (I*e*), significant peaks m/e 227, 200, 199, and 195 (Fig. 3);

component 36 was methyl 14-methyldocosanoate (I*f*), significant peaks m/e 255, 228, 227, and 223 (Fig. 3);

component 39 was methyl 16-methyltetracosanoate (I*g*), significant peaks m/e 283, 256, 255, and 251 (Fig. 3);

component 20 was a mixture of methyl 8-methylhexadecanoate (I*a*) and methyl 10-methylhexadecanoate (II*a*), significant peaks 171, 144, 143, 139, and 199, 172, 171, 167, respectively (Fig. 2).

As shown in Fig. 1, component 30 was but a small sideband of component 31; consequently, the over-all intensity of its mass spectrum was weak. Nonetheless, application of the Ryhage–Stenhagen Rule indicated that it was predominantly methyl 11-methylnonadecanoate (I*d*). A small amount of methyl 10-methylnonadecanoate (II*c*) may also have been present.

Structures were assigned to the monounsaturated fatty acid methyl esters in analogous fashion. Results are shown in Table 2. Intensity ratio measurements (2) were used to demonstrate that the materials had monoenoic rather than the isomeric cyclopropanoid structures. It is hoped that a future study will allow us to determine the position and stereochemistry of the double bond.

The major ions in the mass spectra of the remaining non-fatty acid components of the methylated lipid mixture derived from *M. phlei* are shown in Table 3. No firm assignments of structure have been made as yet to any of these materials, although several appear to be related to the large number of aromatic metabolites produced by this organism (Bentley, R., I. M. Campbell, and A. T. Hudson, unpublished observations).

DISCUSSION

Hitherto, our knowledge of the fatty acid composition of *M. phlei* was based on the observations of Lennarz et al. (1). The materials positively identified by these workers are italicized in Tables 1 and 2. The current investigation shows that the organism produces a more complex mixture of fatty acids than was previously realized.

Apart from the greater range of normal and monoenoic fatty acids, the most striking observation is the occurrence of large numbers of monomethyl substituted fatty acids. Firstly, members of the iso and anteiso series have been encountered. Such substances are not uncommon

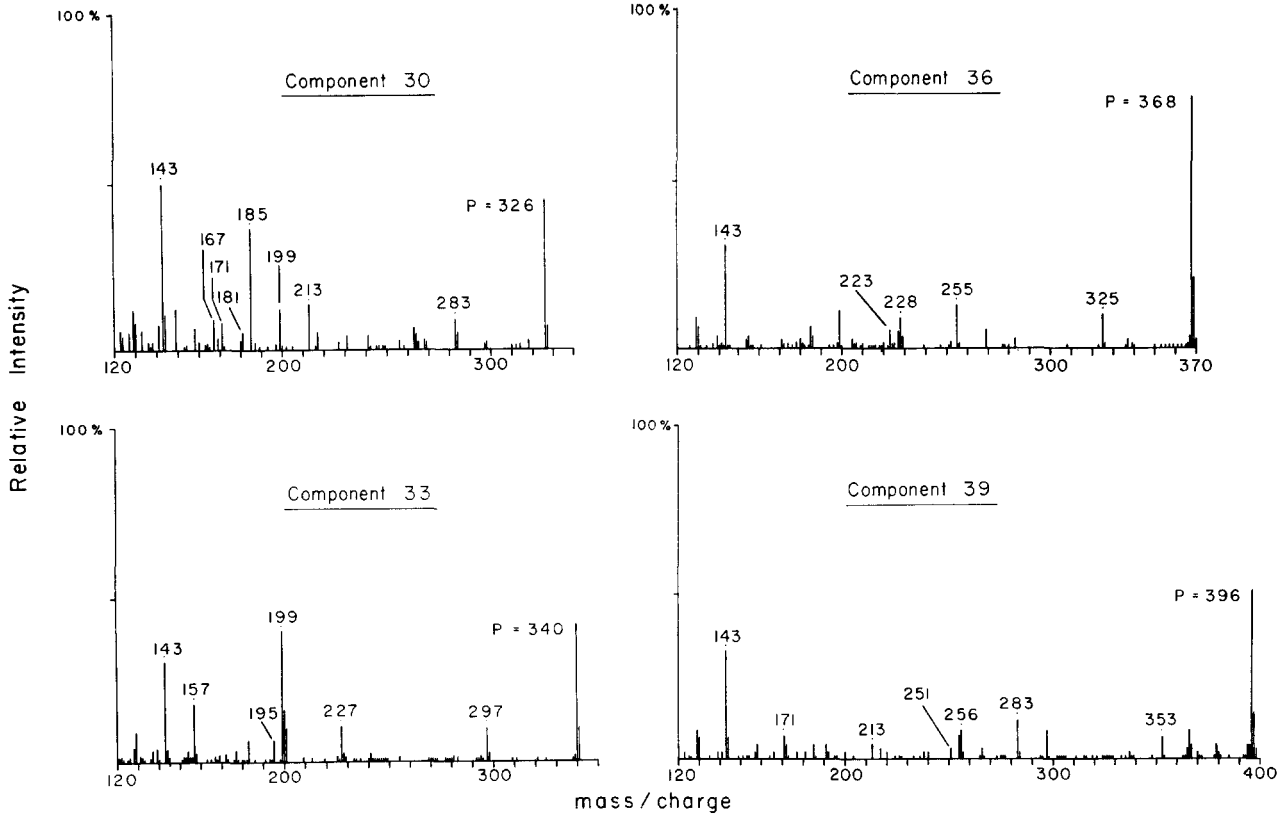


FIG. 3. Mass spectra (17 ev) of components 30, 33, 36, and 39.

TABLE 2 MONOUNSATURATED FATTY ACID METHYL ESTERS OBTAINED FROM *M. phlei*

Component	Parent Ion (m/e)	GLC Retention Time Corresponds to	$P/(P-32)_{17ev}$	Allocated Structure: Methyl
8	240	$C_{14:1}$	34/100	Tetradecenoate
14	254*	$C_{15:1}$	—	Pentadecenoate
18	268	$C_{16:1}$	36/100	*Hexadecenoate*
22	282	$C_{17:1}$	36/100	*Heptadecenoate*
25	296	$C_{18:1}$	27/100	*Octadecenoate*
28	310†	$C_{19:1}$	18/100	Nonadecenoate
34	352	$C_{22:1}$	21/100	Docosenoate
37	384	$C_{24:1}$	20/100	Tetracosenoate
42	408	$C_{26:1}$	17/100	Hexacosenoate

Italicized structures same as reported by Lennarz et al. (1)
* Component contaminated by material of parent ion m/e 186.
† Component contaminated by material of parent ion m/e 296, 312.

TABLE 3 MAJOR IONS IN SPECTRA OF NON-FATTY ACID COMPONENTS

Component	m/e (most intense ion italicized)
1	194, *163*, 135, 133
3	139, 138, *101*, 84
4	*188*
5	*188*, 165, 137
10	184, *143*
11	180, *74*
16	*250*
31	*320*, 282
40	364, 332, *155*
41	378, 239, *155*

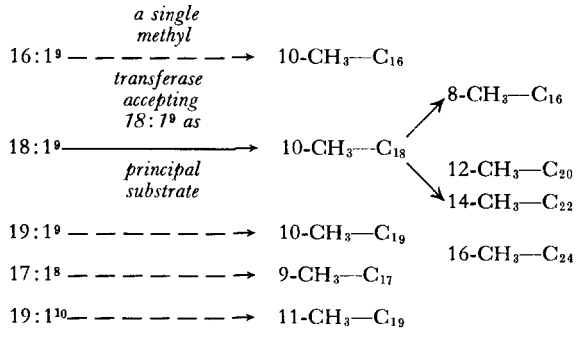
metabolites of the *Eubacteriales* (6). They have been detected in members of the family *Bacillaceae* and in several species of *Nocardia*, *Streptomyces*, and *Microbispora* (6–10). This fact, taken in conjunction with the present finding, may indicate that the ability to use leucine and isoleucine as "starters" in fatty acid biosynthesis is shared by all members of the *Actinomycetales*.

Of even greater significance is the group of seven (*n*–8)-methyl substituted fatty acids. One member of this series, 10-methyloctadecanoic acid, is already known as a cellular constituent of members of the order *Actinomycetales* (6–14) and of the yeast *Torulopsis corallina* (15). Another, 8-methylhexadecanoic acid, has been detected in *M. tuberculosis* (11) and in butter fat (16). The remaining five acids, however, have not been previously encountered in biological material.

Two other fatty acids with methyl substitution at intermediate points along the carbon chain are present in *M. phlei*, namely 10-methylhexadecanoic and 10-methylnonadecanoic. The first of these, the initial object of our quest, has been tentatively identified before in extracts of this organism (1), and is known to occur in *M. tuberculosis* (11), in two species of *Streptomyces* (8), and in butter fat (16). The second acid has not been encountered till now.

The variety of fatty acid structures shown by this study to be present in *M. phlei* raises some intriguing biological questions. Of considerable interest is the manner in which the substances are distributed between the fatty acid-containing components of the cell. Some work has already been done on the phospholipids of mycobacteria (10, 12, 13, 17–19). A portion of this, however, may need to be reexamined in the light of the present findings.

Biosynthetically, 10-methyloctadecanoic acid is formed by methylation of oleic acid (ref. 20, and references therein). A similar methylation of a monoenoic fatty acid may be responsible for the synthesis of each of the other acids. Conversely, materials such as 12-methyleicosanoic, 14-methyldocosanoic, and 16-methyltetracosanoic acids could be formed by chain elongation of 10-methyloctadecanoic; 8-methylhexadecanoic acid could be derived from the same starting material through β-oxidation. This second suggestion appears more reasonable since it could be integrated into a general scheme for the biosynthesis of all the encountered methyl-branched fatty acids. Such a scheme is shown below and assumes that the methyl transferase operates most effectively with octadec-9-enoic acid but is nevertheless able to handle in a limited fashion the closely related materials hexadec-9-enoic, nonadec-9-enoic, heptadec-8-enoic, and possibly nonadec-10-enoic. The



validity of such a scheme could easily be ascertained by tracer studies with appropriately labeled substrates. Such studies are being undertaken.

It is a pleasure to acknowledge the interest and encouragement of Dr. R. Bentley and the valuable technical assistance of Mrs. S. Dreher.

This investigation was supported by Grants AM 09311 and FR 00273 of the U.S. Public Health Service.

Manuscript received 26 March 1969; accepted 4 June 1969.

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