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THE MULTIBRANCHED HIGHER SATURATED ACIDS FROM TUBERCLE BACILLUS[1]

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Abstract—The previously reported levorotatory saturated acid isolated from tubercle bacillus, tentatively classified as a C_{31} acid, has been subjected to structure determination and characterized as 2,4,6,8-trimethyloctacosanoic acid. This is the C_{32}-mycocerosic acid of Stenhagen *et al.* A new sample of this acid, separated by gas chromatography, was admixed with a normal acid and a non-conjugated unsaturated acid. These components could be separated by use of urea adducts and of counter-current extraction of the sodium salts. An acid at the C_{29} level has been isolated and characterized as the 2,4,6-trimethylhexacosanoic acid, hence is the C_{29}-phthianoic acid. Gas chromatography is able to distinguish between a phthienoate (2,4,6-trimethyl-2-alkenoate), a phthianoate (2,4,6-trimethylalkanoate) and a mycocerosate (2,4,6,8-tetramethylalkanoate). Contrary to previous reports based on studies of mixtures, analyses of several of the high-boiling ester fractions have indicated that the C_{29}-phthianoate is the only acid of this type present in significant amounts in the unhydrogenated lipids. Very small amounts of the C_{30}-phthianoate are probably present, but lower homologs could not be detected. The dominant mycocerosate is the C_{32} homolog; however, significant amounts of the C_{31} homolog are present, together with very small amounts of the C_{33} and C_{34} homologs, probably the C_{28}homolog. There is evidence of a trace amount of the previously undetected C_{29}-phthienoate. The significant component with gas chromatography retention time equal that of the C_{29}-mycocerosate, near the C_{28}-phthianoate, proved to be an essentially homogeneous sample of the n-hexacosanoate; these two branched-chain isomers could not be detected.

Recent phases of the long-continuing investigations in these laboratories of the fatty acids from tubercle bacillus have involved the singly-branched acids with less than 20 carbon atoms[3] and the 2,4,6-trimethyl-2-alkenoic acids.[4] The latter type, known as phthienoic acids, have been demonstrated at the C_{22} to C_{28} level. In addition, there occur in this organism numerous higher molecular weight, levorotatory, multibranched saturated acids. These types are the subject of the present report.

In the pioneering investigations of Anderson on the lipids from the tubercle bacillus, a levorotatory fraction was encountered[5] among the variety of component fatty acids. In subsequent investigations,[6,7] several methods for separation of the levorotatory acids were utilized, and there was isolated an acid termed mycocerosic acid.[7] The analytical data indicated a C_{31} saturated acid; however, it proved diffi-

[1] This investigation was supported in part by a research grant (No. AI-00086-13 BIO) from the National Institutes of Health, United States Public Health Service.

[2] Recipient of a Monsanto Chemical Co. Research Fellowship, summer of 1960, and of a Woodrow Wilson Foundation Fellowship, 1961, 62.

[3] A leading reference is J. Cason and W. T. Miller, *J. Biol. Chem.* **238**, 883 (1963).

[4] A summary paper is that by J. Cason, P. Tavs and A. Weiss, *Tetrahedron* **18**, 437 (1962).

[5] R. J. Anderson, *J. Biol. Chem.* **97**, 639 (1932).

[6] C. W. Wieghard and R. J. Anderson, *J. Biol. Chem.* **126**, 515 (1938).

[7] L. G. Ginger and R. J. Anderson, *J. Biol. Chem.* **157**, 203 (1945).

cult to obtain samples whose properties were not altered by further purification procedures. Specific levorotations in the range 4·03–5·7° for the acid, 6·2–7·8° for the ester were reported.

A sample of the methyl ester of mycocerosic acid, obtained from Anderson, was subsequently examined by mass spectrometry,[8] and it was reported to be the ester of a C_{32} acid, with methyl substituents in the 2-, 4- and 6-positions. Synthesis of the methyl 2D,4D,6D-trimethylnonacosanoate was next accomplished;[9] however, the synthetic compound proved to have different properties than the sample of ester which had been isolated by Anderson and co-workers. As a result of this discrepancy, the mycocerosic ester was examined by gas chromatography[9] and found to contain two major and four minor components. The principal component (about 65% of the total) apparently was not obtained in sufficient quantity for examination of properties; however, mass spectra of it and the alcohol obtained by reduction were interpreted to arrive again at the C_{32} formula, but with substituent methyls in the 2-, 4-, 6- and 8-positions. In retention of the name first used by Anderson, this acid was called C_{32}-mycocerosic acid.[10] Gas chromatographic data were published which required that the ester of this acid have essentially the same retention time as the ester of a C_{31} acid substituted with methyl groups in the 2-, 4- and 6-positions. Other acids, detected in two strains of tubercle bacillus, were regarded as the 2,4,6-trimethyl-alkanoic (phthianoic)[10] acids at the C_{25}, C_{27}, and C_{29} levels, together with the C_{30} and C_{34} mycocerosic acids.

In independent investigations at Oxford, a fraction of levorotatory acids was isolated from the tubercle bacillus and termed mycoceranic acid.[10,11,12] It was degraded by use of the sequence: α-bromination, dehydrohalogenation and permanganate oxidation. From examination of the degradation products, this acid was described as a 2,4,6-trimethylalkanoic acid, with the total number of carbons probably 31. Subsequent to the report[9] of the Swedish and French investigators, examination of this material by gas chromatography led to the conclusion[13] that seven or eight acids were present, ranging from C_{25} to C_{32}. By degradation of the total mixture and gas chromatography of the products, it was concluded that there were present 2,4,6-trimethylalkanoic acids at C_{25}, C_{26}, C_{27} and C_{29}, together with 2,4,6,8-tetramethylalkanoic acids at C_{30}, C_{31} and C_{32}. Although the C_{29} trimethyl acid and the

[8] J. Asselineau, R. Ryhage and E. Stenhagen, *Acta Chem. Scand.* **11**, 196 (1957).

[9] C. Asselineau, J. Asselineau, R. Ryhage, S. Ställberg-Stenhagen and E. Stenhagen, *Acta Chem. Scand.* **13**, 822 (1959).

[10] The plethora of names applied to acids (or mixtures of them) from the tubercle bacillus seems unfortunate. In reports by us and by the Swedish investigators, the prefix indicating molecular weight has been used, together with a generic name for a type of acid, in order to avoid a multiplicity of names in describing the abundance of acids elaborated by the tubercle bacillus. Since phthienoic has been used to designate the 2,4,6-trimethyl-2-alkenoic acids, the 2,4,6-trimethylalkanoic acids may be appropriately termed phthianoic acids. In view of the occurrence in the tubercle bacillus of acids of the phthianoic and mycocerosic types, it would be expected, on biogenetic grounds, that 2,4-dimethyl-alkanoic acids might well occur at somewhat lower molecular weight ranges. The search for this type of acid is the subject of a subsequent communication from this laboratory, and the generic name, mycosanoic acid, is proposed. Consistent use of this type of nomenclature should make the literature much more intelligible.

[11] N. Polgar, *J. Chem. Soc.* 1011 (1954).

[12] G. S. Marks and N. Polgar, *J. Chem. Soc.* 3851 (1955).

[13] N. Polgar and W. Smith, *Chem. & Ind.* 1958 (1961); *J. Chem. Soc.* 3081 (1963).

C_{32} tetramethyl acid were synthesized,[14] it was stated[13] that "with the chromatographic apparatus employed it was not possible to differentiate between a C_n-tetramethyl-substituted and a C_{n-1}-trimethylsubstituted ester". There was no mention of the possibility of occurrence of unsaturated acids. Major components in the mixture were described as the 2,4,6-trimethylhexacosanoic acid (C_{29}) and the 2,4,6,8-tetramethyloctacosanoic acid (C_{32}). Thus, the two groups of investigators agreed on the structure and molecular weight of the C_{32} acid and described it as a major component of the mixture. One group depended on degradation of a mixture of several acids, while the other depended on the fragmentation pattern in mass spectrometry from a component separated by gas chromatography.

Investigation of the tubercle acids in our laboratories has placed basic emphasis on isolation and characterization of pure components representative of the several types of acids. Although several fractions of the levorotatory acids have been examined,[15] only one has been described as probably a homogeneous substance. This was termed C_{31}-mycosanoic acid,[16] m.p. 19–20° and 28–29·5° (polymorphic), $[\alpha]_D^{25}$ −7·8°. No structure determination was attempted, since 320 mg seemed insufficient for the methods then available to us. The molecular weight was assigned on the basis of an equivalent weight by titration.[17] A Kuhn-Roth analysis indicated three or four branching methyl groups in the acid, with the evidence somewhat in favor of four such groups.[18] It might be reasoned that we had simply isolated a different acid than that to which the other proposals of structure applied, since a pure compound had not been isolated in the other investigations; however, our "C_{31}-mycosanoic acid" was a major component, no major higher-boiling component was observable, and a C_{32} acid was described by the other investigators[9,13] as a major component of the mixtures. Thus, investigation of the structure of our isolated acid seemed in order.

C_{32} *Mycocerosic acid*

The previously isolated[15] sample of "C_{31}-mycosanoic acid" was obtained by simple crystallization of the acid obtained by saponification of the highest-boiling

[14] N. Polgar and W. Smith, *Chem. & Ind.* 1959 (1961); *J. Chem. Soc.* 3085 (1963).

[15] J. Cason and G. J. Fonken, *J. Biol. Chem.* **220**, 391 (1956).

[16] Since the Swedish investigators used a different name for this acid, and our originally reported molecular weight has proved in error, it seems best to refer henceforth to this substance as C_{32}-mycocerosic acid, and to use the term, mycosanoic, for a different type of acid (see footnotes 10 and 17).

[17] It was later noted[8] that our equivalent weight was midway between the values for C_{31} and C_{32}, actually slightly closer to the C_{32} value, hence not in conflict with the value determined by mass spectrometry on the sample of mycocerosic acid from Anderson (actually a mixture of at least 6 components, but C_{32} was the highest molecular weight present in substantial amount). Our assignment of the C_{31} formula was based on the persistent experience that complete removal of traces of neutral materials from such high molecular weight acids is so very difficult that we have rarely been able to obtain neutral equivalents less than 4–8 units too high. In addition, titration of such insoluble substances involves great experimental difficulties, which tend to leave traces of acid not neutralized; this also leads to high values for the equiv. wt.

[18] Two Kuhn-Roth oxidations yielded 3·21 and 3·24 moles of acetic acid per mole of "mycosanoic acid", about 80·5% of theory for three branches (total of four methyls) or 64% of theory for four branches. In seven analyses[15] on C_{27}-phthienoic acid, its ester and its dihydro derivative, wherein three branching methyls are well established, the lowest value for acetic aid was 67% of 4 moles, the highest 82%, and the average 75%.

ester fraction. This sample has now been subjected to structure determination, as has another sample isolated by different methods from different lots of bacteria. It may be stated at the outset that mass spectrometric determination of the molecular weight of our acid proves the C_{32} formula to be the correct one.[19]

Examination of the several high-boiling levorotatory fractions on hand from earlier investigations (see below) revealed that fractions distilling above 250°/2 mm,

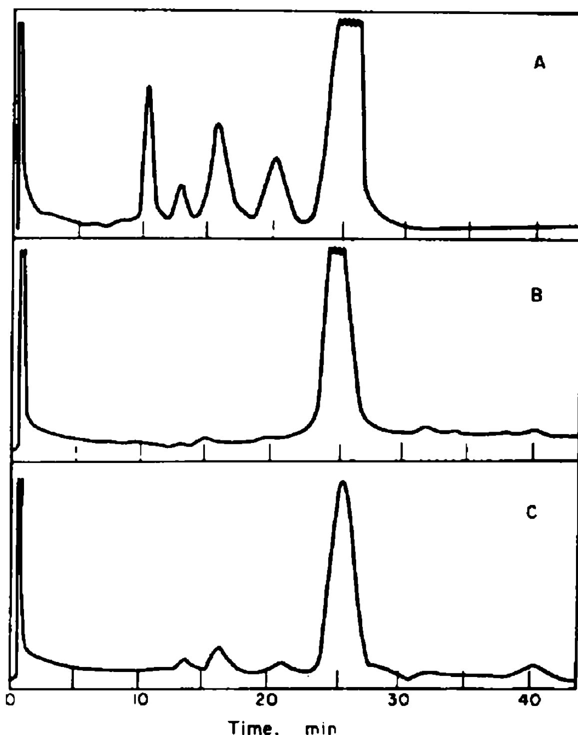


FIG. 1. Gas chromatography of high-boiling methyl ester fractions from a combined lot of lipids from tubercle bacillus. Chromatography in a ¼ in × 5-ft column, containing 10% dispersion of high vacuum silicone grease[21] on Chromosorb P; temp 276–277°; helium flow rate 60 ml/min. The initial off-scale bands are from solvent. Curve A: esters of b.p. 250–260°/2 mm (cf. next to last item, Table 3). Curve B: esters of b.p. 260–265°/2 mm. Curve C: material stripped from column after distillation up to 265°/2 mm (cf. last item, Table 3).

from a lot of esters from a systematic fractional distillation,[20] contained high percentages of a single component (cf. Fig. 1). The highest-boiling material, distilling above 260°/2 mm, was almost exclusively this ester, so far as may be judged from gas chromatography on silicone grease[21] (Fig. 1, Curves B and C). Since this ester

[19] We are indebted to Mr. Donald C. Damoth, The Bendix Corp., Cincinnati, Ohio, for this valuable item of information. The molecular weight of the methyl ester was determined as 495 ± 1, on the time-of-flight mass spectrometer; $C_{31}H_{63}CO_2CH_3$ is 494.

[20] C. F. Allen and J. Cason, *J. Biol. Chem.* **220**, 407 (1956).

[21] A low percentage (10% or less) of high vacuum silicone grease is especially well adapted to chromatography of these high molecular weight materials. Preparation of column packing utilizing this partitioning agent has been described by J. Cason and W. T. Miller, *J. Org. Chem.* **24**, 1814 (1959).

has the longest retention time of anything present in significant quantity, and was such a dominant component of the mixture, it was expected to be the same component as previously isolated[15] from the highest-boiling ester fractions by simple crystallization of the acid.

Gas chromatography of the methyl ester of the previously isolated "C_{31}-mycosanoic acid" showed only one band, and this band was of the same retention time as the major component appearing in Fig. 1. Furthermore, construction of a standard plot of log retention time *vs.* number of carbon atoms, for the bands in Fig. 1, Curves A and C, revealed that all the retention times except one fall on a single line (Fig. 2).

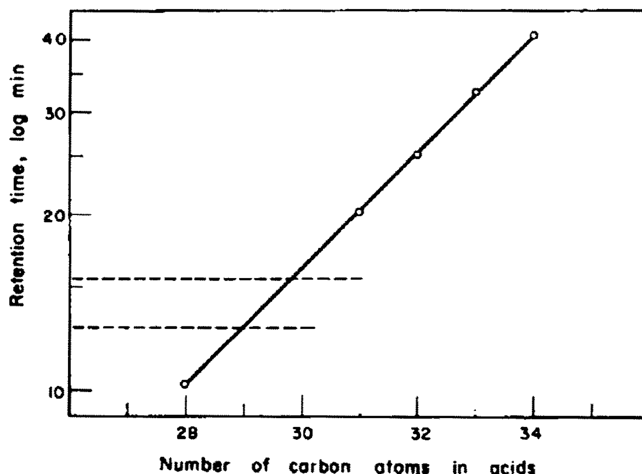


FIG. 2. Semi-log plot of retention times recorded in the gas chromatographies shown in Fig. 1, Curves A and C.

Since the major constituent separated from the band collected at 25 min was established by mass spectrometry[19] as the methyl ester of a C_{32} acid, the other points on this line were assigned accordingly, except for the point at 12·8 min. The material in the 12·8-min band, in contrast with the other fractions, crystallized readily in the receiver. Identification of this material, as well as that in the 15·5-min band which falls off the homologous series line, will be described below, after consideration of the abundant C_{32} acid. It may be noted at this point, however, that the points on the homologous series line in Fig. 2 are essentially identical with the retention times for the normal isomers with three less carbon atoms, so either normal or highly branched structures are indicated.

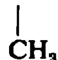
Examination of the material in the 25-min band, Fig. 1, showed the mixture of tubercle acids to be even more complex than indicated by gas chromatography, and suggests that the material previously examined by mass spectrometry[9] was a mixture of at least three components. Attempted recrystallization of the acid, obtained by saponification of the ester separated by gas chromatography, gave small amounts of a relatively high-melting (up to 81°) material. Since treatment with urea removed this material, it is judged to be a normal isomer (C_{29}). Counter-current extraction of the sodium salt of the acids, using hexane and water-ethanol in a Kies apparatus,[22] divided the material into approximately equal parts. The salt in the hexane phase

[22] M. W. Kies and P. L. Davis, *J. Biol. Chem.* **189**, 637 (1951).

yielded an acid containing about 30% (by UV absorption) of a non-conjugated unsaturated component, while the acid recovered from the aqueous phase was essentially free of the unsaturated component. This saturated acid, m.p. 31·0–32·5°, $[\alpha]_D^{22}$ −3·4°, exhibited an IR spectrum and other properties the same as those reported for "C_{31}-mycosanoic acid",[15] except for the lower rotation. The lower rotation is attributed to partial racemization at the α-carbon during one or more of the alkaline saponifications. Rather variable rotations previously observed in the levorotatory acids[7] are probably caused by the major contribution to the rotation of the α-position, which is subject to racemization by way of enolate ion formation. The previously reported sample[15] of "C_{31}-mycosanoic acid" has $[\alpha]_D^{22}$ −7·8°, in reasonable agreement with the value of −7·2° reported[9] for synthetic methyl 2D,4D,6D-trimethylnonacosanoate.[23]

The magnitude of the optical rotation of the C_{32} acid demonstrates a substituent in the 2-position, and the rate of amide hydrolysis, as previously developed,[24] may be used to show whether this substituent is methyl or a larger group. A part of each sample of the acid was converted to the amide, which was saponified under the conditions defined[24] for rate determination. The apparent second order rate constant, in each instance, proved to be in agreement with that for 2-methyloctadecanamide, nearly ten times that for a 2-ethyl amide; therefore, the 2-methyl substituent is established. Presence of a 4-methyl group is indicated by the optical rotation; however, the IR spectrum furnishes additional evidence. In his classical study of the IR spectra of branched-chain acids, Freeman[25] showed that the double band near 8 μ (usually about 7·8 and 8·1 μ) is of considerable diagnostic value. In particular, the longer-wavelength component is ordinarily the weaker of the two, frequently a shoulder, except in the case of 2-methyl acids, in which instances the intensity of the second component becomes the greater. In the case of one type of structure, the 2,4-dimethyl acids, the two bands were of nearly equal intensity, and this feature is prominent in the phthianoic acids. Since our C_{32} acid also gives the double band of about equal intensities at 7·78 and 8·10 μ, 2,4-dimethyl substitution is indicated.

A substituent at the 8-position was located by application of the previously developed oxidation with chromyl acetate.[4] In this process, the chain is cleaved at a branch (tertiary carbon atom) at a considerably higher rate than at secondary carbon atoms, except that a branch at the 4-position is attacked relatively slowly, and branches at the 2- and 3-positions are not selectively attacked. In the degradation of each sample of the C_{32} acid (or its amide), the only major band observed on gas chromatography of the neutral products (Fig. 3) exhibited the retention time of 2-docosanone. It follows[26] that the hydrocarbon end of the molecule has the structure, CH_3—$(CH_2)_{19}$—C—. Assignment of the fourth branching methyl to the 6-position,



[23] The principal contributions to this rotation are from the 2- and 4-positions.

[24] J. Cason, C. Gastaldo. D. L. Glusker, J. Allinger and L. B. Ash, *J. Org. Chem.* **18**, 1129 (1953).

[25] N. K. Freeman, *J. Amer. Chem. Soc.* **74**, 2523 (1952).

[26] It should be noted that ketones have a shorter retention time as the keto group moves away from the end of the chain. Since this degradation product had the retention time of the methyl ketone, the unlikely possibility that the branch is larger than methyl is eliminated.

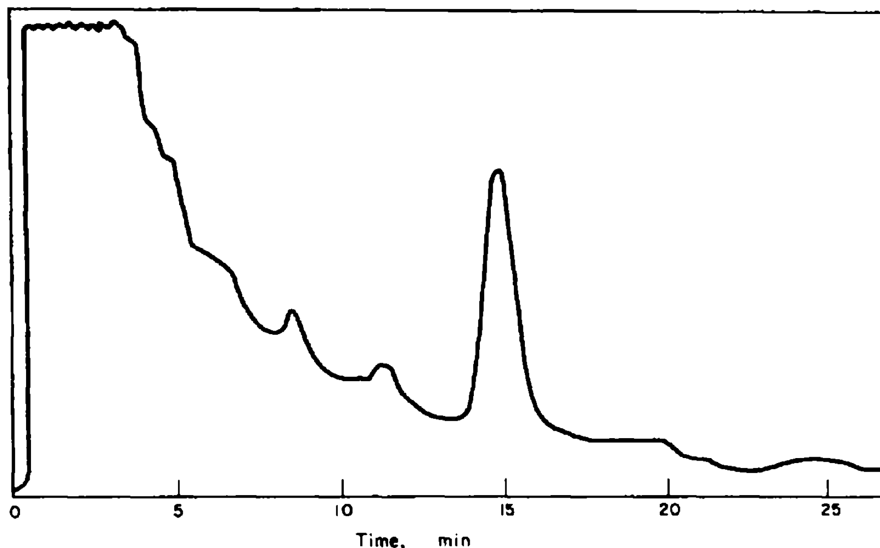


FIG. 3. Recorder tracing for gas chromatography of neutral degradation products from chromyl acetate oxidation of 10 mg of the C_{32} amide. The initial enormous band is from solvent (100–200 μl) used to gather the trace of degradation product. Chromatography column: ⅛ in × 5 ft, 10% high vacuum silicone grease on Chromosorb P. Temp, 225°. Sequential chromatography of a mixture of 2-alkanones gave a homologous series line showing values: C_{21} 11·6 min, C_{22} 14·9 min, C_{23} 19·7 min.

expected on biogenetic grounds, gives to our C_{32} acid the structure of C_{32}-mycocerosic acid.[9]

n-Hexacosanoic acid

Although the 12·8-min band, Fig. 2, falls on the homologous series line for the mycocerosic acids, within the usual precision of gas chromatography, the crystalline nature of the collected ester precludes a highly branched structure. The ester of the normal C_{26} acid is the most likely candidate, and this identity has been established. Collection of this component from gas chromatography of another fraction of distilled esters (see below) in which it was more abundant, followed by two crystallizations from acetone, yielded white crystals, m.p. 63·0–64·0°. Probably the most reliable recorded m.p. for methyl n-hexacosanoate is 63·5°.[27] A single crystallization of the acid obtained by saponification of the ester collected from chromatography gave material of m.p. 88·0–89·0° (lit.[27] 87·7°), no depression on admixture with an authentic sample of n-hexacosanoic acid.

Since the sample of hexacosanoate collected by gas chromatography was essentially homogeneous, and the crude fraction had a negligible rotation (cf. Table 1), there appear to be present in the tubercle acids *no significant quantities of the* C_{28}-*phthianoate or the* C_{29}-*mycocerosate.*

C_{29}-Phthianoic acid

Since the 15·5-min band, Fig. 2, was slightly but definitely off the homologous series line for the mycocerosates (and n-alkanoates), this fraction was isolated and

[27] F. Francis and S. H. Piper, *J. Amer. Chem. Soc.* **61**, 577 (1939).

examined. For this purpose there were selected[28] cuts of fractionally distilled esters (cf. Fig. 4 and Table 1) whose dominant component was the desired material. Fractions collected in gas chromatography are shown in Table 1, and semi-log plots of retention times are shown in Fig. 5. In order to secure the precision necessary for the distinctions appearing in Fig. 5, chromatographies were made of adroitly

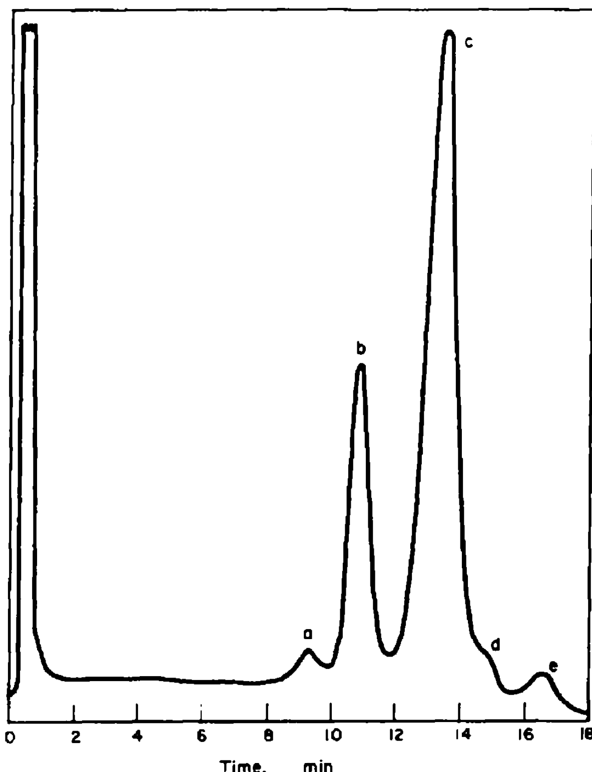


FIG. 4. Gas chromatography of methyl ester fraction, b.p. 240–247°/2 mm, from a combined lot of lipids from tubercle bacillus (fifth item, Table 3). Chromatography on column described for Fig. 1; temp 267°; helium flow rate, 70 ml/min; initial off-scale band from solvent. Material collected from the lettered bands is described in Table 1 (except for Band *e*, assigned to methyl C_{30}-phthianoate), and semi-log plots of the retention times are included in Fig. 5.

chosen mixtures. For example, the fraction shown in Fig. 4 was chromatographed immediately before a mixture of even-carbon n-isomers and odd-carbon phthianoates. This gave cleanly resolved bands for establishing homologous series lines

[28] Since a rather extensive homologous series of phthienoic acids has been reported[4] in tubercle bacillus, the present investigation of saturated multibranched acids has been directed to material that had not been hydrogenated during the isolation procedure. This factor is of significance in connection with the mass spectrometric investigation of the mycocerosate fraction from Anderson,[9] for Anderson's isolation procedure involved hydrogenation. The object of this hydrogenation was conversion of n-alkenoic acids to the saturated analogs, which give ether-insoluble lead salts. Although phthienoic acids are resistant to hydrogenation in ethanol, the solvent used, slow uptake of hydrogen does occur.

for the n-alkanoates and phthianoates under precisely the same chromatographic conditions. Furthermore, identity of the retention times for n-C_{26} in Fig. 4 and in the known mixture established the validity of comparisons with the homologous series lines. These experiments and the data in Fig. 2 make possible the presenting

TABLE 1. ESTER FRACTIONS FROM GAS CHROMATOGRAPHY REPRESENTED IN FIG. 4[a]

Chromat. band	Wt., mg	$[\alpha]_D^{26}$ ($CHCl_3$)	α,β-Unsat.[b]	Identity[c]
a	18	$+6{\cdot}3°$	40%	C_{27}-phthienoate[d]
b	120	$+0{\cdot}15°$	<5%	*n*-hexacosanoate[e]
c	150	$-3{\cdot}6°$	<5%	C_{29}-phthianoate[f]
d	6·7	$-1{\cdot}2°$	10%	[g]

[a] The material available for chromatography was about 0·6 g of the fourth item and about 0·2 g of the fifth item in Table 3. Column used for the separation was $\frac{1}{4}$ in. × 5ft. packed with 20% GE-SF-96 silicone fluid on Chromosorb P; temp. 265–270°; helium flow rate 150 ml/min; about 15 μl used per injection.

[b] None of these fractions showed significant end-absorption (rising absorption with decrease of wavelength below 220 mμ), thus no significant amounts of non-conjugated unsaturated esters are present. α,β-Unsaturation is estimated from absorption at 215 mμ, assuming ϵ_{215} of about 14,000 for a pure α-methyl-α,β-unsaturated ester.

[c] Identity is based on position in gas chromatography and the further examinations which are described. Extent of heterogeneity of each cut is estimated from the recorded physical properties and rechromatography of each band as reported in footnotes *d*–*g*.

[d] Contains several per cent of the adjacent hexacosanoate, but other components of different retention time were not noted. Both rotation and UV absorption indicate less than 50% concentration of the phthienoate.

[e] Contains a few per cent of the adjacent phthienoate (responsible for the rotation) and a trace of the C_{29}-phthianoate.

[f] Contains 7% of n-hexacosanoate and a trace of Band *d*.

[g] The majority of the cut collected at this shoulder is the C_{29}-phthianoate; no more than 20% of the collected material has retention time at position of the shoulder, Band *d*. Since the majority of the material is the levorotatory phthianoate, it is likely that the low levorotation is caused by presence of the small amount (indicated by UV) of a highly dextrorotatory α,β-unsaturated acid.

of the relative retention times shown in Table 2. It is of particular interest that an n-isomer has a significantly longer retention time than the neighboring phthianoate but is the same as the mycocerosate.

Thus, the types of acids represented in Table 2 may be detected by gas chromatography in presence of each other, except for the n-alkanoates and the mycocerosates. These components may be separated by use of urea adducts. A low percentage of mycocerosate or phthianoate in presence of a larger amount of the other probably would escape detection.

The data in Table 1 strongly suggest that Band *d*, Fig. 4, should be ascribed to presence of traces of C_{29}-phthienoic acid. In the previous report[4] on this type of acid, the series from C_{22} to C_{28} was detected, but not the C_{29} isomer.

The plot in Fig. 5 places Point *e* precisely on the homologous series line for C_{30}-phthianoate, off the line for n-alkanoates. This differs from the situation depicted in Fig. 2, wherein the closely located point lies precisely on the n-alkanoate-mycocerosate line, and the C_{29}-phthianoate lies off this line. The cited differences

in retention times are small; however, they have been noted in chromatography of several fractions (cf. Table 3) and in chromatography of the same fraction at different temperatures. There seems little doubt that small amounts of the C_{31}-mycocerosate may be detected in the higher-boiling fractions, while traces of the

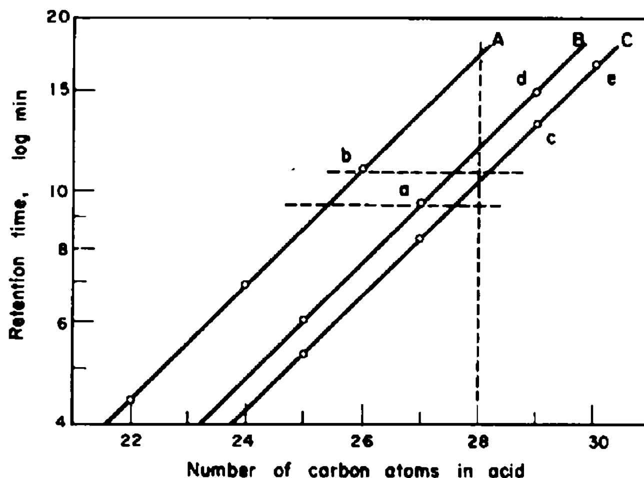


FIG. 5. Retention times recorded in precise gas chromatography of groups of methyl esters. Lettered points correspond to the bands in FIG. 4. Other points represent known compounds used to establish homologous series lines: A, n-alkanoates; B, phthienoates; C, phthianoates.

TABLE 2. RELATIVE RETENTION TIMES OF ESTERS OF FOUR TYPES OF ACIDS IN TUBERCLE BACILLUS

Type of acid	Relative number of carbons	Increment of retention time[a]
Phthianoate	C_{x+s}	−0·23
n-Alkanoate	C_x	0
Mycocerosate	C_{x+s}	0
Phthienoate	C_{x+s}	0·41
Phthianoate	C_{x-s}	0·77
n-Alkanoate	C_{x+1}	1·0

[a] An increment of unity represents the difference in retention time of the esters of adjacent homologs.

C_{30}-phthianoate become detectable in the lower-boiling fractions. Since the phthianoate in question has one less carbon than the mycocerosate, concentration of the former in the lower-boiling fractions from elaborate fractional distillation would be expected. On the other hand, occurrence of these compounds at nearly the same retention times in gas chromatography was quite unexpected by us, but is well

established by our structure proofs of the C_{32}-mycocerosate, described above, and the C_{29}-phthianoate, described below.

Although the principal component of Band *c*, Fig. 4, is rather well classified by the physical data as the C_{29}-phthianoate, it appears to be the only acid of this type present in prominent amounts in the unhydrogenated lipids; therefore, a sample of the pure acid was isolated and subjected to more rigorous structure proof. By a sequence of crystallizations and sublimations there was isolated from Band *c* (cf Fig. 4 and Table 1) 28 mg of a waxy white acid of m.p. 45·5–48·0°, $[\alpha]_D^{26}$ $-6·1 \pm 0·3°$. The UV spectrum of this acid showed neither α,β-unsaturation nor end absorption (non-conjugated unsaturation). The IR spectrum, analyzed according to Freeman[25] was quite revealing. Immediately prominent was the nearly equal intensity of absorption at 7·78 and 8·10 μ, the double carboxyl band, which indicates 2,4-dimethyl substitution. The intensity of the methyl absorption at 7·25 μ was measured quantitatively against an arbitrary background[25] and compared with other branched-chain acids. Optical densities were determined as follows: 2-methyloctadecanoic acid, 0·037; 2,4-dimethyldocosanoic acid, 0·066; 2,4,6-trimethyltetracosanoic (C_{27}-phthianoic) acid, 0·078; acid from Band *c*, 0·081. Thus, the presence of three branching methyl groups becomes rather well established. Since absorption at the methyl band is stronger for substituents more remote from carboxyl, the near-identity of the absorption of the Band *c* acid and C_{27}-phthianoate makes the 2,4,6-trimethyl structure a prime candidate. The optical rotation also suggests 2,4-dimethyl substitution, for synthetic 2D,4D,6D-trimethylnonacosanoate has been reported[9] as having a specific rotation of $-7·2°$. The NMR spectrum also showed four methyl groups (thus three branches), including a doublet displaced downfield from the normal position for methyl, as is characteristic for the 2-methyl. Presence of the 6-methyl was confirmed by chromyl acetate oxidation.[4] Gas chromatography of the neutral components from the oxidation gave a tracing similar to that shown in Fig. 3, with the major band at the retention time for 2-docosanone. Thus, all the evidence is in support of the C_{29}-phthianoate structure for the acid isolated from Band *c*, Fig. 4.

Composition of the high-boiling ester fractions

Since there has been acquired the information necessary for gas chromatographic analysis of the higher-boiling levorotatory fractions, several of these fractions, previously obtained by fractional distillation, were subjected to analysis (cf Table 3). There were observed in the several fractions only bands which may be assigned to the types of acids which have been characterized (except a single minor band in the third entry). Major constituents from the several lots of the organism are the n-hexacosanoate, the C_{29}-phthianoate and the C_{32}-mycocerosate.

Except for the probable occurrence of a sub-trace of the C_{29}-phthienoate and of a trace of the C_{28}-mycocerosate, the saturated acids with three or more branching methyls have not been detected in the molecular weight range (C_{22} to C_{28}) where the α,β-unsaturated phthienoates have been found. The two major saturated acids (C_{29}-phthianoate and C_{32}-mycocerosate) differ from each other by the propionate residue, as might be expected on biogenetic grounds; however, they do not so differ from the major unsaturated component, C_{27}-phthienoate. The latter acid would result after addition of three propionate residues to stearate (or oleate); however,

TABLE 3. GAS CHROMATOGRAPHIC ANALYSIS OF HIGH-BOILING ESTER FRACTIONS FROM THE LIPIDS OF TUBERCLE BACILLUS

Fractions Chromatographed[a]	Acids assigned to chromatography bands Observed retention times, min (% in fraction)[b]					
	C_{27}-Phthie.	n-C_{24}	C_{28}-Phthia.	C_{30}-Phthia.	C_{31}-Myco.	C_{32}-Myco.
B.P. 241–249°/2 mm	11·0 (24·7)	12·8 (45)	15·7[c] (27·3)		19·9 (1·9)	24·5 (1·1)
B.P. 249–256°/2 mm	11·0 (7·7)	12·9 (24·2)	15·7[c] (20·1)		20·1 (13·5)	25·0 (34·5)
Column stripped after b.p. 260°/3 mm[d]		12·7 (19·3)	15·5 (5·7)		20·0 (13·1)	24·6 (30·3)
B.P. 238–240°/2 mm $[\alpha]_D^{25}$ − 0·61	10·9 (5·7)	12·8 (43·6)	15·3 (50·7)	19·3 (tr.)		
B.P. 240–247°/2 mm	11·0 (tr.)	12·7 (18·6)	15·6[c] (80)	19·5 (1·2)		
Column stripped after b.p. 247°/2 mm	10·9 (4·6)	12·8 (15·2)	15·5[c] (62·3)	19·6 (8·3)		24·3 (9·6)
B.P. 250–260°/2mm	*e*	12·8 (2·3)	15·5[c] (10·4)		20·1 (9·3)	25·0 (69·4)
Column stripped after b.p. 256°/2 mm[f]		13·1 (1·5)	15·8 (4·9)		20·5 (2·1)	25·3 (85·7)

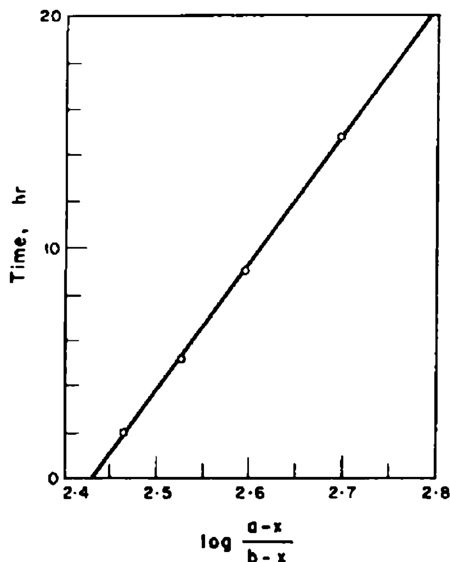


FIG. 6. Plot for determination of slope in calculation of rate constant for hydrolysis of C_{32}-mycocerosamide. For this run on 25·3 mg amide, $a = 0{\cdot}5325$, $b = 0{\cdot}00218$, slope = 56.

Footnotes to Table 3.

[a] In all instances, methyl esters were chromatographed. The first two fractions are from lipids of a human strain of tubercle bacillus grown on Long's medium; cf Fig. 1 of a previous report.[20] The third fraction (cf Table 2 in the same report)[20] is from strain H-37 grown on Long's medium. The last five fractions are from systematic redistillation[20] of esters originating in four different lots of virulent strains of the organism. The final two fractions are the highest boiling material in Fig. 2, Profile I, in ref. 20 (these fractions were not redistilled, as were the lower-boiling fractions in this Profile).

[b] The percentages, reported in parentheses, are percentages of total area under all bands, with no allowance for possible small differences in ratio of weight to area, as a function of molecular weight. In the case of areas estimated as less than 1% of the total (e.g., cf Fig. 4, Band *a*), this is reported as tr. (trace). The chromatographies were carried out on a 5-ft column, ¼ in diam., packed with 10% high vacuum silicone grease[21] dispersed on Chromosorb P; temp. 276–277°; rate of helium flow, 60 ml/min.

[c] In these fractions, there was a shoulder on the 15·5-min band at about 17·3 min, which represented a barely detectable amount of material (e.g., cf Fig. 4, Band *d*). The data (cf Table 1) indicate that this shoulder is due to C_{29}-phthienoate.

[d] In this chromatography there were three additional broad bands at longer retention times: 10% of area at 33 min (C_{32}-mycocerosate), 11·5% at 36 min, 10% at 40 min (C_{34}-mycocerosate). These three bands are almost continuous, so both retention times and areas are inaccurate.

[e] The band for C_{27}-phthienoate was absent from this tracing, and there was a band at 10·2 min (8·6% of area; see Fig. 1, Curve A). This band falls on the homologous series line for C_{28}-mycocerosate (cf Fig. 2) or n-pentacosanoate.

[f] In this chromatography there were additional bands for C_{32}-mycocerosate at 32·2 min (tr. of area) and for C_{34}-mycocerosate at 40 min (5·8%); cf Fig. 1, Curve C, and Fig. 2.

[20] J. Cason, G. Sumrell, C. F. Allen, G. A. Gillies and S. Elberg, *J. Biol. Chem.* **205**, 435 (1953).

palmitate is especially abundant in tubercle bacillus, while C_{25}-phthienoate[4] is not especially abundant in most lots of the tubercle bacillus. There is the possibility that the C_{18} and C_{20} normal acids are no more abundant in the tubercle lipids because they are depleted in formation of the abundant C_{27}, C_{29} and C_{32} branched-chain acids. Finally, it should be noted that there is no evidence yet available that the 10-methyl acids with less than 20 carbon atoms engage in the chain extension process to yield the multibranched higher molecular weight acids.

EXPERIMENTAL[30]

Isolation of C_{32}*-mycocerosic acid.* The large band shown in Fig. 1 was collected by gas chromatography on a 15 mm o.d. × 3 m spiral hard glass column; partitioning agent, 10% high vacuum silicone grease[31] on Chromosorb P; helium flow rate, 150 ml/min at pressure of 23 cm of mercury. The desired component, which had a retention time of about 64 min at a column temp of 278°, was collected in a glass tube whose outlet was lightly plugged with solvent-extracted cotton moistened with methanol. The material was injected as a 50% solution in benzene, in 100-μl quantities. From a total of 220 μl of ester in one fraction (Fig. 1, Curve B) and 200 μl in the other (Curve C), there was collected 309 mg in the major band.

The total chromatographed ester was saponified by heating under reflux for 5 hr in 20 ml ethanol containing 10% by weight KOH. The cooled and acidified reaction mixture was extracted with three 100-ml portions hexane, then the extracts were washed with water and passed through a 4-stage Kies extraction.[22] The four extraction tubes were charged respectively: (1) 100 ml 33% aqueous ethanol (2:1 water–ethanol by volume) containing 321 mg NaOH, (2) 100 ml 33% aqueous ethanol containing 200 mg NaOH, (3) 100 ml 33% aqueous ethanol, (4) 100 ml water. After all the hexane extract had been passed through the extraction train, it was followed by 400 ml hexane. Migration of insoluble sodium salt from the first tube into subsequent tubes was no disadvantage so long as it did not pass the last tube. Any insoluble salt was worked up with the aqueous layers. Acidification and work-up of the aqueous alkaline phases yielded 121 mg yellow-white semi-solid product, while the hexane effluent yielded 133 mg of material of similar appearance. The latter 133 mg material was dissolved in 100 ml hexane and subjected to a second, similar Kies extraction. This yielded 105 mg of material from the hexane and 28 mg from the aqueous phases. The different distribution of material in the second extraction demonstrates presence of at least two components.

The acid recovered from the aqueous phases of the first and second Kies extractions proved to be almost completely saturated (ϵ_{206} 320); however, crystallization from about 1 ml acetone yielded a first crop of 3 mg, m.p. 80–81°; subsequent crops melted near 30°. For separation of straight-chain material as the urea clathrate, by use of urea in columns as described previously,[29,31] a 31·6-mg sample of the saturated acids, dissolved in 5 ml isooctane containing 1% methanol, was applied to 1·04 g of dried 200-mesh urea in a 6-mm i.d. tube. An additional 25 ml isooctane-methanol solvent was applied for elution. The urea column was dissolved in water, acids were recovered by extraction with $CHCl_3$, and the recovered acids were applied to a second column of urea in the manner described. The combined eluants from the two urea columns were filtered to remove a small precipitate (urea ?); solvent was distilled through a column; and the residue was crystallized from acetone at 0° to yield white, waxy C_{32}-mycocerosic acid, m.p. 31–32·5°, $[\alpha]^{25}_{D}$ −3·3° ($CHCl_3$). From a total of 140 mg of the crude saturated acid that was processed, 79·6 mg of pure C_{32}-mycocerosic acid was obtained.

The IR spectrum of this sample was identical to that of the previously isolated sample;[18] a prominent feature of the spectrum is the double band of about equal intensity at 7·78 and 8·10 μ.

The unsaturated fraction. The material in the hexane phase from the Kies extraction consisted entirely of a salt (nothing through the column in gas chromatography); so it was shaken out with hexane and 1 N H_2SO_4, and the acid (95 mg) was recovered from the washed hexane solution.

[30] IR spectra were recorded on a Perkin–Elmer Infracord, using matched 0·1-mm NaCl cells. M.p.'s were determined in capillary tubes, with use of a standardized thermometer. Acids of known structures used for comparison were synthesized in previous investigations in these laboratories.

[31] During the course of the present investigation, it was dsicovered that the *esters* of normal acids of C_{18} or higher do not form clathrates in passage through a urea column in the manner described, whereas the *acids* are retained essentially quantitatively.

Crystallization of this product from 2 ml acetone yielded successive fractions: (1) 1·2 mg, m.p. 67–68°; (2) 30·9 mg, m.p. 29–30°; (3) 22 mg, m.p. 29–30°. The UV spectrum of the last fraction, in heptane, showed steeply rising end-absorption: ϵ_{210} 1250, ϵ_{200} 1690, ϵ_{190} 4250, no indication of an imminent maximum at 290 mμ. Since a sample of methyl oleate showed ϵ_{200} 5510, there is indicated about 31% non-conjugated unsaturated acid with no substituent on the double bond. For the same fraction: $[\alpha]_D^{22}$ −3·8° ($CHCl_3$); IR spectrum identical with that of the pure C_{32}-mycocerosic acid (cf above). This material has not been investigated further at this time.

C_{32}-*Mycocerosamide.* A solution of 77 mg pure C_{32}-mycocerosic acid and 1 ml purified thionyl chloride in 4 ml dry benzene was allowed to stand overnight at room temperature. Solvent and excess thionyl chloride were removed at red. press., the residue was dissolved in 4 ml dry purified dioxane, and the solution was added dropwise with swirling to 20 ml ice-cold ammonium hydroxide. After the mixture had stood for 5 min at 0°, the white precipitate was collected and crystallized from 4 ml acetone to yield 67·6 mg (87·8%) white amide, m.p. 54–57°.

Rate of hydrolysis of C_{32}-*mycocerosamide.* The method used for hydrolysis with KOH in boiling n-propanol has been described in detail.[32] In preliminary runs on 2-methyloctadecanamide, 2-methyleicosanamide and 2-ethyloctadecanamide, it was found that a precision of 20–30% could be realized on samples of 15–20 mg, although the apparent second-order rate constant decreases slowly with molarity as previously noted.[32] In runs on the 2-methyl amides, rate constants × 10^3 (molarity × 10^2 in parentheses) were: 59 (6·70), 58·3 (4·43), 62·0 (1·02), 61·2 (0·69), 62·5 (0·41), 70·7 (0·21). Constants for the 2-ethyl amide differ by almost a power of ten: 7·85 (1·44), 10·5 (0·50). Thus precision on the small samples is more than adequate.

The data were handled as previously described,[32] with the equation

$$t = \frac{2 \cdot 303}{k(a-b)} \left(\log \frac{b}{a} + \log \frac{a-x}{b-x} \right)$$

where a is the initial molarity of alkali, b is the initial molarity of amide, and x is the molar concentration of amide which has reacted in time t. By plotting $\log (a - x)/(b - x)$ vs. t in hr the equation is reduced to the form

$$k = \frac{2 \cdot 303}{a-b} \times \frac{1}{\text{slope}}$$

In Fig. 4 is shown the plot for determination of slope[33] in hydrolysis of a 25·3-mg sample of C_{32}-mycocerosamide isolated in the present work. The apparent second-order rate constant, k_2, is calculated as 77·0 × 10^{-3} liters $moles^{-1}$ hr^{-1}. The hydrolysis was about 65% complete after 25 hr, and evolution of ammonia had nearly ceased after 3·6 days, at which point about 96% of the theoretical amount of ammonia had been titrated.

In a similar run on 34·6 mg of amide, m.p. 50–52°, prepared from the previously reported[15] sample of "C_{31}-mycosanoic acid", k was determined as 50·1 × 10^{-3}. A parallel run, with the same reagents and at similar concentration, on 2-methyleicosanamide, gave k of 58·3 × 10^{-3}.

Chromyl acetate oxidation of C_{32}-*mycocerosic acid.* The previously described method[4] was used for oxidation of two samples[34] of the C_{32}-mycocerosamide described above and two samples of the C_{32}-mycocerosic acid previously described.[15] Results of the four oxidations were similar, and a characteristic tracing of gas chromatography of the neutral products is shown in Fig. 3. The only major band corresponds precisely with the retention time for 2-docosanone.

C_{29}-*Phthianoic acid.* A 141-mg sample from the third entry in Table 1 was saponified as described for the C_{32}-mycocerosate, but the ether extract of the acids was simply washed with water, dried and evaporated to leave 125 mg of acids. Crystallization of this product from acetone at 0° gave a solid which was then crystallized from acetone at room temperature. Recrystallization of this second crystallizate from acetone yielded 5 mg, m.p. 87·0–88·0°, of n-hexacosanoic acid (shown by gas chromatography to be present in this fraction, cf Table 1). The mother liquor from the first crystallization at room temperature was cooled to 0° to yield 47 mg of acid which was sublimed at

[32] J. Cason and H. J. Wolfhagen, *J. Org. Chem.* **14**, 155 (1949).

[33] Detailed calculations for these hydrolyses may be found in the Ph.D. dissertation of W. T. Miller, University of California, 1961.

[34] Several trial oxidations of 6-ethyloctadecanamide showed that results are the same as obtained by oxidation of the acid.

200°/2mm to give 42 mg of waxy acid m.p. 44–48°. A subsequent crystallization from acetone, followed by sublimation at 180°/2 mm, yielded 28 mg of C_{29}-phthianoic acid, m.p. 45·5–48·0°, $[\alpha]_D^{26}$ −6·1 ± 0·3° (22 mg/ml $CHCl_3$).

The IR spectrum was recorded on a solution of 4·4 mg/0·1 ml CCl_4, in 0·1-mm cells; spectra of compounds of known structure were determined for quantitative comparison at 7·25 μ in the same cells at the same molar concentration.

The methyl ester of the acid, prepared with methanol and sulfuric acid, was used for chromyl acetate oxidation by the previously described method.[4] The ester was also used for determination of the NMR spectrum, so that the sharp, isolated band for methyl in methoxyl might be used for reference as the area resulting from three hydrogen atoms. On account of overlap of the methylene and methyl bands, especially the 2-methyl band, estimation of the area under the methyl band is subject to substantial error, depending on the system used for allocation of area between the overlapping bands; however, comparison with the known C_{25}-phthianoate,[4] using the same system of extrapolation, gave essentially identical results. In two determinations on the C_{29}-phthianoate, number of methyls was estimated as 5·12, 4·86; for C_{25}-phthianoate, 4·80.