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The Structure of an Ornithine-containing Lipid from *Thiobacillus thiooxidans**

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SUMMARY

The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans* has been elucidated. Methanolysis of the lipid released methyl *cis*-11,12-methylene-2-hydroxyoctadecanoate. Acid hydrolysis of the residue yielded ornithine and a mixture of fatty acids, the major components of which were 3-hydroxyhexadecanoic and 2-hexadecenoic acids. Identification of the 3-hydroxy fatty acid was based on the thin layer chromatographic mobilities of the acid, its methyl ester, the methyl ether, and acetate derivatives of its methyl ester, on the equivalent chain lengths of the derivatives of the acid and the acid obtained by oxidation of the natural acid with permanganate, and on mass spectral studies. Similar techniques were used for the identification of 2-hexadecenoic acid. The minor fatty acids and the 2-hexadecenoic acid were found to be degradation products of the 3-hydroxy acid.

A 1:1:1 molar ratio was determined for the three lipid components, ornithine, *cis*-11,12-methylene-2-hydroxyoctadecanoic acid, and 3-hydroxyhexadecanoic acid. Evidence for an ester linkage between the carboxyl group of the 2-hydroxy fatty acid and the hydroxyl group of the 3-hydroxy acid was obtained by infrared spectra, degradation of ^{14}C-labeled lipid, and reactions of the lipid with $NaOCH_3$ and NH_2OH. The 3-hydroxyhexadecanoic acid was determined to be attached to the α-amino group of ornithine through an amide bond. This conclusion was supported by the conditions necessary to achieve hydrolysis, infrared spectra, end group analysis with dansyl chloride, and nonaqueous titrations. Data obtained in this investigation indicated that the δ-amino group and the carboxyl group of ornithine were free and that the extracted lipid existed as a zwitterion. Elemental analysis and unit weight determinations were consistent with the proposed structure.

The isolation of an ornithine-containing lipid from *Thiobacillus thiooxidans* and the identification of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid as a component of the lipid have been reported (1, 2). The purpose of this paper is to report the identification of 3-hydroxyhexadecanoic as a third component of the lipid and to present evidence that the structure of the lipid is that shown in Fig. 1.

EXPERIMENTAL PROCEDURE

Methanolysis, acetylation, and oxidation of hydroxy fatty acids with $KMnO_4$ have been described (2). Methyl esters were prepared with diazomethane (3). Both 5- and 12-foot columns packed with diethylene glycol succinate on Anakrom AB were utilized for gas-liquid chromatography. These procedures and the determination of equivalent chain lengths were reported earlier (2). Silica Gel G was used as the adsorbent for thin layer chromatography. The preparation of plates (4) and recovery of samples from the adsorbent (4) have been described. Unless otherwise specified, the development of plates was performed with a solvent of heptane-diethyl ether-glacial acetic acid (50:50:1, v/v/v).

Acid hydrolyses were performed by suspending the sample in 0.3 to 1.0 ml of 6.7 N HCl. After evacuation the tubes were

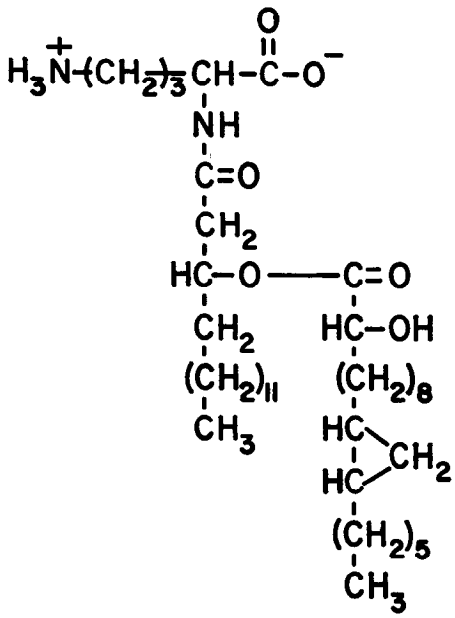

FIG. 1. The proposed structure of an ornithine-containing lipid from *T. thiooxidans*.

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sealed and heated at 100° overnight. Methyl ether derivatives were prepared with CH_3I and Ag_2O (5). Quantitative determination of esterified fatty acids was according to Hill's method which utilizes the colored ferric hydroxamate complex (6). Methyl 2-hydroxyhexadecanoate was used to prepare the standard curves for the colorimetric assay, although there was little difference between the curves prepared with the 2-hydroxy ester and those prepared with methyl hexadecanoate except that the 2-hydroxy ester curves deviated from linearity at higher concentrations. With the following modifications, the method of Wilcox (7) was used for quantitative assays of methyl ester groups. The saponification reaction was performed with 1.0 ml of 1 N NaOH which contained 15 mg of sodium dodecyl sulfate, and the mixture was gently heated and stirred for 40 min. With methyl stearate as a standard the modified method yielded results that were 90 to 95% of the expected values.

Dansyl[1] chloride was utilized for end group analysis (8). The method was modified to accommodate a lipid sample. To 0.1 ml of a $CHCl_3$ solution of the lipid was added 0.1 ml of 0.5 M $NaHCO_3$ and 0.2 ml of 2.5% (w/v) solution of dansyl chloride in acetone. The tube was sealed and shaken for 24 hours at room temperature and then heated for 2 hours at 50°. After evaporating the solvent under a stream of nitrogen, the sample was hydrolyzed with 6.7 N HCl at 100° overnight. The HCl was removed in a vacuum over NaOH and the residue was dissolved in acetone-water (1:1, v/v) then applied to high voltage electrophoresis paper. The preparation of derivatives of ornithine was performed as described by Gray (8) except that the ratio of reactants were varied to achieve the desired degree of derivatization. For high voltage electrophoresis, a pH 1.9 buffer consisting of 10 ml of 88% formic acid and 40 ml of glacial acetic acid diluted to 1 liter with water was used in place of the pH 1.9 buffer described by Gray (8). A Savant, 8-Kv power supply was used.

Nonaqueous titrations were performed in a solvent of $CHCl_3$-CH_3OH (2:1, v/v) with 0.036 N HCl in the same solvent and 0.103 N $NaOCH_3$ in CH_3OH as titrants. A microburet and a Radiometer pH meter 26 equipped with a GK2321C combination electrode (Radiometer, Copenhagen) were utilized. Addition of small quantities of water (1%) did not appear to alter the titration characteristics of acetic acid or *N*,*N*-dimethylethanolamine.

For the quantitative analysis of ornithine in acid hydrolyzates a Hitachi Perkin-Elmer KLA-3B ligand exchange amino acid analyzer was utilized. A Perkin-Elmer IR model 621 was used to obtain spectra of samples, which were incorporated into KBr discs. Mass spectra were obtained with a Hitachi model RMU-6D double focusing instrument with an ionizing potential of 80 e.v. and a maximum temperature of 150° for the inlet oven. Radioactive counting was accomplished with a Packard Tri-Carb model 314 EX-2, with conditions reported previously (4). Elemental analyses and attempted molecular weight determinations were performed by the Clark Microanalytical Laboratory, Urbana, Ill.

The culture of the organism and isolation of the lipid have been reported (1). In these studies, larger quantities of the lipid were obtained and therefore as an additional step in the purification, the lipid was precipitated twice from a diethyl ether-acetone solution at −15°. Samples were stored under vacuum at −20°.

Lipid, labeled with ^{14}C, was isolated from cultures grown under $^{14}CO_2$ (9). The ^{14}C-lipid was assumed to be uniformly labeled and was isolated as described for the nonlabeled lipid except the precipitation steps were omitted.

Methanolysis of the lipid followed by the addition of a drop of glacial acetic acid, evaporation of methanol, and partition of the residue between diethyl ether and water yielded an ether-soluble compound identified as methyl *cis*-11,12-methylene-2-hydroxyoctadecanoate (2). The remainder of the lipid was not appreciably soluble in water or diethyl ether and it remained at the solvent interface. This material was called lyso-lipid and was purified by thin layer chromatography with a $CHCl_3$-CH_3OH-H_2O (66:30:4, v/v/v) solvent. The lyso-lipid was eluted from the adsorbent with the same solvent and then precipitated from a warm $CHCl_3$-CH_3OH solution by the addition of diethyl ether and cooling.

In experiments involving U-^{14}C-lyso-lipid, the material was isolated from the methanolysis reaction mixture as described above but was not further purified by thin layer chromatography or precipitation.

Sources of the fatty acids, esters, and other reagents were the same as reported previously (1, 2).

RESULTS AND DISCUSSION

The lipid, isolated by precipitation from a diethyl ether-acetone mixture, appeared to be hygroscopic. After being stored under vacuum with P_2O_5 for several days then being exposed to the atmosphere for 2 days, the lipid was dried by high vacuum at 50°. The drying caused an approximate weight loss of 4%. With softening at 98°, the lipid melted between 156–159°, and a definite transition was observed near 157°. Elemental analysis of the lipid indicated a composition of C, 69.83; H, 11.09; N, 4.00 and O, 15.30%. The melting point and elemental composition of the lyso-lipid were not determined. Removal of the 2-hydroxy fatty acid appeared to make the lipid more hygroscopic.

Components of Lipid—The identification of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid as a component of the lipid has been reported (2).

Previously, we reported that acid hydrolysis of the lipid yielded ornithine (1). The presence of other water-soluble or volatile components has now been rigorously excluded. Samples of U-^{14}C-lipid were subjected to acid hydrolysis. After extraction with ether, the aqueous phase was vacuum distilled and the distillate was found to be devoid of appreciable radioactivity. The residue from the distillation was dissolved in water and small quantities of ornithine, glycerol, ethylene glycol, and 1,2-propanediol were added. When aliquots of this solution were subjected to paper chromatography (1) followed by autoradiography, essentially all of the radioactivity was associated with the ornithine, which was detected on paper with ninhydrin. No radioactivity was observed in the areas of the chromatograms corresponding to the glycols, which were detected with $KMnO_4$ and HIO_4. Aliquots of the aqueous solution were assayed for radioactivity and others were applied to the amino acid analyzer. The effluent corresponding to the ornithine peak was collected from the analyzer. When ninhydrin was allowed to react with the column effluent, approximately 80% of the radioactivity applied to the column was

[1] The abbreviation used is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

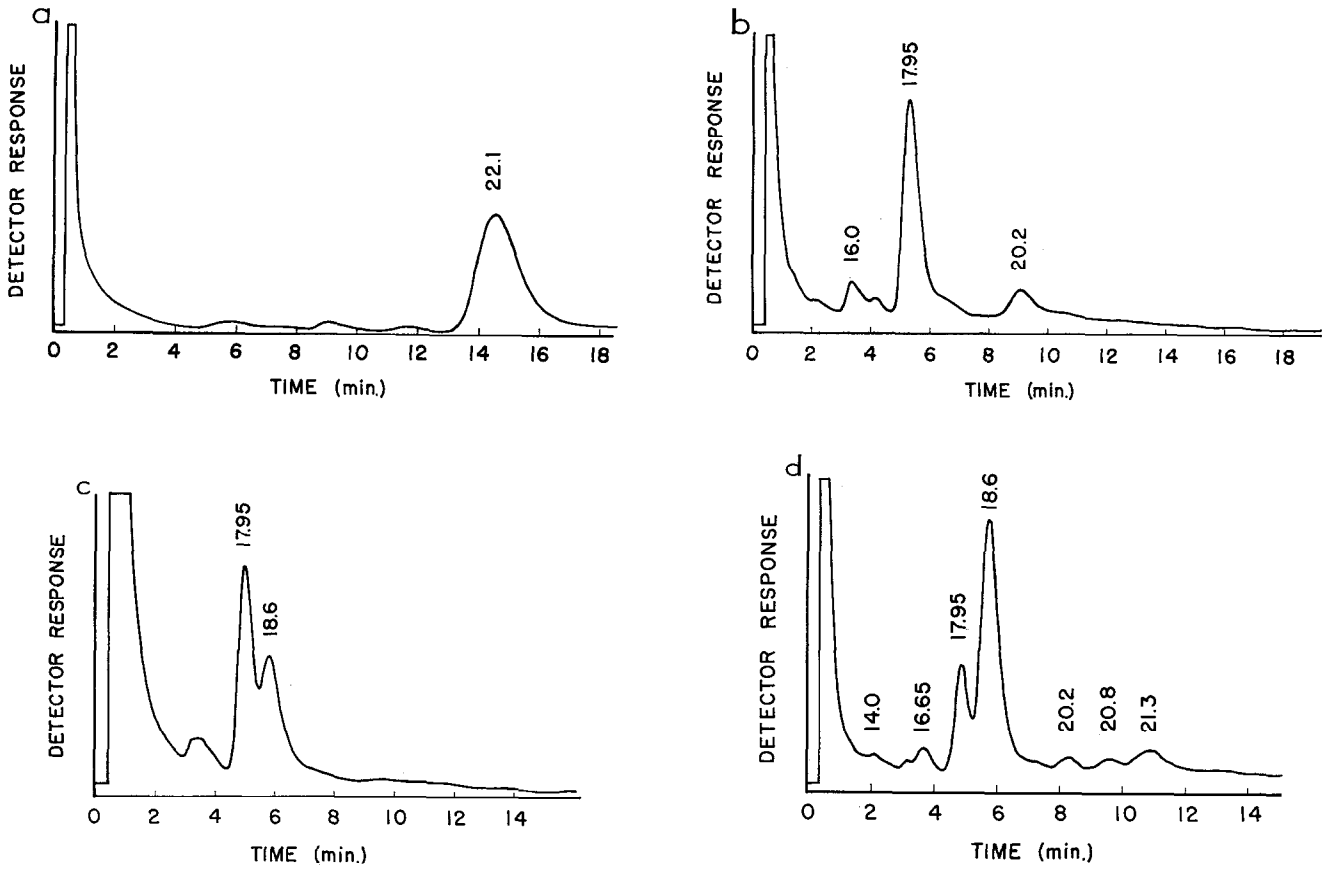


FIG. 2. Gas-liquid chromatography tracings of fatty acid derivatives obtained from the lyso-lipid which was prepared by methanolysis of the intact ornithine-containing lipid to remove the *cis*-11,12-methylene-2-hydroxyoctadecanoic moiety. A 5-foot column, with diethylene glycol succinate as the liquid phase, was used. Other chromatography details are indicated under "Experimental Procedure." Equivalent chain lengths are indicated by the *numbers* above the peaks. *a*, the acetylated methyl ester of the hydroxy fatty acid (3-hydroxyhexadecanoic acid) released by HCl hydrolysis of the lyso-lipid. The acid was separated from the nonpolar fatty acids, which were also released by HCl hydrolysis, prior to gas-liquid chromatography. *b*, the methyl esters of the nonpolar fatty acids released by HCl hydrolysis of the lyso-lipid. Thin layer chromatography was used to separate the nonpolar fatty acids from the hydroxy fatty acids prior to gas-liquid chromatography. Methyl 2-hexadecenoate was identified as the major component (equivalent chain length 17.95). *c*, the methyl esters and methyl ether derivatives of the mixture of fatty acids released by HCl hydrolysis of the lyso-lipid. CH_3I and Ag_2O were used for derivatization. Major components were identified as methyl 2-hexadecenoate (17.95) and methyl 2-methoxyhexadecanoate (18.6). *d*, the methyl esters of the acids released by HCl hydrolysis of the lyso-lipid which previously had been treated with CH_3I and Ag_2O. The major components were identified as in Fig. 2*c*.

present in the colored effluent. Knowing the retention time of ornithine, the same operation could be performed with the ninhydrin pump off, thus preventing the decarboxylation of ornithine in the effluent. In such experiments 96 to 98% of the radioactivity applied to the column could be recovered in that portion of the effluent that corresponded to ornithine. Therefore, it was concluded that ornithine was the only water-soluble product released by acid hydrolysis of the ornithine-containing lipid.

A sample of U-^{14}C lyso-lipid was subjected to HCl hydrolysis. The diethyl ether extract of the hydrolyzate was treated with diazomethane and subjected to thin layer chromatography. Autoradiography showed several radioactive bands, and one of the major bands exhibited the same R_F as methyl esters of nonpolar fatty acids, such as methyl stearate. The other major band had the same R_F as methyl esters of monohydroxy fatty acids. The minor bands were faint by comparison to the major bands and were not as distinct. These components exhibited mobilities which were between the two major components.

Identification of 3-Hydroxyhexadecanoic Acid—Using nonlabeled lyso-lipid, the fatty acids released by acid hydrolysis were treated with diazomethane and subjected to thin layer chromatography. Extraction of the adsorbent corresponding to the monohydroxy fatty ester band yielded an ester which was then acetylated, rechromatographed, extracted from the adsorbent, saponified, and finally chromatographed again with a diethyl ether-heptane-glacial acetic acid (70:30:1, v/v/v) solvent. Thin layer chromatographic mobilities of the polar ester, its acetate derivative, and the resulting acid were identical with analogous forms of 3-hydroxyoctadecanoic acid. In the chromatographic system employed for the free acids, all monohydroxy fatty acids migrate at the same rate except those with the hydroxyl in position 2 (2). Therefore the possibility that this component was a 2-hydroxy fatty acid was eliminated.

Another sample of the lyso-lipid was hydrolyzed and the methyl ester of the polar monohydroxy acid isolated. A portion of the ester was acetylated and another portion treated with CH_3I and Ag_2O. Both derivatives were isolated by thin layer

chromatography and analyzed by gas-liquid chromatography. Fig. 2*a* is a recorder tracing obtained by the chromatography of the acetate derivative. The methyl ether and acetate derivatives of this ester exhibited equivalent chain lengths of 18.6 and 22.1 units, respectively. Equivalent chain lengths for the methyl ether and acetate derivatives of methyl 3-hydroxyoctadecanoate were determined to be 20.6 and 24.1, respectively. Since the contribution of a particular functional group to the equivalent chain length of an ester depends upon the position of the group in the ester (particularly if it is near one of the ends of the chain) (10) these results indicated that the polar acid was 3-hydroxyhexadecanoic acid. A portion of the acetate derivative of the polar ester was saponified and oxidized with $KMnO_4$. After treating the resulting acids with diazomethane they were analyzed by gas-liquid chromatography. The principal component (about 75% of total esters) exhibited an equivalent chain length of 14.0 while the minor components appeared to be shorter homologues of the primary product, methyl tetradecanoate. These results would be expected if the acid oxidized was 3-hydroxyhexadecanoic acid. A mass spectrum of the acetate derivative of the hydroxy ester was obtained and is shown in Fig. 3*A*. Although a molecular ion peak (328 *m/e*) was not observed, a peak at 269 *m/e* (M-59) which represents the loss of an acetate group or the ester functions was readily apparent. Because of the positions of the functional groups, extensive fragmentation of the molecule would be expected. Other features of the spectrum appear to be consistent with a structure of methyl 3-acetoxyhexadecanoate. Except for differences, which could be attributed to the larger homologous carbon chain, a similar spectrum was obtained for methyl 3-acetoxyoctadecanoate. Thus, the polar acid released by acid hydrolysis of the lyso-lipid was identified as 3-hydroxyhexadecanoic acid.

Identification of 2-Hexadecenoic Acid—Because of the instability of 3-hydroxy fatty acids it was believed that the other acids obtained by acid hydrolysis of the lyso-lipid were degradation products of 3-hydroxyhexadecanoic acid. Therefore, 2-hexadecenoic acid was suspected as being the primary nonpolar fatty acid. Gas-liquid chromatography of the nonpolar fatty acids, as their methyl esters, yielded the recorder tracing shown in Fig. 2*b*. The major component, with an equivalent chain length of 17.95, appeared to be an unsaturated fatty acid when analyzed by thin layer chromatography with $AgNO_3$-impregnated Silica Gel G as the adsorbent and a solvent of diethyl ether-heptane (70:30, v/v). A sample of the ester was isolated by trapping from the gas-liquid chromatograph (2, 4) and analyzed by mass spectrometry. The spectrum (Fig. 3*B*) revealed a molecular ion (268 *m/e*) and a base peak of 87 *m/e*. Other features of the spectrum are consistent with the structure that was proposed to be methyl *trans*-2-hexadecenoate (10a).

Evidence for Degradation of 3-Hydroxyhexadecanoic Acid—When the fatty acids released by acid hydrolysis of the lyso-lipid were treated with CH_3I and Ag_2O and then analyzed by gas-liquid chromatography, the recorder tracing (Fig. 2*c*) showed that methyl 2-hexadecenoate (equivalent chain length, 17.95) was the predominate ester. Acid hydrolysis of methyl 3-hydroxyoctadecanoate and analysis of the resulting products by the same procedures yielded a recorder tracing that was quite similar to Fig. 2*c* except that the equivalent length of the components was 2 units greater. The primary components had equivalent chain lengths of 19.95 and 20.6. When the products of the hydrolysis were first separated by thin layer chromatography according to the procedures described for the acids obtained from the lyso-lipid, the major nonpolar ester was found to have had an equivalent chain length of 19.95, and the acetate derivative of the polar ester (24.1) was identical with the acetate derivative of the starting material, methyl 3-hydroxyoctadecanoate. In an attempt to avoid degradation during hydrolysis, the lyso-lipid was allowed to react with CH_3I and Ag_2O. The product was hydrolyzed with HCl, and the released acids were treated with diazomethane then analyzed by gas-liquid chromatography. Fig. 2*d* is a recorder tracing from the chromatography of the products. While methyl 3-methoxyhexadecanoate (equivalent chain length 18.6) was the primary product, a considerable amount of methyl 2-hexadecenoate was also present. Incomplete methylation of the hydroxyl groups in the deacylated lipid could explain these results, although some degradation of the methyl ethers may have occurred also. However, it appears that the primary fatty acid linked to ornithine is 3-hydroxyhexadecanoic acid and that the other components obtained by acid hydrolysis of the lyso-lipid arise by degradation of this acid. This conclusion is furthered by evidence to be presented later in sections concerning the molar ratio of components and their linkages.

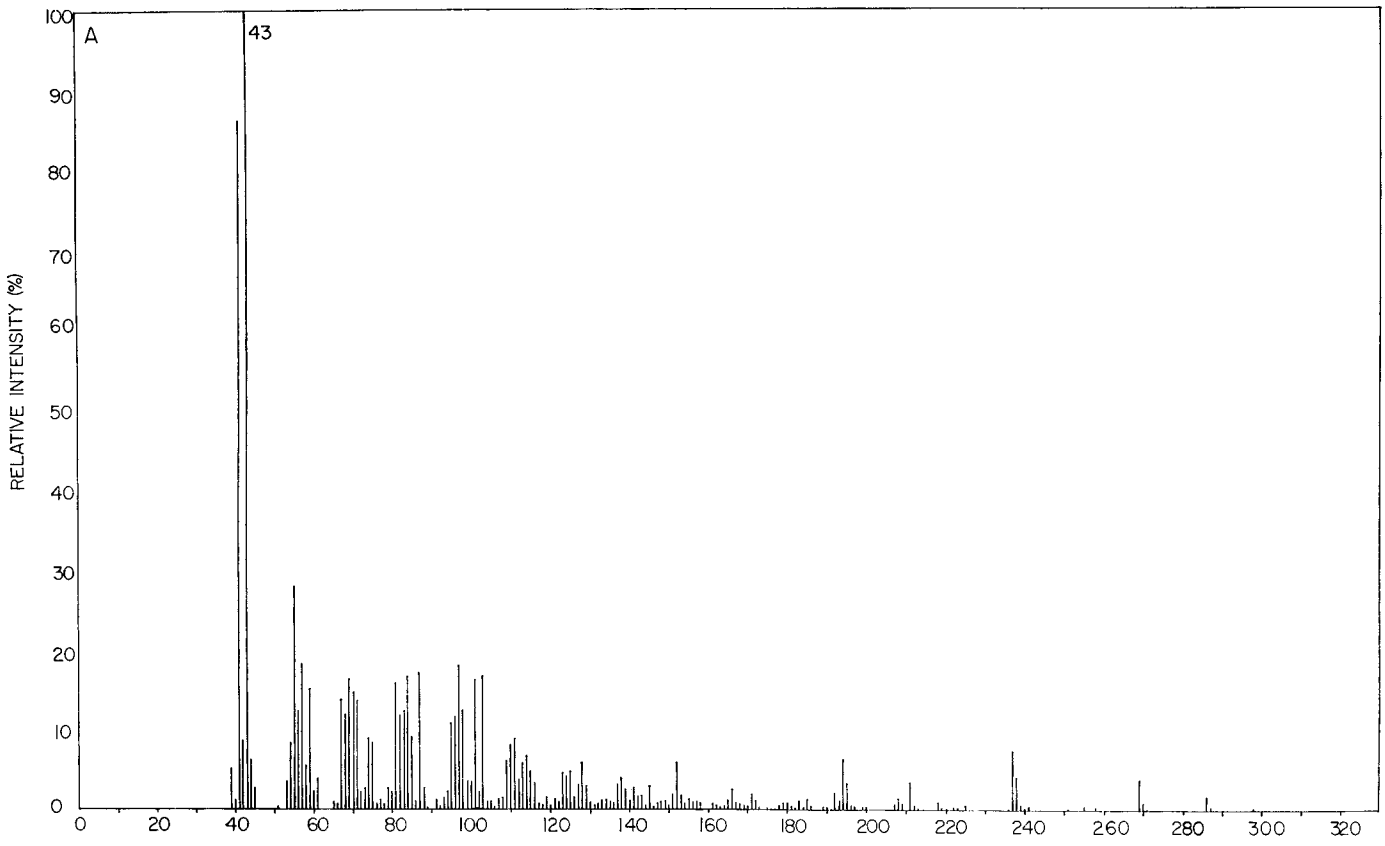
Molar Ratio of Components—According to the formula, $C_{40}H_{76}N_2O_6 \cdot 0.5H_2O$, the calculated elemental composition (C, 69.62; H, 11.25; N, 4.06; O, 15.07) agrees well with the composition found. Evidence for the actual degree of hydration is tenuous. However, when considering the 4% weight loss by drying (performed before elemental analysis) and the probable presence of 0.5 mole of water associated with the lipid at the time of analysis, it may be calculated that the lipid, when exposed to air, probably existed as a dihydrate. Since the total number of carbon atoms in the lipid components is 40, it seemed likely that the three components were present with a molar ratio of 1:1:1.

When U-^{14}C lipid was subjected to methanolysis and the released methyl 11,12-methylene-2-hydroxyoctadecanoate was extracted, the percentage of radioactivity released was determined to be 45.8 and 43.4% in two trials. These values compare favorably to the value expected (47.5%) if a molar ratio of 1:1:1 is assumed.

Molecular Weight—Unit weight determinations were performed by hydrolyzing small quantities (5 to 10 mg) of the lipid and quantitatively determining ornithine in the hydrolyzate. In three successive experiments the unit weight was determined to be 691, 679, and 689. The unit weight of the proposed formula is about 690.

Attempts to determine the molecular weight by boiling point elevation methods indicated that the molecular weight was quite large for no elevation in boiling point was observed when either benzene or isopropyl alcohol were used as solvents. Some superheating was observed, but the temperature was not elevated once the liquid actively boiled. Tough films formed at gas-liquid interfaces when solutions of the lipid were evaporated under a stream of N_2. Also the rate of dissolution in $CHCl_3$-CH_3OH or diethyl ether increased sharply as the lipid dissolved. Therefore the possibility that the lipid was highly associated in solutions was considered to be a plausible explanation for the results. Although the results suggested a polymeric structure, the molecular weight was shown to be the same as the unit weight by the experiments described below.

Linkages of Components—The fact that methyl *cis*-11,12-methylene-2-hydroxyoctadecanoate was released by methanolysis


A
43
RELATIVE INTENSITY (%)
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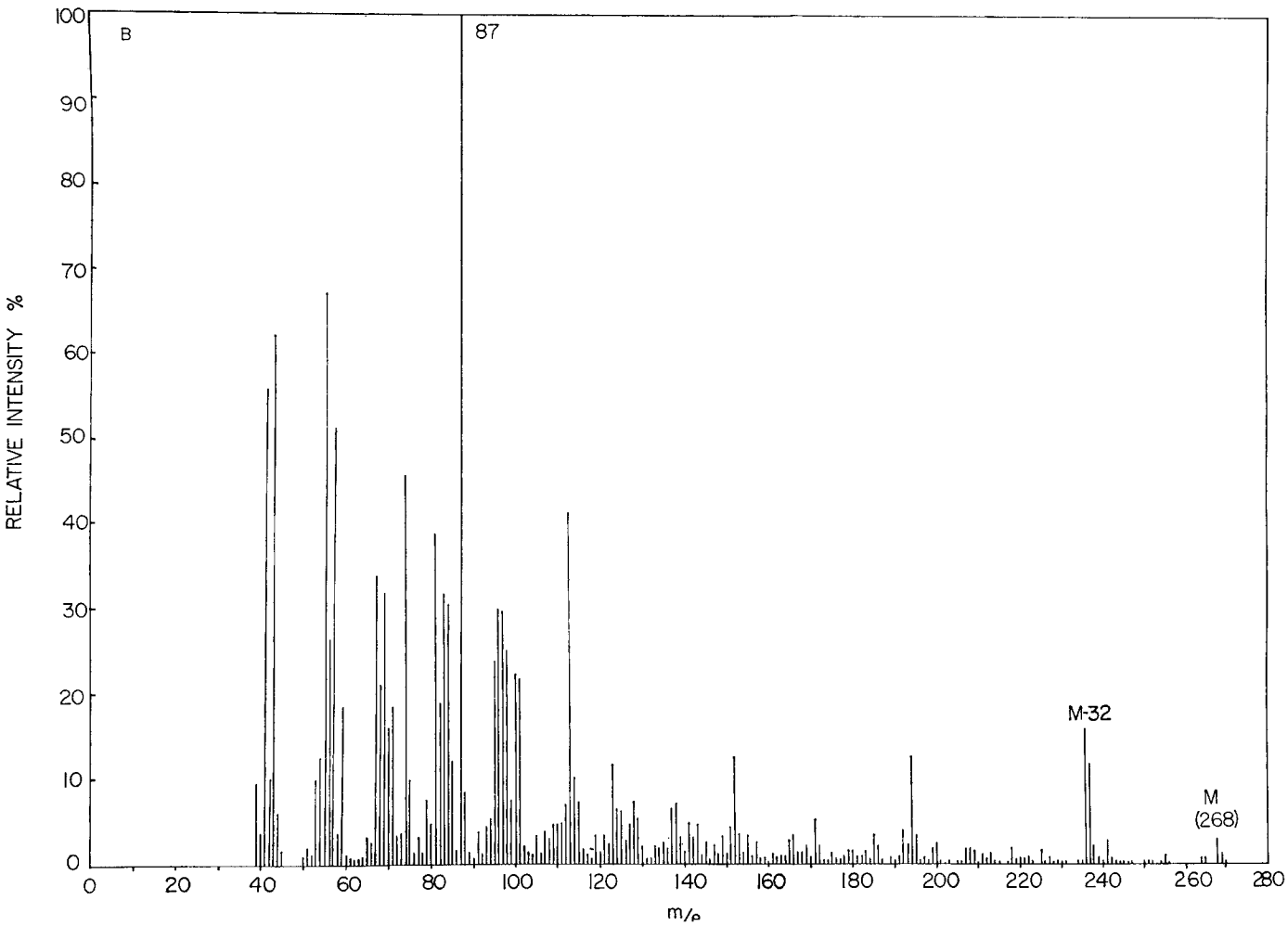
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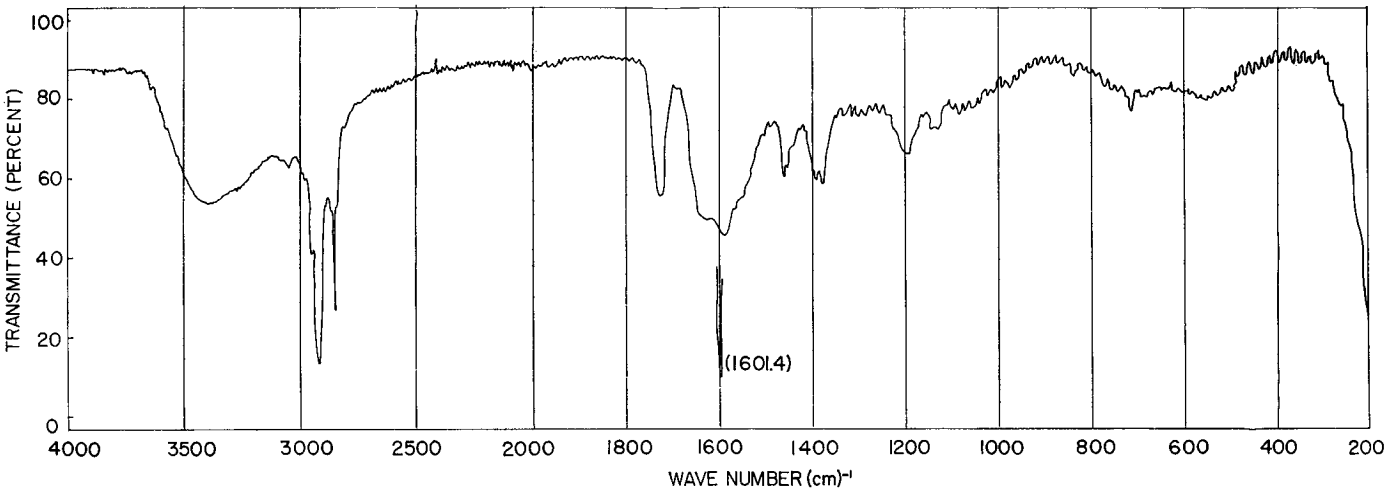

FIG. 4. An infrared spectrum of the ornithine-containing lipid from *T. thiooxidans*. The lipid was incorporated into a KBr disc.

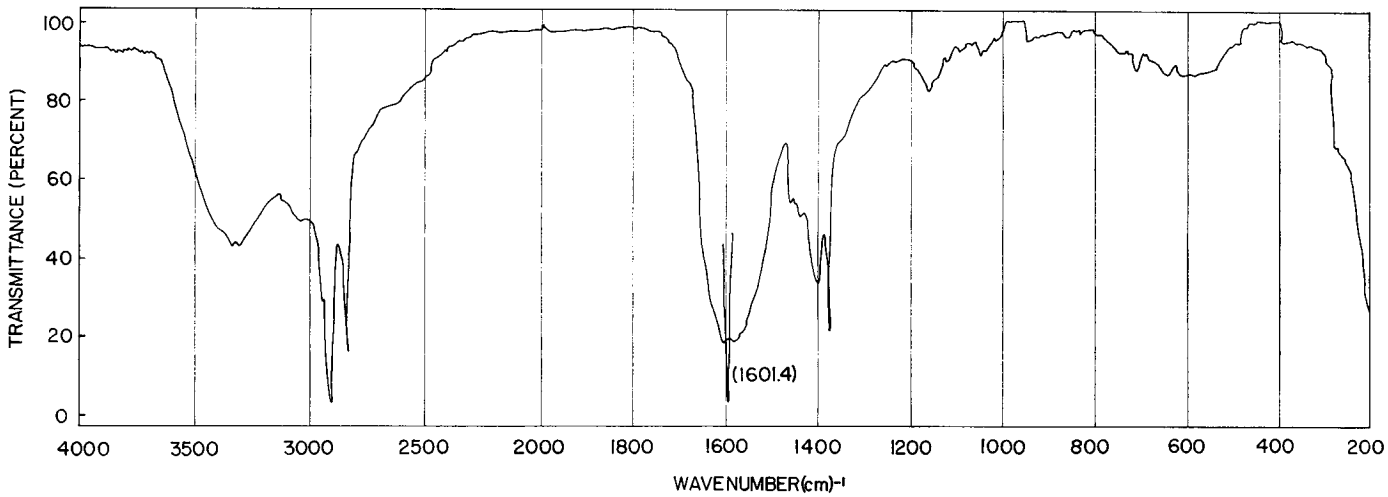

FIG. 5. An infrared spectrum of the lyso-lipid which was obtained by methanolysis of the intact ornithine-containing lipid to remove the *cis*-11,12-methylene-2-hydroxyoctadecanoic acid moiety. The sample was incorporated into a KBr disc.

of the lipid suggested that the carboxyl group of that acid was esterified. To exclude the possibility that the lipid contained a methyl ester function, U-^{14}C-lipid was subject to methanolysis. After neutralizing the $NaOCH_3$ with acetic acid, the methanol was vacuum distilled, distilled at atmospheric pressure, and assayed for radioactivity. No appreciable radioactivity was observed, thus indicating the lack of a methyl ester group in the natural lipid molecule.

The infrared spectrum of the lipid (Fig. 4) shows a strong absorption band near 1730 cm^{-1} and a weaker band at 1195 cm^{-1} both of which were indicative of an ester function. Similar ester absorption bands were absent in the spectrum of the lyso-lipid (Fig. 5). If the carboxyl group of ornithine had been esterified the methanolysis procedure should have converted the group to a methyl ester that would have been observable in the spectrum of the lyso-lipid. It was believed that the carboxyl group of the 3-hydroxyhexadecanoic acid was not involved in an ester linkage because strong acid was necessary to hydrolyze the lyso-lipid, infrared spectroscopy indicated that amide bonds were present in both the lipid and lyso-lipid, and other evidence, to be presented later, showed that the carboxyl group of ornithine was free. Assuming that the molecule was not polymeric and consisted of 1 molecule of each of the components, it was concluded that the 11,12-methylene-2-hydroxyoctadecanoic acid was esterified with the hydroxyl group of the 3-hydroxyhexadecanoic acid moiety. Further evidence to support this conclusion was obtained by determining the quantity of esterified fatty acids in the lipid. Aliquots of a $CHCl_3$ solution of the lipid were analyzed for esterified fatty acids by the Hill procedure (6). Ornithine was quantitatively determined in other aliquots. The data obtained (Table I) indicated a 1:1 ratio of esterified fatty acid to ornithine.

Evidence for amide bonds was obtained from both of the infrared spectra. While the region 1680 to 1500 cm^{-1} is poorly resolved, presumably due to overlapping of amide, carboxylate, and amine salt bands, a shoulder near 1640 to 1610 cm^{-1} was

FIG. 3. Mass spectra of derivatives of the major fatty acids released by HCl hydrolysis of the lyso-lipid which was prepared by methanolysis of the intact ornithine-containing lipid to remove the *cis*-11,12-methylene-2-hydroxyoctadecanoic acid moiety. *A*, a spectrum of the acetylated methyl ester shown in Fig. 2*a*. The compound, isolated by trapping it from the gas-liquid chromatograph, was identified as methyl 3-acetoxyhexadecanoate. *B*, a spectrum of the major component shown in Fig. 2*b*. The compound, isolated by gas-liquid chromatography was identified as methyl 2-hexadecenoate.

TABLE I

Analyses of ornithine-containing lipid of T. thiooxidans for ornithine and esterified fatty acid

The quantity of esterified fatty acid was determined by a colorimetric method in which a ferric hydroxamate complex was the chromophore (6). Using separate aliquots, ornithine was assayed with an amino acid analyzer.

Trial	Ornithine	Fatty ester
	μmoles	
1	2.87	2.59
2	2.90	2.86
3	2.90	2.60

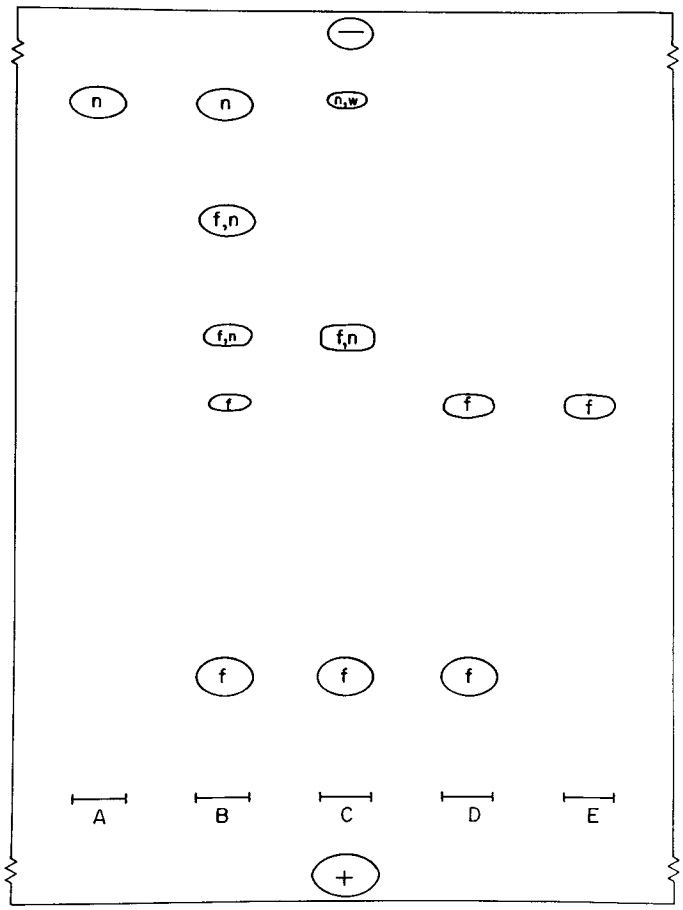

FIG. 6. A composite diagram of high voltage electrophoretic strips used for end group analysis of the ornithine-containing lipid of *T. thiooxidans* with dansyl chloride. *A*, authentic ornithine. *B*, components obtained when an excess of ornithine was allowed to react with dansyl chloride; the components were, in order of decreasing mobility, unreacted ornithine, α-dansyl ornithine, δ-dansyl ornithine, α,δ-didansyl ornithine, and dansyl-OH. *C*, products obtained when the lipid was allowed to react with an excess of dansyl chloride; besides a trace of free ornithine, the components were δ-dansyl ornithine and dansyl-OH. *D*, products obtained when ornithine was allowed to react with an excess of dansyl chloride; the components were α,δ-didansyl ornithine and dansyl-OH. *E*, authentic α,ϵ-didansyl lysine. Fluorescent components are indicated by *f* and ninhydrin-reactive spots by *n*. Weak or faint ninhydrin-positive spots are denoted by *n,w*. The starting lines and polarity are indicated. Electrophoresis was performed with a pH 1.9 buffer. Other conditions and reaction details are given under "Experimental Procedure."

indicative of an amide linkage. One of the shoulders between 1560 to 1530 cm^{-1} and absorption in the region 3300 to 3030 cm^{-1} may have been due to an amide bond also. While it seemed certain that the 3-hydroxy acid was involved in this type of linkage the possibility of the molecule having a polyornithine skeleton, to which the 3-hydroxy acid was attached, was considered.

Since the lipid and lyso-lipid reacted positively with ninhydrin, one of the amino groups of the ornithine moiety was believed to be free. The infrared spectra indicated the presence of a free amino group (as NH_3^+) by absorption bands or shoulders near 1550 and 3300 to 3000 cm^{-1}. However, hydroxy groups were also indicated by the broad absorption band near 3400 cm^{-1} and a weaker band 1130 to 1160 cm^{-1}. Because of overlapping with carboxylate and amide bands in the 1500 to 1650 cm^{-1} region, definite assignments could not be made.

To determine which amino group of ornithine was free, the lipid was subjected to end group analysis with dansyl chloride. Since authentic dansyl derivatives of ornithine were not readily available, the electrophoretic mobilities of α-dansyl, δ-dansyl, and α,δ-didansyl ornithine were deduced as follows. Ornithine was allowed to react with an excess of dansyl chloride, and after HCl hydrolysis, the products were subjected to high voltage electrophoresis. As shown in the composite diagram of electrophoretic strips (Fig. 6), two bright fluorescent spots were observed. One corresponded to the hydrolysis product of dansyl chloride, 1-dimethylaminonaphthalene-5-sulfinic acid (dansyl-OH), and the other (α,δ-didansyl ornithine) had a mobility which was indistinguishable from authentic α,ϵ-didansyl lysine. No ninhydrin-positive spots were apparent. When an excess of ornithine was allowed to react with dansyl chloride, electrophoresis of the hydrolyzed reaction mixture revealed the presence of ornithine, dansyl-OH, α,δ-didansyl ornithine, and two fluorescent components which were also ninhydrin-positive. Assuming the three dansyl derivatives of ornithine would exhibit the same mobilities, relative to one another, as the analogous derivatives of lysine in a pH 1.9 buffer (8), the fluorescent, ninhydrin-positive component with the greatest mobility was identified as α-dansyl ornithine and the other as δ-dansyl ornithine.

Electrophoresis of the hydrolyzed products obtained by the reaction of the ornithine-containing lipid with dansyl chloride revealed one fluorescent, ninhydrin-positive component, which had the same mobility as δ-dansyl ornithine (Fig. 6). In addition, dansyl-OH and a trace of free ornithine were observed. The small quantity of ornithine was probably due to hydrolysis of some of the δ-dansyl ornithine (8) or to incomplete derivatization of the lipid. These results indicated that essentially all of the ornithine residues in the lipid possessed a free δ-amino group and that the 3-hydroxyhexadecanoic acid moiety was bound through the α-amino group.

With only one of the amino groups involved in an amide linkage, a polyornithine structure would be impossible. Therefore the carboxyl group of ornithine would have to be free since its involvement in an ester linkage had been ruled out. It was reasoned that, with the carboxyl group of ornithine free and a molar ratio of 1:1:1 for the components, a covalent polymeric structure could be eliminated as a possibility.

Additional evidence was sought to support the conclusions that the carboxyl group of ornithine was free. An absorption shoulder near 1585 cm^{-1} and a band near 1400 cm^{-1} suggested the existence of a carboxylate anion. While overlapping absorption bands

hindered a definite assignment, the bands near 1390 or 1400 cm^{-1} provided evidence for such a group in the lipid and lyso-lipid.

Analyses for free carboxyl groups were performed by washing a $CHCl_3$ solution of the lipid with dilute HCl and then with cold water. Aliquots of the solution were taken. After evaporating the solvent, the samples were redissolved in 10% methanol in diethyl ether and treated with diazomethane. To reduce the possibility of transesterification occurring, the reaction period (about 5 min) was terminated when the first sign of excess diazomethane was observed. Although exhaustive treatment had been found to produce some *N*-alkylation of the lipid, as determined by thin layer chromatography, this was not considered to be disadvantageous for this procedure. After evaporating the solvent *in vacuo*, the lipid was saponified and the resulting methanol trapped and quantitatively determined. Ornithine analysis of aliquots of the washed lipid solution was also performed. In three experiments the quantities of ornithine and methanol were compared for equivalent aliquots. The data obtained are presented in Table II. While the values for free carboxyl groups are low compared to ornithine, these results indicate that at least 80% of the ornithine residues existed with a free carboxyl group.

The infrared spectra of the lipid and lyso-lipid suggested that the free carboxyl and amino groups of the lipid were ionized. To investigate this possibility and to support the molecular weight assignment, a nonaqueous titration of the lipid was performed in $CHCl_3$-CH_3OH (2:1, v/v) with HCl and $NaOCH_3$ as titrants. Exactly 25.82 mg (corresponding to 37.4 μmoles of the lipid if a molecular weight of 690 is used), was titrated with HCl, then with $NaOCH_3$. The resulting data were plotted and are shown in Fig. 7. Two ionizable groups were present. The inflection points, corresponding to pK_a values, appear to be near −65 and +185 mv, with the lower value being approximately 125 mv below the analogous value observed for the titration of acetic acid. Titration of *N*,*N*-dimethylethanolamine yielded an inflection point within 20 mv of the value obtained for the δ-amino group of the ornithine residue. The limits of the titration curve on the millivolt scale correspond to the limits of the electrode potential that were observed when solutions of HCl were titrated with $NaOCH_3$ and vice versa. Due to a lack of solubility in the solvent system, ornithine could not be titrated. From the equivalents of titrant used and the quantity of lipid titrated, it may be calculated that, in a $CHCl_3$-CH_3OH solution, all of the lipid molecules exist with free, ionized carboxyl, and amino groups. By existing as a zwitterion, a high degree of molecular association would be expected in a nonaqueous solvent, a situation which could account for the fact that the boiling points of benzene and isopropyl alcohol were not significantly elevated when the lipid was dissolved or dispersed in them.

The conclusions that the molar ratio of components is 1:1:1, the carboxyl and δ-amino group of the ornithine residue are free, the 2-hydroxy acid is linked through an ester bond to the hydroxyl group of 3-hydroxyhexadecanoic acid moiety, which, in turn, is bonded to the α-amino group of the ornithine residue, and that the molecule is not polymeric lead to the conclusion that the hydroxyl group of the 11,12-methylene-2-hydroxyoctadecanoic acid moiety is free. As mentioned earlier, the infrared spectrum (Fig. 4) supports this conclusion. Considering the evidence presented here we concluded that the only structure that is consistent with the data obtained is the structure presented in Fig. 1.

TABLE II

Analyses of ornithine-containing lipid of T. thiooxidans for ornithine and free carboxyl groups

The quantity of free carboxyl groups was determined by treating the lipid with diazomethane, saponifying the ester, and determining the amount of methanol liberated by $K_2Cr_2O_7$ oxidation. Using separate aliquots, ornithine was assayed with an amino acid analyzer.

Trial	Ornithine	Carboxyl group
	μmoles	
1	1.73	1.35
2	0.87	0.71
3	0.86	0.62

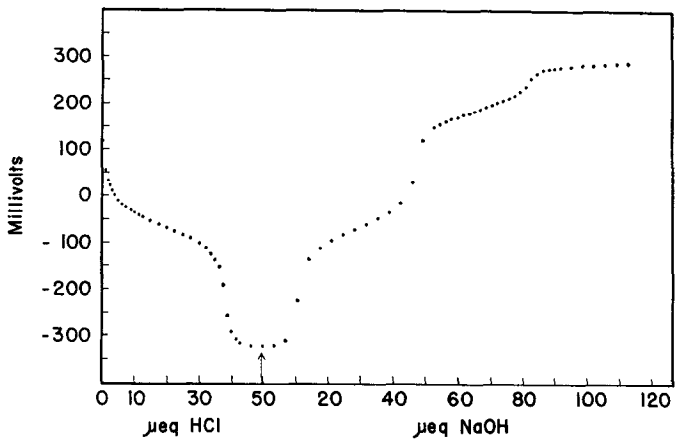

FIG. 7. The curve obtained by the nonaqueous titration of the ornithine-containing lipid of *T. thiooxidans* with HCl and $NaOCH_3$, successively. The solvent was $CHCl_3$-CH_3OH (2:1, v/v). Microglass and calomel electrodes were utilized. In the diagram, NaOH should read $NaOH_3$.

The structures of other lipids which contain basic amino acids, but not phosphorus, have been reported (11–14). However, this lipid appears to be more polar. Like the lipid described here, the ornithine-containing lipids of *Brucella melitensis* and *Mycobacterium bovis* (11), as well as the lysine-containing lipid of *Streptomyces sioyaensis* (12, 13), possess 3-hydroxy fatty acids which form amide linkages with the α-amino groups of the amino acid moieties. The ornithine-containing lipid of *Rhodopseudomonas sphereoides* apparently lacks this feature as a normal saturated fatty acid is linked to the α-amino group (14). Within each of the lipids from *T. thiooxidans*, *B. melitensis*, and *M. bovis*, the chain lengths and functional groups of the fatty acid moieties are unusually constant when the lipids are compared to other amphipathic lipids. All amino lipids mentioned above possess ester functions although the nature of these groups vary between lipids. Interesting is the fact that of the esterified hydrocarbon chains, which have been studied in detail (alcohol or fatty acid), all contain either a cyclopropane function or a methyl branch.

While the biological function of the lipid reported here is yet to be determined, it appears that the lipid is ideally suited for a role in membranes. Its structural similarity to phospholipids, particularly sphingamine derivatives, is striking.

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