

Direct Incorporation of Fatty Acids into the Halosulfatides of *Ochromonas danica*[†]

Carolyn L. Mooney, Eileen M. Mahoney,[‡] Manuel Pousada, and Thomas H. Haines*

ABSTRACT: The lipids of the phytoflagellate, *Ochromonas danica*, contain large amounts of docosane 1,14-disulfates and tetracosane 1,15-disulfates with from 0 to 6 hydrogens on the chain replaced with chlorine atoms. [¹⁴C]Acetate, [¹⁴C]octanoate, [¹⁴C]laurate, [¹⁴C]palmitate, [¹⁴C]stearate, and [¹⁴C]oleate were incorporated into these sulfatides from the media. Each of these precursors was utilized for the biosynthesis of the fourteen disulfates investigated with a virtually identical labeling pattern. Incorporation was con-

firmed by degradation of the product. Palmitate was most efficiently incorporated into the sulfatide fraction and stearate was least efficiently utilized for sulfatide biosynthesis. These data show that (1) the carbon chain is biosynthesized using the normal fatty acid pathway; (2) the secondary hydroxyl group is put on the chain after the chain is fully synthesized *via* the hydration of a *cis* double bond; (3) the fatty acid chains are chlorinated after the chains are fully synthesized.

A unique series of lipids has been characterized in the photosynthetic protozoan, *Ochromonas danica*. These polar lipids are polychlorodocosane 1,14-disulfates and tetracosane 1,15-disulfates with from 0 to 6 chlorine atoms replacing hydrogens on the aliphatic chain (Elovson and Vagelos, 1969, 1970; Haines, 1965, 1971; Haines *et al.*, 1969; Mayers and Haines, 1967; Mayers *et al.*, 1969; M. Pousada, B. Das,

and T. H. Haines, personal communication). The lipids are unique as polar lipids because they (a) are alkyl sulfates, (b) contain polar (charged) groups at both ends of the molecule, and (c) contain up to six chloro groups on the chain. Each series may be considered as derivative of the hexachlorosulfatides 2,2,11,13,15,16-hexachloro-1,14-docosanediol 1,14-disulfate (Elovson and Vagelos, 1970) and 2,2,12,14,16,17-hexachloro-1,15-tetracosanediol 1,15-disulfate (Haines, 1971). Other compounds in the series have been shown to contain chlorine atoms on the respective alkyl disulfates in various combinations of the positions described above (Haines, 1971).

The chlorosulfolipids constitute approximately 3% of the dry weight of the cells (Elovson and Vagelos, 1969) or 10–20% of the lipids. The occurrence of such large amounts of a polar lipid in a microbe suggests that the lipid is present in membrane. The location of these compounds in the cell and their function is as yet unknown. The study of the biosynthesis

[†] From the Department of Chemistry, The City College of the City University of New York, New York, New York 10031. Received June 30, 1972. This work has been supported by grants from the donors of the Petroleum Research Fund, administered by the American Chemical Society (5727-AC1) and the City University of New York Research Foundation (1002, 1213). A major portion of the data is taken from the dissertation of C. L. M. to the City University of New York in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[‡] Present address: The Rockefeller University, New York, N. Y. 10021.

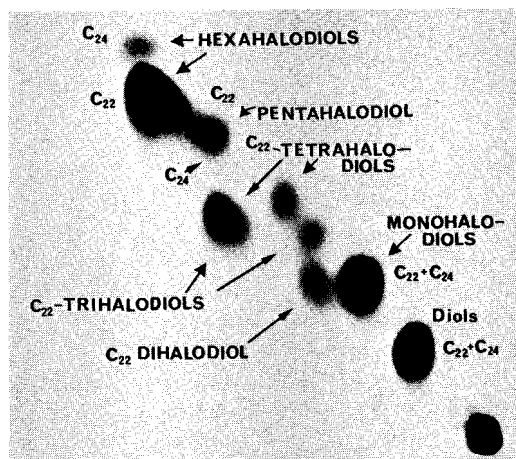


FIGURE 1: Autoradiogram of diols obtained after $[1-^{14}\text{C}]$ laurate feeding. The extracted sulfatide fraction was solvolysed to diols. The chromatogram was developed vertically in ether-hexane (3:7, v/v), then horizontally in benzene-chloroform-methanol (50:40:1, v/v), and then vertically again in the ether-hexane solvent.

of these substances is of interest in that it may suggest the design of specific inhibitors which would help elucidate the role of the chlorosulfolipids in the cell.

Materials and Methods

$[1-^{14}\text{C}]$ Acetate (specific activity 28.0 Ci/mole), $[1-^{14}\text{C}]$ octanoate (specific activity 1.99 Ci/mole), and $[1-^{14}\text{C}]$ laurate (specific activity 28.8 Ci/mole) were purchased from Calbiochem (Los Angeles, Calif.). The radioactive purity of each was >99, >98, and >99%, respectively, as demonstrated by thin-layer chromatography (tlc) and gas-liquid chromatography (glc). $[16-^{14}\text{C}]$ Palmitate (specific activity 51 Ci/mole), $[1-^{14}\text{C}]$ stearate (specific activity 46 Ci/mole), and $[18-^{14}\text{C}]$ stearate (specific activity 55 Ci/mole) were purchased from New England Nuclear (Boston, Mass.). The radioactive purity of each was >98, >99, and >99%, respectively, as demonstrated by TLC and glc. $[1-^{14}\text{C}]$ Oleate (specific activity 52.2 Ci/mole) was purchased from Applied Science Laboratories, Inc. (State College, Pa.). Its radioactive purity was >99%, as demonstrated by TLC and glc.

Dioxane, reagent grade, was distilled over lithium aluminum hydride and stored at 4° under N_2 . Ether, hexane, and benzene were redistilled before use. TLC was conducted on silica gel F-254 on either glass plates or plastic sheets purchased from Brinkmann Instruments, Inc. Two-dimensional chromatograms were developed vertically in ether-hexane (3:7, v/v), then horizontally in benzene-chloroform-methanol (50:40:1, v/v), and then vertically again in the ether-hexane solvent.

Radiocounting was conducted on a Nuclear-Chicago automatic planchet counting system with a D-47 Geiger Muller counter or on a Nuclear-Chicago scintillation counter. Autoradiograms of TLC's were obtained using Picker No-Screen X-ray film.

All other chemicals were reagent grade and obtained commercially.

Incubations. *Ochromonas danica* was grown axenically in a chemically defined medium (Aaronson and Baker, 1969) (pH 4.5) at 26° under constant illumination of 125–150 foot-candles of light.

The fatty acids were added dissolved in 80% ethanol. The

final concentration of ethanol in the media was 1%. Final concentrations of fatty acids in the media were as follows: $[1-^{14}\text{C}]$ acetate (20 μCi), 0.071 mM; $[1-^{14}\text{C}]$ octanoate (20 μCi), 0.112 mM; $[1-^{14}\text{C}]$ laurate (50 μCi), 0.179 mM; $[16-^{14}\text{C}]$ palmitate (50 μCi), 0.098 mM; $[1-^{14}\text{C}]$ stearate (50 μCi), 0.109 mM; $[18-^{14}\text{C}]$ stearate (50 μCi), 0.091 mM; and $[1-^{14}\text{C}]$ oleate (50 μCi), 0.0087 mM. Radioactive acids were added to the medium preceding inoculation and incubated for 5 days, except in the cases of acetate and oleate, in which the acids were added to cultures of approximately 2-days growth.

Isolation of Chlorodiols. Cells were harvested by centrifugation at 9000g for 10 min at 4°. The cells were washed twice with deionized water by suspension and centrifugation as before. The pellet was then extracted with chloroform-methanol, by the method of Folch *et al.* (1957). The entire procedure was conducted under nitrogen to protect the polyunsaturated fatty acids from oxidation. The sulfolipids appear in the upper phase of the partitioned Folch extract. These lipids were recovered from this fraction and solvolysed in aqueous dioxane to yield diols (Mayers *et al.*, 1969). When solvolysis is conducted on micro amounts of sulfate esters it is difficult to maintain the water concentration at appropriate levels. It was found desirable to add a very small amount of acid (approximately 0.01 M or less, final concentration) to reliably obtain solvolysis products (Goren, 1971). Diols were separated by two-dimensional chromatography. The monochlorodiols were scraped from the chromatogram and cleaved as described below.

Degradation of Labeled Chlorohydrins. The procedure used is essentially that of Kusamran and Polgar (1971). The chlorohydrin was allowed to stand overnight in 2 ml of 0.5 M KOH in methanol. After addition of 2 ml of water, the epoxide was extracted with ether, back-washed with water, dried over anhydrous sodium sulfate, and evaporated under a stream of N_2 .

A suspension of periodic acid in ether was made by stirring 100 mg of H_5IO_6 in 10 ml of dry ether vigorously for 1 hr. This suspension (5 ml) was added to the labeled epoxide in ether and stirred vigorously for 1 hr. An equal volume of water was added and separated. The ether was back-washed, dried, and evaporated as before.

Separation of Aldehyde Products. The resulting aldehydes were separated on TLC using ether-hexane (3:7, v/v) as the solvent system. Standards were run on the same plate and visualized using 1% 2,4-dinitrophenylhydrazine (w/v) in 2 N HCl in 75% ethanol (v/v). The corresponding unvisualized areas were then scraped from the plate and counted in a liquid scintillation counter.

Results

All of the $[^{14}\text{C}]$ fatty acids were incorporated into the lipids of *O. danica* after 5-days incubation in the medium. This was confirmed in each case by a two-dimensional autoradiogram of the solvolysed disulfates (diols). An example of the pattern obtained in this procedure is shown in Figure 1. Similar autoradiograms were obtained by incubating each of the labeled precursors with cells and following the same procedure.

The relative distribution of the radioactivity incorporated into the various lipid fractions after the $[1-^{14}\text{C}]$ acetate feeding is summarized in Table I. The lower phase of the Folch extract contains the bulk of the radioactive material. Approximately 20% of the label occurs in the upper phase of the Folch extract, the sulfolipid fraction. After hydrolysis of the upper

TABLE I: Incorporation of [1-¹⁴C]Acetate.

Fraction	Total Counts	Percentage
Total lipid	371,500	100
Lower-phase Folch	245,900	66
Upper-phase Folch	77,300	21
Solvolysis		
H ₂ O layer	0	0
Ether layer	82,000	22

 TABLE II: Incorporation of [1-¹⁴C]Laurate.

Fraction	Total Counts	Percentage
Total lipid	22,000,000 ^a	100
Lower-phase Folch (phospholipids, etc.)	19,360,000	88.0
Upper-phase Folch	2,156,000	9.8
Solvolysis		
H ₂ O layer	324,000	1.5
Ether layer	1,450,000	6.6

^a Value based on total of upper- and lower-phase Folch.

phase, all the radioactivity is found with the diols in the ether phase. The distribution data obtained from the [1-¹⁴C]laurate feeding is summarized in Table II. The incorporation data are similar to that of the acetate feeding, although the amount of radioactivity in the upper phase of the Folch extract is 10%. The radioactivity in the aqueous layer after solvolysis is due to incomplete solvolysis of the sulfolipid. Similar incorporation data are obtained from the [16-¹⁴C]palmitate feeding (Table III), and from the [1-¹⁴C]stearate and [18-¹⁴C]stearate feedings (Table IV). A pattern similar to that of the laurate feeding is found for these feedings, although the values show quantitative differences.

Earlier work (Haines, 1965; Haines and Block, 1962) had indicated that the sulfatides are excreted into the medium of the culture. The culture medium was therefore acidified with hydrochloric acid to form a 1 N solution, refluxed 2 hr, and extracted with ether. In each case a pattern similar to that in Figure 1 was obtained, confirming that the sulfatides were synthesized from the labeled precursor and excreted into the medium.

 TABLE III: Incorporation of [16-¹⁴C]Palmitate.

Fraction	Total Counts	Percentage
Total lipid	2,407,200	100
Lower-phase Folch (phospholipids, etc.)	2,052,750	85.3
Upper-phase Folch	368,350	15.3
Solvolysis		
H ₂ O layer	43,500	1.8
Ether layer	187,500	7.8

 TABLE IV: Incorporation of [1-¹⁴C]Stearate and [18-¹⁴C]Stearate.

Fraction	Sample	Total Counts	Percentage
Total lipid	[1- ¹⁴ C]Stearate	2,819,000	100
	[18- ¹⁴ C]Stearate	9,753,000	100
Lower-phase Folch (phospholipids, etc.)	[1- ¹⁴ C]Stearate	2,401,000	85.2
	[18- ¹⁴ C]Stearate	8,969,000	92.0
Upper-phase Folch	[1- ¹⁴ C]Stearate	143,000	5.1
	[18- ¹⁴ C]Stearate	345,000	3.5
Solvolysis			
H ₂ O layer	[1- ¹⁴ C]Stearate	45,000	1.6
	[18- ¹⁴ C]Stearate	40,000	0.4
Ether layer	[1- ¹⁴ C]Stearate	91,000	3.2
	[18- ¹⁴ C]Stearate	266,000	2.7

Table V shows the results obtained from the degradation of the monochlorodiols after the incorporation of various fatty acid precursors. In each case, the radioactivity was found in the appropriate cleavage product expected for incorporation of the intact fatty acid chain (Scheme I). The data for the degradation of monochlorodiols obtained from the laurate feeding show that the maximum amount of random incorporation of acetate units into the chain was under 10% after 5-days incubation. The low value of counts obtained in the [18-¹⁴C]stearate experiment is presumably due to the volatility of nonanal (I) and to the low level of activity in the isolated chlorohydrin.

Discussion

In order to establish the biosynthetic pathway for the chlorosulfatides, one must first determine the route used for the formation of the carbon chain. The incorporation of acetate and octanoate into the sulfolipids implies that the chain is synthesized by the usual fatty acid synthesizing enzymes. The label pattern shown in Figure 2 is that expected for the sulfolipid after incorporation of [1-¹⁴C]acetate or [1-¹⁴C]octanoate.

Where it has been investigated, the mechanism for the biosynthesis of all sulfate esters to date has involved the trans-

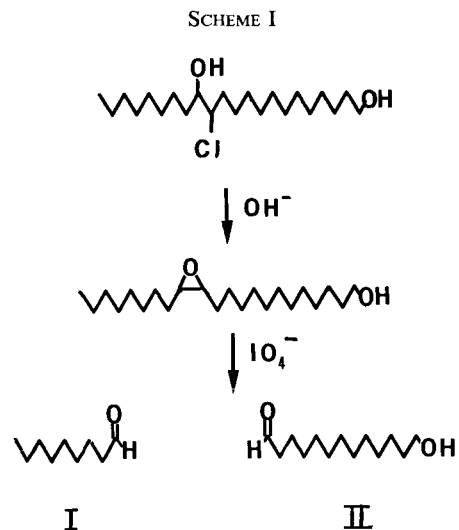


TABLE V: Cleavage of ^{14}C -Labeled Monochlorodiols Obtained from Four Radioactive Studies.

Fragment ^a	Incorp of [$1\text{-}^{14}\text{C}$]Acetate			Incorp of [$1\text{-}^{14}\text{C}$]Laurate		Incorp of [$18\text{-}^{14}\text{C}$]Stearate		Incorp of [$1\text{-}^{14}\text{C}$]Oleate	
	Counts	Actual Percentage	Expected Percentage	Counts	Actual Percentage	Counts	Actual Percentage	Counts	Actual Percentage
I	205	28	36	125	6	100	100	10	2
II	525	72	64	2300	94	0	0	409	98

^a Fragment I is nonanal or decanal; fragment II is 13-hydroxytridecanal or 14-hydroxytetradecanal.

fer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to a hydroxyl group. Assuming this reaction occurs here, the next step in elucidating the biosynthesis of the chlorosulfolipids would be that of establishing the mechanism for placing the secondary hydroxyl group on the chain. The primary hydroxyl group is assumed to be obtained by a reduction of the carboxyl group on the fully synthesized alkyl chain. The secondary hydroxyl group can be added to the chain as it is being synthesized or it can be placed on the chain after the synthesis of the chain is completed.

Figure 3 shows the location of the ^{14}C label in the sulfolipid if the long-chain fatty acids were incorporated intact. This route would imply that the entire chain, or at least a major part of it, is synthesized before the hydroxyl group is placed on the chain. An incorporation of this sort rules out an anaerobic mechanism for the introduction of the hydroxyl group. Long-chain saturated fatty acids were fed to the organism and were found to be incorporated into the sulfolipids. A question remained, however, as to whether the incorporation occurred *via* elongation or *via* degradation and *de novo* synthesis. The distributions of radioactivity in the various lipid fractions were virtually identical in the carboxyl-labeled stearate feeding and the methyl-labeled stearate feeding (Table IV). The chlorodiols patterns obtained on two-dimensional chromatograms were also identical. These data, together with that obtained by Gellerman and Schlenk (1972) on the elongation and conversion of stearic acid and other fatty acids in *O. danica*, suggest that the fatty acids are in-

corporated intact. In their experiments the fatty acids were shown to be converted directly by elongation and desaturation to other acids such as arachidonic acid. This was demonstrated by ozonolysis, oxidation, and radiocounting of the isolated fragments. Their conditions of incubation of the ^{14}C precursors were virtually identical with ours (their 3-day incubations were somewhat shorter than our 5-day incubations).

To unequivocally establish the intact incorporation of the long-chain fatty acid into the sulfolipid, a degradation of the monochlorodiols from four radioactive feedings was performed. The location of the [$1\text{-}^{14}\text{C}$]laurate label, as predicted on the basis of intact incorporation, may be followed through the degradation of 13-chloro-1,14-docosanediol in Scheme I. Note that this label, in addition to the [$1\text{-}^{14}\text{C}$]stearate and [$1\text{-}^{14}\text{C}$]oleate labels should have appeared in II, whereas, ideally, no radioactivity should have been found in I. In con-

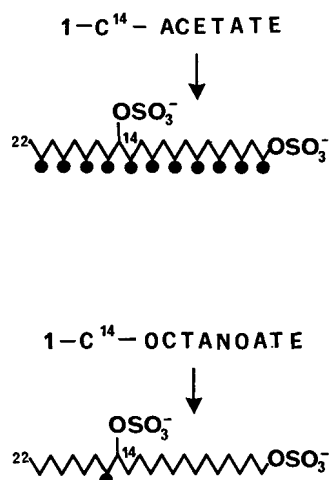


FIGURE 2: Diagram of labeled acetate and octanoate incorporation into sulfolipid.

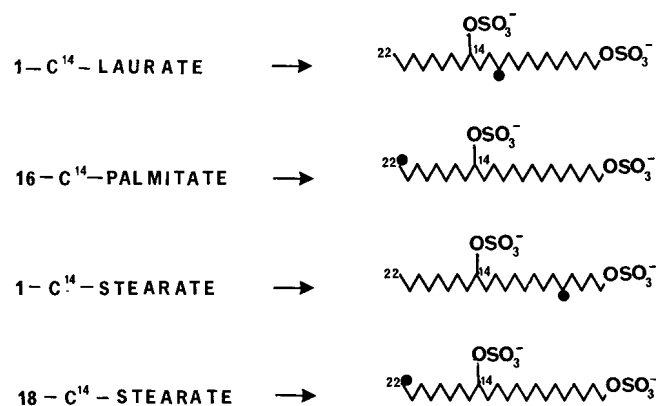


FIGURE 3: Diagram of labeled palmitate and stearate incorporation into sulfolipid.

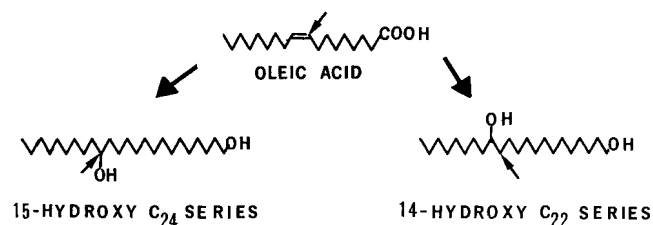


FIGURE 4: Diagrammatic representation of the proposed incorporation of oleic acid into the two series of chlorosulfatides. Incorporation occurs by hydration of the double bond of oleic acid. The small arrows indicate the position of C-9 of oleic acid in each diol after elongation of the chain to 22-24 carbons.

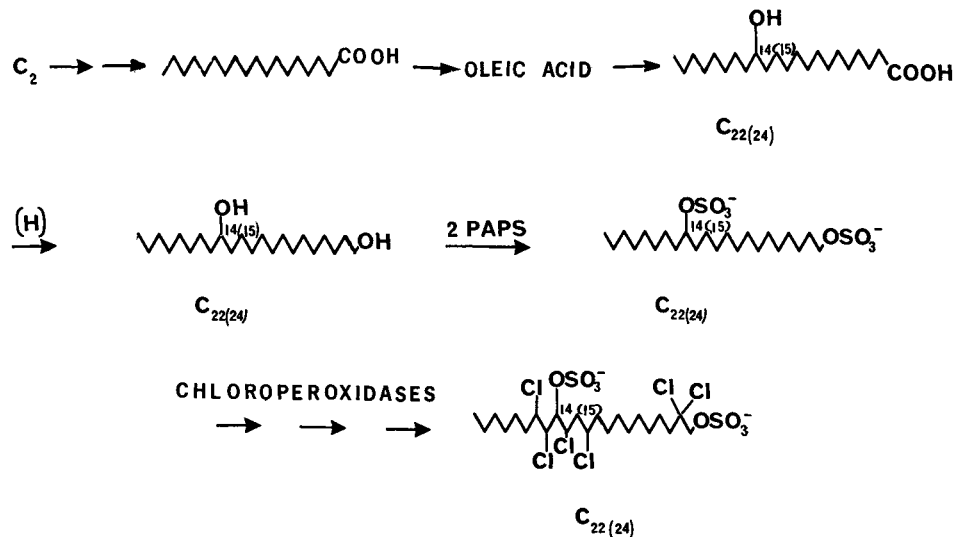


FIGURE 5: Proposed biosynthetic pathway for the chlorosulfolipids.

trast, the [1-¹⁴C]octanoate, [16-¹⁴C]palmitate, and [18-¹⁴C]stearate labels should have appeared in I, whereas II should have been devoid of radioactivity. Approximately ⁴/₁₁ (or 36%) of the combined radioactivities of the two fragments should have been present in I and ⁷/₁₁ (or 64%) in II after degradation of the monochlorodiols of cells grown in [1-¹⁴C]acetate. The results obtained from the degradations are shown in Table V and intact incorporation of all of the fatty acids tested into the sulfolipids is indicated.

The capacity of the organism to incorporate the labeled fatty acids into chlorosulfolipids is summarized in Table VI. A decrease in the incorporation of labeled fatty acids into sulfolipid as chain length increased might be expected. It is interesting to note that [¹⁴C]palmitate is incorporated efficiently, while the labeled stearates are incorporated to a smaller extent. A difference in the metabolism of these fatty acids normally present in the cell would explain the results. Stearate was found by Gellerman and Schlenk (1972) to be a poorer precursor for conversion into the polyunsaturated fatty acids than palmitate.

There are two groups of chlorosulfatides in the mixture. The dominant series is that of the 1,14-docosanediol 1,14-disulfates. The second series is a mixture of 1,15-tetracosanediol 1,15-disulfates. Since the fatty acid synthesizing enzymes are utilized to make the chain, it is somewhat surprising that the difference in the location of the secondary sulfate in these two series should be one carbon. Thus the length of the methylene chain for the tetracosane series, both proximal and distal to the secondary sulfate is greater by one carbon than that for the docosane series. Furthermore these two series are the only ones found in the organism. It would appear from this structural pattern that the hydroxyl group is introduced on the saturated chain. The introduction might occur by direct hydroxylation or by desaturation followed by hydration, with the hydroxyl on the distal carbon in the docosane series and on the proximal carbon in the tetracosane series. The latter was found to be the case by degradation of the monochlorodiols obtained from the [¹⁴C]oleate feeding. The addition of a molecule of water to the cis double bond might occur in either of two ways (Figure 4). The substitution of the OH on the ninth carbon from the methyl end of the fatty acid would be appropriate for the formation of the sulfate ester

at C-14 in the C₂₂ series of sulfolipids. The substitution of the OH on the tenth carbon from the CH₃ terminus would be appropriate for the formation of the sulfate ester at C-15 in the C₂₄ series of sulfolipids. It is not known whether elongation to C₂₂ or C₂₄ occurs before hydration or after it. Work is currently being carried out to establish the correct order of these events.

The autoradiogram of the chlorodiols obtained after feeding [¹⁴C]laurate is shown in Figure 1. The pattern is essentially identical with that obtained when the crude sulfatide mixture is extracted from the cells and hydrolyzed, and the diols were chromatographed two dimensionally. Since all the chlorodiols appear to be equally labeled, it seems likely that the chlorines are put on a fully synthesized chain. Similar autoradiograms of the chlorodiols were obtained from the individual feedings of the other labeled precursors. The chlorinating system of *O. danica* may be similar to that operative in *Caldariomyces fumago*, studied by Hager *et al.* (1970). Hollenberg *et al.* (1972) propose that the halogenating intermediate is an OCl ligand on heme iron. It is not known whether chlorination occurs prior or subsequent to sulfation.

The proposed biosynthetic pathway is shown in Figure 5. The results obtained allow the following conclusions to be drawn about the biosynthesis of the chlorosulfolipids: (1)

TABLE VI: Incorporation of Labels into Diols.

Fraction	Sample	Total Counts	Total Lipid (%)
Upper-phase Folch	[1- ¹⁴ C]Laurate	2,156,000	9.8
	[16- ¹⁴ C]Palmitate	368,350	15.3
	[1- ¹⁴ C]Stearate	143,000	5.1
	[18- ¹⁴ C]Stearate	345,000	3.5
Solvolysis: ether layer	[1- ¹⁴ C]Laurate	1,450,000	6.6
	[16- ¹⁴ C]Palmitate	187,500	7.8
	[1- ¹⁴ C]Stearate	91,000	3.2
	[18- ¹⁴ C]Stearate	266,000	2.7

the carbon chain is biosynthesized using the normal fatty acid pathway; (2) the secondary hydroxyl group is put on the chain after the chain is fully synthesized *via* the hydration of a cis double bond; and (3) the fatty acid chains are chlorinated after the chains are fully synthesized.

References

- Aaronson, S., and Baker, H. (1959), *J. Protozool.* 6, 282.
 Elovson, J., and Vagelos, P. R. (1969), *Proc. Nat. Acad. Sci. U. S.* 62, 957.
 Elovson, J., and Vagelos, P. R. (1970), *Biochemistry* 9, 3110.
 Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem.* 226, 497.
 Gellerman, J. L., and Schlenk, H. (1972), *Lipids* 7, 51.
 Goren, M. B. (1971), *Lipids* 6, 40.
 Hager, L. P., Thomas, J. A., and Morris, D. R. (1970), in *Biochemistry of the Phagocytic Process*, Schultz, J., Ed., Amsterdam, North Holland Publishing, p 67.
 Haines, T. H. (1965), *J. Protozool.* 12, 665.
 Haines, T. H. (1971), *Progr. Chem. Fats Other Lipids* 11, 297.
 Haines, T. H., and Block, R. J. (1962), *J. Protozool.* 9, 33.
 Haines, T. H., Pousada, M., Stern, B., and Mayers, G. L. (1969), *Biochem. J.* 113, 565.
 Hollenberg, P. F., and Hager, L. P. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 31, Abstract 1581.
 Kusamran, K., and Polgar, N. (1971), *Lipids* 6, 962.
 Mayers, G. L., and Haines, T. H. (1967), *Biochemistry* 6, 1665.
 Mayers, G. L., Pousada, M., and Haines, T. H. (1969), *Biochemistry* 8, 2981.