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THE BIOSYNTHETIC PATHWAY OF THE CHLOROSULFOLIPIDS OF *OCHROMONAS DANICA*

by

CAROLYN L. MOONEY

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1973

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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INTRODUCTION

Ochromonas danica is a phytoflagellate, belonging to the Chrysophyta. A large ribbon-shaped chloroplast surrounds a leucosin vacuole and the cell's nucleus.[1] It is capable of living photosynthetically using CO_2 as its sole carbon source,[2] provided biotin is available.[3] It can also live heterotrophically, absorbing its food directly from its aqueous environment or phagotrophically, by consuming bacteria, for example.

A wide variety of lipids are found in *Ochromonas danica*. These include phospholipids, most of the common fatty acids (myristic, palmitic, linoleic, γ-linoleic, and arachidonic acids, in particular),[4] chlorophyll a,[5] β-carotene,[6] six sterols,[7] and two unique series of sulfatides which have been designated chlorosulfolipids. The chlorosulfolipids comprise 10 - 20% of the cell's lipids and 3% of the dry weight of the cell;[8] they contain up to 60% of the sulfur content of the cell.[9]

There are at least fourteen different sulfolipids in *Ochromonas danica*[8,10,11,12,13,14] (Fig. 1), comprising the two series. The first series has a C_{22} *n*-alkyl chain possessing sulfate esters at the first and fourteenth positions. The second series has a C_{24} *n*-alkyl chain, with sulfate esters at the first and fifteenth positions. As many as six chlorines may be substituted for hydrogens in members of each series to form the chlorinated

Chlorosulfolipids of Ochromonas danica

OSO_3^- OSO_3^-
22 14

OSO_3^- OSO_3^-
24 15

OSO_3^- OSO_3^-
Threo Cl

OSO_3^- OSO_3^-
Threo Cl

OSO_3^- OSO_3^-
Erythro Cl Cl

OSO_3^- OSO_3^-
(Cl_1) (Cl_1)

OSO_3^- OSO_3^-
(Cl_1) (Cl_2)

OSO_3^- OSO_3^-
(Cl_1) (Cl_2)

OSO_3^- OSO_3^-
(Cl_2) (Cl_2)

OSO_3^- OSO_3^-
(Cl_1) (Cl_3)

Cl OSO_3^- Cl Cl OSO_3^-
Cl Cl

Cl OSO_3^- OSO_3^-
Cl Cl Cl Cl

Cl OSO_3^- Cl Cl OSO_3^-
Cl Cl Cl

Cl OSO_3^- OSO_3^-
Cl Cl Cl Cl Cl

Figure 1.

derivatives of the parent docosanediol disulfate or tetracosanediol disulfate. In each series the chlorination sites are localized in the vicinity of the substituent sulfates.

The chlorosulfatides that have been isolated represent the only natural class of alkyl sulfates to date. Other types of sulfate esters have been found in natural sources, however. Some are sulfate esters of polysaccharides and other organic molecules. Compounds, including steroid sulfates, aryl sulfates and bilirubin sulfate, although lipoidal,are not generally considered sulfatides.

According to Haines,[14] the term "sulfolipid" applies to any lipid that contains sulfur. A sulfatide is a sulfolipid in which the sulfur is in the form of a sulfate ester; and a sulfonolipid is one in which the sulfur occurs in the sulfonic acid form. Of the sulfatides, only five others have been characterized at present. These are cerebroside sulfate in brain,[15,16] lactosyl ceramide sulfate of kidney,[17] ganglioside sulfate in hard tissues,[18] the diphytyl diether glycolipid sulfate of *Halobacterium cutirubrum*,[19] and the 2,3,6,6 -tetraester of 2 -trehalose sulfate.[20,21,22]

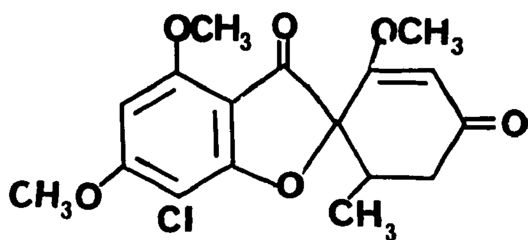
There is also another class of sulfolipids--the sulfonolipids. At the present time, the only sulfonolipids that have been characterized are deoxyglycosulfonates.

A chloroplast sulfonolipid, 6-sulfo-D-α-quinovopyranosyl-(1,1')-2',3',-di-O-acyl-D-glycerol is found in Ochromonas danica when grown under phototrophic conditions.[2]

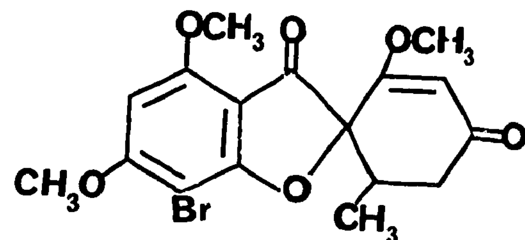
The uniqueness of the chlorosulfatides from Ochromonas danica also derives from the presence of chlorines on the alkyl chain. Although chlorinated compounds were considered to be rare natural products a few years ago, it now appears that the number of chlorine-containing metabolites is quite large. Over 170 chlorinated compounds have been identified, mainly from microbes. The actinomycetes are the richest source of covalently bound chlorine compounds. Included in the actinomycetes metabolites are many potent antibiotics such as chloramphenical, griseofulvin, and chlorotetracycline. The structures of these compounds are given in Figure 2. The minimum numbers for each known organo-halogen compound are: 7 for fluorine, 62 for bromine, and 14 for iodine. There are at least 9 compounds containing both bromine and chlorine. In addition to these, the bromine analogs of at least 20 structures are known.[29] Most alkaloids with chlorinated aliphatic side chains that have been reported from higher plants are suspected of being artifacts rather than natural products.[30] Recently, however, a chlorinated alkaloid has been isolated and identified and it is apparently a naturally occurring compound.[31]

In the cases mentioned above, the various chlorinated compounds find their chlorines substituted on relatively active positions, such as on phenolic rings and

GRISEOFULVIN
(PENICILLIUM)

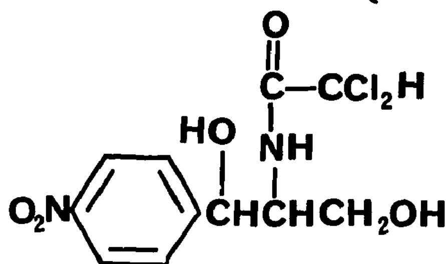


OXFORD, RAISTRICK and ŠIMONART, 1939 [23]

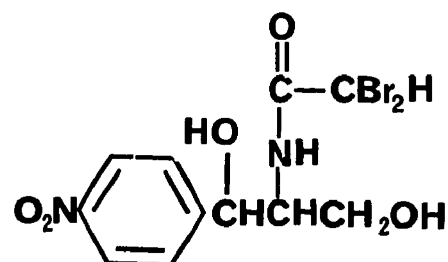


MACMILLAN, 1954 [24]

CHLORAMPHENICOL
(STREPTOMYCES)

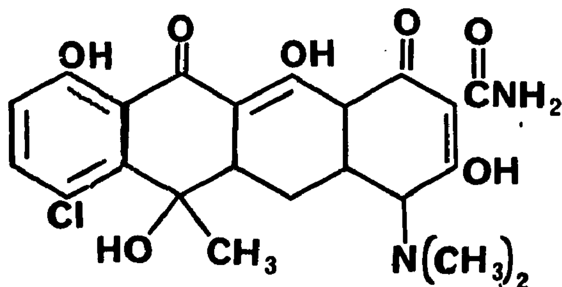


BARTZ, 1948 [25]

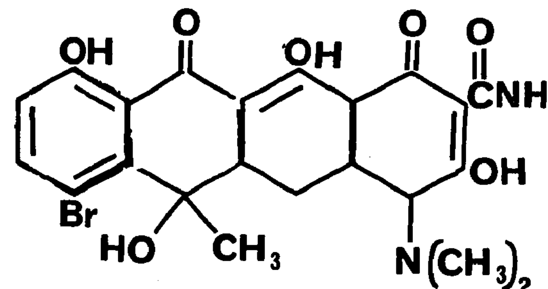


SMITH, 1958 [26]

TETRACYCLINE
(STREPTOMYCES)



DUGGAR, 1948 [27]



SENSI, DE FERRARI, GALLO and ROLLAND, 1955 [28]

Fig. 2. Chlorinated compounds found in nature.

on carbons adjacent to carbonyl groups. In contrast, the chlorosulfolipids have the chlorines located on relatively isolated positions.

While the chlorinated disulfates are the naturally occurring compounds, it is possible to grow the organism under relatively halide-free conditions and obtain the non-chlorinated disulfates as the only disulfates present in the cell. The lack of chloride ion in the media prevented chlorophyll synthesis in the cell. The stimulation of photosynthesis by chloride ion is a well-known effect.[32,33,34] Under chlorine-free conditions, the cells are white and contain no chloroplasts. The chlorinated disulfates, however, are probably not connected to the photosynthesizing apparatus of the cell. When _Ochromonas danica_ is grown in the dark, it loses its chloroplast and becomes yellowish-white.[35] Provided that chlorine is freely present in the media of dark grown cells, the amount of chlorinated disulfates remains constant.[36]

It is also possible to substitute one halogen for another, that is, to substitute bromide ion for chlorine ion in the media. When bromide ion is present in the media, one obtains the corresponding brominated disulfates.[37]

Another unusual aspect of the chlorosulfolipids is their marked difference from other straight chain lipids. Unlike the phospholipids, the chlorosulfolipids possess substituent polar groups, not

merely at one end of the molecule, but close to the opposite end as well.

The function of these compounds in the cell is, at present, unknown. It might seem that a lipid, synthesized in such large amounts, should be present in membrane or associated with it in some manner. Indeed, the sulfolipids have been found to associate with a membrane fraction of _Ochromonas danica_.[38] These compounds cannot be incorporated into the bilayer theory of membrane structure without difficulty. Because of this, the presence of these compounds in membrane is indeed surprising.

An investigation of the biosynthesis of these unique compounds may provide an insight into the function of these lipids in the cell. Understanding of the biosynthetic pathway may allow the choice of a selective inhibitor of the production of chlorosulfolipids. The systematic analysis of any variations in _O. danica_ subsequent to its growth in the presence of an inhibitory substance may establish the role of the chlorosulfolipids in cellular processes or structures.

A knowledge of the biosynthetic pathway of these compounds may be helpful in elucidating the biogenesis of membranes. That the chlorosulfatides are present in membrane is consistent with their high concentration in the cells and their occurrence in the medium into which

the cells extrude vesicles of membrane.[39,40] By manipulating the biosynthesis of these compounds, one may in turn manipulate membrane formation.

Furthermore, this system is an attractive one for the study of hydroxy fatty acid formation, and of sulfation and chlorination of long chain compounds. The enzymes which are invovled in the biosynthesis of the compounds are interesting in their own right.

A general outline of the biosynthesis of the sulfatides is described in the work reported here. The carbon chain is biosynthesized using the normal fatty acid pathway. Saturated, even-numbered, straight-chain fatty acids are elongated and incorporated into the sulfolipids. The secondary hydroxyl group is put on the chain after the chain is fully synthesized, _via_ the hydration of a cis-double bond.

It is presumed that the sulfate is derived from 3'-phosphoadenosine-5'-phosphosulfate (PAPS), as this intermediate has been reported to be the intermediate in the biosynthesis of all the sulfate esters where the biosynthesis has been investigated.[41] Furthermore, it has been shown that PAPS is an intermediate in the _in vitro_ synthesis of the sulfatides.[42] The _in vitro_ work supplements _in vivo_ studies in which molybdate was shown to inhibit the biosynthesis of the sulfatides.[43] Wilson and Bandurski had originally shown that the PAPS synthesizing

enzymes (in particular, ATP sulfurylase) were effectively blocked by molybdate.[44]

Both nonhalogenated and hexahalogenated diols were found to be incorporated into the sulfatides of the organism, which is the expected result since PAPS sulfates hydroxyl groups.

The chlorination of the nonhalogenated sulfatide was shown to occur. The enzyme responsible for this chlorination is markedly different from the chloroperoxidase isolated by Hager *et al*.[45] The chloroperoxidase, isolated from the mold, *Caldariomyces fumago*, is the only enzymatic chlorinating system that has been investigated in depth to date. This system does not use a free-radical intermediate, since it chlorinates carbons adjacent to carboxyl groups, which are therefore activated. The enzyme operating in *O. danica* places chloro groups on a saturated carbon chain, a process which requires considerable energy. This suggests a more energetic free radical intermediate, possibly a hydroperoxide.

Intact incorporation of the various precursors was shown by degradation of the resulting monochlorodiol (sulfolipid). The procedure for degradation of the diol was carefully worked out and a method for visualization of the degradative products was established.

EXPERIMENTAL

Materials

1-^{14}C-Acetate (specific activity 28.0 mCi/mmole), 1-^{14}C-octanoate (specific activity 1.99 mCi/mmole), and 1-^{14}C-laurate (specific activity 28.8 mCi/mmole) were purchased from Calbiochem (Los Angeles, California). 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}C-Palmitate (specific activity 51 mCi/mmole), 1-^{14}C-myristate (specific activity 5mCi/mmole), 1-^{14}C-stearate (specific activity 46 mCi/mmole), and 18-^{14}C-stearate (specific activity 55 mCi/mmole) were purchased from New England Nuclear (Boston, Massachusetts). The radioactive purity of each was $>98\%$, $>99\%$, $>98\%$, and $>99\%$ respectively, as demonstrated by TLC and GLC. 1-^{14}C-Oleate (specific activity 52.2 mCi/mmole) was purchased from Applied Science Labs., Inc. (State College, Pennsylvania). 1-^{14}C-Oleate (specific activity 56 mCi/mm) was also purchased from New England Nuclear (Boston, Mass.). Their radioactive purity was $>99\%$, as demonstrated by TLC and GLC. 14-^{14}C-Erucic acid (specific activity 47.1 mCi/mm) was purchased from Schwartz/Mann and was $>98\%$ as demonstrated by TLC and GLC. 1-^{14}C-Laurate (specific activity 5.28 mCi/mmole) was purchased from ICN, Irvine, California. Its radioactive purity was $>99\%$ as

demonstrated by TLC.

All solvents were reagent grade; each was redistilled before use. In addition, dioxane, redistilled over $LiAlH_4$, was stored at 4°C under nitrogen.

All other reagents were analytical grade and were obtained commercially.

Erucinol and brassidol were synthesized by $LiAlH_4$ reduction of the corresponding acids, following the procedure of Nystrom and Brown.[46]

A Sorvall SS RC-2 Automatic Refrigerated Centrifuge was used for all centrifugation.

Cultures

Ochromonas danica was grown axenically in a chemically defined medium (see below) at 26° under constant illumination of 125 - 150 ft-candles of light. In the case of chloride-free media, fifteen generations of growth in fresh media were necessary before a good preparation of halogen-free sulfatides could be obtained.

Media

The cells were grown on a chemically defined medium[47] (pH = 4.5). Unless otherwise stated, the chloride ion concentration of the medium was 0.0133M. This is the normal chloride ion concentration. For some experiments,

the chloride ion concentration was increased to 0.11M. This concentration was chosen to maximize the amount of hexachloro-sulfatides produced while the growth rate was normal. At the higher chloride ion concentrations, the growth was somewhat slower than normal.

A halogen-free medium was developed to obtain a preparation of halogen-free sulfatide. The composition of this medium is given in Table I. It is essentially the medium of Aaronson and Scher,[48] with chloride ion removed. The usual 0.0133M chloride ion concentration was replaced by sulfate ion to maintain the ionic strength.

Incubations

The radioactive 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}C-acetate (20 μCi) - 0.071 mM; 1-^{14}C-octanoate (20 μCi) - 0.112 mM; 1-^{14}C-laurate (50 μCi) - 0.179 mM; 1-^{14}C-myristate (50 μCi) - 0.143 mM; 16-^{14}C-palmitate (50 μCi) - 0.098 mM; 1-^{14}C-stearate (50 μCi) - 0.091 mM; 1-^{14}C-oleate (50 μCi) - 0.0087 mM; and 14-^{14}C-erucate (16 μCi) - 0.033mM.

Octanoate, laurate, palmitate, and stearate were added to the medium preceding inoculation and incubated for five days. For the other acids, each 10 ml test tube was inoculated with 1 drop of *O. danica* in the logrithmic

TABLE I: Defined media for the ahalo growth of *Ochromonas danica*.

KH_2PO_4	3.00g
$MgSO_4 \cdot 7H_2O$	10.00g
$MgCO_3$	4.00g
(Ethylenedinitrilo)-tetraacetic acid	2.00g
$CaCO_3$	0.50g
L-Glutamic Acid	30.00g
Thiamine mononitrate	0.01g
Dextrose	100.00g
L-Histidine	3.22g
L-Arginine	3.33g
Biotin	0.1 mg
Metals* mix	0.10g
$(NH_4)_2SO_4$	6.14g
Na_2SO_4	2.84g
pH	4.5
deionized water to 10 liters	

* Metals mix: contains the following achloro salts - $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 19.9992g; $ZnSO_4 \cdot 7H_2O$, 9.9991g; $MnSO_4 \cdot H_2O$, 5.0001g; $CuSO_4 \cdot 5H_2O$, 0.7988g; $CoSO_4 \cdot 7H_2O$, 1.0010g; H_3BO_3, 1.0002g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4978g; $Na_3VO_4 \cdot 16H_2O$, 0.1006g.

phase of growth and allowed to grow for two days before addition of precursor, after which it was allowed to grow for 3 days.

In the time study using 1-^{14}C-laurate, three simultaneous inoculations were performed. Eleven hundred ml of medium were divided equally among three Fernbachs, and each was inoculated with 30 ml of Ochromonas danica. The concentration of 1-^{14}C-laurate in each Fernbach was 0.017 μM.

For the tolerance studies, the compounds were dissolved in either absolute ethanol, or a 10% solution of Tween 80 in absolute ethanol (v/v). Detergents similar to Tween 80 were found to have no effect on the growth of O. danica, in the concentrations used.[49]

The diols and nonhalogenated sulfatides were added dissolved in 80% ethanol. The final maximum concentration of ethanol in the media was 1%. The diols and nonhalogenated sulfatides were added to cultures of approximately two days' growth. These compounds were incubated for three days.

Isolation of alkyl diols and alkyl disulfates

The isolation of the chlorodiols and the chlorosulfatides were conducted as described below. C^{14}-labeled diols were further purified by TLC before incubation with cells. C^{14}-Labeled sulfatides were checked for purity by solvolysis of the sulfatides and TLC of the resulting diols.

Folch Extraction (Figure 3).

Cells were harvested by centrifugation at 9000g for ten minutes at 4°C. The medium was decanted and stored frozen. The cells were washed twice with deionized water by suspension and centrifugation as before. Aqueous washes were stored frozen. The cells were then extracted with 20 volumes chloroform-methanol (2:1, v/v) by the method of Folch *et al.*,[50] and centrifuged. The extracts were decanted and partitioned against 0.2 volumes of deionized water. The entire procedure was conducted under nitrogen to protect the polyunsaturated fatty acids from oxidation. The lower phase contains most of the lipid material of the cells. The sulfolipids appear in the *upper phase* of the partitioned Folch extract. The lower phase was stored under nitrogen in the cold. The contents of the upper phase were taken to dryness *in vacuo*. The sulfate esters of the crude sulfolipids in the residue were then cleaved by solvolysis, or hydrolysis.

Solvolysis

The procedure used is essentially that of Mayers *et al.*[11] 10 ml of 99% aqueous dioxane was added to the crude sulfolipid obtained from 10 ml of log phase *O. danica*, and the solution refluxed for ½ hour. Then enough water to make a 98% aqueous dioxane solution was added and reflux continued for another ½ hour. At this point, the excess water was removed as the 82.8°C constant-boiling azeotrope

FOLCH EXTRACTION OF HALOSULFOLIPIDS

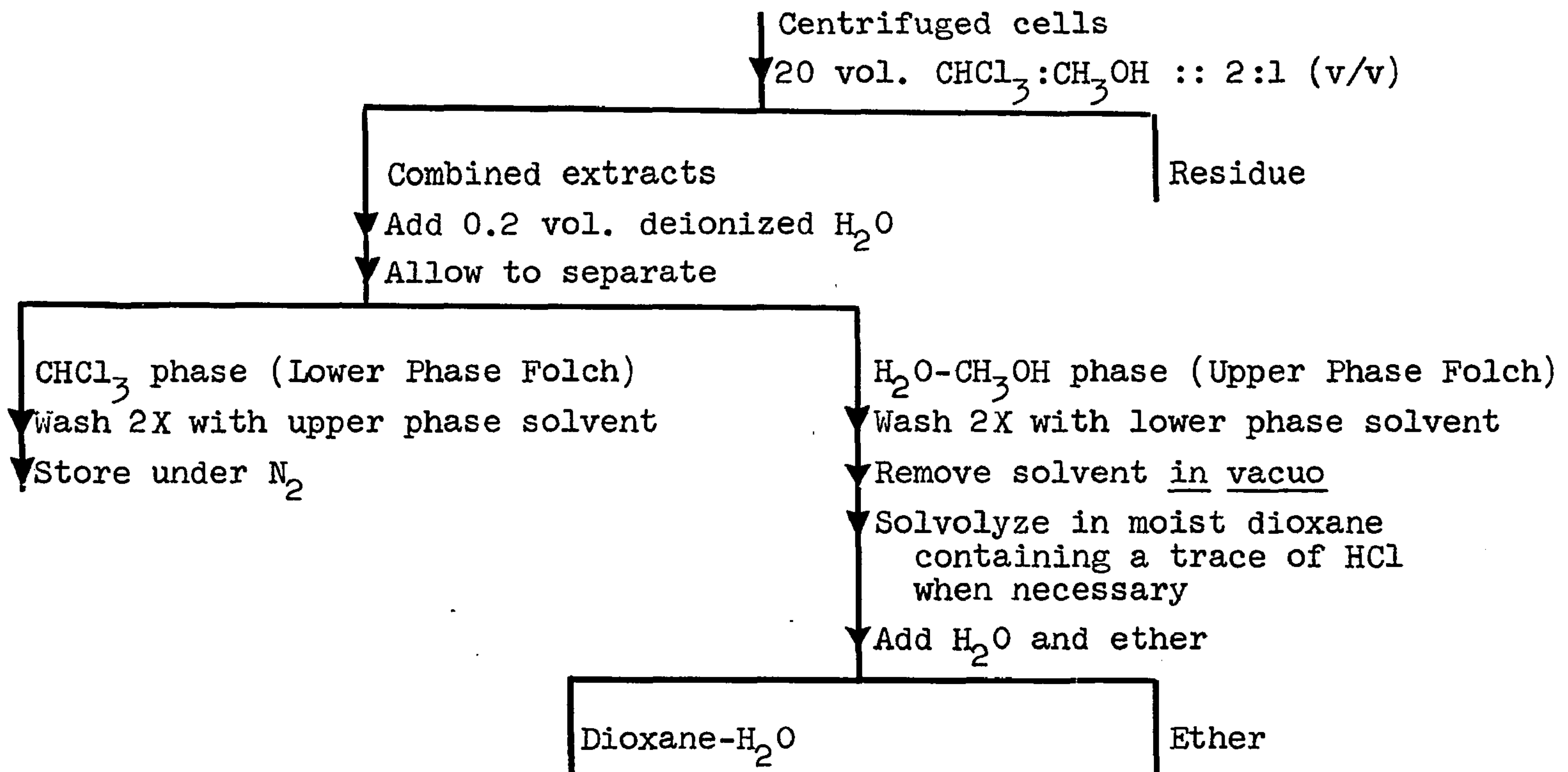


Figure 3.

of dioxane/water. The reaction mixture was cooled and 10 ml of water added. The water solution was extracted 5 times with 10 ml of diethyl ether. The combined ether extracts were washed 3 times with 5 ml of water. The ether solution was dried over anhydrous magnesium sulfate and the solvent removed *in vacuo*.

When solvolysis was conducted on micro amounts of sulfate esters, it was 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 obtain consistent solvolysis products.[51]

Hydrolysis

The residue to be hydrolyzed was placed in 25 ml of 1N hydrochloric acid and the mixture was refluxed for 2 hours. The solution was cooled and made basic with 6N potassium hydroxide. The basic reaction mixture was extracted with diethyl ether. The diethyl ether solution was washed with water until neutral, dried over anhydrous magnesium sulfate, and evaporated *in vacuo*.

A second method which proved useful for obtaining diols was the following: The residue of crude sulfolipids was hydrolyzed at 100°C for 60 min in a mixture of 3 ml of water, 10 ml of dioxane, and 1.5 ml of concentrated hydrobromic acid, per gram or less of residue. After cooling,

the mixture was transferred to a separatory funnel, diluted with one-half volume water, and extracted three times with one-half volumes of ether. The ether extracts were washed until neutral with distilled water, dried over magnesium sulfate, and taken to dryness to yield the crude chlorodiol mixture.

Radiocounting

A Nuclear Chicago Scintillation Counter Model No. 724 and a Nuclear Chicago Automatic Planchet Counting Gas Flow System with a D-47 Geiger-Muller counter were used for radiocounting.

Aliquots of 5 µl and 10 µl were taken at each step in the Folch extraction and solvolysis procedures. Some aliquots were spotted on planchets for Geiger-Muller counting. Others were dissolved in solutions of Spectraflor and toluene prepared according to manufacturer's specifications for liquid scintillation counting. For more aqueous solutions, Bray's solution[52] was used to effect complete solubility.

Chromatography and autoradiography

Thin-layer chromatography was conducted in two dimensions using Silica Gel F-254 on either glass plates or plastic sheets purchased from Brinkmann Instruments, Inc. For one-dimensional TLC, the chromatograms were prepared using Supelcosil 12B purchased from Supelco, 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/v), and then vertically again in the ether-hexane solvent.

The developing solvent for the sulfolipid was chloroform-methanol-water (65:33:1, v/v/v).

The cleavage products from the degradation procedure were separated on TLC using ether:hexane (3:7, v/v) as the developing solvent.

For autoradiography, each chromatogram was sprayed with Omnispray Image Intensifier for Autoradiography (2,5-diphenyloxazole PPO in isopropanol), and then exposed to X-ray film in the dark at $-16^{o}C$ or less, for a period of several days to several weeks, depending on the amount of radioactivity spotted on the thin-layer plate. The autoradiograms were developed using standard photographic techniques.

Compounds were visualized in various ways. One method involved the charring of the compound. A solution of 25% sodium bisulfate (w/v) containing 3% H_2SO_4 (v/v) was sprayed on the plate. The plate was then heated slowly on a hot plate until charring occurred. Compounds were also visualized by spraying with 0.1% 2,7-dichlorofluorosceine in methanol and viewing under ultraviolet light. For aldehyde products, a solution of 1% 2,4-dinitrophenylhydrazine (w/v) in 2N hydrochloric acid in

75% ethanol (v/v) was used for visualization. The chromatograms were also visualized with iodine vapors.

When necessary, the appropriate visualized zones were either scraped directly into scintillation vials or into scintered glass filter funnels for elution with ether or methanol.

Degradation of labeled chlorohydrins

The procedure used is essentially that of Kusamran and Polgar.[53] 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, backwashed 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 hour. Five ml of this suspension were added to the labeled epoxide in ether and stirred vigorously for one hour. An equal volume of water was added and separated. The ether was backwashed, dried, and evaporated as before.

Ozonolysis

Brassidol was ozonized in order to obtain standards for the periodate cleavage reaction. The procedure of Schlenk and Gellerman was followed.[54] Samples of 5 - 40 mg were dissolved in 2 ml of methanol, in a rimless test tube

which fit into the oxidation vessel of the Bonner semi-micro ozonizer.[55] The ozonizer was flushed with O_2 for 1 hour at a flow rate of 80 ml/min before turning on the high voltage. Sample and outer tube were cooled to -10°C. The tubes were attached to the ozonizer after it had been equilibrated for more than 5 min. The substance was ozonized for approximately 1 min/20 mg/double bond. The sample tube was removed and excess O_3 was immediately carried away by N_2 at 0°C. About 200 mg of Lindlar's catalyst was added.[56,57] The suspension was bubbled for 10 min at 0°C with H_2. The catalyst was removed by filtration.

RESULTS

Biosynthesis of the alkyl chain

All of the ^{14}C-fatty acids were incorporated into the sulfolipids of *O. danica*. This was confirmed in each case by a two-dimensional autoradiogram of the solvolyzed disulfates (diols). An example of the pattern obtained in this procedure is shown in Figure 4. Similar autoradiograms were obtained by incubating each of the labeled precursors with cells and following the same procedure.

Only acetate was incorporated into the sterols. Radioactivity from the long-chain fatty acids which was incorporated into the sterols was probably there because of breakdown and *de novo* synthesis. Acetate, as well as intermediate and long-chain fatty acids were incorporated into the sulfolipid fraction of the cell's lipids.

The relative distribution of the radioactivity incorporated into the various lipid fractions after the 1-^{14}C-acetate feeding was summarized in Table II. 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 phase, all the radioactivity was found with the diols in the ether phase.

The distribution data obtained from the

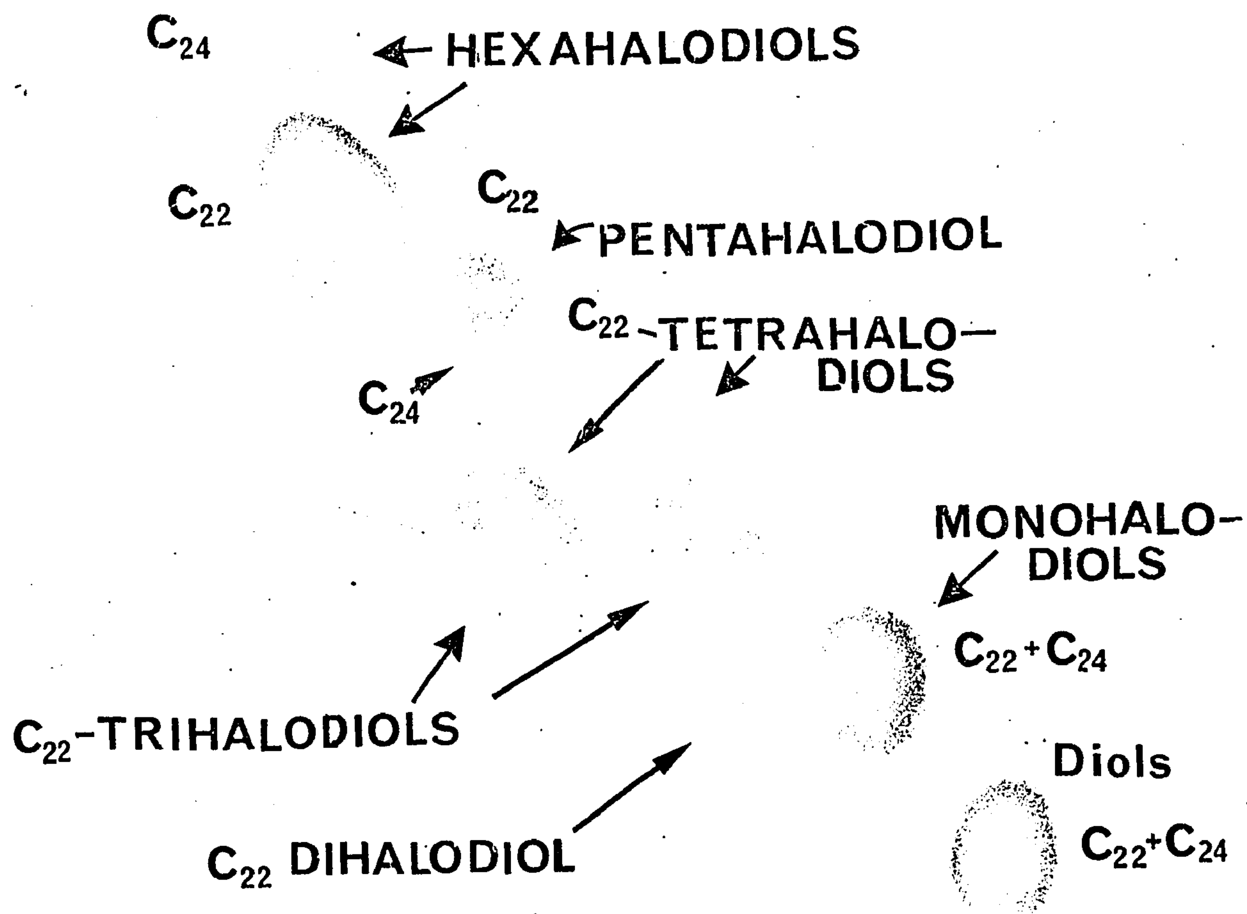


Fig. 4. Autoradiogram of halodiols obtained from solvolysis of sulfolipids in the 1-^{14}C-laurate study.

TABLE II: Incorporation of $1-^{14}C$-acetate

Fraction	Total Counts	Percentage
Total Lipid	371,500	100%
Lower-phase Folch (Phospholipids, etc.)	245,900	66%
Upper-phase Folch	77,300	21%
Solvolysis: H_2O Layer	0	0%
Solvolysis: Ether Layer	82,000	22%

1-^{14}C-laurate feeding is summarized in Table III. The incorporation data was 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 was due to incomplete solvolysis of the sulfolipids. Repeated solvolyses and hydrolyses were necessary for complete recovery of diols. The dioxane-water layer was checked by TLC each time and found to contain only radioactive sulfolipid.

The distribution of radioactivity in the lipids of *Ochromonas danica* after growth in media supplemented with 16-^{14}C-palmitate is summarized in Table IV. The pattern was somewhat similar to that observed for the 1-^{14}C-laurate feeding. A higher percentage (12.9%) of radioactivity appeared in the sulfolipids of cells from the palmitate study, when compared to the laurate feeding. The palmitate feeding had incorporation data similar to that of the laurate feeding which indicated that the general trend of incorporation is correct, and that palmitate may perhaps be better incorporated than laurate into the sulfolipid.

The distribution of radioactivity in the lipids of *O. danica* after growth in the presence of 1-^{14}C-stearate was compared with that after growth in the presence of 18-^{14}C-stearate in Table V. Both sets of data exhibited the same general trend observed for the laurate and palmitate feedings. Significantly, the extent of incorporation of the differently labeled stearates into the sulfolipids was

TABLE III: Incorporation of 1-^{14}C-laurate

Fraction	Total Counts	Percentage
Total Lipid	22,000,000*	100%
Lower-phase Folch (Phospholipids, etc.)	19,360,000	88.0%
Upper-phase Folch	2,156,000	9.8%
Solvolysis: H_2O Layer	324,000	1.5%
Solvolysis: Ether Layer	1,450,000	6.6%

* Value based on total of upper and lower phase Folch.

TABLE IV: Incorporation of 16-^{14}C-palmitate

Fraction	Percentage
Total Lipid	100%
Lower-phase Folch (Phospholipids, etc.)	86.0%
Upper-phase Folch	12.9%
Solvolysis: H_2O layer	0.9%
Solvolysis: Ether layer	7.7%

TABLE V: Incorporation of 1-^{14}C-stearate and 18-^{14}C-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_2O Layer	1-^{14}C-Stearate	45,000	1.6%
	18-^{14}C-Stearate	40,000	0.4%
Solvolysis: Ether Layer	1-^{14}C-Stearate	91,000	3.2%
	18-^{14}C-Stearate	266,000	2.7%

essentially equivalent in the two independent feedings. This finding lent support to the hypothesis that saturated, even-numbered, straight-chain fatty acids are incorporated intact into the sulfolipids, without degradation of the compound fed and subsequent _de novo_ synthesis of a new aliphatic chain. Proving the hypothesis, however, required the degradation and analysis of a sulfolipid.

Table VI shown the results obtained from the degradation of the monochlorodiols after the incorporation of various saturated 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 shows that the maximum amount of random incorporation of acetate units into the chain was under 10% after five days of incubation. The low value of counts obtained in the 18-^{14}C-stearate experiment is presumably due to the volatility of nonanal (I) and to the low level of activity in the isolated chlorohydrin.

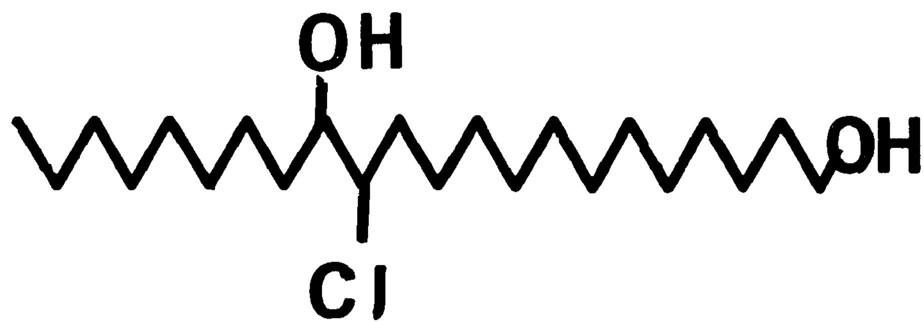
Occurrence of the sulfatides in the medium and in the cell residue after extraction.

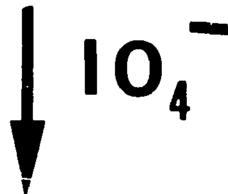
Earlier work[9,58] had indicated that the sulfatides are excreted into the medium of the culture. The culture medium was therefore hydrolyzed and extracted with ether.

TABLE VI: Cleavage of ^{14}C-labeled monochlorodiols obtained from three radioactive studies.

	Fragment[a]	Incorporation of 1-^{14}C-acetate			Incorporation of 1-^{14}C-laurate			Incorporation of 18-^{14}C-stearate		
			Percentage			Percentage			Percentage	
		Counts	Actual	Expected	Counts	Actual	Expected	Counts	Actual	Expected
First Experiment	I	205	28%	36%	0	0%	0%	100	100%	100%
	II	525	72%	64%	2870	100%	100%	0	0%	0%
Second Experiment	I				125	0%	6%			
	II				2300	100%	94%			

[a]Fragment I is nonanal; Fragment II is 13-hydroxytridecanal.





I

II

In each case a pattern similar to that in Figure 4 was obtained, confirming that the sulfatides were synthesized from the labeled precursor and excreted into the medium.

Furthermore, the residue which was left after the extraction of cells according to the method of Folch[50] was also subjected to the hydrolysis procedure. The ether layer obtained from the hydrolysis was chromatographed and the autoradiogram obtained showed a typical diol pattern, indicating that some small portion of sulfolipid remains tightly bound to the cell residue even after lipid extraction.

Hydroxylation Mechanism

When 1-^{14}C-oleate was fed to the organism, the sulfatide fraction became labeled. This result was first obtained from the work of M. Pousada. Since this monounsaturated fatty acid can be activated and incorporated into the sulfolipids, it appears that the hydroxylation occurs by the hydration of a double bond rather than by the direct incorporation of oxygen into the fully formed alkyl chain.

In view of the results with oleate, it was of interest to know whether, when hydroxylation occurs, the chain is at its final length or at some shorter stage. The results obtained when ^{14}C-oleate was fed to the organism in the presence of other long-chain saturated and unsaturated fatty acids are listed in Table VII. It appears that the incubation period is not sufficiently lengthy for the cells

TABLE VII: Incorporation of 1-^{14}C-oleate in the presence of other fatty acids.

Non-radio-active fatty acid	Erucic		Behenic		Linoleic	
	CPM	%	CPM	%	CPM	%
Total Lipid	998,000	100	1,585,000	100	2,640,000	100
Lower-phase Folch	859,000	86.0	1,291,000	81.5	2,284,000	86.4
Upper-phase Folch	27,900	2.8	70,000	4.4	86,000	3.3
Solvolysis:						
H_2O layer	3,200	0.3	5,300	0.3	2,300	0.1
Ether layer	23,100	2.3	60,000	3.8	35,400	1.3

to regulate the biosynthesis of the various compounds involved to such a degree, that the differences in incorporation will be sufficiently large enough to be discernable.

The results obtained when 14-^{14}C-erucate was fed to the organism are shown in Table VIII. Various time periods of incubation were used. The extent of incorporation into the sulfolipids does not change over the time periods used (one, three, and five day periods). The data in Table VIII represents the mean of the three experiments. The actual incorporation into the sulfatides is small. This may reflect the inaccessibility of the enzymatic site to the long chain fatty acid. A degradation of the labeled monochlorodiols obtained from two-feedings was performed and the results are listed in Table IX, along with the results from the oleate feeding. Some degradation does occur, since the label does appear in the longer fragment. This amount represents approximately 35% breakdown, which is high. However, it still indicates that part of the chain is incorporated intact.

Sulfation of diols

Table X indicates the extent of incorporation of diols, both chlorinated and halogen-free, into the sulfatide fraction. The distribution of radioactivity into the various fractions seems to indicate that the diols do not serve as good precursors to the sulfatides. Most of the radioactivity remained in the lower phase for both the

TABLE VIII: Incorporation of 14-^{14}C-erucate

Fraction	Total Counts	Percentage
Total Lipid	10,000,000	100%
Lower-phase Folch (Phospholipids, etc.)	9,500,000	95.0%
Upper-phase Folch	200,000	2.0%
Solvolysis: H_2O Layer	4,000	0.04%
Solvolysis: Ether Layer	170,000	1.7%

TABLE IX: Cleavage of ^{14}C-labeled monochlorodiols obtained from radioactive unsaturated fatty acid studies.

1-^{14}C-Oleate

Fragment[a]	Counts	Actual Percentage	Expected Percentage
I	10	2	0
II	409	98	100

14-^{14}C-Erucate

	3-day			5-day	
	Counts	Actual Percentage	Expected Percentage	Counts	Actual Percentage
I	910	72	100	650	77
II	360	28	0	190	23

[a]Fragment I is nonanal; Fragment II is 13-hydroxytridecanal.

TABLE X: Incorporation of non-halogenated and hexahalogenated-diols.

Fraction	Sample	Total Counts	Percentage
Total lipid	ahalo diols	282,000	100
	hexachloro diols	140,000	100
Lower-phase Folch (diols)	ahalo diols	247,000	88
	hexachloro diols	135,000	96
Upper-phase Folch	ahalo diols	19,000	7
	hexachloro diols	12,500	8.9
Solvolysis:			
H_2O layer	ahalo diols	0	0
	hexachloro diols	0	0
Ether layer	ahalo diols	15,000	5
	hexachloro diols	4,500	3.2

achloro and hexachloro diols. The results indicate that a portion of the diols was sulfated by the organism. The sulfolipid fraction, the upper phase, was hydrolyzed to diols which were chromatographed on TLC (Figure 5). The lower phase of the Folch extract was also chromatographed and showed a diol pattern identical to that of the compounds fed to the organism. Furthermore, no evidence was found indicating conversion of either diol to fatty acids or sterols. Nor was evidence obtained which suggested direct chlorination of the diols.

The results listed in Table XI show the amount of diol that remains associated with the cell after washing. The hexahalogenated diol does not appear to be transported well.

<u>Chlorination of the nonhalogenated sulfatide</u>

In order to see if the nonhalogenated sulfatide could become chlorinated, it was necessary to prepare the labeled nonhalogenated sulfatide. The approach used to do this involved isolation of the labeled sulfatide from the organism. Special growth conditions were employed to avoid halogen contamination of the media. When the halide concentration in the media was at a minimum level, 1-^{14}C-myristate was fed to this chloride-free culture. The data of incorporation of 1-^{14}C-myristate into the various lipid fractions listed in Table XII indicate normal incorporation into the various fractions. The labeled sulfatides were

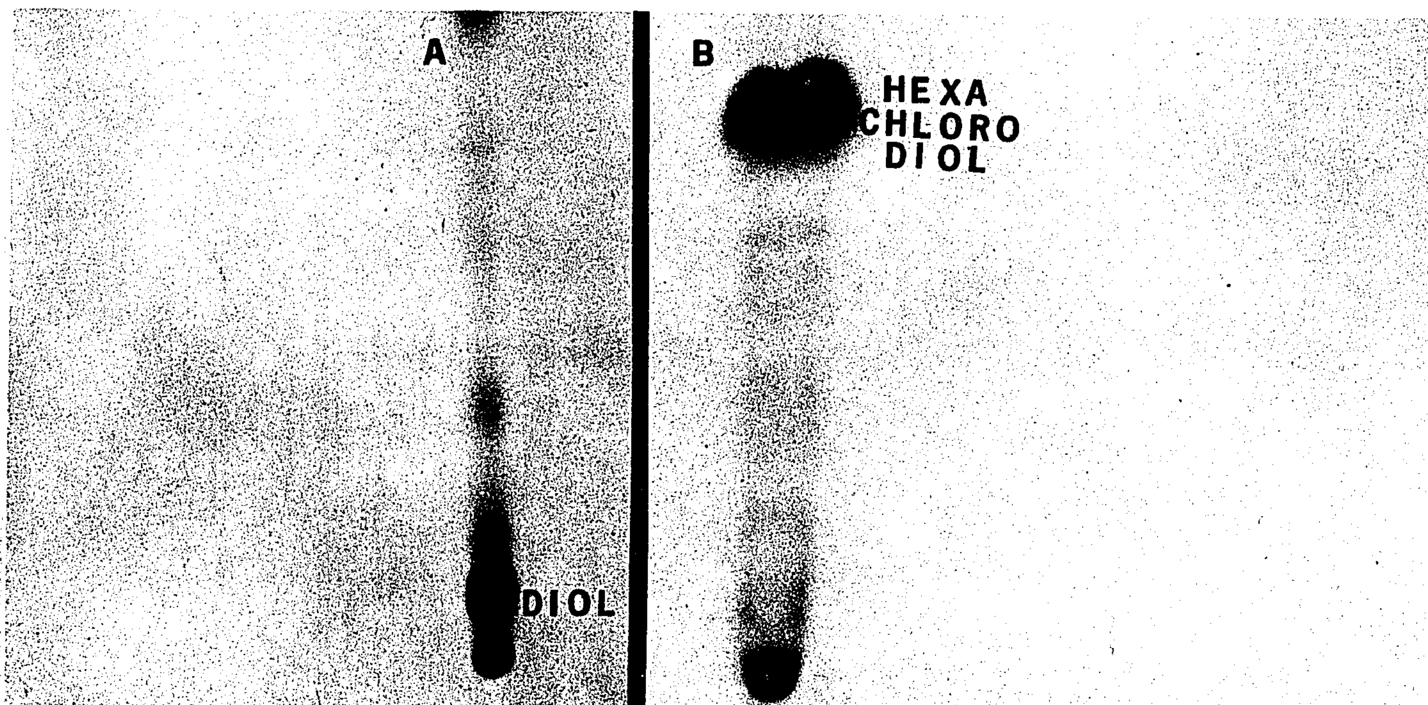


Fig. 5. Autoradiogram of a thin layer chromatogram of diols obtained by the hydrolysis of sulfatides isolated from O. danica after two separate feedings. (a) [^{14}C] Labeled achloro diols were incubated with the organism cultured in "normal" (with respect to halogen concentration) medium. (b) [^{14}C] Labeled hexachlorodiols were incubated with the organism grown under normal conditions.

TABLE XI: Incorporation of diol into the cell.

Diol		Total fed to cells (cpm)	Associated with cells (cpm)	% of total
Nonhalogenated	1.	85,000	45,000	53
"	2.	900,000	625,000	69
"	3.	480,000	250,000	52
"	4.	400,000	280,000	70
Hexahalogenated	1.	200,000	40,000	20
"	2.	780,000	140,000	18

TABLE XII: Incorporation of 1-^{14}C-myristate

Fraction	Total Counts	Percentage
Total lipid	88,651,000	100
Lower-phase Folch (phospholipids, etc.)	58,844,000	66.4
Upper-phase Folch	8,315,000	9.4
Portion of Upper-phase Folch used for hydrolysis	1,072,900	100
Hydrolysis-Ether layer	659,250	64.8

extracted following the usual procedure checked for the absence of halogenated sulfatides (Figure 6A). The data in Table XIII show the incorporation pattern obtained when the sulfatide fraction was extracted. Hydrolysis was performed on the sulfatide fraction. The autoradiogram (Figure 6) indicates that the labeled sulfatide fed to the organism was essentially nonhalogenated and that the resulting sulfatide obtained was indeed chlorinated. Furthermore, the incorporation data of labeled sulfatide into the various lipid fractions indicate an overwhelming preference for the upper phase (the sulfatide fraction). The counts present in the lower phase could be explained by: 1.) the free fatty acid that was adsorbed onto the sulfatide when it was fed to the organism (Figure 6A); 2.) the small amount of sulfolipid that partitions itself in the lower phase; and 3.) the amount of non-halogenated sulfolipid that was hydrolyzed by the acidic medium during the refeeding experiment. The autoradiogram indicates dramatically that chlorination can occur after sulfation in the biosynthesis of the chlorosulfolipids.

Effect of fatty acids and alcohols on the growth of *Ochromonas danica*.

It was also of interest to study the maximum toleration levels of various long-chain fatty acids and alcohols. The data is listed in Table XIV. It appears that at least mMolar concentrations of the various compounds

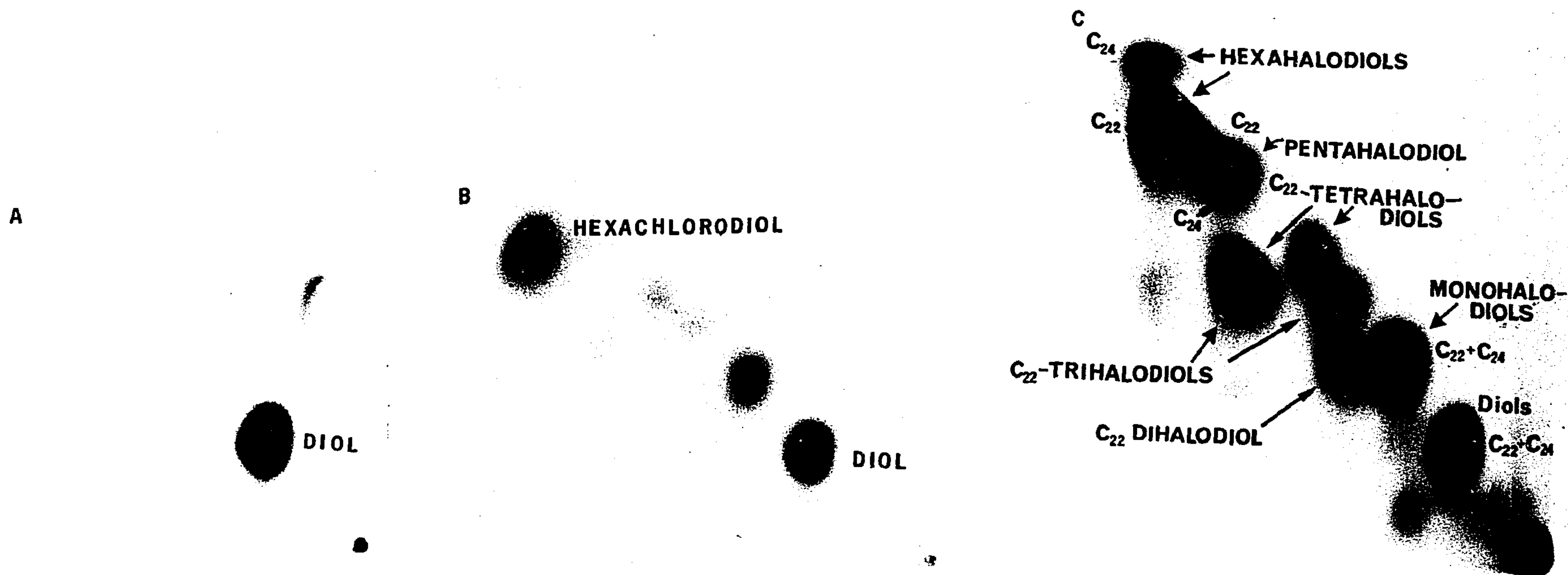


Fig. 6. Autoradiograms of 2-dimensional thin layer chromatograms of diols obtained by the hydrolysis of sulfatides isolated from O. danica. (a) [1-^{14}C] Myristate was incubated with the phytoflagellate cultured in halogen-free medium. (b) The sulfatide obtained from the above C^{14}-myristate feeding was incubated with O. danica cultured in enriched halide medium. (c) [1-^{14}C] Laurate was fed to the organism grown in normal halide medium. This is the normal diol pattern obtained from O. danica and is shown for comparison purposes.

TABLE XIII: Incorporation of 9-^{14}C-1,14-docosanediol 1,14-disulfate and 11-^{14}C-1,15-tetracosanediol 1,15-disulfate

Fraction	Total Counts	Percentage
Total lipid	986,000	100
Lower-phase Folch (Phospholipids, etc.)	77,000	7.8
Upper-phase Folch	811,400	83.2
Solvolysis:		
H_2O layer	0	0
Ether layer	527,900	53.5

TABLE XIV: Tolerance levels of various compounds in *Ochromonas danica*.

Compound	Toleration level(mM)	Toxic level(mM)
Lauric acid	30	60
Stearic acid	8.8	17.6
Behenic acid	0.3	*
Erucic acid	3.5	7.3
1,12-dihydroxyoctadecane	5.25	10.5
Docosanol	1.53	*
Erucinol	0.60	*
Brassidol	1.26	2.5

* not established

can be present in the culture before any non-specific, perhaps detergent, effects are seen. This is important because in adding radioactive compounds one wants to be sure that the cells are able to grow normally, so that normal biosynthetic pathways are used by the organism. The main problem with the fatty acid incubations is the difficulty in adding the compounds aseptically in a form in which they are soluble. Even if the compound is dissolved in the inoculum solution, it often precipitates out when added to the culture medium. Fatty acids, such as stearic and lauric acid, can be tolerated in very high concentrations.

Time study of incorporation of ^{14}C-laurate.

Table XV lists the extent of incorporation of 1-^{14}C-laurate incubated for three different time periods. The data indicates that extensive changes of incorporation patterns do not occur over this extended time course.

TABLE XV: Time study of incorporation of 1-^{14}C-laurate.

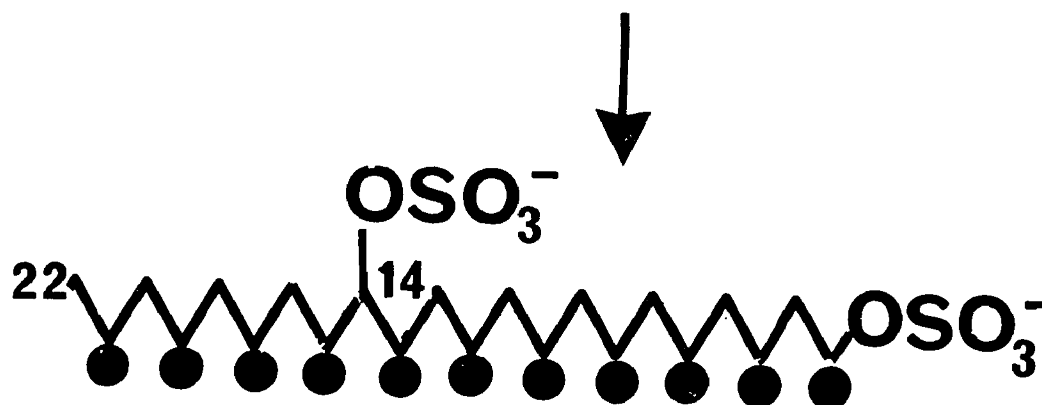
	Percentage		
Incubation period	1 day	3 day	5 day
Total lipid	100	100	100
Lower Phase Folch	92.0	88.8	75.0
Upper Phase Folch	5.5	8.3	12.0
Solvolysis: Ether Layer	5.0	7.1	10.0
H_2O Layer	0.2	1.1	2.1

DISCUSSION

The determination of the biosynthetic pathway of the chlorosulfolipids of *Ochromonas danica* involves establishing (1) how the alkyl chain is formed; (2) how the sulfate esters are synthesized; and (3) how the various chlorine substitutions on the alkyl chain occur. In addition, the sequence of these reactions must be elucidated in order to establish the biosynthetic pathway of the chlorosulfolipids.

When cells were grown in the presence of 1-^{14}C-acetate and 1-^{14}C-octanoate, the labels appeared in all the chlorodiols, and in the same relative distributions as the naturally occurring compounds, and therefore had been incorporated into all of the sulfolipids. Figure 7 shows the location of the acetate and octanoate labels in the C_{22} sulfolipid. The label was expected to appear on every second carbon in the sulfolipids of cells grown in the presence of 1-^{14}C-acetate, since the acetate molecule could serve both as the starting point for the synthesis of the chain and as a source of the C_2 fragments used in the sequential elongation of the chain.

The label should have appeared only on the eighth carbon from the distal terminus of the alkyl chain in the sulfolipids of cells grown in the presence of 1-^{14}C-octanoate, since the acetate units added on for elongation of the



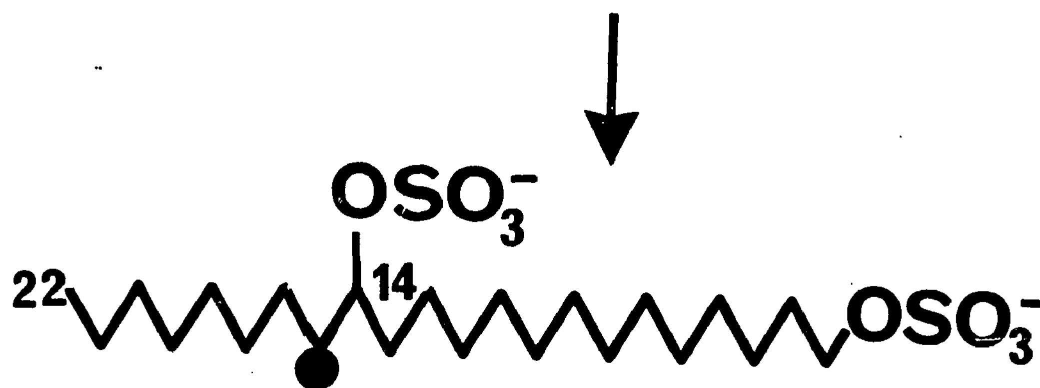


Fig. 7. Locations of 1-^{14}C labels of short chain precursors in 1,14-docosanediol-1,14-disulfate.

chain should not be labeled, assuming that the compound was not degraded and incorporated randomly.

Figure 8 shows the expected locations of the labels in the sulfolipids of cells grown in the presence of 1-^{14}C-laurate, 16-^{14}C-palmitate, 1-^{14}C-stearate and 18-^{14}C-stearate, assuming that longer chain saturated fatty acids are incorporated intact with the sulfatides. This route implies 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. The data presented in Tables III, IV, and V demonstrate that the label does appear in the sulfatides. Long-chain saturated fatty acids 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 V). The chlorodiol patterns obtained on two-dimensional chromatograms were also identical. These data, together with that obtained by Gellerman and Schlenk[59] on the elongation and conversion of stearic acid and other fatty acids in _O. danica_, suggest that the fatty acids are incorporated intact. In their experiments the fatty acids were shown to be converted directly by elongation and desaturation to

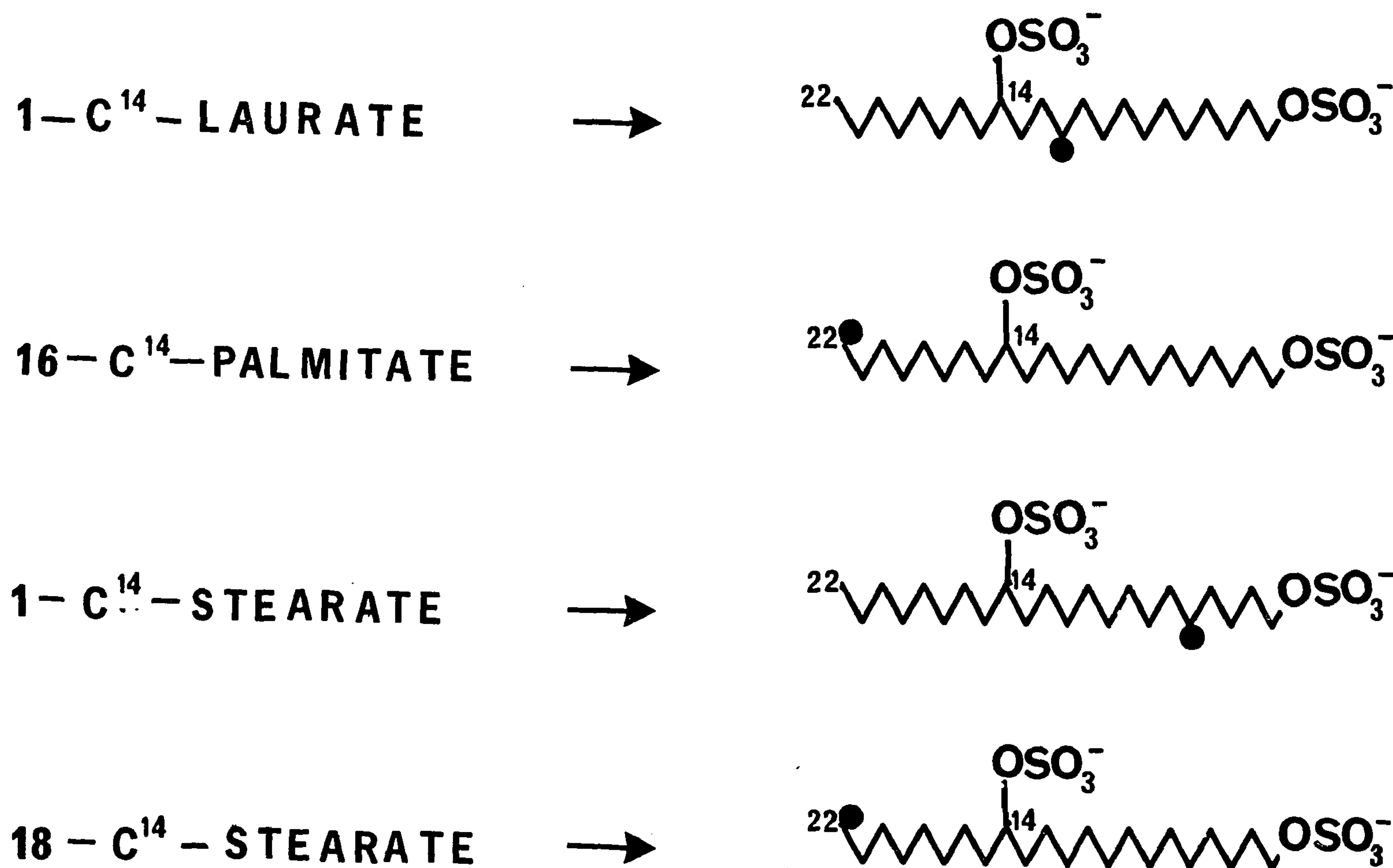


Fig. 8. Locations of ^{14}C labels of long chain fatty acid precursors in 1,14-docosanediol-1,14-disulfate.

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 to ours (their three-day incubations were somewhat shorter than our five-day incubations).

To unequivocally establish the intact incorporation of the long chain fatty acid into the sulfolipid, a degradation of the monochlorodiols from the radioactive feedings was performed. The location of the 1-^{14}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-^{14}C-stearate label should have appeared in II, whereas, ideally, no radioactivity should have been found in I. In contrast, the 1-^{14}C-octanoate, 16-^{14}C-palmitate, and the 18-^{14}C-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-^{14}C-acetate. The results obtained from the degradations are shown in Table VI 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 is summarized in Table XVI.

TABLE XVI: Incorporation of labels into diols.

Fraction	Sample	Percentage of Total Lipid
Upper-phase Folch	1-^{14}C-Laurate	9.8
	16-^{14}C-Palmitate	12.9
	1-^{14}C-Stearate	5.1
	18-^{14}C-Stearate	3.5
Solvolysis: Ether Layer	1-^{14}C-Laurate	6.6
	16-^{14}C-Palmitate	7.7
	1-^{14}C-Stearate	3.2
	18-^{14}C-Stearate	2.7

Differences in the extent of incorporation of the different labels into the sulfolipid are noticed. The normal free fatty acid composition of O. danica may account for the variations observed.

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 ^{14}C-oleate feeding (Table IX).

The mechanism operative in O. danica whereby the double bond of the fatty acid is converted to the secondary hydroxyl group may be similar to that operative in a

pseudomonad, as outlined by Schroepfer and his group.[60] In this organism, oleic acid is converted to 10-D-hydroxystearic acid. The mechanism involves a stereospecific addition of water across the double bond of oleic acid. It is reasonable to propose a mechanism for the formation of the diols which involves the enzymatic addition of water to a *cis*-double bond present in the correct position in a fatty acid.

With this hypothesis a study on the mechanism of hydroxylation of the fatty acids destined to be sulfolipids was made. It was of interest to know whether, when hydroxylation occurred, the chain was at its final length or at some shorter stage. It was known that oleic acid could be incorporated into the sulfatides but whether the hydroxylation occurred at this length or after chain elongation occurred was not known. It was reasoned that, if the double bond was hydrated at the erucic acid level, addition of erucic acid in the presence of labeled oleate should decrease the amount of radioactivity incorporated into the sulfatide fraction relative to a normal labeled oleic acid feeding. On the other hand, if the hydration of oleic acid occurs, the addition of linoleic acid to the culture should increase the incorporation of oleic into the sulfatide fraction. This would result because the oleic acid that might be diverted into the biosynthesis of the polyunsaturated fatty acids would now be available for sulfolipid biosynthesis instead, since linoleic acid can now

serve as the substrate for further desaturations. If erucic acid is the acid that is hydroxylated, its presence should decrease the extent of incorporation of oleic acid into sulfolipid. If oleic acid is hydroxylated, then the presence of linoleic in the culture should increase the oleic acid incorporation. Behenic acid (a C-22 acid) was added as the control culture, since it is a long chain fatty acid and yet is not involved in sulfolipid biosynthesis. It appears, however, that the desired results are unobtainable using this approach. Perhaps if the cultures had been preincubated with the respective non-radioactive fatty acid, to allow the cells to adjust their biosynthetic apparatus, the results may have been different.

To finally establish whether elongation to C_{22} or C_{24} occurs before or after hydration, a feeding of labeled erucic acid was done. The data in Table VIII indicates that erucate can indeed be activated and incorporated into the sulfatides.

Assuming that the hydroxy compound is formed by the addition of water to the _cis_-double bond, there are two possible modes of addition which would yield the final product. 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 the fourteen carbon in the C_{22} series of sulfolipids. The substitution of the -OH on the tenth carbon from the $-CH_3$ terminus would be appropriate for the formation of the sulfate ester

at the fifteen carbon in the C_{24} series of sulfolipids. Elongation to C_{24} can occur either before hydration or after it. This mechanism is shown in Figure 9.

The final steps in the biosynthetic route of the chlorosulfolipids involve the sulfation and chlorination reactions followed by _O. danica_.

All the sulfolipids contain a sulfate at the proximal terminus of the alkyl chain--the last segment to be added to the chain. Hence, the sulfate ester on the 1-carbon of a sulfolipid is formed only after synthesis of the carbon skeleton has reached either twenty-two or twenty-four carbons. The appearance of radioactivity in the entire series of chlorodiols in each study indicated that elongation of the _n_-alkyl chain is completed, or nearly so, before any substitution on the chain is made. In addition to the sulfate ester in the latter half of the chain, a second sulfate ester and as many as four chlorines appear as substitutions at sites within the first half of the chain synthesized. None of the 1-^{14}C-laurate, 16-^{14}C-palmitate, 1-^{14}C-stearate, and 18-^{14}C-stearate labels would be expected to appear in the unchlorinated disulfates if the sulfate ester in the first half of the chain was formed during the reactions incorporating C_2 fragments into that part of the chain. Similarly, none of the labels would be expected to appear in the chlorinated disulfates if chlorination of the distal portion of the chain occurred

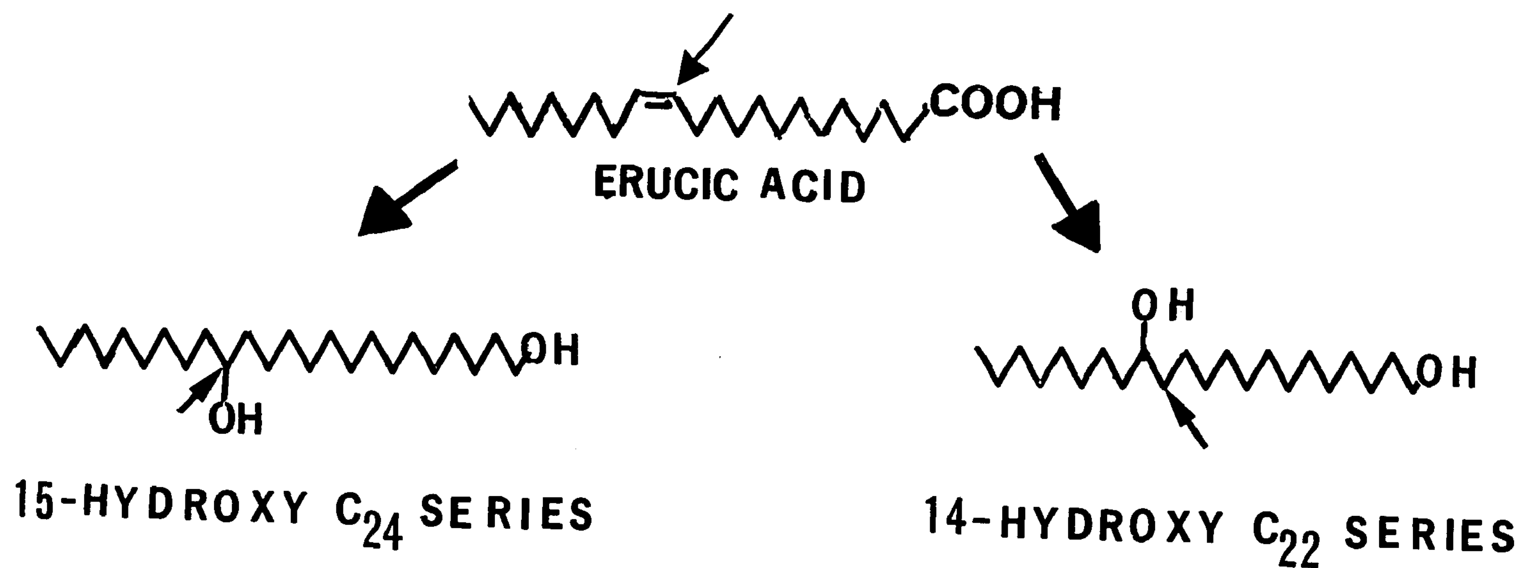


Fig. 9. Mechanism for placing secondary hydroxyl on alkyl chains of C_{22} and C_{24} sulfolipids. Arrows indicate hydroxylation site in the precursor erucic acid.

during the reactions incorporating C_2 fragments into the first half of the chain. However, the detection of radioactivity in all the aliphatic diols solvolyzed with retention of configuration[11] from the sulfolipid mixtures in each of the radioactive studies proved that the full-length alkyl chain is the substrate in sulfation and chlorination.

The reactions entailed in sulfation of the alkyl chain are postulated to involve hydroxy intermediates. The formation of the secondary hydroxyl is discussed above. The COO^- group of the last segment incorporated into the chain is probably reduced to $--CH_2OH$ before the sulfate ester on the 1-carbon is formed. The mechanism of sulfation probably involves 3'-phosphoadenosine 5'-phosphosulfate (PAPS), since, in other organisms, sulfate is transferred from PAPS to a hydroxyl group. Work in this laboratory on molybdate inhibition of sulfolipid biosynthesis seems to implicate PAPS as the sulfating agent used in the biosynthesis.[43] Therefore, sulfation presumably occurs by the enzyme-mediated transfer of the sulfate group of PAPS to the primary and secondary hydroxyl groups of the fully formed alkyl chain. Because of the differences in a terminal hydroxyl and a secondary hydroxyl located in the middle of a chain, the possibility exists of there being two different sulfating enzymes.

The amount of diol transported into the cell depends upon the extent of chlorination of the chain (Table XI). At least half of the nonhalogenated diols

supplied in the medium are transported into the cell. The ease of incorporation of nonhalogenated diol approaches that of fatty acid incorporation into _O. danica_. The hexa-halogenated diol was not incorporated well. At most, only 20% of that which was supplied in the medium was incorporated into the lipids of _O. danica_.

It appears, however, that both hexachlorodiol and the halogen-free diol can be sulfated (Table X). In some cases, radioactive diol was found in the upper phase. This presumably is due to the detergent effects of the sulfo-lipid on the diol. The actual amount of diol involved is small, however.

Sulfation also occurs to the same extent on both diols. Therefore the extent of chlorination of the alkyl chain seems to have little or no effect on the sulfate transfer. While halogenated diols can be converted to sulfate esters, it does _not_ appear that this is the major biosynthetic route. The poor conversion of diol to sulfa-tide implies that the diol did not reach the site of sulfation within the cell. It is easy to see how the presence or absence of chloro groups on the aliphatic chain would have a minimal effect on the substrate specificity of the sulfating enzymes.

It is of interest to note that hydroxy fatty acids are not normally found in _O. danica_. Free diols have also never been present in the lipids of the organism. This

implies that once the precursors reach a certain stage, they are converted efficiently to the sulfatides. This suggests a multi-enzyme complex in the final stages of the biosynthesis. Furthermore, since the sulfolipids are such strong detergents, it is not likely that they are ever free-floating in the cytoplasm of the cell. They are probably membrane-associated during their formation and remain so after synthesis is complete. The sulfolipid has been found to be associated with a membrane-band on a density gradient.[38] It has also been found (Table XIV) that the organism cannot tolerate as high a concentration of either natural or synthetic diol as that of fatty acid. This is reasonable since fatty acid can be metabolized in many ways, whereas diols can only be converted to sulfolipid.

The conversion of halogen-free sulfatide (Figure 6A) to the normal pattern of halogenated sulfatides (Figure 6C) shows that chlorination of the sulfated chain occurs in a pattern identical to the original composition of the halogenated sulfatides. At least 50% of the sulfatide that was incorporated into the cell was found to be chlorinated. The chromatograph of the chlorodiols obtained from the labeled sulfatide feeding shows that all of the chlorodiols are labeled, including the hexachloro diol. It is important to note that it has been established that cleavage of the sulfate esters under these growth conditions does not occur.[52] In those experiments it was shown that S^{35}-sulfatide incubated

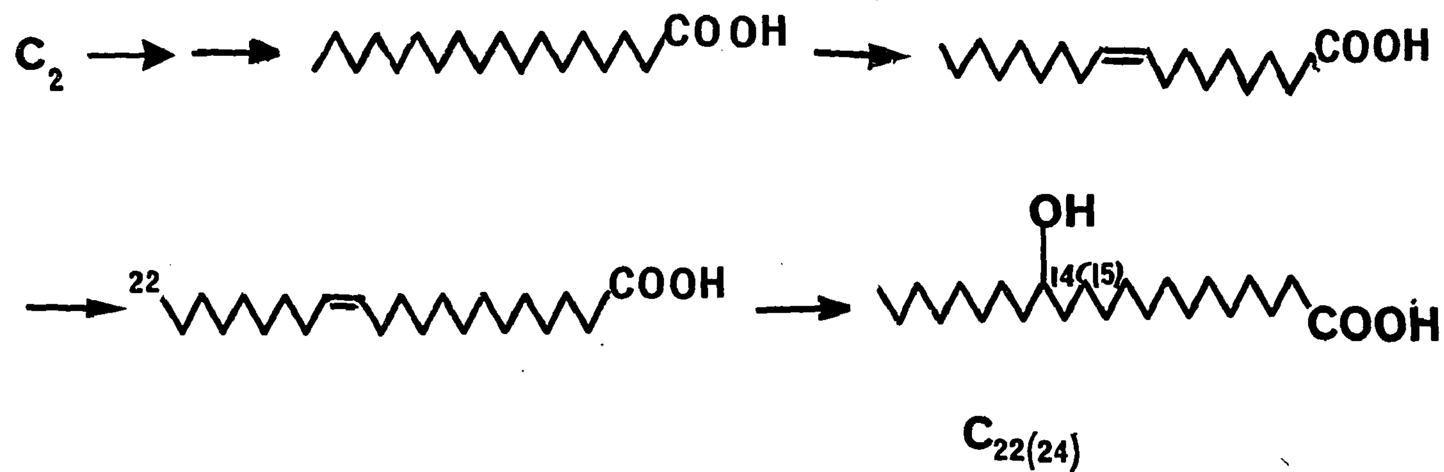
with the organism was not converted to S^{35}-cystine or S^{35}-methionine whereas S^{35}-sulfate was rapidly converted to these amino acids. Additionally, the results in Table XIII show very little or no diol found in the cell extracts of these experiments, which further suggests the organism is incapable of cleaving the sulfate esters.

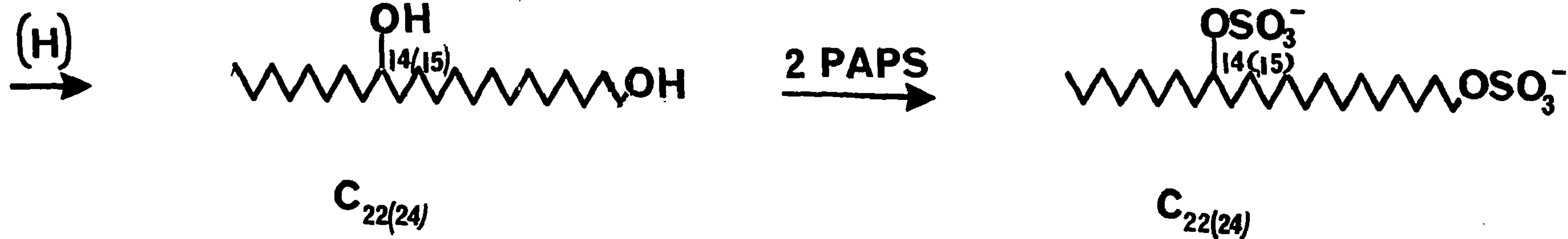
The inability of the organism to chlorinate the halogen-free diol and its rapid conversion of the achloro alkyl disulfate to the usual pattern of halogenated diols implies that the sulfate esters are the substrates for the chlorinating enzymes. It further suggests that the halogenating enzymes have the expected substrate specificity.

It is remarkable that the halogenation reaction occurs on carbon atoms that are not activated. Thus chlorination almost surely occurs *via* a free-radical intermediate. This is in contrast to the chlorinating system of *Caldariomyces fumago*, studied by Hager *et al.*[45] This is the only enzymatic halogenating system explored in depth to date. The system does not utilize a free-radical intermediate. Recent evidence indicates that the halide anion binds close to the heme site but the actual chemical structure of the active site is not known.[61] The difference that exists between *C. fumago* enzyme and that of *O. danica* lies in the type of substrate to be chlorinated. The energy requirements for halogenation of the aliphatic chain of the sulfatides in *O. danica* are far in excess of

those of *C. fumago*. It is for this reason that we postulate a free-radical mechanism of chlorination for this system. All of the halogenated natural products to date likewise contain the halogen on activated carbons and may be more similar to the *C. fumago* system than to the *Ochromonas* enzyme system. It is curious that the chlorinations in *Ochromonas* are clustered around the sulfate groups.

The proposed biosynthetic pathway is shown in Figure 10. The following conclusions can be drawn about the biosynthesis of the chlorosulfolipids: (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; and (3) the alkyl chains are first sulfated and then chlorinated to form the chlorosulfatides.





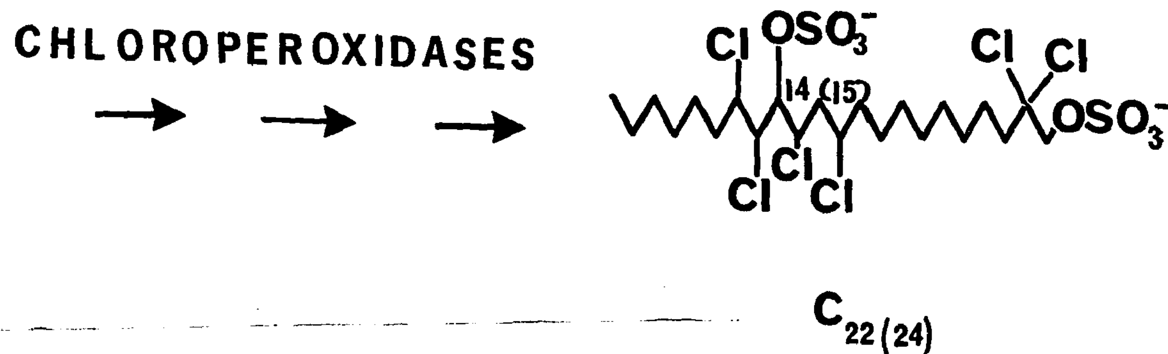


Fig. 10. Biosynthetic pathway for the chlorosulfolipids of *Ochromonas danica*.

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