Chlorination and Sulfation Reactions in the Biosynthesis of Chlorosulfolipids in *Ochromonas danica, in Vivo*†

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ABSTRACT: The phytoflagellate, *Ochromonas danica*, produces large amounts of chlorosulfolipids. These compounds are derivatives of docosane 1,14-disulfate and tetracosane 1,15-disulfate, in which one to six chlorine atoms have been substituted for the equivalent number of hydrogens. ^{14}C-Labeled docosanediol 1,14-disulfate and ^{14}C-labeled tetracosanediol 1,15-disulfate were incorporated into the chlorinated sulfatides directly. Since the sulfatide is not degraded or desulfated by the organism, this demonstrates that chlorination of the chain occurs without cleavage of the sulfate group. Thus, chlorination occurs after sulfation in the *in vivo* biosynthesis of the chlorosulfolipids. The positions at which chlorination occurs are not chemically activated. The chlorination reaction almost surely occurs *via* a free-radical intermediate. Enzymatic halogenations described to date occur on activated carbons and these chlorination reactions have considerably lower energy requirements.

The phytoflagellate *Ochromonas danica* synthesizes an unusual group of compounds—the chlorosulfolipids. They may be divided into two series—the polychlorodocosane 1,-14-disulfates and the polychlorotetracosane 1,15-disulfates with from zero to six 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). Each series may be considered to be derivatives 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, 1973). These lipids are the only natural alkyl sulfates isolated to date. They are unique in that they have chlorines on an aliphatic chain, and in that they are essentially polar at both ends of the molecule.

The chlorosulfolipids are present in large amounts in the cell, comprising 10–20% of the cell's lipids and 3% of the cell's dry weight (Elovson and Vagelos, 1969). They appear to be localized in a single membrane fraction (L. L. Chen and T. H. Haines, manuscript in preparation). The function of these compounds in the organism has not been established. Biosynthetic studies may shed some light on the role of these compounds in the cell. Regulation of the biosynthesis of these compounds may also provide a tool for studying the biogenesis of membrane in this organism. Furthermore, this system is an attractive one for the study of hydroxy fatty acid formation and of sulfation and chlorination of long chain compounds. Previous work has established that the alkyl chain was biosynthesized using the normal fatty acid synthesizing system. It was also shown that the secondary hydroxyl resulted from hydration of a double bond after the chain was synthesized (Mooney *et al.*, 1972). This paper reports *in vivo* studies of incorporation of: (1) 1(*S*),14-docosanediol and 1,-15-tetracosanediol; (2) 2,2,11,13,15,16-hexachloro-1,14-[11-^{14}C]docosanediol and 2,2,12,14,16,17-hexachloro-1,15-[13-^{14}C]tetracosanediol; and (3) 1(*S*),14-[9-^{14}C]docosanediol 1,-14-disulfate and 1,15-[11-^{14}C]tetracosanediol 1,15-disulfate into the chlorosulfatides. The incorporation data indicate that the chlorination reaction follows that of sulfation and clearly establishes the final steps in the biosynthesis of the chlorosulfatides.

Materials and Methods

[1-^{14}C]Laurate (specific activity 5.28 Ci/mol) was purchased from ICN TracerLab (Irvine, Calif). The radioactive purity was 99%, as demonstrated by thin-layer chromatography (tlc) and gas–liquid chromatography (glc). [1-^{14}C]Myristate (specific activity 5.0 Ci/mol) was purchased from New England Nuclear Corp. (Boston, Mass.). Its radioactive purity was 97%, 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 over $LiAlH_4$ 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 first on ether–hexane (3:7, v/v), then in benzene–chloroform–methanol (50:40:1, v/v/v), and then again in the ether–hexane solvent.

Radiocounting was conducted on a Nuclear-Chicago scintillation counter Model No. 724. Bray's (1960) solution was used for more aqueous samples. All other samples were dissolved in solutions of Spectraflor and toluene prepared according to manufacturer's specifications for liquid scintillation counting. Autoradiograms of tlc's were obtained using Picker No-Screen X-ray film.

All other chemicals were reagent grade and obtained commercially.

Cultures. Ochromonas danica was grown axenically in a

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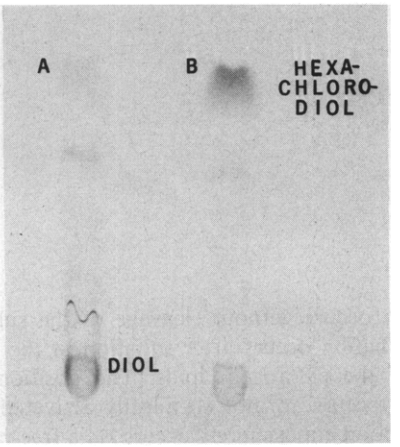

FIGURE 1: Autoradiogram of a thin-layer chromatogram of diols obtained by the hydrolysis of sulfatides isolated from *O. danica* after two separate feedings. (a) ^{14}C-Labeled achlorodiols were incubated with the organism cultured in "normal" (with respect to halogen concentration) medium. (b) ^{14}C-Labeled hexachlorodiols were incubated with the organism grown under normal conditions. The chromoatogram was developed in ether–hexane (3:7, v/v).

chemically defined medium (see below) at 26° under constant illumination of 125–150 ft-candles of light. In the case of chloride-free media, 15 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 (Aaronson and Baker, 1959) (pH 4.5). Unless otherwise stated, the chloride ion concentration of the medium was 0.0133 M. This is the normal chloride ion concentration. For some experiments, the chloride ion concentration was increased to 0.11 M. This concentration was chosen to maximize the amount of hexachlorosulfatides produced while keeping the growth 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 (1960), with chloride ion removed. The usual 0.0133 M chloride ion concentration was replaced by sulfate ion to maintain the ionic strength.

TABLE I: Defined Media for the Ahalo Growth of *Ochromonas danica*.

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

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

TABLE II: Incorporation of Achloro- and Hexachlorodiols.

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

Incubations. All compounds were added dissolved in 80% ethanol. The final maximum concentration of ethanol in the medium was 1%. Final concentrations of the fatty acids in the media were as follows: [1-^{14}C]laurate (1 mCi), 0.017 μM; [1-^{14}C]myristate (50 μCi), 0.143 mM. [1-^{14}C]Laurate was added to the medium preceding inoculation and incubated for 5 days. [1-^{14}C]Myristate was added to a culture of achloro cells which had been growing for 3 days. This culture was then incubated for 4 days. The diols and achlorosulfatides were added to cultures of approximately 2 days growth. These compounds were incubated for 3 days.

Isolation of Alkyl Diols and Alkyl Disulfates. The isolations of the chlorodiols and the chlorosulfatides were conducted as previously described (Mooney *et al.*, 1972). ^{14}C-Labeled diols were purified by tlc before incubation with cells. ^{14}C-Labeled sulfatides were checked for purity by solvolysis of the sulfatides and tlc of the resulting diols.

Results

The percentage incorporation of diols, both chlorinated and halogen-free (achloro), into the sulfatides is listed in Table II. The use of the Folch *et al.* (1957) procedure allows separation of the sulfatides from the diols. The diols remain in the lower phase and about 95% of the sulfatides remain in the upper phase (Mooney *et al.*, 1972). Most of the radioactivity remained in the lower phase for both the achloro- and hexachlorodiols. 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 1). The lower phase of the Folch extract was also chromatographed and showed a diol pattern identical with 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 distribution of radioactivity into the lipids of *O. danica*, grown under minimal halide concentration in the presence of [1-^{14}C]myristate, is summarized in Table III. There is a large incorporation of label into the phospholipids of *O. danica*. About 10% of the label appears in the upper phase of the Folch extract, the sulfolipid fraction. This is in agreement

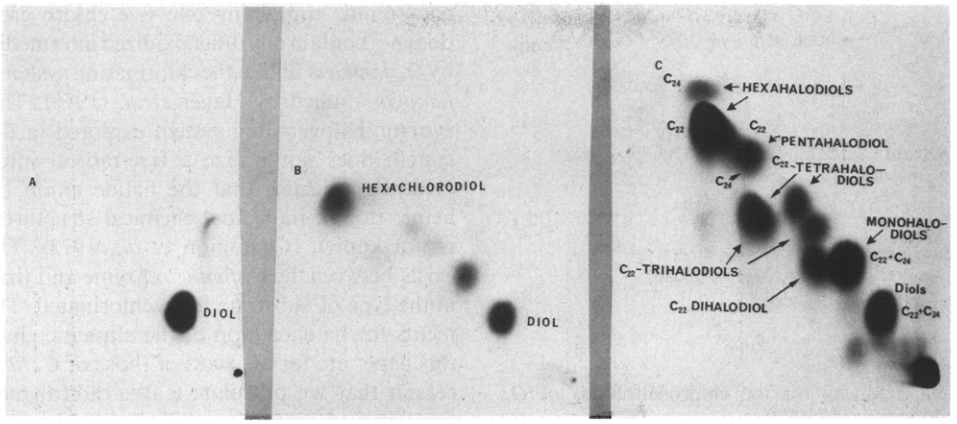

FIGURE 2: Autoradiograms of two-dimensional thin-layer chromatograms of diols obtained by the hydrolysis of sulfatides isolated from *O. danica*. (a) [1-^{14}C]Myristate was incubated with the phytoflagellate cultured in halogen-free medium. (b) The sulfatide obtained from the above [1^{14}C]myristate 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. The 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 again vertically in the ether–hexane solvent.

with work published previously, on incorporation of fatty acids into the lipids of *O. danica* grown in normal chloride medium (Mooney *et al.*, 1972). The cells grow at a slower rate in minimal halide media. The pattern of incorporation of [1-^{14}C]myristate, however, appears to be identical with that of cells grown on the "normal" defined media (Mooney *et al.*, 1972). A portion of the upper phase was subjected to hydrolysis to obtain the alkyl diols. An autoradiogram of a tlc of these diols (Figure 2A) allowed a confirmation of the absence of halogenated diols.

The labeled sulfatides obtained in the above experiment were incubated with the organism, now grown in an intermediate chloride medium (0.11 M). This medium was used to maximize chlorination of the substrate, should chlorination occur. The data in Table IV indicate the labeling pattern obtained from the extraction procedure. Most of the label appears in the upper phase (the sulfatide fraction). The counts present in the lower phase could be explained by (1) the small amount of sulfolipid that partitions itself in the lower phase (about 5%); (2) the free fatty acid that was adsorbed onto the sulfatide when it was fed to the organism (1–2%); and (3) the amount of sulfolipid that was hydrolyzed by the acidic medium (pH 4.5). Figure 2A shows the diols obtained from the sulfolipids of the achloro medium labeled with [1-^{14}C]myristate. Figure 2B shows the diols obtained from the sulfolipid fraction of the cells fed radioactive sulfolipid obtained from the above myristate feeding. It indicates that the labeled halogen-free sulfatide was converted to halosulfatides in the same proportion in which these halosulfatides are found in the organism (Figure 2C).

Discussion

The proposed biosynthetic pathway (Mooney *et al.*, 1972) of the chlorosulfolipids of *O. danica* is shown in Figure 3. It has been established that long-chain fatty acids are incorporated intact into the sulfatides (Mooney *et al.*, 1972). Oleic acid is a key intermediate in the biosynthetic pathway. The hydroxylation of the chain presumably occurs by addition of water to its cis double bond. The hydroxy compound is then chain lengthened, reduced to a diol, sulfated, and chlorinated.

The mechanism of sulfation probably involves 3′-phosphoadenosine 5′-phosphosulfate, since, in other organisms, sulfate is transferred from 3′-phosphoadenosine 5′-phosphosulfate to a hydroxyl group. Work in this laboratory on molybdate inhibition of sulfolipid biosynthesis seems to implicate the 5′-phosphosulfate as the sulfating agent used in the biosynthesis (L. L. Chen and T. H. Haines, manuscript in preparation). Therefore, sulfation presumably occurs by the enzyme-mediated transfer of the sulfate group of the 5′-phosphosulfate to the primary and secondary hydroxyl groups of the fully formed alkyl chain.

Both hexachlorodiol and the halogen-free diol can be sulfated (Table II). Furthermore, it appears that sulfation occurs to the same extent on both diols. It would appear therefore that the extent of chlorination of the alkyl chain has 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

TABLE III: Incorporation of [1-^{14}C]Myristate.

Fraction	Total Counts	%
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

TABLE IV: Incorporation of 1,14-[9-^{14}C]Docosanediol 1,14-Disulfate and 1,15-[11-^{14}C]Tetracosanediol 1,15-Disulfate.

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

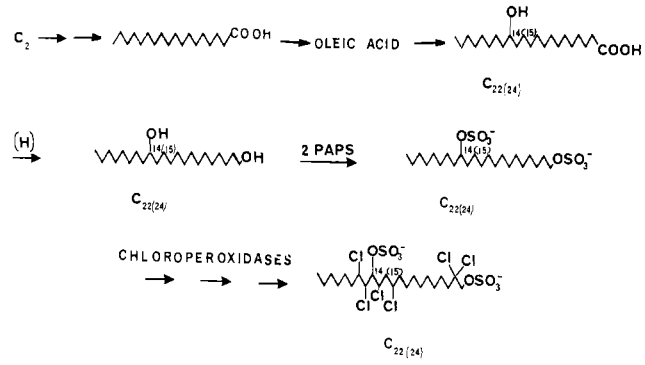

FIGURE 3: Biosynthetic pathway for the chlorosulfolipids of *O. danica* proposed by Mooney *et al.* (1972).

diol to sulfate implies either that the diol did not penetrate the cell efficiently or that the diol did not reach the site of sulfation within the cell. In view of the ease of incorporation of the fatty acids into *O. danica* (Gellerman and Schlenk, 1972; Mooney *et al.*, 1972), it would appear that the latter was the problem. 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.

The specificity of the chlorinating enzymes, however, is a different problem entirely. The difference between the diol and the alkyl disulfate with two charged sulfate groups is dramatic. The conversion of halogen-free sulfatide (Figure 2A) to the normal pattern of halogenated sulfatides (Figure 2C) shows that chlorination of the sulfated chain occurs in a pattern identical with 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 hexachlorodiol. It is important to note that it had been established that cleavage of the sulfate esters under these growth conditions does not occur (Haines, 1965). In those experiments it was shown that [^{35}S]sulfatide incubated with the organism was *not* converted to [^{35}S]cystine or [^{35}S]-methionine, whereas [^{35}S]sulfate was rapidly converted to these amino acids. Additionally, the results in Table IV 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 the rapid conversion of the achloro alkyl disulfate to the usual pattern of halogenated diols imply 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. The possibility of prior oxidation of the saturated chain is not completely excluded, but it would appear unlikely since the group of compounds containing one–five chloro groups on the chain does not contain any other oxidized intermediates. Chlorination by *O. danica* is unlike the chlorinating system of *Caldariomyces fumago*, studied by Hager *et al.* (1970). 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 (Champion *et al.*, 1973). The difference that exists between the *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 as described in this paper 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 shown in Figure 3 is thus further demonstrated by this paper. The results obtained allow the following conclusions to 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 give the final product—a chlorosulfolipid.

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