Biosynthesis of Chlorosulfolipids in *Ochromonas danica*. Origin of Primary and Secondary Hydroxyl Groups Determined by ^{18}O Incorporation *in Vivo*†

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ABSTRACT: The unusual chlorosulfolipids in *Ochromonas danica* are derivatives of docosane-1,14-diol and tetracosane-1,15-diol. Since their structure obviously suggests that their hydrocarbon chains derive from long-chain fatty acids, the question arises how a ω-9 (ω-10) secondary hydroxyl group is introduced during their biosynthesis. This study reports results using ^{18}O incorporation experiments *in vivo*, which show that the secondary hydroxyl oxygen derives from molecular oxygen, not from water; the reverse is found for the primary hydroxyl group. These results exclude hydration of an ω-9-unsaturated intermediate, such as oleic acid, as a mechanism in (chloro)diol synthesis in *O. danica*. It is suggested that the secondary hydroxyl is formed by hydroxylation of a saturated intermediate; the primary hydroxyl would be formed by reduction of a carboxyl group derivative which exchanges oxygen with medium water during biosynthesis.

The chrysomonad *Ochromonas danica* is unique in that it elaborates long-chain aliphatic disulfates (Mayers and Haines, 1967; Mayers *et al.*, 1969) which subsequently (Elovson and Vagelos, 1969) were shown to contain up to six chlorine atoms per molecule. The structure of the major chlorosulfolipids have been described (Elovson and Vagelos, 1969, 1970). The parent diols all have a docosane-1,14-diol or tetracosane-1,15-diol structure, and as a first approach to the question of their biogenesis it was deemed of interest to establish the mode of introduction of the secondary hydroxyl group in these compounds. This report describes the results obtained using ^{18}O incorporation *in vivo*, which establish that this secondary hydroxyl derives from molecular oxygen rather than from medium water.

Experimental Section

Materials

O. danica was obtained from the American Type Culture Collection. Normalized $H_2{}^{18}O$ (20.63 atom % ^{18}O, 0.165 atom % ^{17}O) was purchased from Miles-Yeda, Ltd. $H^{36}Cl$ (0.368 Ci/mol) was obtained from Amersham-Searle. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals. Needox 1114 was a gift from Ashland Chemical Co., Columbus, Ohio 43216.

Sources of other reagents and materials have been previously described (Elovson and Vagelos, 1969, 1970).

Methods

The previously described growth medium for *O. danica* was used with omission of the histidine supplement; the final concentration and specific activity of $^{36}Cl^-$ are indicated in the text.

Growth in $^{18}O_2$ (Experiment III). Sterile culture under electrolytically generated $^{18}O_2$ was performed in a simple system essentially as described by Thorpe and Sweeley (1967). The electrolysis U tube (Cleland and Johnson, 1954) had an inside diameter of 6 mm and contained 0.050 ml of concentrated sulfuric acid in 5 ml of oxygen isotope enriched water; electrolysis was performed using 110-V ac rectified with a diod. During the initial high output generation of $^{18}O_2$ to equilibrate the system, as described by Thorpe and Sweeley, the generator was cooled by immersion in a beaker of ice. The flask plus medium was first equilibrated with $^{18}O_2$ (200 ml of medium, 1 mM Cl^-, ^{36}Cl, specific activity 80,500 dpm/μmol), and then inoculated to give an optical density of 0.05 at 600 nm. The flask was wrapped in aluminum foil and growth was continued with slow mechanical stirring for 4 days, to an optical density of 9.1, at which time the cells were harvested. $H_2{}^{18}O$ remaining in the electrolysis tube was distilled into a trap cooled with liquid nitrogen, and used in the following experiment, after dilution.

Growth in $H_2{}^{18}O$ (Experiment II). Cells were grown in the dark to stationary phase in 4 ml of medium (Cl^-, 0.05 mM, specific activity 80,500 dpm/μmol) containing oxygen-isotope enriched water (10.1% ^{18}O, 0.08% ^{17}O).

Preparation of (Chloro)diols. Chlorosulfolipids were extracted essentially as described by Bligh and Dyer (1959); owing to their detergent nature, sulfolipids partition into the aqueous portion of the resultant two-phase system, from which they are recovered by extraction with butanol. Cells were harvested by centrifugation, washed once with water, and resuspended in a small volume of 0.01 M EDTA. After addition of 3.75 volumes of chloroform–methanol (1:2, v/v), the cell debris was centrifuged down and reextracted twice by the same procedure. The pooled lipid extracts were separated into two phases by addition of chloroform and 0.01 M EDTA to give a final ratio of chloroform–methanol–aqueous EDTA of 1:1:1. The upper aqueous phase was further washed with small amounts of chloroform until colorless. The pooled chloroform washes were reextracted with one-half volume of methanol–water (1:1), the upper phase was again washed with a small amount of chloroform, and the combined aqueous extracts were taken to dryness *in vacuo*. The residue was washed into a separatory funnel with a small amount of water and the chlorosulfolipids were recovered by two extractions with two volumes of 1-butanol. The combined butanol extracts were washed several times with small amounts of water and taken

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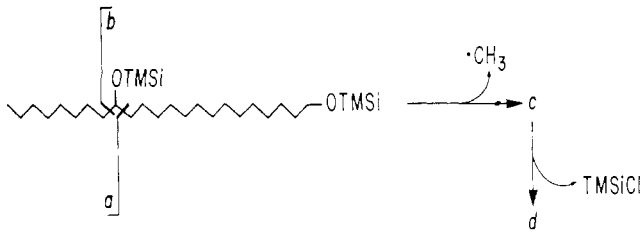

FIGURE 1: Fragmentation patterns of (chloro)docosane-1,14-diol Me_3Si ethers. Fragment d is only formed from species having a 13(15)-chlorine substituent.

to dryness *in vacuo*. The residue was taken up in dry methanol, insoluble material was removed by high-speed centrifugation in the cold, and the crude chlorosulfolipids were recovered by removing the methanol *in vacuo*. Sulfate esters were cleaved by acid hydrolysis (1 N HCl in 50% aqueous methanol, 1 hr at 90°, expt I) or by solvolysis (dioxane–water, 99:1, 1 hr at 100°, Mayers *et al.* (1969), expt II and III). The diols were recovered and separated into subclasses by thin-layer chromatography as previously described (Elovson and Vagelos, 1969, 1970). Me_3Si derivatives of these resolve into single species in the gas chromatography–mass spectrometry procedure. In some cases pure individual diols were separated preparatively by reversed-phase partition chromatography on hydroxyalkylated Sephadex LH-20 (Ellingboe *et al.*, 1968) using heptane-saturated 90% aqueous methanol as mobile phase. Details of this procedure will be published elsewhere. Procedures for preparation of Me_3Si ethers, gas–liquid chromatography (glc), and glc mass spectrometry on the LKB 9000 have been described (Elovson and Vagelos, 1969, 1970). For direct probe analysis of the Me_3Si ethers a suitable aliquot of the whole reaction mixture was transferred to the sample holder and excess reagents were removed under nitrogen.

Mass Spectrometric Analysis of Oxygen-Isotope Content. The important oxygen-containing fragment ions from (chloro)-diol Me_3Si ethers have been previously described (Elovson and Vagelos, 1969, 1970). To simplify presentation, these products (a–d, Figure 1 and Table I) are here referred to as fragments. Each fragment appears in the spectrum as a cluster of peaks, the shape of which depends on the abundance of heavy isotopes in the component elements. Each such peak is here referred to as an ion, with a mass, $m/e = m$, $m + 1$, etc., where m is the mass of the ion containing no heavy isotope. The relative abundance, N_{m+i}, of each ion $m + i$ is expressed as per cent of the total abundance in the ion cluster produced by the fragment. Experimental values for N_{m+i} in Tables II–IV are the means of at least four scans of each cluster. To obtain the theoretical distribution a program was constructed to compute N_{m+i} for the ion cluster at $m/e = m$, $m + 1$, $m + 2$, $m + 3$, $m + 4$, $m +$ (5 and higher) for any fragment ($C_xH_ySi_zO_uCl_v$), using different atom per cent enrichment of ^{18}O combined with the natural isotopic abundance of the other elements. For fragments containing two oxygens results were computed assuming that either one or both oxygens were enriched with ^{18}O. The contribution of ^{17}O was included in the calculations. The program is available on request.

Since substitution of ^{18}O for ^{16}O shifts an ion away from $m/e = m$ (to $m/e = m + 2$) a simple relationship permits direct estimation of the approximate ^{18}O abundance in a fragment which contains only one labeled oxygen, *i.e.*

$$\%\ ^{18}O\ \text{found} = \frac{(N_m)_{\text{normal}} - (N_m)_{\text{enriched}}}{(N_m)_{\text{normal}}} \times 100$$

where subscripts refer to results from normal (*i.e.*, unenriched) and enriched material, respectively. These results are listed in Tables II through IV as "% ^{18}O found."

TABLE I: Major Oxygen-Containing Fragments of Diol Me_3Si Ethers.

Fragment	Diol[a]	$m/e = m$	Formula
a	22:0 22:1	215	$C_{12}H_{27}OSi$
	22:6	283	$C_{12}H_{25}OSiCl_2$
b	22:0	373	$C_{20}H_{45}O_2Si_2$
c	22:0	471	$C_{27}H_{59}O_2Si_2$
	22:1	505	$C_{27}H_{58}O_2Si_2Cl$
d	22:1	397	$C_{24}H_{49}O_2Si$

[a] Abbreviations used are: 22:0, docosane-1,14-diol; 22:1, 13-monochlorodocosane-1,14-diol; 22:6, 2,2,11,13,15,16-hexachlorodocosane-1,14-diol.

Results and Discussion

The backbone common to all TMS ethers of the docosane-1,14-diols found in *O. danica* chlorosulfolipids is shown in Figure 1. Other than the parent compound docosane-1,14-diol (22:0) the major components analyzed here are 13-chlorodocosane-1,14-diol (22:1) and 2,2,11,13,15,16-hexachlorodocosane-1,14-diol (22:6). The fragmentation patterns of the Me_3Si ethers of these compounds have been previously

TABLE II: ^{18}O Isotope Analysis of Fragment a of 22:0, 22:1, and 22:6 Me_3Si Ethers.[a]

	I[b]		II[b]		III[b]	
	$^{16}O_2$[c]/$H_2{}^{16}O$[c]		$^{16}O_2/H_2{}^{18}O$[c]		$^{18}O_2$[c]/$H_2{}^{16}O$	
m/e	Exp	Comp	Exp	Comp	Exp	Comp
			22:0, 22:1			
215	78.3[d]	80.2	79.6	72.2	64.0	63.6
216	15.1	15.2	14.8	13.8	11.9	12.2
217	4.2	4.1	4.5	11.6	19.7	19.7
218	0.5	0.5	0.5	1.9	3.3	3.5
219				0.3	0.6	0.6
% ^{18}O found[e]			0		20.2	
Source of oxygen			$^{16}O_2$		$^{18}O_2$	
			22:6			
283	45.0	45.3			36.6	36.0
284	8.7	9.0			7.5	7.2
285	30.4	31.9			34.3	34.6
286	7.2	6.2			6.5	6.8
287	7.9	5.9			11.7	11.0
% ^{18}O found					18.7	
Source of oxygen					$^{18}O_2$	

[a] See Figure 1 and Table I. [b] Experiments described in Methods. [c] Normal samples assumed to contain 0.203 atom % ^{18}O. $H_2{}^{18}O$ in expt II was 10.0 atom %; $^{18}O_2$ in expt III was 20.6 atom %. [d] Tabulated values are N_{m+i}; relative abundance of ion at $m + i$ as % of total in cluster. See Methods. [e] Calculated as described in Methods.

TABLE III: ^{18}O Isotope Analysis of Fragments b and c of 22:0 Me_3Si Ether.[a]

	I[b]		II[b]			III[b]		
	$^{16}O_2/H_2{}^{16}O$[c]		$^{16}O_2/H_2{}^{18}O$[c]			$^{18}O_2/H_2{}^{16}O$[c]		
				Comp			Comp	
m/e	Exp	Comp	Exp	1[f]	2[g]	Exp	1[f]	2[g]
			Fragment b					
373	68.1[d]	67.4	61.2	60.7	54.6	54.0	53.4	42.4
374	21.9	22.5	20.8	20.3	18.2	17.6	17.9	14.3
375	8.2	8.2	13.8	14.0	18.7	20.3	20.4	27.2
376	1.6	1.9	3.6	3.9	5.6	5.6	6.2	8.6
377	0.2	0.1	0.9	0.5	1.6	1.5	1.0	4.4
% ^{18}O found[e]		—		10.1			20.7	
Source of oxygen				$^{16}O_2$ and $H_2{}^{18}O$			$^{18}O_2$ and $H_2{}^{16}O$	
			Fragment c					
471	62.7	62.2	56.5	56.1	50.4	49.2	49.4	39.1
472	25.1	25.8	23.5	23.3	21.0	20.7	20.5	16.4
473	9.1	9.4	14.4	14.6	18.7	20.3	20.2	26.2
474	2.1	2.4	4.9	4.7	6.6	6.9	7.2	10.0
475	0.2	0.1	0.5	0.5	1.5	1.4	1.0	4.1
% ^{18}O found[e]		—		9.9			21.6	
Source of oxygen				$^{16}O_2$ and $H_2{}^{18}O$			$^{18}O_2$ and $H_2{}^{16}O$	

[a–e] See footnotes, Table II. [f] Computed assuming that one oxygen derives from the enriched medium component. [g] Computed assuming that two oxygens derive from the enriched medium component.

described (Elovson and Vagelos, 1969, 1970). The origin of major oxygen-containing fragment ions are also shown schematically in Figure 1; their elemental composition and $m/e = m$ for the three different diols are shown in Table I. Fragment d appears only in spectra of chlorine-containing diols.

The (chloro)diols analyzed in this report came from three different sources. "Normal" samples (expt I in tables) were previously obtained from a number of cultures grown in $^{16}O_2/H_2{}^{16}O$ with the natural abundance of heavy oxygen isotopes. In expt II cells were cultured in $^{16}O_2/H_2{}^{18}O$ (10.0 atom % ^{18}O). In expt III cells were cultured in $^{18}O_2$ (20.6 atom % ^{18}O)/$H_2{}^{16}O$. Owing to the high cost of $H_2{}^{18}O$, expt II had to be run on a much smaller scale and with less ^{18}O enrichment than expt III. The chloride concentration in the medium was therefore also reduced in order to simplify the pattern of the small amounts of

TABLE IV: ^{18}O Isotope Analysis of Fragments c and d of 22:1 Me_3Si Ether.[a]

	I[b]		II[b]			III[b]		
	$^{16}O_2/H_2{}^{16}O$[c]		$^{16}O_2/H_2{}^{18}O$[c]			$^{18}O_2$[c]$/H_2{}^{16}O$		
				Comp			Comp	
m/e	Exp	Comp	Exp	1[f]	2[g]	Exp	1[f]	2[g]
			Fragment d					
397	69.1[d]	69.7	63.2	63.0	56.6	55.4	55.5	43.9
398	22.8	22.9	20.0	20.7	18.7	17.8	18.3	14.6
399	6.7	6.2	11.6	12.3	17.4	19.2	19.2	26.7
400	1.4	1.1	2.9	3.2	5.0	5.4	5.5	8.2
401			0.5	0.3	1.1	1.3	0.5	3.8
% ^{18}O found[e]		—		8.6			19.8	
Source of oxygen				$^{16}O_2$ and $H_2{}^{18}O$			$^{18}O_2$ and $H_2{}^{16}O$	
			Fragment c					
505	45.8	46.7	41.5	42.2	37.9	36.6	37.1	29.4
506	19.3	19.5	17.6	17.7	15.9	14.7	15.6	12.4
507	22.2	22.4	25.3	24.8	26.5	28.5	27.4	29.4
508	7.4	8.2	9.2	9.3	10.1	9.6	10.5	11.6
509	2.3	1.2	3.9	2.8	4.7	7.2	4.7	8.7
% ^{18}O found[e]		—		9.4			20.1	
Source of oxygen				$^{16}O_2$ and $H_2{}^{18}O$			$^{18}O_2$ and $H_2{}^{16}O$	

[a–g] See footnotes, Tables II and III.

diols produced; consequently, the amount of 22:6 in that experiment was insufficient for adequate analysis.

To eliminate the unlikely complication of acid-catalyzed oxygen exchange between diols and water in the usual acid hydrolysis procedure, the diols in expt II and III were released by solvolysis of the chlorosulfolipid in dioxane–water (99:1). In a control experiment "normal" chlorosulfolipids were solvolyzed in this mixture containing $H_2{}^{18}O$ (20.6 atom % ^{18}O). Isotope analysis of the 22:0 and 22:1 so liberated showed that they contained only the natural isotope abundance of ^{18}O; thus, it could be concluded that solvolysis did not produce artifacts due to oxygen exchange.

Tables II–IV summarize the results of the isotope analysis. For each fragment the relative abundance, N_{m+i}, of the ions at $m/e = m, m + 1, \ldots, m + 4$ is shown for material from the three experiments. For expt I, without ^{18}O enrichment, these experimental results are compared to results computed using the natural abundance of ^{18}O; for expt II and III the experimental results are compared to those computed using the known ^{18}O enrichment in the $H_2{}^{18}O$ and $^{18}O_2$, respectively, which were employed in these experiments. For fragments containing two oxygens the tables show the distribution which was computed assuming that either one, or both, oxygens becomes enriched. The tables also show the "% ^{18}O found" (see Methods) in fragments from diols isolated in expt II and III. From these results unambiguous conclusions may be drawn about the origin of the oxygen atom(s), and they are so identified in the tables.

The conclusions are straightforward. Table II shows the analysis for the distal fragment a (C-14 to C-22), which carries only the secondary hydroxyl in the diols. In expt III, for all three diols, this fragment achieves the same ^{18}O enrichment as the $^{18}O_2$ employed in that experiment. Conversely, from the data on 22:0 and 22:1 in expt II it is seen that this fragment does not incorporate any ^{18}O from $H_2{}^{18}O$. Thus, the secondary hydroxyl derives from molecular oxygen, and not from water. 22:0 and 22:1 give identical a, m/e 215; the isotope data for the two are also indistinguishable and have been combined in Table II.

The other fragments analyzed contain both the primary and secondary hydroxyl oxygens. Results for 22:0 are given in Table III, those for 22:1 in Table IV. In each case it is seen that the ^{18}O enrichment is that expected only if one atom derived from molecular oxygen and one from the medium water: the data in Tables III and IV clearly exclude the alternatives where both oxygens are derived from either O_2 or H_2O. Since the results in Table II show that the secondary hydroxyl derives exclusively from molecular oxygen it follows that the primary hydroxyl in fragments b, c, and d must derive from water.

O. danica also elaborates a series of tetracosane-1,15-diols (Elovson and Vagelos, 1969). The parent compound and the 14-chlorotetracosane-1,15-diol were also analyzed completely in these experiments with results identical to those shown for the 22-carbon diols, *i.e.*, labeling of the secondary hydroxyl occurs exclusively from molecular oxygen and that of the primary hydroxyl exclusively from water.

Although a low-resolution spectrometer was used the overall agreement between computed and experimental values in Tables II–IV is quite good, since the fragments analyzed are major ions in the spectra and contain at most two chlorines. However, as the number of chlorine atoms in a fragment increases the clusters become more spread out, the absolute abundances of the fragments decrease and the fragmentation modes increase; taken together, these complicating factors become sufficiently troublesome in the case of the 22:6-diol to discourage isotope analysis of the b–d fragments for that species. The data in the tables were usually obtained by glc mass spectrometry. In several cases the diols were also preparatively purified by reversed-phase partition chromatography and the Me_3Si ethers introduced into the ion source with the direct probe. No difference in results was observed between the two methods.

The good agreement between computed and experimental results in Tables II–IV also shows that there is no appreciable dilution of the labeled oxygens in these experiments where dark-grown cells were used. In the light, however, *O. danica* has a sufficiently vigorous Hill reaction to substantially dilute the oxygen in the gas phase. Thus, in a preliminary experiment in the light, the enrichment from $^{18}O_2$ in the diols was only about one-half of that shown in Tables II–IV; this was also the case for the 3-OH oxygen in the poriferasterol from this culture.

The structures of the (chloro)docosanediols in *O. danica* obviously suggest their origin from long-chain fatty acids. Reduction of the carboxylic group, presumably as the coenzyme A derivative, such as that which affords the long-chain alcohols in *Euglena gracilis* (Kollatukudy, 1970), is consistent with the finding here that the primary hydroxyl has the same oxygen isotope enrichment as the water in the growth medium, since activated acyl oxygens appear to equilibrate rapidly with water (Thorpe and Sweeley, 1967). For the origin of the secondary hydroxyl of carbon-14, corresponding to the ω-9 position in a hypothetical 18-carbon precursor acid, two possibilities suggest themselves: it could be introduced by hydration of a ω-9 double bond in an unsaturated precursor, such as oleic acid, or it could be formed in a mixed function oxygenase hydroxylation of a saturated precursor, such as stearic acid. ^{18}O incorporation experiments, such as those undertaken in this study, have long provided the first approach to such questions in other areas of lipid metabolism. In the present instance the results unambiguously exclude the first alternative: the secondary hydroxyl derives from molecular oxygen, not from water. This result does not in itself prove the alternative direct hydroxylation of a saturated precursor. Another mechanism could involve epoxidation of an unsaturated precursor, with subsequent reductive opening to the hydroxyl compound. Indeed, Mooney *et al.* (1972) recently claimed that *O. danica* incorporated oleic acid into 22:1 *in vivo*, along with a number of saturated acids up to 18-carbon chain length. These authors in fact assumed, on this evidence, that diol synthesis proceeds *via* hydration of an ω-9-unsaturated intermediate, either oleate or a higher homolog. Since our results clearly exclude this pathway this reported incorporation of oleic acid into a chlorodiol could suggest an epoxidation–reduction pathway or possibly simply a preliminary reduction to a saturated fatty acid. However, it must be pointed out that Mooney *et al.* (1972) do not provide any data by which the efficiency of oleate incorporation may be compared to that of the saturated acids and at this point further speculation about the actual precursor is unwarranted; a full discussion of this point will be included with presentation of other data from this laboratory on *in vivo* incorporation of labeled precursors, which establish the sequence octadecanoic acid → → docosanoic acid → 14-hydroxydocosanoic acid → docosane-1,14-diol for chlorosulfolipid diol synthesis (J. Elovson, in preparation).

Direct oxygenation rather than desaturation–hydration is perhaps the more common hydroxylation mechanism, particularly in eucaryotes. Other than the numerous instances in steroid biochemistry the direct incorporation of molecular

oxygen into ricinoleic acid by hydroxylation of oleic acid in castor beans (Galliard and Stumpf, 1966; Morris, 1967), the hydroxylation of fatty acids by *Torulopsis*, described by Heinz *et al.* (1969, 1970) and the formation of hydroxy fatty acids in plant cutins (Kolattukudy and Walton, 1972) are examples analogous to the reaction studied here. However, the alternative sequence also does occur: formation of ricinoleic acid in *Claviceps purpurea* presumably occurs by hydration of linoleic acid (Morris *et al.*, 1966) although direct incorporation of H_2-^{18}O has not been demonstrated. Another very well-documented case is the reversible hydration of oleic acid to 10*D*-hydroxystearic acid in *Pseudomonas* sp. (Neihaus *et al.*, 1965, 1970). Unlike the present instance, however, there are also cases where a straightforward assignment of mechanism has not been possible. Formation of the C-4 hydroxyl in phytosphingosine in *H. ciferii* is a case in point where evidence from ^{18}O incorporation (Thorpe and Sweeley, 1967) cannot be simply reconciled with either a hydroxylation or hydration mechanism, although other evidence (Polito and Sweeley, 1971) seems to exclude the latter alternative. We are now studying the hydroxylation reaction in *O. danica in vitro* to determine if it indeed is a mixed-function oxygenase.

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