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- (11) For a complete discussion, see J. B. Covington, Ph.D. Thesis, University of Illinois, 1978, available from University Microfilms, Ann Arbor, Mich. The errors for each term in the expressions follow: for eq 2, second term  $\pm 0.3$ , third term  $\pm 0.5$ , fourth term  $\pm 0.3$ , fifth term  $\pm 1$ ; for eq 3, second term  $\pm 0.6$ , third term  $\pm 1.2$ , fourth term  $\pm 1.2$ , fifth term  $\pm 1.9$ .
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- (14) This value is obtainable by extrapolation to  $\epsilon = 1$ .
- (15) This term is  $u_x^2/a_x^3 - u_y^2/a_y^3$  from the Onsager reaction field model.
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- (17) Variation of the dipoles with changes in solvent and different energies of hydrogen bonding for different isomers could prove important on further analyses.

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### Sphingolipid Base Metabolism. Concerning the Origin of the Oxygen Atom at Carbon Atom 4 of Phytosphingosine<sup>1</sup>

Sir:

Sphingolipid bases are major constituents of a variety of biologically important classes of complex lipids of eukaryotes including ceramides, sphingomyelins, gangliosides, and other complex glycosphingolipids. One of the major sphingolipid bases, from which these complex sphingolipids are derived, is phytosphingosine<sup>2</sup> (I). The great majority of studies of the biosynthesis of this compound have utilized *Hansenula ciferrii* (II), a yeast which excretes large quantities of I in the form of its tetraacetyl derivative.<sup>3,4</sup> The precise nature of the enzymatic reactions leading to the formation of I are unclear. Some possible modes of formation of I<sup>5</sup> are illustrated in Figure 1. Greene et al.<sup>6</sup> have demonstrated the incorporation of the label of [9,10-<sup>3</sup>H]palmitic acid (III) and of [3-<sup>14</sup>C]serine into I by II. Under the same conditions, the label of [9,10-<sup>3</sup>H]- $\alpha$ -hydroxypalmitic acid was not efficiently incorporated into I. The enzymatic formation of I in a cell-free preparation has not been reported. However, it has been shown that microsomal preparations of II catalyze the pyridoxal phosphate-dependent condensation of serine and palmitoyl coenzyme A to yield 3-ketosphinganine which is then reduced, in an NADPH-dependent reaction, to give dihydrosphingosine<sup>7,8</sup> (IV). While the formation of sphingosine<sup>9</sup> (V) has been shown to occur in this system when 2,3-*trans*-hexadecenoyl coenzyme A was used as the substrate, no formation of I could be detected when

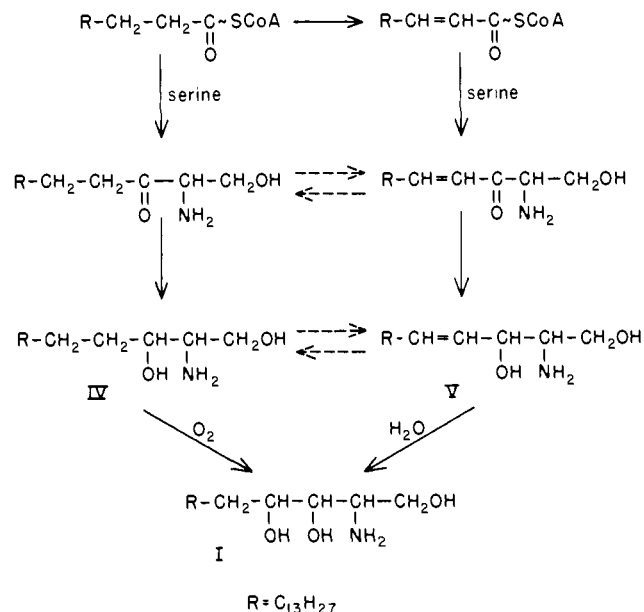


Figure 1.

$\alpha$ -hydroxypalmitoyl coenzyme A was used as the substrate.<sup>8</sup> It has been suggested that I of II is formed via a hydroxylation of IV.<sup>10</sup> The combined results of two laboratories<sup>10,11</sup> have indicated that the major, if not exclusive, pathway involved in the incorporation of III, by II, into I occurs with the loss of only one hydrogen atom (from C-2) of III and that this hydrogen has the *pro R* configuration. These findings appear to preclude a mechanism which involves the formation of I by hydration of the double bond of V (or an unsaturated precursor of V) but are compatible with a mechanism which involves a stereospecific hydroxylation at C-4 of IV (or a precursor of IV). However, the latter mechanism has appeared to have been excluded by the results of two laboratories which have indicated that the oxygen at C-4 of I was not derived from molecular oxygen.<sup>11,12</sup> The purpose of this communication is to report the results of a reinvestigation of the possible incorporation of molecular oxygen into the hydroxyl function at C-4 of I in its formation in II.

The tris(*O*-trimethylsilyl)-*N*-acetyl derivative of I was chosen as a suitable compound on which to base the assay of the extent of incorporation and localization of labeled oxygen in I. This selection was based upon the results of mass spectral analyses of the above derivative of authentic I, the same compound containing perdeuterated trimethylsilyl functions, and of [(4*R*)-<sup>18</sup>O]-I. The latter compound was synthesized from tribenzoyl-V by a modification of an approach described by two laboratories<sup>13,14</sup> (Figure 2). This synthesis involves epoxidation of tribenzoyl-V, reduction of the resulting product with lithium aluminum hydride, and subsequent hydrogenolysis of the resulting *N*-benzyl derivative to give the free base. Our results indicate that the procedure yields four compounds (as shown in Figure 2) which could be resolved from each other by medium-pressure chromatography of the *N*-acetyl derivatives on silicic acid columns.<sup>15</sup> These compounds were characterized by melting point determination and low and high resolution mass spectral, infrared, optical rotation, chromatographic, and chemical degradation studies<sup>15</sup>. The corresponding compounds labeled with <sup>18</sup>O in either the 4 or 5 positions were prepared as outlined in Figure 2 except that <sup>18</sup>O-labeled *m*-chloroperbenzoic acid<sup>16</sup> was used.

The mass spectrum of the tris(*O*-trimethylsilyl)-*N*-acetyl derivative of I did not show a significant molecular ion but did show an ion of high abundance at *m/e* 560. The results of high resolution mass spectral analysis of this derivative of I and the results of low resolution analyses of the corresponding M<sub>3</sub>Si-*d*<sub>9</sub>

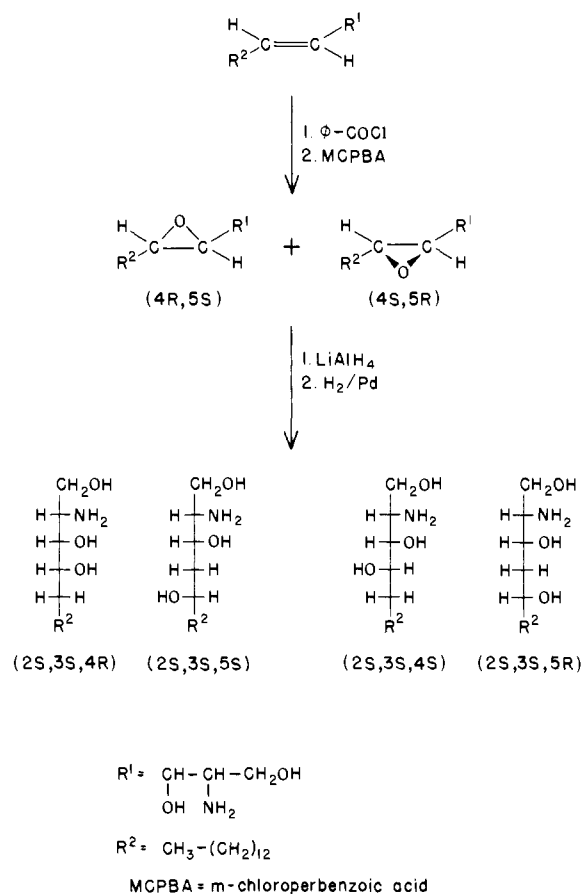


Figure 2.

**Table I.** Mass Spectral Analyses of Isotopic Composition of Phytosphingosine (I) Formed by *H. ciferrii* in atmosphere of  $^{18}\text{O}$  gas

No. of atoms of $^{18}\text{O}$ /molecule	$[\text{M} - \text{CH}_3]^+$	$[\text{CH}_3-(\text{CH}_2)_{13}-\text{CH}-\text{O}-\text{Si}(\text{CH}_3)_3]^+$
	Expt 1	
0	17.3 ± 2.4 <sup>a</sup>	19.7 ± 1.0 <sup>b</sup>
1	79.8 ± 4.2	80.3 ± 1.0
2	2.9 ± 3.0	0.0 ± 0.9
	Expt 2	
0	16.8 ± 1.7 <sup>a</sup>	17.8 ± 0.5 <sup>b</sup>
1	81.4 ± 2.0	82.2 ± 0.8
2	1.9 ± 1.4	0.0 ± 0.4

<sup>a</sup> Mean ± SD (six determinations). <sup>b</sup> Mean ± SD (five determinations).

derivative and of the  $[4\text{-}^{18}\text{O}]$ -labeled compound indicated that this ion corresponds to  $\text{M} - \text{CH}_3$ , the concerned methyl being derived from a trimethylsilyl function. This ion was used as the basis of the estimation of total  $^{18}\text{O}$  content. The ion at  $m/e$  299 was shown by similar analyses to correspond to  $\text{CH}_3-(\text{CH}_2)_{13}-\text{CH}-\text{O}-\text{Si}(\text{CH}_3)_3$ , the oxygen of which was derived from the oxygen function at C-4 of I. This ion was therefore used as the basis of estimation of the  $^{18}\text{O}$  content at C-4 of I.<sup>17</sup>

It was grown in an atmosphere of  $^{18}\text{O}$  gas (99%  $^{18}\text{O}$ ) for 42 h.<sup>18</sup> The polyacetylated I was obtained from the incubation media by extraction with hexane. Mild alkaline hydrolysis<sup>19</sup> gave *N*-acetyl-I which was analyzed, in the form of its tris(*O*-trimethylsilyl) derivative, by combined gas-liquid chromatography-mass spectrometry. The results of this analysis indicated that ~80% of the molecules contained one atom of  $^{18}\text{O}$  which was exclusively located at carbon atom 4;

~20% of the molecules contained no  $^{18}\text{O}$ . A second independent experiment gave similar results (Table I). It is concluded that molecular oxygen represents the major source of the oxygen of the hydroxyl function at C-4 of I. These findings, coupled with those cited above, strongly suggest that the major mode of formation of I in II involves an oxygen-dependent hydroxylation of IV (or a precursor or derivative of IV). The less than quantitative incorporation of the  $^{18}\text{O}$  of oxygen gas into I observed in our experiments<sup>20</sup> and the slight (5.7, 17.7, and 16.5%),<sup>12,21</sup> but significant, incorporation of  $^{18}\text{O}$  at C-4 of I in its formation in II grown in the presence of  $\text{H}_2^{18}\text{O}$  suggest the possibility that the formation of I in II may proceed by two independent routes: (1) a quantitatively more important pathway involving a hydroxylation at C-4 of IV (or a precursor or derivative of IV) and (2) a quantitatively less important route involving a stereospecific hydration of the double bond of V (or a precursor or derivative of V).<sup>22</sup> Further studies of the mechanisms involved in the formation of I in II are in progress.

## References and Notes

- (1) Supported in part by grants from the National Institutes of Health (HL-15376), the National Multiple Sclerosis Society, and the Robert A. Welch Foundation (C-583).
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- (16) Prepared by a modification of the method of P. A. A. Van der Beek (*Recl. Trav. Chim. Pas-Bays*, **47**, 286 (1928)) using *m*-chlorobenzaldehyde and  $^{18}\text{O}$ -labeled oxygen gas.
- (17) Thorpe and Sweeley<sup>12</sup> utilized the ion at  $m/e$  145 in the mass spectrum of tetraacetylphytosphingosine to quantitate the extent of  $^{18}\text{O}$  labeling at carbon atom 4 of I formed in an atmosphere enriched in  $^{18}\text{O}$  gas. Our studies of the mass spectrum of the tetraacetyl derivative of  $[(4*R*)-^{18}\text{O}]\text{-I}$  indicate that this ion (a doublet) contained negligible  $^{18}\text{O}$ . In experiments involving the study of the incorporation of the oxygen of  $\text{H}_2^{18}\text{O}$  into I, Thorpe and Sweeley<sup>12</sup> utilized analysis of the spectrum of the tris(*O*-trimethylsilyl) derivative of *N*-acetyl-I to locate and quantitate the  $^{18}\text{O}$ .
- (18) The culture medium was composed of dextrose (6%), yeast extract (0.3%), malt extract (0.3%), and bacto-peptone (0.5%).
- (19) 1 N KOH (0.5 mL) in methanol (5 mL), room temperature, 18 h.
- (20) In independent experiments (not presented herein) we have established that the less than quantitative incorporation of  $^{18}\text{O}$  at carbon atom 4 of I was not due to dilution by endogenous, unlabeled I.
- (21) C. C. Sweeley and K. Krisnangkura, personal communication.
- (22) The possibility of small amounts of I being formed from V is not totally excluded by the results of the studies of the incorporation of perdeuterated palmitate into I.<sup>10</sup> Polito and Sweeley<sup>10</sup> estimated that 95% or more of I synthesized from the perdeuterated palmitate lost only one deuterium atom in the conversion. However, the fact that the labeled palmitic acid used was not fully deuterated introduces uncertainties in the precise quantitation of small amounts of I containing 29 atoms of deuterium atoms in the presence of molecules containing 30 atoms of deuterium.

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