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Chemistry and Metabolism of Sphingolipids. On the Biosynthesis of Phytosphingosine by Yeast*

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ABSTRACT: A gas chromatographic method was developed for the determination of extracellular tetraacetylphytosphingosine produced by *Hansenula ciferrii* grown aerobically in shake culture. Studies on the origin of the oxygen atoms in this product were carried out with ^{18}O-labeled water and molecular oxygen, using combined gas chromatography–mass spectrometry for the differential determination of isotopic abundance in various oxygen atoms in the molecule. *None* of the oxygen atoms of tetraacetylphytosphingosine was derived from molecular oxygen. All but one of the oxygen atoms were derived from, or exchanged with, oxygen of water molecules in the medium.

The hydroxyl group on C-4 of phytosphingosine was very slightly labeled with ^{18}O from water; it has been concluded, however, that this hydroxyl group is derived from some unknown hydroxyl donor in the medium.

The yeast *Hansenula ciferrii* has been found by Stodola and co-workers to produce fully and partially acetylated sphingolipid bases in relatively large amounts (Stodola and Wickerham, 1960; Stodola *et al.*, 1962). The principal sphingolipid products, which were found in abundance in the cells and in the culture medium, were tetraacetylphytosphingosine (TAPS)[1] and triacetyldihydrosphingosine. The production of TAPS by this organism was shown to occur during aerobic growth and to closely parallel glucose dissimilation (Maister *et al.*, 1962). In later studies on the biosynthesis of phytosphingosine by *H. ciferrii*, Greene *et al.* (1965) found that [3-^{14}C]serine and [9,10-^{3}H]-palmitic acid were incorporated equally well into extracellular TAPS, suggesting an enzymatic mechanism not unlike that previously proposed for the synthesis of sphingosine and dihydrosphingosine in animals (see, for example, Zabin, 1957; Brady *et al.*, 1958; Fujino and Zabin, 1962). Although a pathway involving the condensation of a C_{16} chain with serine is clear from the studies of Greene *et al.* (1965), the source of oxygen for the secondary hydroxyl group on C-4 in phytosphingosine, and the exact step in the sequence when this OH group is introduced, are two points which remain to be determined. Of several plausible mechanisms, the following three reactions appeared to be the most likely possibilities.[2] Incorporation of α-

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[1] Abbreviations used: TAPS, tetraacetylphytosphingosine; $OSiMe_3$ in chemical structures, *O*-trimethylsilyl.

[2] These schemes ignore the fact that both phytosphingosine and dihydrosphingosine are isolated as the acetylated derivatives; thus, precursors shown above may or may not be fully or partially acetylated.

hydroxypalmitate into TAPS by whole cells of *H. ciferrii* was shown by Greene *et al.* (1965) to be only

sphingosine + water → phytosphingosine (1)

dihydrosphingosine + oxygen → phytosphingosine (2)

α-hydroxypalmitate + serine → phytosphingosine (3)

very slight, as compared with yields from labeled palmitate and serine. Although this might be considered sufficient evidence to eliminate pathway 3 above, the authors pointed out that a permeability problem may have prevented efficient conversion of the hydroxy acid.

This paper describes the results of studies on the incorporation of ^{18}O from water and molecular oxygen into extracellular TAPS synthesized by *H. ciferrii*. Simple pathways to phytosphingosine, such as pathways 1 and 2 above, are excluded by the findings that molecular oxygen does not serve as a precursor for the hydroxyl group on C-4 of phytosphingosine, and oxygen from labeled water is only slightly incorporated into this hydroxyl group. All the other oxygen atoms in TAPS were either derived from, or freely exchanged with, the oxygen of water in the medium.

Experimental Section

Materials. A culture of *H. ciferrii* (mating type F-60-10, strain NRRL-Y-1031) was kindly provided by Dr. F. H. Stodola and Dr. L. J. Wickerham. Samples of pure TAPS, triacetyldihydrosphingosine, and crude yeast sphingolipids were gifts of Dr. J. H. Law. The following ^{18}O compounds were obtained from Yeda Research and Development Co., Ltd., Rehovoth, Israel: 100 cc of oxygen gas (93% ^{18}O and 0.348% ^{17}O) and 5 g of water (20.1% ^{18}O, 0.88% ^{17}O) for the electrolysis experiment, and two 5-g batches of water (21.75% ^{18}O and 0.21% ^{17}O, normalized for ^{2}H content) for the growth of yeast in isotopic-labeled water. Solvents were reagent grade and were used as supplied unless otherwise noted.

Gas Chromatography. Analyses for yields of TAPS in media and cells were made routinely by gas chromatography of concentrated extracts of crude TAPS on an F & M Model 402 chromatograph with a hydrogen flame ionization detector. The column was a glass U tube, 6 ft in length and 3-mm i.d., that was packed with 1.5% ECNSS-S[3] on 100–120 mesh, acid-washed and silanized Gas Chrom S. The liquid phase and support were furnished by Applied Science Laboratories, Inc., State College, Pa.; the packing was prepared on a small scale by the solution-coating technique (Horning *et al.*, 1959). After preconditioning the column in the usual fashion, TAPS determinations were made isothermally at 212–215°, with the injection heater at 260° and the detector block approximately 15° above column temperature. The retention time for TAPS under these conditions was about 24 min. Yields of TAPS were calculated by comparing the areas of TAPS peaks with those given by known quantities of pure material.

Mass Spectrometry. Mass spectra were obtained with an LKB 9000 (LKB Produkter, Stockholm, Sweden), consisting of a gas chromatograph and a single-focusing, 60° magnetic sector mass spectrometer, coupled directly with molecule separators of the Becker–Ryhage type (Ryhage, 1964). The gas chromatographic column was of coiled glass, 6 ft in length by 4-mm i.d., and was packed with 2.5% SE-30 on 100–120 mesh, acid-washed and silanized Gas Chrom S. Determinations of ^{18}O abundance in TAPS and other derivatives of phytosphingosine and dihydrosphingosine were made with crude extracts; approximately 2 μg of material was usually injected into the gas chromatographic portion of the instrument. Spectra were recorded at 20 (TAPS) and 70 ev (*N*-acetyltri-*O*-trimethylsilyl-phytosphingosine), with an accelerating voltage of 3.5 kv, an ion source temperature of 250°, and a filament current of 60 μa. Calculations of isotopic abundance of various fragment ions included corrections for normal isotopic abundance and for column "bleeding," as described in greater detail in the Appendix. Empirical formulas of selected fragment ions in the mass spectrum of TAPS were calculated from data obtained with the high-resolution MS-9 mass spectrometer (Associated Electronic Industries, Ltd.).

Growth of Yeast and Analysis of TAPS. H. ciferrii was grown in shake culture at 28–30°, using a previously defined yeast maintenance medium (Wickerham and Stodola, 1960). Vigorous aeration was achieved by shaking the flasks on a Model G-10 New Brunswick gyratory shaker at a speed setting of 9 or 10. Growth was estimated from readings of optical density at 660 mμ on a Coleman Junior spectrophotometer. Cells were harvested by centrifugation for 5–10 min at three-fourths speed in an International Model HN centrifuge. The supernatant solution was decanted and extracted three times with 10-ml portions of low-boiling petroleum ether (bp 30–60°). When emulsions formed during these extractions, they were broken with small volumes of added methyl alcohol. The combined extracts were evaporated under a stream of nitrogen, and the residue was dissolved in a known volume of chloroform. Aliquots were analyzed for yield of TAPS by gas chromatography and for isotopic abundance of ^{18}O by combined gas chromatography–mass spectrometry.

Cellular lipids were extracted from the paste with 20 ml of acetone, with stirring for 30 min at room temperature. It is likely that extraction of the lipids was not quantitative with acetone, but recoveries of TAPS were not determined. After removing the residue by filtration, the extract was evaporated *in vacuo*. A solution of the extracted material in chloroform was examined as described above.

Incorporation of ^{18}O from Water. Two experiments (I and II) were carried out with medium containing

[3] A chemically combined polymer consisting of ethylenesuccinate and a low percentage of cyanoethylsilicone.

^{18}O-enriched water. In each case an appropriate weighed quantity of mixed solids comprising the yeast maintenance medium was poured directly into 5 ml of labeled water. After gentle shaking to dissolve the solids, the solution was filtered with suction through a 0.22-μ Millipore filter into a sterile 125-ml suction flask. A small volume (1 ml) of distilled water ($H_2{}^{16}O$) was used to rinse the filter, then a small volume (0.1 and 0.4 ml in two experiments) of an overnight growth of yeast was added to the flask as an inoculum. A control flask with the same total volume of medium and the same volume of added inoculum was prepared and used to measure initial optical density and TAPS concentration. The flask containing labeled water was incubated with shaking for 22 hr, after which the final optical density was recorded and TAPS was extracted for analysis. The final estimated isotopic abundance in the water, expressed as atom per cent excess ^{18}O, was 17.5 $\pm$ 0.5%.

Incorporation of ^{18}O from Molecular Oxygen. In the first study with labeled oxygen (expt III), an actively growing yeast culture was exposed for a brief time to 93% $^{18}O_2$ in a completely closed system. After placing 21 ml of sterile medium in a sterile 250-ml erlenmeyer flask, 1.3 ml of an overnight growth of *H. ciferrii* was added as inoculum. An aliquot (2.7 ml) was removed immediately for determinations of initial optical density and TAPS concentration. The open flask was shaken aerobically for 6 hr, after which 3 ml was used for optical density measurement to assure active growth of the yeast. An extract of this aliquot was analyzed to establish the concentration of extracellular TAPS just before exposure of the yeast to labeled oxygen. The remaining 16.6 ml of growing yeast was transferred by sterile technique to a special gas flask (Figure 1) which was then alternately evacuated for 60 sec with a water aspirator, then flushed for 5 sec with nitrogen. The evacuations and nitrogen equilibrations were controlled by stopcock A, with stopcock C closed (Figure 1). After the tenth evacuation and with both stopcocks closed, the seal of a glass cylinder of $^{18}O_2$ (attached to joint B in Figure 1) was broken with a magnet and stopcock C was opened for 5 sec, then closed. To approximate atmospheric pressure in the flask, stopcock A was opened to nitrogen at slightly above atmospheric pressure for 5 sec. With all stopcocks closed, the cylinder of labeled oxygen was removed and the gas flask was shaken for 2 hr. Optical density was recorded and TAPS was extracted from the extracellular medium for analysis.

To permit a longer exposure of growing yeast to labeled oxygen gas, a second study (expt IV) was designed to utilize oxygen generated continuously by electrolysis of ^{18}O-enriched water. A mixture of 5 ml of labeled water and 0.14 ml of concentrated sulfuric acid was transferred to an electrolysis U tube with platinum plates inserted for electrodes (Cleland and Johnson, 1954). A current of about 13 amp was sufficient to produce adequate volumes of oxygen. An overnight growth of yeast (1 ml) was mixed with 15 ml of medium in the gas flask used in expt III. A carbon dioxide trap, consisting of a closed tube filled with Ascarite, was attached to the joint in the center of the flask. The flask was alternately evacuated and flushed with nitrogen as described above; after the tenth evacuation, oxygen from the U-tube generator was introduced through joint B by opening stopcock C. After 30 min, electrolysis was interrupted briefly while the system was brought approximately to atmospheric pressure by exposure of the system to nitrogen for 1 sec. The oxygen generator was then turned on again and the flask was placed on the rotary shaker. An equilibrium was soon established in which oxygen depletion in the flask (giving a temporary slight vacuum) was met by generation of oxygen in the electrolysis tube (until a slight overpressure caused electrolysis to stop). When the experiment was terminated after 50 hr of incubation, 0.6 ml of labeled water had been consumed in the electrolysis U tube. Optical density was determined and the medium was extracted for determinations of TAPS yield and isotopic labeling. The water used in the electrolysis tube was 20.1% ^{18}O.

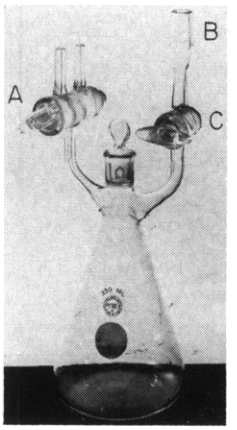


FIGURE 1: Flask used for growth of *H. ciferrii* in the presence of labeled molecular oxygen.

Alkaline Hydrolysis of TAPS and Reacetylation. Labeled TAPS from the extracellular medium of expt I was converted to free phytosphingosine by alkaline hydrolysis. Approximately 100 μg of TAPS was mixed with 0.5 ml of dioxane and 0.5 ml of half-saturated barium hydroxide; the solution was refluxed for 5 hr under a condenser fitted with an Ascarite trap. After cooling, the solvents were removed *in vacuo* and a mixture (0.5 ml) of pyridine and acetic anhydride (2:1, v/v) was added to the residue. The stoppered flask was allowed to stand overnight at room temperature, then the solvents were evaporated with a stream of nitrogen. Regenerated TAPS was extracted from the residue with chloroform, and submitted for mass spectral analysis.

Conversion of TAPS to N-Acetyl-1,3,4-tri-O-trimethylsilylphytosphingosine. Approximately 75 μg of crude TAPS from yeast paste (expt I) was dissolved in 0.5 ml of dry (distilled from $LiAlH_4$) dioxane and 12 mg of lithium borohydride was added. After standing overnight at room temperature, excess $LiBH_4$ was destroyed

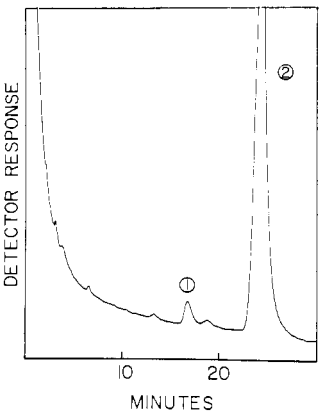


FIGURE 2: Gas chromatographic separation of crude extract from medium, containing triacetyldihydrosphingosine (1) and TAPS (2), on 1.5% ECNSS-S at 212.°

by addition of a few drops of dilute HCl. The clear solution was evaporated to dryness *in vacuo*, and *N*-acetylphytosphingosine was recovered from the residue by extraction five times with 1-ml portions of diethyl ether. The trimethylsilyl derivative of *N*-acetylphytosphingosine was prepared by addition of 25 μl of pyridine–silanes reagent (Gaver and Sweeley, 1965), consisting of hexamethyldisilazane, trimethylchlorosilane, and pyridine (2:1:10, v/v), to a residue from the combined ether extracts.

A preparation of crude extracellular TAPS (expt II) was converted to *N*-acetyl-*O*-trimethylsilyl derivative in the same way. In this preparation, triacetyldihydrosphingosine was converted to *N*-acetyl-*O*-trimethylsilyldihydrosphingosine and mass spectral analyses of ^{18}O were made with both of the trimethylsilyl derivatives.

An alternative route to *N*-acetylphytosphingosine was used in the preparation of *N*-acetyl-*O*-trimethylsilylphytosphingosine from extracellular TAPS of expt I. About 100 μg of crude TAPS was dissolved in 1 ml of 0.1 M sodium hydroxide in aqueous methyl alcohol. Selective hydrolysis of *O*-acetyl groups was achieved by heating at 60° for 1 hr. Recovered *N*-acetylphytosphingosine was converted to the trimethylsilyl derivative as described above.

Conversion of Labeled TAPS to Pentadecanol. *N*-Acetylphytosphingosine was prepared from 100 μg of extracellular labeled TAPS (expt II) by $LiBH_4$ reduction as described above. A solution of the crude product in 1 ml of methyl alcohol was mixed with 0.2 ml of 0.2 M sodium metaperiodate (pH 6.8). After 90 sec 12 mg of sodium borohydride was added. The reaction mixture was allowed to stand at room temperature for 30 min with occasional shaking, 0.4 ml of water was added, and pentadecanol was isolated by extraction three times with 2-ml portions of chloroform. A concentrate of the crude extract was submitted for mass spectral analysis of the pentadecanol by combined gas chromatography–mass spectrometry. The yield of pentadecanol obtained under these conditions was only 10–30% of theory; extracts contained a substantial proportion of unreacted *N*-acetylphytosphingosine.

Results

Determination of TAPS in Cellular and Extracellular Lipids. A simple and direct method for the determination of TAPS was developed, utilizing gas chromatography to separate the acetylated sphingolipid bases from contaminants in the crude extracts. The chromatogram in Figure 2 is typical of the peaks observed; an abnormally large amount was injected in this case so that triacetyldihydrosphingosine (peak 1) would be more clearly visible. Nearly symmetrical peaks were obtained with the liquid phase used in this determination (a copolymer of nitrile silicone and polyethylene glycol succinate), whereas a few other liquid phases tested gave moderate to severe tailing of TAPS. Determinations of TAPS were made with reasonable accuracy by comparing the peak area for 4 μg of pure TAPS with those observed from 0.2 to 5 μg of product in crude yeast extracts.

Mass Spectral Analyses of Reference Compounds. The mass spectrum of an authentic sample of TAPS.

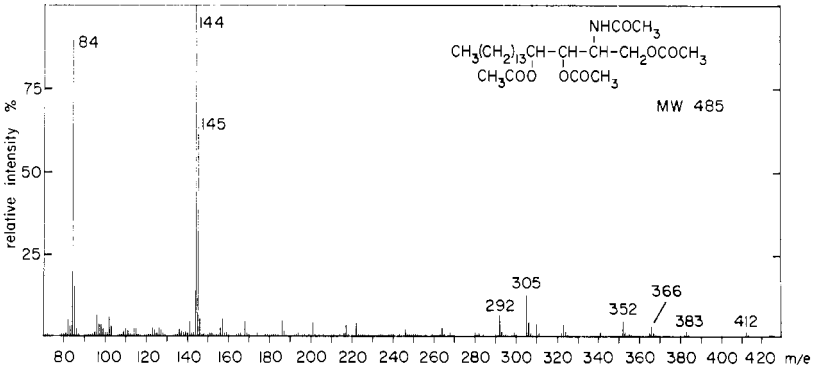
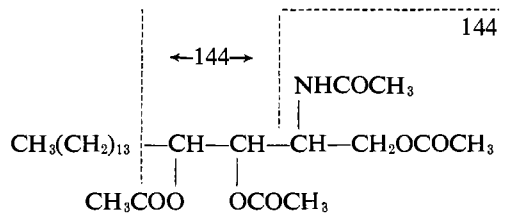


FIGURE 3: Mass spectrum of TAPS.

TABLE I: Exact Masses and Empirical Formulas of Selected Fragment Ions from TAPS.

Nominal *m/e* Ratio	Empirical Formula	Exact Mass		$\Delta \times 10^3$	Possible Structure
		Calcd	Found		
84	C_4H_6ON	84.0449	84.0439	−1.0	$[CH_3CONHCH{=}CH]^+$
144	$C_6H_{10}O_3N$	144.0661	144.0653	−0.8	$[CH(NHCOCH_3)CH_2OCOCH_3]^+$
145 (doublet)	$C_6H_9O_4$	145.0501	145.0494	−0.7	$[CH(OCOCH_3)CHOCOCH_3 + H]^+$
	$C_5{}^{13}CH_{10}O_3N$	145.0697	145.0688	−0.9	Mono-^{13}C of *m/e* 144
	$C_6H_{11}O_3N$	Not found in spectrum			
292	$C_{19}H_{34}ON$	292.2640	292.2649	+0.9	$[CH_3(CH_2)_{12}CH{=}CHCH{=}C(CH_3CONH)]^+$
305	$C_{20}H_{35}ON$	305.2719	305.2739	+2.0	M −3 × acetic acid
352	$C_{21}H_{38}O_3N$	352.2851	352.2846	−0.5	$CH_3(CH_2)_{13}CH{=}CC^+HNHCOCH_3$ ($OCOCH_3$)

recorded by combined gas chromatography–mass spectrometry, is shown in Figure 3. Most of the significant ions of higher mass, from *m/e* 292 to 412, were undoubtedly formed by fragmentation processes which included losses of acetate groups; these ions were not suitable, therefore, for measurements of isotopic abundance following experiments with labeled water and molecular oxygen. Furthermore, measurements could not be made with the molecular ion, since none was observed in the spectrum. The three major peaks at *m/e* 84, 144, and 145 turned out to be most useful in the mass spectral interpretation of labeled TAPS, since they were shown to represent unique fragment ions characteristic of different portions of the molecule. The exact masses of these three ions and several of the fragment ions of higher mass were determined by high-resolution mass spectrometry; the exact masses and empirical formulas are given in Table I. The large peak at *m/e* 84 was attributed to $[C_4H_6ON]^+$. Although the structure shown in Table I is an arbitrarily chosen one, there can be no doubt that *m/e* 84 is formed from the *N*-acetyl group and two carbon atoms from the chain of TAPS. Two portions of TAPS molecules might give rise to *m/e* 144, as indicated below, but it



was clear from high-resolution mass spectral data that *m/e* 144 was exclusively from a fragment ion from the terminal part of the molecule $[CH_3CONHCHCH_2OCOCH_3]^+$. The strong peak at *m/e* 145 was shown to be a doublet, consisting mainly of an ion $[CH_3COOCHCHOCOCH_3 + H]^+$ from C-3 and C-4 of TAPS along with a neighboring hydrogen atom. The minor ion at *m/e* 145 was the mono-^{13}C isotopic form of *m/e* 144, as expected.

There was no information in the mass spectrum of TAPS that could be used to distinguish labeling in the oxygens on C-3 and C-4. When it became necessary to compare the extent of incorporation of ^{18}O from water into these two positions, another derivative of phytosphingosine was used. In the mass spectrum of *N*-acetyl-1,3,4-tri-*O*-trimethylsilylphytosphingosine (shown in Figure 4), there were fragment ions that could be used for this purpose. As observed earlier in mass spectra of *N*-acetyl-*O*-trimethylsilyl derivatives of sphingosine and dihydrosphingosine (Gaver and Sweeley, 1966), a prominent peak at M −174 results from simple cleavage between C-2 and C-3 with positive charge retention on the long-chain fragment. With the phytosphingosine derivative, this fragment ion $[CH_3(CH_2)_{13}CH(OSiMe_3)CHOSiMe_3]^+$ is observed at *m/e* 401. Cleavage between C-3 and C-4 gives a fragment ion $[CH_3(CH_2)_{13}CHOSiMe_3]^+$ which occurs at *m/e* 299 (M −276). This latter cleavage is unique with phytosphingosine since there is no analogous M −276 peak in the mass spectra of sphingosine or dihydrospningosine derivatives. Comparisons of the relative ^{18}O abundance in the two fragment ions at *m/e* 299 and 401 were therefore considered to be of diagnostic value in distinguishing ^{18}O incorporation into the hydroxyl groups on C-3 and C-4 of phytosphingosine.

Incorporation of ^{18}O from Gaseous Oxygen. In expt III, an actively growing yeast culture was exposed to highly enriched molecular oxygen in a closed system for a period of 2 hr. After this time, the yeast stopped growing in prototype and actual isotope experiments, apparently because of oxygen depletion. During the 2-hr period, however, yeast continued to grow and produced extracellular TAPS (Table II). The concentra-

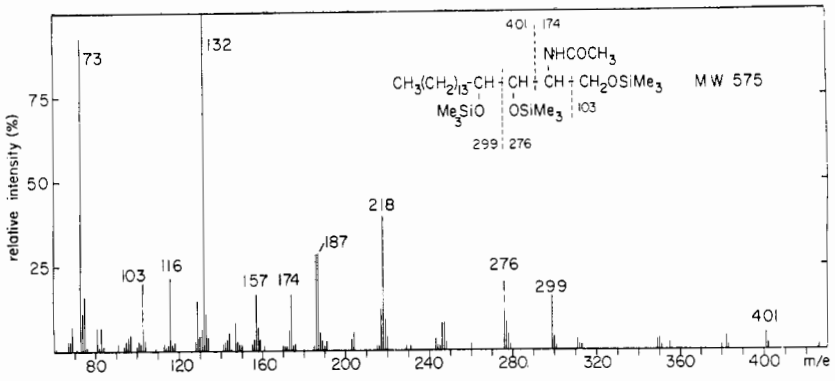


FIGURE 4: Mass spectrum of *N*-acetyl-1,3,4-tri-*O*-trimethylsilylphytosphingosine.

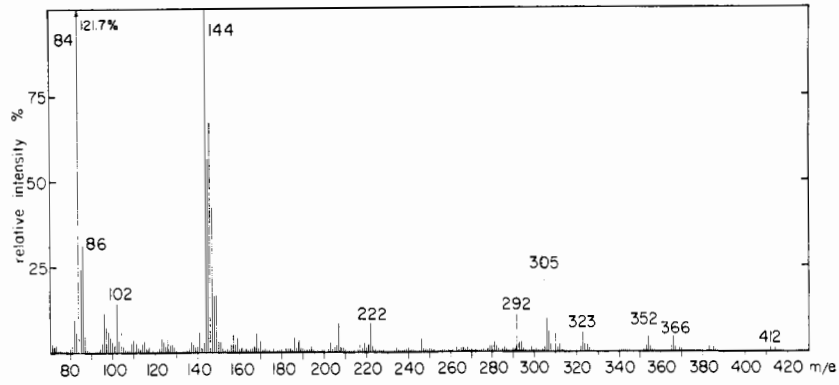


FIGURE 5: Mass spectrum of TAPS labeled with ^{18}O from water (expt I).

tion of TAPS increased from about 11 μg/ml in the medium to a final level of about 29 μg/ml. Incorporation of molecular oxygen into any single oxygen atom of TAPS would increase *m/e* 146 or 147, depending on the location of the ^{18}O, and the observed ratio of ion intensities, 146/(144 + 146) or 147/(145 + 147), would be 0.58 after correction for normal isotopic abundance. There was *no* increase in the relative intensities of *m/e* 146 or 147 in the mass spectrum of TAPS isolated in expt III.

In expt IV the yeast was exposed to labeled oxygen for a much longer time, during which the concentration of TAPS in the medium increased approximately 50-fold (see Table II). In this case, dilution by unlabeled TAPS could be ignored and the expected level of ^{18}O in any one position would be the same as that in the oxygen from electrolyzed water (20.1% ^{18}O). The mass spectrum of TAPS from this experiment was identical with that of authentic, unlabeled TAPS. It was concluded, therefore, that none of the oxygen atoms of TAPS is derived from molecular oxygen.

TABLE II: Determinations of Yeast Growth and TAPS Production.

Expt	Labeled Compd	Optical Density		TAPS (μg/ml)		Total Yield (μg)
		Initial	Final	Initial	Final	
I	$H_2{}^{18}O$	0.12	1.2	0.6	74.5	471
II	$H_2{}^{18}O$	0.16	2.0	0.7	22.0	132
III	$^{18}O_2$	0.4	0.6	10.9	29.1	483
IV	$^{18}O_2$	0.2	1.6	0.2	10.9	174

Incorporation of ^{18}O from Water. As shown in Table II, the extracellular concentration of TAPS increased from 0.6 to 74.5 μg/ml during exposure of the yeast to ^{18}O-enriched water for 22 hr in expt I. A mass spectrum of the product, reproduced in Figure 5, indicated that ^{18}O was incorporated into several positions in TAPS, as judged by the increases in intensity of *m/e* 86, 146, and 147. Comparisons of the predicted and observed isotopic abundance of ^{18}O in these three fragment ions are summarized in Table III. The percentage ratio of corrected intensities of *m/e* 84 and 86 (20.3%) was slightly higher than the predicted abundance but nevertheless provided clear evidence for incorporation of ^{18}O into the carbonyl oxygen of the *N*-acetyl group (or free exchange of this oxygen atom with the aqueous medium at some point). An alternative method of correcting *m/e* 86 values for background abundance of isotopes, involving simple subtraction of the percentage

TABLE III: Incorporation of ^{18}O from Water into Extracellular Tetraacetylphytosphingosine.

Structure	*m/e* Pair	Atom Per Cent Excess ^{18}O			Regenerated TAPS[a]
		Predicted (%)[b]	Isolated TAPS (%) Expt I	Expt II	Expt I (%)
$[CH_3CONHCH{=}CH]^+$	84 and 86	17.5 ± 0.5 (1)	20.3	22.2	2.5
$[CH_3CONHCHCH_2OCOCH_3]^+$	144 and 146	29.7 ± 0.7 (2) 38.8 ± 0.8 (3)	38.5	37.1	15.7
$[CH_3COOCHCHOCOCH_3 + H]^+$	145 and 147	38.8 ± 0.8 (3) 45.8 ± 0.8 (4)	43.0	43.0	17.7

[a] Isolated TAPS converted to phytosphingosine with $Ba(OH)_2$; product reacetylated with acetic anhydride. [b] Based on calculated atom per cent excess ^{18}O in water (17.5 ± 0.5%). Values are predicted for incorporation of from 1 to 4 atoms of ^{18}O (number in parentheses) into the fragment ion shown.

TABLE IV: Abundance of ^{18}O from Water in *N*-Acetyl-*O*-trimethylsilyl Derivatives from Extracellular Acetylated Bases.

Compound	Fragment Ion	*m/e* Pair	Atom Per Cent Excess ^{18}O (%)		
			Predicted	Expt I[a]	Expt II[a]
Phytosphingosine	$[CH_3(CH_2)_{13}\underset{\displaystyle OSiMe_3}{\underset{\vert}{C}}HCHOSiMe_3]^+$	401 and 403	17.5 ± 0.5 (1) 29.8 ± 0.7 (2)	15.7	14.4
	$[CH_3(CH_2)_{13}CHOSiMe_3]^+$	299 and 301	17.5 ± 0.5 (1)	1.0	3.1
Dihydrosphingosine	$[CH_3(CH_2)_{14}CHOSiMe_3]^+$	313 and 315	17.5 ± 0.5 (1)		11.8

[a] *N*-Acetylphytosphingosine was prepared from TAPS by mild alkaline hydrolysis in aqueous methanolic NaOH (expt I) and by $LiBH_4$ reduction in dioxane (expt II).

ratio of *m/e* 84 and 86 observed in unlabeled TAPS, gave an observed abundance (18.0%) much more nearly identical with the predicted level of 17.5 ± 0.5%.

The corrected percentage ratio of *m/e* 144 and 146 in expt I (38.5%) was almost exactly the same as that predicted if all three oxygen atoms in the *N,O*-diacetylethanolamine moiety of TAPS were derived from or exchanged with water of the medium.

The determination of isotopic abundance in the fragment ion from C-3 and C-4 of TAPS, at *m/e* 145 and 147 in the spectrum from labeled TAPS, was not so precise, since the corrected percentage of ^{18}O (43%) was about halfway between the predicted levels for three (38.8%) or four (45.8%) labeled oxygen atoms. Since the arithmetic manipulations required to correct *m/e* 145 and 147 were considerably more complex than those used in the correction of *m/e* 146, it was concluded that an appreciable error in either direction might be expected in the calculated isotopic abundance in this fragment ion. An alternative explanation was that 3 of the 4 atoms of oxygen in this fragment ion of TAPS were derived completely from water, whereas one was only partially labeled with oxygen from water. The results of a duplicate experiment with labeled water were in very good agreement (Table III, expt II) with those found in expt I.

The acetyl groups were removed from labeled TAPS with hot barium hydroxide and the crude free phytosphingosine was reacetylated. A mass spectrum of the regenerated TAPS was taken by combined gas chromatography–mass spectrometry, and corrected values for isotopic abundance (Table III) showed conclusively that the terminal OH group and one of the two secondary OH groups on C-3 and C-4 of phytosphingosine were labeled from water. The small percentage of isotope in the fragment ion at *m/e* 84 and 86 was attributed to survival of a slight amount of *N*-acetylphytosphingosine in the alkaline hydrolysis.

To provide evidence for which of the secondary OH groups was labeled from water, *N*-acetylphytosphingosine was oxidized with periodate, and the resulting aldehyde was quickly reduced with sodium borohydride. Mass spectral examination of the pentadecanol showed no evidence of ^{18}O in the molecule, suggesting that the labeled secondary OH group of

phytosphingosine must be on carbon three. Negative results in this experiment must be considered inconclusive, however, since labeled oxygen in the intermediate aldehyde might exchange with solvents at a very high rate, leading to unlabeled pentadecanol.

The results of mass spectral determinations of isotopic abundance in the *N*-acetyl-*O*-trimethylsilyl derivatives of phytosphingosine and dihydrosphingosine are presented in Table IV. In the case of phytosphingosine, the corrected percentage ratios of observed ion intensities at *m/e* 401 and 403 agreed well in expt I and II and the observed values (15.7 and 14.4%) were close to the known abundance of ^{18}O in the water. These data support the conclusion drawn previously that one of the two secondary OH groups remains without ^{18}O when the yeast is grown in the presence of labeled water. Furthermore, the results were found to be independent of the method used to deacetylate TAPS. The fragment ion at *m/e* 299 and 301 in the mass spectrum of labeled *N*-acetyl-*O*-trimethylsilylphytosphingosine was only slightly labeled (1.0 and 3.1%) in the two experiments with $H_2{}^{18}O$. It was, therefore, concluded that water was a poor source of oxygen for the secondary OH group on C-4 of phytosphingosine, but that the other secondary OH group (on C-3) was derived from water.

The secondary OH group on C-3 of dihydrosphingosine was also derived from water, since the fragment ion at *m/e* 313 was accompanied by appropriately increased intensity at *m/e* 315 in the mass spectrum of labeled *N*-acetyl-*O*-trimethylsilyldihydrosphingosine (Table IV). This result is consistent with and strengthens the conclusions drawn from mass spectra of labeled *N*-acetyl-*O*-trimethylsilylphytosphingosine.

Discussion

Although sphingosine has never been found in the extracellular medium or in the cells of *H. ciferrii*, a possible pathway for the biosynthesis of phytosphingosine from palmitate and serine in this organism might involve hydration of the olefinic group in sphingosine. The newly formed secondary hydroxyl group on C-4 would be derived from water in this case. A second possibility, which does not implicate sphingosine as an intermediate, involves the direct hydroxylation of dihydrosphingosine with insertion of one oxygen atom derived from molecular oxygen. Still other possibilities might be those in which either hydration or hydroxylation occurs at some step much earlier in the biosynthetic sequence. It is apparent that determination of the source of oxygen for the secondary hydroxyl group on C-4 of phytosphingosine is of crucial importance to the further elucidation of a pathway for its synthesis by *H. ciferrii*.

Mass spectral analyses of extracellular TAPS afforded conclusive evidence for the incorporation of oxygen from water into the terminal hydroxyl group of phytosphingosine and the acetyl groups on C-1 and C-2 of TAPS. These conclusions were drawn from the accurate and unambiguous corrected isotopic abundances found in fragment ions *m/e* 84 (86) and 144 (146) in mass spectra of labeled extracellular TAPS. The data do not, however, imply that these three oxygen atoms were necessarily derived from water during the course of the synthetic reactions in the cell. It is equally possible, and probable in some cases, that labeled oxygen atoms arise from exchange at some step in the reaction sequence. For example, any or all of the carbonyl oxygens of the acetyl groups in TAPS *may* have derived from glucose but equilibrated with water in some intermediate such as pyruvate or acetyl-CoA. These isotopic experiments with whole cells cannot distinguish between the two alternatives.

It was difficult to interpret the observed values for corrected isotopic abundance in the fragment ion of TAPS at *m/e* 145 (147). The results suggested that all, or perhaps all but one, of the oxygen atoms in this fragment ion were derived from or equilibrated with water. Assuming that the acetyl groups of C-3 and C-4 would be labeled the same as those of carbons one and two, these results were interpreted to mean that one of the two secondary OH groups on C-3 and C-4 of phytosphingosine *might not* be derived from water. This assumption was confirmed by the results of mass spectral examination of regenerated TAPS, containing no ^{18}O in the acetyl groups. There were only two labeled oxygen atoms in this compound, when derived from TAPS of expt I, and it was clear from the observed ratios of ^{18}O and ^{16}O in the fragment ions at *m/e* 144 (146) and 145 (147) that the terminal OH and *one* of the two secondary OH groups of phytosphingosine was labeled.

Comparisons of isotopic abundance in the fragment ions at *m/e* 299 (301) and *m/e* 401 (403) from labeled *N*-acetyl-*O*-trimethylsilylphytosphingosine afforded clear evidence for incorporation of isotope from water into the oxygen atom on C-3. This OH group in phytosphingosine was derived originally from the C_{16} intermediate that condensed with serine; it is reasonable, therefore, that it becomes labeled when the synthesis of TAPS is conducted in $H_2{}^{18}O$. This finding is consistent in the triacetyldihydrosphingosine as well, since the analogous fragment ion from *N*-acetyl-*O*-trimethylsilyldihydrosphingosine, at *m/e* 313 (315), was also appropriately labeled with ^{18}O from water. Oxygen from water was incorporated into the OH group on carbon four of phytosphingosine only to a slight extent, amounting to about 15% of the expected isotopic abundance.

Of some concern in the mass spectral analyses of isotopic abundance in regenerated TAPS and *N*-acetyl-*O*-trimethylsilylphytosphingosine (expt I) was the possibility that the unlabeled oxygen atom may have resulted from an abnormal mechanism of alkaline hydrolysis. For example, abnormal cleavage of the secondary *O*-acetyl ester on C-4 as shown below (a type $B_{A1}1$ mechanism described by Gould, 1959) would lead to exchange of a labeled hydroxyl oxygen with the solvent. To evaluate this possibility, *N*-acetylphytosphingosine was prepared from TAPS by a route that did not involve

$$RO^*COCH_3 + OH^- \rightarrow ROH + (CH_3COO^*)^-$$

alkaline hydrolysis of the esters. The same low percentage of isotope was found in the fragment ion at *m/e* 299 (301) from the *N*-acetyl-*O*-trimethylsilyl derivative, regardless of whether alkaline hydrolysis or lithium borohydride reduction was used. It was concluded from these results that the low concentration of isotope in the OH group on C-4 could not be attributed to isotopic exchange.

The data obtained in experiments with labeled water were consistent with a hypothesis that the secondary OH group on C-4 of phytosphingosine is derived from a source other than water, even though a small proportion of oxygen from water was found in this position. These results implied a mechanism in which molecular oxygen served as a precursor for this OH group, and we undertook to prove this point in two experiments with $^{18}O_2$. In a completely closed system containing a relatively small head volume of highly enriched $^{18}O_2$ mixed with nitrogen, growth of *H. ciferrii* proceeded for a short time, during which the concentration of extracellular TAPS approximately tripled. Isolated TAPS was completely unlabeled in all positions. Because of the importance of this finding, a second experiment with labeled molecular oxygen was designed to provide a greater degree of reliability. With a system that generated oxygen on demand by electrolysis of $H_2{}^{18}O$, the yeast grew well for more than 2 days, during which time the concentration of extracellular TAPS increased 50-fold and was accompanied by the utilization of about 33 μmoles of water in the electrolysis tube. Again, the isolated TAPS contained no labeled oxygen atoms.

These studies eliminate those pathways to phytosphingosine involving either water or molecular oxygen as a precursor of the hydroxyl group at C-4. The exact source of this oxygen is, in fact, an elusive problem that still remains to be settled. A low percentage of this OH group of phytosphingosine was labeled in both experiments with $H_2{}^{18}O$, but the observed level was hardly sufficient to conclude that the OH group was derived from water. It is rather more likely that some unknown compound provided in the yeast maintenance medium serves as an oxygen donor in the biosynthesis of phytosphingosine by *H. ciferrii*. If such a hypothesis were correct, the compound would have to be in a chemical form resistant to solvent exchange. A finding by Maister *et al.* (1962) that TAPS is formed by *H. ciferrii* only during the dissimilation of glucose is of some interest in this connection. When the glucose content of the medium was exhausted, they noted that TAPS was no longer produced by the yeast. It is intriguing that the high pool of glucose initially available to the growing cells represents a source of hydroxyl groups which would not be subject to immediate exchange with the water. Whether such a substance or one of its metabolites might serve exclusively as the precursor for the elusive hydroxyl group of phytosphingosine is highly speculative but worthy of active consideration.

Appendix

An implicit assumption in the mass spectral determinations, and their interpretations, is that isotopic and normal molecules of water or oxygen are used randomly by yeast in the biosynthesis of TAPS. The possibility of an isotope effect in the fragmentation of TAPS was ignored. Total probabilities, expressed as per cent, for heavier fragment ions resulting from normal isotopic abundance of ^2H, ^{13}C, etc., in the compounds were taken from Beynon and Williams (1963), who used the following assumed normal isotopic abundances: ^{13}C, 1.069%; ^2H, 0.016%; ^{15}N, 0.380%; ^{17}O, 0.039%; ^{18}O, 0.200%. Normal isotopic abundance in fragment ions from the *O*-trimethylsilyl derivatives was found from mass spectra of unlabeled compound, rather than from probability theory based on empirical formulas.

Normal Total Isotopic Abundance in Fragment Ions of TAPS

$$C_4H_6NO: \quad \frac{85}{84} = 4.839\%; \frac{86}{84} = 0.294\%$$

$$C_6H_{10}NO_3: \quad \frac{145}{144} = 7.142\%; \frac{146}{144} = 0.821\%$$

$$C_6H_9O_4: \quad \frac{146}{145} = 6.784\%; \frac{147}{145} = 0.997\%$$

Equations for Calculations of Per Cent ^{18}O

C_4H_6NO

$$\%\ ^{18}O = \frac{86_{cor}}{84_{obsd} + 86_{cor}} \times 100$$

$$86_{cor} = 86_{obsd} - 0.00294 \times 84_{obsd}$$

For TAPS of expt I

$$86_{cor} = 37.9 - 0.00294 \times 146.9 = 37.5$$

$$\%\ ^{18}O = \frac{37.5}{146.9 + 37.5} \times 100 = 20.3\%$$

$C_6H_{10}NO_3$

$$\%\ ^{18}O = \frac{146_{cor}}{144_{obsd} + 146_{cor}} \times 100$$

$$146_{cor} = 146_{obsd} - 0.00821 \times 144_{obsd} - 0.06784(145_{obsd} - 0.07142 \times 144_{obsd})$$

For TAPS of expt I

$$146_{cor} = 80.7 - 0.00821 \times 120.7 - 0.06784(68.0 - 0.07142 \times 120.7) = 75.7$$

$$\%\ ^{18}O = \frac{75.7}{120.7 + 75.7} \times 100 = 38.5\%$$

$C_6H_9O_4$

$$\%\ ^{18}O = \frac{147_{cor}}{145_{cor} + 147_{cor}} \times 100$$

$$147_{cor} = 147_{obsd} - 0.07142(146_{obsd} - 0.00821 \times 144_{obsd} - 0.06784(145_{obsd} - 0.07142 \times 144_{obsd})) - 0.00997(145_{obsd} - 0.07142 \times 144_{obsd})$$

$$145_{cor} = 145_{obsd} - 0.07142 \times 144_{obsd}$$

For TAPS of expt I

$$147_{cor} = 50.8 - 0.07142(80.7 - 0.00821 \times 120.7 - 0.06784(68.0 - 0.07142 \times 120.7)) - 0.00997(68.0 - 0.07142 \times 120.7) = 44.8$$

$$145_{cor} = 68.0 - 0.07142 \times 120.7 = 59.4$$

$$\%\ ^{18}O = \frac{44.8}{59.4 + 44.8} \times 100 = 43.0\%$$

Predicted Percentage of Isotopic Oxygen in Mono-^{18}O-Labeled Species

Composition of water = 82.5% $H_2{}^{16}O$, 0.21% $H_2{}^{17}O$, 17.5% $H_2{}^{18}O$ (low level of ^{17}O is ignored in these calculations)

C_4H_6NO

$$\text{mono-}^{18}\text{O-labeled species} = 17.5\%$$

$C_6H_{10}NO_3$ and $C_6H_9O_4$

For one labeled position

$$1 \times 1 \times 1 \times 0.825 = 0.825 \text{ unlabeled}$$

$$1 \times 1 \times 1 \times 0.175 = 0.175 \text{ labeled}$$

$$\text{mono-}^{18}\text{O-labeled species} = \frac{0.175}{0.825 + 0.175} \times 100 = 17.5\%$$

For two labeled positions

$$1 \times 1 \times 0.825 \times 0.825 = 0.681 \text{ unlabeled}$$

$$1 \times 1 \times 0.825 \times 0.175 = 0.144 \text{ singly labeled}$$

$$1 \times 1 \times 0.175 \times 0.825 = 0.144 \text{ singly labeled}$$

$$1 \times 1 \times 0.175 \times 0.175 = 0.031 \text{ doubly labeled}$$

$$\text{mono-}^{18}\text{O-labeled species} = \frac{2 \times 0.144}{0.681 + 0.288} \times 100 = 29.7\%$$

For three labeled positions

$$1 \times 0.825 \times 0.825 \times 0.825 = 0.562 \text{ unlabeled}$$

$$1 \times 0.175 \times 0.825 \times 0.825 = 0.119 \text{ singly labeled}$$

$$1 \times 0.825 \times 0.175 \times 0.825 = 0.119 \text{ singly labeled}$$

$$1 \times 0.825 \times 0.825 \times 0.175 = 0.119 \text{ singly labeled}$$

$$1 \times 0.175 \times 0.175 \times 0.825 = 0.025 \text{ doubly labeled}$$

$$1 \times 0.175 \times 0.825 \times 0.175 = 0.025 \text{ doubly labeled}$$

$$1 \times 0.825 \times 0.175 \times 0.175 = 0.025 \text{ doubly labeled}$$

$$1 \times 0.175 \times 0.175 \times 0.175 = 0.005 \text{ triply labeled}$$

$$\text{mono-}^{18}\text{O-labeled species} = \frac{3 \times 0.119}{0.562 + 3 \times 0.119} \times 100 = 38.8\%$$

For four labeled positions

$$0.825 \times 0.825 \times 0.825 \times 0.825 = 0.463 \text{ unlabeled}$$

$$0.175 \times 0.825 \times 0.825 \times 0.825 = 0.098 \text{ singly labeled}$$

$$0.825 \times 0.175 \times 0.825 \times 0.825 = 0.098 \text{ singly labeled}$$

$$0.825 \times 0.825 \times 0.175 \times 0.825 = 0.098 \text{ singly labeled}$$

$$0.825 \times 0.825 \times 0.825 \times 0.175 = 0.098 \text{ singly labeled}$$

$$0.175 \times 0.175 \times 0.825 \times 0.825 = 0.021 \text{ doubly labeled}$$

etc. for other doubly, triply, and fully labeled species

$$\text{mono-}^{18}\text{O-labeled species} = \frac{4 \times 0.098}{0.463 + 4 \times 0.098} \times 100 = 45.8\%$$

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Preferential Formation of Antibodies Specific toward D-Amino Acid Residues upon Immunization with Poly-DL-peptidyl Proteins*

Israel Schechter and Michael Sela

ABSTRACT: Precipitating antibodies reacting with poly-DL-alanyl determinants were produced in rabbits upon immunization with poly-DL-alanyl proteins. The specificity of these antibodies was directed mainly toward the D-alanine sequences in the poly-DL-alanyl determinants, as concluded from cross-precipitation reactions, absorption experiments, immunodiffusion tests, and inhibition studies with alanine peptides. The preferential immune response to determinants composed of D-amino acids was also observed when poly-DL-phenylalanyl and poly-DL-tyrosyl proteins were used for immunization.

The above findings may be interpreted as antigenic competition between sequences composed of L-, D-, or DL-amino acids, with the D sequences being the most efficient.

Polypeptidyl proteins are proteins to which peptide chains are attached. From the point of view of their immunological properties, the attached peptides may be considered haptens. Polypeptidyl protein antigens have been used in immunological studies concerned with immunogenicity, antigenic specificity, and immunological tolerance (Sela, 1966).

Investigations of antibodies to poly-L-alanyl and poly-D-alanyl determinants were reported recently (Sage *et al.*, 1964; Schechter and Sela, 1965a; Schechter *et al.*, 1966). The extent of formation of antibodies with specificity directed toward either determinant was of the same order of magnitude. The antibodies formed were strictly stereospecific as expected. When immunoglobulin G preparations devoid of proteolytic activity (Schechter *et al.*, 1966) were used, the size of the specific combining region of the antibodies was found such as to accommodate a maximum of three to four alanine residues, and the region of the antigenic determinant farthest removed from the protein carrier was of paramount importance in determining the specificity of the antibodies formed, as concluded from inhibition experiments with alanine peptides (Schechter *et al.*, 1966).

In view of the above results it was of interest to elucidate the specificity of the anti-polyalanyl antibodies formed upon immunization with poly-DL-alanyl pro-

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