STUDIES ON THE BIOSYNTHESIS OF SPHINGOLIPID BASES

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
KANIT KRISNANGKURA
1974

This is to certify that the

thesis entitled

STUDIES ON THE BIOSYNTHESIS
OF SPHINGOLIPID BASES

presented by

KANIT KRISNANGKURA

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Charles C. Sweeley
Major professor

Date 9/12/74

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ABSTRACT

STUDIES ON THE BIOSYNTHESIS OF SPHINGOLIPID BASES

by

Kamit Kriangkrai

The biosynthesis of sphingolipid bases was studied in rat liver microsomes and whole cells of yeast, Hansenula saturnus, using stable isotopes. Condensation of serine and palmitoyl CoA was accompanied with the initial loss of a hydrogen atom at C-2 of serine through the formation of serine-PLP Schiff's base. The product intermediate, 2-amino-3-ketoacid-PLP Schiff'base, underwent decarboxylation, followed by addition of a proton from the medium to yield 3-ketosphinganine after hydrolysis. The overall process occurred with retention of configuration since 3-ketosphinganine was formed from L-serine as the L-serine substrate with the stereospecific loss of the pro-R hydroxyl group of serine. A kinetic isotope effect was observed in the incubation of [2,3,3-$^{2}H_{3}$]serine by microsomal enzyme preparations.

4-Hydroxysphinganine could be derived from sphinganine by 1) reduction of 3-ketosphinganine, followed by stereospecific hydroxylation of 3-ketosphinganine, followed by reduction. The mechanism of the hydroxylation step is still unknown. Water and molecular oxygen were ruled out as the direct oxygen donors to the hydroxyl group on C-4 of 4-hydroxysphinganine (Thorpe and Sweeley, 1967). [1-^{18}O]palmitate, [3-^{18}O]serine, [1-^{18}O]sphinganine, [3-^{18}O]sphinganine,

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ABSTRACT

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The biosynthesis of sphingolipid bases was studied in rat liver microsomes and whole cells of yeast, Hansenula ciferri, using stable isotopes. Condensation of serine and palmitoyl CoA was accomplished with the initial loss of a hydrogen atom on C-2 of serine through the formation of serine-PLP Schiff's base. The proposed intermediate, 2-amino-3-ketoacid-PLP Schiff'base, underwent decarboxylation, followed by addition of a proton from the medium to yield 3-ketosphinganine after hydrolysis. The overall process occurred with retention of configuration since 3-ketosphinganine has the same configuration as the L-serine substrate with the palmitoyl group replacing the carboxyl group of serine. A kinetic isotope effect was observed in utilization of [2,3,3-$^{2}H_{3}$]serine by microsomal enzyme system from rat liver.

4-Hydroxysphinganine could be derived from 3-ketosphinganine by: 1) reduction of 3-ketosphinganine, followed by hydroxylation; and 2) hydroxylation of 3-ketosphinganine, followed by reduction. The mechanism of the hydroxylation step is still unknown. Water and molecular oxygen were ruled out as the direct oxygen donors to the hydroxyl group on C-4 of 4-hydroxysphinganine (Thorpe and Sweeley, 1967). [1-^{18}O]Palmitate, [3-^{18}O]serine, [1-^{18}O]sphinganine, [3-^{18}O]sphinganine,

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[2-^{18}O]glucose, glucose and possibly all the glycolytic intermediates except those present in the yeast extract were all ruled out as the oxygen donors to the hydroxyl group on C-4 of 4-hydroxysphinganine. The oxygen donor was heat-stble and did not exchange its oxygen with water in the medium at autoclave temperature.

By

Kanit Krisnangkura

A DISSERTATION

Submitted to

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in partial fulfillment of the requirements

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I would like to express my sincere gratitude to Dr. Charles C. Sweeley for his guidance and constant encouragement during the course of this study. Sincere appreciation is also expressed to the other members of my Ph.D. guidance committee: Dr. William W. Wells, Dr. Loran L. Bieber, Dr. Robert S. Bandurski and Dr. John C. Speck, Jr.

Special acknowledgements are made to Mr Bernd Soltmann and Jack E. Harten for their expert in high and low resolution mass spectrometry studies, respectively. Finally, I would like to thank for the helps I have recieved from Dr. Roger A. Laine, Dr. Ray K. Hammond, Dr. Mark A. Bieber and other associates from this lab and the Department of Biochemistry.

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AVA	a
Bu	l
Cbz	c
Cer	c
DNP	c
DTT	c
Et	e
Gal	g
GalNAc	N
Glc	g
GLC	g
LM-1	l
LM-2	l
LM-3	l
Me	m
MS	m
NANA	N
Ph	P
PLP	P
Pro	P
TLC	t
TMSi	t

LIST OF ABBREVIATIONS

Ac	acetyl
AVA	accelerating voltage alternator
Bu	butyl
Cbz	carbobenzoxycarbonyl
Cer	ceramide
DNP	dinitrophenyl
DTT	dithiothreitol
Et	ethyl
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GLC	gas-liquid chromatography
LM-1	liquid medium-1
LM-2	liquid medium-2
LM-3	liquid medium-3
Me	methyl
MS	mass spectrometry
NANA	N-acetylneuraminic acid
Ph	phenyl
PLP	pyridoxal phosphate
Pro	propyl
TLC	thin-layer chromatography
TMSi	trimethylsilyl

A. Structur

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REVIEW OF LITERATURE ON THE SPHINGOLIPID BASES

A. Structure and Stereochemistry.

In studies on the chemical composition of human brain, Thudichum (1884) isolated an alkaloid-like compound which he called "sphingosin" (from Greek, sphingein, which means to bind tightly). The empirical formula ($C_{17}H_{35}NO_2$) given by Thudichum was incorrect but remarkably close for that time, especially using the free base for analysis. Worner and Thierfelder (1900) reported that sphingosine absorbed bromine, indicating that it was an unsaturated compound. Levene and Jacobs (1919) characterized sphingosine as a monoaminodihydroxy unsaturated compound by hydrogenation and preparation of triacetyl derivative of sphingosine and the reduced product. Chromic acid oxidation of sphingosine and dihydrosphingosine yielded myristic acid and palmitic acid, respectively (Klenk, 1929; Klenk and Diebold, 1931), suggesting that sphingosine was a C_{18}-straight chain compound with a double bond between C-4 and C-5. Ozonolysis of sphingosine or its triacetyl derivative yielded a nitrogen fragment containing four carbon atoms (Levene and West, 1914; Klenk and Diebold, 1931), providing another piece of evidence that the double bond was at C-4. Dihydrosphingosine was reported to consume 2 moles of periodate whereas its N-acetyl or N-benzoyl derivative were not attacked by periodate under a variety of conditions (Carter et al., 1942 and 1947), suggesting that the two hydroxyl groups were not adjacent. Accordingly, 1,3-dihydroxy-2-aminooctadec-4-ene

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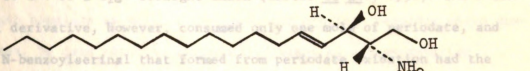
was proposed for the structure of sphingosine.

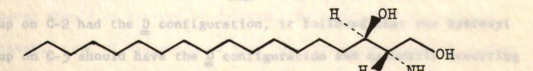
Naturally occurring sphingosine, cerebrosides and sphingomyelins absorbed in the infrared region around 975 cm^{-1} (or 10.3 mμ) which was characteristic of the trans double bond (Mislow, 1952; Marinetti and Stotz, 1954). Kiss et al. (1954) correlated the structure of sphingosine to D-erythro-2-amino-3,4-dihydroxybutyric acid and suggested that naturally occurring sphingosine was D-erythro-1,3-dihydroxy-2-aminooctadec-4-ene, as shown below.



Sphingosine

(1)

During studies on the chemistry of sphingosine, Carter and Norris (1942) usually detected a small amount of dihydrosphingosine. The structure and stereochemistry of this compound was subsequently shown to be D-erythro-1,3-dihydroxy-2-aminooctadecane (Carter and Shapiro, 1953).



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Sphingolipid bases which occurred predominantly in the plant kingdom were found to contain phytosphingosine (Carter et al., 1954), although this compound, phytosphingosine, was first detected in mushroom by Zellner (1911). Several structures had been proposed for yeast cerebrin before the correct structure was proposed by Carter et al. (1954) and Carter and Hendrickson (1963). Phytosphingosine consumed 3 moles of periodate with a concomitant production of formaldehyde and pentadecanal, suggesting that 4 polar functional groups were on C-1 to C-4 of a C_{18}- straight chain (Carter et al., 1954). The N-benzoyl derivative, however, consumed only one mole of periodate, and the N-benzoylserinal that formed from periodate oxidation had the same configuration as N-benzoylserinal derived from periodate oxidation of N-benzoylglucosaminitol. The amino group on C-2 was, therefore, assigned the D configuration (Carter et al., 1954). Partial periodate oxidation of phytosphingosine followed by Ag_2O oxidation yielded 2D-hydroxypalmitate (Carter and Hendrickson, 1963). This experiment established that the hydroxyl group on C-4 had the D configuration. The ease of acyl migration from the amino group on C-2 to the hydroxyl group on C-3 of anhydrophytosphingosine suggested that the amino and the hydroxyl groups were in the cis configuration. Since the amino group on C-2 had the D configuration, it followed that the hydroxyl group on C-3 should have the D configuration and naturally occurring phytosphingosine was then assigned as D-ribo-1,3,4-trihydroxy-2-amino-octadecane (Carter and Hendrickson, 1963).

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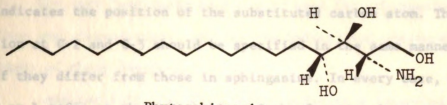

Phytosphingosine

(3)

Periodate oxidation of sphingolipid bases (Sweeley and Moscatelli, 1959) revealed, besides the three major long-chain bases (sphingosine, dihydrosphingosine and phytosphingosine), the occurrence of chain-length homologs as well as the iso- and anteiso- branched chain compounds. To date about 64 sphingolipid bases have been detected (Karlsson, 1970; Weiss and Stiller, 1972 and 1973).

B. Nomenclature.

The Commission of Biochemical Nomenclature of IUPAC-IUB (1967) recommended the name sphinganine (2) for the compound previously named dihydrosphingosine (2D-aminooctadecane-1,3-diol or D-erythro-2-aminooctadecane-1,3-diol or (2S,3R)-2-aminooctadecane-1,3-diol).

The name, sphinganine, may be modified to indicate additional substituents or higher or lower homologs. The prefixes to designate homologs should be derived by deleting the terminal "ne" from the systematic name of hydrocarbons that have the same number of carbon atoms as the principal chains of the long-chain bases.

Examples. Eicosasphinganine for C_{20}-homolog; sphinganine for C_{18}-homolog; hexadecasphinganine for C_{16}-homolog.

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specified by the prefixes "D-" or "L-" following the number that indicates the position of the substituted carbon atom. The configuration at C-2 and C-3 should be specified in the same manner, but only if they differ from those in sphinganine. In every case, the prefixes D or L refer to the orientation of the functional group to the right or left, respectively, of the carbon chain written vertically in Fischer projection with C-1 on top. If the configuration is unknown, the term "X-" should be used as prefix to the name.

Example. 4D-hydroxysphinganine for the compound previously named phytosphingosine.

The names of unsaturated compounds are derived from the names of the corresponding saturated compounds by replacing the ending "ane"* with the appropriate ending denoting unsaturation such as "ene", "adiene",, "yne". A double bond is presumed to have the trans orientation of the carbon chain unless cis or unknown geometry is specified by the term "cis-" or "x-" preceeding the number that indicates the position of the double bond.

Examples. 4-Sphingenine for the compound previously called sphingosine; cis-4-sphingenine for the geometric isomer of sphingosine.

The trivial name "sphingosine" may be retained. If trivial names other than sphingosine are used, they should be defined in each paper in terms of this nomenclature, or of the general nomenclature of organic chemistry.

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* "ane" is not the correct ending of sphinganine, it should be "anine" ....."enine", adienine", "ynine".

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The term long-chain base may be used for sphinganine, its homologs and stereoisomers, and for the hydroxy and unsaturated derivatives of these compounds;

-sphingolipid, for any lipid containing a long-chain base;

-glycosphingolipid, for any lipid containing a long-chain base and one or more sugars;

-ceramide, for N-acyl long-chain base;

-cerebroside, for a monoglycosylceramide;

-ganglioside, for a glycosphingolipid containing a neuraminic acid.

Certain disadvantages of the aforementioned nomenclature were pointed out by Karlsson (1970), who said "Several authors have used synonyms of the terms sphingolipid long-chain base(s), not including specific information on chain length, stereochemistry, etc., for example, sphingosine bases, sphinganines, sphingosines. This does not follow the proposal given, but may indicate a need for a general term in addition to long chain bases". Accordingly, the term sphinganine will be used here to represent 2-aminooctadecane-1,3-diol, ignoring the stereochemistry on C-2 and C-3. Similarly, 4-sphingenine will represent 2-aminooctadec-4-ene-1,3-diol. The terms "erythro-", "threo-","ribo-", "arabino-", "lyxo-" and "xylo-" which were frequently encountered in the literature will also be used here to designate the stereochemistry of the whole molecule of long-chain bases.

Examples. D-Erythro-sphinganine for the recommended sphinganine; D-erythro-4-sphingenine for sphingosine; D-ribo-4-hydroxysphinganine

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The term sphingolipid base will be used interchangeably with the term long-chain base;

-dihydroxy long-chain base will be used for sphingolipid base which has two hydroxyl groups;

-trihydroxy base, for sphingolipid base which has three hydroxyl groups, on C-1, C-3 and C-4.

C. Isolation and Separation.

Although free long-chain bases were recently found in nature (Karlsson, 1970), they represented a very small amount in comparison with the total long-chain bases in that tissue, most of which are bound with fatty acids and sugars or phosphorylcholine. Alkaline hydrolysis or acid hydrolysis can be used to liberate the long-chain bases from these complex molecules. Alkaline hydrolysis gave poor yields of sphingolipid bases (Robbin et al., 1956). Anhydrous methanolic acid hydrolysis, however, tended to give 3-O-methyl-4-sphingenine and 5-methoxy-3-deoxy-3-sphingenine by-products of 4-sphingenine (Weiss, 1964). If the long-chain base used in the study was 4-hydroxysphinganine, anhydro-4-hydroxysphinganine was formed in considerable amounts (O'Connell and Tsein, 1959). Gaver and Sweeley (1965) observed that a trace amount of water in methanol could suppress the amount of ether by-products. Optimal conditions for the hydrolysis of complex sphingolipids were pursued and reported by Gaver and Sweeley (1965). Stoffel and Assmann (1972) claimed that the conditions used by Gaver and Sweeley (1965) were not strong

*Table 1. Chromatographic Separation of Long-chain Bases**

Separation of Dihydroxy and Trihydroxy Long-chain Bases.

Table 1. Chromatographic Separation of Long-chain Bases*

Separation of Dihydroxy and Trihydroxy Long-chain Bases.		
GLC		
SE-30	N-Ac and O-TMSi	Thorpe and Sweeley (1967)
	O-TMSi	Gaver and Sweeley (1965)
		Karlsson (1965)
TLC		
Silica gel G,		
$CHCl_3$-MeOH-H_2O, 65:25:4	Free bases	Braun and Snell (1967)
$CHCl_3$-MeOH-2N NH_4OH, 40:10:1	Free bases	Sambasivaroa and McCluer (1963)
Petroleum ether-$CHCl_3$-MeOH, 7:3:1	N- and O-Ac	Gaver and Sweeley (1965)
$CHCl_3$-MeOH, 8:1	N-Ac	Stoffel et al. (1968b)
Silica Gel G and 2% NaB_2O_7,		
Hexane-$CHCl_3$-MeOH, 5:5:2	DNP	Karlsson (1968)
Column		
Silica gel S		
$CHCl_3$-MeOH and 2N NH_4OH Gradient	Free bases	Barenholz and Gatt (1968a)
Acetylcellulose, benzene	Acetone or p-Nitro-bezaldehyde	Weiss and Stiller (1970)

Abbreviations: TMSi, trimethylsilyl; Ac, acetyl; DNP, 2,4-dinitrophenyl; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

Table 1 (cont'd)

Counter-current Distribution		
Petroleum ether-Acetonitrile	N- and O-Ac	Stodola and Wickerham (1960)

Table 1 (cont'd)

Counter-current Distribution		
Petroleum ether-Acetonitrile	N- and O-Ac	Stodola and Wickerham (1960)
Separation of Saturated and Unsaturated Long-chain Bases.		
GLC		
SE-30	N-Ac and O-TMSi	Gaver and Sweeley (1966)
		Polito and Sweeley (1971)
		Hammond and Sweeley (1973)
	O-TMSi	Gaver and Sweeley (1965)
XE-60	N-Ac and O-TMSi	Polito and Sweeley (1971)
OV-17	N-Ac and O-TMSi	Polito and Sweeley (1971)
TLC		
Silica gel G,		
$CHCl_3$-Acetone, 2:1	Free bases	Fujino and Zabin (1962)
$CHCl_3$-MeOH, 4:1	Free bases	Fujino and Zabin (1962)
$CHCl_3$-MeOH-H_2O, 65:25:4	Free bases	Braun and Snell (1967)
$CHCl_3$-MeOH-2N NH_4OH, 40:10:1	Free bases	Sambasivarao and McCluer (1963)
Silica gel G and 20% $AgNO_3$,		
$CHCl_3$-MeOH, 94:6	N-Ac	Renkonen and Hirvisalo (1969)
$CHCl_3$-MeOH, 98:2	N- and O-Ac	Hammond and Sweeley (1973)
Silica gel G and 2% $Na_2B_2O_7$,		
$CHCl_3$-MeOH-Hexane, 50:15:50	DNP	Karlsson (1960)

Table 1 (cont'd)

Alumina G, *$CHCl_3$-MeOH, 100:1 or 100:2*	DNP	Michalec (1965)

Table 1 (cont'd)

Alumina G,		
$CHCl_3$-MeOH, 100:1 or 100:2	DNP	Michalec (1965)
Paper		
Pyridine	Free bases	Brady and Koval (1958)
Reverse Phase		
Paper-Tetralin,		
MeOH-Et_3N-H_2O, 85:5:10	DNP	Karlsson (1960)
Chain Length Separation.		
GLC		
SE-30	N-Ac and O-TMSi	Gaver and Sweeley (1966)
	O-TMSi	Gaver and Sweeley (1965)
Reverse Phase		
Al_2O_3-Tetralin,		
Tetralin-MeOH-H_2O, 90:10:10	DNP	Michalec (1965)
Separation of Threo and Erythro Isomers		
GLC		
SE-30	N-Ac and O-TMSi	Gaver and Sweeley (1966)
		Carter and Gaver (1967)
	O-TMSi	Gaver and Sweeley (1965)

Table 1 (cont'd)

TLC		
Silica gel G,		
$CHCl_3$-MeOH-H_2O, 65:25:4	Free bases	Braun and Snell (1967)

Table 1 (cont'd)

TLC		
Silica gel G,		
$CHCl_3$-MeOH-H_2O, 65:25:4	Free bases	Braun and Snell (1967)
$CHCl_3$-MeOH-2N NH_4OH, 40:10:1	Free bases	Sambasivaroa and McCluer (1963)
$CHCl_3$-MeOH, 4:1	Free bases	Fujino and Zabin (1962)
$CHCl_3$-Acetone, 2:1	Free bases	Fujino and Zabin (1962)
Silica gel G and Borate,		
$CHCl_3$-MeOH-Hexane, 50:10:50	DNP	Karlsson et al. (1973)

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enough to cleave the phosphate diester linkage in sphingomyelin and that sphingenyl-1-phosphorylcholine was formed in almost quantitative amount. Michalec (1967) and Karlsson (1968) treated sphingomyelins with phospholipase C to hydrolyze the phosphate diester linkage prior to alkaline hydrolysis and these authors reported a quantitative amount of 4-sphingenine. Separation of long-chain bases into different classes or individual compounds can be achieved by chromatographic techniques, shown in Table 1. Optical isomers were resolved with optically active organic acids (Ellner et al., 1970; Kisic et al., 1971; Sticht et al., 1972).

D. Chemical Synthesis.

1. Conversion of 4-Sphingenine to Sphinganine and 4-Hydroxysphinganine.

4-Sphingenine was converted to sphinganine by catalytic hydrogenation (Weiss and Stiller, 1967). When tritium gas was used, radioactivity was not evenly distributed. About 47% was on C-4, 37% was on C-5 and 16% was on C-6 to C-18. Reduction of unsaturated long-chain bases to saturated long-chain bases could be accomplished by using aqueous hydrazine (Renkonen and Hirvisalo, 1969; Hammond and Sweeley, 1973). The use of hydrazine had certain advantages over catalytic hydrogenation in that it did not cause isomerization or migration of double bond (Aylward and Sawisloka, 1962).

Conversion of 4-sphingenine to 4-hydroxysphinganine was independently reported by Weiss and Stiller (1965) and Prostenik et al. (1965). Tribenzoyl-4-sphingenine was oxidised with perbenzoic acid

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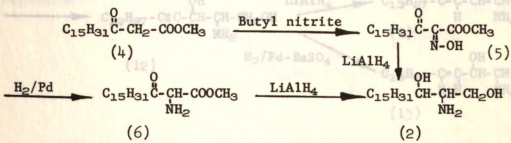
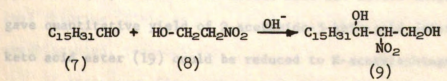
and the epoxide intermediate was then reduced to 4-hydroxysphinganine derivative.

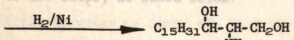
2. Synthesis.

Sphinganine was first chemically synthesized by Gregory and Malkin (1951). 3-Ketoacid ester was oximinated with butyl nitrite and the oxime intermediate (5) was catalytically hydrogenated to amino ester (6) which was further reduced to sphinganine with $LiAlH_4$. The oxime intermediate (5) could be directly reduced to sphinganine with $LiAlH_4$ (Fisher, 1952).



Grob and Jenny (1952) and Egerton et al. (1952) independently reported the condensation of 2-nitroethanol and palmitaldehyde, yielding 2-nitro-1,3-dihydroxyoctadecane (9). The nitroalcohol (9) was catalytically hydrogenated to sphinganine.





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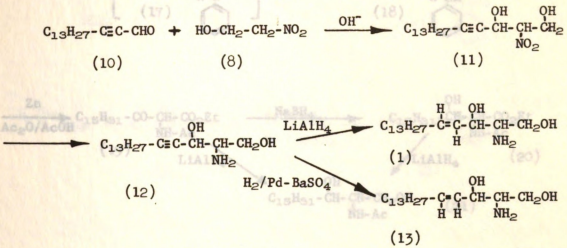
A similar synthetic sequence was reported by Grob and Gadient (1957), who used 2-hexadecynal in place of palmitaldehyde for the synthesis of 4-sphingenine. The nitro-diol intermediate (11), which could not be hydrogenated in this case, was reduced to an amino-diol (12) either by aluminium amalgum or zinc in hydrochloric acid.



A different approach for the synthesis of sphinganine was reported by Shapiro and Segal (1954) and Shapiro et al. (1958). Acylation of ethyl acetoacetate anion (15) with palmitoyl chloride yielded diketo-acid ester (16). Diazotization of diketo-acid ester (16), in the presence of ammonium salt, gave hydrazone (18). Reductive acetylation of hydrazone (18) with zinc in acetic acid and acetic anhydride mixture gave quantitative yield of 2-acetamido-3-keto-acid ester (19). This keto acid ester (19) could be reduced to N-acetylsphinganine (21) in one or two steps, as shown below.

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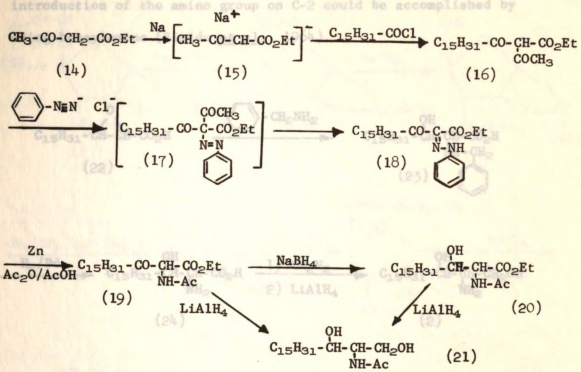

This synthetic procedure has been used repeatedly for the synthesis of radioactive sphinganine for biological studies (DiMari et al., 1971; Stoffel and Sticht, 1967b).

Substitution of palmitoyl chloride with 2-hexadecenoyl chloride (Shapiro and Segal, 1954; Shapiro et al., 1958a) and 2-methoxypalmitoyl chloride (Kisic et al., 1971), yielded 4-sphingenine and 4-hydroxysphinganine, respectively.

A stereospecific synthesis of threo- and erythro-sphinganine was first reported by Jenny and Grob (1953). The cis- and trans-2,3-epoxide (22) were treated with ammonia, and threo- and erythro-2-hydroxy-3-amino acids were formed in equal amount. Specific

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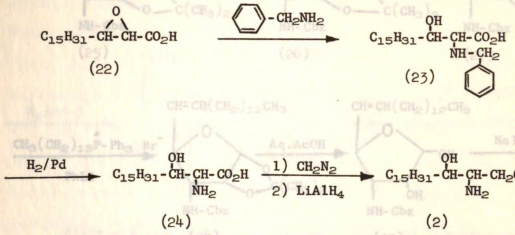
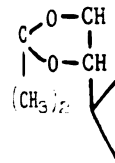
introduction of the amino group on C-2 could be accomplished by using benzylamine (Sisido et al., 1964).



The synthesis of D-erythro-sphinganine and 4-sphingenine was reported by Reist and Christie (1970). Selective removal of the 5,6-isopropylidene blocking group of (25) yielded (26). Periodate oxidation of (26) gave aldehyde (27) which was allowed to react with tetradecyltriphenyl phosphonium bromide in strong basic solution (Wittig reaction). A mixture of cis- and trans-olefins (28) was obtained. Varying the reaction conditions, especially the concentration of the phenyl lithium, the trans-isomer could be obtained in 60% yield. The mixture (cis and trans) might be hydrogenated, in the last step, to saturated derivative with simultaneous deblocking of the protecting (benzyloxycarbonyl) group.



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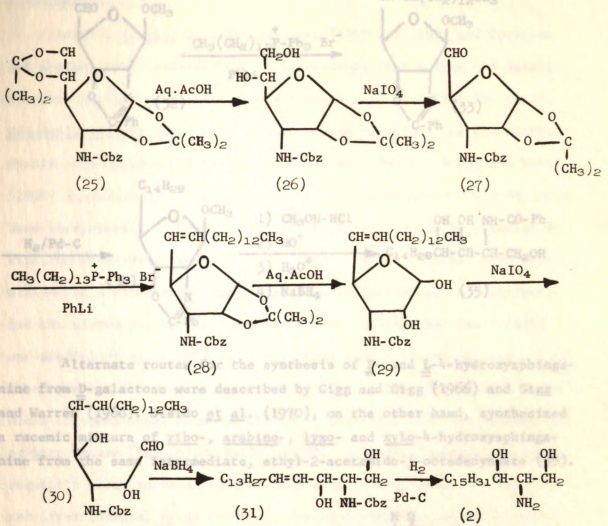

Stereospecific synthesis of D-ribo-4-hydroxysphinganine from D-glucosamine was described by Gigg et al. (1966). The aldehyde (32) which is a derivative of 2-amino-2-deoxy-D-allose, was synthesized from D-glucosamine. This aldehyde (32) was condensed with Wittig reagent (triphenyltridecylphosphonium bromide or -pentadecylphosphonium bromide). The olefin product (33) was saturated by catalytic hydrogenation. Hydrolysis, reduction with $NaBH_4$ and deblocking yielded D-ribo-4-hydroxysphinganine derivative (35).

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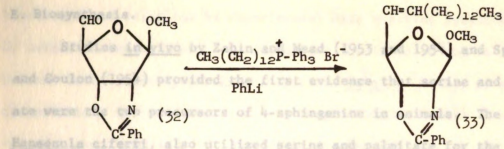
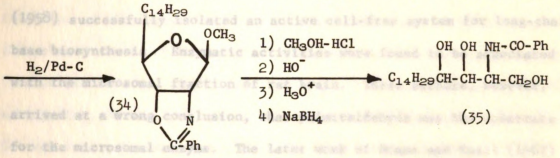
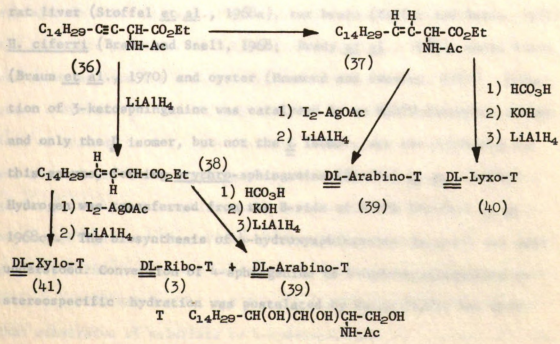




Alternate routes for the synthesis of D- and L-4-hydroxysphinganine from D-galactose were described by Gigg and Gigg (1966) and Gigg and Warren (1966). Sisido et al. (1970), on the other hand, synthesized a racemic mixture of ribo-, arabino-, lyxo- and xylo-4-hydroxysphinganine from the same intermediate, ethyl-2-acetamido-3-octadecynoate (36).



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E. Biosynthesis.

Studies in vivo by Zabin and Mead (1953 and 1954) and Sprinson and Coulon (1954) provided the first evidence that serine and palmitate were the two precursors of 4-sphingenine in animals. The yeast, Hansenula ciferri, also utilized serine and palmitate for the biosynthesis of 4-hydroxysphinganine (Green et al., 1965). Brady and Koval (1958) successfully isolated an active cell-free system for long-chain base biosynthesis. Enzymatic activities were found to be associated with the microsomal fraction of rat brain. These authors, however, arrived at a wrong conclusion, that palmitaldehyde was the substrate for the microsomal enzyme. The later work of Braun and Snell (1967) and Stoffel et al. (1968a) indicated that palmitoyl CoA was the actual substrate for the condensing enzyme. 4-Sphingenine formed with rat brain homogenate was shown on TLC to be identical with the natural erythro isomer (Fujino and Zabin, 1962). 3-Ketosphinganine was subsequently shown to be an intermediate with the microsomal system of rat liver (Stoffel et al., 1968a), rat brain (Kanfer and Bates, 1970), H. ciferri (Braun and Snell, 1968; Brady et al., 1969), mouse brain (Braun et al., 1970) and oyster (Hammond and Sweeley, 1973). Reduction of 3-ketosphinganine was catalysed by an NADPH-dependent enzyme and only the D isomer, but not the L isomer, was the substrate for this enzyme, forming erythro-sphinganine (Stoffel et al., 1968a). Hydrogen was transferred from the B-side of NADPH (Stoffel et al., 1968c). The biosynthesis of 4-hydroxysphinganine is still not well understood. Conversion of 4-sphingenine to 4-hydroxysphinganine by stereospecific hydration was postulated by Weiss (1965) but this

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hypothesis was ruled out by experimental data reported subsequently by several authors: that 1) 4-sphingenine itself could not be incorporated into 4-hydroxysphinganine (Stoffel et al., 1968b), 2) perdeuteropalmitate was converted to 4-hydroxysphinganine with loss of only one (obligatory) deuterium atom on C-2 (Polito and Sweeley, 1971), suggesting that there was no unsaturated intermediate in the formation of 4-hydroxysphinganine from palmitate, and 3) $H_2^{18}O$ was not the primary source of the hydroxyl group on C-4 of 4-hydroxysphinganine (Thorpe and Sweeley, 1967). With yeast grown in the presence of [4,5-^{3}H]-sphinganine, Weiss (1965) found that about 50% of the radioactivity on C-4 was lost relative to that on C-5. Thus, it was concluded that sphinganine was directly transformed to 4-hydroxysphinganine. This experiment, however, does not exclude the possibility that 3-ketosphinganine might be the immediate precursor of 4-hydroxysphinganine. Using [1-^{14}C,3-^{3}H] sphinganine as the substrate, Stoffel et al. (1968b) found that $^3H/^{14}C$ ratio of 4-hydroxysphinganine dropped to 1/7 that of the sphinganine substrate and it was concluded that 3-ketosphinganine was the immediate precursor of 4-hydroxysphinganine. A contradictory result was reported from the same laboratory (Stoffel and Binczek, 1971), that yeast converted [3-^{3}H,3-^{14}C] sphinganine to 4-hydroxysphinganine without loss of tritium on C-3. The isotopic ratio in 4-hydroxysphinganine was the same as that of sphinganine substrate, suggesting that sphinganine (but not 3-ketosphinganine) was the immediate precursor of 4-hydroxysphinganine. Regardless of whether 3-ketosphinganine or sphinganine was the immediate precursor of 4-hydroxysphinganine, Polito and Sweeley (1971) and Stoffel and Binczek (1971) independently reported that conversion of palmitate to 4-hydroxysphinganine involved the loss

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of the pro-R hydrogen atom on C-2 of palmitate or C-4 of sphinganine, whereas the pro-S hydrogen was retained. These findings together with the known stereochemistry of 4-hydroxysphinganine led these authors to conclude that the hydroxylation step proceeded with retention of configuration. The origin of the hydroxyl group on C-4 is still an elusive problem. $H_2^{18}O$ and molecular oxygen were both ruled out as the source of this hydroxyl group, although water was poorly incorporated (Thorpe and Sweeley, 1967).

The sequence of steps in 4-sphingenine synthesis is still controversial. Introduction of the double bond between C-4 and C-5 was shown to occur at 1) fatty acid level, in yeast (DiMari et al., 1971), 2) at the 3-ketosphinganine level (Fujino and Nakano, 1971; Hammond and Sweeley, 1973) and 3) after the reduction of the 3-ketosphinganine intermediate (Stoffel et al., 1971a; Ong and Brady, 1973). The stereochemical course of dehydrogenation was shown to proceed via a trans elimination in yeast, where the pro-R hydrogens on C-2 and C-3 of palmitate were removed (Polito and Sweeley, 1971) and via a cis elimination in rat, in which the pro-R hydrogen on C-2 and the pro-S hydrogen on C-3 of palmitate were removed (Stoffel et al., 1971).

Current knowledge of long-chain bases biosynthesis is summarized in Fig.1.

F. Degradation.

Studies on the biodegradation of long-chain bases were initiated by Barenholz and Gatt (1967), who injected [9,10-^{3}H]-4-hydroxysphinganine into the tail vein of rats and characterized the degradation product in liver. Pentadecanoic acid was the major radioactive component.

Serine + Palmitoyl CoA

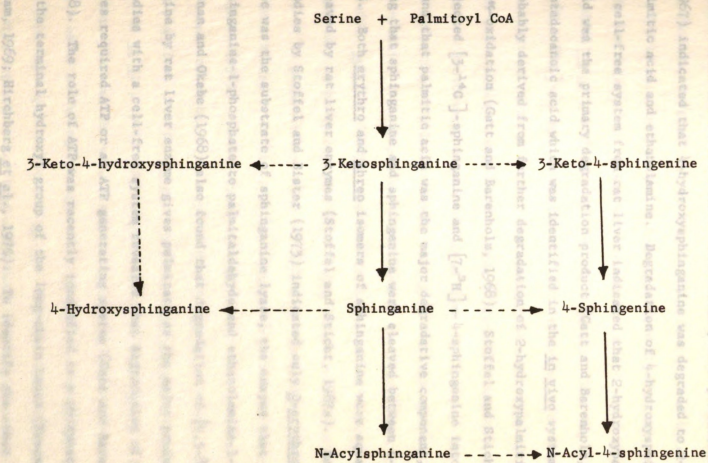

Fig.1. Intermidiary metabolism of long-chain bases (dotted lines are used where conclusive evidence for an individual step is not available).

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A small amount of heptadecanoic acid, the elongation product of pentadecanoic acid, was also detected. The authors tentatively concluded that 4-hydroxysphinganine was probably cleaved between C-3 and C-4 in an aldol-like mechanism. Studies in yeast by Karlsson et al. (1967) indicated that 4-hydroxysphinganine was degraded to 2-hydroxypalmitic acid and ethanolamine. Degradation of 4-hydroxysphinganine in cell-free system from rat liver indicated that 2-hydroxypalmitic acid was the primary degradation product (Gatt and Barenholz, 1968). Pentadecanoic acid which was identified in the in vivo system, was probably derived from further degradation of 2-hydroxypalmitic acid by α-oxidation (Gatt and Barenholz, 1968). Stoffel and Sticht (1967a) injected [3-^{14}C]-sphinganine and [7-^{3}H]-4-sphingenine into rats and found that palmitic acid was the major degradative component, suggesting that sphinganine and sphingenine were cleaved between C-2 and C-3. Both erythro and threo isomers of sphinganine were equally cleaved by rat liver enzymes (Stoffel and Sticht, 1967a). Recent studies by Stoffel and Bister (1973) indicated only D-erythro-sphinganine was the substrate of sphinganine lyase, the enzyme that cleaves sphinganine-1-phosphate to palmitaldehyde and ethanolamine-1-phosphate. Keenan and Okabe (1968) also found that degradation of [4,5-^{3}H]sphinganine by rat liver enzyme gives palmitate as the major product. Studies with a cell-free system revealed that degradation of long-chain bases required ATP or an ATP generating system (Gatt and Barenholz, 1968). The role of ATP was recently identified as a phosphate donor to the terminal hydroxyl group of the long-chain bases (Keenan and Maxam, 1969; Hirchberg et al., 1970). To identify the other fragment

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of the degradation product Stoffel et al. (1968d) injected [1-^{3}H]-4-sphingenine into a rat. The phospholipid fraction had the highest amount of radioactivity. After treatment of the phospholipids with phospholipase C, most of the radioactivity resided in ethanolamine and choline, indicating that 4-sphingenine was cleaved between C-2 and C-3 in an aldolase-like mechanism. Besides palmitate, palmitaldehyde was detected in an appreciable amount in the cell-free system (Stoffel et al., 1968b). Palmitaldehyde derived from degradation of [3-^{3}H,3-^{14}C]sphinganine in the cell-free system was found to have the same isotope ratio as that of the sphinganine substrate, indicating that palmitaldehyde was not a secondary product from reduction of palmitate. Conversely, it was concluded that palmitate was derived from the oxidation of palmitaldehyde. Using [1-^{14}C]sphinganine-1-phosphate, ethanolamine-1-phosphate was identified as the degradation product (Stoffel et al., 1968d). The authors suggested that long-chain bases might thus be phosphorylated at C-1 by a kinase enzyme and subsequently cleaved to palmitaldehyde and ethanolamine-1-phosphate. The properties of this kinase from rat liver was recently reported by Keenan and Haegelin (1969). This enzyme, sphinganine kinase, has been detected in human and rabbit erythrocytes (Stoffel et al., 1970a), human and pig platelets (Stoffel et al., 1973) and Tetrahymena pyriformis Stoffel et al., 1974). Sphinganine kinase (Stoffel et al., 1973) from erythrocytes was shown to catalyze phosphorylation of a variety of long-chain bases including sphinganine, 4-sphingenine, 4-hydroxysphinganine and 3-ketosphinganine (Stoffel et al., 1970a). The next step in the enzymatic degradation of long-chain bases was catalysed by a

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PLP-dependent enzyme (Keenan and Maxam, 1969). The enzyme catalysed the cleavage of sphinganine-1-phosphate to palmitaldehyde and ethanolamine-1-phosphate and was found to be bound to endoplasmic reticulum and mitochondrial membrane of liver, heart, brain, muscle, kidney and lung of rat (Stoffel et al., 1969b). Whether there is only one enzyme which is responsible for the degradation of all the long-chain bases or whether there are different enzymes which are specific for individual long-chain bases is not known. The reaction catalysed by this enzyme, sphinganine-1-phosphate lyase, probably proceeds in a manner analogous to that described for cleavage of threonine by threonine aldolase (Keenan and Maxam, 1969). Sphinganine-1-phosphate lyase was shown to catalyse the cleavage of a sphinganine of shorter chain-length, C_7-sphinganine (Stoffel et al., 1969a). The D-erythro-sphinganine but not the other three optical antipods was a substrate of this enzyme (Stoffel and Bister, 1973). The mechanism and stereochemistry of sphinganine-1-phosphate lyase was recently reported by Akino et al. (1974), who showed that cleavage of sphinganine-1-phosphate-PLP complex involved transfer of a proton from the medium to C-2 of ethanolamine-1-phosphate with retention of configuration.

G. Biological Properties.

4-Sphingenine was identified by Hecht (1951 and 1953) as the active component in delaying blood-clotting. After adding 4-sphingenine to chicken plasma Hecht and Shapiro (1957) observed that the clotting times were prolonged to 4 hours or more (clotting time for the control was 30 min.). Both erythro and threo isomers of sphinganine were weak anticoagulants compared to erythro-4-sphingenine.

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N-Benzoylsphinganine, triacetyl-4-sphingenine and pure sphingomyelin were inactive.

Mycostatic properties of long-chain bases were mentioned by Karlsson (1966). A small amount of free long-chain base could arrest the growth of yeast. This report was not confirmed. Conversely, several authors had added free long-chain bases in the growth medium for biological studies (Weiss and Stiller, 1967; Stoffel et al., 1968b). These authors did not comment on any adverse effects of long-chain bases on the growth of the yeast. A more thorough study on the effect of free long-chain base on growth was reported by Thorpe (1968) she found that the concentration of long-chain base in the medium could be as high as 50 μg/ml without having a deleterious effect on growth. A growth depression effect of long-chain base, however, was observed in higher animals (Carroll, 1960). Rats which were fed with a diet containing 1 to 2% of weight as free bases lost weight rapidly and usually died after about two weeks. The sulfate salt of long-chain base could also depress growth but the effect was less pronounced than that of the free base. Fully acetylated 4-sphingenine was inactive. Carroll suggested that growth inhibition was probably associated with the free amino group. To strengthen his hypothesis, several long-chain amines were fed to rats. Similar effects were observed for amines of C_{10}- or longer chain length but amines of C_8- or shorter chain length were inactive in growth depression. Psychosine, at a 1% level in the diet, was also toxic whereas ceramides, at a 5% level in the diet, did not depress growth.

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Psychosine has been reported to have hemolytic activity (Taketomi and Nishimura, 1964) which was comparable to that of lysolecithin. When the free amino group was blocked by benzoylation, the hemolytic activity was abolished.

4-Sphingenine was reported to have antituberculin allergy properties (Fisher et al., 1951). The activity was of the same order of magnitude as that of cortisone. N-Acetyl-4-sphingenine was also active. The antiallergic activities of long-chain bases were unlike those of cortisone in that they were diet independent.

Sphingolipid bases were degraded in an animal to palmitaldehyde and ethanolamine-1-phosphate which were effectively incorporated into plasmalogen and phospholipids (Henning and Stoffel, 1969; Stoffel et al., 1970), implicating this pathway as one of the possible roles of long-chain bases in phospholipids and plasmalogen metabolism.

Sphingolipid bases occur in nature as complex molecules. Only small amounts of free long-chain bases have recently been detected (Karlsson, 1970) in human kidney autopsy sample. Accordingly, the biological functions of the sphingolipid bases are probably determined by the type of compounds they are associated with. Immunological activity of glycosphingolipids has been known for many years (Rapport, 1961; Martensson, 1969). The role of ganglioside in synaptic transmission was discussed by Lapetina et al. (1967 and 1968) and Martensson (1969). Sulfatide was postulated to be involved in a corticosteroid-dependent sodium transport system (Karlsson et al., 1969). Although the physiological functions of this heterogeneous group of

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sphingolipids are not well understood, accumulation of these substances in the body are pathogenic. Abnormal metabolism, leading to the accumulation of one of a variety sphingolipids is indicated in the metabolic pathway shown in Fig.2.

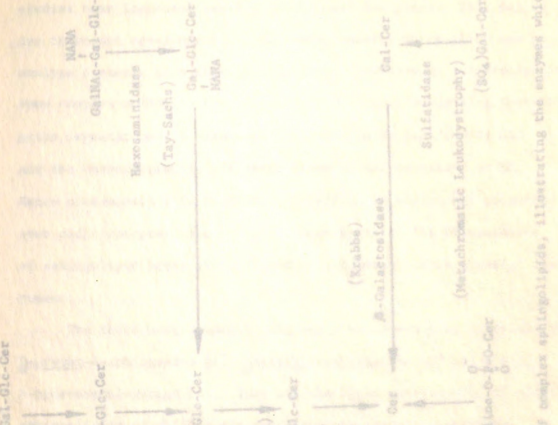

Fig.2. Degradation of complex sphingolipids, illustrating the enzymes which are characteristic of human disease if their activities are attenuated.

GalNAc-Gal-Gal-Glc-Cer

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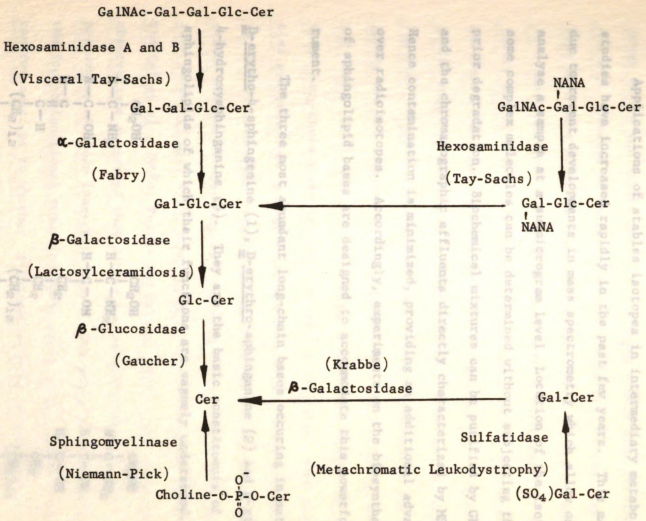

Fig.2. Degradation of complex sphingolipids, illustrating the enzymes which are characteristic of human disease if their activities are attenuated.

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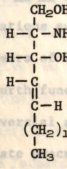
INTRODUCTION

Applications of stables isotopes in intermediary metabolic studies have increased rapidly in the past few years. This may be due to recent developments in mass spectrometry which allow one to analyse a sample at a submicrogram level. Location of the isotope in some complex molecules can be determined without subjecting them to prior degradation. Biochemical mixtures can be purified by GLC and the chromatographic effluents directly characterized by MS. Hence contamination is minimized, providing an additional advantage over radioisotopes. Accordingly, experiments on the biosynthesis of sphingolipid bases are designed to accommodate this powerful instrument.

The three most abundant long-chain bases occuring in nature are D-erytho-4-sphingenine (1), D-erythro-sphinganine (2) and D-ribo-4-hydroxysphinganine (3). They are the basic constituents of all the sphingolipids of which their functions are vaguely understood.



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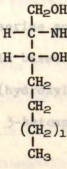

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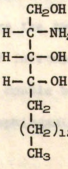

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Structurally, they differ from each other only at C-4 and C-5. Sphinganine possesses three basic functional groups in the form of 2-amino-1,3-diol, 4-hydroxysphinganine has an additional hydroxyl group on C-4 and 4-sphingenine has a trans double bond between C-4 and C-5. Because of their structural similarity, several hypotheses have been made about the metabolic relationship. 4-Sphingenine might be transformed into 4-hydroxysphinganine by a stereospecific hydration (Weiss and Stiller, 1965; Barenholz and Gatt, 1967); alternatively, Karlsson (1964) and Carter et al.(1966) suggested that 4-hydroxysphinganine might be dehydrated to 4-sphingenine. Experimental results reported subsequently indicated that 4-hydroxysphinganine and 4-sphingenine are not interconvertible (Stoffel and Sticht, 1967a; Keenan and Okabe, 1968). At present, the metabolic relationship of these three long-chain bases is still not well established; some possibilities are presented in Fig. 1 (in the literature review section). Conversion of serine and palmitate to 3-ketosphinganine, 3-keto-4-sphingenine and 4-hydroxysphinganine has been reported from many laboratories (Brady et al., 1958; Green et al., 1965; Braun and Snell, 1967; Stoffel et al., 1968a; Fujino and Nakano, 1971). The aforementioned observations suggested that serine and palmitate are the two common precursors of these long-chain bases. The stage of introduction of the fourth functional group (hydroxyl group or the double bond) is still controversial as to whether 3-ketosphinganine or sphinganine is the immediate precursor.

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Therefore, one objective of this study was directed to these two problems. Using deuterium label on C-1 and C-3 of sphinganine would be a promising solution to both problems. If 3-ketosphinganine was the immediate precursor of 4-hydroxysphinganine or 4-sphingenine, loss of deuterium on C-3 would be expected On the other hand, if sphinganine was the immediate precursor, all three deuterium atoms would be incorporated into 4-hydroxysphinganine or 4-sphingenine

The origin of oxygen on C-4 of 4-hydroxysphinganine is still an elusive problem Water and molecular oxygen, the two most likely oxygen donors, have been ruled out as the primary donors (Thorpe and Sweeley, 1967). Inorganic phosphate was also unlikey to be the oxygen donor (Thorpe, 1968). Another possibility for this oxygen donor is one of a variety of oxygen-containing compounds (Hayaishi, 1969). Sphinganine itself has two hydroxyl groups one at C-1 and another at C-3. Transfer of a hydroxyl group from one of these two hydroxyl groups to C-4 via a cyclic ether intermediate is plausible. Glucose and phosphoenolpyruvate, both of which have hydroxyl groups that are not exchangable with water in the medium (Thorpe, 1968), are also considered to be possible donors. Yeast grown on ethanol as the sole carbon source might give an indirect solution to this problem.

Finally, studies were focused on the mechanism of PLP-dependent condensation of serine and palmitoyl CoA. At least two broad mechanisms for the formation of 3-ketosphinganine are possible (Fig. 3). These two different mechanisms, discussed by Braun and Snell (1968) are that

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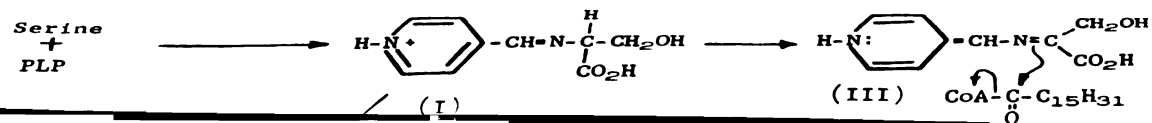
the Schiff's base complex I may undergo decarboxylation to yield complex II, after which 3-ketosphinganine is formed by the addition of the palmitoyl group followed by hydrolysis; or the α-hydrogen atom of serine is lost to form complex III and 3-ketosphinganine is then formed via complexes IV and V. Using tritium label on C-2 and C-3 of serine, Weiss (1963) noted that isotope ratios of C-1/C-2 of 4-sphingenine isolated from rat brains were similar to C-3/C-2 of serine substrate, although some variations of the isotope ratios were observed. Preliminary studies on yeast grown in the presence of [2,3,3-$^{2}H_{3}$] serine indicated that deuterium on C-2 was lost during the formation of 4-hydroxysphinganine. Thus, it is desirable to reinvestigate the biosynthesis of sphinganine in the animal system.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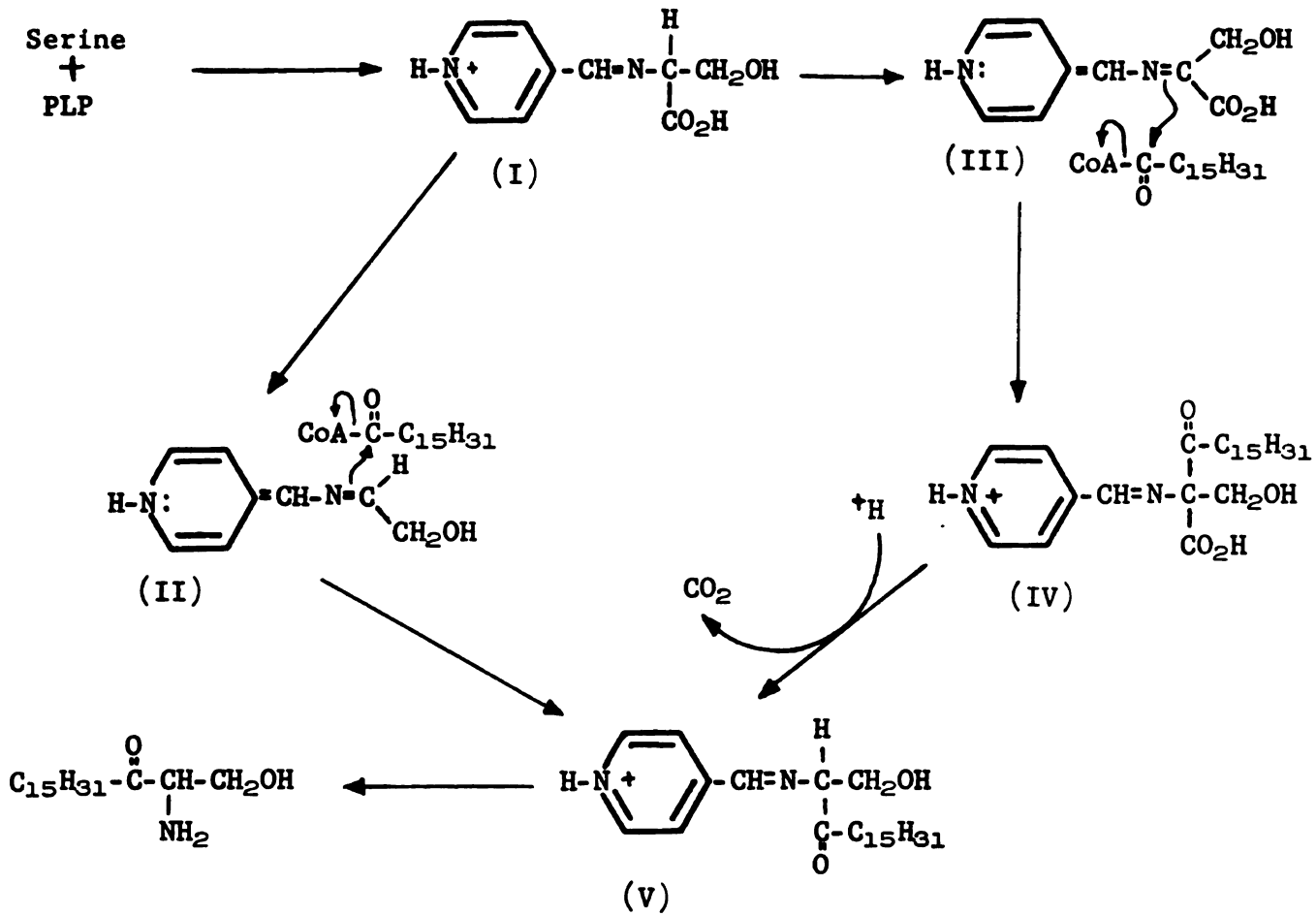

Fig. 3. Two broad hypothetical mechanisms of 3-ketosphinganine biosynthesis (Braun and Snell, 1968).

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MATERIALS AND METHODS

A. Materials.

Chemicals

Palmitoyl chloride	Pfaltz and Bauer Inc., Flushing, New York. Palmitoyl chloride was double redistilled under reduced pressure.
Ethyl acetoacetate	Aldrich Chem. Co. Inc., Milwaukee, Wisconsin. Ethyl acetoacetate was redistilled before use.
Mannosamine-HCl	Sigma Chem. Co., St. Louis, Missouri.
N-Acetylglucosamine	Sigma Chem. Co.
DL-Erythro-sphinganine	Sigma Chem. Co.
DL-Erythro-4-sphingenine sulfate	Sigma Chem. Co.
Hexamethyldisilazane and trimethylchlorosilane	Analabs, Inc., North Haven, Connecticut.
Yeast extract, malt extract and peptone	Difco Laboratories, Detroit, Michigan.

Solvents

General solvents	All solvents were redistilled before use.
Diethyl ether	Mallinckrodt Chem. Works, St. Louis, Missouri. Diethyl ether was stored over small pieces of sodium metal.

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CH_3O^2H, ^2H_2O, $N_2^2H_4.^2H_2O$, $LiAl^2H_4$, NaB^2H_4, d_3-serine and d_8-acetic anhydride	Merck Sharp and Dohme of Canada, Ltd., Montreal, Canada. Distributed in the United States by Merck and Co. Inc., Rahway, New Jersey.
Bis(trideuteromethylsilyl)trifluoroacetamide-d_{18}	Regis Chem. Co., Morton Grove, Illinois.
$H_2^{18}O$ (normalized)	Miles Labs., Inc., Elkhart, Indiana. Monsanto Research Corp.
Chromatographic Supplies	
3% SE-30 on Supelcoport (80-100 mesh)	Supelco, Inc., Bellefonte, Pennsylvania.
Silica gel G	EM Reagents Division, Brinkmann Instruments, Inc., Westbury, New York.
Amberlite MB-3	Mallinckrodt Chem. Works.
Silicic acid (Unisil, 100-200 mesh)	Clarkson Chem. Co., Inc., Williamport, Pennsylvania.
Animals	
Rats (10-14 day-old)	Spartan Research Animals, Haslett, Michigan.
Live oysters	City Fish Co., Lansing, Michigan.
Yeast	
Hansenula ciferri (mating type F-60-10)	Gift of Dr. Kurztman,C.P.

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Liquid medium-1 (LM-1)	yeast extract (0.3%), malt extract (0.3%), peptone (0.5%) and glucose (1.0%) in water. The medium was sterilized by millipore (0.8 μ) filtration.
Liquid medium-2 (LM-2)	the same as liquid medium-1 excepted that sterilization was accomplished by autoclaving at 120°C for 25 min.
Liquid medium-3 (LM-3)	yeast extract (0.3%), $MgSO_4.7H_2O$ (0.07%), $(NH_4)_2SO_4$ (0.12%), NaCl (0.05%), KH_2PO_4 (0.5%).

B. Methods.

1. Gas-Liquid Chromatography (GLC)

Gas chromatography was carried out on a Hewlett-Packard Model F and M 402 gas chromatograph equipped with a flame ionization detector. A 6 ft. glass column packed with 3% SE-30 on Supelcoport, 80-100 mesh (Supelco Inc., Bellefonte, Pa.), was used throughout the studies. Nitrogen was the carrier gas. The flash heater and detector were set about 20°C above the column temperature, which was set as described elsewhere.

2. Mass Spectrometry (MS).

Mass spectra were recorded with an LKB 9000 combined gas chromatograph-mass spectrometer. Conditions for gas chromatography were the same as those described for gas chromatography except that

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helium was used as the carrier gas. Mass spectra were recorded at 70 eV, with an accelerating voltage of 3.5 KV and filament current of 60 μA. The mass spectrometer was interfaced to a PDP-8/I computer for on-line, real-time data collection and reduction as described by Sweeley et al. (1970). The deuterium content of labeled compounds was analysed by the accelerating voltage alternator (AVA) technique as described by Holland et al. (1973).

3. Preparation of [2-^{18}O] Glucose

[2-^{18}O] Glucose was synthesized from mannosamine hydrochloride by treating the aminosugar with nitrous acid in $H_2^{18}O$ according to the procedure of Horton and Philips (1972) for synthesis of the unlabeled compound, with a slight modification as described below. Mannosamine hydrochloride (216 mg) was dissolved in 5 ml of $H_2^{18}O$ (8.8%). The solution was stirred magnetically in an ice-bath. When the temperature reached 0°C, sodium nitrite (278 mg) was added in small portions. Glacial acetic acid (0.3 ml) was added slowly and stirring was continued for another 4 hours at 0°C. Nitrogen was then bubbled through the solution for 5 min. and the solution was deionized by passing through a column packed with 10 g. of amberlite MB-3 mixed-bed resin. The column was allowed to run dry and $H_2^{18}O$ was recovered by lyophilization. The column was washed once with 20 ml of distilled water. The second eluent and the lyophilized glucose were combined and then passed through a second column packed with 30 g. of the mixed-bed resin. The column was washed with 20 ml of distilled water and eluent was concentrated by lyophilization to give a pale yellow syrup. TLC of this syrup on silica gel G (developed with butanol-pyridine-water, 70:15:15) showed a minor by-product at the origin and glucose was at Rf = 0.2 (mannose

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was at Rf = 0.28). GLC analysis of TMSi derivative showed about 1% impurity. This syrup was diluted to a proper concentration and used for growing yeast. No growth inhibition of yeast was observed. The yield, determined by GLC, was 93 mg (52%) of 6.43% ^{18}O enrichment on C-2 of glucose.

4. Synthesis of [3-^{18}O]Serine

N-Acetylglucosamine (2 g.) was dissolved in 5 ml of $H_2^{18}O$ (20% enrichment). The solution was left at 4°C for 10 days, after which time $H_2^{18}O$ was recovered by lyophilization. The aminosugar residue was redissolved in 20 ml of cold water and 1M $NaBH_4$ in 0.05N NaOH (10 ml) was added. The mixture was kepted at 4°C overnight. Excess $NaBH_4$ was removed by careful acidification with 0.1N HCl. Boric acid so formed was removed under reduced pressure as its methyl ester by repeated addition of absolute methanol to the dried solid residue. The N-acetylglucosaminitol residue was used in the next step without purification.

Water (50 ml) was added to the solid residue, followed by 50 ml of $NaIO_4$ (pH 4.5). The solution was kept in the dark at room temperature overnight. Methanol (50 ml) was added and the inorganic precipitate was removed by filtration. The filtrate was reduced to half of the original volume. $KMnO_4$ (40 mmoles in 80 ml of water) was added and the solution was kept at room temperature overnight. Excess $KMnO_4$ was removed by addition of a dilute solution of oxalic acid. The precipitate was filtered and the filtrate was treated with decolorizing carbon, refiltered and lyophilized. The solid residue was extracted

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three times with 10 ml of absolute methanol. The combined extracts were dried under reduced pressure and the residue was determined to be about 95% pure by GLC of its TMSi derivative. The major peak in the chromatogram co-chromatographed with di-O-TMSi-N-acetylserine. The mass spectrum of the TMSi derivative of this residue was identical to that of di-O-TMSi-N-acetylserine (Fig.7).

The solid residue was dissolved in 20 ml of 3N HCl and heated at 80°C for 6 hours. The solution was neutralized with dilute Na_2CO_3 and evaporated to dryness under reduced pressure. Serine was extracted three times with 10 ml of 90% methanol. The combined extracts were dried under reduced pressure, yielding 0.5 g. with 6.94% of ^{18}O enrichment on C-3. TLC on silica gel G (developed with butanol-acetic acid-water, 60:20:20 and with phenol-water, 75:25) showed only one nihydrin- and H_2SO_4-positive spot at the same Rf value as that of reference serine. Supplementing yeast medium with this labeled serine did not inhibit the growth of the yeast, Hansenula ciferri.

5. Preparation of [1-^{18}O]Palmitate.

Palmitoyl chloride (100 mg) was added to 2 ml of $H_2^{18}O$ (9.5% enrichment). The mixture was sonicated for 5 min. and two drops of Triton X-100 were added. The mixture was then sonicated for another 15 min. The tube was sealed and left at room temperature for three days. Fatty acid was extracted with chloroform and dried under a stream of nitrogen. For analysis of ^{18}O enrichment, about 1 mg of the fatty acid was treated with freshly prepared diazomethane in ether containing 10% methanol. Excess diazomethane and solvents were removed under a stream of nitrogen, hexane (5 ml) was added to the residue, and the

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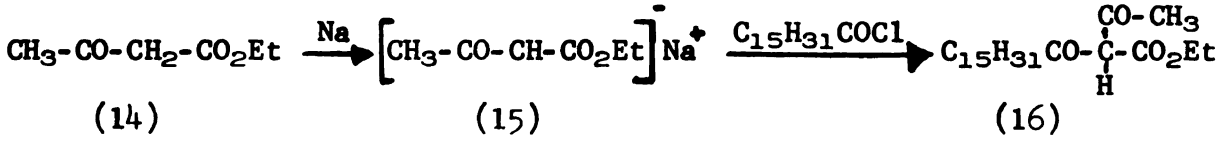
6. Synthesis of Deuterated Sphinganines.

Ethyl 2-acetamido-3-ketooctadecanoate (19) was synthesized from palmitoyl chloride and ethyl acetoacetate as described by Shapiro *et al.* (1958) and Shapiro (1969) as outlined below.



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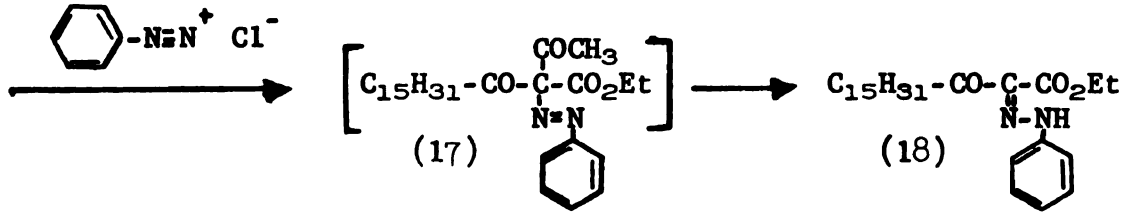

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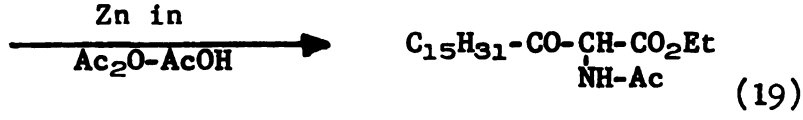

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a. [1,1,3-$^2H_3$] N-Acetylsphinganine.

Ethyl 2-acetamido-3-ketoactadecanoate (500 mg) was dissolved in 50 ml of anhydrous diethyl ether-tetrahydrofuran (1:1). The solution was cooled in an ice-bath and lithium aluminum deuteride (15 mg) was then added. The solution was refluxed gently for one hour, after which sodium hydroxide (0.2N, 50 ml) was added; the phases were separated and the aqueous phase was extracted with ether (10 ml). The combined

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b. [3-^{2}H] N-Acetylsphinganine.

Ethyl 2-acetamido-3-ketooctadecanoate (100 mg) was dissolved in 10 ml of methanol. The solution was cooled in an ice-bath and sodium borodeuteride (10 mg) was added. The solution was left in the cold room for 24 hours after which time it was acidified with dilute HCl. The solvents were evaporated under reduced pressure. Boric acid that formed in the reaction was removed as its methyl ester by repeated addition and removal in vacuo of anhydrous methanol to the dry residue left after each evaporation. Diethyl ether (5 ml) and tetrahydrofuran (5 ml) were added to the dry residue and the suspension was sonicated briefly in a sonic oscillator to help dissolve organic materials. The solution was then reduced with lithium aluminum hydride and N-acetylsphinganine was recovered as described in the synthesis of [1,1,3-$^2H_3$] N-acetylsphinganine.

c. [1,1-$^2H_2$] N-Acetylsphinganine.

[1,1-$^2H_2$] N-Acetylsphinganine was synthesized by the procedure described for [3-^{2}H] N-acetylsphinganine except that sodium borohydride and lithium aluminum deuteride were used in place of sodium borodeuteride and lithium aluminum hydride, respectively.

d. [1,1,2,3,4,4-$^2H_6$] N-Acetylsphinganine.

Sodium metal (about 0.2 g) was added in small pieces to CH_3O^2H (10 ml). When the evolution of deuterium gas had subsided, deuterium

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oxide (0.5 ml) was added, followed by ethyl 2-acetamido-3-ketooctadecanoate (100 mg). The solution was left at room temperature for 24 hours, after which time acetic anhydride (2 ml) was added. Chloroform (20 ml) and water (3 ml) were added, the two phases were separated by centrifugation, and the lower phase was washed once with 10 ml of Folch's upper phase (Folch et al., 1957) and then evaporated under reduced pressure. Traces of water were removed by azeotropization with absolute ethanol. The residue was then reduced with lithium aluminum deuteride as described in the synthesis of [1,1,3-$^2H_3$]N-acetylsphinganine.

e. [N—$^2H_3$]-Acetylsphinganine.

N-Acetylation of sphinganine was carried out as described by Gaver and Sweeley (1966). Hexadeutero-acetic anhydride (0.1 ml) was added to the solution of sphinganine (1 mg in 1 ml of methanol) with a slight shaking to help distribute the anhydride evenly. The solution was left at room temperature for 10 min., after which chloroform (2 ml) and water (0.5 ml) were added with thorough mixing. The lower phase was washed once with Folch's upper phase (Folch et al., 1957) and the lower phase was evaporated to dryness under a stream of nitrogen.

f. [4,5-$^2H_2$]Sphinganine.

This compound was a biosynthetic product isolated from oyster microsomes incubated with serine and [2,3-$^2H_2$]palmitate as described by Hammond and Sweeley (1973).

7. Preparation of Microsomal Enzymes.

Microsomes were prepared from liver of 10-14 day-old rats as

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described by Brady et al. (1965). The tissues were homogenized in 5 volumes of 0.25 M sucrose with a teflon homogenizer at 2-4°C. The homogenate was centrifuged at 8600 x g for 15 min. Supernatant was ultracentrifuged at 100,000 x g for 45 min. The microsomal sediment was suspended in 0.1 M potassium phosphate buffer (pH 7.5), containing 1 mM PLP and 1 mM dithiothreitol (DTT), equal to 0.2 of the original volume.

8. Isolation and Purification of Sphinganine.

At the end of incubation (1 hour at 37°C), sodium hydroxide (1 N, 1 ml) was added and the lipids were extracted 3 times with 10 ml of diethyl ether. The combined ether extracts were washed once with 10 ml of water, dried over anhydrous sodium sulfate, and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in a minimal volume of chloroform and then applied on a silica gel G thin layer plate with a sphinganine standard applied on the side lane. After developing with chloroform-methanol-water, 65:25:4 (Braun and Snell, 1967) the plate was exposed to iodine vapor briefly and the area at the same Rf value as that of the standard was scraped off. Sphinganine was extracted from the silica gel with three portions (10 ml each) of chloroform-methanol 1:1. Solvents were evaporated in vacuo.

9. Preparation of Volatile Derivative of Sphingolipid Bases.

Sphingolipid bases were N-acetylated by the procedure of Gaver and Sweeley (1965). Methanol (2 ml) was added to the dry long-chain bases. The mixture was sonicated briefly and acetic anhydride (0.2 ml) was added. The reaction mixture was left at room temperature for 10 min.

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Chloroform (4 ml) and water (1.0 ml) were then added. The two phases were mixed thoroughly and then separated by centrifugation. The lower phase was evaporated to dryness under a stream of nitrogen. TMSi-donor agent, consisting of pyridine-hexamethyldisilazane-trimethylchlorosilane, 10:2:1 (Gaver and Sweeley, 1965), was added about 10 min. prior to analysis by GLC.

10. Growth of Yeast and Isolation of Tetraacetyl-4-hydroxy-sphinganine.

Hansenula ciferri was grown aerobically at 26-28°C in different liquid media (10 ml). Growth was initiated by adding 0.1 ml of yeast previously grown to stationary phase and kept at 4°C overnight. Cells were harvested 48 hours after growth by centrifugation or lyophilization. Tetraacetyl-4-hydroxysphinganine in the cell paste or dry cells was extracted twice with 10 ml of acetone, that from the medium was extracted two times with 10 ml of petroleum-ether. The combined extracts were dried under reduced pressure.

11. Hydrolysis of Sphingolipids.

Complete hydrolysis of sphingolipids was accomplished by the procedure described by Gaver and Sweeley (1965) with a slight modification. The sphingolipids were dissolved in 10 ml of methanol followed by the addition of HCl (6 N; 2 ml). The mixture was heated at 80°C for 16 hours in a Teflon-lined screw-capped tube. Chloroform (20 ml) and water (4 ml) were added, the lower phase was washed once with Folch's upper phase (Folch et al., 1957), and was dried in vacuo.

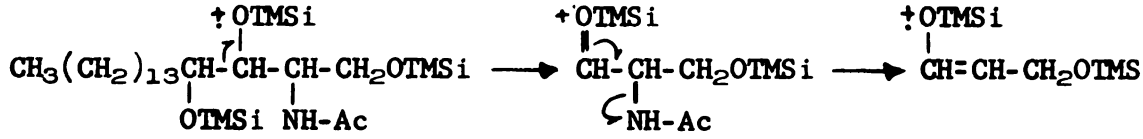
Partial hydrolysis was used to remove O-acetyl groups selectively. The crude tetraacetyl-4-hydroxysphinganine was dissoved in 2 ml of 0.1 N sodium hydroxide in methanol and the solution was kept at 60° for

1 hour (Thorpe and Sweeley, 1967). Chloroform (4 ml) and water (1 ml) were added, the chloroform layer was washed once with Folch's upper phase (Folch *et al.*, 1957) and dried under a stream of nitrogen.

RESULTS

A. Mass Spectrum of Tris-O-TMSi-N-acetyl-4-hydroxysphinganine.

The mass spectrum of tris-O-TMSi-N-acetyl-4-hydroxysphinganine (Fig.4) was reported previously by Thorpe and Sweeley (1967). The molecular ion (m/e 575) was not present in the spectrum but could be deduced from the ion at M-15 (m/e 560), which is probably derived from the loss of a methyl residue from one of the TMSi groups. Direct cleavage between C-3 and C-4 yields ions at m/e 276 and 299 with the positive charge retained on C-3 and C-4, respectively. The ion at m/e 218 is possibly derived from further cleavage of the C-N bond of the former ion with the loss of nitrogen containing fragment, as shown below.



m/e 276 m/e 218

Cleavage between C-2 and C-3 gives rise to ions at m/e 401 and 174, depending on which fragment charge retention occurs. Homolytic cleavage of C-1 and C-2 bond yields two ions at m/e 103 and m/e 472. The ion at m/e 103 was shown to be derived from both C-1 and C-3 of TMSi-N-acetylsphinganine, and may be derived from other fragments of TMSi-N-acetyl-4-hydroxysphinganine. Supporting evidence for the above statement is the presence of ions at both m/e 103 and m/e 105 in the spectrum of TMSi-[1,1-$^2H_2$]N-acetyl-4-hydroxysphinganine (Fig. 20).

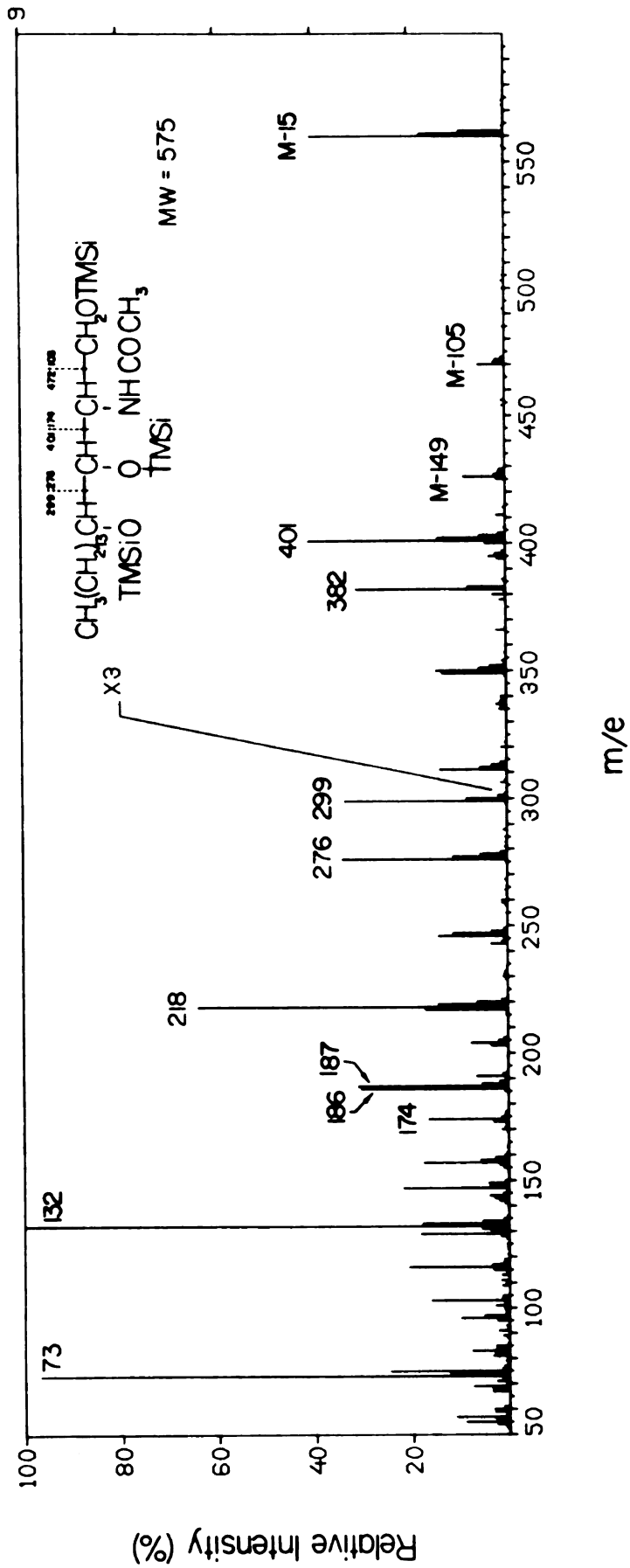


Fig. 4. Mass spectrum of tris-O-TMSi-N-acetyl-4-hydroxysphinganine.

The ion at m/e 472 is unstable and loses TMSiOH to give a more stable ion at m/e 382. The ion at m/e 299 is useful for the analysis of labeled oxygen on C-4 (Thorpe and Sweeley, 1967) since substitution of ^{18}O will shift this ion to m/e 301. It contains only one oxygen atom, thus, the increment of isotope ratio of m/e 301/299 from the reference (natural isotope) value can be used directly for the calculation of isotopic enrichment on C-4. The origin of this oxygen is of considerable interest, and can be studied using oxygen on C-1 and C-3 as experimental controls in case of negative incorporation into C-4. Therefore, analysis of ^{18}O on C-1 and C-3 is usually necessary. Analysis of ^{18}O on C-1 and C-3 was therefore carried out by stepwise subtraction. The ion at m/e 401 consists of oxygen on C-3 and C-4. Subtraction of isotopic enrichment on C-4 (from m/e 301/299) will give the value of isotopic enrichment on C-3.

The intense ion at m/e 218 is suitable for the analysis of ^{18}O on C-1 and C-3. Isotopic enrichment on C-1 is then obtained by subtraction of that calculated for C-3.

B. Studies on The Incorporation of [1,1,3-$^{2}H_3$]Sphinganine into 4-Hydroxysphinganine.

[1,1,3-$^{2}H_3$]Sphinganine (100 μg) and Triton X-100 (40 mg) were dispersed in 10 ml of LM-2. The medium was innoculated with 0.1 ml of viable yeast and was shaken at 26-28°C for 48 hours. Tetraacetyl-4-hydroxysphinganine was isolated and converted to the TMSi-N-acetyl-4-hydroxysphinganine as described in Methods. Deuterium contents in 4-hydroxysphinganine were analysed by GLC-AVA, using intensity of the ions at m/e 218, 220 and 221 for the analysis. The ion at m/e 218 is

Table 2. AVA Analysis of [1,1-$^2H_2$]-4-Hydroxysphinganine and [1,1,3-$^2H_3$]-4-Hydroxysphinganine from Yeast Grown in the Presence of [1,1,3-$^2H_3$]Sphinganine.

Determinations	m/e 220/218		m/e 221/218	
	Reference	Sample	Reference	Sample
1	0.0919	0.1590	0.0227	0.1414
2	0.0915	0.1588	0.0225	0.1405
3	0.0926	0.1589	0.0226	0.1390
Average	0.0920	0.1589	0.0226	0.1403
*	-	0.0669	-	0.1177

* is the difference between sample and reference values.

m/e 218, $CH{=}CH{-}CH_2OTMSi$ (OTMSi); m/e 220, $CH{=}CH{-}CD_2OTMSi$ (OTMSi);

m/e 221, $CD{=}CH{-}CD_2OTMSi$ (OTMSi).

derived from C-1, C-2 and C-3 with two TMSi groups (Results, A). Substitution of deuterium on these three carbons would be expected to increase the mass of this ion by an amount corresponding to the number of deuterium atoms incorporated. Therefore, direct hydroxylation of sphinganine to 4-hydroxysphinganine will be expected to involve retention of all three deuterium atoms an C-1 and C-3 in 4-hydroxysphinganine and the ion at m/e 218 should be shifted to m/e 221. On the other hand, if the process occurs via a 3-ketosphinganine intermediate, an obligatory loss of deuterium on C-3 will be expected and the ion at m/e 218 will be shifted to m/e 220. Results are normalized to the ion at m/e 218 and are summarized in Table 2. An increase in the isotope ratio of m/e 221/218 (11.77% above the reference sample) suggested that sphinganine was incorporated into 4-hydroxysphinganine without loss of deuterium; thus it is inferred that hydroxylation proceeded at the sphinganine level rather than 3-ketosphinganine. An increase in isotope ratio of m/e 220/218 (6.7%) is of interest since it suggests that 3-ketosphinganine is also a direct precursor of 4-hydroxysphinganine. Loss of deuterium due to degradation and resynthesis by utilization of the degradation product is unlikely. Degradation of sphinganine would result in loss of all the three deuterium atoms rather than just one of them. Hence it was concluded that hydroxylation of sphingolipid bases probably occurs at both 3-ketosphinganine and sphinganine level (Fig. 5).

C. Incorporation of [1,1,3-$^{2}H_{3}$]Sphinganine into 4-Sphingenine.

Although sphingolipid bases are not soluble in water, several

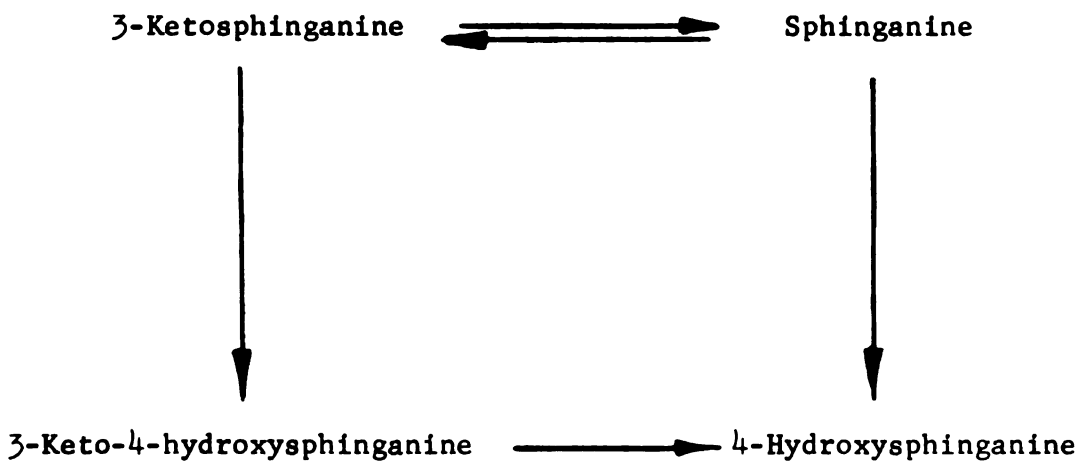


Fig. 5. Proposed mechanism of 4-hydroxysphinganine biosynthesis.

methods can be used to disperse them in water. Kanfer and Gal (1966) dissolved 4-sphingenine in 0.1 M acetate buffer, pH 5.0 (20 μg/ml). Barenholz and Gatt (1968) dispersed the free bases in saline (1 mM), but 4-sphingenine did not yield a clear solution at this concentration. Egg lecithin was then added (10 times the sphingolipid bases weight). Bovine serum albumin (5% solution) was used as a dispersing agent by Stoffel and Sticht (1967), who obtained a clear solution of 5 mg/ml. Keenan and Okabe (1968), on the other hand, dissolved the sphingolipid bases in dimethyl sulfoxide (1 mM) instead of water.

Stoffel et al. (1971) used 17% Triton WR-1339 in saline to solubilize the sphingolipid base (100 mM) for intracerebral studies. This is the highest dispersing power (to my knowledge) for in vivo studies of sphingolipid bases metabolism. [1,1,3-$^{2}H_{3}$]Sphinganine was therefore prepared in this solution at a concentration of 100 mM. Each rat received 5 μl by injection into the brain via the frontal sagittal suture. Brains were removed by decapitation after 24 hours. 4-Sphingenine was isolated and converted to TMSi-N-acetyl derivative as described in Methods and deuterium content was analysed by GLC-AVA.

The mass spectrum of TMSi-N-acetyl-4-sphingenine (Fig.6) was reported previously by Gaver and Sweeley (1966). Cleavage of the bond between C-2 and C-3 gives ions at m/e 174 and 311. Substitution of deuterium on C-1 and C-3 will shift the mass of these ions to m/e 176 and 312 respectively.

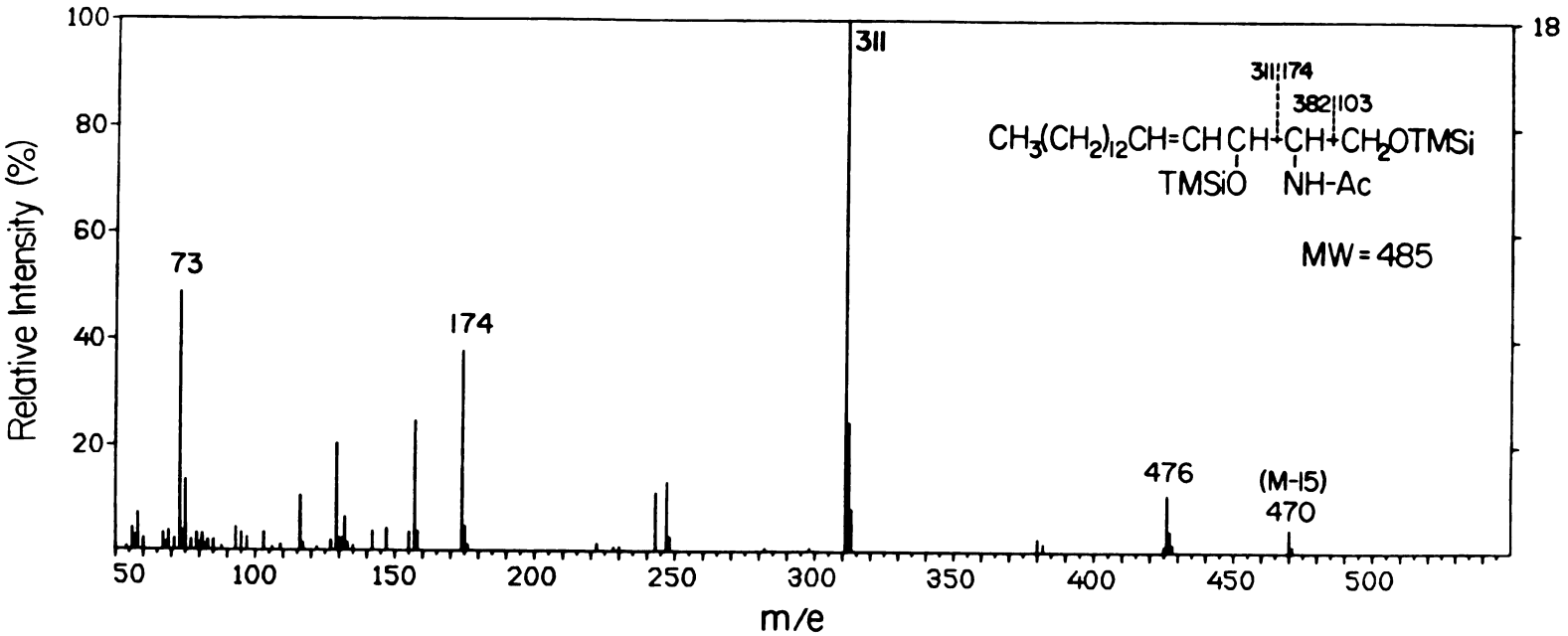
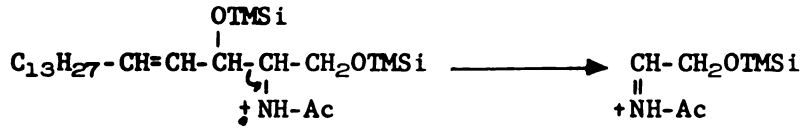
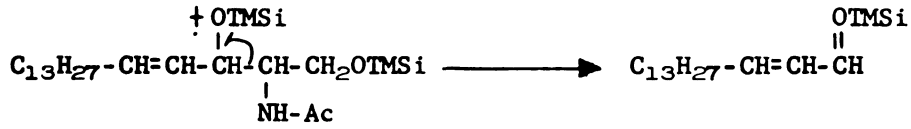


Fig. 6. Mass spectrum of bis-O-TMSi-N-acetyl-4-sphingenine.



m/e 174



m/e 311

Analysis of ions at m/e 174 and 176 indicated that there was no deuterium enrichment in 4-sphingenine. GLC analysis did not indicate any accumulation of sphinganine. The failure to detect deuterated 4-sphingenine in rat brain might be due to rapid degradation of injected sphingolipid bases (Stoffel et al., 1968b) and a high endogenous 4-sphingenine in rat brain.

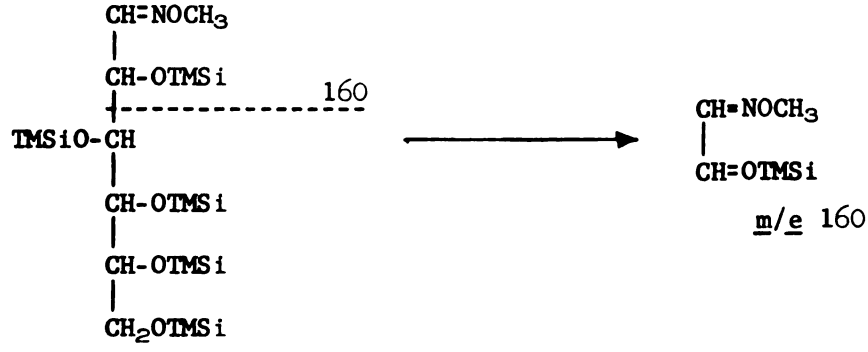
Attempts to inject higher doses (25 µl each) into rat brains were not successful. Severe bleeding was observed in several rats and those which were not bleeding only survived for a few hours. The dead frozen rats and the survivors were pooled. There was no deuterium detected in the isolated 4-sphingenine. This problem was not further investigated.

D. Studies on Yeast Grown on [2-^{18}O]Glucose.

1. Determination of ^{18}O Enrichment on C-2 of Glucose.

The mass spectrum of TMSi-methoxime of [2-^{18}O]glucose synthesized from mannosamine-HCl was identical to that of TMSi-methoxime glucose which was reported by Laine and Sweeley (1971). The

spectrum is easily interpreted and isotopic substitution on each carbon can be distinguished. The ion at m/e 160 is derived from direct cleavage between C-2 and C-3 with the charge retained on C-2 fragment. Since oxygen on C-1 is removed during the formation of the methoxime derivative, this ion can be used directly for determination of isotopic abundance on C-2 without complication. Substitution of ^{18}O on C-2 will shift this ion to m/e 162. Therefore, the increment of ratio at m/e 162/160 from the natural isotopic abundance will be the net incorporation of ^{18}O into this position. The result from AVA analysis (Table 2) shows that isotopic enrichment at this position is 6.43%.



2. 4-Hydroxysphinganine Produced by Yeast Grown on [2-^{18}O]Glucose.

It was speculated by Thorpe (1968) that phosphoenolpyruvate might be an oxygen donor in 4-hydroxysphinganine synthesis. Experiments with phosphoenolpyruvate itself may suffer from rapid hydrolysis and complication by poor transport across cell membrane. In this experiment it was hoped that some answers might be obtained about whether phosphoenolpyruvate is a donor or not, using [2-^{18}O]glucose, since the hydroxyl

Table 3. AVA Analysis of ^{18}O on Carbon-2 of Synthetic Glucose.

Determinations	m/e 162/160	
	Reference	Sample
1	0.0520	0.1156
2	0.0508	0.1155
3	0.0510	0.1153
4	0.0507	0.1151
5	0.0512	0.1149
6	0.0505	0.1154
Average	0.0510	0.1153
Δ*	-	0.0643

Δ* is the difference between sample and reference values.

m/e 160, $\begin{matrix} CH{=}N\text{-}OCH_3 \\ | \\ CH{=}OTMSi \end{matrix}$; m/e 162, $\begin{matrix} CH{=}N\text{-}OCH_3 \\ | \\ CH{=}{}^{18}OTMSi \end{matrix}$.

group on C-2 of phosphoenolpyruvate can be derived from hydroxyl groups on either C-2 or C-5 of glucose from glycolytic pathway (Thorpe, 1968).

Yeast was grown on LM-3 in the presence of 1% [2-^{18}O]glucose (6.43% isotopic enrichment). Tetraacetyl-4-hydroxysphinganine obtained from the medium and intracellular sources were combined and partially deacetylated with 0.1 N methanolic NaOH. After trimethylsilylation it was analysed by GLC-AVA. The ratio of ions m/e 301/299 was the same as that of the reference sample, indicating that there was no isotopic enrichment into C-4 of 4-hydroxysphinganine. It was concluded that the hydroxyl group of C-4 of 4-hydroxysphinganine is not derived from oxygen on C-2 of glucose, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate.

It is important to note that partial or complete loss of ^{18}O on C-2 of glucose may happen at the following steps.

1) This hydroxyl group was transformed into the carbonyl oxygen in fructose-6-phosphate, fructose-1,6-diphosphate and dihydroxyacetone-phosphate, hence loss of some ^{18}O by exchanging with water in the medium might occur. Heron and Caprioli (1973) reported loss of about 50% of the isotope from C-2 of fructose-1,6-diphosphate at room temperature for 20 hours. However, the actual loss of ^{18}O on C-2 of glucose might be much less than the value given by Heron and Caprioli, since fructose 1,6-diphosphate formed in the cell might be subjected to further metabolism and the time of exposure to the medium should be less than 20 hours.

2) Aldolase, which catalyses the cleavage of fructose-1,6-diphosphate to two molecules of triose phosphate, may cause a complete loss of isotope on C-2. Such a mechanism requires PLP-Schiff's base formation, as shown with rabbit muscle aldolase (Horecker et al., 1961). Yeast aldolase, on the other hand, does not require PLP for catalytic activity (Rutter, 1964). Accordingly, yeast aldolase converts fructose-1,6-diphosphate to dihydroxyacetone phosphate without loss of isotope on C-2 (Heron and Caprioli, 1973).

E. Studies on Yeast Grown in the Presence of [3-^{18}O]Serine

1. Determination of ^{18}O on C-3 of Serine.

Isotopic abundance on C-3 of serine was determined by GLC-AVA of the N-acetyl-TMSi derivative. A mass spectrum of this derivative is shown in Fig.7. Molecular ion (m/e 291) is not present in the spectrum but can be deduced from the ion at M-15 (m/e 276) which may be derived from the loss of a methyl group from one of the TMSi groups. Loss of trimethylsilanol from this ion and that from the molecular ion give ions at m/e 186 and 201, respectively. Cleavage between C-1 and C-2 with charge retention on the nitrogen containing fragment gives an ion at m/e 174. Cleavage between C-2 and C-3 with the charge retained on the latter fragment gives an ion at m/e 103. This ion contains only one oxygen of C-3 and its intensity is strong, thus it is used for the analysis of isotopic enrichment in this position. Results in Table 4 show an enrichment of 6.94%. The ion at m/e 261 (M-30) is probably derived from loss of formaldehyde from C-3 by transfer of TMSi to the nitrogen atom as outlined below.

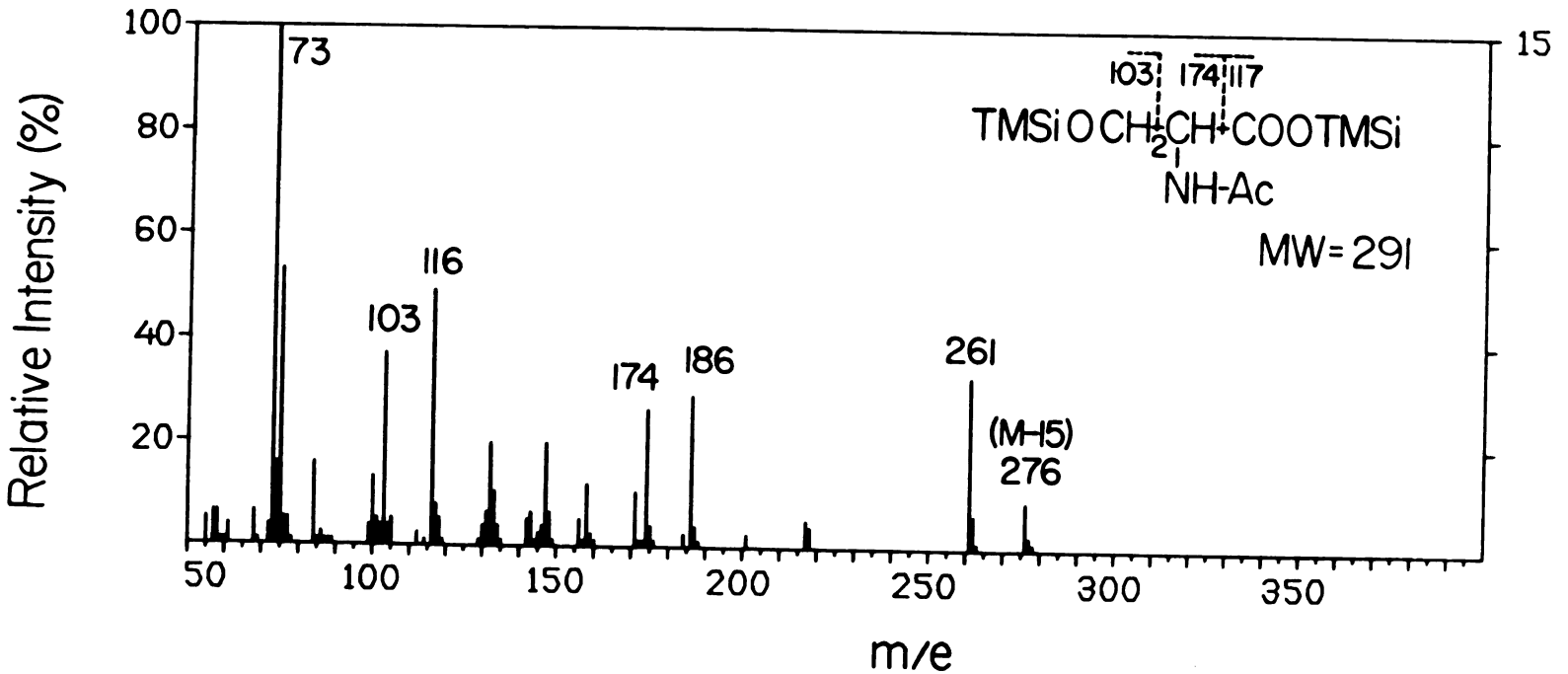


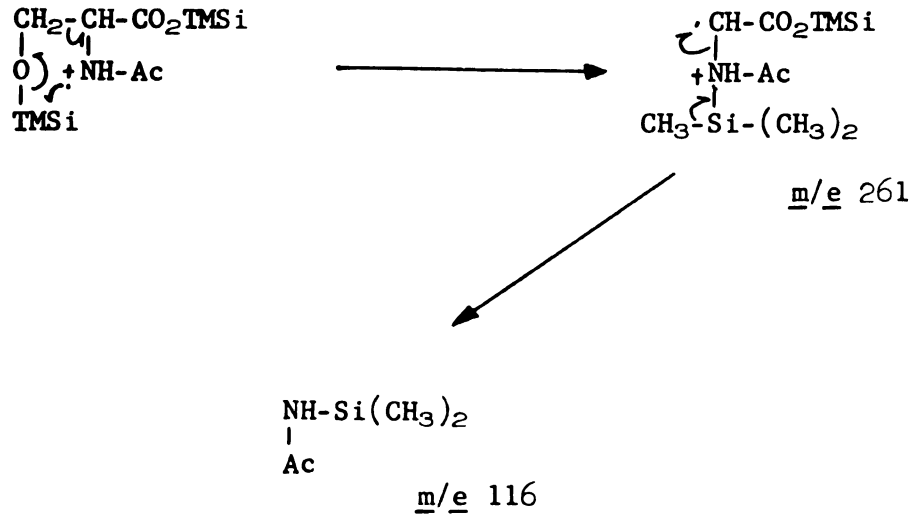
Fig. 7. Mass spectrum of bis-O-TMSi-N-acetylserine.

Table 4. AVA Analysis of ^{18}O on C-3 of Synthetic Serine.

Determinations	m/e 105/103	
	Reference	Sample
1	0.0520	0.1212
2	0.0504	0.1201
3	0.0504	0.1207
4	0.0518	0.1203
Average	0.0512	0.1206
$\triangle^*$	-	0.0694

* is the difference between sample and reference values.

m/e 103, CH_2=OTMSi; m/e 105, CH_2^{18}=OTMSi.



This type of mechanism has been illustrated for the fragmentation of the sphinganine derivative (Hammarström et al., 1970). This ion (m/e 261) may then undergo 1,2-elimination, involving the loss of a methyl group of TMSi and C-1 and C-2 fragment as shown above, to give an ion at m/e 116.

2. 4-Hydroxysphinganine from Yeast Grown in the Presence of [3-^{18}O]Serine.

Yeast grown in LM-3 in the presence of 1% glucose and 0.5% [3-^{18}O] serine. Tetraacetyl-4-hydroxysphinganine from medium and intracellular sources were combined and N-acetylated. GLC-AVA analysis of ^{18}O enrichment in 4-hydroxysphinganine is summarized in Table 5. The ion at m/e 299, which is derived from cleavage of C-3 and C-4 bond, is used for analysis of isotope abundance on C-4. Substitution of ^{18}O will shift the ion to m/e 301. Subtraction of the ratio of m/e 301/299 from that of the reference sample gave a value of 0.003 or .3%. Since [3-^{18}O]serine used in this study was 6.94% enriched with ^{18}O, the

Table 5. AVA Analysis of ^{18}O in 4-Hydroxysphinganine from Yeast Grown in LM-3 in the Presence of 1% Glucose and 0.5% [3-^{18}O]Serine (6.94%).

Determinations	m/e 301/299		m/e 403/401		m/e 220/218	
	Reference	Sample	Reference	Sample	Reference	Sample
1	0.0713	0.0735	0.1312	0.1326	0.0919	0.1489
2	0.0714	0.0730	0.1297	0.1343	0.0924	0.1496
3	0.0713	0.0744	0.1310	0.1310	0.0920	0.1491
4	0.0714	0.0777	0.1321	0.1348	0.0920	0.1499
Average	0.0714	0.0747	0.1310	0.1333	0.0921	0.1493
Δ*	-	0.0033	-	0.0023	-	0.0572

Δ* is the difference between sample and reference values.

Incorporation of ^{18}O into C-4 is 4.8%, C-3 is 0% and C-1 is 82.4%.

m/e 299, $C_{14}H_{27}$-CH=$\overset{+}{O}$TMSi; m/e 301, $C_{14}H_{27}$-CH$^{18}\overset{+}{O}$TMSi; m/e 401, $C_{14}H_{27}$-CH(OTMSi)-$\overset{+}{C}$H-CH=OTMSi;

m/e 403, $C_{14}H_{27}$-CH(OTMSi)-CH=$^{18}\overset{+}{O}$TMSi and $C_{14}H_{27}$-CH(^{18}OTMSi)-CH=$\overset{+}{O}$TMSi; m/e 218, CH=CH-CH$_2$-OTMSi;
$\overset{+}{}$OTMSi

m/e 220, CH=CH$_2$ with ^{18}OTMSi and $\overset{+}{}$OTMSi; CH=CH-CH$_2$-OTMSi
$^{18}\overset{+}{}$OTMSi

theoretical percent incorporation was (.3/6.94)x100 or 4.8%. Total incorporation of isotope into C-3 and C-4 was (.23/6.94)x100 or about 3.3%, which is slightly less than that of C-4, thus it is concluded that there is no net incorporation of isotope into C-3 of 4-hydroxy-sphinganine. Analysis of isotope on C-1 and C-3 (the ratio of m/e 220/218) shows an incorporation of 81.5%. Since there is no incorporation of isotope into C-3, this incorporation is then attributed to that of C-1 only. This is slightly less than the theoretical value (100%), probably due to dilution from synthesis from non-isotope endogeneous serine. Although a 4.8% incorporation into C-4 of 4-hydroxy-sphinganine has been observed, this value may be insignificant in this study because the precision of the instrument has been reported to be 1% (Holland et al.,1973) and the net isotopic enrichment on C-4 was only 0.3% which is probably beyond the precision of the instrument. Therefore, [3-^{18}O]serine and [1-^{18}O]sphinganine, which can be derived from [3-^{18}O]serine in situ, are ruled out as the hydroxyl donors.

F. Studies on Yeast Grown in the Presence of [1-^{18}O]Palmitate.

Isotopic enrichment on C-1 of palmitic acid was analysed by GLC-AVA as its methyl ester derivative. The mass spectrum of methyl palmitate has been reported elsewhere (Budzikiewicz et al.,1967). The molecular ion is relatively intense and has been used for the analysis of total ^{18}O in the molecule. The ion at m/e 239 (M-31), which arose from the loss of the methoxyl group was used for determination of isotopic abundance on the carbonyl oxygen. The result in Table 6 shows that enrichment on the whole molecule was 11.1%, and that on the carbonyl group was 8.3%.

Table 6. AVA Analysis of ^{18}O in Methyl Palmitate.

Determinations	m/e 272/270		m/e 241/239	
	Reference	Sample	Reference	Sample
1	0.0237	0.1352	0.3623	0.4426
2	0.0240	0.1368	0.3622	0.4463
3	0.0255	0.1355	0.3633	0.4464
4	0.0252	0.1360	0.3682	0.4467
Average	0.0246	0.1359	0.3625	0.4455
Δ^*	-	0.1113	-	0.0830

Δ^* is the difference between sample and reference values.

m/e 270, $C_{15}H_{31}-\overset{\overset{O^{+\cdot}}{\|}}{C}-OCH_3$; m/e 272, $C_{15}H_{31}-\overset{\overset{^{18}O^{+\cdot}}{\|}}{C}-OCH_3$ and

$C_{15}H_{31}-\overset{\overset{O^{+\cdot}}{\|}}{C}{}^{18}OCH_3$; m/e 239, $C_{15}H_{31}-\overset{\overset{O^{+}}{\|}}{C}$; m/e 241,

$C_{15}H_{31}-\overset{\overset{^{18}O^{+}}{\|}}{C}$.

Table 7. AVA Analysis of ^{18}O in 4-Hydroxysphinganine from Yeast Grown in LM-1 in the Presence of [1-^{18}O]-Palmitate (1 mg/ml).

Determinations	m/e 301/299		m/e 403/401	
	Reference	Sample	Reference	Sample
1	0.0681	0.0672	0.1246	0.1779
2	0.0680	0.0669	0.1249	0.1766
3	-	-	0.1269	0.1774
4	-	-	0.1278	0.1786
Average	0.0681	0.0671	0.1260	0.1776
Δ^*	-	-	-	0.0516

Δ^* is the difference betwee sample and reference value.

^{18}O on C-3 of 4-hydroxysphinganine is 62.17%.

m/e 299, $C_{14}H_{29}$-CH=$\overset{+}{O}$TMSi; m/e 301, $C_{14}H_{29}$-CH=$\overset{+}{^{18}O}$TMSi;

m/e 401, $C_{14}H_{29}$-CH(OTMSi)-CH=$\overset{+}{O}$TMSi; m/e 403, $C_{14}H_{29}$-CH(OTMSi)-CH=$\overset{+}{^{18}O}$TMSi

and $C_{14}H_{29}$-CH(^{18}OTMSi)-CH=$\overset{+}{O}$TMSi.

Adding 1 mg of this [1-^{18}O]palmitate, dispersed in 20 mg of Triton X-100, into LM-1 (10 ml) resulted in an incorporation of ^{18}O into C-3 of 4-hydroxysphinganine after incubating with viable yeast for 48 hours at 26-28°C. About 62% (100 x 5.2/8.2) enrichment was found on C-3 (Table 7). None was observed on C-4 of 4-hydroxysphinganine. It was concluded that [1-^{18}O]palmitate and [3-^{18}O]sphinganine, which can be derived from [1-^{18}O]palmitate in situ, were not the oxygen donor on the hydroxyl group on C-4 of 4-hydroxysphinganine.

G. Incorporation of $H_2^{18}O$ into 4-Hydroxysphinganine by Yeast Grown on Differrent Media.

1. Yeast Grown on LM-1 and LM-2.

Table 8 shows the incorporation of $H_2^{18}O$ into various hydroxyl groups of 4-hydroxysphinganine by yeast grown on LM-1, containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose in 10 ml of $H_2^{18}O$ (30.13%). Incoporation of ^{18}O into C-4 of 4-hydroxysphinganine (16.5%) was comparable to that reported by Thorpe and Sweeley (1967). When the medium was autoclaved at 120°C for 25 minutes (LM-2, $H_2^{18}O$ used was 19.1%), incorporation of ^{18}O from $H_2^{18}O$ into C-4 of 4-hydroxysphinganine was not affected by heat (Table 9, 13.9%). The slightly lower value (2.6%) is probably due to experimental errors in the determination of low isotopic abundance in 4-hydroxysphinganine product or due to slight variations of harvesting time which is not known whether it is a critical factor for isotopic incorporation. The hydroxyl group on C-3 of 4-hydroxysphinganine was fully derived from water in both experiments (LM-1 and LM-2). This hydroxyl

Table 8. AVA Analysis of ^{18}O in 4-Hydroxysphinganine from Yeast Grown in LM-1 in $H_2{}^{18}O$ (30.13%).

Determinations	m/e 301/299		m/e 403/401		m/e 220/218	
	Reference	Sample	Reference	Sample	Reference	Sample
1	0.0677	0.1177	0.1216	0.5049	0.0947	0.5998
2	0.0686	0.1183	0.1211	0.5013	0.0949	0.5997
3	0.0668	0.1180	0.1214	0.4941	0.0935	0.5998
4	0.0693	0.1177	0.1170	0.5038	0.0940	0.5952
Average	0.0681	0.1177	0.1210	0.5010	0.0942	0.5986
Δ*	-	0.0496	-	0.3800	-	0.5044

Δ* is the difference between sample and reference values.

Incorporation of ^{18}O into C-4 is 16.46%, C-3 is 109.66% and C-1 is 57.74%.

m/e 299, $C_{14}H_{27}$-CH-$\overset{+}{O}$TMSi; m/e 301, $C_{14}H_{27}$-CH-$\overset{+}{O}{}^{18}$TMSi; m/e 401, $C_{14}H_{27}$-CH(OTMSi)-CH=$\overset{+}{O}$TMSi;

m/e 403, $C_{14}H_{27}$-CH(OTMSi)-CH=$\overset{+}{O}{}^{18}$TMSi and $C_{14}H_{27}$-CH(^{18}OTMSi)-CH=$\overset{+}{O}$TMSi; m/e 218, CH($\overset{+}{O}$TMSi)-CH=CH-CH_2-OTMSi;

m/e 220, CH($\overset{+}{O}$TMSi)-$CH_2{}^{18}$OTMSi and CH($^{18}\overset{+}{O}$TMSi)-CH=CH-CH_2-OTMSi

Table 9. AVA Analysis of ^{18}O in 4-Hydroxysphinganine from Yeast Grown in LM-2 in $H_2^{18}O$ (19.10%).

Determinations	m/e 301/299		m/e 403/401		m/e 220/218	
	Reference	Sample	Reference	Sample	Reference	Sample
1	0.0677	0.0939	0.1216	0.3641	0.0947	0.4549
2	0.0686	0.0949	0.1210	0.3548	0.0945	0.4507
3	0.0668	0.0946	0.1214	0.3551	0.0935	0.4594
4	0.0693	0.0943	0.1199	0.3581	0.0940	0.4562
Average	0.0681	0.0946	0.1210	0.3575	0.0942	0.4553
Δ^*	-	0.0265	-	0.2365	-	0.3611

Δ^* is the difference between sample and reference values.

Incorporation of ^{18}O into C-4 is 13.87%, C-3 is 109.95% and C-1 is 79.10%.

m/e 299, $C_{14}H_{27}$-CH=$\overset{+}{O}$TMSi; m/e 301, $C_{14}H_{27}$-CH=$^{18}\overset{+}{O}$TMSi; m/e 401, $C_{14}H_{27}$-$\underset{\text{OTMSi}}{\text{CH}}$-$\overset{+}{\text{CH}}$=OTMSi;

m/e 403, $C_{14}H_{27}$-$\underset{\text{OTMSi}}{\text{CH}}$-CH=$^{18}\overset{+}{O}$TMSi and $C_{14}H_{27}$-$\underset{^{18}\text{OTMSi}}{\text{CH}}$-CH=$\overset{+}{O}$TMSi; m/e 218, $\underset{\overset{+}{\text{OTMSi}}}{\text{CH}}$=CH-$CH_2$-OTMSi;

m/e 220, $\underset{\overset{+}{\text{OTMSi}}}{\text{CH}}$=$CH_2$ ^{18}OTMSi and $\underset{^{18}\overset{+}{\text{OTMSi}}}{\text{CH}}$=CH-$CH_2$-OTMSi

group was derived from palmitate; exchange with water in the medium somewhere after palmitate synthesis cannot account for complete exchange and therefore the exchange with water must take place prior to the incorporation into palmitate.

Incorporation of $H_2^{18}O$ into the primary hydroxyl group on C-1 of 4-hydroxysphinganine was increased from 57.7% in LM-1 to 79.1% in LM-2. This increase is expected because an increase in temperature would increase the rate of oxygen exchange between water and the aldehydic oxygen on C-1 of glucose. This oxygen is transformed into the hydroxyl group on C-3 of 3-phosphoglyceric acid during glycolysis then to the hydroxyl group on C-3 of serine (Umbarger and Umbarger, 1962). This hydroxyl group of serine was shown in the earlier study to be the precursor of the hydroxyl group on C-1 of 4-hydroxysphinganine.

2. Yeast Grown on Ethanol as the Principal Carbon Source.

Since over 95% of the dry weight of malt extract was found to be anthrone-positive compounds (calculated as glucose), it was necessary to omit this nutrient in order to minimize the source of carbon other than ethanol. Peptone contained about 1.5% of anthrone-positive material and was also omitted in this study. Yeast extract, however, contained about 7% of anthrone positive substances, but could not be eliminated without affecting growth of the yeast.

Yeast was grown on LM-3, containing 0.12% ammonium sulfate, 0.07% magnesium sulfate, 0.05% sodium chloride, 0.5% potassium phosphate (dibasic), 0.5% yeast extract and 2% ethanol in 10 ml of $H_2^{18}O$ (13.7%). To utilize ethanol as the building block for other biological substances, yeast must oxidise it to acetate. Oxygen of acetate is assumed to be

Table 10. AVA Analysis of ^{18}O in 4-Hydroxysphinganine from Yeast Grown in LM-3 and 2% Ethanol in $H_2^{18}O$ (13.7%).

Determinations	m/e 301/299		m/e 403/401		m/e 220/218	
	Reference	Sample	Reference	Sample	Reference	Sample
1	0.0691	0.0900	0.1215	0.2343	0.0947	0.2993
2	0.0682	0.0908	0.1211	0.2363	0.0945	0.2989
3	0.0707	0.0910	0.1199	0.2368	0.0940	0.2963
4	0.0702	0.0915	0.1214	0.2322	0.0935	0.2985
Average	0.0696	0.0906	0.1210	0.2334	0.0942	0.2982
Δ^*	-	0.0210	-	0.1124	-	0.2040

Δ^* is the difference between sample and reference values.

Incorporation of ^{18}O into C-4 is 15.32%, C-3 is 66.72% and C-1 is 82.19%.

m/e 299, $C_{14}H_{27}$-CH=$\overset{+}{O}$TMSi; m/e 301, $C_{14}H_{27}$-CH=$\overset{+}{^{18}O}$TMSi; m/e 401, $C_{14}H_{27}$-$\underset{\overset{|}{\text{OTMSi}}}{\text{CH}}$-$\underset{+}{\text{CH=OTMSi}}$;

m/e 403, $C_{14}H_{27}$-$\underset{\overset{|}{\text{OTMSi}}}{\text{CH}}$-$\underset{+}{\text{CH=}^{18}\text{OTMSi}}$ and $C_{14}H_{27}$-$\underset{\overset{|}{^{18}\text{OTMSi}}}{\text{CH}}$-$\underset{+}{\text{CH=OTMSi}}$; m/e 218, $\underset{\overset{|}{\text{OTMSi}}}{\text{CH}}$=CH-$CH_2$-OTMSi;

m/e 220, $\underset{\overset{|}{\text{OTMSi}}}{\text{CH}}$=$CH_2$-^{18}OTMSi and $\underset{\overset{|}{^{18}\text{OTMSi}}}{\text{CH}}$=CH-$CH_2$-OTMSi

Table 11. AVA Analysis of ^{18}O on C-2 of Glucose from Yeast Grown in LM-3 and 2% Ethanol in $H_2^{18}O$ (13.7%).

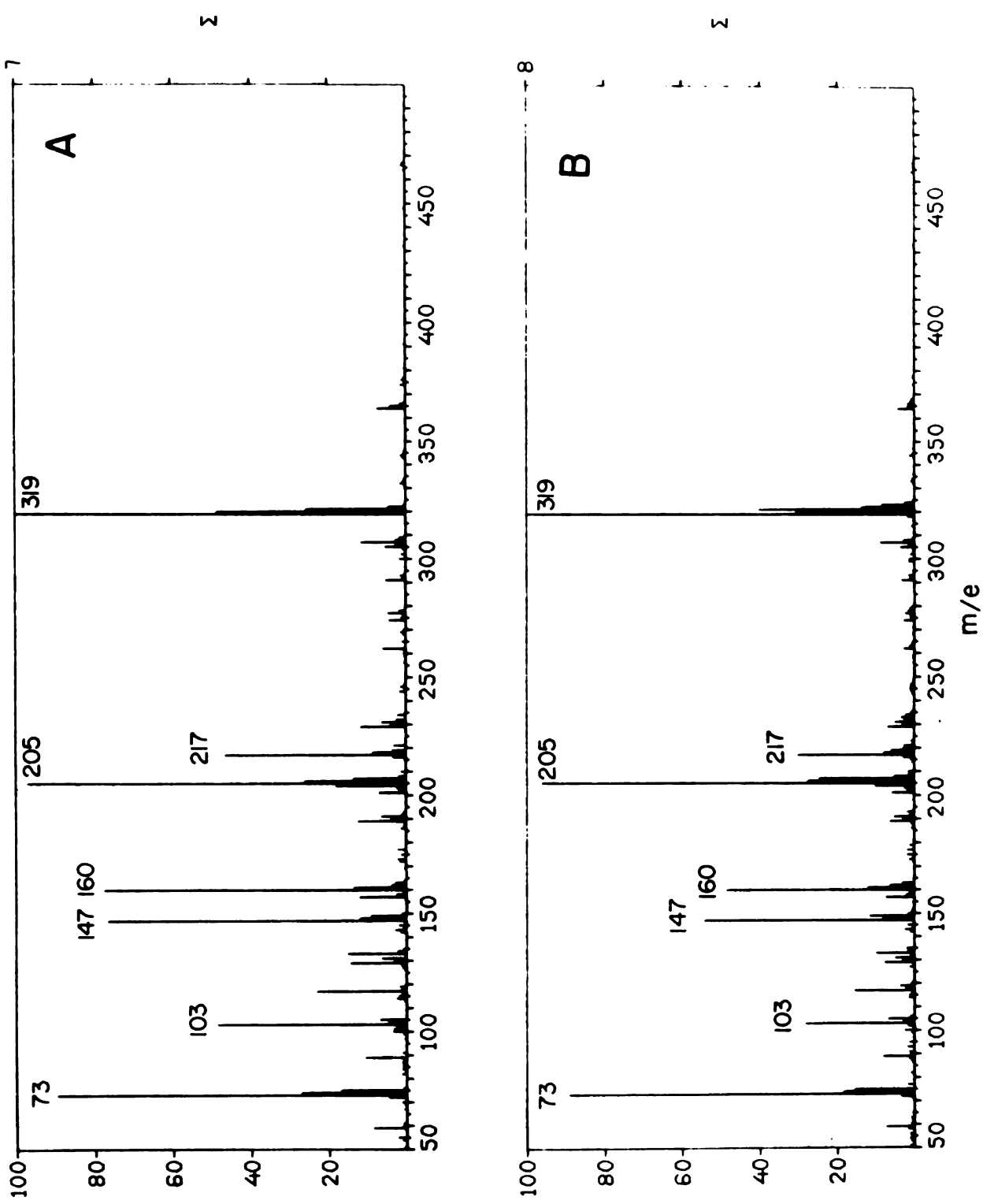
Determinations	m/e 162/160	
	Reference	Sample
1	0.0529	0.1475
2	0.0529	0.1477
3	0.0530	0.1473
4	0.0535	0.1460
Average	0.0531	0.1471
Δ^*	-	0.0940

Δ^* is the difference between sample and reference values.

Incorporation of ^{18}O into C-2 of glucose is 68.61%.

m/e 160, $\begin{matrix} CH{=}N{-}OCH_3 \\ | \quad + \\ CH{-}OTMSi \end{matrix}$; m/e 162, $\begin{matrix} CH{=}N{-}OCH_3 \\ | \quad + \\ CH{=}{}^{18}OTMSi \end{matrix}$.

Fig. 8. Mass spectra of TMSi-glucose methoxime; A) reference and B) isolated from yeast grown on ethanol as the principal carbon source.

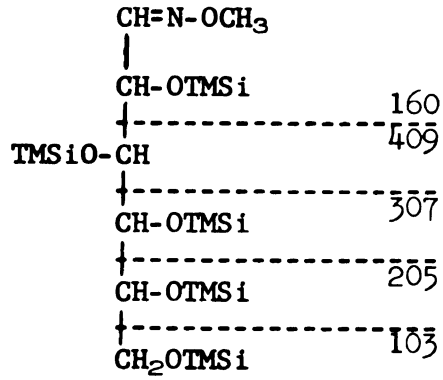

A
B
Σ
73
103
147
160
205
217
319
50
100
150
200
250
300
350
400
450
20
40
60
80
100
m/e
Relative Intensity (%)

easily exchanged with water in the medium and most biological compounds would, therefore, be expected to bear oxygen isotope in the molecule. Table 10 shows that incorporation of $H_2^{18}O$ into C-4 of 4-hydroxysphinganine was not increased compared to the results with yeast grown in LM-1 and LM-2. The only increase was that on the primary group on C-1 which originated from serine. By either pathway, glyceric acid (Umbarger and Umbarger 1962) or glycine and formaldehyde (Kislink and Sakami, 1954), an increase in the ^{18}O abundance on C-3 of serine, relative to that of LM-1 and LM-2, would be expected.

It was a surprise that the isotopic abundance of the hydroxyl group on C-3 of 4-hydroxysphinganine was less than that in yeast grown in LM-1 and LM-2. This difference in ^{18}O incorporation might be due to the fact that acetyl CoA, the oxidation product of ethanol, might be used directly for the synthesis of palmitoyl CoA. Thus, unhydrolysed acetyl CoA might be responsible for incorporation of the ethanol oxygen and would give a lower level of ^{18}O from water.

Analysis of glucose isolated from the cell paste (Table 11) indicates that 68.6% of oxygen on C-2 was derived from water in the medium. The spectrum of TMSi-glucose methoxime from the cell paste (Fig.8B) indicated the incorporation of ^{18}O into various positions of glucose, thus glucose was tentatively ruled out as the possible oxygen donor to the hydroxyl group of 4-hydroxysphinganine. Interpretation of the mass spectrum of TMSi-glucose methoxime (Fig.8A) has been published (Laine and Sweeley, 1971). The major ions are shown below.

The ions at m/e 409 and 307 are unstable and subsequent loss of TMSiOH yields an ion at m/e 319 and 217, respectively.



(42)

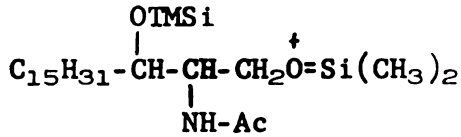
H. Mass Spectra of Bis-O-TMSi-N-Acetylsphinganine.

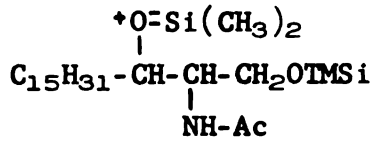
The mechanism of electron-induced fragmentation of TMSi-N-acetylsphinganine was studied with the aid of deuterium labelling and exact mass measurement. The ions formed on electron impact ionization at 70 eV were divided into two main categories with respect to electron abstraction from one of the oxygen atoms or the nitrogen atom of sphinganine.

1. Ions Derived by Electron Abstration from Oxygen Atoms.

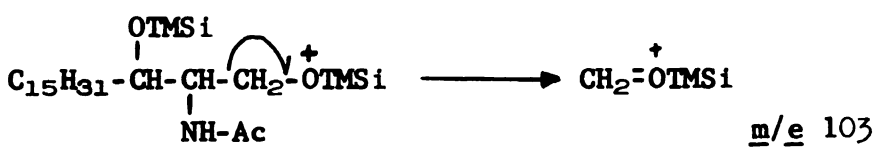
The mass spectrum of TMSi-N-acetylsphinganine (Fig.9) was previously reported (Gaver and Sweeley, 1966). The molecular ion was not detected, but could be deduced from other ions in the spectrum, especially the ion at M-15 (m/e 472). This ion, at m/e 472 ($C_{25}H_{54}NO_3Si_2$; calc. 472.3641; observed, 472.3651), is derived from loss of a methyl group from one of the TMSi groups. Substitution on TMSi with d_9-TMSi on N-acetylsphinganine resulted in a shift from the

loss of 15 amu to 18 amu (Fig.10), suggesting the loss of a d_3-methyl from one of the d_9-TMSi groups. However, which of the two TMSi groups (on C-1 or C-3) the methyl was lost from was not ascertained. It is assumed to be a mixture of the following two structures:





It is possible to differentiate these two ions, if selective derivatization of one of the hydroxyl groups with d_9-TMSi is achieved. This technique has been used successfully with steroids which contain a hindered hydroxyl group on C-17 and a nonhindered hydroxyl group on C-3 or C-20 (Vouros and Harvey, 1973). The two hydroxyl groups of sphinganine appear to be equally reactive to TMSi-donor agent, however, and they are probably trimethylsilylated instantaneously. Attempt was not made to pursue this problem, therefore. The ion at m/e 103 ($C_4H_{11}OSi$; calc., 103.0579; observed, 103.0579) is probably formed by cleavage of C-1 and C-2 bond.

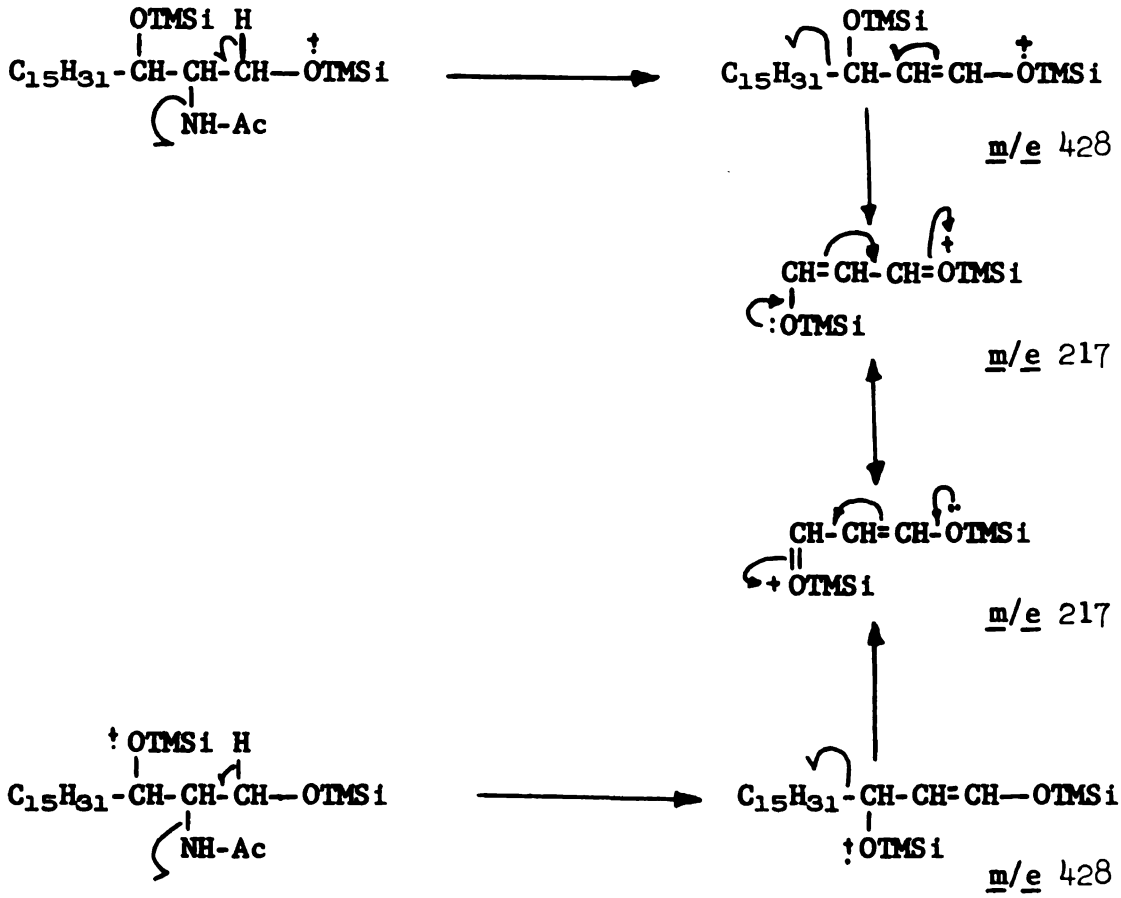


Substitution of two deuterium atoms on C-1 shifted the ion to m/e 105 (Fig.12 and 15) regardless of isotopic substitution at other positions

(Fig.11 and 13) and it was observed at m/e 112 in the spectrum of d_9-TMSi derivative (Fig.10), indicating that the ion is composed of one TMSi group and the methylene group on C-1.

This ion also derived partially from C-3 of sphinganine by a more complicated mechanism, however, since substitution of deuterium on position-3 shifted a significant proportion of the intensity at m/e 103 to m/e 104 (Fig.11). Cleavage of both C-C bonds of C-3, accompanied by transfer of hydrogen to the positively charged ion, is necessary to account for this component of m/e 103; the origin of the hydrogen transfer cannot be ascertained in this study.

The ion at m/e 217 ($C_9H_{21}O_2Si_2$; calc., 217.1079; observed, 217.1085) may be derived from the following two pathways.



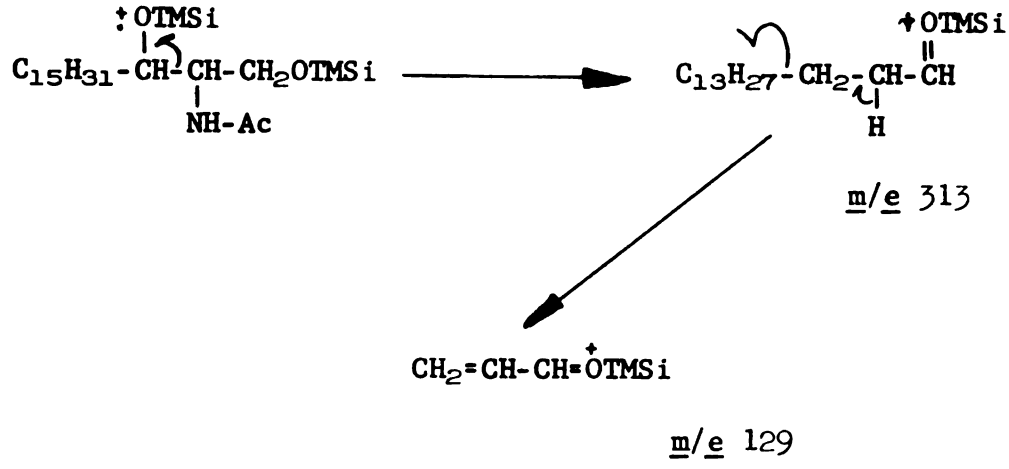
It is assumed that this ion can be derived from the molecular ion which has charge retention on the oxygen atom of either C-1 or C-3. The initial loss of the elements of neutral acetamide by 1,2 elimination gives an ion at m/e 428, which is very weak in the spectrum of the protium species (Fig.9) but for an unknown reason is enhanced (at m/e 429) in the spectrum of [3-^{2}H]-sphinganine (Fig.11). The intensity of this ion is suppressed to an undetectable level by further substitution of deuterium atoms at other positions.

Homolytic cleavage of the ion at m/e 428 gives a more stable ion at m/e 217. A reversal of the sequence leading to this ion, involving initial cleavage of the bond between C-3 and C-4, followed by loss of elements of neutral acetamide, is also possible. However, the ion at m/e 276, which is presumably derived from cleavage of the C-3 and C-4 bond initially, has never been detected in the spectra of protium or deuterium forms of sphinganine. The ion at m/e 217 is, therefore, more likely to be derived from ion at m/e 428 rather than from m/e 276.

Substitution of two deuterium atoms on C-1 of sphinganine shifted the ion by only one mass unit (Fig.12), suggesting that one of the deuteriums is lost during the formation of the ion at m/e 428. As would be predicted from the mechanism, the ion at m/e 217 is not affected by deuterium atom on C-4 and C-5 (Fig.13) or on the acetyl group (Fig.14). It is shifted by 18 mass units in the spectrum of the bis-O-d_9-TMSi derivative (Fig.10). Deuterium substitution on C-1, C-2, C-3 and C-4 shifted the ion by three mass units (Fig.16). Since

substitution of deuterium on C-1 and C-3 shifted the ion by two mass units (Fig. 15) whereas substitution on C-4 had no effect (Fig. 13), it can be inferred that deuterium on C-2 could contribute one mass increment to this ion. The structure and mechanism of the formation of this ion, shown above, is therefore consistent with the spectra shown in Figs. 9-16, and the composition of the ion (given above) is exactly the same as that of ion at m/e 217 derived from penta-O-TMSi-α-β-D-glucopyranose (DeJongh et al., 1969), which was observed in the spectra of TMSi derivatives of a variety of sugars (DeJongh et al., 1969; Laine and Sweeley, 1973).

The ion at m/e 313 ($C_{19}H_{41}OSi$; calc., 313.2926; observed, 313.2934) is believed to be derived directly by simple cleavage between C-2 and C-3 with charge retention on the trimethylsilyloxy group, as shown below. Substitution of deuterium on C-3 (Fig. 11), C-4 and C-5 (Fig. 13) and on the trimethylsilyl residue (Fig. 10) shifted the mass of this ion by 1,2 and 9 mass units, respectively. Substitution of deuterium on C-1 and on the acetyl group did not affect its mass, as predicted by the proposed mechanism. Further fragmentation of this ion with loss of elements of tridecane, by a mechanism shown below, gives an ion at m/e 129 ($C_6H_{13}OSi$; calc., 129.0735; observed, 129.0736). This ion was affected, as predicted by the proposed pathway, when deuterium was substituted on C-3 (m/e 130; Fig. 11), C-4 and C-5 (m/e 131; Fig. 13) and on the TMSi groups (m/e 138; Fig. 10), whereas substitution of deuterium on C-1 (Fig. 12) and on the acetyl group (Fig. 14) had no effect.

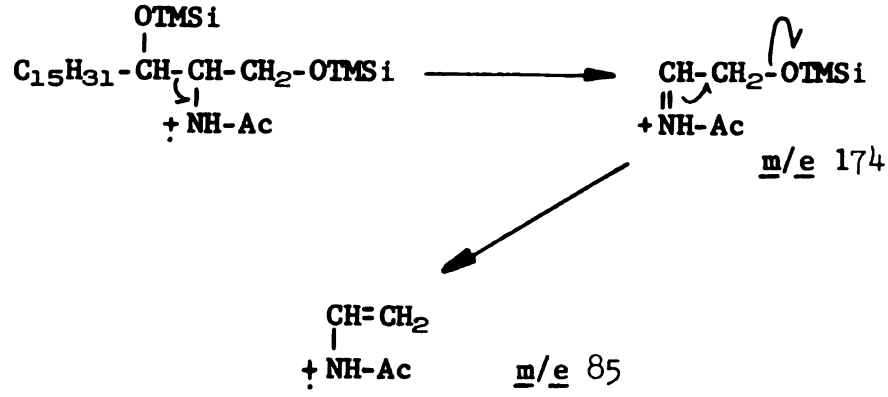


m/e 313

m/e 129

2. Ions Derived by Electron Abstration from the Nitrogen Atom.

The ion at m/e 174 ($C_7H_{16}NO_2Si$; calc., 174.0950; observed, 174.0945) arises by direct cleavage between C-2 and C-3 with charge retention on the nitrogen containing fragment, as show below. Substitution of deuterium on C-1 (Fig.12), on the acetyl group (Fig.14), and on the TMSi group (Fig.10) shifted the ion to m/e 176, 177 and 183, respectively.

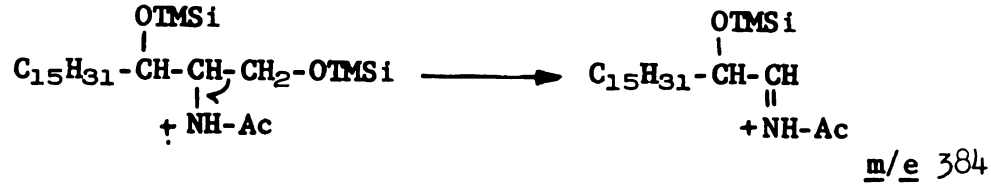


m/e 174

m/e 85

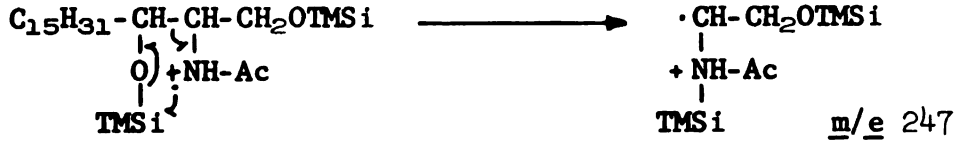
Substitution of deuterium on C-3 (Fig.11) and on C-4 and C-5 (Fig.13) did not affect the mass of the ion but deuterium substitution on C-1, C-2, C-3 and C-4 shifted the mass by three mass units (Fig.16), which is assumed to be accounted for by the two deuterium atoms on C-1 and that on C-2, rather than the deuteriums on carbons-3,4 and 5. Further fragmentation, with a loss of TMSi radical, yields an ion at m/e 85 (C_4H_7NO; calc., 85.0528; observed, 85.0530). This ion is expected to be affected by the same deuterium substitution pattern as the ion at m/e 174 except for that due to the TMSi residue, which is lost in the conversion of m/e 174 to 85.

The ion at m/e 384 ($C_{22}H_4NO_2Si$; calc., 384.3297; observed, 384.3275) is presumably derived from covalent bond cleavage between C-1 and C-2 with loss of the C-1 containing fragment as shown below. Deuterium substitution on any position other than C-1 would therefore be expected to affect the mass of the ion, and the results of deuterium labelling on various positions (Figs.9-16) are consistent with the structure.



A cyclic transition mechanism has been proposed by Hammarström et al. (1970) to account for the formation of an equivalent ion of m/e 247 in the mass spectra of long-chain fatty acyl sphinganine ceramides. Data obtained here on the spectra on N-acetyl derivatives

of sphinganine labeled with deuterium at various positions agree with this type of fragmentation mechanism, which is shown below. The ion ($C_{10}H_{25}NO_2Si_2$; calc., 247.1423; observed, 247.1424) is at m/e 247 in the protium species (Fig. 9) and occurs at m/e 249, 250, 250 and 265 in [1,1-$^2H_2$]sphinganine (Fig. 12), [1,1,2,3,4,4-$^2H_6$]sphinganine (Fig. 16), N-[$^2H_3$]acetylsphinganine (Fig. 14) and d_9-TMSi-sphinganine (Fig. 10), respectively.



The ions at m/e 157 and 116 are presumably derived from further fragmentation of the ion at m/e 247, as shown below. Loss of TMSiOH gives a companion ion at m/e 157 ($C_7H_{15}NOSi$; calc., 157.0925; observed, 157.0922). The sphinganine labeled with deuterium on C-1 and C-2 (Fig. 16) and on the acetyl moiety (Fig.14) did not loss 91 amu instead of 90, indicating that the hydrogen atom involved in this elimination process is from a source other than C-1, C-2 or on the acetyl group. The hydrogen atom involved in this elimination process was tentatively assigned to that attached to the nitrogen atom. A concomitant loss of one methyl radical and a carbene from the ion at m/e 247 is suggested to account for the ion at m/e 116 ($C_4H_{10}NOSi$; calc., 116.0531; observed, 116.0532). This ion is not affected by deuterium substitution on the sphinganine moiety, but shifts to m/e 119 and 122 in N-[$^2H_3$]acetyl sphinganine (Fig. 14) and d_9-TMSi species (Fig. 10), respectively,

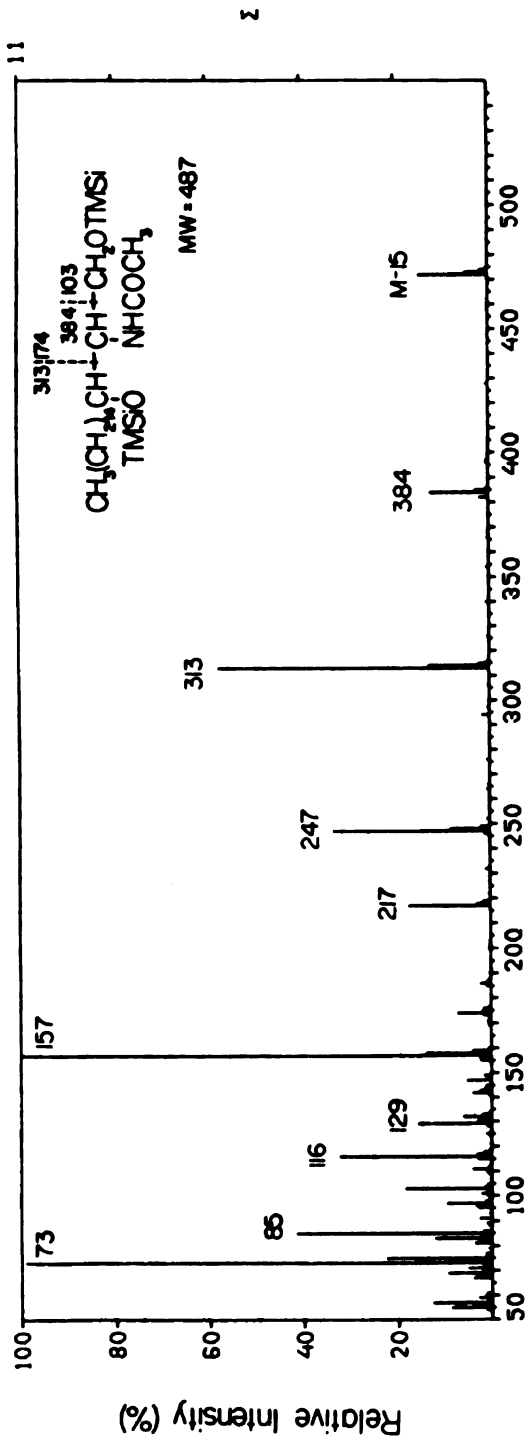
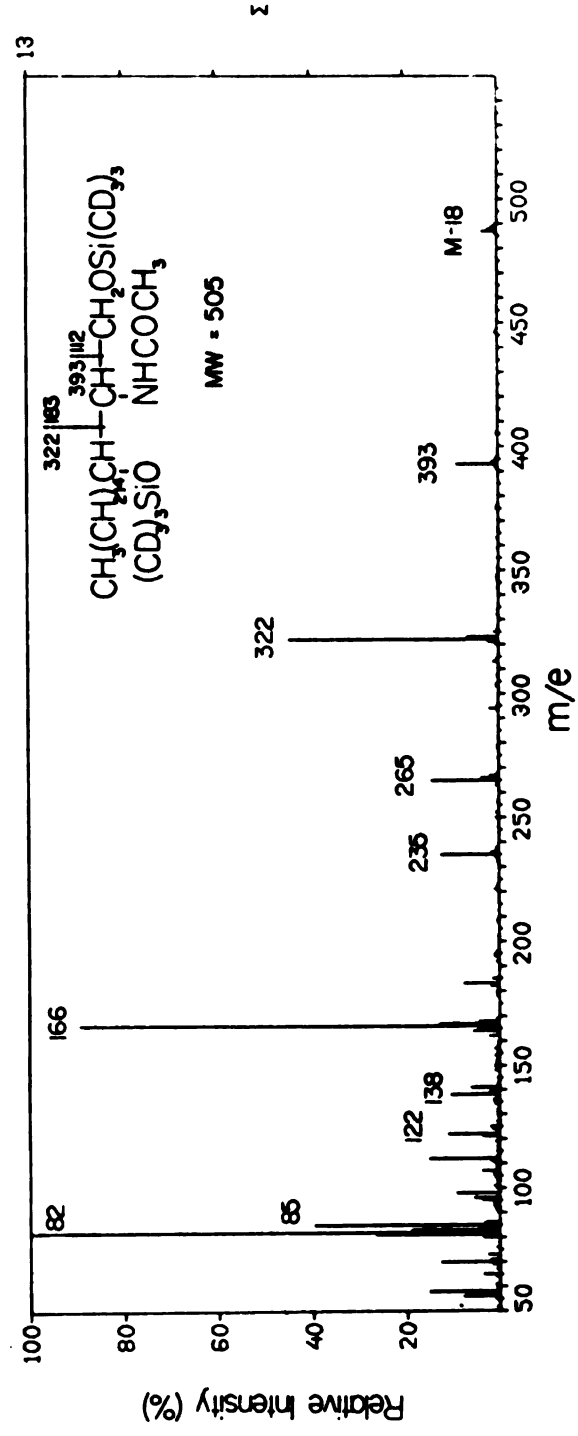


Fig. 9 (above). Mass spectrum of bis-O-TMSi-N-acetylsphinganine.

Fig.10 (below). Mass spectrum of bis-O-[$^2H_9$]TMSi-N-acetylsphinganine.



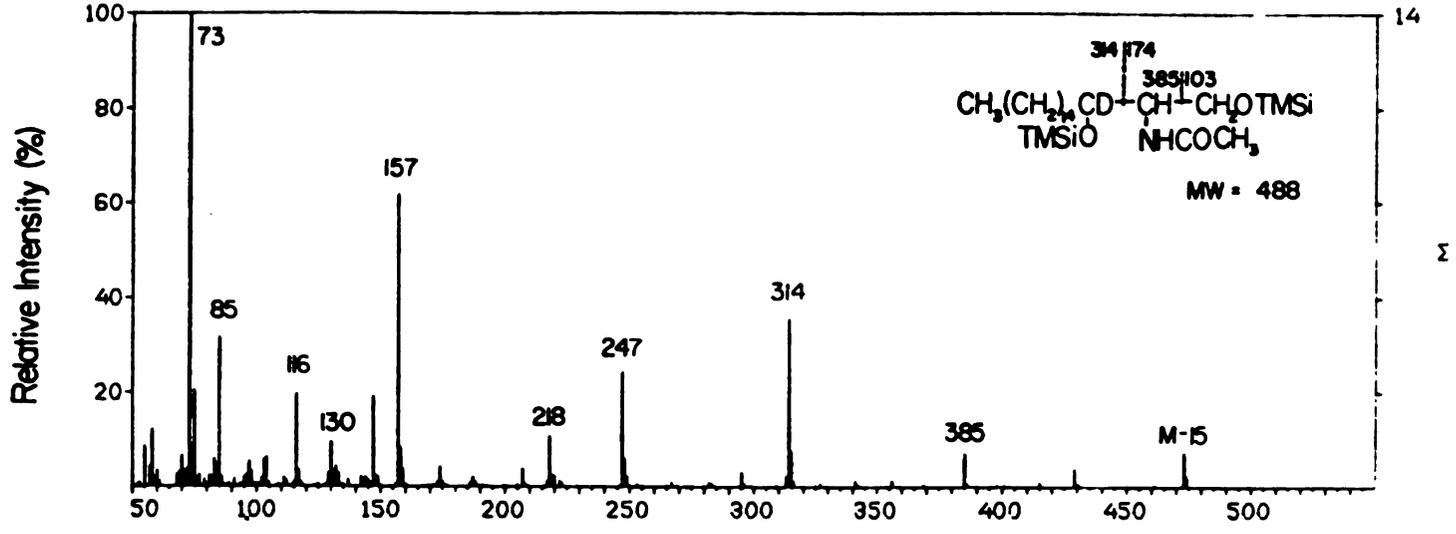
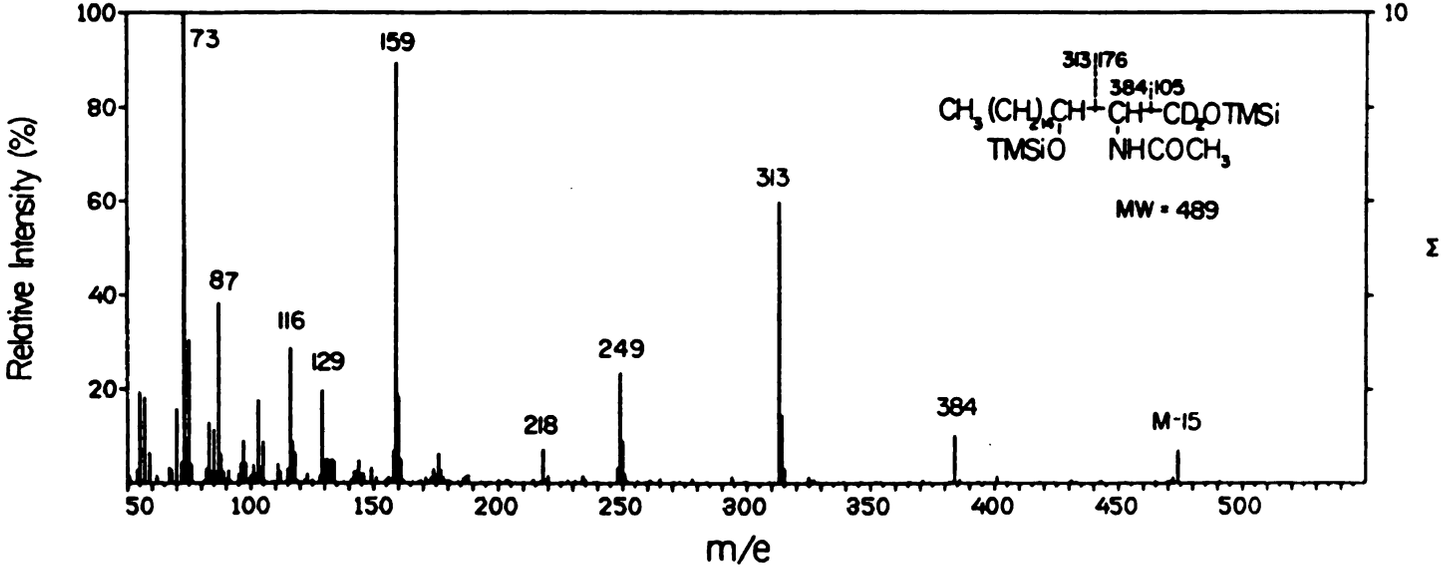


Fig. 11 (above). Mass spectrum of bis-O-TMSi-[3-^{2}H]N-acetylsphinganine.

Fig. 12 (below). Mass spectrum of bis-O-TMSi-[1,1-^{2}H]N-acetylsphinganine



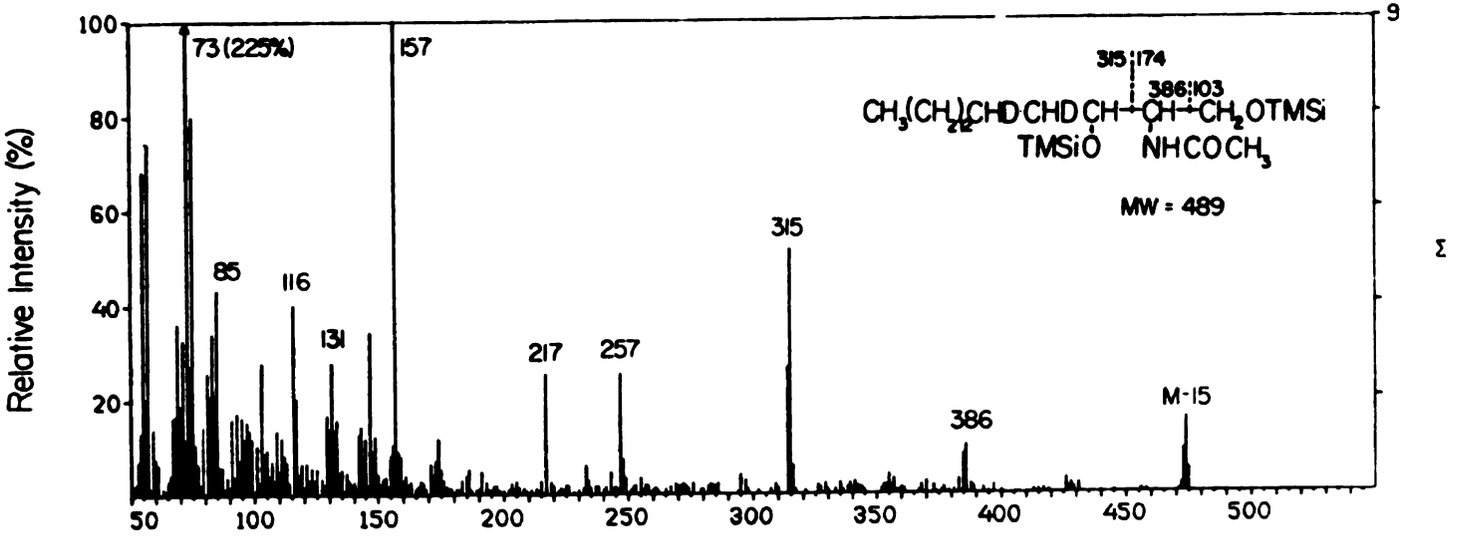
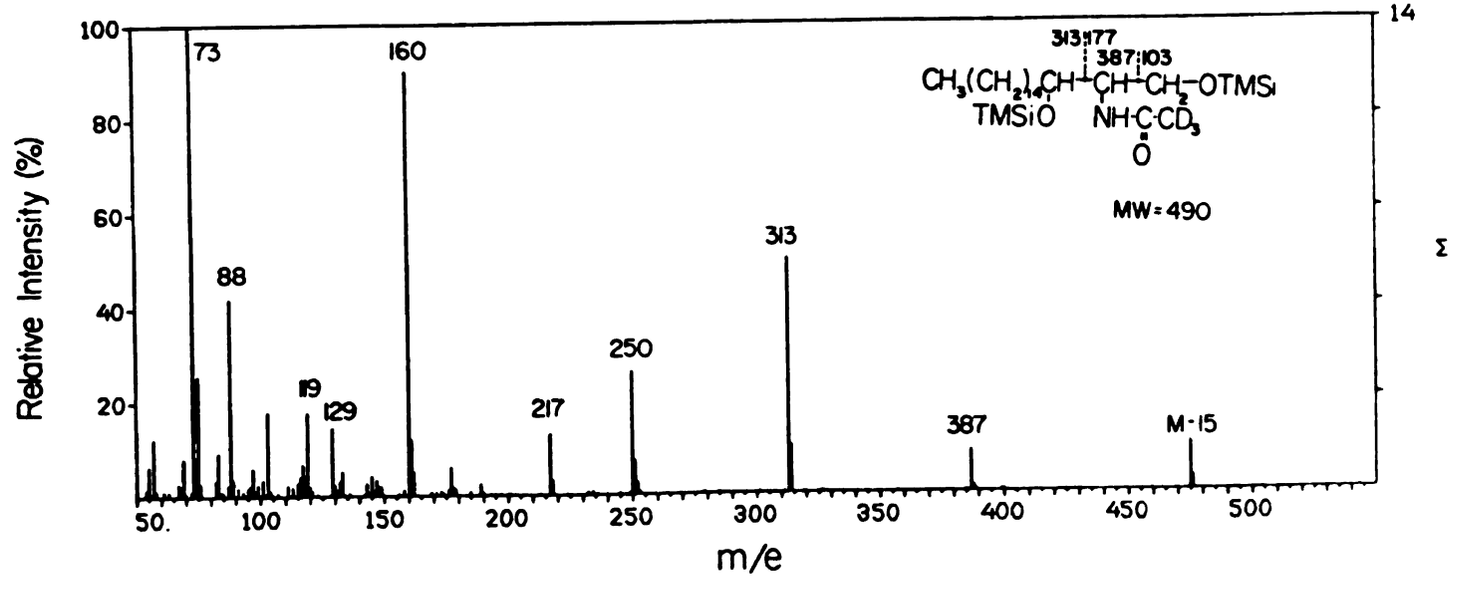


Fig. 13 (above). Mass spectrum of bis-O-TMSi-[4,5-$^2H_2$]N-acetylsphinganine.

Fig. 14 (below). Mass spectrum of bis-O-TMSi-N-[$^2H_3$]acetylsphinganine.



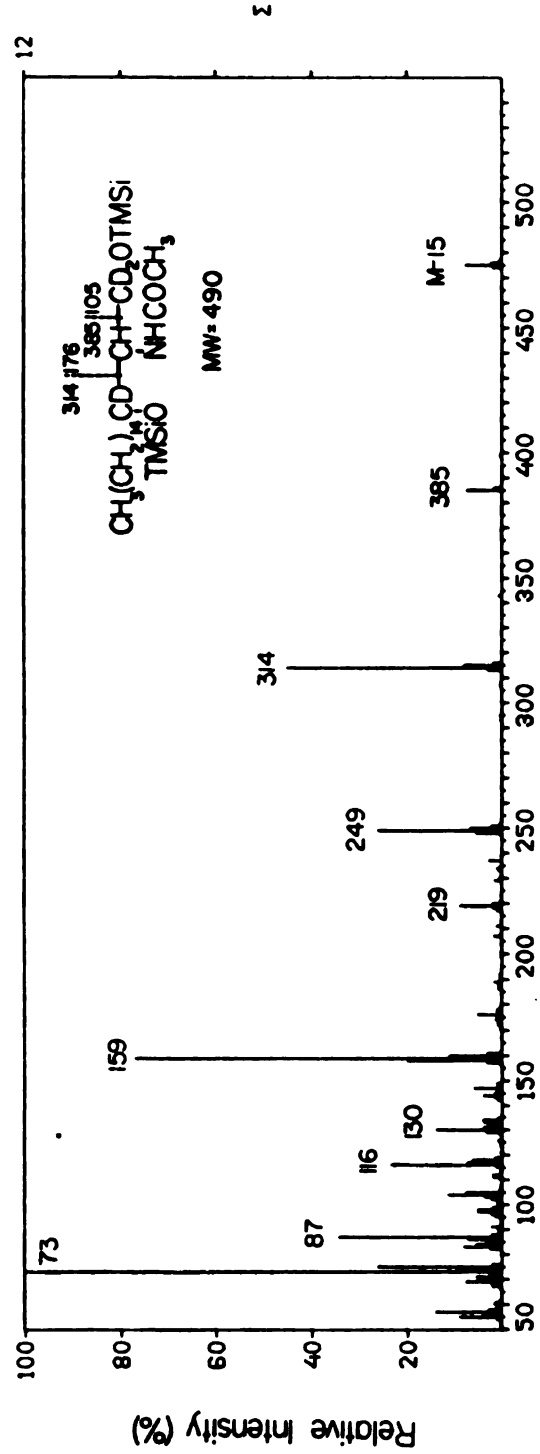
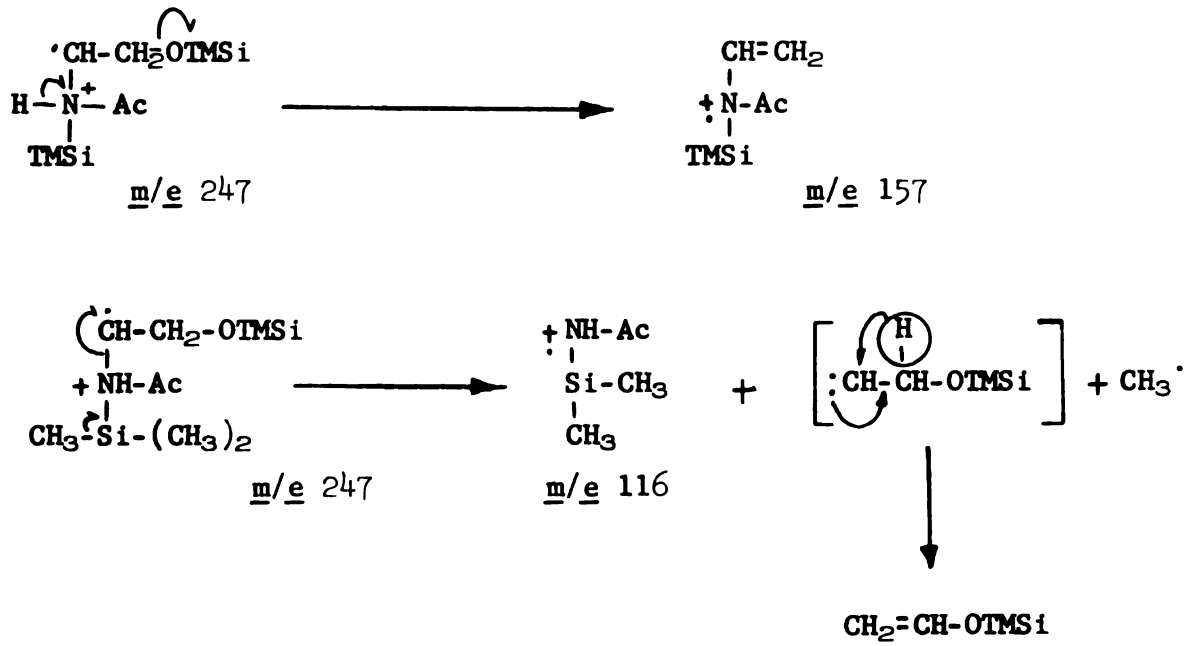


Fig. 15 (above). Mass spectrum of bis-O-TMSi-[1,1,3-$^2H_3$]N-acetylsphinganine.

Fig. 16 (below). Mass spectrum of bis-O-TMSi-[1,1,2,3,4,4-$^2H_6$]N-acetylsphinganine.

indicating the presence of an acetyl group and a dimethylsilyl residue. The structure and the fragmentation mechanism for its formation from the ion at m/e 247 are as follows.



I. Incorporation of [2,3,3-$^2H_3$] Serine into Sphingolipid Bases.

1. Characterization of [2,3,3-$^2H_3$] Serine by Mass Spectrometry.

[2,3,3-$^2H_3$] Serine was characterized by GLC-MS as the TMSi-N-benzalidine derivative. This derivative was prepared by adding TMSi-donor agent (Gaver and Sweeley, 1965) to the dry solid serine (1 mg/ml). When all the solid had dissolved, benzaldehyde (0.2 volume) was added. The solution was left at room temperature for 30 min. before analysis by GLC-MS.

The mass spectrum of TMSi derivative of benzaldehyde adduct of

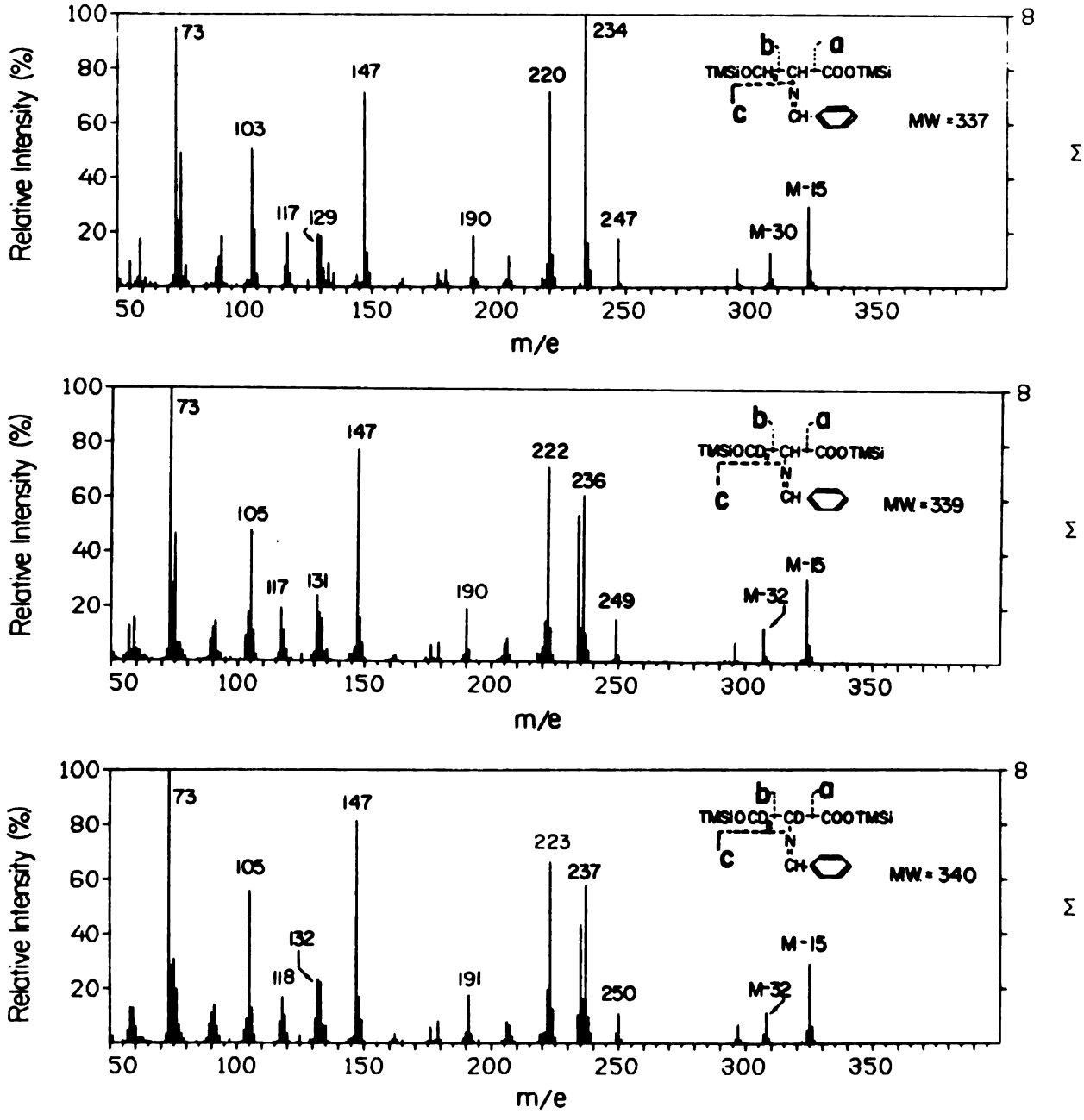
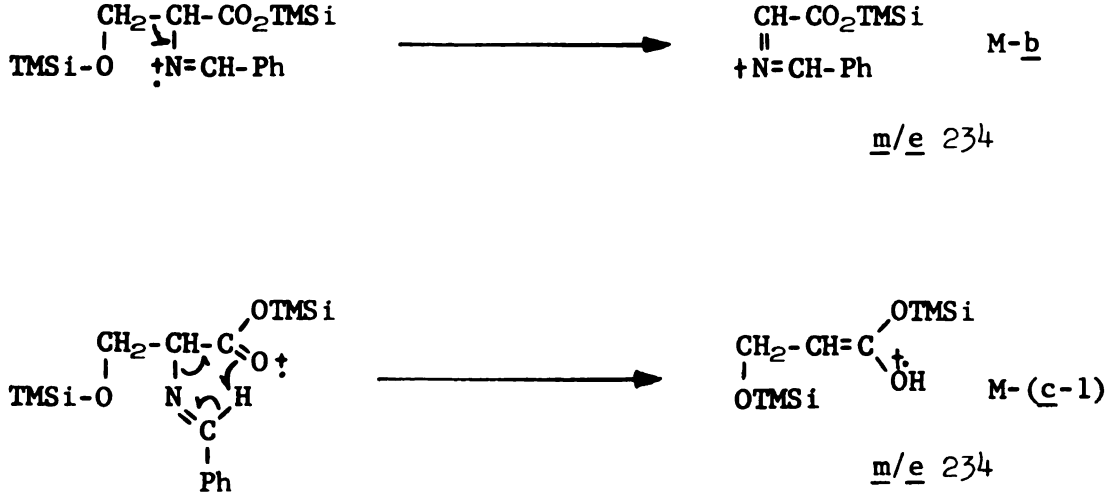


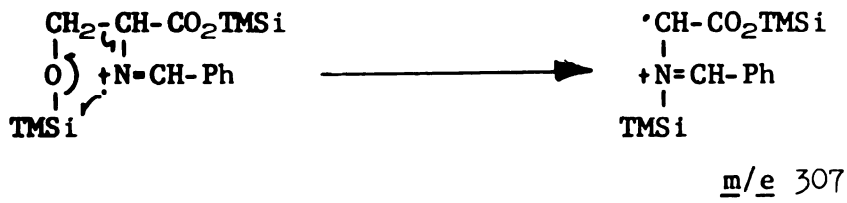
Fig. 17. Mass spectra of bis-O-TMSi-N-benzylidineserine; A) serine B) [3,3-$^{2}H_2$]serine and C) [2,3,3-$^{2}H_3$]serine.

serine is shown in Fig. 17A. The molecular weight could be calculated from ion at M-15 (m/e 322) and M-90 (m/e 247), which resulted from loss of a methyl group and TMSiOH, respectively. These two ions were shifted to m/e 325 and 250 in the spectrum of the [2,3,3-$^2H_3$]-serine derivative (Fig. 17C), proving there are three deuterium atoms in the molecule. The ion at m/e 324 is probably derived from loss of a methyl group from one of the TMSi groups of dideuterioserine derivative impurity. The intensity at m/e 324 was about 10% at m/e 325. The base peak at m/e 234 is probably derived from loss of ion b or ion c-1 by the following mechanisms.



These two ions were split into two ions at m/e 235 and 237 in the spectrum of the [2,3,3-$^2H_3$]serine derivative (Fig. 17C), indicating that two deuterium atoms are on C-3 and one on C-2. The ion at M-a-b (m/e 190 in Fig. 17B; 191 in Fig. 17C) provides unequivocal evidence that there is one deuterium atom on C-2. The ion at M-30 (m/e 307; Fig. 17A) is probably derived from a cyclic transition by cleavage of C-2 and C-3 bond with a simultaneous transfer of TMSi on C-3 to the

functional group C-2, accompanied by expulsion of formaldehyde:



m/e 307

The loss of 32 amu from [2,3,3-$^2H_3$] serine (Fig. 17C) and [3,3-$^2H_3$]serine derivatives (Fig. 17B) is consistent with this type of mechanism. A cyclic transition with transfer of TMSi to the vicinal nitrogen atom has been observed in the spectrum of TMSi-N-acylsphinganine (Hammarström et al., 1970) and TMSi-N-acetylsphinganine.

2. Incorporation of [2,3,3-$^2H_3$] Serine into Sphinganine by Rat Liver Microsomes.

The first step of sphingolipid base biosynthesis involves the PLP-dependent condensation of serine and palmitoyl CoA, yielding 3-ketosphinganine. There are at least two broad mechanisms for the formation of 3-ketosphinganine (Braun and Snell, 1968), depending on whether the initially formed serine-PLP Schiff's base complex I undergoes decarboxylation to furnish complex II or loss of the α-hydrogen atom to form complex III (Fig.3). To differentiate between these two mechanisms [2,3,3-$^2H_3$]serine was incubated with a crude rat liver microsomal system, in the presence of the necessary cofactors (Table 12), at 37°C for one hour. Since 3-ketosphinganine has been reported to be very unstable (Mendershausen and Sweeley, 1969), it was reduced to sphinganine with NADPH-dependent 3-ketosphinganine reductase which

Table 12. Composition of Reaction Mixture for the Incorporation of [2,3,3-$^2H_3$]Serine into Sphinganine by Rat Liver Microsomes.

Palmitic acid	20 μmoles
Triton X-100	20 mg
$MgCl_2$	25 μmoles
CoA	7.5 μmoles
ATP	10 μmoles
NADPH	1 μmole
PLP	10 μmoles
DTT	10 μmoles
d_3-serine	40 μmoles
Microsomal enzymes	2 ml
Phosphate buffer (pH 7.5)	10 ml
Final volume	12 ml

was present in the microsomal fraction (Stoffel et al., 1968c). The reaction was terminated by adding 1.0 ml of 1.0N sodium hydroxide followed by ether extraction. Sphinganine was partially purified by TLC on silica gel G as described in Methods (Section 8). The partially purified sphinganine was converted to the TMSi-N-acetyl derivative and characterized by GLC-MS. It eluted from a 3% SE-30 column at the same retention time as authentic TMSi-N-acetyl-DL-erythro-sphinganine, suggesting that the sphinganine formed in the microsomal preparation has the erythro configuration (Gaver and Sweeley, 1966). The mass spectrum of the TMSi-N-acetyl derivative is shown in Fig.18. The locations of the major ions are consistent with the characteristic ions of TMSi-N-acetyl-sphinganine (Fig. 9) except that some of these ions are shifted to higher mass due to the presence of deuterium atoms in these fragments. The ions at m/e 472, 247 and 157 are shifted to m/e 474, 249 and 159, respectively, indicating that there are two deuterium atoms in these ions. The ion at m/e 474 arises from the molecular ion by loss of a methyl group from one of the TMSi groups. The ions at m/e 249 and 159 are derived from cleavage of the bond between C-2 and C-3 with transfer of TMSi on C-3 to the nitrogen atom by a cyclic transition as shown in the Section on Mass Spectra of Bis-O-TMSi-N-Acetylsphinganine, suggesting that both deuterium atoms are on C-1 and/or C-2. When fragmentation involves charge retention on the C-3 fragment, homolytic cleavage of the bond between C-2 and C-3 results in loss of both deuterium atoms and yields an ion at m/e 313, providing additional indirect evidence that both deuterium atoms are on the C-1 and C-2 fragment. Homolytic cleavage of the bond between C-1 and C-2

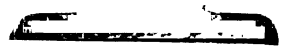
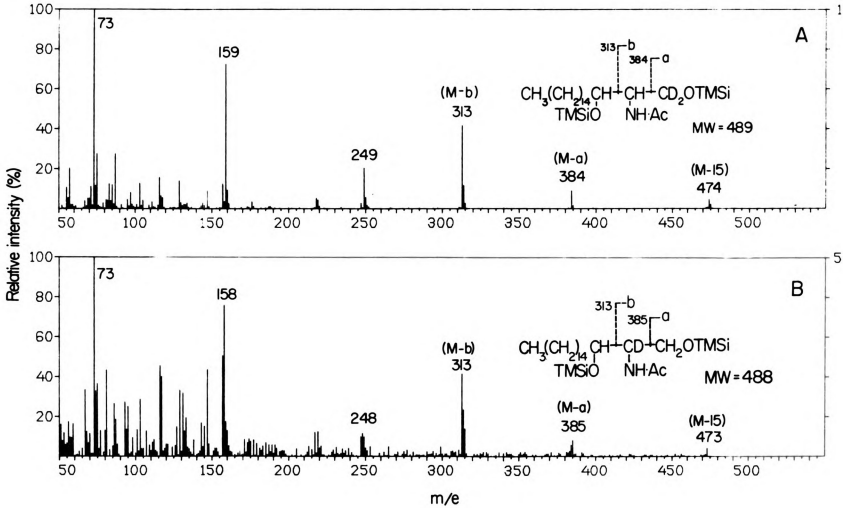


Fig. 18 (Top). Mass spectrum of TMSi-N-acetylsphinganine isolated from microsomal reaction incubated with [2,3,3-$^{2}H_3$]serine.

Fig. 19 (bottom). Mass spectrum of TMSi-N-acetylsphinganine isolated from microsomal reaction carried out in $^{2}H_2O$.


A
73
159
249
313
384
474
(M-b)
313
(M-a)
384
(M-15)
474
MW = 489
B
73
158
248
313
385
473
(M-b)
313
(M-a)
385
(M-15)
473
MW = 488
Relative intensity (%)
m/e

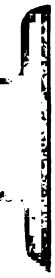
with charge retention on the C-2 fragment and liberation of the C-1 fragment as a free radical yields an ion at m/e 384, indicating both deuterium atoms are on C-1. Thus it was concluded that the microsomal preparation from rat liver converted 2,3,3-trideuteroserine to sphinganine with loss of the deuterium on C-2.

J. Incorporation of Deuterium from ^2H_2O into Sphinganine.

To determine whether the hydrogen atom lost from serine during sphinganine synthesis can be replaced be a proton from the medium, rat liver microsomes were incubated with serine in ^2H_2O. The composition of the reaction mixture is given in Table 13. The reaction mixture was shaken in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicaco, Ill) for one hour at 37°C. Sphinganine formed in the reaction carried out in ^2H_2O was about half of that formed in H_2O. The mass spectrum of the TMSi-N-acetyl derivative is shown in Fig. 19. The ion at M-15 was located at m/e 473, suggesting the presence of deuterium atom in the molecule. The characteristic ions of TMSi-N-acetylsphinganine, m/e 247 and 157, are shifted to m/e 248 and m/e 158, but the ion at m/e 313 is unaffected, thus this deuterium should be on either C-1 or C-2. Homolytic cleavage of the bond between C-1 and C-2 with charge retention on the C-2 fragment yielded an ion at m/e 385 which was shifted from the ion at m/e 384, in the protium form (Fig.9), by one mass unit, suggesting that deuterium is on either C-2 or on the nitrogen atom. Hydrogen on the N-atom is labile, however, since sphinganine and N-acetylsphinganine obtained after exchange equilibration with deuterium in ^2H_2O was completely lost during GLC analysis. Thus, it was concluded that the deuterium atom was on C-2.

Table 13. Composition of Reaction Mixture for the Incorporation of Deuterium from ^2H_2O into Sphinganine by Rat Liver Microsomes.

Palmitic acid	20 μmoles
Triton X-100	20 mg
$MgCl_2$	25 μmoles
CoA	7.5 μmoles
ATP	10 μmoles
NADPH	1 μmole
PLP	10 μmoles
DTT	10 μmoles
Serine	40 μmloes
Microsomal enzymes	2 ml
Phosphate buffer in ^2H_2O (pD 7.5)	10 ml
Final volume	12 ml



Although isotopic purity of $^{2}H_2O$ used in this study was over 99%, it was diluted by microsomal solution to about 80%. Incorporation of deuterium on C-2 of sphinganine was about 55% (judged from comparisons of the ions at m/e 158, 248 and 385 with those at m/e 157, 247 and 384, respectively). The decrease in incorporation of deuterium into C-2 of sphinganine might be due to a slow rate of exchange between hydrogen and deuterium around the active site of the enzyme, 3-ketosphinganine synthetase. Thus, a relatively high initial rate of incorporation of hydrogen would be expected. The slow rate of exchange between hydrogen and deuterium may involve the exchange of hydrogen attached to a nitrogen atom (such as an amino or amide nitrogen) in a hindered site or in a relatively hydrophobic region of the enzyme active site.

K. Incorporation of Serine/2,3,3-Trideuteroserine into Sphinganine.

Substitution of deuterium (^{2}H) or tritium (^{3}H) for an ordinary hydrogen often causes appreciable variation in the rate of an enzyme-catalysed reaction, particularly when the reaction involves breaking the bond to the labeled atom (Gould, 1959). This isotope effect is usually expected if 1), the bond to the labeled atom is broken at the rate-limiting step or 2), a rapid and reversible reaction preceeding the rate-limiting step may involve cleavage of this bond. If the bond to the labeled atom is broken after the rate-limiting step, little or not isotope effect will be observed. To determine whether there is any isotope effect involved in the breaking of the ^{2}H-C bond of serine during the conversion to sphinganine, 2,3,3-trideuterioserine and serine were allowed to compete for the microsomal enzymes for one

Table 14. Composition of Reaction Mixture for the Incorporation of [2,3,3-$^2H_3$]Serine/Serine into Sphinganine by Rat Liver Microsomes.

Palmitic acid	20 μmoles
Triton X-100	20 mg
$MgCl_2$	25 μmoles
CoA	25 μmoles
CoA	7.5 μmoles
ATP	10 μmoles
NADPH	1 μmole
PLP	10 μmoles
DTT	10 μmoles
[2,3,3-$^2H_3$]Serine/Serine (1.17)	0.3 mg*
Microsomal enzymes	2 ml
Phosphate buffer (pH 7.5)	10 ml
Final volume	12 ml

*second experiment employed 3 mg.

hour at 37°C. The composition of the reaction mixture is given in Table 14. The exact ratio of d_3-serine/serine was determined by GLC-AVA of the TMSi-N-acetyl derivative, using ions at m/e 174 and 177 for the determination: The ion at m/e 174 is derived from simple cleavage between C-1 and C-2 with charge retention on the nitrogen-containing fragment. Substitution of three deuterium atoms on C-2 and C-3 shifted the mass of this ion to m/e 177. Results are shown in Table 15.

The deuterium content in the sphinganine product was analysed by GLC-AVA as the TMSi-N-acetyl derivative. The ions at m/e 157 and 159 were used for the analysis. The ion at m/e 157 was shown in the previous section (Mass spectra of bis-O-TMSi-N-acetylsphinganine) to be derived from the first two carbons. Substitution of two deuterium atoms on C-1 shifts the mass of this ion to m/e 159 (Fig. 18). If there was no isotope effect, the same proportion of 2,3,3-trideuterio-serine and serine would be expected to be converted to [1,1-$^2H_2$]sphinganine and sphinganine and the ratio of d_2-sphinganine/sphinganine should be the same as that of the d_3-serine/serine substrate. On the other hand, if there was an isotope effect in breaking the ^2H-C bond, incorporation of d_3-serine into d_2-sphinganine should be less than that of serine into sphinganine. Results in Table 16 show that the ratio of d_2-sphinganine/sphinganine is 0.2767 when the concentration of d_3-serine and serine mixture is 0.3 mg/12 ml. The ratio of d_2-sphinganine/sphinganine is raised to 0.6735 when the concentration of d_3-serine and serine mixture is 3.0 mg/12 ml. These two ratios of d_2-sphinganine/sphinganine are still lower than that of d_3-serine/serine substrate (1.17). The change in d_2-sphinganine/sphinganine ratio is indicative

Table 15. Determination of the Amount of [2,3,3-$^2H_3$]-Serine/Serine in the Mixture by AVA.

Determinations	m/e 177/174	
	Reference	Sample
1	.0105	1.1764
2	0.0104	1.1823
3	0.0090	1.1887
4	0.0090	1.1613
Average	0.0093	1.1772
Δ*	-	1.168

Δ*is the difference between sample and reference values.

m/e 174, $\underset{+\,NH\text{-}Ac}{\overset{\|}{CH}}\text{-}CH_2\text{-}OTMSi$; m/e 177, $\underset{+\,NH\text{-}Ac}{\overset{\|}{CD}}\text{-}CD_2\text{-}OTMSi$.

of the presence of endogenous serine. The amount of endogenous serine is then calculated as follows.

Assuming that there is no isotope effect and the amount of endogenous serine is X μg,

$$\frac{d_3\text{-serine}}{X - \text{serine}} = 0.2767$$

$$\frac{300\ (1.17/2.17}{X - 300/2.17} = 0.2767$$

$$X = 446\ \mu g.$$

Substitution of this X value into the 3 mg mixture give a value of d_3-serine/serine of 0.88 as shown below.

$$\frac{d_3\text{-serine}}{X - \text{serine}} = \frac{3000(1.17/2.17)}{446 - 3000/2.17}$$

$$= 0.88$$

The calculated value of d_3-serine/serine (0.88) is still higher than the observed value of d_2-sphinganine/sphinganine (0.6735). This difference is ascribed to an isotope effect. The calculated value for endogenous serine (446 μg) might not represent the exact amount of endogenous serine because the calculation was based on the assumption that there was no isotope effect, which is not true. Correction for the isotope effect should give a lower value for endogenous serine than 446 μg. Accordingly, the ratio of d_2-sphinganine/sphinganine should be higher than 0.88. It was therefore concluded that serine was incorporated into sphinganine faster than d_3-serine into d_2-sphinganine.

Table 16. Determination of the Amount of Sphinganine and [1,1-$^2H_2$]-Sphinganine Formed in the Microsomal Reaction Incubated with [2,3,3-$^2H_3$]Serine/Serine Mixture.

Determination	m/e 159/157		
	Reference	0.3 mg Mixture	3.0 mg Mixture
1	0.0608	0.3225	0.7330
2	0.0594	0.3339	0.7385
3	0.0601	0.3283	0.7357
4	0.0586	0.3610	0.7147
Average	0.0597	0.3364	0.7330
$\triangle$*	-	0.2767	0.6733

* is the difference between sample and reference values.

m/e 157, $^{\bullet}CH(+NH\text{-}Ac\text{-}TMSi)\text{-}CH_2\text{-}OTMSi$; m/e 159, $^{\bullet}CH(+NH\text{-}Ac\text{-}TMSi)\text{-}CD_2\text{-}OTMSi$.

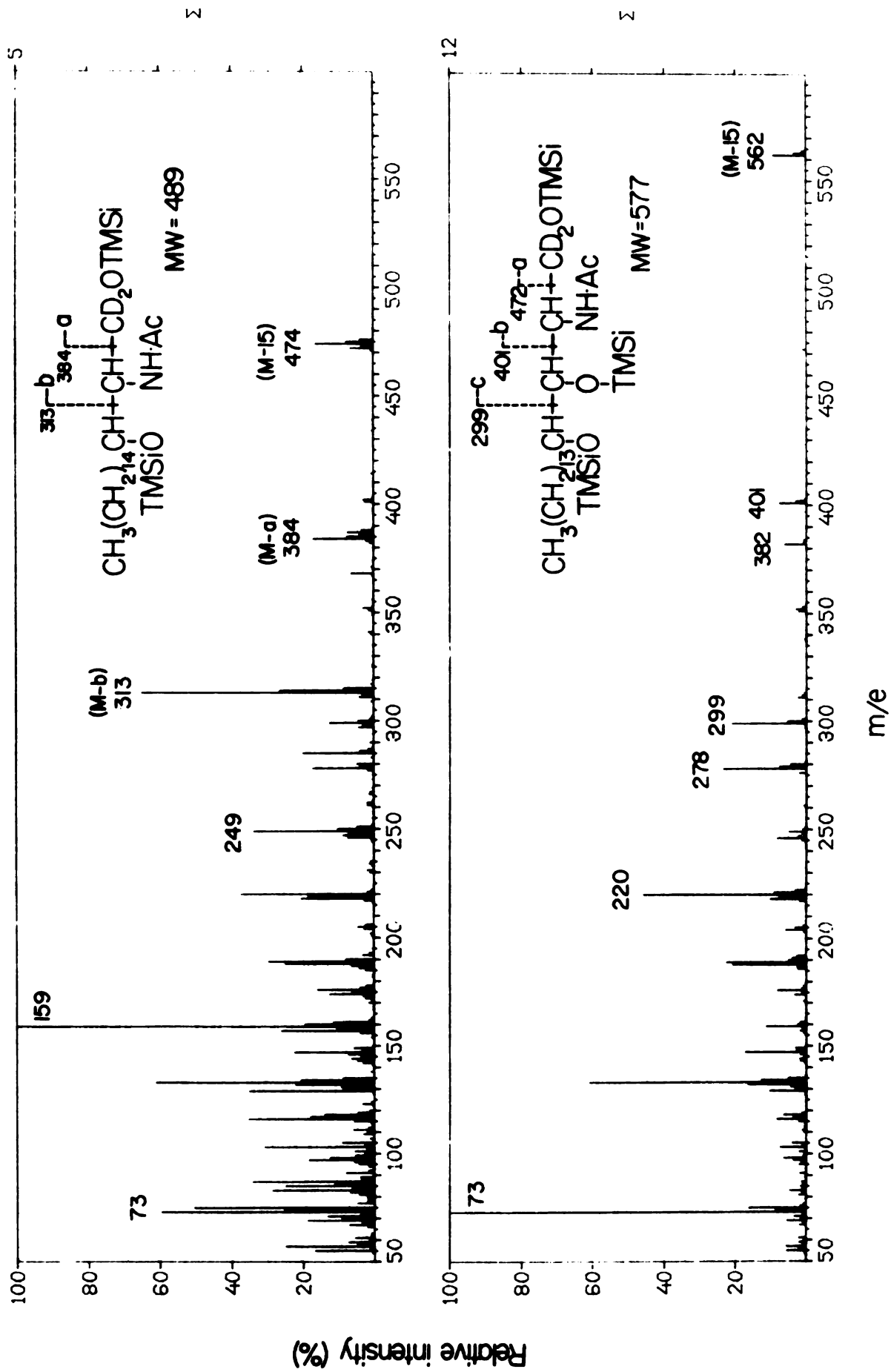
L. Incorporation of [2,3,3-$^2H_3$]Serine into Sphingolipid Bases by Yeast.

It was demonstrated in the previous section that serine was transformed into sphinganine with the complete loss of the α-hydrogen atom by rat liver microsomes. The yeast, Hansenula ciferri, was shown by Stodola and Wickerham (1960) to produce a relatively large amount of acetylated 4-hydroxysphinganine and a minor amount of acetylated sphinganine (Stodola et al., 1962). To study whether this strain of yeast synthesizes sphingolipid bases by the same mechanism as that demonstrated in the mammalial system, [2,3,3-$^2H_3$]serine (0.5% weight/-volume) was incubate with viable yeast in LM-3 in the presence of 1% glucose at 26-28°C for 48 hours. Sphingolipid bases from the medium and cell paste were combined, partially deacetylated and trimethylsilylated as described in Methods. The mass spectra of TMSi-N-acetylsphinganine and TMSi-N-acetyl-4-hydroxysphinganine are shown in Figs. 20A and 20B, respectively. The spectrum of TMSi-N-acetylsphinganine from yeast grown in the presence of [2,3,3-$^2H_3$]serine was identical to that of TMSi-N-acetylsphinganine from rat liver microsomes incubated with [2,3,3-$^2H_3$]serine (Fig. 18), suggesting that the yeast, H.ciferri, svnthesizes sphinganine by the same mechanism as the rat microsomal system.

The spectrum of TMSi-N-acetyl-4-hydroxysphinganine (Fig. 20B) is consistent with [1,1-$^2H_2$]-4-hydroxysphinganine derivative. The ion at M-15 was shifted from m/e 560, in the protium form (Fig. 4), to m/e 562, indicating the presence of two deuterium atoms in the molecule. Loss of fragments c, b and a are accompanied by loss of both

deuterium atoms, thus both deuterium atoms must be on C-1. This is consistent with the finding that sphinganine isolated from the same source also contained two deuteriums on C-1.

Fig. 20. Mass spectra of TMSi-N-acetylsphinganine (Top) and TMSi-N-acetyl-4-hydroxysphinganine (Bottom) isolated from yeast grown in the presence of [2,3,3-$^{2}H_{3}$]serine.


Relative intensity (%)
m/e
MW = 489
MW=577
CH3(CH2)14CH-CH-CH-CD2OTMSi
TMSiO NHAc
CH3(CH2)13CH-CH-CH-CD2OTMSi
TMSiO O NHAc
TMSi
(M-b) 313
(M-a) 384
(M-15) 474
(M-15) 562
73
159
220
249
278
299
382
401
333-b
384-a
299-c
401-b
472-a

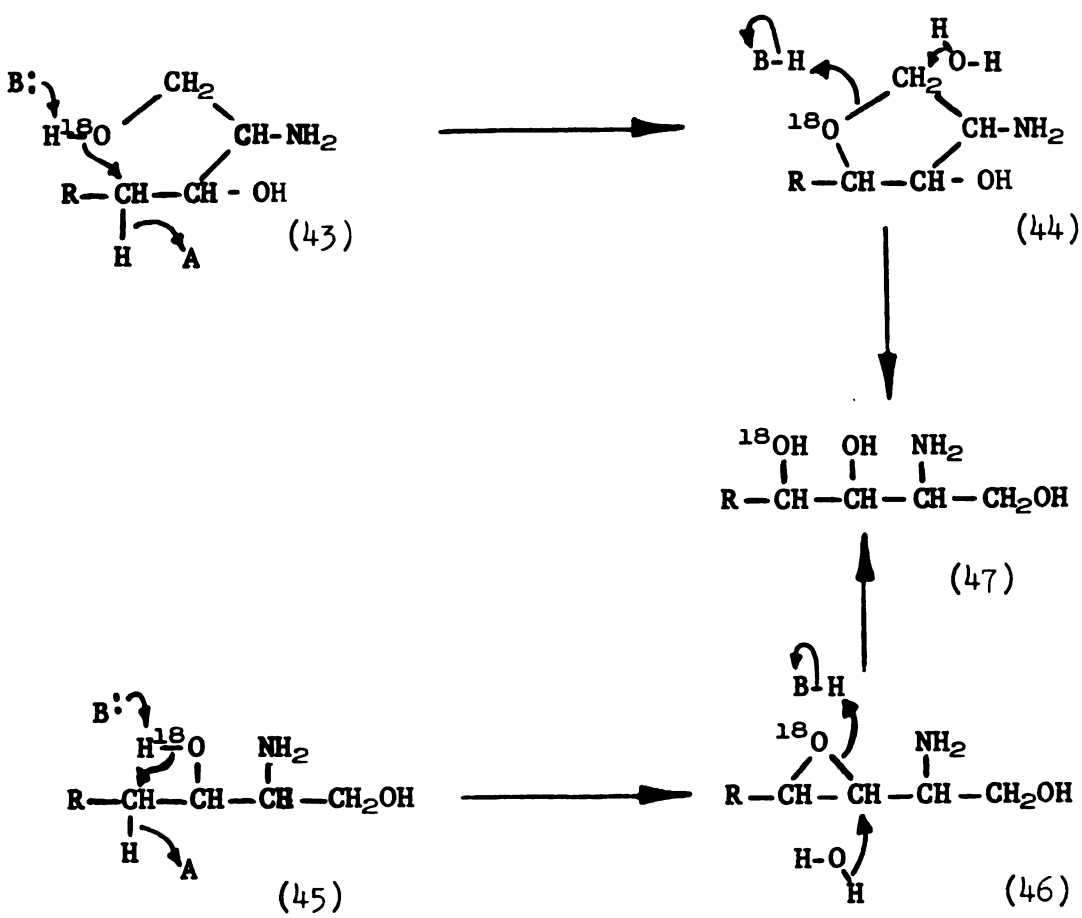
DISCUSSION

The yeast, Hansenula ciferri, was found by Wickerham and Stodola (1960) to accumulate a relatively large amount of acetylated base in the medium. The acetylated base can be extracted without prior breaking of the cell and need not be subjected to extensive purification. There were important reasons for the choice of this strain of yeast for extensive studies of the metabolism of 4-hydroxysphinganine (Green et al., 1965; Braun and Snell, 1967; Thorpe and Sweeley, 1967; Stoffel et al., 1968b). Experiments on yeast grown in the presence of [1,1,3-$^{2}H_{3}$]sphinganine indicated that 4-hydroxysphinganine, from the medium and cell paste, consisted of 11.77% trideuterated species, 6.7% dideuterated species and the remainder was non-deuterated species. The presence of trideuterated species suggested that sphinganine can serve as a direct precursor of 4-hydroxysphinganine. However, the presence of dideuterated 4-hydroxysphinganine is of interest since this species probably results from loss of a deuterium atom during the conversion of trideuterosphinganine to 4-hydroxysphinganine. Loss of one of the two deuteriums on C-1 is unlikely because the experiment on yeast grown on trideuteroserine did not indicate any loss of deuterium from that position (Fig. 20). Loss of deuterium on C-3 by degradation and reutilization of the degradation products is also unlikely because this process would result in the loss

of all three deuterium atoms instead of only that on C-3. One of the degradation product would be ethanolamine containing two deuterium atoms; it is not a precursor in the synthesis of sphingolipid bases (Sprinson and Coulon, 1954). It is, therefore, concluded that dideutero-4-hydroxysphinganine must have been synthesized by oxidation of sphinganine to 3-ketosphinganine followed by hydroxylation and reduction to dideutero-4-hydroxysphinganine. It therefore appears that 4-hydroxysphinganine can be derived by hydroxylation of both sphinganine and 3-ketosphinganine. The data presented in Table 2 indicate that sphinganine is converted to 4-hydroxysphinganine much faster than 3-ketosphinganine; however, these do not represent physiological conditions. Addition of trideuterosphinganine into the growth medium made it much more readily available to the enzyme than 3-ketosphinganine, which must have been present in negligible concentration at the time of addition. Under physiological conditions, on the other hand, 3-ketosphinganine is the first biosynthetic product and its rate of conversion to 4-hydroxysphinganine might be significantly greater than that of sphinganine.

The nature of the hydroxylase is still obscure. The two most likely oxygen donors, water and molecular oxygen, have been ruled out as the primary source of the hydroxyl group on C-4 of the 4-hydroxysphinganine (Thorpe and Sweeley, 1967). Condensation of 2-hydroxypalmitoyl CoA and serine seems also to be unlikely (Green et al., 1965; Braun and Snell, 1968). Another possible oxygen donor is one of a

variety of oxygen-containing compounds (Hayaishi, 1969). Some preliminary experiments were carried out to test whether oxygen was transferred intramolecularly, from either C-1 or C-3 to C-4, as shown below. 3,4-Epoxysphinganine (46) was listed as one of the possible intermediates of 4-hydroxysphinganine by Thorpe (1968). Attack of water on C-3 would result in transfer of oxygen from C-3 to C-4. Another cyclic ether is anhydro-4-hydroxysphinganine (44). This cyclic ether has been detected in acid hydrolysates during isolation of 4-hydroxysphinganine (Carter et al., 1954; O'Connel and Tsien, 1959). Attack of water on C-1 of this ether (44) would result in transfer of oxygen to C-4. Using sphinganine labeled with ^{18}O on



C-1 or C-3) to evaluate these possibilities has certain disadvantages. The compounds are difficult to synthesize and incorporation of sphinganine into 4-hydroxysphinganine is low. [1-^{18}O]Palmitate and [3-^{18}O]-serine were therefore used for the studies. These two compounds are converted to sphinganine with ^{18}O on C-1 and C-3, respectively. 4-Hydroxysphinganine isolated from yeast grown in the presence of [1-^{18}O]-palmitate and [3-^{18}O]serine was found to contain ^{18}O on the expected positions but negligible amounts of the isotope were found on C-4. Thus it was concluded that [1-^{18}O]palmitate, [3-^{18}O]serine, [3-^{13}O]-sphinganine and [1-^{18}O]sphinganine were not oxygen donors to the hydroxyl group on C-4 of 4-hydroxysphinganine.

Experiments carried out in LM-1 (containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1.0% glucose) in $H_2^{18}O$ indicated that incorporation of ^{18}O from water into C-4 of 4-hydroxysphinganine was about 16%, confirming the experiment of Thorpe and Sweeley (1967) and indicating that water is not the primary source of the oxygen donor. The donor must be present in the medium and its oxygen must not be easily exchanged with that of water in the medium. Carbon dioxide, which can easily exchange its oxygen with water, was ruled out as the possible donor (Thorpe, 1968). Yeast which was grown on [2-^{18}O]-glucose as the principal carbon source failed to incorporate ^{18}O into C-4 of 4-hydroxysphinganine.

Glycolysis of [2-^{18}O]glucose would yield several products which bear ^{18}O in the molecule, as outlined in Fig. 21. Weaknesses in this experiment were that exchange of ^{18}O with water in the medium might have caused a partial loss of isotope on C-2 of fructose-1-diphosphate,

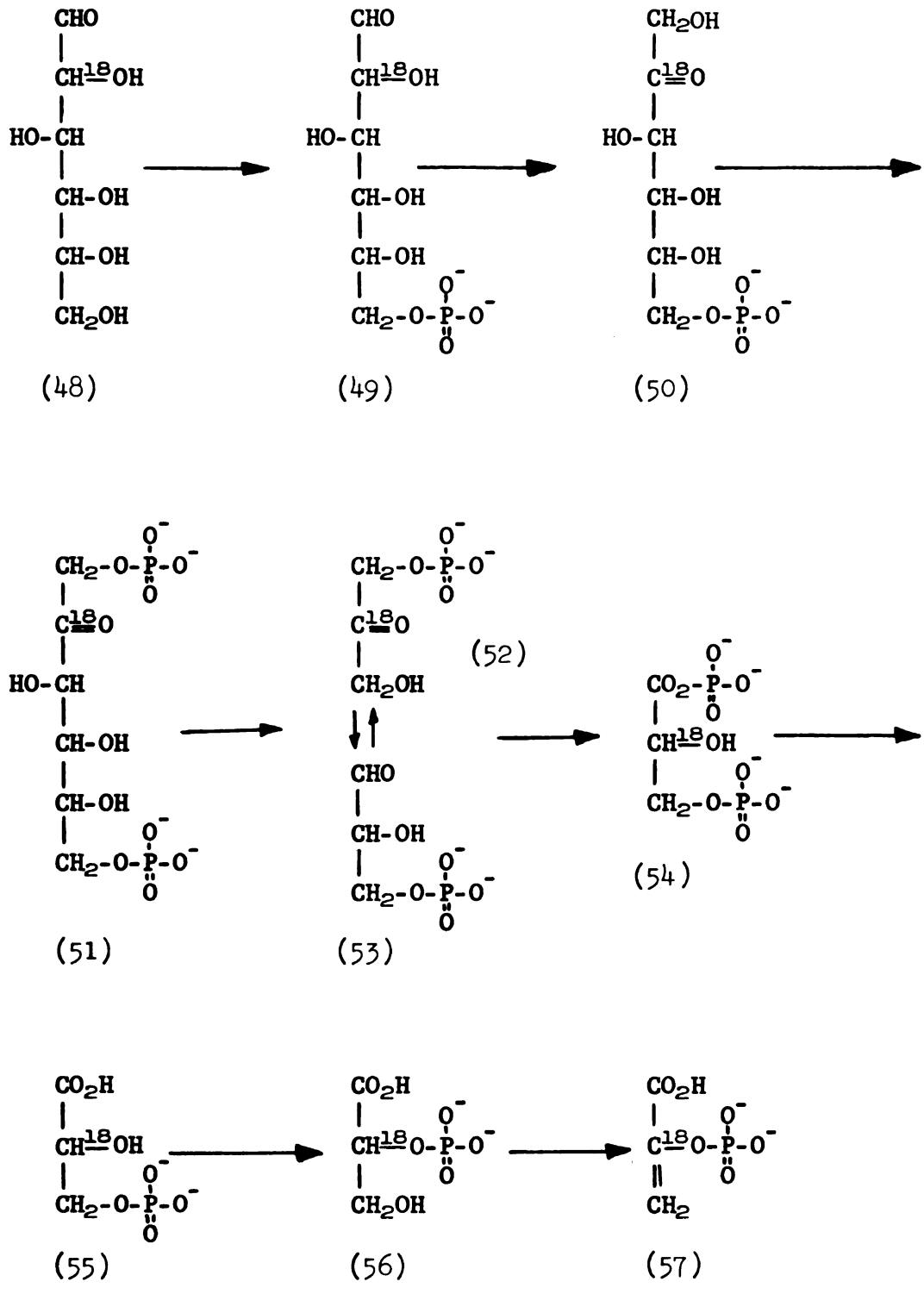


Fig. 21. Glycolysis of [2-^{18}O]glucose (enzymes and cofactors were omitted).

fructose-6-phosphate and dihydroxyacetone phosphate, and complete loss of ^{18}O of fructose-1,6-diphosphate to the medium might have happened during transformation to two moles of triose phosphate (Heron and Caprioli, 1973). If one of the [2-^{18}O]glucose metabolites listed in Fig. 21 was the oxygen donor and negative incorporation into C-4 of 4-hydroxysphinganine was due to complete or partial loss of isotope in one of the aforementioned steps, however, it would be expected that incorporation of ^{18}O from $H_2^{18}O$ into this hydroxyl group would be high. Thus it seems to be possible to rule out all of the [2-^{18}O]glucose metabolites outlined in Fig 21 as oxygen donors.

Experiments on yeast grown on ethanol as the principal carbon source indicated no increase in the incorporation of ^{18}O from water into the hydroxyl group on C-4 of 4-hydroxysphinganine. Malt extract and peptone were omitted in this study. The only organic substances in the medium were yeast extract and ethanol. It was tentatively concluded that glucose was not the oxygen donor. In gluconeogenesis from ethanol, yeast must oxidise ethanol to acetate which exchanges its oxygen with water in the medium. Therefore, most of the oxygen in glucose should be labelled. Glucose isolated from the growth medium and cell paste was characterized by GLC-MS as methoxime-TMSi derivative (Fig. 8B) and ^{18}O on C-2 was analysed by GLC-AVA (Table 11). About 68% of the oxygen on C-2 was shown to be derived from water in the medium. The increase in the intensity of various ions, m/e 321, 219 and 207, in the spectrum of glucose methoxime-TMSi (Fig.8B) indicated that assimulation of ^{18}O into these ions occurred to a certain

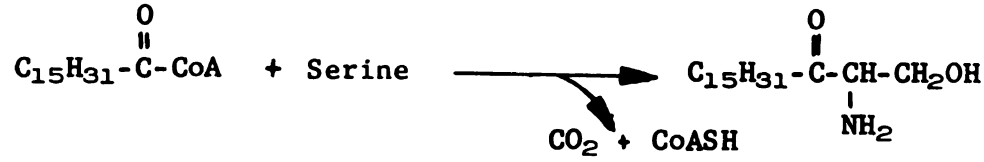
extent. Since peptone and malt extract were omitted in this experiment, it was logical to conclude that the oxygen donor is in the yeast extract. Inorganic phosphate and sulfate oxygen are exchangable with water, particularly at high temperature (Hayaishi, 1969); they were also ruled out, therefore, as the oxygen donor. This was in good agreement with the experiment of Thorpe (1968) that inorganic ^{18}O phosphate was not the oxygen donor of 4-hydroxysphinganine.

Experiments on yeast grown in autoclaved medium (LM-2) did not indicate any increase in the incorporation of ^{18}O from water into the hydroxyl group on C-4 of 4-hydroxysphinganine, suggesting that the donor is heat-stable (not destroyed and no exchange of its oxygen at elevated temperature). Thus, carboxyl oxygen and carbonyl oxygen which can exchange their oxygen with water, especially at elevated temperature, might be ruled out as the possible oxygen donors.

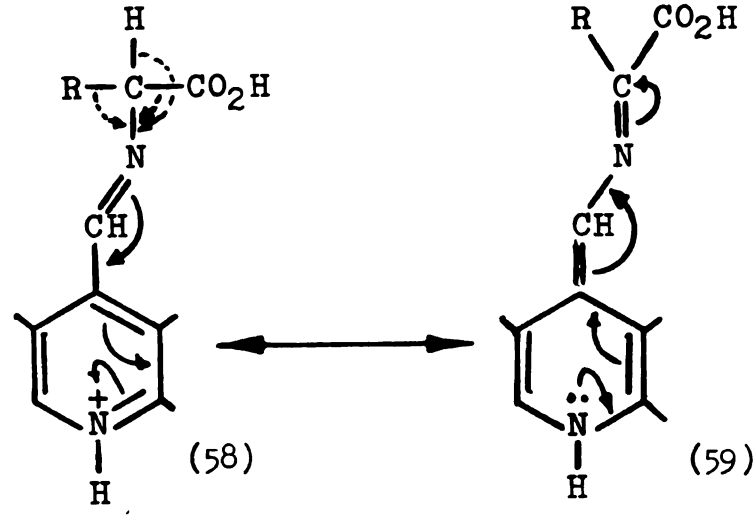
The incorporation of water into the hydroxyl group on C-4 of 4-hydroxysphinganine is not completely negative, however, as found by both Thorpe and Sweeley (1967) and myself. About 11-17% incorporation was observed in most cases, suggesting that the oxygen donor can be synthesized by yeast and that the biosynthetic sequence must proceed through at least one intermediate which can partially exchange its oxygen with that of water or else incorporates water into the molecule. Although this problem was not pursued further, it is important to reemphasize Thorpe's comments (1968) on the failure to obtain an active cell-free system for 4-hydroxysphinganine synthesis, that there might be an unidentified oxygen donor absent in the <u>in vitro</u> system.

If the comments were correct, adding yeast extract should enable one to obtain an active cell-free system for 4-hydroxysphinganine biosynthesis.

It is generally accepted that the initial step in the biosynthesis of sphingolipid bases involves PLP-dependent condensation of serine and palmitoyl CoA. 3-Ketosphinganine is formed with the concomitant release of CO_2 and CoA.



Activation of serine involves the formation of Schiff's base with PLP (Braun and Snell, 1968). Studies in a nonenzymatic system, by Metzler et al. (1954), suggested that the strong electron withdrawal effect of the nitrogen atom in the heterocyclic ring of PLP is responsible for electron displacement from the bond to the α-carbon atom in the amino acid, thus giving a conjugated system of double bonds extending from the electron attraction group to the site of reaction, as shown below.



This general mechanism accounted satisfactorily for all of the known nonenzymatic PLP-catalysed reactions of amino acids and also the corresponding enzymatic reactions that were catalysed by PLP-dependent enzymes (Snell, 1958). Thus PLP-dependent condensation of serine and palmitoyl CoA may be approximately represented in Fig. 22. Loss of the deuterium atom on C-2 of 2,3,3-trideuteroserine was observed both in rat liver microsomes and whole cells of yeast. Nonenzymatic loss of a deuterium atom on the α-position of a carbonyl group at the 3-ketosphinganine or its PLP Schiff's base II (Fig. 3) is possible but it is unlikely that this phenomenon will account for the complete loss of the isotope in 1 hour at 37°C and pH 7.5. Loss of tritium (2S) on the C-2 of glycine due to exchange with protons in the medium during conversion to 5-aminolevulinic acid was reported to be somewhere between 13 and 25% (Akhtar and Jordan, 1968). The biosynthesis of 5-aminolevulinic acid from glycine and succinyl CoA (Fig. 26) is analogous to that of 3-ketosphinganine from serine and palmitoyl CoA, thus loss of isotope on C-2 of serine due to exchange should be comparable to that of [2S-^{3}H]glycine.

If loss of deuterium on C-2 of serine was due to exchange with a proton in the medium at the level of 3-ketosphinganine, both sides of the carbonyl group would be expected to be approximately equally exchanged. Experiments carried out in deuterium oxide indicated that only one deuterium was incorporated, into C-2 of sphinganine. A very small amount of deuterium was detected on C-3 to C-18, but it represented a negligible amount compared to that on C-2 (Fig.19). It is

concluded, therefore, that loss of deuterium on C-2 of serine is an obligatory step in 3-ketosphinganine biosynthesis. This observation is consistent with the report of DiMari *et al.* (1971) that decarboxylation of serine in sphingolipid base biosynthesis is palmitoyl CoA dependent.

Loss of deuterium on C-2 of serine due to rapid equilibrium of complexes I and III (Fig. 3) on the enzyme surface while the actual sequence of 3-ketosphinganine synthesis goes via complexes II and V is unlikely because such a process requires reorientation of groups on the enzyme surface. Reorientation of groups on the enzyme surface is unlikely since the specificity of PLP-catalysed enzymatic reaction is achieved by binding two of the three groups (N-atom is already covalently bonded to PLP) bonded to the α-carbon atom of amino acid (Snell, 1958). Free rotation around the C_α-N bond is then limited to a single orientation in which the bond to be broken lies in a plane perpendicular to that of the conjugated pi system of the heterocyclic ring (Dunathan, 1966).

The rate of utilization of 2,3,3-trideuteroserine is slower than that of serine. This isotope effect suggests that breaking the H-C bond is probably the rate limiting step (Gould, 1959), unless this selective effect was due to secondary isotope effect (Richards, 1970) or to the fact that the two bulky deuterium atoms on C-3 of trideuteroserine cause a steric effect in the enzyme-substrate complex.

Whether the aldimine complex (60 in Fig. 22) loses the hydrogen atom before or simultaneously with condensation with palmitoyl CoA cannot be established from the data presented in this thesis. The

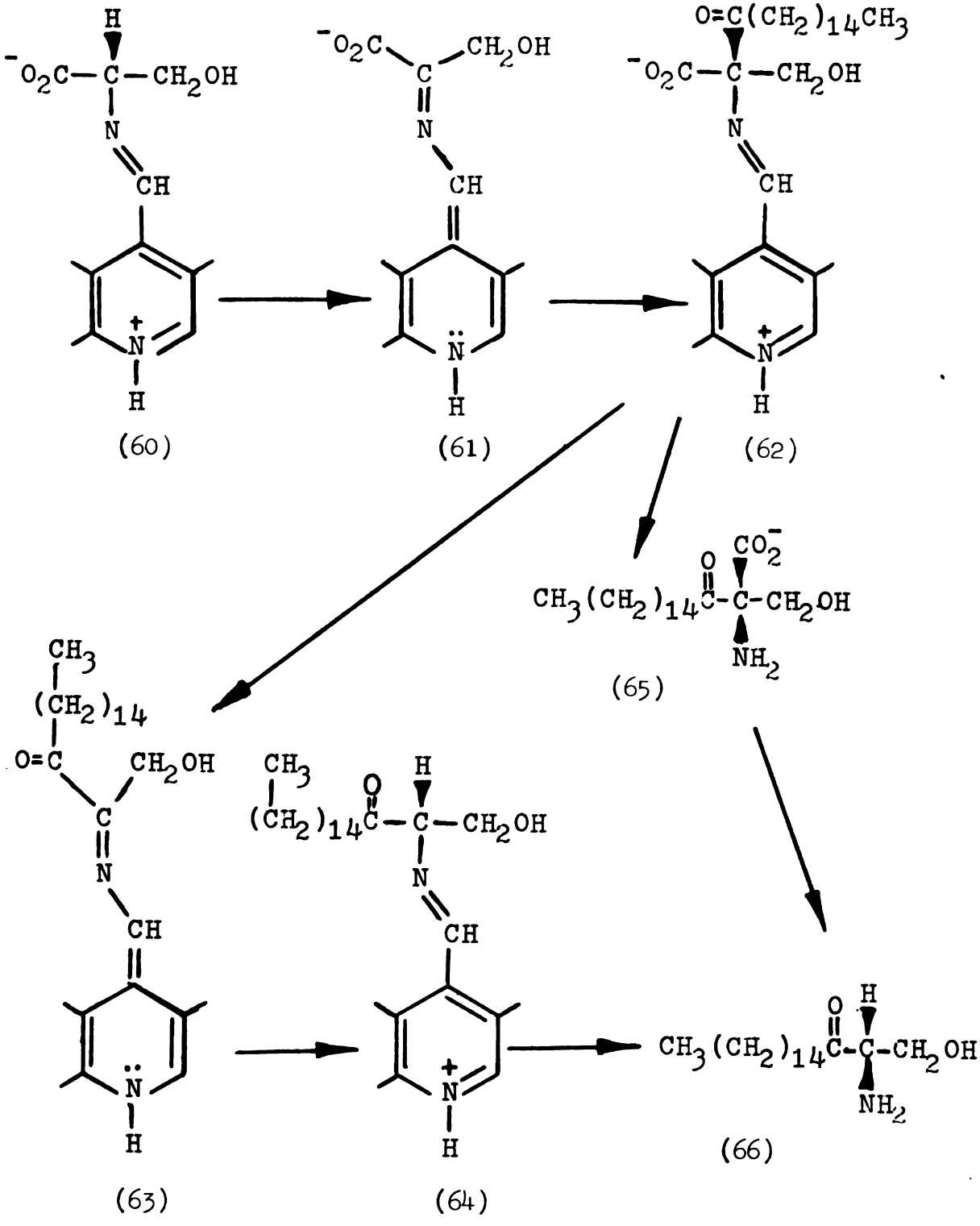


Fig. 22. Probable mechanisms of 3-ketosphinganine biosynthesis.

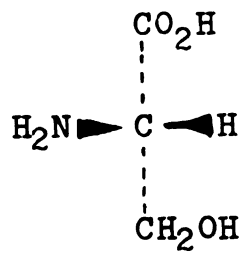
latter possibility requires that the incoming (palmitoyl) group approaches from the opposite side of the carbon bearing the (hydrogen) atom, and the reaction would proceed with inversion of configuration. This type of mechanism is contradictory to most of the known examples of stereospecificity of PLP-dependent enzymatic reactions, shown in Table 17.

The mechanism of 3-ketosphinganine synthesis proposed in Fig. 22 involves two electrophilic substitutions, by the palmitoyl group for hydrogen and by a proton for the carboxyl group. Each of them may proceed either with retention or inversion of configuration. Therefore, there are four possible sequences: 1) both steps proceed with retention of configuration, 2) both steps proceed with inversion of configuration, 3) the first step proceeds with retention of configuration and is followed by inversion of configuration and 4) the first step proceeds with inversion of configuration and is followed by retention of configuration. Since the configuration of the serine substrate and the 3-ketosphinganine product are known (Fig. 23), the consistency of these four reaction sequences can be evaluated. The first two reaction sequences can be ruled out since they lead to the wrong stereoisomer of 3-ketosphinganine. Isolation and characterization of one of the intermediates (62) or its hydrolytic product (65) will give a straight-forward decision about the remaining possibilities. However, in a recent review on the stereochemical aspects of PLP catalysis, Dunathan (1971) noted that "results with a broad spectrum of PLP enzymes reinforce the generalization of 'one-side' chemistry in which all bond making and breaking takes place on one

Table 17. Some Examples of PLP-Dependent Enzymatic Reactions which Are Accompanied by Retention of Configuration.

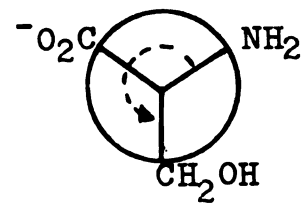
Substrates	Enzymes	Products
L-Serine	Serine hydroxymethylase in ^3H_2O	S[2-^{3}H]Glycine[1]
L-Threonine	Serine hydroxymethylase in ^3H_2O	S[2-^{3}H]Glycine[1]
Aminomalonate	Aspartate-β-decarboxylase in ^3H_2O	S[2-^{3}H]Glycine[2]
L-Tyrosine	Tyrosine decarboxylase in ^2H_2O	R[1-^{2}H]Tyramine[3]
[2-^{2}H]Tyrosine	Tyrosine decarboxylase in H_2O	S[1-^{2}H]Tyramine[3]
Glycine	Serine hydroxymethylase in ^3H_2O	S[2-^{3}H]Glycine[4]
RS[2-^{3}H]Glycine	Serine hydroxymethylase in H_2O	R[2-^{3}H]Glycine[4]
Sphinganine-1-phosphate	Sphinganine-1-phosphate lyase in ^3H_2O	R[2-^{3}H]Ethanolamine-1-phosphate[5]

1, Jordan and Akhtar (1970); 2, Palekar *et al.* (1970 and 1971) 3, Belleau and Burba (1960); 4, Akhtar and Jordan (1969); 5, Akino *et al.* (1974).



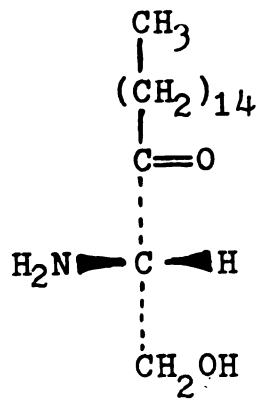
L-Serine

(67)



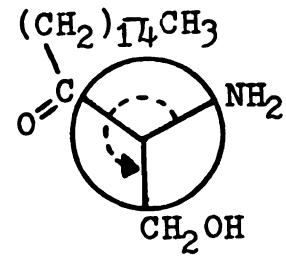
2S-Serine

(68)



L-3-Ketosphinganine

(69)



2S-3-Ketosphinganine

(70)

Fig. 23. Structural relationship of L-serine and L-3-keto-sphinganine.

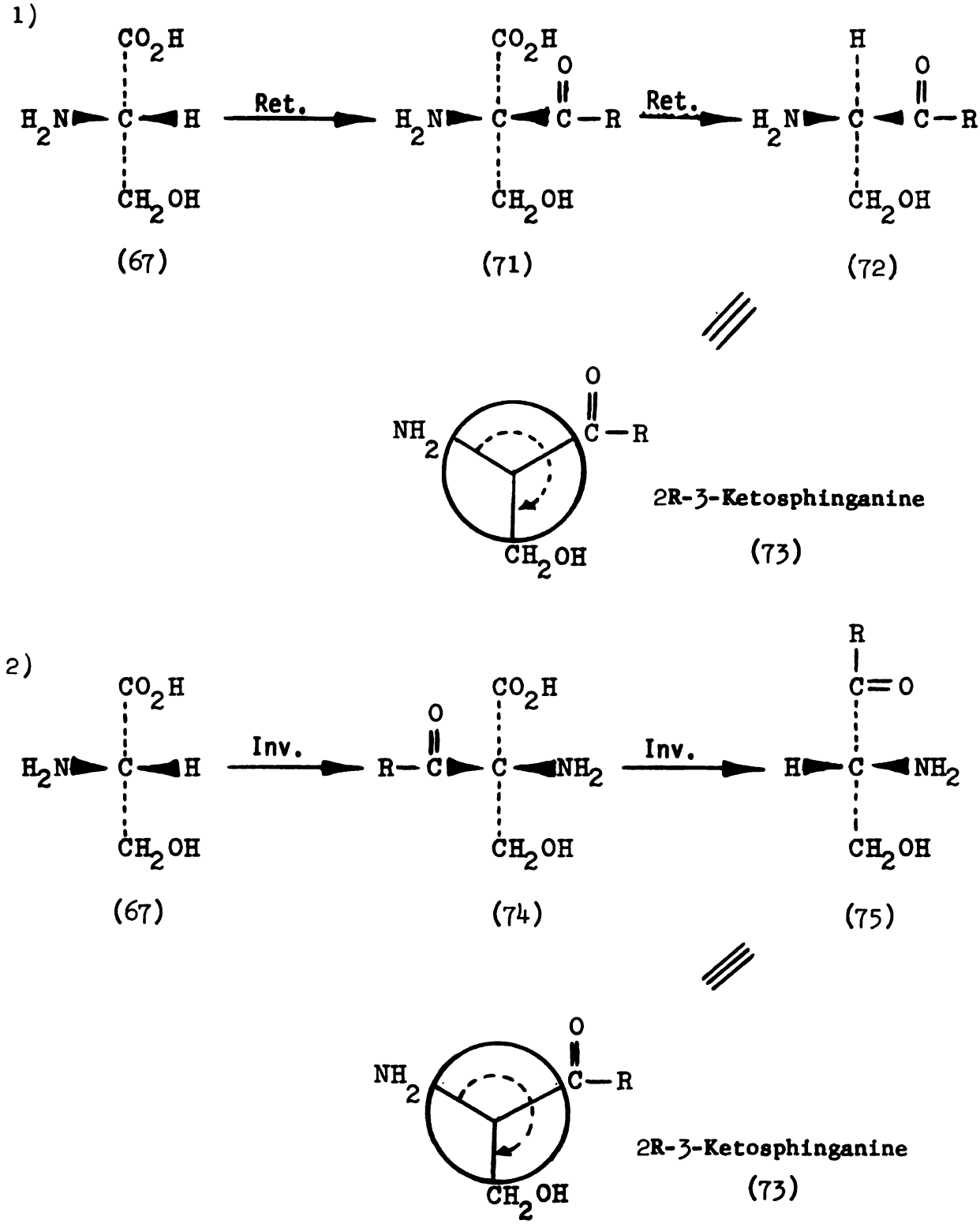

1)
Ret.
Ret.
(67)
(71)
(72)
2R-3-Ketosphinganine
(73)
2)
Inv.
Inv.
(67)
(74)
(75)
2R-3-Ketosphinganine
(73)

Fig. 24a.

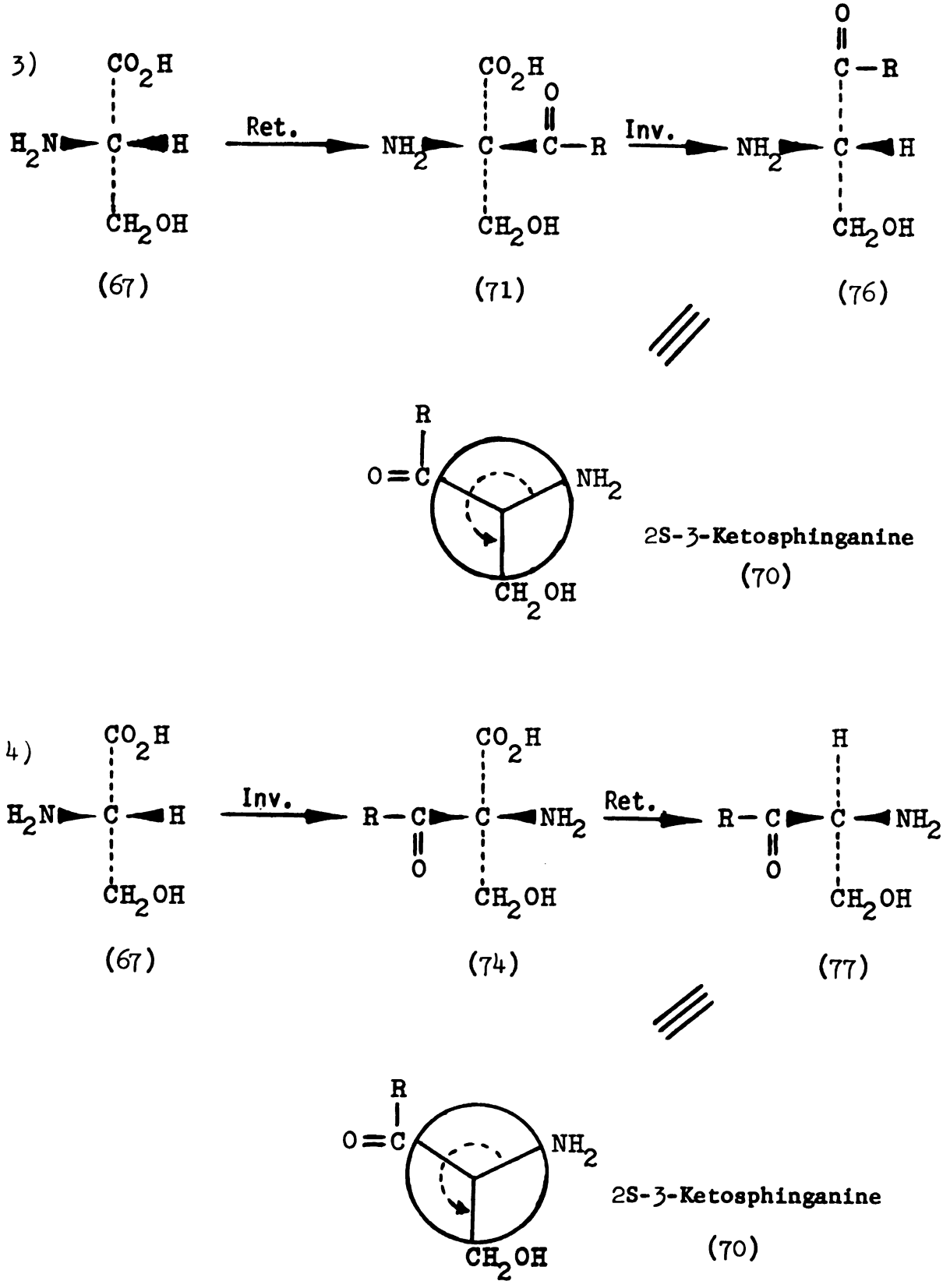


Fig. 24b.

Fig. 24. Possible reaction sequences of 3-ketosphiganine biosynthesis.

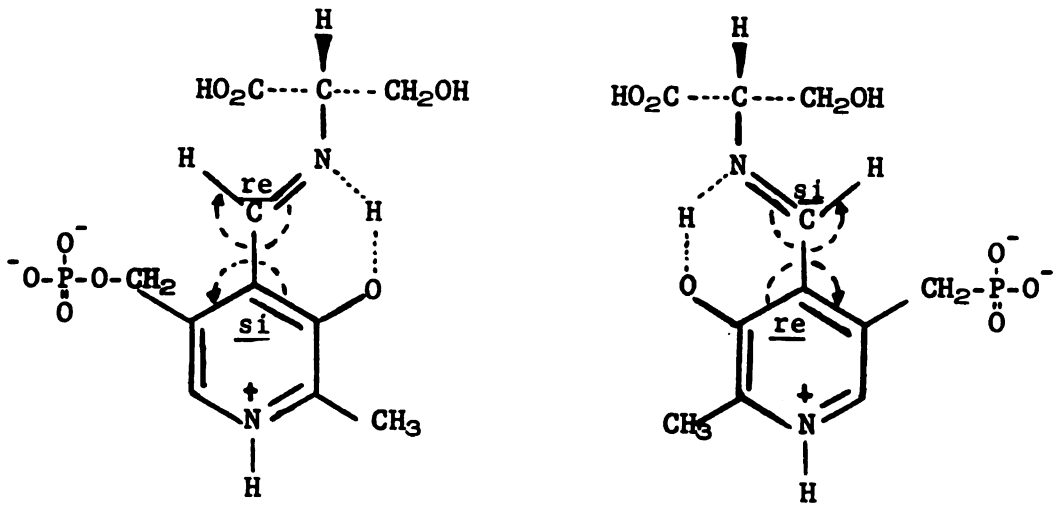
side of the cofactor-substrate imine." Some of the PLP-dependent enzymatic reactions which proceed with retention of configuration were listed in Table 17. If 3-ketosphinganine synthetase falls into one of the above generalizations of one-side chemistry, replacement of the palmitoyl group for a hydrogen atom will proceed with retention of configuration and intermediate (62) or (65) will have the S configuration. Intermediate (62) may be decarboxylated with or without a decarboxylase. In the presence of a decarboxylase the reaction would be expected to proceed with retention of configuration and lead to the improper stereoismer of 3-ketosphinganine. Thus participation of a decarboxylase is not favored by this analysis. The unstable nature of 2-amino-3-ketoacids are, however, well documented. Laver et al. (1959) reported that 2-amino-3-keto-butyric and -adipic acids undergo decarboxylation with a half-life of less than one minute at pH 7.0. PLP is also known to increase the rate of decarboxylation of α-amino acids (Metzler et al., 1954). The reinforcement of the electron withdrawal effect of PLP would, therefore, be expected to accelerate the rate of decarboxylation of intermediate (62) to an extent that a decarboxylase may not be necessary. The reaction would possibly yield a planar intermediate (63) with the palmitoyl group occupying that where the carboxyl group was located. Addition of a proton from the less hindered side would give 3-ketosphinganine after hydrolysis. This type of mechanism would fulfill the stereochemical requirements of the 3-ketosphinganine product as well as the 'one-side' chemistry of the PLP enzyme. However, in the absence of more definitive evidence, the fourth reaction sequence (Fig. 24b) and decarboxylation of

intermediate (65) cannot be ruled out.

The serine-PLP Schiff's base (60) may have several conformations due to 1), free rotation around the C_{α}-N bond; 2), cis and trans configuration of the C_4'=N double bond; and 3), free rotation around the C_4'-C_4 bond of the cofactor. However, some of these factors have been limited to only a single variable. For example, the C_4'=N double bond has been defined as trans since the cis geometry results in serious steric hindrance to planarity (Dunathan, 1971); free rotation around the C_4-C_4' bond of the cofactor has been restricted by metal chelation (Metzler et al., 1954) or hydrogen bonding (Metzler, 1957) between the phenolic oxygen and nitrogen of the substrate. Free rotation around the C_{α}-N bond has been postulated to be limited by binding two of the three groups surrounding the α-carbon atom (Snell, 1958; Dunathan, 1966).

The aforementioned factors limit the several variable conformations of serine-PLP Schiff's base complex to two possible conformations, shown in Fig. 25. Since the hydrogen on C-2 of serine is first labilized by the enzyme, 3-ketosphinganine synthetase, the C_{α}-H bond should lie in a plane perpendicular to the plane of the heterocyclic ring, either on the re-si face (78) (Hanson, 1966) or si-re face (79) of the N=C_4'-C_4 plane.

It is interesting to point out that in all the five cases where absolute stereochemistry is known: glutamic-aspartic transaminase (Besmer and Arigoni, 1969), pyridoxine-pyruvate transaminase (Ayling et al., 1968), dialkyl amino acid transaminase (Bailey et al., 1970) glutamic decarboxylase (Sukhareva et al., 1971; Voet et al., 1973),

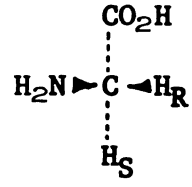


(78) (79)

Fig. 25. Probable conformations of PLP-serine Schiff's base in the transition state.

the conformation is similar to that of (79) with the si-re face of the $N{=}C_4^1\text{-}C_4$ being exposed to the medium.

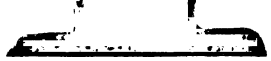
The biosynthesis of 5-aminolevulinic acid from glycine and succinyl CoA (Akhtar and Jordan, 1968 and 1969; Jordan and Shermin, 1972) is analogous to that of 3-ketosphinganine from serine and palmitoyl CoA. Activation of glycine was reported by Akhtar and Jordan (1968) to be accomplished through the loss of hydrogen on C-2 of glycine. Interestingly, the pro-R hydrogen which is removed by 5-aminolevulinic acid synthetase has the same configuration as the H-atom on C-2 of L-serine.



(Glycine)

(80)

It was predicted (Dunathan, 1971) that condensation of glycine-PLP complex (81) and succinyl CoA should proceed with retention of configuration and that the product (83) should have the R configuration. This is analogous to the proposed mechanism of 3-ketosphinganine synthesis. As discussed earlier, the first substitution (palmitoyl group for the hydrogen atom) would lead to a retention of configuration. In addition, the stereochemistry of 5-aminolevulinic acid is expected to be analogous to that of 3-ketosphinganine; that is, the second substitution step (hydrogen atom for the carboxyl group) would proceed with inversion of configuration and a proton from the medium should occupy the pro-R position.



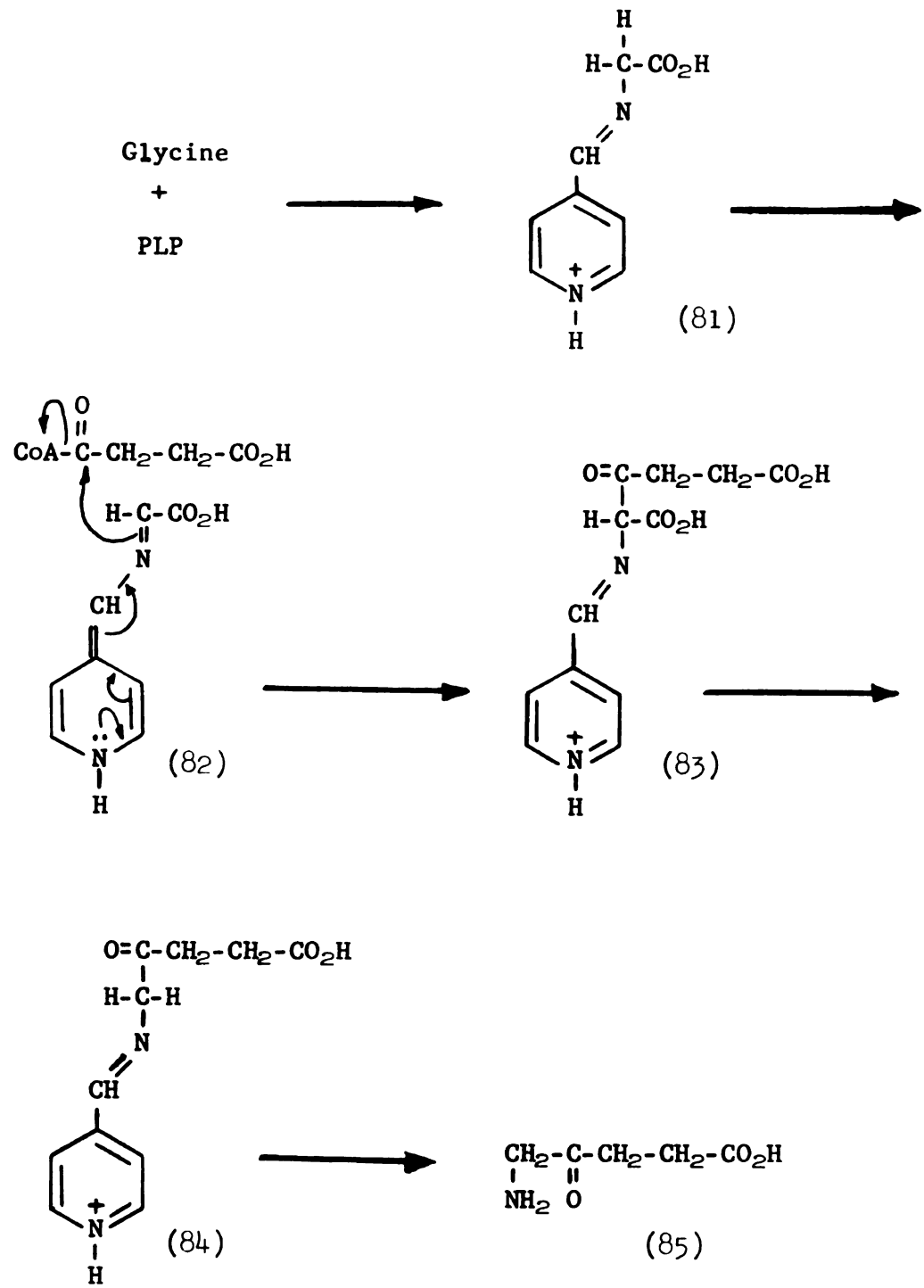


Fig. 26. Biosynthesis of 5-aminolevulinic acid.

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