RICE UNIVERSITY

THE CHEMISTRY AND METABOLISM OF SPHINGOLIPID LONG CHAIN BASES

by

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OBJECTIVES

The purpose of the research described herein was to study some aspects of the chemistry and biochemistry of sphingolipid long chain bases. In order to study in detail the metabolic fate of labeled dihydrosphingosine, a very pure sample of $[4,5\text{-}^{3}H_{2}]$-dihydrosphingosine of high specific activity was needed. The availability of this compound as a substrate permitted detailed studies (utilizing improved chromatographic techniques) of the distribution of radioactivity in rat liver lipids after intraportal injection. Studies of the substrate specificity of dihydrosphingosine kinase were proposed. In order to conduct these studies, it was necessary first to prepare a crude enzyme preparation from rat liver and second, to prepare potential substrates. In addition, it was proposed to chemically synthesize phytosphingosine-1-phosphate. This could be used in subsequent studies to identify labeled phytosphingosine-1-phosphate formed enzymatically from labeled phytosphingosine. The chemical synthesis of phytosphingosine-1-phosphate requires large amounts of pure phytosphingosine which could be prepared from yeast. The chemical synthesis of dihydrosphingosine-1-phosphate by similar procedures would provide an analogous system for comparison.

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ABBREVIATIONS

ATP	Adenosine Triphosphate
bp	Boiling Point
C	Centigrade
cm	Centimeter
cm^{-1}	Reciprocal Centimeter
Ci	Curie
CPM	Counts Per Minute
DPM	Disintigrations Per Minute
uCi	Microcurie
ul	Microliter
mCi	Millicurie
Me	Methyl
mg	Milligram
mm	Millimeter
mmole	Millimole
mp	Melting Point
NAD(P)	Oxidized Nicotinamide Adenine Dinucleotide (Phosphate)
NAD(P)H	Reduced Nicotinamide Adenine Dinucleotide (Phosphate)
NM	Nanometer
nmole	Nanomole
NMR	Nuclear Magnetic Resonance
Ph	Phenyl
PPM	Parts Per Million
TMS	Tetramethylsilane
UL	Uniformly Labeled
v	Volume
w	Weight

INTRODUCTION

Significant advances have been achieved in understanding the metabolism of sphingolipid long chain bases. However, as will become evident from this review, the literature abounds in observations and conclusions that are both confusing and contradictory.

As many as sixty varieties of natural long chain bases are found in nature[53]. The biological synthesis of all of the bases appears to involve the formation of the basic carbon skeleton by a common sequence of reactions. The results of early *in vivo* studies indicated the incorporation of labeled acetate in a head to tail fashion with nearly all of the activity residing in carbons three through eighteen of both sphingosine and dihydrosphingosine[121,122]. Results from *in vivo* rat studies reported that labels from [2-^{14}C][75] or [1,2-^{14}C][122]-ethanolamine and [1-^{14}C]-glycine[75] were not incorporated into either of these bases. Carbon atoms two and three of serine proved to be a source for carbon atoms two and one, respectively, of sphingosine[75]. The label from [2-^{14}C]-glycine was incorporated into both carbons two and one of sphingosine[75]. [^{14}C]-Formate was incorporated into carbon atom one of brain and spinal sphingosine *via* the *de novo* synthesis of [3-^{14}C]-serine.

The results of *in vitro* studies[11] with rat liver confirmed that serine provided base carbons one and two and that [UL-^{14}C]-glucose (*via* its catabolic formation of acetate) provided for base carbons three through eighteen. For maximal incorporation of the label in [UL-^{14}C]-L-serine into sphingosine with minced liver, spleen and brain slices $MnCl_2$, $MgCl_2$, NAD, NADPH, pyridoxal phosphate, ATP and coenzyme

A were needed[11]. The latter two components could be replaced by palmitoyl coenzyme A and a NADPH generating system[11]. Neither palmitaldehyde nor 2t-hexadecenal served as a precursor for the long chain bases in this system[11]. It was noted during these studies that, as a function of time, the amount of dihydrosphingosine formed would first increase and later decrease as the amount of sphingosine rose[11]. It was later found that palmitaldehyde could serve as a precursor in the above system if serum albumin or a detergent such as Tween 20 was used[12]. Rat brain homogenates[36] were also found to catalyze the incorporation of the label of [3-^{14}C]-DL-serine into erythro-sphingosine and erythro-dihydrosphingosine. Weiss[106] injected rats subcutaneously with [2,3-$^{3}H_2$]-DL-serine. He reported that the recovered sphingosine of brain complex sphingolipids had a tritium ratio on carbon two to carbon one identical with that of carbon two to carbon three of serine. Thus, the alpha-hydrogen of serine was retained with retention of configuration[89]. However, Krisnangkura and Sweeley using DL-[2,3,3-$^{2}H_3$]-serine with rat liver microsomes reported a total loss of the alpha-hydrogen in the formation of dihydrosphingosine[59].

The yeast Hansenula ciferrii[76,114] contains long chain bases which are over 90% phytosphingosine, about 6% dihydrosphingosine and a small amount of sphingosine[41]. All are in partially or fully acetylated forms[41]. Whole cells [41] or their microsomal fractions[14] were found to condense serine with sodium palmitate in the presence of ATP and coenzyme A or palmitoyl-coenzyme A forming sphingosine and dihydrosphingosine. Pyridoxal phosphate was found to be absolutely required for this step[14]. Using the yeast microsomal fraction, palmitaldehyde was not converted into dihydrosphingosine when NAD, NADH, NADP or NADPH

were present but did when ATP, coenzyme A and a NADPH generating system were present[14]. This finding implies that oxidation of the palmitaldehyde must first occur and the palmitic acid thus formed then activated with ATP and coenzyme A. Using mouse brain[16], rat liver[89], yeast[13,15,89] or oyster microsomes[45], the absolute need for NADPH was established for the reduction of the 3-keto-bases formed by the condensation of serine and palmitoyl-coenzyme A. With palmitoyl-coenzyme A and serine as substrates in these microsomal systems, a lack of NADPH resulted in the accumulation of 3-keto-dihydrosphingosine[13,15,16,45,89] and 3-keto-sphingosine[13]. When 2t-hexadecenoyl-coenzyme A and serine were used as substrates, yeast microsomes without added NADPH produced only 3-keto-sphingosine[29].

If 3-keto-dihydrosphingosine was incubated with NADPH and microsomes from yeast[13,89], mouse brain[16] or rat liver[89], only dihydrosphingosine was produced. In the mouse brain microsomes[16] some N-acyl-sphingosine and N-acyl-dihydrosphingosine were also formed, but no free sphingosine was observed. Incubation of 3-keto-sphingosine with yeast microsomes produced only sphingosine[13,29]. The results of *in vivo* rat studies[88] showed the incorporation of the label from [3-^{14}C]-3-keto-dihydrosphingosine into both dihydrosphingosine and sphingosine of liver ceramides and sphingomyelins. The NADPH dependent 3-keto-dihydrosphingosine reductase was partially purified from beef liver microsomes. Using this partially purified enzyme, Stoffel, *et. al.*[91], demonstrated that the D-isomer of 3-keto-dihydrosphingosine could be converted to dihydrosphingosine in a reduction involving a transfer of the B-hydrogen of NADPH. Little or no transfer of the A-hydrogen occured[91]. The L-isomer was not a substrate[91]. These authors also

found similar enzymatic activity in the microsomal fractions of rat liver, kidney, heart muscle, skeletal muscle, brain and spleen[91].

Both the microsomal system[15,29] and whole cells[41] of yeast have been used in incubations where the label in 2t-hexadecenoic acid was incorporated into both sphingosine and dihydrosphingosine. In the same microsomal system[29] the label from palmitic acid was also incorporated into both bases. This microsomal yeast fraction was also found to incorporate the label from ammonium palmitate into 2t-hexadecenoic acid[29]. Stearoyl-coenzyme A condensed with serine to form C-20 long chain bases[16] while 2-hydroxy-palmitic acid was not incorporated into any of the bases, particularly phytosphingosine[15,29,41].

As stated above, only yeast could incorporate unsaturated fatty acids directly into unsaturated long chain bases. In other systems the desaturation must occur at some point(s) subsequent to the condensation of palmitoyl-coenzyme A and serine. Mouse brain microsomes were used in incubations to convert 3-keto-dihydrosphingosine to dihydrosphingosine, N-acyl-dihydrosphingosine and N-acyl-sphingosine[16]. However, since no free sphingosine (which was the only product from 3-keto-sphingosine)[13,29] was detected, it was assumed that 3-keto-dihydrosphingosine was not desaturated to 3-keto-sphingosine as the first step of sphingosine synthesis[16]. Further information on this point was reported by Stoffel *et al.*[81,97] When [3-^{3}H], [3-^{14}C]-D-*erythro*-dihydrosphingosine was injected intravenously into rats, the sphingosine recovered from the liver ceramides and sphingomyelins showed no loss of tritium. Loss of the labeled hydrogen at carbon atom 3 of the dihydrosphingosine would be expected to occur if 3-keto-dihydrosphingosine were an intermediate. *In vivo* studies in rat brain

gave identical results[63]. However, Fujino and Nakano[35] reported findings suggesting the direct desaturation of 3-keto-dihydrosphingosine to form 3-keto-sphingosine in rat liver microsomes. This system was fortified with flavin adenine dinucleotide or flavin mononucleotide (but not with NADPH). These authors assumed that there was a negligible amount of endogenous NADPH in the unwashed microsomes. Hammond and Sweeley reported that, upon incubation of labeled 3-keto-dihydrosphingosine in the presence of oyster microsomes fortified with NADPH, the label was recovered in both 3-keto-sphingosine and 3-keto-sphing-4,8-diene[45]. Neither sphingosine nor dihydrosphingosine served as a precursor of the 3-keto-diene in this system; however, 3-keto-sphingosine did. Fujino and Nakano[34,61] suggested an isomerization pathway. If palmitoyl-coenzyme A and serine were incubated with unwashed rat liver microsomes with no added NADPH, both 3-keto-dihydrosphingosine and free sphingosine could be found[34]. If 3-keto-dihydrosphingosine were incubated in the same system, sphingosine was produced in about 6% yield[61]. It should be noted that in other *in vitro*[16,81,97] and *in vivo*[63] mammalian systems no free sphingosine could be detected.

Stoffel *et al.*[97] injected palmitic acids labeled stereospecifically with tritium into the brains of young rats. These workers reported that the 4R and 5S hydrogens of dihydrosphingosine were stereospecifically removed with the removal of the 4R hydrogen as the rate limiting step. These results differ from those of Polito and Sweeley[66]. These workers incubated palmitic acid samples labeled stereospecifically with deuterium with a yeast microsomal system and reported findings compatible with the loss of the 4R and 5R hydrogens

of dihydrosphingosine.

Phytosphingosine occurs widely in nature including soybeans[24], corn[24], protozoa[102], fungi[68], yeast[67], mutant yeast H. ciferrii[76,111,114], rat kidney[19] and bovine kidney[19] among other sources. The precursor of phytosphingosine, the source of the C-4 oxygen and the mechanism of that oxygen's insertion have been active areas of investigation with few concrete results.

As stated earlier, 2-hydroxy-palmitic acid does not condense with serine to produce phytosphingosine[15,29,41]. However, in vivo conversion of labeled palmitate or dihydrosphingosine to form phytosphingosine in the yeast Hansenula ciferrii has been reported[41,66,89,90,110]. Sphingosine was not a precursor in this system[90]. No in vitro system for production of phytosphingosine has yet been reported.

To determine the source of the oxygen at carbon atom four of phytosphingosine, H. ciferrii was grown with either an $^{18}O_2$ atmosphere[80,104] or in $H_2{}^{18}O$ media[104]. The results showed that none of the oxygen at carbon atom 4 was derived from molecular oxygen. Only a small amount of the label from $H_2{}^{18}O$ was observed to be incorporated at carbon atom four. The researchers concluded that the oxygen in question must be derived from an unknown hydroxyl donor which does not exhange readily with water.

To test whether or not 3-keto-dihydrosphingosine might be an intermediate in the enzymatic formation of phytosphingosine, Stoffel et al.[81] incubated [3-^{3}H], [1-^{14}C]-dihydrosphingosine with yeast. The isolated phytosphingosine retained only 1/7 of the tritium activity. They concluded that the label was lost due to the forma-

tion of the 3-keto-dihydrosphingosine which was subsequently hydroxylated and reduced to produce phytosphingosine. However, Weiss and Stiller[110] incubated [4,5-$^{3}H_{2}$]-dihydrosphingosine (with 47% of the activity on carbon atom four and 37% on carbon atom five) with H. ciferrii. The phytosphingosine recovered retained all of the activity at carbon atom five and lost about half of the activity at carbon atom four. Later Stoffel and Binczek[89] repeated their experiment and also reported no loss of tritium at carbon atom three of dihydrosphingosine upon conversion to phytosphingosine. Using perdeutero- and stereospecifically labeled deutero-palmitic acids[66] and stereospecifically labeled tritio-palmitic acids[89,97], the stereospecific loss of the 4R hydrogen was confirmed. This supported a pathway involving direct hydroxylation as opposed to a mechanism involving 3-keto-dihydrosphingosine, 4-keto-dihydrosphingosine or sphingosine as an intermediate.

With one exception, the results of previous investigators have not demonstrated the in vivo formation of phytosphingosine in mammals. Assmann and Stoffel[4] reported no conversion of either sphingosine or dihydrosphingosine to phytosphingosine in in vivo experiments with rats. They reported that either [5,6-$^{3}H_{2}$]- or [UL-^{14}C]-phytosphingosine, when given orally or intravenously, was found apparently unchanged in ceramides, cerebrosides, sphingomyelin and phytosphingosine-1-phosphate[4]. No activity was found in sphingosine or dihydrosphingosine. Only pentadecanoic, heptadecanoic and a variable but small amount of 2-hydroxy-palmitic acids were found labeled. Hirschberg[50] reported incorporation of the label of dihydrosphingosine into phytosphingosine of the ceramides, cerebrosides/free bases (which were not separated by the procedures utilized) and sphingomyelins of the kidney and liver of rats after

intravenous injection into the tail vein. The fatty acids of these lipids contained considerable activity in 2-hydroxy-palmitic acid and its oxidation product, pentadecanoic acid. These acids are known biodegradation products of phytosphingosine[4,5,6,38,54,90]. However, consistent incorporation of the label of [4,5-$^3H_2$]-dihydrosphingosine into phytosphingosine and into the above mentioned acids was not observed.

Each of the three major bases appears to follow the same general catabolic pathway. The first step involves a phosphorylation of the base at carbon atom one. Keenan and Maxam[57] found an ATP dependent kinase in rat liver mitochondria which could phosphorylate dihydrosphingosine. Such kinases were also observed in kidney mitochondria[56] and in the soluble portion of rat liver[49]. Using human or rabbit erythrocytes (the activity was later found to be localized in the platelets)[99] the three labeled bases were reported to be converted to their 1-phosphate esters[95]. Sphingosine and dihydrosphingosine were converted to their phosphate esters with microsomal preparations of Tetrahymena pyriformis[55,100] and the soluble fraction of human and pig platelets[99]. All three of the bases were found in the blood platelets of pigs[46]. N-Acyl-dihydrosphingosine was not phosphorylated by a 40,000 X g pellet (microsomes and mitochondria) from lyophilized T. pyriformis[55]. Schneider and Kennedy reported that a diglyceride kinase of E. coli could phosphorylate ceramides[69]. Ceramides are not normal constituents of E. coli[69].

The next step involves a lyase-type cleavage between carbons two and three. Using either sphingosine or dihydrosphingosine labeled in carbons three through eighteen in in vivo experiments in rats, sub-

stantial amounts of labeled palmitic acid and its elongation product, stearic acid, were detected[6,58,86,87,100]. Phytosphingosine *in vivo*[4,5,6,54,90] in rats and *in vitro*[38] gave 2-hydroxy-palmitic acid and its oxidation product, pentadecanoic acid. Phytosphingosine, when incubated with intact *H. ciferrii*, yielded 2-hydroxy-palmitic acid and free ethanolamine[48]. Labeling in the first two base carbons of each of the three bases and their phosphate esters was used in reports that the immediate cleavage product was ethanolamine-1-phosphate[47,77,81,90,92,94,100], which was often incorporated into phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin[47,84,87,92,93]. However, characterization of the labeled ethanolamine-1-phosphate was not rigorous and consisted of a paper electrophoresis[92,93]. It was reported in *in vitro* studies that the primary long chain cleavage product of dihydrosphingosine (*via* its phosphate ester) was not the acid, but rather hexadecanal [57,77,79,92,93,94]. Sphingosine-1-phosphate yielded 2t-hexadecenal and phytosphingosine-1-phosphate yielded 2-hydroxy-palmitaldehyde[77,79,100]. Using [3-^{3}H], [3-^{14}C]-dihydrosphingosine, palmitaldehyde of an identical isotope ratio was isolated [92]. This result indicated that the C-3 hydrogen was retained and that the lyase reaction does not involve a 3-keto-intermediate[92]. However, results in which palmitaldehyde were found must be viewed with suspicion since the aldehyde was either identified and isolated by thin layer chromatography in petroleum ether-ether-acetic acid (90:15:1.5) or identified and isolated as its dimethyl acetal by treatment of the crude lipid extracts with methanolic HCl[92]. Under these conditions, acid labile plasmalogens readily release aldehydes which could have

been mistaken for the immediate cleavage product of the lyase. The need for pyridoxal phosphate, ATP and Mg^{+2} in the degradation of dihydrosphingosine was reported[57,92]. Akino et al.[2] showed that ethanolamine-1-phosphate incorporated tritium from ^3H_2O into the pro-R position of ethanolamine-1-phosphate in the enzymatic degradation by rat liver microsomes of D-erythro-dihydrosphingosine-1-phosphate. The lyase has been reported to be localized in mitochondrial membrane and is present in all organs of mammals studied and in H. ciferrii[92].

The long chain aldehyde released by the lyase has two major fates. First, as stated above, it may be oxidized to the corresponding acid which can be used for complex lipid and sphingolipid synthesis or can be degraded to carbon dioxide[86,87]. A second pathway involved reduction to the corresponding alcohol[77,83,96,100]. The soluble fraction of Tetrahymena pyriformis[100] and of rat liver, heart, kidney and lungs[83] reduced 2t-hexadecenal to hexadecanal. This finding may explain why, in earlier studies, sphingosine was found to be degraded to palmitic acid. The hexadecanol is then incorporated into plasmalogens (1-O-alkyl-1'-en-2-acyl-glycerophospholipids). In vitro systems from Ehrlich ascites cells, preputial gland tumors[974,118], guinea pig livers[42], mouse brains[42], rat brains[10,51], Tetrahymena pyriformis[100] and Clostridium butyricum[48] synthesized plasmalogens. The long chain alcohols condensed at carbon one on a glyceryl-acceptor molecule with retention of the alcoholic oxygen atom of the long chain alcohol[100]. Both dihydroxyacetone phosphate (DHAP)[33,42,43,73,74,116-119] and glyceraldehyde-3-phosphate[72,73] served as precursors of plasmalogens. Glyceraldehyde-3-phosphate did not serve as an acceptor if triose-phosphate isomerase was inhibited[73,116]. DHAP did not serve as a precursor in Cl. butyricum since [2-^{3}H]-glycerol has been reported

to be incorporated into plasmalogens without loss of tritium[48]. It has been reported that DHAP condenses with acyl-coenzyme A to give 1-acyl-DHAP[44,119]. The 1-acyl-DHAP then condenses with a long chain alcohol without cofactors to form 1-O-alkyl-DHAP in tumor, guinea pig liver or mouse brain microsomes[42,44,119]. The 1-O-alkyl-DHAP was then reduced with either NADH or NADPH to 1-O-alkyl-glycerol phosphate with tumor microsomes[119]. 1-O-Alkyl-glycerol phosphate[117, 119] or 1-O-alkyl-glycerol (probably *via* its phosphate) was once again acylated to form 1-O-alkyl-2-acyl-glycerol phosphate. Finally, a desaturase which involves a mixed function oxidase using cytochrome b_5[65,120] then stereospecifically removed the 1'S and 2'S hydrogens in a *cis* elimination to form plasmalogens[1,10,51,115].

To summarize the confusion which exists concerning substrate specificity for many of the above reactions, the following Table I is provided. In general, natural lipids are better substrates than synthetic. This table does not reflect relative K_m or rate values but only positive or negative observations.

Some work has been reported concerning the stability of the bases under various conditions. Sphingosine[20,106] or sphingomyelin[103], cerebroside[103] or psychosine[103] containing the base sphingosine when treated with acid in methanol formed 3-O-methyl- and 5-O-methyl-sphingosines *via* a carbonium intermediate. A small amount of L-*threo*-sphingosine was also formed. Carter and Fujino[17] observed that acid hydrolysis of N-acyl-dihydrosphingosine caused degradation by a mechanism earlier proposed by Welsh[113]. This mechanism involves an N to O acyl shift with inversion of the hydroxyl groups which occurs *via* a cyclic intermediate. The same process occured in the acetolysis of

TABLE I
ISOMER SPECIFICITY

DIHYDROSPHINGOSINE ISOMER	IN VIVO	IN VITRO	SPHINGOSINE SYNTHESIS	CERAMIDE SYNTHESIS	SPHINGOMYELIN SYNTHESIS	CEREBROSIDE SYNTHESIS	KINASE	LYASE
D(+)-*erythro*-	X		+(81)	+(81,100)	+(81)	+(81)	+(55,81,100)	+(55,81,100)
		X		+(100)	+(81,93*)	+(81)	+(99,100)	+(100)
L(-)-*threo*-	X		+(81)	+(81,93*,100)	+(81,93*)	+(81)	+(55,81,87,93*,100)	+(55,87,93*) -(81,100)
		X		+(100)			+(99,100)	-(100)
D(+)-*threo*-	X		-(81)	+(81,93*,100)	+(93*)	-(81)	+(55,81,93*) -(100)	+(55,93*) -(81,100)
		X		+(100)			+(100) -(99)	-(100)
L(-)-*erythro*-	X		-(81)	+(81,100)	-(81)	-(81)	+(81) -(100)	-(81,100)
		X		+(100)			-(99,100)	-(100)

* A *threo*-(D,L ?)-dihydrosphingosine was used as the substrate.

D-erythro-dihydrosphingosine forming D-threo-dihydrosphingosine and in the acetolysis of D-ribo-phytosphingosine yielding 3L, 4D; 3D, 4L and 3L, 4L isomers[53]. Acid treatment of phytosphingosine also resulted in dehydration forming 2-tetradecyl-3-hydroxy-4-amino-tetrahydrofurane (anhydrophytosphingosine)[62]. Quantitive yields of natural free bases were obtained from ceramides by alkaline hydrolysis[25]. Acid-water-methanol mixtures[39] were most widely used in the literature and gave near quantitative hydrolysis of ceramides and cerebrosides with minimal side product formation. Unfortunately, this method hydrolyzed only about one half of the sphingomyelins[60]. Finally, it was observed that bases decompose when either stored or chromatographed as free amines but were stable as their HCl salts[108].

MATERIALS AND METHODS

SOLVENTS: Hexane was a fraction of hexanes (85% n-hexane; Mallinckrodt; bp 66.1° C) which was distilled at 68-69° C. Pyridine was distilled from barium oxide and stored over potassium hydroxide. Acetonitrile obtained from Matheson was distilled prior to use. All organic solvents were analytical grade unless otherwise noted. Glass distilled deionized water was used in all enzymatic incubations.

THIN LAYER CHROMATOGRAPHY: Thin layer plates were hand made to 0.5 (or 1.25) mm in thickness by slurrying for each 20 by 20 cm plate 10 (or 25)grams of adsorbent in 21 (or 51) ml of glass distilled deionized water. After application of the slurry, the plates were dried in air overnight and heat activated at 105° C for two hours. Silica Gel G was used for analytical and radioanalytical chromatography. In the latter case, one cm sections were scraped directly into the scintillation solvent and the vials gently swirled. After about 15 minutes the vials were counted. A one cm section below the origin was used as a blank. Silica Gel PF 254 + 366 was used for both analytical and preparative thin layer chromatography. Both adsorbents were products of E. Merck, Darmstadt.

THIN LAYER VISUALIZING REAGENTS:

Molybdic Acid: This general lipid reagent was a slight modification of the one described by Clark[27]. Ammonium molybdate (20 grams) was dissolved in concentrated sulfuric acid (25 ml) with heating on a steam bath. After cooling, the volume was brought to 400 ml with the addition of water. After the thin layer plate was sprayed, charring at

130° C for 5 minutes generally brought a blue spot on a white background. Unless otherwise stated, this reagent was used.

Ninhydrin[71]: On thin layer plates, primary amines appeared red-violet when sprayed with 0.2% (w/v) ninhydrin (National Biochemicals Corporation) in n-butanol-saturated water followed by heating at 130° C for 10 minutes.

Dittmer's Reagent[36]: At room temperature, most (not all) phospholipids produced an immediate blue spot on a white background when sprayed with this reagent. It was prepared by dissolving MoO_3 (40.11 grams) in 25 N sulfuric acid (one liter) with heating over a flame. To 500 ml of this was added Mo powder (1.78 grams) with continued heating. The latter solution was then mixed with the remaining 500 ml of the former and the volume brought to 2 liters with the addition of water.

COLUMN CHROMATOGRAPHY: Columns were packed under gravity in a continuous slurry using the first solvent of elution or one less polar. Unisil, a product of Clarkson Chemical Company, Inc., was 100-200 mesh acid-washed silicic acid. Silicic acid, 100 mesh, was a product of Mallinckrodt that was stirred three times in four volumes of methanol and, after settling for 15 minutes, the supernatant was poured off to remove the "fines." The silicic acid so obtained was filtered under suction and allowed to air dry. Finally, it was heat activated overnight at 135° C.

GAS-LIQUID CHROMATOGRAPHY: When determining purity or identity of samples, a Hewlett-Packard Model 402 High Efficiency Gas Chromatograph

was used. Areas under curves and exact retention times were determined by a Hewlett-Packard Model 3370B integrator. Columns were "U"-shaped, six foot long glass columns with an inside diameter of 4 mm.

Radio-gas-liquid chromatographic analyses were performed on a Barber Colman Selecta-System 5000 which used a 4 or 8 foot, 4 mm inside diameter "U"-shaped glass column. The effluent stream was fitted with a metal "T" splitter to permit simultaneous mass tracings and fraction collecting. Fractions of one minute each were collected using a one foot by 2.5 mm inside diameter glass tube which fitted about 3 cm over the splitter's collection port which extended about 3 mm from the detector bath. The tubes were then rinsed with scintillation fluid (10 ml) directly into vials for counting. Approximately 50% recovery of the injected radioactivity could thus be obtained.

Unless other wise stated, radio-gas-liquid chromatographic analyses of long chain bases were run on a 4 foot, 1% SE 30 column at 212° C and , after a 20 minute delay, the temperature was increased 5° C/minute to a limit of 270° C. The trimethylsilyl derivatives of the alcohols derived by the periodate-borohydride treatment of long chain bases were run on an 8 foot, 3% OV 17 column at 175° C, and , after a 25 minute delay, the temperature was increased 30° C/minute to a limit of 215° C. Fatty acid methyl esters were run on a 4 foot, 1% SE 30 column at 165° C and, after a 12 minute delay, the temperature was increased 3° C/minute to a limit of 250° C.

Both gas chromatographs used a flame ionization detector and the injector and detector baths were kept a minimum of 10-20° C above

the temperature of the column bath. All liquid phases were coated on Gas Chrom Q (100-120 mesh) and were obtained from Applied Science Laboratories. The carrier gas was nitrogen with a flow rate of 66 ml/minute.

LIQUID SCINTILLATION COUNTING: All radioactive analyses were performed using a Beckman LS 250 liquid scintillation spectrometer. The scintillation solution consisted of 4 grams of 2,5-diphenyl-oxazole (Beckman) per liter of scintillation toluene (Beckman)-absolute ethanol (2:1). The volume of the scintillation solution in each of the low potassium glass vials (Beckman) was 10 ml. Counting efficiencies reanged between 20-30% for tritium and 80-90% for carbon-14.

MASS SPECTROMETRY: Low resolution mass spectrometric analyses of probe or gas-liquid chromatographic samples were performed on an LKB Gas Chromatograph-Mass Spectrometer 9000S at 70 electron volts; 60 microamperes and a source temperature of 270° C. The chromatograph used coiled glass columns with an inside diameter of 3 mm. The carrier gas was helium with a flow rate of 30 ml/minute.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: Samples for 60 mega-Hertz NMR spectra were dissolved in 99.8% deuterochloroform containing 1% TMS as a reference and were recorded on either a Varian Associates Model A-60A Analytical NMR Spectrometer or a Perkin-Elmer R12 NMR Spectrometer.

INFRARED SPECTROSCOPY: Infrared spectra were recorded as KBr micro-

pellets on a Beckman IR-9 Infrared Spectrometer adapted with a micro-condensor attachment. Carbon dioxide absorbances were observed frequently at 2360 cm^{-1}.

ULTRAVIOLET SPECTROSCOPY: Samples for ultraviolet spectra were dissolved in absolute ethanol and the spectra were recorded on a Cary 118 Spectrophotometer or a Beckman Model DB-GT Grating Spectrophotometer.

MELTING POINTS: Melting points were recorded in open capillary tubes using a Thomas-Hoover Capillary Melting Point Apparatus which was standardized with crystallized diphenylamine, diphenyl carbamoyl-chloride, p-nitroaniline, succinic acid and anthracene.

SILYLATING REAGENTS: Carter's "improved reagent for silylation"[18] was used on all lipids except for glycol phosphate and was prepared by adding trimethylchlorosilane (1.6 ml; Sigma) to a mixture of hexamethyldisilazane (2.6 ml; Applied Science Laboratories) and dry pyridine (2.0 ml). After centrifugation, an aliquot of the supernatant was then allowed to react at least 5 minutes at room temperature with the desired lipid.

Glycol phosphate was silylated at least 5 minutes at room temperature with Duncan's Reagent[31] which was prepared by mixing together dry pyridine (50 ul), N,N-bis (trimethylsilyl) trifluoroacetamide (50 ul; Sigma) and trimethylchlorosilane (1 ul).

PERIODATE OXIDATIONS: Unless otherwise noted, periodate oxidations

were performed as described by Sweeley and Moscatelli[101]. The substrate (1 mg or less) was dissolved in chloroform-methanol (1:1; 0.6 ml). Freshly prepared sodium metaperiodate (0.2 M; 0.1 ml; G. Frederick Smith Chemical Company) was added and the solution left in the dark for 90 minutes at room temperature. Methanol-water (1:1; 1.0 ml) and chloroform (1.3 ml) were added and mixed. The aldehydes in the lower Folch layer were dried under nitrogen at 40°C or less and reduced by dissolving them in methanol (0.5 ml) and adding 10% (w/v) sodium borohydride (Ventron) in 0.04 N aqueous sodium hydroxide (0.5 ml) After 30 minutes at room temperature, 0.054 N HCl (0.55 ml), methanol (0.3 ml) and chloroform (1.6 ml) were added successively to form a Folch partition. After the alcohols in the lower layer were dried under a stream of nitrogen at 35° C, water (0.5 ml) was added and the alcohols were extracted with three portions of ether (2 ml each). After careful drying in vacuo over phosphorous pentoxide, the alcohols were silylated with Carter's reagent and analyzed by gas-liquid chromatography on a 3% OV 17 column.

FATTY ACID ESTERIFICATION: Diazomethane was formed[3] by adding N-nitrosomethylurea (K & K Laboratories) in ether to a 60% (w/v) aqueous sodium hydroxide solution. A stream of nitrogen was bubbled through the lower layer and then passed through the fatty acids which were dissolved in 10% methanol in ether. When a yellow color persisted, the solution was allowed to stand in a hood for 30 minutes and the ether then was removed under a stream of nitrogen at 35° C. The dried methyl esters were dissolved in a small portion of hexane for gas-liquid chromatographic analysis.

ELEMENTAL ANALYSES: Elemental analyses were performed by Alfred Bernhardt Mikroanalytisches Laboratorium, 5251 Elback über Engelskirchen, Fritz-Pregl Strasse, West Germany.

PROTEIN ANALYSIS: Concentrations of protein were determined using biuret reagent as described by Gornall *et al.*[40] $CuSO_4 \cdot 5H_2O$ (1.50 grams) and $NaKC_4H_4O_6 \cdot 4H_2O$ (6.0 grams) were added with mixing to 10% sodium hydroxide (w/v; 300 ml) and the total volume was brought to 1 liter with the addition of water. This reagent (3 ml) was mixed with the protein solution (2 ml). After 30 minutes at room temperature, the absorbance was read at 540 nanometers on a Coleman Junior II-A, linear absorbance spectrophotmeter. A standard curve was drawn using bovine serum albumin in known concentrations.

THE PREPARATION OF [4,5-$^3H_2$]-DIHYDROSPHINGOSINE

CATALYTIC REDUCTION OF N-BENZOYL-SPHINGOSINE: The starting material was a sample prepared in the laboratory of Dr. H. E. Carter.[21,22] The material was analyzed by its trimethylsilyl derivative on gas-liquid chromatography and was shown to consist of N-benzoyl-sphingosine and N-benzoyl-dihydrosphingosine in the ratio of 5:8 (Fig. 1). The actual reduction was performed by New England Nuclear as follows: The mixture (41 mg) was dissolved in dry dioxane (2 ml) and 5% palladium on carbon (41 mg) and tritium gas (5 Ci) were added. After stirring for 20 minutes, hydrogen was added to attain a pressure of 860 mm of mercury and the stirring then continued overnight at room temperature. Labile tritium was removed in vacuo from a mixture of benzene, methanol and water (1:1:2) by distilling ten times. After filtration of the catalyst, the final product was taken up in 10 ml of benzene-methanol (1:1). Assay of the [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine showed a yield of 160 mCi; 3.9 mCi/mg; 0.256 mg/mCi and 1.58 Ci/mmole.

ALKALINE HYDROLYSIS OF [4,5-$^3H_2$]-N-BENZOYL-DIHYDROSPHINGOSINE: [4,5-$^3H_2$]-N-Benzoyl-dihydrosphingosine (1 mCi; 0.256 mg in 62 ul of benzene-methanol (1:1)) was added to pure dihydrosphingosine (0.75 mg) in a mixture of absolute ethanol (13 ml) and aqueous potassium hydroxide (1.5 ml; 2 N). After 22 hours of refluxing, thin layer radioanalysis (Fig. 2) showed completed hydrolysis leaving only labeled dihydrosphingosine and its non-polar decomposition products. The solution was cooled to room temperature and the ethanol removed under a stream of nitrogen at 40° C. Water (3 ml) was added and the solution

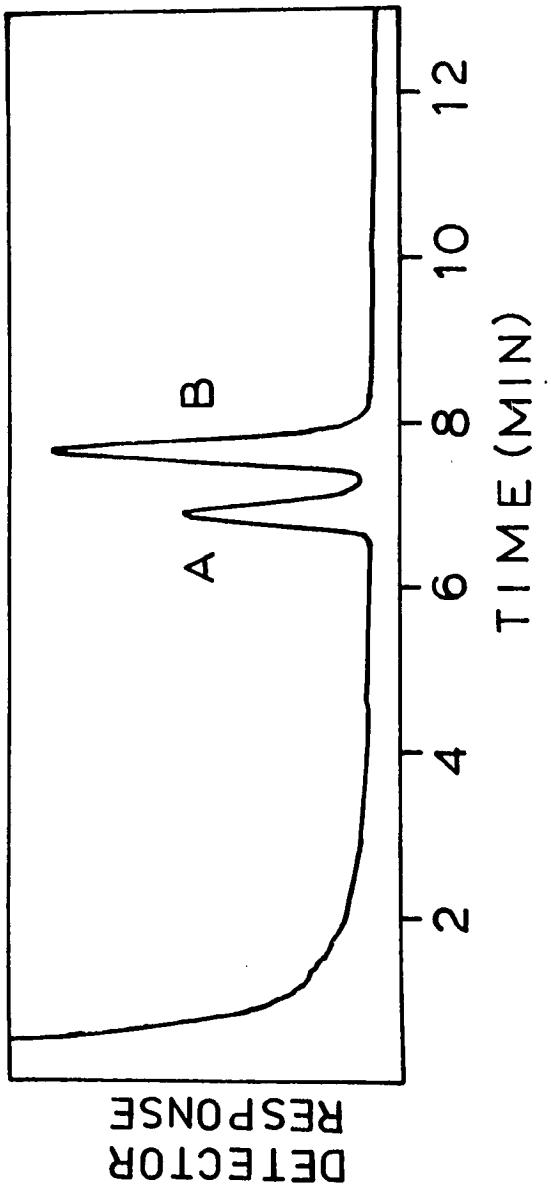


Figure 1. Gas-liquid chromatogram of the silylated derivatives of a mixture of N-benzoyl-sphingosine (A) and N-benzoyl-dihydrosphingosine (B) on a 6 foot, 1% SE 30 column at 259° C.

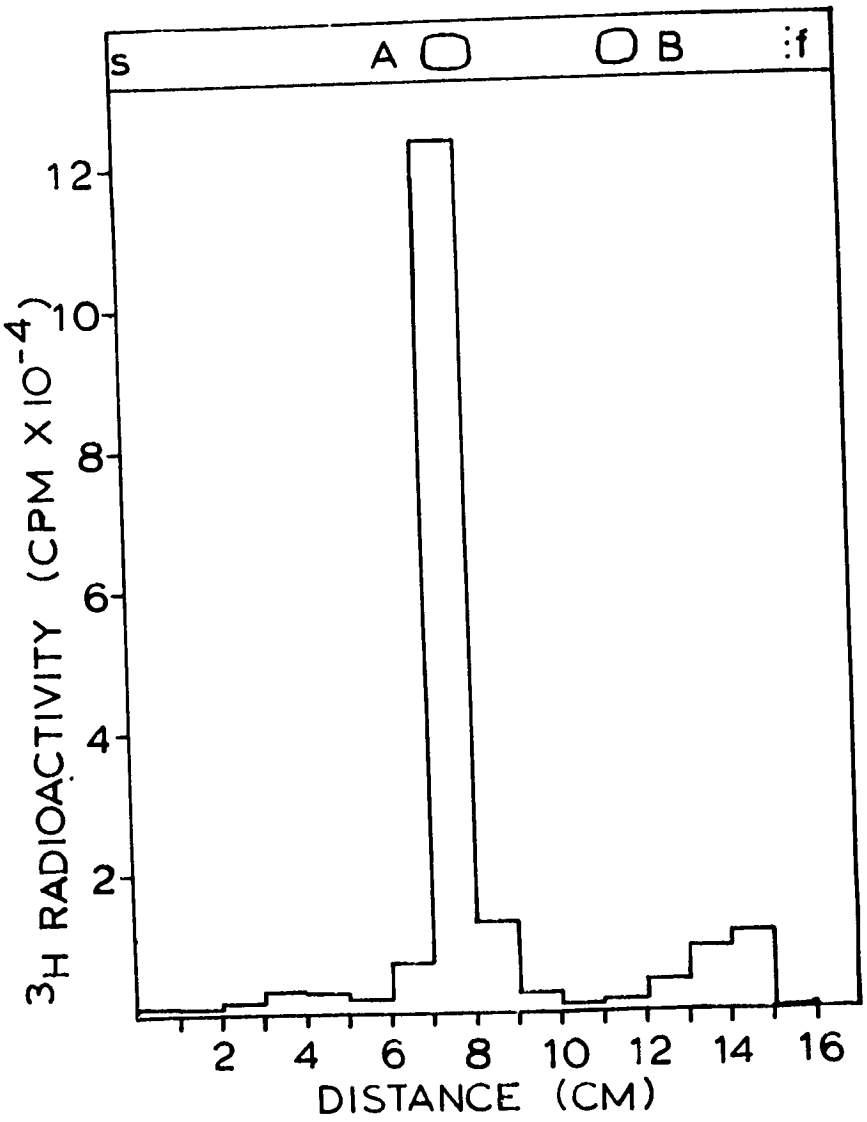


Figure 2. Thin layer radiochromatogram of the alkaline hydrolysate of [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine and N-benzoyl-sphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

extracted four times with ether (8 ml/extraction). The combined organic layers were washed once with water (2 ml) and the solvent was removed under nitrogen at 40° C and the residue dried *in vacuo* over phosphorous pentoxide. This residue was redissolved in absolute ethanol-chloroform (1:99; 10.0 ml) and an aliquot counted. The final yield was 1.85 X 10^9 DPM with a specific activity of 1 mCi/mg.

PURIFICATION OF [4,5-$^3H_2$]-DIHYDROSPHINGOSINE:

Preparation I: The solution of labeled dihydrosphingosine in 10 ml of 1% ethanol in chloroform was applied to a 2 gram silicic acid column packed in chloroform (0.9 X 8.5 cm). The column was then eluted with 3% ethanol in chloroform (150 ml; about 2.5 ml/fraction) followed by 100% ethanol (25 ml; about 1.5 ml/fraction). Care was taken so as to avoid having chloroform in the first ethanol fraction. A portion of each fraction was counted to provide a radio-profile (Fig. 3). Fractions 38 and 39 were pooled (about 1.2 X 10^9 DPM) and analyzed by thin layer chromatography (Fig. 4). Fraction 5 was also analyzed by thin layer chromatography (Fig. 5). Less than 1.8% of the total activity in Fig. 4 had a mobility equal to or greater than that of N-benzoyl-dihydrosphingosine. A sample was analyzed as its trimethylsilyl derivative on gas-liquid chromatography and a homogeneous bell-shaped radio-profile was observed (Fig. 6). The volume of ethanol was reduced under a stream of nitrogen at 30° C to about ¼ ml so that it could be used directly for *in vivo* metabolic studies. The resultant concentration was 2 ug/ul and the specific activity was 1 uCi/ug.

Preparation II: [4,5-$^3H_2$]-Dihydrosphingosine (88% pure by thin layer chromatography with a specific activity of 6 X 10^9 DPM/mg)

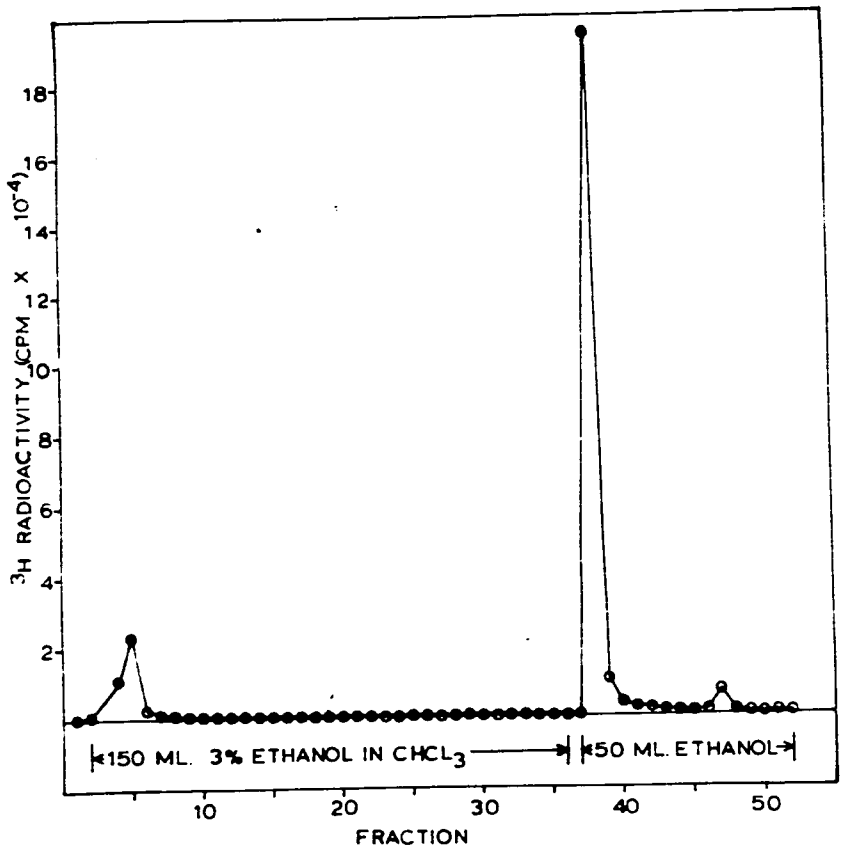


Figure 3. Radio-profile of silicic acid column chromatography of $[4,5\text{-}^3H_2]$-dihydrosphingosine (Preparation I) after alkaline hydrolysis of $[4,5\text{-}^3H_2]$-N-benzoyl-dihydrosphingosine.

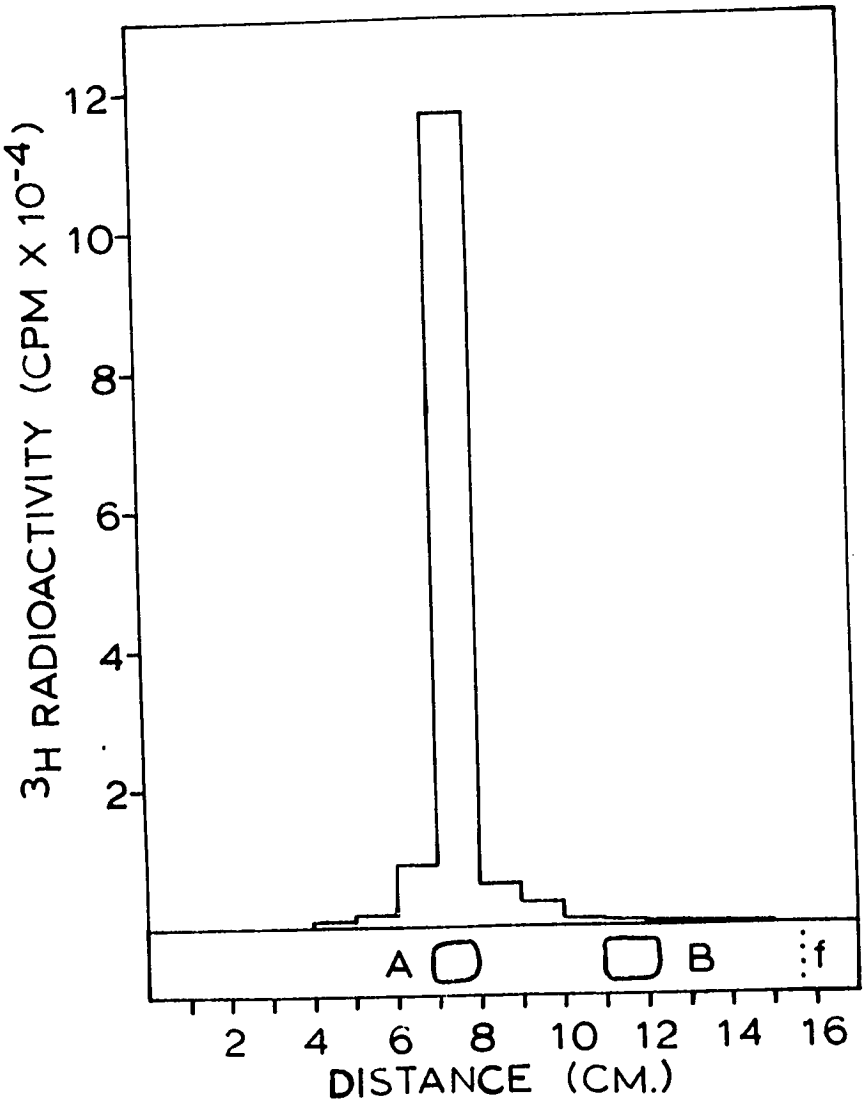


Figure 4. Thin layer radiochromatogram of pooled fractions 38 and 39 from silicic acid column chromatography of [4,5-^{3}H$_2$]-dihydrosphingosine (Preparation I) after alkaline hydrolysis of [4,5-^{3}H$_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

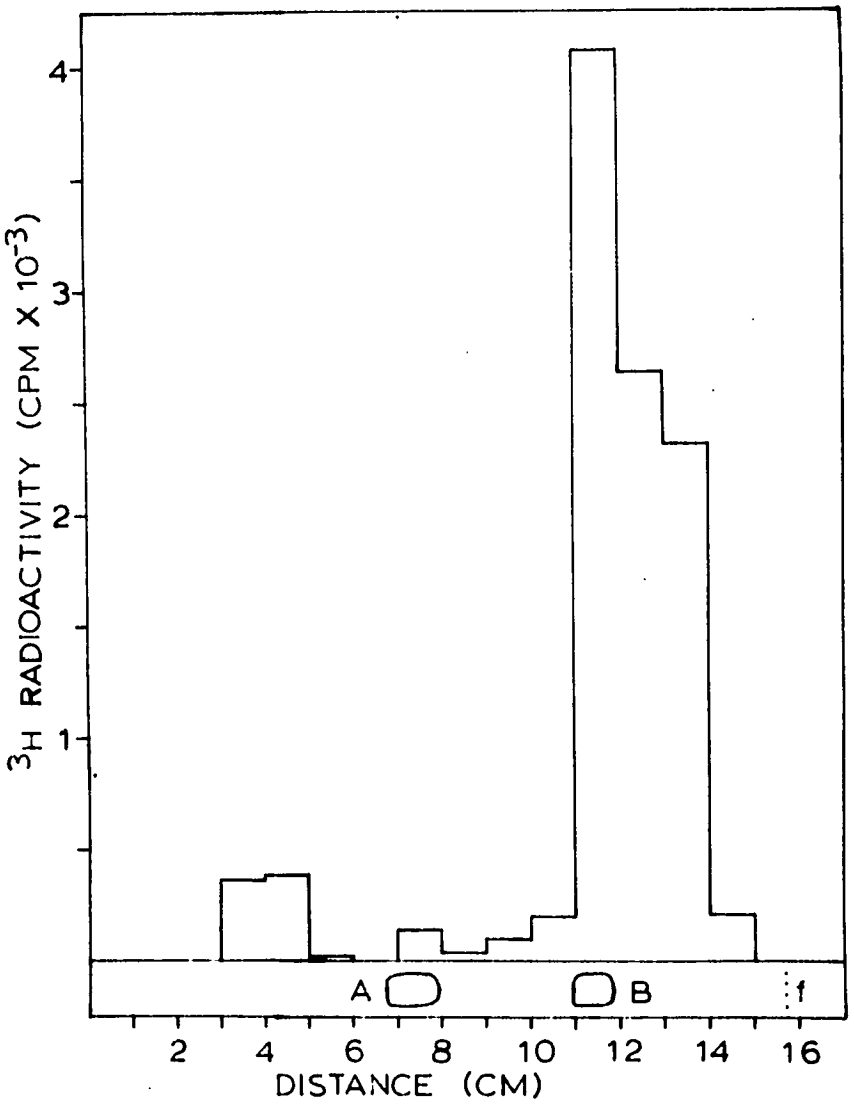


Figure 5. Thin layer radiochromatogram of fraction 5 from silicic acid column chromatography of [4,5-$^3H_2$]-dihydrosphingosine (Preparation I) after alkaline hydrolysis of [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

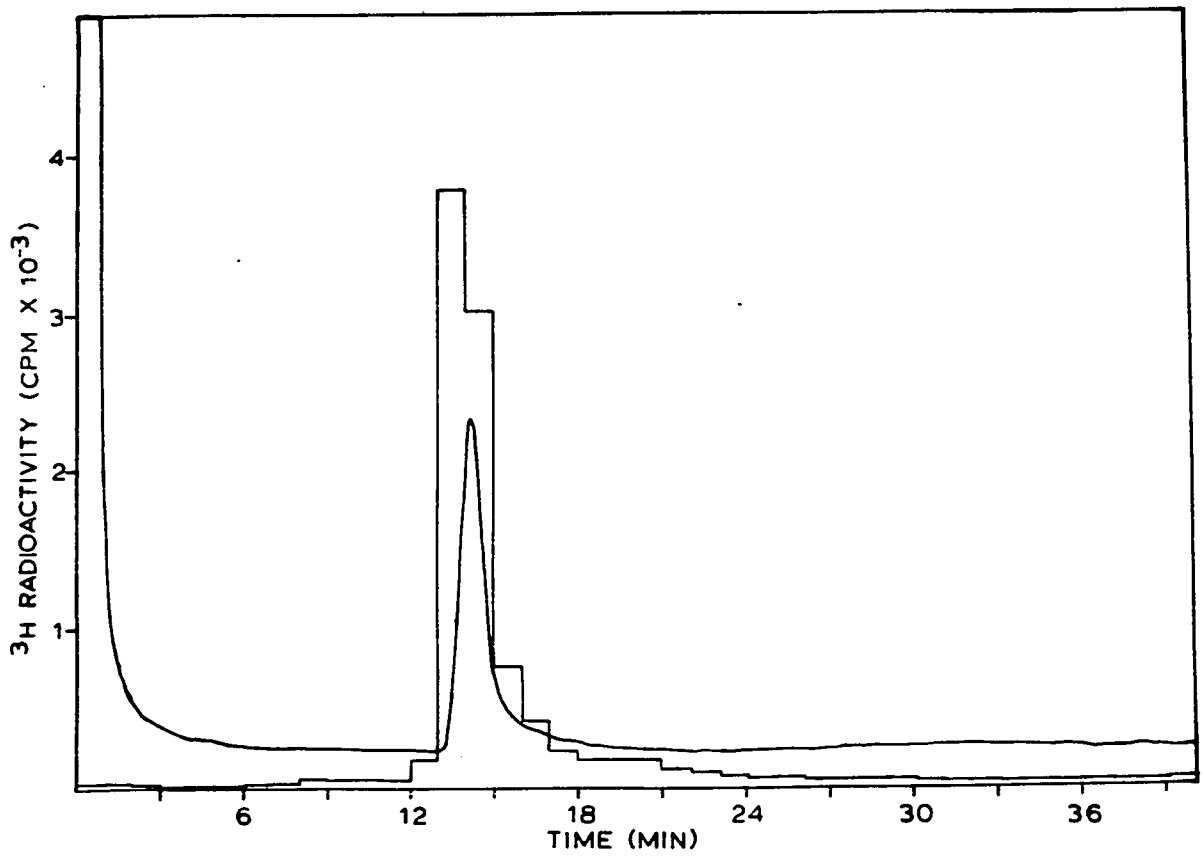


Figure 6. Gas-liquid radiochromatogram of the silylated derivatives of $[4,5\text{-}^3H_2]$-dihydrosphingosine (Preparation I) and dihydrosphingosine on a 4 foot, 1% SE 30 column at 212° C.

was applied in 3% methanol in chloroform (1.0 ml) to a 2 gram silicic acid column (0.9 X 8.5 cm; packed in chloroform). This was followed by two washes (1 ml each) of 3% methanol in chloroform. The column was then eluted with more of the same solvent (97 ml) followed by methanol (50 ml). An aliquot of each fraction was counted to provide a radio-profile (Fig. 7). Fractions 39 and 44 were analyzed by thin layer chromatography (Figs. 8 & 9, respectively). Fractions 39-45 were pooled and the volume reduced to 3.0 ml under nitrogen at 40° C and again analyzed (Fig. 10). Purity of this material was 98.5% as judged by thin layer chromatography and 99% by gas-liquid chromatography of its trimethylsilyl derivative (Fig. 11).

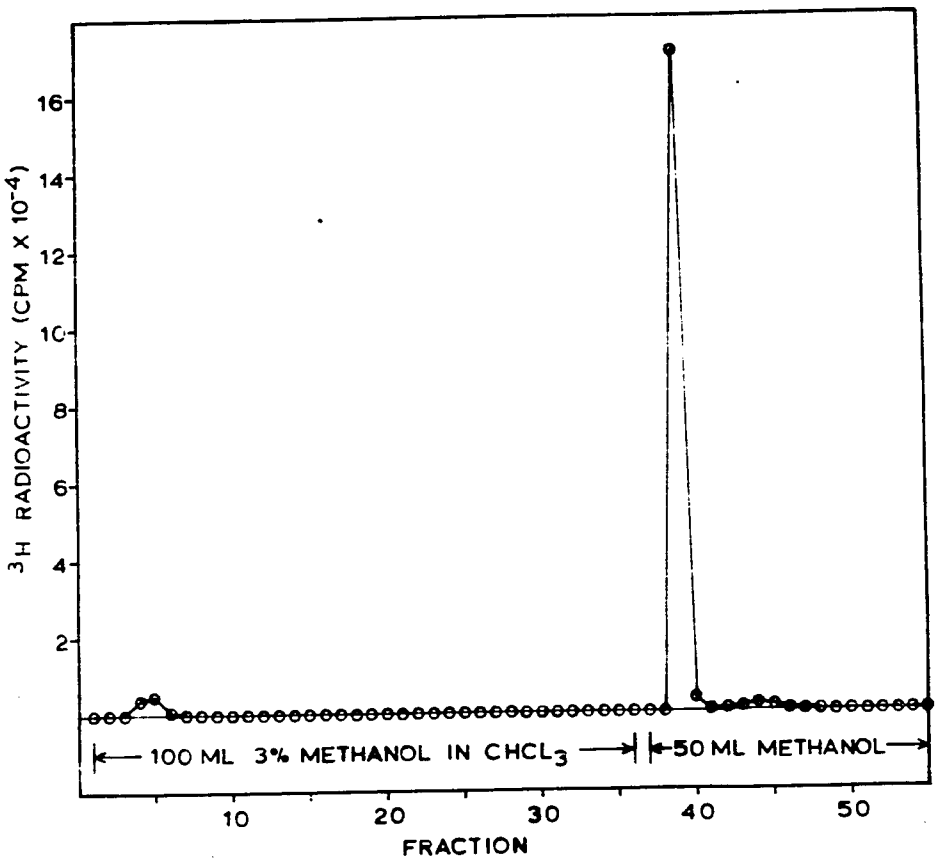


Figure 7. Radio-profile of silicic acid column chromatography of $[4,5\text{-}^3H_2]$-dihydrosphingosine (Preparation II) after alkaline hydrolysis of $[4,5\text{-}^3H_2]$-N-benzoyl-dihydrosphingosine.

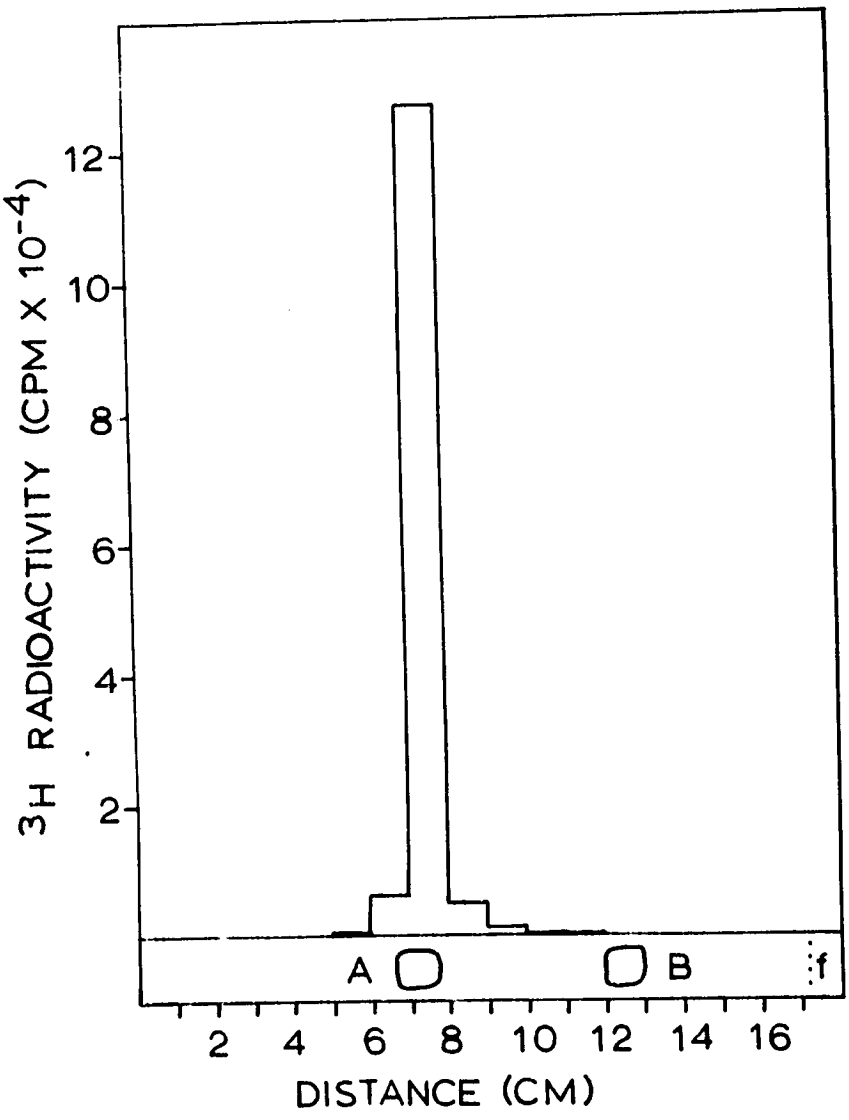


Figure 8. Thin layer radiochromatogram of fraction 39 from silicic acid column chromatography of [4,5-^{3}H$_2$]-dihydrosphingosine (Preparation II) after alkaline hydrolysis of [4,5-^{3}H$_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

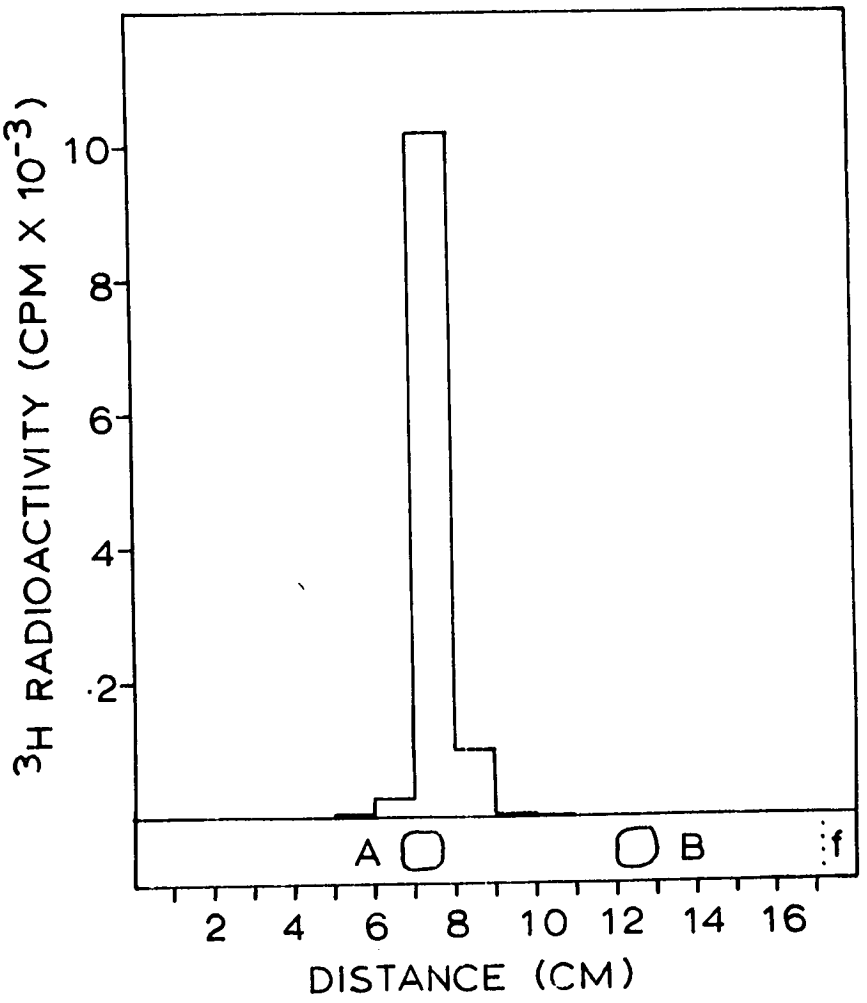


Figure 9. Thin layer radiochromatogram of fraction 44 from silicic acid column chromatography of [4,5-$^3H_2$]-dihydrosphingosine (Preparation II) after alkaline hydrolysis of [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

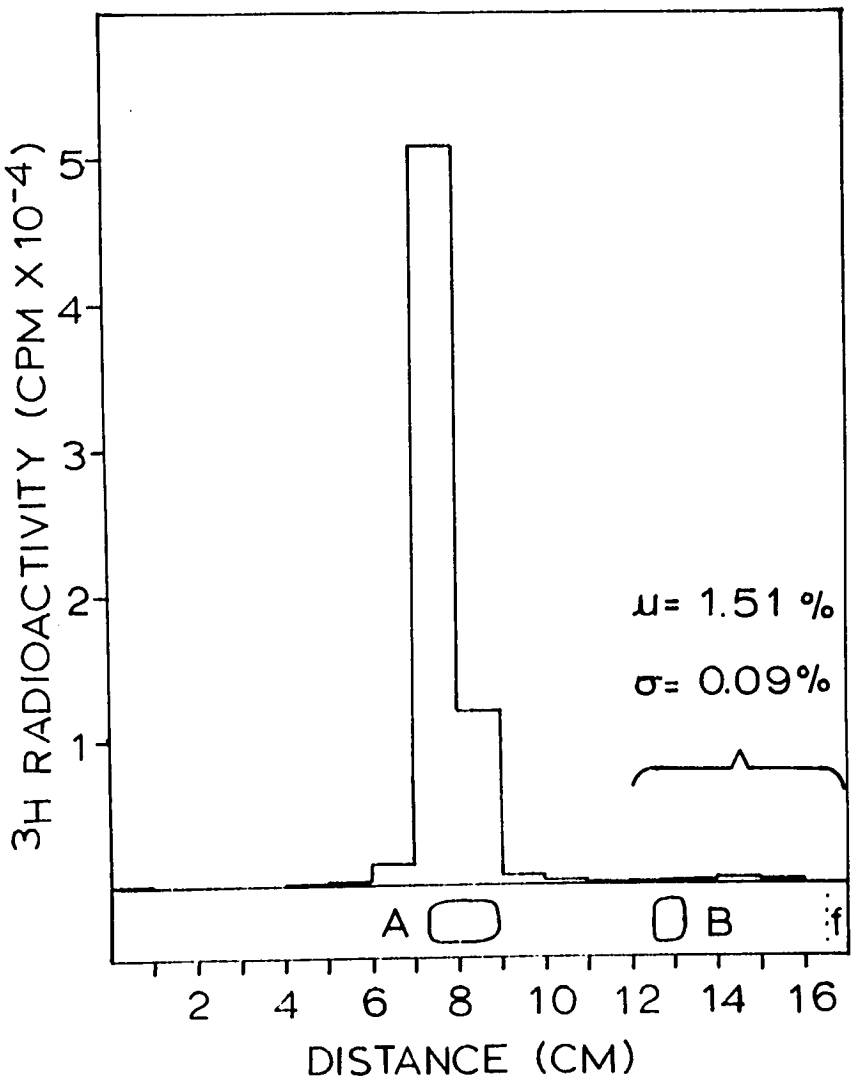


Figure 10. Thin layer radiochromatogram of pooled fractions 39-45 from silicic acid column chromatography of [4,5-$^3H_2$]-dihydrosphingosine (Preparation II) after alkaline hydrolysis of [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

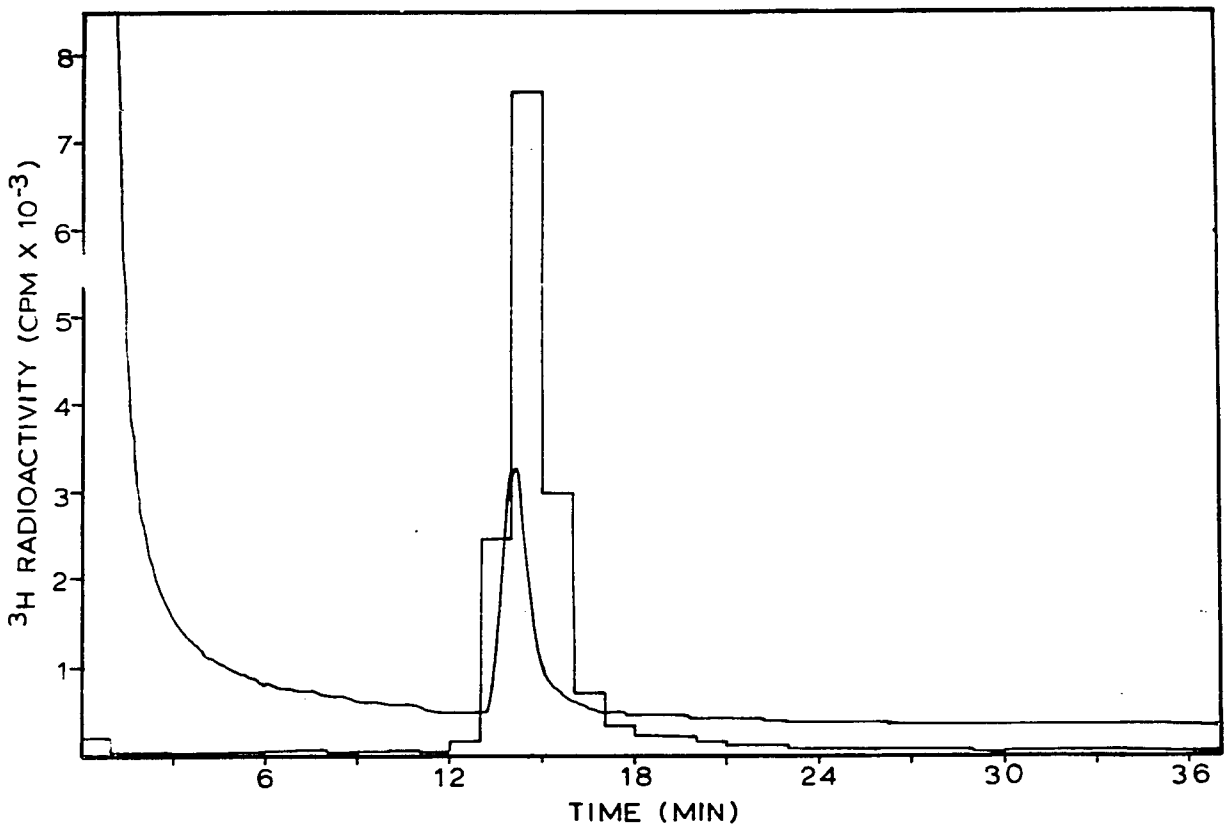


Figure 11. Gas-liquid radiochromatogram of the silylated derivatives of [4,5-$^3H_2$]-dihydrosphingosine (Preparation II) and dihydrosphingosine on a 4 foot, 1% SE 30 column at 212° C.

STABILITY OF [4,5-$^3H_2$]-DIHYDROSPHINGOSINE

It became evident during preliminary studies that small quantities of labeled dihydrosphingosine had a tendency to decompose to radioactive species having higher mobilities on thin layer analysis. On occasion the mass of the decomposition products could be observed on thin layer chromatography. The phenomenon appeared to be reduced when larger masses were involved. In this section these observations were quantitated and the best method for purification and storage determined.

Thin layer plates were prepared using a slurry of Silica Gel G in deionized water spread to a thickness of 0.5 mm on glass plates (which were washed with Alconox and rinsed with tap water and deionized water, dried in air and wiped with a hexane-soaked tissue) and air dried. Prior to use, the plates were heat activated for one hour at 120° C. Each plate was divided into 2 cm wide channels, spotted, developed in fresh chloroform-methanol-ammonia (100:25:2.5) and air dried. Analytical plates were scraped in 1 cm sections directly into vials containing scintillation solution. Desired sections of preparative plates were scraped into a Pasteur pipet containing a plug of Pyrex brand glass wool. Elution with ether-methanol (1:1) then recovered about 88% of the activity present. These samples were then dried under a stream of nitrogen at 50° C and the residue transferred in three portions of the eluting solvent (300 ul each) to a small culture tube and dried as above. The residue was once again dissolved in a very small portion of the eluting solvent (about 100 ul) and the

resulting solution then used for further preparative or analytical thin layer chromatography.

The weighted mean amount of impurity (μ) and its uncertainty (σ) were determined from two countings of the fractions from the thin layer chromatograms as shown below in the example for Experiment #1A.

FRACTION	FIRST COUNT (CPM)	STATISTICAL ERROR (CPM)	SECOND COUNT (CPM)	STATISTICAL ERROR (CPM)
1	210	6	204	14
2	107	5	107	11
3	125	6	118	12
4	178	9	172	12
5	228	7	200	14
6	375	11	367	18
7	1593	24	1454	44
8	50960	255	49391	247
9	12087	60	11611	116
10	781	16	747	37
11	402	12	410	21
12	195	10	196	14
13	201	6	211	15
14	285	9	270	19
15	436	13	488	22
16	257	8	259	18
17	92	5	99	10
RAW TOTAL:	68512	$\pm$ 462	66264	$\pm$ 644

	FIRST COUNT (CPM)	STATISTICAL ERROR (CPM)	SECOND COUNT (CPM)	STATISTICAL ERROR (CPM)
A = Background	51	4	55	8
B = A X 17	867	68	935	136
C = RAW TOTAL - B*	67645	530	65329	780
D = Raw Sum of Fractions 13 through 17	1271	41	1287	84
E = A X 5	255	20	275	40
F = D - E*	1016	61	1012	124

*Statistical errors were added.

The percent impurity measure from the first fraction which co-chromatographed with N-benzoyl-dihydrosphingosine (fraction 13 in this case) to the front (fraction 17 in this case) was computed as follows:

G = percent impurity = F/C X 100 .

For the first count G = 1.50% and for the second count G = 1.55%. To find the greatest possible error in the value of G, the following calculation was performed:

$$H = \frac{F - \text{its statistical error}}{C + \text{its statistical error}} \times 100 \ .$$

For the first count H = 1.40% and for the second count H = 1.34%. The uncertainty in the value of G was then:

I = uncertainty in G = G - H .

For the first count I = 0.10% and for the second count I = 0.21%. The weighted mean amount of impurity (μ) was computed as follows:

$$\mu = \frac{\sum G/I^2}{\sum 1/I^2} = \frac{\dfrac{1.50}{(0.10)^2} + \dfrac{1.55}{(0.21)^2}}{\dfrac{1}{(0.10)^2} \quad \dfrac{1}{(.21)^2}} = 1.51\% \ .$$

The uncertainty of the mean (σ) was determined as follows:

$$\sigma = \sqrt{\frac{1}{\sum 1/I^2}} = \sqrt{\frac{1}{\dfrac{1}{(0.10)^2} + \dfrac{1}{(0.21)^2}}} = 0.09\% \ .$$

EXPERIMENT #1: A) Freshly purified [4,5-$^3H_2$]-dihydrosphingosine from Preparation II (hereafter called the stock solution) was analyzed by thin layer chromatography as 98.5% pure (Fig. 10).

B) The labeled substrate was stored at -17° C with occasional thawing for the removal of aliquots for the following experiments. After 15 days, the stock solution was again analyzed by thin layer chromatography (Fig. 12). Under these conditions, the purity of the stock solution was maintained.

EXPERIMENT #2: A) An aliquot (20 ul; 6 ug; 872,000 CPM in methanol) was spotted on each of two channels of a thin layer plate, air dried and developed. One channel was used for analysis (Fig. 13).

B) The desired section of the remaining channel corresponding to pure dihydrosphingosine was scraped, eluted and applied to two channels of a second plate. After developed, one channel was used for analysis (Fig. 14).

C) The desired section of the remaining channel

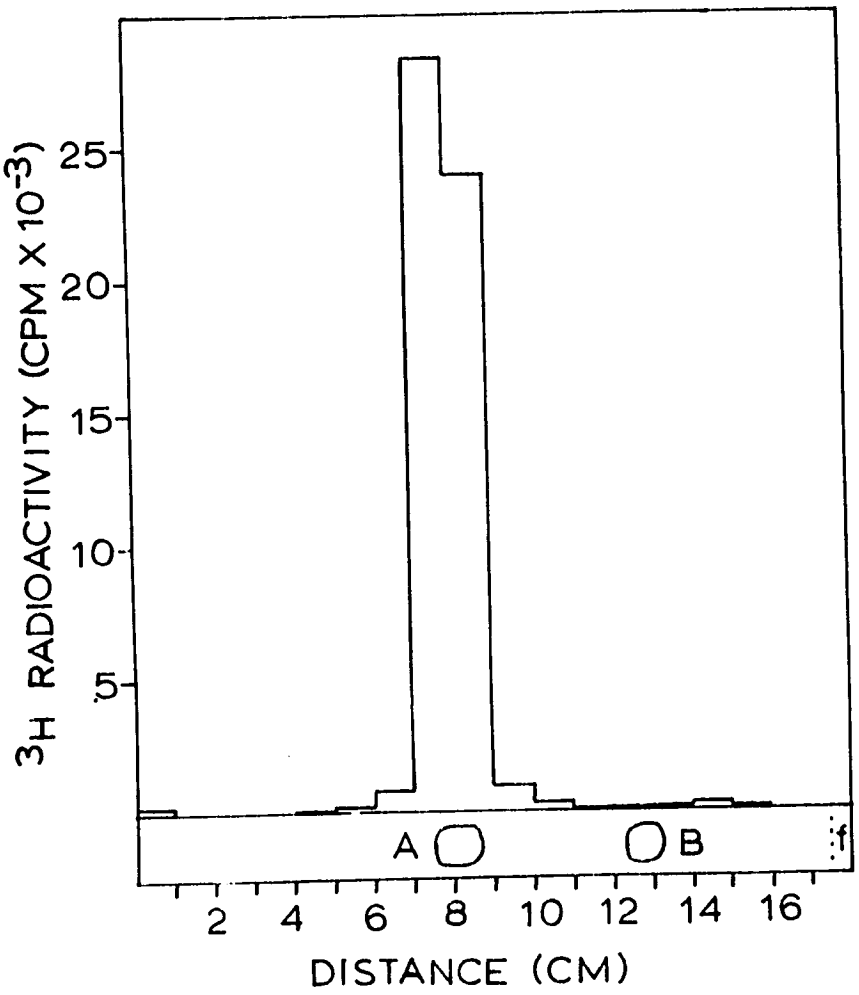


Figure 12. Thin layer radiochromatogram of Experiment #1B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

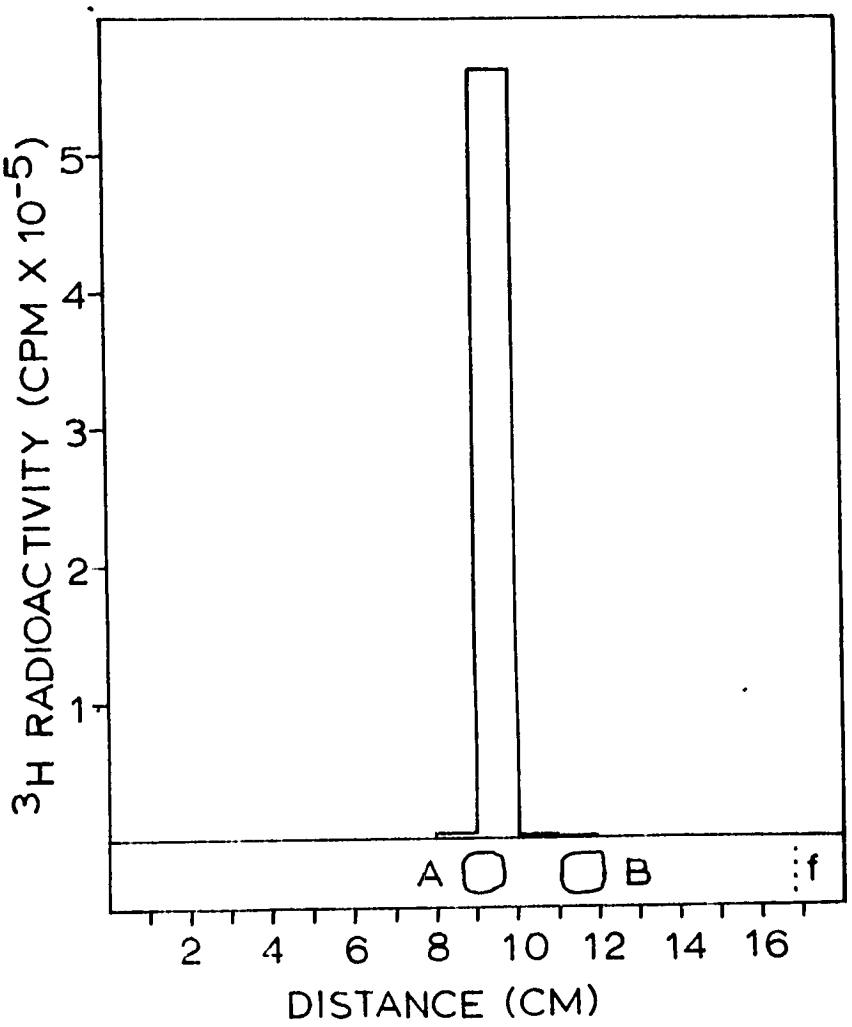


Figure 13. Thin layer radiochromatogram of Experiment #2A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

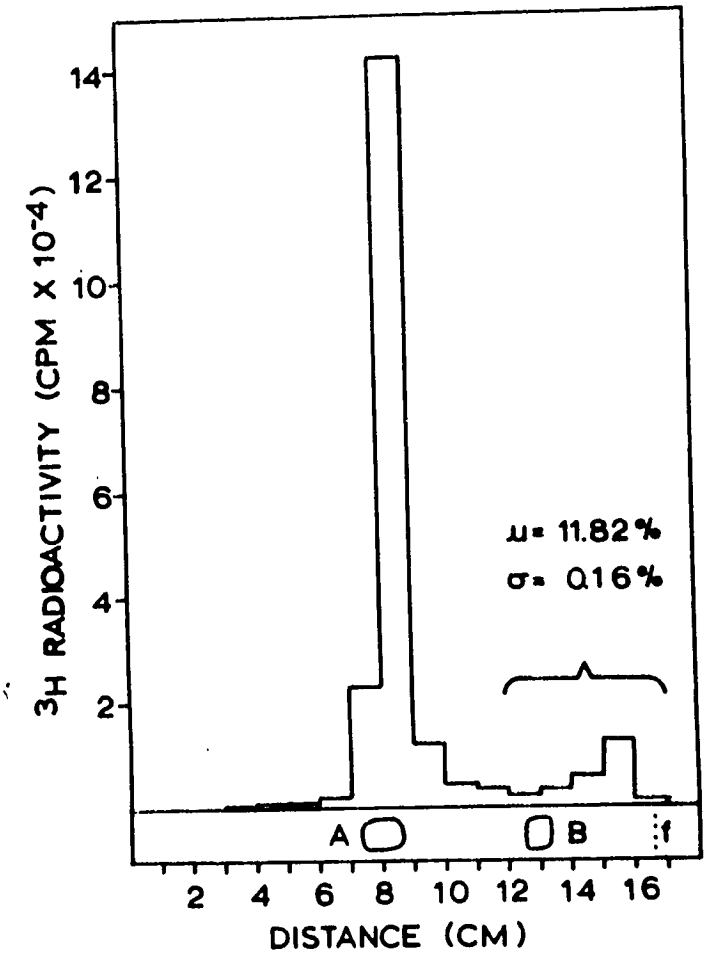


Figure 14. Thin layer radiochromatogram of Experiment #2B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

was eluted and applied to a third plate for analysis (Fig. 15).

EXPERIMENT #3: A) The stock solution (20 ul) along with an additional amount of unlabeled dihydrosphingsosine (40 ug) was spotted on each of two channels of a thin layer plate, air dried and developed. One channel was used for analysis (Fig. 16).

B) The desired section corresponding to pure dihydrosphingosine of the remaining channel was scraped, eluted and applied to two channels of a second plate. After development, one channel was used for analysis (Fig. 17).

C) The desired section of the remaining channel was eluted and applied to a third plate for analysis (Fig. 18).

The results of these experiments showed that with each preparative procedure, the substrate exhibited a greater tendency to form the less polar impurity. This effect was smaller when large amounts of carrier were present. Sufficiently pure $[4,5\text{-}^{3}H_{2}]$-dihydrosphingosine of high specific activity could not be prepared by this method.

The following studies were performed so as to further understand the process by which decomposition occurred. The vessels used were Kimax tubes (14 X 125 mm) that were washed with a solution of Alconox and rinsed successively with tap water, deionized water and distilled, deionized water. The tubes were covered with Teflon-lined screw caps.

EXPERIMENT #4: A) The stock solution (10 ul) was added to a test tube, dried under a stream of nitrogen at 24° C, capped and stored

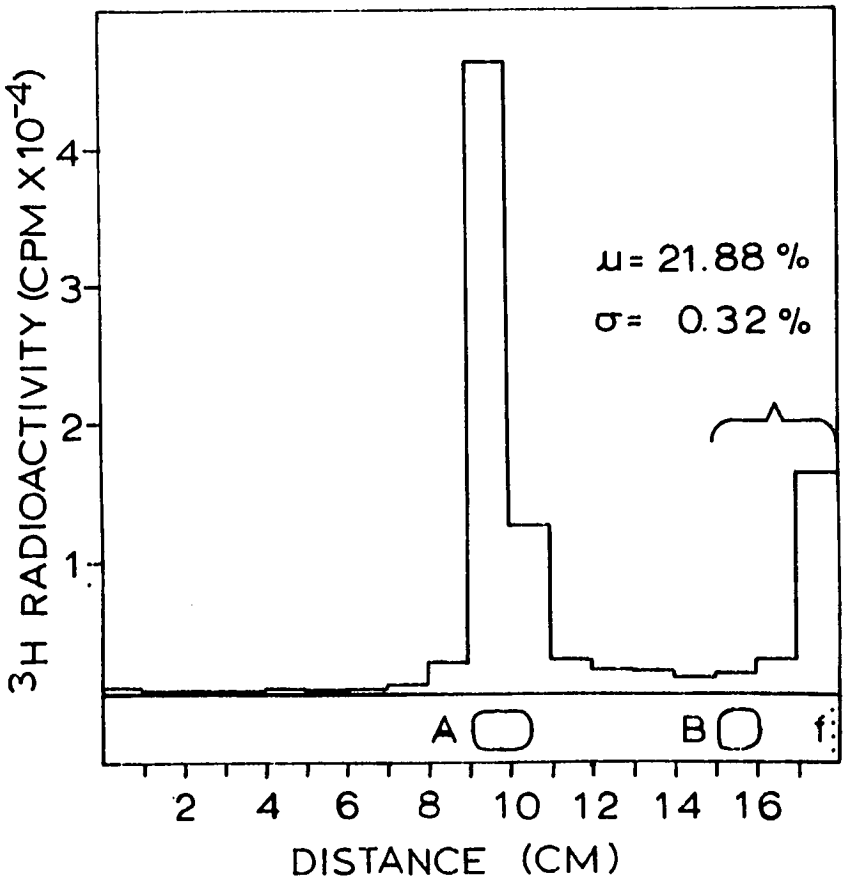


Figure 15. Thin layer radiochromatogram of Experiment #2C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

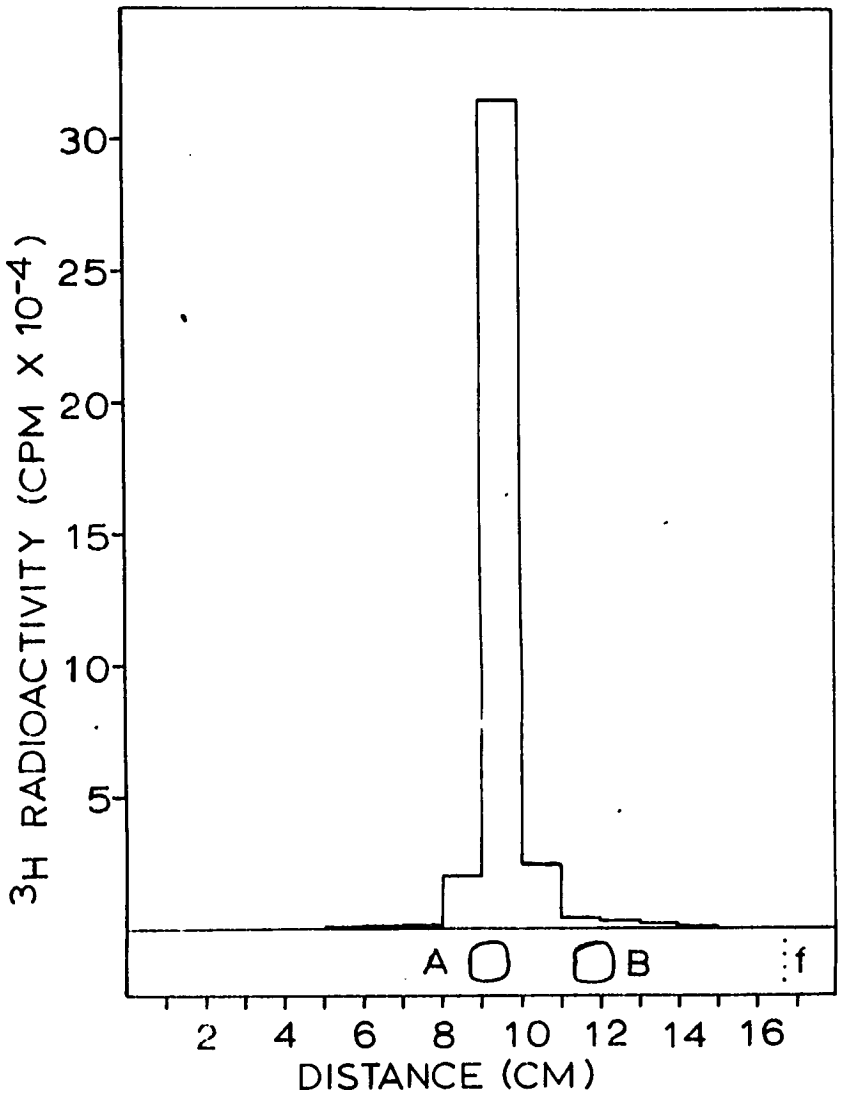


Figure 16. Thin layer radiochromatogram of Experiment #3A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

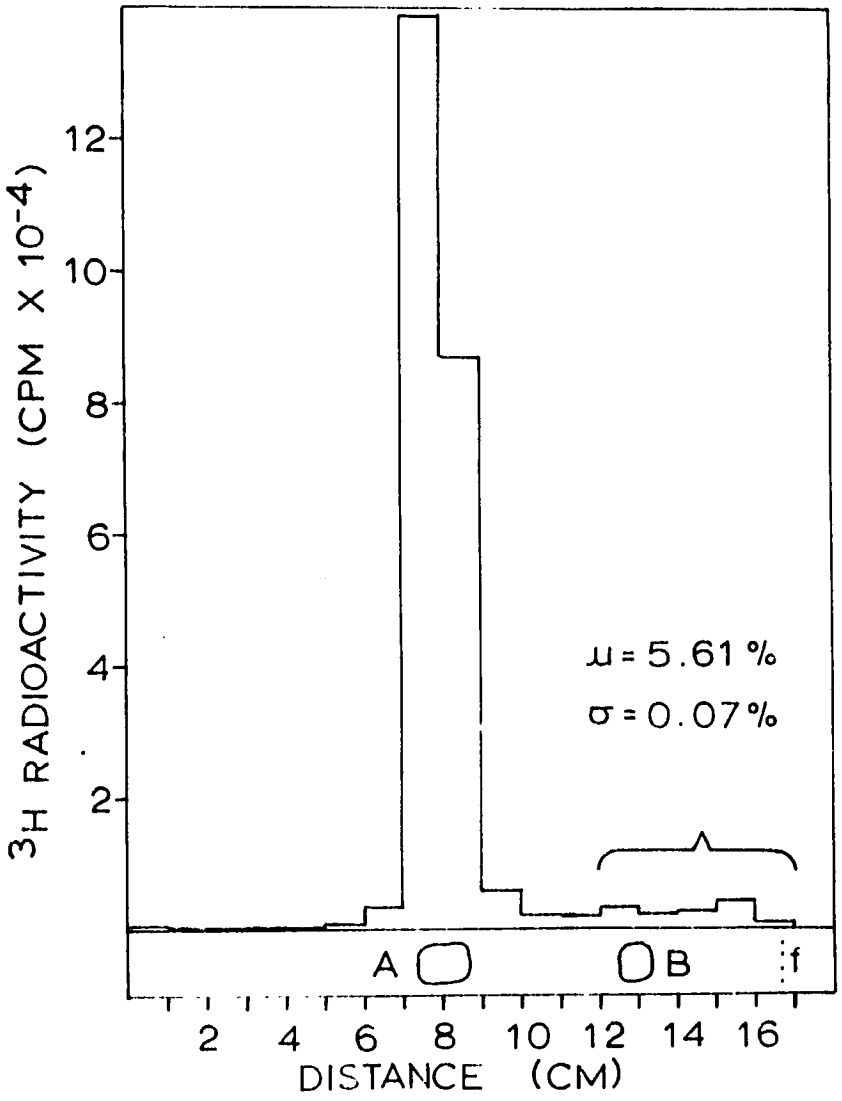


Figure 17. Thin layer radiochromatogram of Experiment #3B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

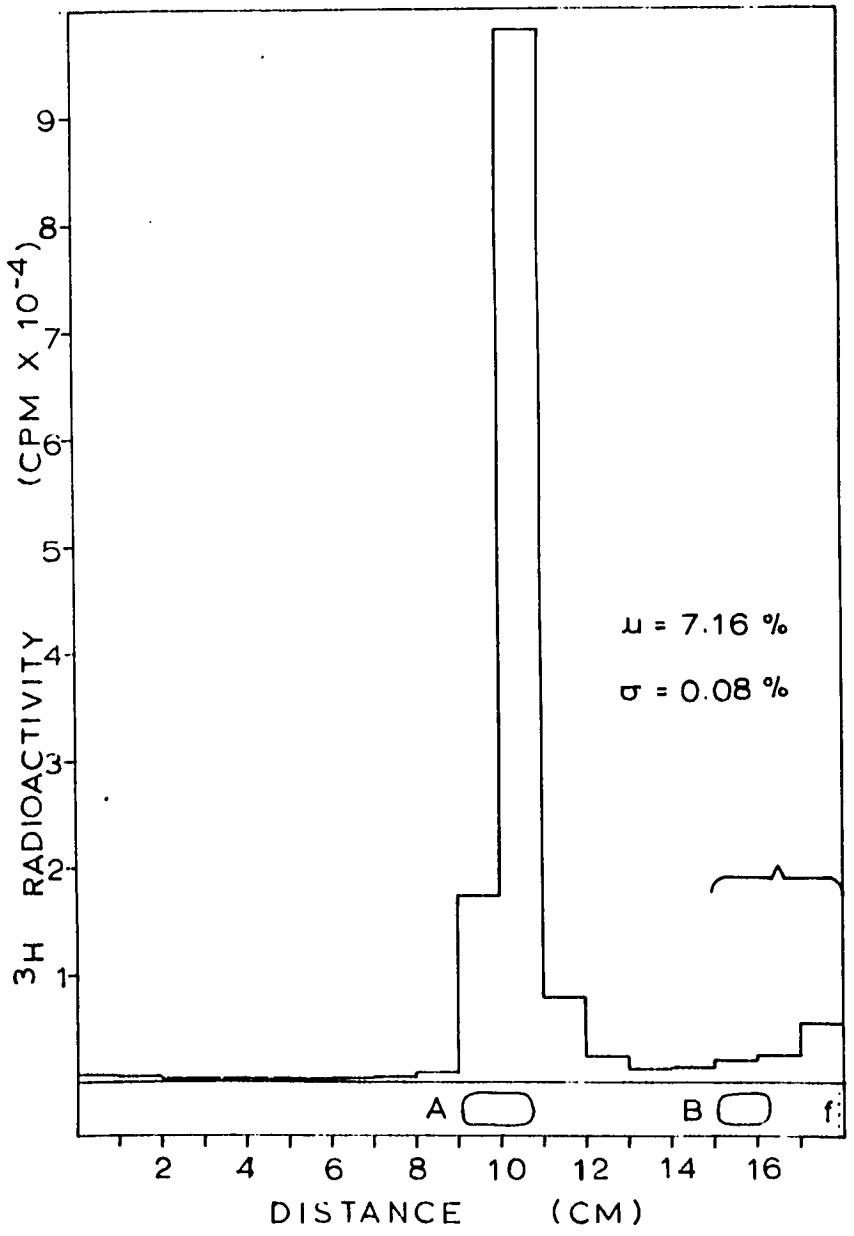


Figure 18. Thin layer radiochromatogram of Experiment #3C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

for 4 hours at 23° C. This was redissolved in ether-methanol (1:1; 500 ul) and an aliquot (100 ul) was spotted on a thin layer plate for analysis (Fig. 19).

B) The remainder of the solution was dried under a stream of nitrogen at 40° C, capped and stored for 3 days at 23° C. The sample was then redissolved in ether-methanol (1:1; 500 ul) and analyzed (Fig. 20).

EXPERIMENT #5: Noting that in Experiment #4 the sample was taken to dryness twice and that the volume differed each time, the procedure was modified as follows:

A) The stock solution (10 ul) was placed in a test tube, dried under a stream of nitrogen at 23° C, stored for 5 minutes at 23° C and redissolved in ether-methanol (1:1) for analysis (Fig. 21).

B) The stock solution (10 ul) was placed in a test tube, dried under a stream of nitrogen at 23° C, stored for 3 days at 23° C and redissolved in ether-methanol (1:1) for analysis (Fig. 22).

C) The stock solution (10 ul) was placed in a test tube, dried and stored <u>in vacuo</u> for 5 minutes and redissolved in ether-methanol (1:1) for analysis (Fig. 23).

D) The stock solution (10 ul) was placed in a test tube, dried and stored <u>in vacuo</u> for 3 days and then redissolved in ether-methanol (1:1) for analysis (Fig. 24).

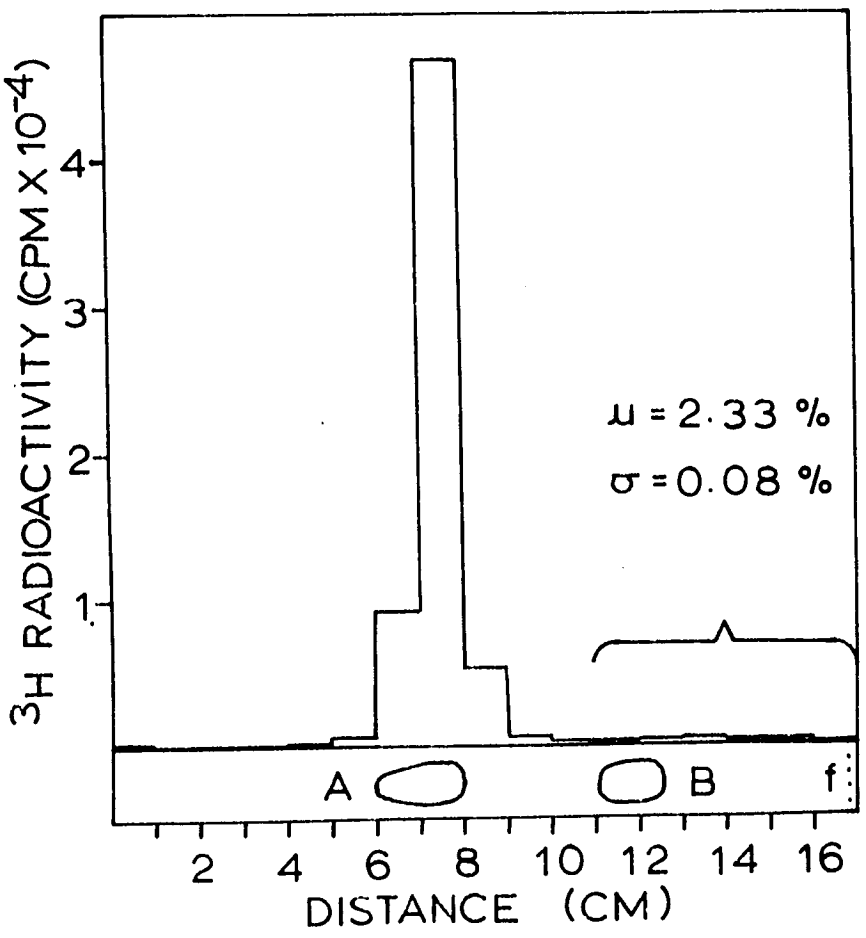


Figure 19. Thin layer radiochromatogram of Experiment #4A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

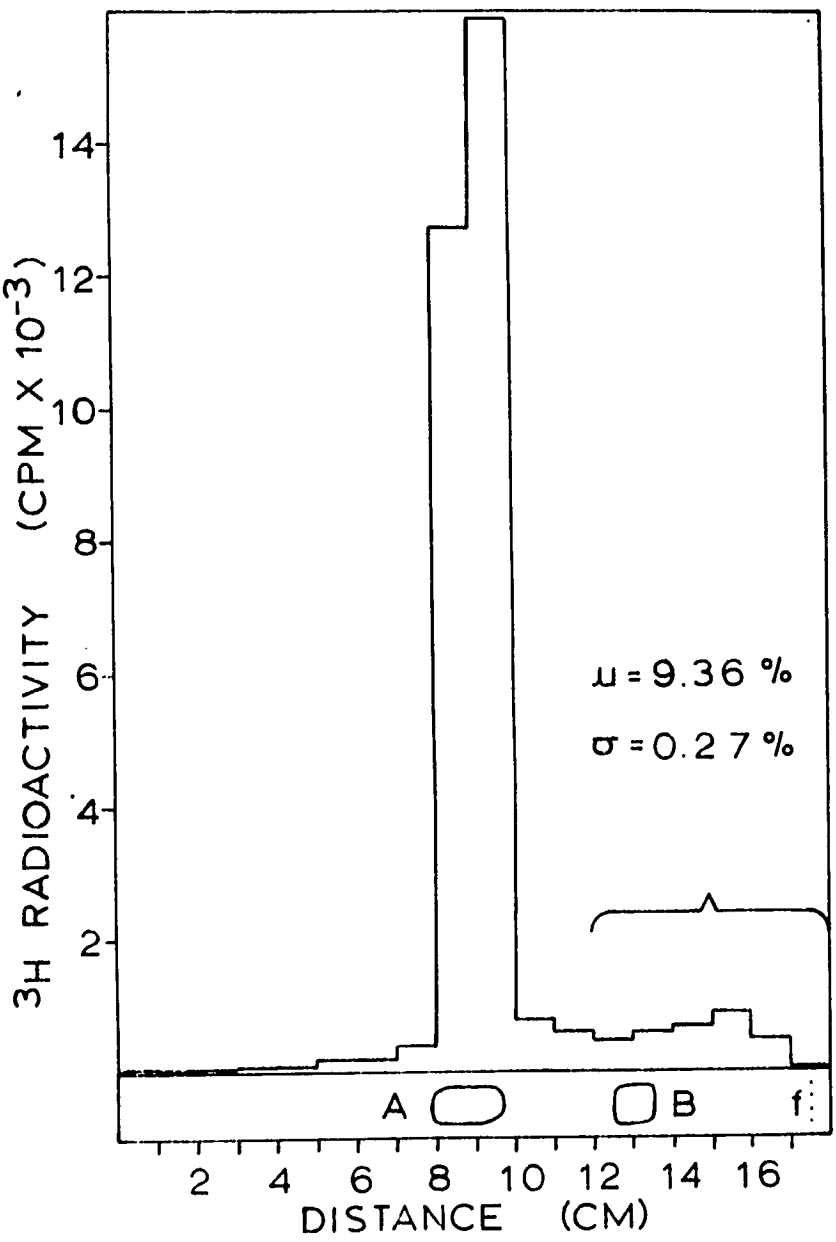


Figure 20. Thin layer radiochromatogram of Experiment #4B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

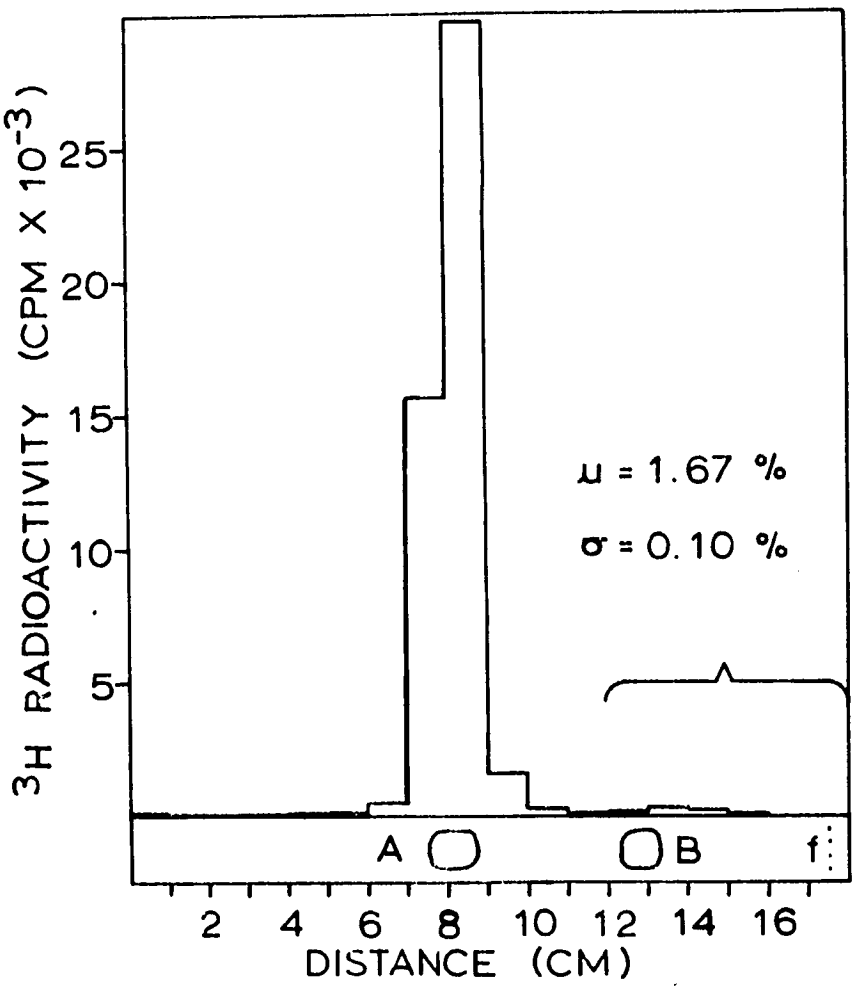


Figure 21. Thin layer radiochromatogram of Experiment #5A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

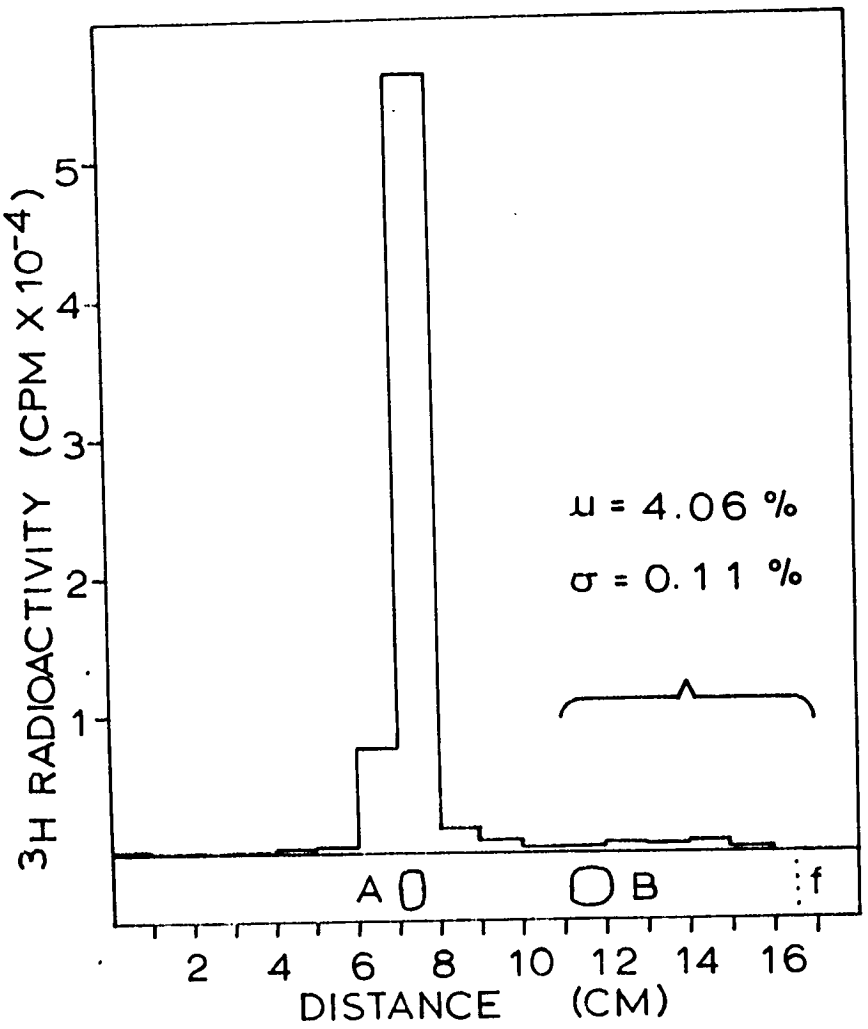


Figure 22. Thin layer radiochromatogram of Experiment #5B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25 2.5).

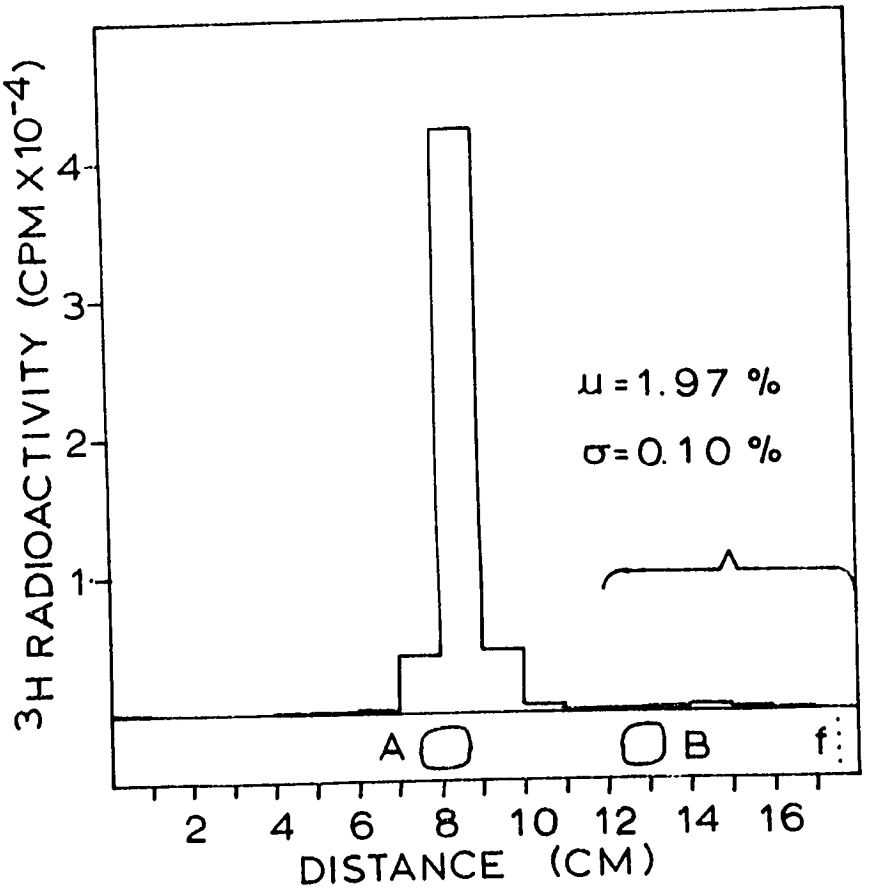


Figure 23. Thin layer radiochromatogram of Experiment #5C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

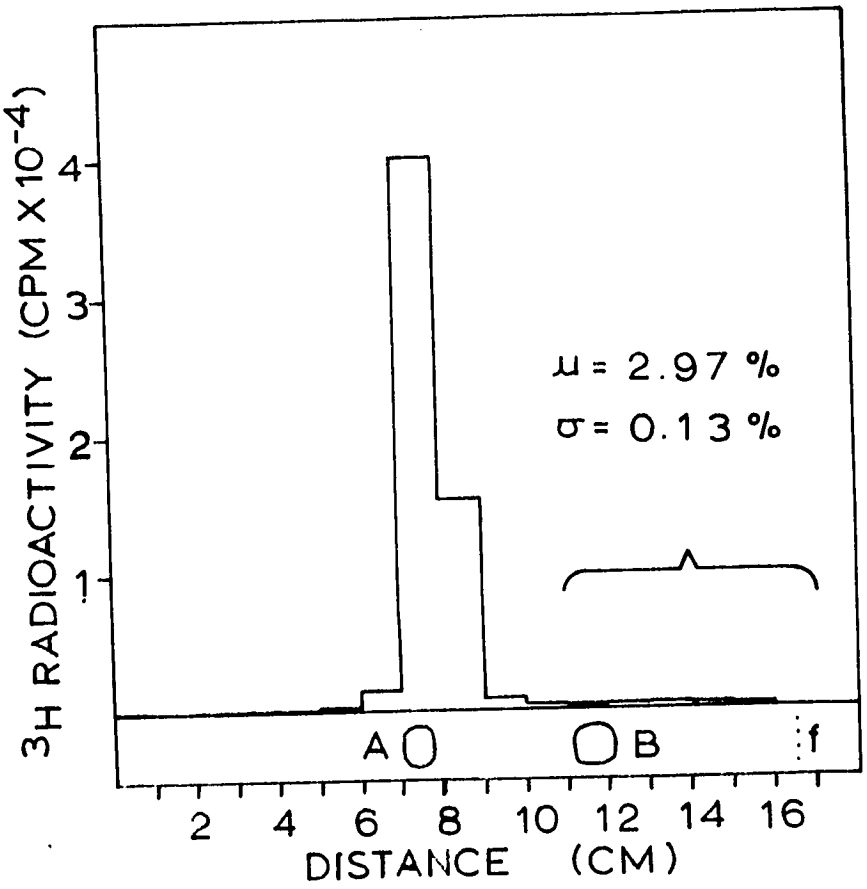


Figure 24. Thin layer radiochromatogram of Experiment #5D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

EXPERIMENT #6: A) The stock solution (10 ul) was added to ether-methanol (1:1; 3 ml), mixed and dried under a stream of nitrogen at 43° C. The residue was redissolved in ether-methanol (1:1) for analysis (Fig. 25).

B) The stock solution (10 ul) was added to chloroform (freshly distilled)-methanol (92:8; 3 ml), mixed and dried under a stream of nitrogen at 43° C. The residue was redissolved in ether-methanol (1:1) for analysis (Fig. 26).

The results of Experiments #6A and #5A indicated that larger volumes (and, therefore, larger surface areas) promoted more decomposition upon being taken to dryness. Experiments #5A, #5B, #5C, #5D and #4A indicated that decomposition increases with time when stored in a dry state. The combination of these two principles explained why the decomposition was so large in Experiment #4B. Experiments #5C and #5D indicated that drying and storing <u>in vacuo</u> may be slightly more advantageous only when samples were stored in a dry state over a long period of time. Experiments #6A and #6B showed that the use of chloroform made no significant difference in stability.

Together these results showed that whenever [4,5-$^{3}H_{2}$]-dihydrosphingosine in a relatively large volume of solvent (as when eluted from a column or from a thin layer adsorbent) was taken to dryness, a significant decomposition must have occurred.

The following experiments were intended to determine if chromatographic adsorbents added a further factor to the decomposition process.

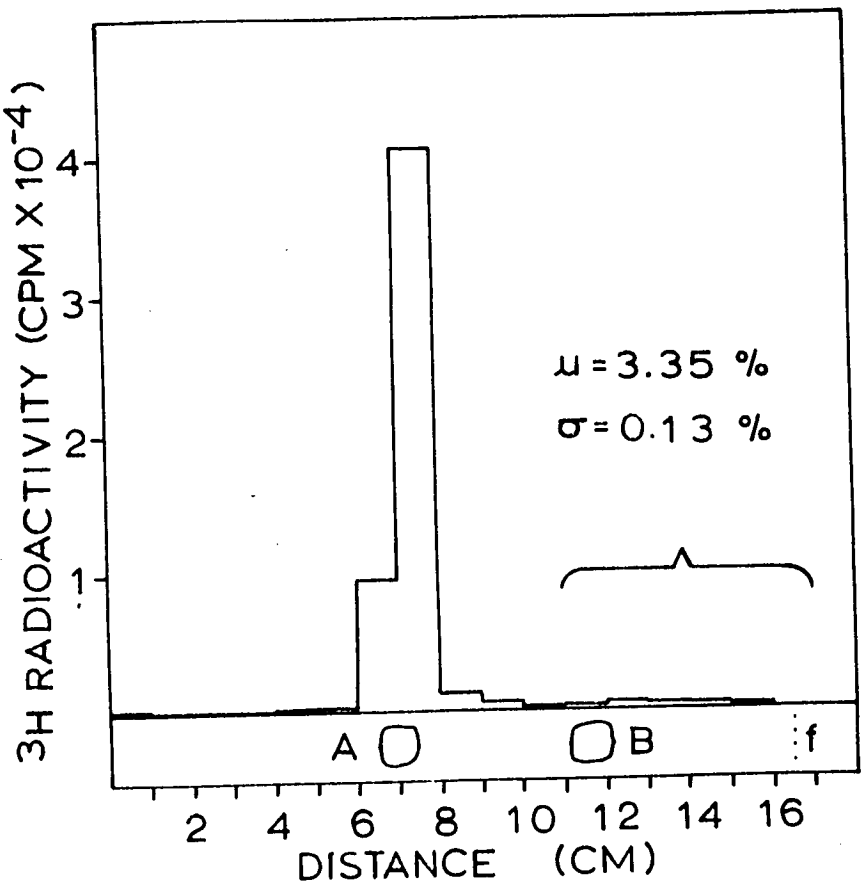


Figure 25. Thin layer radiochromatogram of Experiment #6A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

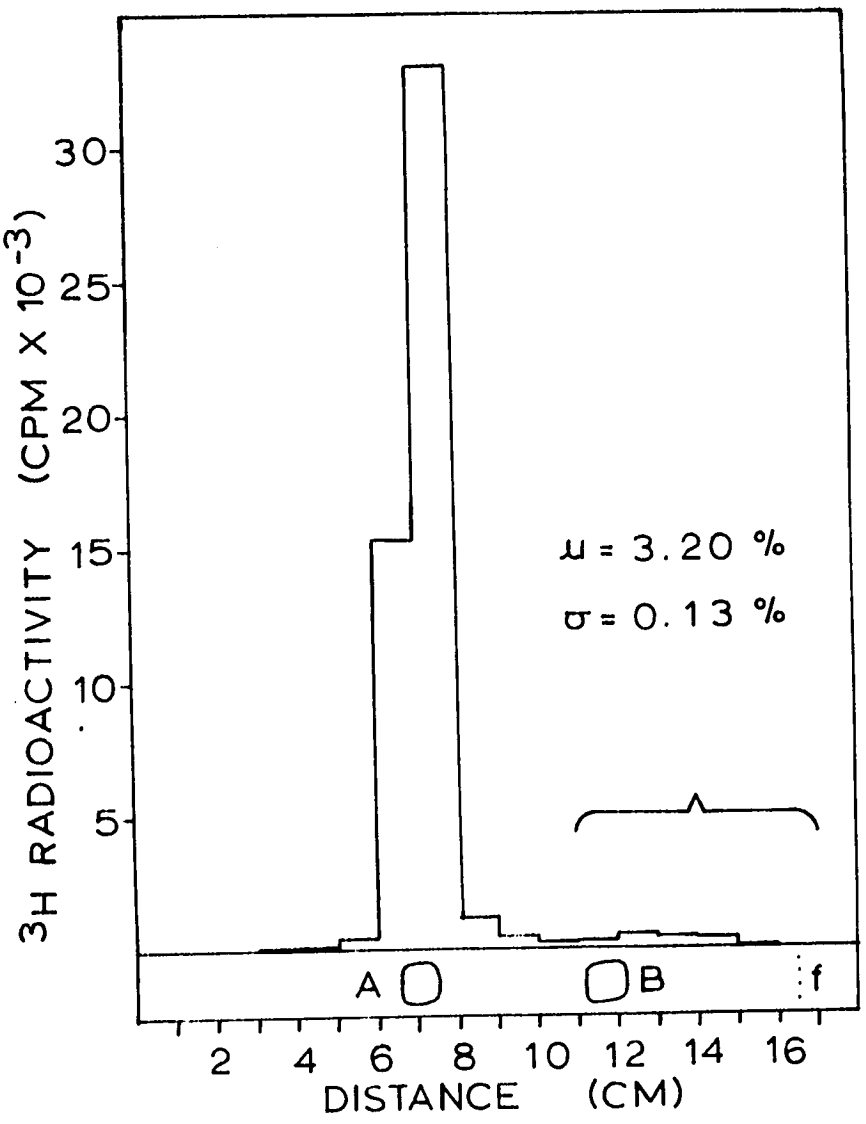


Figure 26. Thin layer radiochromatogram of Experiment #6B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

EXPERIMENT #7: A) The stock solution (20 ul) was added to ether-methanol (1:1; 3 ml) and shaken vigorously in an incubator at 23° C for 4 hours and and the resulting solution analyzed (Fig. 27).

B) After a total of three days of incubation, the solution was once again analyzed (Fig. 28).

C) The stock solution was added to chloroform (freshly distilled)-methanol (92:8; 3 ml) and shaken vigorously at 23° C for 4 hours and analyzed (Fig. 29).

D) After a total of three days of incubation, this solution was once again analyzed (Fig. 30).

EXPERIMENT #8: A) The stock solution (20 ul) was spotted on a Silica Gel G plate, allowed to dry and then scraped along with the adsorbent (0.5 gram) into a test tube. To this was added ether-methanol (1:1; 3 ml) and the mixture was incubated for 4 hours at 23° C. An aliquot was then analyzed (Fig. 31).

B) After a total of three days of incubation, the solution was once again analyzed (Fig. 32).

C) The stock solution (20 ul) was added to a mixture of Silica Gel G (0.5 gram) in ether-methanol (1:1; 3 ml) and incubated for 4 hours at 23° C and then analyzed (Fig. 33).

D) After a total of three days of incubation, this solution was once again analyzed (Fig. 34).

EXPERIMENT #9: A) The stock solution (20 ul) was added to a mixture of silicic acid (0.5 gram) in ether-methanol (1:1; 3 ml) and incubated for 4 hours at 23° C and analyzed (Fig. 35).

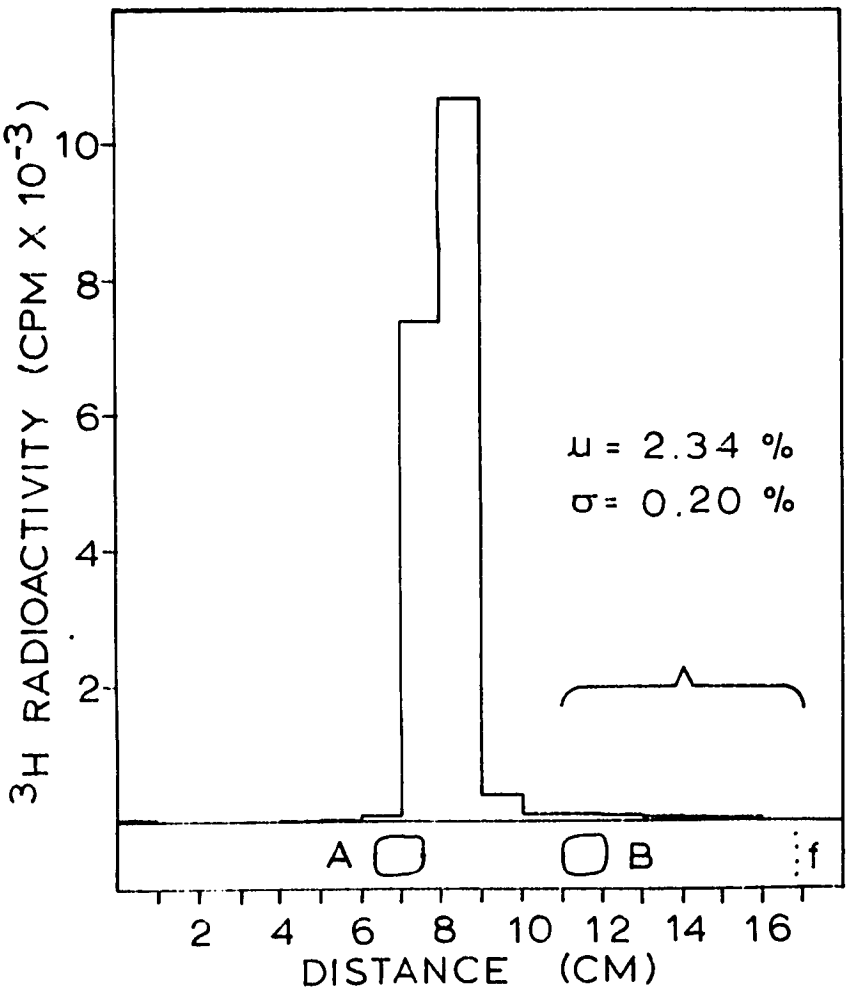


Figure 27. Thin layer radiochromatogram of Experiment #7A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

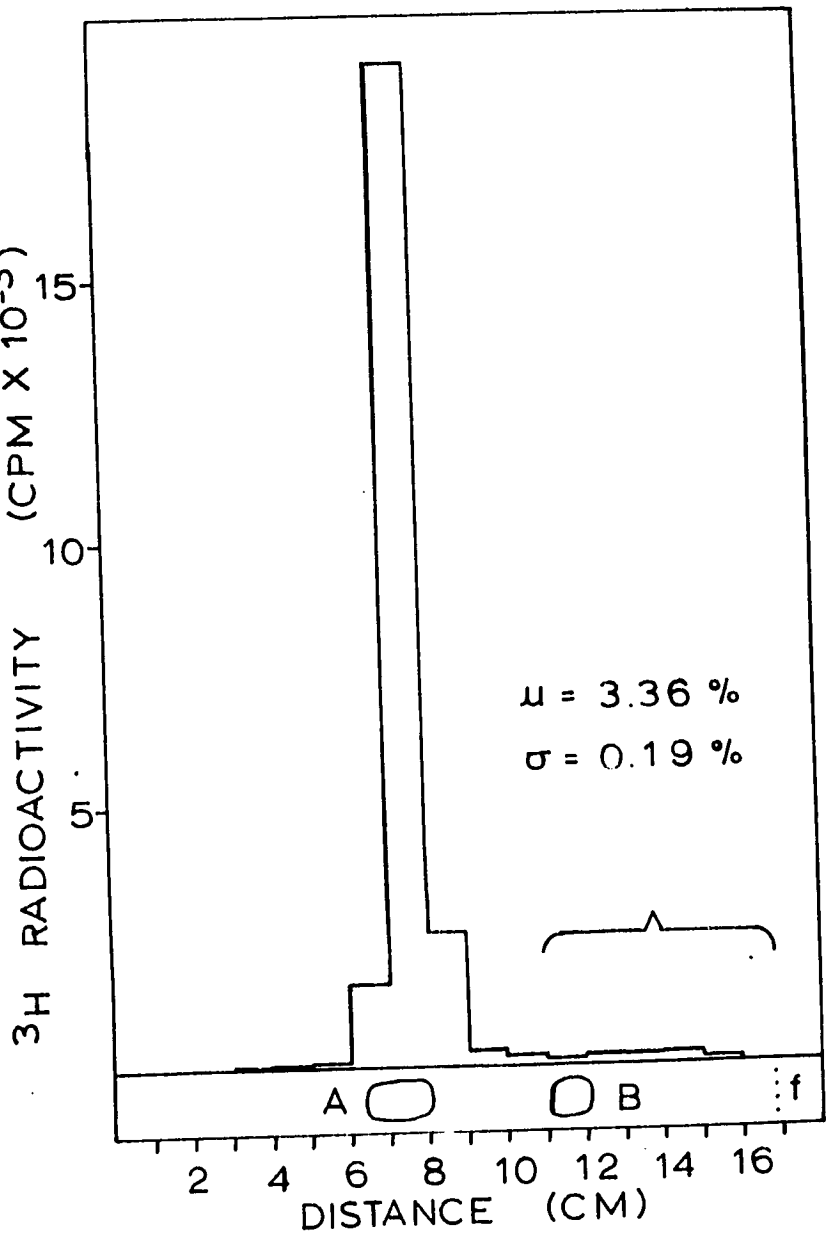


Figure 28. Thin layer radiochromatogram of Experiment #7B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

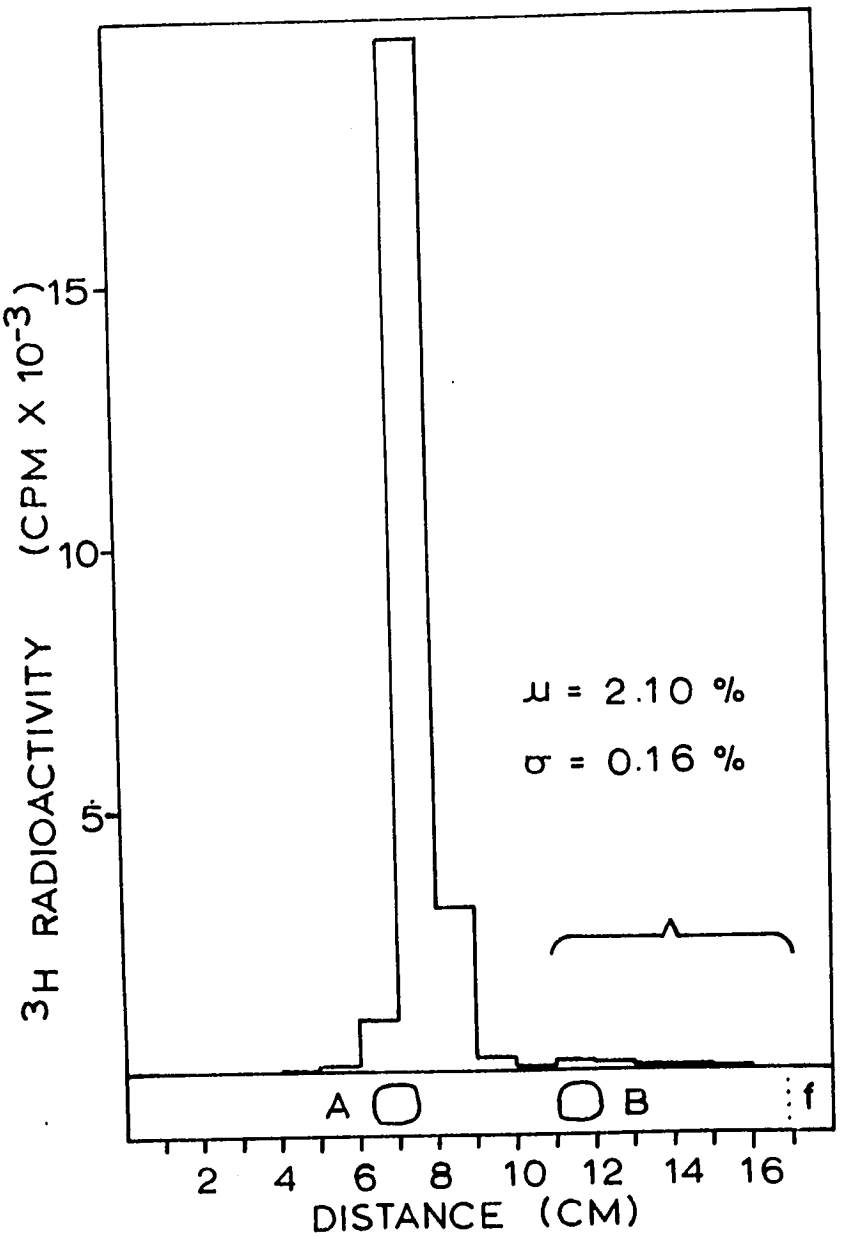


Figure 29. Thin layer radiochromatogram of Experiment #7C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

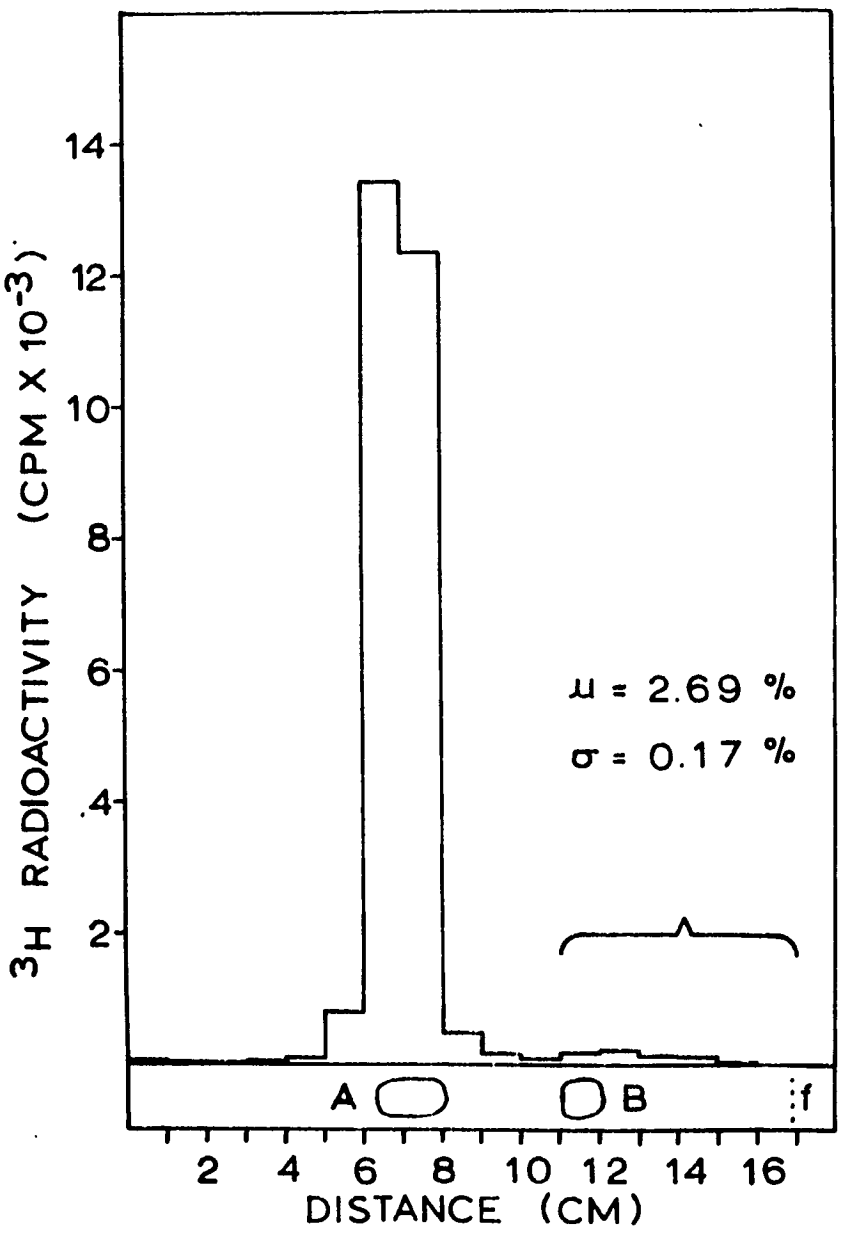


Figure 30. Thin layer radiochromatogram of Experiment #7D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

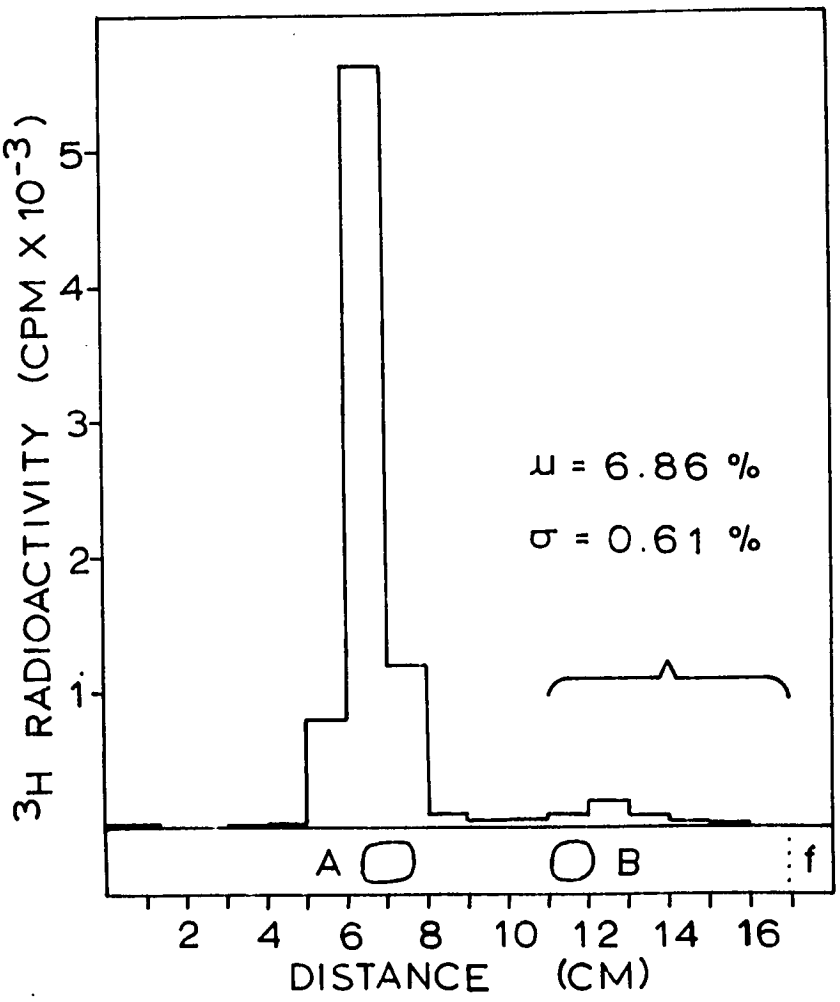


Figure 31. Thin layer radiochromatogram of Experiment #8A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

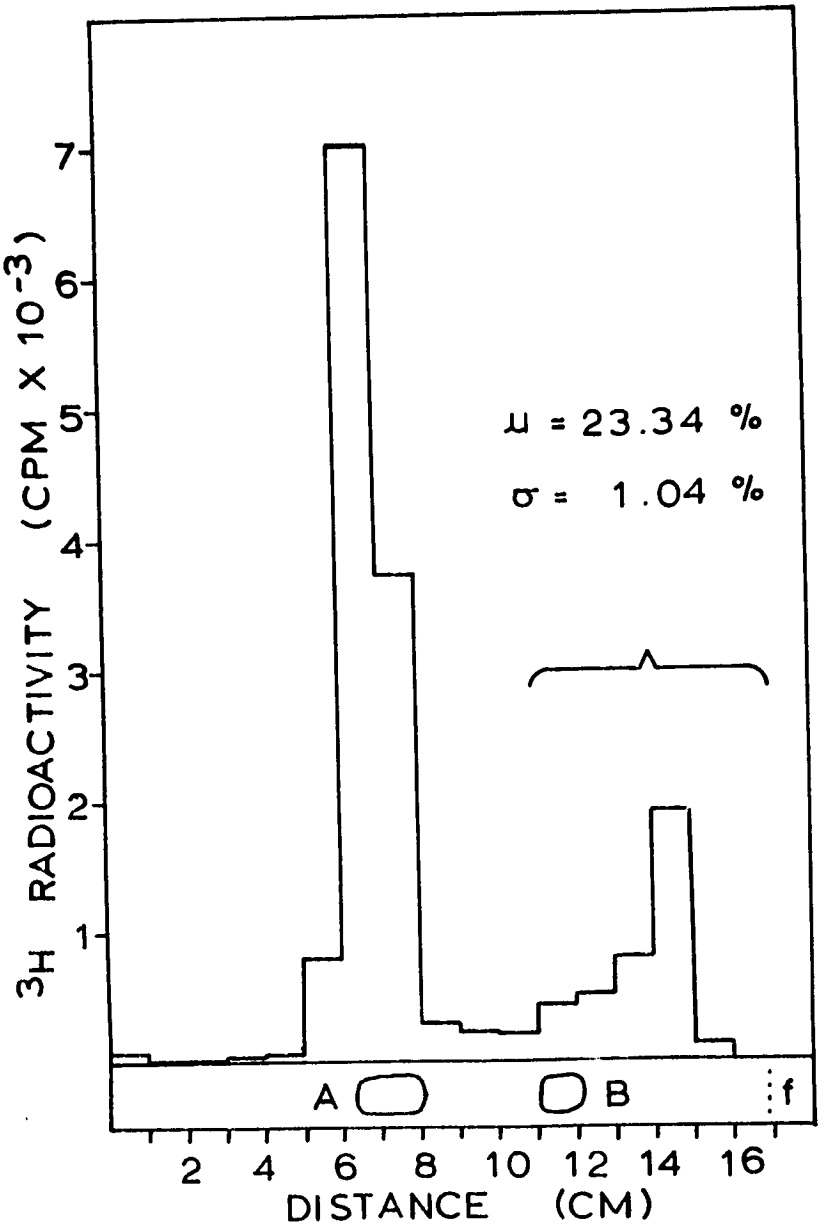


Figure 32. Thin layer radiochromatogram of Experiment #8B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

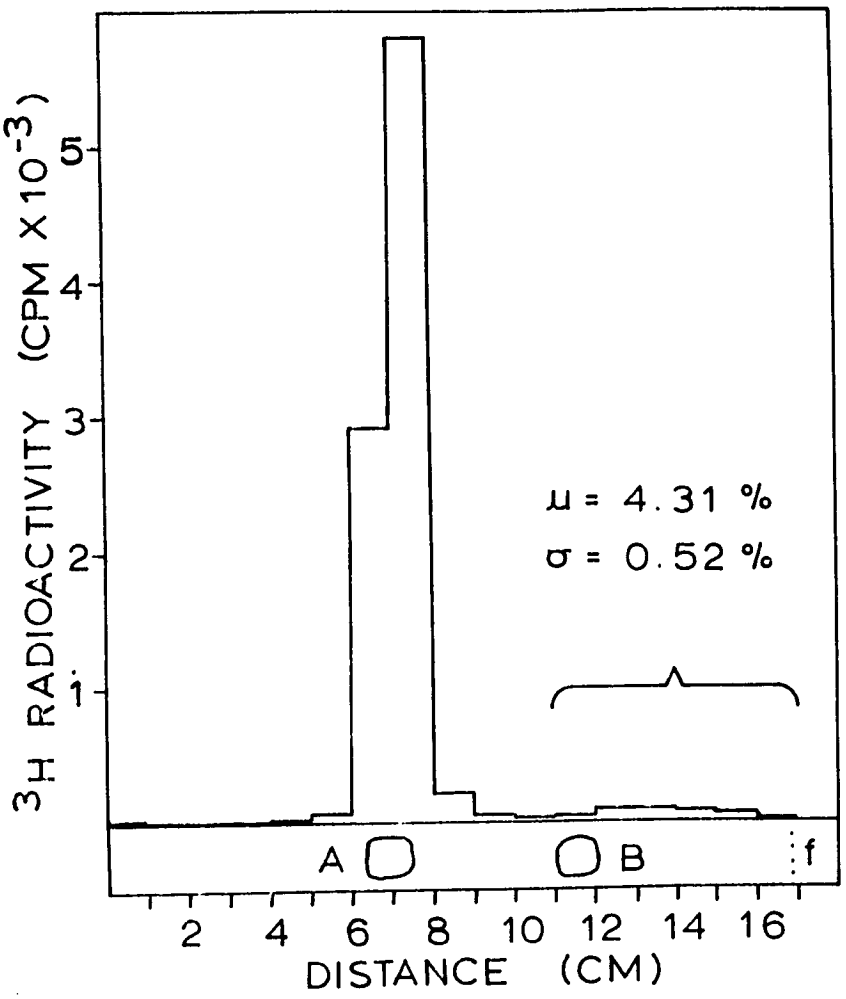


Figure 33. Thin layer radiochromatogram of Experiment #8C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

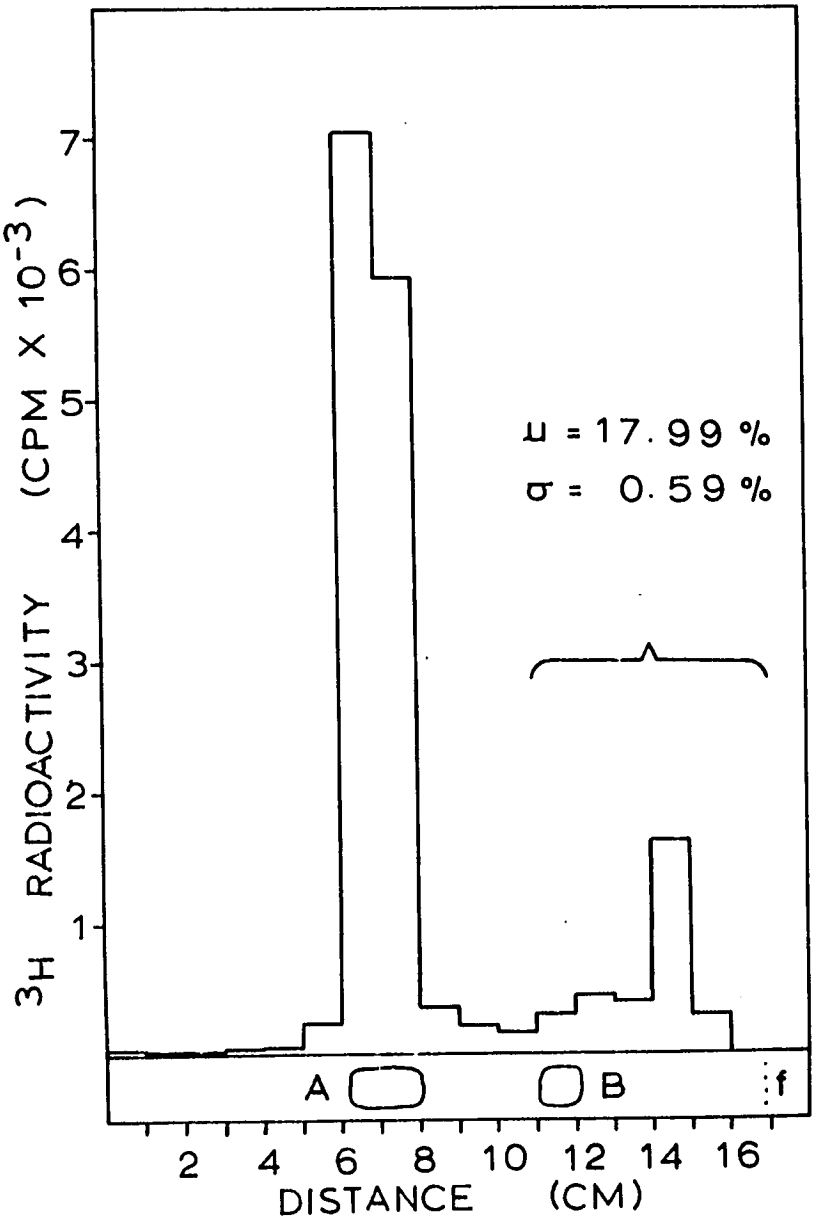


Figure 34. Thin layer radiochromatogram of Experiment #8D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

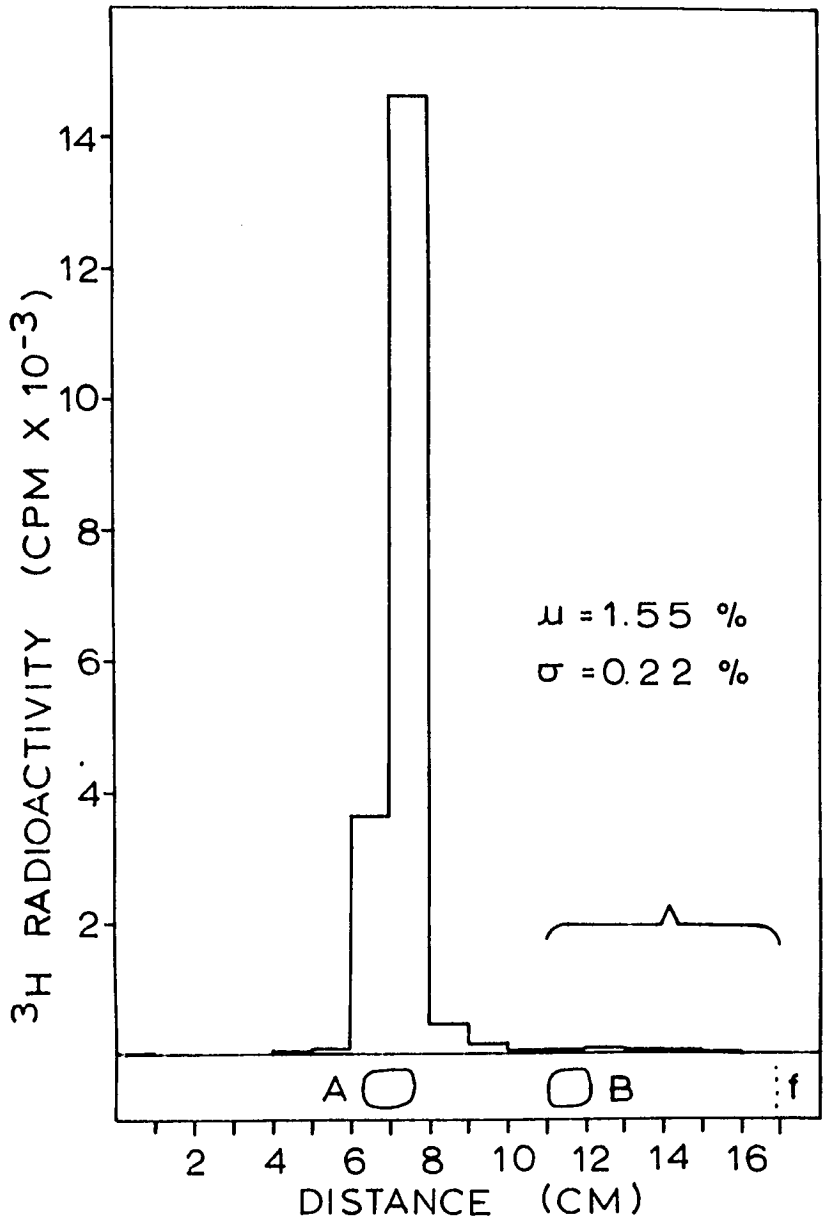


Figure 35. Thin layer radiochromatogram of Experiment # 9A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

B) After a total of 3 days of incubation, the solution was once again analyzed (Fig. 36).

C) The stock solution (20 ul) was added to a mixture of Unisil (0.5 gram) in ether-methanol (1:1; 3 ml) and incubated for 4 hours at 23° C. An aliquot of this was analyzed (Fig. 37).

D) After a total of 3 days of incubation, this solution was once again analyzed (Fig. 38).

EXPERIMENT #10: A) The stock solution (20 ul) was added to silicic acid (0.5 gram) in chloroform (freshly distilled)-methanol (92:8; 3 ml) and incubated for 4 hours at 23° C. An aliquot of this solution was analyzed (Fig. 39).

B) After a total of 3 days of incubation, this solution was once again analyzed (Fig. 40).

C) After 5 days of incubation, the ratio of chloroform to methanol was brought to 1:1 by the addition of methanol (2.52 ml) and the supernatant was analyzed (Fig. 41).

D) The supernatant of #10C was decanted and filtered through Whatman #1 paper. This was dried under a stream of nitrogen at 50° C and redissolved in ether-methanol (1:1) for analysis (Fig. 42).

EXPERIMENT #11: A) The stock solution (20 ul) was added to Unisil (0.5 gram) in chloroform (freshly distilled)-methanol (92:8; 3 ml) and incubated for 4 hours at 23° C (Fig. 43).

B) After a total of 3 days of incubation, the solution was once again analyzed (Fig. 44).

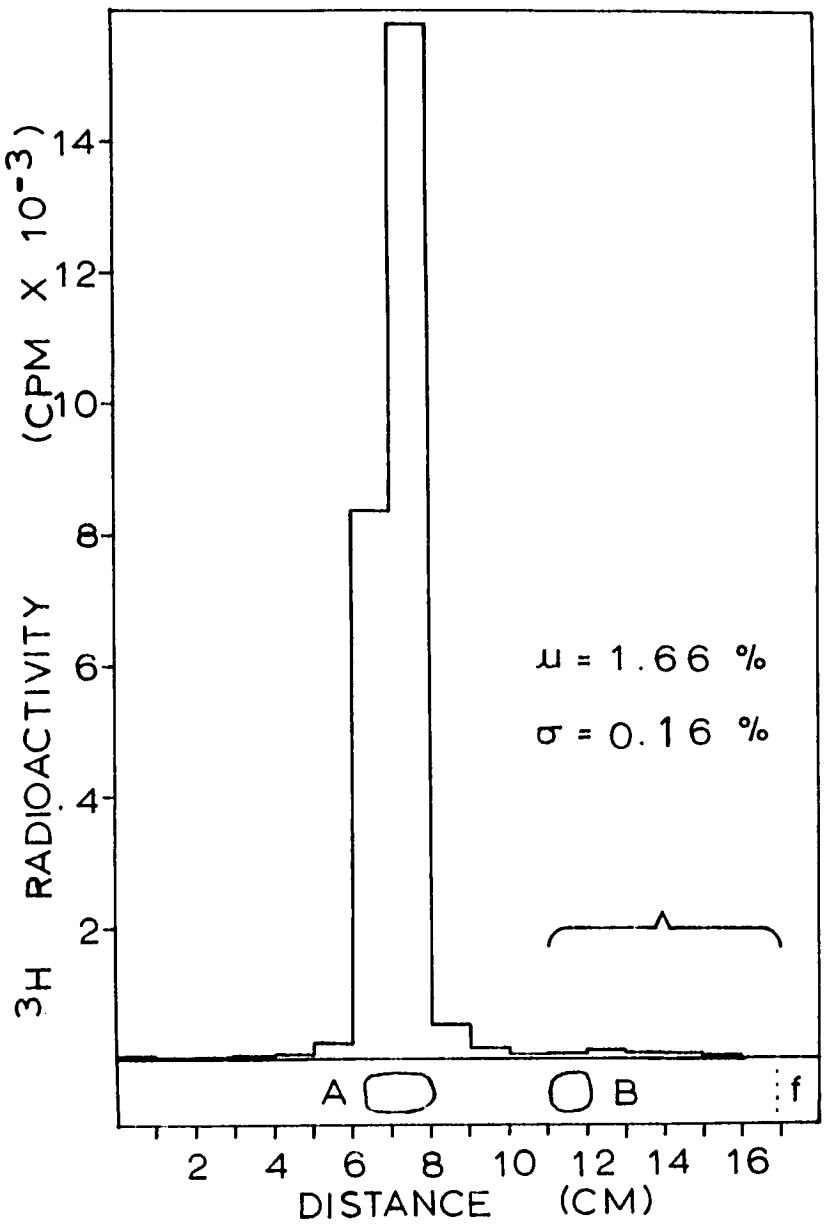


Figure 36. Thin layer radiochromatogram of Experiment #9B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

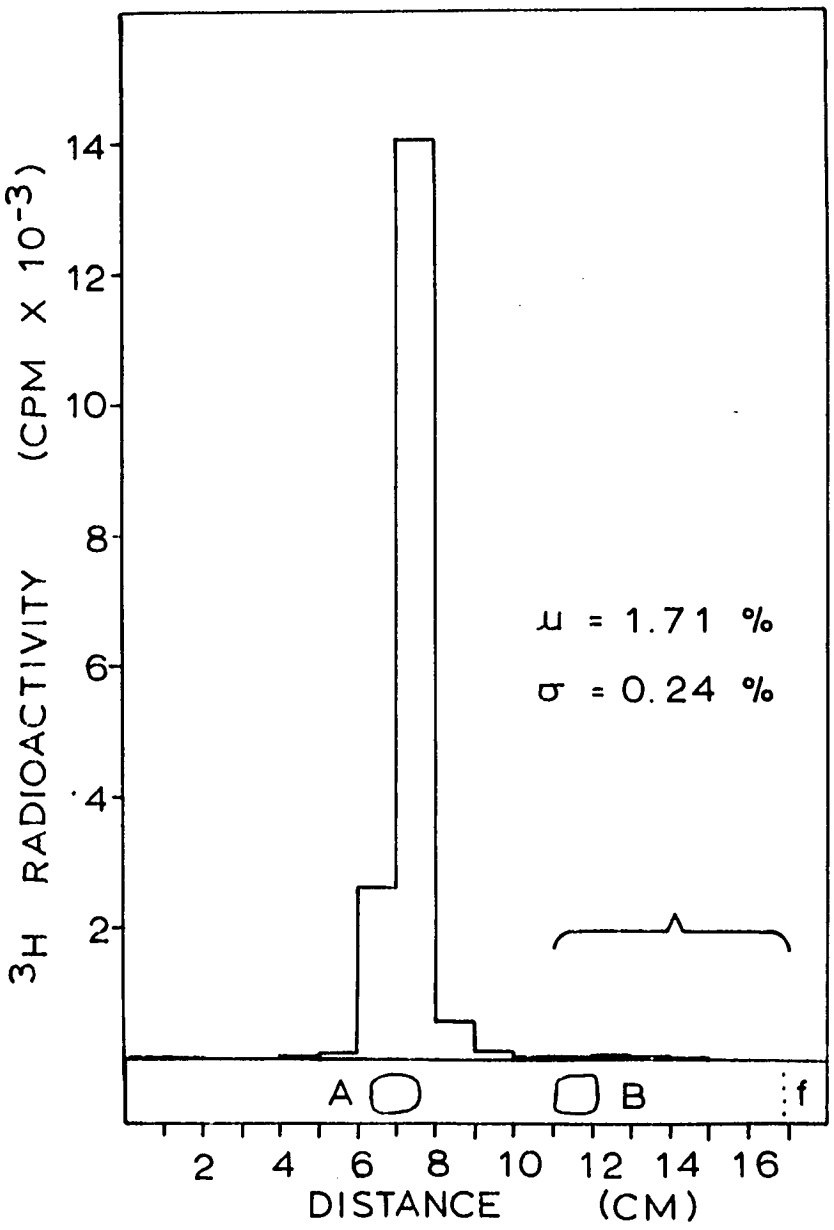


Figure 37. Thin layer radiochromatogram of Experiment #9C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

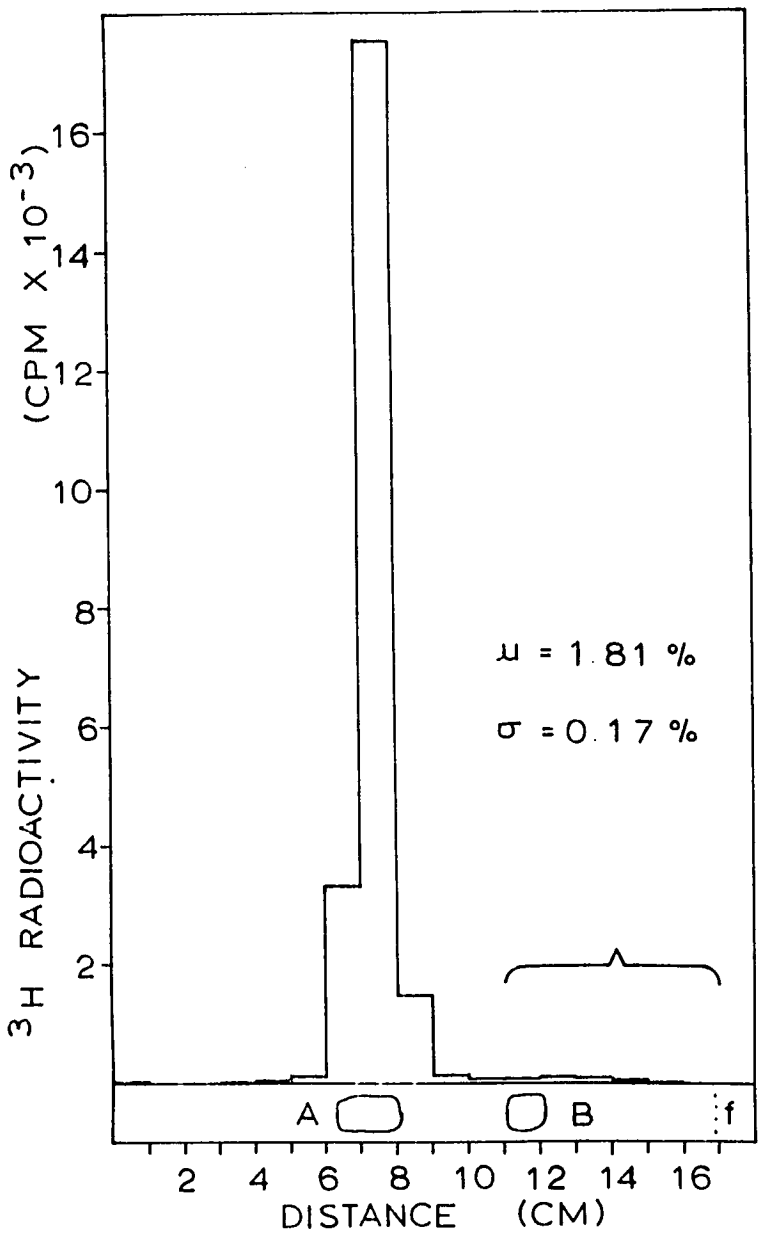


Figure 38. Thin layer radiochromatogram of Experiment #9D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

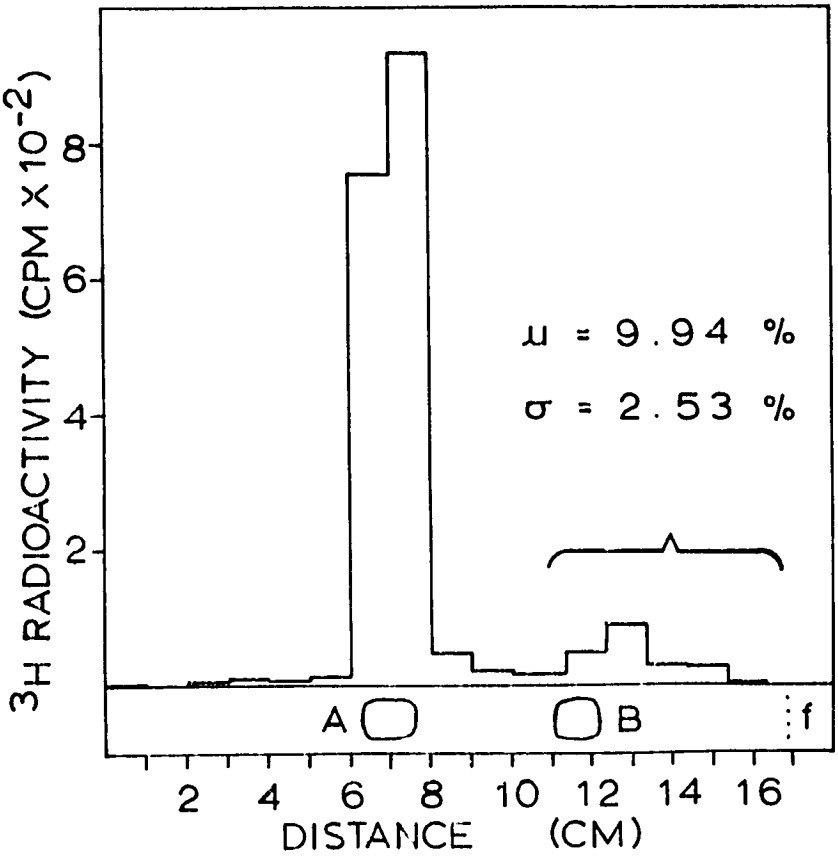


Figure 39. Thin layer radiochromatogram of Experiment #10A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

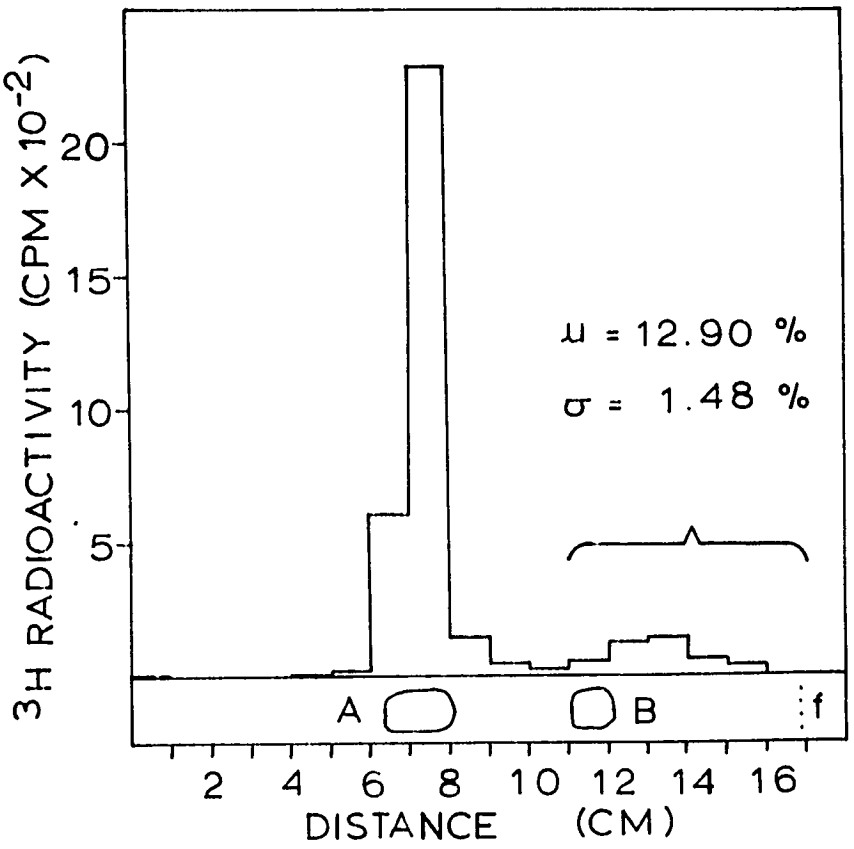


Figure 40. Thin layer radiochromatogram of Experiment #10B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

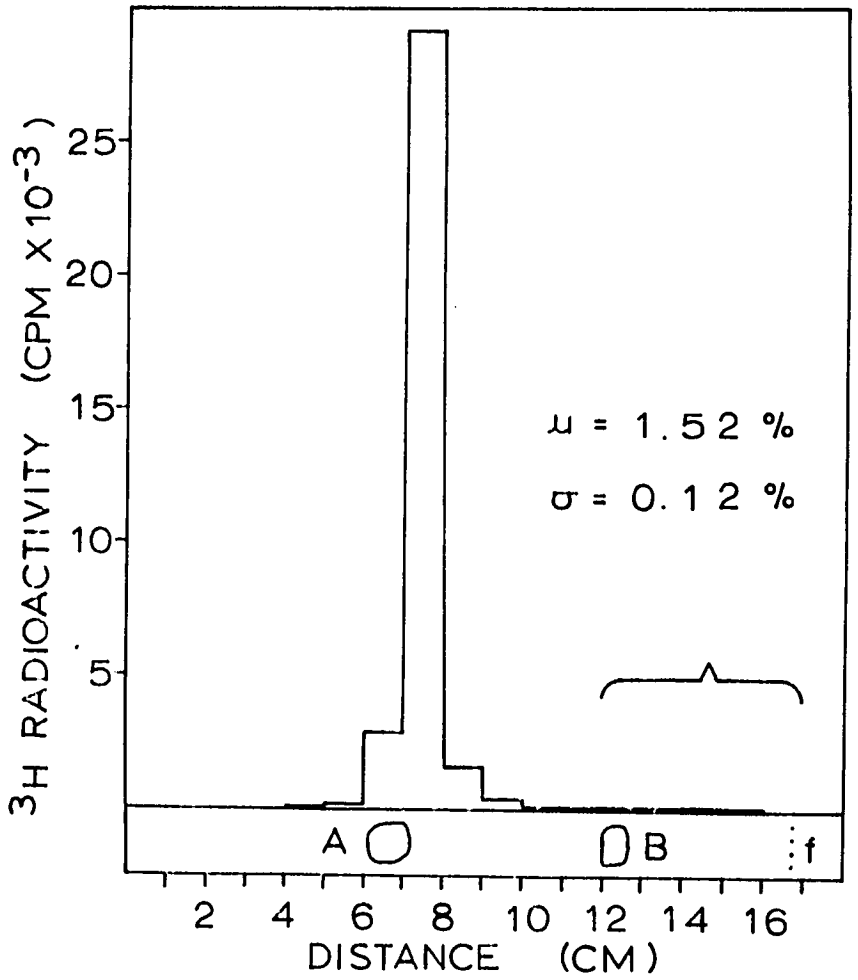


Figure 41. Thin layer radiochromatogram of Experiment #10C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

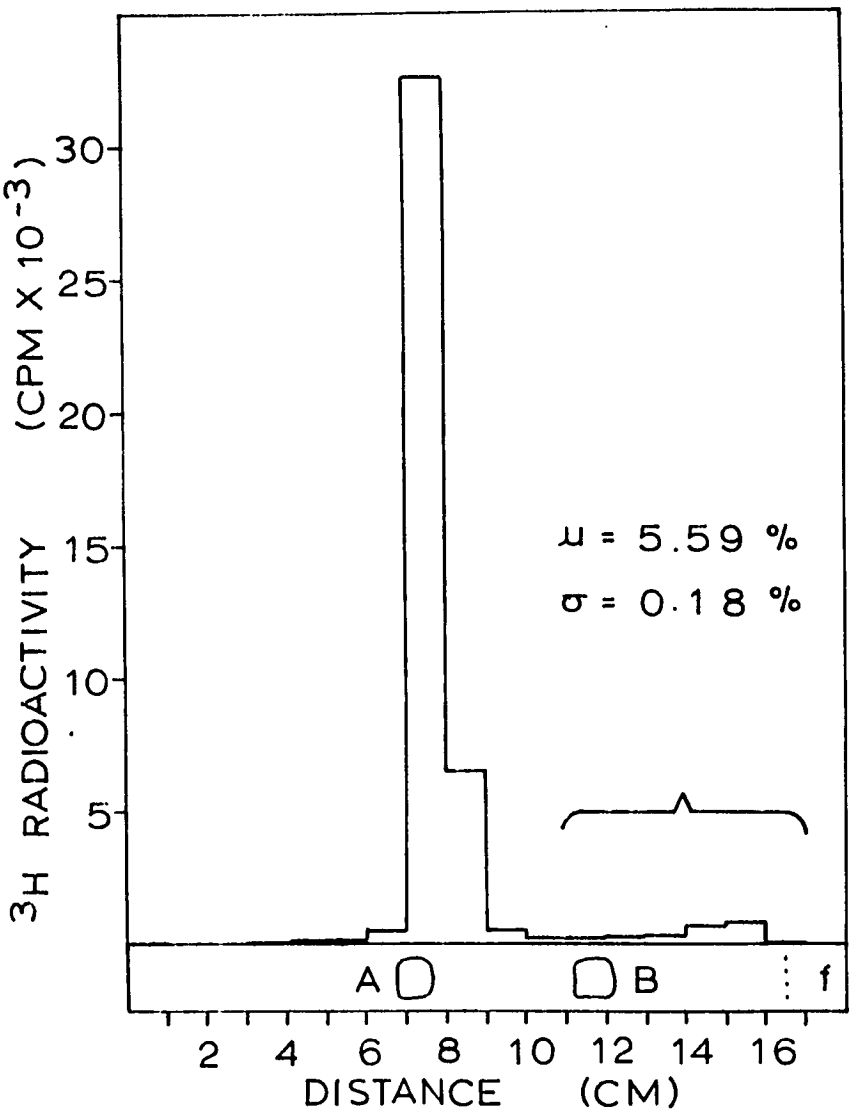


Figure 42. Thin layer radiochromatogram of Experiment #10D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

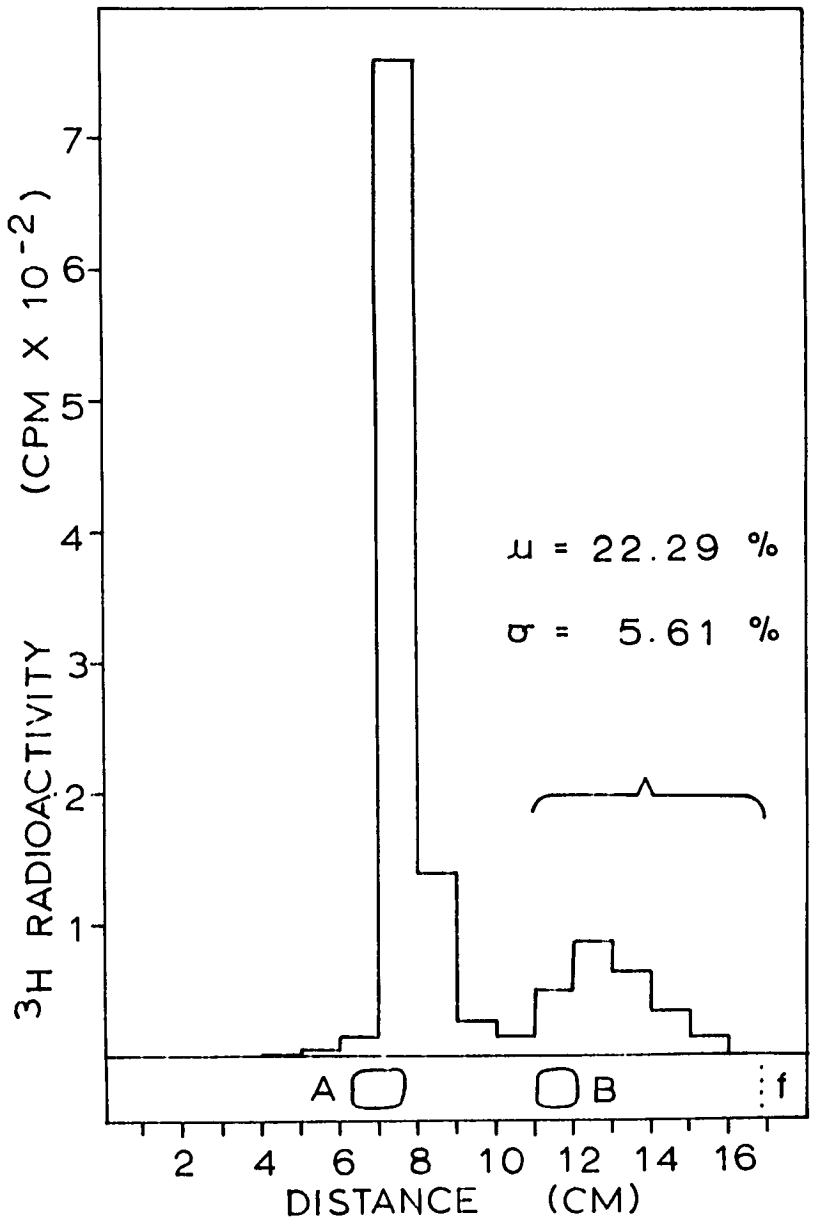


Figure 43. Thin layer radiochromatogram of Experiment #11A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

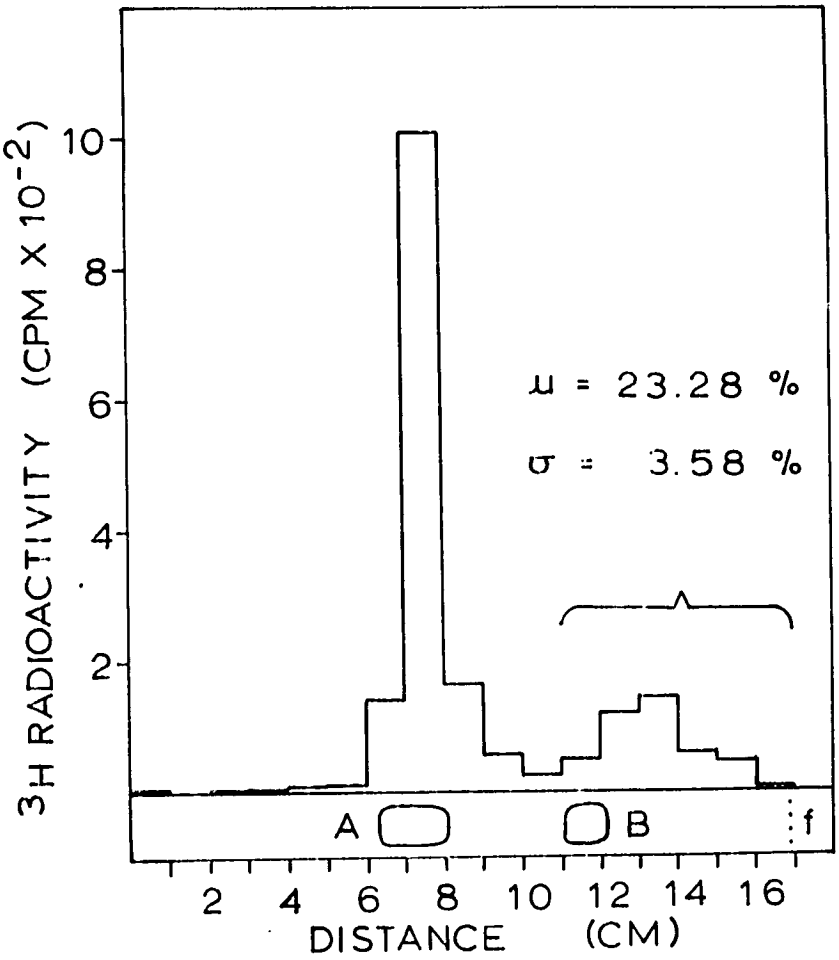


Figure 44. Thin layer radiochromatogram of Experiment #11B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

C) After 5 days of incubation, the ratio of chloroform to methanol was brought to 1:1 by the addition of methanol (2.52 ml) and the supernatant was then analyzed (Fig. 45).

D) The supernatant of #11C was decanted and filtered through Whatman #1 paper. This was dried under a stream of nitrogen at 50° C and redissolved in ether-methanol (1:1) for analysis (Fig. 46).

EXPERIMENT #12: A) Pyrex brand glass wool (0.111 gram; dated 1969; pH of 0.216 gram in 30 ml of glass distilled water = 7.6) was placed in ether-methanol (1:1; 3 ml) and the stock solution (20 ul) was added and incubated for 4 hours at 23° C. An aliquot of this solution was analyzed (Fig. 47).

B) After a total of 3 days of incubation, the solution was once again analyzed (Fig. 48).

C) Pyrex brand glass wool (0.213 gram; a fresh box dated January 13, 1972) was placed in ether-methanol (1:1; 3 ml) and the stock solution (20 ul) was added and incubated for 4 hours at 23° C. An aliquot of this solution was analyzed (Fig. 49).

D) After a total of 3 days of incubation, the solution was once again analyzed (Fig. 50).

Experiment #7 indicated that the different solvents used made little overall difference in stability of the substrate. However, it was obvious in this experiment that some decomposition had occurred compared to the original stock solution. This may have been the re-

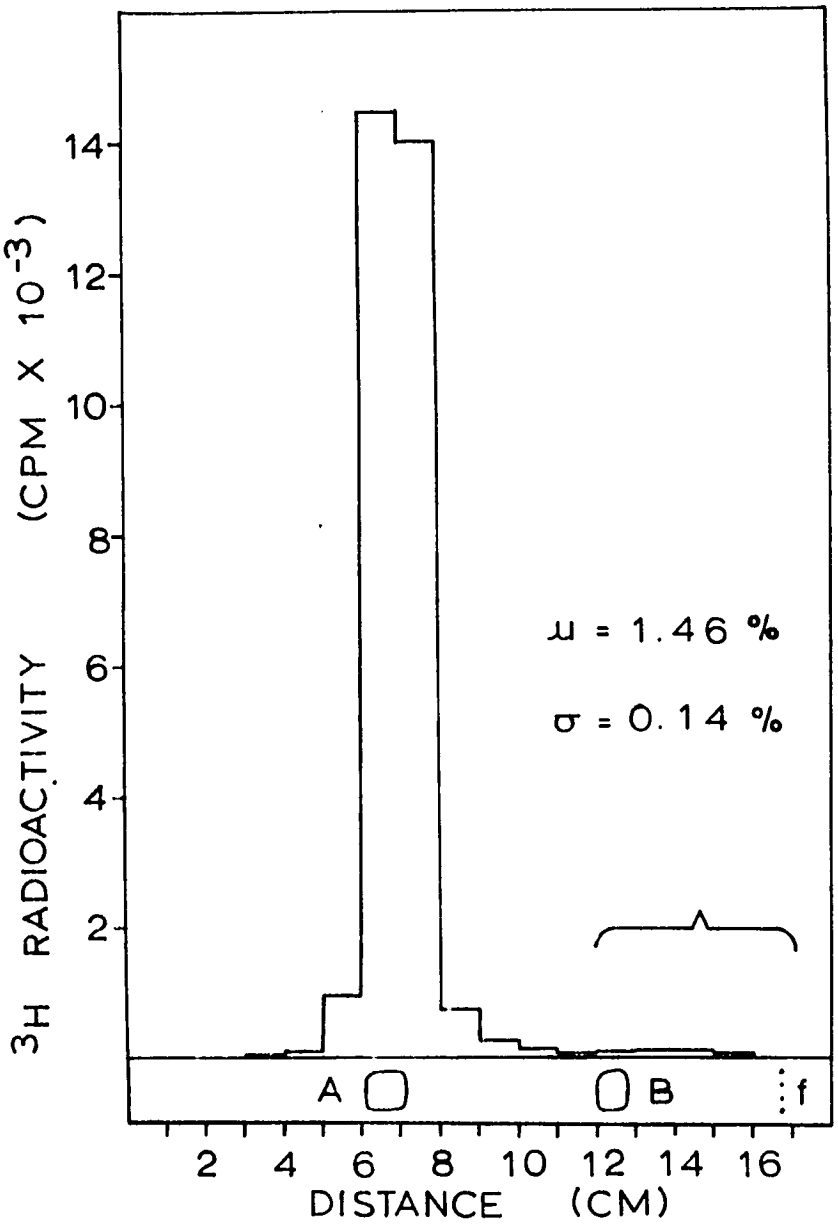


Figure 45. Thin layer radiochromatogram of Experiment #11C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

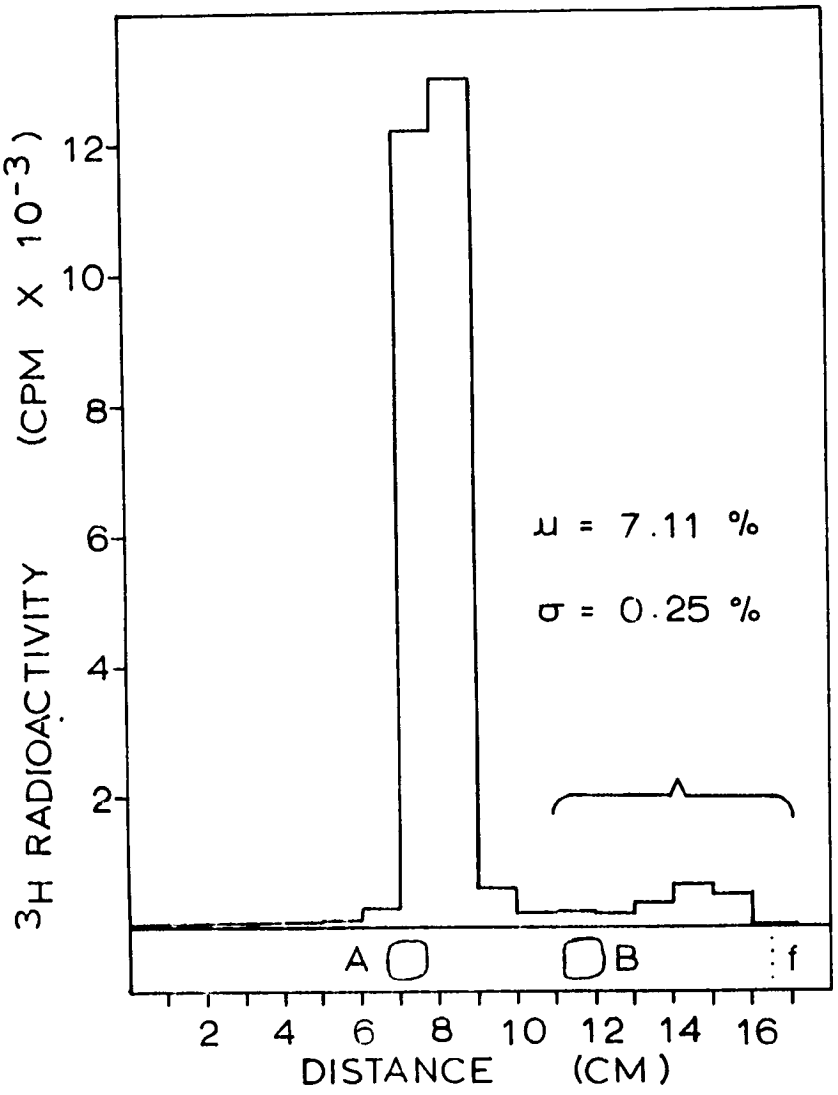


Figure 46. Thin layer radiochromatogram of Experiment #11D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

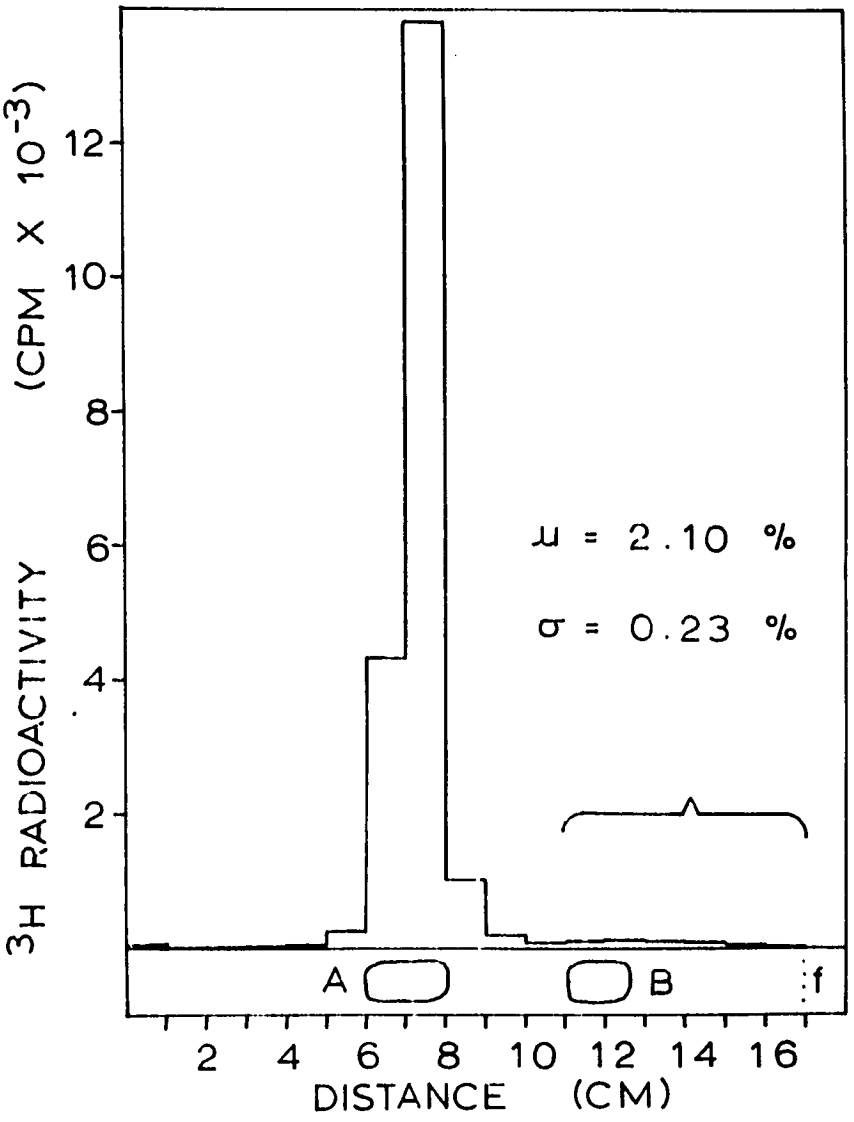


Figure 47. Thin layer radiochromatogram of Experiment #12A. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

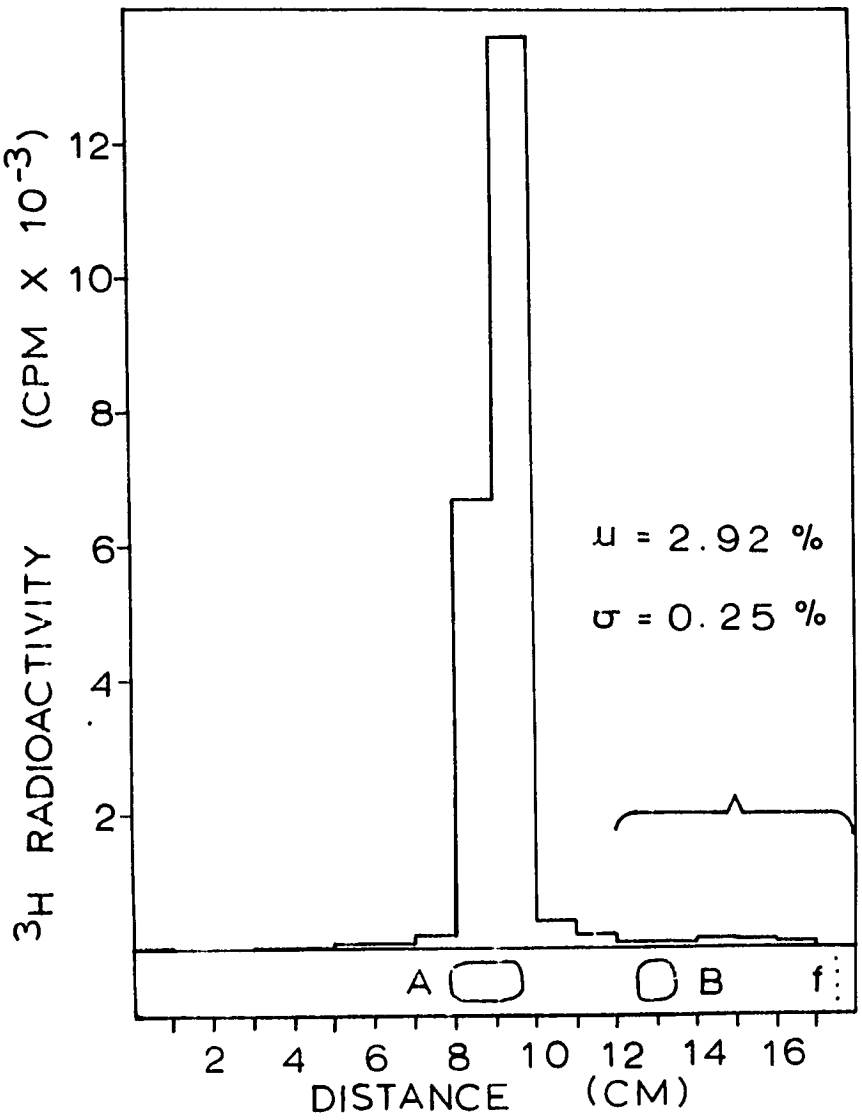


Figure 48. Thin layer radiochromatogram of Experiment #12B. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

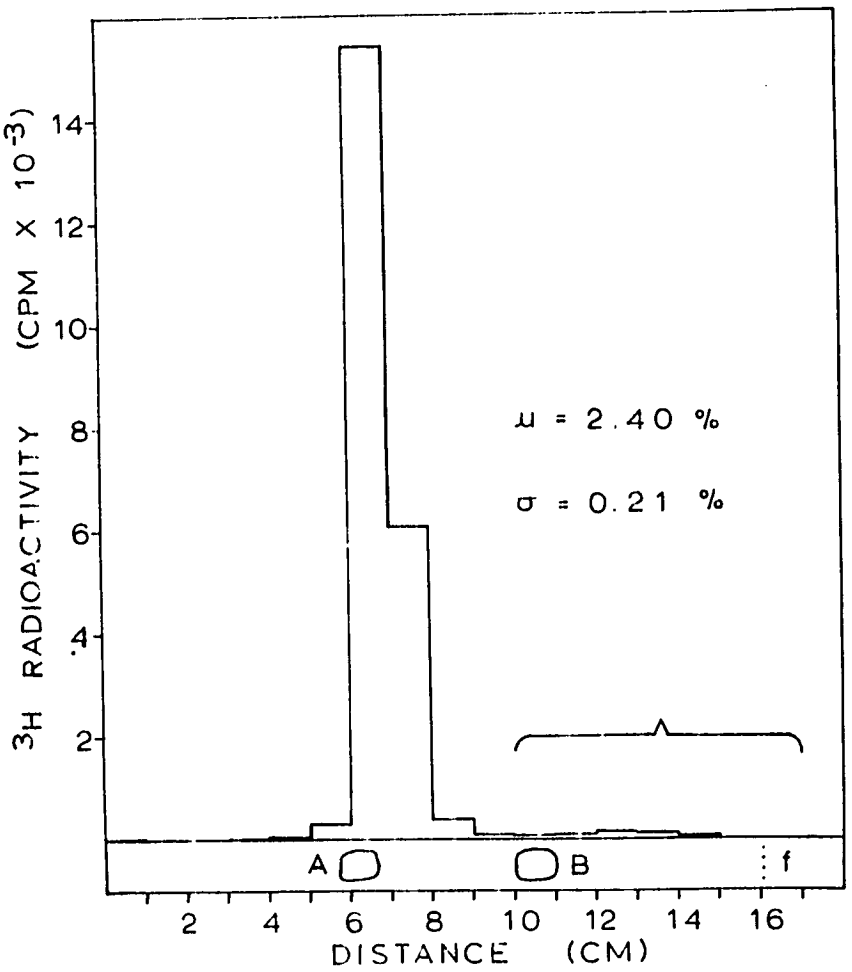


Figure 49. Thin layer radiochromatogram of Experiment #12C. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

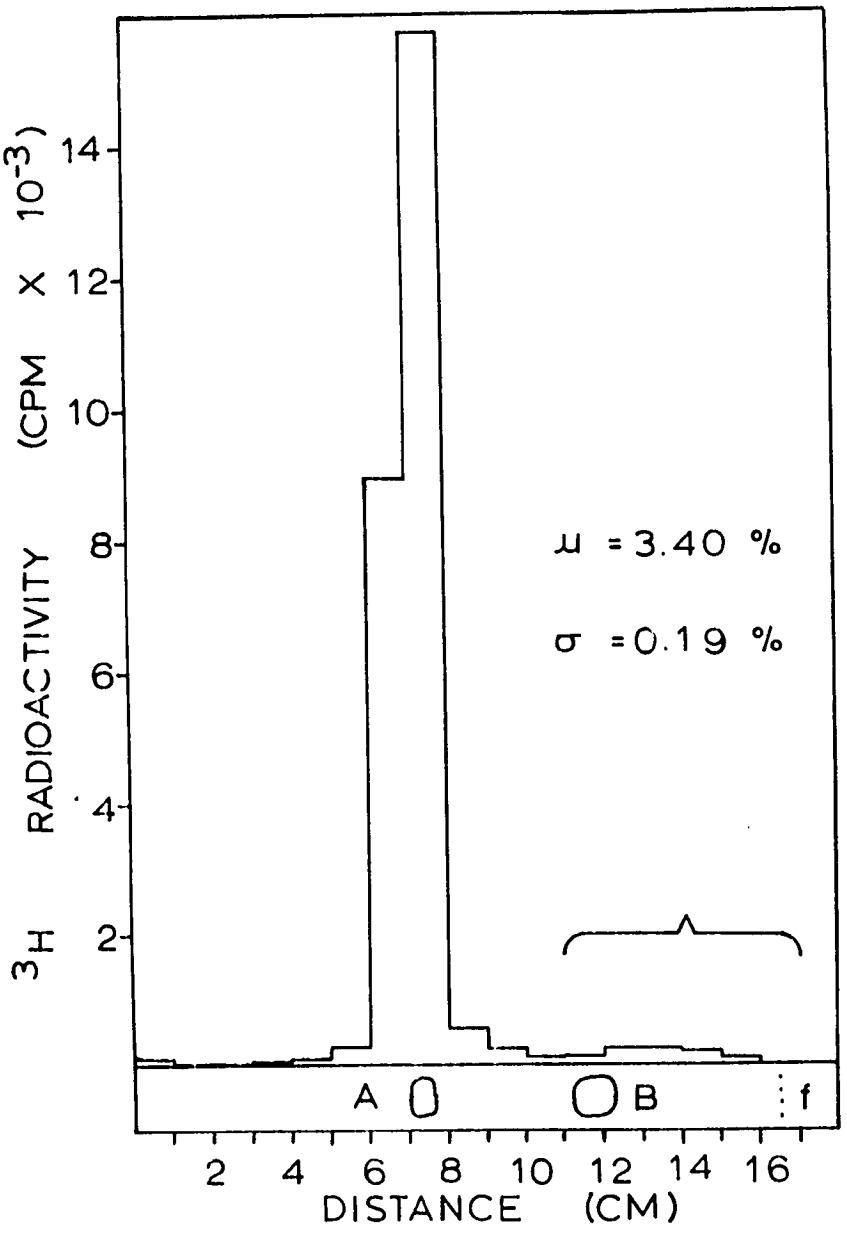


Figure 50. Thin layer radiochromatogram of Experiment #12D. A: Dihydrosphingosine. B: N-Benzoyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

sult of minute amounts of the substrate drying repeatedly on the sides of the test tubes during agitation.

Experiment #8 showed that the presence of Silica Gel G used in thin layer chromatography caused considerable decomposition far above the control level in Experiment #7. Also, it was noted that permitting the substrate to dry on the adsorbent before incubation caused a small, but detectable, increase in decomposition. In reference to running analytical plates, this may have accounted, in part, for the original 1.5% impurity observed in the stock solution. this figure could be used as an outside error limit for thin layer analysis. In preparative thin layer chromatography, a second drying occurred at the end of the plate development which may have caused further decay.

In Experiments #9, #10C and #11C, both silicic acid and Unisil seemed to protect the substrate from even the decomposition seen in control Experiment #7. However, as shown in Experiments #10D and #11D, when the substrate was in a polar solvent which was in contact with either these two adsorbents (which may have partially dissolved) and that solution was then taken to dryness, a greater decomposition occurred than if just the substrate in a similar volume of solvent was taken to dryness (as in Experiment #6B). This process, too, may have accounted for part of the 1.5% impurity of the stock solution since the labeled substrate was originally prepared by silicic acid chromatography.

As shown in Experiments #10 and #11, the radio-impurities were preferrentially eluted in chloroform-methanol (92:8). Experiment #12

showed that glass wool causes little or no decomposition over a short period of time compared to control Experiment #7.

For a graphic summary of all of the above results, please see Fig. 51.

The best method of purification indicated by the above data would be the use of column chromatography on silicic acid. The eluate containing the substrate should be very concentrated so that, when necessary, only small volumes of the solution need be withdrawn and used or taken to dryness. Finally, the eluate should be stored at reduced temperature without ever being taken to dryness. All of these facets have been incorporated into the preparation of the stock solution used for these and all subsequent experiments.

Samples should be stored in Teflon-capped glass vials as shown by the following experiment: Pure dihydrosphingosine (10 mg) was added along with [4,5-$^{3}H_{2}$]-dihydrosphingosine (1 ul of Preparation I; about 2 uCi; 2 ug) to each of three glass vials containing chloroform-methanol (2:1; 2.0 ml). One vial was sealed with a Kimble #60975-L polyethylene cap and the other two vials with a Teflon-lined screw cap. Each was stored at room temperature. In a fourth vial was added phytosphingosine (10 mg; 98% pure) from yeast which contained a trace of dihydrosphingosine. Chloroform-methanol (2:1; 2.0 ml) was added and the vial was sealed with a polyethylene cap. After 37 days at room temperature, only the dihydrosphingosine capped with polyethylene showed any significant change as observed by thin layer visualization (Fig. 52) or radio-analysis (Fig. 53 & Fig. 54). Note that even after 96 days at room temperature, phytosphingosine did not appear to undergo the same decomposition (at least, not at the same rate) as

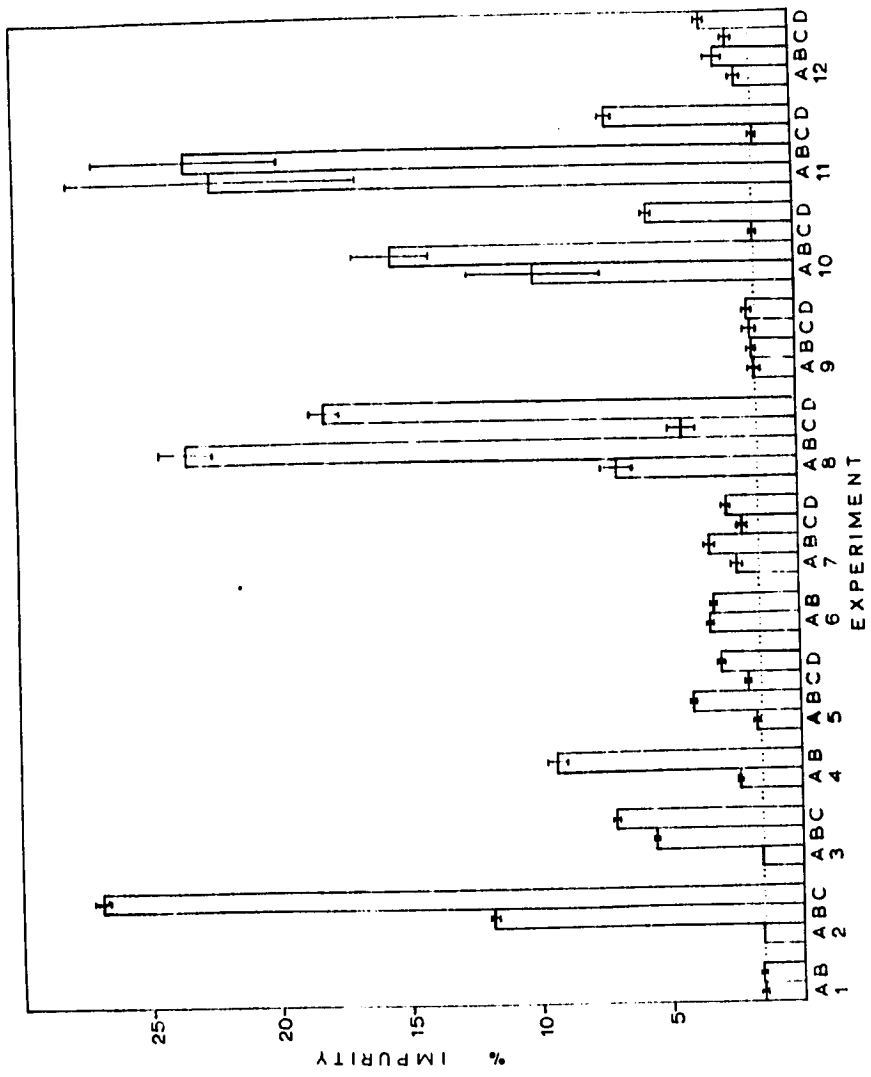


Figure 51. Summary of the percent impurities found in Experiments #1 through #12.

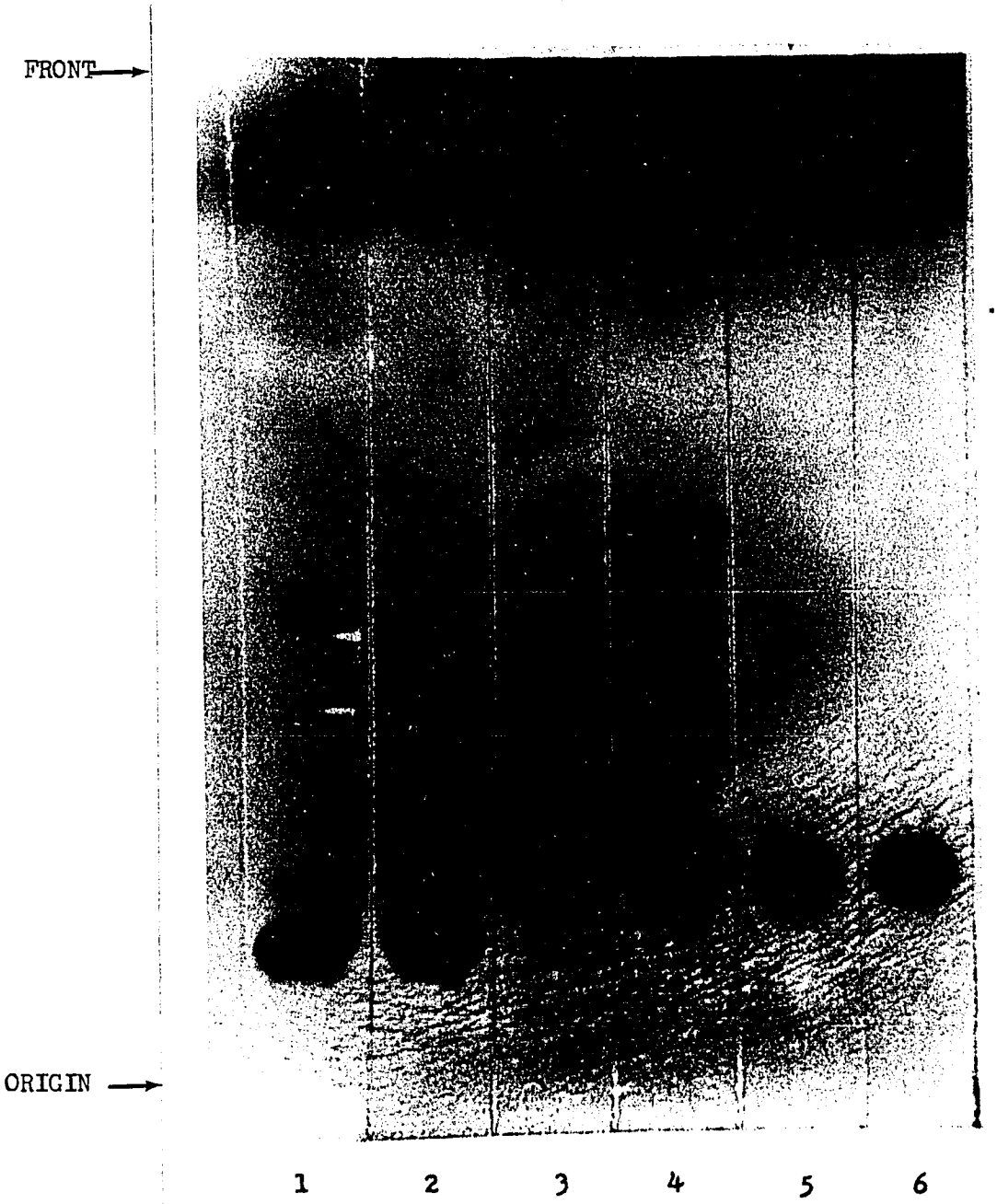


Figure 52. Thin layer chromatography on Silica Gel PF 254 + 366, molybdic acid stained. Channel 1: Fresh phytosphingosine. Channel 2: Phytosphingosine after 37 days (23° C) in chloroform-methanol (2:1) with a plastic cap. Channel 3: Fresh dihydrosphingosine. Channel 4: Dihydrosphingosine after 37 days (23° C) in chloroform-methanol (2:1) with a plastic cap. Channels 5 & 6: Two samples of dihydrosphingosine after 37 days (23° C) in chloroform-methanol (2:1) with Teflon caps. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

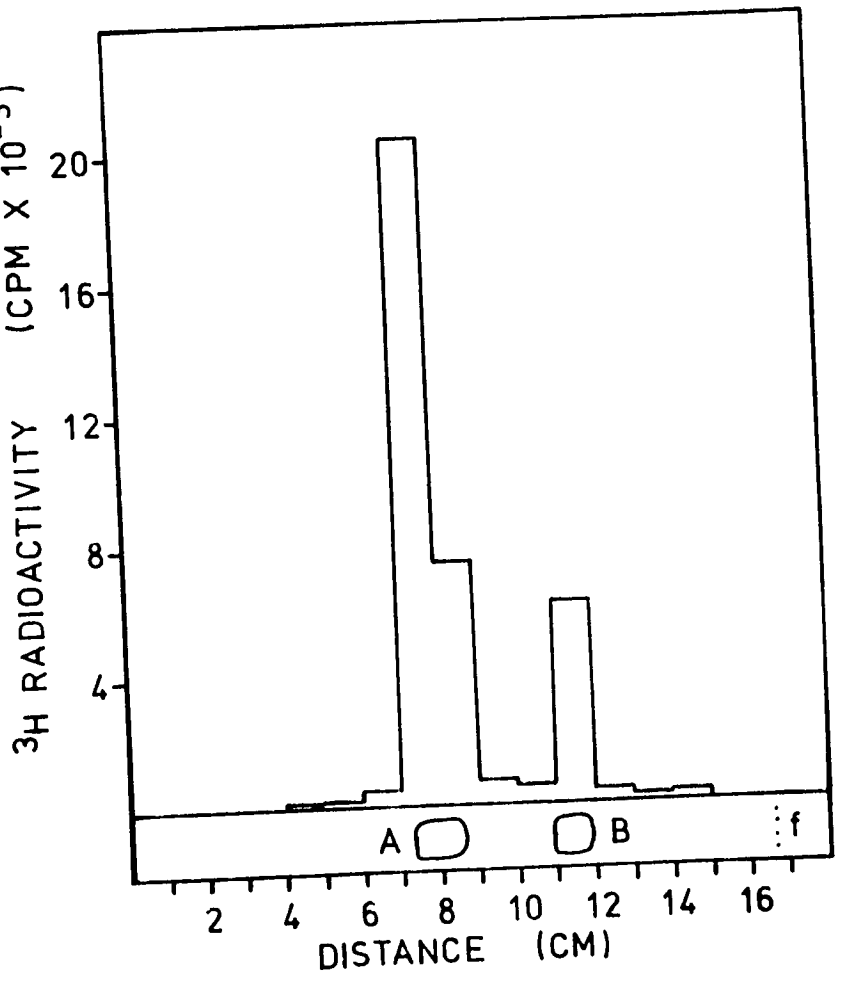


Figure 53. Thin layer radiochromatogram of [4,5-$^3H_2$]-dihydrosphingosine after 37 days (23° C) in chloroform-methanol (2:1) and sealed with a polyethylene cap. A: Dihydrosphingosine. B: Mass of the decomposition product. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

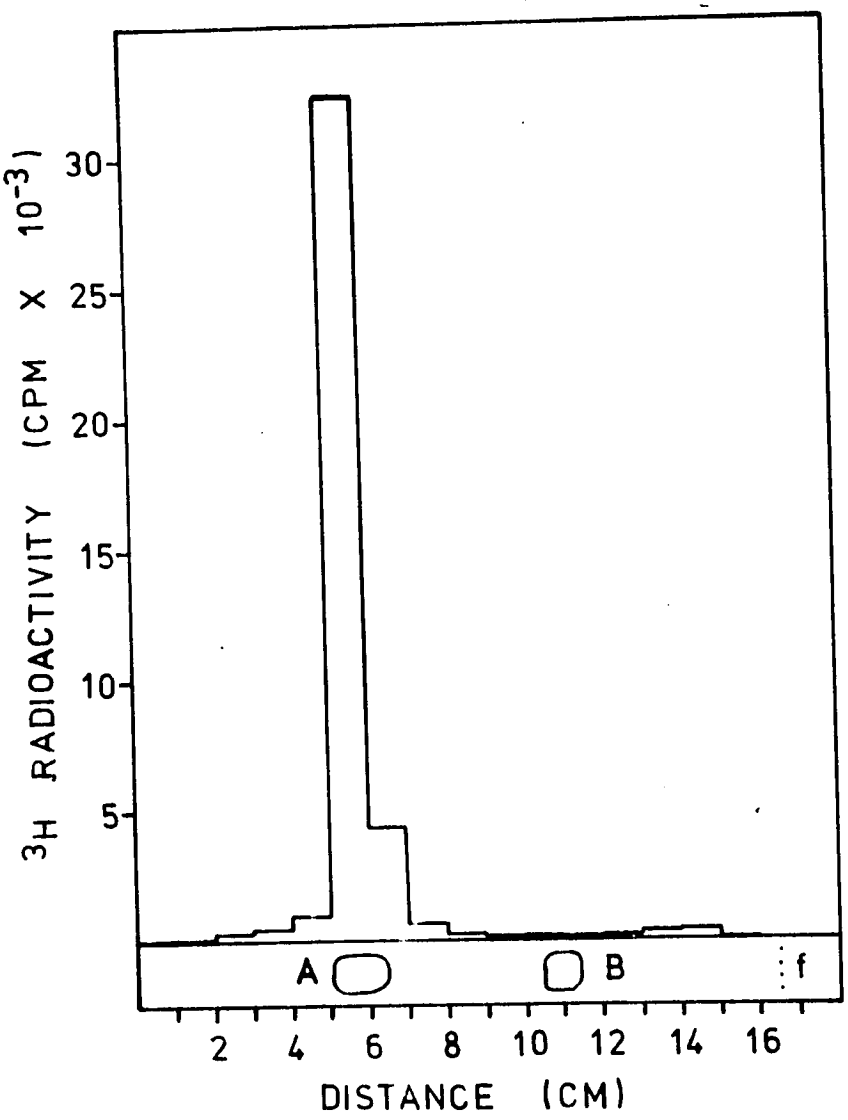


Figure 54. Thin layer radiochromatogram of $[4,5\text{-}^3H_2]$-dihydrosphingosine after 37 days (23° C) in chloroform-methanol (2:1) and sealed with a Teflon cap. A: Dihydrosphingosine. B: Mass of the decomposition product from the sample sealed with a polyethylene cap. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

the dihydrosphingosine which was capped with polyethylene (Fig. 55). This dihydrosphingosine decomposition product (as opposed to that of the previous experiments) of an unlabeled sample was applied in chloroform (5 ml; fraction 1) to a 2 gram silicic acid column (0.9 X 7.5 cm; packed in chloroform). The column was eluted with the following solvents (5 ml/fraction): 0.5% methanol in chloroform (10 ml), 1.0% methanol in chloroform (15 ml) and 2.0% methanol in chloroform (5 ml). Fractions 5 and 6 were pooled and dried under nitrogen at 27° C. The residue (5.9 mg) formed feathery white crystals (mp 126-128° C). The infrared spectrum (Fig. 56) showed a strong carbonyl absorption at 1705 cm^{-1}. The mass spectrum (Fig. 57) showed the formation of a substantially higher molecular weight species than the original dihydrosphingosine at molecular weight 301 grams/mole. When a portion of the sample was burned on a copper wire, no green color was observed in the flame which would have indicated a halogen (arising from a reaction with chloroform).

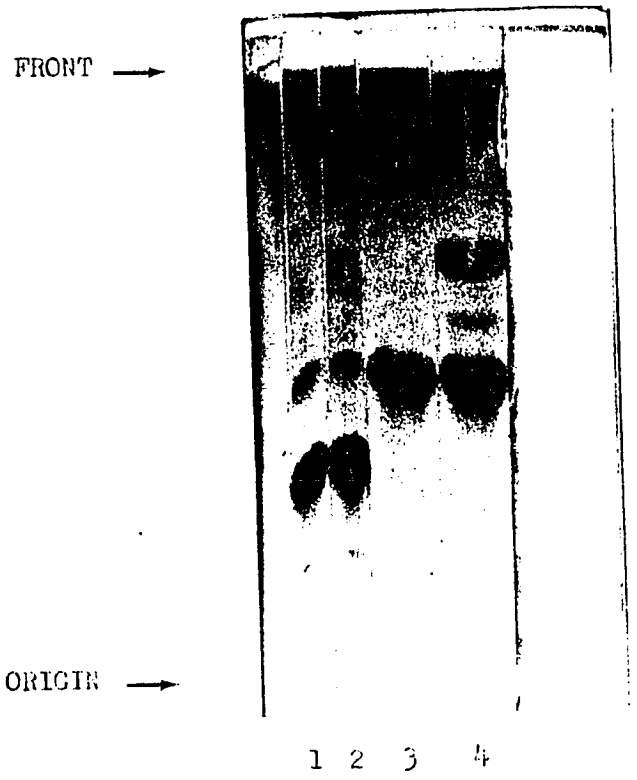


Figure 55. Thin layer chromatogram on Silica Gel PF 254 + 366, molybdic acid stained. Channel 1: Fresh phytosphingosine. Channel 2: Phytosphingosine after 96 days (23° C) in chloroform-methanol (2:1) sealed with a plastic cap. Channel 3: Fresh dihydrosphingosine. Channel 4: Dihydrosphingosine after 96 days (23° C) in chloroform-methanol (2:1) and sealed with a plastic cap. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

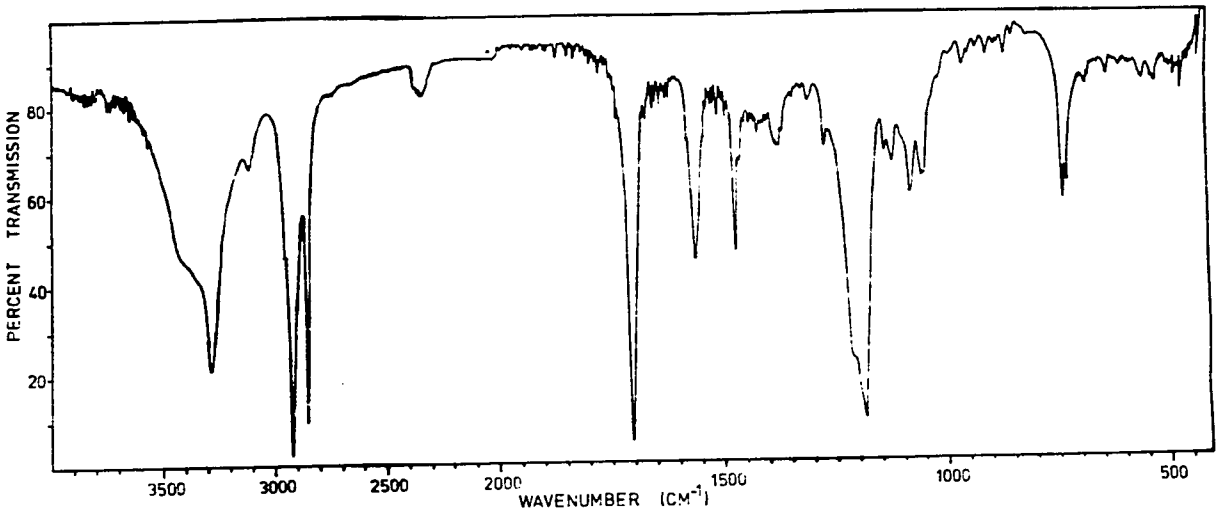


Figure 56. Infrared spectrum of the decomposition product formed from dihydrosphingosine in chloroform-methanol (2:1) which was sealed with a polyethylene cap. The product was purified by preparative thin layer chromatography as described in the text.

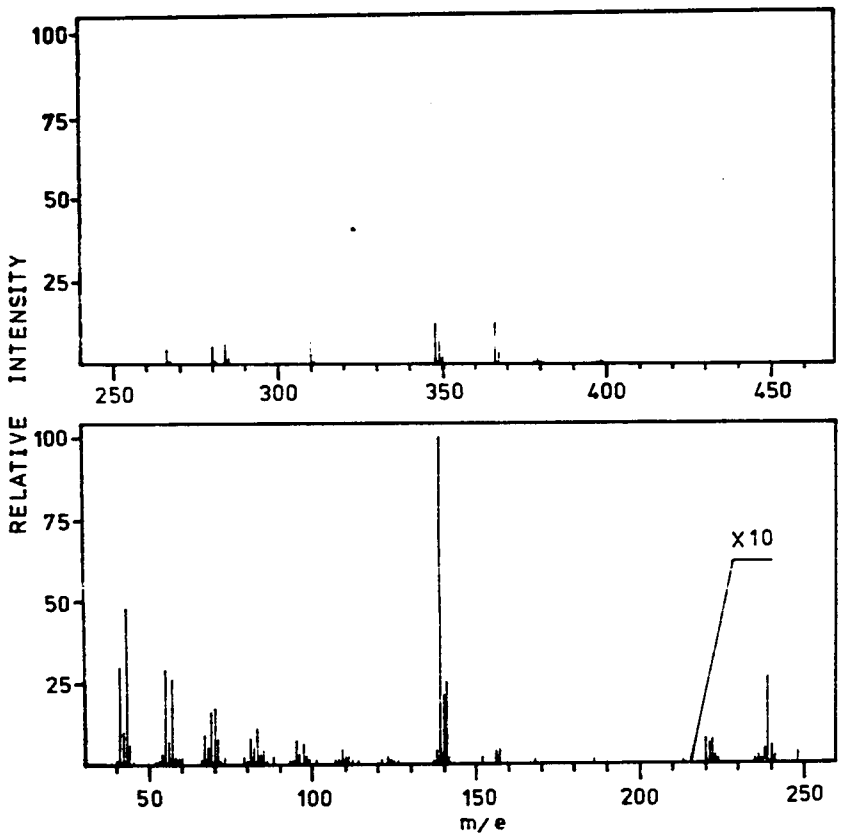


Figure 57. Mass spectrum of the decomposition product formed from dihydrosphingosine in chloroform-methanol (2:1) which was sealed with a polyethylene cap. The product was purified by preparative thin layer chromatography as described in the text.

IN VIVO STUDIES OF [4,5-$^3H_2$]-DIHYDROSPHINGOSINE

The study of liver metabolism of dihydrosphingosine was conducted on female Sprague-Dawley white rats obtained from Texas Inbred Mice Company (raised on Wayne's Feeds--Lab Blox) and fed on Ralston's Rat Chow ad libitum for a few days prior to injection. Axenic (free of other living organisms) rats (47-51 days old) were obtained from Charles Rivers Mouse Farms, rehydrated on sterile water one day before injection and fed ad libitum on the accompanying sterile food. Rats #1-3, 5-7, 8-10 and 11-13 were sacrificed as groups and each group sacrificed between 9 AM and 1 PM.

Each rat was placed under light absolute ether (Mallinckrodt) anesthesia and [4,5-$^3H_2$]-dihydrosphingosine (Preparation I; 1 uCi/ug; 10-20 ul; 2 uCi/ul) in absolute ethanol was injected into the portal vein of the liver. Isotonic saline was used to prevent the body cavity from drying out. One hour later, each rat was sacrificed, the liver excised, frozen in liquid nitrogen and lyophilized. The ground liver was stirred for 20 minutes in methanol (50 ml) after which chloroform (100 ml) was added and the stirring was continued for an additional 20 minutes. The suspension was filtered under suction through Whatman #1 paper and the residue washed with chloroform-methanol (2:1; 30 ml). Then 0.9% saline solution (45 ml) was added, the solution was mixed and the layers allowed to separate overnight at 4° C. The clear lower layer was removed and the solvent was removed at reduced pressure at 35° C. The residue was dried in vacuo over phosphorous pentoxide (Table II). The residue was redissolved in

TABLE II

Synopsis of lipids recovered after injection of [4,5-$^{3}H_{2}$]-dihydrosphingosine into the portal vein of rats. The livers were excised, lyophilized and the lipids extracted by Folch partitioning.

RAT	RAT WEIGHT (GM)	LIPID WEIGHT (MG)	MG LIPID/GM LIVER	INJECTION (uCi)	ACTIVITY RECOVERED (DPM X 10^{-6})	(%)
1	272	548.1	186	20	23.6	54
2	266	567.9	168	20	23.9	54
3	255	549.6	180	20	29.1	66
4	203	402.9	174	24	2.6	5
5	195	415.8	170	20	21.3	51
6	195	364.8	179	40	51.7	59
7	176	351.8	138	20	10.2	23
8 Axenic	173	343.9	164	20	26.4	60
9 Axenic	115	204.4	172	20	27.1	62
10 Axenic	106	179.4	150	20	27.2	62
11 Starved	107	178.6	165	20	31.1	71
12 Starved	105	184.5	169	20	29.7	68
13 Starved	102	188.5	187	20	24.3	55

chloroform-methanol (2:1; 5.0 ml) to which 1 N sodium hydroxide (5 ml; 95% methanol, 5% water) was then added. After 30 minutes of occasional stirring at room temperature, a Folch partition was formed by adding successively 0.37 N HCl (15 ml), chloroform (10 ml) and chloroform-methanol (2:1; 40 ml). After mixing and storing overnight at 4° C, the solvent from the clear lower layer was removed under reduced pressure at 35° C and the resulting residue was dried in vacuo over phosphorous pentoxide. This residue was then redissolved in chloroform (5.0 ml). These mild alkali stable lipids (Table III) were applied in chloroform (5.0 ml) to a 5 gram silicic acid column (0.9 X 18 cm; packed in chloroform) or, in the case of rat #4, a 5 gram Unisil column (0.9 X 18.5 cm; packed in chloroform) and eluted batchwise with chloroform (100 ml; non-polar, mild alkali stable lipids of Table IV) and methanol (100 ml; polar, mild alkali stable lipids of Table IV). Each polar fraction was applied in chloroform (5 ml) to a 4 gram silicic acid column (or, in the case of rat #4, a 4 gram Unisil column, each 0.9 X 15 cm; packed in chloroform) and eluted with increasing concentrations of methanol in chloroform as shown in Fig. 58. Peaks B, C and D were analyzed by thin layer chromatography (Fig. 59 I, II and III, respectively). In order of elution, the major peaks represented ceramides, cerebrosides, free bases and sphingomyelins. Total recoveries of activity from this column are shown in Table V. Tables VI-IX describe in detail the recoveries for each class of lipids.

Sphingosine, dihydrosphingosine and phytosphingosine (approximately 120 ug of each) were added to each class of lipids isolated by the above column. One-half of the free bases was analyzed as its silylated derivative on gas-liquid chromatography and the other half treated with

TABLE III

Analyses of total mild alkali stable lipids. The lipids from Table II were treated with mild alkali and extracted by Folch partitioning.

RAT	LIPID WEIGHT (MG)	ACTIVITY RECOVERED FROM HYDROLYSIS (DPM X 10^{-6})	(%)
1	390.9	20.1	85
2	438.4	22.0	92
3	453.9	26.2	90
4	330.7	2.4	94
5	348.2	21.6	102
6	301.5	49.7	96
7	322.8	9.9	97
8 Axenic	287.9	26.1	99
9 Axenic	158.4	24.7	91
10 Axenic	145.6	25.4	93
11 Starved	143.1	29.0	93
12 Starved	145.8	28.7	96
13 Starved	145.4.	22.9	94

TABLE IV

Recovery of mild alkali stable lipids after column chromatography. Lipids from Table III were separated on column chromatography into polar and non-polar fractions.

RAT	NON-POLAR LIPIDS			POLAR LIPIDS			PERCENT OF APPLIED ACTIVITY RECOVERED FROM COLUMN
	LIPID WEIGHT (MG)	ACTIVITY RECOVERED (DPM X 10^{-6})	(%)#	LIPID WEIGHT* (MG)	ACTIVITY RECOVERED (DPM X 10^{-6})	(%)#	
1	377	12.7	61	29	8.20	39	104
2	411	14.1	63	31	8.37	37	102
3	426	14.2	50	24	14.0	50	108
4	321	1.3	40	20	2.0	60	136
5	289	10.0	45	19	12.4	55	104
6	284	30.0	53	19	27.1	47	115
7	278	2.38	23	20	8.19	77	107
8 Axenic	234	12.5	49	41	12.9	51	97
9 Axenic	105	11.3	46	45	13.4	54	100
10 Axenic	95	9.6	38	41	15.4	62	98
11 Starved	130	6.8	26	10	21.0	74	96
12 Starved	130	12.0	44	11	15.5	56	97
13 Starved	131	10.9	50	10	10.8	50	95

* These data may include the weight of some silicic acid which dissolved in the eluting solvent.

\# Values are with respect to the total activity recovered from the column.

TABLE V

Percent of the applied activity recovered from column chromatography. The polar lipids of Table IV were separated into classes by column chromatography.

RAT	PERCENT RECOVERY
1	88
2	88
3	71
4	52
5	86
6	87
7	84
8 Axenic	91
9 Axenic	93
10 Axenic	82
11 Starved	89
12 Starved	82
13 Starved	89
MEAN:	83
STANDARD DEVIATION:	11

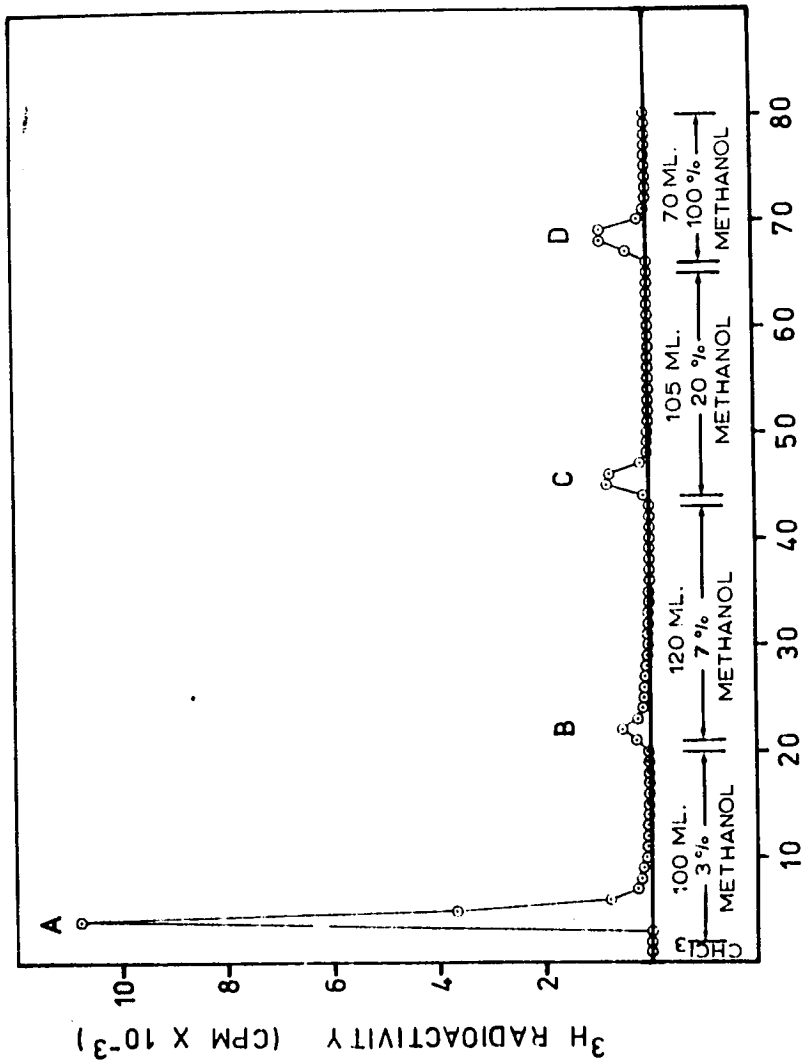


Figure 58. Radio-profile of silicic acid column chromatography of the polar, mild alkali stable lipids of Table IV.

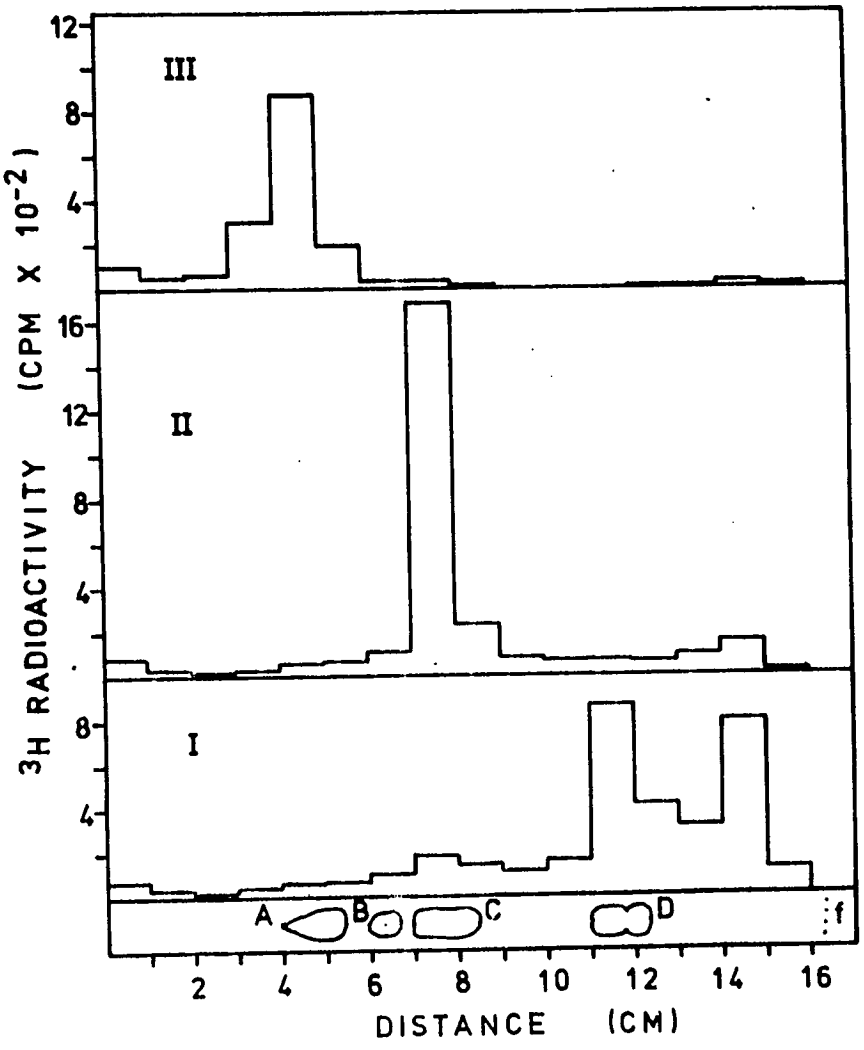


Figure 59. Thin layer radiochromatography of the eluted activity from silicic acid column chromatography of the polar, mild alkali stable lipids of Table IV. Aliquots of pooled activity corresponding to peaks B (I), C (II) and D (III) of figure 58. A: Phytosphingosine and egg yolk sphingomyelin. B: Dihydrosphingosine. C: Sphingosine. D: Rat spleen gluco and bovine spinal cord galacto-cerebrosides. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

TABLE VI

Recoveries of ceramides obtained from column chromatography of the polar lipids of Table IV.

RAT	LIPID WEIGHT (MG)	PERCENT OF TOTAL WEIGHT RECOVERED	CERAMIDE ACTIVITY (DPM X 10^{-6})	PERCENT OF ACTIVITY RECOVERED FROM THE COLUMN
1	0.6	3	4.89	68
2	0.4	2	5.12	70
3	0.7	4	6.08	65
4	3.3	-	0.53	51
5	2.7	15	7.54	71
6	3.6	21	16.8	71
7	2.9	21	5.04	73
8 Axenic	24.2	61	9.46	81
9 Axenic	30.7	69	10.2	82
10 Axenic	27.5	69	10.7	85
11 Starved	1.2	9	6.74	36
12 Starved	1.2	9	8.19	55
13 Starved	1.3	19	7.01	73
MEAN:				68
STANDARD DEVIATION:				16

TABLE VII

Recoveries of the cerebrosides obtained from column chromatography of the polar lipids of Table IV.

RAT	LIPID WEIGHT (MG)	PERCENT OF TOTAL WEIGHT RECOVERED	CEREBROSIDE ACTIVITY (DPM X 10^{-6})	PERCENT OF ACTIVITY RECOVERED FROM THE COLUMN
1	1.1	6	0.37	5
2	0.9	4	0.46	7
3	0.9	5	0.84	8
4	---	-	0.32	31
5	2.0	11	0.91	9
6	2.4	14	1.80	8
7	2.2	16	0.30	4
8 Axenic	4.3	11	0.67	6
9 Axenic	4.6	10	0.58	5
10 Axenic	4.8	12	0.82	6
11 Starved	2.1	17	2.55	14
12 Starved	0.2	2	0.84	7
13 Starved	0.2	3	0.32	3
MEAN:				9
STANDARD DEVIATION:				7

TABLE VIII

Recoveries of the free bases obtained from column chromatography of the polar lipids of Table IV.

RAT	LIPID WEIGHT (MG)	PERCENT OF TOTAL WEIGHT RECOVERED	FREE BASE ACTIVITY (DPM X 10^{-6})	PERCENT OF ACTIVITY RECOVERED FROM THE COLUMN
1	2.8	15	0.49	7
2	7.0	32	0.61	8
3	1.3	8	2.62	24
4	---	--	0.06	6
5	0.5	3	0.78	7
6	0.5	3	2.20	9
7	0.4	3	1.07	16
8 Axenic	3.4	9	0.53	5
9 Axenic	3.1	7	0.30	2
10 Axenic	2.6	6	1.06	8
11 Starved	3.6	28	8.55	46
12 Starved	2.8	22	3.67	29
13 Starved	0.4	6	1.07	11
MEAN:				14
STANDARD DEVIATION:				12

TABLE IX

Recoveries of the sphingomyelins obtained from column chromatography of the polar lipids of Table IV.

RAT	LIPID WEIGHT* (MG)	PERCENT OF TOTAL WEIGHT RECOVERED	SPHINGOMYELIN ACTIVITY (DPM X 10^{-6})	PERCENT OF ACTIVITY RECOVERED FROM THE COLUMN
1	14.3	76	1.41	20
2	13.8	62	1.22	17
3	14.4	83	1.33	12
4	----	--	0.12	12
5	13.1	72	1.44	13
6	10.4	62	2.91	12
7	8.0	59	0.45	7
8 Axenic	7.9	20	1.00	9
9 Axenic	6.0	14	1.31	11
10 Axenic	5.2	13	0.67	5
11 Starved	5.8	46	0.80	4
12 Starved	8.6	67	1.12	9
13 Starved	4.8	72	1.21	13
MEAN:				11
STANDARD DEVIATION:				4

* These values may reflect the weight of some silicic acid which was partially soluble in the eluting solvent.

periodate-borohydride as described in Materials and Methods. The ceramides and cerebrosides were suspended in fresh 1 N methanolic HCl (10 M in water) and the sphingomyelins in fresh 1 N methanolic HCl (20 M in water) to a concentration of about 1 mg/ml. After 18 hours at 80° C, the solvent was removed under nitrogen at 40° C and methanol (1.8 ml) and water (0.2 ml) were added. The fatty acids and their methyl esters were extracted with three portions of petroleum ether (5 ml each; bp 30-60° C). The lower layer was dried under a stream of nitrogen at 40° C and 2 N potassium hydroxide (2 ml) was added. The free bases were extracted with three portions of ether (5 ml each) (Tables X-XII). One-half of these bases were analyzed as their silylated derivatives on gas-liquid chromatography and the other half treated with periodate-borohydride, as described in Materials and Methods, and the silylated derivatives of the alcohols thus formed were analyzed by gas-liquid chromatography. The fatty acid-fatty acid methyl ester fraction was completely esterified with diazomethane as described in Materials and Methods and were then analyzed along with NIH F fatty acid methyl ester mixture (Applied Science Laboratories; Fig. 60) as an internal standard.

The expected general trend of a larger amount of lipids being extracted from larger rats was observed in Table I. However, no general trend in the weight of lipids per gram of liver was observed. Recoveries of 50-70% of the injected radioactivity were common. This was excellent for an *in vivo* experiment and permitted careful analysis of the lipids from a single target organ. As shown in Table IV, the radioactivity was partitioned more or less evenly between the non-polar and polar lipids. It should be noted that the methanol eluted

TABLE X

Recoveries of the fatty acids and free bases from the acid hydrolyses of the ceramides of Table VI.

RAT	FATTY ACIDS		FREE BASES		PERCENT ACTIVITY RECOVERED FROM HYDROLYSIS
	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	
1	0.37	10	3.2	90	73
2	0.43	14	2.5	86	58
3	0.27	7	3.9	93	68
4	0.38	9	3.8	91	--
5	----	--	---	--	--
6	----	--	---	--	--
7	----	--	---	--	--
8 Axenic	3.9	57	3.0	43	72
9 Axenic	3.4	44	4.4	56	77
10 Axenic	2.1	32	4.4	68	61
11 Starved	1.4	30	3.4	70	72
12 Starved	0.43	7	5.7	93	75
13 Starved	0.51	9	4.9	91	78
MEAN:		22		78	70
STANDARD DEVIATION:		18		19	7

TABLE XI

Recoveries of the fatty acids and free bases from the acid hydrolyses of the cerebrosides of Table VII.

RAT	FATTY ACIDS		FREE BASES		PERCENT ACTIVITY RECOVERED FROM HYDROLYSIS
	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	
1	0.056	9	0.60	91	--
2	0.053	14	0.34	86	85
3	0.14	31	0.30	69	52
4	0.41	9	4.3	91	--
5	0.065	14	0.40	86	51
6	0.18	17	0.84	83	57
7	0.034	21	0.13	79	54
8 Axenic	0.056	15	0.33	85	58
9 Axenic	0.047	13	0.30	87	61
10 Axenic	0.088	15	0.49	85	70
11 Starved	-----	--	----	--	--
12 Starved	0.064	10	0.55	90	73
13 Starved	0.025	10	0.22	90	76
MEAN:		15		85	64
STANDARD DEVIATION:		6		6	12

TABLE XII

Recoveries of the fatty acids and free bases from the acid hydrolyses of the sphingomyelins of Table IX.

RAT	FATTY ACIDS		FREE BASES		PERCENT ACTIVITY RECOVERED FROM HYDROLYSIS
	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	ACTIVITY (DPM X 10^{-6})	PERCENT OF RECOVERED ACTIVITY	
1	0.13	30	0.29	70	30
2	0.10	23	0.34	77	36
3	0.12	24	0.38	76	37
4	0.027	26	0.075	74	--
5	0.096	26	0.28	74	26
6	0.22	32	0.46	68	23
7	0.024	24	0.076	76	23
8 Axenic	0.021	15	0.12	85	14
9 Axenic	0.027	14	0.17	86	15
10 Axenic	0.025	17	0.13	83	22
11 Starved	0.037	29	0.091	71	16
12 Starved	0.11	20	0.43	80	47
13 Starved	0.042	13	0.29	87	27
MEAN:		23		77	26
STANDARD DEVIATION:		6		6	10

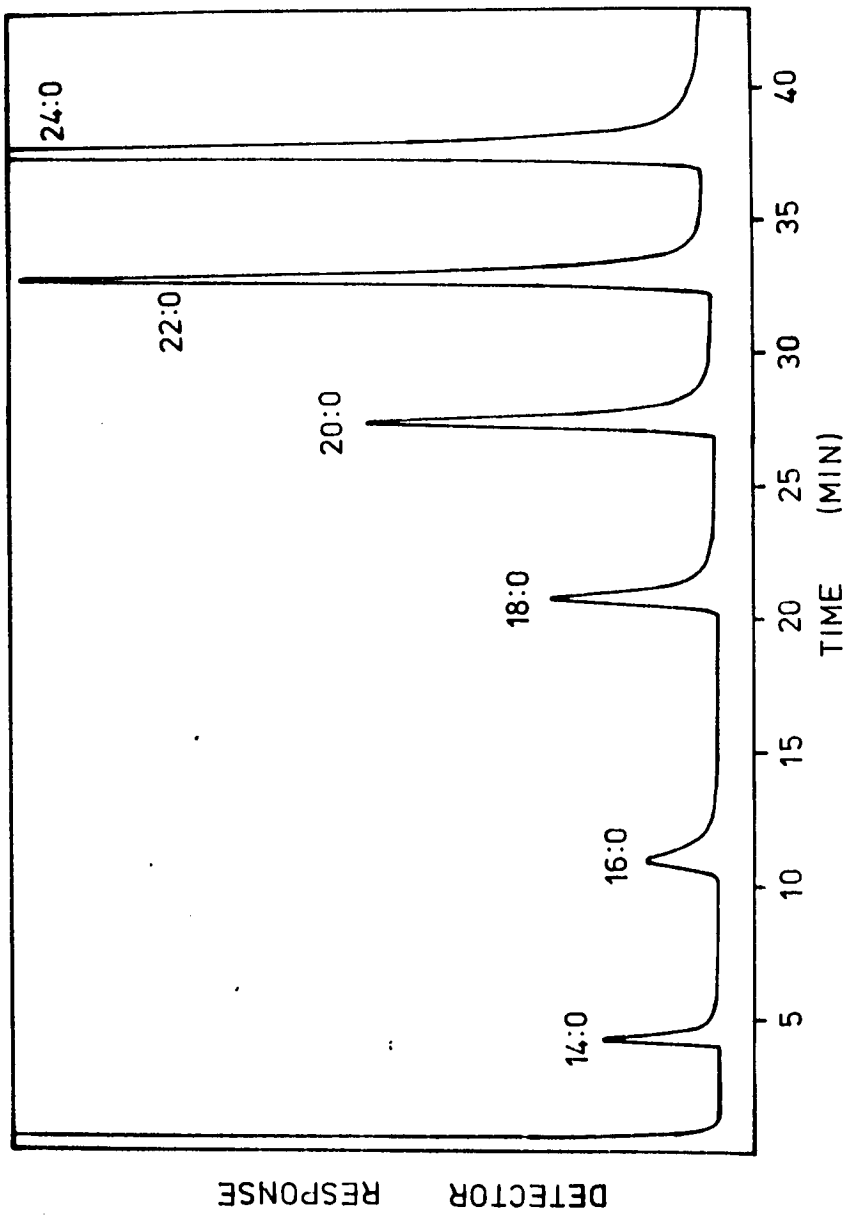


Figure 60. Gas-liquid chromatogram of the saturated NIH F fatty acid methyl ester standards under conditions described in Materials and Methods.

small amounts of silicic acid and that the weight of the polar lipids was, therefore, not truly accurate. If these figures were accepted as an upper limit, then the specific activity of the polar lipids calculated were at least 3-20 times higher than that of the non-polar lipids. Also, the mass of the polar lipids of the axenic rats was significantly larger, especially when the relative weight of these rats (Table II) or the mass of the total lipids of Tables II and III were considered.

The recovery of the total activity of the ceramides, cerebrosides, free bases and sphingomyelins from the columns averaged 83% of the activity of the polar lipids applied to the columns (Table V). The separation of classes, particularily the free bases from the cerebrosides, was remarkable. Usually, these two classes were eluted together[50]. The largest portion of activity was found in the ceramides (about 68%) with the remainder of the activity divided about equally among the cerebrosides (9%), free bases (14%) and sphingomyelins (11%).

Hirschberg[50] reported the recoveries of activity in the Folch extract of the homogenized livers after injection of labeled [4,5-$^{3}H_{2}$] -dihydrosphingosine into the tail vein of rats. These were 3.8% one hour after injection and 2.7% four hours after injection. The classes were separated batchwise on silicic acid columns into ceramides (eluted with 3% methanol in chloroform), cerebrosides and free bases (eluted with 20% methanol in chloroform) and sphingomyelins (eluted with 40% methanol in chloroform). The activities recovered in each of these fractions were 35% (1 hour) to 44% (4 hours), 21% (1 hour) to 22% (4 hours) and 43% (1 hour) to 33% (4 hours), respectively, of the total activity of all of the classes recovered from the column.

Distribution of the activity between the fatty acid and base moieties after acid hydrolysis was not reported.

Assmann and Stoffel[4] injected [5,6-$^{3}H_{2}$]-phytosphingosine into the tail vein of rats and recovered only 5% (after 24 hours) of the activity in the liver lipids. Reported recoveries of activity in the liver lipids of rats injected intravenously into the tail vein with [3-^{14}C][86,87]; [1-^{3}H], [1-^{14}C][87] and [1-^{14}C][93] -dihydrosphingosine were 17% (after 6 hours)[86], 25% (after 6 hours)[87] and 18% (after 3 hours)[93]. In each of these cases, the phospholipids were not destroyed by mild alkali treatment as in the present work. These workers separated the classes of lipids by silicic acid chromatography. Examination of the elution profile revealed, however, that free bases were not separated from the phospholipids[86,87,93]. Cerebrosides were not usually isolated as a separate class[86,87,93]. The reported activities recovered in the separate classes do not relate directly to those reported in the present work. In the literature reports, recoveries of activity from each class were relative to total sphingolipid and phospholipid activity recovered from the column chromatography. In the present work, values were related to the activity recovered after column chromatography of the mild alkali stable polar lipids.

It was reported that after acid hydrolysis, the recoveries of activity in the fatty acid moieties from ceramides and sphingomyelins were 10%[86] and 45%[87] with palmitic acid as the major component (80%) in each case. The free base liberated from the acid hydrolysis of the ceramides and sphingomyelins accounted for 90%[86] and 55%[87] of each of

the classes. In ceramides, sphingosine accounted for 13% and dihydrosphingosine accounted for 87% of the radioactivity of the bases as determined by thin layer chromatography[93]. In sphingomyelins, sphingosine accounted for 35% and dihydrosphingosine accounted for 65% of the radioactivity of the bases[93].

Stoffel and Bister[82] injected N-([1-^{14}C]-palmitoyl)-[3-^{3}H]-dihydrosphingosine or N-acyl-1-([UL-^{14}C]-choline)-[3-^{3}H]-dihydrosphingosine into the tail vein of rats and recovered 23% of the administered activity after 6 hours in the liver lipids. These lipids were treated with mild alkali and applied to a silicic acid column for further separation into neutral lipids, a ceramide-cerebroside-free base mixture (called ceramides by these authors) and sphingomyelins. These "ceramides" were composed of 95% dihydrosphingosine and 5% sphingosine in the base moiety. The sphingomyelins were composed of 37% dihydrosphingosine and 63% sphingosine in the base moiety. With the exception of the studies of Hirschberg[50], none of the other above studies reported in the literature used [4,5-$^{3}H_2$]-dihydrosphingosine and, therefore, did not encounter a loss of any activity in the formation of sphingosine from their labeled labeled dihydrosphingosines.

In Tables VI-IX it was important to realize that the percent of the total weight recovered for each class included an error due to some silicic acid which was eluted along with the sphingomyelins. Even so, it was clear (Table VI) that the axenic rats had at least three times the mass of ceramides as any other rat examined both in an absolute sense and in relation to the total mass recovery of all of the classes of lipids.

The recoveries of the radioactivity from the acid hydrolyses of the

ceramides (Table X), cerebrosides (Table XI) and sphingomyelins (Table XII) averaged 70%, 64% and 26%, respectively. The data derived from the procedure was taken only qualitatively as there was no guarantee that different species within each class were equally hydrolyzed. In ceramides, cerebrosides and sphingomyelins 78%, 85% and 77%, respectively, of the recovered activity resided in the base moieties. The activity in the fatty acids of the ceramides of axenic rats averaged 22% above the average for 10 rats. This may be significant. However, the ceramide fatty acids of rat #11 were also about 8% above the average.

The analysis of the alcohols obtained after periodate-borohydride treatment of the bases provided an important analytical tool. A standard sample was analyzed to determine the efficiency of this reaction. A sample of [4,5-$^3H_2$]-dihydrosphingosine (Preparation I) was mixed with sphingosine, dihydrosphingosine and phytosphingosine (120 ug of each) and the mixture was treated with periodate-borohydride as described in Materials and Methods. The alcohols derived were 2t-hexadecenol, hexadecanol and pentadecanol, respectively. The major portion of the activity from this treatment co-chromatographed with the silylated derivative of hexadecanol (Fig. 61). It should be noted that both activity and extra mass peaks came off the gas-liquid chromatographic column when the programming to higher temperature was used. At times during this study, these extra products became quite significant and the activity recovered along with them represented a large portion of the total recovered activity. In this standard case, the activity eluted from 26 to 39 minutes after injection accounted for 10% of the total recovered activity (Fig. 61). These areas have not

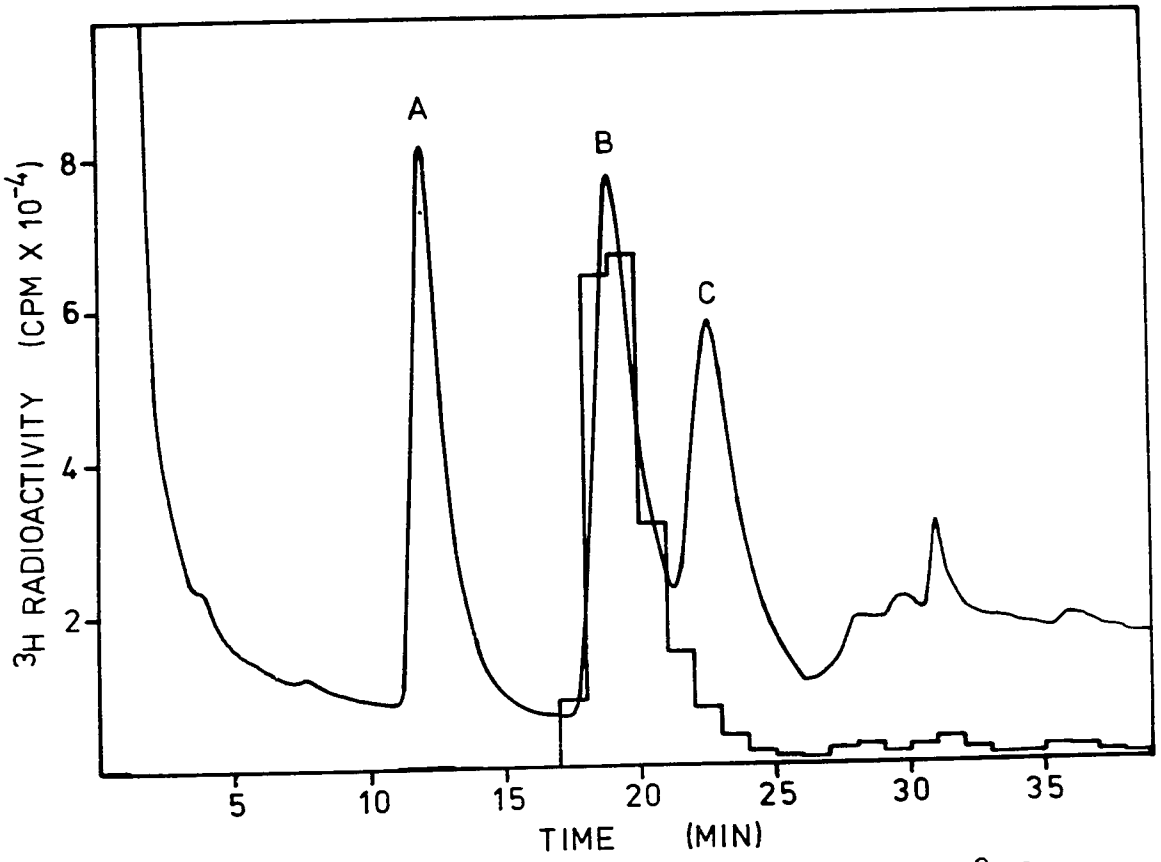


Figure 61. Gas-liquid radiochromatogram of the silylated derivatives of [1,2-$^3H_2$]-hexadecanol, pentadecanol (A), hexadecanol (B) and 2t-hexadecenol (C) derived from the periodate-borohydride treatment of [4,5-$^3H_2$]-dihydrosphingosine (Preparation I), phytosphingosine, dihydrosphingosine and sphingosine, respectively.

been shown in other chromatograms in this section. Silylated derivatives of periodate-borohydride alcohols derived from 3-O-methyl-sphingosine and 3-O-methyl-dihydrosphingosine formed in the acid hydrolyses were also eluted with the final temperature programming. Sweeley and Moscatelli[101] had described the original procedure as useful only for qualitative analyses. It was also noted that little or none of the activity from hexadecanol was oxidized further to pentadecanol.

With a few major exceptions, the analyses of the long chain bases and fatty acid methyl esters showed no qualitative differences among the different rats. Rat #8 offered a representative sample. The alcohols (Fig. 62) derived from the base moiety of the ceramides showed the presence of approximately equal amounts of radioactivity corresponding to the silylated derivatives of 2t-hexadecenol and hexadecanol (Table XII). In 10 rats, the mean ratio of the activity under each mass peak was approximately 0.83:1, respectively for the two alcohols. This did not take into account the tailing from the peak corresponding to the silylated derivative of hexadecanol into the peak corresponding to the silylated derivative of 2t-hexadecenol. Polito and Sweeley[66] reported a trans elimination of hydrogens in the formation of sphingosine from dihydrosphingosine when it was incubated with yeast microsomes. Stoffel et al.[97] reported a cis elimination in intact rats. In either case, one-half of the activity of the non-stereospecifically cis labeled [4,5-$^3H_2$]-dihydrosphingosine would be lost. Therefore, approximately twice the amount of sphingosine was formed than the amount of activity which was recovered in the silylated derivative of 2t-hexadecenol indicates. This assumes that all of the activity of [4,5-$^3H_2$]-dihydrosphingosine

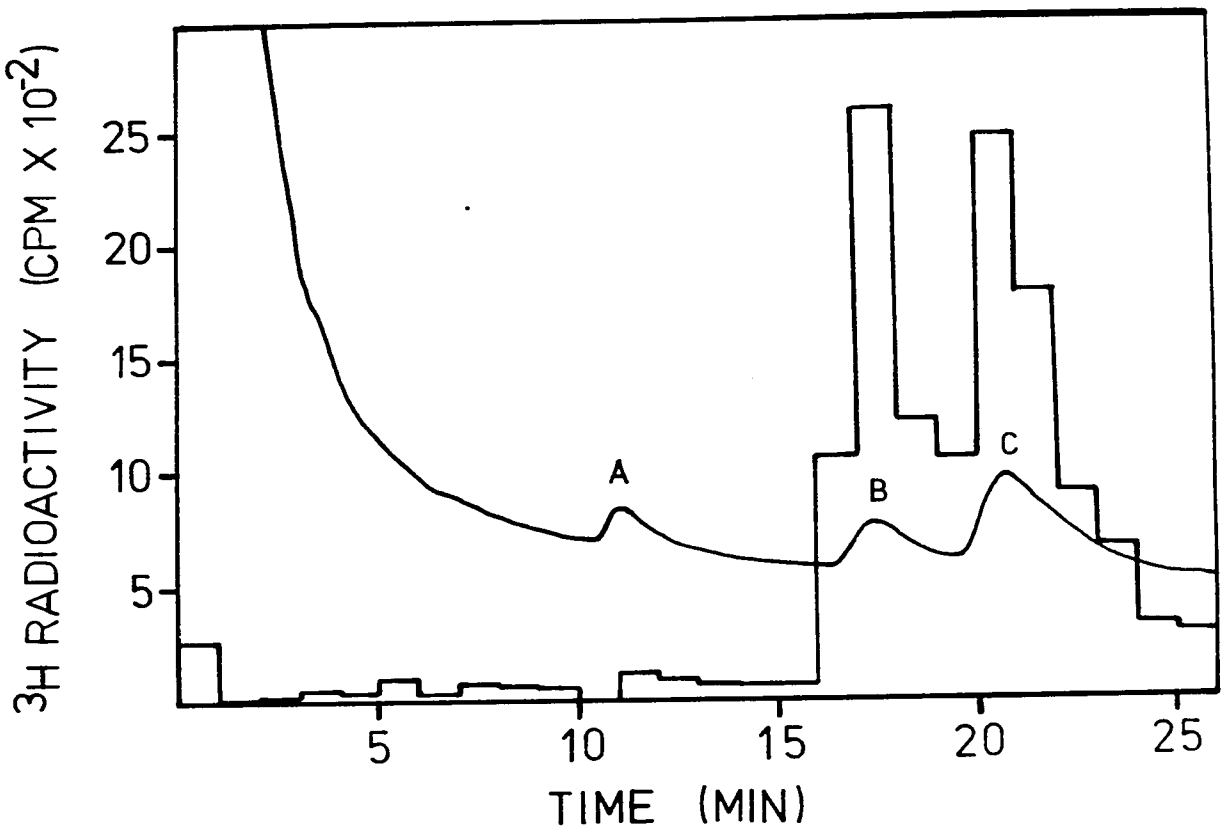


Figure 62. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the ceramides of rat #8 in Table X. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

TABLE XIII

Approximate ratios of the amount of activity of sphingosine to dihydrosphingosine in ceramides as determined by the silylated derivatives of the periodate-borohydride derived alcohols.

RAT	SPHINGOSINE CPM/DIHYDROSPHINGOSINE CPM
1	0.71
2	0.78
3	1.19
4	----
5	0.16
6	----
7	----
8 Axenic	1.48
9 Axenic	0.95
10 Axenic	0.57
11 Starved	0.28
12 Starved	1.04
13 Starved	1.17
MEAN:	0.83
STANDARD DEVIATION:	0.37

resided on carbon atoms four and five. This is not usually this case in catalytic hydrogenations[110]. A small amount of activity corresponded to the silylated derivative of pentadecanol presumably derived from phytosphingosine. This was common in most classes and in most rats. Clearer examples of this were seen in the alcohols derived from the long chain bases derived from the ceramides (Fig. 63), cerebrosides (Fig. 64) and sphingomyelins (Fig. 65) of rat #1 (as well as in other rats).

The analysis of the long chain bases derived from the cerebrosides (Fig. 66) of rat #8 showed significant amounts of activity corresponding to both sphingosine and dihydrosphingosine. Mass and activity was observed corresponding to the 3-O-methyl derivatives of sphingosine and/or dihydrosphingosine[17] which were formed during the hydrolysis. In the chromatograms of the trimethylsilyl derivatives of the bases, small amounts of phytosphingosine, if, indeed, there was any, were lost in the tailing from the silylated derivative of dihydrosphingosine. The alcohols (Fig. 67) derived from these bases reflect significant activity in hexadecanol, less in 2t-hexadecenol and a trace in pentadecanol (which was easier to detect here since the order of elution was reversed). Table XIV shows that for 11 rats, the mean ratio of activity of the silylated derivatives of 2t-hexadecenol to hexadecanol was 0.49:1, respectively. A comparison of the mass tracing of this chromatogram with those of the long chain bases they were derived from (Fig. 66) and of the standard alchohols (Fig. 61) revealed again the irreproducible, non-quantitave nature of the periodate-borohydride reaction. This effect was seen often in this study and it was assumed

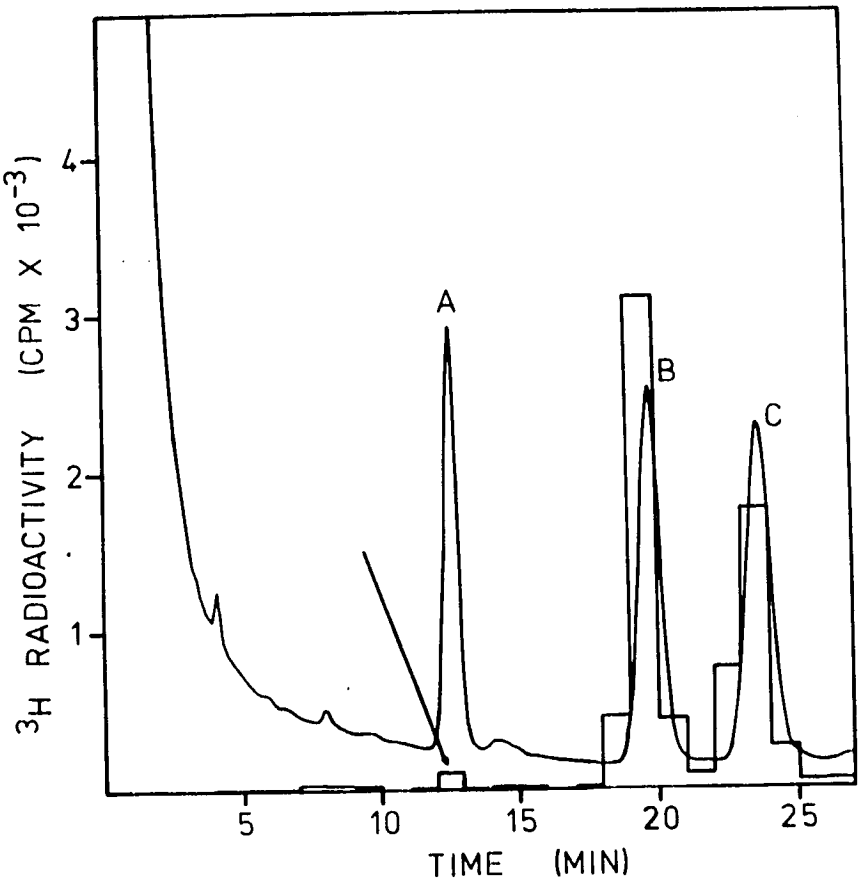


Figure 63. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the ceramides of rat #1 in Table X. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

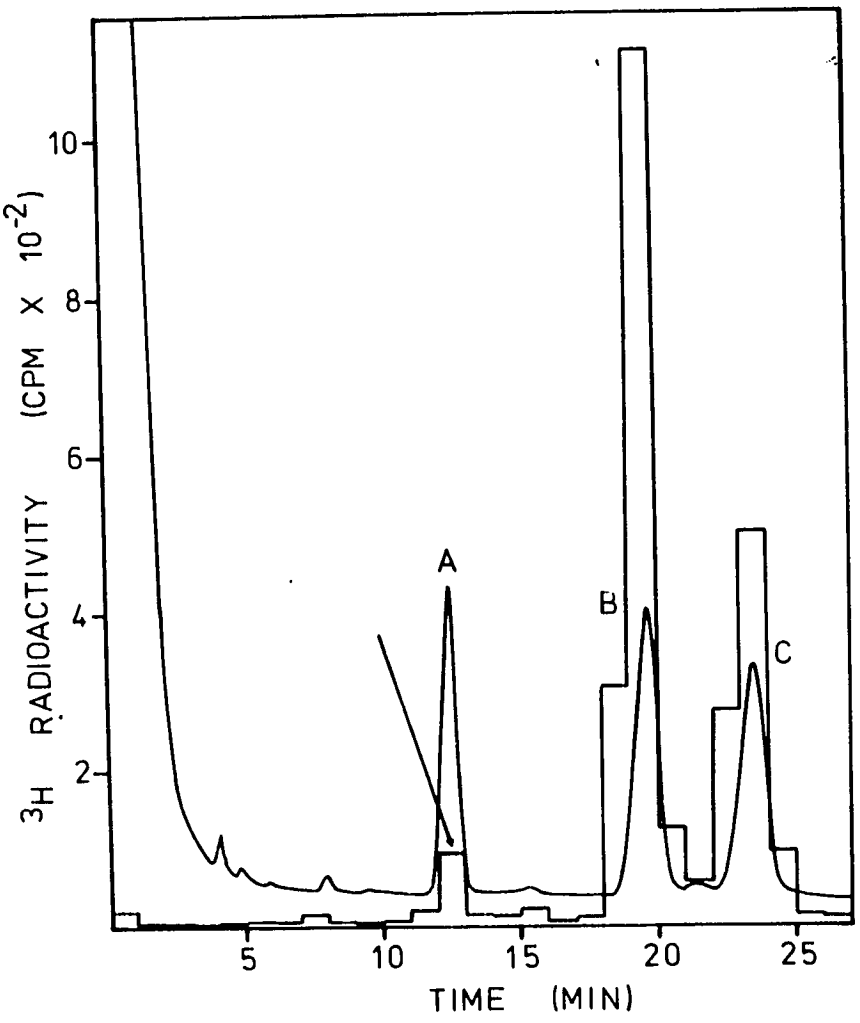


Figure 64. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the cerebrosides of rat #1 in Table XI. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

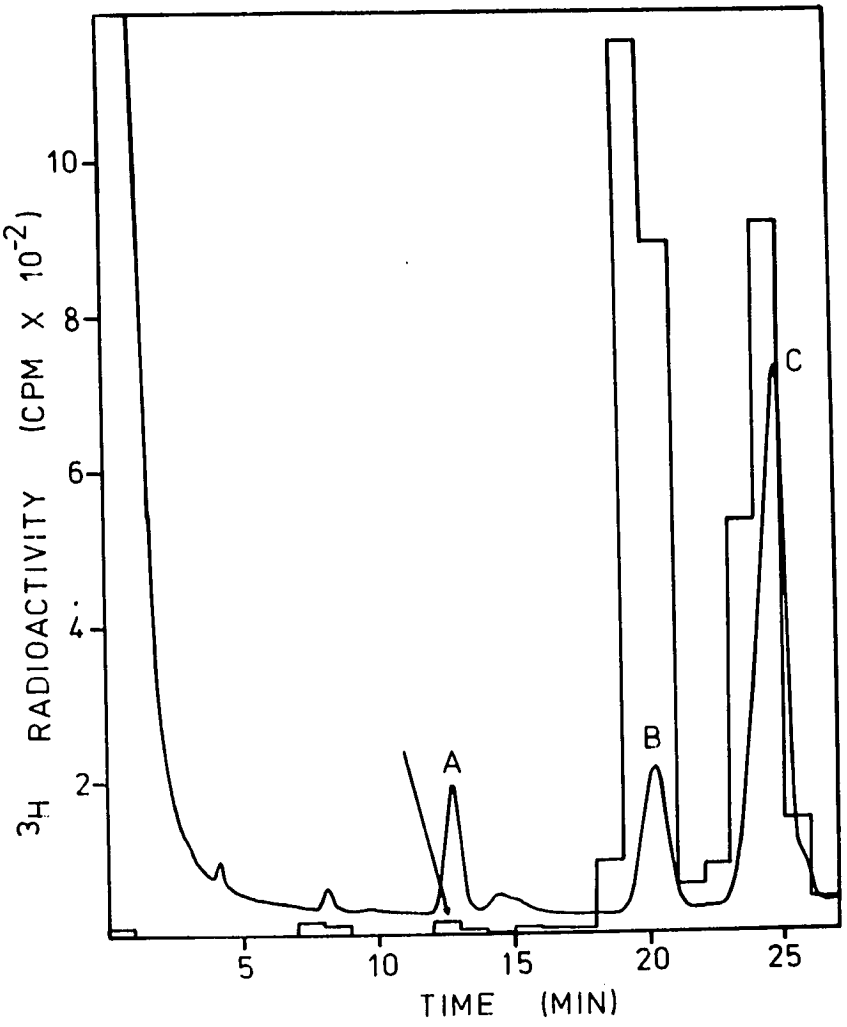


Figure 65. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the sphingomyelins of rat #1 in Table XII. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

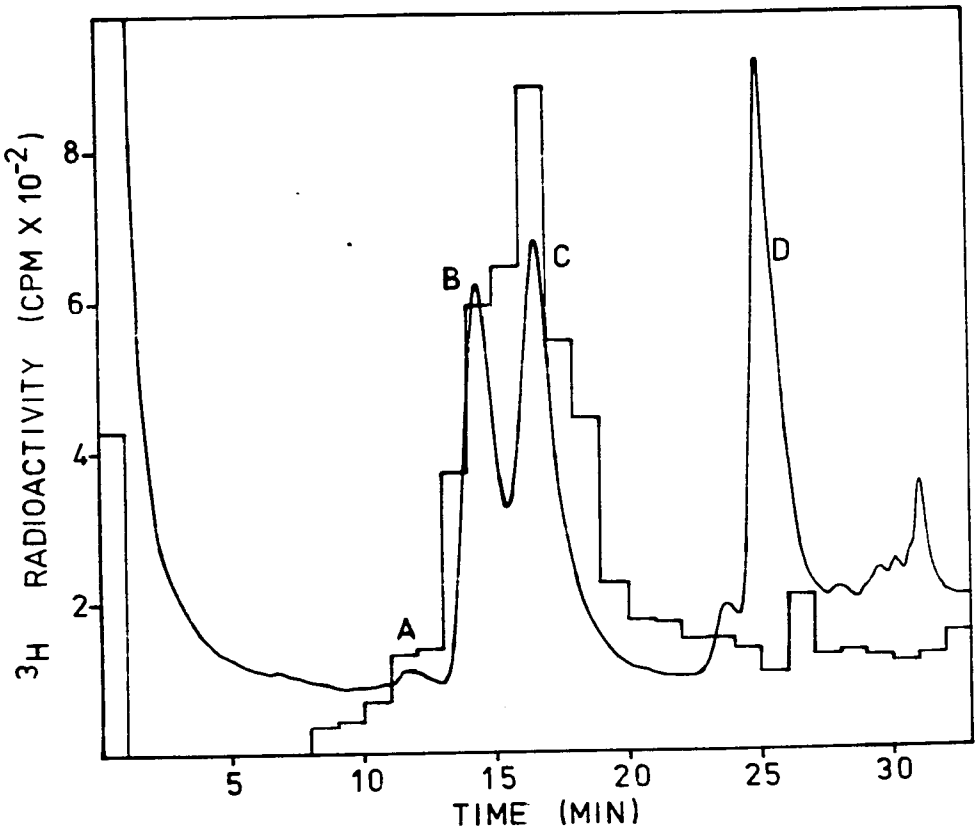


Figure 66. Gas-liquid radiochromatogram of the silylated derivatives of the base moiety of the cerebrosides of rat #8 in Table XI. A: Silylated derivative of 3-O-methyl-sphingosine and/or 3-O-methyl-dihydrosphingosine. B: Silylated derivative of sphingosine. C: Silylated derivative of dihydrosphingosine. D: Silylated derivative of phytosphingosine.

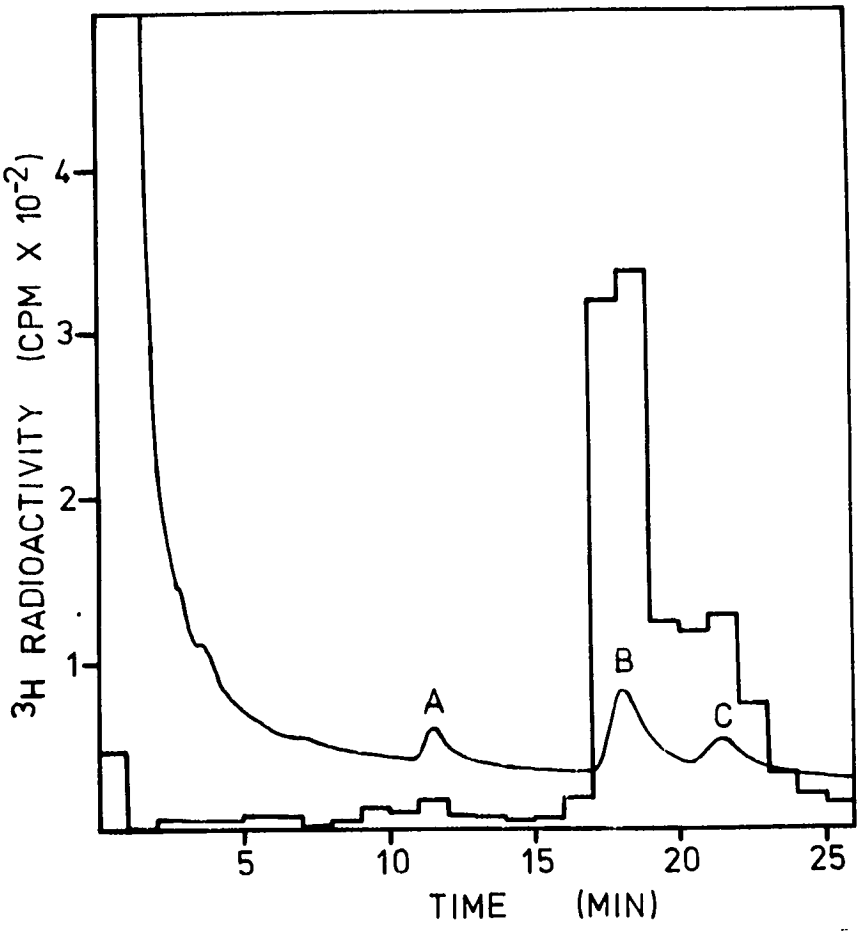


Figure 67. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the cerebrosides of rat #8 in Table XI. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

TABLE XIV

Approximate ratios of the amount of activity of sphingosine to dihydrosphingosine in cerebrosides as determined by the silylated derivatives of the periodate-borohydride derived alcohols.

RAT	SPHINGOSINE CPM/DIHYDROSPHINGOSINE CPM
1	0.65
2	0.65
3	0.47
4	----
5	0.69
6	0.90
7	0.10
8 Axenic	0.47
9 Axenic	0.53
10 Axenic	0.17
11 Starved	----
12 Starved	0.33
13 Starved	0.46
MEAN:	0.49
STANDARD DEVIATION:	0.23

that the radioactive recoveries paralleled that of the mass recoveries.

The silylated free bases (Fig. 68) of rat #8 and the silylated alcohols (Fig. 69) derived from the free bases showed activity corresponding almost totally to dihydrosphingosine with, perhaps, a small amount corresponding to sphingosine (Table XV). The latter point was not crystal clear from these chromatograms.

The silylated long chain base moiety (Fig. 70) of the sphingomyelins of rat #8 and the silylated alcohols (Fig. 71) derived from the base moiety showed large amounts of activity (and endogenous mass) corresponding to sphingosine (Table XVI). A slightly smaller amount of activity corresponded to dihydrosphingosine. In 11 rats, the mean ratio of the activity of the silylated derivatives of 2t-hexadecenol to hexadecanol was 1.39:1, respectively.

While the relative amounts of the activity of sphingosine to dihydrosphingosine was highest in sphingomyelin followed by the ceramides and then the cerebrosides, the absolute amount of labeled sphingosine formed was greatest in the ceramides (Table XVII; 3.6 nmoles, average for 10 rats) followed by the sphingomyelins (Table XIX; 0.80 nmole, average for 11 rats) and then by the cerebrosides (Table XVIII; 0.26 nmole, average for 11 rats). Several assumptions were made in these calculations. First, it was assumed that there was no preferential degradation or loss of one base relative to the other due to the acid hydrclysis of each class or due to the periodate-borhydride treatment of the bases thus obtained. Second, calculations of the relative amount of activity in sphingosine (as determined by its periodate-borohydride derived alcohol, Tables XII-XVI) did not

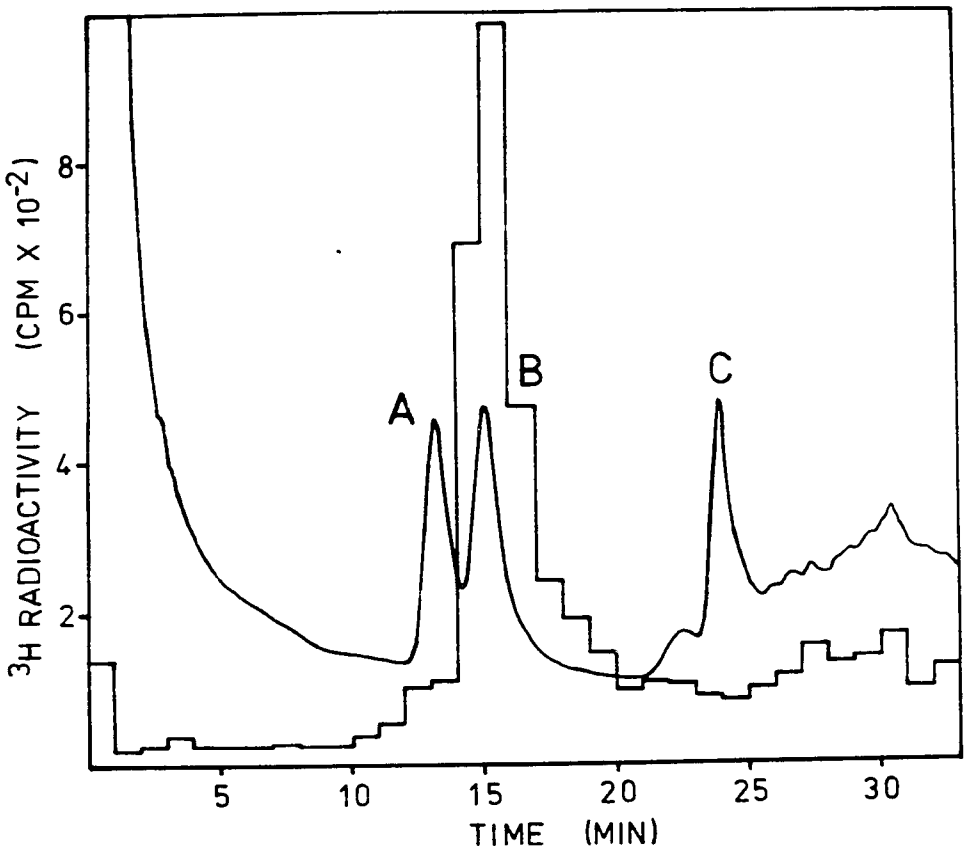


Figure 68. Gas-liquid radiochromatogram of the silylated derivatives of the free bases of rat #8 in Table VIII. A: Silylated derivative of sphingosine. B: Silylated derivative of dihydrosphingosine. C: Silylated derivative of phytosphingosine.

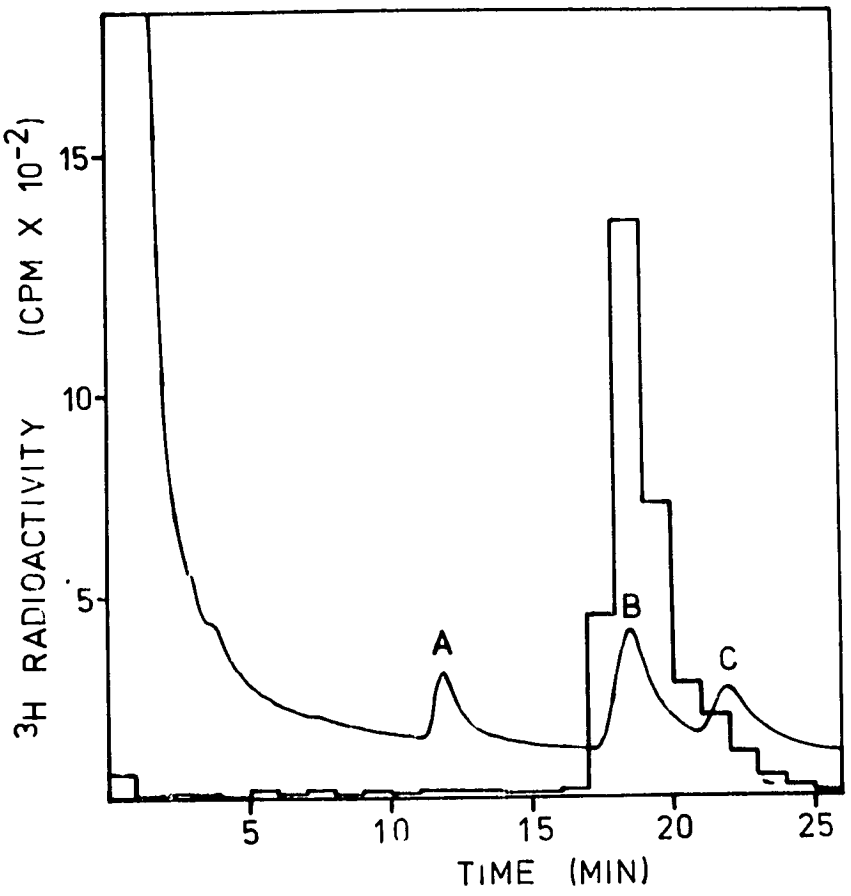


Figure 69. Gas-liquid raliochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the free bases of rat #8 in Table VIII. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

TABLE XV

Approximate ratios of the amount of activity of sphingosine to dihydrosphingosine in the free bases as determined by the silylated derivatives of the periodate-borohydride derived alcohols.

RAT	SPHINGOSINE CPM/DIHYDROSPHINGOSINE CPM
1	0.29
2	0.36
3	0.03
4	----
5	0.07
6	0.05
7	0.02
8 Axenic	0.15*
9 Axenic	0.18*
10 Axenic	0.12*
11 Starved	0.14*
12 Starved	0.17*
13 Starved	----
MEAN:	0.14
STANDARD DEVIATION:	0.11

* These values were due mostly to the tailing of the activity from the silylated derivative of hexadecanol into the region of the silylated derivative of 2t-hexadecenol.

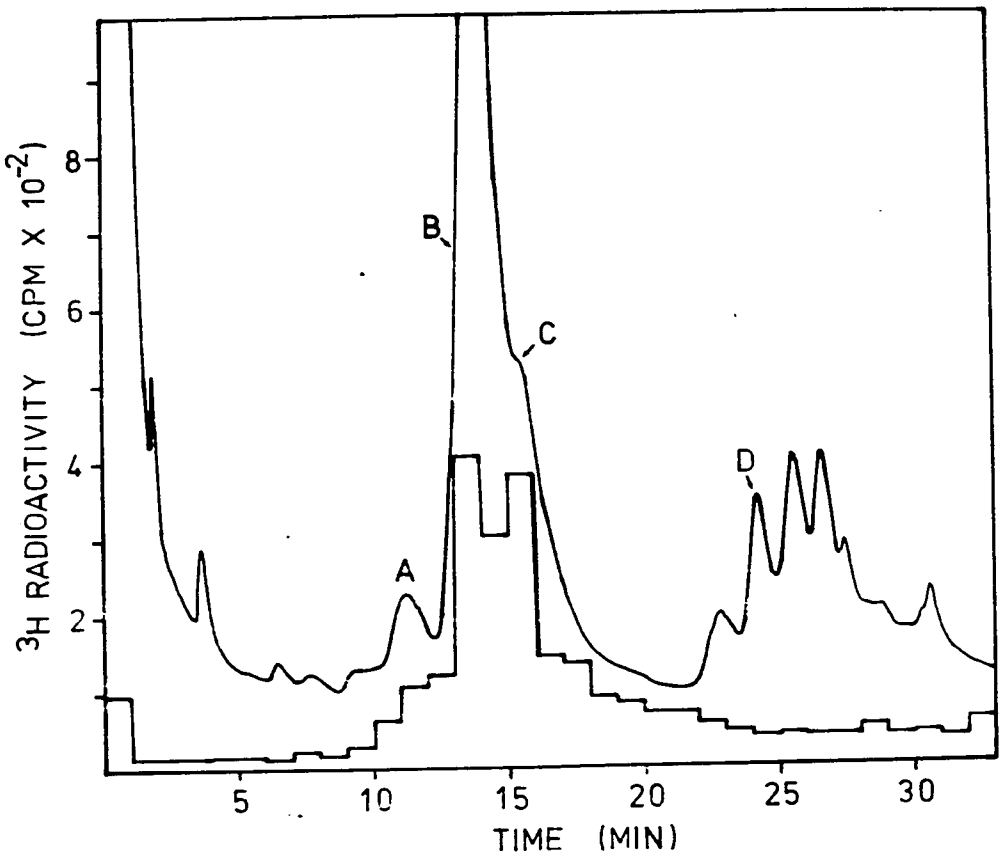


Figure 70. Gas-liquid radiochromatogram of the silylated derivatives of the base moiety of the sphingomyelins of rat #8 in Table XII. A: Silylated derivative of 3-O-methyl-sphingosine and/or 3-O-methyl-dihydrosphingosine. B: Silylated derivative of sphingosine. C: Silylated derivative of dihydrosphingosine. D: Silylated derivative of phytosphingosine.

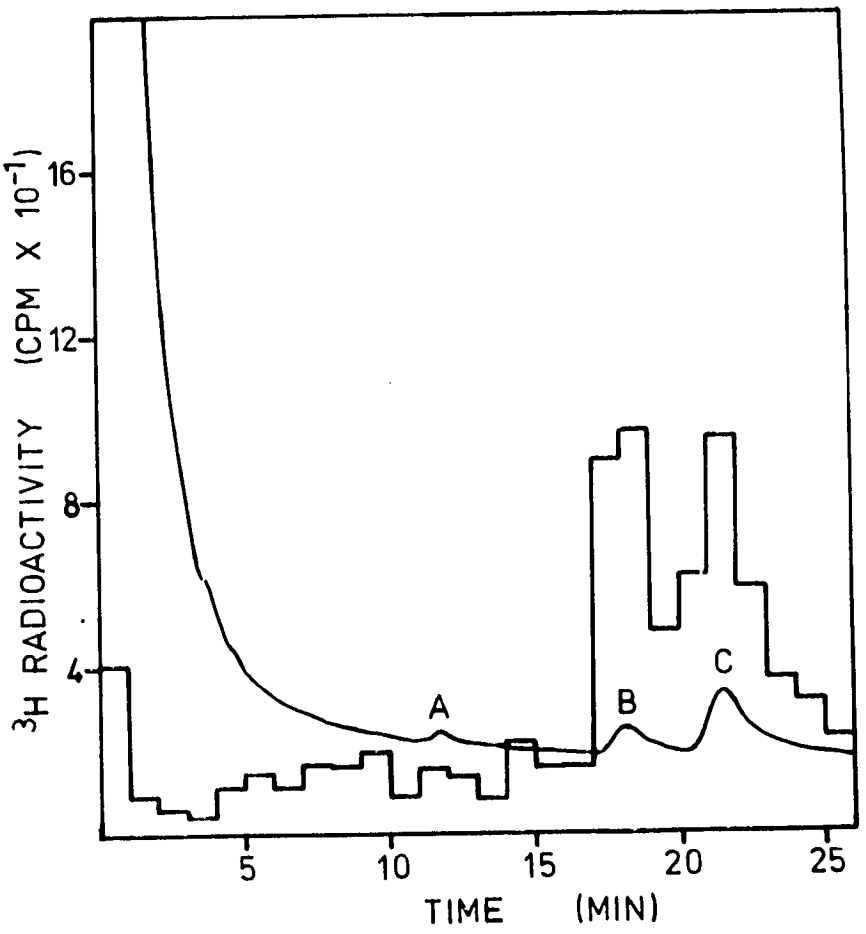


Figure 71. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the base moiety of the sphingomyelins of rat #8 in Table XII. A: Silylated derivative of pentadecanol. B: Silylated derivative of hexadecanol. C: Silylated derivative of 2t-hexadecenol.

TABLE XVI

Approximate ratios of the amount of activity of sphingosine to dihydrosphingosine in sphingomyelins as determined by the silylated derivatives of the periodate-borohydride derived alcohols.

RAT	SPHINGOSINE CPM/DIHYDROSPHINGOSINE CPM
1	0.83
2	1.70
3	1.38
4	----
5	1.91
6	1.77
7	----
8 Axenic	1.21
9 Axenic	1.39
10 Axenic	0.63
11 Starved	2.23
12 Starved	1.33
13 Starved	1.77
MEAN:	1.39
STANDARD DEVIATION:	0.48

TABLE XVII

Approximate amounts of labeled sphingosine isolated in the base moiety of liver ceramides. [4,5-$^{3}H_{2}$]-Dihydrosphingosine was injected into the portal vein of rats. Calculations are corrected for the loss of one-half of the activity of the substrate in the formation of sphingosine. Values are normalized for a 20 uCi (66 nmoles) injection.

RAT	LABELED SPHINGOSINE ISOLATED (nmoles)
1	2.8
2	2.9
3	4.6
4	---
5	1.4*
6	---
7	---
8 Axenic	3.7
9 Axenic	4.3
10 Axenic	4.0
11 Starved	1.6
12 Starved	5.8
13 Starved	5.2
MEAN:	3.6
STANDARD DEVIATION:	1.5

* The mean value of 78% (from Table X) of the activity of the ceramides being composed of the base moiety was used in this calculation.

TABLE XVIII

Approximate amounts of labeled sphingosine isolated in the base moiety of liver cerebrosides. [4,5-$^{3}H_{2}$]-Dihydrosphingosine was injected into the portal vein of rats. Calculations are corrected for the loss of one-half of the activity of the substrate in the formation of sphingosine. Values are normalized for a 20 uCi (66 nmoles) injection.

RAT	LABELED SPHINGOSINE ISOLATED (nmoles)
1	0.20
2	0.23
3	0.28
4	----
5	0.49
6	0.53
7	0.03
8 Axenic	0.28
9 Axenic	0.27
10 Axenic	0.16
11 Starved	----
12 Starved	0.29
13 Starved	0.14
MEAN:	0.26
STANDARD DEVIATION:	0.14

TABLE XIX

Approximate amounts of labeled sphingosine isolated in the base moiety of liver sphingomyelins. [4,5-$^{3}H_{2}$]-Dihydrosphingosine was injected into the portal vein of rats. Calculations are correction for the loss of one-half of the activity of the substrate in the formaion of sphingosine. Values are normalized for a 20 uCi (66 nmoles) injection .

RAT	LABELED SPHINGOSINE ISOLATED (nmoles)
1	0.68
2	0.90
3	0.89
4	----
5	1.07
6	0.96
7	----
8 Axenic	0.57
9 Axenic	0.99
10 Axenic	0.33
11 Starved	0.60
12 Starved	0.78
13 Starved	1.02
MEAN:	0.80
STANDARD DEVIATION:	0.23

take into account activity which tailed from the silylated derivative of hexadecanol into the region of the silylated derivative of 2t-hexadecenol. Third, it was assumed that all of the activity of [4,5-$^{3}H_{2}$]-dihydrosphingosine resided on carbon atoms 4 and 5 and that the amount of sphingosine formed from the substrate was exactly twice the amount that was indicated by the activity. All calculations were normalized for an injection of 20 uCi (66 nmoles) of the substrate.

The fatty acid methyl esters derived from the ceramides (Fig. 72), cerebrosides (Fig. 73) and sphingomyelins (Fig. 74) of rat #8 were all qualitatively similar with the largest amount of activity corresponding to methyl palmitate.

Some very significant and unmistakable observations were noted. First, analyses of the silylated derivatives of the alcohols derived from the free bases of rat #1 (Fig. 75) and rat #2 (Fig. 76) showed the presence of radioactivity in the silylated derivative of 2t-hexadecenol derived from sphingosine. Only in two previous *in vitro*[34,61] studies was the formation of free sphingosine observed. All other *in vitro*[15,70,86] and *in vivo*[54] studies failed to make this observation. Stoffel and Bister[82] even based, in part, their hypothesis of desaturation of dihydrosphingosine forming sphingosine at an N-acylated level upon the lack of evidence of the formation and occurrence of free sphingosine.

A second remarkable observation was noted in rat #4. The silylated derivative of the long chain bases derived from the ceramides (Fig. 77) reveals that almost 63% of the recovered activity co-chromatographed with the silylated derivative of phytosphingosine on gas-liquid chromatography. The actual amount formed was approximately 25% greater

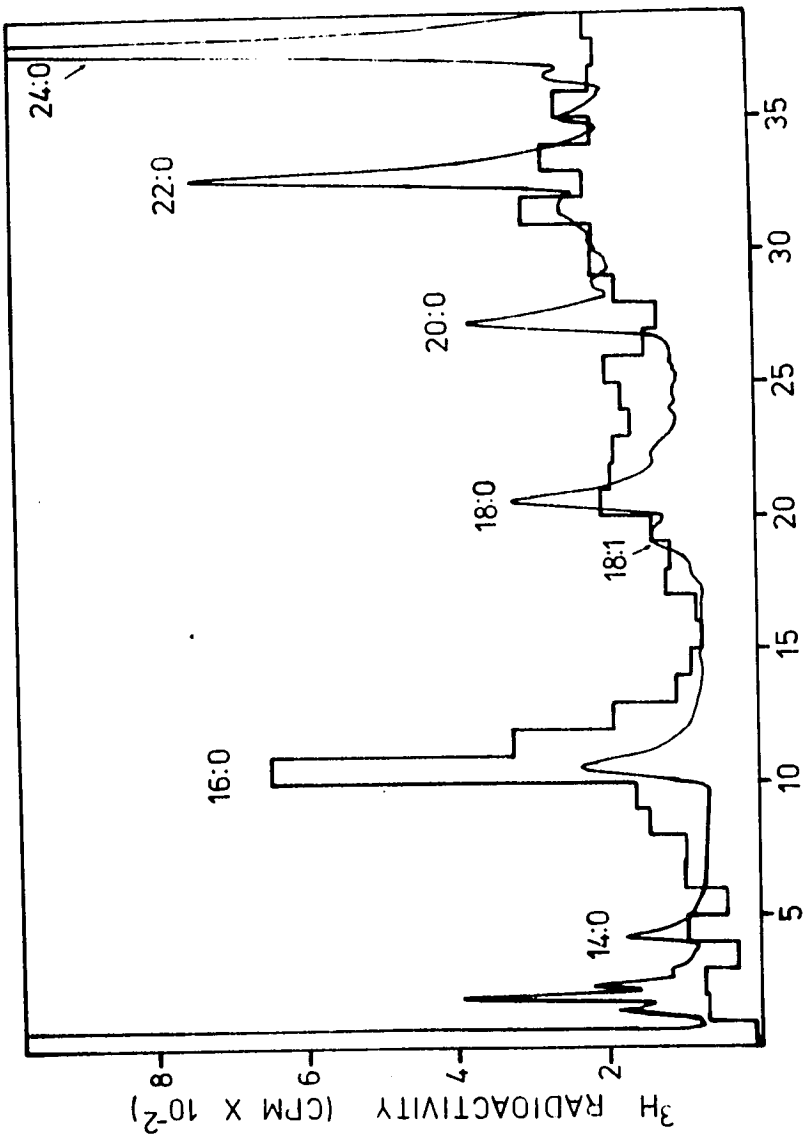


Figure 72. Gas-liquid radiochromatogram of the methyl esters of the fatty acids from the ceramides of rat #8 in Table X.

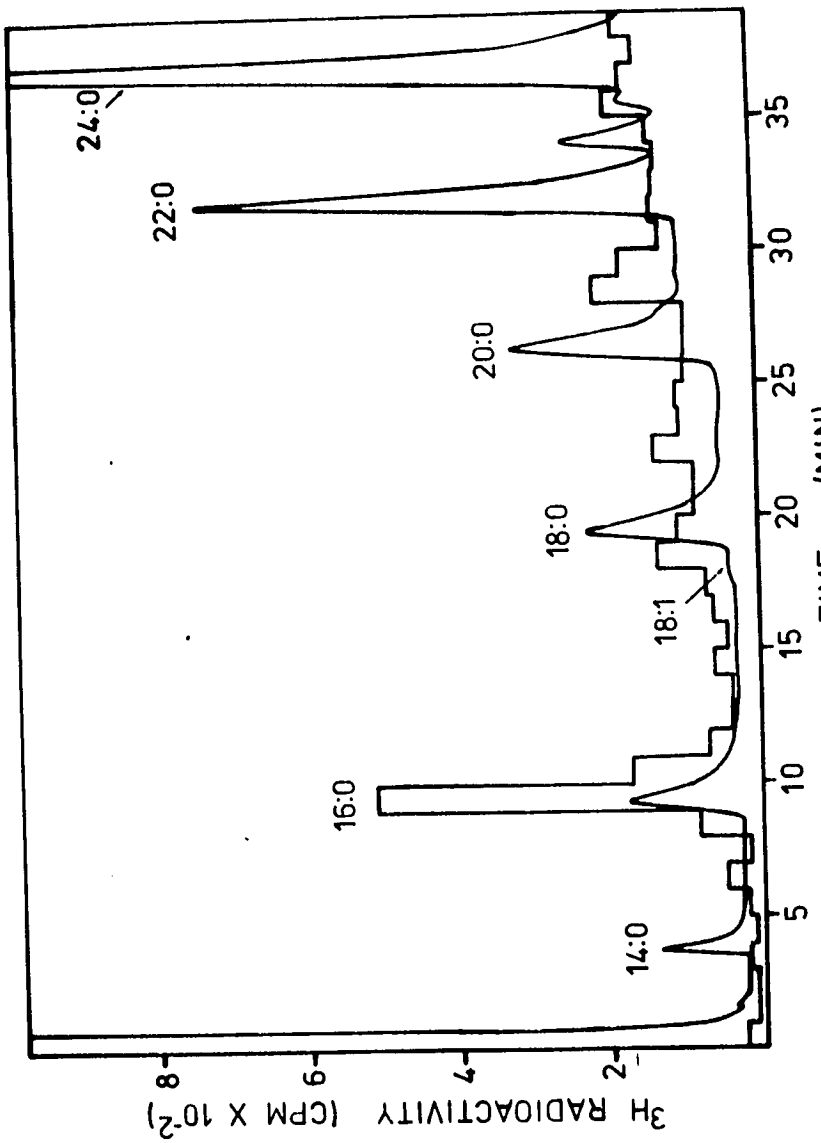


Figure 73. Gas-liquid radiochromatogram of the methyl esters of the fatty acids from the cerebrosides of rat #8 in Table XI.

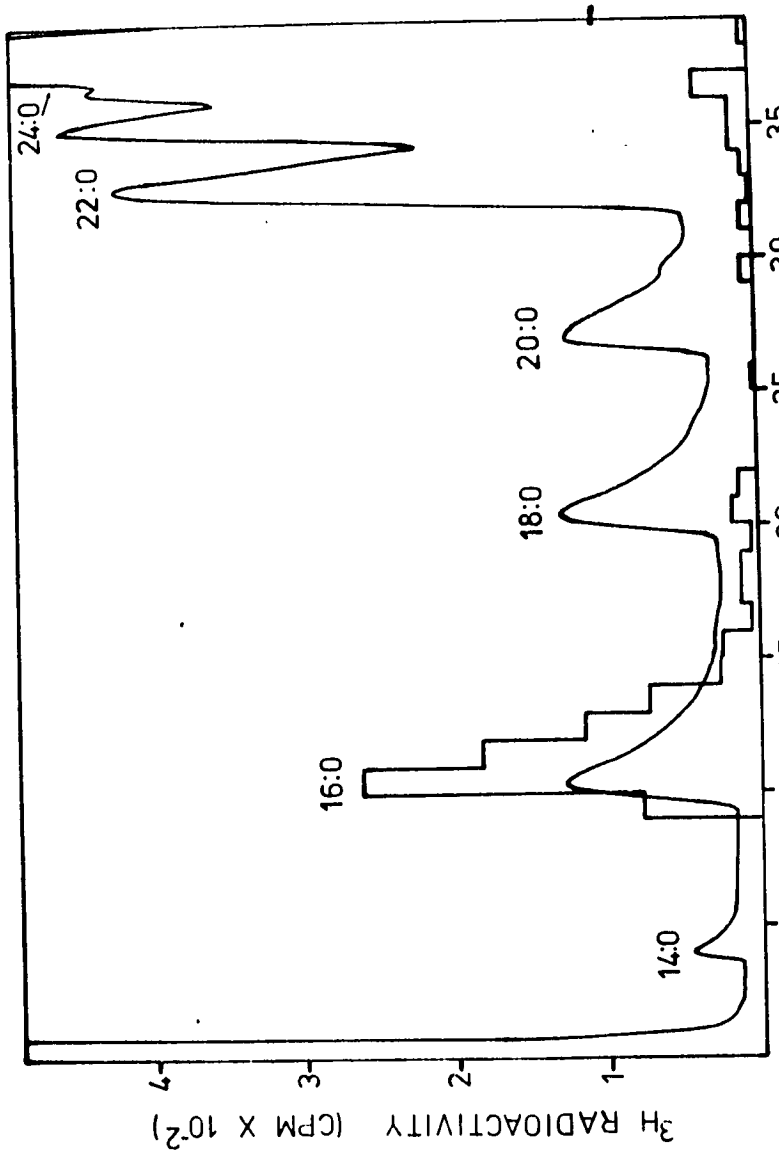


Figure 74. Gas-liquid radiochromatogram of the methyl esters of the fatty acids from the sphingomyelins of rat #8 in Table XII.

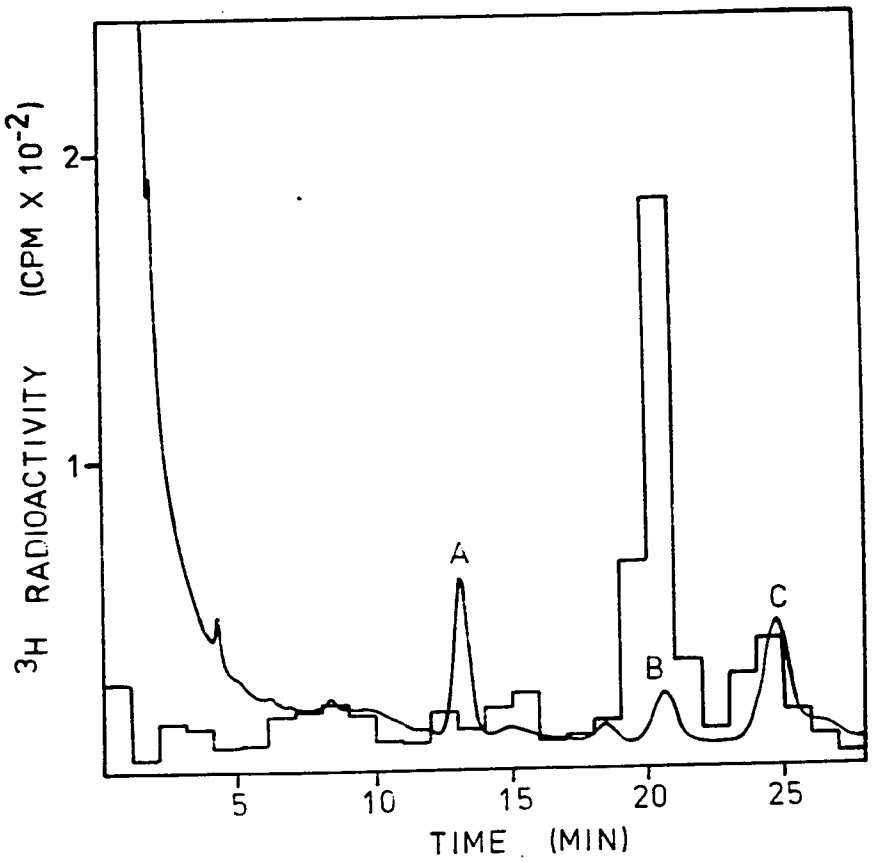


Figure 75. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the free bases of rat #1 in Table VIII.

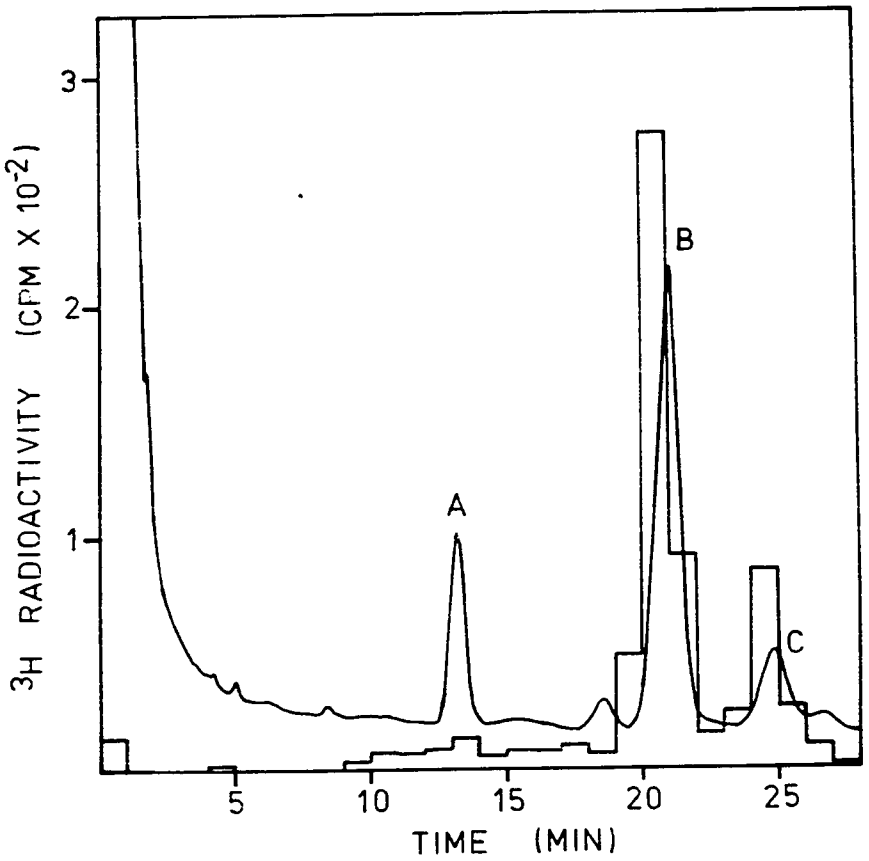


Figure 76. Gas-liquid radiochromatogram of the silylated derivatives of the alcohols derived from the periodate-borohydride treatment of the free bases of rat #2 in Table VIII.

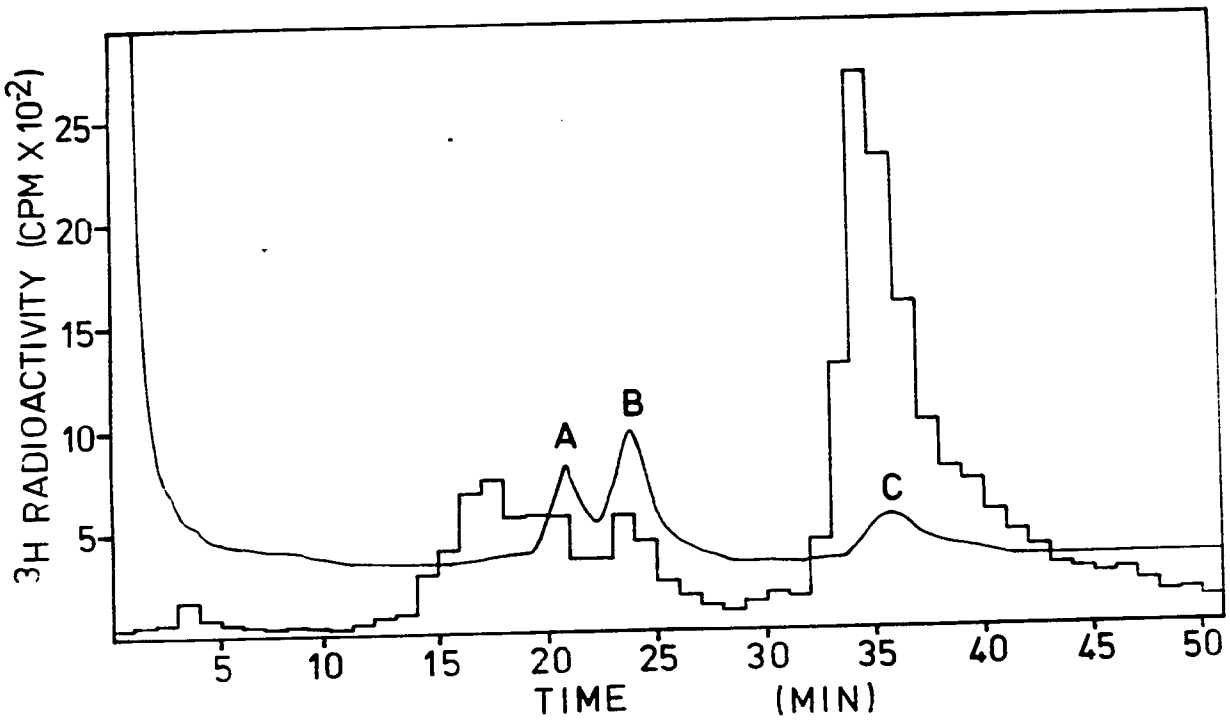


Figure 77. Gas-liquid radiochromatogram of the silylated derivatives of the base moiety of the ceramides of rat #4 in Table IV on a four foot, 1% SE 30 at 209° C. A: Silylated derivative of sphingosine. B: Silylated derivative of dihydrosphingosine. C: Silylated derivative of phytosphingosine.

due to the stereospecific loss of the 4R hydrogen from [4,5-$^3H_2$]-dihydrosphingosine in the formation of a [5-^3H]- and [4,5-$^3H_2$]-phytosphingosine mixture. A significant amount of activity corresponded to anhydrophytosphingosine[17] (retention time: 18 minutes in Fig. 77) which was formed during the acid hydrolysis. Some activity was also observed in sphingosine and dihydrosphingosine.

The activity and mass tracings of the fatty acid methyl esters of rat #4 ceramides (Fig 78) and cerebrosides (Fig. 79) were different from those of any other rat. Endogenous peak B of figure 78 was analyzed by combined gas-liquid chromatography-mass spectrometry (Fig. 80) and was identical to the mass spectrum of methyl-2-hydroxy-palmitate[32]. Characteristic ions observed were m/e 286 (the molecular ion), m/e 254 (M-32; M - CH_3OH), m/e 227 (M-59; M - $COOCH_3$) and m/e 90 (M-196; the McLafferty rearrangement product shown in figure 80)[32]. It should be noted that only in this rat was there enough endogenous 2-hydroxy-palmitic acid present to be observed in the chromatogram. Endogenous peak A of figure 78 had a relative retention time corresponding to methyl pentadecanoate. Peak A and peak B each had a large amount of corresponding activity. As stated in the introduction, the catabolic products of phytosphingosine usually observed were 2-hydroxy-palmitic acid and its oxidation product, pentadecanoic acid. Peak D of figure 78 may have been the two carbon elongation product of 2-hydroxy-palmitic acid. Unfortunately, instrumental failure precluded the further analysis of the lipids from this rat. However, these results parallel those of Hirschberg[50].

It was reasonable to ask why rat #4 differed from all of the

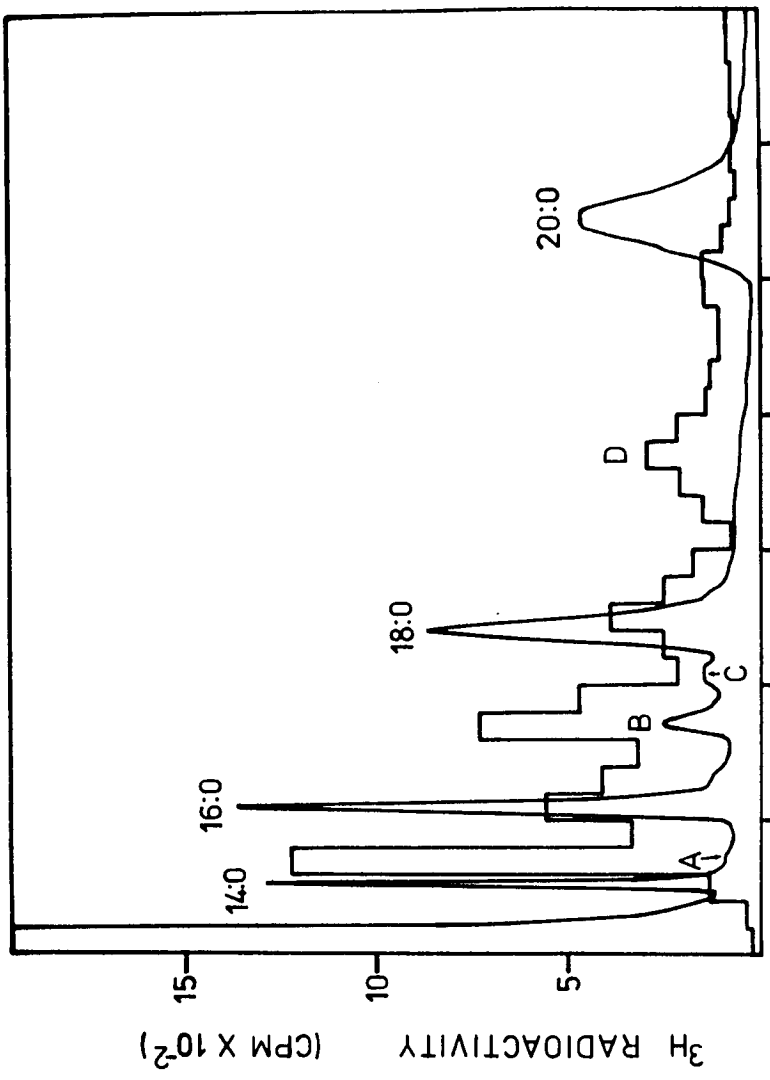


Figure 78. Gas-liquid radiochromatogram of the methyl esters of the fatty acids from the ceramides of rat #4 in Table X on a four foot, 1% SE 30 column at 185° C.

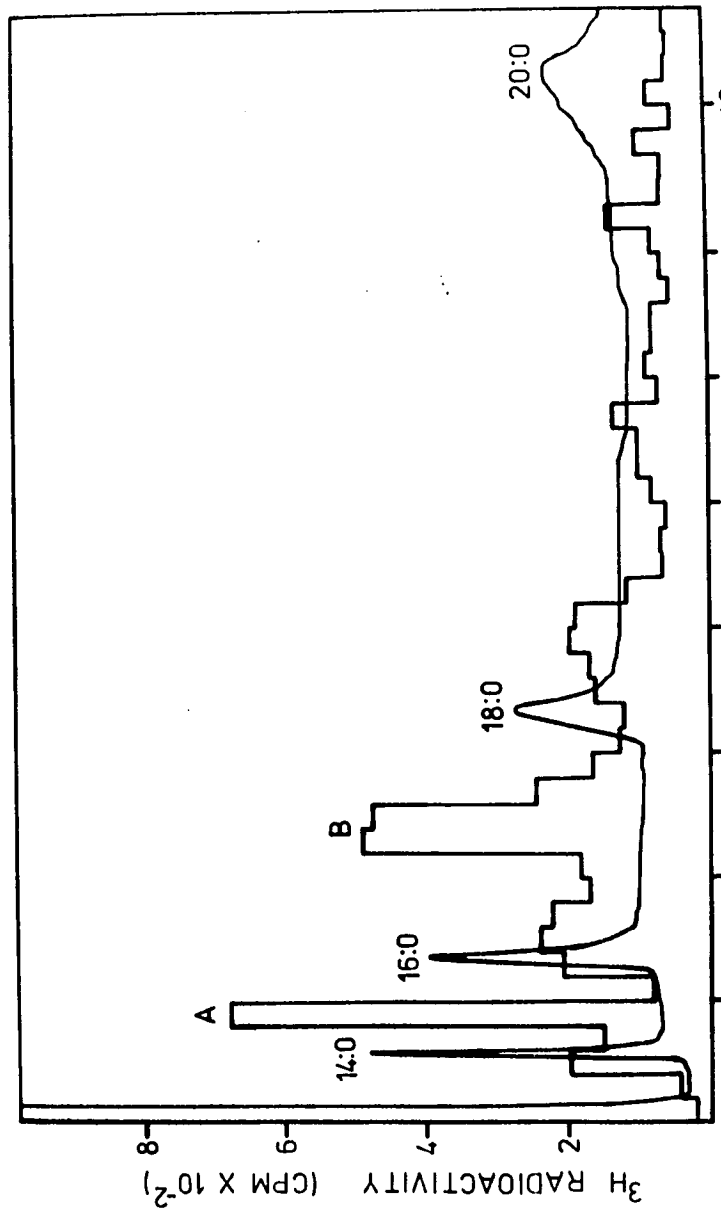


Figure 79. Gas-liquid radiochromatogram of the methyl esters of the fatty acids from the cerebrosides of rat #4 in Table XI on a four foot, 1% SE 30 column at 165°C.

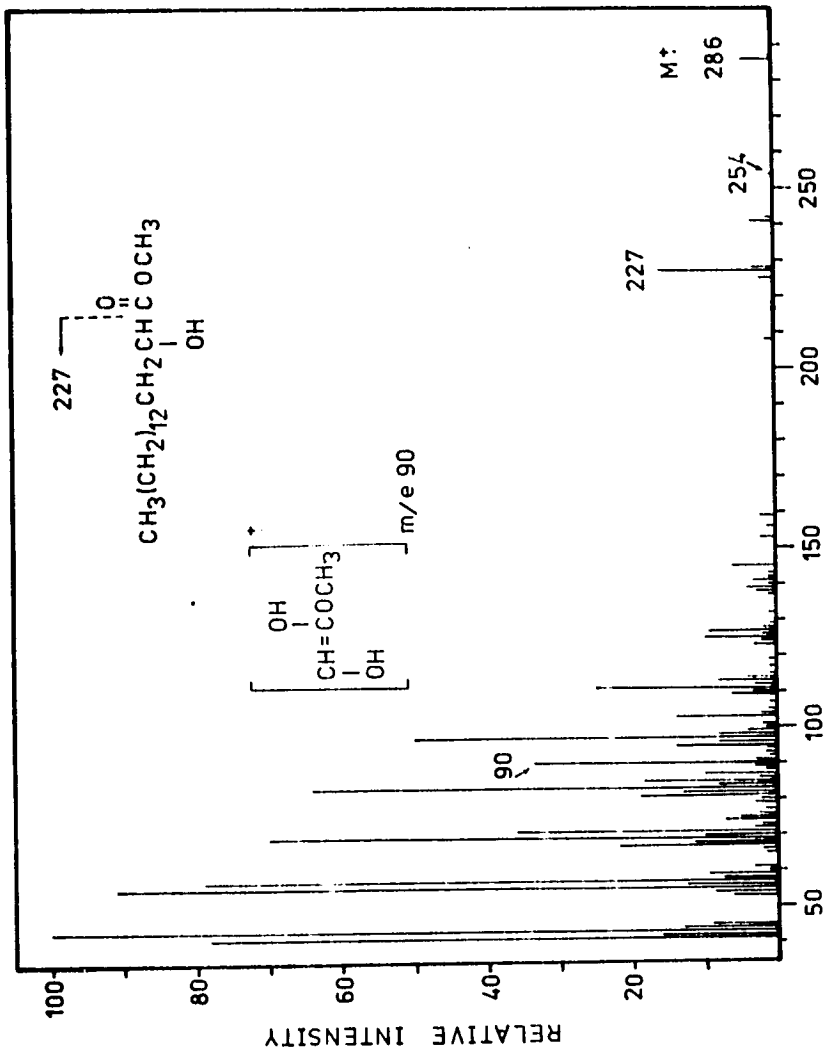


Figure 80. Combined gas-liquid chromatography-mass spectrum of peak B of figure 78 on a six foot, 1% OV 1 column at 185° C.

other rats. Experience in this laboratory ahd shown that very small amounts of unlabeled or labeled phytosphingosine decomposed at an incredible rate in a fashion similar to that of dihydrosphingosine (which was studied in detail earlier in this work). Yet, it appeared that this cannot be the sole explanation for the failure to observe labeled phytosphingosine in the other twelve rats in significant amounts. First, rat #4 showed beyond a doubt that, at least in that case, labeled phytosphingosine in the presence of 120 ug of the unlableed phytosphingosine (which was added prior to hydrolysis) could survive the procedures. Second, and of more importance, the occurrence of 2-hydroxy-palmitic and pentadecanoic acids would be expected regardless of any possible degradation of the base moiety during the workup. Rat #4 was, therefore, unique in this series of experiments.

An important point was that previously unreported observations were seen to occur within sets of rats in a single experiment. Hirschberg[50] noted that the conversion of the label in dihydrosphingosine to phytosphingosine occurred only in one experiment where a set of three rats were worked up simultaneously. Repeated efforts to reproduce these results failed. In the present work, the observation of the label in free sphingosine occurred in two of the three rats in a single experiment. Rat #4 was worked up as a separate experiment. This implied that some yet unknown external variable may be operable.

Dietary variations were still possible as orally ingested sphingolipids may have depressed enzymatic activity. However, the results of

the experiments with the 24 hour-starved rats were similar to those of rats fed ad libitum. Hormonal variation of the female rats may have played some part. Age (or weight) seemed to have no role. Another matter to be considered is that of diurnal variation. These experiments were carried out during the morning hours (although the precise times of Hirschberg's experiments is unknown). Each of these possibilities derserves further study.

SUBSTRATE SPECIFICITY OF DIHYDROSPHINGOSINE KINASE

In order to test the substrate specificity of rat liver dihydrosphingosine kinase, the following labeled materials were synthesized:

[4,5-$^3H_2$]-N-BENZOYL-DIHYDROSPHINGOSINE: [4,5-$^3H_2$]-N-Benzoyl-dihydrosphingosine (3.9 mCi/mg) was prepared as described earlier in the synthesis of [4,5-$^3H_2$]-dihydrosphingosine, and was about 94% pure by thin layer analysis (Fig. 81).

[4,5-$^3H_2$]-N-ACETYL-DIHYDROSPHINGOSINE: Dihydrosphingosine (200 ug) was added to a flask along with about 3 uCi of [4,5-$^3H_2$]-dihydrosphingosine (Preparation II, 2.7 mCi/mg). Freshly prepared methanol-acetic anhydride (4:1; 100 ul) was added and after standing overnight at room temperature, water (100 ul) was added and the mixture lyophilized one hour later. This method quantitatively acetylated the primary amine of the long chain base[18]. The sample was analyzed by thin layer chromatography (Fig. 82).

[1-$^3H_2$]-DL-ALPHA-AMINO-STEARYL-ALCOHOL: Methyl-DL-alpha-amino-stearate-hydrochloride (mp 109-111° C, clear liquid at 158° C; 200 mg; 0.572 mmole; a sample prepared in the laboratory of Dr. H. E. Carter [23]) was added to 5% sodium bicarbonate (20 ml) and shaken. Ether (40 ml) was added and the mixture shaken vigorously. The aqueous solution was brought to pH 12 by the addition of 1 N sodium hydroxide. The mixture was extracted three times with ether (40 ml each). The pooled ether layers were then washed to neutrality with water, filtered

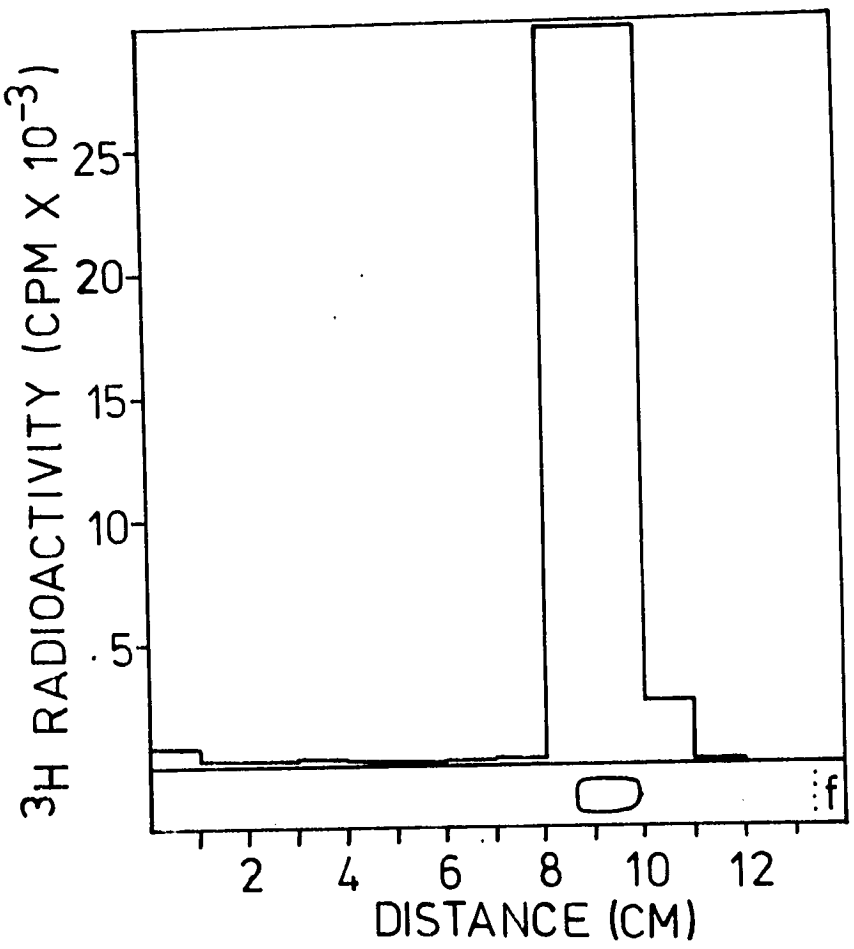


Figure 81. Thin layer radiochromatogram of $[4,5\text{-}^3H_2]$-N-benzoyl-dihydrosphingosine. Standard: N-Benzoyl-dihydro-sphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

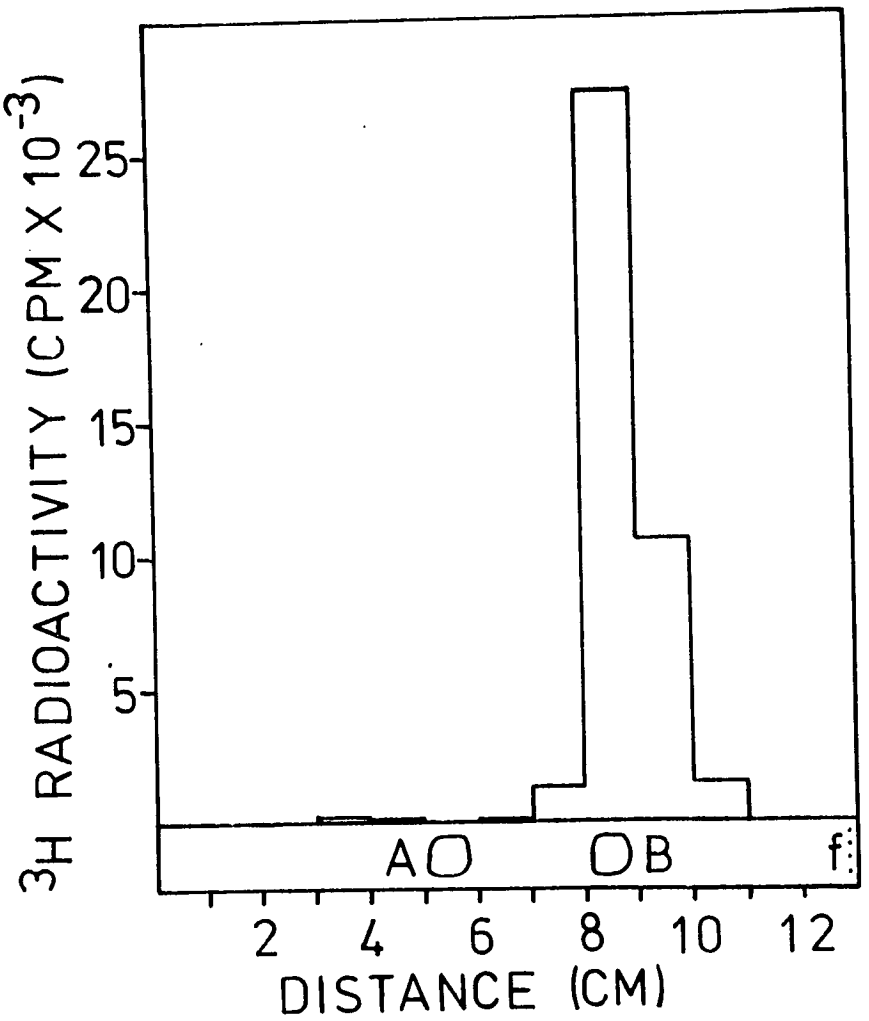


Figure 82. Thin layer radiochromatogram of [4,5-$^3H_2$]-N-acetyl-dihydrosphingosine. A: Dihydrosphingosine. B: N-Acetyl-dihydrosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

through Hyflo Super Cel (Johns-Mansville) and dried under reduced pressure at 35° C. The theoretical yield of methyl-DL-alpha-amino-stearate was 179 mg. The actual yield was 160 mg (89%) of white solid (mp 47.5-50° C). This was recrystallized two times from methanol-water. Upon cooling slowly to room temperature with stirring an oil formed. Cooling slowly with stirring to 11° C solidified the product which was then filtered with suction and washed with methanol-water (1:1) and dried *in vacuo* over phosphorous pentoxide to give 128 mg (71% overall yield) of methyl-DL-alpha-amino-stearate which melted sharply at 49-50° C, but the liquid was not clear until 190° C. Thin layer analysis on Silica Gel PF 254 + 366 in both chloroform-methanol (99:1) and (92:8) showed that a very small amount of impurity was present. About 100 mg of the crystallized material was applied to a 20 gram silicic acid column (2 X 12.5 cm packed in hexane). The sample was washed on to the column with two portions (3 ml each) of hexane-chloroform (2:8) and the column was developed (about 15 ml/fraction/32 minutes) with the same solvent (750 ml). Fractions 21-29 (82 mg) were pooled as pure methyl-DL-alpha-amino-stearate as analyzed by thin layer (Fig. 83) and gas-liquid (Fig. 84) chromatography. This material melted at 47.5-50° C (Lit.[23] mp 48-49° C) forming a clear liquid.

The infrared spectrum (Fig. 85) showed a weak, sharp assymetric N-H stretching absorbance at 3395 cm^{-1}; an ester carbonyl at 1721 cm^{-1}; methyl and methylene deformation at 1472 cm^{-1} and methoxy single bond stretching at 1179 cm^{-1} and 1208 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford *et al.*[28] and Jones[52].

The NMR spectrum (Fig. 86) showed both the methyl protons (3.68 PPM; 3 protons; singlet) and the C-2 proton (3.40 PPM; 1 proton;

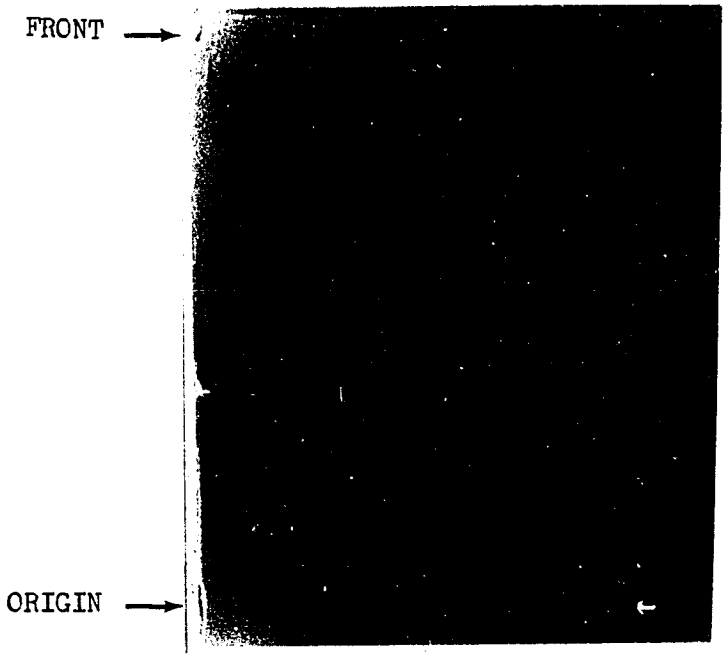


Figure 83. Thin layer chromatogram of methyl-DL-alpha-amino-stearate on Silica Gel PF 254 + 366. Solvent: Chloroform-methanol (97.5:2.5). Stain: Molybdic acid.

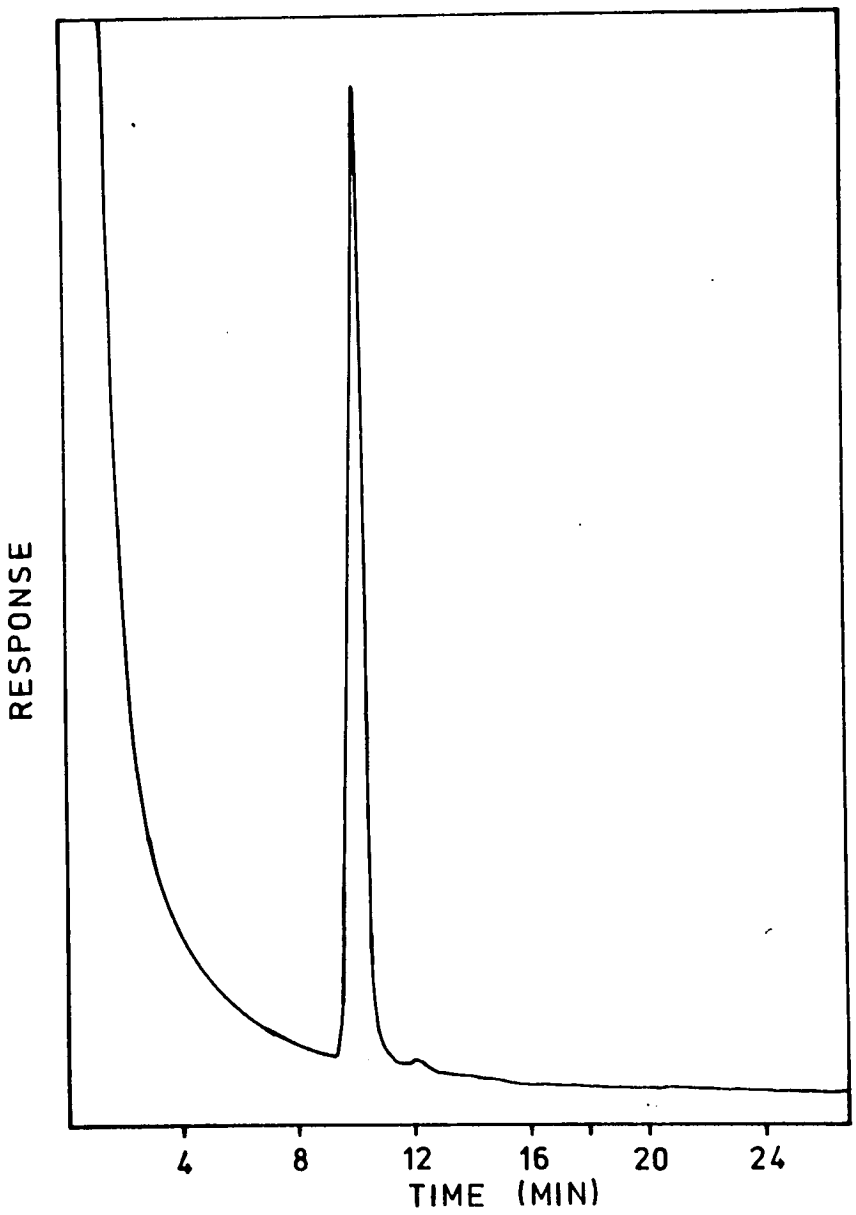


Figure 84. Gas-liquid chromatogram of the silylated derivative of methyl-DL-alpha-amino-stearate on an eight foot, 1% OV 17 column at 200°C.

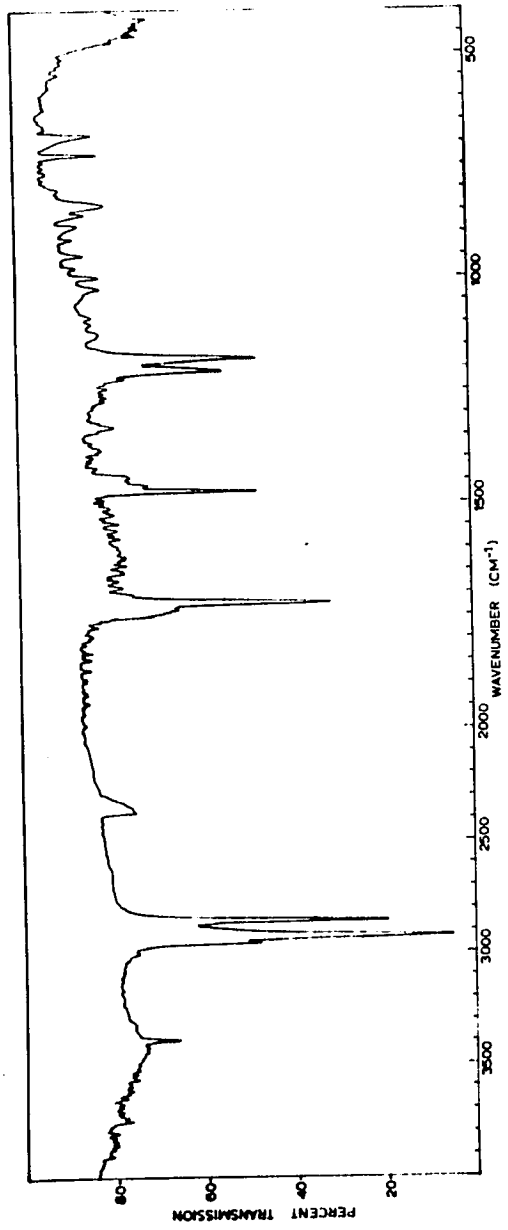


Figure 85. Infrared spectrum of methyl-DL-alpha-amino-stearate.

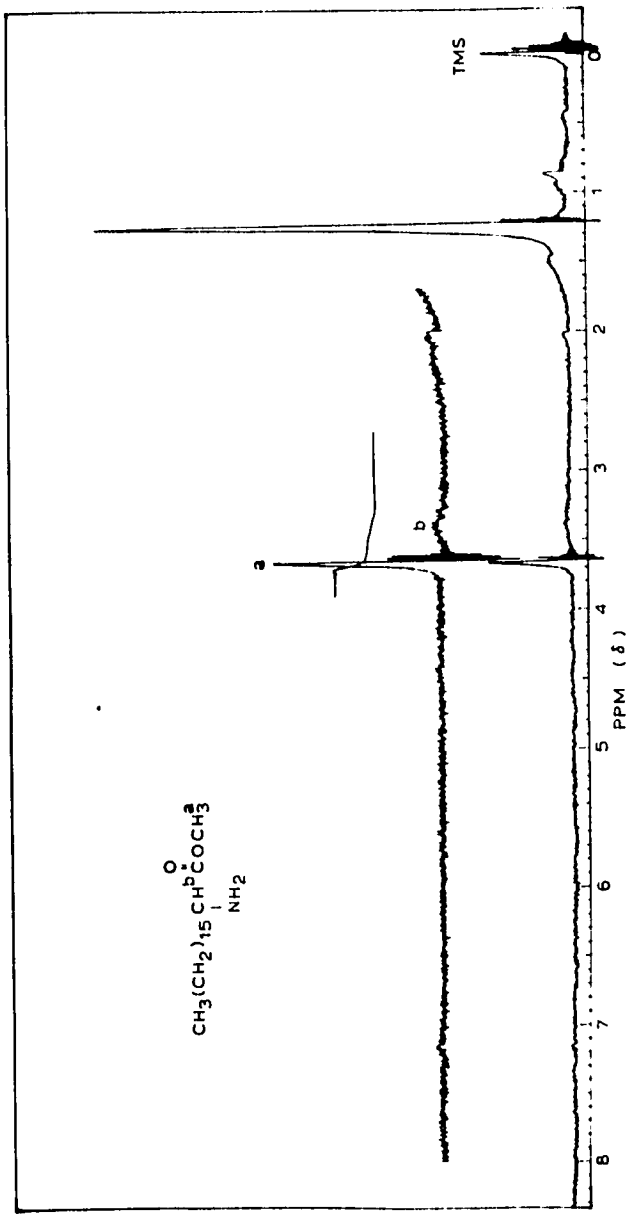


Figure 86. NMR spectrum of methyl-DL-alpha-amino-stearate.

multiplet).

The major ions and their tentative assignments in the mass spectrum (Fig. 87) were m/e 313 (the molecular ion, $M^{+}\cdot$), m/e 298 (M-15; M - CH_3), m/e 282 (M-31; M - CH_3O), m/e 254 (M-59; M - CH_3OCO) and m/e 88 (M-225; $CH_3OCOCH(NH_2)$). The major ions and their tentative assignments in the mass spectrum (Fig. 88) of N-trimethylsilyl-methyl-DL-alpha-amino-stearate were m/e 385 (the molecular ion, $M^{+}\cdot$), m/e 370 (M-15; M - CH_3), m/e 326 (M-59; M - CH_3OCO), m/e 254 (M-131), m/e 160 (M-225; M - $CH_3(CH_2)_{14}CH_2$), m/e 128 (M-257; M - $CH_3(CH_2)_{14}CH_2$ - CH_3OH) and m/e 73 ($SiMe_3$).

Methyl-DL-alpha-amino-stearate (31 mg; 0.099 mmole) in ether (10 ml) was reduced by adding $LiAlH_4$ (Matheson, Coleman & Bell; 25.6 mg; 0.674 mmole) with mixing followed by refluxing for one hour. Water (25 ul), 15% sodium hydroxide (25 ul) and water (75 ul) were added successively with stirring. The solution was filtered and the precipitate washed with ether (10 ml). The theoretical yield of DL-alpha-amino-stearyl-alcohol was 28 mg; the actual yield was 25 mg (87%). A small amount of non-polar impurities were observed on thin layer chromatography on Silica Gel PF 254 + 366 developed with chloroform-methanol-ammonia (100:25:2.5). The reaction product was applied in chloroform (1 ml) to a 4 gram silicic acid column (0.9 X 15 cm; packed in chloroform) and washed on to the column with chloroform (1 ml). The column was then eluted (about 10 ml/fraction/64 minutes) with chloroform (25 ml; fractions 1-3), 2% methanol in chloroform (150 ml; fractions 4-21) and 4% methanol in chloroform (450 ml; fractions 22-66). Fractions 29-65 were pooled (25.7 mg) and crystallized three times from

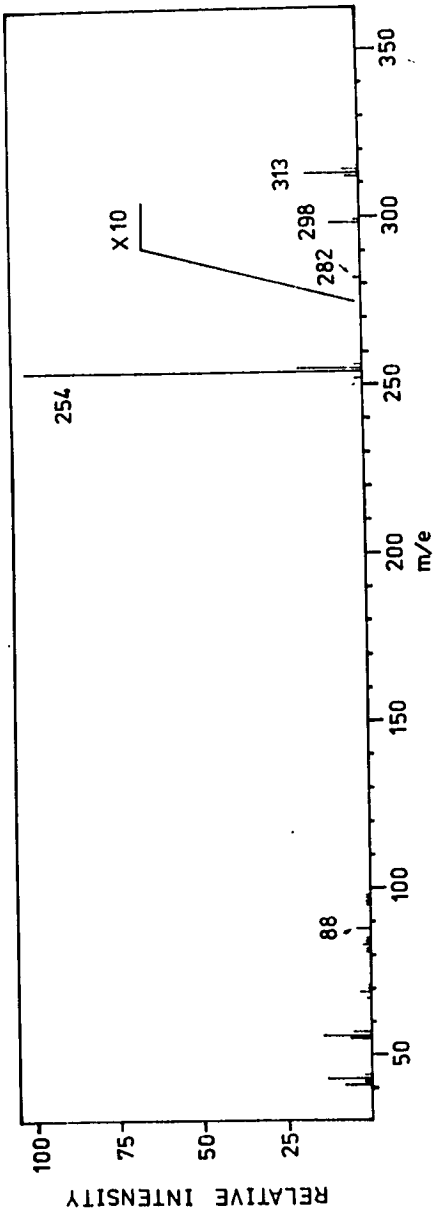


Figure 87. Mass spectrum of methyl-DL-alpha-amino-stearate.

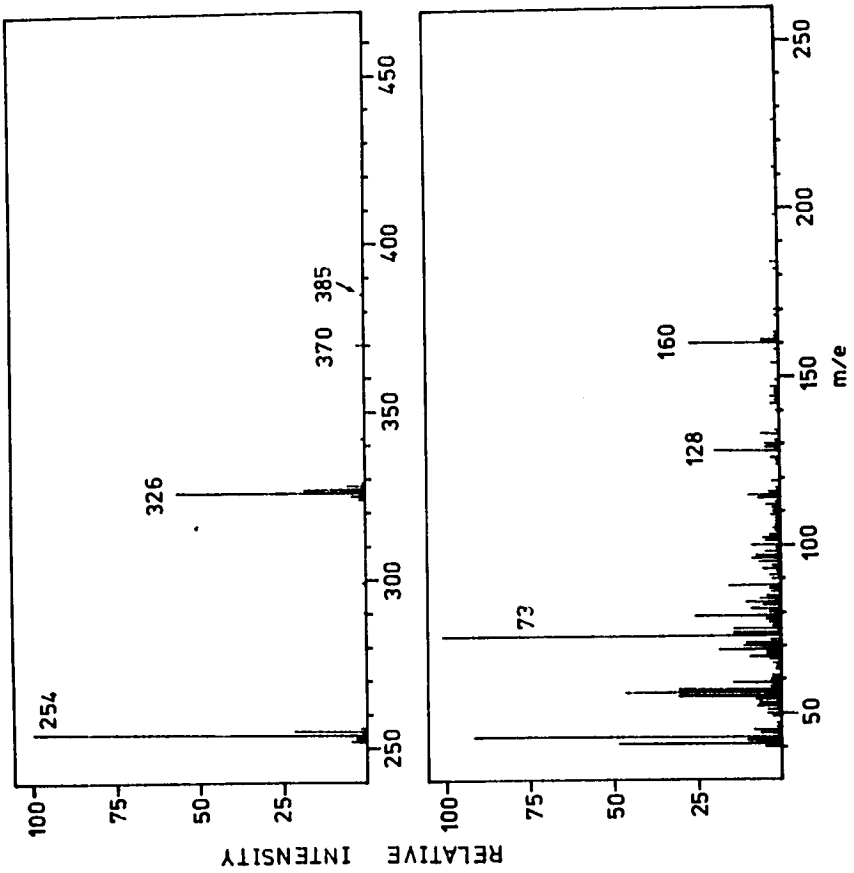


Figure 88. Combined gas-liquid chromatography-mass spectrum of the silylated derivative of methyl-DL-alpha-amino-stearate on an eight foot, coiled 1% SE 30 at 200° C.

hot hexane. The first crop was 9.4 mg of feathery white crystals (mp 81.5-83° C (Lit.[23] mp 81-81.5° C); Channel 1, Fig. 89) and the second crop 2.5 mg of feathery white crystals (mp 81.5-83° C; Channel 2, Fig. 89).

The infrared spectrum (Fig. 90) was rather uninformative and showed only the methylene absorptions at 2860 cm^{-1} and 2925 cm^{-1}. The primary hydroxyl and amino groups gave weak absorbances.

The NMR spectrum (Fig. 91) showed the C-1 protons (3.3 PPM; 2 protons; multiplet) and the C-2 hydrogen (2.75 PPM; 1proton; multiplet).

The major ions and their tentative assignments in the mass spectrum (Fig. 92) were m/e 285 (the molecular ion, $M^{+\cdot}$), m/e 268 (M-17; M - NH_3[70b]), m/e 266 (M-19; M - H - H_2O) and m/e 254 (M-31; M - CH_2OH). The major ions and their tentative assignments in the combined gas-liquid chromatography-mass spectrum (Fig. 93) of the silylated derivative were m/e 356 (M-73; M - $SiMe_3$), m/e 342 (M-87), m/e 175 (M-254) and m/e 103 (M-326; CH_2OSiMe_3).

Absolute ether (10 ml) was distilled in oven dried glassware into a flask containing methyl-DL-alpha-amino-stearate (dried <u>in vacuo</u> over phosphorous pentoxide for 18 hours; 7 mg; 0.022 mmole). When the solid had dissolved with stirring, $LiAl^3H_4$ (New England Nuclear, lot # 885-008; 135.36 mCi/mmole; 1.4 mg of pale yellow solid; 0.0369 mmole; 5 mCi; molar ratio 3.35:1) was added and stirred at reflux for one hour. An additional portion of $LiAlH_4$ (10 mg) was added and the refluxing continued for one hour. Added successively were water (11.4 ul), 15% sodium hydroxide (11.4 ul) and water 34.2 ul) and the precipitate filtered under suction, washed with ether (10 ml) and the filtrate transferred to a round-bottom flask and dried under nitrogen

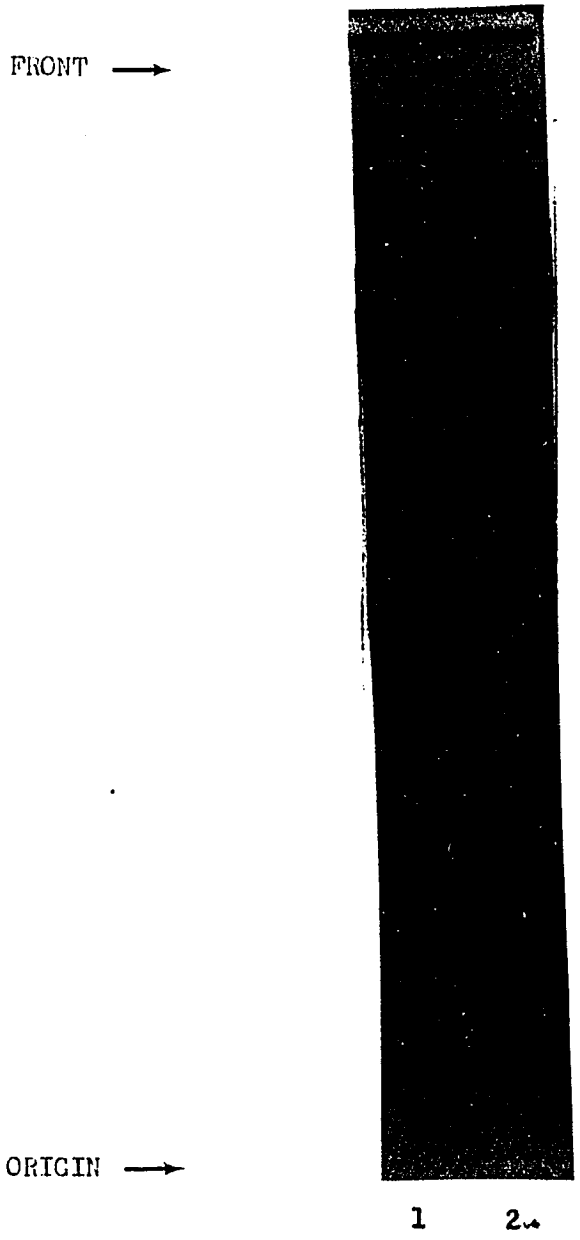


Figure 89. Thin layer chromatogram of DL-alpha-amino-stearyl-alcohol on Silica Gel PF 254 + 366. Channel 1: First crop from crystallization. Channel 2: Second crop. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Molybdic acid.

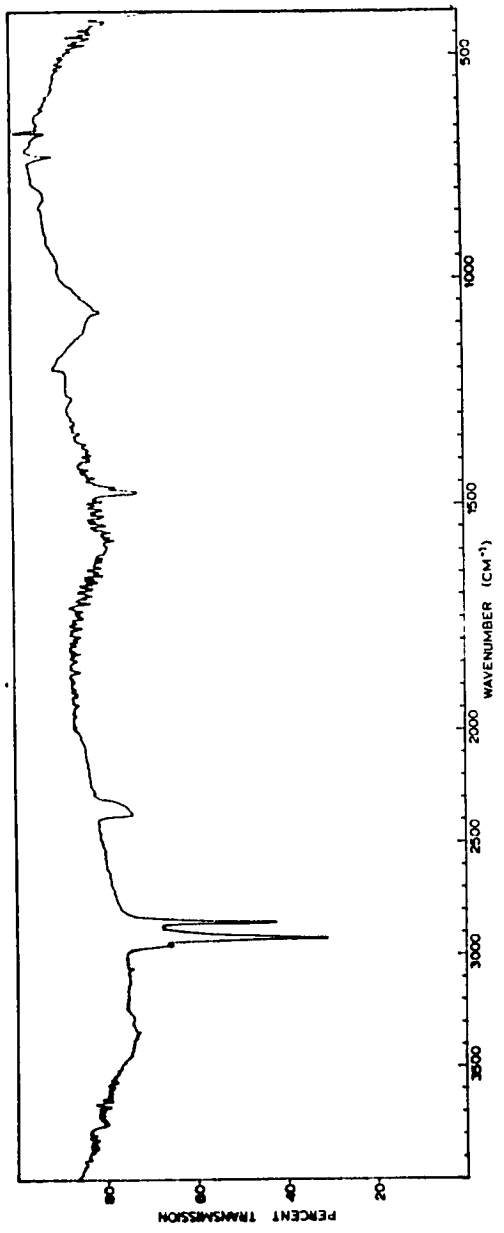


Figure 90. Infrared spectrum of DL-methyl-alpha-amino-stearyl-alcohol.

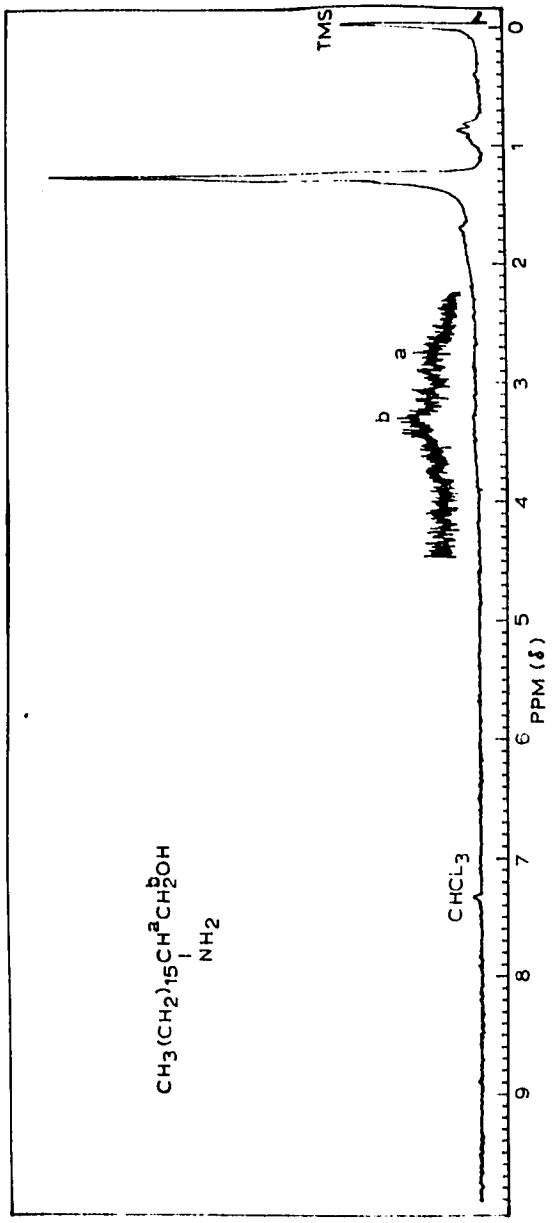


Figure 91. NMR spectrum of DL-methyl-alpha-amino-stearyl-alcohol.

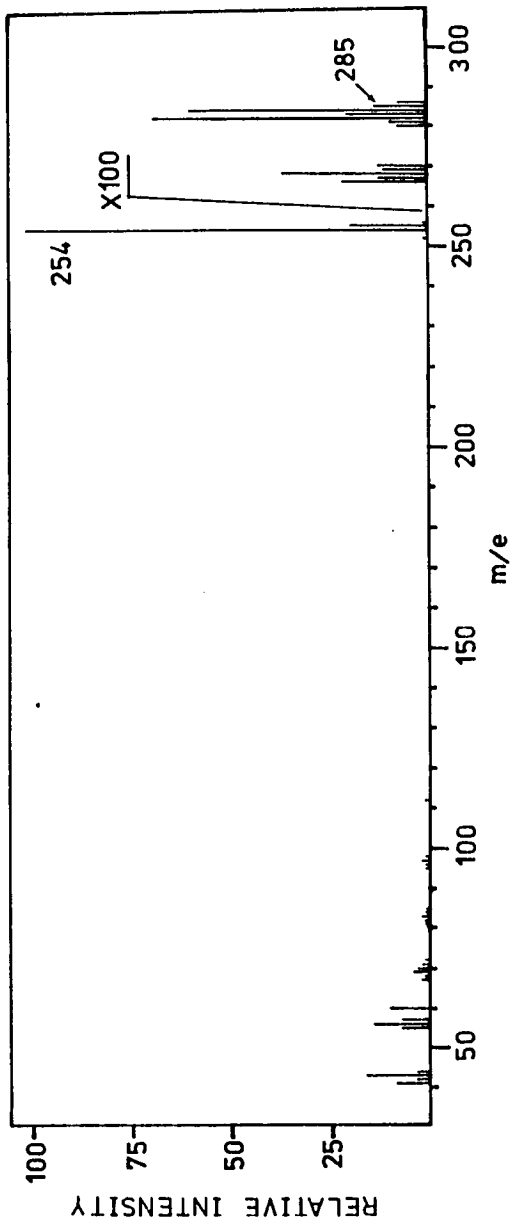


Figure 92. Mass spectrum of DL-methyl-alpha-amino-stearyl-alcohol.

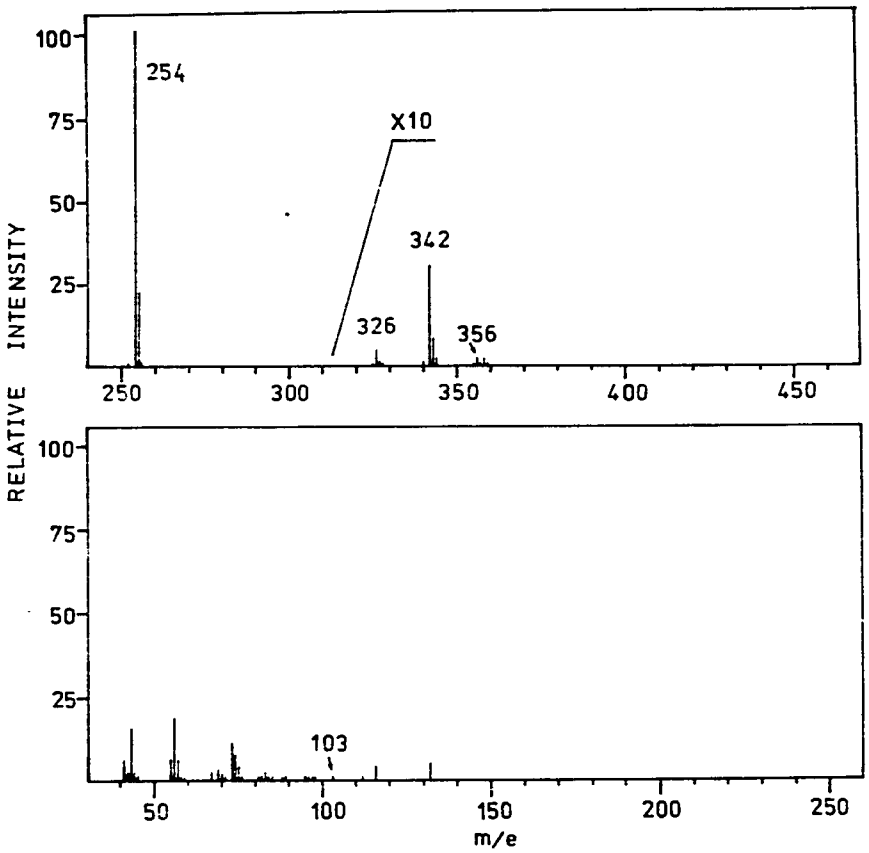


Figure 93. Combined gas-liquid chromatography-mass spectrum of the silylated derivative of DL-methyl-alpha-amino-stearyl-alcohol on an eight foot, coiled 1% SE 30 at 200° C.

at 30° C. A mixture of water-benzene-methanol (1:5:5) was distilled two times under vacuum from the residue with gentle heating. The residue was dried under nitrogen and in vacuo over phosphorous pentoxide. The theoretical yield of [1-$^3H_2$]-DL-alpha-amino-stearyl-alcohol was 6.4 mg; the actual yield was 6.8 mg (107%). This was dissolved in benzene (10.0 ml) and an aliquot counted showing a total of 8.64 X 10^9 DPM (3.93 mCi; 264% radioactive yield; 0.57 mCi/mg; 162.5 mCi/mmole). The high radioactive yield was probably due to the fact that the specific activity of the LiAl^3H_4 was innaccurately determined by the supplier. The residue was applied in benzene (10 ml) to a 4 gram silicic acid column (0.9 X 15 cm; packed in chloroform) and washed onto the column with two portions of chloroform (2.5 ml each). The column was then eluted with the following solvents (about 6.2 ml/fraction/32 minutes): 2% methanol in chloroform (250 ml), 3.5% methanol in chloroform (200 ml) and methanol (150 ml; 5 ml/fraction/64 minutes). Care was taken to avoid chloroform in the first methanol fraction. Fractions 75-100 were pooled and the solvent removed at reduced pressure at 30° C leaving a residue of about 3.8 mg. This was transferred in benzene to a tared centrifuge tube and the solvent removed under nitrogen at 30° C and dried in vacuo over phosphorous pentoxide. This was crystallized from warm hexane (1 ml) by cooling very slowly to 0° C. After standing overnight in the mother liquor, the precipitate was collected by centrifugation and dried in vacuo over phosphorous pentoxide leaving about 1.9 mg which was then redissolved in benzene (10.0 ml) and an aliquot counted. The total activity was 3.02 X 10^9 DPM (1.59 X 10^9 DPM/mg; 0.723 mCi/mg; 206 mCi/mmole). This material was at least 96% radiopure as analyzed by thin

layer (Fig. 94) and gas-liquid (Fig. 95) chromatography. The specific activity of the material used as a substrate in enzymatic incubations was 1.18 X 10^6 DPM/mg.

[UL-^{14}C]-1,2-DIGLYCERIDES: Egg lecithin (Sigma; 1 mg) and [UL-^{14}C]-lecithin (1.4 uCi; New England Nuclear; from Chlorella vulgaris with fatty acids consisting mostly of linoleic, oleic and palmitic acids; lot # 666-059; 1.846 Ci/mmole) were added to a screw cap test tube. These were dissolved in ether-95% ethanol (98:2; 1 ml) and 0.08 M $CaCl_2$ (20 ul) and phospholipase C (Sigma; 0.5 mg in 0.5 ml of 0.05 M tris-maleate buffer, pH 7.3) were then added. The solution was mixed vigorously on a vortex mixer for two hours at room temperature while sealed tightly under nitrogen. Water (1 ml) was added and the solution was extracted several times with ether (2 ml each). The 1,2-diglycerides thus formed were purified by preparative thin layer chromatography on Silica Gel PF 254 + 366 using pet. ether (bp 30-60° C)-ether-methanol (60:40:2) as the developing solvent and ether as the eluting solvent. The purified sample was then analyzed by thin layer chromatography (Fig. 96). This material was used immediately after preparation.

Each of the above substrates was suspended in propylene glycol with stirring at 80° C for 5 minutes and used in that form for the following experiments unless otherwise stated.

All steps for the enzyme preparations were carried out at 0-4° C. The livers (40 grams) of five female, one-day-fasted rats (200 grams each, Sprague-Dawley) were homogenized for one minute in 0.1 M

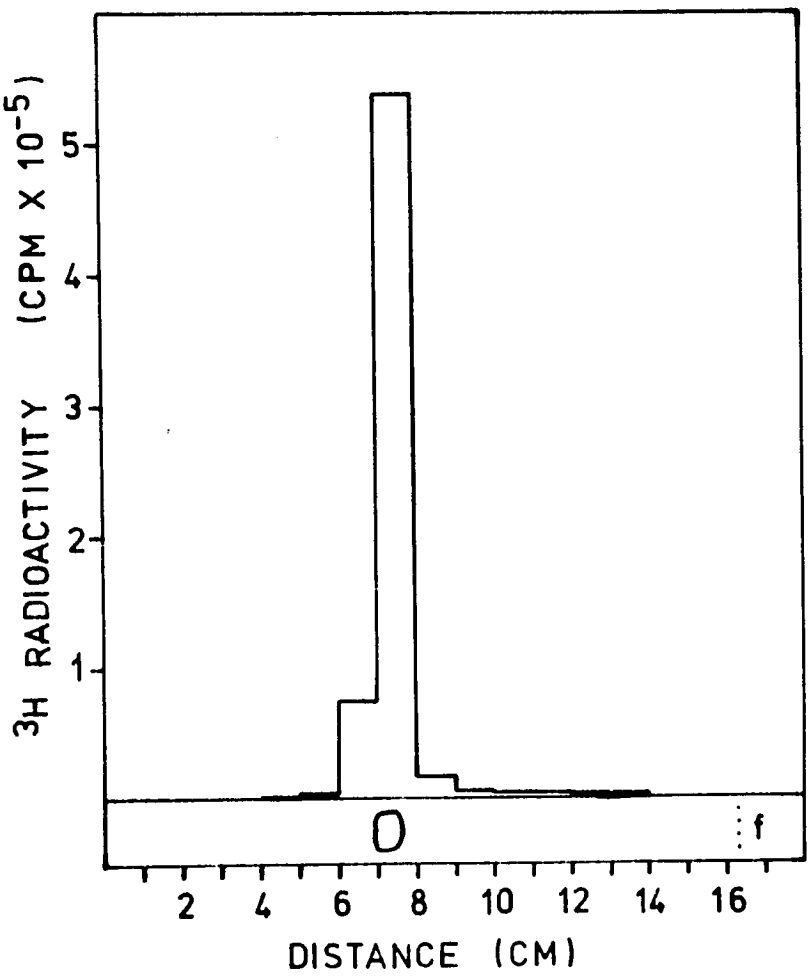


Figure 94. Thin layer radiochromatogram of [1-$^3H_2$]-DL-methyl-alpha-amino-stearyl-alcohol. Standard: DL-methyl-alpha-amino-stearyl-alcohol. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

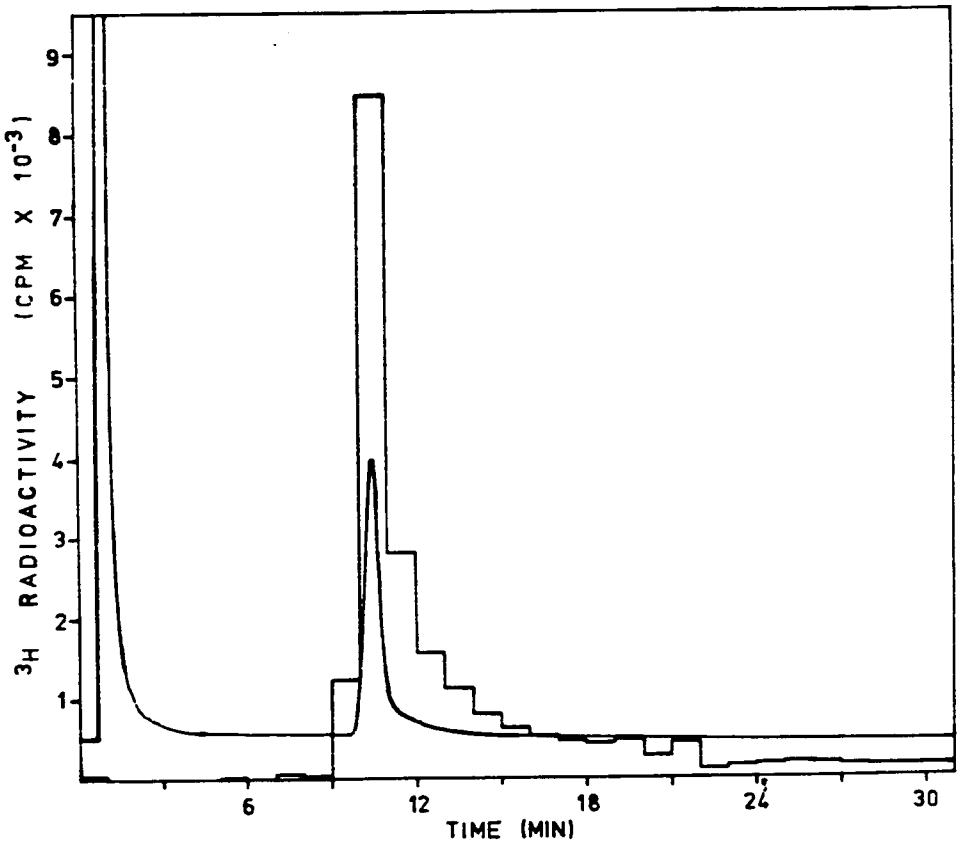


Figure 95. Gas-liquid radiochromatogram of the silylated derivatives of $[1\text{-}^3H_2]$-DL-methyl-alpha-amino-stearyl-alcohol and of DL-methyl-alpha-amino-stearyl-alcohol on a four foot, 1% SE 30 at 231° C.

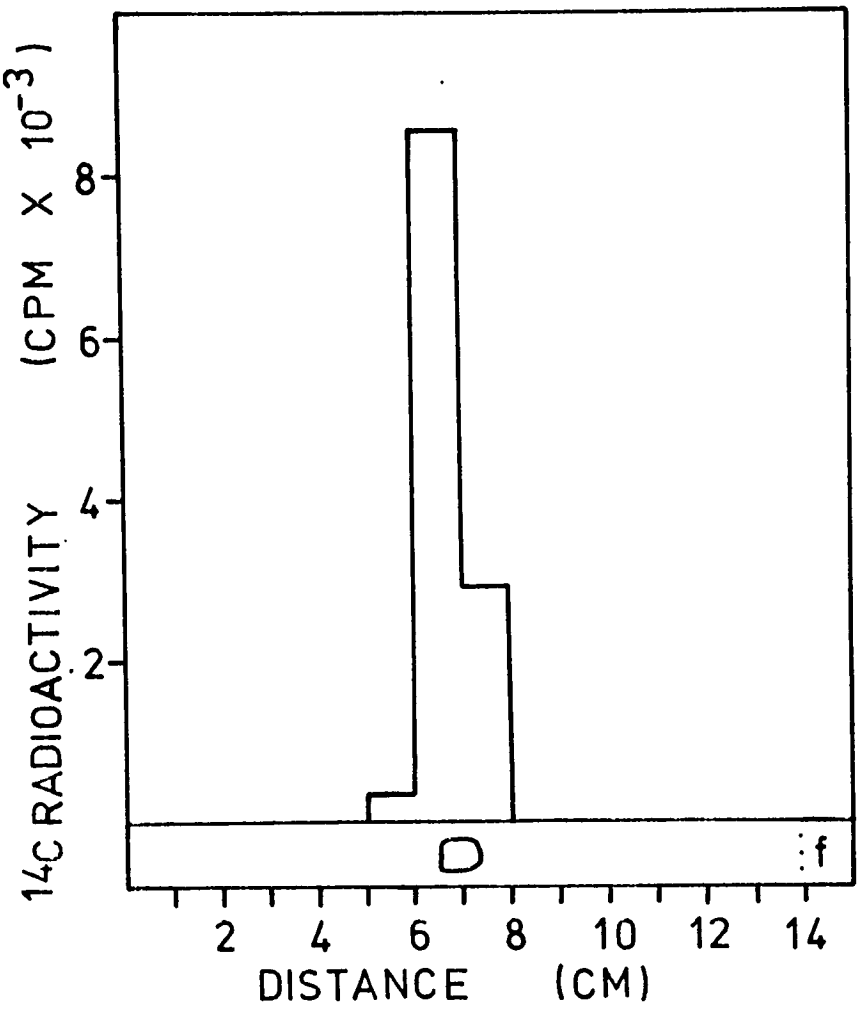


Figure 96. Thin layer radiochromatogram of [UL-^{14}C]-1,2-diglycerides. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

phosphate buffer (pH 7.4; 80 ml; hereafter known as Buffer A) and centrifuged for 30 minutes at 10,000 X g. The resulting supernatant fraction was again centrifuged for 60 minutes at 105,000 X g and the pellet discarded. The protein concentration of the resulting supernatant fraction was about 20 mg/ml after removal of the floating fatty layer. This supernatant (60 ml) was brought to 30% ammonium sulfate saturation by adding ammonium sulfate (10.56 grams; Schwarz/Mann; ultra pure; special enzyme grade) and adjusted to pH 7.4 with dilute ammonia. After centrifugation for 15 minutes at 20,000 X g, the resulting supernatant was brought to 60% ammonium sulfate saturation by adding ammonium sulfate (12.8 grams) and again adjusting to pH 7.4 with dilute ammonia. Centrifugation at 20,000 X g for 15 minutes separated the resulting 30-60% supernatant fraction (34 mg protein/ml) from the precipitate. To the precipitate was added Buffer A (15 ml). A portion of this preparation, the 30-60% cut (66 mg protein/ml), was dialyzed overnight against Buffer A (2 liters). The final protein concentration in this preparation was 47 mg/ml. Each enzyme preparation was stored at 4° C.

All incubations were carried out at 37° C for $2\frac{1}{2}$ hours, unless otherwise noted. The total incubation volume was 1.0 ml in Buffer A (unless otherwise noted) which contained 5 umoles of ATP and 3 umoles of $MgCl_2$ as described by Hirschberg *et al.*[42,43] The reactions were then terminated by adding 3 drops of concentrated ammonium hydroxide followed by chloroform-methanol (2:1; 4 ml). After mixing and centrifuging in a clinical centrifuge for 10 minutes, aliquots of the upper and lower layers were counted. Activity partitioning into the upper phase was used as an indication of the formation of a polar, water

soluble phosphorylated substrate[42,43]. Reactions involving the diglycerides were terminated by adding chloroform-methanol (2:1; 4 ml) and the lower layer, after mixing and centrifugation, was analyzed by thin layer chromatography.

The experiments carried out as described in Table XX indicated that most of the activity present in the 105,000 X g supernatant resided in the 30-60% cut and not in the 30-60% supernatant. Neither [4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine nor [1-$^3H_2$]-DL-alpha-amino-stearyl-alcohol formed a water soluble product. Schneider and Kennedy[69] observed that in E. coli diglycerides could be phosphorylated, but only in the presence of lecithin, phosphatidyl ethanolamine or cardiolipin. The experiment described in Table XXI tested the effect of egg yolk L-alpha-lecithin (Sigma) on the previous two substrates. Using the dialized enzyme preparation, no significant activity above the control levels partitioned into the aqueous phase. Note that the undialized 30 - 60% cut seemed to have a slightly higher activity than that of the dialized enzyme. At this point this may have been due to a difference in the enzyme concentration.

On the fourth day after the enzyme preparation, the experiment described in Table XXII tested the ability of [4,5-$^3H_2$]-N-acetyl-dihydrosphingosine to act as a substrate for the kinase. Results showed that little or no activity above the control levels partitioned into the aqueous phase. Note, however, that by the fourth day the undialyzed enzyme had a far greater activity than that of the dialyzed enzyme. In experiments with the three substrates mentioned above, substrate solubility may have been a reason for the lack of the observation of an enzymatically formed, water soluble product.

TABLE XX

Test of various substrates and enzyme fractions of dihydrosphingosine kinase in Buffer A.

SUBSTRATE	*ENZYME	ENZYME BOILED 30 MINUTES	AQUEOUS CPM / ORGANIC CPM
[4,5-$^3H_2$]-dihydrosphingosine (0.3 ug)	I		0.29
"	I		1.31
"	-		0.01
"	-		0.01
"	II		0.22
"	II	X	0.04
"	III		0.06
"	III	X	0.07
[4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine (50 ug)	I		0.04
"	I	X	0.02
"	-		0.02
[1-$^3H_2$]-DL-alpha-amino-stearyl-alcohol (700 ug)	I		0.04
"	I	X	0.04
"	-		0.03

* I = undialyzed, 30-60% cut (6.6 mg protein); II = 105,000 X g supernatant (2.0 mg protein); III = 30-60% supernatant (3.4 mg protein).

TABLE XXI

Effect of lecithin on substrate specificity of dihydrosphingosine kinase.

SUBSTRATE	DIALYZED ENZYME (4.7 mg protein)	LECITHIN (1 mg in 100 ul of propylene glycol)	UNDIALYZED ENZYME (6.6 mg protein)	AQUEOUS CPM / ORGANIC CPM
[4,5-$^3H_2$]-dihydrosphingosine (0.3 ug)	X			0.25
"			X	0.35
"	X	X		0.20
"	—		—	0.01
[4,5-$^3H_2$]-N-benzoyl-dihydrosphingosine (50 ug)	X			0.05
"	X	X		0.03
"	—		—	0.01
[1-$^3H_2$]-DL-alpha-amino-stearyl-alcohol (700 ug)	X			0.06
"	X	X		0.04
"				0.03

TABLE XXII

Effect of dihydrosphingosine kinase on N-acetyl-dihydrosphingosine.

SUBSTRATE	DIALYZED ENZYME (4.7 mg protein)	UNDIALYZED ENZYME (6.6 mg protein)	ENZYME BOILED 30 MINUTES	AQUEOUS CPM / ORGANIC CPM
[4,5-$^3H_2$]-dihydrosphingosine (0.3 ug)		X		0.52
"	X			0.20
	X		X	0.06
"	———	———		0.01
[4,5-$^3H_2$]-N-acetyl-dihydrosphingosine (50 ug)		X		0.02
"	X			0.02
"	X		X	0.01
"	———	———		0.00

After seven days the dialyzed enzyme was almost totally inactive. With the [4,5-$^3H_2$]-dihydrosphingosine as a substrate, the aqueous CPM/organic CPM was 0.06. However, the ratio obtained using the undialyzed enzyme was 0.30. The diglyceride when incubated with the undialyzed (now 9 days old) formed a more polar product (Fig. 97) which accounted for about 14% of the activity. When the diglyceride was treated with the 7 day old dialyzed enzyme, only about 3% of the total activity migrated more slowly on thin layer chromatography (Fig. 98). The loss of enzymatic activity on the diglycerides paralleled the loss of activity of the kinase. In such a crude enzyme system, it was impossible to tell if the activities were related.

The polar activity derived from the diglycerides did not co-chromatograph with phosphatidic acid which remained at the origin in the above solvent system. The activity ran just slightly higher than did alpha- or beta-mono-palmitin (Sigma) or alpha- or beta-mono-stearin (Sigma), all of which had an R_f = 0.05 when chromatographed in this system.

A fresh undialyzed enzyme was prepared and the following incubations were carried out for 10 minutes using 0.01 ml of the enzyme solution (0.7 mg of protein). The effect of cardiolipin (from bovine heart; Sigma) was then tested. Diglyceride (about 25 ug in 0.05 ml of propylene glycol) was incubated in the presence of cardiolipin (1 mg; approximately a 1 mM solution). Thin layer analysis of the Folch extract (Fig. 99) showed about 12% of the activity migrating more slowly than did the diglycerides but still not co-chromatographing with phosphatidic acid.

Schneider and Kennedy[69] used a 0.05 M tris-phosphate buffer (pH

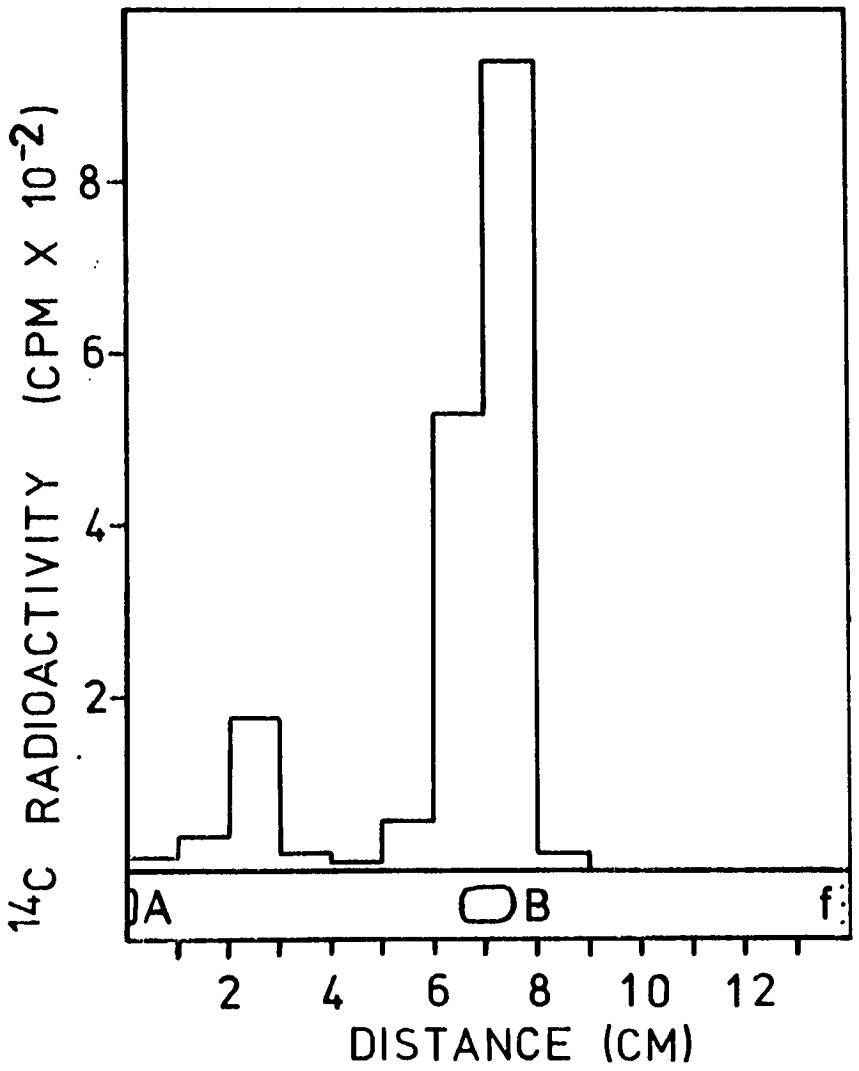


Figure 97. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer A of [UL-^{14}C]-1,2-diglycerides in propylene glycol with a 9 days old preparation of undialyzed, crude dihydrosphingosine kinase from rat liver. A: Phosphatidic acid (Pierce Chemicals). B: 1, 2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

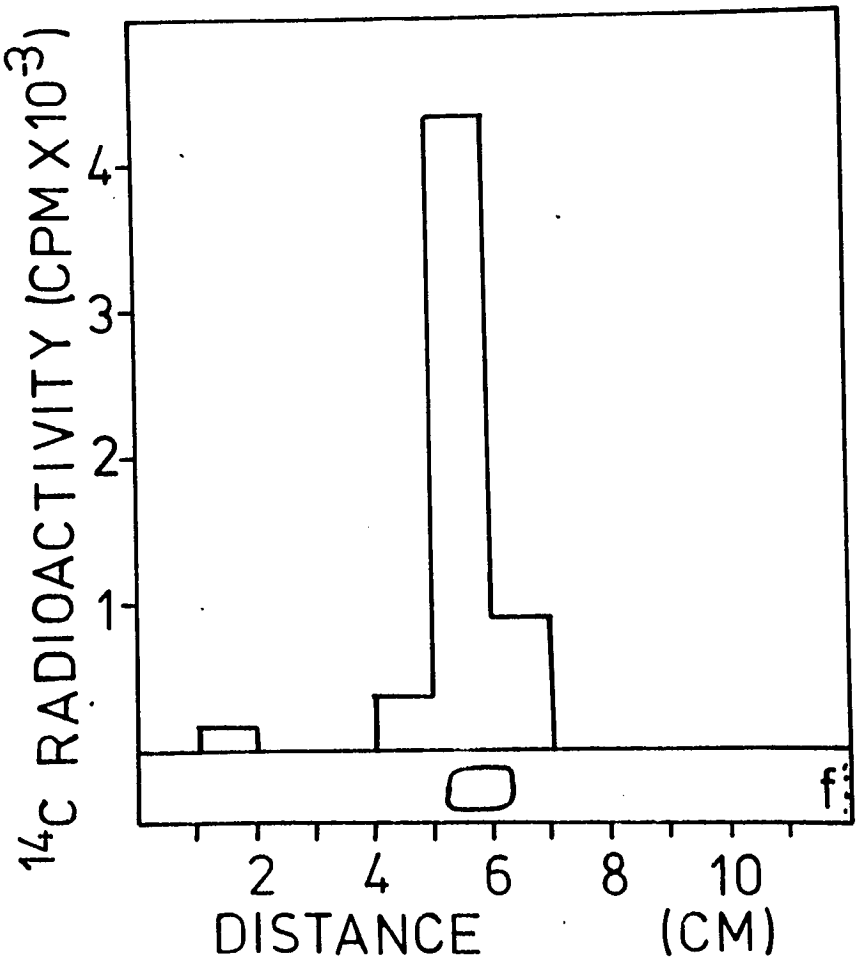


Figure 98. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer A of [UL-^{14}C]-1,2-diglycerides in propylene glycol with a 7 days old preparation of dialyzed, crude dihydrosphingosine kinase from rat liver. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

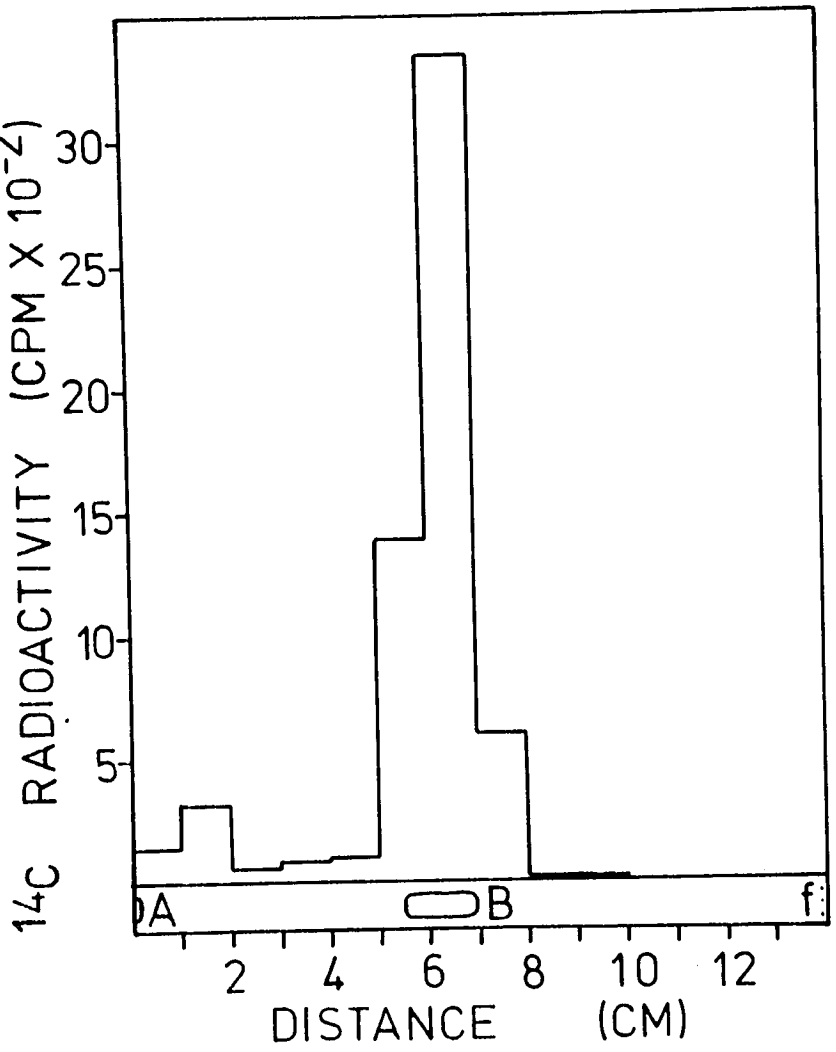


Figure 99. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer A of [UL-^{14}C]-1,2-diglycerides in propylene glycol with undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. A: Phosphatidic acid. B: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

6.6; 1 mM NaN_3; 100 mM NaCl; 10 mM $MgSO_4$; 10 ul mercaptoethanol/10 ml and 0.77% Triton X-100 detergent; hereafter known as Buffer B). The diglyceride (25 ug) in propylene glycol (50 ul) was incubated in Buffer B (0.90 ml) in the presence of cardiolipin (1 mg in 50 ul of propylene glycol) and the undialyzed enzyme (0.7 mg). The lower Folch layer showed little activity chromatographing with phosphatidic acid in Fig. 100 and no activity chromatographing with phosphatidic in Fig. 101. The above authors had used as a solubilizer only Triton X-100 and not propylene glycol. In the following experiment, cardiolipin (1 mg) was added to Buffer B along with the diglyceride (25 ug) which had first been mixed with Triton X-100 and then added to Buffer B (with the detergent omitted) so that the final concentration was 0.77% in Triton X-100. The lower Folch layer of this incubation (Fig. 102) showed the same distribution of activities as in the previous systems. A boiled enzyme control (Fig. 103) showed only labeled diglycerides. The same experiment experiment using Triton X-100 and Buffer A gave the same results for the experimental (Fig. 104) and boiled enzyme (Fig. 105) incubations.

Under these conditions, no conversion of diglycerides to phosphatidic acid was observed. The identity of the more polar activity remains unknown.

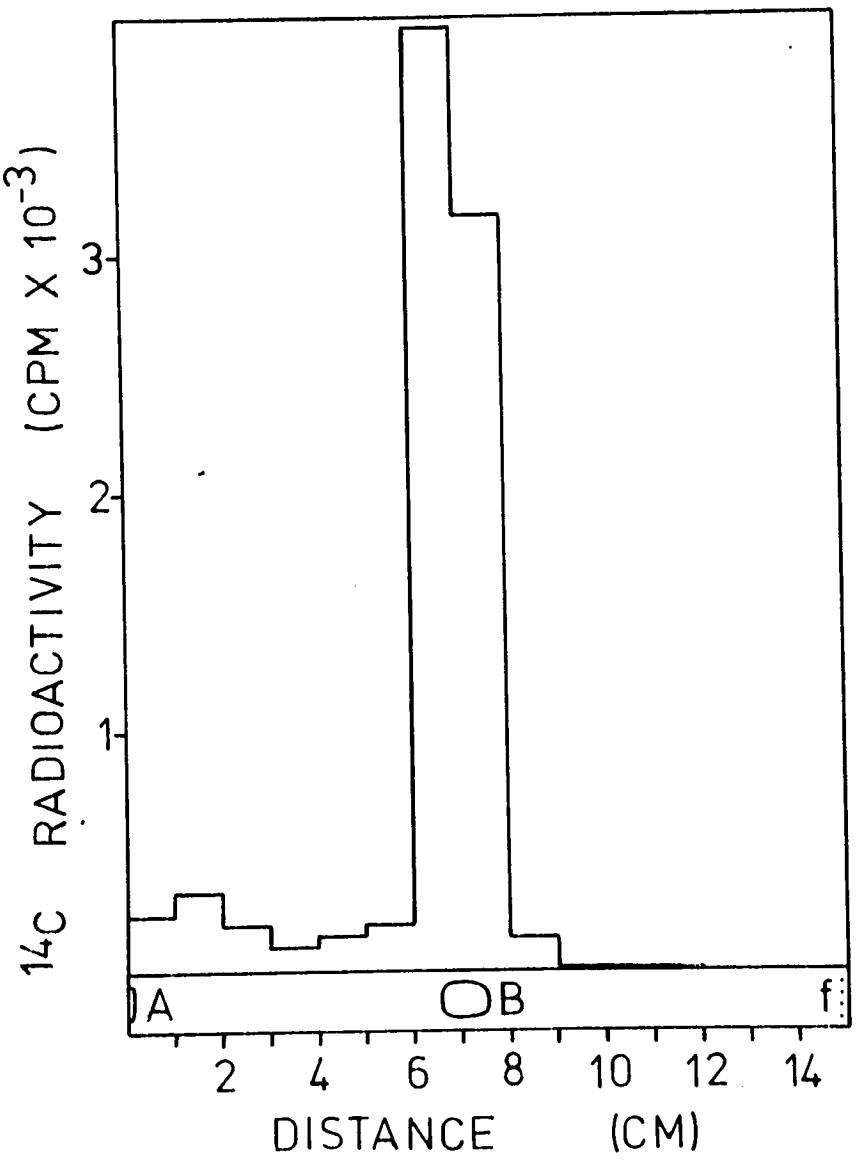


Figure 100. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer B of [UL-^{14}C]-1,2-diglycerides in propylene glycol with undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. A: Phosphatidic acid. B: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

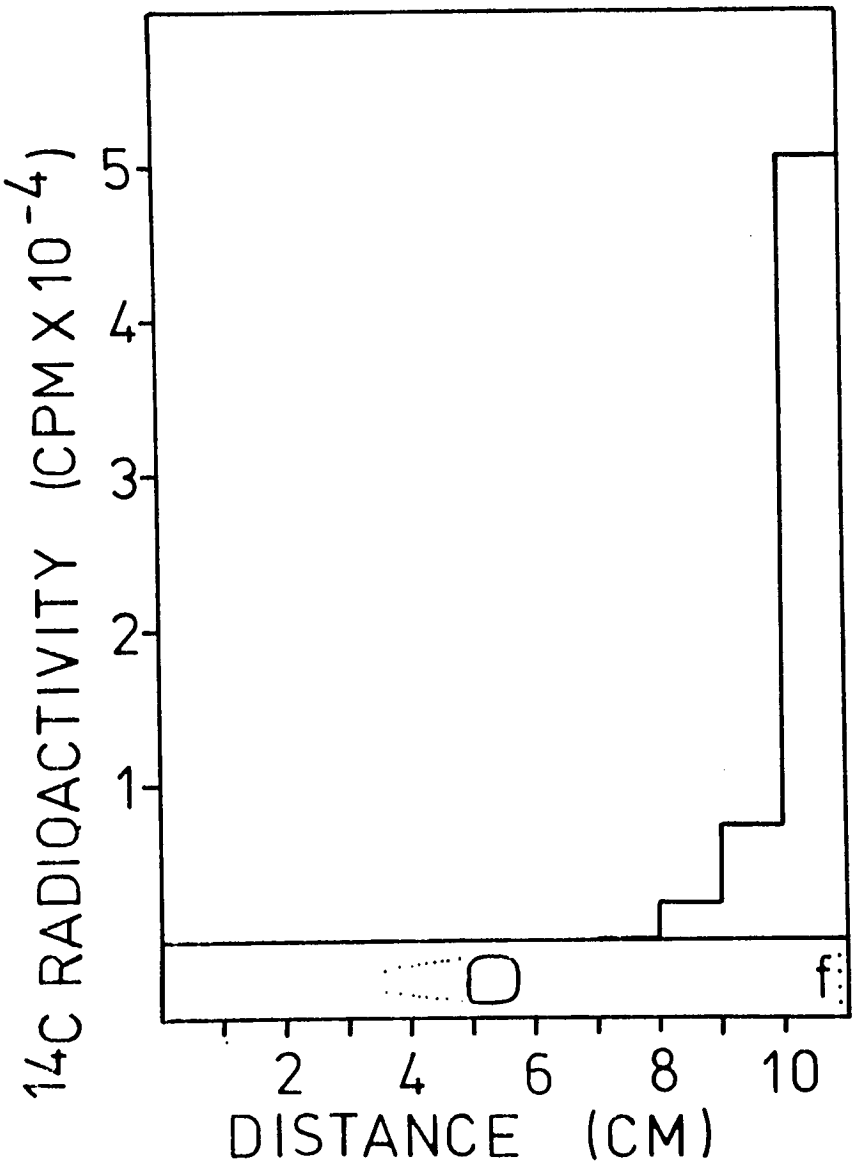


Figure 101. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer B of [UL-^{14}C]-1,2-diglycerides in propylene glycol with undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipid. Standard: Phosphatidic acid. Solvent: Chloroform-pyridine-88% formic acid (50:30:7).

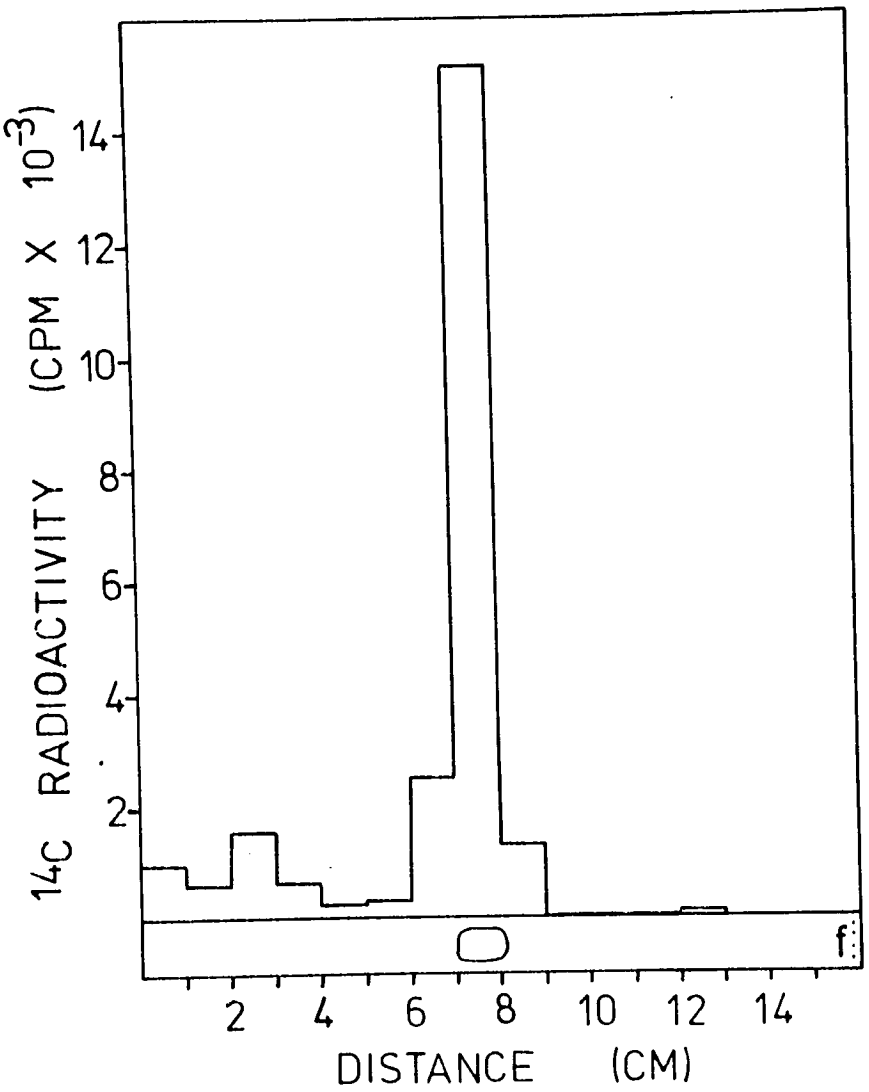


Figure 102. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer B of [UL-^{14}C]-1,2-diglycerides in Triton X-100 with undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

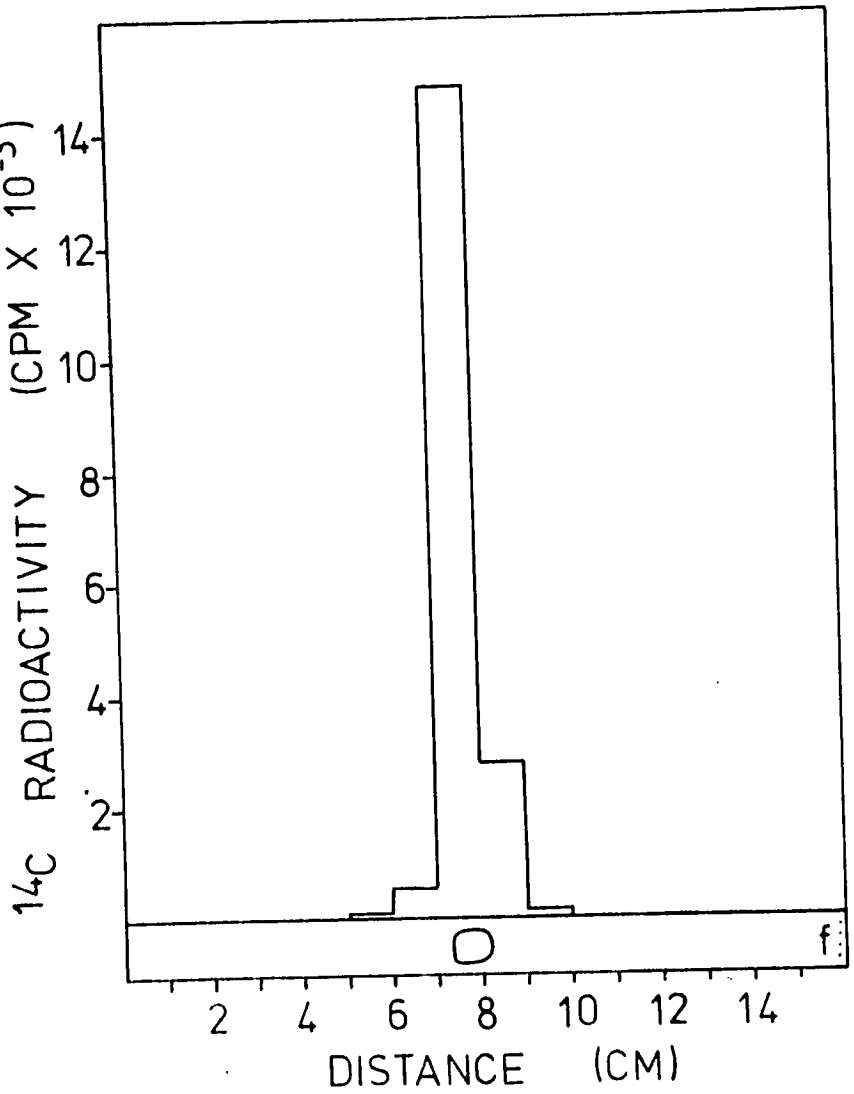


Figure 103. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer B of [UL-^{14}C]-1,2-diglycerides in Triton X-100 with boiled (30 minutes) undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

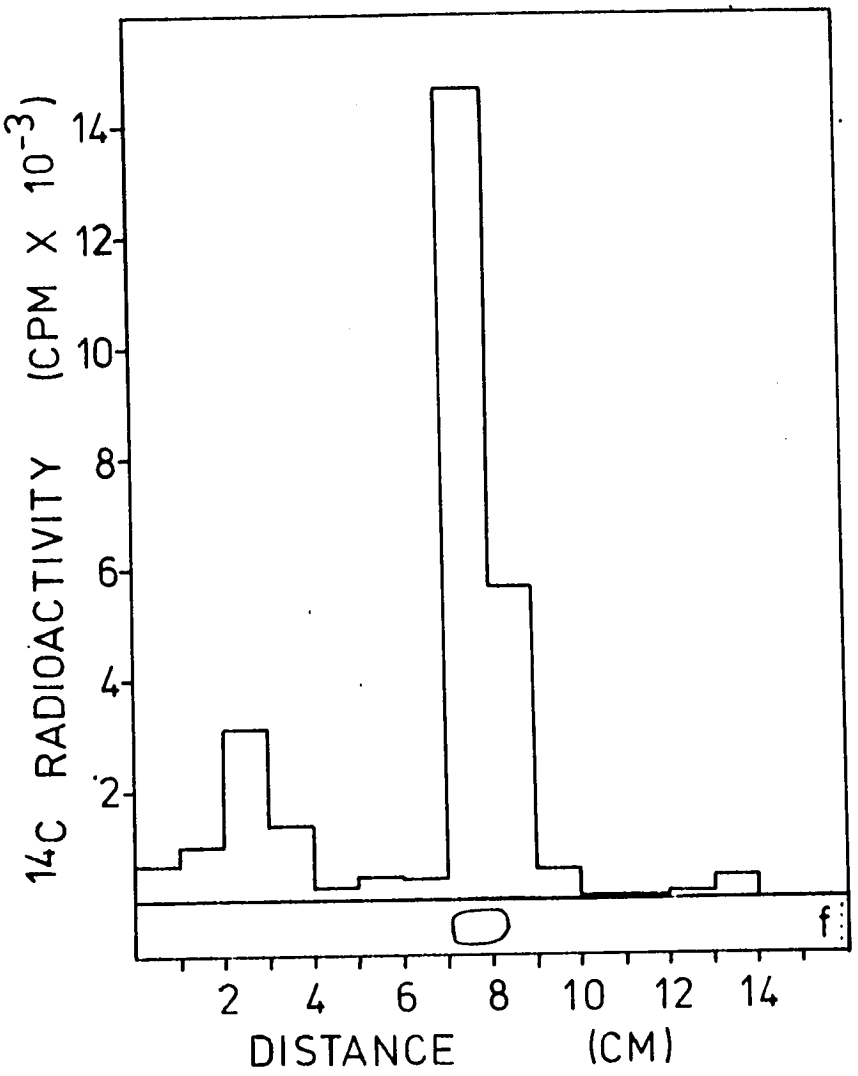


Figure 104. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer A of [UL-^{14}C]-1,2-diglycerides in Triton X-100 with undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

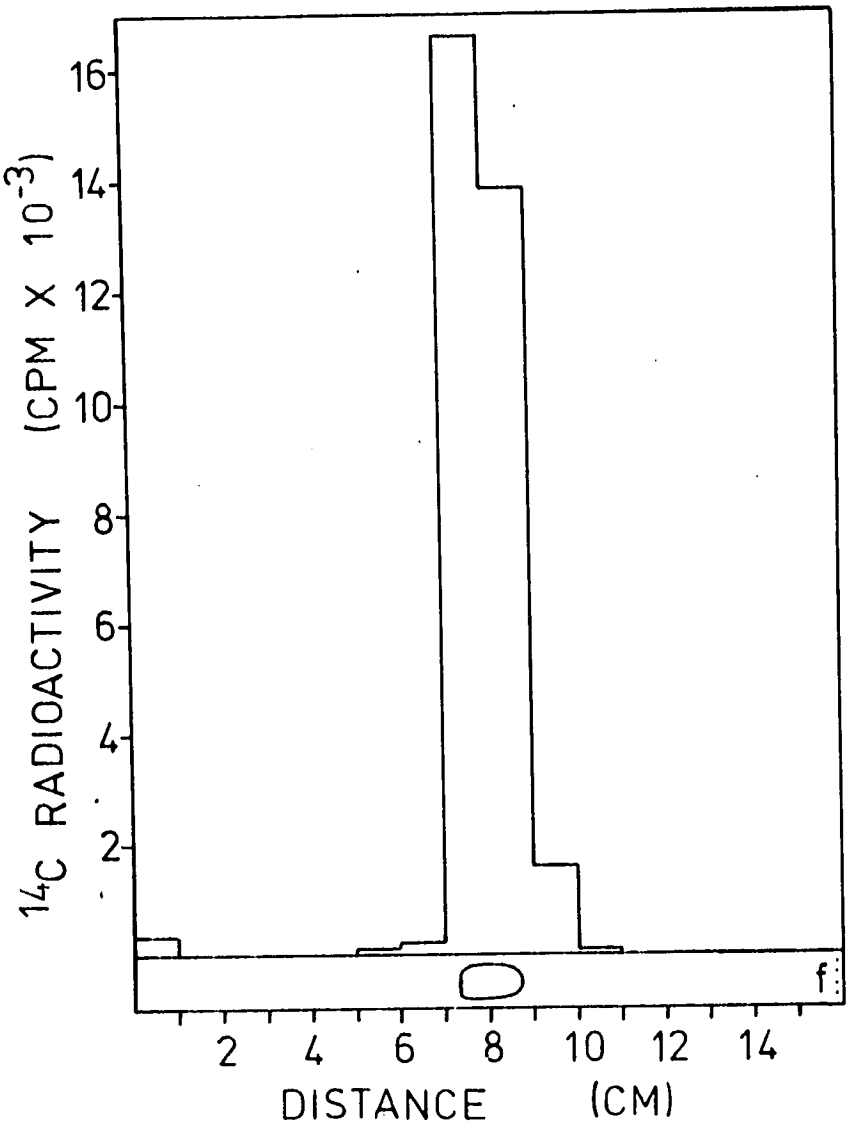


Figure 105. Thin layer radiochromatogram of the Folch extract of an incubation in Buffer A of [UL-^{14}C]-1,2-diglycerides in Triton X-100 with boiled (30 minutes) undialyzed, crude dihydrosphingosine kinase from rat liver in the presence of cardiolipin. Standard: 1,2-Diglycerides formed by phospholipase C action on egg lecithin. Solvent: Pet. ether (bp 30-60° C)-ether-methanol (60:40:2).

ISOLATION AND PURIFICATION OF PHYTOSPHINGOSINE

Large amounts of phytosphingosine were desired so that the chemical synthesis of phytosphingosine-1-phosphate might be attempted by the general method of Weiss[105]. The large scale preparation of phytosphingosine utilized a modified method of Weiss and Stiller[111].

Preparation I: Lyophilized Hansenula ciferrii (NRRLY-1031; mating type F-60-10; a generous gift of C. P. Kurtzman, U. S. Dept. of Agriculture, Agricultrual Research Service, Peoria, Ill.) was suspended in sterile yeast medium (YM; 7 ml) containing 60 grams of dextrose, 3 grams each of yeast and malt extract and 5 grams of bacto-peptone (all products of Difco Laboratories, Detroit, Michigan) per liter of deionized water. Several drops were transferred to each of six agar yeast medium (AYM) slants (containing 25 grams of agar/liter of YM) which then were incubated for 3 days at room temperature (23° C). These slants were then used to innoculate a 3 liter batch of YM which was aerated vigorously for 3 days at room temperature. This innoculum was then added to 100 liters of YM (containing 80 grams of Dow Anti-foam A) and the resulting mixture was agitated vigorously and aerated vigorously for 3 days. The temperature varied between 24° C and 32° C due to a deficient cooling system. The cells (1809 grams wet weight) were collected by flow-through centrifugation and then stirred for 15 hours at room temperature in a mixtrue of methanol (7 liters) and 10 N KOH (70 ml). The cells were collected by centrifugation in 250 ml bottles and washed once with methanol. After filtering the combined supernatants through Whatman #1 paper, the volume was re-

duced at reduced pressure with the aid of Antifoam A (Dow) to about 550 ml. An equal volume of saturated sodium chloride solution was added and the mixture extracted three times with ether (500 ml each). The organic phases were pooled and dried over anhydrous magnesium sulfate, filtered through Hyflo Super Cel and the solvent was removed at reduced pressure at 35° C. The residue (7.18 grams) was crystallized from acetonitrile to give 1.719 grams of crude N-acetyl-bases. This was applied in warm chloroform (75 ml) to a 30 gram silicic acid column (2 X 20.5 cm; packed in chloroform) with the aid of a heating tape (about 40° C) wrapped around the top of the column during the application process. Two major yellow bands (455.5 mg) were eluted in a single batch of chloroform (300 ml). Thereafter, approximately 5.5 ml fractions were collected. The column was then eluted successively with 2% methanol in chloroform (100 ml), 3% methanol in chloroform (500 ml) and 20% methanol in chloroform (100 ml). Fractions 38-126 were pooled (919 mg) and reapplied in warm chloroform (50 ml) to an identical column as above and eluted successively with 2% methanol in chloroform (200 ml), 3% methanol in chloroform (400 ml) and 10% methanol in chloroform (100 ml). Fractions 50-65 (297 mg) were pooled and an aliquot was analyzed after formation of the trimethylsilyl derivative by gas-liquid chromatography. The analysis indicated that the mixture was composed of 84.5% N-acetyl-phytosphingosine and 14.0 % N-acetyl-dihydrosphingosine. Fractions 66-74 (143 mg) contained 98.9% N-acetyl-phytosphingosine and fractions 75-141 (260 mg) was analyzed as 100% N-acetyl-phytosphingosine. The purest sample of N-acetyl-phytosphingosine had a melting point of 114.8-116.5° C.

The infrared spectrum (Fig. 106) of N-acetyl-phytosphingosine exhibited a strong, broad absorbance at 3200-3500 cm^{-1} due to both the hydroxyls and the amide proton. The weak, sharp peak at 3095 cm^{-1} represented the first overtone of the N-H in plane deformation of a secondary amide; the doublet at 1640 cm^{-1} resulted from the amide carbonyl; the peak at 1560 cm^{-1} from the N-H in plane bend; the peak at 1160 cm^{-1} from the secondary hydroxyls and the peak at 1030 cm^{-1} from the primary hydroxyl C-O stretch. Assignments were determined with the aid of Bellamy[7], Crawford *et al.*[28] and Jones[52].

Major ions and their tentative assignments in the mass spectrum (Fig. 107) of N-acetyl-phytosphingosine were m/e 341 (M-18; M - H_2O), m/e 340 (M-19; M - H_2O -H), m/e 328 (M-35; M - H_2O - OH[70a]), m/e 323 (M-36; M - $2H_2O$), m/e 310 (M-49; M - H_2O - CH_2OH), m/e 294 (M-65; M - H_2O - CH_2OH - CH_3), m/e 268 (M-91), m/e 132 (M-227; $\overset{OH}{CH}-\overset{NHCOCH_3}{CH}-CH_2OH$), m/e 115 (M-244; $\overset{OH}{CH}-\overset{NHCOCH_3}{CH}-CH_2OH$ - OH[70a]), m/e 102 (M-257; $\overset{NHCOCH_3}{CH}-CH_2OH$), m/e 85 (M-274; $\overset{NHCOCH_3}{CH}-CH_2OH$ - OH[70a]) and m/e 60 (M-299; $\overset{NH_2}{CH}-CH_2OH$) which was formed by the elimination of CH_2CO[70c] after the simple cleavage between C-2 and C-3. The combined gas-liquid chromatograph-mass spectrum (Fig. 108) of the silylated derivative (compare with Ref. 104) exhibited major ions at m/e 560 (M-15; M - CH_3), m/e 299 (M-276; $CH_3(CH_2)_{13}\overset{OSiMe_3}{CH}$), m/e 276 (M-299; $\overset{OSiMe_3}{CH}-\overset{NHCOCH_3}{CH}-CH_2OSiMe_3$), m/e 401 (M-174; $CH_3(CH_2)_{13}\overset{OSiMe_3}{CH}-CH$), m/e 174 (M-401; $\overset{NHCOCH_3}{CH}-CH_2OSiMe_3$), m/e 103 (M-472;

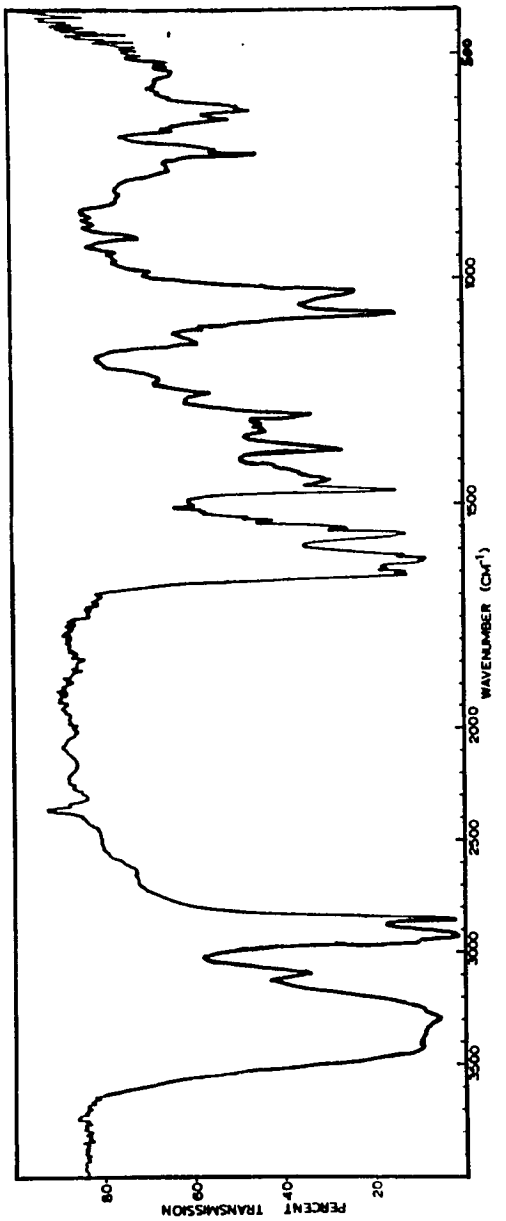


Figure 106. Infrared spectrum of N-acetyl-phytosphingosine.

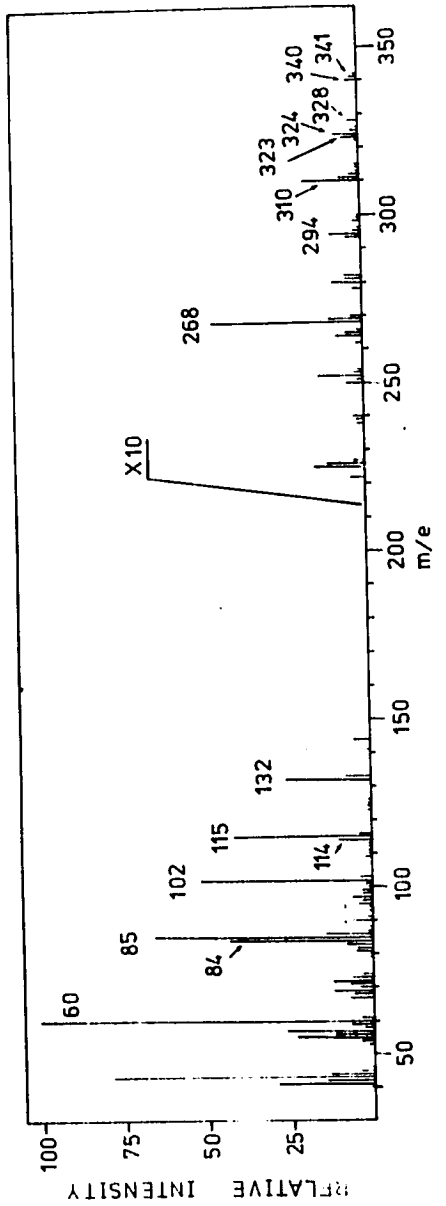


Figure 107. Mass spectrum of N-acetyl-phytosphingosine.

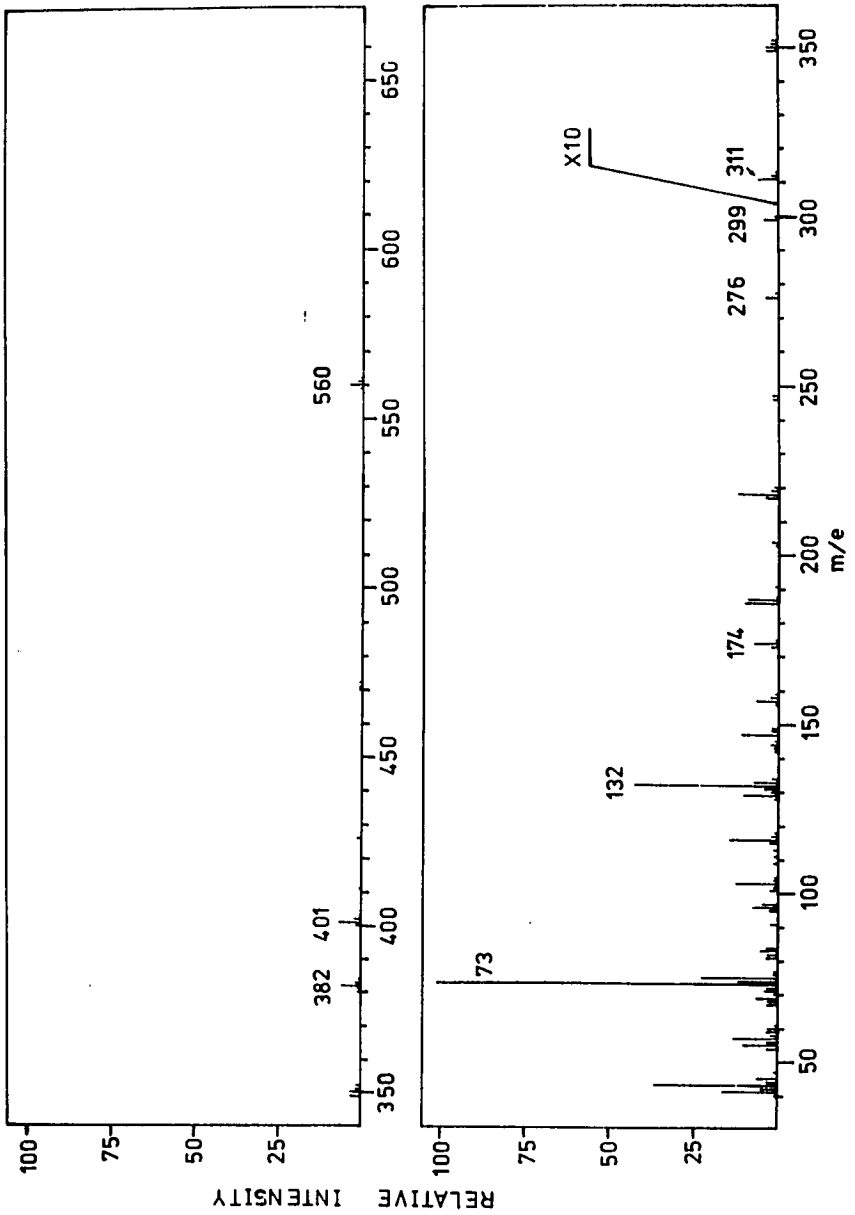


Figure 108. Combined gas-liquid chromatography-mass spectrum of the silylated derivative of N-acetyl-phytosphingosine on an eight foot, coiled 1% SE 30 column at 240° C.

CH_2OSiMe_3), m/e 472 (M-103; $CH_3(CH_2)_{13}$CH(OSiMe$_3$)-CH(NHCOCH$_3$)-CH(OSiMe$_3$)), m/e 382 (M-193; $CH_3(CH_2)_{13}$CH(OSiMe$_3$)-CH(NHCOCH$_3$)-CH(OSiMe$_3$) - Me_3SiOH), m/e 311 (M-264; $CH_3(CH_2)_{13}$CH(OSiMe$_3$)-CH(OSiMe$_3$) - Me_3SiOH) and m/e 132 (M-443; $CHCH_2OSiMe_3$ with NH_2)[70c].

(Compare the mass spectrum of the silylated derivative with that in Ref. 104 and with that of a deuterated sample in Ref. 66).

Preparation II: A lyophilized sample of H. ciferrii was suspended in sterile YM (7 ml) and 8-10 drops were transferred to 6 AYM slants to incubate at room temperature for 3 days. These were transferred in sterile YM to three liters of YM and aerated vigorously for 3 days. This was transferred to 100 liters of YM (containing 80 grams of Dow Antifoam B; temperature 27.5° C; pH 5.1) which was agitated and aerated vigorously for 3 days. The temperature varied between 25.5° C and 29.5° C. A total of 1741 grams of wet cells were suspended in a mixture of methanol (7 liters) and 10 N KOH (70 ml) and were stirred at room temperature for 2½ days. The cells were centrifuged and washed with two portion of methanol. The total volume was reduced to about 500 ml at reduced pressure with the aid of Dow Antifoam A at 35° C. An equal volume of water was added and the mixture was extracted three times with ether (500 ml each). The residue after removal of the solvent was crystallized from acetonitrile. A total of 12.97 grams of the crude N-acetyl-phytosphingosine was then purified by silicic acid chromatography similar to that in

Preparation I yielding 5.239 grams of purified (98-99+%) N-acetyl-phytosphingosine and about 360 mg of purified (85%) N-acetyl-dihydro-sphingosine.

ALKALINE HYDROLYSIS OF N-ACETYL-PHYTOSPHINGOSINE: Purified N-acetyl-phytosphingosine (11.95 mmoles) was refluxed for 48 hours in a mixture of 95% ethanol (130 ml) and 2 N KOH (15 ml). After cooling to room temperature, an equal volume of water was added and the mixture was extracted three times with ether (300 ml each). The ether was washed once with water (100 ml) and was dried over anhydrous sodium sulfate. The solvent was removed at reduced pressure at 35° C and the resulting residue was dried *in vacuo* over phosphorous pentoxide. The residue (3.34 grams) was applied in 2% methanol in chloroform (250 ml) to a Unisil column (40 grams; 2 X 30 cm; packed in chloroform) with the eluate being collected in a single batch. Then, while collecting approximately 10 ml fractions, the column was developed successively with 10% methanol in chloroform (200 ml), 13% methanol in chloroform (200 ml), 16% methanol in chloroform (200 ml) and 20% methanol in chloroform (200 ml). Fractions 11-45 and 46-75 were pooled and the solvent removed and the residue dried as above. The residues were washed once with hot hexane. By gas-liquid chromatographic analysis of the trimethylsilyl derivatives, the first residue was 98.9% and the second 99.6% pure phytosphingosine (Fig. 109). Each residue had a melting point of 102-103.7° C (Lit.[109] mp 102-104° C). The final yield was 3.033 grams (78%).

The infrared spectrum (Fig. 110) of phytosphingosine showed a broad absorption for the hydroxyls at 3340 cm^{-1}; hydrocarbons at

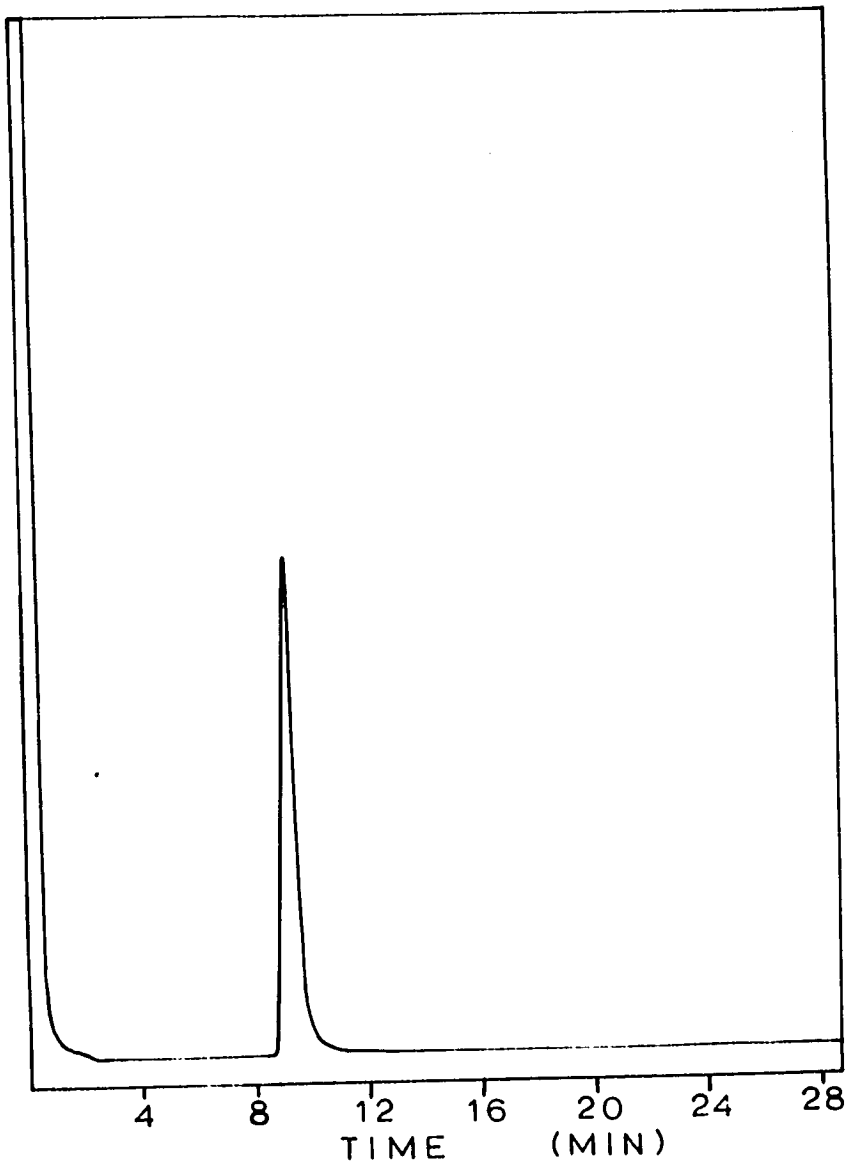


Figure 109. Gas liquid chromatogram of the silylated derivative of phytosphingosine on an eight foot, 1% SE 30 column at 222° C.

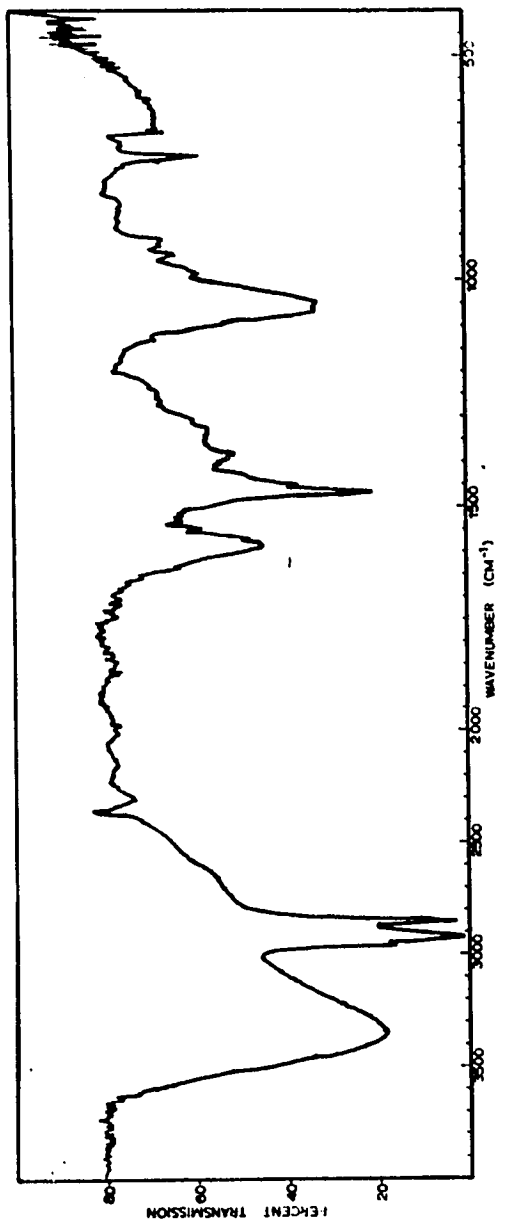


Figure 110. Infrared spectrum of phytosphingosine.

2860 cm^{-1} and 2925 cm^{-1}; NH_2 "scissors" bend at 1600 cm^{-1}; hydrocarbon at 1470 cm^{-1} (CH_2 "scissors"); C-O stretching at about 1050 cm^{-1} and a weak, sharp $(CH_2)_n$ rocking mode at 720 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford *et al.*[28] and Jones[52].

Major ions and their tentative assignments in the mass spectrum (Fig. 111) were m/e 286 (M-31; M - CH_2OH), m/e 270 (M-47; M - CH_2OH - NH_2[70a]), m/e 268 (M-49; M - CH_2OH - H_2O), m/e 252 (M-65; M - CH_2OH - H_2O - NH_2[70a]), m/e 60 (M-257; $\overset{\overset{NH_2}{|}}{CH}-CH_2OH$) and m/e 90 (M-227; $\overset{\overset{OH}{|}}{CH}-\overset{\overset{NH_2}{|}}{CH}-CH_2OH$). The mass spectrum (Fig. 112) of the *tetrakis* (trimethylsilyl) phytosphingosine obtained by combined gas-liquid chromatography-mass spectrometry exhibited ions at m/e 590 (M-15; M - CH_3), m/e 515 M-90; M - Me_3SiOH), m/e 502 (M-103; $CH_3(CH_2)_{13}\overset{\overset{OSiMe_3}{|}}{CH}-\overset{\overset{OSiMe_3}{|}}{CH}-\overset{\overset{NHSiMe_3}{|}}{CH}$), m/e 412 M-193; M - CH_2OSiMe_3 - Me_3SiOH), m/e 401 (M-204; M - $\overset{\overset{NHSiMe_3}{|}}{CH}-CH_2OSiMe_3$), m/e 340 (M-265), m/e 299 (M-306; $CH_3(CH_2)_{13}\overset{\overset{OSiMe_3}{|}}{CH}$), m/e 306 (M-299; M - $CH_3(CH_2)_{13}$), m/e 204 (M-401; $\overset{\overset{NHSiMe_3}{|}}{CH}-CH_2OSiMe_3$) and m/e 103 (M-502; CH_2OSiMe_3).

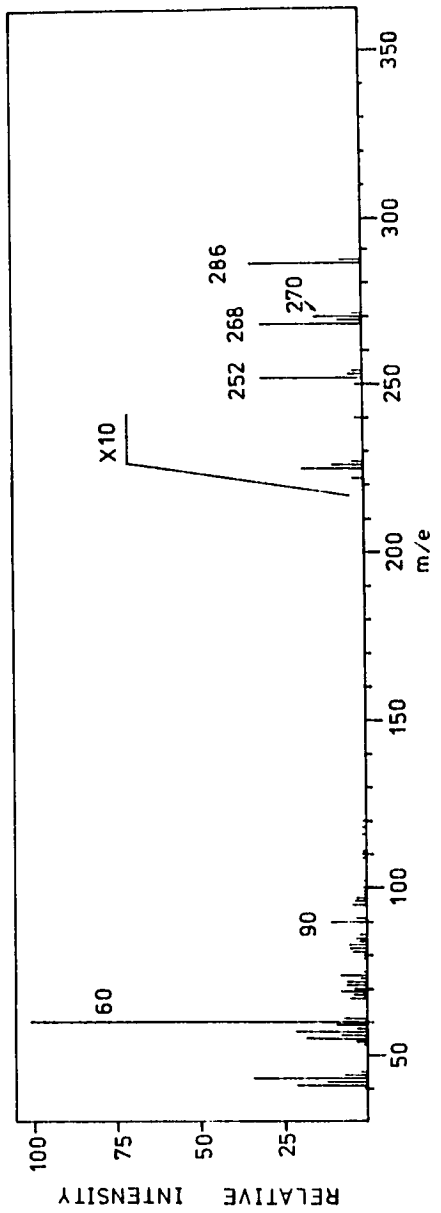


Figure 111. Mass spectrum of phytosphingosine.

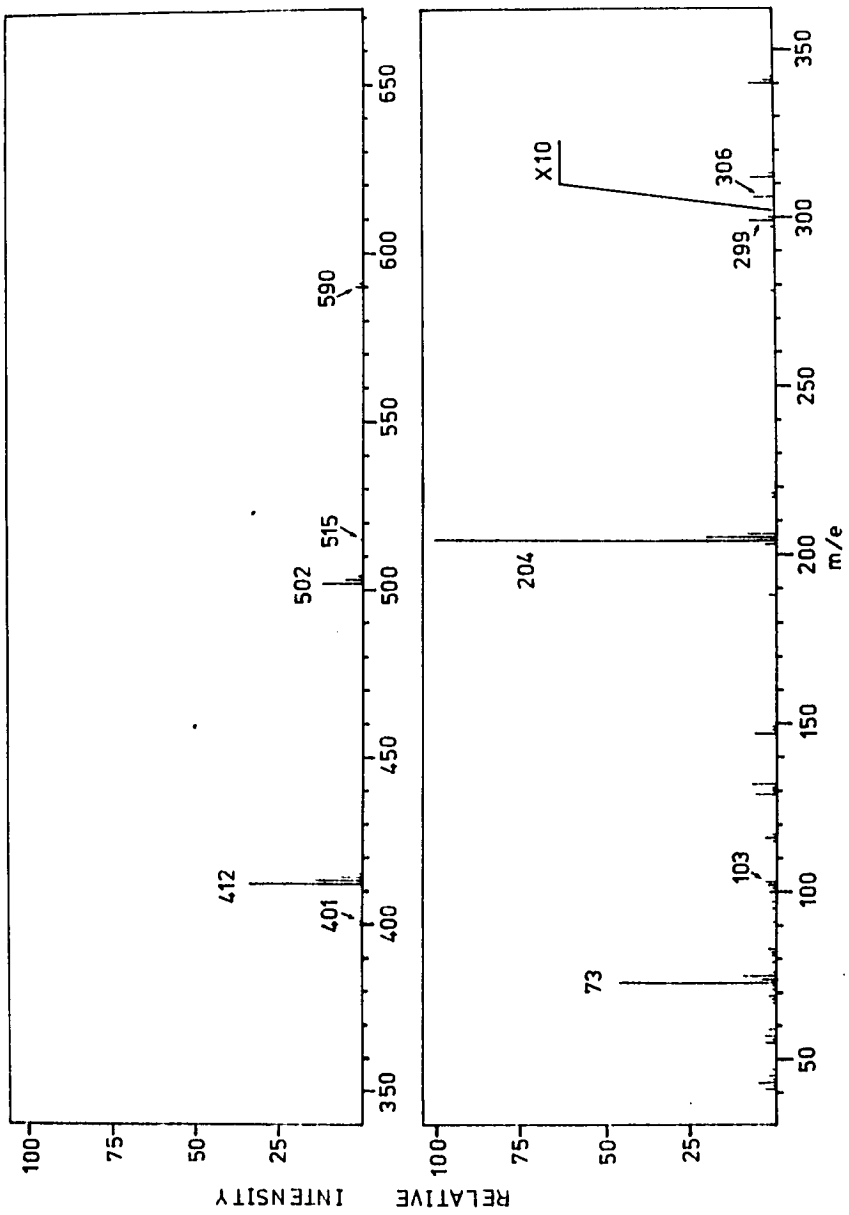


Figure 112. Combined gas-liquid chromatography-mass spectrum of the silylated derivative of phytosphingosine on an eight foot, 1% SE 30 column at 220° C.

THE ANALYSIS OF THE WEISS AND STILLER[111] METHOD OF PURIFICATION OF PHYTOSPHINGOSINE

Weiss and Stiller[111] described a method for isolation of phytosphingosine from yeast. This involved treating free base mixtures with p-nitro-benzaldehyde. The authors reported that only phytosphingosine formed an oxazolidene derivative with this reagent. They stated specifically that dihydrosphingosine did not react in a similar fashion. They further reported that only the phytosphingosine derivative could pass through through a Woelm cellulose acetate column and was therefore used to isolate the phytosphingosine derivative. Phytosphingosine sulfate could then be obtained by treatment of the derivative with dilute sulfuric and gentle warming.

This method was employed to isolate phytosphingosine from H. ciferrii. However, in the hands of this author, the final samples of base-sulfate always contained about 5% dihydrosphingosine as analyzed by thin layer and gas-liquid chromatography.

In order to evaluate the effectiveness of Weiss and Stiller's[111] method of purification of phytosphingosine from dihydrosphingosine contamination, the following experiments were conducted. [4,5-$^3H_2$]-Dihydrosphingosine (Preparation II; 98.5% pure) was used to quantitate the analysis. Both hexane and benzene were distilled and the p-nitrobenzaldehyde (Matheson) was crystallized two times from 10% ethanol in water. Cellulose acetate columns were prepared by adding a slurry of either ICN-Woelm (Lot #217, mesh 140/150) or Eastman (Acetyl 40.0%, ASTM Visc. 25) cellulose acetate (8 grams) in benzene to a column (1.6 X 60 cm) under slight nitrogen pressure. The column was then

washed successively under slight nitrogen pressure with benzene (75 ml), absolute ethanol (75 ml) and benzene (125 ml). Each column was used only once.

EXPERIMENT #1: A) A mixture of dihydrosphingosine (10 mg; 33 umoles), phytosphingosine (10 mg; 32 umoles) and labeled dihydrosphingosine (9,290,000 DPM; 1.5 ug) was dried in vacuo over phosphorous pentoxide. Hexane (5 ml) and p-nitro-benzaldehyde (11 mg; 81 umoles) were added and the resulting mixture was heated carefully in a 60° C bath for about 15 minutes until all solid had dissolved. After cooling to room temperature, the precipitate was collected by centrifugation and resuspended in boiling hexane (5 ml). After cooling to room temperature, the collected precipitate (1,870,000 DPM) was dried in vacuo over phosphorous pentoxide. The residue was dissolved in benzene (5.0 ml) and analyzed by thin layer chromatography in two solvent systems (Fig. 113 & Fig. 114). The remainder was applied to an Eastman cellulose acetate column and developed with benzene (125 ml).

The eluate was dried under reduced pressure at 30° C and the residue (8 mg) was redissolved in benzene for thin layer analysis (Fig. 115 & and Fig. 116). A total of 1,470,000 DPM or 78% of the activity applied to the column was eluted from it.

B) A mixture of dihydrosphingosine (5 mg; 17 umoles), phytosphingosine (5 mg; 16 umoles) and labeled dihydrosphingosine (5,080,000 DPM; 0.8 ug) were dried as above. p-Nitrobenzaldehyde (6 mg; 43 umoles) and hexane (2.5 ml) were added and the resulting mixture was heated in a 70° C bath for 10 minutes. After

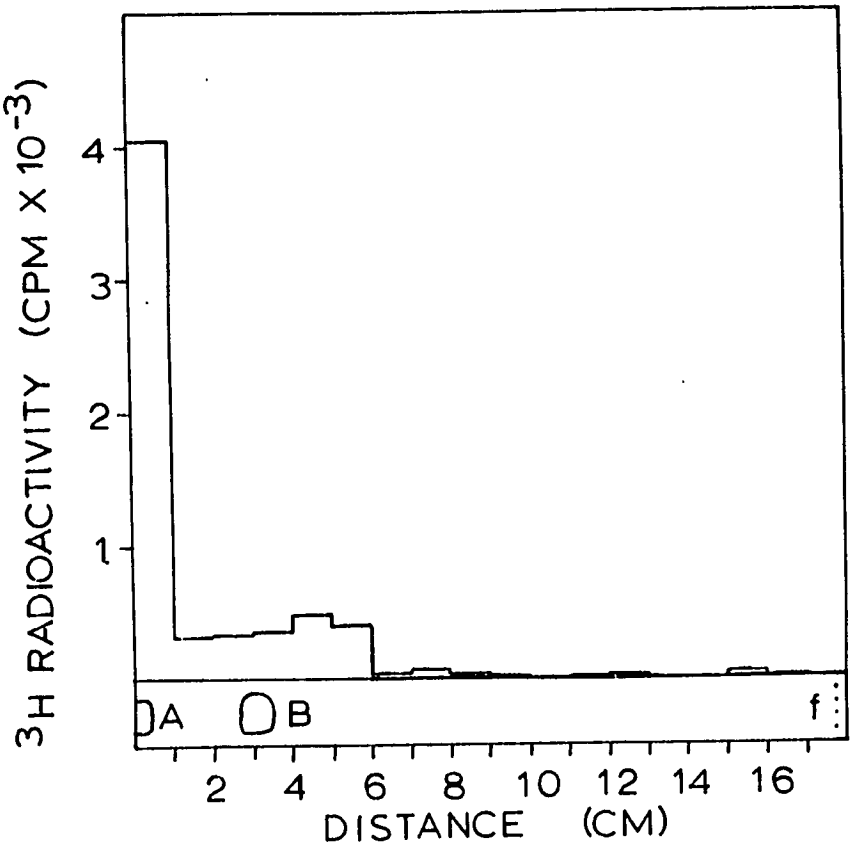


Figure 113. Thin layer radiochromatogram of the crude reaction products formed by heating a mixture of [4,5-$^3H_2$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 60° C. A: Dihydrosphingosine. B: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Hexane-ether (15:85).

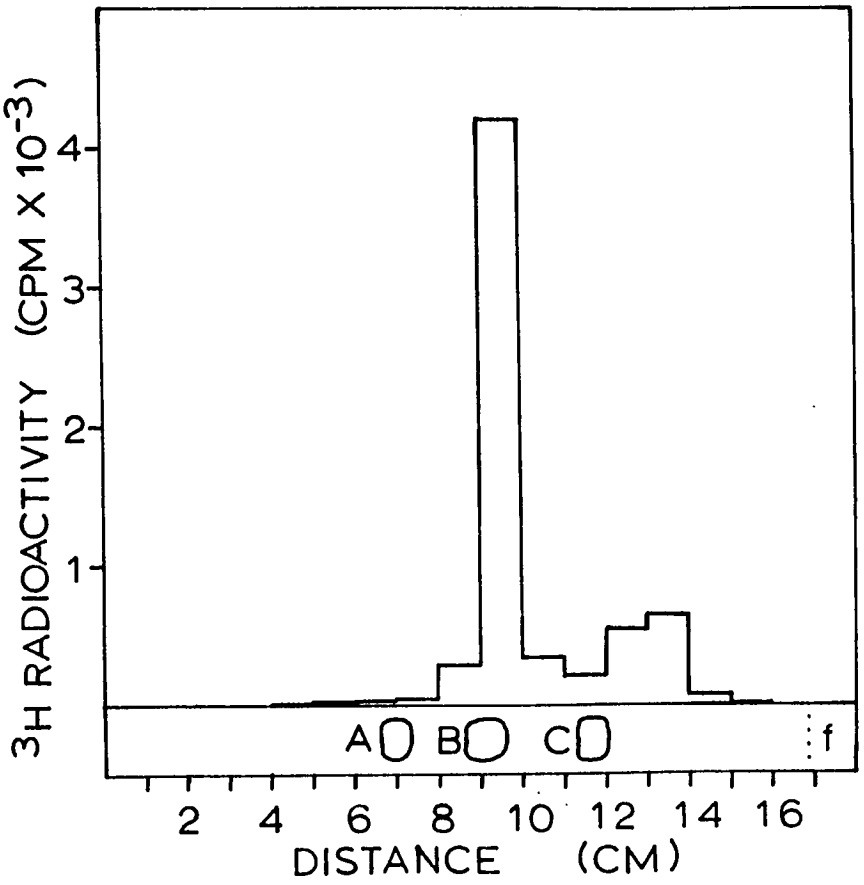


Figure 114. Thin layer radiochromatogram of the crude reaction products formed by heating a mixture of [4,5-$^3H_2$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 60° C. A: Phytosphingosine. B: Dihydrosphingosine. C: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

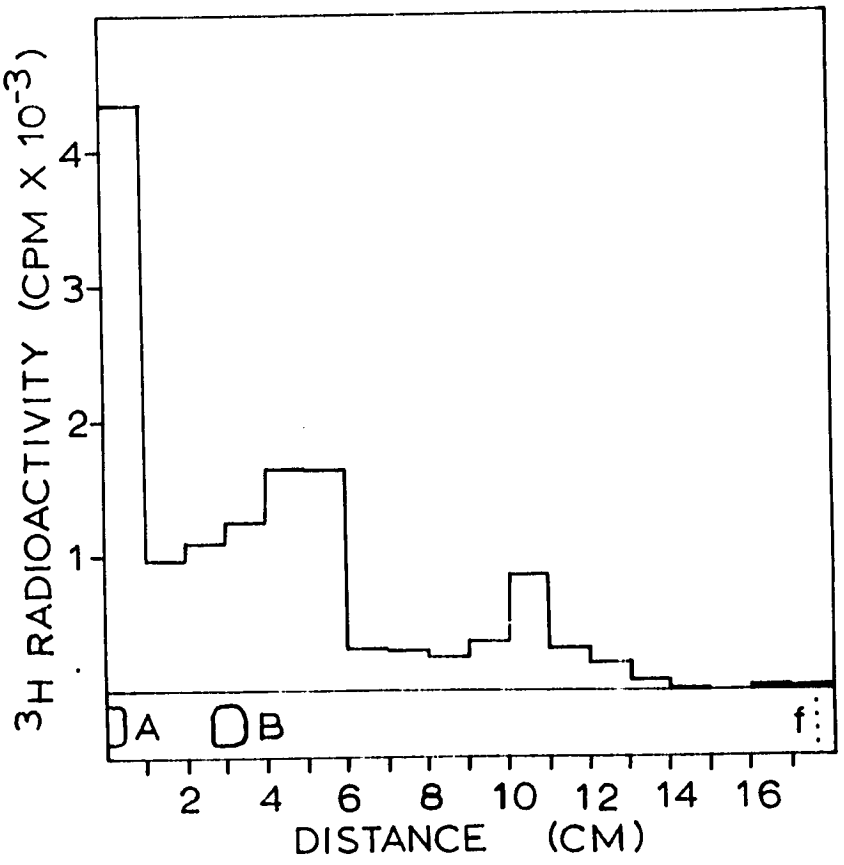


Figure 115. Thin layer radiochromatogram of the eluate from Eastman cellulose acetate column chromatography of the crude reaction products formed by heating a mixture of [4,5-$^3H_2$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 60° C. A: Dihydrosphingosine. B: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Hexane-ether (15:85).

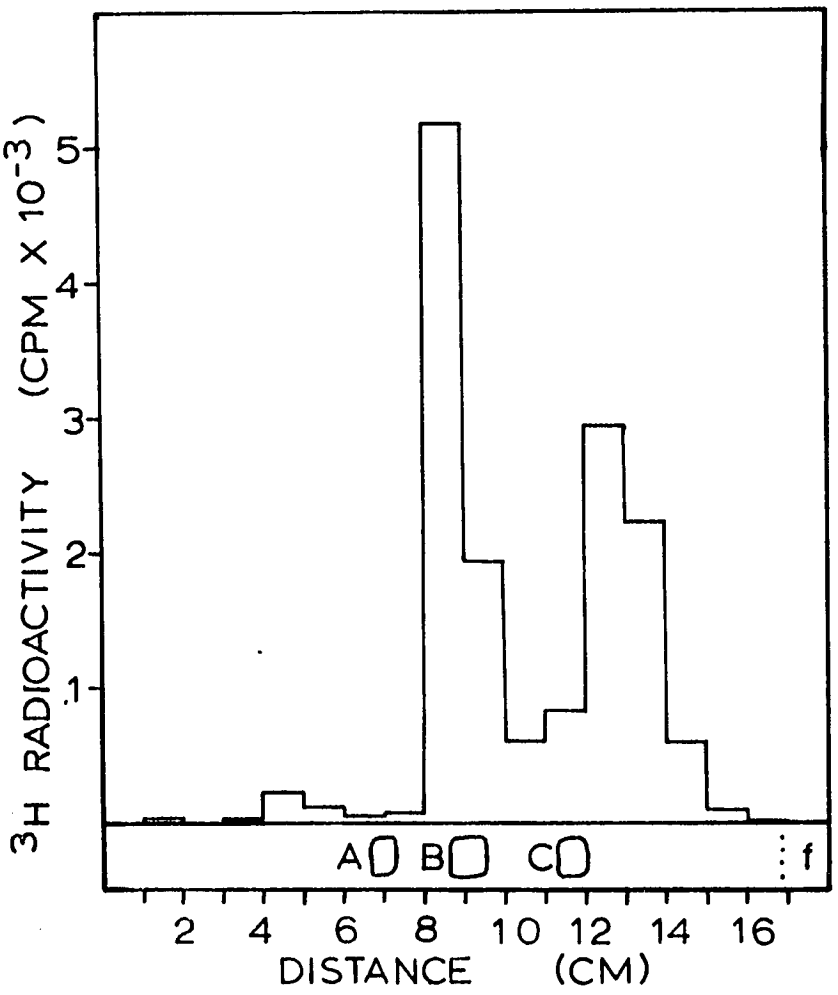


Figure 116. Thin layer radiochromatogram of the eluate from Eastman cellulose acetate column chromatography of the crude reaction products formed by heating a mixture of [4,5-$^3H_2$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 60° c. A: Phytosphingosine. B: Dihydrosphingosine. C: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

cooling to room temperature, the precipitate was collected by centrifugation and washed once with hot hexane (2.5 ml). After cooling, the precipitate (1,150,000 DPM) was dried in vacuo over phosphorous pentoxide, dissolved in benzene (1.0 ml) and applied to a Woelm cellulose acetate column. The column was developed with benzene (125 ml) and the solvent removed from the eluate under reduced pressure at 35° C. The residue (6 mg; 810,000 DPM) was redissolved in benzene and analyzed by thin layer chromatography in two solvents (Fig. 117 & Fig. 118).

EXPERIMENT #2: A) Dihydrosphingosine (10 mg; 32 umoles) and 10,700,000 DPM of labeled dihydrosphingosine were dissolved in benzene (5.0 ml) with gentle warming and applied to an Eastman cellulose acetate column. The column was developed with benzene (125 ml) and the solvent removed from the eluate at reduced pressure at 35° C leaving a residue of 4 mg and 4,110,000 DPM.

B) Dihydrosphingosine (5 mg; 16 umoles) and 5,080,000 DPM of the labeled dihydrosphingosine in benzene (5.0 ml) was applied to a Woelm cellulose acetate column and develed with benzene (125 ml) The solvent was removed from the eluate at reduced pressure at 35° C leaving a residue of 5 mg and 2,500,000 DPM.

The conclusions drawn from these experiments are very straightforward. First, as shown in Experiment #1, the procedure did not provide a means of separation of phytosphingosine from dihydrosphingosine as reported by Weiss and Stiller[111]. In that same report they also stated that dihydrosphingosine did not form a derivative with p-

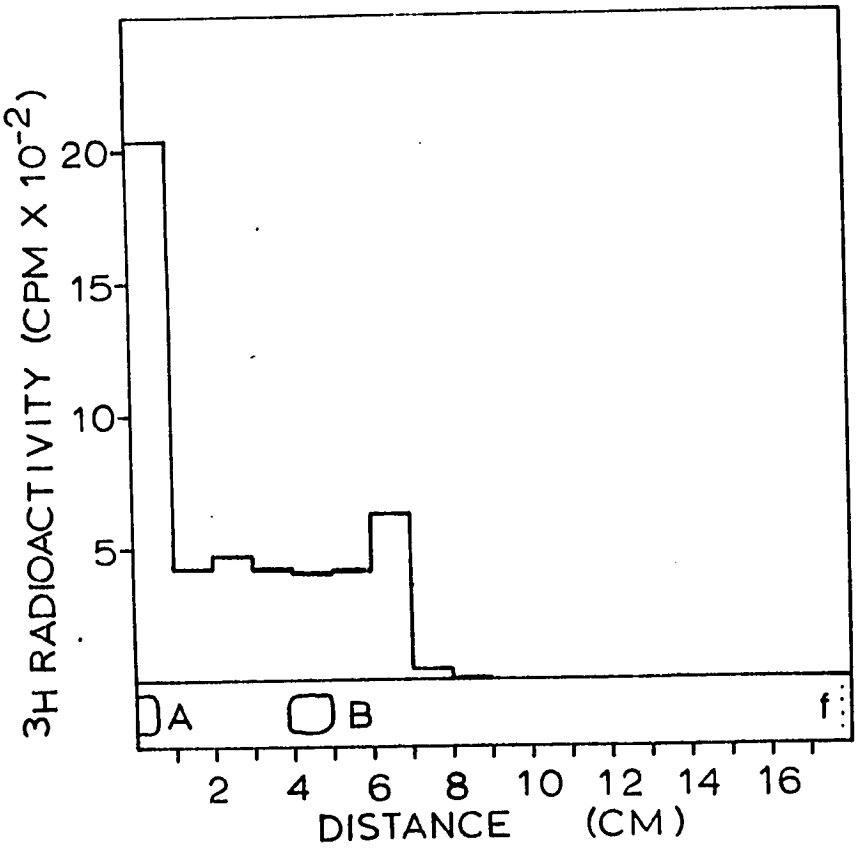


Figure 117. Thin layer radiochromatogram of the eluate from Woelm cellulose acetate column chromatography of the crude reaction products formed by heating a mixture of [4,5-$^{3}H_{2}$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 70° C. A: Dihydrosphingosine. B: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Hexane-ether (15:85).

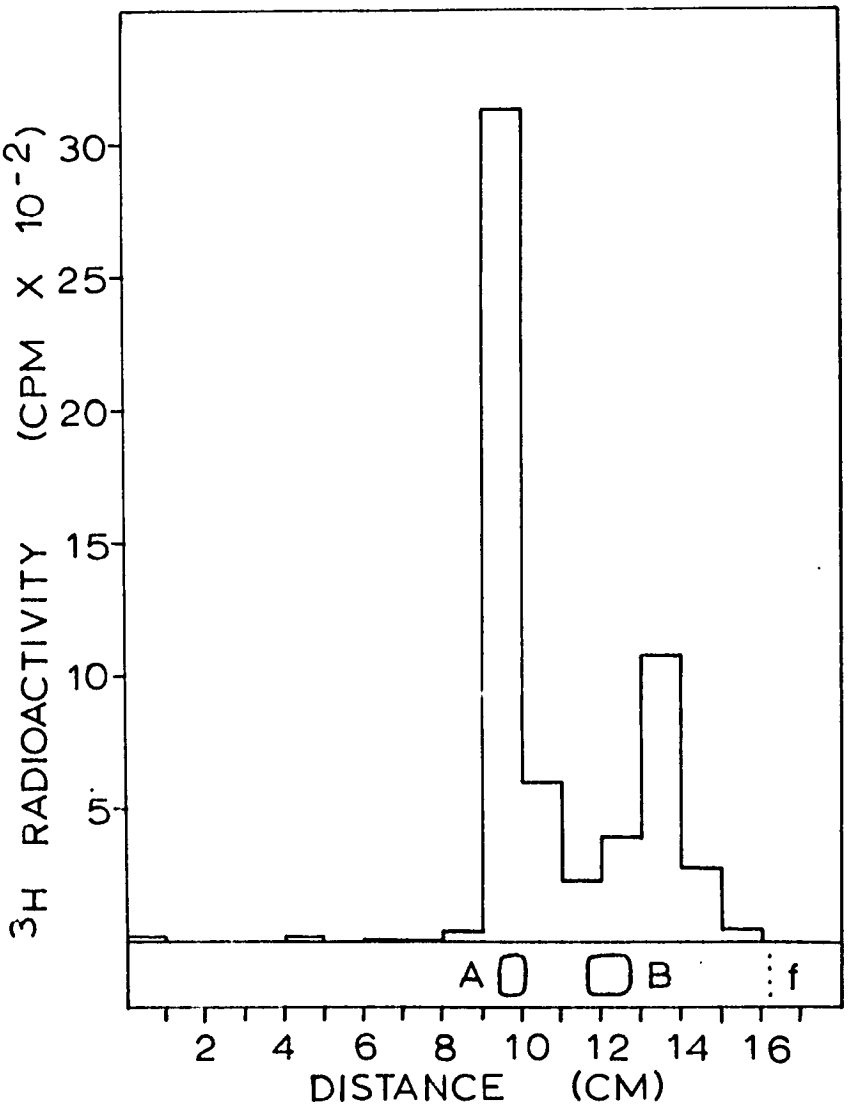


Figure 118. Thin layer radiochromatogram of the eluate from Woelm cellulose acetate column chromatography of the crude reaction products formed by heating a mixture of [4,5-$^3H_2$]-dihydrosphingosine, dihydrosphingosine, phytosphingosine and p-nitro-benzaldehyde in hexane at 70° C. A: Dihydrosphingosine. B: D-ribo-2-(p-Nitro-phenyl)-4-(1,2-dihydroxy-hexadecyl)-oxazolidene. Solvent: Chloroform-methanol-ammonia (100:25:2.5).

nitro-benzaldehyde as did phytosphingosine. However, thin layer analyses in Experiment #1 clearly showed that dihydrosphingosine did form a less polar derivative or derivatives. If, in fact, dihydrosphingosine formed a derivative similar to that of phytosphingosine, one may have expected to see activity of slightly higher mobility than the mobility of the phyto-adduct. This was observed. The smear of activity between that point of highest mobility and that of dihydrosphingosine may have been due to additional products or to decomposition on the chromatogram. As shown in the next section, dihydrosphingosine formed at least six different derivatives with p-nitrobenzaldehyde under the conditions described here and by Weiss and Stiller[111].

Secondly, as shown in Experiment #2, the cellulose acetate column did not separate the phyto-adduct from the dihydro-adduct(s) nor did it even seem to retard the unreacted dihydrosphingosine.

Thirdly, two sources of cellulose acetate were used and the results were the same in each case. Unless the particular unspecified lot of Woelm cellulose acetate used by Weiss and Stiller had peculiar characteristics, the column itself was not a factor in the differences observed between these experiments and those of Weiss and Stiller.

PREPARATION OF DIHYDROSPHINGOSINE

Beef spinal cord sphingosine (1.184 grams; 98.8% pure by gas-liquid chromatography; 3.91 mmoles of sphingosine prepared in this laboratory by the method of Carter, et. al.[21,22]) was dissolved in absolute ethanol (150 ml). Platinum oxide (J. Bishop and Co.; 40 mg) was added and the mixture was hydrogenated for 24 hours at room temperature in a Parr hydrogenation apparatus at 40 pounds per square inch. After removal of the catalyst by filtration, the solvent was removed under reduced pressure at 40° C. The residue (1.255 grams) was applied in 50 ml of chloroform to a 30 gram Unisil column (2.4 X 18 cm; packed in chloroform). The column was eluted with the following solvents: chloroform (150 ml), 3% methanol in chloroform (200 ml), 8% methanol in chloroform (300 ml), 10% methanol in chloroform (400 ml), 13% methanol in chloroform (200 ml), 15% methanol in chloroform (200 ml) and 20% methanol in chloroform (100 ml), successively. Fractions about 10 ml in volume were collected. Fractions 46-132 were pooled and the residue after removal of the solvent under reduced pressure at 40° C was crystallized from a minimum (about 10 ml) of hot ethyl acetate two times yielding 732 mg (98% dihydrosphingosine by gas-liquid chromatography). An additional 99 mg was obtained from the mother liquor by repeated recrystallizations (99% dihydrosphingosine). The total yield was 70%. The melting point of the purer sample was 70-73° C (Lit.[22] mp 84-86° C).

The infrared spectrum (Fig. 119) of the dihydrosphingosine was very similar to that of phytosphingosine. Absorbances due to hydrogen bonded hydroxyls were seen at 3370 cm^{-1}; NH_2 "scissors" at 1585 cm^{-1};

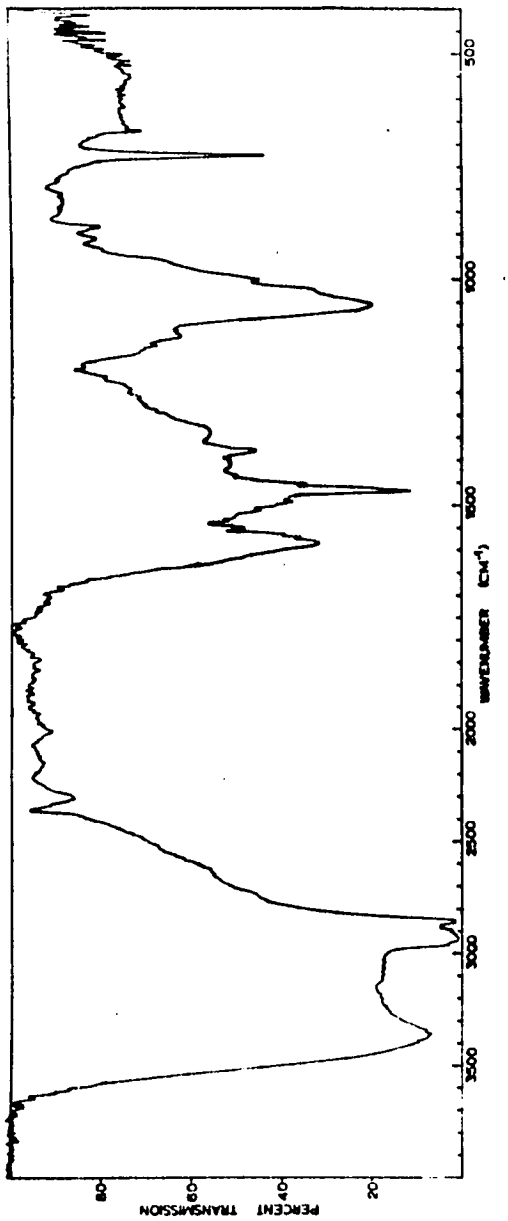


Figure 119. Infrared spectrum of dihydrosphingosine.

CH_2 "scissors" at 1468 cm^{-1}; CH_3 symetric deformation at 1375 cm^{-1}; C-O stretches at about 1050 cm^{-1} and the $(CH_2)_n$ rock at 721 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford, et. al.[28] and Jones [52].

Major ions and their tentative assignments in the mass spectrum (Fig. 120) of dihydrosphingosine were m/e 270 (M-31; M - CH_2OH), m/e 252 (M-49; M - CH_2OH - H_2O), m/e 60 ($\overset{\overset{|}{NH_2}}{CH}-CH_2OH$) and m/e 90 ($\overset{\overset{|}{OH}}{CH}-\overset{\overset{|}{NH_2}}{CH}-CH_2OH$).

The combined gas-liquid chromatography-mass spectrum (Fig. 121) of the tris (trimethylsilyl) dihydrosphingosine exhibited major ions (and their tentative assignments) at m/e 517 ($M^{+\cdot}$), m/e 502 (M-15; M - CH_3), m/e 414 (M-103; M - CH_2OSiMe_3), m/e 342 (M-175), m/e 204 (M-313; $\overset{\overset{|}{NHSiMe_3}}{CH}-CH_2OSiMe_3$) and m/e 132 (M-385).

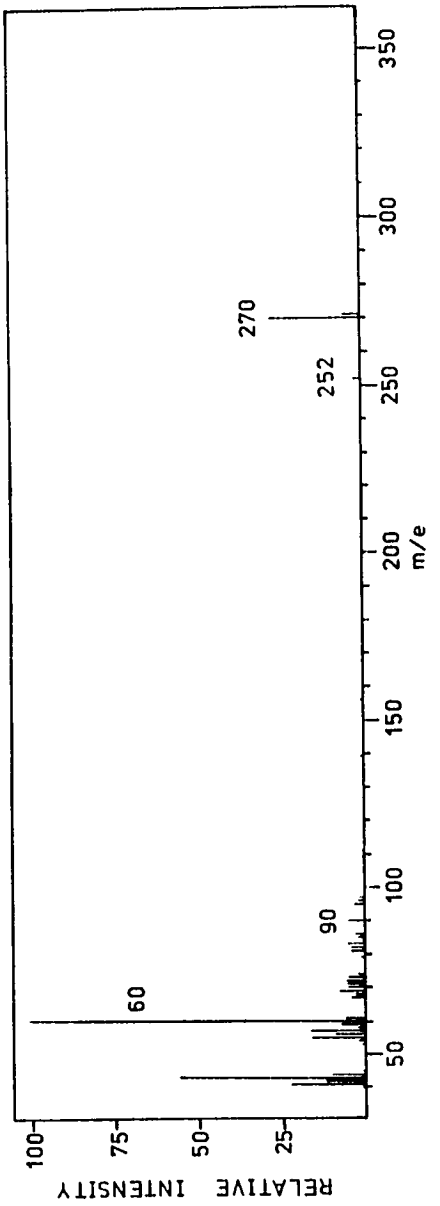


Figure 120. Mass spectrum of dihydrosphingosine.

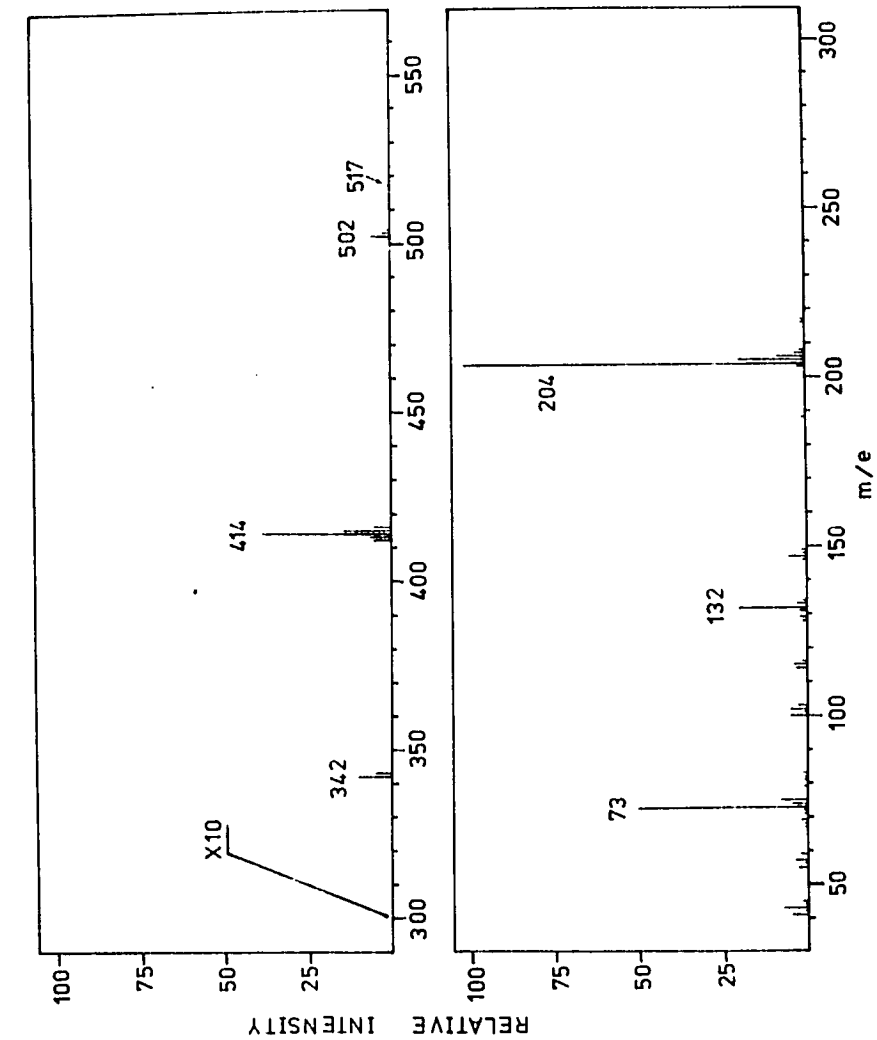


Figure 121. Combined gas-liquid chromatography-mass spectrum of the silylated derivative of dihydrosphingosine on an eight foot, 1% SE 30 column at 220° C.

ISOLATION AND CHARACTERIZATION OF THE DIACETYL DERIVATIVES OF THE p-NITRO-BENZALDEHYDE ADDUCTS OF DIHYDROSPHINGOSINE

Dihydrosphingosine (100 mg; 0.332 mmole) and p-nitro-benzaldehyde (54 mg; 0.358 mmole; Matheson; crystallized two times from 10% ethanol in water prior to use) were added to hexane (13.5 ml) and heated carefully in a 70° C bath until all of the material dissolved (about 15 minutes). The solvent was removed under nitrogen at 35° C and the residue was dried *in vacuo* over phosphorous pentoxide. Previous attempts to isolate the individual products at this stage by column or thin layer chromatography were unsuccessful due to the similarities in their chromatographic properties and their tendency to revert back to the starting materials. These problems were overcome by dissolving the above dried residue in dry pyridine (2 ml) and adding acetic anhydride (1.2 ml). After 22 hours at room temperature, water (6 ml) was added and the mixture was lyophilized one hour later. The acetylated products were relatively stable and were separable on thin layer chromatography when developed in hexane-ether (17:83) on Silica Gel PF 254 + 366 (Fig. 122). From the top of the plate, they were named D_1 (R_f = 0.75), D_2 (R_f = 0.69), D_3 (R_f = 0.59), D_4 (R_f = 0.51), D_5 (R_f = 0.36) and D_6 (R_f = 0.31). D_2 did not stain with molybdic acid as did the others, but like the others was clearly visible when viewed under short wavelength ultraviolet light on the fluorescent plate. Qualitatively, D_5 was present in largest amount followed by D_1; however, usually mass recoveries of D_1 were rather low. The compounds were separated by preparative thin layer chromatography using multiple developments in hexane-ether (17:83) and eluted with ether. Some

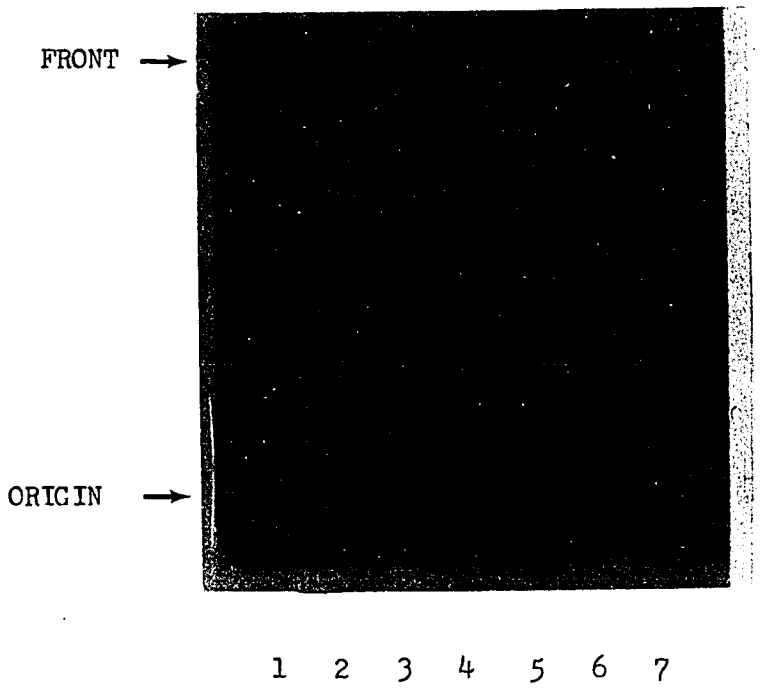


Figure 122. Thin layer chromatogram of p-nitro-benzaldehyde derivatives of dihydrosphingosine on Silica Gel PF 254 + 366 plates. Channels 1 & 7: Crude reaction products formed by heating dihydrosphingosine and p-nitro-benzaldehyde in hexane at 70° C. Channel 2: Crude D_1. Channel 3: Crude D_3. Channel 4: Crude D_4. Channel 5: Crude D_5. Channel 6: Crude D_6. Solvent: Hexane-ether (16-84). Stain: Molybdic acid.

partially purified samples appear in Fig. 122; however, samples homogeneous both by thin layer and gas-liquid chromatography were obtained for each product. Typical yields from several of the above preparations were 6 mg of D_1 (oil, 3% yield), 16 mg of D_2 (pale yellow, feather-like crystals, 9% yield), 15 mg of D_3 (white solid, 9% yield), 7 mg of D_4 (oil, 4% yield), 55 and 76 mg of D_5 (oil, 32 and 44% yield) and 5 and 10 mg of D_6 (oil, 3 and 6% yield). These yields were calculated assuming that the molecular weight for each of the compounds was 518 grams/mole. At 285° C on a 4 foot, 1% SE 30 column, the relative retention times on gas-liquid chromatography with respect to D_5 were: D_1 = 0.69, D_2 = 0.69, D_3 = 1.12, D_4 = 1.07, D_5 = 1.00 and D_6 = 1.23. The retention time for D_5 was about 20 minutes.

The combined gas-liquid chromatography-mass spectra appeared to occur in pairs of patterns, probably due to the fact that each of the six samples was one member of a diastereomeric pair differing only by the chirality of what was the carbonyl carbon of p-nitro-benzaldehyde. If there were a diastereomeric pair for each of the possible addition products (cyclic 1,2; 1,3 and 2,3), that would account for the six products isolated. The trapping of intermediate hemi-acetals by the acetylation procedure was discounted as the observed mass spectra did not in any way correspond to such structures.

The mass spectra of D_5 (Fig. 123) and D_3 (Fig. 124) are compatible with the following tentative structure (I):

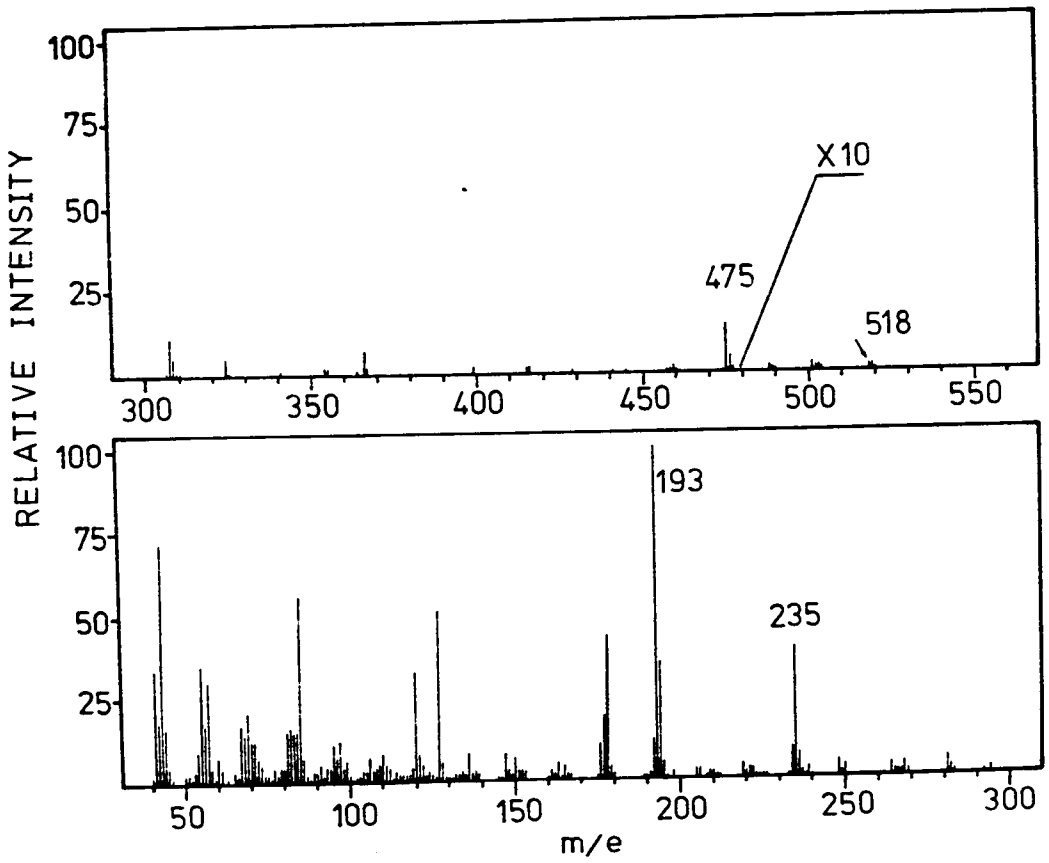


Figure 123. Combined gas-liquid chromatography-mass spectrum of D_5 on a six foot, 1% SE 30 column at 285° C.

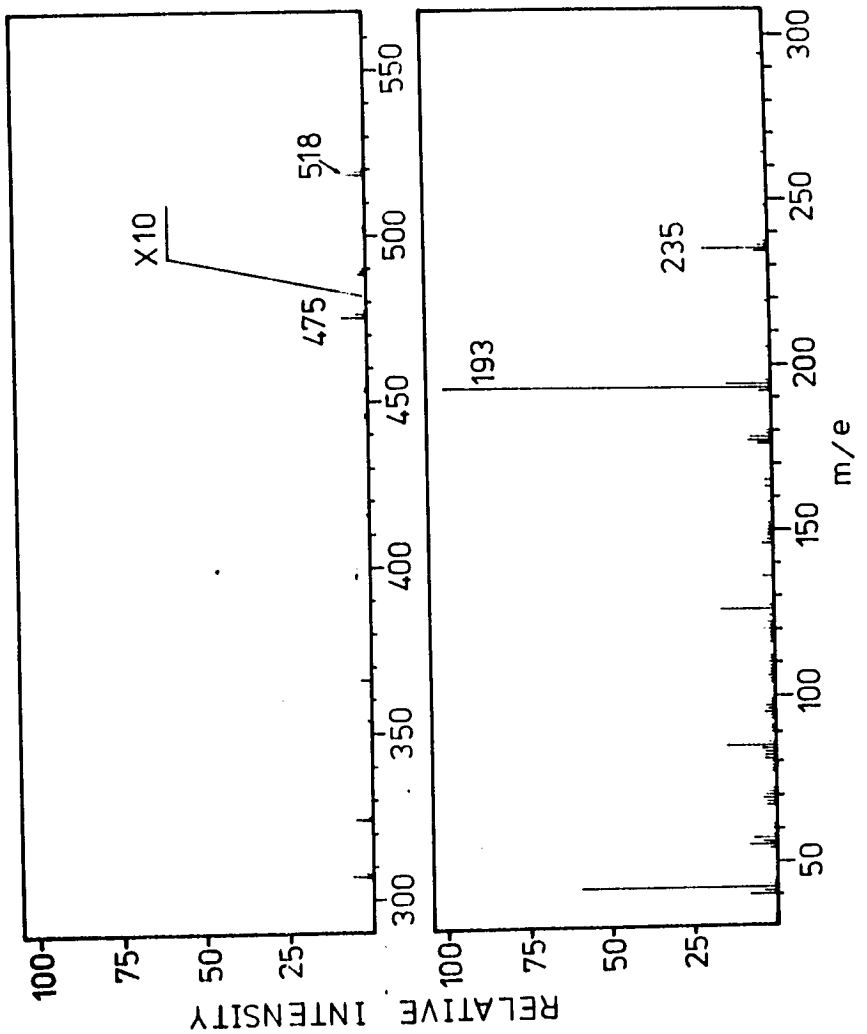
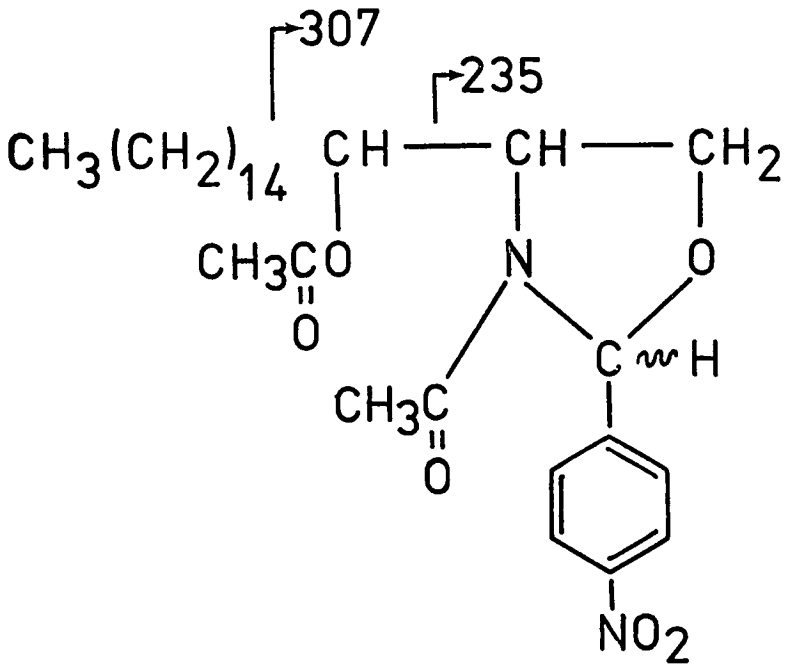


Figure 124. Mass spectrum of D_3.

(I)



Important ions and their tentative assignments were m/e 518 (the molecular ion), m/e 475 (M-43; M - CH_3CO), m/e 307 (M-211; M - $CH_3(CH_2)_{14}$), m/e 235 (M-283; see above) and the loss of ketene[70c] from this ion to give m/e 193 (M-325). The metastable ion (m^*) which resulted by the formation of a daughter ion (M_2) from the parent ion (M_1) can be calculated by the formula:

$$m^* = \frac{(M_2)^2}{M_1} .$$

In this case, $m^* = \frac{(193)^2}{235} = 158.5$ and, indeed, D_3 did exhibit a metastable peak at m/e 158.5.

The NMR spectrum (Fig. 125) of D_5 confirms that the sample did, indeed, have two acetyl groups. The methyl protons of the acetyl groups were found as singlets at 1.66 and 2.16 PPM and integrated for about 3 protons each. The three hydrogens on skeletal carbons 1 and 2 were observed as a broad singlet at 4.15 PPM. The skeletal hydrogen

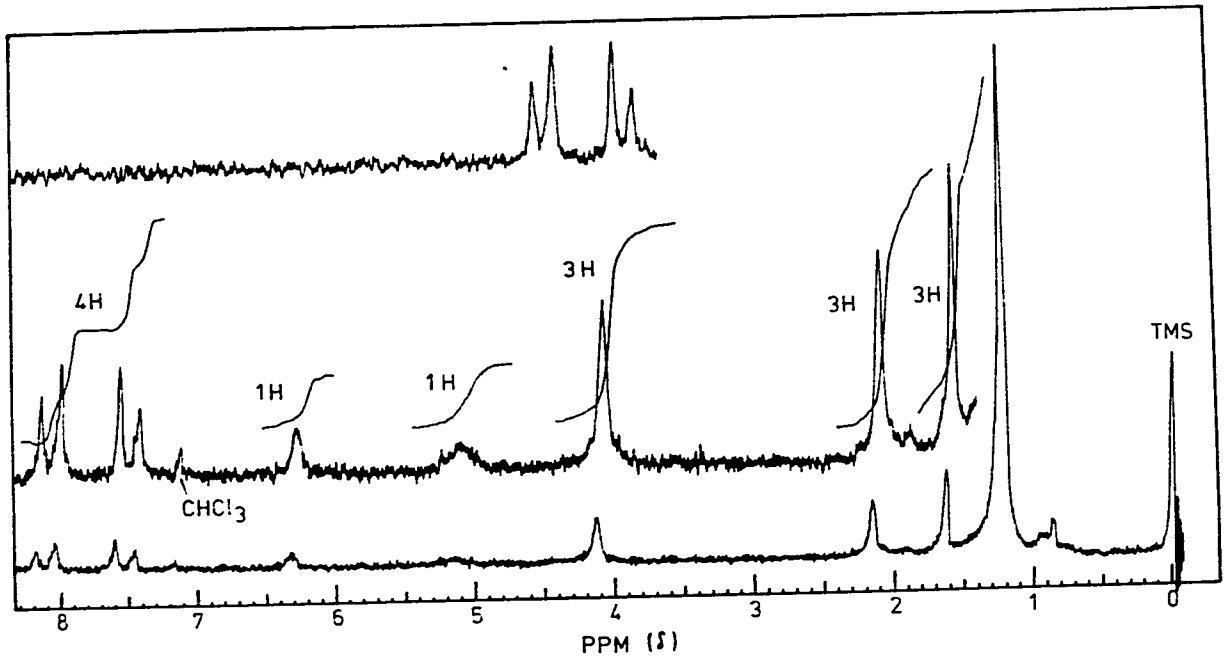
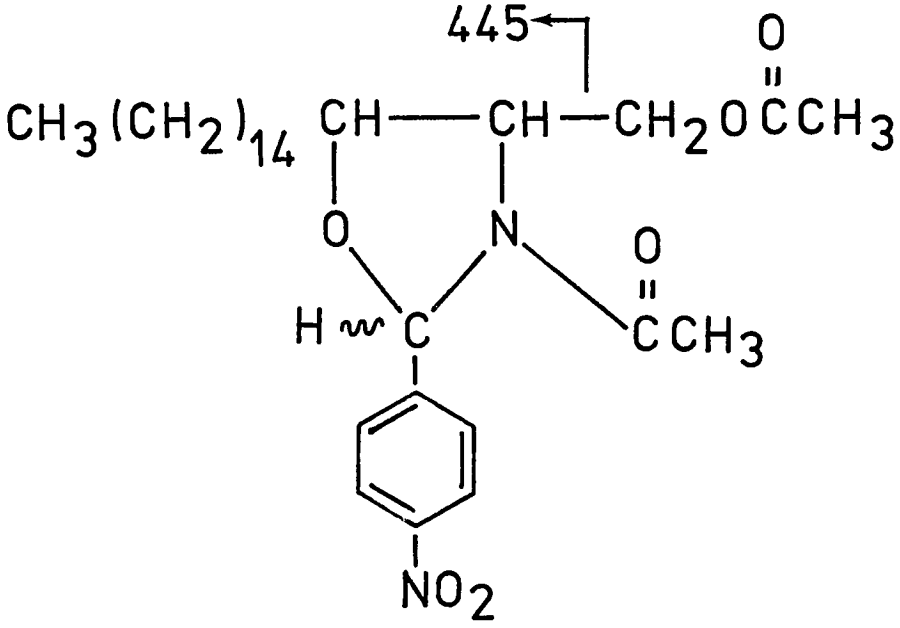


Figure 125. NMR spectrum of D_5.

at carbon atom 3 accounted for the broad multiplet at 5.16 PPM. The hydrogen which was alpha to the aromatic ring and also on the same carbon as the hetero-atoms of nitrogen and oxygen was found at 6.32 PPM. Finally, the classic aromatic splitting of a para-disubstituted benzene was centered about 7.8 PPM.

The mass spectra of D_4 (Fig. 126) and D_6 (Fig. 127) were compatible with the following tentative structure (II):

(II)



Important ions and their tentative structures were m/e 518 (the molecular ion), m/e 475 (M-43; M - CH_3CO), m/e 445 (M-73; M - CH_2OCOCH_3) and an an elimination of ketene[70c] (CH_2CO) from this ion gave m/e 403 (M-115; M - CH_2OCOCH_3 - CH_2CO).

The final pair of mass spectra of D_1 (Fig. 128) and D_2 (Fig. 129) were compatible with the unusual, tentative structure below (III):

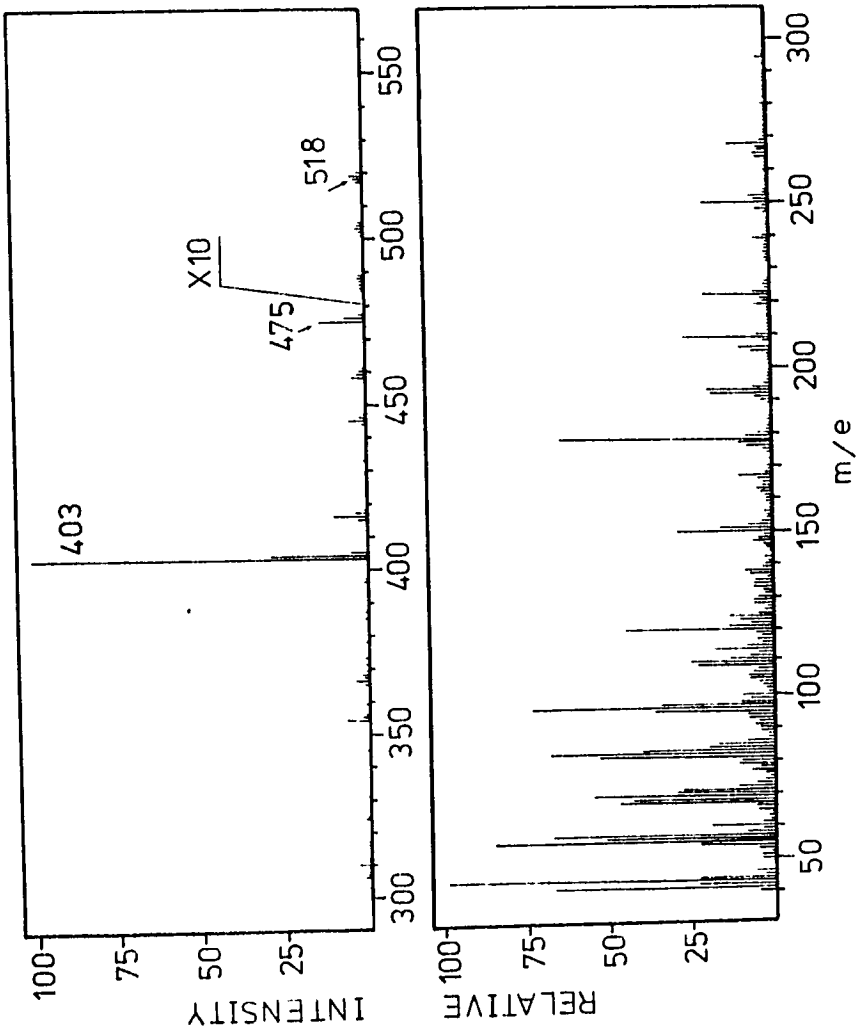


Figure 126. Combined gas-liquid chromatography-mass spectrum of D_4 on a six foot, 1% SE 30 column at 285° C.

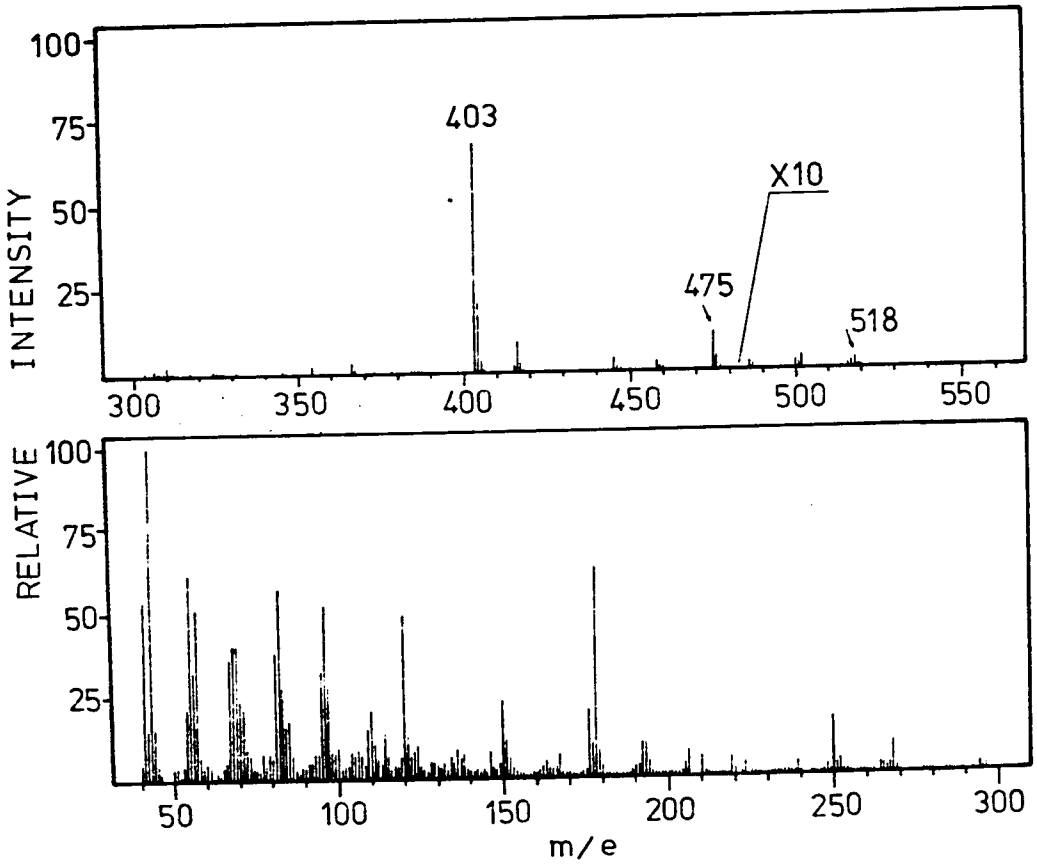


Figure 127. Combined gas-liquid chromatography-mass spectrum of D_6 on a six foot, 1% SE 30 column at 285° C.

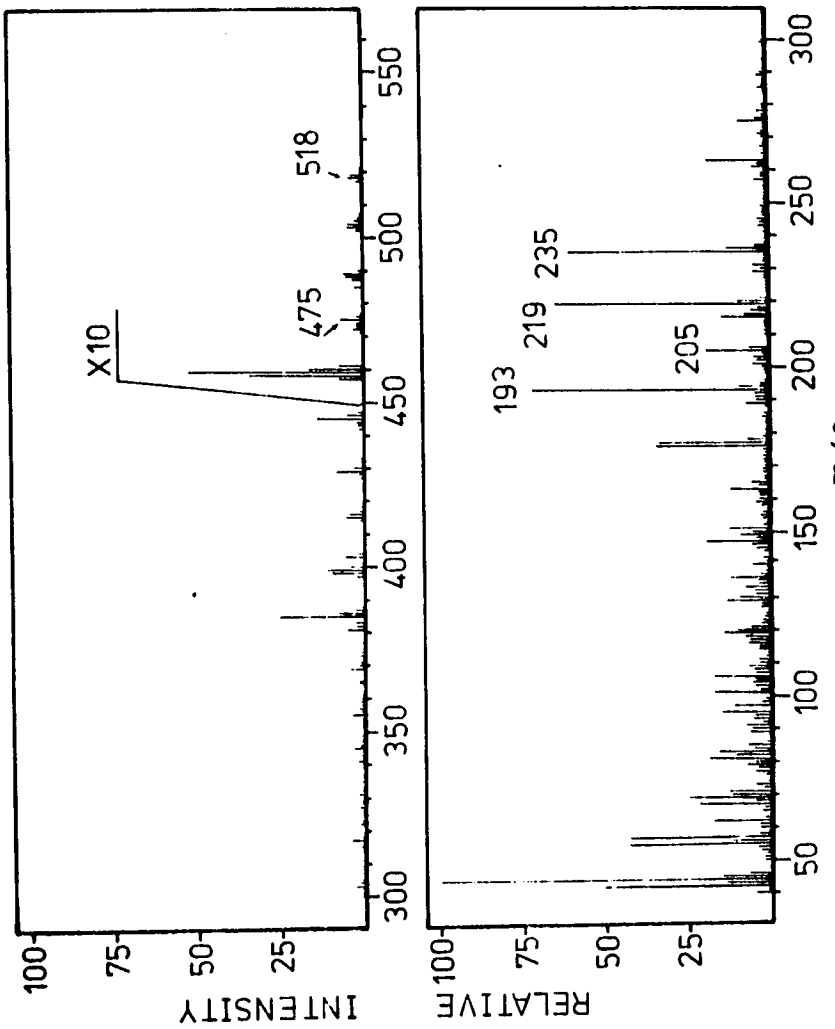


Figure 128. Combined gas-liquid chromatography-mass spectrum of D_1 on a six foot, 1% SE 30 column at 285° C.

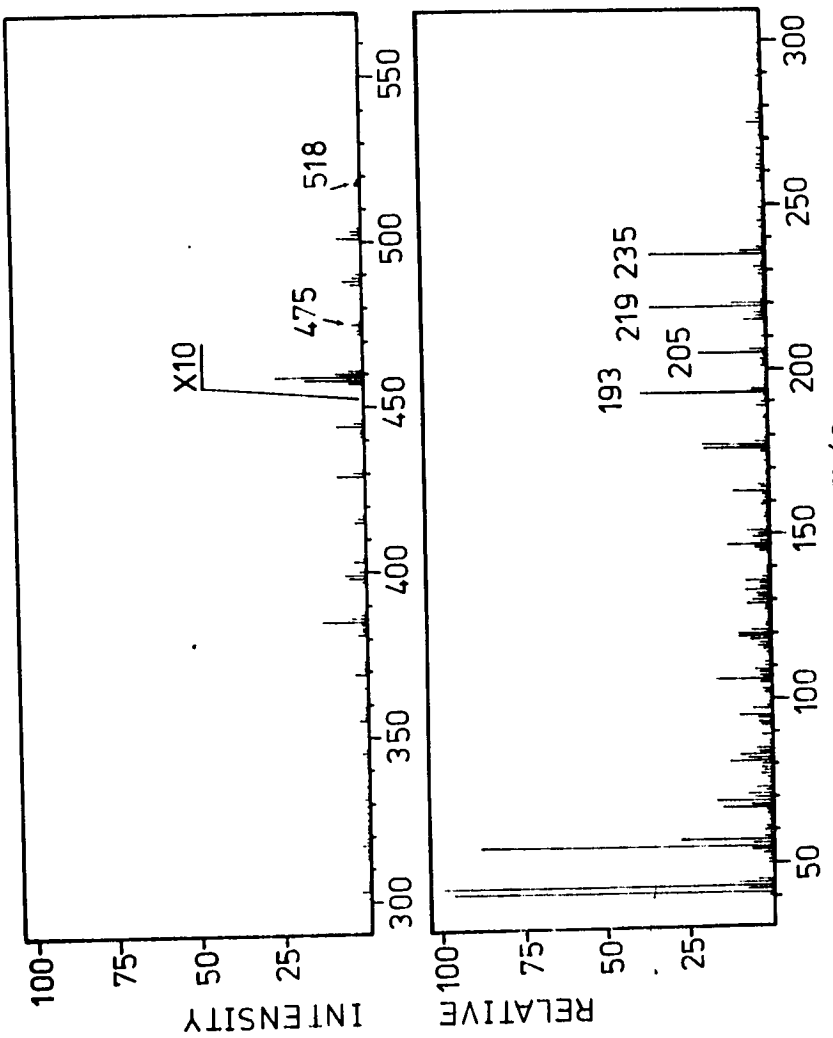
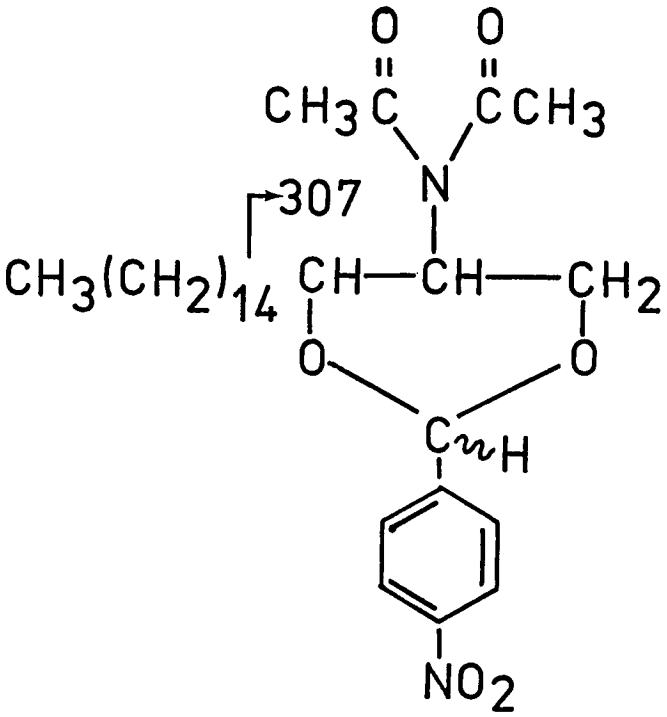


Figure 129. Combined gas-liquid chromatography-mass spectrum of D_2 on a six foot, 1% SE 30 column at 285° C.

(III)



The major ions and their tentative assignments were m/e 518 (the molecular ion), m/e 475 (M-43; M - CH_3CO), m/e 235 (M-283), m/e 219 (M-299), m/e 205 (M-313) and m/e 193 (M-325). The ions at m/e 219 and m/e 205 differentiated this pair of compounds from D_3 and D_5. It was speculated that m/e 205 arose from an alpha cleavage between skeletal carbons 3 and 4 to form an unstable ion of molecular weight 307 followed by an elimination of $(CH_3CO)_2NH$. Both D_1 and D_2 exhibited a metastable ion at m/e 158.8 corresponding to the loss of 42 atomic mass units (ketene[70c]) from m/e 235 to form m/e 193. If the proposed structure was, indeed, correct; then the molecule underwent a complicated rearrangement to form a structure not unlike those of D_3 and D_5. The proposed structure had serious deficiencies since it was unusual under the mild acetylation conditions to diacetylate a primary amine.

A sample of D_5 (30 mg; 0.058 mmole) was dissolved in methanol

(0.9 ml) and 2 N KOH (0.1 ml) was added. An immediate white precipitate formed. After one hour at room temperature, water (4 ml) was added and the mixture was extracted with ether (5 ml; leaving behind a small interface layer). The residue after removal of the solvent under nitrogen at 30° C was crystallized two times from warm hexane to give 11 mg (40% yield) of pale, yellow solid, mp 61.5-62.5° C. On thin layer chromatography with ether as the solvent on Silica Gel PF 254 + 266 plates, D_5 had an R_f = 0.43 and the hydrolysis product gave a single spot of R_f = 0.25. Hydrolyses of this type usually cleave only ester linkages while leaving those of amides untouched.

The infrared spectrum (Fig. 130) of the partially deacetylated D_5 showed a free hydroxyl absorption at 3320 cm^{-1}; an amide carbonyl absorption at 1630 cm^{-1} and an asymmetric nitro stretching mode at 1525 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford, et. al.[28], and Jones[52].

The ultraviolet spectrum of the partially deacetylated D_5 showed a single broad absorption at 265.5 nm with an extinction coefficient of 7,900 (nitrobenzene had an absorption maximum at 265.8 nm and an extinction coefficient of 7,800).

The mass spectrum (Fig. 131) of the partially deacetylated D_5 exhibited the molecular ion at m/e 476. Major ions were m/e 235 and m/e 193. Their tentative assignments are the same as for those ions in the mass spectrum of D_5.

D_3 and D_4 were treated with alkali as above. The products thus formed were crystallized from hexane. These were more polar on thin layer chromatography in ether than their respective substrates. However, the molybdic acid stained plate exhibited considerable streaking

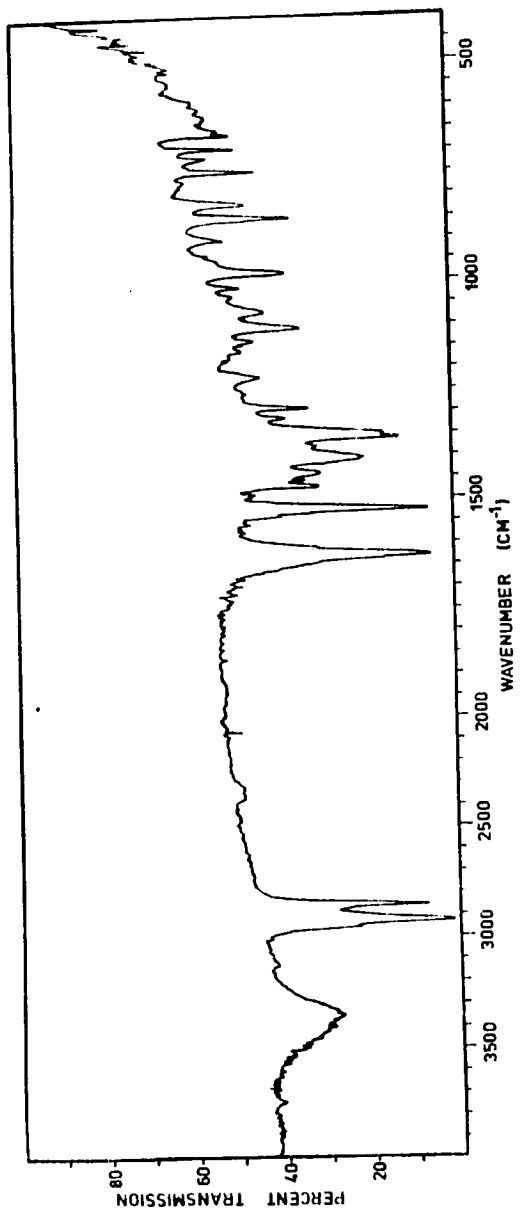


Figure 13C. Infrared spectrum of the partially deacetylated D_5 which was formed upon mild alkali hydrolysis of D_5.

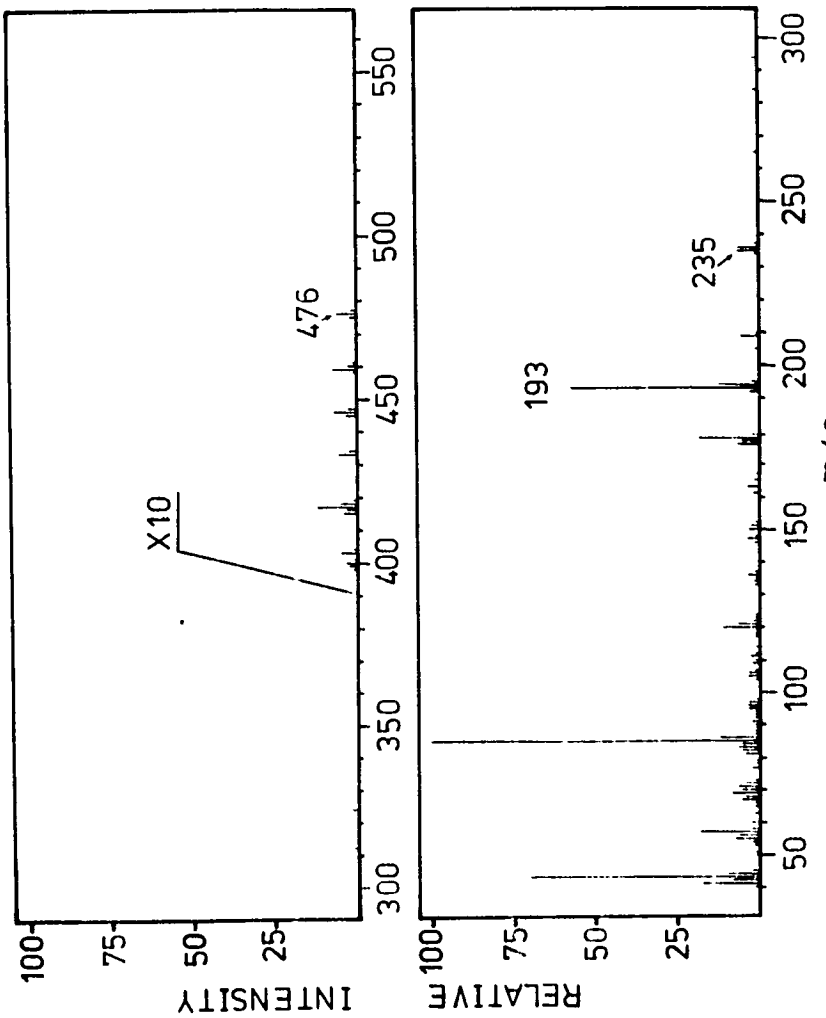


Figure 131. Mass spectrum of the partially deacetylated D_5 which was formed upon mild alkali hydrolysis of D_5.

toward the origin where dihydrosphingosine chromatographed. An ultraviolet visible streaking toward a spot where the non-staining p-nitrobenzaldehyde chromatographed was also observed on the fluorescent plate. This explained why the unacetylated derivatives could not be isolated in a stable form by thin layer or column chromatography.

CHEMICAL SYNTHESIS OF DIHYDROSPHINGOSINE-1-PHOSPHATE

A modification of the method of Weiss[105] was developed for the synthesis of dihydrosphingosine-1-phosphate on a much smaller scale. Dihydrosphingosine (100 mg; 0.333 mmole) was suspended in ether (15 ml) and 0.5 N sodium hydroxide (1.8 ml). Benzyl chloroformate (73 ul; 0.511 mmole; Chemetron Corporation) in ether (1 ml) was added dropwise over 5 minutes with stirring at room temperature. After 30 minutes of continued stirring, the mixture was extracted three times with ether (15 ml each). The combined ether layers were washed with water (20 ml). The solvent was removed under reduced pressure at 35° C. The residue was crystallized three times from hexane (about 20 ml) to yield N-carbobenzoxy-dihydrosphingosine (122 mg; 84%; Lit.[105] yield 72%; mp 105.5-106° C; Lit.[105] mp 106-107° C). The purity of the sample was confirmed by thin layer chromatography (Fig. 132). Elemental analysis showed:

	C	H	N
Calculated:	71.68	10.41	3.22
Found	71.46	10.42	3.30 (%)

The infrared spectrum (Fig. 133) indicated the formation of an amide by the absorption at 1695 cm^{-1} for the carbonyl and the amide N-H in plane bend at 1550 cm^{-1}. Aromatic hydrogen stretches appeared at 3060 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford, et. al.[28], and Jones[52].

The NMR spectrum (Fig. 134) gave absorptions for the aromatic hydrogens at 7.30 PPM (5 protons; singlet); the amide proton at 5.56 PPM (1 proton; doublet); the benzyl hydrogens at 5.08 PPM (2 protons; singlet); the skeletal hydrogens of carbons 1,2 and 3 at 3.8 PPM (4

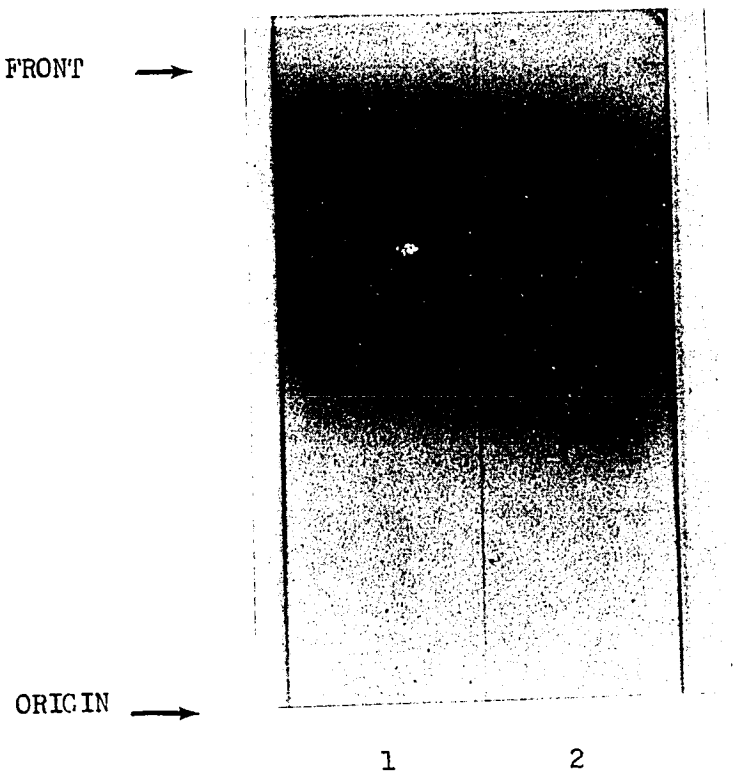


Figure 132. Thin layer chromatogram of N-carbobenzoxy-dihydrosphingosine (Channel 1) and N-carbobenzoxy-phytosphingosine Channel 2) on a Silica Gel PF 254 + 366 plate. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Molybdic acid.

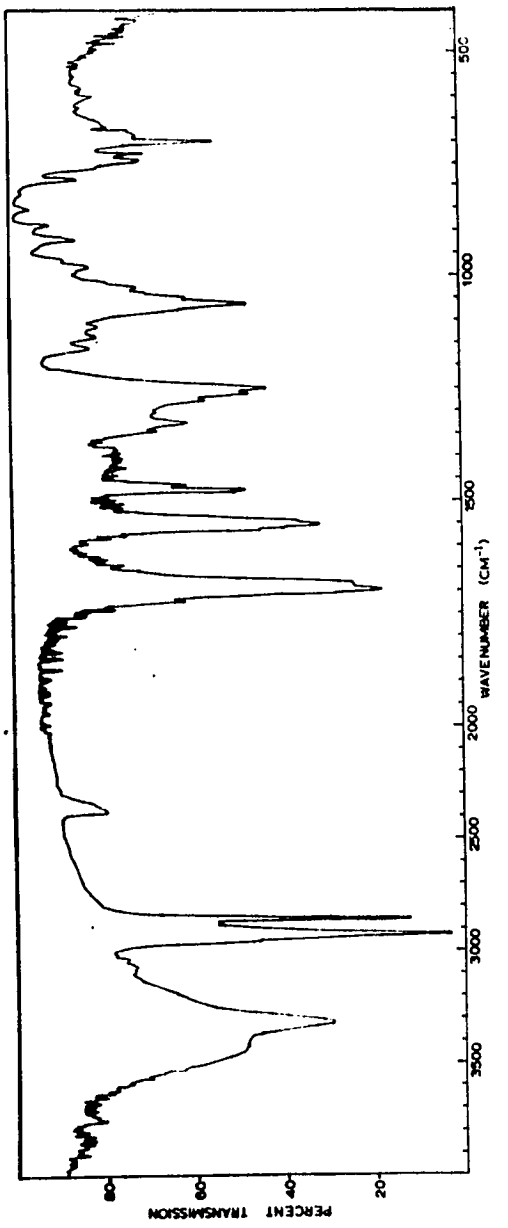


Figure 133. Infrared spectrum of N-carbobenzoxy-dihydrosphingosine.

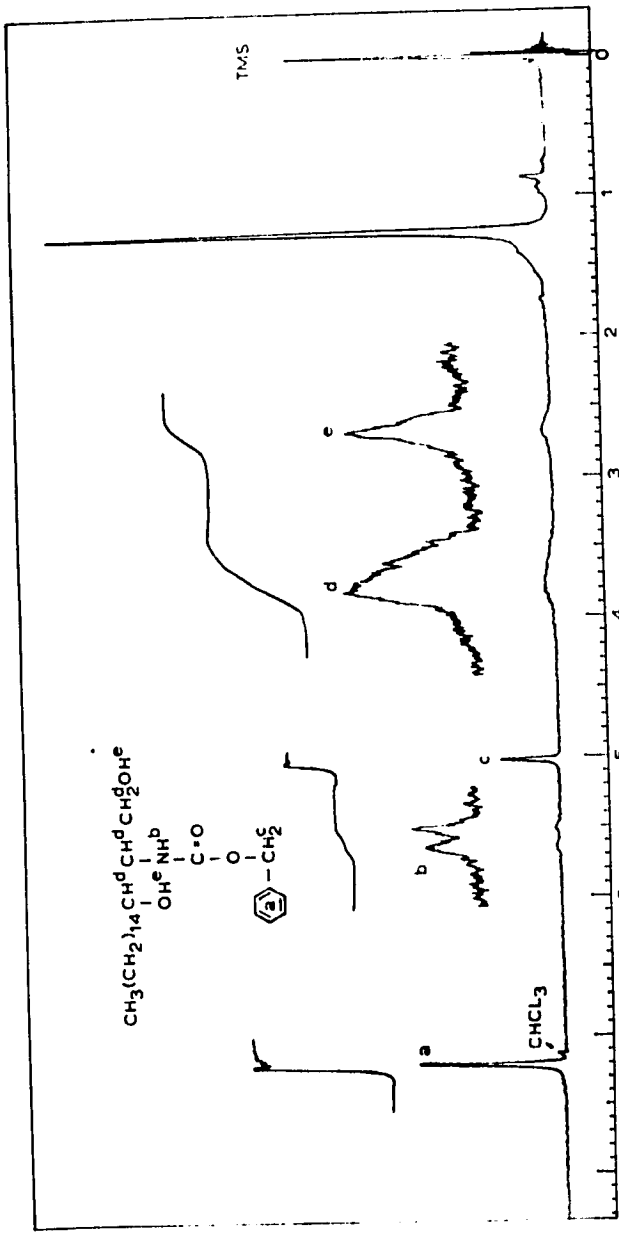


Figure 134. NMR spectrum of N-carbobenzoxy-dihydrosphingosine.

protons; multiplet) and the hydroxyl protons at 2.67 PPM (2 protons; multiplet).

The ultraviolet spectrum, maxima and extinction coefficients are shown in Fig. 135 along with an ethanol vs ethanol blank.

The major ions and their tentative assignments in the mass spectrum (Fig. 136) were m/e 327 (M-108; M - $PhCH_2OH$), m/e 296 (M-139; M - $PhCH_2OH$ - CH_2OH), m/e 252 (M-183; M - $PhCH_2OCONH_2$ - CH_2OH), m/e 107 (M-328; $PhCH_2O$), m/e 108 (M-327; $PhCH_2OH$) and m/e 87 (M-348; $\begin{matrix}CH\text{-}CH_2OH\\ |\\ NH\\ |\\ C{=}O\end{matrix}$). The probe mass spectrum of the bis (trimethylsilyl) N-carbobenzoxy-dihydrosphingosine (Fig. 137) showed ions at m/e 563 (M-16), m/e 476 (M-103; M - CH_2OSiMe_3), m/e 456 (M-123; M - $PhCH_2OH$ - CH_3), m/e 313 (M-266; $CH_3(CH_2)_{14}\overset{OSiMe_3}{\overset{|}{C}}H$), m/e 103 (M-476; CH_2OSiMe_3), m/e 91 (M-488; $PhCH_2$) and m/e 73 ($SiMe_3$).

Diphenylphosphochloridate was obtained from Eastman and was vacuum distilled at 147-150° C at 1.3-1.4 mm of mercury prior to use.

Its mass spectrum (Fig. 138) gave ions at m/e 268 (molecular ion), m/e 270 (Cl^{37} isotope peak), m/e 232 (M-36; M - HCl), m/e 94 (M-174; PhOH) and m/e 77 (M-191; Ph). This material (113 ul; 139 mg; 0.577 mmole was slowly added through a capillary tube to N-carbobenzoxy-dihydrosphingosine (122 mg; 0.28 mmole) which was dissolved in dry pyridine and cooled to 0° C in an ice bath. The reaction mixture was stirred at 0° C for 24 hours and an aliquot was then analyzed by thin layer chromatography and sprayed with Dittmer's phospholipid reagent (Fig. 139, Channel 1). The immediate blue spot which occurred at R_f = 0.20 was the ammonium salt of diphenylphosphoric acid. After the

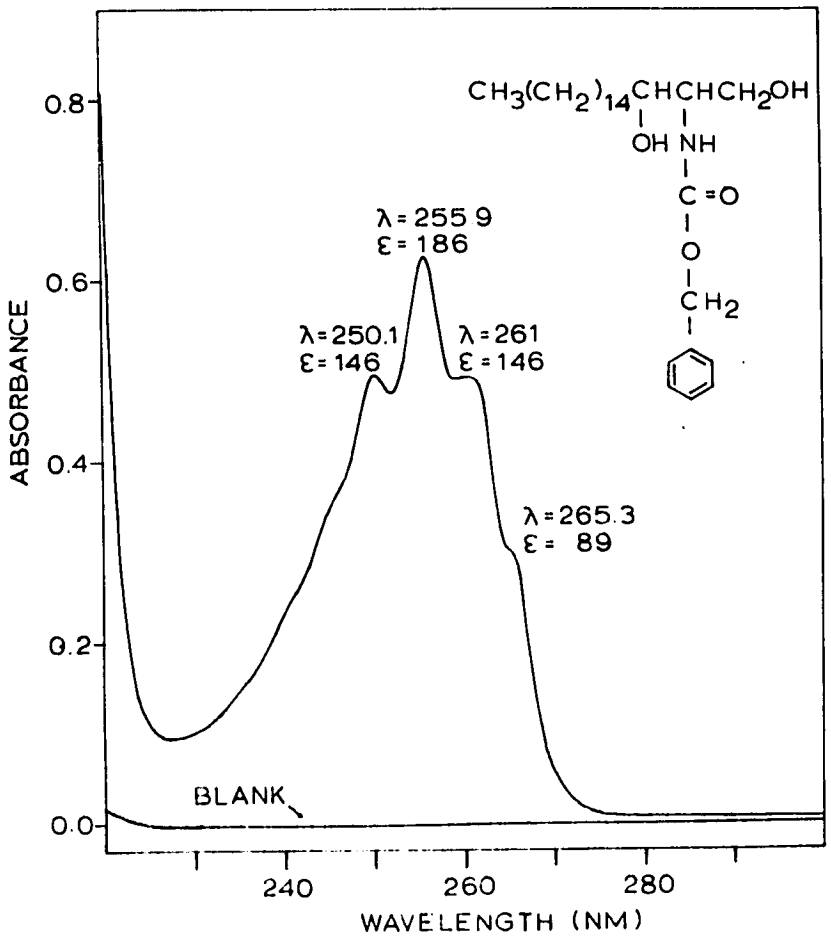


Figure 135. Ultraviolet spectrum of N-carbobenzoxy-dihydrosphingosine.

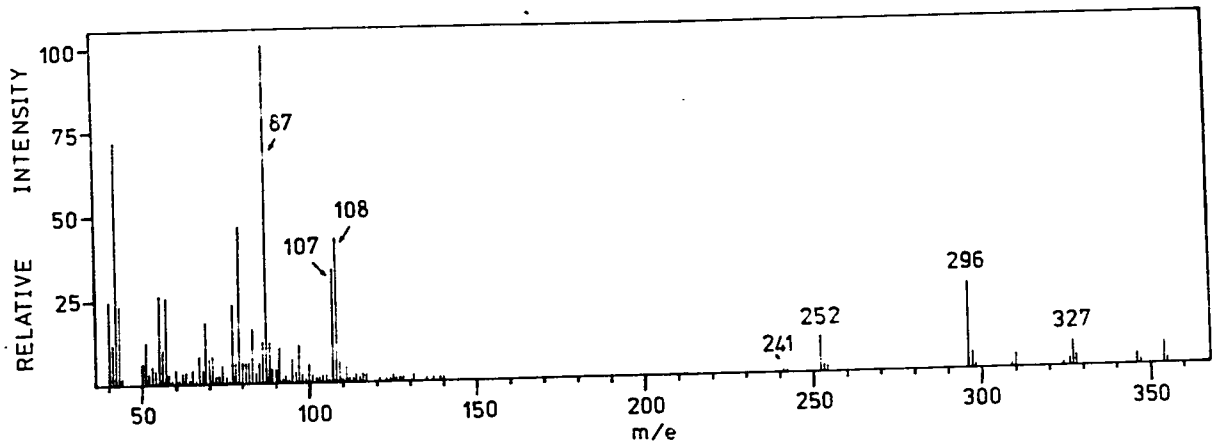


Figure 136. Mass spectrum of N-carbobenzoxy-dihydrosphingosine.

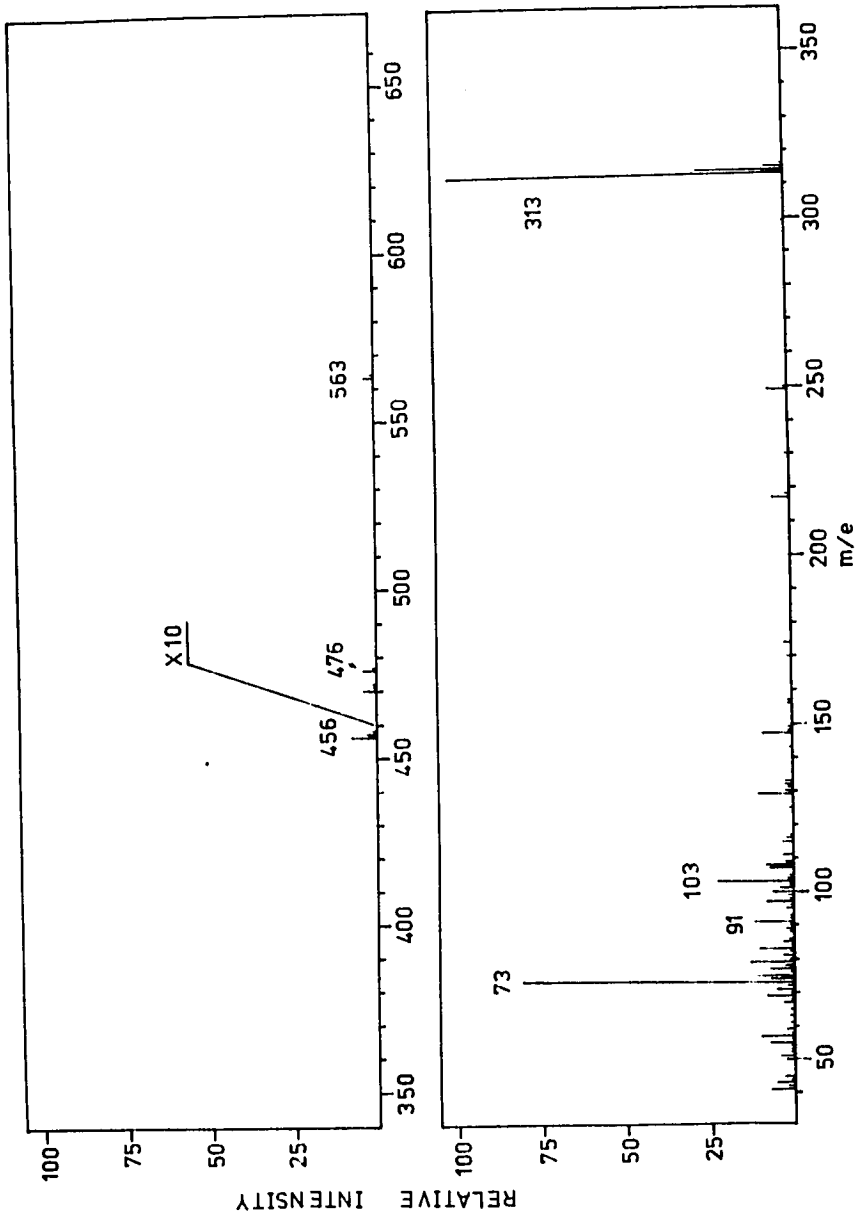


Figure 137. Mass spectrum of the silylated derivative of N-carbobenzoxy-dihydrosphingosine.

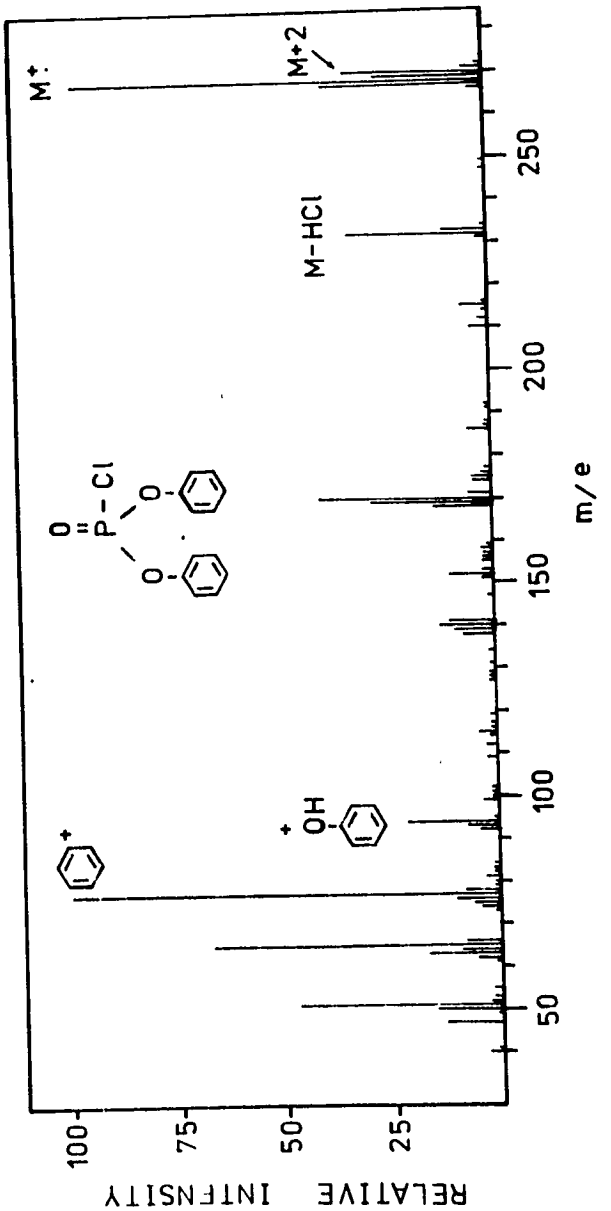


Figure 138. Mass spectrum of diphenylphosphochloridate.

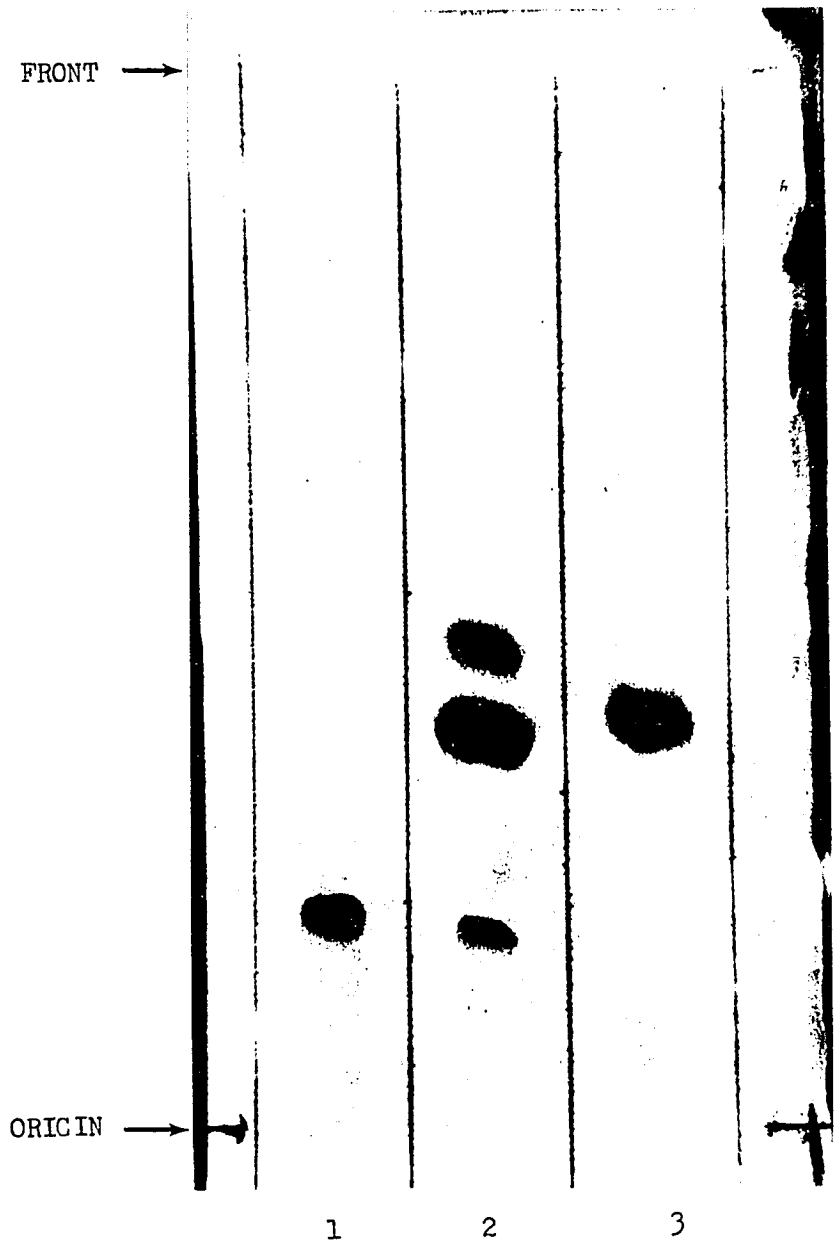


Figure 139. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate. Channel 1: Crude reaction mixture after adding diphenylphosphochloridate to N-carbobenzoxy-dihydrosphingosine in pyridine at 0° C. Channel 2: Crude reaction mixture after adding diphenylphosphochloridate to N-carbobenzoxy-phytosphingosine in pyridine at -30° C. Channel 3: Polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Dittmer's reagent at room temperature.

same plate was charred at 130° C for 15 minutes (Fig. 140, Channel 1), a brown spot of R_f = 0.81 which corresponded to the mass of the desired 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine appeared. It should be noted that the Dittmer's reagent did not give a positive response in this case. Also, the reaction product was less polar than the starting material (N-carbobenzoxy-dihydrosphingosine; R_f = 0.72 in Fig. 132). These two observations were important when compared with a parallel synthesis using phytosphingosine.

The reaction mixture was added dropwise with vigorous stirring to ice-water (25 ml). A white precipitate formed and the solution was stirred for another one-half hour. The precipitate was collected by centrifugation, washed with water (5 ml) and dried *in vacuo* over phosphorous pentoxide. The residue was crystallized from hexane yielding 93 mg (50%; Lit.[105] yield 82%). This had a melting point of 59.5-62.5° C (Lit.[105] mp 55° C). The material was pure as indicated by thin layer chromaotgraphy (Fig. 141). Elemental analysis showed:

	C	H	N	
Calculated:	68.34	8.15	2.10	(%)
Found:	68.16	8.20	2.18	

The infrared spectrum (Fig. 142) indicated that a free hydroxyl still existed by the peak at 3370 cm^{-1}; aromatic hydrogens appeared at 3065 cm^{-1}; The amide carbonyl doublet at 1710 cm^{-1}; dativ P-O at 1289 cm^{-1} and the ester P-O at 969 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford, *et. al.*[28], and Jones[52].

The NMR spectrum (Fig. 143) showed the benzyl aromatic hydrogens at 7.21 PPM (5 protons; singlet); the aromatic ester hydrogens at 7.13 PPM (10 protons; singlet); carbon atom 1 hydrogens at 4.35 PPM

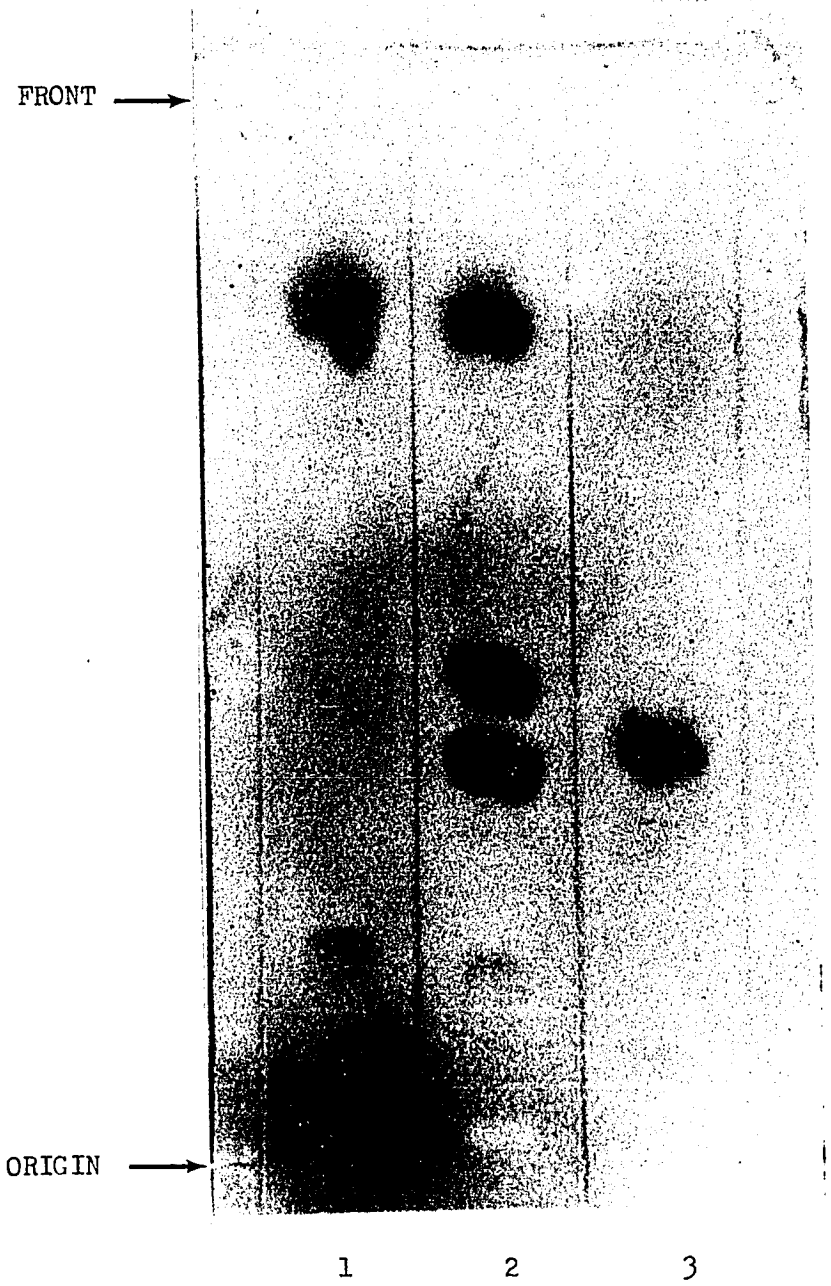


Figure 140. The thin layer chromatogram of figure 139 after charring at 130° for 15 minutes.

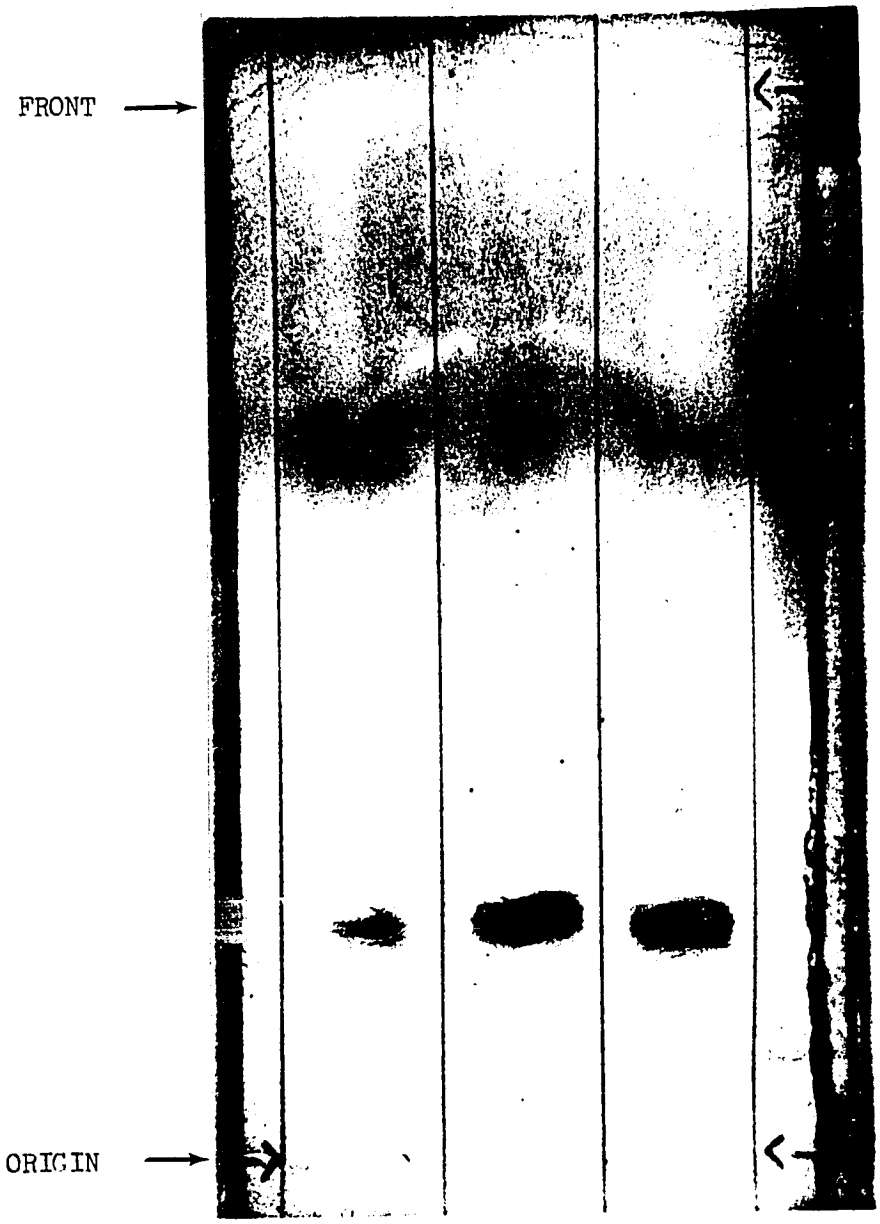


Figure 141. Thin layer chromatogram of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine on a Silica Gel PF 254 + 366 plate. Solvent: Chloroform-ether (85:15). Stain: Molybdic acid.

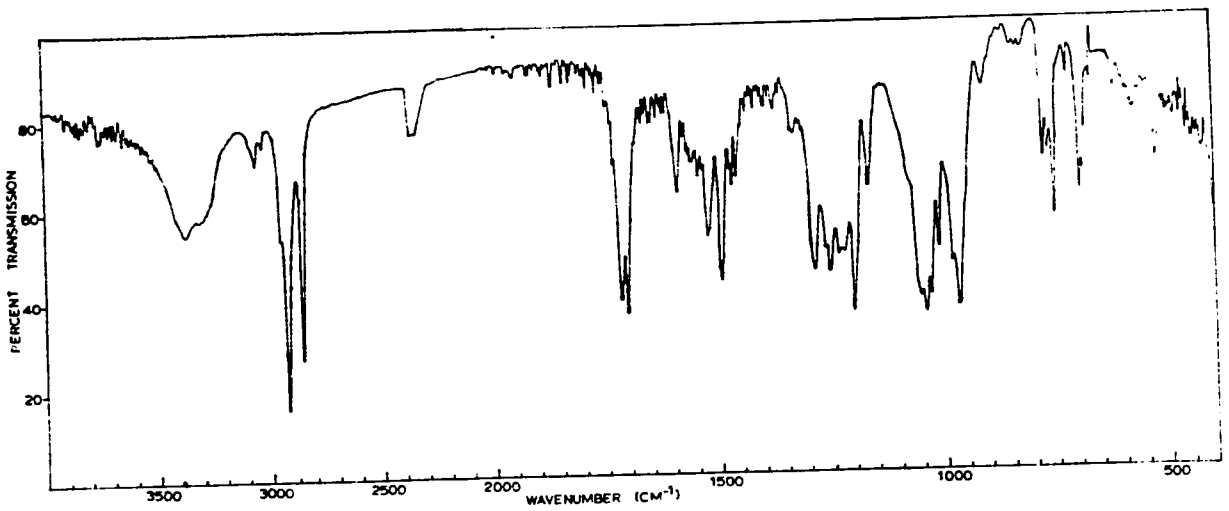


Figure 142. Infrared spectrum of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine.

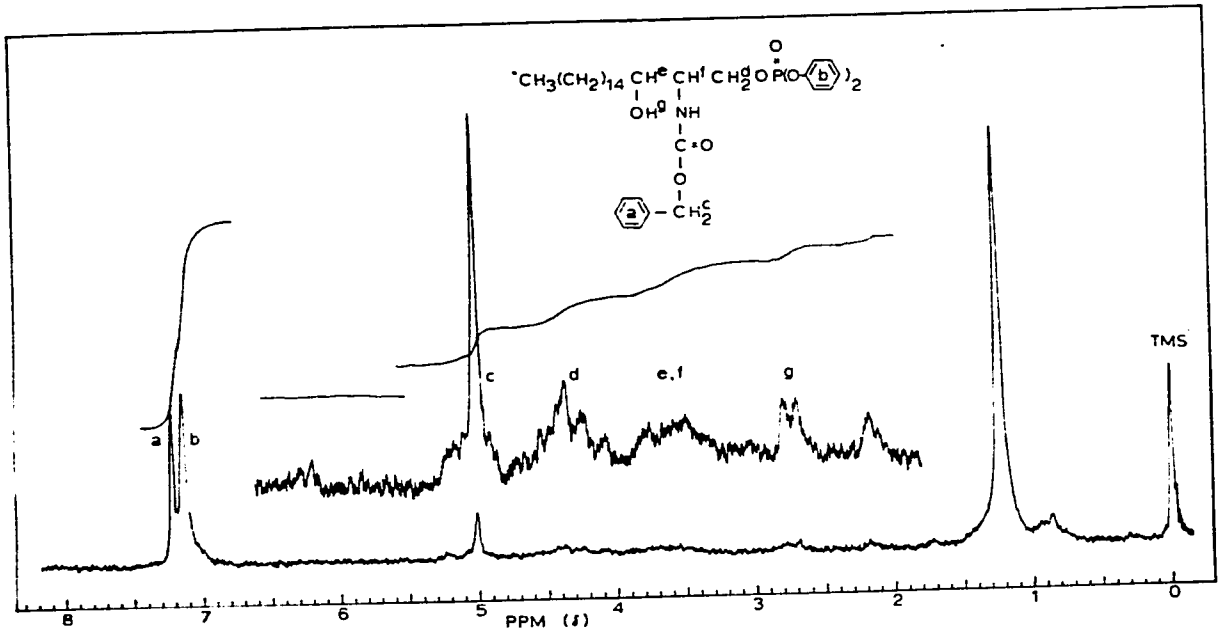


Figure 143. NMR spectrum of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine.

(2 protons; multiplet); hydrogens from carbon atoms 2 and 3 at 3.5 PPM (2.3 protons; multiplet); and the hydroxyl proton at 2.71 PPM (1 proton; doublet). The amide proton may have been present weakly at 5.1 PPM but was not fully accounted for by the integration due to hydrogen exchange.

The ultraviolet spectrum, maxima and extinction coefficients are shown along with an ethanol vs ethanol blank in Fig. 144. It should be noted that the extinction coefficients were nearly four times those of N-carbobenzoxy-dihydrosphingosine due to the presence of the two extra aromatic rings.

The major ions and their tentative assignments in the mass spectrum (Fig. 145) were m/e 573 (M-94; M - PhOH), m/e 482 (M-185; M - PhOH - $PhCH_2$) m/e 466 (M-201: M - PhOH - $PhCH_2O$), m/e 427 (M-240), m/e 388 (M-279; M - 2PhOH - $PhCH_2$), m/e 340 (M-327), m/e 296 (M-371), m/e 250 (M-417; $(PhO)_2POOH$), m/e 249 (M-418; $(PhO)_2POO$), m/e 94 (PhOH), m/e 107 (M-560; $PhCH_2O$) and m/e 91 (M-576; $PhCH_2$). The probe mass spectrum (Fig. 146) of the silylated compound gave a prominent ion at m/e 313 which could have corresponded to $CH_3(CH_2)_{14}\underset{\underset{OSiMe_3}{|}}{CH}$ and suggested the presence of a free hydroxyl group at C-3 in the unsilylated material. The specificity of the diphenylphosphochloridate reagent for primary hydroxyl groups was also noted by Weiss[105] under these conditions.

Platinum (black; 205 mg; Matheson) and 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine (79.5 mg; 0.119 mmole) were added to glacial acetic acid (10 ml) and the mixture was then hydrogenated at 30 psi in a Parr hydrogenation apparatus at room temperature for 23 hours. Thin layer analysis of the crude reaction mixture (after

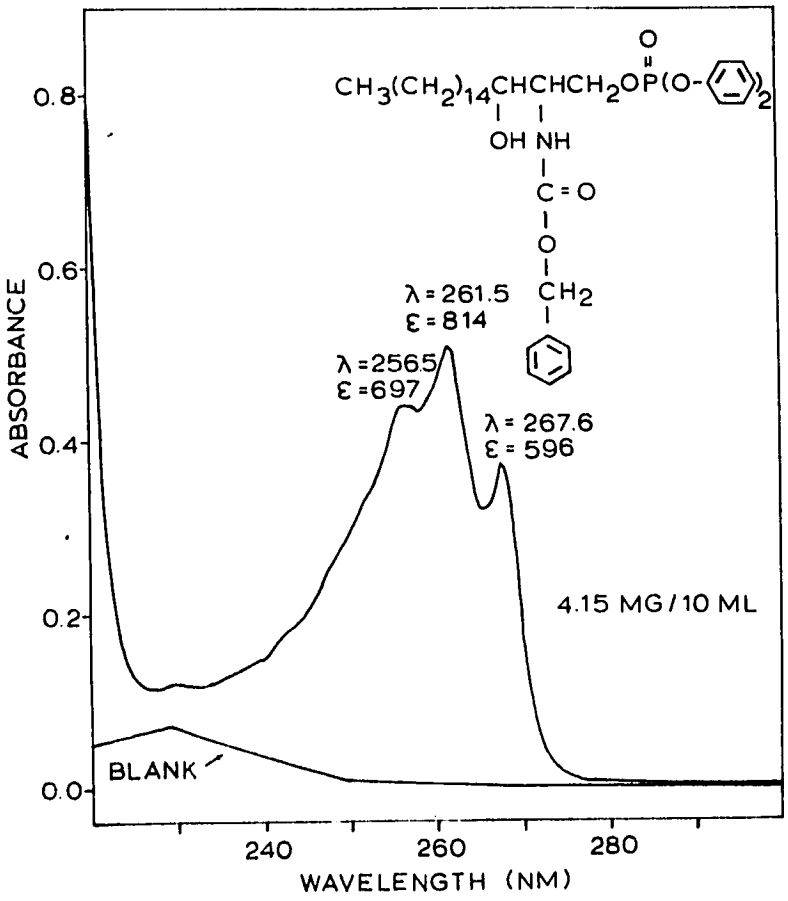


Figure 144. Ultraviolet spectrum of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine.

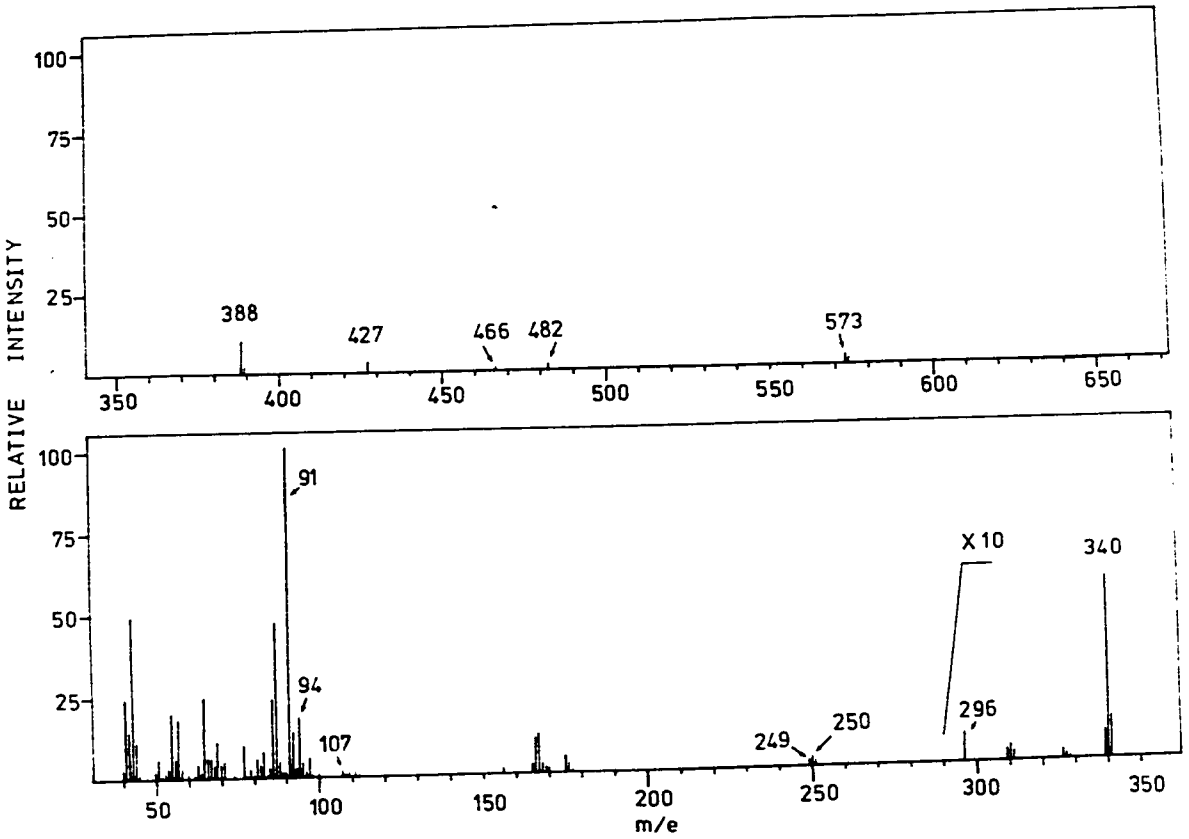


Figure 145. Mass spectrum of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine.

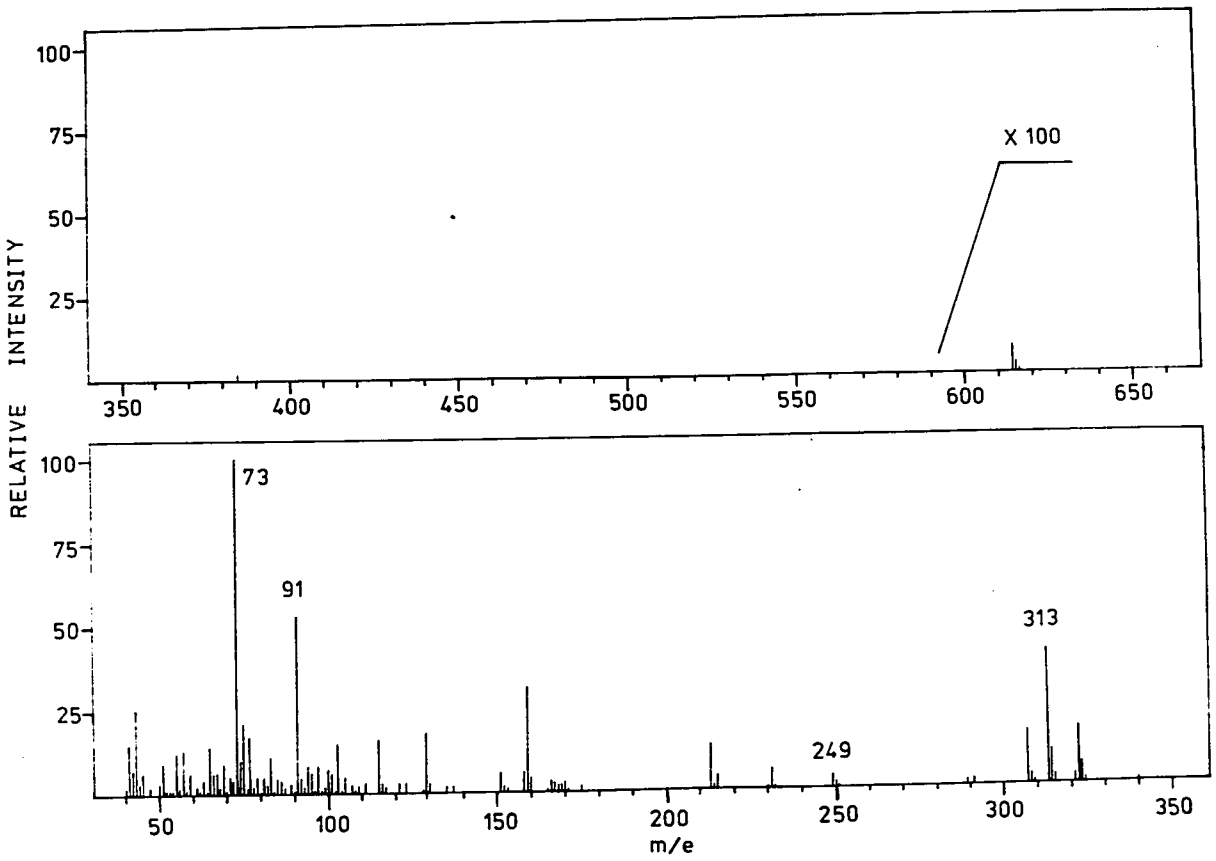


Figure 146. Mass spectrum of the silylated derivative of 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine.

removal of the catalyst by filtration through Hyflo Super Cel) showed two major Dittmer's positive spots at R_f = 0.55 and R_f = 0.45 when developed in n-butanol-acetic acid-water (60:20:20) on Silica Gel PF 254 + 366. The crude reaction solution was applied to a Silica Gel PF 254 + 366 plate and developed in the above solvent system. The lower spot (which co-chromatographed with a slightly impure sample of authentic dihydrosphingosine-1-phosphate prepared in this laboratory by the method of Weiss[105]) was eluted with a mixture of methanol-88% formic acid (99:1; 100 ml). The solvent was removed at reduced pressure at 44° C and dried <u>in vacuo</u> over phosphorous pentoxide. In order to remove any eluted silica gel, the residue was redissolved in hot glacial acetic acid (5 ml) and the solution was then centrifuged. The supernatant was removed at reduced pressure at 41° C and dried as before yielding 10.4 mg of needle-like crystals (23%; Lit. yield[105] 78%). This was finally washed in a centrifuge tube with acetone (1 ml) and ether (1 ml). The Dittmer's positive, ninhydrin positive product was pure by thin layer analysis (Fig. 147) and co-chromatographed with a crude sample of enzymatically formed[49] [4,5-$^3H_2$]- dihydrosphingosine-1-phosphate in the above solvent system.

The method of purification used here differed from that of Weiss[105]. He "crystallized" the product by dissolving gram quantities of it in hot glacial acetic acid and then precipitated the product by adding an equal volume of water to the solution. The collected precipitate was then washed with water, acetone and ether. On small quantities such as used in the present work, that method failed to separate the desired product from the less polar impurity (R_f = 0.55). Preparative thin layer chromatography was, therefore, used to achieve this goal.

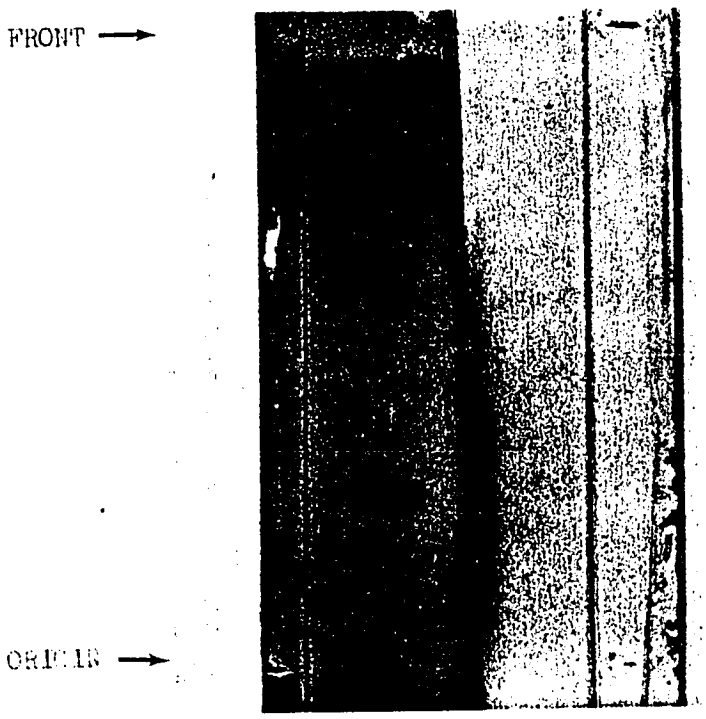


Figure 147. Thin layer chromatogram of dihydrosphingosine-1-phosphate. Channel 1: Molybdic acid stain. Channel 2: Ninhydrin stain. Solvent: n-butanol-acetic acid-water (60:20:20).

ATTEMPTED CHEMICAL SYNTHESIS OF PHYTOSPHINGOSINE-1-PHOSPHATE

Phytosphingosine (1.5104 grams; 4.764 mmoles) was suspended in a mixture of ether (50 ml) and 0.5 N sodium hydroxide (18 ml). Benzyl chloroformate (1.321 grams; 7.74 mmoles) in ether (10 ml) was added dropwise with vigorous stirring over 10 minutes at room temperature. The stirring was continued for 30 minutes and the mixture was then extracted three times with ether (50 ml each). The combined ether extracts were washed with water (20 ml) and dried over anhydrous sulfate. The solvent was removed at reduced pressure at 35° C and dried in vacuo to yield 2.728 grams of residue. This was crystallized two times from warm hexane (about 30 ml) to give 1.858 grams (86.4%) of very pure N-carbobenzoxy-phytosphingosine as analyzed by thin layer chromatography (Fig. 132, Channel 2). Elemental analysis showed:

	C	H	N	
Calculated:	69.14	10.04	3.10	(%)
Found:	69.04	10.02	3.17	

The white solid softened at 92° C and melted from 94.5-96.5° C leaving a clear liquid.

The infrared spectrum (Fig. 148) exhibited absorbances due to the free hydroxyls at 3400 cm^{-1}; aromatic hydrogen stretching at 3050 cm^{-1}; amide carbonyl at 1668 cm^{-1} and the amide N-H in plane bend at 1550 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford, et. al.[28], and Jones[52].

The NMR spectrum (Fig. 149) had resonances due to the aromatic hydrogens at 7.36 PPM (4.9 protons; singlet); amide hydrogen at 5.85 PPM (1.0 proton; doublet); benzylic hydrogens at 5.09 PPM (2.0 protons;

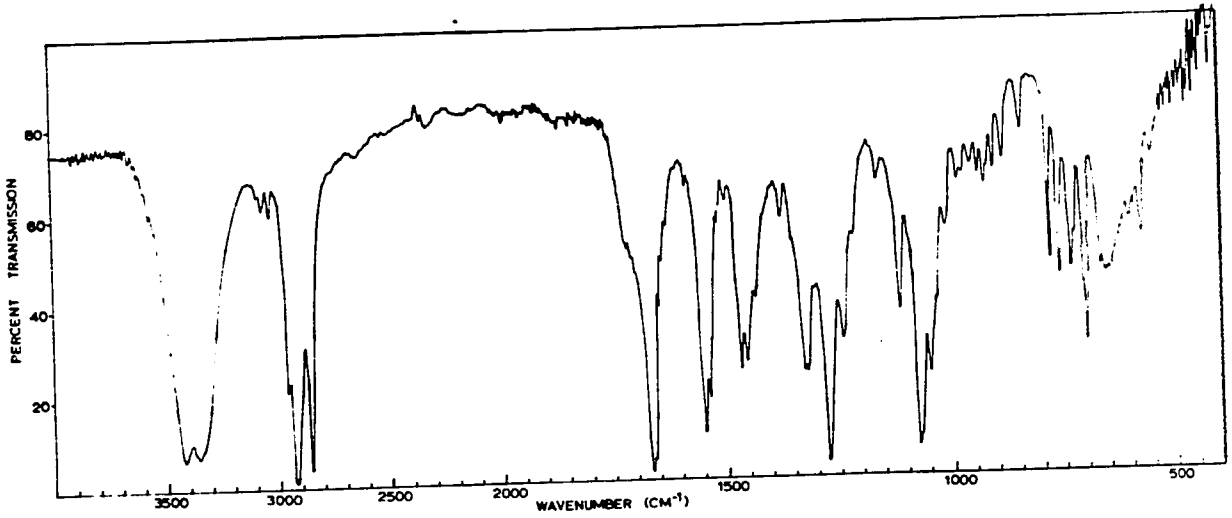


Figure 148. Infrared spectrum of N-carbobenzoxy-phytosphingosine.

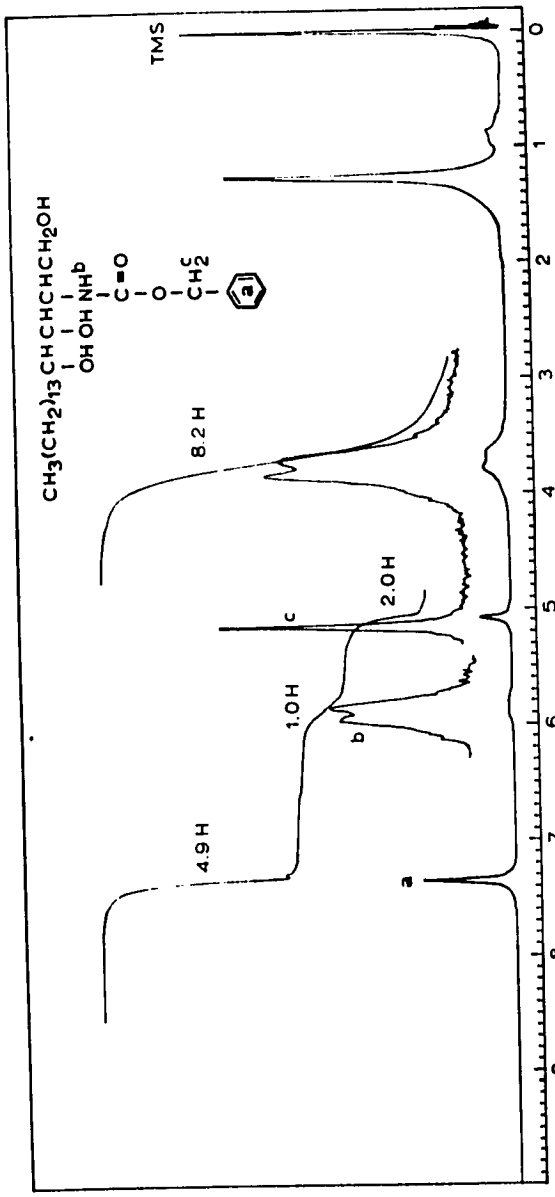


Figure 149. NMR spectrum of N-carbobenzoxy-phytosphingosine.

singlet) and two overlapping multiplets at 3.75 PPM which integrated for 8.2 protons. This would have accounted for both the skeletal hydrogens on carbon atoms one through four and the three hydroxyl hydrogens. In N-carbobenzoxy-dihydrosphingosine, the absorption due to the hydroxyl hydrogens was upfield of the absorption due to the skeletal hydrogens. In order to test if that relationship held in this case, the sample was shaken with D_2O (Fig. 150) to remove exchangeable hydrogens. The absorption due to the amide hydrogen at 5.85 PPM disappeared almost totally with 75 minutes. The absorption at 3.70 PPM (the upfield absorption) was reduced slightly indicating that this resonance corresponded to the hydroxyl hydrogens while that at 3.80 PPM corresponded to the skeletal hydrogens (compare with Fig. 134). The final integration of the peaks at 3.75 PPM showed 6.4 protons. This indicated that at least one of the hydroxyl protons was not readily exchangeable.

The ultraviolet spectrum, maxima and extinction coefficients are shown in Fig. 151 along with an ethanol vs ethanol blank.

Major ions and their tentative assignments in the mass spectrum (Fig. 152) were m/e 326 (M-125; M - $PhCH_2O$ - H_2O), m/e 325 (M-126; M - $PhCH_2OH$ - H_2O), m/e 312 (M-139; M - $PhCH_2OH$ - CH_2OH), m/e 299 (M-152; M - $PhCH_2OCONH_2$ - H), m/e 268 (M-183; M - $PhCH_2OCONH_2$ - H - CH_2OH), m/e 86 (M-365; M - $CH_3(CH_2)_{13}\underset{OH}{CH}\text{-}\underset{OH}{CH}$ - $PhCH_2OH$) and m/e 60 (M-391; $\underset{NH_2}{CH}\text{-}\ CH_2OH$). The probe mass spectrum (Fig. 153) of the tris (trimethylsilyl) N-carbobenzoxy-phytosphingosine exhibited major ions at m/e 740 (M+73; M + $SiMe_3$), m/e 667 (the molecular ion), m/e 652

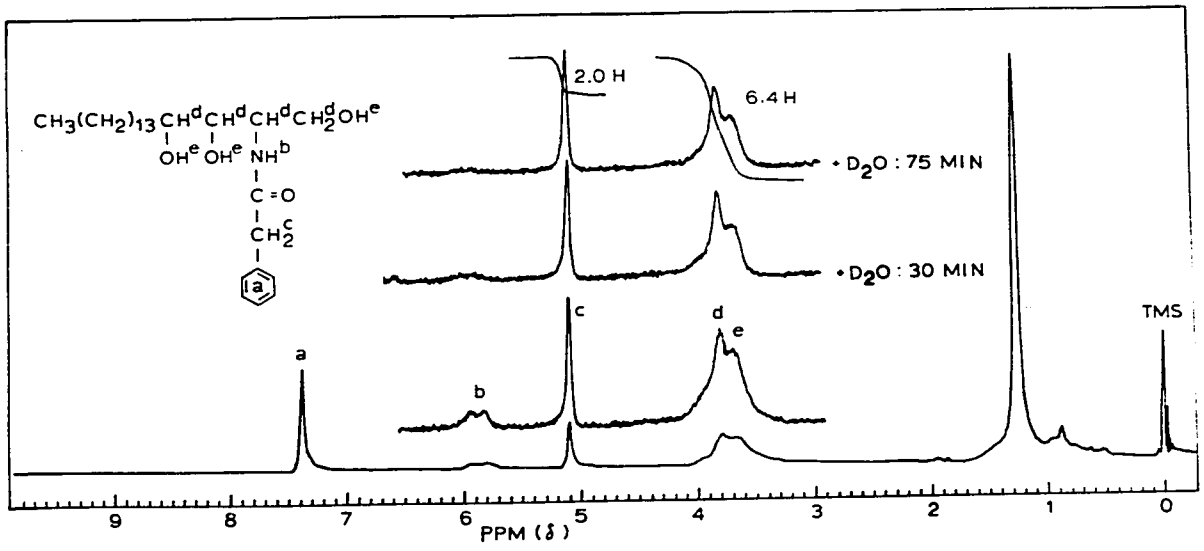


Figure 150. NMR spectrum of N-carbobenzoxy-phytosphingosine before and after shaking with D_2O for 30 and 75 minutes.

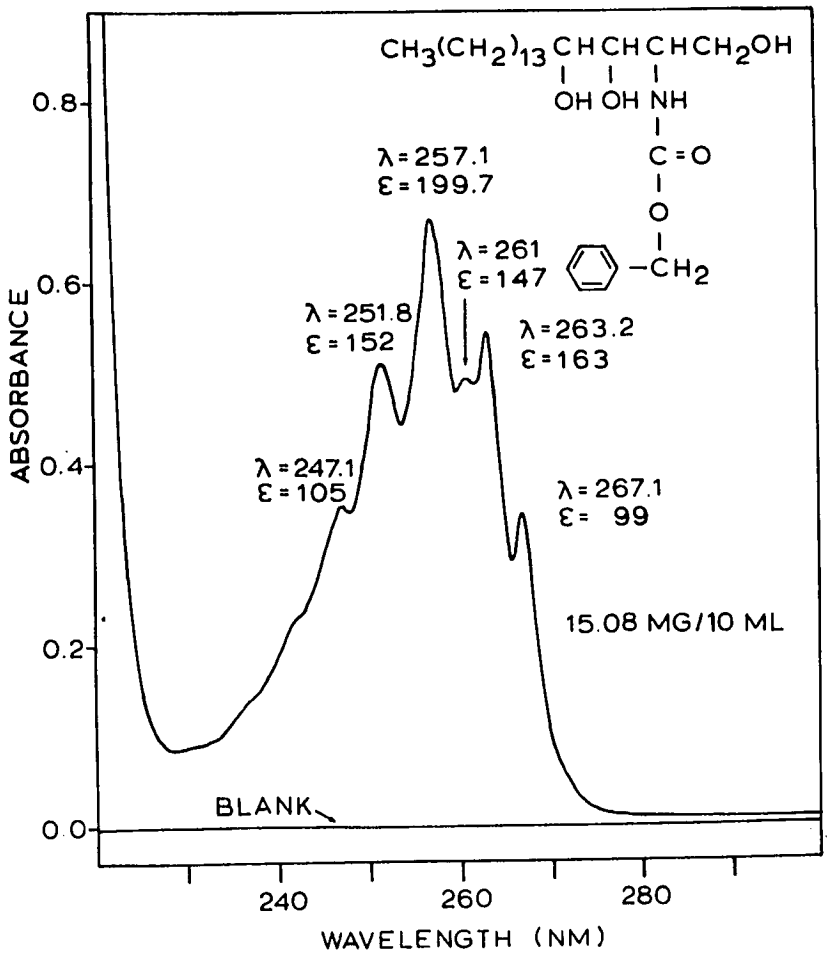


Figure 151. Ultraviolet spectrum of N-carbobenzoxy-phytosphingosine.

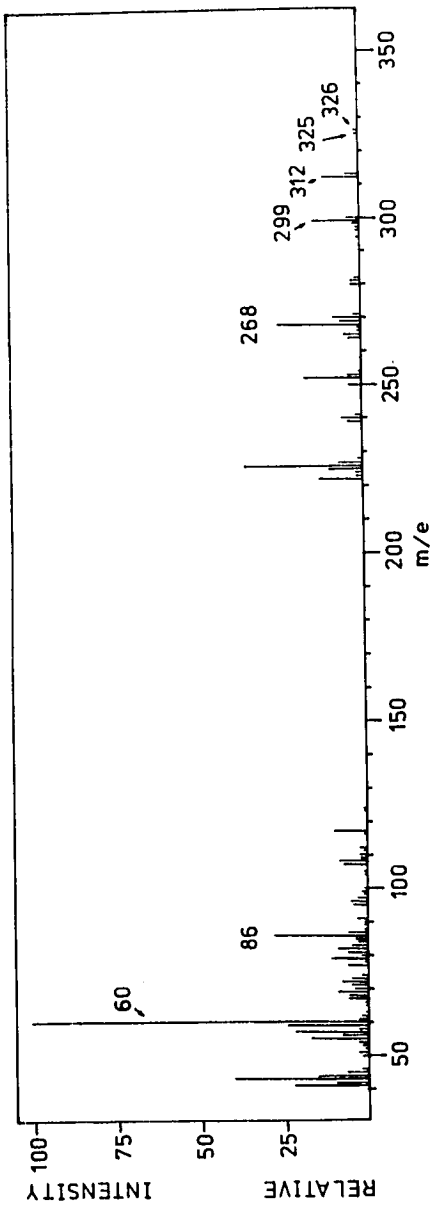


Figure 152. Mass spectrum of N-carbobenzoxy-phytosphingosine.

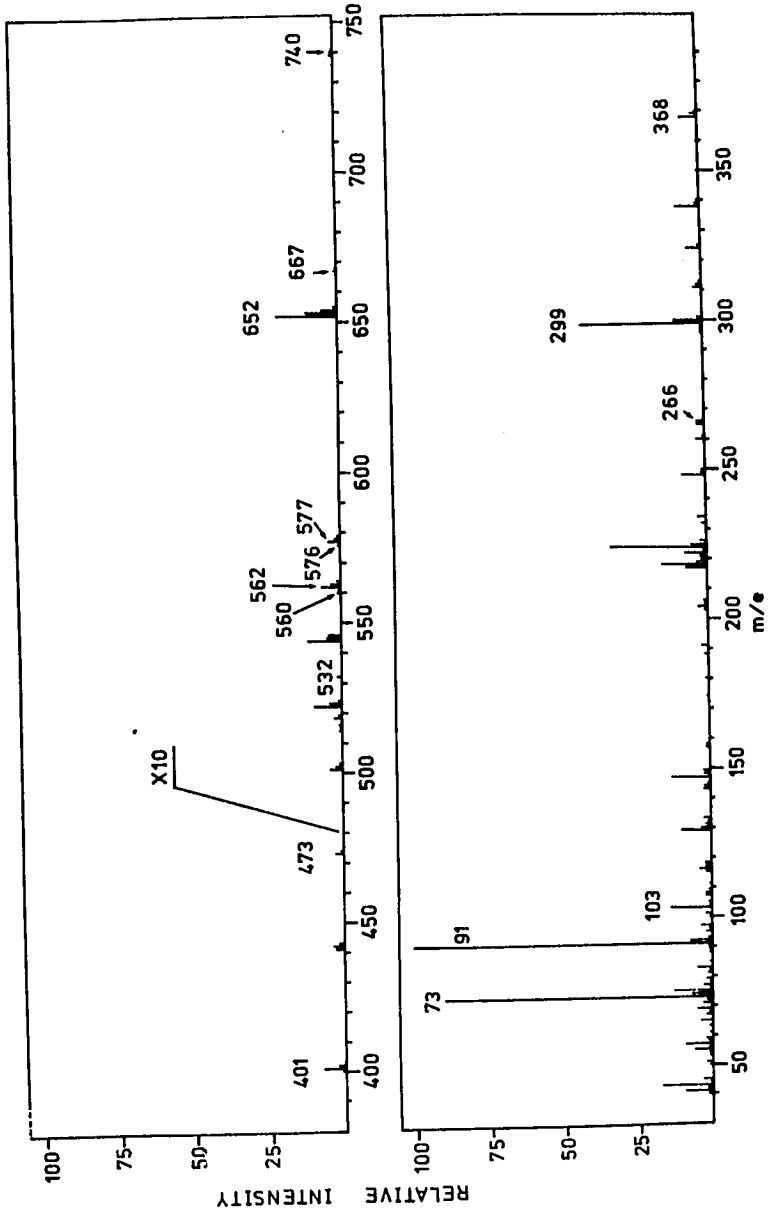


Figure 153. Mass spectrum of the silylated derivative of N-carbobenzoxy-phytosphingosine.

M-15; M - CH_3), m/e 577 (M-90; M - Me_3SiOH), m/e 562 (M-105; M - CH_3 - Me_3SiOH), m/e 576 (M-91; M - $PhCH_2$), m/e 532 (M-135; M - $PhCH_2OCO$), m/e 473 (M-194; M - $PhCH_2$ - CH_2OSiMe_3), m/e 401 (M-266; $CH_3(CH_2)_{13}CH(OSiMe_3)-CH(OSiMe_3)$), m/e 368 (M-299; M - $CH_3(CH_2)_{13}CH(OSiMe_3)$), m/e 299 (M-368; $CH_3(CH_2)_{13}CH(OSiMe_3)$), m/e 266 (M-401; M - $CH_3(CH_2)_{13}CH(OSiMe_3)-CH(OSiMe_3)$), m/e 103 (M-564; CH_2OSiMe_3) and m/e 91 (M-576; $PhCH_2$).

Upon the periodate-borohydride treatment described in Materials and Methods, N-carbobenzoxy-phytospingosine released pentadecanol.

N-Carbobenzoxy-phytosphingosine (130 mg; 0.288 mmole) was dissolved in dry pyridine (1.0 ml) and cooled in an acetone bath to -30° C with the use of added dry ice. (Reaction results were identical when cooled in an ice bath. In this particular case, the purpose of the lower temperature was to favor attack of the phosphoryl chloride at the primary hydroxyl group.) Diphenylphosphochloridate (130 ul; 160 mg; 0.597 mmole) was added slowly from a capillary tube over 5 minutes. The resulting mixture was stirred for 30 minutes at -30° C and then stored at -17° C overnight. The solution was added dropwise to ice-water (25 ml) with vigorous stirring and an oil formed. The mixture was lyophilized one hour later. When the oily residue was analyzed by thin layer chromatography and sprayed with Dittmer's phospholipid reagent (Fig. 139, Channel 2), two major blue spots were observed at R_f = 0.38 and R_f = 0.46. After charring the same plate at 130° C for 10 minutes, a brown (Dittmer's negative reaction) spot appeared at R_f = 0.79 (Fig. 140, Channel 2). The residue was applied to Silica

Gel PF 254 + 366 thin layer plates and developed in chloroform-methanol-ammonia (100:25:2.5). The bands were visualized under short wave length ultraviolet light and eluted with ether-methanol (1:1). The Dittmer's negative, non-polar spot (from a similar reaction which used 401 mg (0.889 mmole) of N-carbobenzoxy-phytosphingosine and 504 mg (1.876 mmole) of diphenylphosphochloridate mixed at 0° C) was rechromatographed in chloroform-ether (97.5:2.5) and the major band (R_f = 0.15) was eluted with methanol-ether (5:95). After removal of the solvent at reduced pressure at 35° C, the residue was crystallized from hexane (about 30 ml) to give 60 mg (15.5% yield) of what was probably 1,4-anhydro-N-carbobenzoxy-phytosphingosine (molecular weight 433 grams/mole). The white solid had a melting point of 93.5-95° C (clear liquid) and was pure by thin layer chromatography (Fig. 154, Channel 2).

The infrared spectrum (Fig. 155) showed the amide carbonyl at 1685 cm^{-1} but no P-O absorbances.

The shape, maxima and extinction coefficients of the ultraviolet spectrum (Fig.156) were remarkably similar to that of N-carbobenzoxy-phytosphingosine (Fig. 151). The blank consisted of ethanol vs ethanol.

The NMR spectrum (Fig. 157) showed aromatic protons at 7.25 PPM (5 protons; singlet); amide hydrogen at 5.31 PPM (1 proton; doublet); benzylic hydrogens at 5.02 PPM (2 protons; singlet); skeletal hydrogens at 3.3-4.2 PPM (5.5 protons; multiplet) and the hydroxyl hydrogen at 2.67 PPM (1 proton; broad singlet).

Major ions and their tentative assignments in the mass spectrum (Fig. 158) were m/e 325 (M-108; M - $PhCH_2OH$), m/e 282 (M-151; M -

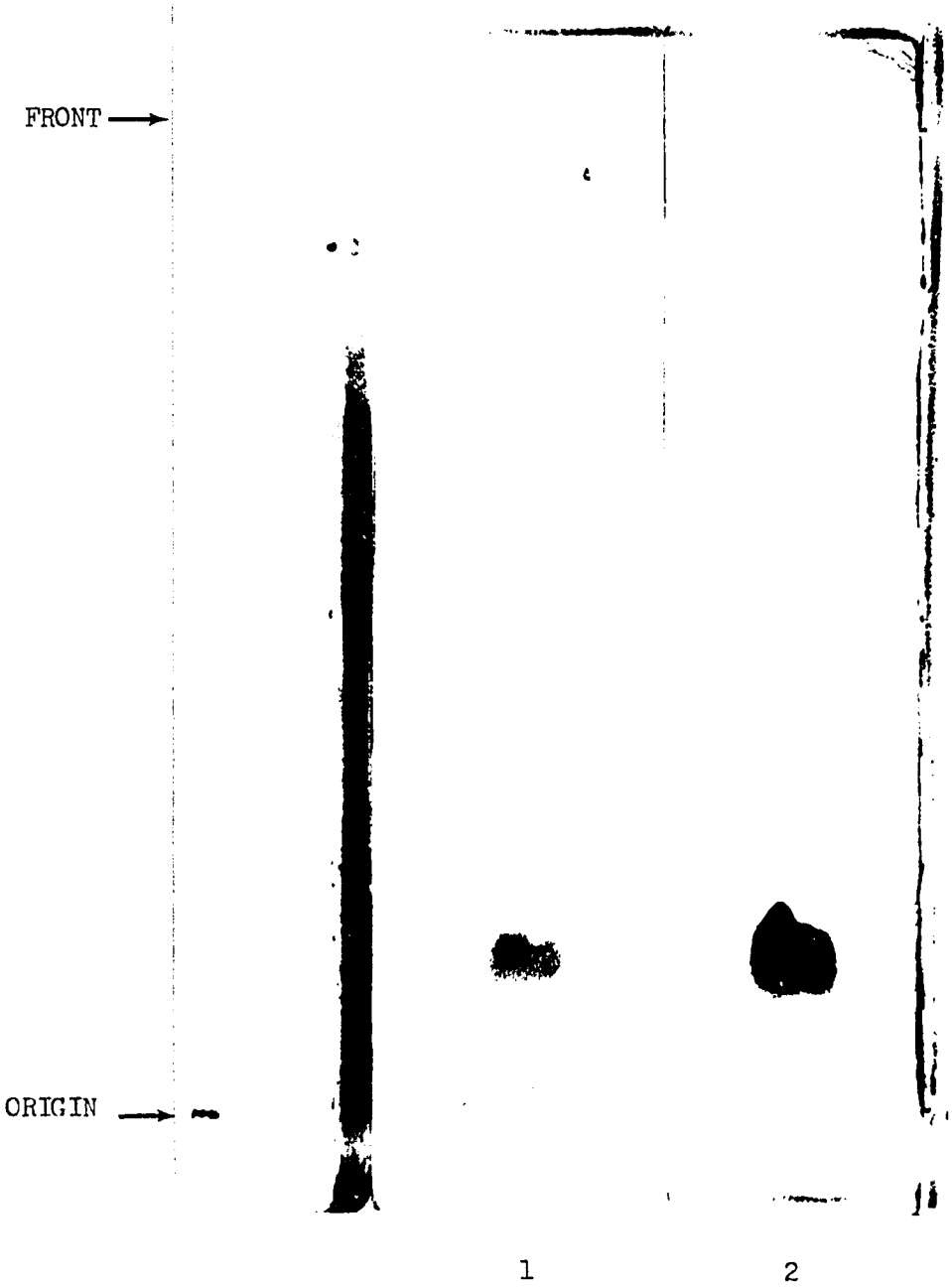


Figure 154. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate. Channel 1: 1-diphenylphosphoryl-N-carbobenzoxy-dihydrosphingosine. Channel 2: Anhydro-N-carbobenzoxy-phytosphingosine. Solvent: Chloroform-ether (97.5:2.5). Stain: Molybdic acid.

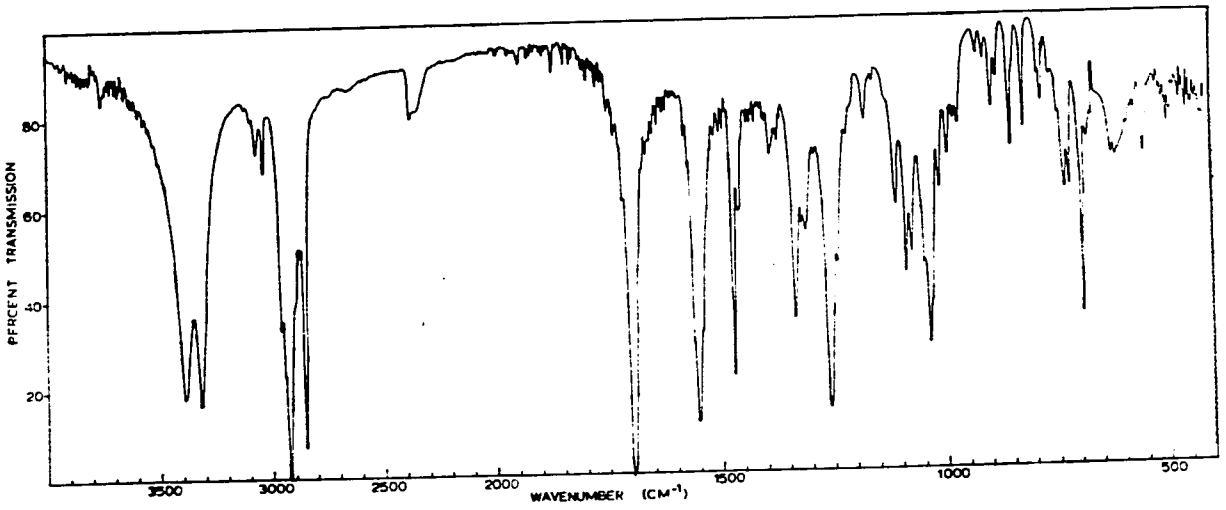


Figure 155. Infrared spectrum of anhydro-N-carbobenzoxy-phytosphingosine.

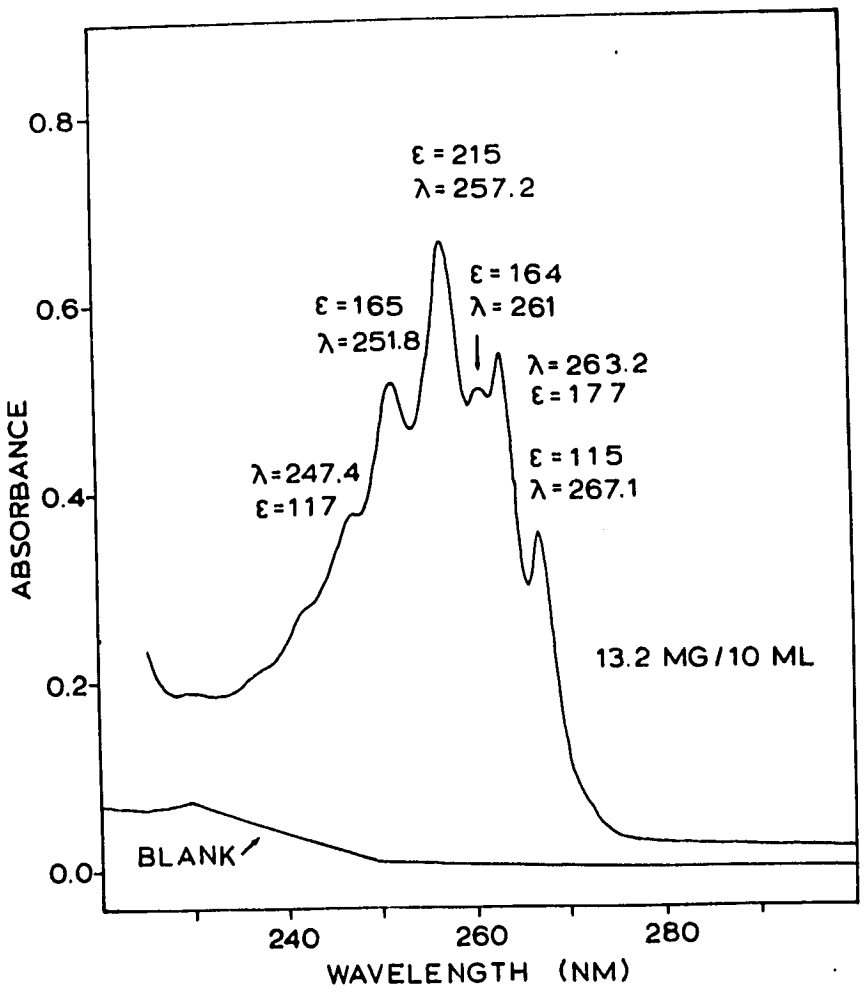


Figure 156. Ultraviolet spectrum of anhydro-N-carbobenzoxy-phytosphingosine.

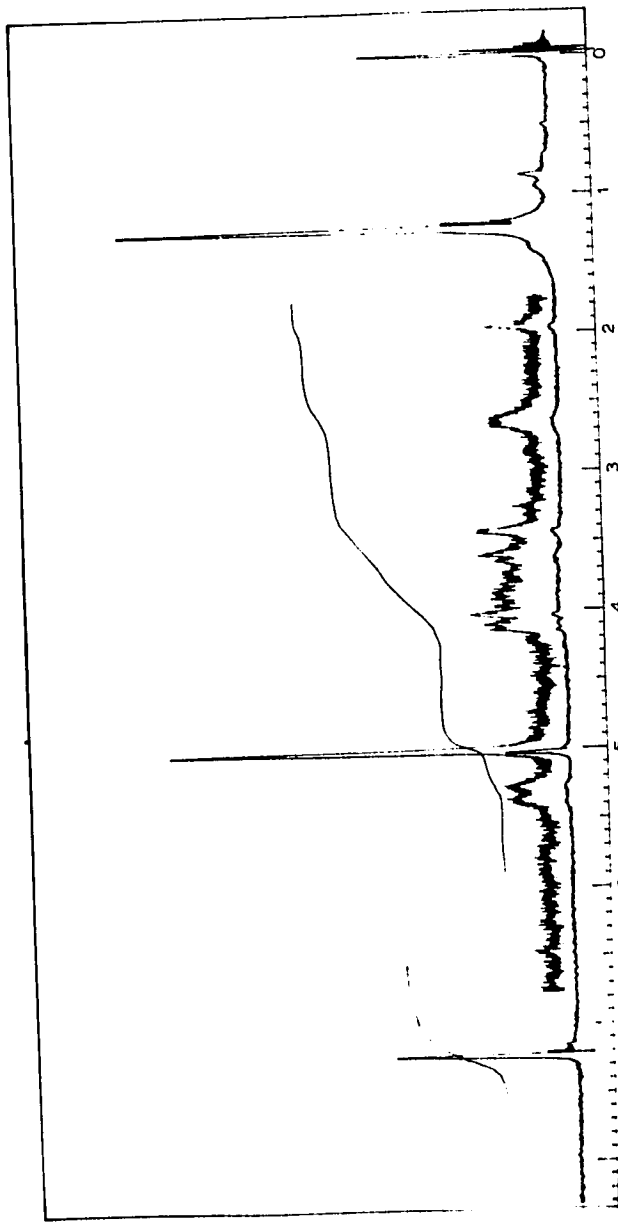


Figure 157. NMR spectrum of anhydro-N-carbobenzoxy-phytosphingosine.

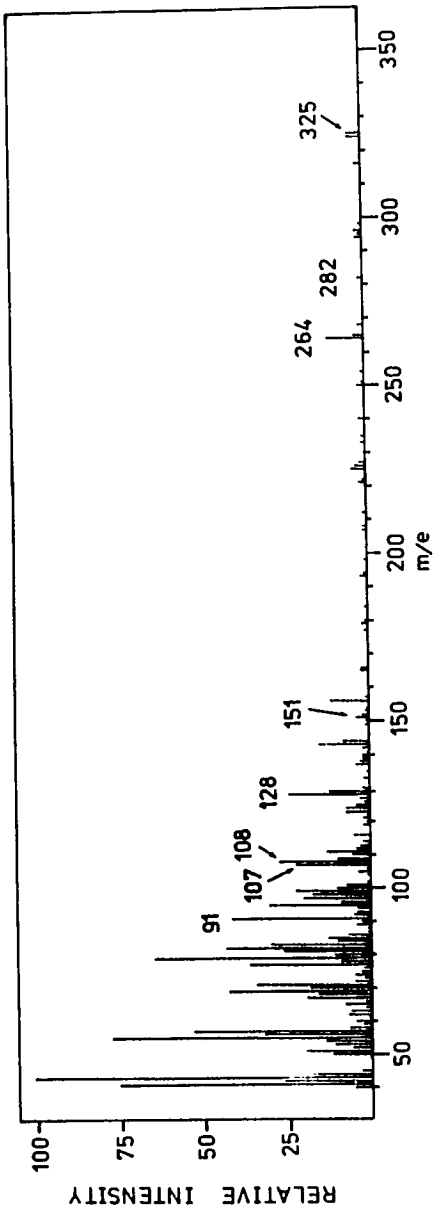


Figure 158. Mass spectrum of anhydro-N-carbobenzoxy-phytosphingosine.

$PhCH_2OCONH_2$), m/e 264 (M-169; M - $PhCH_2OCONH_2$ - H_2O), m/e 151 (M-282; $PhCH_2OCONH_2$), m/e 128 (M-305; M - $PhCH_2OH$ - $CH_3(CH_2)_{13}$), m/e 108 (M-325; $PhCH_2OH$), m/e 107 (M-326; $PhCH_2O$) and m/e 91 (M-342; $PhCH_2$).

The ultraviolet, NMR and mass spectra were consistant with the dehydration of N-carbobenzoxy-phytosphingosine with the formation of a 1,4 (or perhaps a 1,3) ether as did phytosphingosine in the formation of anhydrophytosphingosine when treated with acids[62]. When only a 1:1 molar ratio of diphenylphosphochloridate to N-carbobenzoxy-phytosphingosine was used, only anhydro-N-carbobenzoxy-phytosphingosine and N-carbobenzoxy-phytosphingosine were observed by thin layer chromatography of the reaction mixture.

To test if this sample would react further to form the more polar Dittmer's positive spots; the anhydro-N-carbobenzoxy-phytosphingosine (2 mg; 0.005 mmole) was dissolved in dry pyridine (60 ul) and cooled in an ice bath. Diphenylphosphochloridate (2 ul; 2.5 mg; 0.009 mmole) was added and the mixture was stirred for $\frac{1}{2}$ hour at 0° C. The reaction mixture was analyzed by thin layer chromatography (Fig. 159, Channel 2). If anything, a slightly less polar product was formed.

The less polar (R_f = 0.46) Dittmer's positive material was analyzed by thin layer chromatography (Fig. 160, Channels 3 and 4) and was found to consist of two components which may have corresponded to the two other mono-phosphorylated products. In this case the yield was not determined. However, in an identical workup starting with N-carbobenzoxy-phytosphingosine (100 mg; 0.22 mmole) and diphenylphosphochloridate (126 mg; 0.45 mmole) the yield of the less polar (R_f = 0.46), Dittmer's positive mixture was 19.7 mg (13%) and of the

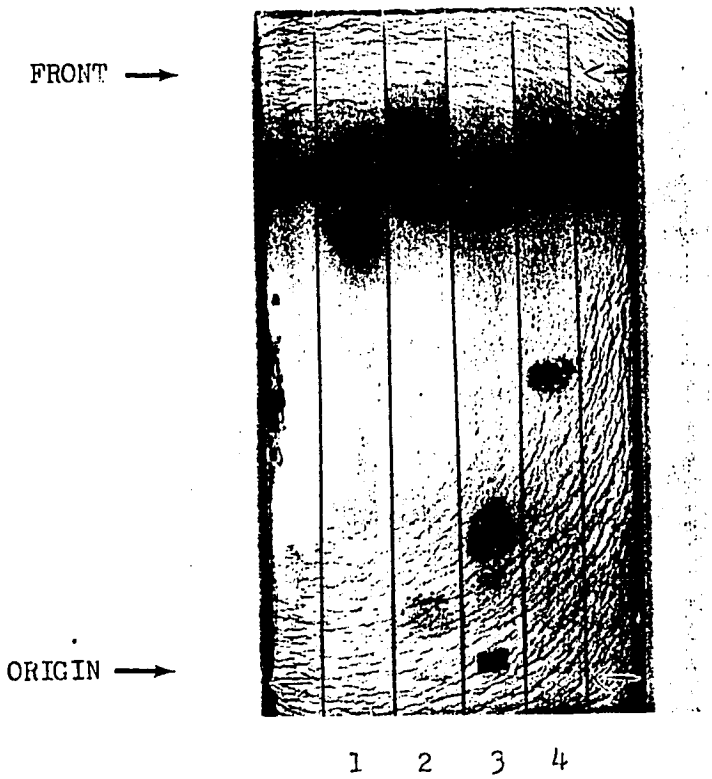


Figure 159. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate. Channel 1: Anhydro-N-carbobenzoxy-phytosphingosine. Channel 2: Crude reaction mixture after treatment of anhydro-N-carbobenzoxy-phytosphingosine in pyridine at 0° C with excess diphenylphosphochloridate. Channel 3: Polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine. Channel 4: N-carbobenzoxy-phytosphingosine. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Molybdic acid.

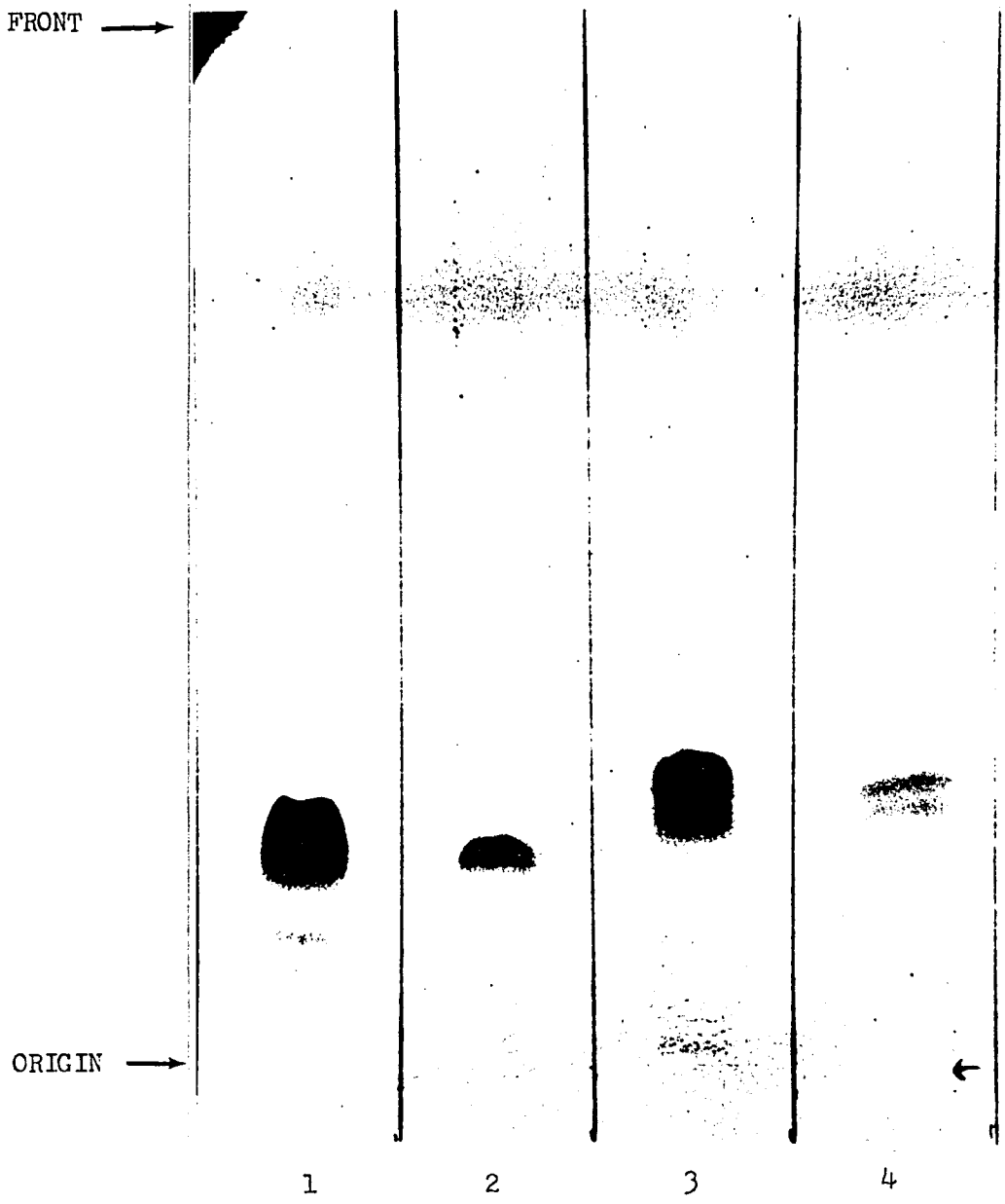


Figure 160. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate. Channels 1 & 2: Polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine (partially purified). Channels 3 & 4: Less polar Dittmer's positive phophorylated products. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Dittmer's reagent followed by charring at 130° for 15 minutes.

more polar (R_f = 0.38), Dittmer's positive compound was 40 mg (26%).

The more polar (R_f = 0.38), Dittmer's positive material (Fig. 160, Channels 1 and 2) from the originally described reaction was purified further by preparative thin layer chromatography on Silica Gel PF 254 + 366 in chloroform-methanol-ammonia (100:25:2.5) to give an oil (54.7 mg; 27.7%) which upon lyophilizing from benzene gave a sticky, white solid which became an oil again after exposure to air for ½ hour. On thin layer chromatography, this residue gave a single spot (Fig. 161). This material was analyzed in detail and used for the next step in the synthesis of phytosphingosine-1-phosphate for two reasons: First, it was formed in the highest yields. The phosphorylation occurred mainly at the primary hydroxyl of the dihydrosphingosine analogue under these conditions[105]. Therefore, it was assumed that the major product was the desired 1-phosphoryl product. Secondly, it was assumed that a primary phosphate would, perhaps, be more polar on thin layer chromatography than either of the possible secondary esters.

Major differences from the dihydrosphingosine analogue were that the products here were Dittmer's positive and were also more polar than the starting N-carbobenzoxy-phytosphingosine.

None of the Dittmer's positive compounds gave any reaction with periodate-borohydride as described in Materials and Methods. Thin layer chromatographic analysis of the Folch extract of the periodate oxidation showed only unreacted substrate. Gas-liquid chromatographic analysis of the silylated residue after borohydride reduction produced no silylated pentadecanol. This was true even when the intial periodate oxidation was carried out at 37° C for up to 3 days. Weiss and Stiller

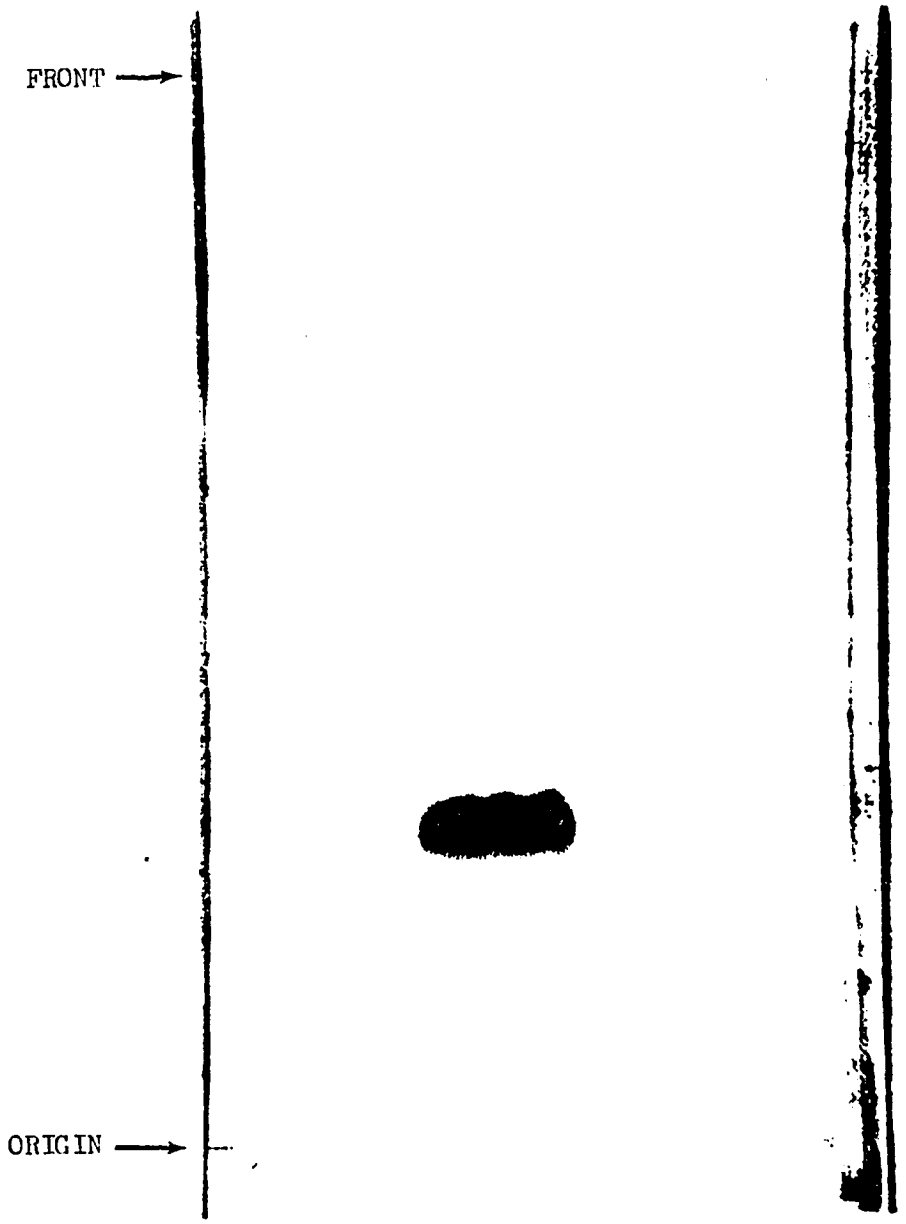


Figure 161. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine after purification by preparative thin layer chromatography. Solvent: Chloroform-methanol-ammonia (100:25:2.5). Stain: Dittmer's reagent followed by charring at 130° for 15 minutes.

noted that threo-4,5-dihydroxy-tribenzoyl-dihydrosphingosine was apparently totally resistant to periodate oxidation[112]. They achieved a non-quantitative oxidation with a periodate-permanganate mixture. The Dittmer's positive compounds (R_f = 0.46 or R_f = 0.38; about 1 mg of either) were dissolved in chloroform-methanol (1:1; 0.6 ml). A solution of 0.2 N sodium periodate and 0.2 N potassium permanganate (titrated to pH 7 with 1 N sodium hydroxide) was added and the mixture was shaken in the dark at 37° C for 24 hours. Chloroform (1.3 ml), methanol (0.5 ml) and 2 N HCl (0.5 ml) were added to form a Folch partition. The lower layer was dried under nitrogen at 35° C and the resulting residue was dried in vacuo over phosphorous pentoxide. The residue was redissolved in dry ether (3 ml) and lithium aluminum hydride (30 mg) was added. After 3 hours at room temperature with stirring, water (30 ul), 1 N sodium hydroxide (30 ul) and water (90 ul) were added successively. The solution was filtered and the solvent was removed under nitrogen at 35° C. The residue was dried in vacuo over phosphorous pentoxide, silylated with Carter's reagent and subjected to gas-liquid chromatography. At most, only a trace of the silylated derivative of pentadecanol was observed.

The infrared spectrum (Fig. 162) of the polar (R_f = 0.38) Dittmer's positive material showed hydrogen bonded hydroxyl hydrogens at 3300 cm^{-1}; aromatic hydrogens at 3050 cm^{-1}; amide carbonyl at 1720 cm^{-1}; aromatic modes at 1590 cm^{-1} and 1490 cm^{-1}; dative P-O at 1285 cm^{-1}; Ph-O at 1230 cm^{-1}; C-O at 1020 cm^{-1} to 1100 cm^{-1} and ester P-O at 960 cm^{-1}. Assignments were made with the aid of Bellamy[7], Crawford et al.[28] and Jones[52].

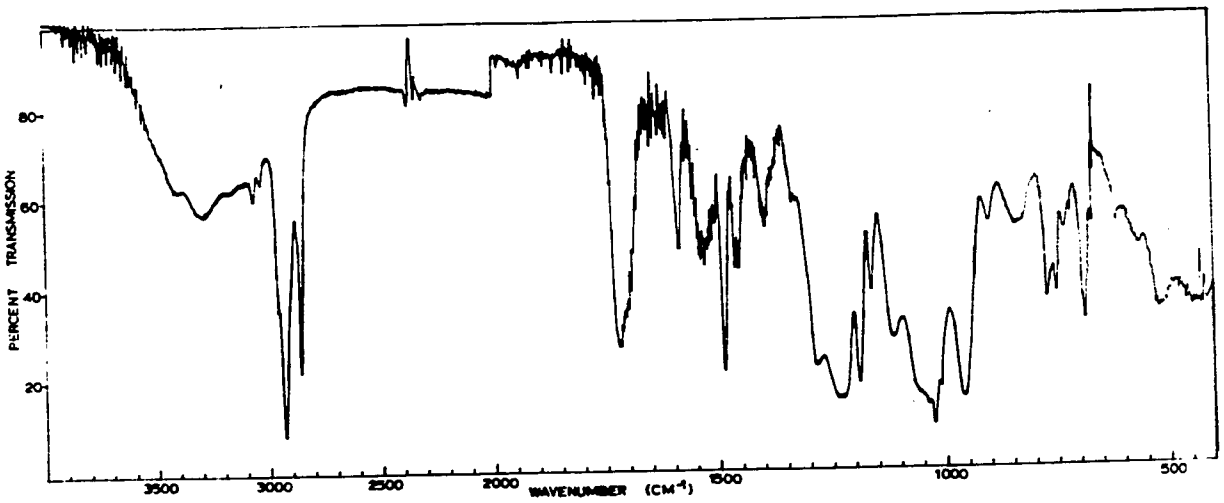


Figure 162. Infrared spectrum of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine.

The shape, maxima and extinction coefficients of the ultraviolet spectrum (Fig. 163) compare favorably with those for the dihydrosphingosine analogue (Fig. 142). The blank consisted of ethanol vs ethanol.

The NMR spectrum (Fig. 164) showed the twin peaks corresponding to the two different types of aromatic rings at 6.99 PPM (15 protons); the benzylic hydrogens at 4.80 PPM (2.2 protons; singlet); the skeletal hydrogens at 4.2 PPM (6.1 protons (a little high); multiplet); and two hydroxyl hydrogens at 4.48 and 3.38 PPM (0.9 proton each; possibly doublets).

Major ions and their tentative assignments in the mass spectrum (Fig. 165) were m/e 495 (M-188; M - 2PhOH), m/e 469 (M-214; M - $CH_3(CH_2)_{12}CH.OH$; possibly an elimination of the carbons 5-18 and the C-4 hydroxyl group); m/e 404 (M-279; M - 2PhOH - $PhCH_2$); m/e 388 (M-295; M - 2PhOH - $PhCH_2O$); m/e 340 (M-343); m/e 250 (M-433; $(PhO)_2POOH$); m/e 259 (M-434; $(PhO)_2POO$); m/e 94 (M-589; PhOH) and m/e 91 (M-592; $PhCH_2$). Unfortunately, none of the ions gave definite proof that the phosphate was linked to carbon atom one. When the compound was treated with Carter's reagent at room temperture or at 80° C for three hours and the resulting mixtures were subjected to mass spectral analysis, only the mass spectrum of the unsilylated material was observed. Acetylation of the compound was attempted using pyridine-acetic anhydride mixtures at room temperature overnight or at 80° C for 3 hours. As observed by thin layer analysis, no reaction occurred. The mass spectrum of the acetylation product was identical to that of the starting material. The NMR, ultraviolet

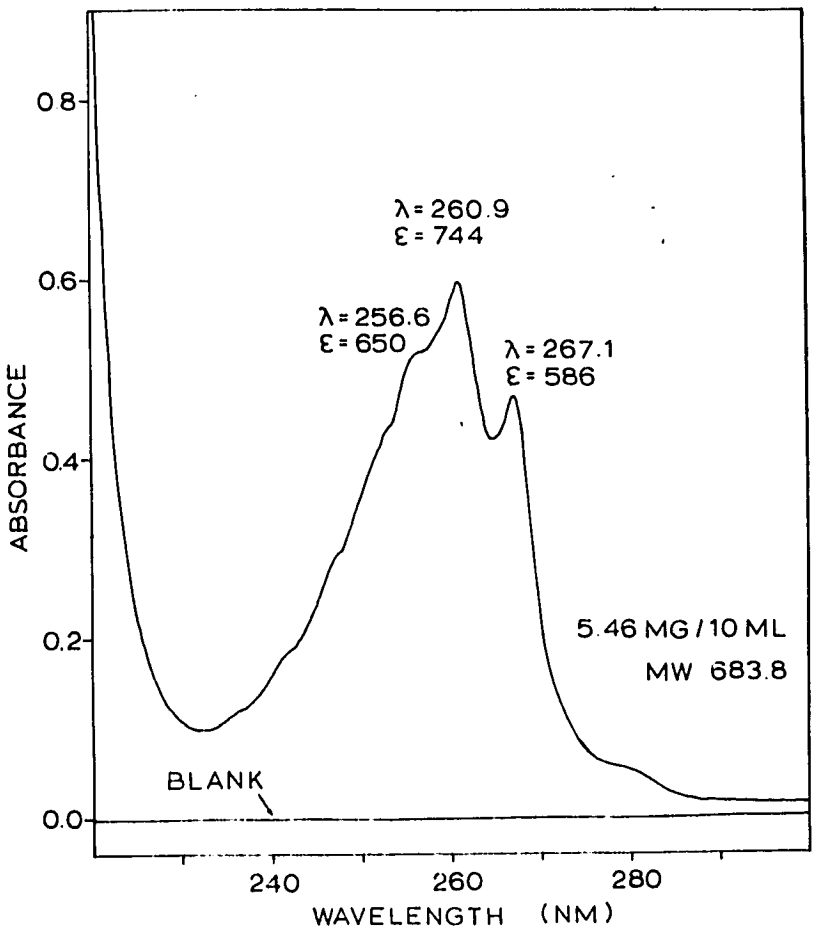


Figure 163. Ultraviolet spectrum of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine.

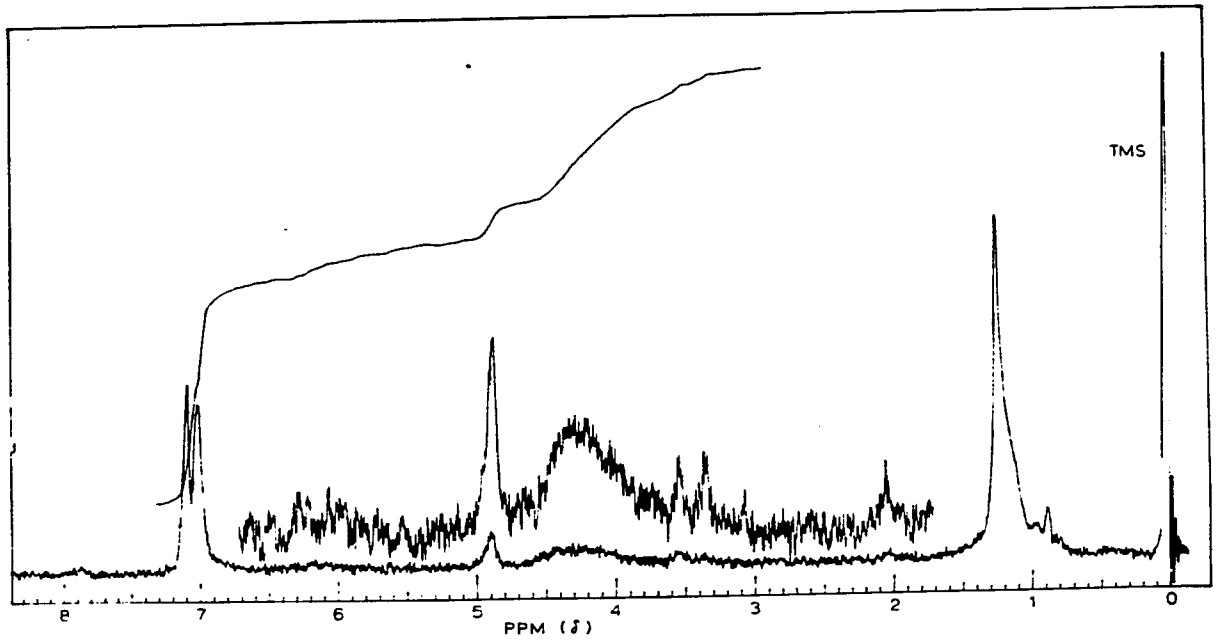


Figure 164. NMR spectrum of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine.

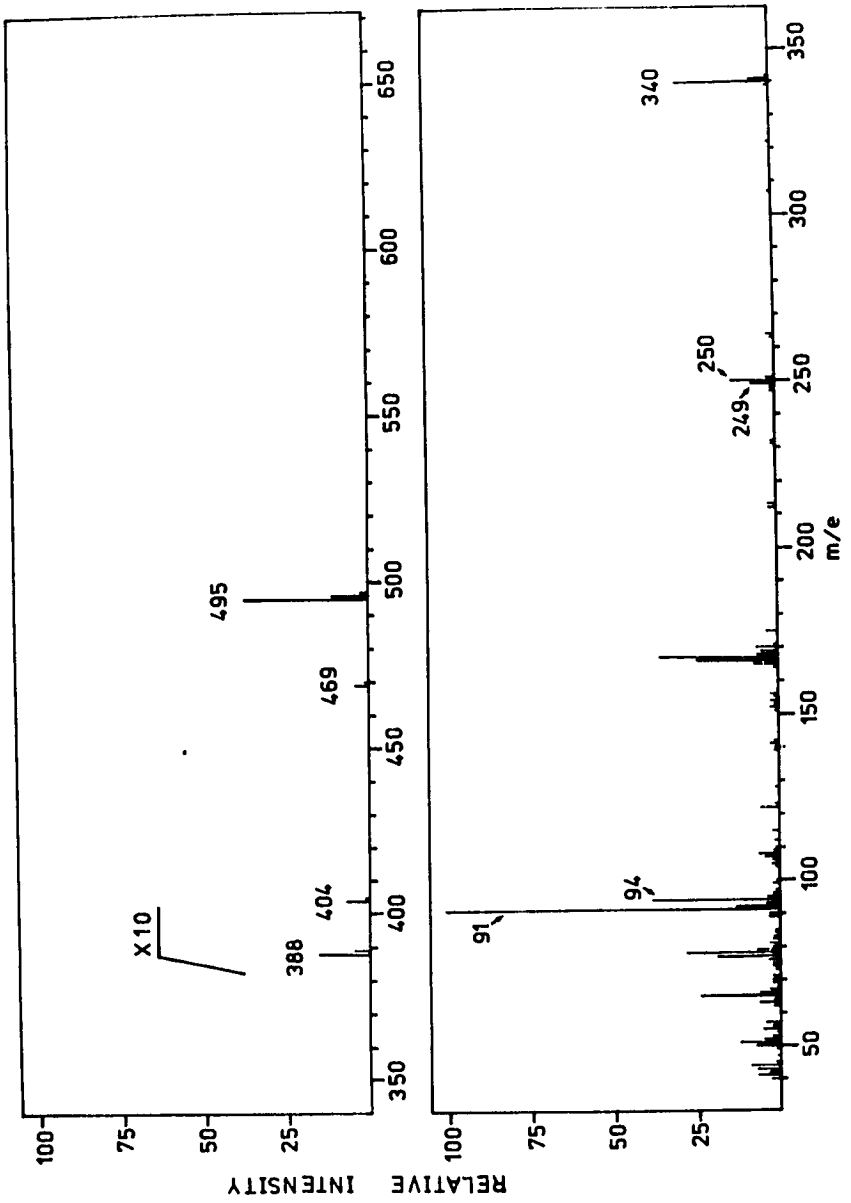


Figure 165. Mass spectrum of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine.

and mass spectra agreed with a structure having an N-carbobenzoxy and mono-diphenylphosphoryl moieties along with two free hydroxyl groups. However, both the periodate oxidations and the attempts at acylation and silylation indicated that the hydroxyls were very unreactive.

Elemental analysis was inconsistent with the spectral data by indicating a lower percentage of carbon and almost twice as much phosphorous as expected:

	C	H	N	P	
Calculated:	66.75	7.96	2.05	4.53	(%)
Found:	57.61	7.46	2.60	8.00	

The Dittmer's positive, polar (R_f = 0.38) compound (166.5 mg; 0.243 mmole) was added to glacial acetic acid (10 ml) along with platinum (black; 400 mg). After hydrogenation for 19 hours at room temperature at 30 psi in a Parr hydrogenation apparatus, the solution was warmed in a steam bath and filtered through Hyflo Super Cel under suction. The catalyst was washed with two portions of warm glacial acetic acid (2 ml each) and the volume of the combined filtrates was reduced at reduced pressure at 40° C to about 1 ml. This was applied to Silica Gel PF 254 + 366 plates and developed in n-butanol-acetic acid-water (60:20:20). The two major spots at R_f = 0.54 and R_f = 0.40 were both Dittmer's positive and ninhydrin positive (Fig. 166). Each band was scraped and was then eluted with methanol-88% formic acid (99:1; 100 ml) and the solvent was removed at reduced pressure at 40° C. The residues were dried <u>in vacuo</u> over phosphorous pentoxide. Each residue was redissolved in warm methanol-88% formic acid (99:1; 5 ml), filtered through Hyflo Super Cel and the solvent removed and the residue dried as above. The upper band (Fig. 167, Channels 1 and 4;

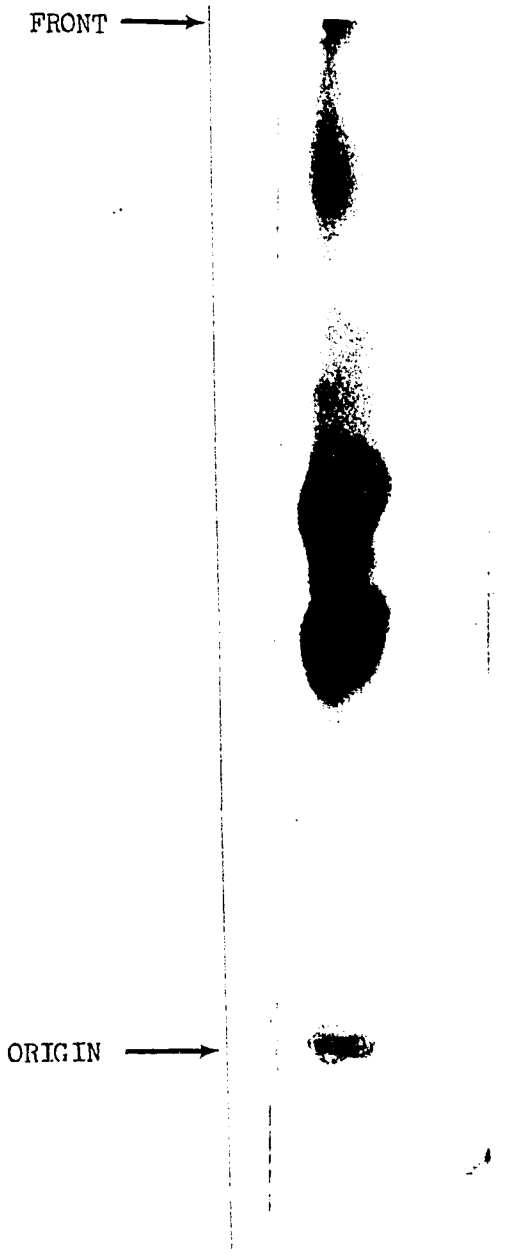


Figure 166. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate of the crude reaction mixture after the catalytic hydrogenolysis of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine. Solvent: n-Butanol-acetic acid-water (60:20:20). Stain: Dittmer's reagent followed by charring at 130° C for 15 minutes.

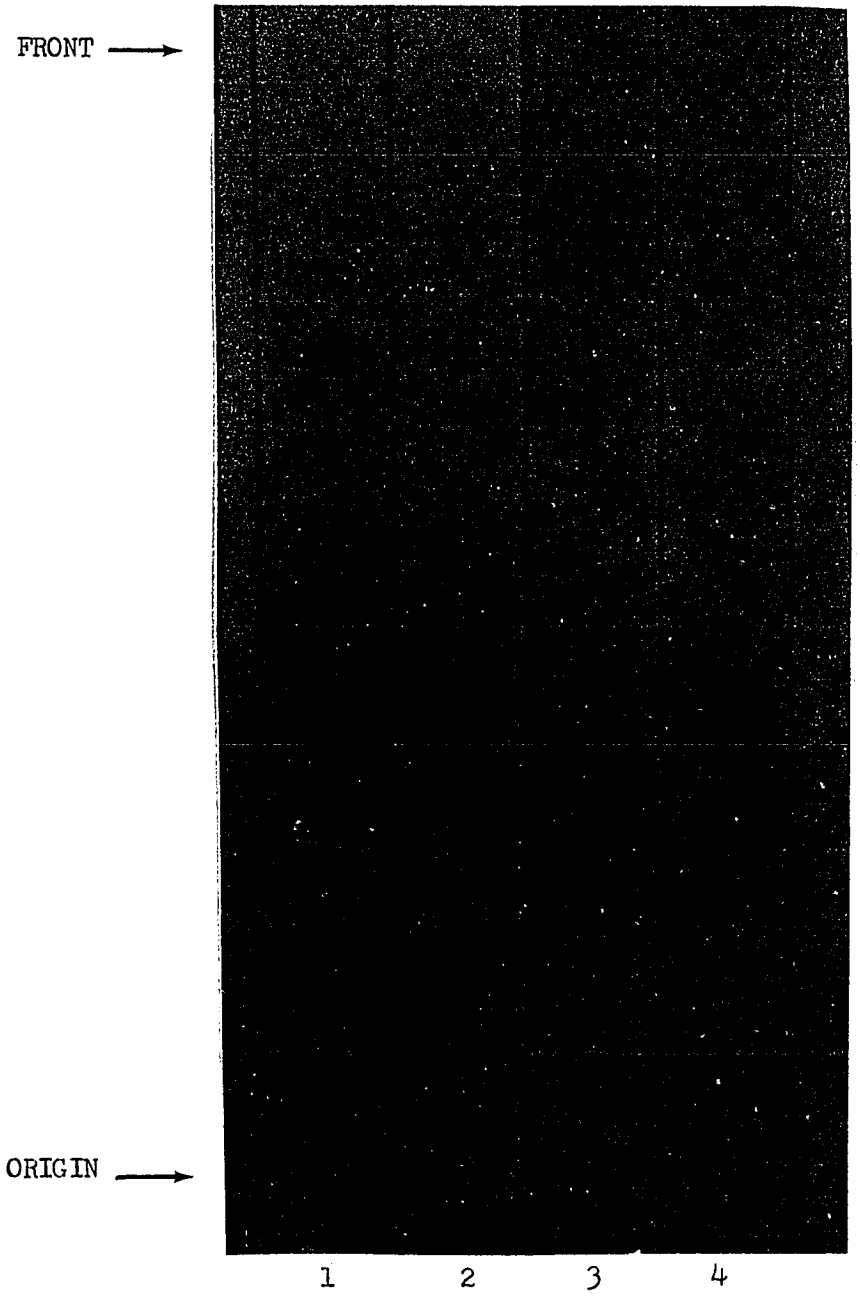


Figure 167. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate of the less polar (Channels 1 & 4) and more polar (Channels 2 & 3) products from the catalytic hydrogenolysis of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine. Solvent: n-Butanol-acetic acid-water (60:20:20). Stain: Dittmer's reagent at room temperature (Channels 1 & 2), ninhydrin reagent (Channels 3 & 4).

R_f = 0.54) had a mass of 66 mg and the lower band (Fig. 167, Channels 2 and 3; R_f = 0.40) had a mass of 86 mg. Each was treated with periodate-borohydride as described in Materials and Methods with the modification[42,43] that the substrates (about 1 mg each) were first dissolved in chloroform-methanol (1:9) and the periodate solution was then added. The reaction was then stored in the dark at 37° C with shaking for 19 hours. After Folch extraction followed by borohydride reduction and silylation of the product, little or no silylated pentadecanol was observed. A sample of crude phytosphingosine-1-phosphate derived from the alkaline hydrolysis of corn phyto-glycolipids (Fig. 168, Channels 1 and 4) showed two major Dittmer's and ninhydrin positive spots on thin layer chromatography. When this material was treated with periodate-borohydride as described above, pentadecanol was liberated. The aqueous layers after Folch extraction of the above periodate oxidations were made alkaline with 8 drops of 1 N sodium hydroxide and sodium borohydride (82 mg) was then added. After 22 hours at room temperature, each solution was acidified with 2 N HCl and then extracted with chloroform until all of the yellow color was removed. This aqueous extract was then passed through a Dowex 50W-X8 (200-400 mesh) cation exchange column (2 X 14 cm which was washed with 2 N HCl followed by water to pH 6) followed with water (150 ml). The water was removed from the eluate under reduced pressure at 50° C and three portions of methanol (10 ml each) were added and then removed at reduced pressure at 50° C. This removed any boric acid. The samples were silylated with Duncan's reagent. Only the corn phytosphingosine-1-phosphate released a product which co-chromatographed with the silylated derivative of authentic glycol-phosphate.

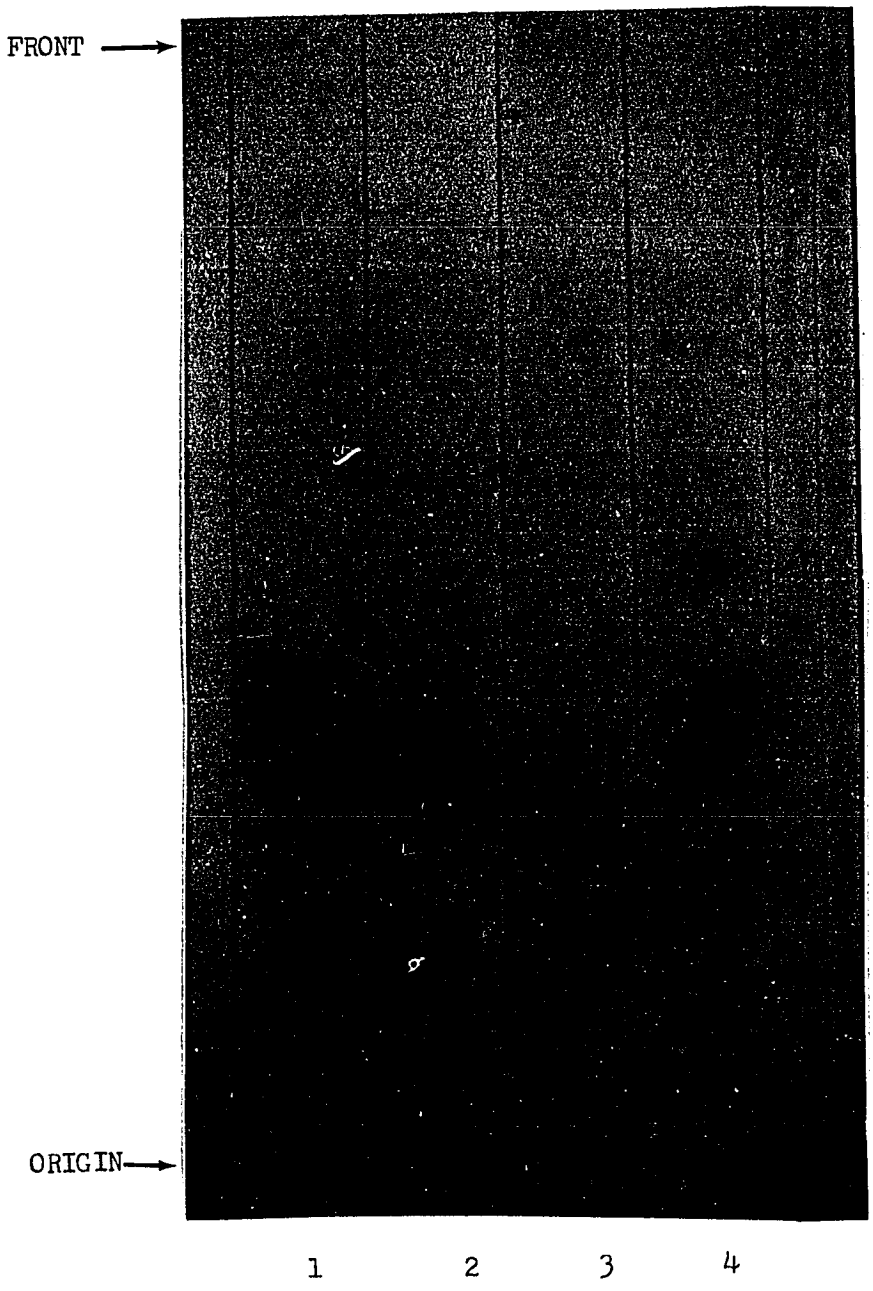


Figure 168. Thin layer chromatogram on a Silica Gel PF 254 + 366 plate of crude corn phytosphingosine-1-phosphate (Channels 1 & 4) and the more polar product from the catalytic hydrogenolysis of the polar Dittmer's positive diphenylphosphoryl-N-carbobenzoxy-phytosphingosine. Solvent: n-Butanol-acetic acid-water (60:20:20). Stain: Dittmer's reagent at room temperature (Channels 1 & 2), ninhydrin reagent (Channels 3 & 4).

The synthesis of phytosphingosine-1-phosphate by this chemical process was, therefore, not achieved. The more polar of the two products may have represented the 3-phosphate if the interpretation of the ion at m/e 469 (Fig. 165) was correct in assuming an elimination of the skeletal carbons 5-18 and the C-4 hydroxyl group.

Other synthetic routes were attempted prior to the one described above, but these proved unpromising. If the 3,4-hydroxyls could have been protected with an acetal linkage, the diphenylphosphochloridate could have reacted only and in high yield with the primary hydroxyl group of the N-carbobenzoxy-acetal of phytosphingosine. The acetal would then have been removed during the acid hydrogenolysis of the phosphorylated compound.

N-carbobenzoxy-phytosphingosine (12.5 mg) was refluxed in acetone (5 ml; distilled from potassium permanganate and stored over calcium chloride) with anhydrous copper sulfate (heated at 110° C overnight) for 4 hours. The solution was filtered and the filtrate was analyzed by thin layer chromatography in 1% methanol in chloroform on Silica Gel PF 254 + 366 plates. The starting material remained at the origin while 4 major (R_f = 0.31, R_f = 0.40, R_f = 0.55 and R_f = 0.60) and 1 minor (R_f = 0.65) spots were observed with higher mobility. Isolation and characterization of each of the low yield products was impractical.

N-carbobenzoxy-phytosphingosine (5 mg) was dissolved in anhydrous ether (5 ml) and anhydrous copper sulfate (10 mg), acetone (1 ml) and p-toluene-sulfonic acid (10 mg) was added. After refluxing for 1½ hours, the mixture showed three less polar spots (R_f = 0.26, R_f = 0.35 and R_f = 0.55) on thin layer chromatography in 1% methanol in chloro-

form. If no acetone was added, no reaction occurred as only the substrate was observed on thin layer chromatography in the above solvent system. If p-nitro-benzaldehyde was substituted for acetone, one major (R_f = 0.57) and two minor (R_f = 0.75 and R_f = 0.85) products were observed on thin layer chromatography in the above solvent system. When the acid catalyst was omitted from the last reaction, one major product (R_f = 0.57) was formed as observed by thin layer chromatography in the above solvent system. While this was hopeful, all attempts to purify this product by column or thin layer chromatography produced incredible decomposition. As analyzed by thin layer chromatography, the product would be present, but there was considerable streaking between that spot and the less polar p-nitro-benzaldehyde and the more polar N-carbobenzoxy-phyto-sphingosine standards. This process was similar to that observed with the p-nitro-benzaldehyde derivatives of dihydrosphingosine before acetylation. This line of work was, therefore, terminated.

SUMMARY

[4,5-$^{3}H_{2}$]-Dihydrosphingosine of high specific activity and high purity has been prepared. The results of careful studies with this compound indicated marked instability when small amounts of the material were subjected to conventional procedures of handling and storage. For example, the compound readily decomposed to form a less polar product (or products) upon being taken to dryness. The magnitude of the decomposition appeared to increase with increasing volumes of the solution when taken to dryness. The magnitude of the process increased with increasing time of storage in the dry state. The magnitude of the decomposition increased significantly when a solution of the labeled substrate came in contact with Silica Gel G, silicic acid or Unisil prior to being taken to dryness. If solutions of the labeled compound in chloroform-methanol (2:1) were stored in glass vials sealed with polyethylene caps, a non-polar product was observed on thin layer chromatography. Vials sealed with Teflon lined caps showed no such product.

The results of studies of the distribution of radioactivity in various lipids isolated from rat liver 1 hour after intraportal injections of labeled dihydrosphingosine into female rats showed that the largest amount of radioactivity (68%) of the polar mild alkali stable lipids resided in the ceramides. The remainder of the radioactivity was found about equally distributed among the cerebrosides, free bases and sphingomyelins. In the acylated classes about 80% of

the radioactivity resided in the long chain base moiety with the remainder of the radioactivity in the fatty acid moiety. The largest amount of activity in the fatty acids was recovered in palmitic acid. The free bases obtained after column chromatography of the polar mild alkali stable lipids generally consisted only of labeled dihydrosphingosine. In two rats, however, labeled free sphingosine was observed. Large amounts of labeled sphingosine were found in the base moieties of each of the acylated long chain bases. The largest ratio of sphingosine to dihydrosphingosine in these classes was found in the sphingomyelins followed by the ceramides and then the cerebrosides. However, a rough calculation of the absolute amount of labeled sphingosine formed from the administered substrate was highest in the ceramides followed by the sphingomyelins and then the cerebrosides. In one rat, labeled phytosphingosine was observed in the ceramides. The fatty acid moieties of the ceramides and cerebrosides of this rat contained radioactivity in palmitic acid, 2-hydroxy-palmitic acid and pentadecanoic acid. The latter two compounds are known catabolic products of phytosphingosine. The irreproducibility of the latter findings suggests that an unknown variable exists in the metabolism of labeled dihydrosphingosine. Possible areas for future exploration are diurnal, feeding and time studies.

The results of <u>in vitro</u> studies using crude dihydrosphingosine kinase from rat liver indicated that N-acetyl- and N-benzoyl-dihydrosphingosines did not serve as substrates for this enzyme. The crude enzyme system did not catalyze the phosphorylation of labeled 1,2-diglycerides. However, it did catalyze the formation of a product more polar than the substrate on thin layer chromatography. The identity of

this product (or products) remains unknown.

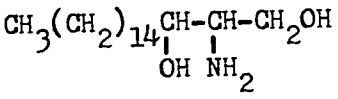
The method of Weiss and Stiller[111] proved an ineffective method for the purification of phytosphingosine. This method has its basis in the formation of a derivative followed by chromatography. The results of the experiments described herein demonstrated that the derivatization is not specific for phytosphingosine as previously reported. Several of the derivatives of dihydrosphingosine have been isolated and studied by combined gas-liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy.

Dihydrosphingosine-1-phosphate was prepared by chemical synthesis. The intermediates in the synthesis were characterized by thin layer chromatography, elemental analyses, melting points, ultraviolet, infrared, NMR and mass spectrometry.

The preparation of phytosphingosine-1-phosphate was attempted. The chemical and physical characteristics of N-carbobenzoxy-phytosphingosine compared favorably with those of the dihydrosphingosine analogue. However, when N-carbobenzoxy-phytosphingosine was treated with diphenylphosphochloridate, none of the several reaction products isolated had characteristics totally analogous to the N-carbobenzoxy-1-diphenylphosphoryl-dihydrosphingosine. The major phosphate positive derivative of phytosphingosine was hydrogenated as described by Weiss[105]. The final products isolated differed in their physical and chemical characteristics from those of crude phytosphingosine-1-phosphate obtained from natural sources.

APPENDIX I

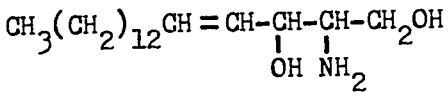
STRUCTURES AND NAMES FOR COMMON SPHINGOLIPIDS



Dihydrosphingosine

Sphinganine

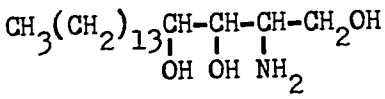
D-erythro-1,3-Dihydroxy-2-Amino-Octadecane



Sphingosine

Sphingenine

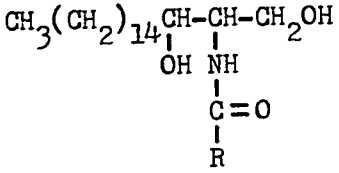
trans-D-erythro-1,3-Dihydroxy-2-Amino-Octadec-4-Ene



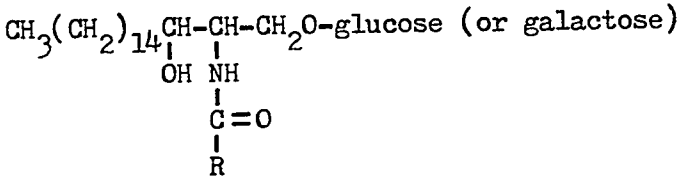
Phytosphingosine

4-Hydroxy-Sphinganine

D-ribo-1,3,4-Trihydroxy-2-Amino-Octadecane



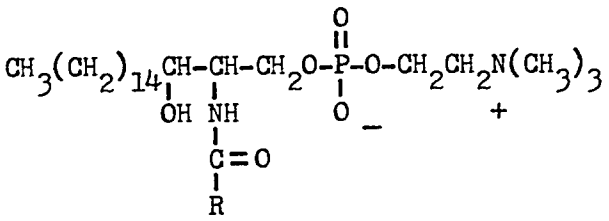
Ceramide



Cerebroside

APPENDIX I

(Continued)



Sphingomyelin

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