Biosynthesis of Unsaturated Sphingolipid Bases by Microsomal Preparations from Oysters*  

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SUMMARY  
A cell-free particulate preparation from oyster viscera incorporated [1-14C]palmitate and [1-14C]serine into the long chain base sphingadienine. Both double bonds of the product were shown to have the trans configuration. trans-2-Hexadecenoic and trans-6-hexadecenoic acids were not effective in decreasing the incorporation of the radioactive label from palmitate, nor was label from palmitate incorporated into these two monounsaturated fatty acids. Excess 2-Hexadecenoic and trans-6-hexadenoic acids were not product were shown to have the trans configuration. trans-chain base sphingadienine. Both double bonds of the incorporation of palmitate and [1-14C]serine into the long

The biosynthesis of sphingolipid long chain bases (reviewed recently by Morell and Braun (1)) in yeast (Hansenula ciferri) and mammals (rat liver and brain) involves the condensation of a fatty acyl coenzyme A and the pyridoxal phosphate complex of serine to yield carbon dioxide and 1-hydroxy-2-amino-3-keto long chain base (8, 10-17). An NADPH-requiring reductase converts the 3-keto group to a hydroxyl thus completing the syn-formation of 1,3-dihydroxy-2-amino long chain base (8, 10-17). The condensation and reduction steps are illustrated by the following reactions:

\[ \text{R} - \text{C} - \text{CH(NH}_2\text{)}\text{CH}_2\text{OH + NADPH + H}^+ \rightarrow \text{RCH(OH)CH(NH}_2\text{)}\text{CH}_2\text{OH + NADP}^+ \]

Presently, more than 60 different sphingolipid long chain bases have been reported, many of which contain one or two double bonds (see Karlsson's review (18)). The point in the biosynthetic pathway at which double bonds are introduced has been determined conclusively, although several possibilities have been suggested, as follows: (a) prior to condensation (desaturation of the fatty acid or CoA derivative); (b) after condensation but prior to reduction (desaturation of the 3-keto long chain base); (c) after reduction of the 3-keto group (desaturation of the sphingolipid long chain base).

Stoффel et al. (8) and Braun and Snell (14) have suggested that the trans double bond of sphing-4-ene is introduced after condensation of palmitoyl-CoA with serine, whereas DiMari et al. (12) demonstrated the conversion of ammonium [1-14C]palmitate to trans-2-hexadecenoic acid by microsomes from H. ciferri, which suggested that the desaturation reaction might occur prior to condensation. Stoффel's group (16, 19) has subsequently provided evidence for the desaturation of sphinganine itself in the formation in vivo of the trans double bond at carbon 4 by rats. Finally, Fujino and Nakana (20) have described results which indicate that 3-keto[1-14C]sphinganine is converted directly to 3-keto[1-14C]sphingadienine in the rat.

Several organisms contain sphingolipid long chain bases with an additional isolated double bond in the aliphatic chain, notably sphinga-4,14-diene of human plasma sphingomyelin (21), sphinga-4,8-diene of oysters (22) and sea anemone (23), and eicosasphinga-4,11-diene of the western scorpion (24). Since nothing is known about the mechanism of formation of the isolated double bond of these dienes, and since there is some controversy about the pathway for the formation of the double bond at carbon 4, we were prompted to investigate these problems. In this paper, we report studies of the biosynthesis of sphingadienine by a cell-free particulate enzyme preparation from oysters, with particular emphasis on the mechanism of double bond formation.

EXPERIMENTAL PROCEDURES  

Materials  
Live oysters were obtained from the City Fish Company, Lansing, Mich., and pregnant rats were supplied by Spartan Research Animals, Haslett, Mich. Scorpions were purchased from...
Lorin Honetschläger, Mess, Ariz. Silicie acid (200–325 mesh Unisil) was purchased from Clarkson Chemical Company, Williamsport, Pa., and Silicie Gel II was obtained from EM Reagents Division, Brinkmann Instruments, Inc., Westbury, N. Y. Thin layers of silica gel were prepared on glass plates using the plate leveling and spreader manufactured by "Quickfit" Instruments of England and distributed by Matheson, Elk Grove Village, Ill. Thin layer chromatographic standards of sphingomyelin, sphing-4-ene, and sphinganine were purchased from Applied Science, State College, Pa. trans-2-Hexadecenoic acid was purchased from Miles Laboratory, Inc., Elkhart, Ind., and chadic acid was purchased from Sigma Chemical Company, St. Louis, Mo. trans-6-hexadecenoic acid was isolated from erne sea turtle depot fat, kindly provided by Dr. R. G. Ackman, Fisheries Research Board of Canada, Halifax, N. S. Sodium [I'-14C]-palmitate was purchased from Tracerlabs, Waltham, Mass., [3-3H]serine from Amersham-Searle, Chicago, Ill., and [l-14C]- and 3-3H]serine from New England Nuclear, Boston, Mass.

Mass spectral analyses were carried out with an LKB 9000 combined gas chromatograph mass spectrometer. Scan times over a mass range from m/z 20 to 600 were about 6 s, the ion source temperature was 250° with an ionizing current of 60 μamp, electron energy of 70 eV and accelerating potential of 3.5 kv. The gas-liquid chromatographic column (1.6 m x 1.9 mm, inner diameter) contained 5% SE-30 on 100 to 120 mesh Gas-Chrom Q (Applied Science, State College, Pa.). For analyses of fatty acid methyl esters the column was maintained at 190°, for the methoxime-O-trimethylsilyl-N-acetyl 3-keto bases the column was at 230°, and for trimethylsilyl-N-acetyl long chain bases the column temperature was programmed from 200–250° at 5° per min.

14C-Lipids were detected on thin layer chromatograms with a Berthold model 6000 radioemitter (Varian, Walnut Creek, Calif.). Infrared spectroscopy was carried out with a Perkin Elmer 621 grating infrared spectrophotometer, using potassium bromide pellets prepared with a Carver Laboratory Press.

To quantitate 14C and 3H in lipid samples, a Beckman LS-150 liquid scintillation spectrometer was employed. For counting 14C alone, the instrument was operated at about 90% efficiency. To quantitate 14C in the presence of 3H, the instrument was adjusted so that the 3H channel was operating at 44% efficiency with respect to 3H, with no overlap of 14C, and the 14C channel was operating at 55% efficiency with respect to 14C and 18% with respect to 3H.

Methods

Preparation of Enzyme System—The oyster particulate system was prepared by a modification of the procedure described by Polito and Sweeney (25). Mined oyster visceras were suspended in 2 volumes of 0.1 M KH2PO4, pH 7.4, containing 2 mM dithiothreitol and 0.2 mM pyridoxal phosphate. The tissue suspension was ground with a porcelain pestle until well homogenised, after which the homogenate was centrifuged at 3,000 x g for 20 min. The pellet was discarded and the supernatant fraction was centrifuged at 14,000 x g for 30 min to remove residual cell debris and mitochondria. The supernatant fraction from this step was centrifuged at 100,000 x g for 90 min to obtain a light tan, gelatinous pellet which was resuspended in the phosphate-dithiothreitol-pyridoxal phosphate buffer using a manually operated glass homogenizer. All manipulations were carried out at 2-4°. For storage of the enzyme system, the microsomal fraction was resuspended in the same buffer, containing 30% (v/v) glycerol (12), and was stored at -20°. Under these conditions the enzyme was stable for up to 3 months and could be used, after thawing, without dialysis to remove glycerol.

Assays of Enzyme Activities—Long chain base synthesizing activity of the particulate enzyme fraction was measured by determining incorporation of [1-14C]palmitate into the bases. The enzyme reaction mixtures were prepared by mixing 1 ml of 0.1 M Tris-HCl, pH 7.4, containing 0.5 mM MgCl2, 8 mM ATP, 2 mM reduced coenzyme A, 1 mM FAD, 0.2 mM palmitic acid, 1.2 mM L-serine, and 1 mg per ml of Triton X-100, with 1 ml of an NAPDI regenerating system composed of 0.1 mM KH2PO4, 1 mM NADP, 1 mM glucose-6-phosphate, and 0.5 mM MgCl2. To this mixture was added 2 x 106 dpm of radioactive precursor, 10 units of glucose-6-phosphate dehydrogenase, and 3 ml of the enzyme suspension for a total of 5 ml. For reactions from which the 3-keto intermediates were to be isolated, the NADPH regenerating system was replaced by 1 ml of 0.1 M KH2PO4 (pH 7.4) containing 0.5 mM MgCl2, and the dehydrogenase was omitted. To study the effects of excess trans-2- or trans-6-hexadecenoic acids, 3-keto-sphinganine, sphinganine, or sphing-4-ene on the incorporation of [1-14C]palmitate, these lipids were added in at least 10-fold excess compared with palmitate concentration. All reaction mixtures were incubated at 35° for 2 hours in a Dubnoff metabolic shaking incubator (Precision Scientific Company, Chicago, Ill.).

In experiments designed to determine the mode of incorporation of serine into sphingoid long chain bases, reaction mixtures containing [3-3H]serine and [1-14C]serine or [3-3H]serine and [3-14C]serine were incubated as described above.

To determine fatty acid-desaturating activity, 1 ml of the oyster particulate system was added to 1 ml of 0.1 M KH2PO4, (pH 7.4) solution of 1 mM MgCl2, 0.4 mM palmitic acid, 2 x 104 dpm of sodium [1-14C]palmitate, and 0.5 mg per ml of Triton X-100. This mixture was incubated at 35° for 1 hour as described above.

Activity in desaturating 3-keto long chain bases was determined by incubating 1 ml of the oyster particulate system with 1 ml of 0.1 M KH2PO4, (pH 7.4) solution of 1 mM FAD, 0.4 mM MgCl2, 0.5 mg per ml of Triton X-100, and either 3-keto-[14C]sphinganine (35,500 dpm) or 3-keto-[14C]sphing-4-ene (8,900 dpm). Dihydroxy long chain base desaturating activity was determined by incubating 1 ml of the oyster particulate system with 1 ml of 0.1 M KH2PO4, (pH 7.4) solution of 1 mM NADH, 1 mM FAD, 0.5 mg per ml of Triton X-100, and either 3-keto-[14C]sphinganine (5,800 dpm) or [14C]sphing-4-ene (11,100 dpm).

Extraction of Lipids from Reaction Mixtures—The lipid extraction procedure was a modification of the Bligh and Dyer (26) method for total lipid extraction from bacterial suspensions. Reactions were terminated by addition of the proper portions of chloroform and methanol to the aqueous reaction mixture (5:12.5:6.25, water-methanol-chloroform) to render the solvents uniphasic after vigorous shaking. After standing for an hour sufficient additional chloroform and water (6.25 parts of each) were added to give a biphasic system after vigorous shaking. The lower phase was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo to yield the total lipid extract.

Isolation of Long Chain Bases or Fatty Acids from Total Lipid Extracts—To isolate dihydroxy long chain bases, the total lipid extract was subjected to methanalysis as described by Gaver and Sweeney (27) (modified aqueous methanolic HCl, 1 N in HCl and 10 M with respect to water; 18 hours at 80°). After neutralization with solid silver carbonate, the methanolyzate was evaporated to dryness under a stream of dry nitrogen, redissolved in a minimum volume of chloroform and applied to a 4-g silicic acid column (25). After washing thoroughly with 150 ml of chloroform, the column
was eluted successively with 150-ml portions of chloroform-methanol 98:2, 90:10, and 80:20. All of the sphingolipid long chain bases were eluted in the 80:20 fraction. Further purification of the long chain bases was achieved by thin layer chromatography as described by Polito and Sweeley (25). The 80:20 fraction was evaporated to dryness in vacuo, redissolved in a minimum volume of chloroform, and applied as a series of adjacent spots on a Silica Gel H thin layer plate. After development with chloroform-methanol-water (65:25:4), the center section of the plate was covered with a smaller glass plate, leaving the outer edges exposed. Ninhydrin spray detected the components of the lipid mixture which cochromatographed with authentic sphing-4-enine (Fig. 1A illustrates a typical chromatogram). The area of the covered section of the plate corresponding to the ninhydrin-positive component was scraped and the silica gel extracted with 8 ml each of chloroform, chloroform-methanol (2:1), and methanol. The combined extracts were evaporated to dryness in vacuo, redissolved in a minimum volume of chloroform, and applied as a series of adjacent spots on a Silica Gel H thin layer plate. After development with chloroform-methanol, 98:2 (28). The lipids were detected by charring a plate covered as described above (Fig. 1B illustrates a typical chromatogram). This combination of silicic acid column and thin layer chromatography (Fig. 1, A and B) was used throughout for purification of long chain bases.

Total lipid extracts containing 3-keto bases were not subjected to methanolation, but rather were N-acetylated in methanol-acetic anhydride (4:1) (29) and applied to a silicic acid column. After thoroughly washing the column with chloroform, the ketones were eluted with chloroform-methanol, 99:1 (20). The ketone fraction was evaporated to dryness in vacuo, redissolved in a minimum volume of chloroform and further purified by thin layer chromatography on Silica Gel H (chloroform-methanol, 95:5). The lipids were detected by exposure of the partially covered plate to iodine vapors. To remove the N-acetyl group from these keto bases, the lipids were hydrolyzed in 5% HCl in methanol (1 hour at 70°) as described by DiMari et al. (12) and Shapiro et al. (30). After recovery of the deacetylated ketones, chromatography on Silica Gel H (chloroform-methanol-2 N ammonium hydroxide, 40:10:1) separated the saturated from the unsaturated 3-keto bases.

To isolate fatty acids, the total lipid extract was methanolyzed in modified aqueous methanolic HCl as described above. After neutralization with silver carbonate, the methanolysate was extracted four times with equal volumes of hexane, and the combined extracts evaporated to dryness in vacuo. The fatty acid methyl esters were trimethylsilylated as described by Carter and Gaver (29) with hexamethyldisilazane - trimethylchlorosilane - dry pyridine, treated with hexamethyldisilazane - trimethylchlorosilane - dry pyridine, and applied to a Silica Gel H thin layer plate containing 4% (w/w) silver nitrate. These plates were prepared from 4% silver nitrate. The plate was developed in petroleum ether-diethyl ether, 95:5. The lipids were detected by exposure of the partially covered plate to iodine vapors. To remove the N-acetyl group from these keto bases, the lipids were hydrolyzed in 5% HCl in methanol, redissolved in a minimum volume of hexane and applied to a Silica Gel H thin layer plate containing 4% (w/w) silver nitrate. The plate was developed in petroleum ether-diethyl ether, 95:5.

Preparation of Volatile Derivatives for Gas Chromatography and Mass Spectrometry—The purified triacyl bases obtained after preparative argentation thin layer chromatography were hydrolyzed to the free bases with the modified aqueous methanolic HCl and the products were re-N-acetylated. N-Acetyl dihydroxy long chain bases and N-acetyl 3-keto long chain bases were trimethylsilylated as described by Carter and Gaver (29) with hexamethyldisilazane - trimethylchlorosilane - dry pyridine, 634.

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2.6:1.6:2.0, or with a 1:1 mixture of bis(trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane (Regisil, Regis Chemical Company, Chicago, Ill.) and dry pyridine. Methoxime derivatives of 3-keto long chain bases were prepared by treating the lipid with methoxylamine hydrochloride in dry pyridine for 2 hours at 80°C before trimethylsilylation with Regisil (31).

Isolation of trans-6-Hexadecenoic Acid—Approximately 30 g of crude depot fat from leatherback turtle (supplied by R. G. Ackman) were dissolved in 500 ml of ethanol-water, 1:1, containing 1.5 moles of KOH. This mixture was refluxed for 12 hours, acidified to pH 2, and extracted three times with equal volumes of petroleum ether. The combined extracts were taken to dryness in vacuo and redissolved in a minimum volume of chloroform. Preparative thin layer chromatography (Silica Gel H developed with petroleum ether-ethyl ether-acetic acid, 90:10:1) was used to purify the fatty acids. The purified product of free fatty acids was then esterified in 10 ml of 1N methanolic HCl at 100°C for 2 hours. Fatty acid methyl esters were extracted into hexane and the combined extracts were concentrated in vacuo and redissolved in 5 ml of hexane.

Preparative argentation thin layer chromatography of these methyl esters (4% silver nitrate on Silica Gel H (w/w) developed with petroleum ether-ethyl ether-acetic acid, 95:5:1) effectively separated the cis- and trans-monoenes in the mixture (Fig. 2). The component cochromatographing with authentic methyl trans-9-octadecenoate was shown by gas chromatography-mass spectrometry to be predominantly the 16-carbon monoene, which was reported previously to be the major trans-monoene in the leatherback turtle lipids (32). The purified methyl trans-6 hexadecenoate was hydrolyzed in 1N aqueous KOH and the free acid recovered by solvent partitioning.

Preparation of Authentic 3-Ketosphinganine—N-Acetylsphinganine was oxidized to the 3-keto base with chromic anhydride exactly as described by Gaver and Sweeley (34). After extraction and purification, a small aliquot of the product was derivatized with methoxylamine hydrochloride (31) and trimethylsilylation reagents (29) and the purity and identity were confirmed by gas chromatography-mass spectrometry (Fig. 3A). N-Acetyl-3-ketosphinganine was hydrolyzed with 5% HCl in dioxane and the deacetylated base hydrochloride was recovered in about 50% yield.

Preparation of [4C]Ketosphinganine and [14C]Ketosphing-4-enine—A particulate fraction isolated from the brains of 14-day-old rats was incubated with [1-4C]palmitate (5 x 10⁶ dpm) and [3-14C]serine (5 x 10⁶ dpm) in the absence of NADPH. After incubation for 1 hour at 35°C the lipids were extracted, N-acetylated (29), and purified by column and thin layer chromatography as described earlier under “Methods.” Immediately prior to incubation with oyster microsomes, the labeled ketones were deacetylated with 5% HCl in dioxane and resolved into labeled 3-ketosphinganine and 3-ketosphing-4-enine on preparative thin layers of Silica Gel H developed in chloroform-methanol-2N ammonium hydroxide, 40:10:1 (11, 12). Gas chromatography-mass spectrometry of the methoxime-trimethylsilyl derivatives of the ketones confirmed their structures (Fig. 3A and B).

Determination of Double Bond Configurations in Dienic Base—Reduction of the dienic base was carried out as described by Renkonen and Hirvisalo (21) for human plasma sphinga-4,14-dienine. The purified triacetylated diene was dissolved in 2 ml of 10% solution of hydrazine hydrate in methanol; the solution was incubated with occasional shaking at 35°C for 2 hours. The reaction was terminated by addition of 1 ml of 1N methanolic HCl, and lipids were extracted from this mixture by addition of 2.3 ml of water and 3.0 ml of chloroform. The lower phase was collected, dried over anhydrous sodium sulfate, and evaporated.
to dryness under a stream of dry nitrogen. Due to partial hydrolysis of the ester-linked acetate groups in the slightly alkaline reduction mixture, it was necessary to reacetylate the bases in dry pyridine-acetic anhydride, 3:2 (21). Resolution of the mixture of fully and partially reduced forms into pure bases for infrared spectroscopy was accomplished as described by Rennen and Hirvivaso (21) by thin layer chromatography. Silver nitrate-silica gel thin layers were developed in chloroform-methanol, 98:2, to resolve the mixture into two zones, one of which contained the diene and the Δ'-monoene and the other the Δ'-monoene and the saturated base. These were collected, deacetate with the modified aqueous methanolic HCl, and chromatographed separately on thin layers of Silica Gel H developed in chloroform-methanol-water, 65:25:4, which effectively separated the diene from the Δ'-monoene and the Δ'-monoene from the saturated base. The two monoenes were trimethylated and prepared for analysis by infrared spectroscopy as potassium bromide pellets.

Preparation of [3H]Sphing-4-enine and [14C]Sphinganine—Each of 50 young western scorpions (24) was injected with 106 dpm of [1-14C]palmitic acid in light mineral oil using a 30-gauge needle and automatic micropipettor (Biotronics, Brookings, S. D.). After 12 hours, the lipids were extracted by homogenizing the scorpions in chloroform-methanol (2:1) in a Sorvall Omni mixer. The total lipid fraction was methanolized and the long chain bases isolated as described earlier under "Methods." 14C-Labeled saturated and monounsaturated bases were purified by thin layer chromatography.

RESULTS

Identification of Biosynthetic Products of Oyster Particulate Preparations—Sphingolipid long chain bases isolated from reaction mixtures containing sodium [1-14C]palmitate and all cofactors contained a ninhydrin-positive component which cochromatographed with authentic sphing-4-enine in Silica Gel H (Fig. 1A). Generally about 19,000 ± 2,000 cpm of 14C were recovered in this component, representing an incorporation efficiency of about 1%. When this radioactive component was collected and the triacetyl derivative chromatographed on 4% silver nitrate-Silica Gel H, two radioactive components were detected, one cochromatographed with authentic triacetyl sphing-4-enine and containing 15 to 25% of the incorporated 14C. The slower moving component contained 75 to 85% of the incorporated 14C (Fig. 1B) and its Rf on thin layer chromatography was coincident with that of sphinga-4,8-diene of oysters. These components were collected, methanolized, and the Δ-acetyl trimethylislyl derivatives were analyzed by combined gas chromatography-mass spectrometry. The mass spectrum of the fast moving component (Fig. 1B) was identical with that of Δ-acetyl-di-O-trimethylsilylsphing-4-enine and the mass spectrum of the slow moving component was identical with that of Δ-acetyl-di-O-trimethylsilylsphinga-4,8-diene as reported by Hayashi and Matsubara (22). In addition, the fast moving component was shown by gas chromatography-mass spectrometry to contain some Δ-acetyl-di-O-trimethylsilylsphinga-4,8-diene as previously observed by Hayashi and Matsubara (22).

Mode of Incorporation of Serine into Sphingolipid Long Chain Bases—The sphingolipid long chain bases isolated from reaction mixtures containing [3-H] and [14C]serine were labeled as shown in Table I. The [3-H]:14C ratios in bases isolated from reaction mixtures containing [1-14C]serine and [3-H]serine were greatly increased over initial ratios, as expected if carbon 1 of the serine was lost during the condensation reaction. When the incubation mixtures contained [3-14C]serine and [3-3H]serine, the [3-H]:14C ratios were relatively unchanged. The oyster particulate enzyme preparation was therefore assumed to carry out long chain base synthesis via the same basic pathway as yeast and mammalian systems.

Identification of Biosynthetic Products of Oyster Microsomal Incubations in Absence of NADPH—An N-acetyl 3-keto long chain base fraction, obtained from [14C]palmitate, was eluted from a silicic acid column with chloroform-methanol, 99:1, and gave three spots on thin layer chromatography. Half of this fraction was reduced with sodium borohydride as described by Gaver and Sweeley (34). Aliquots of the reduction product and the unreduced N-acetyl 3-keto base fraction were chromatographed on 4% silver nitrate-Silica Gel H, and their RF on thin layer chromatography was coincident with that of sphinga-4,8-diene of oysters. These components were collected, methanolyzed, and the N-acetyl trimethylsilyl derivatives chromatographed on 4% silver nitrate-Silica Gel H, and their RF on thin layer chromatography was coincident. with that of sphinga-4,8-diene of oysters. These components were collected, methanolized, and the N-acetyl trimethylsilyl derivatives were analyzed by combined gas chromatography-mass spectrometry. The mass spectrum of the fast moving component (Fig. 1B) was identical with that of N-acetyl-di-O-trimethylsilylsphing-4-enine and the mass spectrum of the slow moving component was identical with that of N-acetyl-di-O-trimethylsilylsphinga-4,8-diene as reported by Hayashi and Matsubara (22). In addition, the fast moving component was shown by gas chromatography-mass spectrometry to contain some N-acetyl-di-O-trimethylsilylsphinga-4,8-diene as previously observed by Hayashi and Matsubara (22).

Table I

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<td>Added</td>
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FIG. 4. Thin layer chromatography and radio scanning of 3-keto bases and their borohydride reduction products. A, Silica Gel H developed with chloroform-methanol, 95:5 (v/v). Lane 1 is authentic N-acetyl-3-keto sphinganine (NAS); Lane 2 is a mixture of unresolved reference N-acetyl sphingamine and N-acetyl sphinga-4,8-diene (NAS); Lane 3 is oyster microsomal N-acetyl 3-keto bases from incubations in the absence of NADPH, separated by silicic acid chromatography (chloroform-methanol, 99:1, fraction); and Lane 4 is the reduction product of the major radioactive component of Lane 3. The radio scans of Lane 3 and 4 are shown in A and B, respectively. B, 4% silver nitrate-Silica Gel H developed in chloroform-methanol, 98:2 (v/v). Lane 1 is reference triacetylsphingamine and triacetyl sphinga-4,8-diene (TAS); Lane 2 is a mixture of triacetyl derivatives from the major radioactive zone of Lane 4 in A. The radio scan (2') is of Lane 2. The lipids were detected by charring.
Hydrazine reduction of oyster sphinga-4,8-dienine, isolated from the rat brain particulate system, are shown in Fig. 4A, and the monounsaturated 3-keto base, both of which were mass spectrometry of the methoxime-O-trimethylsilyl-N-acetyl mixtures lacking NADPH was provided by gas chromatography-mass spectrometry of the N-acetyl-0-trimethylsilyl derivative to be a sphinga-4,8-dienine.

Further evidence for the accumulation of 3-keto bases in reaction mixtures lacking NADPH was provided by gas chromatography-mass spectrometry of the authentic saturated 3-keto base and the mixture of the mono- and diunsaturated 3-keto bases. This labeled compound was shown by gas chromatography-mass spectrometry of the N-acetyl-0-trimethylsilyl derivative to be a sphingadine.

**Gas Chromatography-Mass Spectrometry of Methoxime-Tri methylsilyl Derivatives of N-Acetyl Keto Long Chain Bases—**Further evidence for the accumulation of 3-keto bases in reaction mixtures lacking NADPH was provided by gas chromatography-mass spectrometry of the methoxime-O-trimethylsilyl-N-acetyl 3-keto bases. Mass spectra of the authentic saturated 3-keto base and the monounsaturated 3-keto base, both of which were isolated from the rat brain particulate system, are shown in Fig. 3, A and B. Fig. 3C illustrates the mass spectrum of the major radioactive component isolated after incubation of [14C]palmitate with the oyster particulate system in the absence of NADPH. It is evident from the mass spectral data that the product contained a mixture of monounsaturated and diunsaturated bases, as expected because these bases are not resolved on the 3% SE-30 column.

The locations of some of the major ions in these mass spectra are characteristic of sphingolipid bases, and others are indicative of the ketomethoxime group. The 3-ketosphinganine derivative has a molecular ion at m/e 442, and ions resulting from elimination of a methyl group (m/e 427), a methoxyl group (m/e 411), trimethylsilanol (m/e 352), trimethylsilanol plus a methyl group (m/e 337), and trimethylsilanol plus a methoxyl group (m/e 321) are prominent. An ion is also observed at m/e 339 and represents loss of the terminal —CH2OTMS. Similar ions are observed in the mass spectra (Fig. 3, B and C) of the monounsaturated 3-keto base and the mixture of the mono- and diunsaturated 3-keto bases, respectively. The locations of these ions indicate the number of double bonds and chain length of the unsaturated bases.

**Stereochemistry of Double Bonds of Sphing-a-4,8-dienine—**The infrared spectra of the two triaeryl monounsaturated bases, isolated after partial hydrazine reduction of oyster sphingadine, are shown in Fig. 5. The absorption band at 965 to 970 cm⁻¹ in the spectra of both monoenes (Fig. 5, B and C) indicate that the Δ4 and the Δ8 double bonds have the trans-cis configuration.

This is in agreement with the sphinga-4,8-dienine isolated from sea anemone by Karlsson (23).

**Fatty Acid Desaturating Activity in Oyster Particulate Preparation—**If the desaturating steps in the biosynthesis of sphingadine in the oyster were to occur prior to condensation, the dienic fatty acyl precursor would presumably be trans-2,6-hexadecadienoate. Assuming stepwise desaturation, either trans-2- or trans-6-hexadecenoate would be the first desaturation product. The oyster particulate system was therefore incubated aerobically with [14C]palmitate in the presence of NADH and other cofactors. Fatty acid methyl esters from the total lipid fraction were chromatographed on 4% silver nitrate-Silica Gel H, as shown in Fig. 6. The only radioactive component was assumed to be palmitate, although the reference spot for methyl palmitate had a slightly different mobility than the radioactive material. No 14C was incorporated into the components chromatographing with authentic trans-monoenoic methyl esters. The radioactivity was operated under conditions appropriate for detection of 1000 cpm in any component of the mixture. This sensitivity would represent 0.05% incorporation of the added 2 × 10⁶ dpm of [14C]palmitate, an incorporation efficiency far below the ob-

---

**Table II**

Effect of excess unlabeled trans-2- and trans-6-hexadecenoate on incorporation of [14C]palmitate into sphingolipid bases

In incubation mixtures were prepared as described in Table I and "Methods" and were supplemented with 2.0 mM levels of unlabeled trans-2- or trans-6-hexadecenoic acid; each incubation contained 2 × 10⁶ dpm of [1-14C]palmitic acid. The mixtures were incubated at 35°C for 2 hours.

<table>
<thead>
<tr>
<th>Fatty acid added</th>
<th>[14C] recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sphing-a-4-enine</td>
</tr>
<tr>
<td>None</td>
<td>1,650</td>
</tr>
<tr>
<td>trans-6-Hexadecenoate</td>
<td>1,260</td>
</tr>
<tr>
<td>trans-2-Hexadecenoate</td>
<td>1,430</td>
</tr>
</tbody>
</table>

**Table III**

Effect of excess sphinganine and sphing-a-4-enine on incorporation of [14C]palmitate into sphingolipid bases

In incubation mixtures, prepared as described in Table I and under "Methods," were supplemented with 2.0 mM levels of unlabeled sphinganine or sphing-a-4-enine and contained 2 × 10⁶ dpm of [1-14C]palmitic acid. The mixtures were incubated at 35°C for 2 hours.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[14C] recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1,250</td>
</tr>
<tr>
<td>Sphinganine</td>
<td>1,250</td>
</tr>
<tr>
<td>Sphing-a-4-enine</td>
<td>1,600</td>
</tr>
<tr>
<td>800</td>
<td>17,700</td>
</tr>
<tr>
<td>950</td>
<td>16,100</td>
</tr>
</tbody>
</table>

---
Fig. 6 (left). Silver ion thin layer chromatography and radio-
scan of oyster fatty acid methyl esters. Thin layers of 4% silver
nitrate-Silica Gel H were developed with petroleum ether-diethyl
ether, 95:5 (v/v). A, authentic methyl cis-9-octadecenoate (c9); B,
authentic methyl trans-9-octadecenoate (t9) contaminated with
some c9; C, oyster methyl esters after microsomal incubation of
[14C]palmitate, ATP, reduced CoA, and NADH as described under
"Methods"; D, authentic methyl trans-2-hexadecenoate (t9); and
E, authentic methyl palmitate. The radio scan (C) is of Lane C.
Lipids were detected by charring.

Fig. 7 (right). Silver ion thin layer chromatography and
TABLE IV

<table>
<thead>
<tr>
<th>Conditions</th>
<th>14C recovery</th>
</tr>
</thead>
</table>
|                              | Sphing-4-ene| Sphing-4,8-
                                      | enine       | dienine    |
| Control                      | 860         | 11,400      |
| After preincubation with 3-keto-| 490         | 7,630       |

served conversion of [14C]palmitate to sphingolipid long chain
bases.

Further evidence against the direct involvement of trans-
omonoenoic fatty acids in the pathway of biosynthesis of long
chain bases by oysters is illustrated in Table II. When excess
amounts of the unlabeled monoenoic fatty acids were added to
incubation mixtures containing [14C]palmitate and all cofactors,
neither trans-2- nor trans-6-hexadecenoic acid had an effect on
the incorporation of palmitate into sphingadienine, as shown in
Table II.

Sphingolipid Long Chain Base Desaturating Activity of Oyster
Particulate Preparation—Samples of [14C]sphing-4-ene (11,100
dpm) and [14C]sphinganine (5,500 dpm) were incubated sepa-

dantly with the oyster particulate system in the presence of
NADH and FAD. The triacetate derivatives of unsaturated
bases were separated by argentation thin layer chromatography
and monitored with the Berthold scanner. There was no meas-
urable conversion of either substrate to sphingadienine. Fur-
thermore, the addition of unlabeled sphing-4-ene and sphinga-
nine to incubations containing [14C]palmitate had little effect on
the incorporation of label into sphingadienine (Table III).

3-Keto Long Chain Base Desaturating Activity in Oyster
Particulate Preparation—Unlabeled 3-ketosphinganine was added in
large excess along with [14C]palmitate to a reaction mixture lack-
ing NADPH. A partially purified 3-keto base mixture was
isolated and completely separated from the [14C]palmitate, and
was reincubated with oyster particulate system in the presence
of NADPH for 1 hour at 35°. A control experiment was carried
out without the unlabeled 3-ketosphinganine. Table IV shows
that incubation in the presence of excess unlabeled 3-ketosphinga-
nine inhibited the net incorporation of [14C]palmitate into sphing-
dienine by as much as 33% as compared to the control.

Incubation of 3-keto[14C]sphinganine (33,300 dpm) or 3-keto-
[14C]sphing-4-ene (8,900 dpm) was carried out with the oyster
particulate system in the presence of FAD, as described by Fu-
jino and Nakano (20). An argentation chromatogram of the
borohydride-reduced enzymatic products is illustrated in Fig. 7
and shows that the 3-keto forms of both sphinganine and sphing-4-
enine were converted in relatively high yields to sphingadienine.
There is a remote possibility that this conversion was nonen-
zymatic; insufficient labeled substrate precluded an experiment
with boiled enzyme.

Discussion

Our results indicate that oysters biosynthesize sphingolipid
long chain bases by the same general steps as yeast (H. ciferri)
and mammals (rat liver and brain) (1-17), since palmitate and
serine (except the carboxyl group) were incorporated effective-
ly into unsaturated bases and production of dihydroxy bases was
dependent on added NADPH. It is assumed that the major
product formed from labeled palmitate is the same dienic base described by Hayashi and Matsubara (22), sphinga-4,8-diene, but the position of the remote double bond was not determined on the biosynthetic product. The primary concern of this study, however, was to determine the point in the pathway at which double bonds are introduced, since previous studies indicated the possibility of desaturation at the stage of fatty acyl-CoA (12), a 3-keto intermediate (8, 14, 15, 20), and the long chain bases themselves (16, 19).

DiMari et al. (12) suggested that fatty acyl-CoA is desaturated and that trans-2-hexadecenoate is utilized in the biosynthesis of sphing-4-ene in yeast. If one assumes that similar monoenoic and dienoic acyl-CoA derivatives are precursors of the sphing-4-ene and sphinga-4,8-diene, respectively, the oyster enzyme system should be capable of desaturating palmitate to trans-2-hexadecenoate, trans-trans-2,6-hexadecadienoate, and perhaps trans-6-hexadecenoate. However, trans-[14C]monoenoic fatty acids could not be detected after incubation of [14C]palmitate and cofactors with oyster microsomes, indicating that this enzyme preparation may not be capable of desaturating palmitate to the necessary unsaturated fatty acids. Neither trans-2- nor trans-6-hexadecenoic acid was available in a labeled form to test directly whether they are precursors of the oyster long chain bases. However, the unlabeled compounds were available and were used in incubation mixtures as “swamping” agents, with no detectable dilution of the incorporation of [14C]palmitate into sphingadienine. This experiment has validity providing the rate-limiting step in the conversion of palmitate to the long chain bases is not the reaction forming palmitoyl-CoA. Separate experiments were not performed to determine whether the CoA derivative could be converted to trans unsaturated acids. The dilution experiments provide only tentative evidence, therefore, but it appears that the desaturation steps do not occur at the fatty acyl-CoA level.

The existence of a sphinganine desaturating system in rats has been suggested by results reported by Stoffel et al. (16, 19) who used doubly labeled sphinganine with H2 on carbon 4 to exclude formation of 3-ketosphinganine as an intermediate. Our results with oyster microsomal preparotions are not in agreement with this mechanism, however, since we were not able to demonstrate the direct conversion of [14C]sphinganine or [14C]sphing-4-ene into sphingadienine. Furthermore, excess unlabeled sphinganine had little effect on the incorporation of [14C]palmitate into either the mono- or diunsaturated base. Excess unlabeled sphing-4-ene did not decrease the incorporation of [14C]palmitate into sphingadienine but the label in sphing-4-ene was only about 60% of control values, perhaps as a result of some product inhibition by the sphing-4-ene added to the incubations. These results indicate that oysters are unable to carry out direct desaturation of dihydroxy long chain bases.

Evidence supporting desaturation of 3-keto bases has been reported (8, 14, 15), and recently Fujino and Nakano (20) have demonstrated the conversion of 3-keto[14C]sphinganine to labeled 3-ketosphing-4-ene with a particular fraction from rats incubated with FAD but not NADPH. If a similar mechanism were involved in the reactions carried out by oyster microsomes, one would expect to find olefinic 3-keto bases in incubation mixtures lacking NADPH. Purification of the 3-keto bases from such incubations and analysis of the N-acetyl methoxime-trimethylsilyl derivatives by gas chromatography-mass spectrometry indicated that both monoene and diene bases were indeed present as the 3-keto forms. In addition, incubations with [14C]palmitate in the absence of NADPH gave labeled monoene and diene 3-keto bases. Further evidence for their involvement was the finding that a large excess of unlabeled 3-ketosphinganine decreased the incorporation of [14C]palmitate into these unsaturated 3-keto bases in the absence of NADPH. Finally, the oyster particulate preparation converted both 3-keto[14C]sphinganine and 3-keto[14C]sphing-4-ene to labeled 3-ketosphingadienine in relatively high yields. We have concluded from these studies that both of the trans double bonds of sphingadienine in the oyster are introduced in reactions involving the 3-keto intermediates.

The desaturation that yields a trans-D-3-keto base is quite analogous to the formation of the trans double bond at carbon 2 in the β oxidation of fatty acids, as shown below:

\[
\begin{align*}
&\text{H} & \text{H} & \text{O} & \rightarrow & \text{H} & \text{H} & \text{O} \\
&\text{R} & & & & & & &\text{R} \\
&\text{X} & & & & & & &\text{X}
\end{align*}
\]

In fatty acid oxidations the double bond is introduced by a flavin-requiring dehydrogenase and oxygen is not required. Similarly, a dehydrogenase is assumed to be involved in forming the trans-Δ4-olefine group in sphing-4-ene and sphinga-4,8-diene, and previous studies indicate that a flavoprotein is involved in the reaction (5, 6, 16, 19, 20).

The mechanism for the introduction of the isolated trans double bond at carbon 8 in sphingadienine of oysters does not presently have a close analogy in fatty acid desaturations. The conversion of stearate to oleate in microorganisms (35, 36) as well as higher animals (37–40) and plants (41, 42), requires molecular oxygen and some form of electron acceptor and transport chain that has not been completely defined. The newly formed double bond in the unsaturated fatty acid has the cis configuration. Unsaturated fatty acids formed by anaerobic microorganisms have cis double bonds, and are introduced at the level of C18 by a specific dehydratase which catalyzes the conversion of 3-hydroxydecanoate to trans-2- and cis-3-decanoate, and the latter compound is chain elongated to cis-9-hexadecenoate and cis-11-octadecenoate (43, 44). It is not likely that either of these mechanisms will be involved in the formation of sphingadienine of oysters although it has not been determined whether molecular oxygen is required for the reaction. We did not attempt to determine whether FAD was obligatory for the conversion of 3-ketosphing-4-ene to 3-ketosphinga-4,8-diene, and cannot therefore be certain that a flavoprotein dehydrogenase is involved.

It has been observed in several laboratories that the monoenoic fatty acids of some sphingolipids might consist of a mixture of cis and trans stereoisomers (45, 46). Perhaps the trans constituents are synthesized by a pathway similar to that involved in the introduction of isolated trans double bonds in the marine sphingadienines. It is interesting to note, in this connection, that the sphingadienines do not universally have trans double bonds. The Δ4 double bond in sphinga-4,14-diene from human plasma sphingomyelin has the cis configuration (21). The mechanism of biosynthesis of this compound has not been studied to our knowledge. If the cis double bond is formed at the fatty acyl-CoA stage by a typical oxygen-requiring fatty acid desaturase, a heretofore unreported fatty acid, cis-12-hexadecenoate, or perhaps trans-2-cis-12-hexadecadienoate, would be involved. Alternatively, if desaturation of the 3-keto intermediates or sphing-4-ene occurs, it is likely to be a somewhat different
mechanism than that of oysters to account for the different stereochemistry of the newly formed double bond.

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Biosynthesis of Unsaturated Sphingolipid Bases by Microsomal Preparations from Oysters
Ray K. Hammond and Charles C. Sweeley


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