*THE BIOSYNTHESIS OF DIHYDROSPHINGOSINE IN CELL-FREE PREPARATIONS OF HANSENULA CIFERRI**

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Studies of the biosynthesis of sphingosine and dihydrosphingosine in whole animals[1-4] and in brain tissue homogenates[5-8] indicate that the first two carbon atoms of these compounds are derived from serine while the remainder of the molecule is derived from palmitic acid or palmitaldehyde. Brady, Formica, and Koval[6] reported that pyridoxal phosphate (PLP), Mn^{++}, nicotinamide, cytidine 5′-diphosphate (CDP)-choline, and several other cofactors stimulated the synthesis of sphingosine from serine in cell-free particulate preparations of rat brain. In subsequent experiments by these and other investigators, PLP and Mn^{++} were routinely added to the reaction mixtures.[9] Even though several PLP-dependent mechanisms for the condensation of serine with a 16-carbon derivative have been suggested,[4-6] a requirement for this coenzyme in this specific reaction has never been clearly demonstrated.

The very low rate of sphingolipid base synthesis in brain extracts has directed attention to the yeast *Hansenula ciferri* as a potential source of an enzyme system capable of synthesizing sphingolipids at a higher rate.[10] This yeast is able to produce large quantities of acetylated phytosphingosine and, to a lesser extent, acetylated dihydrosphingosine.[11-14] In experiments with intact cells and radioactive precursors, Green, Kaneshiro, and Law[10] showed that phytosphingosine and dihydrosphingosine are derived from serine and palmitic acid, presumably by a pathway resembling that found in brain tissue. These investigators, however, were unable to demonstrate the formation of these lipids *in vitro*. More recently, Haskell and Snell[15] showed that the vitamin B_6-deficient yeast, *Hanseniaspora valbyensis*, contained lowered amounts of phytosphingosine, thus providing nutritional evidence for a role of this vitamin in sphingolipid biosynthesis.

The postulated role for pyridoxal phosphate in the synthesis of sphingolipids is unusual for PLP-enzymes in that the serine carboxyl is replaced by a carbon chain derived from palmitate rather than by a hydrogen atom. We have therefore investigated the enzymatic aspects of this reaction, and present herein experiments that demonstrate synthesis of dihydrosphingosine from palmityl-CoA and serine by a cell-free particulate fraction of *H. ciferri*, and that PLP is required in the condensation reaction.

Experimental Procedures.—Materials: Substrates and cofactors not specified below were commercially available products. Palmityl-CoA was synthesized according to the procedure of Seubert.[16] Chromatographic samples of authentic sphingolipid bases were gifts from Dr. H. E. Carter.

Growth of organisms and preparation of enzyme: Hansenula ciferri (NRRL Y-1031, mating type F-60-10), provided through the courtesy of Dr. L. J. Wickerham, was grown in the New Brunswick fermentor on yeast maintenance medium[11, 14] containing 20 gm of glucose per liter. An aeration rate of 5 liters per minute and a temperature of 25° provided an optimal enzyme yield when the cells were harvested at 36 hr. The fresh cells were washed twice with 10 volumes of cold, distilled water, then suspended in 2 volumes of 0.05 *M* potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) and disrupted in an Aminco pressure cell at a pressure of 20,000 psi. Whole

cells, debris, and the mitochondrial fraction were sedimented by centrifugation at 14,500 × *g* for 30 min.[17] The supernatant solution was centrifuged at 100,000 × *g* for 90 min to sediment the "particulate" fraction which contained nearly all of the sphingolipid-synthesizing ability of the cell. The well-drained pellet was suspended in the phosphate-dithiothreitol solution (described above) with the aid of a manual glass homogenizer. The final protein concentration was approximately 15 mg per ml.

Assay for sphingolipid synthesis: Sphingolipid synthesis was followed by the incorporation of radioactive serine into ether-extractable, nonsaponifiable lipids. The composition of the enzyme reaction mixtures is given in the tables. After shaking for 1 hr at 30° in a rotatory shaking bath, the reaction was terminated by the addition of an equal volume of 1 *N* methanolic KOH and the mixture was saponified for 10 min at 50°. Sphingolipids were removed by three extractions with an equal volume of ether. Ether extracts were washed until neutral and dried over anhydrous $MgSO_4$. Volumes were reduced to approximately 0.2 ml by shaking the tubes in warm water. Scintillation fluid was added and the samples were counted in a Packard Tri-Carb counter. A correction for "background" counts, determined from a control reaction mixture containing boiled enzyme, was applied to all data. The values varied from 300 to 1000 dpm, depending on the amount of ether-extractable impurity in various lots of serine-C^{14}. Counting efficiency was determined with an internal standard.

Chromatography and autoradiography: Ether-extractable lipids were chromatographed on activated (100°, 30 min) thin layers of silica gel G (Merck) using solvent systems consisting of $CHCl_3$—CH_3OH—H_2O (65:25:4) and $CHCl_3$—CH_3OH—2 *N* NH_4OH (40:10:1).[18] Both systems gave adequate separation of the sphingolipid bases. Spots were visualized by spraying with ninhydrin[18] or with 50% H_2SO_4 and charring in a hot oven. Radioactive compounds were detected by autoradiography on Kodak No-Screen X-ray film.

Results and Discussion.—Location and stability of the sphingolipid-synthesizing system: The enzyme system which catalyzes incorporation of serine-3-C^{14} into sphingolipids was confined largely to a particulate fraction of the cell extract which sedimented between 14,500 × *g* and 100,000 × *g* (Table 1). Very low activity was found in an extract from which only whole cells and cell wall material had been removed, possibly as a consequence of other reactions involving the substrates used. The active particulate enzyme could be demonstrated only in freshly harvested yeast cells. When the washed cell paste was stored for only a few hours at 2° or in the frozen state prior to disruption, we were unable to obtain enzymatically active preparations. Once separated from other cellular components, however, the particulate cell fraction retained its enzymatic activity for at least five days when stored at 0–2° in the presence of 1 mM dithiothreitol.

Under optimal conditions, the most active particulate enzyme fraction was able to incorporate 7.2 mμmoles of serine-3-C^{14} per mg of protein per hour, or in terms

TABLE 1

INCORPORATION OF SERINE INTO ETHER-EXTRACTABLE SPHINGOLIPIDS BY CELL FRACTIONS FROM *H. ciferri*

Cell Fraction		C^{14}-Incorporation into sphingolipid (dpm/mg protein)
Centrifugal force	Fraction	
3300 × *g*	Supernatant fluid	25
14,500 × *g*	Pellet (mitochondrial fraction)	200
14,500 × *g*	Supernatant fluid	65
100,000 × *g*	Pellet	2330
100,000 × *g*	Supernatant fluid	0

Fresh cells were disrupted as described in *Methods*. Unbroken cells and large fragments were removed by centrifuging at 3300 × *g* for 10 min. The supernatant solution was centrifuged at 14,500 × *g* (30 min), then at 100,000 × *g* (90 min). The pellets were carefully drained and uniformly suspended (see *Methods*).

Each assay tube contained, in a total volume of 2.0 ml: DL-serine 3-C^{14} (6 mM, 6 μc), palmityl-CoA (0.25 mM), dithiothreitol (0.5 mM), Cutscum (isooctylphenoxypolyoxyethanol) (0.5 mg), NaCl (0.05 *M*), glucose 6-phosphate (0.5 mM), TPN^+ (0.5 mM), MgCl (0.4 mM), glucose 6-phosphate dehydrogenase (4 units), phosphate buffer (0.1 M), and protein (2 to 7 mg). The pH was 7.4.

of radioactivity, 8000 dpm per mg of protein per hour. This activity is approximately 10 to 20 times greater than that reported in brain microsomal preparations.[6]

Nature of the sphingolipid formed: Ether extracts containing the C^{14}-labeled product (10^4 to 2×10^4 dpm) of the enzymatic reaction were applied to thin layers of Silica Gel G. The chromatograms were developed and autoradiograms prepared (see *Methods*). After exposure for 72 hours, two radioactive spots were evident—a prominent spot corresponding in R_f value to dihydrosphingosine, and a spot which moved slightly ahead of N-acetyl sphingolipid base on the chromatogram. Although the faster-moving component has not yet been identified, an N-palmityl base might be expected to chromatograph in this fashion in the two solvent systems used.

A similar C^{14}-sphingolipid extract was hydrolyzed in aqueous methanolic HCl, a procedure known to cleave N-acyl groups of cerebrosides and ceramides.[19] Chromatography on silica gel and radioautography showed a single dark spot which had an R_f value identical to that of dihydrosphingosine. A ninhydrin-positive spot of the same size and shape was observed on the chromatogram. These observations suggest that dihydrosphingosine is the only sphingolipid base formed under the reaction conditions cited and that this compound may be rapidly acylated. It is pertinent to note that dihydrosphingosine and phytosphingosine are excreted by *H. ciferri* in an acetylated form. Experiments are in progress to characterize these products more fully and to determine the configuration of the base.

Substrate and coenzyme requirements for sphingolipid synthesis: Palmityl-CoA appeared to be the preferred substrate in the condensation reaction, although sodium palmitate in the presence of ATP and coenzyme A was nearly as effective (Table 2, expt. 1). Palmitaldehyde, alone or in the presence of DPN^+, DPNH, TPN^+, or TPNH, was unable to promote the incorporation of serine-C^{14} into sphingolipids. However, in the presence of TPNH, ATP, and CoA, this aldehyde was as efficient as palmitate in stimulating C^{14}-incorporation (Table 2, expt. 2). That this effect was not due to ATP or CoA alone is evident from experiment 3. It is difficult to visualize a biochemical reaction other than the formation of a thioester in which palmitaldehyde and both ATP and CoA are involved. Moreover, TPN^+ accelerated the reaction with palmitaldehyde nearly threefold (expt. 4), an observation one might expect if the aldehyde is first dehydrogenated to the corresponding acid, then activated to yield palmityl-CoA. If such a reaction occurs, the TPNH formed could satisfy the nucleotide requirement for the synthesis of dihydrosphingosine. Although Brady *et al.*[6] reported that palmitaldehyde served as the primary long-chain substrate for the synthesis of dihydrosphingosine and sphingosine in brain extracts, our data strongly suggest that palmityl-CoA is the more direct precursor of dihydrosphingosine in yeast.

The requirement for TPNH in the conversion of palmityl-CoA to dihydrosphingosine is specific (Table 2, expt. 5); DPNH is ineffective.

The incorporation of serine into the ether-extractable sphingolipids was proportional to palmityl-CoA at low concentrations (Fig. 1). Under the conditions used here, palmityl-CoA inhibited the condensation reaction at concentrations of 0.25–0.4 mM. This effect was not unexpected in view of the frequently observed inhibition of enzymes by micellar substrates. A similar inhibitory effect of palmityl-CoA on phosphatidic acid synthesis in yeast has been reported by Kuhn and Lynen.[20]

TABLE 2

EFFECT OF PALMITYL-CoA, SODIUM PALMITATE, PALMITALDEHYDE, AND VARIOUS COFACTORS ON THE CONVERSION OF SERINE-C^{14} TO SPHINGOLIPIDS

Experiment	Additions to reaction mixtures	C^{14}-Incorporation into sphingolipids (dpm/mg protein)
1	Palmityl-CoA (0.35 mM) + TPNH-GS*	8310
	Sodium palmitate (0.5 mM) + TPNH-GS	135
	+ ATP (1 mM)	252
	+ CoA (0.25 mM)	440
	+ ATP (1 mM) + CoA (0.25 mM)	6125
2	Palmitaldehyde (0.25 mM)	52
	+ TPNH (1 mM)	56
	+ DPNH (1 mM)	40
	+ DPN^+ (0.5 mM)	28
	+ TPN^+ (0.5 mM)	52
	+ TPNH (1 mM) + $MnCl_2$ (0.5 mM)	74
	+ TPNH-GS + ATP (1 mM) + CoA (0.25 mM)	6218
3	Palmitaldehyde (0.25 mM) + TPNH-GS	—
	+ ATP (1 mM)	560
	+ CoA (0.25 mM)	980
	+ ATP (1 mM) + CoA (0.25 mM)	4406
4	Palmitaldehyde (0.25 mM) + ATP (1 mM) + CoA (0.25 mM)	1382
	+ TPN^+ (0.5 mM)	3742
	+ TPNH-GS	4406
5	Palmityl-CoA (0.25 mM)	130
	+ TPNH-GS	1607
	+ TPN^+ (0.5 mM)	337
	+ DPN^+ (0.5 mM)	236
	+ DPNH (1 mM)	140

* The TPNH-generating system (TPNH-GS) was composed of TPN^+ (0.5 mM), glucose 6-phosphate (0.5 mM), and glucose 6-phosphate dehydrogenase (6 units).

The incubation mixtures (2.0 ml) contained substrates and cofactors as indicated, together with potassium phosphate buffer (0.1 *M*), dithiothreitol (0.5 mM), $MgCl_2$ (0.4 mM), DL-serine-3-C^{14} (6 mM, 6 μc), and 4–5 mg protein (100,000 × *g* pellet; see *Methods*). When palmitaldehyde was present, the reaction mixtures also contained 0.5 mg Cutscum. The pH was 7.5. All tubes were incubated for 1 hr at 30°.

The dependence of enzyme activity on the serine concentration appeared to follow first-order kinetics; a saturating concentration (6 mM) was used in all experiments described here. The incorporation of serine occurred optimally at pH 7.3–7.5 in phosphate buffer. The presence of a sulfhydryl reducing agent was mandatory.

Requirement for pyridoxal phosphate: The addition of 0.5 mM PLP to the reaction mixture frequently, but not always, resulted in 10 to 40 per cent stimulation of enzyme activity (Table 3). Cysteine and L-penicillamine form thiazolidine derivatives with PLP,[21] and these compounds have previously been used as inhibitors of various PLP-enzymes.[22] When the active fraction from yeast was treated with 0.05 *M* cysteine for two hours in the cold, then washed to remove free cysteine, 80 per cent of its activity was lost (Table 3). Addition of PLP (0.5 mM) restored enzyme activity almost to control values.

These data demonstrate that the role of vitamin B_6 in formation of sphingolipid bases in yeast cells, previously indicated by nutritional studies,[16] is a direct one. As a hypothesis for further testing, we visualize PLP as being required for the direct enzymatic condensation of palmityl-CoA and serine, with displacement of the carboxyl group of serine and the CoA moiety of palmityl-CoA to yield, either free or enzyme-bound, a 3-keto intermediate which is reduced by TPNH to form dihydrosphingosine. The condensation reaction *per se* would thus be entirely analogous to the formation of δ-amino-levulinic acid from succinyl-CoA and glycine.[23, 24] Weiss[4] has also suggested the possible occurrence of a long-chain keto intermediate in formation of sphingosine in rats.

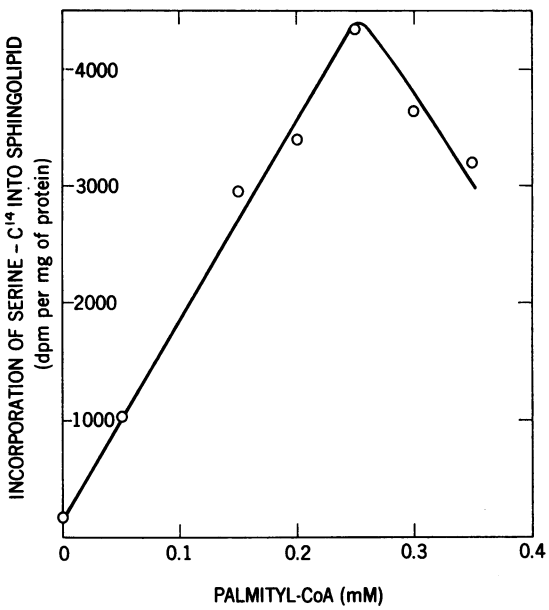

FIG. 1.—Effect of palmityl-CoA concentration on the incorporation of serine-C^{14} into sphingolipid by a particulate fraction of *H. ciferri*. The reaction mixture consisted of DL-serine-3-C^{14} (6 mM, 6 μc), palmityl-CoA, TPN^+ (0.5 mM), glucose 6-phosphate (0.5 mM), glucose 6-phosphate dehydrogenase (6 units), $MgCl_2$ (0.4 mM), dithiothreitol (0.5 mM), and phosphate buffer (0.1 *M*, pH 7.4) in a final volume of 2.0 ml.

The data reported here indicate that *H. ciferri* is a suitable organism from which to obtain enzyme preparations for studies on the reaction sequence and the mechanism of sphingolipid base formation. The origin of the extra hydroxyl group in phytosphingosine remains an interesting question. Investigation concerned with these several problems are in progress.

Summary.—Procedures are described for preparation of a cell-free particulate fraction of *Hansenula ciferri* which synthesizes appreciable quantities of dihydrosphingosine from serine and palmityl-CoA. Palmityl-CoA, rather than palmitaldehyde, appears to be the substrate for the enzymatic condensation reaction.

TABLE 3

REQUIREMENT FOR PYRIDOXAL PHOSPHATE IN THE ENZYMATIC SYNTHESIS OF DIHYDROSPHINGOSINE

Additions	C^{14}-Incorporation into sphingolipid (dpm/mg protein)
Untreated enzyme	3100
Untreated enzyme + PLP (0.5 mM)	4840
Cysteine-treated enzyme	716
Cysteine-treated enzyme + PLP (0.5 mM)	3900

Particulate enzyme (30 mg) in 3 ml of 50 mM potassium phosphate, pH 7, containing 1 mM dithiothreitol was mixed with 0.3 ml of 0.5 *M* cysteine (pH 7) and held for 2 hr at 0°. The enzyme was removed from the cysteine solution by centrifuging at 190,000 × *g* for 1 hr. The pellet was drained well and resuspended in phosphate-dithiothreitol buffer and centrifuged. This washing procedure was repeated once to remove the remaining cysteine and the enzyme was resuspended for assay. The reaction mixture was the same as that described in Fig. 1; palmityl-CoA was present in a concentration of 0.25 mM.

TPNH is required specifically for the reaction, but no other added cofactor appears to be essential. The enzyme preparation is inactivated by treatment with cysteine. Activity is restored by addition of pyridoxal phosphate, thus demonstrating conclusively the requirement for pyridoxal phosphate in the enzymatic synthesis of a sphingolipid base.

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