Biosynthesis of the sphingoid bases: a provocation

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Although the investigation of sphingolipid biosynthesis began over 35 years ago, there are still fuzzy spaces in our list of enzymes required for the process. The enzymes that *have* been uncovered have received only the simplest of characterizations. One goal of this review is to indicate to uncommitted young biochemists who are casting about for a research field—and to mature biochemists—that intermediary metabolism can still be a fruitful area.

A warning to the beginner: lipoidal bases react relatively readily with active metabolites (such as CoA thio esters) and it is essential to run boiled enzyme incubations at more than one level, or poisoned incubations, to prove that an enzyme reaction is indeed occurring. Try to find the blank values in published works!

A list of real or possible enzyme reactions in the biosynthesis of the sphingols is given in **Table 1.** (The name "sphingol" is proposed as a less redundant replacement for "sphingoid base.") No one questions that a fatty acyl-CoA condenses with serine to form a 3-keto sphingol (*reaction* 2, 4, or 6). What *is* uncertain is how the 4,5-double bond gets in. Phytosphingosine poses an additional puzzle, i.e., the mechanism of insertion of the C-4 oxygen atom. Probably the most recent reviews on this topic are by Kanfer (1) and Kishimoto (2).

Condensation with serine (*reaction* 2)

Most studies have used L-serine and palmitoyl-CoA (or a similar fatty acyl thioester). Carbon dioxide is lost from the serine and a keto amino fatty alcohol is formed, with retention of the C-2 configuration of serine. The 18-carbon sphingol is commonly named 3-ketodihydrosphingosine (KDS, 3-ketosphinganine, 3-dehydrosphinganine, or 3-keto-2-amino-octadecanol-1). The ketone has been thoroughly characterized chemically, although no one has shown directly that it has the natural D-configuration. The possible existence of a racemase has to be eliminated.

The enzyme catalyzing this condensation contains pyridoxal phosphate and has been purified more than 100-fold from an anaerobic bacterium (3). The synthase has been found in the very small particles of a yeast (4, 5), liver (6), and brain (7).

Vitamin K_1 was needed by the bacteria during growth in order to make the enzyme (8). Adding the vitamin to depleted cells rapidly induced formation of the enzyme. At present, the only known role for this vitamin is in the gamma-carboxylation of certain glutamic acid side chains in certain proteins. These proteins bind calcium strongly, due to the presence of the malonic acid groups. It is possible that *1*) the synthase needs this kind of amino acid residue for activity, or *2*) vitamin K_1 is needed to carboxylate palmitoyl-CoA. For the first possibility, one would expect that calcium ions are needed for KDS synthesis, but no one has noticed this need (perhaps for lack of trying). To support the second possibility, I point to the importance of carboxylation in the first step of fatty acid biosynthesis, the formation of malonyl-CoA. It would surely be interesting to test labeled or unlabeled 2-carboxy palmitoyl-CoA, as well as the effect of bicarbonate and calcium.

Another corollary thought: does the rodenticide, warfarin, act in any way through a blockade of KDS synthesis? This coumarin derivative is a known interfering agent in vitamin K metabolism, acting to cause fatal internal bleeding by blocking carboxylation of prothrombin and Factor X precursors. The relevant association is this: antithrombin III, an inhibitor of blood clotting, was reported to contain a significant amount of glucosyl ceramide (9). If warfarin-treated rodents are unable to synthesize glucocerebroside, the plasma level of antithrombin III might be decreased, reducing its inhibitory control of clotting. Thus vitamin K may act on the clotting phenomenon in two ways. (Of course this possibility also bears on anticoagulant therapy with warfarin in humans.)

This hypothesis could be tested by measuring the level of antithrombin III, and its cerebroside, in warfarin-treated animals. In fact, the initial observation that cerebroside is present in the protein ought to be confirmed.

Abbreviations: KDS, 3-ketodihydrosphingosine; TLC, thin-layer chromatography.

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TABLE 1. Routes of sphingoid base biosynthesis

(1) $R–CH_2–CH_2–CO–SCoA$ (I) $- 2H \rightarrow R–CH=CH–CO–SCoA$ (II)
(2) I + $HOOC–CH(NH_2)–CH_2OH \rightarrow CO_2 + R–CH_2–CH_2–C(=O)–CH(NH_2)–CH_2OH$ (KDS)
(3) I + $CO_2 \rightarrow$ $R–CH_2–CH(COOH)–CO–SCoA$ (III)
(4) III + serine $\rightarrow 2CO_2$ + KDS
(5) KDS $- 2H \rightarrow R–CH=CH–C(=O)–CH(NH_2)–CH_2OH$ ketosphingosine (IV)
(6) II + serine $\rightarrow$ IV
(7) KDS + NADPH + $H^+ \rightarrow$ dihydrosphingosine or sphinganine (V)
(8) IV + NADPH + $H^+ \rightarrow$ sphingosine or sphingenine (VI)
(9) fatty acyl-CoA + IV or V or KDS or VI $\rightarrow$ keto ceramide or ceramide
(10) keto ceramide + NADPh $\rightarrow$ ceramide (saturated or unsaturated)
(11) N-acyl V $- 2$ H $\rightarrow$ N-acyl VI (acyl sphingosine)
(12) ceramide + H_2O (ceramidase) $\rightarrow$ sphingoid base + fatty acid
(13) $R–CH_2–CH(OH)–CH(NH_2)–CH_2OH$ (V) + $O_2 \rightarrow R–CH(OH)–CH(OH)–CH(NH_2)–CH_2OH$ phytosphingosine
(14) $R–CH=CH–CH(OH)–CH(NH_2)–CH_2OH$ (VI) + H_2O

Williams, Wang, and Merrill (10) have developed a simplified (but expensive) assay for KDS synthesis in rat liver microsomes and have compared different fatty acyl-CoA's as substrates.

Reduction of the ketone group (*reaction* 7)

The reduction of DL-KDS with NADPH has been found to produce primarily or only the 2D, 3D (*erythro*) diol (11). From this, from the fact that L-serine is utilized for the condensation, and from other stereochemical experiments, one might conclude that KDS is formed by the synthase only as the 2D-compound. However, a direct proof has not been offered. This might be done by treating biosynthetic and racemic KDS with an optically active acyl chloride and separating the diastereoisomers by TLC. The chemically synthesized KDS should yield two spots and the naturally formed KDS should yield only one.

Two other possible reduction steps, *reactions* 8 and 10, have not been demonstrated but seem very plausible.

Dehydrogenation of 3-keto sphinganine (*reaction* 5)

A study (12) seemed to show that a flavoenzyme is involved in the direct conversion of KDS to ketosphingenine. After incubating rat liver microsomes with radioactive KDS, Triton, Mg^{2+}, and FAD or FMN, the authors added "a small amount of" carrier sphingenine and other sphingolipids and acetylated the total lipids to convert the sphingols to amides. Fractionation of the lipids with a silica gel column yielded several radioactive peaks. The N-acetyl keto bases eluted with chloroform–methanol 99:1 and labeled ceramides (naturally occurring long chain acyl sphingols) eluted as a subsequent peak with a surprisingly polar solvent, chloroform–methanol 95:5. Normally, ceramides elute with chloroform–methanol 99:1. If these lipids were indeed ceramides, they could have been made from labeled sphingols (not ketosphingenine) and endogenous fatty acids. Another possible explanation is that the "ceramide" peak was actually N-acetyl sphingenine, from labeled sphingenine that could have been formed by reduction of ketosphingenine with endogenous NADPH. An accurate TLC examination should have distinguished between long chain ceramide and acetyl sphingenine.

Examination of the ketone fraction by TLC and radioautography showed, by comparison of standards, that both acetyl KDS and acetyl ketosphingenine were radioactive (11). If a flavin was omitted from the incubation system, the latter was not labeled. The identity of the N-acetyl ketosphingenine was confirmed additionally by reduction of the carbonyl group with borohydride and radiochromatography of the product, which seemed to contain only labeled acetyl sphingenine. Standard spots from sphingenine and sphinganine amides were well separated. An unexpected feature of the TLC plate is that it showed no spot for the *threo* acetyl bases, which presumably form with the *erythro* compounds during the borohydride reduction. However it is possible that the acetylated bases could not separate with the TLC system used here.

One possible explanation for the findings is that the labeled KDS was first acylated by an endogenous fatty acid donor, forming N-acyl KDS ("keto ceramide," *reaction* 9). This could then have been reduced by endogenous NADPH to ordinary ceramide (*reaction* 10). This may have been dehydrogenated at the 4,5-position (*reaction* 11), then hydrolyzed by ceramidase to sphingenine (*reaction* 12). I admit that this seems like a rather large number of steps, made additionally improbable by the absence of added NADPH in the incubation medium.

More evidence for direct dehydrogenation of the ketone came from a study of the biosynthesis of oyster sphingadienine, a sphingol with *two* double bonds (13).

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Oyster microsomes converted labeled palmitate into mono- (4*t*) and di- (4*t*,8*t*) unsaturated keto bases. It would be useful to look for an effect of flavins on this system.

Braun and Morell showed in my laboratory (7) that brain microsomes, with serine, [^{14}C]palmitoyl-CoA, and an NADPH-generating system, produced not only sphinganine, but also the two kinds of ceramides, N-acyl sphingenine and N-acyl sphinganine. The former amide appeared much faster than the latter during the early part of the incubation. This observation may indicate that KDS was reduced to sphinganine, a small part of which was acylated by the exogenous palmitoyl-CoA. Another part of the KDS was dehydrogenated to ketosphingenine, which was then reduced to sphingenine and rapidly acylated. An unfortunate weakness of this interpretation is that the experiment was not done also with labeled serine, so the possibility remains that the labeled acyl sphingenine actually was derived from labeled palmitoyl-CoA and endogenous sphingenine, liberated by ceramidase from microsomal ceramide.

Dehydrogenation of ceramide

We found that KDS is readily acylated enzymatically in vitro by stearoyl-CoA to form "keto ceramide" (14) and raised the possibility that KDS is normally acylated immediately after formation, then reduced at the ketone site to acyl sphinganine (*reactions* 9 + 10). Shoyama and Kishimoto (15) investigated this idea by injecting rats intracardially with [^{14}C]lignoceroyl [^{3}H]ketosphingenine. The (nonhydroxy) ceramides isolated from liver contained both isotopes in the original ratio, indicating that a direct reduction of keto ceramide (*reaction* 10) had occurred. In view of the ease with which the reduction of KDS can be demonstrated in vitro (5, 16), it should not be difficult to demonstrate the reduction of the keto ceramide. Indeed, both reductions might involve the same enzyme.

Ong and Brady (17) injected [^{14}C]stearoyl [^{3}H]sphinganine directly into rat brains. Isolation of the brain N-acyl sphingenines produced material with almost the original isotopic ratio, indicating that direct dehydrogenation of the saturated ceramide had occurred (*reaction* 11). A very similar study and observation was made by Stoffel and Bister (18).

It might be worth repeating these studies with stearoyl sphinganine labeled in both moieties with deuterium, and isolating the stearoyl sphingenine by reverse phase high performance liquid chromatography. Examination of the ceramide by mass spectrometry should show a molecular ion only two units lighter if the linkage between the two moieties truly remained intact.

The desaturatase should be sought in subcellular preparations with labeled ceramide. The existence of ceramidases certainly makes interpretations difficult, but perhaps they can be blocked with inhibitors.

Dehydrogenation prior to condensation (*reactions* 1 + 6)

It is possible that the double bond of sphingenine enters through dehydrogenation of palmitoyl-CoA. Yeast enzyme particles (19) readily converted labeled palmitate to *trans*-2-hexadecenoate, and utilized both acids in the CoA form for synthesizing a mixture of sphinganine and sphingenine. Can mammalian microsomal particles dehydrogenate palmitoyl-CoA similarly? This sequence of reactions seems to have dropped from favor, but further study is warranted with purified KDS synthase.

Inhibitors of sphingoid base synthesis

This topic has been virtually unstudied, although it is obvious that analogs of serine or acyl-CoA might block KDS synthesis. Very recently, a report has appeared showing that cycloserine is a potent, irreversible inhibitor of the bacterial and mouse brain synthases (20). Cycloserine, a naturally occurring antibiotic, is 4-amino-3-isoxazolidinone, a cyclic hydroxylamine derivative which presumably reacts, by ring opening, with the aldehyde group in pyridoxal phosphate (the cofactor in KDS synthase). While both the L- and D-isomers had similar inactivating power against microbial KDS synthase, the L-form was far more effective with the microsomes from brain (20).

Potentially important for the treatment of sphingolipidosis patients was the observation that the L-form was effective in vivo; a dose of 100 mg/kg produced an 85% loss of synthase activity in brain within 2 hr. Multiple injections with the inhibitor produced a decrease in the brain sphingolipids, gangliosides and galactocerebroside + sulfatide (analyzed together). Recovery of activity after a single injection was relatively fast and it would be interesting to discover whether this was due to normal synthesis of new enzyme molecules or to replacement of the derivatized coenzyme with pyridoxal phosphate from body stores.

Other serine analogs are available commercially and they ought to be tried too. One must hope for a substance that has a stronger affinity for the synthase than for the other serine-using enzymes. Cysteine is a competitive inhibitor versus serine (3) but also acts to remove the pyridoxal phosphate from the enzyme (10). Thiocyanate is a modest inhibitor of the bacterial synthase (3); if this is true of the mammalian enzyme too, salivary glands (which produce relatively concentrated thiocyanate ions) may have a low level or turnover of sphingolipids.

If my hypothesis about the action of warfarin is correct (see above), cycloserine ought to act as a synergist

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for the inhibitor in rodent poisoning and anticoagulant therapy.

Analogs of sphingols might be useful in blocking other enzymatic reactions of these lipids.

A recent paper on sphingomyelin formation in fibroblasts has shown that serum low density lipoprotein inhibits synthesis of this sphingolipid (21). Unfortunately, the authors refer to sphingol TLC spots as "free" long chain bases, so the casual reader might conclude from this paper that KDS synthase is affected by the lipoprotein. However, the data do not actually show this. This system ought to be studied by isolating KDS and ceramide.

Phytosphingosine biosynthesis

This sphingol (4-hydroxysphinganine) occurs in animals, as well as in plants. Because of its name and limited distribution in animal tissues, there was some question as to whether it is actually synthesized in animals. Assmann and Stoffel (22) showed that rats could incorporate labeled phytosphingosine into their sphingolipids when fed or injected with the base. Since a similar experiment (unpublished) with labeled sphingenine and sphinganine showed no conversion to phytosphingosine, they concluded that the base is not synthesized in the animal and that it must be obtained from the diet. However, Crossman and Hirschberg (23) injected germ-free rats with labeled sphinganine and found that the animals did indeed produce the hydroxylated base. Barring a nonenzymatic hydroxylation, this would seem to prove that there is an enzymatic conversion in animals. However, as these authors point out, the reaction may not be a direct hydroxylation of the intact base, since their injected [4,5-^{3}H]sphinganine may have been degraded in part to [2,3-^{3}H]palmitic acid (24), which could be incorporated into phytosphingosine by a different mechanism.

Experiments with ^{18}O water and oxygen in intact yeast (25, 26) seem to have demonstrated a direct hydroxylation step as the primary synthetic route, possibly a hydroxylation of sphinganine or a related compound (*reaction* 13). However, some oxygen entered phytosphingosine from labeled water, so there may be an addition of water to the double bond of sphingenine (*reaction* 14). Several workers have noted a preponderance of *2-hydroxy* fatty acids on the nitrogen atom of phytosphingosine lipids; it may be that the hydroxylating enzyme acts on both the base and the acid while they are in amide linkage.

OVERALL COMMENT: There seems to be a plethora of biosynthetic routes waiting for in vitro studies!

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