Enzymic Synthesis of Sphingosylphosphorylcholine

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SPC* was reported to be a precursor in the biosynthesis *in vitro* of sphingomyelin (Brady, Bradley, Young & Kaller, 1965). We confirmed the finding and demonstrated that *erythro*-SPC, the natural type of sphingolipid, and *threo*-SPC, the unnatural one, were both active as lipid acceptor in the enzymic reaction (Fujino & Negishi, 1968). Though the natural occurrence of SPC has not been clearly established (Zabin, 1960), it seemed of interest, in light of the above results, to investigate its biosynthesis. The present paper describes the synthesis of SPC by an enzymic reaction of sphingosine with CDP-choline in the particulate fractions of chicken liver.

erythro-Sphingosine was prepared by hydrolysis with methanolic 0.5N-KOH of triacetyl-sphingosine (m.p. 101–102°) (Fujino & Zabin, 1962), which had been derived from cerebrosides of ox brain by the procedures of Carter, Norris, Glick, Phillips & Harris (1947). A part of the preparation of *erythro*-sphingosine was then treated with acetic acid–10% H₂SO₄ (9:1, v/v) (Klenk, 1926) to produce a mixture of *N*-acetyl-*erythro*- and *N*-acetyl-*threo*-sphingosine, which was hydrolysed with methanolic 0.5N-KOH. Thin-layer chromatography of the resultant sphingosine bases with chloroform–methanol (4:1, v/v) on a silica gel G plate gave two spots, corresponding respectively to *erythro*-sphingosine and *threo*-sphingosine (Fujino & Zabin, 1962). *threo*-Sphingosine was recovered from the plate by scraping off the appropriate area and extracting it with methanol. *N*-Acetyl-*erythro*-sphingosine and *N*-acetyl-*threo*-sphingosine were chemically synthesized from the respective free sphingosine isomer by the methods of Carter *et al.* (1947).

Radioactive CDP-choline was enzymically prepared from CTP and phosphoryl[Me-¹⁴C]choline by the procedure of Kennedy & Weiss (1956). Standard SPC was prepared from sphingomyelin of horse spinal cord (Fujino & Negishi, 1959) by hydrolysis with butanolic HCl by a modification of Kaller's (1961) method.

A 150-day-old White Leghorn chicken was killed, and the liver was chilled to be minced. The tissue was homogenized in 10 vol. of 0.25M-sucrose containing 1mM-EDTA with a Potter–Elvehjem

homogenizer. Nuclei and debris were removed by centrifugation at 1000g for 10 min. Two particulate fractions, mitochondrial and microsomal, were obtained, the former by centrifuging the supernatant fluid at 10000g for 20 min. and the latter by centrifuging the supernatant suspension of the preceding fraction at 100000g for 30 min. Each particulate fraction was resuspended in two-thirds the original tissue volume of 0.25M-sucrose containing 1mM-EDTA, and kept frozen at –20°.

Sphingosine, labelled CDP-choline, the particulate fraction and cofactors were mixed and incubated as shown in Table 1. At the end of incubation, a small amount of SPC was added as carrier to the mixture, which was heated at 100° for 30 sec. to stop the enzymic reaction. The lipids in the mixture were extracted with portions of methanol, and subjected to mild alkaline hydrolysis followed by mild acidic hydrolysis according to the methods of Dawson (1960). The alkali- and acid-stable lipids were repeatedly extracted with chloroform, and the combined extracts were thoroughly washed with 2M-KCl and finally with water. Samples of the chloroform phase were applied to a thin-layer silica gel G plate. The

Table 1. *Enzymic formation of sphingosylphosphorylcholine from sphingosine and CDP-[Me-¹⁴C]choline*

Each tube contained 50 μmoles of tris-potassium phosphate (2:3) buffer, pH 7.0, 10 μmoles of MgCl₂, 1 mg. of Tween 20, 2 μmoles of sphingosine acceptor, 1.7 μmoles of CDP-[Me-¹⁴C]choline (2300 counts/min.) and 0.5 ml. of particulate fraction prepared from chicken liver in a final volume of 1.4 ml. The tubes were incubated at 37° for 2 hr.

Acceptors	Sphingosylphosphorylcholine synthesized (mμmoles)	
	Mitochondrial fraction	Microsomal fraction
<i>erythro</i> -Sphingosine	16.4	40.3
<i>N</i> -Acetyl- <i>erythro</i> -sphingosine	5.2	10.5
<i>threo</i> -Sphingosine	32.5	67.2
<i>N</i> -Acetyl- <i>threo</i> -sphingosine	10.2	17.0
No sphingosine, control	1.6	—
Sphingosine, boiled control	1.5	—

* Abbreviation: SPC, sphingosylphosphorylcholine.

chromatogram, obtained by development with chloroform-methanol-water (65:25:4, by vol.) and subsequent treatment with iodine vapour, was examined with the Radio Thin-Layer Chromatograph (Japan Radio Co. Ltd., Tokyo, Japan). The radioactivity of the SPC area on the plate was determined. The R_F value of 0.15 distinguished this area from those of the other choline-containing lipids that in fact had been eliminated from the chloroform phase after treatment with alkali and acid.

In a typical experiment (Table 1) both the substrates, *erythro*-sphingosine and *threo*-sphingosine, were active for the enzymic synthesis of SPC under the conditions used in the mitochondrial as well as in the microsomal fraction of chicken liver. Comparatively speaking, *threo*-sphingosine was more active than the *erythro* isomer as substrate, and the microsomal fraction possessed a higher activity than the mitochondrial as enzyme system. *N*-Acetylsphingosine isomers were in every case less active as substrates than the corresponding sphingosine isomers, suggesting that ceramide would be hydrolysed to sphingosine (Gatt, 1966) to become a precursor of SPC. The present findings, together with earlier work indicating that SPC may be converted into sphingomyelin (Brady *et al.* 1965; Fujino & Negishi, 1968), imply that the reaction sequence ceramide $\rightarrow$ sphingosine $\rightarrow$ SPC $\rightarrow$ sphingomyelin may have proceeded at least in the enzymic system tested.

It was confirmed by the following observations that the labelled substance was SPC: (1) when the radioactive product extracted from the plate was hydrolysed with methanolic 5% H_2SO_4 , and the hydrolysates were partitioned between water and ether, most of the initial radioactivity was recovered in the aqueous phase and no appreciable radioactivity appeared in the organic phase; (2) the labelled alkali- and acid-stable lipid, which was extracted without addition of carrier SPC from a large-scale incubation mixture, gave an infrared spectrum identical with that of the authentic sample of SPC.

These findings show that SPC was formed enzymically by the reaction of sphingosine with CDP-choline, conceivably according to the following equation, which is analogous to those known in the biosynthesis of choline-containing phospholipids (Kennedy & Weiss, 1956; Sribney & Kennedy, 1958; Kiyasu & Kennedy, 1960):

The enzyme that catalyses the synthetic reaction would be called, systematically, CDP-choline-sphingosine cholinephosphotransferase in accordance with recommendations of IUB Enzyme Commission, and phosphorylcholine-sphingosine transferase by analogy with designations by Kennedy & Weiss (1956) and Sribney & Kennedy (1958). The enzyme has a wide distribution in animal tissues, among which the liver has the highest activity.

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