Enzymic Synthesis of Cerebroside from Ceramide and Uridine Diphosphate Galactose

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It was reported by Brady (1962) that the formation of cerebroside (ceramide-galactose) occurred via a reaction between psychosine (sphingosine-galactose) and acyl-CoA in a microsomal preparation obtained from young rat brain. Two pathways are now known for the biosynthesis of sphingomyelin (ceramide-phosphorylcholine): one by transfer of acyl group from acyl-CoA to sphingosyl-phosphorylcholine (Brady, Bradley, Young & Kaller, 1965; Fujino & Negishi, 1968) and the other through transfer of the phosphorylcholine moiety from CDP-choline to ceramide (*N*-acylsphingosine) (Sribney & Kennedy, 1958; Fujino, Nakano, Negishi & Ito, 1968). Since the known pathway for the synthesis of cerebroside is analogous to the former synthetic reaction for sphingomyelin, it was decided to test whether an alternative pathway might exist for cerebroside formation. The present paper describes experiments indicating that cerebroside is synthesized from ceramide and UDP-galactose by an enzyme system prepared from animal tissue, particularly rat brain.

erythro-Sphingosine and *threo*-sphingosine were fractionated and purified by silicic acid column chromatography of the mixture of sphingosine bases, which had been obtained by methanolysis of phrenosine (Gaver & Sweeley, 1965). *N*-Acetyl-*erythro*- and *N*-acetyl-*threo*-sphingosine were prepared from *erythro*- and *threo*-sphingosine respectively by the acetylation procedure of Gaver & Sweeley (1966). *N*-Acetyl-*erythro*- and *N*-acetyl-*threo*-dihydrosphingosine were respectively derived from the corresponding unsaturated compounds by the hydrogenation technique described by Gatt & Rapport (1966). *N*-Palmitoyl-*erythro*- and *N*-palmitoyl-*threo*-sphingosine were synthesized from the respective free bases by the acylation method of Carter, Glick, Norris, Phillips & Harris (1947). Natural ceramide was isolated from ox spleen by solvent fractionation (Fränkel & Bielschowsky, 1932) and purified by Florisil column chromatography (Makita, 1964).

UDP-galactose and UDP-[U-¹⁴C]galactose (62500 c.p.m./ μ mole) were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and from New England Nuclear Corp. (Boston, Mass., U.S.A.) respectively.

The enzyme system was prepared from the brains of 15-day-old rats, largely according to the pro-

cedure of Brady *et al.* (1965). The tissue was homogenized with 4 vol. of 0.25 M-sucrose in a Potter-Elvehjem homogenizer. Nuclei and debris were removed by sedimentation at 600g for 12 min. Two particulate fractions, the mitochondrial and the microsomal, were separated, the former by centrifuging at 8600g for 12 min. and the latter by centrifuging the supernatant liquid from the preceding fraction at 100000g for 30 min. The particulate material from each fraction was resuspended in 0.1 M-potassium phosphate buffer, pH 7.8, equal to 0.2 vol. of the original homogenate.

Ceramide, labelled UDP-galactose, the tissue fraction and cofactors were mixed, emulsified and incubated, essentially as described by Fujino *et al.* (1968). At the end of incubation methanol was added to the mixture to stop the reaction, and a small amount of authentic cerebroside was also added as carrier. The lipids were repeatedly extracted from the mixture with chloroform-methanol (2:1, v/v), and the combined extracts were kept at 37° for 2 hr. with methanolic KOH (final concn. 0.4 M). Alkali-stable lipids were extracted with portions of chloroform from the incubated solution. The combined extracts were washed with 0.1 M-KCl and with water by the procedure of Kampine, Martensson, Yankee & Kanfer (1968). Samples of the chloroform phase were then introduced into a gas-flow counter to determine the radioactivity. As shown in Table 1, all ceramides tested, the synthetic and the natural, were active as substrate to form alkali-stable radioactive lipids. The *erythro*-ceramides (including natural ceramide), unsaturated ceramides and bound sphingosines were respectively more reactive than *threo*-ceramides, saturated ceramides and free sphingosines, especially in the microsomal fraction, which gave a higher enzymic activity than the mitochondrial fraction.

The following observations confirmed that the labelled substances were glycolipids, with ceramide-galactose dominating. (1) The alkali-stable radioactive lipids formed from natural ceramide were subjected to hydrolysis with HCl in methanol, and the hydrolysate was partitioned between light petroleum, diethyl ether and methanol. More than 80% of the initial radioactivity was found to be in the methanolic fraction containing methyl esters of the component sugar. (2) The alkali-stable lipids

Table 1. *Incorporation of UDP-[U-¹⁴C]galactose into alkali-stable lipids*

Each tube contained 100 μ moles of potassium phosphate buffer, pH 7.8, 4 μ moles of MnCl_2 , 20 μ moles of cysteine, 4 μ moles of ATP, 3 mg. of Tween 20, 2 mg. of Triton X-100, 4 μ moles of ceramide, 1 μ mole of UDP-[U-¹⁴C]galactose (625 000 c.p.m./ μ mole) and 0.25 ml. of particulate fraction prepared from rat brain homogenates in a final volume of 1.0 ml. The tubes were incubated at 37° for 2 hr.

Acceptor	Radioactivity incorporated (c.p.m.)	
	Mitochondrial fraction	Microsomal fraction
No acceptor (control)	—	215
<i>erythro</i> -Sphingosine	325	894
<i>threo</i> -Sphingosine	206	637
<i>N</i> -Acetyl- <i>erythro</i> -sphingosine	781	1565
<i>N</i> -Acetyl- <i>threo</i> -sphingosine	526	837
<i>N</i> -Acetyl- <i>erythro</i> -dihydro-sphingosine	459	974
<i>N</i> -Acetyl- <i>threo</i> -dihydro-sphingosine	367	661
<i>N</i> -Palmitoyl- <i>erythro</i> -sphingosine	462	819
<i>N</i> -Palmitoyl- <i>threo</i> -sphingosine	400	781
Natural ceramide from ox spleen	564	1045

highly labelled material in question was applied to a borate-impregnated silica-gel G plate (Kean, 1966), developed with chloroform-methanol-water (24:7:1, by vol.) and scanned for radioactivity. Two spots were detected on the plate: a highly radioactive one, moving slower, and a slightly radioactive one, moving faster. Comparison with the reference preparations showed that the slower-moving substance corresponded to ceramide-galactose and the faster-moving one to ceramide-glucose. (5) The most highly labelled material in question was collected from a larger-scale experiment and brought to the methanolysis stage. The sugar fraction obtained was developed with butanol-pyridine-water (6:4:3, by vol.) on paper (Suzuki & Chen, 1967), treated with AgNO_3 in alkali and examined by the radioscanner. Two spots appeared: one, strongly coloured and highly labelled, had R_F 0.32 corresponding to that of methylgalactose, and the other, faintly coloured and little labelled, possessed R_F 0.43 consistent with that of methylglucose.

These results show that, under the conditions used, the enzymic reaction of ceramide with UDP-galactose yielded mainly ceramide-galactose, together with small quantities of ceramide-glucose and possibly also complex glycolipids, presumably according to the following equations (Basu, Kaufman & Roseman, 1965, 1968; Hauser, 1967):

were applied to a silica-gel G plate, developed with chloroform-methanol-water (65:25:4, by vol.) and sprayed with the anthrone reagent. Spots of cerebroside and the other glycolipids were located on the plate. Radioscanning of the chromatogram showed several peaks, the largest one, equivalent to almost 70% of the total radioactivity, coinciding with the cerebroside location. (3) The most highly labelled material on the plate was scraped off and extracted with methanol. The extracts were applied to a silicic acid column, eluted with chloroform, chloroform-methanol (49:1, 19:1, 9:1 and 4:1, v/v) and methanol as described by Makita (1964) and Kampine *et al.* (1968), and the radioactivity of the fractions was determined. The elution curve for radioactivity gave two adjacent peaks, one remarkably large and the other fairly small, and the pattern was similar to that obtained from the column chromatography of the mixture of ceramide-galactose and ceramide-glucose. (4) The most

The enzyme system catalysing the synthetic reaction was also found in rat liver and chicken liver, but with a lower activity than that of rat brain. It is thus possible that the biosynthesis of cerebroside occurs in animal tissue either via acylation of psychosine (Cleland & Kennedy, 1960; Brady, 1962) or by way of reaction between ceramide and UDP-galactose, although which route is preferred is not yet clear.

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