THE ENZYMATIC SYNTHESIS OF CERAMIDE LACTOSIDE FROM CERAMIDE GLUCOSIDE AND UDP-GALACTOSE

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Ceramide lactoside occurs in a variety of normal and pathological tissues, such as spleen (Rapport et al., 1960; Makita and Yamakawa, 1962; Wagner, 1964; Suomi and Agranoff, 1965), kidney (Makita and Yamakawa, 1964; Martinsson, 1963, 1966), liver (Svennerholm and Svennerholm, 1963a), blood elements (serum (Svennerholm and Svennerholm, 1963b), erythrocytes (Klenk and Wolter, 1952; Klenk and Lauenstein, 1953; Yamakawa et al., 1962) and leucocytes (Miras et al., 1966)), Tay-Sachs brain (Suzuki and Chen, 1967), human epidermoid carcinoma (Rapport et al., 1959, 1961) and ascites sarcoma cells (Gray, 1965). It is one of a variety of sphingoglycolipids with different numbers of carbohydrate residues, the most complex of which are the gangliosides, themselves a heterogeneous group of lipids. Although investigations designed to elucidate the metabolic interrelationships between these compounds have demonstrated the existence of enzyme systems capable of catalyzing the degradation of ceramide lactoside (Gatt and Rapport, 1966) and the addition of either N-acetylneuraminic acid (Basu and Kaufman, 1965) or N-acetylgalactosamine (Handa and Burton, 1966) to the molecule, the step leading to its formation from the immediate precursor has not been reported. This communication provides evidence that the incorporation of radioactivity from UDP-galactose-1-^{3}H into a rat spleen homogenate is stimulated by ceramide glucoside and that the resulting product is ceramide lactoside.

METHODS- Whole homogenates of rat spleen in 0.3 M sucrose served as the enzyme source. Proteins were determined by the method of Lowry et al. (1951).

Ceramide glucoside was prepared by fractionation on Florisil of a washed total lipid extract from spleen tissue of a patient with Gaucher's disease, kindly made available by Dr. M. J. Malone. The lipids were eluted with chloroform, chloroform-methanol 19:1, 9:1, 4:1 and 2:1 (v/v) and fractions examined by thin-layer chromatography on plates of silica-gelG, prepared with sodium tetraborate (Young and Kanfer, 1965; Kean, 1966). Solvent systems used for glycolipid separation were chloroform-methanol-water (65:25:4), chloroform-methanol-acetic acid (4:1:0.1) and n-propanol-ammonia-water (7:2:1). Plates were sprayed with anthrone reagent, H_2SO_4 or ammonium molybdate (Wagner et al., 1961) or exposed to iodine vapors. Fractions containing only ceramide glucoside were combined and suitable aliquots dried directly in the tubes used in the incubations.

Two samples of ceramide lactoside were used. One, prepared by fractionation of the hydrolysis products of beef brain gangliosides, was the generous gift of Dr. A. Stoffyn, the other, obtained from human erythrocytes, was kindly donated by Dr. S. Hakomori. UDP-galactose and UDP-galactose-1-^{3}H were obtained from Calbiochem and New England Nuclear Corp. respectively.

Standard incubation mixtures contained 53 mM tris buffer, pH 6.9, 15 mM $MnCl_2$, 0.4% of the nonionic detergent cutscum and 26 µM UDP-galactose-1-^{3}H (1 µc) in a total volume of 0.5 ml. The dried acceptor was suspended in the medium by vigorous agitation with a Vortex mixer. Incubations were carried out for time periods up to 2 hrs in a 37° waterbath and stopped by the addition of 10.0 ml of chloroform-methanol (2:1,v/v). 250 µg of ceramide lactoside were added as carrier and the solution washed with 0.2 vol. 0.88% KCl, containing 100 µg UDP-galactose. Two additional washes with theoretical upper phase containing 0.375% KCl (Folch et al., 1957) were done, the extract made to volume and aliquots counted in a Packard Tri-carb liquid scintillation spectrometer. A 25% loss of added carrier occurred in the washing process.

Ceramide lactoside was isolated on small Florisil columns together with ceramide glucoside by eluting the glycolipids with chloroform-methanol (2:1,v/v)

according to Radin, Lavin and Brown (1955). Further separation was achieved on borate-impregnated silica-gel G thin-layer plates as outlined above. Commercially obtainable plates, which could be broken into strips (Analtech, Inc., Wilmington, Del.), were sprayed with the borate solution and the strips scanned, after development, with a Packard radiochromatogram scanner, Model 7201. In later experiments the total washed extract was chromatographed directly on thin-layer plates.

The ceramide lactoside bands were removed from the plates, combined and eluted with chloroform-methanol (2:1,v/v) and this material was used for rechromatography or degradation. For isolation of the carbohydrate and lipid components, methanolysis, separation of the methylglycosides from the sphingosine and fatty acid methyl esters on charcoal-Celite 535 columns, hydrolysis with 2N H_2SO_4 and deionization on Amberlite CG-45 columns in the acetate form were carried out, (Stoffyn, in preparation). The free sugars were separated on Whatman 3 MM paper by descending paper chromatography for 20 hrs in ethyl-acetate-pyridine-water (12:5:4, by vol.) and visualized by silver nitrate-NaOH treatment (Trevelyan, Procter and Harrison, 1950).

RESULTS AND DISCUSSION- Incorporation of radioactivity from UDP-galactose-1-^{3}H was obtained with homogenates of rat brain, kidney, spleen and liver, although liver homogenates gave very low activity. Since the highest incorporation per mg of protein occurred with spleen homogenates, this tissue was used in all subsequent experiments. Incorporation was virtually maximal in 30 min, increasing only 27% in a 2 hr incubation. Roughly comparable enzymatic activities were found with spleens from young rats between 2 and 40 days, somewhat lower ones with adult spleens. Approximately linear incorporation was achieved with amounts of homogenate protein from 1 to 5 µg.

Fairly high concentrations of Mn^{++} were essential and could not be replaced by Mg^{++} (Table I), analogous to the requirement for the galactosyltransferase described by Basu et al. (1965), which did not utilize ceramide glucoside as substrate. Water could be substituted for sucrose as the homogenizing medium

and no protection for sulfhydryl groups was required. The addition of glucose as exogenous energy source did not stimulate the incorporation of radioactivity, but either ATP or UTP prevented it completely and changed the physical appearance of the homogenate both during the incubation and after chloroform-methanol addition.

TABLE I

Incorporation of UDP-galactose-1-^{3}H into the chloroform-methanol extract

Control values were obtained under standard incubation conditions (see Methods) which included 250 µg ceramide glucose as acceptor and 100 µl of a 10% sucrose homogenate of rat spleen. Since data from several experiments with rats of different ages are included, results are expressed as percentages of incorporation in the relevant control.

Changes in incubation conditions		% of incorporation in control experiments
Water homogenate		89.0
Cutscum	0.8%	111.5
Mn^{++}	6 mM	67.6
No Mn^{++}; Mg^{++}	15 mM	4.2
Na_2EDTA	2 mM	81.3
Dithiothreitol	2 mM	94.3
Glucose	10 mM	91.3
ATP	5 mM	0.6
UTP	5 mM	3.2

Appreciable amounts of label appeared in the chloroform-methanol extract in the absence of ceramide glucoside as acceptor (Table II). Incorporation was stimulated in the presence of ceramide glucoside and was dependent on the quantity present. About 2 µg of ceramide glucoside / 10 mg spleen are present in the homogenate and may be more readily accessible to the enzyme than added acceptor in a heterogeneous system. Presumably the ratio of enzyme to acceptor is a critical factor. It has not yet been determined into what compound the endogen-

ous incorporation occurred. On scans of thin-layer chromatograms of the entire extract a radioactive peak is seen in a lipid which runs slower than ceramide lactoside in chloroform-methanol-water (65:25:4, by vol.) and which contains P. UDP-galactose may have been hydrolyzed and the labeled sugar metabolized further.

TABLE II

Stimulation of incorporation by added acceptor

Standard incubation conditions for 1 hr with 150 µl of a 20% homogenate of spleen from 32 day old rats.

Ceramide glucoside added	Incorporation
µg	counts/min
0	4570
8.6	6180
43	5940
129	9520
258	10920

The glycolipid fraction from incubations with ceramide glucoside gave one radioactive peak after thin-layer chromatography on neutral or borate-impregnated plates in three different solvent systems, in each case coincident with the ceramide lactoside location, (Fig. 1). The fact that carrier had been added was not deemed objectionable, since endogenous ceramide lactoside (about 8 µg per 10 mg of spleen tissue used, by analogy with human spleen (Suomi and Agranoff, 1965)) also acts as carrier for the minute quantities formed de novo. The radioactivity could not be separated from ceramide lactoside on rechromatography and appears to have been incorporated into this lipid.

In order to determine the distribution of label among the lipid and carbohydrate moieties of the product, these were isolated and counted. Although the radioactivity data are not rigidly comparable because of different counting

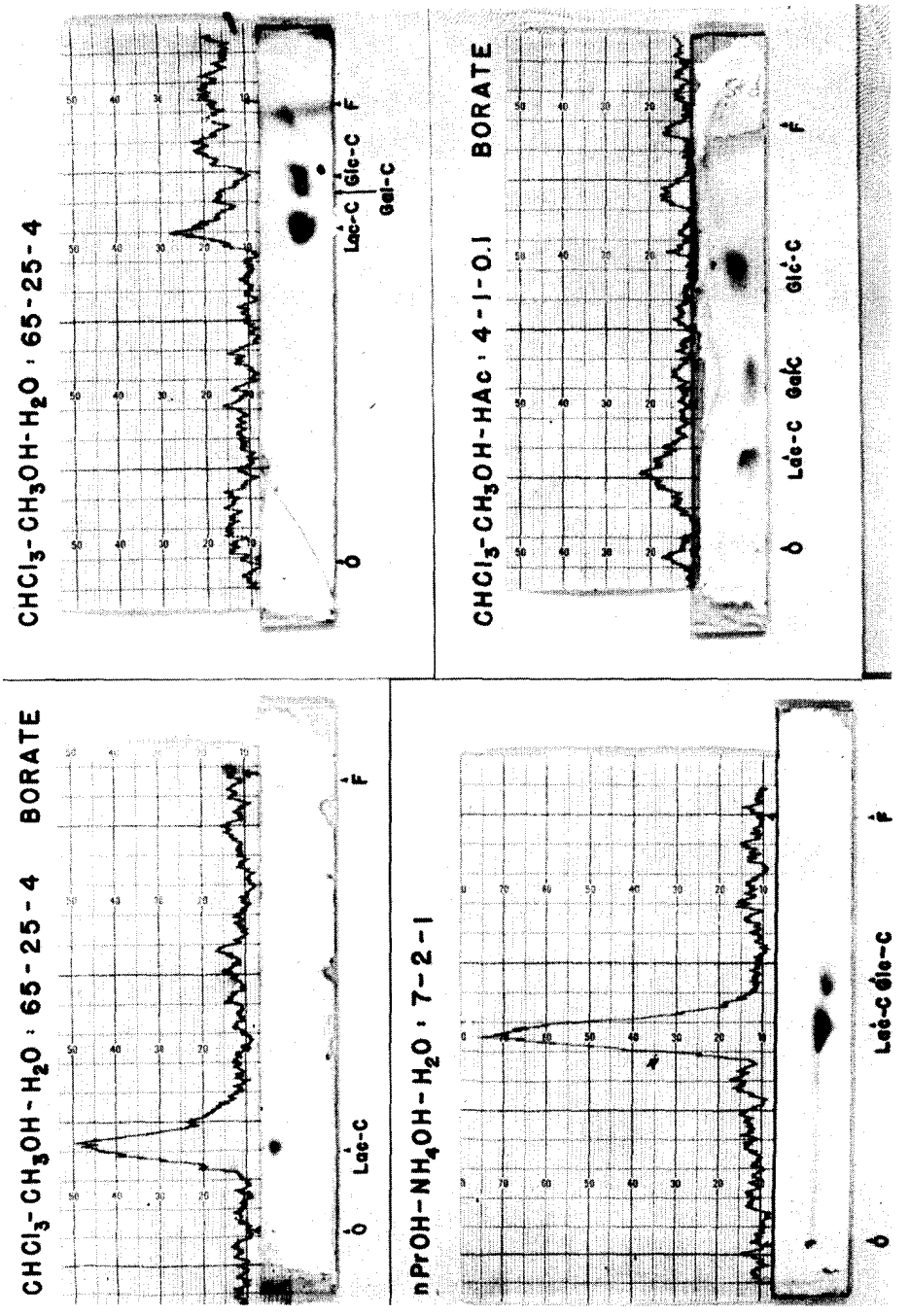

Fig. 1-Radioactivity scans of thin layer chromatograms of biosynthetically produced ceramide lactoside on neutral or borate impregnated plates. O=origin; F=front; Lac-C=ceramide lactoside; Gal-C=ceramide galactoside; Glc-C=ceramide glucoside.

conditions, the fatty acid methyl ester plus sphingosine fraction contained virtually no label (less than 5% of the intact ceramide lactoside). After chromatography of the free sugars, radioactivity was found only in the galactose, glucose being inactive.

From this evidence it is concluded that rat spleen homogenates contain UDP-galactose:ceramide glucoside galactosyl transferase activity, which is responsible for the formation of the labeled product. Indirect evidence for similar activity in leucocytes has been obtained from the incorporation of labeled galactose into ceramide dihexoside, which may, however, be the digalactoside compound (Martensson et al., 1967). Although the analogous product synthesized in brain preparations has not yet been characterized, it is likely that it is identical with that reported here and that, consequently, ceramide glucose lies on the pathway for the stepwise synthesis of gangliosides. The failure of labeled ceramide glucoside to give rise to gangliosides in vivo (Kanfer, 1965) may be due to its rapid cleavage (Brady et al., 1965a b; Gatt, 1966) or to slow penetration to the site of synthesis.

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