THE ENZYMATIC SYNTHESIS OF GANGLIOSIDES

I. The Incorporation of Labeled N-acetylneuraminic Acid into Monosialoganglioside

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The biosynthesis of gangliosides has been investigated in rats by Moser and Karnovsky (1959) and Burton, et al. (1963) by following the incorporation of labeled sugars in vivo. Korey, et al. (1963) have presented evidence for the incorporation of labeled glucose into these compounds in vitro. We wish to report the incorporation of labeled N-acetylneuraminic acid (NANA) into gangliosides catalyzed by a cell-free preparation of rat kidney tissue.

Materials and Methods

The synthesis of NANA-C^{14} (labeled in the acetyl portion) and its conversion to CMP-NANA-C^{14} was accomplished by previously published methods (Warren and Blacklow, 1962). Monosialoganglioside was prepared from a Sigma Chemical Corp. preparation Lot #81B623 and mixed beef brain gangliosides as previously described (Somers, et al.). Gangliosides from the brains of patients with Tay-Sachs disease were prepared by the method of Gatt and Berman (1963). The brains were kindly provided by Dr. Bruno Volk. Aminoglycolipid and asialoganglioside were prepared by acid hydrolysis of beef brain ganglioside (Somers, et al.) and yielded a single spot on thin layer chromatography. Cutscum was purchased from Fisher Scientific Company.

Kidneys were removed from 14 day old Sprague-Dawley rats and were immediately chilled. They were homogenized in a loose fitting teflon homogenizer in 1 volume of a solution containing 0.1 M potassium phosphate buffer (pH 7.2), 0.01 M magnesium acetate, and 0.01 M 2-mercaptoethanol. To demonstrate the incorporation of NANA-C^{14}, the incubation vessels containing the materials listed in Table I were incubated for three hours at 37°C. The incubation was terminated by the addition of 10 ml of chloroform-methanol (2:1).

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The insoluble material was filtered off and the filtrate equilibrated with 0.2 volumes of 0.1 M KCl. The tubes were centrifuged. After the upper phase was removed, the lower phase was equilibrated with 0.4 volumes of "theoretical upper phase" (Folch, et al., 1957), centrifuged, and the upper phase removed. The pooled upper phases were concentrated to dryness in vacuo, dissolved in 1.5 ml water and dialyzed against 1000 volumes of distilled water overnight at 4°C. Aliquots of the dialyzed samples were plated and counted.

Hexose was determined by the anthrone method (Hassid and Abraham, 1957), NANA by the thiobarbaturic acid (Warren, 1959) or resorcinol (Svennerholm, 1957) methods, and hexosamine by the method of Rondle and Morgan (1955).

TABLE I

INCORPORATION OF NANA-C^{14} INTO GANGLIOSIDE BY RAT KIDNEY HOMOGENATE

Except as indicated, the reaction mixture contained 640 μgrams of Cutscum, 1μmole Mg acetate, 2.5 μmoles glutathione, 25 mμmoles CMP-NANA-C^{14} (containing 11,000 total counts), 400 μgrams aminoglycolipid, 0.3 μmoles each of UTP, UDPG, UDPGal, UDP-N-acetylglucosamine, and 0.3 ml of the kidney homogenate in a final volume of 0.44 ml. The tubes were gassed with nitrogen for 1 minute, tightly stoppered and incubated with shaking at 37°C for 3 hours.

	Total Counts
Complete system	2,300
" " , non-incubated control	54
" " , boiled enzyme control	60
" " , Cutscum omitted	703
" " , aminoglycolipid omitted	703
" " , 250 mμmoles non-radioactive NANA added	2,300
" " , aminoglycolipid omitted, asialoganglioside (400 μg) added	930

Results

The data presented in Table I indicate that the cell-free kidney preparation contains enzymes which catalyze the incorporation of NANA-C^{14} from CMP-NANA-C^{14} into gangliosides. This incorporation is increased approximately three-fold by the addition of aminoglycolipid. The presence of Cutscum was required to obtain this stimulation by aminoglycolipid. A requirement for the nucleotide sugars and UTP could not be shown with certainty. However, small increases in the incorporation of NANA-C^{14} were occasionally obtained

when the incubation vessels were supplemented with these materials. In no instance was more than 1% of the radioactivity found in the lower ($CHCl_3$) phase. This finding suggests that Tay-Sachs' gangliosides were not being formed since it has been demonstrated (Rosenberg and Chargaff, 1959; Gatt and Berman, 1963) that the extraction technique employed in these experiments does not remove all the Tay-Sachs' ganglioside from the organic phase.

Maximal synthesis was obtained after 3 hours of incubation. There was no further increase or loss of radioactivity by incubation for an additional 21 hours. The optimum pH appears to be 7.2 with a sharp decrease at either more alkaline or acidic pH's.

In order to isolate and characterize the product of the reaction to the pooled chloroform-methanol extract from 15 incubation flasks containing 56,800 total counts were added 10 mg of carrier monosialoganglioside, 20 mg of mixed beef brain gangliosides and 10 mg of Tay-Sachs' ganglioside. This mixture was then equilibrated with KCl. The lower phase was washed once with "theoretical upper phase-KCl" and then with "theoretical upper phase-water" (Gatt and Berman, 1963). The combined upper phases were taken to dryness, dissolved in water, and extensively dialyzed.

The gangliosides were converted to their barium salts after acidification with Dowex-50 (H^+) and neutralization with barium hydroxide. The barium salts were then precipitated with 4 volumes of absolute ethanol (Trams and Lauter, 1962). They were then dried *in vacuo*, converted to the free acids with Dowex-50 (H^+) and lyophilized. The gangliosides were then dissolved in a small amount of hot methanol. Aliquots were removed for counting and recrystallized to constant specific activity, (Table II). From the amount of carrier added and the radioactivity present in the chloroform-methanol extract a theoretical value of 1500 cpm/mg was anticipated.

Table II

RADIOACTIVITY IN THE METABOLIC PRODUCT

	cpm/mg
Free acid	1630
Recrystallization 1	1600
Recrystallization 2	1560

In order to determine which of the various gangliosides was formed, a sample of the metabolic product was subjected to thin layer chromatography

using chloroform-methanol-formic acid (Trams and Lauter, 1962) and n-propanol-water (Kuhn and Weigandt, 1963) as the solvents. Standards of monosialoganglioside and Tay-Sachs' ganglioside were employed. In both instances more than 80% of the applied radioactivity co-chromatogramed with monosialoganglioside.

The molecule of NANA in monosialoganglioside is not susceptible to hydrolytic cleavage by neuraminidase (Trams and Lauter, 1962; Kuhn and Weigandt, 1963; Svennerholm, 1962). The radioactive product was subjected to hydrolysis by neuraminidase for 5 hours and an equal portion treated in the same manner in the absence of the enzyme. After dialysis aliquots were then removed for radioactivity determination and colorimetric analysis for NANA. There were no differences between the control and the neuraminidase-treated preparations.

It has been demonstrated that at least 90% of the NANA molecules in monosialoganglioside are removed by heating in 0.05 - 0.1 N H_2SO_4 at 80°C for 2 hours. Such treatment of the metabolic product and subsequent dialysis resulted in the complete loss of radioactivity from the dialysis sac. The dialysate was then lyophilized and aliquots subjected to paper chromatography using both n-butanol-pyridine-water (6:4:3) and n-butanol-n- propanol-0.1 N HCl (1:2:1) as the solvent systems (Gottschalk, 1960). In both cases all of the radioactivity co-chromatogramed with authentic samples of NANA-C^{14} submitted to the same acid hydrolysis procedure.

Discussion

Roseman, et al. have described two systems in which CMP-NANA is a sialyl donor. One of these is a mammary gland preparation which actively synthesizes sialyl-lactose (Jourdian, et al., 1963) and the other a bacterial system that makes colominic acid (Aminoff, et al., 1963). The kidney system reported here is therefore another example of a "sialyl transferase." The demonstration that there was no decrease of radioactivity incorporated in the presence of a 10-fold excess of non-radioactive NANA indeed indicates that CMP-NANA and not free NANA is involved.

Since aminoglycolipid (I) is a more effective stimulator for the incorporation of NANA than asialoganglioside, one is, therefore, tempted to speculate that the product of the transfer reaction is Tay-Sachs' ganglioside (II). This compound might conceivably be the substrate for another enzyme that attaches a galactose unit on the terminal position to yield the normal monosialoganglioside (III). Such a scheme is depicted as follows:

Ceramide-glucose-galactose-N-acetylgalactosamine (I) + CMP-NANA →

ceramide-glucose-galactose-N-acetylgalactosamine (II) + CMP

NANA

Ceramide-glucose-galactose-N-acetylgalactosamine (II) + UDPGal →

NANA

ceramide-glucose-galactose-N-acetylgalactosamine-galactose (III) +

NANA

UDP

Experiments designed to test this hypothesis are under investigation.

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