

The Biosynthesis of Gangliosides

THE INCORPORATION OF GALACTOSE, N-ACETYLGALACTOSAMINE AND N-ACETYLNEURAMINIC ACID INTO ENDOGENOUS ACCEPTORS OF SUBCELLULAR PARTICLES FROM RAT BRAIN *IN VITRO*

By A. ARCE, H. J. MACCIONI AND R. CAPUTTO

Departamento de Química Biológica, Instituto de Ciencias Químicas, Ciudad Universitaria, Córdoba, Argentina

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Gangliosides bound to subcellular particles from rat brain were labelled by incubation of the particles (i) with CMP- N [^3H]-acetylneuraminic acid and (ii) simultaneously, with CMP- N [^3H]-acetylneuraminic acid and UDP- N -acetyl-[$^{14}\text{C}_1$]galactosamine or with CMP- N [^3H]-acetylneuraminic acid and UDP-[U- ^{14}C]-galactose. Analysis of the labelled gangliosides showed that in (i), (a) the labelling was mostly in the neuraminidase-labile sialyl groups, (b) rigid relationships exist between the enzymes and the sialyl acceptors; the enzymes are not free to interact with all the specific substrates present in the preparation and (c) the precursor of the trisialoganglioside was the major disialoganglioside with a sialyl 2 \rightarrow 8 sialyl group. In (ii), (a) precursor-product relationships between the main pools of each ganglioside apparently do not exist, (b) for the labelling of Tay-Sachs ganglioside the amount formed from hematoside was at least 2.5 times that from aminoglycolipid and (c) the major monosialoganglioside was the precursor for the major disialoganglioside with a sialyl 2 \rightarrow 8 sialyl group.

The gangliosides are membrane constituents; however, very few membrane models have been proposed in which these compounds are considered (Lehninger, 1968) and we know of no report in which their synthesis is studied as part of a growing or turning-over membrane. A pathway for the biosynthesis of the carbohydrate chain of the gangliosides was proposed by Kaufman, Basu & Roseman (1966, 1968) and a different one by Kanfer, Blacklow, Warren & Brady (1964), Yip & Dain (1969) and Handa & Burton (1969). These pathways have been worked out from reactions occurring between water-soluble donors of glycosyl groups and lipid-soluble acceptors, catalysed by particulate preparations in a medium containing detergents which presumably altered the structures of the membranes. By using this method, possible steps for the synthesis are shown, but the natural relationships of the enzymes with their substrates are disregarded. The medium surrounding the membranes *in vivo* may convey the different substrates into contact with the enzymes as in the experiments with solubilized acceptors, but these also may be built attached to the membranes so that there are no interchanges with the pools of free intermediates. These different situations are important in determining the ratio of labelling of pools of metabolites related as precursor and product. If the

precursor was obtained from a free pool, typical precursor-product relationship should be demonstrated; if the precursor was built and remained bound to the same particle that builds the product, non-typical relationship may be obtained. In the biosynthesis of neuraminyl-lactose, discrepancies between results of experiments *in vitro* and *in vivo* were explained because the synthesis obtained with free lactose, as carried out *in vitro*, is very small compared with the synthesis occurring when the synthesis of lactose and the binding of the sialyl group is carried out by the same enzyme complex (Barra, Cumar & Caputto, 1969).

The present paper describes kinetic studies on the relationships between the enzymes and their endogenous substrates. It also deals with the incorporation of sialyl, galactosaminyl and galactosyl groups into endogenous acceptors of intact brain particles, to complete most of their different types of gangliosides. Distribution of labelling in endogenous Tay-Sachs ganglioside and multisialoganglioside in particles exposed to labelled donors in the supernatant are given. An important feature of the system used is that, similar to what occurs *in vivo* (Suzuki & Korey, 1964), the pools of different gangliosides are built largely independently of each other; this probably reflects the similarity of conditions in which the acceptors are *in vivo* and in

the particles. Another important observation *in vivo*, namely that in rats that had been given [^{14}C]glucose the neuraminidase-resistant sialyl group is more labelled than the neuraminidase-labile sialyl group (Suzuki & Korey, 1964; A. Arce, H. J. Maccioni & R. Caputto, unpublished work), has not been reproduced. No explanation can be offered at this moment for this discrepancy between labelling *in vivo* and all the pathways worked out from experiments *in vitro*. In any case, the relative labelling of the different endogenous ganglio sides, and their components, are bound to be important in determining the pathway of synthesis of these compounds.

EXPERIMENTAL

Preparation of particles. Brains from 15-day-old rats killed by decapitation were washed twice with several volumes of 50 mM-tris-HCl buffer (pH 7.4)–0.1 M-KCl at 4°C and homogenized in 10 vol. of the same buffer. The homogenate was centrifuged at 800g for 10 min; the pellet was discarded and the supernatant was centrifuged at 100 000g for 30 min. The sediment from this last centrifugation was homogenized with 10 vol. of the same buffer as above and re-centrifuged; the pellet was finally suspended in 0.1 M-potassium phosphate buffer, pH 6.8, to a protein concentration of 2.5 mg/ml.

In cases where the distribution of radioactivity in different gangliosides was studied, larger amounts of particles were needed; better yield of particles was obtained when the brains were homogenized in 0.32 M-sucrose and centrifuged at 1000g for 10 min, followed by the procedure described above but with 0.32 M-sucrose in place of the tris-HCl-KCl solution. The particles were suspended in 0.32 M-sucrose–20 mM-potassium phosphate buffer, pH 6.8; the total volume was 1 ml/g of brain used as starting material.

The method of Eichberg, Whittaker & Dawson (1964) was followed for the preparation of subcellular organelles used to study the distribution of sialyltransferase.

Determination of sialyltransferase activity. The incubation system contained, in a total volume of 0.5 ml, 40 μmol of potassium phosphate buffer, pH 6.8; 5 μmol of MgCl_2 ; 10 nmol of CMP- N [^{14}C]-acetylneuraminic acid (sp. radioactivity, 9×10^6 c.p.m./ μmol) and enzyme preparation equivalent to 1 mg of protein. The incubation was carried out at 37°C in test-tubes covered with waxed paper. To stop the reaction, 20 vol. of chloroform-methanol (2:1, v/v) was added and the mixture vigorously shaken; the mixture was filtered through paper and the filtrate was passed through a column containing 2 g of Sephadex G-25 equilibrated with chloroform-methanol-water (12:6:0.9, by vol.) by the method of Wells & Dittmer (1963). The column was washed with 2 ml of the same solvent mixture, the eluates and the washing were dried into vials and the radioactivity was measured.

Isolation and characterization of labelled products. For the study of the distribution of radioactivity into each ganglioside, the particles prepared in sucrose (see above) were incubated with CMP- N [^3H]-acetylneuraminic acid or with CMP- N [^3H]-acetylneuraminic acid and UDP- N -

acetyl[^{14}C]-galactosamine or with CMP- N [^3H]-acetylneuraminic acid and UDP-[U- ^{14}C]galactose under conditions that will be stated for each case. After inactivation of the incubation system by addition of 20 vol. of chloroform-methanol (2:1, v/v) the suspension was filtered and the filtrate was mixed with 0.2 vol. of water by the method of Folch, Lees & Sloane-Stanley (1957). The upper phase was concentrated in a rotary evaporator at room temperature and dialysed for 96 h against tap water and then for 24 h against distilled water.

The dialysed upper phase was freeze-dried and the residue dissolved in approx. 10 ml of chloroform-methanol-water (12:6:0.9, by vol.); any insoluble material was washed twice with the same solvent mixture and then eliminated by centrifugation. The soluble fractions were pooled and the freeze-drying and dissolving procedures repeated twice. The solution of the crude ganglioside mixture was passed through a Sephadex G-25 column (Wells & Dittmer, 1963) to eliminate any remaining non-lipid contaminants. The ganglioside mixture was subjected to t.l.c. on silica gel G (Merck) with chloroform-methanol–20% NH_3 (60:35:8, by vol.) as solvent. To locate the various gangliosides a strip of about 2 cm was cut at the border of the plate and stained with the resorcinol reagent (Svennerholm, 1957); the rest of the plate was exposed to iodine vapour and the zones positive to both the resorcinol and iodine reagents were scrapped; six zones were separated each of which migrated to the same position as standard samples of respectively G Lac 1 (G_{M_3}),* G GNTr 1 (G_{M_2}), G GNT 1 (G_{M_1}), G GNT 2a ($\text{G}_{\text{D}_{1a}}$), G GNT 2b ($\text{G}_{\text{D}_{1b}}$) and G GNT 3 (G_{T_1}). Penick, Meisler & McCluer (1966) found, with the solvent used, that G Lac 2 runs slightly below G GNT 1 (G_{M_1}), and G GNTr 2 between G GNT 2b ($\text{G}_{\text{D}_{1b}}$) and G GNT 2a

* Abbreviations: NANAc, *N*-acetylneuraminic acid. For gangliosides, the abbreviation system based on structural considerations (Wiegandt, 1968) has been used. The more commonly used nomenclature suggested by Svennerholm (1964) based on chromatographic mobility has been added in parentheses: hematoside G Lac 1 (G_{M_3}), *N*-acetylneuraminyl-(2 \rightarrow 3)-galactosyl-(1 \rightarrow 4)-glucosylceramide; G Lac 2, *N*-acetylneuraminyl-(2 \rightarrow 8)-*N*-acetylneuraminyl-(2 \rightarrow 3)-galactosyl-(1 \rightarrow 4)-glucosylceramide; Tay-Sachs ganglioside G GNTr 1 (G_{M_2}), *N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide; G GNTr 2, *N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 8)-*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide; monosialoganglioside G GNT 1 (G_{M_1}), galactosyl-(1 \rightarrow 3)-*N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide; disialoganglioside G GNT 2a ($\text{G}_{\text{D}_{1a}}$), *N*-acetylneuraminyl-(2 \rightarrow 3)-galactosyl-(1 \rightarrow 3)-*N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide; disialoganglioside G GNT 2b ($\text{G}_{\text{D}_{1b}}$), galactosyl-(1 \rightarrow 3)-*N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 8)-*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide; trisialoganglioside G GNT 3 (G_{T_1}), *N*-acetylneuraminyl-(2 \rightarrow 3)-galactosyl-(1 \rightarrow 3)-*N*-acetylgalactosaminyl-(1 \rightarrow 4)-[*N*-acetylneuraminyl-(2 \rightarrow 8)-*N*-acetylneuraminyl-(2 \rightarrow 3)]-galactosyl-(1 \rightarrow 4)-glucosylceramide. Aminoglycolipid, G GNTr, *N*-acetylgalactosaminyl-(1 \rightarrow 4)-galactosyl-(1 \rightarrow 4)-glucosylceramide.

(G_{D1a}). The silica from the zones where G Lac 2 and G GNT 2 were expected was not taken. The silica from each of the other zones was suspended in chloroform-methanol-water (10:10:3, by vol.) (Ledeen, Salsman & Cabrera, 1968), placed in a column and washed with about 20 vol. of the same solvent mixture. Each eluate was evaporated to dryness in a rotary evaporator at 35°C; the residue was again subjected to t.l.c., 3 μ mol or less of ganglioside NANAc being placed on a plate (20 cm \times 20 cm; 0.27 mm thick). In some cases some gangliosides were chromatographed three or four times before they appeared satisfactorily homogenous when sprayed with the resorcinol reagent. Authentic G GNT 1 (G_{M2}) was isolated from the brain of a patient with Tay-Sachs disease as follows. Total gangliosides were obtained as described above; the upper phase was made 0.15M with respect to KCl and partitioned twice with theoretical lower phase (Gatt, 1965); from the pooled lower phases G GNT 1 (G_{M2}) was purified by t.l.c.

Determination of the specific radioactivities of the neuraminidase-labile and resistant N[³H]-acetylneuraminic acid. Each of the fractions isolated as described above, corresponding to G Lac 1 (G_{M3}), G GNT 1 (G_{M2}), G GNT 1 (G_{M1}), G GNT 2a (G_{D1a}), G GNT 2b (G_{D1b}) and G GNT 3 (G_{T1}), was treated as follows: the total of G Lac 1 (G_{M3}) and G GNT 1 (G_{M2}) obtained (about 0.1 and 0.05 μ mol respectively) and 0.3–1 μ mol of the major gangliosides were dried in test tubes; to each tube 66 μ mol of sodium acetate-acetic acid buffer, pH 5.2, and 40 μ g of *Clostridium perfringens* neuraminidase (Sigma, type VI) were added (Burton, 1963). The total volume was 0.25 ml. The mixtures were incubated for 3 h at 37°C. After incubation the mixtures were transferred to cellophan tubes and dialysed for a total of 12 h against three successive portions of 4 ml of water. The diffusates containing the neuraminidase-labile *N*-acetylneuraminic acid were stored. To isolate the monosialogangliosides formed during the incubation with neuraminidase, the dialysis residues were run on t.l.c. under the same conditions as before. The amounts isolated showed that at least 90% of multisialogangliosides were converted into G GNT 1 (G_{M1}) whereas the original G GNT 1 (G_{M1}) was recovered unchanged; G GNT 1 (G_{M2}) was not run on t.l.c. but passed through a Sephadex G-25 column (Wells & Dittmer, 1963).

Each purified monosialoganglioside was hydrolysed for 2.5 h at 80°C in 0.25 ml of 0.05 M-H₂SO₄. After this treatment the neuraminidase-resistant NANAc was separated by dialysis. The fractions containing free NANAc were passed through columns (0.5 cm \times 5 cm) of, successively, Dowex 50 (H⁺ form) and Dowex 1 (formate form). After washing the columns with 30 ml of water, NANAc was eluted from the Dowex-1 columns with m-formic acid; the formic acid was removed by freeze-drying and the residue was dissolved in water for NANAc determinations and radioactivity counting. All the radioactivity recovered from the columns migrated together with authentic NANAc in co-chromatography experiments. To determine the specific radioactivities of the different labile NANAc moieties of G GNT 3 (G_{T1}), the observation of Svennerholm (1963) on their different susceptibility to neuraminidase was applied; G GNT 3 (G_{T1}) was incubated with neuraminidase in the same conditions described above, but with a tenfold diluted enzyme preparation and for 15 min only. The reaction was stopped by adding 4 ml

of chloroform-methanol (2:1, v/v); the solvent was evaporated and the residue was subjected to t.l.c.; the G GNT 2b (G_{D1b}) obtained from G GNT 3 (G_{T1}) was isolated from the plate and treated again with neuraminidase, by using the standard incubation system. The specific radioactivity of the NANAc bound to the terminal galactose was determined by the difference of specific radioactivities between total labile NANAc from G GNT 3 (G_{T1}) and labile NANAc from their corresponding G GNT 2b (G_{D1b}).

Determinations of specific radioactivities of double-labelled compounds. When a ganglioside was doubly labelled the specific radioactivities were determined directly on the intact compound isolated as described above.

Preparation and purification of labelled CMP-N-acetylneuraminic acid and lactosylceramide. CMP-N-acetylneuraminic acid was prepared by the method of Roseman (1962) with the modification of Arce, Maccioni & Caputto (1966); CMP-N-acetylneuraminic acid was isolated free from NANAc, proteins and CTP by passing the preparation through a DEAE-cellulose column and eluting the CMP-N-acetylneuraminic acid with an increasing concentration of LiCl (Warren & Blacklow, 1962). To eliminate LiCl, the dried eluate, containing approx. 3.5 μ mol of CMP-N-acetylneuraminic acid, was taken up in the minimal amount of ethanol (15 ml) and the salt suspension deposited on the top of a column (2.5 cm \times 46 cm) of Whatman cellulose powder that had been washed with 0.1 M-tris-acetate buffer, pH 7.6, and then equilibrated with ethanol. The column was washed with ethanol until no more Cl⁻ could be detected in the washing when it was tested with AgNO₃ (approx. 600 ml of ethanol was needed); after this, CMP-N-acetylneuraminic acid was eluted from the column with 0.2 M-tris-acetate buffer, pH 7.6; fractions (20 ml) were collected and checked for radioactivity and extinction at 270 nm. The fractions containing radioactivity and absorbing at 270 nm were pooled and freeze-dried. Determination of free NANAc in the pool of nucleotide indicated that no more than 8–10% of the nucleotide was hydrolysed. The absorption spectrum was typical for cytidine and the cytidine/NANAc molar ratio was 1.23.

Lactosylceramide was prepared as described by Cumar, Barra, Maccioni & Caputto (1968).

Chemical determinations. Free sialic acid was determined by the method of Warren (1959). In some cases the resorcinol reaction was used to determine bound NANAc; it was carried out by the method of Svennerholm as modified by Miettinen & Takki-Luukkainen (1959). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951); bovine serum albumin was used as standard, unless indicated.

Measurement of radioactivity. Determinations of radioactivity were carried out in a Beckman model LS 200 B liquid-scintillation spectrometer equipped with external standard. Determinations of radioactivity of double-labelled compounds were carried out by using the channel windows for counting ³H and ¹⁴C radioactivities separately. The samples were evaporated in vials, the residues dissolved in 0.1 ml of water and 5 ml of dioxan containing 10% of naphthalene and 0.5% of 2,5-diphenyl-oxazole was added. The error in the radioactivity counting was less than 5%; the efficiency was, for ³H, 30–35%, for

^{14}C , 92% as determined by external standardization, and for ^{14}C above ^3H , 58–62%.

RESULTS

Sialyltransferase activity of different subcellular organelles. In a previous report of the distribution of sialyltransferase activity in different subcellular organelles, the crude mitochondrial fraction was used (Arce *et al.* 1966). Since the difference in the transferase activity between mitochondria and synaptosomes can be meaningful from the physiological point of view, an experiment was run to measure the activity in both fractions. The results showed (Table 1) that the specific activity toward endogenous acceptors was about two- to three-fold higher in the mitochondria than in the other fractions whereas the specific activity with exogenous lactosylceramide as acceptor was somewhat higher in myelin than in the other fractions. In any case, the differences were small enough to justify the conclusion that the sialyltransferase activity is rather evenly distributed in all the subcellular particles.

Kinetic studies with endogenous acceptors. (a) Time curve of $N[^{14}\text{C}]$ -acetylneuraminic acid incorporation. Fig. 1 shows that the transference of NANAc from CMP- N -acetylneuraminic acid to acceptors attached to brain subcellular particles occurred at two distinct rates. During the initial period the velocity was relatively high, but at 8–10 min an apparent inactivation occurred and the reaction proceeded, until the experiment was terminated, at a rate 5 times lower than in the initial period. Three causes may have possibly been responsible for this apparent inactivation, namely enzyme inactivation, destruction of CMP- $N[^{14}\text{C}]$ -acetylneuraminic acid or depletion of acceptors. Enzyme inactivation was discounted since the enzymic activity of particles preincubated for

15 min at 37°C in the absence of CMP- $N[^{14}\text{C}]$ -acetylneuraminic acid (but in otherwise identical conditions as in the incubation system) did not show any significant difference of activity with particles of the same batch maintained at 0 – 4°C . Destruction of CMP- $N[^{14}\text{C}]$ -acetylneuraminic acid was discounted because determination of NANAc by the method of Warren & Blacklow (1962) showed that after 15 min of incubation approx. 96% of $N[^{14}\text{C}]$ -acetylneuraminic acid was still bound in a linkage

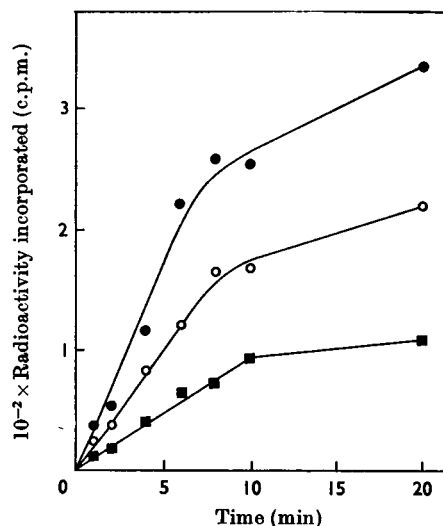


Fig. 1. Incorporation of $N[^{14}\text{C}]$ -acetylneuraminic acid into endogenous acceptors from particulate enzyme preparations as a function of time. The assay mixtures contained: ■, 0.2 mg of protein; ○, 0.4 mg of protein; ●, 0.6 mg of protein. Other assay conditions were as described in the Experimental section.

Table 1. *Sialyltransferase activity in various subcellular particles of rat brain*

The incubation system contained, in a total volume of 0.5 ml, $40\ \mu\text{mol}$ of potassium phosphate buffer, pH 6.8, 50 nmol of CMP- $N[^{14}\text{C}]$ -acetylneuraminic acid (specific radioactivity 1×10^6 c.p.m./ μmol) and particulate enzyme preparation equivalent to 0.5 mg of tyrosine. The incubation was carried out at 37°C for 1 h. For this experiment radioactivity was measured in a gas-flow counter. Particles were prepared by the method of Eichberg *et al.* (1964). The values in the presence of added lactosylceramide ($0.1\ \mu\text{mol}$) are corrected for the endogenous values.

| Particles | Addition | Incorporation (c.p.m./mg of tyrosine) |
|--------------|------------------|--|
| Light myelin | None | 50 |
| | Lactosylceramide | 208 |
| Synaptosomes | None | 82 |
| | Lactosylceramide | 123 |
| Mitochondria | None | 180 |
| | Lactosylceramide | 134 |
| Microsomes | None | 73 |
| | Lactosylceramide | 93 |

whose stability was identical with that of CMP-*N*-acetylneuraminic acid. After incubation of CMP-*N*[¹⁴C]-acetylneuraminic acid with brain particles for 15 min approx. 2% of this compound was converted into *N*-acetylmannosamine; this was identified by paper chromatography with two different solvents and it was evaluated by radioactivity counting.

Since enzyme inactivation and CMP-*N*-acetylneuraminic acid destruction could be discounted as the causes of inactivation of the system, depletion of these acceptors or any other mechanism by which they become unaccessible remained as the most probable cause. Provided that this is correct, it was notable that the reaction followed a linear curve up to the breaking point (zero-order reaction) indicating that the enzyme was saturated with the endogenous acceptor at any concentration available. The nature of the substrate for the slow reaction of the final period has not been determined; this reaction probably was due to renewal of NANAc on the same gangliosides completed in the first period but it has not been excluded that it measured the transference of NANAc to a slow-reacting acceptor.

(b) Enzyme-concentration curve. In the transference of sialyl groups to endogenous acceptors of brain particles (Fig. 2) the plot of initial velocity versus enzyme concentration was linear; this showed that in our particulate system each particle was a

compartment in the sense that the enzymes from one particle did not interact with the substrates of others. If this were not so the compound effect of a free enzyme and substrate system (Reiner, 1959) should have been observed.

(c) Heat-inactivation. Test tubes (0.5 cm diam.) containing particles obtained as stated in the Experimental section were immersed in a 52°C bath for periods of from 15 to 70 s; after these periods the tubes were immersed immediately in an ice-water bath where they were kept until used. The resulting preparations were tested by incubating for 6 min in the presence of CMP-*N*[¹⁴C]-acetylneuraminic acid; results showed that the inactivation amounted to 13, 42 and 57% after 15, 45 and 70 s in the 52°C water bath, respectively. A preparation inactivated for 60 s at 52°C was compared with the original unheated preparation; it was found that the percentage of activity measured in the partially inactivated preparation relative to the original preparation was the same at 10, 20 and 60 min of incubation with CMP-*N*-acetylneuraminic acid as donor and that the period at which the breaking point of the incorporation was reached was the same for the heated and the original preparations (Fig. 3). Further, provided that the incorporation at 10 min measured the velocity of reaction whereas the incorporation at 60 min measured the total acceptor capacity of the preparation, it appeared that the decrease in the total amount of NANAc incorporated

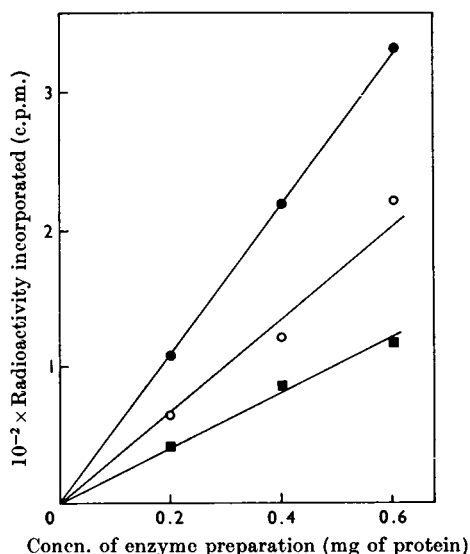


Fig. 2. Incorporation of *N*[¹⁴C]-acetylneuraminic acid into endogenous acceptors as a function of concentration of the particulate enzyme preparation. Incubation times were: ■, 4 min; ○, 6 min; ●, 20 min. Other assay conditions were as described in the Experimental section.

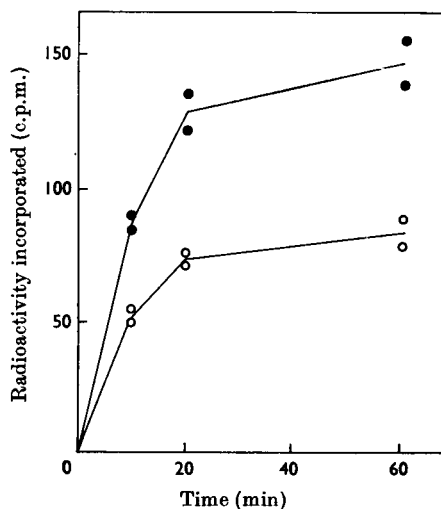


Fig. 3. Incorporation of *N*[¹⁴C]-acetylneuraminic acid into endogenous acceptors from heat-inactivated particulate enzyme preparation. ●, Unheated control; ○, heated at 52°C for 60 s. Particulate preparation contained 0.5 mg of protein/ml. Other assay conditions were as described in the Experimental section.

into the heated preparation was proportional to the decrease in the enzyme activity. This was noteworthy because it showed that the heat inactivation of the enzyme produced a proportional elimination of acceptor molecules even if these are not heat-labile substances.

(d) Effect of exogenous acceptors on the incorporation of *N*-acetylneuraminic acid into endogenous acceptors. It is known that rat brain (Arce *et al.* 1966) and embryonic chicken brain (Kaufman *et al.* 1966) preparations catalyse the transference of NANAc from CMP-*N*-acetylneuraminic acid to lactosylceramide or lactose to form, respectively, hematoside or neuraminyl-lactose. In the present work it was investigated if in the presence of exogenous acceptors the incorporation of NANAc by the endogenous acceptors was inhibited. The specific radioactivity of each ganglioside after 5 and 15 min incubation was determined by the method of Suzuki & Chen (1967). Addition of lactosylceramide (0.4 mM) or lactose (16 mM) had no effect on the rate of incorporation of NANAc to form the major mono-, di-, or tri-sialogangliosides from endogenous acceptors. Conversely, attempts were made to elucidate whether the activity of the enzyme towards endogenous acceptors interfered with its activity towards exogenous lactosylceramide. The acceptor capacity of a sample of particles was exhausted by incubation for 10 min with CMP-*N*[¹⁴C]-acetylneuraminic acid and the activity of these particles was compared with the activity of another sample preincubated in the absence of CMP-*N*[¹⁴C]-acetylneuraminic acid. The incorporation of *N*[¹⁴C]-acetylneuraminic acid into exogenous lactosylceramide (0.2 μmol

dissolved in 0.5 mg of Tween 20) was measured at different times from 5 to 20 min. No difference was found between the two differently treated particles.

(e) *K_m* values for CMP-*N*-acetylneuraminic acid in the reactions with endogenous acceptors. It is not known whether or not the enzymes that catalyse the transference of sialyl groups to endogenous acceptors are the same as those that work with exogenous acceptors. Different *K_m* values were found for CMP-*N*-acetylneuraminic acid when the different types of acceptors were used. With lactosylceramide as exogenous acceptor the *K_m* value was 4×10^{-5} M (Arce, 1968); with endogenous acceptors and 1 mg of particulate enzyme preparation for an incubation period of 3 min, the *K_m* value was 3×10^{-6} M. It is uncertain whether these *K_m* values are comparable. In the determination of *K_m* with exogenous acceptor this was at saturating concentration. For the reasons given above it was considered that the endogenous acceptors were saturating the enzyme, but in working with gangliosides in various stages of complexity, it is possible that different enzymes were competing for the donor CMP-*N*-acetylneuraminic acid, whereas only lactosylceramide was present as exogenous acceptor.

Distribution of labelled components incorporated into endogenous acceptors. (a) General distribution. Three experiments were run in which the particles were incubated with (a) CMP-*N*[³H]-acetylneuraminic acid, (b) simultaneously with CMP-*N*[³H]-acetylneuraminic acid and UDP-*N*-acetyl[¹⁴C]-galactosamine and (c) simultaneously with CMP-*N*-[³H]-acetylneuraminic acid and UDP-[U-¹⁴C]galactose. In the experiment with CMP-*N*[³H]-acetyl-

Table 2. *Distribution of N[³H]-acetylneuraminic acid in gangliosides formed in vitro from endogenous acceptors*

The incubation system contained: particles from 21 g of 15-day-old-rat brain; CMP-*N*[³H]-acetylneuraminic acid (specific radioactivity, 6×10^7 c.p.m./μmol), 0.3 μmol; MgCl₂, 75 μmol; potassium phosphate buffer, pH 6.8, 600 μmol; final volume, 15 ml; temperature, 37°C. The gangliosides were isolated and purified as described in the Experimental section.

| Ganglioside | Incubation time (min) | Neuraminidase-labile NANAc | | Neuraminidase-resistant NANAc* | |
|---|-----------------------|---------------------------------|----------------------------|---------------------------------|----------------------------|
| | | Sp. radioactivity (c.p.m./μmol) | Amount incorporated (nmol) | Sp. radioactivity (c.p.m./μmol) | Amount incorporated (nmol) |
| G Lac 1 (G _{M3}) | 15 | 24 933 | 62.3 | — | — |
| G GNT _r 1 (G _{M2}) | 15 | — | — | 11 400 | 66 |
| G GNT 1 (G _{M1}) | 5 | — | — | 180 | 17 |
| | 15 | — | — | 100 | 9 |
| G GNT 2a (G _{D1a}) | 5 | 11 704 | 1311 | 140 | 15 |
| | 15 | 16 092 | 1802 | 130 | 14 |
| G GNT 2b (G _{D1b}) | 5 | 7 442 | 434 | 180 | 10 |
| | 15 | 23 292 | 1359 | 140 | 8 |
| G GNT 3 (G _{T1}) | 5 | 8 941 | 834 | 140 | 6 |
| | 15 | 17 352 | 1619 | 130 | 6 |

* Approximate values.

neuraminic acid it became evident that most of the NANAc incorporation was into a neuraminidase-labile position (Table 2). In a neuraminidase-resistant position only the sialyl group of the Tay-Sachs ganglioside was substantially labelled; even in this case the results were not inconsistent with the possibility that this incorporation could have come from the addition of endogenous *N*-acetyl-galactosamine to G Lac 1 (G_{M3}) (24 933 c.p.m./ μ mol) to form Tay-Sachs ganglioside (11 400 c.p.m./ μ mol) but this cannot be considered proven from this experiment. G GNT 2a (G_{D1a}) was labelled in the neuraminidase-labile NANAc only, indicating that it was synthesized from G GNT 1 (G_{M1}) pre-existing in the particles. However, the specific radioactivity of the *N*-acetyl-galactosamine (Table 3) or galactose (Table 5) moieties of G GNT 1 (G_{M1}), G GNT 2a (G_{D1a}), G GNT 2b (G_{D1b}) and G GNT 3 (G_{T1}) indicate that the pools of these gangliosides are built independently between each other since no dilutions were observed in those moieties in passing from the pool of the monosialoganglioside to the pool of any of the disialogangliosides or from these to the trisialoganglioside.

(b) Pathway to Tay-Sachs ganglioside. The discrepancies in the pathway of synthesis of gangliosides apparent in experiments *in vitro* (see the introduction) appeared to be centred around the sequence of incorporation of the sialyl and galactosaminyl groups in G GNTr 1 (G_{M2}); the double

labelling of endogenous acceptors may contribute to solving this problem provided that a favourable combination of concentrations of the acceptors was found. After simultaneous incubation of the particles with UDP-*N*-acetyl[$^{14}C_1$]galactosamine and CMP-*N*[3H]-acetylneuraminic acid, the $^{14}C/^3H$ molar ratio in the Tay-Sachs ganglioside formed during the incubation period should be 1:1, 1:0 or 0:1, when the starting materials for its synthesis were lactosylceramide, hematoside or aminoglycolipid respectively. Results in Table 4 were calculated two ways since substantial amounts of sugar nucleotides were found in the particles and at present it is not known whether a part or all of the particle nucleotides interchange with the labelled nucleotides present in the incubation medium. Whether the incorporations were calculated assuming that only added nucleotides ($^{14}C/^3H$ molar ratio 2.5:1) or that all the recovered nucleotides were active donors in the system ($^{14}C/^3H$ molar ratio 37.7:1), the molar ratios of incorporation of *N*-acetyl[$^{14}C_1$]galactosamine and *N*[3H]-acetylneuraminic acid indicated that G Lac 1 (G_{M3}) was a precursor quantitatively more important than G GNTr for the synthesis of G GNTr 1 (G_{M2}).

(c) Labelling of the disialoganglioside G GNT 2b (G_{D1b}). The double-labelling experiments apparently solved the problem of whether or not the labile sialyl group attached by a 2→8 linkage to the sialyl group resistant to neuraminidase enters before

Table 3. Incorporation of *N*[3H]-acetylneuraminic acid and *N*-acetyl[$^{14}C_1$]galactosamine into endogenous gangliosides

The incubation system contained: particles from 35 g of 15-day-old-rat brain; CMP-*N*[3H]-acetylneuraminic acid (sp. radioactivity 12×10^7 c.p.m./ μ mol), 0.5 μ mol; UDP-*N*-acetyl[$^{14}C_1$]galactosamine (sp. radioactivity 8.8×10^7 c.p.m./ μ mol), 0.05 μ mol; $MgCl_2$, 125 μ mol; $MnCl_2$, 125 μ mol; potassium phosphate buffer, pH 6.8, 1 mmol; final volume, 25 ml; incubated at 37°C for 15 min. CMP-*N*[3H]-acetylneuraminic acid (sp. radioactivity 7.2×10^7 c.p.m./ μ mol) was isolated, after the reaction was stopped with ethanol, at 37°C, pH 7.6 for 10 min. The precipitate was centrifuged, the supernatant was evaporated and CMP-*N*-acetylneuraminic acid was isolated from the residue as described by Warren & Blacklow (1962). UDP-*N*-acetyl[$^{14}C_1$]galactosamine (sp. radioactivity 3.6×10^6 c.p.m./ μ mol) was isolated as described by McGarrah & Maley (1962) from the upper phase of the Folch's partition, after elimination of KCl by passing the solution through a column of Dowex-50 (H^+ form) and precipitation of Cl^- with Ag_2CO_3 . Since no satisfactory constant radioactivities were obtained for sialyl groups of very low specific radioactivities in gangliosides in which sialyl groups with high specific radioactivities were present no attempt has been made to compare these sialyl groups with other components of low specific radioactivities.

| Ganglioside | Sp. activity (nmol incorporated/ μ mol of NANAc) | | | | | | |
|------------------------|--|--------|--------|--------------------------------|---------------------------------------|--------|--------------------------------|
| | Calculated from added nucleotides | | | | Calculated from recovered nucleotides | | |
| | NANAc | | GalNAc | GalNAc/NANAc (labile) ratio | Labile NANAc | GalNAc | GalNAc/NANAc (labile) ratio |
| | Resistant | Labile | | | | | |
| G GNT 1 (G_{M1}) | 3.1 | — | 1.46 | — | — | 35.8 | — |
| G GNT 2a (G_{D1a}) | 2.2 | 135 | 3.73 | 0.028:1 | 225 | 91.1 | 0.405:1 |
| G GNT 2b (G_{D1b}) | 4.5 | 356 | 0.62 | 0.002:1 | 593 | 15.3 | 0.026:1 |
| G GNT 3 (G_{T1}) | 3.8 | 435 | 2.16 | 0.005:1 | 709 | 52.8 | 0.074:1 |

Table 4. *Incorporation of N[³H]-acetylneuraminic acid and N-acetyl[¹⁴C]₁galactosamine into endogenous Tay-Sachs ganglioside*

The incubation system was as described in Table 3. Tay-Sachs ganglioside was added as carrier to the inactivated incubation mixture; the ganglioside that migrated chromatographically as Tay-Sachs ganglioside was then isolated, treated with neuraminidase and purified to constant specific radioactivity as described in the Experimental section.

| Incubation time (min) | Amount incorporated into Tay-Sachs ganglioside* (nmol) | | | | | |
|--------------------------|--|--------|--|---------------------------------------|--------|--|
| | Calculated from added nucleotide | | | Calculated from recovered nucleotide† | | |
| | NANAc | GalNAc | $\frac{\text{GalNAc}}{\text{NANAc}}$ ratio | NANAc | GalNAc | $\frac{\text{GalNAc}}{\text{NANAc}}$ ratio |
| 5 | 37.2 | 93 | 2.5:1 | 59.2 | 2322 | 37.7:1 |
| 15 | 48.4 | 126 | 2.6:1 | 77.4 | 3024 | 39:1 |

* The NANAc of Tay-Sachs ganglioside was resistant to the neuraminidase preparation used.

† The specific radioactivities of recovered nucleotides are indicated in Table 3.

Table 5. *Incorporation of N[³H]-acetylneuraminic acid and [U-¹⁴C]galactose into endogenous gangliosides*

The incubation system contained: particles from 37 g of 15-day-old-rat brain; CMP-N[³H]-acetylneuraminic acid (sp. radioactivity 12×10^7 c.p.m./ μ mol), 0.4 μ mol; UDP-[U-¹⁴C]galactose (sp. radioactivity 4.9×10^7 c.p.m./ μ mol), 0.4 μ mol; MgCl₂, 125 μ mol; MnCl₂, 125 μ mol; potassium phosphate buffer, pH 6.8, 1 mmol, final volume, 25 ml; incubated at 37°C for 7 min. The gangliosides were isolated, treated with neuraminidase and purified as described in the Experimental section. Since no satisfactory constant radioactivities were obtained for sialyl groups of very low specific radioactivities in gangliosides in which sialyl groups with high specific radioactivities were present, no attempt has been made to compare these sialyl groups with other components of low specific radioactivities.

| Ganglioside | Sp. activity (nmol incorporated/ μ mol of ganglioside) | | | | |
|---------------------------------------|--|--------|-----------|-----------------------------------|---|
| | NANAc | | Galactose | Galactose/NANAc (labile) ratio | |
| | Resistant | Labile | | | |
| G Lac 1 (G _{M3}) | — | 1166 | 571 | — | — |
| G GNT _r (G _{M2}) | 370 | — | 91 | — | — |
| G GNT 1 (G _{M1}) | 14.4 | — | 16.6 | — | — |
| G GNT 2a (G _{D1a}) | 10.8 | 275 | 20.9 | 0.08:1 | |
| G GNT 2b (G _{D1b}) | 8.8 | 191 | 16.4 | 0.09:1 | |
| G GNT 3 (G _{T1}) | 15.3 | 496 | 23.0 | 0.05:1 | |

the galactosaminyl group. Table 3 shows that the N-acetyl[¹⁴C]₁galactosamine/labile N[³H]-acetylneuraminic acid molar ratio calculated both from added nucleotides or from recovered nucleotides was lower than unity, indicating that most of the synthesis of G GNT 2b (G_{D1b}) did not occur via G Lac 2. Table 5 shows that in G GNT 2b (G_{D1b}) the sialyl group entered more labile NANAc than galactose (galactose/labile NANAc molar ratio 0.09:1), which indicated that G GNT 1 (G_{M1}) was a precursor of G GNT 2b (G_{D1b}).

(d) Labelling of trisialoganglioside. To obtain information on the sequence of incorporation of the two labile sialyl groups of G GNT 3 (G_{T1}), this ganglioside was isolated from the products of the incubation of particles with CMP-N[³H]-acetylneuraminic acid. A sample of this preparation was

incubated with neuraminidase in conditions designed to release completely the labile sialyl groups (total labile; for details see the Experimental section). Another sample of the same preparation was incubated with diluted neuraminidase so that the maximum possible amount of G GNT 2b (G_{D1b}) was obtained. In the experiment described in Table 6, 33% of the theoretical amount of G GNT 2b (G_{D1b}) was obtained; this was treated with neuraminidase under the standard conditions to release the labile NANAc attached through a 2→8 linkage to the resistant NANAc residue. The specific radioactivity of the released NANAc was determined (labile 2→8). Table 6 shows that most of the labelling was in the sialyl group attached to position 3 of the terminal galactose, showing that in the conditions of our experiment most of

Table 6. *Distribution of labile N[³H]-acetylneuraminic acid in the trisialoganglioside formed in vitro from endogenous acceptors*

The incubation system contained: particles from 30g of 15-day-old-rat brain; CMP-N[³H]-acetylneuraminic acid (sp. radioactivity 10×10^6 c.p.m./ μ mol), 0.4 μ mol; MgCl₂, 100 μ mol; potassium phosphate buffer, pH 6.8, 800 μ mol; final volume, 20ml; incubated for 15min at 37°C. G GNT 3 (G_{T1}) was isolated, purified and treated with neuraminidase as described in the Experimental section.

| Labile NANAc | Incorporation (c.p.m./ μ mol of NANAc) |
|-------------------------------------|--|
| Total | 15004 |
| 2→8 linkage (to resistant NANAc) | 600 |
| 2→3 linkage (to terminal galactose) | 30374 |

the G GNT 3 (G_{T1}) was synthesized from G GNT 2b (G_{D1b}).

DISCUSSION

The presence of sialyltransferases for gangliosides in all the subcellular organelles (Arce *et al.* 1966) indicated that each organelle synthesizes its own gangliosides; this was supported by experiments *in vivo* in which no sequential but simultaneous labelling of the gangliosides in different organelles was found (Rubiolo de Maccioni & Caputto, 1968; Harzer, Jatzkewitz & Sandhoff, 1969). In addition, in the present work it was shown that rat brain particles are able to complete the formation of all the major gangliosides by using endogenous precursors attached to the particles and added water-soluble CMP-N-acetylneuraminic acid.

Some of the experiments in this work were devised to gain a first insight on the problem of the synthesis of gangliosides as membrane constituents. For this reason any treatment damaging the integrity of the particles, as, for instance, addition of detergents, was avoided, and so the system was expected to be essentially compartmentalized. That this was obtained was indicated by the linear curve of activity versus concentration of particles. This showed that the particles were active in discrete compartments; if interaction between substrates and enzymes from different particles had played an important part in the measured reactions, an exponential curve would have been obtained; any substrate from a given particle is in exogenous position to any enzyme from another particle, and there was no reason to assume that under the conditions of exogenous substrates they were at saturating concentrations all along the range of particle concentrations tested. In a system in which the largest possible compartments were the particles

themselves, it was notable that the curve of activity versus time was that of a zero-order rather than a first-order reaction. On average, this means that in each particle the pools of acceptors are at saturating concentrations for the catalytic units in the same particle. But at the same time, the heat-inactivation experiments showed that activity and acceptor capacity of the preparations decreased simultaneously. One possible explanation is that a substrate is such for one enzyme or catalytic centre only and once this is inactivated the substrate cannot be used by another enzyme. We suggest that this observation fits well with those models of membrane in which enzymes and lipids are interdispersed in such a way that the lipids are distributed between the enzyme molecules (Sjöstrand, 1968). In these models relatively small pools may be saturating the enzyme with which it is in almost continuous contact but once that particular enzyme is inactivated the substrate cannot get in contact with another enzyme.

From the specific radioactivity of common moieties of gangliosides (such as N-acetylgalactosamine and galactose), no indications were found of precursor-product relationships between the mono- and di-sialogangliosides or between these and the trisialoganglioside, since there was no definite radioactive dilution between them. This does not necessarily mean that in building up a complex ganglioside it does not pass through the stages of less complex compounds of the same group; but evidently, provided that this is the mechanism of synthesis, only a small part of each pool is formed of stages in the formation of the more complex compounds. Whether the different stages travel on the surface of the corresponding enzymes or the acceptor is in a set position and in it the total compound is built up, there is no way of deciding at present. Our attempts to demonstrate reciprocal interference between endogenous and exogenous acceptors on the incorporation of NANAc failed; because of the uncertainties as to the conditions of the endogenous substrate no definite conclusion can be drawn from these experiments but they indicate that the enzymes working with acceptors in these two positions are different.

The rigid arrangement suggested by our findings provides an explanation for the independent biosynthesis of each of the different gangliosides found by Suzuki & Korey (1964) in experiments *in vivo* and confirmed by us (A. Arce, H. J. Maccioni & R. Caputto, unpublished work). However, contrary to what has been reported in those experiments the labelling *in vitro* was found almost exclusively in the neuraminidase-labile sialyl groups. The results suggest that most of the incorporation of sialyl groups was by addition to hematoside or to more complex stages in the building up of gangliosides.

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