The Biosynthesis of Brain Gangliosides

SEPARATION OF MEMBRANES WITH DIFFERENT RATIOS OF GANGLIOSIDE-SIALYLATING ACTIVITY TO GANGLIOSIDES

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(Received 22 March 1977)

Brain subcellular fractions were analysed for ganglioside-sialylating activity by measuring the incorporation of *N*-[³H]acetylneuraminic acid from CMP-*N*-[³H]acetylneuraminic acid into endogenous ganglioside acceptors (endogenous incorporation) and into exogenous lactosylceramide (haematoside synthetase activity). The ratios of endogenous incorporation to gangliosides and of haematoside synthetase to gangliosides for the synaptosomal and mitochondrial fractions from a washed crude mitochondrial fraction were lower than those obtained for other membrane fractions. The differences appear to reflect intrinsic characteristics of each membrane fraction. The results of labelling *in vitro* and the time course of labelling of gangliosides of the different subcellular fractions *in vivo* after injection of *N*-[³H]acetylmannosamine are consistent with the possibility of a subcellular site for synthesis of gangliosides different from that of ganglioside deposition.

Work *in vitro* with endogenous acceptors (Arce *et al.*, 1971, 1972; Maccioni *et al.*, 1972*a*) and *in vivo* (Maccioni *et al.*, 1971, 1972*b*) provided evidence for the pathways for the biosynthesis of gangliosides and also showed the presence of at least two pools of gangliosides in rat brain (Caputto *et al.*, 1974, 1976). One of these, the pool of end products, consists of most of the gangliosides and is not involved in further synthesis, whereas the other pool of gangliosides is very much smaller and consists of identical compounds that are intermediates in the biosynthesis of gangliosides of increased complexity.

Studies on these pools may provide information not only on the synthesis of gangliosides but also on the formation of membranes in the brain. In brain microsomal preparations, the pool of end-product gangliosides is more accessible to neuraminidase than is the pool of intermediates (Maccioni *et al.*, 1974). The object of the present work was to find out whether these distinct pools are in the same membrane but in different positions, or in fractions that can be separated by centrifugation.

Materials and Methods

Chemicals

N-[³H]Acetylmannosamine (200 mCi/mmol) was prepared from D-mannosamine hydrochloride and

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[³H]acetic anhydride (400 mCi/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) by the procedure of Brunetti *et al.* (1962). Other materials were as previously described (Maccioni *et al.*, 1974).

Fractionation of brain tissue

Brains from 15-day-old rats were homogenized in 10 vol. of cold 0.32 M-sucrose and centrifuged at 1000g for 10 min. To obtain the crude mitochondrial fraction, the supernatant was centrifuged as described by Whittaker (1969) at either 17000g for 60 min (procedure A) or at 10000g for 20 min (procedure B). Where stated, the pellet obtained at 10000g for 20 min was washed three times by suspending it in 0.32 M-sucrose and centrifuging at 10000g for 20 min (washed crude mitochondrial fraction; Whittaker, 1969). The supernatant plus the washing fluids were centrifuged at 17000g for 30 min and the pellet was resuspended in 0.32 M-sucrose. The supernatant suspensions remaining after the crude mitochondrial fractions were obtained (by either procedure A or B) were centrifuged at 100000g for 1 h to obtain the microsomal fractions and the cytosols.

For preparation of the synaptosomal fraction, the crude mitochondrial pellet was resuspended in 8 ml of 0.32 M-sucrose and layered on a discontinuous gradient consisting of 12 ml of 0.8 M- and 12 ml of 1.2 M-sucrose. After centrifugation at 53000g for 2 h in the SW25 rotor of the Spinco ultracentrifuge, three fractions were obtained, which were termed, as designated by Eichberg *et al.* (1964):

A, the small myelin fragments found on top of 0.8M-sucrose; B, the synaptosomal fraction found on top of 1.2M-sucrose; C, the mitochondrial fraction found as a pellet at the bottom. In other cases, the crude mitochondrial pellet obtained at 17000g for 60min was subjected to hypo-osmotic shock by suspending it in cold water (2ml/g of original tissue; Whittaker *et al.*, 1964). The water suspension (*W*) was centrifuged at 10000g for 20min to remove the larger mitochondrial and myelin fragments (*W_p*), leaving a supernatant (*W_s*), from which 5ml was then placed on a discontinuous gradient consisting of 5ml each of 0.4, 0.6, 0.8, 1.0 and 1.2M-sucrose. After centrifugation at 53000g for 2h in the SW25 rotor of the Spinco ultracentrifuge, the membranes floating at the different sucrose densities were collected by suction. These fractions were named, according to Whittaker *et al.* (1964): O, soluble; D, vesicles; E, membranes; F, synaptosome 'ghosts'; G, synaptosome 'ghosts'; H, partially disrupted synaptosomes; I, mitochondria.

To assay AcNeu* incorporation, each fraction from the gradient was diluted with water to a sucrose concentration of 0.32M and pelleted by centrifugation at 100000g for 90min. The pellet was resuspended in 0.32M-sucrose to a final protein concentration of 5–10mg/ml. For colorimetric determination of AcNeu from gangliosides and measurement of radioactivity incorporated *in vivo*, the fractions were processed without previous pelleting at 100000g (see below).

Measurement of ganglioside-sialylating system

From our previous studies of the pool of ganglioside intermediates (Arce *et al.*, 1971), the endogenous acceptors for ganglioside synthesis and the glycosyltransferase activities are found to have a close relationship. In the present work, total ganglioside-accepting capacity for sialyl groups and the enzymic activity for synthesis of haematocide (a monosialo-ganglioside) from exogenous lactosylceramide were determined as indications of the presence of the pool of ganglioside intermediates. These determinations were carried out as follows:

(a) *Total capacity for incorporation of [³H]AcNeu into endogenous ganglioside acceptors.* The incubation system contained, in a final volume of 25 μ l, 0.16M-sucrose, 8 μ M-CMP-*N*-[³H]AcNeu (8×10^8 c.p.m./ μ mol), 5mM-MnCl₂, 25mM-cacodylate/HCl buffer, pH6.8, and membrane protein (100–300 μ g). Unless otherwise stated, the incubation time was 1h at a temperature of 37°C. Under these conditions a plateau was reached after 25min and the maximal incorporation was proportional to the protein added. Since neither CMP-[³H]AcNeu nor the catalytic activity of the membranes was the

limiting factor for the maximal incorporation, it was considered that the endogenous acceptor capacity of the preparation was saturated (Maccioni *et al.*, 1974).

(b) *Determination of haematocide synthetase activity (CMP-AcNeu-lactosylceramide N-acetylneuraminyl-transferase, EC 2.4.9.9) (Arce et al., 1966).* The incubation system contained, in a final volume of 25 μ l, 0.16M-sucrose, 20 μ M-CMP-[³H]AcNeu (8×10^8 c.p.m./ μ mol), 80 μ M-lactoceramide, 15mM-MnCl₂, 25mM-cacodylate/HCl buffer, pH6.8, membrane protein (100–300 μ g), and a Triton CF-54/Tween 80 (2:1, w/w) mixture in the amount required to give a protein/detergent ratio of 1:1 (w/w). Unless otherwise stated the incubation time was 60min and the temperature 37°C. Under these conditions the reaction rate was proportional to protein concentration and the incorporation was linear with time up to 120min of incubation. The endogenous incorporation obtained under the same conditions but in the absence of lactosylceramide was subtracted in all determinations.

Radioactivity incorporated into endogenous gangliosides or exogenous lactosylceramide was measured as previously described by Maccioni *et al.* (1974).

Labelling of gangliosides *in vivo*

The precursor used was *N*-acetylmannosamine (Kolodny *et al.*, 1973); 10 μ l of a solution containing 30 μ Ci of *N*-[³H]acetylmannosamine was injected intracisternally into 15–18-day-old rats. After receiving the injection the animals were returned to their mothers, until the moment when they were killed by decapitation. Subcellular fractions obtained as described above were made 5% and 0.5% (w/v) in trichloroacetic acid and phosphotungstic acid respectively, and centrifuged at 300g for 5min. The lipid extract was obtained from the precipitate as previously described by Maccioni *et al.* (1974). After hydrolysis of the lipid extract in 0.05M-H₂SO₄ at 80°C for 2h, the behaviour of most of the radioactive material of the extract was identical with authentic [¹⁴C]AcNeu in a column (0.6cm $\times$ 12cm) of Dowex (X8; formate form), eluted with a gradient of 0–0.5M-formic acid.

Determination of gangliosidic AcNeu

The lipid extract was evaporated under nitrogen and the residue was resuspended in 0.05M-H₂SO₄ (usually 0.2ml). After heating at 80°C for 2h, the hydrolysate was neutralized with NaOH and partitioned against chloroform. The upper phase was collected and the chloroform layer was washed once with water. The mixture of the upper phase plus washing was freeze-dried and the residue redissolved in water for colorimetric determination

* Abbreviation: AcNeu, *N*-acetylneuraminic acid.

of AcNeu. The recovery obtained after a standard sample of total gangliosides, measured by the resorcinol reaction, was submitted to the entire procedure was 80–85%, so that a correction factor of 1.2 was applied to each determination.

Chemical determinations

AcNeu was usually determined by the resorcinol method of Svennerholm (1963) as modified by Miettinen & Takki-Luukkainen (1959). Occasionally the thiobarbituric acid assay (Aminoff, 1961) was used. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Radioactivity measurements were carried out as previously described by Maccioni *et al.* (1974).

Results

Subcellular distribution of gangliosides and the sialylating system

The distribution of gangliosides and proteins among the brain subcellular fractions is shown in Table 1. More than 75% of the gangliosides, the endogenous ganglioside acceptors and the sialyltransferase activity were in the microsomal fraction. Most of the remainder was in the crude mitochondrial fraction. The values for any of these three determinations were very low in the nuclear fraction and below the limits of detection in the cytosol. These results confirmed that the concentration of gangliosides in the microsomal fraction is higher

than in the other fractions (Seminario *et al.*, 1964; Lapetina *et al.*, 1967; Avrova *et al.*, 1973). It was also noted that in the microsomal fraction the concentration of endogenous ganglioside acceptors and sialyltransferase activity relative to the ganglioside content was somewhat higher than in the crude mitochondrial fraction.

A clear difference in the distribution of gangliosides and the sialylating system was found when the crude mitochondrial fraction was subjected to washing steps (Table 2). The washed crude mitochondrial fraction contained 39.6% of the gangliosides present in the unwashed fraction and only 4.3% and 8.8% of endogenous acceptors and sialyltransferase respectively, whereas the washing fluids contained 60.3% of the gangliosides but more than 70% of the sialylating activity. According to Raghupathy *et al.* (1972), this washing procedure leaves a crude mitochondrial fraction that is also very low in glycoprotein glycosyltransferase activity.

The washed crude mitochondrial fraction obtained by centrifuging at 10000g for 20 min and the washing fluid were centrifuged separately in a discontinuous sucrose gradient to obtain their subfractions *A*, *B* and *C* (Whittaker, 1969). In subfractions *B* and *C* the contents of gangliosidic AcNeu in nmol/mg of protein were approximately equal whether the pellet obtained at 10000g for 20 min or the washing fluid was the starting material (Table 3). On the other hand, the sialylating activity showed clear differences. The activities in fractions *A* and *B* of the gradient from the washing fluid were 5–10-fold higher than in the fractions *B* and *C* obtained from the washed crude mitochondria. Since, according to our previous work (Caputto *et al.*,

Table 1. Gangliosides, endogenous gangliosidic acceptors of sialyl groups and haematoside synthetase activity in fractions from rat brain homogenate

Values are means for three experiments and are expressed as percentages of those in the total homogenate. Absolute values per g of brain were: protein, 85.7mg; gangliosidic AcNeu, 1404nmol; endogenous acceptor capacity, 245616c.p.m. of [³H]AcNeu incorporated into endogenous ganglioside acceptors; haematoside synthetase activity, 3380951c.p.m. of [³H]AcNeu incorporated into lactosylceramide. For gangliosidic AcNeu, the concentration factor represents percentage of recovered AcNeu/percentage of recovered protein. For endogenous gangliosidic acceptors and haematoside synthetase activity, the concentration factor represents percentage of recovered AcNeu incorporation (c.p.m.)/percentage of recovered gangliosidic AcNeu. Determination of gangliosidic AcNeu, endogenous acceptor capacity, haematoside synthetase activity and subcellular fractionation by procedure B were as described in the Materials and Methods section. N.D., not detectable.

Subcellular fraction	Protein (% of total)	Gangliosidic AcNeu		Endogenous gangliosidic acceptor capacity		Haematoside synthetase activity ([³ H]AcNeu incorporation into lactosylceramide)	
		(% of total)	(Concn. factor)	(% of total)	(Concn. factor)	(% of total)	(Concn. factor)
Nuclear	9.0	3.0	0.33	4.1	1.36	3.3	1.10
Crude mitochondrial	23.2	32.9	1.41	25.8	0.78	23.2	0.70
Microsomal	41.6	76.2	1.83	81.8	1.07	75.8	0.99
Cytosol	26.3	N.D.	—	N.D.	—	N.D.	—

Table 2. *Percentage distribution of gangliosides, endogenous gangliosidic acceptors of sialyl groups and haematoside synthetase activity in the washed crude mitochondrial fraction and in the washing fluids*

The crude mitochondrial fraction obtained by centrifugation at 10000g for 20min (procedure B) was washed three times as described in the Materials and Methods section. The washing fluids were combined, centrifuged at 17000g for 30min and the pellet was homogenized in 0.32M-sucrose. Expression of results and determinations are as in Table 1. The percentage values of each determination were calculated by taking as 100% the corresponding values for the crude mitochondrial fraction shown in Table 1.

Fraction	Gangliosidic AcNeu (% of total)	Endogenous gangliosidic acceptor capacity		Haematoside synthetase activity ([³ H]AcNeu incorporation into lactosylceramide)	
		(% of total)	(Concn. factor)	(% of total)	(Concn. factor)
Washed pellet	39.6	4.3	0.11	8.8	0.22
Washing fluids	60.3	92.7	1.53	73.8	1.22

Table 3. *Gangliosides, endogenous gangliosidic acceptors of sialyl groups and haematoside synthetase activity in subfractions from the washed crude mitochondrial fraction and from the washing fluids*

The crude mitochondrial fraction obtained by procedure B was washed, and both the washed fraction and the washing fluids were layered on sucrose gradients for preparation of fractions A, B and C. After centrifugation, the material banding at each interphase was collected by aspiration, diluted to approx. 0.32M-sucrose with water and centrifuged at 100000g for 90min. The pellets were gently homogenized in 0.32M-sucrose for determination of gangliosidic AcNeu, endogenous gangliosidic acceptor capacity and haematoside synthetase activity. For details see the Materials and Methods section.

Subfraction ...	Washed fraction			Washing fluids		
	A*	B	C	A	B	C
Gangliosidic AcNeu (nmol/mg of protein)	—	23.0	10.0	18.8	20.9	8.8
Endogenous gangliosidic acceptor capacity (c.p.m./mg of protein)	—	1010	188	5200	8600	1200
Haematoside synthetase activity (c.p.m./mg of protein)	—	7040	4186	46150	46920	8391

* Insufficient material for assay purposes.

Table 4. *Gangliosides, endogenous gangliosidic acceptors of sialyl groups and haematoside synthetase activity in fractions from crude mitochondria after hypo-osmotic shock*

The crude mitochondrial fraction obtained by centrifugation at 17000g for 1h (procedure A) was disrupted hypo-osmotically. The W_s fraction was layered on a sucrose-density gradient and centrifuged as described in the Materials and Methods section. The membranes floating at the different densities were collected and analysed as in Table 3. Fraction O (soluble) was analysed without previous centrifugation. Values are averages $\pm$ S.E.M. for the numbers of determinations indicated in parentheses. N.D., Not detectable.

Fraction	Gangliosidic AcNeu (nmol/ mg of protein)	Endogenous ganglio- sidic acceptor capacity (c.p.m./mg of protein)	Haematoside synthetase activity (c.p.m./mg of protein)	Ratio of averages	
	(a)	(b)	(c)	(b):(a)	(c):(a)
<i>W</i>	20.6 $\pm$ 0.58 (6)	3004 $\pm$ 100 (6)	18726 $\pm$ 1737 (3)	145	909
<i>W_p</i>	18.3 $\pm$ 0.80 (6)	2340 $\pm$ 295 (9)	13634 $\pm$ 2992 (3)	127	745
<i>W_s</i>	20.6 $\pm$ 0.58 (6)	3000 $\pm$ 131 (6)	17064 $\pm$ 748 (3)	145	828
Recovery (% of <i>W</i>) ...	81.3 $\pm$ 3.10 (3)	71.7 $\pm$ 3.7 (6)	70.7 $\pm$ 3.0 (3)		
<i>O</i>	0.7 $\pm$ 0.14 (3)	N.D.	N.D.		
<i>D</i>	22.2 $\pm$ 2.19 (2)	990 $\pm$ 400 (2)	2200	44	99
<i>E</i>	58.3 $\pm$ 3.30 (8)	3206 $\pm$ 559 (9)	4218 $\pm$ 538 (3)	55	72
<i>F</i>	45.3 $\pm$ 3.30 (9)	10359 $\pm$ 895 (8)	22589 $\pm$ 500 (3)	228	498
<i>G</i>	41.6 $\pm$ 2.15 (8)	12410 $\pm$ 746 (9)	34756 $\pm$ 6515 (3)	298	835
<i>H</i>	29.7 $\pm$ 2.06 (9)	2262 $\pm$ 247 (9)	19326 $\pm$ 2340 (3)	76	650
<i>I</i>	8.8 $\pm$ 1.57 (8)	522 $\pm$ 33 (8)	2593 $\pm$ 345 (3)	59	294
Recovery (% of <i>W_s</i>) ...	73.4 $\pm$ 7.6 (6)	83.7 $\pm$ 5.8 (6)	63.3 $\pm$ 3.7 (3)		

1974) the amount of gangliosides represents the pool of end products, and the endogenous acceptors together with the glycosyltransferase activities are

commensurate with the pool of intermediate gangliosides, results in Tables 2 and 3 indicated the separation of both pools.

Distribution of gangliosides and the sialylating system after hypo-osmotic shock

Membranes enriched in the intermediate pool or in the supposed end-product pool could also be separated from the crude mitochondrial fraction obtained at 17000g for 60min. This crude mitochondrial fraction contained more than 80% of the endogenous ganglioside acceptors and sialyltransferase activity present in the total homogenate. The separation was obtained by subjecting the crude mitochondrial fraction to hypo-osmotic shock and centrifuging in a sucrose-density gradient as described by Whittaker *et al.* (1964). In agreement with the results of Dekirmenjian & Brunngraber (1969), the highest ganglioside concentrations were found in fractions *E*, *F* and *G* (Table 4). Fractions *D*, *E* and *I* showed much lower ratios of endogenous ganglioside acceptors and sialyltransferase activity to gangliosides than fractions *F* and *G*. The recoveries in these experiments were within the experimental error, so that the differences in the ganglioside-sialylating activity per mg of protein among some membrane fractions were not due to a special distribution within the gradient of factors (inhibitors or activators) that could interact freely with the sialylating system.

Subcellular distribution of labelled gangliosides

It was considered possible that, in fractions with a relatively low activity for the sialylating system, this was due to selective inactivation during the approx. 7h required to prepare those fractions. To test this possibility, experiments were carried out in which the initial transfer of AcNeu from CMP-AcNeu to endogenous gangliosides was measured by incubation of the total homogenate immediately after it was obtained (Table 5). Comparing the ganglioside radioactivity among the fractions labelled *in vitro*, it was found that the specific radioactivities of the gangliosides from the washed crude mitochondrial fraction and from the fractions obtained from it were the lowest. For comparison, Table 5 shows the labelling of the gangliosides from the same subcellular fractions from rats that were injected with *N*-[³H]acetylmannosamine 48h before death, in which no substantial differences were found between the different fractions. In another experiment, a total homogenate was labelled *in vitro* by incubation with CMP-[³H]AcNeu for 2, 4, 6, 12 and 24min and the fractions obtained after hypo-osmotic shock of crude mitochondria were analysed. At all the periods tested, fractions *D* and *E* showed the lowest and fractions *F* and *G* the highest labelling in relation to their ganglioside content. Since the results of these experiments were in agreement with those obtained when the fractions were isolated first and then tested for

Table 5. Labelling of gangliosides of different subcellular fractions after incubation of total homogenate with CMP-[³H]AcNeu or after injection of *N*-[³H]acetylmannosamine to rats

Labelling of endogenous gangliosides *in vitro* was carried out by incubation of a total homogenate from one 15-day-old rat brain with CMP-[³H]AcNeu (5μM; 15 × 10⁶c.p.m.) at 37°C for 15min. To avoid clumping of the membranes, no MnCl₂ was added to the incubation system. Labelling of gangliosides *in vivo* was carried out by injection of *N*-[³H]acetylmannosamine into 15-day-old rats as indicated in the Materials and Methods section. The rats were killed 48h after the injection. As a control for changes in the sedimentation properties of the different subcellular fractions from the homogenate labelled *in vitro* caused by the incubation at 37°C, one-half of the homogenate labelled *in vivo* was also incubated for 15min at 37°C and the other half was kept at 4°C for the same time. Subcellular fractions were obtained from the three different homogenates as described in the Materials and Methods section (procedure B). Since no significant differences were found between the amounts of ganglioside radioactivity in the subcellular fractions obtained from the homogenates labelled *in vivo* and kept at either 4°C or 37°C, only the values at 37°C are presented. For determination of gangliosidic AcNeu content and ganglioside radioactivity, each subcellular fraction was made 5% (w/v) in trichloroacetic acid and 0.5% (w/v) in phosphotungstic acid, and processed as described in the Materials and Methods section. N.D., Not detectable.

Subcellular fraction	Labelling (c.p.m./nmol of AcNeu)	
	<i>In vitro</i>	<i>In vivo</i>
Total homogenate	83.3	168.6
Nuclear	75.8	168.7
Crude mitochondrial	52.0	136.1
Washed crude mitochondrial	4.7	160.3
Washing fluids	100.2	218.5
Microsomal	98.4	157.6
Fractions from washed crude mitochondria		
<i>A</i>	N.D.	N.D.
<i>B</i>	4.4	186.6
<i>C</i>	10.2	203.6

their ganglioside-sialylating activities, we concluded that the differences in the endogenous ganglioside acceptor capacity and sialyltransferase activity in the membranes are not due to differential inactivations during the fractionation procedures.

Time course of the labelling in vivo

Since no significant differences in the specific radioactivities of gangliosides from different subcellular fractions were noted 48h after injection of *N*-[³H]-

Fig. 1. Labelling *in vivo* of gangliosides from fractions of crude mitochondria after hypo-osmotic shock

Rats (15 days old) were injected with N -[^3H]acetylmannosamine as described in the Materials and Methods section. At the times indicated, groups of four rats were killed and their brains were pooled. The pooled brains were homogenized and fractionated by procedure A. The crude mitochondrial fraction was subjected to hypo-osmotic shock and processed as in Table 4. ○, Microsomal fraction; △, fraction E; □, fraction F; ●, fraction G; ▲, fraction H.

acetylmannosamine (see Table 5), the course of labelling was examined at shorter incorporation times. Animals that received intracerebral injections of N -[^3H]acetylmannosamine were killed from 6 to 48 h after injection. Fractions E, F, G and H from crude mitochondria subjected to hypo-osmotic shock were chosen for comparison. Fig. 1 shows that the specific radioactivities of gangliosides in fractions F and G increased until they reached a maximum at 12 h after injection, then decreased rapidly in the next 12–24 h period, and more slowly after 24 h. The specific radioactivities of the gangliosides in fractions E and H, on the other hand, increased at a lower rate but continuously up to 24 h, and then followed essentially the same course as fractions F and G. Fig. 1 also shows that the time course of labelling of the gangliosides from the microsomal fraction obtained by procedure A is essentially identical with that of gangliosides from fraction E. This probably indicates that, as observed by Whittaker *et al.* (1964), microsomal membranes contaminating the crude mitochondrial fraction float at the 0.4 M-/0.6 M-sucrose interphase of the gradient (fraction E). Three experiments of this type were run with essentially the same results. It was previously shown by Maccioni *et al.* (1977) that 1 h after the injection

of N -[^3H]acetylmannosamine the specific radioactivity of gangliosides from fraction G was approximately twice that of fraction B from a washed crude mitochondrial fraction, but after 48 h they became equally labelled.

Discussion

The results presented here support the hypothesis of the existence in rat brain of two pools of gangliosides, one of which is very small and is part of the synthesizing system (pool of ganglioside intermediates) and the other, a very much larger pool, that cannot be utilized to synthesize gangliosides of higher complexity (end-product pool). The first evidence for the existence of the two pools was obtained by comparing results of studies *in vitro* and *in vivo*. Whereas the studies *in vitro* showed that gangliosides were built by the stepwise addition of single glycosyl groups to the corresponding acceptors (Arce *et al.*, 1966, 1971; Roseman, 1970; Maccioni *et al.*, 1972a), the studies *in vivo* failed to show precursor-product relationships among brain gangliosides of different complexities (Suzuki & Korey, 1964; Maccioni *et al.*, 1971; Holm & Svennerholm, 1972). Also, the identical specific radioactivities of repeated units in the carbohydrate chain of gangliosides labelled *in vivo* indicated that the synthesis is carried out with a very small pool of precursors, and furthermore that partial turnover of the carbohydrate chain, if it occurs, is a minor event (Maccioni *et al.*, 1971).

Other evidence showed that desialylation of most of the gangliosides in membranes by treatment with neuraminidase did not in practice change the endogenous capacity of the same membranes to accept sialyl groups. It was also shown that the pool of endogenous acceptors is localized in an area more protected from the action of exogenous neuraminidase than are most gangliosides (Maccioni *et al.*, 1974; for reviews see Caputto *et al.*, 1974, 1976; Maccioni *et al.*, 1977).

Finally, in the present work, subcellular fractions were obtained in which the ratios of endogenous acceptors to gangliosides and of sialyltransferase activity to gangliosides varied by factors of between 5 and 10 (Tables 2, 3 and 4). Also, determinations in fractions from animals injected with N -[^3H]acetylmannosamine confirmed the existence of membranes in fractions F and G (designation of Whittaker, 1969) in which the gangliosides reached their highest specific radioactivity before gangliosides in other fractions. These fractions also have higher specific radioactivities than the others and then tend to become equal to them (Fig. 1). The curves are compatible with a precursor-product relationship between components in fractions F and G and the other membrane fractions.

The present results cast some doubts on the concept that each organelle synthesizes its own gangliosides (Arce *et al.*, 1966, 1971; Harzer *et al.*, 1969; Di Cesare & Dain, 1972) and on the concept of the synaptosomal localization of most of the ganglioside glycosyltransferases (Roseman, 1970; Di Cesare & Dain, 1972; Den *et al.*, 1975). In addition, our results suggest that the transient and the end-product pools of gangliosides are in membranes with different sedimentation properties. The possibility of an independent metabolism of these two pools is not excluded, but the possibility that the end-product pool gangliosides were derived from the membranes carrying the intermediate pool should also be considered.

Our previous results showing that no precursor-product relationship can be detected among gangliosides of different degrees of complexity and that repeated units in the molecule had essentially the same specific radioactivity (Maccioni *et al.*, 1971) are compatible with a situation in which the ganglioside pool in the membranes containing the ganglioside-sialylating system is relatively small in size and continuously renewed by transporting the synthesized molecules to the membranes enriched with the end-product pool. The lack of partial turnover of the carbohydrate chain and the inability of endogenous sialyltransferases to resialylate the empty sites of gangliosidic AcNeu created by treatment of membranes with *Clostridium perfringens* neuraminidase (Maccioni *et al.*, 1974) is also compatible with the possibility of a site of synthesis in the cell different from that in which the gangliosides are finally localized and eventually degraded. The mechanism by which the hypothetical intermediate and end-product pools of gangliosides might be interconnected is not known at present. The problem is complicated by the lack of reliable information on the precise localization of the gangliosides. They have been found mainly in the synaptosomal fraction (Seminario *et al.*, 1964; Eichberg *et al.*, 1964; Wiegandt, 1967), but the important question of whether they are pre- or post-synaptic has not been convincingly demonstrated. Axonal transport of gangliosides has been claimed (Forman & Ledeen, 1972) and this interesting possibility has been studied in our laboratories (Maccioni *et al.*, 1977).

Considering the localization of the glycosyltransferases in other organs (Keenan *et al.*, 1974), it is possible that the fraction washed away from the crude mitochondrial fraction obtained at 10000g for 20 min contains Golgi membranes. Unfortunately, we have been unable to find any claim for the separation of Golgi membranes from brain, although Fishman (1974) suggested that Golgi membranes are present in fraction A after fractionation by the procedure of Whittaker (1969). Electron-microscopic examination of the particles found in the

washing fluid from the crude mitochondrial fraction showed structures similar to those observed by Seijo *et al.* (1970) in synaptosomal preparations, which they claim are Golgi-derived membranes. It should be made clear, however, that no claim for purity or enrichment over other fractions can be made at present.

The existence of different pools of gangliosides is undoubtedly a point to consider in dealing with problems of changes in gangliosides brought about by functional or pathological processes.

We thank Dr. E. Hliba for performing the electron-microscopic examination of the different subcellular fractions. This investigation was supported in part by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas and by a grant from the Organization of American States. Part of this work was taken from a thesis by C. A. L. to be submitted to the Facultad de Ciencias Químicas of the Universidad Nacional de Córdoba in partial fulfilment of the requirements for the degree of Ph.D.

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