Rat Brain Microsomal Gangliosides

ACCESSIBILITY TO A NEURAMINIDASE PREPARATION AND THE POSSIBLE EXISTENCE OF DIFFERENT POOLS IN RELATION TO THEIR BIOSYNTHESIS

By H. J. F. MACCIONI, A. ARCE,* C. LANDA and R. CAPUTTO

Departamento de Química Biológica, Facultad de Ciencias Químicas Universidad Nacional de Córdoba, Ciudad Universitaria, Córdoba, Argentina

(Received 23 July 1973)

1. Treatment of rat brain microsomal membranes with a neuraminidase preparation from *Clostridium perfringens* resulted in an almost complete conversion of polysialogangliosides into monosialogangliosides. 2. Neuraminidase treatment of the membranes did not increase the incorporation of *N*-[³H]acetylneuraminic acid from CMP-*N*-[³H]acetylneuraminic acid into the gangliosidic fraction, indicating that a monosialoganglioside is an acceptor of *N*-acetylneuraminic acid in these membranes only if, in addition to having the right chemical structure, it is in a proper position, probably in relation to the endogenous sialyltransferases. 3. These experiments also indicated that no independent turnover of the neuraminidase-labile *N*-acetylneuraminyl groups of gangliosides occurred *in vitro*. 4. *N*-[³H]Acetylneuraminic acid from endogenous polysialogangliosides labelled *in vitro* was released by neuraminidase at a slower rate than *N*-acetylneuraminic acid from unlabelled gangliosides of the same membranes. From this it was concluded that recently synthesized polysialogangliosides (completed *in vitro*) are in the membranes in a position less accessible to neuraminidase than are those synthesized earlier which were present in the membranes at the start of the labelling experiment.

The content of gangliosides in different brain sub-cellular organelles has been established (Eichberg *et al.*, 1964; Lapetina *et al.*, 1967; Avrova *et al.*, 1973) and recently it was reported that in neuronal membranes from adult rat they are not accessible to a purified neuraminidase preparation (Dicesare & Rapport, 1973).

For other membranes, some information pertaining to this problem is also available. In surface membranes from L cells the gangliosides are not accessible to added neuraminidase, whereas this enzyme readily releases AcNeu† from glycoproteins (Weinstein *et al.*, 1970). Results from experiments with antihaematoside antiserum also suggested that gangliosides in cultured fibroblasts and erythrocytes are not exposed at the cell surface but are masked by other membrane constituents such as proteins or glycoproteins (Hakomori *et al.*, 1968; Hakomori, 1969).

In the present work we report results that showed that most of the ganglioside constituents of the membranes of the microsomal fraction of rat brain were

susceptible to the action of a preparation of neuraminidase from *Clostridium perfringens*. However, a small part of these gangliosides was not exposed to the action of this enzyme preparation. These findings together with the inability of the endogenous sialyltransferases to reincorporate sialyl groups into partially desialylated gangliosides remaining attached to the membranes will be interpreted in relation to previously reported results on the biosynthesis of brain gangliosides *in vitro* and *in vivo* (Arce *et al.*, 1971; Maccioni *et al.*, 1971).

Experimental

Materials

Neuraminidase (*N*-acetylneuraminase glycohydrolase, EC 3.2.1.18) from *Cl. perfringens* (type VI, specific activity 2.4 units/mg of protein with sialyllactose as substrate) and AcNeu were from Sigma Chemical Co., St. Louis, Mo., U.S.A. [¹⁴C]AcNeu (10 mCi/mmol) and CMP-[³H]AcNeu (1 Ci/mmol) were prepared as described by Arce *et al.* (1966, 1971). Standard samples of gangliosides were prepared as described by Maccioni *et al.* (1971). Silica gel G was from Merck A.G., Darmstadt, Germany. All solvents were distilled before use.

* Present address: Facultad de Ciencias Exactas, Físicas y Naturales and Instituto de Ciencias Agrómicas, Universidad Nacional de Córdoba, Córdoba, Argentina.

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

Methods

The microsomal fraction was obtained from brains of 16–18-day-old rats by the procedure of Eichberg *et al.* (1964); for enzymic assays it was suspended in 0.32M-sucrose to a final concentration of approx. 10–15 mg of protein/ml.

Enzyme reactions. Labelling of endogenous gangliosides was carried out essentially as previously reported (Arce *et al.*, 1971); the incubation system contained, in a final volume of 0.125 ml, 1 mg of microsomal protein, 16 mM-potassium phosphate buffer, pH 6.8, 0.32 M-sucrose and 2 μ M-CMP-[³H]-AcNeu (250000 c.p.m.) at 37°C.

For treatment of microsomal membranes with *Cl. perfringens* neuraminidase the following incubation system was used: 1 mg of microsomal protein, 10 μ g of neuraminidase and 0.32 M-sucrose in a final volume of 0.2 ml. The pH of the mixture was about 6.4. Unless otherwise stated the temperature was 37°C.

Reactions were stopped by addition of 2 ml of 5% (w/v) trichloroacetic acid containing 0.5% (w/v) phosphotungstic acid (De Vries & Barondes, 1971). The mixture was centrifuged for 5 min and the supernatant solution pipetted off; the pellet was washed successively with 2 and 3 ml of the same trichloroacetic acid–phosphotungstic acid solution and finally with 3 ml of water. Lipids were extracted from the pellet as follows: chloroform–methanol (2:1, v/v) was added, with shaking, until a single phase was obtained (usually 1 ml) and the volume was brought to 3 ml by addition of chloroform–methanol–water (40:20:3, by vol). The mixture was shaken again and left for 10 min. After centrifugation the lipid extract was removed with a Pasteur pipette and the pellet was washed with 3 ml of the same solvent mixture. The pool of extract plus washings was transferred to a vial and evaporated ready for radioactivity measurements. For the colorimetric determination of AcNeu from gangliosides, the extract was partitioned with 0.2 vol. of water, washed once with theoretical upper phase (Folch *et al.*, 1957) without salt and the determination was done on the pool of upper phases. The recovery of gangliosides by this method was 85–90% of that obtained with the method of Folch *et al.* (1957) and no differences were noticed between the chromatographic patterns of the gangliosides isolated by the two methods. For chromatographic analysis and for the determination of the neuraminidase-labile AcNeu content of the gangliosides, the extract was passed through a Sephadex G-25 column (Arce *et al.*, 1971) before the partition step.

Protein-bound AcNeu was determined in the defatted protein residue as follows. The dried residue was dispersed in 0.25 ml of 0.05 M-H₂SO₄ and heated at 80°C for 1 h. The insoluble protein was removed by centrifugation and AcNeu was determined in the

supernatant solution. The AcNeu content of the defatted protein was similar to that found when the protein was defatted by the method of Folch *et al.* (1957).

Determination of N-acetylneuraminic acid released by neuraminidase. Total AcNeu released by the action of neuraminidase on membrane fragments was determined as follows. The neuraminidase reaction was stopped by the addition of 2 ml of the trichloroacetic acid–phosphotungstic acid solution, which also contained 10000 c.p.m. of [¹⁴C]AcNeu (0.5 nmol); the precipitate was separated by centrifugation and the supernatant was used for isolation of AcNeu. Trichloroacetic acid was removed by five extractions with the same volume of ether; phosphotungstic acid was neutralized with cetyltrimethylammonium bromide (Cetavlon) and the entire mixture was applied to a column (0.5 cm $\times$ 3 cm) of Dowex 1 (X8; formate form). The column was washed with water and AcNeu was eluted with 0.5 M-formic acid. The amount of AcNeu was calculated from the isotope dilution of added [¹⁴C]AcNeu. Amounts of [³H]AcNeu and unlabelled AcNeu released from gangliosides by neuraminidase were calculated from the difference between the radioactivity or neuraminidase-labile AcNeu content in the lipid extract obtained from the membrane fragments at zero time and after each period of incubation with neuraminidase. The amount of protein-bound AcNeu released by neuraminidase was calculated from the difference between the amount determined in the defatted protein at zero time and that determined at each time of incubation with neuraminidase.

Chromatographic analysis of gangliosides were done by t.l.c. on silica gel G with chloroform–methanol–20% (v/v) NH₃ (60:35:8, by vol.) as solvent; standard samples of gangliosides GM 3, GM 2, GM 1 and GD 1a (nomenclature of Svennerholm, 1963) were run simultaneously. The different gangliosides were localized and eluted as previously described (Arce *et al.*, 1971).

Chemical determinations. Free or bound AcNeu was usually determined by a micro-adaptation of the resorcinol method of Svennerholm (1963) as modified by Miettinen & Takki-Luukkainen (1959). When the amount of AcNeu to be measured was lower than 15 nmol, a micro-adaptation of the thiobarbituric acid method was used (Aminoff, 1961). The neuraminidase-labile AcNeu content of gangliosides isolated from membranes was determined by the thiobarbituric acid method on samples incubated with neuraminidase under the conditions described by Arce *et al.* (1971). Proteins were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Radioactivity was assayed by liquid-scintillation spectrometry with a Beckman LS-100 liquid-scintillation spectrometer. The scintillation fluid contained 2,5-diphenyloxazole (5 g) and

naphthalene (100g) in dioxan (1 litre). Counting efficiencies were determined by external standardization and in no case were they less than 80% for ¹⁴C and 35% for ³H.

Results

Distribution of N-acetylneuraminic acid and release by neuraminidase

AcNeu from gangliosides accounted for approximately three-quarters of the total AcNeu found in microsomal membrane fragments, the remainder being bound to proteins (Table 1). About 50% of the AcNeu from gangliosides and 70% of that bound to protein were released by incubation with the neuraminidase preparation; gangliosides isolated from microsomal membranes and treated with neuraminidase also lost about 50% of their AcNeu content. A time-course experiment showed that the reaction reached a maximum at 60 min; extending the incubation period to 12h did not increase the amount of AcNeu lost by the proteins, gangliosides or the whole membranes (Fig. 1). Endogenous neuraminidase activity was negligible under the conditions of the assay.

Treatment of membranes with neuraminidase for different periods of time resulted in a progressive degradation of polysialogangliosides to monosialoganglioside (Plate 1). A relative increase of GD 1b at shorter periods of time was also noted, owing, probably, to the relative resistance to *Cl. perfringens* neuraminidase of the AcNeu-(2 → 8)-AcNeu linkage (Svennerholm, 1963). However, a small amount of polysialogangliosides remained undegraded even after incubation for 1 h. It appears that other ganglioside glycosidases were not acting during the assay, as there was no increase in the amount of minor gangliosides (Plate 1) and the total decrease in

AcNeu from ganglioside did not exceed 50% (Fig. 1, Table 1).

Incorporation of [³H]AcNeu from CMP-[³H]AcNeu into gangliosides of intact and neuraminidase-treated membrane fragments

Rat brain subcellular particles contain incomplete gangliosides that readily accept glycosyl residues when incubated with the cognate sugar nucleotides (Arce *et al.*, 1971; Maccioni *et al.*, 1972a). The AcNeu-accepting capacity of membrane fragments treated with neuraminidase was investigated as follows. A microsomal preparation was divided into three portions; two of them were incubated separately for 20 min at 37°C, one with and one without neuraminidase, and the third portion was kept at 4°C. After incubation the membrane fragments were washed twice by addition of 5 ml of 0.32M-sucrose/mg of microsomal protein and centrifuged at 100000g for 1 h. After washing, the incubation of a mixture of neuraminidase-treated and untreated fragments at 37°C for 2 h did not result in the release of detectable amounts of AcNeu (significant neuraminidase activity was found in neuraminidase-treated fragments after washing only once). After incubation with CMP-[³H]AcNeu the incorporation of [³H]AcNeu into endogenous gangliosides of membrane fragments incubated at 37°C with and without neuraminidase followed the same course and reached the same

Table 1. Susceptibility of N-acetylneuraminic acid from rat brain microsomal membranes to *Cl. perfringens* neuraminidase

Microsomal membranes were incubated with neuraminidase under standard conditions for 4h. The reaction was stopped by addition of trichloroacetic acid-phosphotungstic acid mixture containing [¹⁴C]AcNeu (10000 c.p.m., 0.5 nmol). Total and neuraminidase-labile AcNeu were determined as described in the Experimental section.

	AcNeu (nmol/mg of protein)	
	Total	Neuraminidase-labile
AcNeu bound to Gangliosides	66.4	32.8
Protein	22.1	15.8
Whole membranes		48.2

Fig. 1. Release of N-acetylneuraminic acid from microsomal membranes as a function of time of incubation with *Cl. perfringens* neuraminidase

Incubation conditions were as described in the Experimental section. Release from whole membranes (○), from gangliosides (●) and from proteins (Δ) was determined as described in the Experimental section. (▲), AcNeu released from whole membranes incubated without added neuraminidase.

amount, the activity of both preparations being about 50% lower than in particles kept at 4°C (Fig. 2). Since in the neuraminidase-treated membrane fragments about 12nmol of AcNeu/mg of protein was released (Table 2) and because most of the gangliosides remained as GM 1 (Plate 1) a marked increase was expected in the incorporation of AcNeu in treated

Fig. 2. *N*-[³H]Acetylneuraminic acid incorporation into gangliosides of neuraminidase-treated and neuraminidase-untreated microsomal membranes

Incorporation is shown of [³H]AcNeu from CMP-[³H]-AcNeu into endogenous gangliosides of membranes previously kept at 4°C (○), or preincubated for 30min with neuraminidase (20munits/mg of membrane protein) at 37°C (●) or preincubated for 30min without neuraminidase at 37°C (Δ). For details see the Experimental section.

as compared with untreated fragments which incorporated approx. 3.5pmol. The decrease in incorporation into membrane fragments incubated at 37°C, with or without neuraminidase, compared with fragments kept at 4°C, was probably due to partial inactivation of the enzymic system or to partial depletion of endogenous acceptors, filled by transfer from endogenous, unlabelled CMP-AcNeu during the preincubation period (the presence of CMP-AcNeu in brain subcellular organelles was shown by Arce *et al.*, 1971). In any case the inactivation was dependent on the temperature and not on neuraminidase activity, because membrane fragments that had lost AcNeu by treatment with neuraminidase at 4°C and 37°C incorporated amounts of AcNeu comparable with those incubated at the same temperatures but without neuraminidase (Table 2). From Table 2 it appears that in the neuraminidase-treated membrane fragments, less than one ten-thousandth of the total number of sites potentially replenishable with AcNeu were utilized under the conditions of the assay.

Limiting factors for the incorporation of [³H]AcNeu

Destruction of CMP-[³H]AcNeu was not the factor limiting incorporation, as a further addition of this nucleotide did not increase the incorporation (Fig. 3). About 30% of the system was inactivated during preincubation for 90min with hydrolysed CMP-[³H]AcNeu, but this was similar in the membrane fragments preincubated with and without neuraminidase. The enzyme that catalyses the transfer of AcNeu from CMP-AcNeu to lactosylceramide to form GM 3 (Arce *et al.*, 1966) was not damaged by previous incubation of the membrane fragments at 37°C either in the presence or in the absence of neuraminidase (Table 2). However, as more than one sialyltransferase acts in the synthesis of gangliosides (Kaufman *et al.*, 1968), the patterns of labelling of the endo-

Table 2. *GM 3 synthetase activity and incorporation of N-[³H]acetylneuraminic acid in neuraminidase-treated and untreated microsomal membranes*

Microsomal membranes were incubated with or without neuraminidase as indicated and freed from neuraminidase as described in the Experimental section. Samples of the differently treated membranes were taken for determination of gangliosidic AcNeu, [³H]AcNeu-acceptor capacity after 90min of incubation with CMP-[³H]AcNeu under standard conditions, and GM 3 synthetase activity; for this last determination membranes were incubated with CMP-[³H]AcNeu under standard conditions, except for the addition of 40nmol of lactosylceramide, 50μg of Tween 80 and 100μg of Triton CF54. The incubation time was 120min. In a separate experiment determinations were carried out at 30, 60 and 120min and the activity was found to be linear up to 120min. Endogenous incorporation was discounted.

Treatment of membranes	Gangliosidic AcNeu lost (pmol/mg of protein)	[³ H]AcNeu incorporated (pmol/mg of protein)	GM 3 synthetase activity (c.p.m./mg of protein)
4°C (20min)	Not detectable	3.39	4660
4°C, +neuraminidase (50munits, 90min)	4150	3.27	Not assayed
37°C (20min)	500	1.73	4240
37°C, +neuraminidase (20munits, 20min)	12000	1.30	4469

EXPLANATION OF PLATE I

*Thin-layer chromatography of gangliosides isolated from microsomal membranes treated with *Cl. perfringens* neuraminidase over different time-periods*

Membrane fragments (1.5 mg of protein) were incubated with neuraminidase (30 munits). At the times indicated the gangliosides were isolated and separated on t.l.c. as described in the Experimental section. Note the increase in GM 1 and the decrease in polysialogangliosides as a consequence of the activity of neuraminidase.

genous gangliosides from fragments preincubated with or without neuraminidase and from fragments kept at 4°C were compared. In the case of a preferen-

Fig. 3. Demonstration that neither CMP-N-[³H]acetylneuraminic acid nor inactivation of the system was the limiting factor for the incorporation of [³H]AcNeu into endogenous gangliosides of neuraminidase-treated and untreated membranes

Treatment of membranes with neuraminidase at 37°C and determination of their [³H]AcNeu-accepting capacity was as described in Table 2. Arrow indicates supplementation with 0.25 nmol of CMP-[³H]AcNeu and broken lines indicate incorporation in the supplemented systems. Neuraminidase-treated membranes (●); untreated membranes (▲); open symbols indicate incorporation in neuraminidase-treated (○) or untreated membranes (△) after they were incubated for 90 min at 37°C in the presence of hydrolysed (2 h at 22°C in 0.1 M-HCl and freed from acid by freeze-drying) CMP-[³H]AcNeu before addition of CMP-[³H]AcNeu.

tial inactivation of a sialyltransferase by neuraminidase the percentage contribution of its particular product to the ganglioside mixture would be decreased. No significant differences in the patterns of labelling were found, indicating that none of the ganglioside sialyltransferases were preferentially inactivated (Table 3). Since the total incorporation was similar in the neuraminidase-treated membranes to that in those preincubated without neuraminidase (Table 2, Fig. 2), and about half of the incorporation was into endogenous GM 1 to form GD 1a (Table 3), it appears that the GM 1 formed by the action of exogenous neuraminidase cannot be used as an acceptor by the corresponding endogenous sialyltransferase under the conditions of the assay.

Accessibility to neuraminidase of gangliosides labelled *in vitro*

Microsomal membranes were incubated for 90 min with CMP-[³H]AcNeu to label the endogenous gangliosides; excess of radioactive nucleotide was eliminated by addition of 200 vol. of cold 0.32 M-sucrose and homogenization, followed by centrifugation at 100000g for 1 h. The labelled membrane fragments were treated with neuraminidase and the release of labelled and unlabelled AcNeu was compared. The release of gangliosidic neuraminidase-labile AcNeu was complete at 120 min, at which time only 45% of the radioactivity had been released (Fig. 4). Examination of the radioactive gangliosides by t.l.c. after each period of incubation with neuraminidase showed that not all the radioactive gangliosides were degraded at the same rate; a component migrating with GM 1 (possibly GD 3; see also Table 3) had the highest rate of degradation; degradation of the mixture of GD 1b and GT 1 as well as GM 3 was lower, whereas GD 1a appears to be attacked at the lowest rate, as its percentage contribution to the total radioactivity increased with time of neuraminidase treatment (Fig. 5).

Table 3. Distribution of N-[³H]acetylneuraminic acid incorporated into endogenous gangliosides of neuraminidase-treated and untreated microsomal membranes

Membrane fragments were treated with neuraminidase (20 munits/mg of protein) and washed free from neuraminidase as described in the Experimental section. They were then incubated with CMP-[³H]AcNeu for 40 min under standard conditions. Gangliosides were isolated and analysed chromatographically as described in the Experimental section.

Treatment of membranes	Radioactivity (% of total)				
	GT 1 + GD 1b	GD 1a	GM 1*	GM 2	GM 3
4°C	18	66	3	2	9
37°C	16	63	7	2	12
37°C, + neuraminidase	13	64	5	2	15

* Most of this radioactivity belongs to a compound already observed by Arce *et al.* (1971) that runs together with GM 1, whose AcNeu is readily split by neuraminidase and whose relative labelling increased when the [³H]AcNeu incorporation period was extended (cf. Fig. 5).

Two possibilities were considered to explain the sluggish release by neuraminidase of AcNeu incorporated *in vitro*: (a) that [^3H]AcNeu entered into the ganglioside in a position resistant to neuraminidase; (b) that the radioactivity was less accessible to the attack by the enzyme owing to the localization of the gangliosides in the membranes.

The first possibility was discarded because the gangliosides isolated from fragments incubated with CMP-[^3H]AcNeu for 90 min had only 10% of the radioactivity found in the neuraminidase-resistant

Fig. 4. Release by neuraminidase of *N*-acetylneuraminic acid and *N*-[^3H]acetylneuraminic acid from membrane-bound gangliosides labelled *in vitro*

Membranes were labelled *in vitro* and freed from CMP-[^3H]AcNeu as described in Table 4 (Expt. I); treatment with neuraminidase was under standard conditions; at the periods indicated AcNeu, (●) and [^3H]AcNeu, (○) released from gangliosides were determined as described in the Experimental section.

AcNeu; further, no enrichment of the percentage contribution of GM 1 to the radioactivity of the ganglioside mixture was observed when the labelled fragments were incubated with neuraminidase for periods of up to 4 h (Fig. 5). Thus the second possibility appeared as more probable and was confirmed by incubating the labelled fragments with neuraminidase after disruption by sonication or by treatment with a detergent. The release of radioactivity increased from about 10–23% in the undisrupted fragment to about 50–80% in the sonicated or detergent-treated fragments, whereas the total amount of AcNeu released was practically unchanged (Table 4), owing to the negligible contribution of the labelled AcNeu to the total neuraminidase-labile AcNeu (Table 4).

Fig. 5. Accessibility to neuraminidase of membrane-bound gangliosides labelled *in vitro*

Conditions for labelling the membranes and for neuraminidase treatment were as described for Fig. 4. At the different times of neuraminidase treatment gangliosides were isolated and processed as described in Plate 1, except that they were localized by exposure to I_2 vapour, cluted and their radioactivity was determined as indicated in the Experimental section; ○, GM 1 (see Table 3); ▲, GD 1a; △, GD 1b plus GT 1; ●, GM 3.

Table 4. Effect of disrupting the membranes on the accessibility to neuraminidase of recently completed gangliosides

Endogenous gangliosides were labelled under standard conditions during 90 min (Expt. I) or 60 min (Expt. II). The membranes were freed from unchanged CMP-[^3H]AcNeu as described in the Experimental section. The standard incubation medium for neuraminidase treatment contained, in a final volume of 0.2 ml, labelled microsomal membranes (1 mg of protein), *Cl. perfringens* neuraminidase (20 munits) and 0.32 M-sucrose. The time of incubation was 60 min. The AcNeu released from whole membranes was determined as described in the Experimental section. Disruption of membranes by sonication was done by using a Branson model B12 Sonifier for a period of 30 s for Expt. I and 3 min for Expt. II (in twelve 15 s periods separated by 60 s intervals to avoid overheating of the sample).

Modifications to the standard assay	Percentage of ganglioside radioactivity released		Total AcNeu released (nmol/mg of protein) Expt. II
	Expt. I	Expt. II	
None	23.9	10.8	57.2
Membranes previously disrupted by sonication	52.0	78.0	58.1
Addition of Tween 20 (10 μl)	80.3	80.7	58.2

Discussion

The neuraminidase preparation used released approximately the same number of sialyl groups from microsomal gangliosides in their natural position in the membranes as it released from gangliosides after isolation. Since we cannot be certain that the small contamination of the neuraminidase preparation with proteolytic activity was significant under the conditions of our experiments, no definite conclusion can be drawn on the exposure of the sialyl groups of gangliosides or glycoproteins *in situ* at the surface of the membranes.

Chromatographic analysis of the gangliosides from microsomal membranes treated with neuraminidase for different periods of time showed that most of the multisialogangliosides were transformed into GM 1. Endogenous neuraminidase activity was negligible and so was the activity of all other glycosidases that could be acting on gangliosides; although the period of incubation of membranes with neuraminidase was extended to 12 h no indication of further degradation of GM 1 was found. Ohman (1971) and Schengrund & Rosenberg (1970) have observed that the bulk of neuraminidase and glycosidase activities in brain homogenates was in subcellular fractions other than the microsomal fraction; they also reported that the optimum pH for the activity of these enzymes was between 4 and 5, which is considerably lower than the pH used in our work.

The amount of GM 1 found in microsomal membranes could not be noticeably decreased by incubation with CMP-AcNeu despite the fact that GD 1a and GD 1b (formed in these membranes from GM 1; Arce *et al.*, 1971, 1972) were synthesized. Since the CMP-AcNeu present in the system was not the limiting factor for the maximal incorporation of AcNeu, it was concluded that only a small fraction of GM 1 in the particles was available for synthesis of polysialogangliosides, in agreement with conclusions previously obtained from experiments *in vivo* (Maccioni *et al.*, 1971). A further conclusion from the data in the present paper is that GM 1 formed *in situ* by desialylation of polysialogangliosides by the action of exogenous neuraminidase is not available for resialylation; on the assumption that addition of AcNeu to incomplete gangliosides is a part of the mechanism of their biosynthesis, this observation suggested that polysialogangliosides and their related sialyltransferase(s) did not remain complexed after the synthesis finished. These results are in agreement with our previous conclusions, derived from experiments *in vivo* which showed that partial renewal of the carbohydrate chain of the gangliosides did not occur (Maccioni *et al.*, 1971).

Whereas the action of neuraminidase did not change the acceptor capacity of the microsomal

membranes, exposure of these membranes to different temperatures had a strong effect on this capacity (Table 2). This is a confirmation of an earlier observation from this laboratory (Arce *et al.*, 1971) which was attributed to inactivation of enzymes rigidly complexed with the acceptor ganglioside intermediates (Arce *et al.*, 1971). However, the temperature and timing of the earlier experiments (53°C, 3 min) were different from those used in the present work (37°C, 20 min), and that explanation was not quite satisfactory for the present case, as at least one of the enzyme activities involved in the biosynthesis of gangliosides, namely lactosylceramide AcNeu-transferase, was unchanged in the membranes incubated at 37°C. As an alternative mechanism for the decrease in incorporation of AcNeu as a result of incubation we suggest that AcNeu from endogenous CMP-AcNeu (which is known to be present in brain subcellular organelles; Arce *et al.*, 1971) may be transferred to incomplete gangliosides, decreasing as a consequence the acceptor fraction of the preparation. Whether inactivation of the enzymes or partial depletion of available acceptors is the correct explanation, it cannot be decided at present whether the transfer is a direct reaction between the sialyl nucleotide and the final acceptor or if it occurs through an unknown intermediate.

To explain a situation in which smaller gangliosides appeared to be precursors of more complex gangliosides *in vitro* whereas experiments *in vivo* and *in vitro* with endogenous acceptors did not show precursor-product relationships between simpler and more complex gangliosides, the existence of different pools of brain gangliosides was assumed (Arce *et al.*, 1971; Maccioni *et al.*, 1971). The presence of two pools of gangliosides in microsomal membranes may also explain the different rate of release of unlabelled sialyl groups (derived from gangliosides synthesized *in vivo*) and labelled sialyl groups (derived from gangliosides completed *in vitro*) (Fig. 4). If each species of gangliosides occurred in a single pool, unlabelled and labelled AcNeu should have been released at the same rate. A single pool probably existed after disruption of the membranes by detergent or sonication (Table 4). Further, since the gangliosides labelled *in vitro* probably corresponded to those in the pathway to be completed, the two pools resemble, whether or not they were in the same membrane, the pools of 'end products' and 'intermediate gangliosides' mentioned in previous reports (Maccioni *et al.*, 1971, 1972b).

This investigation was supported in part by a Grant from the Consejo Nacional de Investigaciones Científicas y Técnicas and by a Grant from the Ministerio de Salud Pública de la Nación, Argentine Republic. The helpful criticisms of Dr. F. A. Cumar and Dr. H. S. Barra are acknowledged.

References

- Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
- Arce, A., Maccioni, H. J. F. & Caputto, R. (1966) *Arch. Biochem. Biophys.* **116**, 52–58
- Arce, A., Maccioni, H. J. F. & Caputto, R. (1971) *Biochem. J.* **121**, 483–493
- Arce, A., Maccioni, H. J. F. & Caputto, R. (1972) *Asoc. Quim. Argent.* **60**, 185–193
- Avrova, N. F., Chenyakaeva, E. Yu. & Obukhova, E. L. (1973) *J. Neurochem.* **20**, 997–1004
- De Vries, G. H. & Barondes, S. H. (1971) *J. Neurochem.* **18**, 101–105
- Dicesare, J. L. & Rapport, M. M. (1973) *J. Neurochem.* **20**, 1781–1783
- Eichberg, J., Whittaker, V. P. & Dawson, R. M. C. (1964) *Biochem. J.* **92**, 91–100
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Hakomori, S. (1969) *Vox Sang.* **16**, 478–485
- Hakomori, S., Teather, C. & Andrews, H. (1968) *Biochem. Biophys. Res. Commun.* **33**, 563–568
- Kaufman, B., Basu, S. & Roseman, S. (1968) *J. Biol. Chem.* **243**, 5804–5807
- Lapetina, E., Soto, E. & De Robertis, E. (1967) *Biochim. Biophys. Acta* **135**, 33–43
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maccioni, H. J. F., Arce, A. & Caputto, R. (1971) *Biochem. J.* **125**, 1131–1137
- Maccioni, H. J. F., Arce, A. & Caputto, R. (1972a) *FEBS Lett.* **13**, 68–72
- Maccioni, H. J. F., Arce, A. & Caputto, R. (1972b) in *Biochemistry of the Glycosidic Linkage* (Piras, R. & Pontis, H., eds.), pp. 413–418, Academic Press, New York and London
- Miettinen, T. & Takki-Luukkainen, I. T. (1959) *Acta Chem. Scand.* **13**, 856–858
- Ohman, R. (1971) *J. Neurochem.* **18**, 89–95
- Schengrund, C. L. & Rosenberg, A. (1970) *J. Biol. Chem.* **246**, 6196–6200
- Svennerholm, L. (1963) *J. Neurochem.* **10**, 613–620
- Weinstein, D. B., Marsh, J. B., Glick, M. C. & Warren, L. (1970) *J. Biol. Chem.* **245**, 3928–3937