minger, J. L. (1966), *Biochemistry 5*, 3079.
Ludoweig, J., and Dorfman, H. (1960), *Biochim. Biophys. Acta 38*, 212.
Murrell, W. G., and Warth, A. D. (1965), *in* Spores III, Campbell, L. L., and Halvorson, H. O., Ed., Ann Arbor, Mich., American Society for Microbiology, p 1.
Sharon, N., Osawa, T., Flowers, H. M., and Jeanloz, R. W. (1966), *J. Biol. Chem. 241*, 223.
Sharon, N., and Seifter, S. (1964), *J. Biol. Chem. 239*, PC2398.
Tipper, D. J. (1968), *Biochemistry 7*, 1441.
Tipper, D. J. (1969), *Bacteriol Proc. 69*, 24.
Tipper, D. J., and Strominger, J. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 48.
Tipper, D. J., Strominger, J. L., and Ensign, J. C. (1967), *Biochemistry 6*, 906.
Tipper D. J., Tomoeda, M., and Strominger J. L. (1971), *Biochemistry 10*, 4683.
van Heijenoort, J., Elbaz, L., Dezelle, P., Petit, J.-F., Bricas, E., and Ghuysen, J.-M. (1969), *Biochemistry 8*, 207.
Vinter, V., and Stastna, J. (1967), *Fol. Microbiol. 12*, 301.
Warth, A. D. (1965), *Biochim. Biophys. Acta 101*, 315.
Warth, A. D. (1968), Ph.D. Thesis, University of Wisconsin, Madison, Wis.
Warth, A. D., Ohye, D. F., and Murrell, W. G. (1963), *J. Cell. Biol. 16*, 593.
Warth, A. D., and Strominger, J. L. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 528.
Warth, A. D., and Strominger, J. L. (1971), *Biochemistry 10*.

A Natural Inhibitor of Sialyl Transferase and Its Possible Influence on This Enzyme Activity during Brain Development†

Ricardo O. Duffard‡ and Ranwel Caputto*

ABSTRACT: The total activity of cytidine 5′-monophospho-*N*-acetylneuraminic acid lactosyl ceramide sialyl transferase of rat brain increases from the 8- to 40-day-old rat and then remains stable until, at least, the 60th day of life. Similar changes were observed with the activity in each of the different subcellular fractions that contain the enzyme. An inhibitor of the enzyme was found which increases with the age of the animal. It is present in the mitochondrial and microsomal fractions and in the cytosol. It is active on the enzyme from all of the subcellular particles. The inhibitor increases the K_m value of lactosyl ceramide for the enzyme and it is probably the responsible factor for the increase of this value in the adult animal. Gel filtration experiments indicated that the molecular weight of the inhibitor is approximately 70,000–80,000.

The particulate enzyme CMP-NANAc[1] lactosyl ceramide sialyl transferase, or hematoside sialyl transferase, was found by Arce *et al.* (1966) in preparations from rat brain and independently by Kaufman *et al.* (1966) in embryonic chicken brain preparations. The reaction catalyzed by this enzyme is probably a part of the main pathway for the synthesis of the major gangliosides (Kaufman *et al.*, 1966) but this is somewhat controversial (Yip and Dain, 1969); for a complete discussion, see Arce *et al.* (1971).

The rate of synthesis of brain gangliosides *in vivo* increases from the 5th to the 15th day of the life of the rat and then decreases rather rapidly, being about 3-fold higher in the 15- than in the 20-day-old rat (Burton *et al.*, 1963; de Maccioni and Caputto, 1968). On the other hand, the total activity of particulate brain hematoside sialyl transferase measured *in vitro* at the optimal conditions, as will be seen in the present report, increases continually from the 8th to the 40th day of the life of the rat. This apparent contradiction between the results *in vitro* and those expected from the observations *in vivo* indicated that the enzyme is subjected to regulatory influences *in vivo*. In support of this preliminary conclusion a natural inhibitor of the transferase was found which increases as the rat becomes older and which had been partially eliminated from the enzymatic preparations used to determine the total activity.

Materials and Methods

Subcellular Organelles. Mitochondrial, synaptosomal, microsomal, and supernatant fractions were prepared according to Eichberg *et al.* (1964). The fractions obtained by this method have been tested in our laboratories by electron microscopy and by enzymatic methods and they were found satisfactorily pure (for details, see Maccioni *et al.*, 1971).

Enzyme Preparations. Unless otherwise stated, 7 g of brain tissue from 15-day-old rats were dispersed in 16 ml of water in a glass homogenizer with Teflon pestle. The minced tissue was centrifuged successively at 800*g* for 10 min to eliminate cells and debris and at 10,000*g* for 20 min; the supernatant fraction was centrifuged again at 100,000*g* for 40 min. Each precipitate was washed once with 10 ml of water and then

† From Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Córdoba, Argentine Republic, South America. *Received September 28, 1971.* This investigation was supported in part by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentine Republic, and by Grant NB04781 from the National Institutes of Health, U. S. Public Health Service.

‡ Recipient of a scholarship from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentine Republic.

[1] Abbreviation used is: CMP-NANAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid.

suspended separately in 1.2 ml of 0.2 M sodium phosphate buffer (pH 6.8). The whole operation was carried out so that the particulate fractions were maintained below 4° of temperature. The two precipitates obtained by these procedures had approximately the same activity of sialyl transferase per milligram of protein. When kept at −15°, the particles lost about 50% of the activity in 5 days.

Preparation of the Inhibitor. Five grams of cerebral tissue from 40-day-old rats was minced in 10 ml of cold water in a glass homogenizer with Teflon pestle and the minced tissue was centrifuged at 100,000*g* for 45 min. The supernatant fraction was freeze-dried and the dry residue was redissolved in 1.5 ml of 0.2 M sodium phosphate buffer (pH 6.8).

Enzyme Determination. The method used for enzyme determination was essentially that of Arce *et al.* (1966) with slight modifications. In order to obtain a clear inhibition in this method the concentration of lactosyl ceramide used was lower than the K_m for this substrate; 10 nmoles of lactosyl ceramide dissolved in chloroform–methanol (2:1, v/v) was deposited at the bottom of a 6-ml test tube and the solvent evaporated under a current of N_2; 0.01 ml of Tween 20 and 0.04 ml of 0.2 M sodium phosphate buffer (pH 6.8) were added and the mixture was shaken until the lactosyl ceramide was dissolved. The system was completed by adding 11 nmoles of CMP-[^{3}H]NANAc and 0.06 ml of enzyme preparation. The final volume was 0.18 ml. The temperature of incubation was 37°. After 10-min incubation, 3.65 ml of chloroform–methanol (2:1, v/v) was added to end the reaction; the precipitated protein and the soluble material of low molecular weight were eliminated successively by filtration through paper and by passing through a column of Sephadex G-25 (Arce *et al.*, 1966). The material which was not retained by Sephadex G-25 was collected in vials, the solvent evaporated, and the residue suspended in 0.1 ml of 10% Tween 20. Five milliliters of a scintillation fluid made of 2 g of 2,5-diphenyloxazole, 100 g of naphthalene, and dioxane to complete 1000 ml was added. The radioactivity was measured in a Beckman S-200 liquid scintillation spectrometer equipped with external standard. As a blank, a system of identical composition was used but the mixture of chloroform–methanol (2:1, v/v) was added prior to the addition of the enzyme solution. When inhibitor solution was used a corresponding smaller amount of buffer was added.

Materials. CPM-[^{3}H]NANAc was prepared according to Arce *et al.* (1971); in two different batches used, the specific activities were 6×10^8 and 1.6×10^8 cpm per μmole. Lactosylstearoylsphingosine was prepared as described by Cumar *et al.* (1968). Lactosyllignoceroylsphingosine was obtained from the Miles Co., Elkhart, Ind. 46514. All other products were of commercial origin.

Results

Total Sialyl Transferase at Different Ages. This was measured in the particulate fractions obtained after the brains were suspended in distilled water and centrifuged successively at 800*g*, 10,000*g*, and 100,000*g*. The pellet obtained at 800*g* was discarded; the other two were washed once with distilled water. Saturating concentrations of lactosyl ceramide were used in all these experiments (see below). Table I shows that in both particulate fractions the activity increased in the brains between the 8th and 40th day after birth and then remained unchanged at least until they were 60 days of age. Table I also shows that the transferase activity was very similar when substrates with different fatty acid moieties were used (lactosylstearoylsphingosine or lactosyllignoceroylsphingosine).

TABLE I: Sialyl Transferase Activity of Two Particulate Fractions of Brain from Rats at Different Ages.[a]

Age of the Rat	Fatty Acid Moiety of Lactosyl Ceramide	Particulate Fraction (cpm/Particles from 0.3 g of Brain)	
		800*g* to 10,000*g*	10,000*g* to 100,000*g*
8	Stearoyl	1230 ± 98	859 ± 35
15	Stearoyl	1586 ± 136	1582 ± 78
15	Lignoceroyl		1467
30	Stearoyl		2689 ± 127
30	Lignoceroyl		2667
40	Stearoyl	2203 ± 198	2722 ± 124
60	Stearoyl	2300 ± 184	2695 ± 112

[a] Preparation of particles and enzyme determinations were as stated in Materials and Methods, except that 100 nmoles of lactosyl ceramide was added. CMP-[^{3}H]NANAc (specific activity 1.6×10^8 cpm/μmole) was used. Values are the average of four to five experiments ± SEM.

Changes of Sialyl Transferase with Age in Different Subcellular Organelles. Since the results obtained with particles disrupted by treatment with distilled water were apparently in contradiction with those expected from experiments *in vivo*, it was decided to carry out measurements at different ages with organelles obtained by the method of Eichberg *et al.* (1964).

Arce *et al.* (1966, 1971) found that sialyl transferase activity was present in the mitochondrial, synaptosomal, and microsomal fractions and that the activity in these organelles, expressed per milligram of protein, was approximately equal. Figure 1 shows that the activity of the microsomal fraction from brain increased rapidly from the 8th to the 16th day of age and then slowly until the 40th day of life. For the mitochondrial fraction the maximum activity was reached at the 16th day and then remained practically unchanged until the 40th day. Enzyme levels in the synaptosomal fraction were strikingly different, in that the activity decreased continually from the 8th to the 40th day. However if the synaptosomal fractions were suspended in distilled water the activity found in the disrupted particles increased from the 8th to the 40th day of age. The increased activity of disrupted synaptosomes is in a constant proportion to the increasing activity in intact microsomes. It was also noticed (Figure 1) that the activity in the disrupted synaptosomes as related to the intact synaptosomes changed from 2-fold higher in the 8-day-old rats to 5-fold higher in the 40-day-old rats.

Presence of an Inhibitor. A brain homogenate made in distilled water was centrifuged at 100,000*g*. The supernatant resulting from this centrifugation contained an inhibitor of the sialyl transferase. The incorporation of [^{3}H]NANAc was linear from 0 to 30 min without and with the addition of inhibitor. This inhibitor was effective against the sialyl transferase activity of each of the different subcellular particles (Table II). It was less effective on intact synaptosomes, but when these were previously disrupted by suspension in distilled water, the inhibition was similar to that observed on the other organelles. Inhibition of the enzyme preparation by

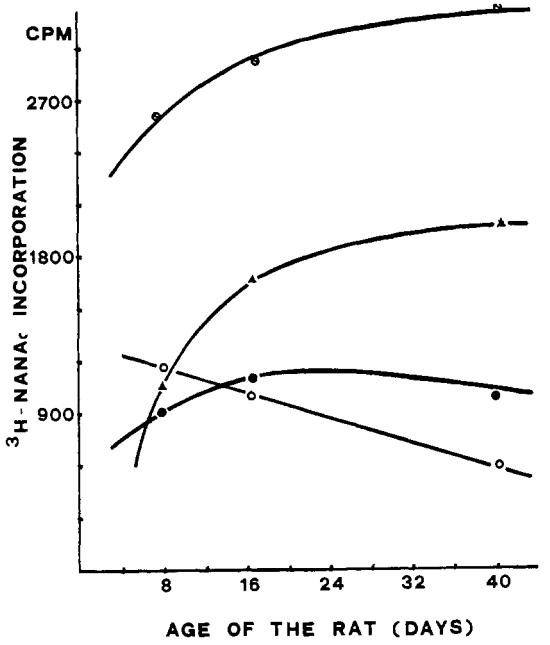

FIGURE 1: Sialyl transferase activity of the subcellular organelles from brains of rats of different ages. The conditions of measurements of the activity are the same described under Material and Methods but with 50 nmoles of lactosyl ceramide. The subcellular organelles were obtained by the method of Eichberg *et al.* (1964). Each determination was carried out with organelles equivalent to 0.12 g of brain. (○) Intact synaptosomes; (⊘) particulate fraction of synaptosomes after these were suspended in distilled water; (●) mitochondria; (▲) microsomes. CPM-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used.

TABLE II: Inhibitory Effect of the Material from the Supernatant Fraction on the Sialyl Transferase from the Different Brain Subcellular Organelles.

Enzyme Prepn[a] Used	Addn to Incubn System	[^{3}H]-NANAc Incorp (cpm)	Inhibn (%)
Standard		1491	
Standard	Inhibitor[b]	714	53
Synaptosomes		613	
Synaptosomes	Inhibitor[b]	566	8
Mitochondria		2750	
Mitochondria	Inhibitor[b]	795	71
Microsomes		6300	
Microsomes	Inhibitor[b]	2590	59
Disrupted synaptosomes		2319	
Disrupted synaptosomes	Inhibitor[b]	591	74

[a] The organelles were prepared by the method of Eichberg *et al.* (1964). Reaction conditions and incubation system for enzyme determination were standard. CMP-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used. [b] The inhibitor was obtained after centrifugation at 100,000*g* of a brain homogenate made in distilled water; the supernatant fraction was freeze-dried and redissolved in sodium phosphate buffer (pH 6.8). The amount used in each tube was from 0.6 g of brain.

inhibitor preparations from rat brain of different ages (Figure 2) showed that the older the animal the less the amount of cell supernatant required to produce 50% inhibition. According to this method of measurement, the relative concentrations of inhibitor in the brains from 8-, 15-, and 40-day-old rats were, respectively, 1:1.5:4.

Inhibition Activity in Homogenates from Rats of Different Ages. Curves of activity *vs.* concentration of the transferase obtained using the 800*g* supernatant from brains of 8-, 15-, or 40-day-old rats prepared in 0.33 M sucrose confirmed the presence of an endogenous inhibitor and showed that its effect is determined by its absolute concentration (Figure 3). For 8-day-old rats no clear indication of the presence of an inhibitor was found in the range of enzyme concentrations used. It should also be noted that in the conditions of experiments depicted in Figure 3 different ratios of enzyme activity between animals of different ages can be obtained depending on the amount of enzyme used. Some of these ratios were similar to those of the rates of renewal of gangliosides observed *in vivo* with animals of corresponding ages (de Maccioni and Caputto, 1968).

K_m Values for Lactosyl Ceramide. Sialyl transferase preparations obtained from particles from rat brains of different ages indicated that K_m values for lactosyl ceramide increased with the increasing of age of the animal, when nonwashed particles were used. Values were somewhat erratic but it was estimated from several experiments that K_m values passed from 0.09 mM for enzyme from animals of 15 days of age to 1.1 mM for preparations from 40-day-old rats. However, similar K_m values for the enzyme from animals of different ages were obtained when the particles were previously washed twice with distilled water. For these particles the values for rats of 15 and 40 days of age were, respectively, 0.09 and 0.11 mM. The inhibitor present in the supernatant fraction of the cell is apparently responsible for increasing the K_m value since it was found that it produces a competitive type of inhibition when the transference of sialyl groups was tested with lactosyl ceramide (Figure 4).

Distribution of the Inhibitor in Different Subcellular Fractions. The different subcellular fractions were prepared according to Eichberg *et al.* (1964) and the different particles washed twice with 0.33 M sucrose. Each of the subcellular particles were subsequently subjected to osmotic shock and centrifuged at 100,000*g* during 45 min. The inhibitor present in the supernatant solution obtained in this procedure was determined using the particulate enzyme preparation described under Methods. Table III shows that the cytosol accounts for 30–40% of the total inhibitory activity detected by this method; about 45% was found in the mitochondrial fraction, and about 15% was in the microsomal fraction. No evidence for the presence of an inhibitor in the synaptosomes was found in these experiments. The failure to find inhibitory activity in the synaptosomes contrasts with the increase of activity of sialyl transferase after these subcellular particles were subjected to disruption by osmotic shock (Figure 1). This indicates that measurements *in vitro* of the sialyl transferase activity in intact synaptosomes from aging rats are hampered by structural reasons rather than by the influence of an inhibitor occurring in this organelle.

On the Mechanism of Action of the Inhibitor. Experiments in which the inhibitor was preincubated with the mixture of substrates gave the same results as when the inhibitor was added directly. Addition of a heat inactivated enzyme preparation to the mixture of substrates and inhibitor during

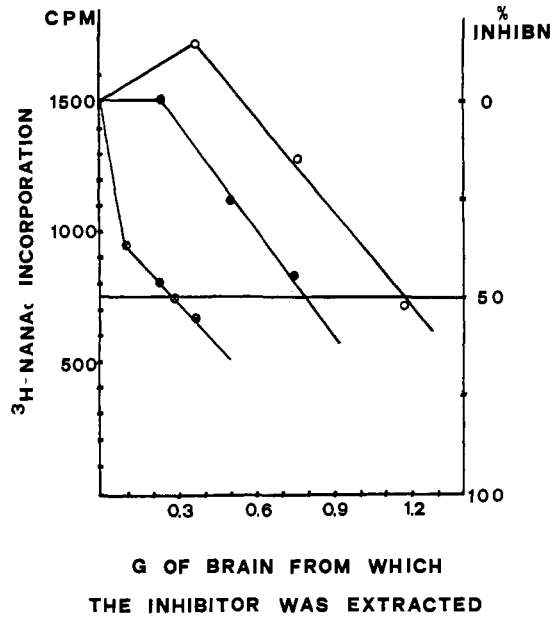

FIGURE 2: Amounts of brain required to produce different degrees of inhibition on a standard sialyl transferase preparation from a 15-day-old rat. A unit of inhibitor was defined as the quantity which produces 50% inhibition on the standard preparation. Reaction conditions were standard. (○) Inhibitor from brains of 8-day-old rats; (●) inhibitor from brains of 15-day-old rats; (⊗) inhibitor from brains of 40-day-old rats. CMP-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used.

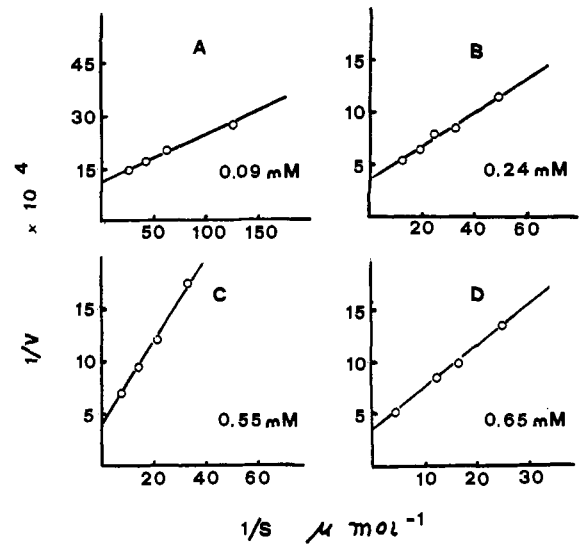

FIGURE 4: K_m variation of lactosyl ceramide for brain sialyl transferase in the presence of a constant amount of supernatant fraction of brain from rats of different ages. (A) Particulate fraction from 15-day-old rat brain. Particles were washed twice with distilled water. (B) Same as part A but for the addition of supernatant fraction of brain from 15-day-old rat. (C) Same as A but for the addition of supernatant fraction of brain from 40-day-old rat. (D) Same as A but for the addition of supernatant fraction of brain from 60-day-old rat. The particulate fraction used was prepared as described in Methods. The inhibitor was prepared as described in Table II. The amount of supernatant fraction used was equivalent to 0.6 g of brain. V is expressed in cpm. The radioactivity incorporated into endogenous acceptors (measured in cpm) was discounted. CMP-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used.

the preincubation period also gave the same results as when the inhibitor was added directly to complete the incubation system. These experiments showed that neither the inhibitor by itself nor in combination with a heat stable factor in the enzyme preparation destroys the substrates of the reaction.

In other experiments, reaction mixtures with and without inhibitor were incubated for 10 min and then inactivated by adding four volumes of chloroform–methanol (2:1, v/v). The precipitated proteins were eliminated by centrifugation and the transference of sialyl group that occurred in both systems was measured in a portion of the supernatant. The rest of the supernatant was dried and then used in a second incubation period after adding fresh enzyme. Results showed that the activity obtained in the second incubation period was equal for the mixtures that originally did or did not contain inhibitor. These results confirm that the action of the inhibitor is not by destroying the substrates.

On the Nature of the Inhibitor. Dialysis experiments showed

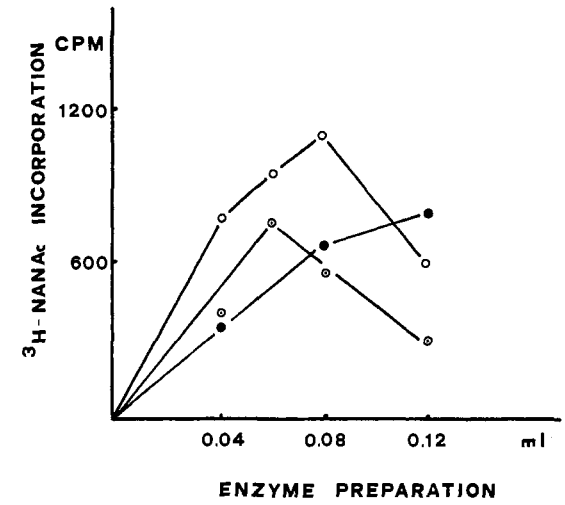

FIGURE 3: Sialyl transferase activity of brain from rats of 8, 15, and 40 days of age as catalyzed by different amounts of cell-free homogenates. Brains were homogenized in five volumes (w/v) of 0.33 M sucrose solution. Reaction conditions for enzyme determination were standard. Enzyme preparations from (●) 8-day-old rat; (○) 15-day-old rat; (⊙) 40-day-old rat. CMP-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used.

TABLE III: Distribution of Inhibitor in Different Subcellular Organelles.[a]

Subcellular Organelles	Inhibitor (Units/g of Brain)[b]
Mitochondria	18.6
Synaptosomes	0.0
Microsomes	7.64
Cytosol	15.0

[a] For preparation of inhibitor each organelle was suspended in about ten volumes of distilled water and after 10 min the disrupted particles were centrifuged at 100,000g and the supernatant freeze-dried and redissolved in sodium phosphate buffer (pH 6.8). [b] A unit is defined as the amount of preparation which produces 50% inhibition in the method described under Enzyme Determination (see Materials and Methods). CMP-[^{3}H]NANAc (specific activity 6×10^8 cpm/μmole) was used.

that the inhibitor did not pass through cellophane membranes. As expected from the dialysis experiments the inhibitor was not retained by Sephadex G-25 or G-50. When passed through Sephadex G-100 it was eluted in the same zone where substances of 70,000–80,000 molecular weight were eluted. The inhibitor was quite heat stable (20% inactivation when immersed 4 min in a boiling-water bath) and was not inactivated by incubation for 3 hr with trypsin. Solubility tests showed that the inhibitor is not soluble in chloroform–methanol (2:1, v/v) but several volumes of chloroform–methanol–water (60:30:4.5, v/v) did not inactivate the inhibitor preparations.

Discussion

A natural inhibitor of the CMP-NANAc lactosyl ceramide sialyl transferase appears in brain during the development of the rat. The concentration of lactosyl ceramide (0.055 mM) used for the determination of the inhibitor was lower than the K_m for this substrate. The low concentration of substrate would naturally help to elicit the inhibitor effect of a competitive inhibitor. However, since the concentration of lactosyl ceramide in the tissue is so small that it has not been measured at present no comparison can be carried out between the inhibition on the exogenous and endogenous lactosyl ceramide. The inhibitor concentration increases with the age of the animal. Since at the same time the total transferase activity at the brain (as measured *in vitro* in the absence of inhibitor) also increases, it is possible that the decrease of ganglioside renewal occurring *in vivo* during development (Burton *et al.*, 1963; de Maccioni and Caputto, 1968) is due to the action of one or several inhibitors like the one described in this work, which regulate the incorporation of glycosidic groups into the gangliosides.

Several lines of evidence indicated that this inhibitor is effective *in vivo*. (a) The inhibitor was found in the mitochondrial and the microsomal fractions, concurrently with the enzyme. (b) The K_m value of lactosyl ceramide for the enzyme increases with the age of the animal but when the particles were thoroughly washed the K_m values of aged and young animals were equal, indicating that an inhibitor attached to the particles was responsible for the changes of K_m values. (c) The inhibitor increases the K_m value of lactosyl ceramide for the enzyme. The inhibitor present in the supernatant fraction would also seem to be effective in the intact cell since when it was added to an incubation medium containing microsomes or mitochondria, it produced an inhibition of enzyme activity in the particles. The observation that synaptosomes do not contain inhibitor indicated that a different type of regulation of the CMP-NANAc lactosyl ceramide transferase operates in these organelles. However, after the organelles were subjected to osmotic shock the enzyme was susceptible to the inhibitor.

References

Arce, A., Maccioni, H. J., and Caputto, R. (1966), *Arch. Biochem. Biophys. 116*, 52.

Arce, A., Maccioni, H. J., and Caputto, R. (1971), *Biochem. J. 121*, 483.

Burton, R. M., García Bunnel, L., Golden, M., and McBride Balfour, Y. (1963), *Biochemistry 2*, 580.

Cumar, F., Barra, H. S., Maccioni, H. J., and Caputto, R. (1968), *J. Biol. Chem. 243*, 3807.

de Maccioni, A. H. R., and Caputto, R. (1968), *J. Neurochemistry 15*, 1257.

Eichberg, J., Whittaker, V. P., and Dawson, R. M. C. (1964), *Biochem. J. 92*, 91.

Kaufman, B., Basu, S., and Roseman, S. (1966), *in* Cerebral Sphingolipidoses, Aronson, S. M., and Volk, B. N., Ed., New York, N. Y., Pergamon Press, p 193.

Maccioni, A. H. R., Giménez, M. S., and Caputto, R. (1971), *J. Neurochem. 18*, 2363.

Yip, M. C. M., and Dain, J. A. (1969), *Lipids 4*, 270.