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THE ENZYMIC SYNTHESIS OF GANGLIOSIDE

II. UDP-GALACTOSE: *N*-ACETYLGALACTOSAMINYL-(*N*-ACETYLNEURAMINYL)GALACTOSYL-GLUCOSYL-CERAMIDE GALACTOSYLTRANSFERASE IN RAT BRAIN

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SUMMARY

This paper describes the developmental pattern, subcellular distribution and general properties of UDP-galactose: *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosyl-glucosyl-ceramide galactosyltransferase (UDP-galactose:G_{M_2} ganglioside galactosyltransferase) in rat brain.

1. The enzyme was detected in fetal rat brain, and the specific activity increased linearly up to 5 days *post partum*. A sharp peak of enzyme activity occurred during the period of active myelination. Significant enzymic activity was detected in adult rat brain.

2. The enzyme from fetal rat brain was localized with the mitochondrial and microsomal particles. The percentage of enzyme activity in the mitochondrial fraction increased after birth, and was found to be localized with this fraction at 10 days of age. Appreciable enzymic activity in the nuclear fraction was found only in the adult brain.

3. General properties of the enzyme: (a) The enzyme was specific for G_{M_2} ganglioside and UDP-galactose. (b) EDTA inhibited the enzyme which also required Mn^{2+} for maximum activity. (c) The K_m value for G_{M_2} ganglioside was 94 μM and 12 μM for UDP-galactose. G_{M_2} ganglioside was inhibitory at a concentration equal to or greater than 0.35 mM. (d) The pH optimum ranged between 6.5 and 7.15.

INTRODUCTION

The developmental pattern of gangliosides from rat brain has been well docu-

Abbreviations: G_{M_2}, *N*-acetylgalactosamyl-(*N*-acetylneuraminyl)-galactosyl-glucosyl-ceramide or Tay-Sachs ganglioside. Abbreviations for the indivual gangliosides are those proposed by SVENNERHOLM[20].

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mented[1-3]. The major monosialoganglioside, G_{M_1}, is present at birth, declines slightly during the first 10 days of life, and then slowly increases[3].

The enzymic synthesis of monosialoganglioside follows the sequence: G_{M_2} ganglioside + UDP-galactose → G_{M_1} ganglioside + UDP (ref. 4). The enzyme, UDP-galactose:G_{M_2} ganglioside galactosyltransferase, which catalyzes this reaction, has been studied in frog brain[5,6], and in embryonic chicken brain[7]. However, attempts to demonstrate enzymic activity in rat brain during development, or in the adult[6] have been unsuccessful.

This paper reports the developmental pattern, intracellular localization, and general properties of UDP-galactose:G_{M_2} ganglioside galactosyltransferase in rat brain.

EXPERIMENTAL PROCEDURE

Materials

Rats of the Sprague–Dawley strain were purchased from Chalres River Co. Uniformly labeled UDP-[^{14}C]galactose was obtained from International Chemical and Nuclear Corp., and unlabeled UDP-galactose from Calbiochem. PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-(5-phenyloxazolyl-2)benzene) were purchased from New England Nuclear Corp. Tween 80 was obtained from Sigma Chemical Co. Triton CF-54 and Triton X-100 were gifts from Rohm and Haas. Glucocerebroside, lactosylceramide, trihexosylceramide (galactosyl-galactosyl-glucosyl-ceramide) were gifts of Dr. J. Kanfer (National Institutes of Health, Bethesda) and hematoside was provided by Dr. T. Yamakawa (University of Tokyo, Tokyo). All other reagents were analytical grade.

Methods

Preparation of substrates

G_{M_2} ganglioside, isolated from the brain of an individual with Tay-Sachs disease by the method of FOLCH *et al.*[8], was purified on a silica gel G column[9] and co-chromatographed as a single spot with authentic G_{M_2} ganglioside in three solvent systems: (1) chloroform–methanol–water (61:32:7, by vol.), (2) chloroform–methanol–water (70:30:4, by vol.), and (3) *n*-propanol–water (70:30, by vol.). Monosialoganglioside (G_{M_1} ganglioside), disialoganglioside (G_{D1a} ganglioside) and trisialoganglioside (G_{T1} ganglioside) were prepared by the above procedure from bovine brain.

Preparation of homogenates

Fresh rat brains were homogenized in 4 vol. of 0.25 M sucrose solution containing 0.11% 2-mercaptoethanol (20% homogenate, w/v). The homogenization was divided into two 1-min periods, and a glass homogenizer was used. The temperature for this operation ranged between 0 and 5°.

Preparation of cellular components

The methods described by DE ROBERTIS *et al.*[10] were used with slight modification. Fresh rat-brain homogenate (20%, w/v) was prepared according to the above procedure except a glass homogenizer with a loose Teflon pestle was used to avoid excessive breakage of the mitochondrial particles. A typical homogenate for the preparation of cellular components contained seventeen 22-day-old fetal rat brains (3.4 g) and 13.6 ml of the sucrose–mercaptoethanol solution. The protein concen-

trations[11] were in the whole homogenate (14.4 mg/ml), 900 × *g* particles (3.47 mg/ml), 13 000 × *g* particles (1.16 mg/ml), 20 000 × *g* particles (1.16 mg/ml), 100 000 × *g* particles (1.98 mg/ml) and the soluble fraction (6 mg/ml). The homogenization was divided into two 1-min periods with an interval for cooling. The total homogenate was centrifuged for 10 min at 900 × *g* in a Sorvall Model RC-2B centrifuge, and the precipitate, after washing once with the sucrose–mercaptoethanol solution, was designated the 900 × *g* particles. The supernatant fluid was pooled and centrifuged at 13 000 × *g* for 20 min, and the once washed precipitate constituted the 13 000 × *g* particles. The combined fluids were subjected to centrifugation at 20 000 × *g* for 30 min, and the resulting precipitate was designated as the 20 000 × *g* particles. The 100 000 × *g* particles were obtained after centrifugation of the 20 000 × *g* supernatant fluid for 1 h at 100 000 × *g* in a Spinco Model G ultracentrifuge. To obtain the 100 000 × *g* supernatant fraction, a portion of the original homogenate was also centrifuged at 100 000 × *g* for 1 h, and the supernatant aspirated off. All the above steps were performed at 0°.

Preparation of reaction mixture

Chloroform–methanol solutions of Tween 80 (100 μg) and Triton CF-54 (200 μg) and aqueous solutions of G_{M_2} ganglioside (7 mμmoles) were mixed and taken to dryness. Cacodylate buffer (10 μmoles, pH 7.0), $MnCl_2$ (1.0 μmole), uniformly labeled UDP-[^{14}C]galactose (140 mμmoles), enzyme and water were added to a final volume of 0.1 ml. The incubation medium was agitated with a Vortex mixer and placed in a water bath at 37° for 4 h.

Enzyme assay

Ascending paper chromatography[4] was used to separate the precursor, UDP-[^{14}C]galactose, from the reaction products. After incubation of the reaction mixture, the enzymic reaction was stopped by the addition of 20 μl chloroform–methanol (2:1, by vol.), and the entire mixture was streaked on Whatman 3 MM filter paper and run in 1% $Na_2B_4O_7$ (pH 9.0). Under these conditions the reaction product remained at the origin after an 8-h run, while the UDP-galactose and its degradation products migrated near the solvent front. The origin *plus* 1 inch was counted in PPO–POPOP fluor by a liquid scintillation spectrometer. The fluor contained 4 g PPO, 50 mg POPOP and 1 l of toluene. Protein was determined according to LOWRY *et al.*[11] or the biuret method[12].

Ornithine aminotransferase assay

The colorimetric method of PERAINO AND PITOT[13] was used with slight modification to determine the mitochondrial marker, ornithine aminotransferase. The reaction mixture in a total volume of 2.0 ml contained 0.5 ml of 0.4 M phosphate buffer (pH 7.6), 0.2 ml of 0.35 M L-ornithine, 0.1 ml of the *o*-aminobenzaldehyde suspension of 0.1 M in 50% hot ethanol, and 0.2 ml of 0.05 M α-ketoglutarate (pH 7.0). The molar extinction coefficient (ε) of $2.71 \cdot 10^3$ (ref. 14) was used to calculate the amount of pyrroline 5-carboxylate produced.

RESULTS

Developmental pattern of rat brain and UDP-galactose:G_{M_2} ganglioside galactosyltransferase

The brain wet weight of the rats studied increased nearly linearly from 0.1 g

in the 15.5-day fetus (6.5 days before birth) to 1.6 g 15 days *post partum*. No significant change in brain weight was observed after 15 days of age.

The activity of UDP-galactose: G_{M_2} ganglioside galactosyltransferase during development is shown in Fig. 1. The enzyme activity, expressed a mμmoles ^{14}C-product/g wet weight or per mg protein per 4 h, follows a similar pattern during development. A sharp rise in enzyme activity was observed from 6.5 days before birth until 5 days *post partum*. The activity remains rather constant until 14 days, then declines moderately. The specific activity of UDP-galactose: G_{M_2} ganglioside galactosyltransferase reached maximum at the point of most active myelination[15].

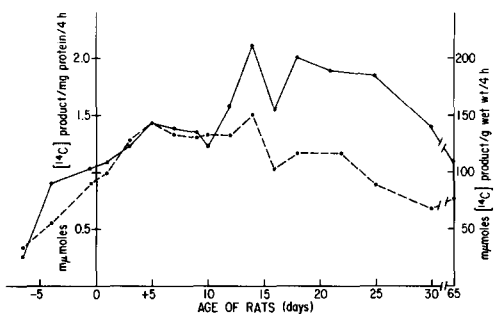

Fig. 1. The activity of UDP-galactose: G_{M_2} ganglioside galactosyltransferase during development. The enzymic activity is expressed as mμmoles [^{14}C] product/g wet wt. per 4 h (solid lines) and mμmoles [^{14}C] product/mg protein per 4 h (broken lines). Aliquots of 20 μl of brain homogenate were assayed as described in EXPERIMENTAL PROCEDURE. The specific activity of UDP-[^{14}C] galactose is $7.1 \cdot 10^5$ counts/min per μmole. The saturating amount of UDP-[^{14}C]galactose, 1.40 mμmoles and G_{M_2} gangliosides, 7 mμmoles were used.

Subcellular distribution of UDP-galactose:G_{M_2} ganglioside galactosyltransferase at different ages

The intracellular localization of the enzyme in fetal rat brain is seen in Table I. Most of the enzymic activity of the homogenate is found with the 13 000 $\times g$ (mitochondria), 20 000 $\times g$ (light microsomes), and 100 000 $\times g$ (heavy microsomes) resuspended particles. No appreciable activity is detected in the 900 $\times g$ particles (nuclear fraction) nor the 100 000 $\times g$ supernatant (soluble fraction). The localization of ornithine aminotransferase in the mitochondrial fraction suggests negligible contaminating mitochondria in the heavy and light microsomal preparations (Table I).

Table II shows the subcellular distribution of UDP-galactose: G_{M_2} ganglioside galactosyltransferase in 1- and 10-day-old and adult rat brain. An increase in enzymic activity with the mitochondrial fraction of the brain cell during development, and drastic decrease in the adult is observed. The percentage of total enzyme activity in the heavy and light microsomal particles is relatively unchanged at the ages examined, except in the adult. In the fetal rat brain (Table I) the sum of the enzymic activity in the heavy and light microsomes approximates that of the mitochondria; however, the percentage of enzyme activity with the mitochondria in 1- and 10-day-old rat brain is greater than that of both microsomal fractions.

The slight activity of the nuclear fraction in the prenatal and young rat brain may be due to mitochondrial contamination as suggested by the subcellular distribution of ornithine aminotransferase (Table I). However, the enzyme activity found

TABLE I

SUBCELLULAR DISTRIBUTION OF UDP-GALACTOSE: G_{M_2} GANGLIOSIDE GALACTOSYLTRANSFERASE AND ORNITHINE AMINOTRANSFERASE IN FETAL RAT BRAIN

Each particulate fraction was resuspended in the homogenizing medium in an amount equal to the original homogenate, and 50-μl aliquotes were assayed for the transferase activity. The method of fractionation and the assay procedures are described in EXPERIMENTAL PROCEDURE. All values are corrected for endogenous values.

Fetal age (days)	*Fraction*	*[^{14}C]product (mμmoles/g wet wt. Per 4 h)*	*Activity (%)*	*Ornithine aminotransferase (units/g)*
21	Homogenate	105.1	100	
	900 × *g* resuspended particle	1.7	2	
	13 000 × *g* resuspended particle	20.8	20	
	20 000 × *g* resuspended particle	18.3	17	
	100 000 × *g* resuspended particle	17.7	17	
	100 000 × *g* supernatant solution	0	0	
22	Homogenate	94.0	100	5.2
	900 × *g* resuspended particle	3.8	4	1.4
	13 000 × *g* resuspended particle	39.2	42	6.0
	20 000 × *g* resuspended particle	16.0	17	0.2
	100 000 × *g* resuspended particle	20.0	22	0.2
	100 000 × *g* supernatant solution	1.4	2	0

in the adult nuclear fraction does not seem to result from contaminating mitochondria, since enzymic activity in this fraction is consistently higher than that of the mitochondrial fraction in three separate experiments. No significant activity was detected in the 100 000 × *g* supernatant at the ages examined.

Characteristics of enzyme

Since the entire activity of the homogenate of 10-day-old rat brain could be recovered in the 13 000 × *g* particles with a 2.5-fold increase in specific activity, this fraction was used as the enzyme source. Attempts to further purify the enzyme were unsuccessful due to instability of the enzyme. Repeated efforts to solubilize the enzyme from the 13 000 × *g* particles has not been fruitful.

Requirements for assays

Table III shows the optimum conditions for assay of rat-brain UDP-galactose: G_{M_2} ganglioside galactosyltransferase. EDTA completely inhibits the system, while Mn^{2+} is required at a concentration of 10 mM. Mg^{2+}, Ni^{2+}, Cu^{2+}, K^{+} and Al^{3+} were tested at 10 mM concentration, and were found to be inactive. This rigid requirement for Mn^{2+} is reported in the particulate enzyme preparation from embryonic chicken

TABLE II

SUBCELLULAR DISTRIBUTION OF UDP-GALACTOSE: G_{M_2} GANGLIOSIDE GALACTOSYLTRANSFERASE IN RAT BRAIN

The assay and procedures are described in EXPERIMENTAL PROCEDURE. Figures in parentheses indicate the number of experiments. All values are corrected for endogenous values.

Cell fraction	*Activity (%) Age of rats (days) after birth*		
	1 (1)	*10 (2)*	*Adult (3)*
Homogenate	100	100	100
900 × *g* resuspended particle	4	5	25
13 000 × *g* resuspended particle	60	103	10
20 000 × *g* resuspended particle	25	18	7
100 000 × *g* resuspended particle	10	11	3
100 000 × *g* supernatant solution	2	1	0

brain[7] and in adult frog brain[16]. The demonstration that there is no significant decrease of radioactivity incorporated in the presence of nonradioactive galactose (Table III) indicates that UDP-galactose is involved in the reaction. Thus, the incorporation of radioactivity is completely inhibited by the addition of unlabeled UDP-galactose.

The galactosyltransferase from 10-day-old rat brain was specific for G_{M_2} ganglioside as lipid acceptor. Individual ganglioside species (G_{M_3}-, G_{M_1}-, $G_{D_{1a}}$-, and G_{T_1} ganglioside), glucocerebroside, lactosylceramide and trihexosylceramide (kidney) at the same molar concentrations, had little or no acceptor activity. Similar results are reported by KAUFMAN *et al.*[4] for the chick enzyme.

Michaelis constants for UDP-galactose and G_{M_2} ganglioside

The approximate K_m values for UDP-galactose and G_{M_2} ganglioside are 12 and 94 μM, respectively (Figs. 2 and 3). The K_m values reported here are lower than those reported for the chick-brain enzyme[7], suggesting a specialization for UDP-

TABLE III

REQUIREMENTS OF UDP-GALACTOSE: G_{M_2} GANGLIOSIDE GALACTOSYLTRANSFERASE IN 10-DAY-OLD RAT BRAIN

Rat brains were suspended in 4 vol. of 0.25 M sucrose containing 0.11% 2-mercaptoethanol, and were homogenized in a glass homogenizer with a loose Teflon pestle. Nuclei and cell debris were removed by centrifugation at 900 × *g* for 10 min, and the supernatant centrifuged at 13 000 × *g* for 20 min. The washed precipitate, resuspended to a final concentration of 6.4 mg of protein per ml in the homogenizing medium, was used as the enzyme source. Aliquots of 20 μl were assayed for the transferase activity as described in EXPERIMENTAL PROCEDURE. The specific activity of UDP-[^{14}C]galactose was $7.94 \cdot 10^5$ counts/min per μmole.

System	[^{14}C]*Galactose incorporated (counts/min)*
Complete system	395
Minus G_{M_2} ganglioside	52
Minus Mn^{2+}	44
Minus detergent mixture	155
Plus 0.7 μmole galactose	368
Plus 0.7 μmole UDP-galactose	54
Plus 5 μmoles EDTA	23

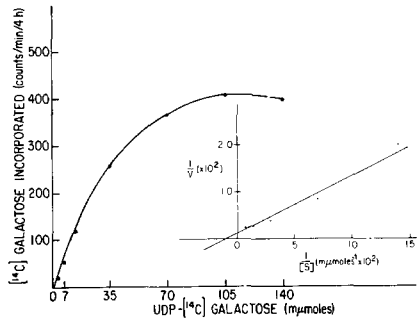

Fig. 2. The activity of UDP-galactose: G_{M_2} ganglioside galactosyltransferase as a function of the concentration of UDP-[^{14}C]galactose. The assay is described in Table III.

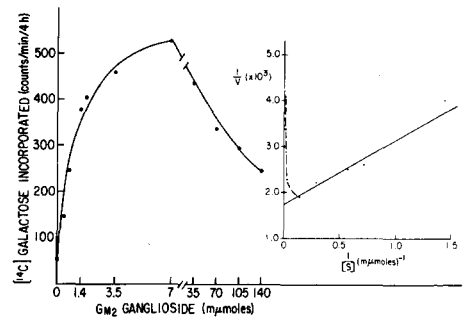

Fig. 3. The activity of UDP-galactose: G_{M_2} ganglioside galactosyltransferase as a function of the concentration of G_{M_2} ganglioside. The assay procedure is as described in Table III.

galactose and G_{M_2} ganglioside by the rat-brain enzyme. Inhibition of the enzymic reaction is observed when the G_{M_2} ganglioside concentration is equal to or greater than 0.35 mM. Similar substrate inhibition has also been reported in adult frog brain[16]. Thus, the optimum concentration of G_{M_2} ganglioside for the chick enzyme is inhibitory in this system[7].

Effect of pH on enzyme activity

The broad pH optimum of rat-brain UDP-galactose: G_{M_2} ganglioside galactosyltransferase is between 6.5 and 7.15 in cacodylate–HCl buffer. Although such data has not been presented by other investigators, optimum pH values of 7.3 and 7.2–7.3 have been reported for the chick and frog enzymes, respectively[7,16].

Other properties of enzyme

The transferase reaction is directly proportional to the reaction time for 6 h and to protein concentration over the range 0.064–0.256 mg per incubation mixture. A lag in reaction velocity observed at low protein concentrations (below 0.064 mg per incubation mixture) may be due to instability of the enzyme in dilute solution.

DISCUSSION

The biosynthesis of gangliosides has been reported to involve the stepwise addition of monosaccharide units from sugar nucleotides to glycolipid acceptors[4]. A key reaction in this sequence is the formation of the monosialoganglioside G_{M_1} from G_{M_2} ganglioside and UDP-galactose catalyzed by a galactosyltransferase. This enzyme, UDP-galactose:G_{M_2} ganglioside galactosyltransferase, has been demonstrated in embryonic chicken brain[7], in adult frog brain[5,6], and, in this report, in developing and adult rat brain. In general, the properties of the rat-brain galactosyltransferase as described in this paper are similar to those of the chick and frog enzymes[7,16].

The activity of UDP-galactose:G_{M_2} ganglioside galactosyltransferase in the rat brain at different ages may reflect the potentiality of the tissue to synthesize gangliosides. Fig. 1 shows a marked increase in enzyme activity from 6.5 days before

birth to 5 days *post partum*, and again from 10 to 14 days. Maximum enzymic activity is observed at 14 days of age. Although a slight decrease of activity is observed at 16 days of age, the general pattern is one of gradual decline after 18 days of age. In the adult, the galactosyltransferase activity approximates that of the 1-day-old. This pattern of UDP-galactose:G_{M_2} ganglioside galactosyltransferase activity during development seems to correlate with the concentration of gangliosides at different rat ages reported by SUZUKI[3]. The total brain gangliosides increase after birth, attain maximum concentration during the period of active myelination, and are relatively constant thereafter.

Incorporation studies involving the transfer of radioactivity from labeled hexoses to the carbohydrate moieties of the ganglioside molecule, *in vivo*, showed the greatest incorporation of radioactive sugars between 8 and 14 days; little incorporation being found in the older animal[17]. Since this paper reports the activity of UDP-galactose:G_{M_2} ganglioside galactosyltransferase in the adult rat brain, this age effect of ganglioside biosynthesis, *in vivo*, must be due to the absence or low activity of other biosynthetic enzymes in the adult. The CMP-*N*-acetylneuraminic acid: glycolipid sialyltransferases have been reported to be extremely low or undetectable in adult chicken and rat brain[4].

The change in subcellular distribution of UDP-galactose:G_{M_2} ganglioside galactosyltransferase during development (Tables I and II) correlates with the maturation of brain tissue. RUBIOLO DE MACCIONI AND CAPUTTO[1] report a 2–2.5-fold increase in the weights of synaptosomes and free mitochondria per g of wet tissue from 3–15-day-old rats. The microsomal fraction, on the other hand, did not show significant changes during this period. These findings are in agreement with the present demonstration of increased galactosyltransferase activity with the 13 000 $\times g$ fraction (which is reported to contain synaptosomes and free mitochondria[70]), and relative constancy of the microsomal enzymic activity (Table III).

The subcellular localization of UDP-galactose:G_{M_2} ganglioside galactosyltransferase in the nuclear fraction of the adult rat brain cell (Table II) cannot be explained at present. The enzymic activity of the adult brain homogenate was consistently greater than the sum of the intracellular fractions in three separate experiments. However, using the same subcellular fractions, ornithine aminotransferase activity was localized mainly in the mitochondrial particles, with complete recovery of enzyme activity.

The inability to detect UDP-galactose:G_{M_2} ganglioside galactosyltransferase activity in the adult rat brain by other investigators[4,6] may be due to the high endogenous incorporation of UDP-[^{14}C]galactose at high protein concentrations. For example, when 1.3 mg of enzyme protein was used for assay, the endogenous incorporation was 80% of the total incorporation, and decreased to 20% when 1.0 mg of protein was used (G. B. YIP AND J. A. DAIN, unpublished data). Therefore, minimal endogenous incorporation of UDP-[^{14}C]galactose can be obtained at an enzyme concentration of less than 1.0 mg.

In light of the recent report by YIP AND DAIN[18] of an alternate pathway for the biosynthesis of gangliosides, the role of the galactosyltransferases in the enzymic synthesis of gangliosides in rat brain merits further investigation. Such studies are in progress in this laboratory.

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