Frog Brain Uridine Diphosphate Galactose-*N*-Acetylglactosaminyl-*N*-acetylneuraminylgalactosylglucosylceramide Galactosyltransferase

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1. The enzyme that catalyses the transfer of galactose from UDP-galactose to *N*-acetylglactosaminyl-(1→4)-*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosylceramide (G_{M2}) was found mainly in the heavy- and light-microsomal fractions of the adult frog brain. 2. The subcellular distribution of the enzyme, UDP-galactose- G_{M2} galactosyltransferase, parallels that of gangliosides in adult frog brain. 3. The enzymic activity was first detected at late gastrulation (Shumway stage 11½) and increased until the completion of the operculum (Shumway stage 25) and then decreased in the tadpoles. 4. In adult frog brain, the enzyme exhibited a pH optimum of 7.2–7.3 in both cacodylate and tris buffers. The enzyme required 10 mM- Mn^{2+} for maximal activity and the K_m for Mn^{2+} was determined as 2.2 mM. The half-maximal velocity was obtained at a G_{M2} concentration of 0.18 mM. Inhibition of the enzymic reaction was found when the G_{M2} concentration was greater than 1.38 mM. 5. The enzymic activity was also inhibited by the products in the pathway of ganglioside synthesis, i.e. either by a mixture of gangliosides or by individual ganglioside components. The most active inhibitor was disialoganglioside. The degree of inhibition is a function of the individual ganglioside concentration. 6. A product-inhibition mechanism for the regulation of ganglioside biosynthesis is discussed.

A stepwise synthesis of gangliosides in adult frog brain has been proposed recently by Yip & Dain (1969a). The pathway involves the addition of nucleotide sugars to the corresponding asialoglycolipid acceptors (reactions 1–4 in Scheme 1).

The synthesis of gangliosides in embryonic chick brain as proposed by Kaufman, Basu & Roseman (1966), differs from reactions 2–4 in that NANA* is first transferred to lactosylceramide by the CMP-NANA-lactosylceramide *N*-acetylneuraminyltransferase (reaction 5). The product of reaction 5,

G_{M3} , is converted into G_{M1} in two enzymic steps catalysed by the UDP-*N*-acetylglactosamine- G_{M3} *N*-acetylglactosaminyltransferase (reaction 6) and UDP-galactose- G_{M2} galactosyltransferase (reaction 7), respectively.

Preliminary studies in our laboratory have demonstrated the presence of UDP-*N*-acetylglactosamine- G_{M3} *N*-acetylglactosaminyltransferase and UDP-galactose- G_{M2} galactosyltransferase in adult frog brains (Dain, Mark, Yiamouyiannis &

*Abbreviations: NANA, *N*-acetylneuraminic acid; Cer, ceramide (2-*N*-acylsphingosine). Ganglioside nomenclature suggested by Svennerholm (1964): G_{M2} , Tay-Sachs ganglioside [*N*-acetylglactosaminyl-(1→4)-*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosylceramide]; G_{M1} , monosialoganglioside [galactosyl-(1→3)-*N*-acetylglactosaminyl-(1→4)-*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosylceramide]; G_{D1a} , disialoganglioside [*N*-acetylneuraminyl-(2→3)-galactosyl-(1→3)-*N*-acetylglactosaminyl-(1→4)-*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosylceramide]; G_{T1} , trisialoganglioside [*N*-acetylneuraminyl-(2→3)-galactosyl-(1→3)-*N*-acetylglactosaminyl-(1→4)-*N*-acetylneuraminyl-(2→8)-*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosyl-(1→1)-(2-*N*-acyl)sphingosine]; G_{M3} , hematoside [*N*-acetylneuraminyl-(2→3)-galactosyl-(1→4)-glucosylceramide]; G_P , polysialoganglioside.

Scheme 1.

Cha, 1967; Yiamouyiannis & Dain, 1968a). This observation suggests the existence of the biosynthetic pathway described by Kaufman *et al.* (1966). Further studies on the individual enzymes involved in the synthesis of gangliosides in adult frog brain have not been reported.

The present paper describes the properties and optimum assay conditions of frog brain UDP-galactose- G_{M2} galactosyltransferase. A possible regulatory role of this enzyme in ganglioside metabolism is discussed.

MATERIALS AND METHODS

Mature male and female specimens of *Rana pipiens* were purchased from J. M. Hazen and Company, Alberg, Vt., U.S.A. A brain from an individual with Tay-Sachs disease was kindly provided by Dr Allen C. Crocker, Department of Pediatrics, Harvard Medical School at the Children's Hospital, Boston, Mass., U.S.A. Hematoside was a gift from Dr T. Yamakawa, Department of Chemistry, The Institute for Infectious Disease, University of Tokyo, Tokyo, Japan. Glucosylceramide was a gift from Dr A. Rosenberg, Hershey Medical Center, Pennsylvania State University, Hershey, Pa., U.S.A. Lactosylceramide, kidney trihexosyl ceramide (Gall $\rightarrow$ 4Gall $\rightarrow$ 4Glc-Cer), tetrahexosyl ceramide (Gall $\rightarrow$ 3GalNAc $\rightarrow$ 4Gall $\rightarrow$ 4Glc-Cer) and G_{M2} were gifts from Dr J. Kanfer, National Institutes of Health, Bethesda, Md., U.S.A. *N*-Acetylneuraminyl-lactose was a gift from Dr Ivan Stern, Baxter Laboratories, Morton Grove, Ill., U.S.A. Fetuin (Fisher, O'Brien & Puck, 1962) was a gift from Dr Harold Fisher, Department of Biophysics, University of Rhode Island, Kingston, R.I., U.S.A. UDP-[U- ^{14}C]galactose, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Tween 80 and NANA were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Triton CF-54 was a gift from Rohm and Haas Co., Philadelphia, Pa., U.S.A.

Preparation of gangliosides. Gangliosides were isolated from ox brain and G_{M2} was extracted from the brain of an individual with Tay-Sachs disease by the method of Folch, Arsove & Meath (1951). The individual gangliosides were isolated from a silica gel G (E. Merck A.-G., Darmstadt, Germany) column by elution with the solvent chloroform-methanol-water (61:32:7, by vol.) (Dain, Weicker, Schmidt & Thannhauser, 1962; Dain, Willis, Sweet, Schmidt & Thannhauser, 1962). An average mol.wt. 1935 for the mixed ox brain gangliosides was assumed in the calculations.

Preparation of cellular components. The methods described by De Robertis, Rodriguez de Lores Arnaiz, Salganicoff, Pellegrino de Iraldi & Zieher (1953) and De Robertis, Alberici, Rodriguez de Lores Arnaiz & Azcurra (1966) were used for fractionation of the frog brain homogenates into their cellular components. Adult frog brains were homogenized in 0.25 M-sucrose solution containing 0.11% (w/v) 2-mercaptoethanol (two brains/ml). The homogenization was divided into two 1 min periods with an interval for cooling. The total homogenate, diluted with an equal volume of the sucrose solution, was

centrifuged for 10 min at 900g. The precipitate was washed twice in sucrose medium and centrifuged to obtain the 900g particle (nuclear) fraction. The supernatant fluid was pooled and centrifuged at 11500g for 20 min.

The precipitate was washed once in the same medium and centrifuged again to obtain the 11500g particle (mitochondrial) fraction. The supernatant fraction was combined and centrifuged at 20000g for 30 min. The precipitate was designated the 20000g particle (heavy microsomes) fraction and the supernatant fluid was centrifuged at 100000g for 1 h. The precipitate was designated the 100000g particle (light microsomes) fraction. To obtain the soluble fraction, a small portion of the original homogenate was centrifuged at 100000g for 1 h and the supernatant was used. All operations were performed at 0°C.

Enzyme assay. The complete incubation mixture (Kaufman *et al.* 1966) for UDP-galactose- G_{M2} galactosyltransferase contained the following components in a final volume of 0.1 ml: G_{M2} , 70 nmol; UDP-[^{14}C]galactose, 70 nmol (7.4×10^5 c.p.m./ μ mol); detergent mixture containing 200 μ g of Triton CF-54 and 100 μ g of Tween 80; cacodylate buffer (pH 7.3), 10 μ mol; $MnCl_2$, 1 μ mol; enzyme preparation (0.1–1.6 mg of protein). The reaction was allowed to proceed for 4 h at 37°C and was terminated by the addition of 0.1 ml of chloroform-methanol (2:1, v/v). The whole reaction mixture was transferred to Whatman 3MM filter paper for ascending paper chromatography in 1% (w/v) $Na_2B_4O_7$ (pH 9.0) for 6–7 h. The UDP-[^{14}C]galactose and its degradation products migrated near the solvent front whereas the reaction products remained at the origin. The radioactivity of the origin plus lin of the paper was counted in fluor in a liquid-scintillation spectrometer. The fluor contained 4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 1 litre of toluene. The counting efficiency was 70%. The reaction blank contained the complete reaction mixture minus G_{M2} . Protein was determined by a biuret method (Gornall, Bardawill & David, 1949). NANA was determined by the method of Svennerholm (1957).

Artificial fertilization. Before use, all frogs were kept at 4°C. To induce ovulation, female frogs were injected intraperitoneally with four to six pituitaries taken from adult females of the same species (Rugh, 1962). The pituitaries suspended in 0.5 ml of water were drawn up into the barrel of a hypodermic syringe and injected through a large-bore hypodermic needle (no. 18). The females were kept at room temperature until they were ready to ovulate (24–48 h). The eggs from each female were stripped and fertilized by mixing with two pairs of minced testes. The testes were dissected in about 10 ml of water kept 10 min at room temperature to allow the spermatozoa to become active. Embryos were allowed to develop in modified Holtfreter's solution (Brown & Caston, 1962) at room temperature and the medium was changed at least three times each week.

RESULTS

Requirements for frog brain UDP-galactose- G_{M2} galactosyltransferase activity. The enzyme required Mn^{2+} for optimum activity and the addition of

Table 1. *Requirements for the UDP-galactose- G_{M2} galactosyltransferase in adult frog brain*

The complete system was as described in the Materials and Methods section. The incubation time was 4 h and 0.6 mg of protein from the whole homogenate was used for the assay.

Incubation mixture	[^{14}C]Galactose incorporated (c.p.m.)
Complete system	2010
Complete system (boiled enzyme)	50
Complete system minus Mn^{2+}	220
minus G_{M2} ganglioside	128
minus detergent mixture (Triton CF-54 and Tween 80)	710
plus 2 μmol of EDTA	55
plus 0.21 μmol of galactose	2090
plus 0.21 μmol of UDP-galactose	620

Table 2. *Substrate specificity of UDP-galactose- G_{M2} galactosyltransferase*

Enzyme protein (1.2 mg) from frog brain homogenate was used for the assay.

Substrate	Concn. (nmol)	[^{14}C]Galactose incorporated (c.p.m.)
None	—	134
G_{M2} ganglioside	70	3479
Ox brain gangliosides	200	153
Trisialoganglioside (G_{T1})	100	148
Disialoganglioside (G_{D1a})	100	107
Monosialoganglioside (G_{M1})	100	158
Glucosylceramide	100	106
Lactosylceramide	100	177
Trihexosylceramide (kidney)	100	169
Tetrahexosylceramide	100	815
Tetrahexosylceramide + NANA	100 (each)	789
Hematoside (G_{M3})	100	297

EDTA completely inhibited the system (Table 1). The requirement for Mn^{2+} was specific; other metal ions, Mg^{2+} , Co^{2+} , Ni^{2+} , Ca^{2+} , Cu^{2+} , Al^{3+} , K^+ , Na^+ or Li^+ , at equimolar concentrations could not substitute for Mn^{2+} . A threefold excess of galactose had no effect on the reaction but the addition of an equal amount of non-radioactive UDP-galactose inhibited 70% of the enzymic activity, suggesting that UDP-galactose and not galactose is involved in the reaction. The rate of the transferase reaction was proportional to the protein concentration and was independent of the time of incubation for at least 4 h.

Substrate specificity of the UDP-galactose- G_{M2} galactosyltransferase activity. G_{M2} was the most

Table 3. *Subcellular distribution of UDP-galactose- G_{M2} galactosyltransferase and gangliosides in adult frog brain*

The fractions were prepared by the methods described in the Materials and Methods section. Gangliosides from each fraction were isolated and the total ganglioside *N*-acetylneuraminic acid was determined by the method of Svennerholm (1957).

	Enzymic activity (%)	Ganglioside NANA (%)
Subcellular fraction		
Whole homogenate	100	100
900g particles	9.4	6.8
11 500g particles	10.6	27
20 000g particles	25.5	29.4
100 000g particles	39.2	29.7
Soluble	10.2	2.6

active substrate for the frog brain enzyme (Table 2). Individual ganglioside species, a mixture of gangliosides, lactosylceramide, kidney trihexosylceramide and G_{M3} had no, or little, acceptor activity. A significant incorporation of [^{14}C]galactose into tetrahexosylceramide was observed. Since one of the frog brain ganglioside species has been reported to contain a penta-hexosylceramide as a molecular skeleton for the attachment of NANA (Tettamanti, Bertona, Gualandi & Zambotti, 1965), the significance of the incorporation of [^{14}C]galactose from UDP-galactose into tetrahexosylceramide remains to be determined. Glucosylceramide exhibited no acceptor activity in the system assayed as described in this study. However, Basu, Kaufman & Roseman (1968) and Hildebrand & Hauser (1969) have demonstrated UDP-galactose-glucosylceramide galactosyltransferase activity in embryonic chick brain and the spleen tissues of young rat respectively.

Subcellular distribution of the frog brain UDP-galactose- G_{M2} galactosyltransferase and gangliosides. The enzyme was distributed in all the subcellular fractions (Table 3). Heavy- and light-microsomal fractions contained most of the UDP-galactose- G_{M2} galactosyltransferase activity (65%). The distribution of the enzymic activity paralleled that of the frog brain gangliosides. Heavy- and light-microsomal particles contained 59% of the total gangliosides in the adult frog brain (Table 3). The remaining portion of the frog brain gangliosides was distributed mainly in the mitochondrial fraction. Gangliosides isolated from each subcellular fraction were qualitatively similar when they were examined by ascending t.l.c. in two solvent systems: propan-1-ol-water (7:3, v/v) and chloroform-methanol-water (61:32:7, by vol.) (Dain *et al.* 1962).

Fig. 1. UDP-galactose- G_{M2} galactosyltransferase activity at different developmental stages. Embryos and tadpoles were homogenized in 0.25 M-sucrose containing 0.11% 2-mercaptoethanol. The enzymic activity was assayed as described in the Materials and Methods section. The temperature was 19–22°C.

Fig. 2. Activity of UDP-galactose- G_{M2} galactosyltransferase as a function of the concentration of G_{M2} . The reaction mixture contained 0.2 mg of protein from the frog brain homogenate.

Appearance of the UDP-galactose- G_{M2} galactosyltransferase activity during frog embryonic development. The enzyme activity was first detectable at the late gastrulation stage, Shumway stage 11½ (Fig. 1). The specific activity after Shumway stage 11½ increased until stage 25, and decreased to a negligible value in the tadpole (Taylor & Kollros stage 9). The observed rapid decrease in specific activity from Shumway stage 25 to tadpole stage 9 is apparently due to a dilution of the enzyme population resulting from the increase in body size. The appearance of the UDP-galactose- G_{M2} galactosyltransferase activity of Shumway stage 11½ correlates with the detectable amount of G_{M1} at the

Fig. 3. Effect of the ox brain gangliosides on the UDP-galactose- G_{M2} galactosyltransferase activity. ▲, Complete system containing the standard incubation mixtures and 1.6 mg of protein; ●, complete system minus substrate; Δ, complete system plus 0.26 μmol of ox brain gangliosides. The enzymic activity was assayed at different times. After 2 h, solutions of various metal ions [$2 \mu\text{mol}$ each of Ca^{2+} (■), Mg^{2+} (□) and Mn^{2+} (○)] were added to different preincubated tubes containing the complete system plus gangliosides. All values were corrected for a zero-time blank.

same developmental stage (Yiamouyiannis & Dain, 1968b).

Fig. 4. Effect of the individual ganglioside components on the UDP-galactose- G_{M2} galactosyltransferase activity. Individual gangliosides were added to the standard incubation mixture and the enzymic activity was assayed as described in the Materials and Methods section. Protein (1mg) from the frog brain homogenate was used in each reaction mixture. ●, G_{D1a} ; ○, G_{T1} ; ▲, G_{M1} .

General characteristic of the frog brain UDP-galactose- G_{M2} galactosyltransferase. The enzyme exhibited a pH optimum of 7.2–7.3 in both cacodylate and tris buffers; boric acid–borax buffer was inhibitory at pH 7.4–8.5. The enzyme required 10mM- Mn^{2+} for maximal activity and the K_m for Mn^{2+} was determined as 2.2mM by the method of Lineweaver & Burk (1934). The half-maximal velocity was obtained at a G_{M2} concentration of 0.18mM (Fig. 2). Inhibition of the enzymic reaction was observed at a G_{M2} concentration equal to or greater than 1.38mM.

Inhibition of the UDP-galactose- G_{M2} galactosyltransferase activity by gangliosides. Since the galactosyltransferase activity is inhibited by high concentrations (1.38mM) of its own substrate, G_{M2} , the effect of a mixture of gangliosides and the individual ganglioside components on the enzymic activity was studied. When 0.26 μ mol of ox brain gangliosides was present in the incubation mixture, the incorporation of [^{14}C]galactose into G_{M2} was greatly inhibited (Fig. 3). The inhibition could be reversed by the addition of Mn^{2+} , Mg^{2+} or Ca^{2+} after 2h. The individual ganglioside components, G_{M1} , G_{D1a} and G_{T1} also inhibited the enzyme activity, the most active inhibitor being G_{D1a} .

Table 4. *Inhibitor specificity of UDP-galactose- G_{M2} galactosyltransferase*

The complete system was as described in the Materials and Methods section. The incubation time was 4 h and 1.2 mg of protein from the whole homogenate was used for the assay.

Incubation mixture	Inhibition (%)
Complete system	0
minus G_{M2}	100
plus 0.2 μ mol of ox brain ganglioside	92
plus 1.2 mg of fetuin	0
plus 0.25 mg of <i>N</i> -acetylneuraminyl-lactose	15
plus 0.5 μ mol of NANA	22
plus 0.5 μ mol of NANA + 0.1 μ mol of tetrahexosylceramide	22
plus 0.1 μ mol of tetrahexosylceramide	10
plus 0.1 μ mol of glucosylceramide	0
plus 0.1 μ mol of lactosylceramide	0
plus 0.1 μ mol of trihexosylceramide (kidney)	0

(Fig. 4). The degree of inhibition was a function of the individual ganglioside concentrations.

Ganglioside seemed fairly specific in inhibiting the enzyme since NANA and NANA-containing analogues (Table 4) had little if any inhibitory effects when added to the assay system.

DISCUSSION

The correlation between the subcellular distribution of the UDP-galactose- G_{M2} galactosyltransferase and the gangliosides in adult frog brain (Table 3) suggests that this enzyme has a role in the synthesis of G_{M1} *in vivo*. The biosynthetic importance of this enzyme is further strengthened by the simultaneous appearance of the galactosyltransferase activity (Fig. 1) and G_{M1} (Yiamouyianis & Dain, 1968b) during embryonic development. However, the relative contribution of the UDP-galactose- G_{M2} galactosyltransferase and the CMP-NANA-Gall $\rightarrow$ 3GalNAc1 $\rightarrow$ 4Gall $\rightarrow$ 4Glc-Cer *N*-acetylneuraminyltransferase (Yip, 1968) in the synthesis of G_{M1} in adult frog brain remains to be determined.

The subcellular distribution of UDP-galactose- G_{M2} galactosyltransferase in adult frog brain differs from that of the embryonic chick brain. Basu, Kaufman & Roseman (1965) and Kaufman *et al.* (1966) have demonstrated that in embryonic chick brain the enzyme is localized mainly in the 39000g particles. By contrast, in the adult frog brain, the heavy- and light-microsomal fractions contain 65% of the total enzymic activity in the homogenate and 11% is found in the mitochondrial

fraction which contains free mitochondria and synaptosomes (Yip, 1968). Recent studies of the UDP-galactose- G_{M2} galactosyltransferase in 21-day-old foetal rat brain by Yip & Dain (1970) have shown that most of the enzymic activity is found in the heavy- and light-microsomal fractions.

The role of Mg^{2+} and Ca^{2+} in the reversal of the inhibition of the UDP-galactose- G_{M2} galactosyltransferase activity deserves further investigation. The fact that Mg^{2+} and Ca^{2+} cannot replace Mn^{2+} in activating enzymic activity but can reverse the inhibition by gangliosides (Fig. 3) suggests that both Mg^{2+} and Ca^{2+} interact with the anionic site of the ganglioside molecule and release the Mn^{2+} for activation of the enzyme. Alternatively, both Mg^{2+} and Ca^{2+} may stimulate the enzymic reaction by modifying the surface charge on the ganglioside micelles.

The inhibition experiments suggest that gangliosides can regulate the activities of their own biosynthetic metalloenzymes by virtue of their affinity for metal ions. G_{D1a} is approximately twice as effective an inhibitor as G_{M1} . The enzymic activity is inversely related to individual ganglioside concentration. Further, the results imply that all residues in G_{M1} and G_{D1a} are equally accessible for interaction with Mn^{2+} whereas the residues of G_{T1} appeared to be less effective. These results appear to be consistent with the data of Meisler & McCluer (1966) who reported that all NANA residues of G_{M1} and G_{D1a} are titratable and a significant proportion of the acid groups in G_{T1} is bound in some form. This observation could explain the less effective inhibition by G_{T1} . The ability of gangliosides to inhibit divalent ion-activated enzymes has been reported (Yip & Dain, 1969b).

An excess of gangliosides in any compartment of the cell may cause a deficiency of the available Mn^{2+} ions locally in the brain with concomitant lowered activity of the UDP-galactose- G_{M2} galactosyltransferase. The subcellular association of the transferase with gangliosides (Table 3) suggests the possibility of a balance between enzyme activity and product concentration.

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