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NEUTRAL HYDROLASES OF RAT BRAIN

PRELIMINARY CHARACTERIZATION AND DEVELOPMENTAL CHANGES OF NEUTRAL β-*N*-ACETYLHEXOSAMINIDASES

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Summary

The bulk of rat brain neutral β-*N*-acetylhexosaminidases (2-acetamido-2-deoxy-β-D-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52) were present in the cytosol fraction. They were not bound by concanavalin A-Sepharose while the acid β-*N*-acetylhexosaminidases were all bound. The neutral β-*N*-acetylglucosaminidase had a pH optimum of 5.2 and K_m of 0.57 mM, while the neutral β-*N*-acetylgalactosaminidase had the highest reaction rate at pH 6.0 with a K_m of 0.12 mM. No divalent ions activated either of the enzymes. The glucosaminidase lost more than 90% of the activity in 30 min at 50°C. The galactosaminidase was heat-stable and lost only 10—20% of its activity after 3 h at 50°C. The neutral glucosaminidase was inhibited by free *N*-acetylglucosamine but not by *N*-acetylgalactosamine. The reverse was found for the neutral β-galactosaminidase. Two enzymes were separated almost completely by hydroxyapatite chromatography. Heat stability of the separated activity peaks suggested that the neutral β-*N*-acetylgalactosaminidase, which was not bound to hydroxyapatite, may be specific to the galactosaminide substrate. The neutral β-*N*-acetylglucosaminidase may, on the other hand, have some activity toward the galactosaminide substrate. Both of the neutral enzyme activities were highest during the first postnatal week in rat brain in contrast to the acidic enzyme which showed peak activities during the second and third weeks. These

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results confirmed and expanded earlier observations by Frohwein and Gatt in calf brain. The relationship of these enzymes to the hexosaminidase C in human tissues is less certain at the present time.

Introduction

Two major categories of β-*N*-acetylhexosaminidase (2-acetamido-2-deoxy-β-D-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52) are present in mammalian tissues. Those with acidic pH optima which are localized in the lysosome have been extensively studied partly because of their involvement in genetic diseases in humans and other mammalian species. They include β-*N*-acetylhexosaminidase A and B [1—4], I_1 and I_2 [5], M [6], P [7], and S [8]. On the other hand, less is known about the second category, the neutral β-*N*-acetylhexosaminidase. Frohwein and Gatt [9,10] described earlier β-*N*-acetylhexosaminidases in calf brain which had more neutral pH optima and were localized primarily in the cytosol fraction. Hooghwinkel et al. [11] and Poenaru and Dreyfus [12] then described a neutral β-*N*-acetylhexosaminidase C in various human organs, including brain. Compared to the acidic β-*N*-acetyl hexosaminidase, hexosaminidase C was localized in the cytosol, rather than in the lysosomal fraction, had a neutral pH optimum, showed a larger molecular weight and very low activity to the *N*-acetylgalactosaminide substrate. It did not cross-react with antisera prepared against β-*N*-acetylhexosaminidase A or B [8]. Unlike the acidic hexosaminidases, hexosaminidase C was not adsorbed by concanavalin A-Sepharose [13].

Within the nervous system, little is known about the natural substrates and possible functional significance of the neutral hydrolases. Systematic explorations would seem desirable. In this report we describe a preliminary characterization of neutral β-*N*-acetylhexosaminidases in developing rat brain. At least two distinct enzymes are present, each predominantly active toward either *N*-acetylglucosaminide or *N*-acetylgalactosaminide.

Materials and Methods

For basic characterization of the enzymes, brains of 30-day-old Sprague-Dawley rats of both sexes were used. For the developmental studies, pregnant rats were purchased and newborn rats maintained in our laboratory to insure accuracy of age.

Preparation of enzyme sources. Rats were killed by decapitation. The following procedures were suitable for a starting brain weight of up to 5 g. The brain was immediately homogenized in 9 vols. (w/v) of 10 mM sodium phosphate buffer, pH 7.0, in a hand-operated Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The homogenate was centrifuged at 105 000 × *g* for 60 min. The supernatant was applied to a concanavalin A-Sepharose 4B column (bed vol. 1 ml) which had been prewashed with 100 ml of 10 mM sodium phosphate buffer, pH 7.0. The fraction not bound by concanavalin A was obtained by eluting the column with the same buffer (6—10 ml). This fraction will be referred to as the post-concanavalin A fraction. Partial purification could be

achieved by DEAE-Sephadex A-50 chromatography. The post-concanavalin A fraction in 10 mM phosphate buffer, pH 7.0, was applied to a DEAE-Sephadex column with a bed vol. 14 ml. It was eluted with the same buffer at 10 ml/h until the DEAE-Sephadex-unadsorbed protein is completely eluted. No hexosaminidase activity was recovered in this fraction. Then a linear gradient of NaCl was introduced into the elution buffer. Neutral hexosaminidase activities were eluted as a single peak with some tailing starting at 0.4 M NaCl. The fractions containing bulk of the enzymatic activity were pooled and applied directly to a column of hydroxyapatite (bed vol. 3 ml). The column was eluted with 10 mM sodium phosphate buffer, pH 7.0, until the absorbance of the eluate at 280 nm reached the baseline. Then the concentration of the eluting phosphate buffer was increased gradually and linearly to 100 mM. A peak of the enzymatic activity was eluted beginning at 50—60 mM phosphate. Both hydroxyapatite-adsorbed and unadsorbed fractions were dialyzed against 10 mM sodium phosphate buffer, pH 7.0. For certain purposes, the hydroxyapatite-adsorbed fraction was eluted batchwise with 100 mM sodium phosphate buffer, pH 7.0, after complete elution of the unadsorbed fraction with the 10 mM buffer.

Subcellular fractionation. When subcellular distribution of the enzymes was tested, rat brain subcellular fractions were prepared in principle according to DeRobertis et al. [14]. Brains were homogenized in 9 vols. (w/v) of ice cold 0.32 M sucrose solution in a Dounce homogenizer. The homogenate was first centrifuged at $900 \times g$ for 10 min. The pellet was resuspended and re-centrifuged three times and the final pellet was designated as the nuclear-cell debris fraction. The combined supernatant was centrifuged at $11\,000 \times g$ for 20 min. The pellet was washed twice to yield the crude mitochondrial fraction. The combined $11\,000 \times g$ supernatants were centrifuged at $105\,000 \times g$ for 60 min. The pellet was washed once. This procedure gave the microsomal (pellet) and the cytosol (supernatant) fractions. The particulate fractions were then subjected to the procedures described above for whole brain homogenate to obtain the enzyme source for subsequent studies. The cytosol fraction could be applied directly to the concanavalin A-Sepharose column after it was adjusted to 10 mM sodium phosphate buffer, pH 7.0.

Enzymatic assays. 4-Methylumbelliferyl β-*N*-acetylglucosaminide and β-*N*-acetylgalactosaminide were used as the substrates. They were kept frozen as 4 mM suspensions in 40 mM sodium citrate/80 mM sodium phosphate buffer, pH 4.5. β-*N*-Acetylhexosaminidases were assayed in a total vol. 0.4 ml containing 0.2 ml of appropriately diluted enzyme source, 0.1 ml substrate suspension, and 0.1 ml of 0.2 M sodium citrate/0.4 M sodium phosphate buffer of appropriate pH. The reaction mixture was incubated for 15 min at 37°C with gentle shaking. The reaction was terminated by the addition of 0.2 M glycine buffer, pH 10.7, and the liberated 4-methylumbelliferone was determined in an Aminco-Bowman SPF-125 spectrofluorometer. Sphingomyelinase, galactosylceramidase and several 4-methylumbelliferyl glycoside hydrolases were determined according to the standard procedures of this laboratory [15]. Arylsulfatases A and B were assayed with *p*-nitrocatechol sulfate as the substrate according to Baum et al. [16]. Acid phosphatase was assayed with *p*-nitrophenyl phosphate as the substrate [17]. The protein content of the enzyme

sources was determined by the method of Lowry et al. [18].

Commercial materials. The following commercial materials were purchased from the indicated sources: all chromogenic or fluorogenic substrates (Research Products International, Elk Grove Village, IL); concanavalin A-Sepharose 4B and DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ); hydroxyapatite (Bio-gel HTP, Bio-Rad, Richmond, CA); [^{14}C]sphingomyelin (New England Nuclear Corp., Boston, MA).

Results

The neutral β-*N*-acetylhexosaminidases were stable for 12 h when the rat brain was left at the room temperature without being removed from the skull. They were stable for 2 weeks at 4°C and for at least 1 month at −70°C in the form of whole brain homogenate. Although not systematically studied, no tendency to increasing instability was observed at any stage of manipulations employed in this series of studies.

The high-speed supernatant of rat brain homogenate showed a bimodal pH curve for both β-*N*-acetylglucosaminidase and galactosaminidase. After the concanavalin A-Sepharose column, the unadsorbed fraction gave a single pH optimum corresponding to the more neutral peak of the supernatant (Figs. 1 and 2). The concanavalin A-adsorbed fraction, which could be eluted essentially quantiatively with additional 10% α-methylglucoside and 0.5 M NaCl

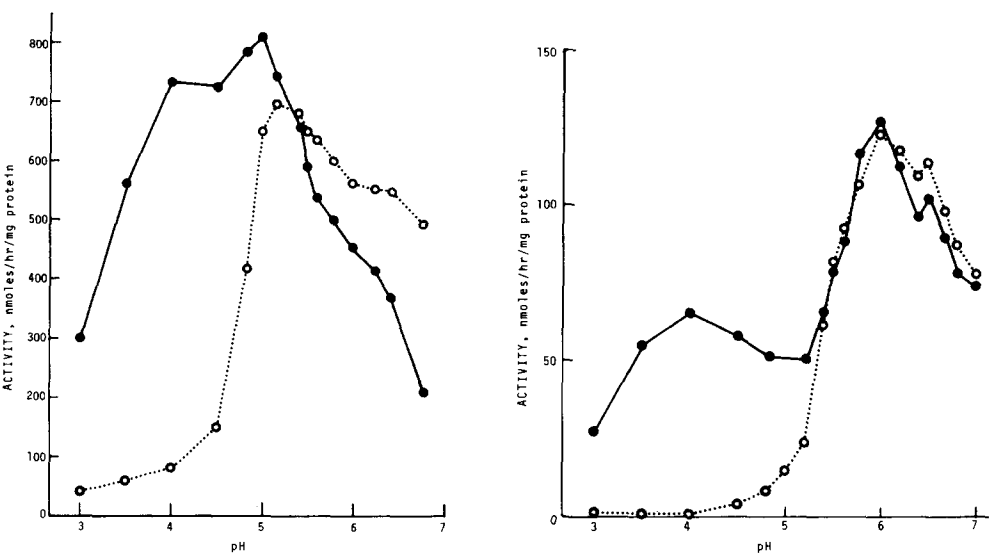

Fig. 1. Effect of pH on the neutral β-*N*-acetylglucosaminidase in the high-speed supernatant and in the post-concanavalin A fraction of young adult brain. The assay procedure was as described in the text except that the pH was varied as indicated. ●———●, 105 000 × *g* supernatant; ○· · · · · ·○, post-concanavalin A fraction. Concanavalin A-Sepharose eliminated the component with an acidic pH optimum.

Fig. 2. Effect of pH on the neutral β-*N*-acetylgalactosaminidase in the high-speed supernatant and in the post-concanavalin A fraction of young adult rat brain. The assay procedure was as described in the text except that the pH was varied as indicated. ●———●, 105 000 × *g* supernatant; ○· · · · · ·○, post-concanavalin A fraction. Concanavalin A-Sepharose eliminated the component with an acidic pH optimum.

[19], on the other hand, exhibited a single acidic pH optimum of 4.0 for both *N*-acetylglucosaminide and *N*-acetylgalactosaminide. Both concanavalin A-adsorbed and unadsorbed fractions could be passed through the concanavalin A-Sepharose column repeatedly with identical behaviors, the same pH optima and quantitative recoveries for the respective fractions. Therefore, the concanavalin A-Sepharose column could effectively separate the neutral β-*N*-acetylhexosaminidases from the acidic enzymes. The concanavalin A-unadsorbed fraction showed some activities of α-mannosidase and acid phosphatase, but little or no activities were found for 4-methylumbellifery β-galactosidase, β-glucosidase, α-fucosidase, arylsulfatase A and B, sphingomyelinase and galactosylceramidase.

With the concanavalin A-unadsorbed fraction as the enzyme source, the pH optimum of the neutral β-*N*-acetylglucosaminidase was 5.2, and that of the neutral β-*N*-acetylgalactosaminidase, 6.0. The reaction was linear for 20 min, and at 60 min it was within 20% of perfect linearity. Similarly the reaction was linear with respect to the amount of protein up to 200 μg per tube. The apparent K_m values were 0.57 ± 0.01 mM ($n = 4$) for *N*-acetylglucosaminide and 0.12 ± 0.02 mM ($n = 4$) for *N*-acetylgalactosaminide. The standard assay systems for the β-*N*-acetylhexosaminidases described in Materials and Methods were developed on the basis of these findings. With these optimized assay procedures, the post-concanavalin A fraction of young adult rat brain had hydrolytic activity approx. 5-times greater for the glucosaminide substrate than for the galactosaminide substrate.

No divalent ion could be found that could activate the neutral β-*N*-acetylhexosaminidases, consistent with the finding that EDTA up to 10 mM showed little effect on the activity of these enzymes (Table I). Some divalent ions, most notably Cu^{2+}, Co^{2+} and Zn^{2+} were inhibitory, particularly at higher con-

TABLE I

EFFECT OF DIVALENT IONS ON RAT BRAIN NEUTRAL β-*N*-ACETYLHEXOSAMINIDASES

The concanavalin A-unadsorbed fraction was used as the enzyme source. The neutral β-*N*-acetylhexosaminidases were measured by the standard assay systems, except that the indicated concentrations of divalent ions were present. They were added as chloride salts. Chloride ion had no effect on these enzymes up to 10 mM when it was added in the form of NaCl. Control activity was the one determined without addition of divalent ions or EDTA. EDTA from 1—10 mM had no effect on *N*-acetylgalactosaminidase and gave only a slight reduction of *N*-acetylglucosaminidase (15%) at the highest concentration.

Divalent ion	Concentration (mM)	*N*-acetylglucosaminidase (% control)	*N*-acetylgalactosaminidase (% control)
Mg^{2+}	5	97	98
	10	84	94
Ca^{2+}	5	98	100
	10	86	98
Mn^{2+}	5	93	95
	10	74	81
Co^{2+}	5	87	93
	10	40	86
Cu^{2+}	5	12	2
	10	12	1
Zn^{2+}	5	92	74
	10	55	52

centrations. Different degrees of inhibition of β-*N*-acetylglucosaminidase (60%) and β-*N*-acetylgalactosaminidase (15%) were consistently noted when 10 mM Co^{2+} were added to the reaction mixture.

When heated at 50°C, the neutral β-*N*-acetylglucosaminidase lost more than 90% of the activity in 30 min, and almost complete inactivation occurred within 60 min. In contrast, the neutral β-*N*-acetylgalactosaminidase was quite stable at 50°C and lost only 15% of the activity in 3 h. The acid β-*N*-acetylhexosaminidase assayed in whole homogenate at pH 4.5, on the other hand, lost 70–75% of the activity at 50°C in 3 h with either *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate (Fig. 3), as is well known for the acidic enzyme [20]. Since the clearly different heat stability was suggestive of distinct neutral β-*N*-acetylhexosaminidases, each active primarily toward the glucosaminide or galactosaminide, the neutral β-*N*-acetylhexosaminidase activities were determined with either free *N*-acetylglucosamine or *N*-acetylgalactosamine as potential inhibitors. The neutral β-*N*-acetylglucosaminidase activity was inhibited by free *N*-acetylglucosamine with 50% inhibition occurring at 7 mM but not by free *N*-acetylgalactosamine (Fig. 4). Conversely, the neutral β-*N*-acetylgalactosaminidase activity was inhibited by free *N*-acetylgalactosamine (50% inhibition at 12 mM) but not by free *N*-acetylglucosamine (Fig. 5). These inhibition studies further suggested distinct neutral β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase. This was in contrast to the similar inhibition studies on the acidic β-*N*-acetylhexosaminidase (Fig. 6). Either 4-methylum-

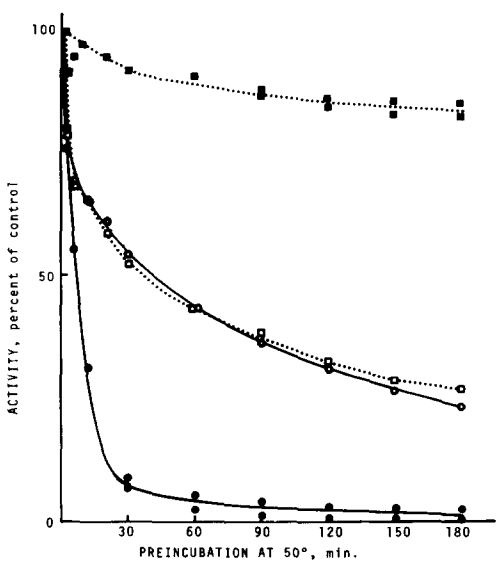

Fig. 3. Heat stability of rat brain β-*N*-acetylhexosaminidases. The enzyme sources were whole homogenate for the acid hexosaminidases, and the post-concanavalin A fraction for the neutral enzymes. They were heated at 50°C for the durations indicated prior to the assays. The heating was done at the pH optimal for the respective enzyme activities; pH 4.5 for acid hexosaminidases, pH 5.2 for the neutral glucosaminidase and pH 6.0 for the neutral galactosaminidase. ●———●, neutral glucosaminidase; ■·····■, neutral galactosaminidase; ○———○, acid glucosaminidase; □·····□, acid galactosaminidase. The heat stability of the neutral enzymes were drastically different while that of the acidic enzyme was identical, irrespective of the substrate used.

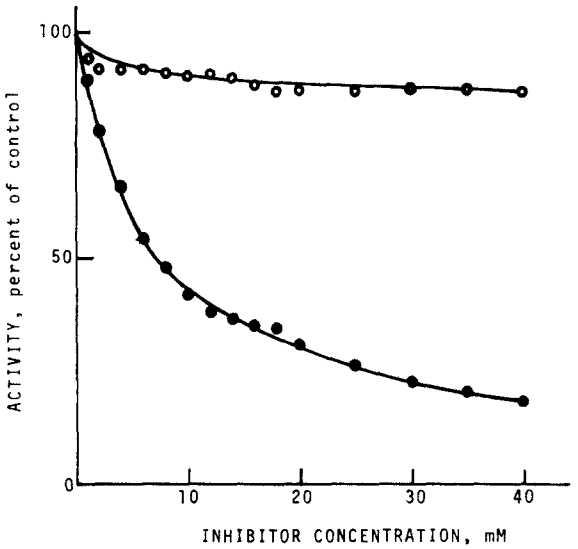

Fig. 4. Product inhibition of rat brain neutral β-*N*-acetylglucosaminidase. The post-concanavalin A fraction was assayed for the neutral glucosaminidase activity according to the standard procedure in the presence of additional *N*-acetylhexosamines, as indicated. The concentration refers to the final concentration in the reaction mixture. ●———●, with free *N*-acetylglucosamine; ○———○, with free *N*-acetylgalactosamine.

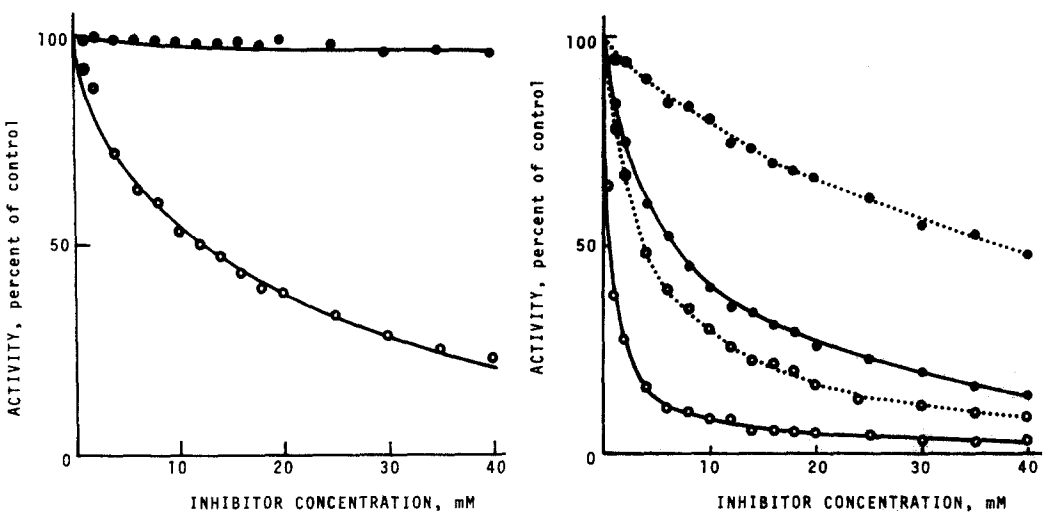

Fig. 5. Product inhibition of rat brain neutral β-*N*-acetylgalactosaminidase. The post-concanavalin A fraction was assayed for the neutral galactosaminidase activity according to the standard procedure in the presence of additional *N*-acetylhexosamines as indicated. The concentration refers to the final concentration in the reaction mixture. ●———●, with free *N*-acetylglucosamine; ○———○, with free *N*-acetylgalactosamine.

Fig. 6. Product inhibition of rat brain acid β-*N*-acetylhexosaminidases. Whole homogenate was assayed for the acid glucosaminidase and galactosaminidase activities in the presence of varying amounts of free *N*-acetylhexosamines as indicated. ●———●, acid glucosaminidase with free *N*-acetylglucosamine; ○———○, acid glucosaminidase with free *N*-acetylgalactosamine; ●·····●, acid galactosaminidase with free *N*-acetylglucosamine; ○·····○ acid galactosaminidase with free *N*-acetylgalactosamine.

belliferyl *N*-acetylglucosaminide or galactosaminide as the substrate, free *N*-acetylgalactosamine was a more potent inhibitor of acid β-*N*-acetylhexosaminidase than *N*-acetylglucosamine. The results were consistent with the acid β-*N*-acetylhexosaminidase being a single enzyme with different affinity to the glucosaminide and galactosaminide substrates (apparent K_m for glucosaminide, 0.40 ± 0.04 mM (n = 4), and for galactosaminide, 0.055 ± 0.006 (n = 4).

The DEAE-Sephadex column did not separate the neutral glucosaminidase and galactosaminidase activities from each other, although the peak of the latter activity tended to emerge from the column slightly later than the former (Fig. 7). The two activities were successfully separated when either the post-concanavalin A fraction or the post-DEAE fraction was subjected to hydroxyapatite chromatography (Fig. 8). The fraction which was not adsorbed by hydroxyapatite contained the bulk of the neutral β-*N*-acetylgalactosaminidase activity with very little activity toward the glucosaminide substrate, while the fraction which was initially adsorbed and then eluted with high concentration of the phosphate buffer contained almost all of the glucosaminidase activity and very low galactosaminidase activity. This procedure thus provided unequivocal evidence for at least two neutral β-*N*-acetylhexosaminidases, one

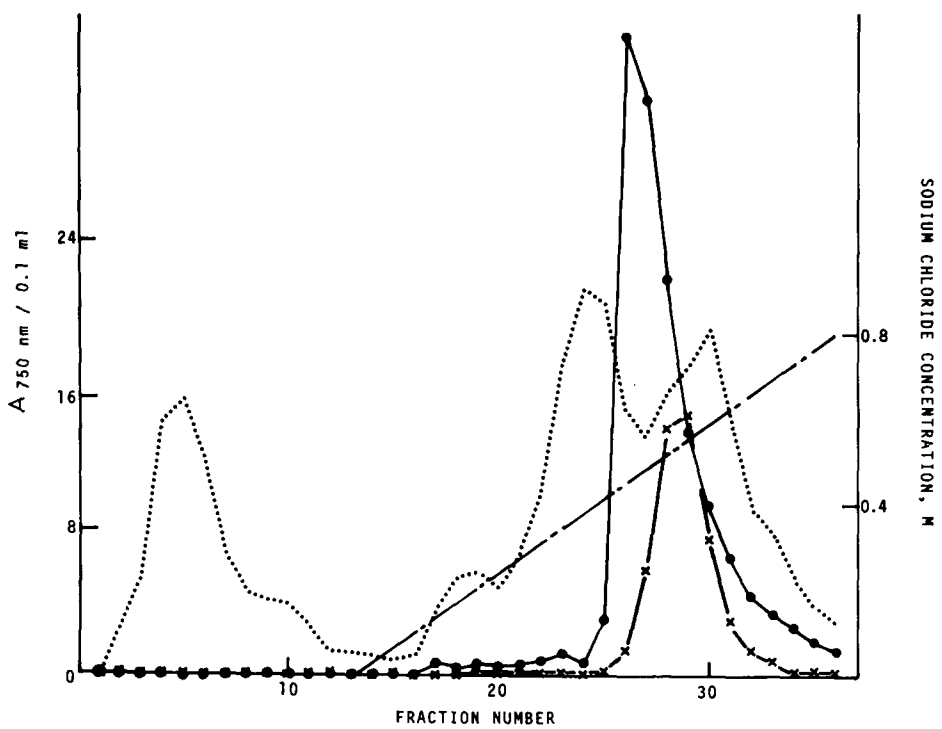

Fig. 7. DEAE-Sephadex chromatography of rat brain neutral β-*N*-acetylhexosaminidases. The post-concanavalin A fraction (6 ml) was applied to a DEAE-Sephadex column (bed vol. 14 ml). The column was washed with 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 9 ml/h. Then a linear gradient of sodium chloride was introduced. The same flow rate was maintained and 3-ml fractions collected. · · · · · ·, protein as determined by the procedure of Lowry et al.; · — · — ·, sodium chloride gradient; ●———●, neutral β-*N*-acetylglucosaminidase; X———X, neutral β-*N*-acetylgalactosaminidase. The enzyme activities are ploted in arbitrary units.

almost exclusively active toward the galactosaminide substrate, and the other primarily active toward the glucosaminide substrate. Heat stability tests of the two fractions separated by hydroxyapatite indicated that the hydroxyapatite-unadsorbed neutral β-*N*-acetylgalactosaminidase activity was heat-stable but the small activity of β-*N*-acetylglucosaminidase associated with this peak was completely inactivated by preincubation at 50°C for 60 min. On the other hand, both β-*N*-acetylglucosaminidase and galactosaminidase activities in the hydroxyapatite-adsorbed fraction were heat-labile. These findings suggested that the hydroxyapatite-unadsorbed fraction might be a specific *N*-acetylgalactosaminidase slightly contaminated by the second neutral β-*N*-acetylhexosaminidase which should have been adsorbed by the column, while the hydroxyapatite-adsorbed enzyme might possess activities toward both β-*N*-acetylglucosaminide and β-*N*-acetylgalactosaminide, although the reaction rates with the respective substrates might be quite different.

Both of the neutral β-*N*-acetylhexosaminidases were distributed approximately equally between the gray and white matters in three young adult rat brains examined. When the total particulate fraction, prepared by homogenization in 0.32 M sucrose and high-speed centrifugation (100 000 × *g*, 60 min), was extracted with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100, centrifuged at 100 000 × *g*, 60 min, and the supernatant passed the concanavalin A column, some activities of the neutral β-*N*-acetylhexosaminidases were recovered. However, the initial cytosol fraction yielded approx. 60% of the total recovered activities of either of the enzymes. Therefore, the concanavalin A-unadsorbed neutral β-*N*-acetylhexosaminidases of rat brain appear to be primarily localized in the cytosol fraction. The bulk of the acid β-*N*-acetylhexosaminidase was recovered in the crude mitochondrial fraction, as expected.

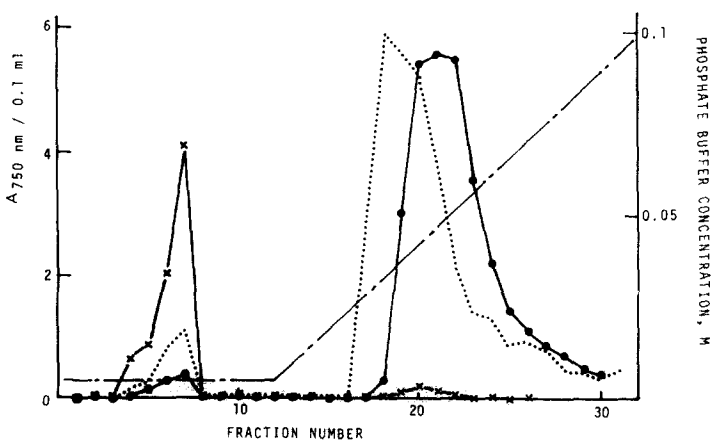

Fig. 8. Hydroxyapatite chromatography of rat brain neutral β-*N*-acetylhexosaminidases. The fraction eluted from the DEAE-Sephadex column (15 ml) was applied to a hydroxyapatite column (bed vol. 3 ml). It was first washed with 10 mM sodium phosphate buffer, pH 7.0 at a flow rate of 5 ml/h and 3-ml fractions collected. Then the concentration of the phosphate buffer was increased linearly to 100 mM. ·····, protein as determined by the procedure of Lowry et al.; ·—·—·, phosphate buffer gradient; ●———●, neutral β-*N*-acetylglucosaminidase; X———X, neutral β-*N*-acetylgalactosaminidase. The enzyme activities are plotted in arbitrary units.

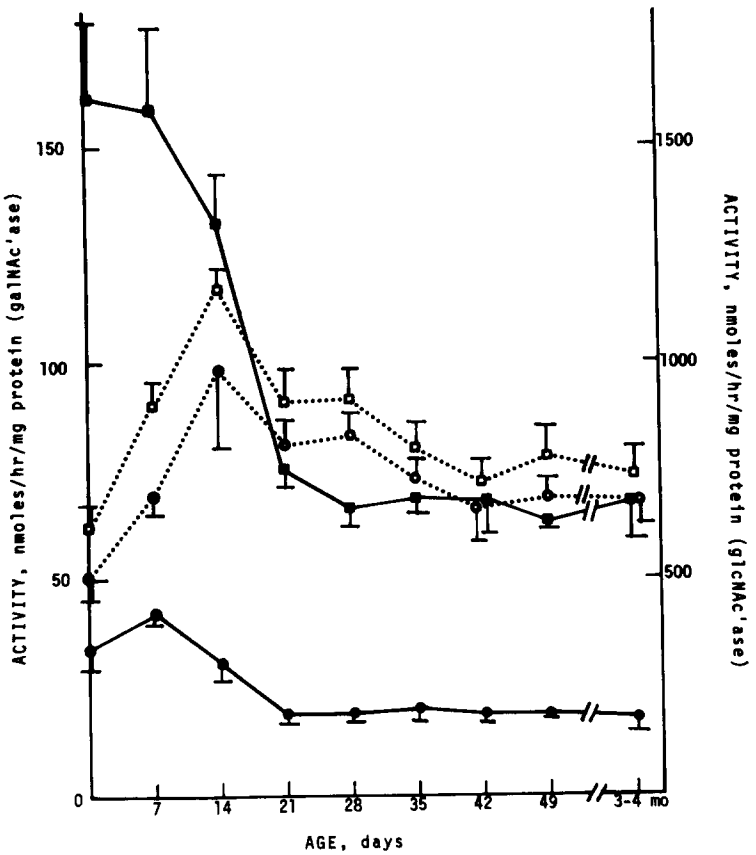

Fig. 9. Developmental changes of rat brain β-*N*-acetylhexosaminidases. The acid hexosaminidases were determined in the whole homogenate and the neutral hexosaminidases in the post-concanavalin A fraction. ●———●, neutral glucosaminidase; ■———■, neutral galactosaminidase; ○·····○, acid glucosaminidase; □·····□, acid galactosaminidase. The statistical variations indicate the S.D. based on up to six animals for each point. GalNAc'ase, *N*-acetylgalactosaminidase; GlcNAc'ase, *N*-acetylglucosaminidase.

In developing rat brain, both of the neutral β-*N*-acetylhexosaminidases showed the highest activities during the first postnatal week (Fig. 9). The activities dropped to slightly less than half of the peak activities by 21—28 days and remained constant thereafter. The developmental changes were similar but not identical between the neutral *N*-acetylglucosaminidase and galactosaminidase. On the other hand, the acid *N*-acetylhexosaminidase activities increased steeply during the first 2 weeks and then gradually declined. Consistent with one enzyme catalyzing both reactions, the activities toward the glucosamine and galactosamine substrates changed identically during development.

Discussion

The results of this initial phase of our studies on rat brain neutral β-*N*-acetylhexosaminidases clearly demonstrated two distinct enzymes. Both are primarily localized in the cytosol, do not bind to concanavalin A, and have pH optima more neutral than that of the acid lysosomal β-*N*-acetylhexosaminidase. One of

the two is possibly a β-*N*-acetylgalactosaminidase which is relatively heat-stable and is not adsorbed by hydroxyapatite under the experimental conditions described. The other is primarily active toward *N*-acetylglucosaminide substrate but probably with minor activity also toward *N*-acetylgalactosaminide and is heat-labile. Both enzymes show highest activities during the first week after birth in developing rat brain.

Our results are not necessarily easy to compare with the brain cytosolic neutral β-*N*-acetylhexosaminidases previously reported. Frohwein and Gatt were the earliest to study this group of enzymes in calf brain [9,10]. They reported the differential inhibitions by free *N*-acetylglucosamine and *N*-acetylgalactosamine, similar to those of our observations, and they were able to partially separate the two activities by a series of conventional procedures. Although in a different species, we consider that the two enzymes reported here are similar to those of Frohwein and Gatt. In addition to the above similarities, the pH optima of their enzymes were similar to ours. The neutral β-*N*-acetylglucosaminidase of Frohwein and Gatt was inhibited by *p*-hydroxymercuribenzoate, silver nitrate and *N*-ethylmaleimide, while the neutral *N*-acetylgalactosaminidase was inhibited by the first two compounds but was activated by *N*-ethylmaleimide. We intend to carry out similar inhibition studies when we obtain our enzymes in higher purity. Our present view is that we are likely to be studying rat brain neutral β-*N*-acetylhexosaminidases similar to those reported by Frohwein and Gatt in calf brain and that we have confirmed and expanded their findings. The use of concanavalin A-Sepharose and hydroxyapatite provide simpler and more effective means to eliminate the acid β-*N*-acetylhexosaminidase and then to separate the two neutral enzymes from each other, than the procedures used by Frohwein and Gatt. When the enzyme fraction from DEAE-Sephadex was subjected to the hydroxyapatite column, the neutral *N*-acetylgalactosaminidase had a relative specific activity of nearly 10, and the neutral *N*-acetylglucosaminidase about 2, relative to the activities in the post-concanavalin A fraction. Since the post-concanavalin A fraction should represent a significant purification over the starting homogenate, the hydroxyapatite-separated fractions appear to be advantageous for further purification attempts.

Comparison of our enzymes with hexosaminidase C is more difficult [11,12,21]. The hexosaminidase C was initially defined by the electrophoretic mobility [11,12]. It shares several properties with the rat brain neutral β-*N*-acetylglucosaminidase, including the cytosolic localization, the neutral pH optimum, heat-lability, primary activity toward the glucosaminide substrate, and no affinity to concanavalin A. There are a few properties, however, which do not correspond to those of ours. The pH optimum of hexosaminidase C is always reported to be 7.0. It could not be recovered from DEAE-Sephadex [21]. These differences could conceivably be due to the different species involved, because most studies on hexosaminidase C have been carried out with human tissues. On the other hand, we always observed a shoulder at pH 6—7 in the pH curve of our neutral β-*N*-acetylglucosaminidase (Fig. 1), which could be the third component corresponding to hexosaminidase C. Further purification and more detailed characterization of the neutral β-*N*-acetylhexosaminidases should provide a more definitive answer.

The relatively high activities of the neutral enzymes during the first postnatal week in rat brain are of interest. Some neutral glycosidases appear to be involved in the process of glycoprotein biosynthesis [22]. Neutral hydrolases in brain could also function in such capacity. Our present approach is to purify these enzymes further and then to search for their natural substrates.

Acknowledgement

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