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N-ACETYL-β-D-GLUCOSAMINIDASE OF NERVE CELLS: A DEVELOPMENTAL STUDY OF TWO MOLECULAR COMPONENTS

O. Z. SELLINGER, JOSEPHINE C. SANTIAGO, M. A. SANDS AND BARBARA FURIN-SLOAT

Laboratory of Neurochemistry, Mental Health Research Institute, University of Michigan Medical Center, Ann Arbor, Mich. 48104 (U.S.A.)

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SUMMARY

1. The development of the lysosomal hydrolase *N*-acetyl-β-D-glucosaminidase was examined in bulk-isolated nerve cell bodies of rat cerebral cortex. Several properties of the enzyme and of two of its molecular components were compared, among them their ease of solubilization, their relative abundance, their heat lability and their behavior on density gradients of sucrose.

2. Repeated freezing–thawing of neuronal particulates in 0.20 M sucrose–0.1 M KCl followed by centrifugation at moderate rather than high speeds resulted in the solubilization of upward of 50% of the glucosaminidase, irrespective of neuronal age.

3. Although the solubilization of *N*-acetyl-β-D-glucosaminidase was dependent on the concentration (mg of protein/ml) of the suspension subjected to freezing–thawing, it was always more effective with the enzyme from the 8- and 18-day-old than with that from the 3- and 5-day-old neurons.

4. Centrifugation of the solubilized neuronal *N*-acetyl-β-D-glucosaminidase on linear density gradients of sucrose resulted, at all ages, in the complete resolution of two activity components a heavy and a light one, H and L respectively. As routinely isolated, the ratio of component L to component H in the neurons of the cerebral cortex was always >1.0 while, in the cerebellar neurons, values <1.0 were also noted.

5. The qualitative and quantitative gradient profiles of *N*-acetyl-β-D-glucosaminidase activity were highly sensitive to pH. Thus, lowering of the pH of frozen–thawed supernatants to 5.4 and below prior to density gradient centrifugation resulted in the virtual disappearance of component H, while adjustment of the pH to 5.6 resulted in L/H ratios <1.0 and in the appearance of additional, smaller peaks of *N*-acetyl-β-D-glucosaminidase activity.

6. About 50% of the activity of component L was lost, irrespective of age, by heating for 20 min at 50 °C and at a pH of 4.1. The heat sensitivity of component H was no greater, except at 5 days, when about 90% of its activity was lost under the same conditions.

7. The molecular weights of the components H and L of neuronal *N*-acetyl-β-D-glucosaminidase were indistinguishable, approximating 158 000. Component H could be readily converted to component L in acid media and this conversion did not require the presence of exogenous neuraminidase.

INTRODUCTION

The lysosomal hydrolase *N*-acetyl-β-D-glucosaminidase[1] (EC 3.2.1.30) exists in nervous tissue[2–8] in at least two distinct molecular forms referred to as components A and B[9–14]. The partial[16,17] or total[12,18] deficiency of component A as well as the total deficiency of components A and B[12] from brain tissue are used, at present, as diagnostic criteria for the clinical characterization of a number of distinct pathological manifestations of cerebral gangliosidosis.

Although it has been known for some time that in brain[2], as in other tissues[19], *N*-acetyl-β-D-glucosaminidase is a lysosomal enzyme, the study of its cellular location in this organ has been made possible only recently thanks to the development of simple and rapid techniques for the bulk isolation of large numbers of nerve cell bodies and glial cells from the same tissue sample[20–22]. Thus, Idoyaga-Vargas *et al.*[7], Idoyaga-Vargas and Sellinger[23] and, more recently, Raghavan *et al.*[24] found closely similar specific activities for neuronal and glial *N*-acetyl-β-D-glucosaminidase in young and adult rat cerebral cortex, while, on the contrary, Sinha and Rose[25], using adult rats and fractions of neuronal cell bodies and of neuropil containing glia together with synaptic elements and capillaries, reported higher specific activities of the enzyme in the former fraction. Hirsch[13] also reported higher values of total *N*-acetyl-β-D-glucosaminidase in individually analyzed anterior horn neurons than in the neuropil surrounding them, as well as in the granular as compared to the molecular layer of the cerebellar cortex.

In a recent publication we reported that the *N*-acetyl-β-D-glucosaminidase of the neuronal cell bodies of the cerebral cortex of the immature rat reaches highest values of specific activity at about 18 days post-natally[7]. We also determined that the hydrolase was lysosome bound as early as 3 days post-natally and, in addition, that it associates, in the 8- and the 12-day-old neuron, with two distinct populations of lysosomal granules which differ markedly in buoyant density[7]. In related studies of the *N*-acetyl-β-D-glucosaminidase of human brain, Harzer and Sandhoff[11] noted that, as the total activity of the enzyme increased from fetal to old age, the ratio of the components B and A also increased. These workers also noted a small but reproducible difference in the B/A ratio of adult cerebrum and cerebellum.

Although most of the studies referred to above have focused on (a) differences in the total activity of *N*-acetyl-β-D-glucosaminidase measured over rather wide time spans of human life[12], (b) the special significance of the deficiency of one or both of its components in human brain disease[16–18] or (c) the development of methodologies for the characterization of tissue-specific *N*-acetyl-β-D-glucosaminidase activity profiles[25–28] for clinical diagnostic use, no systematic study has appeared outlining the properties of the molecular forms of the exclusively neuronal enzyme. In the present report we describe the results of experiments on the characterization of this activity following its solubilization from neuronal lysosomes. We examined several

properties of the two main components of neuronal *N*-acetyl-β-D-glucosaminidase which, on the basis of their sedimentation properties in sucrose density gradients we term as the heavy (H) and the light (L) components. These are: ease of solubilization, relative abundance and stability, sedimentability, heat lability and behavior upon chromatography and electrophoresis. In addition we present comparative data on the components H and L of the *N*-acetyl-β-D-glucosaminidase of the neurons of the developing cerebral and cerebellar cortex.

MATERIALS AND METHODS

The rats were male of the Sprague–Dawley strain. They were generally sacrificed in groups of 10 or 20 between 10 and 11 a.m. Since their day of birth as specified by the shipper (Spartan Farms, Haslett, Mich.) was used to assess chronological age, animals of nominal age of 3 and 5 days old could be off their biological age by as much as 12 h.

The substrate of *N*-acetyl-β-D-glucosaminidase, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was purchased from Sigma Chemical Co., St. Louis, Mo. or from Pierce Chemical Co., Rockford, Ill. 4-Umbelliferyl-*N*-acetyl-β-D-glucosaminide was purchased from Pierce Chemical Co., Rockford, Ill.; polyvinylpyrrolidone (nominal average molecular weight: 40 000; Plasdone C) was obtained from General Aniline and Film, Calvert City, Ky.; bovine serum albumin (Fraction V) was from Miles Laboratories, Kankakee, Ill.; Ficoll and Sephadex G-200 were from Pharmacia, Inc., Piscataway, N.J.; sucrose (enzyme grade) and cytochrome *c* were purchased from Schwarz-Mann, Orangeburg, N.Y.; DEAE-cellulose (type DE 52) was purchased from H. Reeve Angel, Inc., Clifton, N.J.; transferrin and aldolase (5 ×) were products of the Nutritional Biochemical Corp., Cleveland, Ohio; acrylamide (electrophoresis grade) was from Eastman Chemical Co., Rochester, N.Y.; neuraminidase, type VI (*Clostridium perfringens*) and *N*-acetyl-neuramin lactose (type I) were purchased from Sigma Chemical Co., St. Louis, Mo.

The preparation of the neuronal cell bodies from the cerebral and the cerebellar cortices was according to the procedure of Sellinger *et al.*[21] described in somewhat more detail by Johnson and Sellinger[29]. The neuronal cell bodies, isolated as a pellet, were suspended in 0.25 M sucrose and were homogenized with 6 up-and-down strokes of a Potter–Elvehjem glass–Teflon homogenizer rotating at about 1300 rev./min. The approximately 20% (w/v) homogenate was subjected to differential centrifugation. Two schedules of centrifugation were used; in the one used most commonly, the nuclear fraction was first removed by centrifuging the homogenate at 1085 × *g* for 15 min in the SS-34 rotor of the Sorvall RC2B centrifuge, followed by one 12-min wash and Fraction Mit, Ly, Mic was then sedimented at 105 000 × *g* for 2 h in rotor 65 of the Spinco ultracentrifuge. Fraction Mit, Ly, Mic was used in most experiments in which *N*-acetyl-β-D-glucosaminidase was solubilized and its properties examined. In a previous study[7] we showed that between 50 and 65% of the total neuronal *N*-acetyl-β-D-glucosaminidase sediments in this fraction, irrespective of the age of the animal. In experiments in which the aim was to isolate a fraction of purified lysosomes (Fraction Ly), the 5-step centrifugation schedule described by Sellinger and de Balbian Verster[30] was used. For routine solubilization of *N*-acetyl-β-D-glucosaminidase, Fraction Mit, Ly, Mic was suspended in 0.20 M

sucrose–0.1 M KCl and the suspension was frozen–thawed by alternate immersion into a slurry of dry ice and acetone and water at 37 °C. Following the last thaw, the suspension was centrifuged in Spinco rotor 65 for 2 h at 15 000 × *g* and the *N*-acetyl-β-D-glucosaminidase remaining in the supernatant (FT supernatant) was used for further study. Aliquots of the FT supernatant were diluted with 0.1 M KCl and were layered directly on top of 5–25% (w/v) linear sucrose gradients, prepared a short time before use, or were first dialysed against 0.002 M sodium phosphate buffer, pH 7.6, containing 0.08 M NaCl, 0.002 M EDTA and 0.005 M mercaptoethanol. Centrifugation of the 5–25% sucrose gradients was for 14.5 h at 49 000 × *g* in Spinco rotor SW-41.

The two distinct populations of lysosomes characteristic of 8-day-old neurons[7], were isolated from the purified lysosomal fraction (Fraction Ly)[30] on 35–60% (w/v) gradients of sucrose[7] which were also centrifuged for 14.5 h at 49 000 × *g*. Collection of the gradient effluent was done with continuous monitoring of the absorbance at 280 nm.

Chromatography of the *N*-acetyl-β-D-glucosaminidase recovered in the FT supernatant or of its components H and L resolved by means of density gradient centrifugation (see Results) was carried out on Sephadex G-200 columns (2.5 cm × 30 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 7.6, containing 0.4 NaCl and 0.01 M EDTA[31]. Elution of the applied *N*-acetyl-β-D-glucosaminidase was with the same buffer. The column was calibrated using suitable molecular weight markers; the rate of flow was 10 ml/h and 3-ml fractions were collected. Chromatography on DEAE-cellulose columns (1.5 cm × 30 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0[10] was as described by Robinson *et al.*[31] except that the flow rate was 40 ml/h and 10-ml fractions were collected.

Electrophoresis of *N*-acetyl-β-D-glucosaminidase was carried out using both the polyacrylamide disc gel[32] and the cellulose acetate[26] strip techniques. The methodological details will be described more fully in the text. Enzyme activity was determined with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as substrate and was visualized fluorometrically.

Solutions containing *N*-acetyl-β-D-glucosaminidase were routinely concentrated by means of magnetically-stirred cells (Amicon Corp., Lexington, Mass.) provided with PM 30 ultrafilters.

The assay of *N*-acetyl-β-D-glucosaminidase was as previously descr bed[2] at pH 4.1 in 0.15 M citrate–phosphate buffer also containing 24.9 g/l of KCl[33] and in a final volume of 0.93 ml[7]. 1 unit of enzyme activity is defined operationally as the $A_{412\ nm}$/ml per incubation time, which varied from 1 to several hours as specified in detail in the legends. Color development at $A_{412\ nm}$ was linear with respect to time of incubation and concentration of protein under all conditions. The specific activity is expressed in units/mg of protein. Protein was determined according to Lowry *et al.*[34] with crystalline bovine serum albumin as standard.

In some experiments, the activities hydrolyzing *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-galactopyranoside and *p*-nitrophenyl-α- and -β-D-fucopyranoside were also assayed, the former at pH 4.1 and the latter at pH 6.0.

RESULTS

In order to examine the complement of the *N*-acetyl-β-D-glucosaminidase com-

ponents present in the neuronal lysosomes at different early post-natal ages, it was first necessary to solubilize and maintain non-sedimentable as much of the enzyme activity as possible. Recently, Baccino *et al.*[35] found freezing–thawing in 0.25 M sucrose–0.1 M KCl preferable over 7 other different treatments for the solubilization of hepatic *N*-acetyl-β-D-glucosaminidase. These authors reported that centrifugation of frozen–thawed suspensions of the light mitochondrial fraction of adult rat liver at $9 \cdot 10^6$ g/min resulted in the solubilization of about 67% of the enzyme. Of the several treatments listed by Baccino *et al.*[35] and applied to the neuronal Fraction Mit, Ly, Mic, freezing–thawing in 0.20 M sucrose–0.1 M KCl proved most effective, especially if carried out under optimal conditions. The results of a systematic study to attain such conditions are presented in Figs 1–3 and in Table I. The

TABLE I

SOLUBILIZATION OF *N*-ACETYL-β-D-GLUCOSAMINIDASE: EFFECT OF TWO SUCCESSIVE CYCLES OF FREEZING–THAWING AND OF AGE

Homogenates of neuronal cell bodies isolated from the cerebral cortex of 10 3- and 18-day-old rats were centrifuged as described in Materials and Methods to obtain Fraction Mit, Ly, Mic. Suspension of this fraction was in 0.20 M sucrose–0.1 M KCl by means of 6 up-and-down strokes of a Teflon–steel pestle rotating at about 1300 rev./min in a glass homogenizer tube. Freezing–thawing and centrifugation at 15 000 × *g* for 1 h yielded supernatant S_1 and pellet P_1. The concentration of protein in the former was 0.35 mg/ml in the 3-day-old and 0.77 mg/ml in the 18-day-old neurons. The pellets (P_1) were suspended as indicated by the asterisk* in the table and the freezing–thawing process was repeated. FT, frozen–thawed in 0.20 M sucrose–0.1 M KCl three successive times (see Materials and Methods). The recovery of *N*-acetyl-β-D-glucosaminidase: $S_1 + P_1$/Fraction Mit, Ly, Mic was always in excess of 80%; the recovery: $S_2 + P_2/P_1$ was 62.8% at 3 days and 78.7% at 18 days.

Fraction	*3 days*		*18 days*	
	Units	%	*Units*	%
After first freeze–thawing				
FT supernatant$_1$ (S_1)	103.1	31.6	415.0	60.2
FT pellet$_1$ (P_1)	219.9	68.4	276.2	39.8
*After second freeze–thawing**				
FT supernatant$_2$ (S_2)	25.0	18.0	79.8	36.6
FT pellet$_2$ (P_2)	113.4	82.0	130.0	63.4

* Suspensions of pellet P_1 in 0.20 M sucrose–0.1 M KCl (10.9 and 8.9 mg of protein in 16.4 and 17.8 ml at 3 and 18 days) were frozen–thawed three successive times.

study of the effect of neuronal age on the sedimentation of *N*-acetyl-β-D-glucosaminidase from frozen–thawed suspensions at moderate *vs* high *g* forces (Fig. 1) reveals that more than 50% of the enzyme remained non-sedimentable at all of the ages tested, provided the frozen–thawed suspension was centrifuged at 15 000 × *g*. Fig. 1 also shows that the percentage of *N*-acetyl-β-D-glucosaminidase in the 15 000 × *g* supernatant ranged from about 70% at 3 days, to under 60% at 8 days and close to 85% at 18 days. The corresponding values for *N*-acetyl-β-D-galactosaminidase were 43.8% at 3 days, 45.3% at 8 days and 85% at 18 days with *g* forces of 15 000 and 19.7% at 3 days, 32.7% at 8 days and 64.3% at 18 days with *g* forces of 155 000. The pellets obtained by centrifuging 3- and 18-day frozen–thawed suspensions of Fraction Mit, Ly, Mic at 15 000 × *g* were resuspended in 0.20 M sucrose–0.1 M KCl

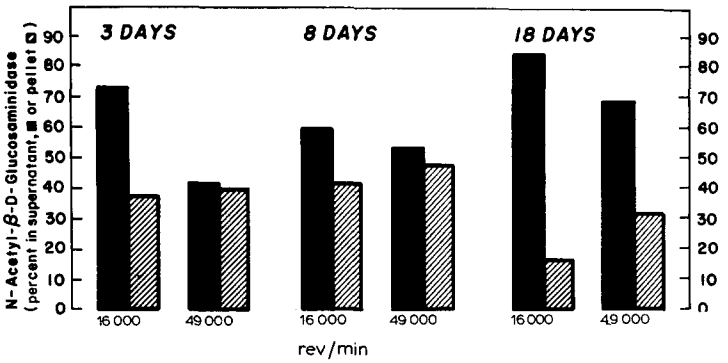

Fig. 1. The effect of centrifugal force and of age on the sedimentability of neuronal *N*-acetyl-β-D-glucosaminidase. Fraction Mit, Ly, Mic was isolated from homogenates of 3-, 8- and 18-day-old neuronal cell bodies and was frozen–thawed as described in Materials and Methods. The resulting suspensions were divided into two equal portions and were centrifuged either at 15 000 × *g* (16 000 rev./min) or 155 000 × *g* (49 000 rev./min) for 1 h. *N*-Acetyl-β-D-gluco- and galactosaminidase activities and protein were determined on the suspensions before freezing–thawing and on the pellets and supernatants obtained by centrifugation. The specific activities ($A_{412\ nm}$/h per mg of protein) of *N*-acetyl-β-D-gluco- and galactosaminidase were 12.9 and 1.82 at 3 days, 12.6 and 2.21 at 8 days and 103.1 and 10.2 at 18 days[7]. The actual recovery averages of two consecutive experiments of the two activities (pellet + supernatant/suspension) at 15 000 × *g* and 155 000 × *g* were: *N*-acetyl-β-D-glucosaminidase, 89.5 and 102.5% at 3 days, 105 and 92% at 8 days and 71.5 and 83.5% at 18 days; *N*-acetyl-β-D-galactosaminidase: 60 and 50.5 at 3 days, 91 and 72.2 at 8 days and 74.8 and 74.6 at 18 days. The average concentration of protein in the frozen–thawed suspensions was 1.02 mg/ml at 3 days, 1.79 mg/ml at 8 days and 0.43 mg/ml at 18 days.

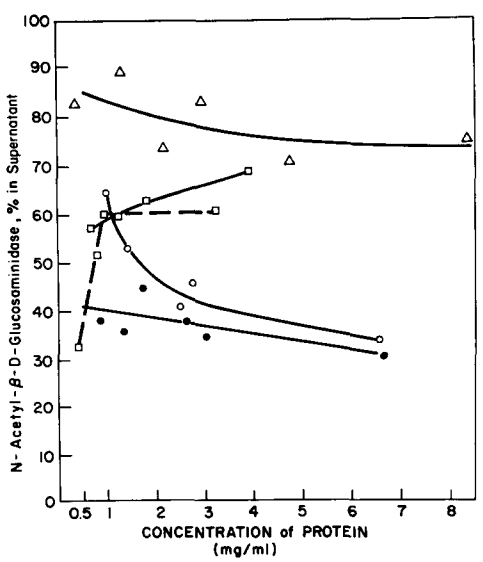

Fig. 2. Effect of the concentration of Fraction Mit, Ly, Mic and of age on the sedimentability of *N*-acetyl-β-D-glucosaminidase. Suspensions of Fraction Mit, Ly, Mic (see Materials and Methods) were either frozen–thawed as prepared or, in a given experiment, the original suspension was further diluted with 0.20 M sucrose–0.1 M KCl before freezing–thawing. At least two concentrations were tested per experiment and at least two experiments were conducted at each age. The curves are thus composites of several concentrations and/or experiments for each age. The activity in the supernatant (ordinate) was calculated taking the sum pellet + supernatant as 100%. ○—○, 3 days; ●—●, 5 days; □—□, 8 days; △—△, 18 days; ----, cerebellar neurons.

and were subjected to a second cycle of freezing–thawing under approximately identical conditions of tissue concentration as for the first cycle (Table I). Following centrifugation at 15 000 × *g* for 1 h, 18% (3 days) and 36.6% (18 days) additional enzyme activity became non-sedimentable, suggesting that *N*-acetyl-β-D-glucosaminidase was more readily extracted from the lysosomes of the older than from those of the younger neuronal cell bodies. The results of a detailed study testing tihs point (Fig. 2) reveal that both age and the concentration of Fraction Mit, Ly, Mic in mg of protein/ml of frozen–thawed suspension combine to determine the solubilization characteristics of the *N*-acetyl-β-D-glucosaminidase. The concentration parameter had the highest effect at 3 days, when substantially more *N*-acetyl-β-D-glucosaminidase appeared in non-sedimentable form from dilute than from concentrated tissue suspensions. Solubilization of the *N*-acetyl-β-D-glucosaminidase from the lysosomes of the 5- and 18-day-old neurons was relatively insensitive to the concentration parameter, in spite of the marked difference in the absolute proportions solubilized, between 75 and 90% at 18 days *vs* about 35–45% at 3 days. At 8 days, on the other hand, when the yield of neuronal perikarya is by far the highest[36], *N*-acetyl-β-D-glucosaminidase appeared to exhibit an intermediate sensitivity to the concentration parameter, with a slight tendency for solubilization to increase with concentration (from about 55% to about 70%). The response of the 8-day cerebellar enzyme (Fig. 2, dashed line) was biphasic, very sensitive to changes in the concentration of the suspension up to about 1 mg of protein/ml and rather constant (at about 60%) at higher concentrations.

The supernatants obtained by centrifuging frozen–thawed suspensions of 3-, 8- and 18-day-old neuronal Fractions Mit, Ly, Mic at 15 000 × *g* *vs* 155 000 × *g* (FT supernatants) for 1 h were also examined by sucrose density gradient centrifu-

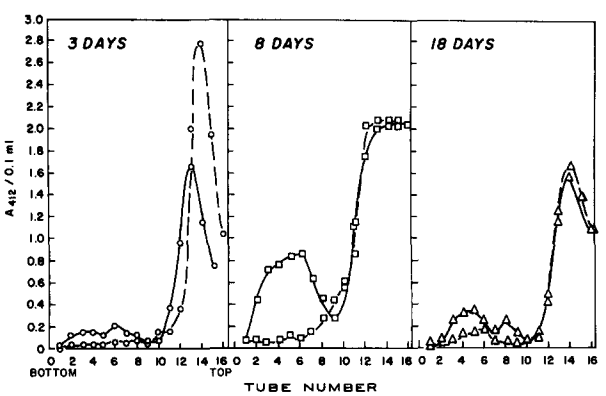

Fig. 3. The effect of centrifugal force and of age on the gradient profile of *N*-acetyl-β-D-glucosaminidase. Fraction Mit, Ly, Mic was prepared as described in Materials and Methods and was frozen–thawed at the concentrations of protein/ml specified in the legend of Fig. 1. The supernatants obtained by centrifuging the frozen–thawed suspensions at 15 000 or 155 000 × *g* for 1 h were layered on 5–25% linear density gradients of sucrose and the gradients were centrifuged for 14.5 h at 49 000 × *g*. The activity of *N*-acetyl-β-D-glucosaminidase was determined in the effluent and is plotted as $A_{412\ nm}$ per 0.1 ml on the ordinate. The dashed lines refer to the supernatants obtained at 155 000 × *g* and the solid lines to those obtained at 15 000 × *g*. Note the virtual absence of component H (tubes 2–7) of *N*-acetyl-β-D-glucosaminidase in the 3- and the 8-day-old profiles and its marked diminution at 18 days after centrifugation of the frozen–thawed suspensions at 155 000 × *g*.

gation. As shown in Fig. 3, more than one *N*-acetyl-β-D-glucosaminidase component was seen at all ages only in the 15 000 × *g* FT supernatants. The middle panel of Fig. 3 clearly demonstrates the absence of the heavy component of the enzyme in the FT supernatant following centrifugation at 155 000 × *g* as against its presence following centrifugation of a separate aliquot of the same tissue suspension at the lower centrifugal speed.

Fig. 4 and Table II illustrate the effects of age and tissue concentration on the ratio of the components H and L of neuronal *N*-acetyl-β-D-glucosaminidase. The sedimentation profiles shown in the 4 panels of Fig. 4 illustrate graphically the results obtained under the extremes of the various experimental conditions used and for which the requisite quantitative data are provided in the accompanying Table II. The low and high ratio values in the cerebral cortex shown in Curves I and II of Fig. 4 are from one experiment each but, as stated in the legend of Table II, between 2 and 6 additional experiments (graphs not shown in Fig. 4) in which intermediate ratio values were obtained, were also carried out. It should also be noted that both the

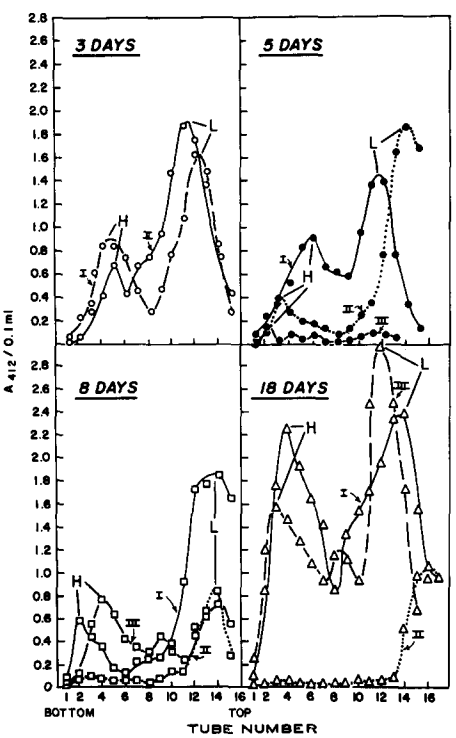

Fig. 4. The gradient profile of *N*-acetyl-β-D-glucosaminidase as a function of age. Following freezing–thawing of Fraction Mit, Ly, Mic isolated from homogenates of neuronal cell bodies of the cerebral and cerebellar cortex, as indicated, the suspensions were centrifuged for 1 h at 15 000 × *g* and the resulting supernatants layered on 5–25% sucrose density gradients and centrifuged for 14.5 h at 49 000 × *g*. The profiles shown were obtained under the conditions specified in more detail in the accompanying Table II. Curves I and II represent the extremes of the cerebral profiles obtained in the study (consult Table II for number of experiments conducted at each age and with each kind of neuronal cell body), while Curve III refers to the cerebellar profiles (5 and 8 days) and to the cerebral profile obtained by freezing–thawing Fraction Ly[30] in lieu of Fraction Mit, Ly, Mic (18 days).

TABLE II

THE COMPONENTS OF *N*-ACETYL-β-D-GLUCOSAMINIDASE IN CEREBRAL AND CEREBELLAR NEURONS: EFFECT OF AGE AND PROTEIN CONCENTRATION

Fraction Mit, Ly, Mic was isolated from homogenates of neuronal cell bodies of the cerebral and the cerebellar cortex of rats of post-natal ages, as indicated. Suspensions of this fraction in 0.20 M sucrose–0.1 M KCl at various concentrations of protein (range, as indicated in first column) were frozen–thawed and centrifuged as described in Materials and Methods to yield FT supernatants which were sedimented on 5–25% sucrose density gradients also as described in Materials and Methods. The range of the protein concentrations in the FT supernatants is indicated in the second column. The activity of *N*-acetyl-β-D-glucosaminidase was determined in the gradient effluents and the L/H ratios were determined by dividing the $A_{412\ nm}$ maximum of the light component, L by the $A_{412\ nm}$ maximum of the heavy component, H. Only gradients in which such maxima were clearly identified were used for this computation. When more than two peaks of activity were noted, the values of the ratio start with the ratio of component L to component H, followed by its ratio(s) to the additional components. The fifth column signifies that, in addition to the 2 or 3 (18 days, cerebral cortex) experiments for which the L/H ratio extremes are given there were others performed for which intermediate L/H ratios were obtained. Their number may be obtained by subtracting 2 or 3 (18 days, cerebral cortex) from the numbers given in the table. The roman numerals (sixth column) refer to the curves in Fig. 4 labelled with the identical numerals.

Post-natal age (days)	*Neuroanatomical origin of neuronal cell bodies*	*Concentration of protein*			*Components H and L of N-acetyl-β-D-glucosaminidase*		
		Suspension (mg/ml)*	*Supernatant** (mg/ml)*	*Ratio of L:H*	*Extreme value of ratio*	*Total number of experiments*	*Gradient profile in Fig. 4*
3	Cerebral cortex	1.49	0.45	1.94	low	6	I
	Cerebral cortex	2.81	0.94	2.72	high		II
5	Cerebral cortex	0.99	0.59	1.53	low	4	I
	Cerebral cortex	1.34	0.70	4.60	high		II
	Cerebellar cortex	0.96	0.43	0.70, 1.60 and 1.47†	low	2	III
	Cerebellar cortex	0.27	0.10	2.46 and 3.72†	high		—
8	Cerebral cortex	3.85	1.28	3.29	low	7	I
	Cerebral cortex	0.35	0.26	10.00	high		II
	Cerebellar cortex	0.87	0.18	0.92 and 1.62†	low	4	III
	Cerebellar cortex	1.48	0.29	2.64 and 3.74†	high		—
18	Cerebral cortex	2.88	2.00	1.06	low	8	I
	Cerebral cortex	0.61	0.21	19.8	high		II
	Cerebral cortex	—	0.84	1.83 and 2.52***,†	n.a.††	4	III

* Fraction Mit, Ly, Mic (see Materials and Methods) in 0.20 M sucrose–0.1 M KCl.
** Obtained by freezing and thawing Fraction Mit, Ly, Mic and centrifuging for 1 h at 15 000 × *g*.
*** Fraction Ly was frozen and thawed (see Materials and Methods).
† Values refer to component H and to one or more components sedimenting between L and H, respectively.
†† n.a., not applicable.

profiles shown in Fig. 4 and the L/H ratios listed in Table II were derived from experiments in which the FT supernatants of Fraction Mit, Ly, Mic, were layered on sucrose gradients either directly (pH 6.0–6.2) or after dialysis for up to 5 h at 4 °C against 0.002 M phosphate buffer, pH 7.6 (see Materials and Methods). The gradient profiles of *N*-acetyl-β-D-glucosaminidase (Fig. 4) demonstrate the presence of two clearly separable components with an additional, less clearly discernible component at 3 days (profile II), 5 days (profiles I and III), 8 days (profiles I and III) and 18 days (profile I). Whereas in the 3- and 5-day-old neurons of the cerebral cortex high L/H ratios necessitated protein concentrations in excess of 1.0 mg/ml, in the 8- and 18-day-old neurons, high L/H ratios were obtained by freezing–thawing suspensions containing less than 0.7 mg of protein per ml. In the neurons of the cerebellar cortex in which three components with *N*-acetyl-β-D-glucosaminidase activity were routinely detected, the ratio of component L to the two heavier ones was quite variable and did not correlate well with the protein concentration of the suspensions from which the corresponding FT supernatants were derived.

Further examination of the modalities governing the number of the molecular components of *N*-acetyl-β-D-glucosaminidase and of their respective quantitative proportions revealed a marked influence of pH. As shown in Fig. 5, adjustment of the pH of the FT supernatants of 8-day-old neurons to 5.6–5.8 prior to their centrifugation on sucrose density gradients unmasked two additional components which could not be discerned at or below pH 5.6 or above pH 6.0. Moreover, at pH 5.6 and 5.8 the L/H ratio approximated unity, a finding which was never noted in the 8-day-old neurons under the commonly used conditions of centrifugation (pH 6.0–6.2 without citrate–phosphate–KCl). As shown by profile III of the 18-day-old neuron,

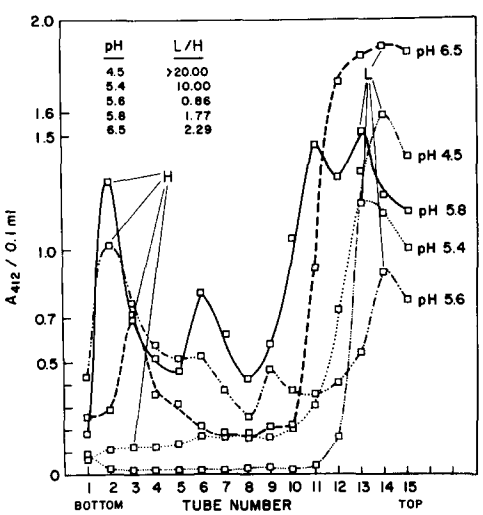

Fig. 5. The effect of pH on the gradient profile of *N*-acetyl-β-D-glucosaminidase of 8-day-old cerebral cortex nerve cell bodies. The pH of the FT supernatants (see Materials and Methods) was adjusted to the value indicated by mixing 1.7 ml with 0.2 ml of citrate–phosphate–KCl buffer (see Materials and Methods) of the appropriate pH and by adding distilled water to 2.5 ml. Samples of 2.3 ml were centrifuged on gradients as described in Materials and Methods and the effluent was collected. The L/H ratios were determined by dividing at each pH the maximum $A_{412\ nm}$ value of tubes 11–14 by the maximum value of tubes 2–4.

TABLE III

HEAVY AND LIGHT COMPONENTS OF *N*-ACETYL-β-D-GLUCOSAMINIDASE IN DIFFERENT POPULATIONS OF NEURONAL LYSOSOMES

The two lysosomal populations, LyA and LyB were prepared from 8-day-old nerve cell bodies of the cerebral cortex by centrifugation of the primary fraction Ly[30] on 35–60% sucrose density gradients[7]. The concentrations of sucrose and of KCl in the pooled fractions containing the two populations of lysosomes were adjusted to 0.20 and 0.1 M, respectively, and the samples were then frozen–thawed three times and dialyzed for 16 h at 4 °C against 500 ml of 0.002 M phosphate buffer, pH 7.6, containing 0.002 M EDTA, 0.08 M NaCl and 0.005 M mercaptoethanol. The dialysis bag contents were lyophilized, and the residues, suspended in 1.5 ml of the above dialysis buffer, were centrifuged for 14.5 h at 49 000 × *g* on 5–25% sucrose density gradients (see Materials and Methods). The gradients were eluted and the enzyme activity determined in the effluent as described in Materials and Methods.

Lysosomal population	*N-Acetyl-β-D-glucosaminidase*		
	Component H ($A_{412\ nm}$)*	*Component L* ($A_{412\ nm}$)*	*Ratio L/H*
LyA**	0.048	0.282	5.8
LyB***	0.037	0.825	22.2

* Absorbance in gradient tube with maximum of *N*-acetyl-β-D-glucosaminidase activity.

** Refers to population of granules sedimenting in a 35–60% (w/v) sucrose gradient to a position close to the bottom of the tube (see Idoyaga-Vargas *et al.*[7]).

*** As for **, except that the lysosomes in this fraction sediment above those in Fraction LyA.

however, (Table II, Fig. 4) centrifugation of FT supernatants derived from the purified Fraction Ly (see Materials and Methods) resulted in the apparent unmasking of an additional *N*-acetyl-β-D-glucosaminidase component with sedimentation rates intermediate between those of the standard components H and L, even though no citrate–phosphate–KCl buffer, pH 5.8, was added to the FT supernatants before gradient centrifugation.

Since our previous study indicated that the 8-day-old neuronal cell body of the cerebral cortex contains two distinct populations of lysosomes differing in buoyant density when centrifuged for 14.5 h at 49 000 × *g* in 35–60% (w/v) sucrose density gradients, it was of interest to determine whether each of these populations contained both the heavy and the light *N*-acetyl-β-D-glucosaminidase components. Affirmative evidence for this point was gained by freezing–thawing separately Fractions LyA and LyB[7] and by subjecting their FT supernatants to 14.5 h of centrifugation at 49 000 × *g* in 5–25% (w/v) sucrose density gradients. The results (Table III) suggest that, while each population indeed appeared to contain both *N*-acetyl-β-D-glucosaminidase components, relatively larger amounts of component L were present in the lighter (LyB) of the two populations.

In an attempt to define some of the molecular characteristics of components H and L, FT supernatants of 18-day-old neurons were applied to a column of Sephadex G-200 equilibrated as described in Materials and Methods and previously calibrated with suitable molecular weight markers. In agreement with the findings of other workers who used chromatography on Sephadex G-200 in rather unsuccessful attempts to separate components A and B of *N*-acetyl-β-D-glucosaminidase in other tissues[37,38], or, more successfully, to separate this glycosidase from related ones[39], no separation

TABLE IV

CHROMATOGRAPHY OF NEURONAL *N*-ACETYL-β-D-GLUCOSAMINIDASE ON SEPHADEX G-200

The void volume of the calibrated column was 42 ml.

Marker or protein	*Molecular weight of calibration standards and of N-acetyl-β-D-glucosaminidase*	*Absorbance or enzyme activity peak (ml effluent)*
Blue dextran	$>2 \cdot 10^6$	63– 66
Aldolase	158 000	108–111
Transferrin	90 000	120–123
Cytochrome *c*	12 400	162–165
N-Acetyl-β-D-glucosaminidase		
unresolved*	≈150 000	105–111
component H**	158 000	108–111
component L***	158 000	108–111

* The sample applied was the supernatant obtained by centrifugation (15 000 × *g* for 1 h) of the frozen–thawed (see Materials and Methods) Fraction Mit, Ly, Mic (FT supernatant) prepared from 18-day-old neuronal perikarya. A total of 1.5 mg of protein containing 47.2 units of enzyme activity were applied. The recovery of *N*-acetyl-β-D-glucosaminidase was 57.5%.

** Obtained by sucrose gradient centrifugation: tubes 3–7 (Fig. 4). The recovery was 88.5%.

*** Obtained by sucrose gradient centrifugation: tubes 11–14 (Fig. 4). This component was also characterized by its failure to adsorb to DEAE-cellulose at pH 7.0. The recovery was 54.5%.

of components H and L was achieved (Table IV). Moreover, chromatography on Sephadex G-200 of the individual components H and L, following their separation on sucrose density gradients (see above), resulted in identical elution profiles for both, namely with activity peaks in coincidence with that of crystalline rabbit muscle aldolase, thus indicating a molecular weight of about 158 000 for each of the two components. In a few separate experiments we determined that component L eluted without retention through DEAE-cellulose at pH 7.0[10] while component H required 0.1 M NaCl for elution, suggesting but not unequivocally proving that under all conditions H is identical with A and L with B. Electrophoresis on polyacrylamide gels and cellulose acetate strips provided an additional means to differentiate between components H and L. The concentration of acrylamide was 5% and that of methylene-bisacrylamide 2.13%. Equal parts of sample gel (about 0.2 mg of protein) were mixed, the sample gel being polymerized for 30 min. Gels were run at 4 °C with a starting potential across the gels of 100 V. Electrophoresis was for 2 h and bromophenol blue was the tracking dye. The procedure of Friedland *et al.*[40] was used to localize enzyme activity. For electrophoresis on cellulose acetate, strips (Sepraphore III, Gelman Co., Inc., Ann Arbor, Mich.) were prepared by soaking in 0.05 M citrate–phosphate buffer, pH 6.0[26]. Sample (5–10 μl) was applied near the anodal end and electrophoresis carried out at 4 ml/strip for 1 h. Enzyme activity was localized as described by Klibansky[26]. Using polyacrylamide gels there was identity of migration between component L but not between that of component H, and one of the fluorescent bands of the FT supernatant. In several runs using cellulose acetate electrophoresis, on the other hand, it was possible to detect at least one fluorescent band migrating, albeit very sluggishly, in the direction opposite to that of authentic component L[26]. No fluorescent bands were seen following heating of enzyme suspensions at 95 °C for 5 min.

A further investigation into the nature of and the differences between, compo-

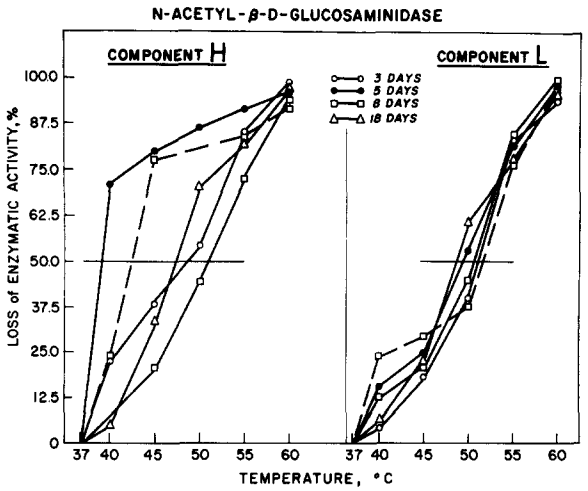

Fig. 6. The heat lability of the components H and L of neuronal *N*-acetyl-β-D-glucosaminidase as a function of age and temperature. Components H and L of *N*-acetyl-β-D-glucosaminidase were isolated by centrifugation of the appropriate FT supernatants on density gradients of sucrose (see Materials and Methods) and by pooling of the contents of the appropriate effluent tubes (see Fig. 4). Heating of the enzymes was for 20 min at the temperatures indicated and at pH 4.1. At the end of this period, substrate was added and the residual activity was determined in the standard assay (see Materials and Methods). Controls consisted of samples preincubated for 20 min and at a pH of 4.1 fat 37 °C. ----, cerebellar neurons

nents H and L included the determination of their relative heat labilities as a function of age. In the first group of experiments the two components, isolated by sucrose density gradient centrifugation were preincubated for 20 min in 0.15 M citrate–phosphate–KCl buffer at pH 4.1 and at temperatures of 37, 40, 45, 50, 55 and 60 °C and the residual enzyme activity was determined by subsequent incubation of the pre-heated enzyme at 37 °C. Fig. 6 reveals a rather age-independent behavior of component L for which half-maximal inactivation values were reached between 48 and 51 °C, irrespective of age and of neuronal origin. On the other hand, the heat lability of component H varied considerably with age and with neuronal origin, *i.e.* cerebral *vs* cerebellar, for while half-maximal inactivation occurred at about 50 °C in the 3-day-old cell bodies, a similar level of activity could be reached at about 39 °C in the 5-day-old cell bodies of the cerebral cortex and at about 42 °C in those of the 8-day-old cerebellar cortex. There was only little difference in the heat lability of components H and L at 8 and 18 days, inasmuch as the activity of both fell to about 50% of control values between 48 and 50 °C. Recently, Crawhall and Banfalvi[41], working with human skin fibroblasts, examined the time course of the heat inactivation of α-galactosidase by preincubating the enzyme at 51 °C and noted a rather rapid rate of inactivation during the first 50 min followed subsequently by a much slower one. Fig. 7 illustrates that preincubation of components H and L of the *N*-acetyl-β-D-glucosaminidase of 3-, 5-, 8- and 18-day-old cell bodies at 45 °C for up to 90 min led to an immediate fall in the activity of component H to values ranging between 30 and 50% of those of non-preincubated controls. This treatment affected component L minimally, the maximal reduction of its activity not exceeding 30% under any of the conditions tested.

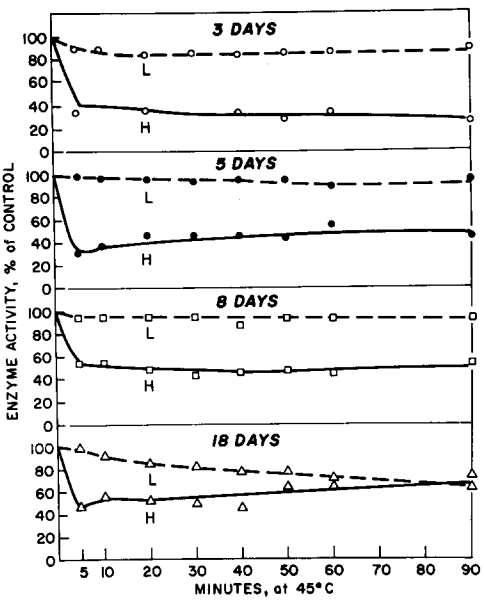

Fig. 7. The heat lability of the components H and L of neuronal *N*-acetyl-β-D-glucosaminidase as a function of age and time of exposure to 45 °C. The separated components H and L were obtained as described in the legend to Fig. 6. They were placed into a bath maintained at 45 °C without adjustment of pH which fluctuated, in the several experiments, between 6.2 and 6.5. Aliquots were withdrawn and added to tubes containing the assay medium, as indicated, and the activity was determined and compared to controls which had not been preincubated at 45 °C.

Many workers[14,37,38,42] have reported that in an acidic medium, component A of *N*-acetyl-β-D-glucosaminidase converts to component B and furthermore that this occurs both in the absence[14], but more effectively yet, in the presence of exogenous neuraminidase and of $CaCl_2$. More recently, Wetmore and Verpoorte[43] purified components A and B from porcine kidney and found 0.3–0.4% sialic acid associated with component A and about 0.2% with component B.

Component H of neuronal *N*-acetyl-β-D-glucosaminidase converts readily into component L, as shown by the results of Table V. In Expt 1, FT supernatants were incubated in acetate buffer within the range of pH 4.1–5.1 in the absence or presence of Zn^{2+} and Cd^{2+}, two cations reported to effectively inhibit cerebral neuraminidase[44] and whose lack of inhibition or inactivation of the neuronal *N*-acetyl-β-D-glucosaminidase was independently confirmed in the present study. A considerable loss of component H without its apparent conversion to component L ensued, as demonstrated by the virtual absence on sucrose density gradients of the former and of no significant increase of the latter. In Expt 2, FT supernatants were frozen and thawed three successive times, a treatment reported to inactivate the soluble neuraminidase of brain tissue[45] and were reexamined by sucrose density gradient centrifugation. This treatment protected somewhat against the loss of component H (Expt 2), but this protection could be readily lifted, as also shown in Expt 2, by adjusting the pH of frozen–thawed FT supernatants to 4.5 before gradient centrifugation, a procedure which again resulted in a virtually total deletion of component H. While this component was shown to be stable at an alkaline pH (Expt 3, a), it could be shown that its

TABLE V

EFFECT OF VARIOUS TREATMENTS ON *N*-ACETYL-β-D-GLUCOSAMINIDASE COMPONENT H AND ON ITS CONVERSION TO COMPONENT L

In Expts 1 and 2 FT supernatants were obtained by freezing–thawing Fraction Mit, Ly, Mic (see Materials and Methods) of nerve cell bodies isolated from the cerebral cortex of 18-day-old rats. In these experiments the quantitative fluctuations of component H of *N*-acetyl-β-D-glucosaminidase were monitored, but no effort was made to evaluate whether and, if so, to what extent, component H converted to component L. In Expt 3 component H was isolated and treated as indicated and the appearance of component L was then determined by sucrose density gradient centrifugation. The percentage ranges refer to results from several experiments. They were derived by adding the $A_{412\ nm}$ of the two gradient tubes in which components H and L peaked and by determining the activity in each peak as a percentage thereof. FT, frozen–thawed.

Treatment or condition	*Component H** (%)	*Component L*** (%)
Expt 1		
FT supernatant diluted with 0.20 M sucrose–0.1 M KCl or with distilled water	100***	—
FT supernatant pre-incubated at 0 or 37 °C for 5–60 min in the pH range 4.1–5.1 with or without Zn^{2+} or Cd^{2+}, $1 \cdot 10^{-3}$ M	6–10	—
Expt 2		
FT supernatant, pH 6.0	100***	—
FT supernatant, frozen–thawed 3×, pH 6.0	62–75	—
FT supernatant, frozen–thawed 3×, at pH 6.0, then pH adjusted to 4.5	10–15	—
Expt 3		
(a) Component H, dialysed at pH 7.4 and rerun on 5–25% sucrose gradient	>85	<15
(b) Component H, purified by Sephadex G-200 chromatography and rerun on 5–25% sucrose gradient	15–100	0–85
(c) Component H, purified by Sephadex G-200 chromatography, then incubated in 0.02 M acetate buffer, pH 5.1 at 37 °C for 15–60 min and rerun on 5–25% sucrose gradient	0–25	75–100
(d) As in (c) + 150 μg of neuraminidase during incubation	0	100

* Refers to the activity of *N*-acetyl-β-D-glucosaminidase determined in tubes 2–6 of 5–25% sucrose gradients (see Materials and Methods and Figs 4 and 5).

** Refers to the activity of *N*-acetyl-β-D-glucosaminidase in tubes 12–15 of 5–25% sucrose gradients (see Materials and Methods and Figs 4 and 5).

*** Refers to the peak $A_{412\ nm}$ in tubes 2–6 of the control 5–25% sucrose gradient. All other values refer to $A_{412\ nm}$ in tubes 2–6 of preparations treated as indicated, expressed as % of control values.

relative instability in acid medium was directly associated with its conversion to component L (Expt 3, b); thus, recentrifugation on a 5–25% sucrose density gradient of a preparation of Sephadex G-200-purified component H resulted in, depending on the experiment, anywhere from zero to 85% conversion to component L. A more effective conversion of component H to component L could be brought about by first adjusting the pH of the Sephadex-purified component H to 5.1, while its complete conversion occurred every time if 150 μg of *Cl. perfringens* neuraminidase were also added to the pH 5.1 medium (Expt No. 3, d).

DISCUSSION

The present study is the first in which the development of lysosomal *N*-acetyl-

β-D-glucosaminidase has been examined in purified nerve cell bodies rather than in subcellular preparations derived from whole brain and which contain lysosomes of mixed cellular origin, *i.e.* from both nerve and glial cells. Since recently we[7,23] and others[13,34,25] reported that lysosomes of glial cells have considerable *N*-acetyl-β-D-glucosaminidase activity and since, on other subcellular levels, the metabolism of cortical nerve cell bodies and glial cells, predominantly astrocytes, differs markedly[36,46,47], being able to study the neuronal *N*-acetyl-β-D-glucosaminidase totally separate from its glial counterpart has obvious advantages for the understanding of its apparent key role in maintaining normal brain function.

In a previous study[23] we showed that while the specific activity of neuronal *N*-acetyl-β-D-glucosaminidase, as measured in homogenates of cell bodies derived from 18-day-old and adult rat cerebral cortex, differed very little, it rose sharply between 3 and 18 days post-natally. Since one of the purposes of the present study was the investigation of the development of the components of neuronal *N*-acetyl-β-D-glucosaminidase activity during this early post-natal period, conditions were sought which would allow their rapid separation. In previous work we noted that centrifugation of homogenates of adult rat cerebral cortex in a hypotonic but ion-rich medium resulted in the maximum recovery of about 40% of total *N*-acetyl-β-D-glucosaminidase in the high-speed soluble supernatant fraction[5], but that repeated freezing and thawing of particulate fractions isolated in 0.25 M sucrose led to solubilizations of the order of 65%[2]. A combination isotonic *plus* ionic medium[35,48] was therefore used in the present study with the results shown in Table I and Figs 1, 2 and 3. Figs 1 and 3 illustrate the importance of the centrifugal force applied to prepare FT supernatants in determining the relative partition of *N*-acetyl-β-D-glucosaminidase between the soluble and particulate phases (Fig. 1) and the consequent appearance of more than one of its molecular components in non-sedimentable form (Fig. 3). Because the experiments depicted in these two figures and in Table I indicated different partitions of *N*-acetyl-β-D-glucosaminidase not only as a function of centrifugal force but apparently also of age, a systematic study was conducted to relate its solubilizations to the concentration of protein of the suspensions actually subjected to freezing–thawing at four different ages. The results indicate that the solubilization of *N*-acetyl-β-D-glucosaminidase was greatest and least concentration-dependent at 18 days, least effective and relatively concentration-independent at 5 days, intermediate and somewhat concentration-dependent at 8 days and, finally, very concentration dependent at 3 days.

Although the separation of molecular components of *N*-acetyl-β-D-glucosaminidase has been accomplished by means of DEAE-cellulose chromatography[10,31,38], isoelectric focusing[11,15,16,38] and electrophoresis on starch[15,31], polyacrylamide[14,40,49,50] and cellulose acetate[18,26,51], we are not aware of reports describing the use of linear density gradients of sucrose to accomplish this end. Figs 4 and 5 and Table II show that, even within neuronal cell bodies of the same age, several experimental factors determine the actual ratio of the two principal molecular components of *N*-acetyl-β-D-glucosaminidase; moreover, one of them, pH, appears to even govern the qualitative spectrum of glucosaminidase components that may be detected on such gradients.

Harzer and Sandhoff[11] studied variations of the ratio of *N*-acetyl-β-D-glucosaminidase A and B as a function of age in brains of human fetuses and of individuals

0–5, 6–40 and 41–85 years old. These workers noted an increase of the B/A ratio which they attributed to the enhancement of the "relative proportion of hexosaminidase B... during life". It should be noted that the brain extracts used by these workers were prepared in 0.05 M citrate–phosphate buffer, pH 4.5, also containing 0.25% Triton X-100. The results of our study indicate that extreme care must be taken before component ratios are compared across a given age span, since a number of factors other than age seriously affect the numerical value of this ratio. As shown in Table II, the absolute extreme values of the L/H ratio varied, for the same age of 18 days, from a low of 1.06 to a high of 19.8 (Fig. 4, lower right panel, profiles I and II), simply as a result of differences in suspension dilution. Yet, in the younger neurons this dilution factor appeared to be of lesser importance since, for a 5-fold difference in supernatant protein (Table II), there was only a 3-fold difference in the L/H ratio in the 8-day-old neuron. In the 5-day-old neuron, 3-fold variations in the L/H ratio were the apparent result of very similar supernatant protein concentrations (Table II), while in the 3-day-old neuron (Table II), virtually identical supernatant protein concentrations yielded closely similar L/H ratios. Two additional factors affected the L/H ratio, namely the neuroanatomical origin of the cell body and the purity of the preparation subjected to freezing–thawing. The influence of both of these factors is indicated (Table II) by the appearance of intermediate *N*-acetyl-β-D-glucosaminidase components in the 5- and 8-day cerebellar neurons and in the 18-day-old cerebral neuron, respectively (Table II and Fig. 4, lower right panel, profile III).

The parameter found to modulate the gradient profile of neuronal *N*-acetyl-β-D-glucosaminidase most strikingly was the pH of the FT supernatant to be layered on the 5–25% sucrose density gradient. A survey of the effect of pH on the L/H ratio, conducted with FT supernatants obtained from 8-day-old neurons (Fig. 5) included the values of 4.5, used by Sandhoff and his associates[11,12,16] with human brain extracts and 5.8, used by Robinson *et al.*[15] with human and calf brain extracts. We noted a more than 20-fold variation in the L/H ratio for less than one unit of change of pH (Fig. 5, compare L/H ratios at pH 4.5 and 5.8). On the basis of these results we conclude that for an assessment of the composition of biological samples in terms of molecular components of *N*-acetyl-β-D-glucosaminidase, monitoring of the pH becomes essential for, as shown in the present study, a given component (component H) may actually not appear if the pH of the sample in which its presence is to be demonstrated is allowed to fall below 5.6.

Our results using chromatography on Sephadex G-200 (Table IV) confirm the relative inseparability of components H and L[38], previously noted with the electrophoretically separated components A and B; on the basis of the overlapping elution patterns of *N*-acetyl-β-L-glucosaminidase and of crystalline aldolase molecular weights for each of the components in the neighborhood of 158 000 are suggested. This value should be compared to the value of 150 000 reported by Robinson *et al.*[15] for the calf brain enzyme, of 120 000–130 000 for the components A and B of human liver[38], and of 118 000 and 151 000 for component A and 158 000 and 181 000 for component B of the hen oviduct[50] and the porcine kidney[51] enzymes, respectively.

The differential response of components A and B of *N*-acetyl-β-D-glucosaminidase to heat has become the chief diagnostic test for the early detection of Tay-Sachs disease[9,12,17]. The test is based on the total loss of the activity of component A following exposure to 50 °C at pH 4.5 for 2 h, with a virtually total preservation of

the activity of component B. Although many workers have confirmed the validity of the original observations of Robinson and Stirling[37] who heated preparations of *N*-acetyl-β-D-glucosaminidase of human spleen, some discrepancies have recently been noted, particularly by Wetmore and Verpoorte[43] who found that both components of porcine kidney *N*-acetyl-β-D-glucosaminidase lost activity when heated at 50 °C and at pH 4.5, both being equally well protected, however, when heating occurred in the presence of 0.01% bovine serum albumin. In the present study the age dependence of the heat sensitivity of the heavy (H) and the light (L) components of neuronal *N*-acetyl-β-D-glucosaminidase was examined by heating at 50 °C and at pH 4.1 for 20 min (Fig. 6) or by preincubating at 45 °C and at pH 6.0–6.5 for up to 90 min[41] (Fig. 7). Fig. 6 illustrates the relatively age-independent effect of heat on component L, about half of its activity being lost at about 50 °C, the severe effect of heat on component H in 5-day-old neurons, about 75% of the activity being lost below 40 °C and the relative insensitivity to heat of component H in the cerebral cortex at the other ages tested, approximately 50% of its activity being lost between 47 and 51 °C. Cerebellar component H reacted to heat to an intermediate extent, half of its activity being lost at about 42 °C. When heating at 45 °C and at pH 6.0–6.5 was applied (Fig. 7) component H was uniformly more sensitive than component L; also, the former was more sensitive in the 3- and the 5-day-old neurons (70% of the activity lost in 5 min) than in the older ones (50% of the activity lost in 5 min).

The facilitation of the conversion of *N*-acetyl-β-D-glucosaminidase component A to component B by the addition of exogenous neuraminidase was first shown by Robinson and Stirling[37] who used the glucosaminidase components of human spleen and was later confirmed for the enzyme from rat kidney[42] and rat, calf and beef brain[14]. Most recently, Robinson *et al.*[15] reported the conversion of the calf brain component A to component B during prolonged vacuum dialysis. Table V lists some conditions leading to the disappearance of component H and also some treatments found to promote its conversion to component L. Although fragmentary, these results reveal that the enzymatic activity associated with component H may be lost both with and without its conversion to component L. In separate experiments we were unable to detect neuraminidase and β-fucosidase activity in either the FT supernatants, the sucrose density gradients or, for that matter, in Sephadex-purified preparations of component H. On the other hand we readily detected α-fucosidase activity, however, only in coincidence with the density gradient peak of component L. (Warshaw, G. and Sellinger, O. Z., unpublished observations). We are presently investigating the apparently subtle nature of the relationship between the heavy and the light components of neuronal *N*-acetyl-β-D-glucosaminidase as well as its age dependence.

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