Neuronal *N*-Acetyl- β -D-glucosaminidase EVIDENCE FOR ITS BIOSYNTHESIS *IN VITRO*

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Neuronal cell bodies, isolated in bulk from 8-day-old rat cerebral cortices, were incubated in the presence of a ^3H -labelled amino acid mixture, and subcellular fractions isolated by differential centrifugation. The particulate fractions were frozen/thawed in 0.20 M-sucrose/0.1 M-KCl [Sellinger *et al.* (1973) *Biochim. Biophys. Acta* 315, 128–146] and the profiles of acid-insoluble radioactivity and *N*-acetyl- β -D-glucosaminidase (glucosaminidase) activity compared in the resulting non-sedimentable fractions by DEAE-cellulose chromatography and cellulose acetate electrophoresis. Radioactivity and glucosaminidase activity co-migrated to a significant extent. Electrophoresis revealed that after 1 min of incubation 42% of the radioactivity of the non-sedimentable microsomal fraction after freezing and thawing co-migrated with an intensely fluorescent band of glucosaminidase activity. Since the pellet fraction obtained on freezing/thawing the microsomal fraction contained up to 75% of the RNA, 95% of the radioactivity and 45% of the glucosaminidase, a detailed study of the association between its radioactivity and nascent glucosaminidase activity was undertaken. After 1 and 2 min of incubation, followed by centrifugation of the microsomal pellet on 35–60% (w/v) sucrose density gradients, radioactivity and glucosaminidase activity exhibited parallel profiles in the region of heavy polyribosomes and at the top of the gradient which contains spontaneously released nascent polypeptide chains. DEAE-cellulose chromatography of these chains revealed glucosaminidase A to be the principal nascent glucosaminidase component, with glucosaminidases B and C as minor peaks. After 2 min of incubation, all of the glucosaminidase components appeared labelled, and glucosaminidase A exhibited two distinct sub-components. The pattern of glucosaminidase labelling in the soluble and microsomal fractions suggested that newly formed glucosaminidase molecules traverse both the cellular sap and the lumen of the endoplasmic reticulum. Only glucosaminidase A reacted specifically with concanavalin A, and radioactive glucosaminidase A could be successfully regenerated by treatment with α -methyl D-glucoside. Glucosaminidase A and a substantial portion of the radioactivity associating with it could be readily converted into glucosaminidase B by re-chromatography on DEAE-cellulose and by reaction of the concanavalin A–glucosaminidase A complex with methyl glucosides.

Neuronal cell bodies isolated from immature brain tissue are capable of incorporating labelled amino acids into protein *in vitro* (Blomstrand & Hamberger, 1969; Tiplady & Rose, 1971; Sellinger *et al.*, 1971a; Johnson & Sellinger, 1971; Kohl & Sellinger, 1972; Hemminki, 1973). We have shown that neuronal protein synthesis is affected by the age of the animal (Johnson & Sellinger, 1971; Sellinger *et al.*, 1971b) as well as by the nature of the amino acid selected for the incorporation (Sellinger *et al.*, 1972; Johnson & Sellinger, 1973a,b). It was therefore decided to investigate the ability of neurons to synthesize a specific, functional protein *in vitro*.

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The lysosomal *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30; glucosaminidase) of nervous tissue (for an extended bibliography, see Sellinger & Santiago, 1975, 1976) exists in at least two molecular forms, which differ in their electrophoretic mobilities and in their chromatographic behaviour; the A form is more acidic (Robinson *et al.*, 1972) and, when sedimented in linear gradients of sucrose, it migrates toward the bottom of the centrifuge tube (Sellinger *et al.*, 1973; Khawaja & Sellinger, 1973). The biosynthesis of glucosaminidase was selected for study because this enzyme protein is an essential determinant of cellular health (Robinson, 1974), its activity is high in isolated nerve cells (Idoyaga-Vargas *et al.*, 1972) and an ultrasensitive fluorimetric assay is available for its detection (Young *et al.*, 1970). Moreover, the study of

its biosynthesis was also highly likely to clarify the relationship between its different molecular forms (Sellinger & Santiago, 1975, 1976) at the level of the neuron, rather than at that of the whole brain (Harzer & Sandhoff, 1971; Hooghwinkel *et al.*, 1972; Robinson *et al.*, 1972; Koenig & Patel, 1976). Preliminary reports of some of the results presented herein have already appeared (Khawaja & Sellinger, 1973; Sellinger *et al.*, 1976).

Experimental

Animals and materials

Male rats of the Sprague-Dawley strain were used. A mixture of ^{20}H -labelled L-amino acids (NET-250) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Ribonuclease-free sucrose was a product of Schwarz/Mann, Orangeburg, NY, U.S.A. 4-Methylumbelliferyl *N*-acetyl- β -D-glucosaminide and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide were obtained from Pierce, Rockford, IL, U.S.A., or Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (DE 52) was from H. Reeve Angel, Clifton, NJ, U.S.A., and concanavalin A from Pharmacia, Piscataway, NJ, U.S.A. All other reagents were of analytical grade; most were obtained from sources cited previously (Kohl & Sellinger, 1972). Ultrafiltration was by means of stirred cells (Amicon Corp., Lexington, MA, U.S.A.) or hollow-fibre devices (Bio-Rad Corp., Richmond, CA, U.S.A.). Cellulose acetate strips were Sepharose III (Gelman Co., Ann Arbor, MI, U.S.A.) and Cellegam (Shandon Scientific Co., Sewickley, PA, U.S.A.). A sample of highly purified jack-bean-meal β -*N*-acetylhexosaminidase was generously given by Dr. S.-C. Li, Delta Regional Primate Center, Covington, LA, U.S.A.

Methods

Isolation of neuronal cell bodies. Neuronal cell bodies from cerebral cortices were prepared as described by Johnson & Sellinger (1971). Isolated cell bodies were homogenized (initially 20%, w/v) in 0.25 M-sucrose (ten up-and-down strokes) in a Potter-Elvehjem glass/Teflon homogenizer driven mechanically at 1300 rev./min.

Incorporation of ^3H -labelled amino acids. This was effected by incubating the neuronal homogenate in system C of Kohl & Sellinger (1972) in the presence of $25\ \mu\text{Ci}$ of the ^3H -labelled amino acid mixture per flask. All glassware and pipettes were sterilized by autoclaving before use. The final volume of the incubation mixture was 5 ml. At the end of the incubation at 37°C , 20 ml of an ice-cold solution containing 20 unlabelled amino acids (0.5 mg/ml) was added to each of several incubation flasks, and their contents were pooled.

Preparation of subcellular fractions. Differential centrifugation was used to separate several subcellular fractions from the incubated neuronal homogenates. In the simpler of the two centrifugation schedules (Sellinger & de Balbian Verster, 1962) the nuclear fraction was first removed by centrifuging at 1100g for 15 min; the resulting supernatant was centrifuged at 150000g for 2 h to sediment a common mitochondrial/lysosomal/microsomal fraction ('fraction Mit-Ly-Mic'). A more elaborate schedule (Sellinger & de Balbian Verster, 1962) permitted the isolation of five distinct fractions. For this, the nuclear fraction was first removed as above and after one 12 min wash, the postnuclear fraction was centrifuged at 7000g for 15 min and washed once at 7000g for 12 min, yielding the mitochondrial fraction, 'fraction Mit'. The postmitochondrial supernatant was centrifuged at 13000g for 15 min, and washed once at 13000g for 12 min, yielding the lysosomal fraction 'fraction Ly'. The postlysosomal supernatant was centrifuged at 150000g for 60 min, yielding the microsomal fraction, 'fraction Mic'. The soluble fraction, 'fraction S', was obtained by carrying a portion of the postnuclear supernatant through the above centrifugation steps without opening the tube until after the final centrifugation step; in this manner, excessive dilution of fraction S was avoided.

Solubilization of *N*-acetyl- β -D-glucosaminidase. Glucosaminidase was solubilized by repeated freezing/thawing in 0.20 M-sucrose/0.1 M-KCl (Sellinger *et al.*, 1973). After the freezing/thawing cycle the suspension was centrifuged at 15000g for 1 h, and the supernatant ('fraction FT-S'), containing the solubilized portion of the glucosaminidase, was removed from the pellet ('fraction FT-P'), which was suspended in 0.018 M-Tris/HCl, pH 7.2, containing 5 mM-magnesium acetate and 25 mM-KCl.

Polyribosomal profiles. A sample (0.8 ml) of the suspension of pellet FT-P was layered on 10 ml of a 35–60% (w/v) linear gradient of sucrose, and the tubes were sedimented at 26500g for 14.5 h in the SW-41 rotor of the Spinco ultracentrifuge. The polyribosomal gradient was eluted through an LKB Uvicord instrument set at 254 nm, and collected in about 18 tubes (0.85 ml/tube).

Analytical studies. Acid-insoluble radioactivity was determined as described previously by retention on GF/C filters (Kohl & Sellinger, 1972). RNA was determined by the method of Fleck & Begg (1965) and protein by that of Lowry *et al.* (1951). For determination of radioactivity, dried filters or liquid samples were placed in scintillation vials; Hyamine or BBS-3 (Beckman, Fullerton, CA, U.S.A.) (0.5–1 ml) was added, and the sealed vials were left at room temperature (23°C) overnight. Then 10 ml of scintillation fluid containing 4 g of PPO (2,5-diphenyloxazole) and 0.1 g of dimethyl POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per litre of

toluene were added and the radioactivity was counted in a Unilux II (Searle Analytic) spectrometer at a counting efficiency of 35–40%. Quenching corrections were carried out by the channels-ratio method. In more recent experiments, Scintisol complete (Isolab, Akron, OH, U.S.A.) or PCS (Amersham/Searle, Arlington Heights, IL, U.S.A.) were used in lieu of the scintillants prepared by ourselves.

Chromatography. Chromatography on DEAE-cellulose columns (1.5 cm × 30 cm) equilibrated with 0.01 M-sodium phosphate buffer, pH 7.0, was carried out as described by Robinson *et al.* (1967). The flow rate was 40 ml/h and the volume of each fraction in the various experiments was between 5 and 10 ml.

Electrophoresis. The detection and resolution of glucosaminidase on strips of cellulose acetate was performed by the method of Suzuki *et al.* (1971). The strips were made to float in 0.05 M-sodium phosphate/citrate buffer, pH 6.5 (Klibansky, 1971), and samples (10–50 μ l) applied near the anodic end. Electrophoresis (2–4 mA/strip) was carried out at room temperature for 60 min. The strips were removed, applied to a glass plate soaked with substrate (7.5 mg of 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide/10 ml of 0.05 M-sodium citrate buffer, pH 4.5) and incubated at 37°C for 60 min. Before detection under a u.v. lamp, they were exposed to ammonia fumes in the dark for 30 s. The position of the fluorescent glucosaminidase bands was marked with pencil and the whole strip cut into segments, which were placed into counting vials.

Enzyme assay. In the colorimetric assay (Idoyaga-Vargas *et al.*, 1972) the concentration of substrate was 4.8 mM. The pH (0.15 M-sodium phosphate/citrate buffer containing 24.9 g of KCl/l) was 4.1 and the final volume was 0.93 ml. A unit of enzyme activity is defined operationally as the E_{412} /ml per incubation time. In the fluorimetric assay (Young *et al.*, 1970; Sellinger & Santiago, 1975) the concentration of 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide was 0.8 mM, and the pH of the sodium phosphate/citrate buffer (0.05 M) was 4.5. A unit of activity is defined as the amount of enzyme necessary to produce a fluorescence reading of 1.0 under the conditions of the assay.

Results

Initially, evidence for the labelling of neuronal *N*-acetyl- β -D-glucosaminidase *in vitro* was sought by incubating 18-day perikarya for 30 min in the amino acid-incorporating system. Fraction Mit-Ly-Mic was isolated (Kohl & Sellinger, 1972), subjected to repeated freeze/thawing and centrifuged to yield fraction FT-S (Sellinger *et al.*, 1973), which contains the solubilized, and fraction FT-P, which contains the structure-bound, enzyme components. Chro-

matographing fraction FT-S on DEAE-cellulose resulted in the elution of one enzyme activity peak and two peaks of radioactivity, one of which co-chromatographed with the enzyme peak. In another experiment, fraction FT-S from neurons of 8-day brain cortices was chromatographed, as above, and, after elution with salt-free buffer, the column was washed with buffer containing 0.4 M-NaCl. The elution profile showed evidence of a second enzyme-activity peak, which eluted with the salt and which contained significant radioactivity. On the basis of these results it was decided to investigate whether a portion of the radioactivity was in the glucosaminidase protein, and an experiment was performed in which radioactive fraction FT-S from 8-day neuronal cell bodies was mixed with 'marker' *N*-acetyl- β -D-glucosaminidase components A and B (Robinson *et al.*, 1972; Sellinger *et al.*, 1973) isolated from non-radioactive 8-day-old neuronal cell bodies. Samples of the mixture were applied to strips of cellulose acetate, and, after electrophoresis (Klibansky, 1971), the strips were monitored for enzyme activity and radioactivity. There was a substantial positional correspondence between the fluorescence of the 'marker'-enzyme bands and radioactivity; in addition, a zone of radioactivity devoid of enzyme activity was also noted. Closely similar results were obtained when the experiment was repeated on fraction FT-S from 18-day-old neuronal cell bodies.

Influence of neuronal postnatal age on protein labelling

Since in a previous study of neuronal protein synthesis *in vitro* (Kohl & Sellinger, 1972) we used only 8-day perikarya, and since neuronal *N*-acetyl- β -D-glucosaminidase attains highest activity at around 18 days (Idoyaga-Vargas *et al.*, 1972), it was decided to compare 8- and 18-day perikarya in terms of their overall incorporative capacity and of the possibility that age influences incorporation into the *N*-acetyl- β -D-glucosaminidase protein. Table 1 reveals comparable specific-radioactivity values (d.p.m./ μ g of RNA) for 8- and 18-day neuronal proteins in all but the soluble fraction S, and also shows that at 8 days the specific radioactivity of fraction FT-P was more than double the 18-day value.

Time-course of neuronal-protein labelling

Short-term protein labelling was examined by incubating 8-day-old neuronal homogenates for 5, 10 and 15 min, and separating the mitochondrial, lysosomal and microsomal fractions. The following were determined in each fraction: the acid-insoluble radioactivity (d.p.m.), the specific radioactivity (d.p.m./mg of protein) and the specific activity (units/mg of protein) of *N*-acetyl- β -D-glucosaminidase. The ratio of the latter two values was taken as

Table 1. *Specific radioactivity of proteins in subcellular fractions of 8- and 18-day-old neuronal perikarya incubated for 30 min*

Homogenates were prepared from neuronal cell bodies of four 8-day-old and eight 18-day-old cerebral cortices and incubated with a mixture of ^3H -labelled amino acids (see under 'Methods') for 30 min at 37°C . The nuclear, Mit-Ly-Mic and soluble S fractions were isolated, and fractions FT-S and FT-P were obtained as described in Sellinger *et al.* (1973) and under 'Methods'. RNA recoveries with respect to the homogenate: 62% at 8 days and 49% at 18 days; with respect to fraction Mit-Ly-Mic: 63% at 8 days and 102% at 18 days. Abbreviations: FT-S and FT-P, supernatant and pellet respectively, after freezing/thawing of fraction Mit-Ly-Mic followed by centrifugation (see under 'Methods'); N.D.: not determined; values marked † were calculated from Johnson & Sellinger (1971).

Fraction	Age (days)	Protein (mg)	RNA (μg)	Specific radioactivity (d.p.m./ μg of RNA)
Homogenate	8	3.4	216	2490
	18	8.8†	190	2045
Nuclei	8	N.D.	9	2820
	18	1.15†	6	2590
Mit-Ly-Mic	8	1.2	100	2490
	18	2.2†	70	1600
FT-S	8	0.5	25	715
	18	N.D.	17	660
FT-P	8	0.6	39	4170
	18	N.D.	54	1900
Soluble S	8	4.0	26	2200
	18	4.0†	16	4180

an indicator of the presumptive labelling of glucosaminidase, and is expressed as 'd.p.m./E' in Table 2. The highest radioactivity and d.p.m./E values were noted in the microsomal fraction, whereas the mitochondrial and lysosomal fractions had closely similar, yet significantly lower, values. Table 2 also reveals that the specific radioactivity and the d.p.m./E values were highest in the mitochondrial and lysosomal fractions after 10, rather than 15 min, of incubation.

The partition of the radioactivity and of glucosaminidase activity between subfractions FT-S and FT-P after 5 min of incubation was examined next. These results (Table 3) show significant differences between the mitochondrial and lysosomal fractions and the microsomal fraction, for subfractions FT-S and FT-P of the former contained between 30 and 40% of the parent radioactivity and glucosaminidase activity, whereas the microsomal subfraction FT-S contained only 6% of the radioactivity but 55% of the glucosaminidase activity of its parent fraction.

Electrophoresis of fractions FT-S

When the lysosomal and microsomal FT-S fractions were electrophoresed, the radioactivity was distributed as shown in Fig. 1, i.e. 26.6 and 34.7% of the radioactivity (d.p.m.) recovered respectively from the lysosomal and microsomal strips were found to associate with the cathodic zone of intense glucosaminidase fluorescence. In subsequent experiments,

incubations were shortened further to 1, 2 and 5 min. Fig. 2 illustrates the significant temporal and qualitative differences between the radioactivity patterns for the mitochondrial and the microsomal FT-S fractions. The electrophoretogram for the microsomal FT-S fraction (Fig. 2a) reveals that, after barely 1 min of incubation, 42% of the radioactivity was associated with the fluorescent glucosaminidase zone and that this proportion decreased to about 19% by 5 min. The pattern for the mitochondrial FT-S fraction (Fig. 2b) was markedly different, inasmuch as the radioactivity associating with the fluorescent enzyme-activity zone decreased from 13% at 1 min to 0% at 5 min.

Labelling of nascent polyribosome-bound proteins

The labelling pattern of the proteins in the microsomal subfraction FT-P was examined to investigate the possibility that the glucosaminidase protein was among them in the form of polyribosome-bound polypeptide chains. To insure that the 1 min incubations reflected accurately the speed and the intensity of nascent-protein labelling, homogenates were also incubated for 2 min. The amount of radioactivity after 2 min was found to be twice that after a 1 min incubation; however, the amount of RNA (about 0.42 mg/g) and of glucosaminidase [about 100 units (as defined above)] remained constant.

The association of the polyribosome-bound, acid-insoluble, radioactivity with glucosaminidase was

Table 2. Time-course of neuronal-protein labelling

Neuronal cell bodies were prepared from 21 8-day-old cerebral cortices and were suspended in 7.5 ml of 0.25M-sucrose. The homogenates were incubated with ³H-labelled amino acids as indicated, and the subcellular fractions isolated by the procedure of Sellinger & de Balbian Verster (1962). The glucosaminidase activity was determined colorimetrically (Idoyaga-Vargas *et al.*, 1972) and the radioactivity (d.p.m.) by standard procedures previously described (Kohl & Sellinger, 1972). The following protein values (mg) were determined (5, 10, 15 min values listed). Homogenate: 22.4, 21.9, 20.1; mitochondrial fraction: 1.01, 0.86, 0.79; lysosomal fraction: 0.48, 0.56, 0.44; microsomal fraction: 4.6, 4.3, 4.4. The corresponding RNA values (μg) were: homogenate: 826, 738, 671; mitochondrial fraction: 58, 50, 29.4; lysosomal fraction: 26.1, 28.8, 18.3; microsomal fraction: 579, 543, 596.

Fraction	Glucosaminidase specific activity (units/mg of protein)	Period of incubation								
		5 min			10 min			15 min		
		Radioactivity (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)	Specific radioactivity/[(d.p.m./mg of protein)/(units/mg of protein)]	Radioactivity (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)	Specific radioactivity/[(d.p.m./mg of protein)/(units/mg of protein)]	Radioactivity (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)	Specific radioactivity/[(d.p.m./mg of protein)/(units/mg of protein)]
Homogenate	41.9	251 200	7860	187	259 800	8430	200	383 600	15 340	372
Mitochondrial	59.4	22 580	22 360	380	24 900	28 660	480	18 780	23 770	400
Lysosomal	71.4	11 240	23 430	337	19 960	36 640	498	10 460	23 770	332
Microsomal	37.2	138 420	30 220	807	142 600	33 470	895	178 760	40 910	1100

Table 3. Partition of radioactivity and of *N*-acetyl- β -D-glucosaminidase between the pellet and supernatant of frozen and thawed microsomal preparations

For details of the preparation of fractions Mit, Ly and Mic, see the legend to Table 2. Fractions FT-S and FT-P were obtained as described in the legend to Table 1. The glucosaminidase activity and radioactivity were determined as described in the legend to Table 2. The protein (mg) and RNA (μ g) values were: supernatant FT-S: fraction Mit, 0.09, 19; fraction Ly, 0.05, 2.2; fraction Mic, 1.31, 81; pellet FT-P: fraction Mit, 0.75, 52.7; fraction Ly, 0.27, 21.1; fraction Mic, 3.09, 510. Recovery is expressed as % of radioactivity (d.p.m.) or enzyme activity in parent fraction. The time of incubation was 5 min.

Fraction	Radioactivity in:						<i>N</i> -Acetyl- β -D-glucosaminidase activity in:					
	Fraction Mit		Fraction Ly		Fraction Mic		Fraction Mit		Fraction Ly		Fraction Mic	
	(d.p.m.)	(%)	(d.p.m.)	(%)	(d.p.m.)	(%)	(units)	(%)	(units)	(%)	(units)	(%)
FT-S	5640	35.2	3260	32	7560	6	16.3	39	5.9	29	107	55
FT-P	10740	64.8	6920	68	113560	94	25.3	61	14.4	71	239	45
Recovery (%)	70.5		90		88		78		59		145

Fig. 1. Comparison of the labelling of fractions Ly FT-S (a) and Mic FT-S (b)

Neuronal cell-body homogenates from 8-day-old cerebral cortices were incubated for 15 min in the presence of the protein-synthesizing system and fractions Ly and Mic were isolated, frozen-thawed and fractions FT-S prepared as described under 'Methods'. Portions (0.020 ml) of fractions Ly and Mic FT-S, containing respectively 1 and 25 μ g of protein, were applied to the cellulose acetate strips as indicated by the arrow, and electrophoresis was performed as described under 'Methods'. ■, Very intense glucosaminidase fluorescence; ▨, moderate glucosaminidase fluorescence. The numerals straddling bars 3 and 4 of the histograms denote the percentages of the radioactivity (d.p.m.) recovered in the area of glucosaminidase fluorescence.

examined by solubilizing the enzyme as described under 'Methods'. Fig. 3 illustrates the polyribosomal profiles and the gradient distribution of the glucosaminidase in the control, un-incubated FT-P pellet (a) and of the nascent radioactivity and glucosaminidase activity after 1 (b) and 2 (c) min of incubation. Figs. 3(a), 3(b) and 3(c) disclose closely similar E_{254} distributions. The radioactivity and glucosaminidase activity were distributed identically in the heavy-polyribosome region of the gradient (tubes 1-7), but somewhat differently in its light regions (tubes 13-16), where strictly coincident peaks were noted only at 1 min (Fig. 3b). This suggests that glucosaminidase activity accompanies all polyribosome-aggregate sizes and that the radioactivity in the heavy-polyribosome region represents polypeptide chains firmly bound to the polyribosomes, presumably because incompletely assembled. The radioactivity peaks in tubes 13-16 of Figs. 3(b) and 3(c) presumably represent nascent polypeptide chains, including *N*-acetyl- β -D-glucosaminidase, which were completely assembled during the first 2 min of incubation, but which became released from the polyribosomes as soluble chains during the 14.5 h of centrifugation.

The nature of the radioactive polypeptide chains and of the glucosaminidase jointly released from the polyribosomes during the overnight centrifugation run was examined by DEAE-cellulose chromatography. Fig. 4(a) reveals that by far the largest portion of the glucosaminidase released from the un-incubated polyribosomes consists of the A component of the enzyme, the B and C (?) components representing a very small percentage of the total recovered activity. Incubation for 1 min (Fig. 4b) resulted in a similar, yet asymmetrical, glucosaminidase pattern and a radioactivity profile consistent with the occurrence of enzyme labelling. Fig. 4(c) discloses the

Fig. 2. Kinetics of the early labelling of fractions Mit FT-S (a) and Mic FT-S (b)

Neuronal cell-body homogenates from 8-day-old cerebral cortices were incubated for (i) 5, (ii) 2 and (iii) 1 min in the protein-synthesizing system, and fractions Mit FT-S (a) and Mic FT-S (b) were isolated as described under 'Methods'. Respectively 1.5–2.0 and 7–13 μg were used for electrophoresis. ■, Glucosaminidase fluorescence. The numerals on top of the histogram bars denote the percentage of the total radioactivity (d.p.m.) recovered in the area of glucosaminidase fluorescence.

appearance of two distinct glucosaminidase peaks in the elution region of the A component in addition to more prominent, albeit small, peaks of the B and C (?) components. More important, however, the radioactivity profile closely accompanied the enzyme activity, there being four distinct peaks corresponding to the four enzyme-activity peaks.

Labelling of glucosaminidase in fraction S

This was investigated to examine the possibility that the enzyme is discharged from polyribosomes directly in the cell sap (fraction S) for subsequent transport to other intracellular locations (Goldstone & Koenig, 1973).

Fig. 5 compares the initial time-course of protein labelling (a) and the specific radioactivity of the labelled proteins in fractions Mic FT-S and S (b). Although incorporation into the proteins of fraction S was severalfold higher than into the solubilized microsomal proteins, the specific radioactivity of the former exhibited values at 1 min which were higher, and values at 5 min which were lower, than those of the

Mic FT-S proteins. The distribution of the radioactivity and of the glucosaminidase A and B in fraction S at 1 and 5 min was analysed by DEAE-cellulose chromatography (Figs. 6a and 6b). The corresponding profiles for fraction Mic FT-S are not shown because they were essentially identical. Fig. 6(a) illustrates the presence of components A and B and of a third component, more acidic than A, which is tentatively and operationally identified as glucosaminidase C. Measurement of the relative contributions of glucosaminidases A and B in fraction S revealed a constant ratio (B/A) of about 30%:60%, and changing ratios of 64%:30% at 1 min and 38%:44% at 5 min, in fraction Mic FT-S (Table 4). Although the radioactivity and the glucosaminidase profiles were totally superimposable for component B (Figs. 6a and 6b), there was significant lack of overlap between the radioactivity, the protein profiles and glucosaminidase A. Re-chromatography of the contents of tubes 1–3 and 12–16 of Fig. 6b yielded the elution profiles shown in Figs. 6(c) and 6(d) respectively. Fig. 6(c) confirms the parallel behaviour of radioactivity and glucosaminidase B, and Fig. 6(d) reveals

Fig. 3. Association of nascent radioactivity and glucosaminidase activity with neuronal polyribosomes

Fraction Mic was isolated from 8-day-old neuronal homogenates incubated in the protein-synthesizing system 0, 1 and 2 min. After freezing/thawing in 0.20M-sucrose/0.1 M-KCl and centrifugation of the suspension at 15000g for 1 h (Sellinger *et al.*, 1973), the pellet (Mic FT-P) was suspended in 1 ml of 0.018M-Tris/HCl buffer, pH7.2, containing 5mM-magnesium acetate and 25mM-KCl, and 0.8 ml of this, containing (a) 2.2, (b) 2.8 and (c) 3.6 mg of protein and (a) 0.29, (b) 0.38 and (c) 0.43 mg of RNA, was centrifuged as described under 'Methods'. The tubes were pierced off-centre to avoid disturbing the pellet and the gradients collected (about 0.85 ml/tube) with continuous monitoring of the E_{254} (—). ○, Glucosaminidase activity; ●, radioactivity. Tube 1 is the bottom and tube 17 the top of the gradient: (a) no incubation; (b) 1 min and (c) 2 min of incubation.

Fig. 4. DEAE-cellulose chromatography of nascent polypeptide chains and of glucosaminidase spontaneously released from neuronal polyribosomes

Fraction Mic was prepared from neuronal cell bodies derived from 20 8-day-old cerebral cortices per experiment and treated as described in the legend to Fig. 3. In these experiments, however, three Mic FT-P pellets were pooled for each gradient run. Separate experiments with three FT-P pellets derived respectively from homogenates either un-incubated (a), or incubated for 1 (b) or 2 min (c) are shown. Tubes 14, 15 and 16 (a), 13, 14 and 15 (b) and 14, 15 and 16 (c) of the Mic FT-P gradients similar to those shown in Fig. 3 were pooled and applied to a column of DEAE-cellulose (see under 'Methods'). Elution was started with 0.01 M-sodium phosphate buffer, pH7.0, and four to six tubes were collected (6.5 ml/tube) before a 0.1–0.1 M-NaCl gradient was applied (where shown by the arrow), and elution continued as indicated. ●, Radioactivity, and ○, glucosaminidase activity were determined in each tube on 1 and 0.5 ml samples respectively.

Fig. 5. Comparison of the kinetics of early labelling of fractions Mic FT-S and S

Fractions Mic and S were prepared by differential centrifugation (Sellinger & de Balbian Verster, 1962) of 8-day-old neuronal homogenates incubated for 1, 2 and 5 min. Samples were taken for determination of acid-insoluble radioactivity (Kohl & Sellinger, 1972) and of protein. (a) Acid-insoluble radioactivity as a function of incubation time; (b) specific radioactivity as a function of incubation time. ●, Fraction Mic; ○, fraction S.

a conversion of glucosaminidase A, together with a substantial portion of the radioactivity accompanying it (Fig. 6b), into glucosaminidase B. The jack-bean-meal enzyme (Figs. 6b and 6c) was passed through the DEAE-cellulose column in a separate run, and therefore the precise superposition of its elution profile with that of glucosaminidase was not achieved; it mainly serves to illustrate (a), that it is eluted only with salt, like glucosaminidase A, and (b), that it is devoid, as obtained, of any activity eluted with the solvent front. The appearance of a small but distinct 'B' peak on its re-chromatography demonstrated, moreover, that freezing and thawing was not required for this transformation (Beutler & Kuhl, 1975).

Interaction of neuronal glucosaminidase with concanavalin A

Bishayee & Bachhawat (1974a) reported the interaction of sheep brain *N*-acetyl- β -D-glucosaminidase with concanavalin A. To assess the relative reactivity

of neuronal glucosaminidases A and B, and of the radioactivity associated with them, toward concanavalin A, two preliminary experiments, shown in Table 5, were performed. First, a portion of the 1 min glucosaminidase B peak of fraction S (Fig. 6a) was freeze-dried and the residue suspended in 1.5 ml of 0.05 M-sodium acetate buffer, pH 5.0, containing 0.5 M-NaCl. To 0.7 ml (1 mg of protein), 300 μ g of concanavalin A (in 0.3 ml of water) was added, and the tube allowed to incubate at 37°C for 60 min. After centrifugation (12000g for 30 min), no pellet could be recovered. Since the inclusion of jack-bean-meal- β -*N*-acetylhexosaminidase (0.5 mg of protein) as carrier did not appreciably change the amount of radioactivity in fraction S1, we conclude that neither the radioactivity associated with the glucosaminidase B peak nor glucosaminidase B itself contains any concanavalin A-sensitive carbohydrate residues. In another experiment, a portion of the pooled, dialysed (against 0.001 M-sodium phosphate buffer, pH 7.0)

Fig. 6. DEAE-cellulose chromatography of fraction S: the association of nascent radioactivity with glucosaminidase

Fraction S was prepared from 8-day-old neuronal homogenates incubated for 1 (a) and 5 (b) min. Its volume was decreased from 30 ml to 3 ml (a), and from 20 ml to 3 ml (b), with the aid of Amicon PM-30 filters. The final protein content in (a) was 30.8 mg, and in (b) 21.5 mg. The samples were applied to DEAE-cellulose columns (see under 'Methods') and elution was carried out as described in the legend to Fig. 4 at a rate of 5.5 ml/tube. The 0.1–0.4 M-NaCl gradient was started where indicated by the arrow. Samples (0.01 and 0.25 ml) were taken from each tube for the determination of glucosaminidase activity (○) and of radioactivity (●) respectively. In (a), protein (Δ) represented 26.7% in tubes 2–4 and 52.2% in tubes 7–14, whereas in (b) this was 18.5% in tubes 1–2 and 52.3% in tubes 11–19. In (b), □ refers to the tubes taken for re-chromatography to yield the profiles shown in (d) and ▼ indicates a separate run in which 0.001 ml (0.7 unit; Li & Li, 1970) of a highly purified jack-bean-meal β -N-acetylhexosaminidase was passed through the column. Note the total absence of a glucosaminidase B-like component. (c) Re-chromatography of glucosaminidase B (○) on DEAE-cellulose. The contents of tubes 1–3 (b) were chromatographed on a DEAE-cellulose column as in (b) and the glucosaminidase activity (B') and the radioactivity (●) determined in 0.025 and 0.2 ml portions of the eluate. (d) Re-chromatography of glucosaminidase A on DEAE-cellulose. The contents of tubes 12–16, corresponding to the glucosaminidase A peak in (b), were subjected to re-chromatography, and the glucosaminidase activity and the radioactivity determined on 0.05 ml and 0.45 ml portions of the eluate collected at a rate of 5.4 ml/tube. Re-chromatography of β -N-acetylhexosaminidase (tubes 18–21 of the ▼ profile of (b)) is again indicated by ▼. Note a small but distinct enzyme-activity peak in tube 3.

Table 4. Association of radioactivity and of protein with glucosaminidases A and B in fractions Mic, FT-S and S

Fractions Mic F-TP and S were prepared from neuronal homogenates incubated for 1 and 5 min by procedures described under 'Methods'. Samples of these fractions were passed through columns of DEAE-cellulose (see legend to Fig. 6). Radioactivity, glucosaminidase activity and protein were determined in each of the eluate tubes and the percentages of the total values under the glucosaminidase B and A peaks (Figs. 6a and 6b) were calculated. An example of the procedure used is given in the legend to Fig. 6 for fraction S. N.D., not determined. The values given in the Table are expressed as percentages of the total recovered item on DEAE-cellulose chromatography. Relative specific radioactivity is defined as the ratio between the radioactivity and the glucosaminidase activity in a given fraction, both being expressed as percentages of the total radioactivity or glucosaminidase activity recovered in that fraction.

Time of incubation ...	1 min				5 min			
Fraction ...	Mic FT-S		S		Mic FT-S		S	
Glucosaminidase ...	B	A	B	A	B	A	B	A
Radioactivity	90	5	25	54	85	14	40	50
Glucosaminidase activity	64	30	34	62	38	44	34	62
Protein	26	27	27	52	N.D.	N.D.	18	52
Relative specific radioactivity	1.40	0.17	0.73	0.87	2.24	0.32	1.17	0.80

Table 5. Interaction of *N*-acetyl- β -D-glucosaminidases A and B and of ^3H -labelled protein with concanavalin A and α -methyl D-glucoside

The preparation of fraction S from neuronal homogenates of 8-day-old cerebral cortices incubated for 1 min. The conditions for the chromatography on DEAE-cellulose and for the interaction of glucosaminidases A and B with concanavalin A are described in the legend to Fig. 6 and in the text.

Fraction	Glucosaminidase A		Glucosaminidase B			
			radioactivity		activity	
	(d.p.m.)	(%)	(d.p.m.)	(%)	(units)	(%)
P1 (Concanavalin A ppt.)	4600	71.5*	no pellet			
S1 (Concanavalin A supernatant)	1820	28.5*	24800	53.2†	4260	81.3†
			21900‡	47.2†	—	—
P2 (Concanavalin A ppt. after Tris wash)	1210	26.2§				
S2 (Tris wash of P2)	1300	28.2§				
S3 (α -Methyl D-glucoside supernatant)	540	44.7				
		11.7§				

* P1+S1 is set as 100%. The actual recovery was 118%.

† As percentage of radioactivity (46500 d.p.m.) and enzyme units (5235) in 0.7 ml of treated sample (see the text).

‡ With jack-bean-meal β -*N*-acetylhexosaminidase as carrier (see the text).

§ As a percentage of P1.

|| As a percentage of P2.

contents of tubes 9–11, corresponding to the glucosaminidase A peak (Fig. 6a), was treated with concanavalin A in the presence of β -*N*-acetylhexosaminidase carrier as above, and the resulting pellet, P1, was washed with 0.01 M-Tris/HCl buffer, pH 7.0. When the concanavalin A- ^3H glycoprotein complex was dissociated in the presence of 0.3 M- α -methyl D-glucoside at pH 4.4 (Bishayee & Bachhawat, 1974a), 11.7% of the radioactivity of pellet P1 was recovered in fraction S3 (Table 5). The results of additional experiments designed to compare and quantify the extent of the common precipitation by concanavalin A of glucosaminidase A and of radio-

activity are shown in Figs. 7 and 8. In these experiments, 0.50 mg of concanavalin A was incubated at pH 5.0, or as indicated (Fig. 8), with 1 ml portions (1.2–1.3 mg of protein) of fractions S and Mic FT-S. The dissociation of the resulting concanavalin A- ^3H -labelled glycoprotein complex was in 0.1 M-Tris/HCl buffer, pH 8.0, rather than in sodium acetate buffer, pH 4.4. When compared with the glucosaminidase peaks A and B in the untreated fraction S (Fig. 6a), which show approximately equal heights (see Table 4 for the percentages of enzymes A and B in fractions S and Mic FT-S), Fig. 7(a) illustrates the selective but incomplete removal of glucosaminidase

Fig. 7. Interaction of nascent radioactivity and of glucosaminidase with concanavalin A

(a) Fraction S was prepared from 8-day-old neuronal homogenates after 1 min of incubation (see Fig. 6a), and to 1 ml portions were added 0.02 ml of 0.05 M-sodium phosphate/citrate buffer, pH 5.0, containing 0.5 M-KCl and 0.1 ml of concanavalin A in water (5 mg/ml). After incubation at 37°C for 1 h, the suspension was centrifuged at 12000g for 30 min, and the supernatant (1.3 ml) passed through a column of DEAE-cellulose (see under 'Methods'), eluted with 0.01 M-sodium phosphate buffer, pH 7.0 (tubes 1–6), followed by, where indicated by the arrow, a 0.1–0.4 M-NaCl gradient in the same buffer. Glucosaminidase activity (○) and radioactivity (●) were determined in 0.25 ml and 1 ml portions of the eluate, collected at a rate of 5.8 ml/tube. (b) The pellet obtained by centrifuging the suspension described above in (a) was suspended in 0.5 ml of 0.1 M-Tris/HCl buffer, pH 8.0, and to 0.4 ml was added 0.4 ml of 0.6 M- α -methyl D-glucoside in 0.1 M-Tris/HCl buffer, pH 8.0. After incubation at 37°C for 1 h and centrifugation, the supernatant was passed through a DEAE-cellulose column as in (a). Glucosaminidase activity and radioactivity (see under a above) were determined in 0.5 ml and 2 ml portions of the eluate respectively. (c) The conditions were as described in (a) and (b) except that fraction Mic FT-S was treated with concanavalin A. □ depicts the glucosaminidase activity profile corresponding to (a), i.e. after reaction of fraction Mic FT-S with concanavalin A, whereas ○ illustrates the glucosaminidase activity profile under conditions prevailing in (b), i.e. after reaction of the concanavalin A-³H-labelled glycoprotein complex with α -methyl D-glucoside. Radioactivity was too low to be accurately determined.

A and its associated radioactivity by concanavalin A, and the resulting decrease in the ratio of the glucosaminidase A to B peak height from values close to unity to a value of about 0.25. The dissociation of the concanavalin A-³H-labelled glycoprotein complex by α -methyl D-glucoside, followed by DEAE-cellulose chromatography of the resulting supernatant, resulted in the elution profiles shown in Fig. 7(b). Glucosaminidase A was released from the complex and was accompanied by substantial amounts of comigrating radioactivity; as shown in Fig. 7(b), glucosaminidase B and C (shoulder in tubes 15–18), each with closely associated radioactivities, were also generated. Fig. 7(c) depicts an identical series of events for fraction Mic FT-S, namely an effective, yet incomplete removal of glucosaminidase A by concanavalin A, followed by its re-appearance after treatment with glucoside; in this case rather small

amounts of glucosaminidase B and C were generated as well. As shown by the results in Table 6, there was a close quantitative agreement between the percentage of the radioactivity and the percentage of the total glucosaminidase activity precipitated by concanavalin A in both fractions S and Mic FT-S.

Since Bishayee & Bachhawat (1974b) reported a high mannose content for cerebral lysosomal hydrolases, including *N*-acetyl- β -D-glucosaminidase, an experiment was designed to test the relative effectiveness of α -methyl D-mannoside in dissociating the concanavalin A-³H-labelled glycoprotein complex. The results (Table 6) revealed that the two glycosides were about equally effective when applied to fraction S, but that the mannoside was about twice as effective as the glucoside when used to release the microsomal glucosaminidase A from its concanavalin A complex.

Discussion

Incubation of neuronal homogenates with ^3H -leucine has previously been shown to result in effective and rapid labelling of neuronal proteins (Hamberger *et al.*, 1971; Kohl & Sellinger, 1972; Hemminki, 1973). At the subcellular level, Kohl & Sellinger (1972) showed that the microsomal proteins of 8-day-old cortical neurons acquired the highest specific-radioactivity values after 5 min of incubation, and Hemminki (1973) reported more intense labelling of the insoluble than of the non-sedimentable proteins when using neurons isolated from the cerebral cortex of adult rats.

A comparison of the specific radioactivities of 8- and 18-day-old neuronal proteins in several subcellular fractions (Table 1) revealed higher values for the former; 8-day-old animals were therefore selected for use in all subsequent experiments.

The results of preliminary experiments suggested that after 30 min of incubation, appreciable acid insoluble radioactivity associated with that portion of glucosaminidase which resided in particulate form, responded to freezing/thawing by becoming non-sedimentable and, in addition, behaved on DEAE-cellulose chromatography and cellulose acetate electrophoresis in a manner suggesting that a significant proportion had become incorporated into the glucosaminidase protein.

The early time-course of labelling of fractions Mit, Ly and Mic (Table 2) revealed steadily increasing specific-radioactivity values for fraction Mic, as against maxima at 10 min for fractions Mit and Ly. The d.p.m./E value also increased over the 15 min period in fraction Mic, and moreover, at 5 min it

exceeded that of the homogenate by more than four-fold. This finding could be interpreted as suggesting that the specific radioactivity of glucosaminidase, defined as the ratio of the (presumed) radioactivity of

Fig. 8. Influence of pH on the reaction of concanavalin A with the nascent radioactivity and the glucosaminidase activity of fraction S

To six 0.4 ml portions of fraction S (3.25 mg of protein/ml) (see legend to Fig. 6a) were added 0.2 ml of 0.01 M-sodium phosphate/citrate buffer, 0.5 M in KCl (pH as indicated) and 0.1 ml of concanavalin A in water (5 mg/ml) and the suspensions were incubated at 37°C for 1 h. After centrifugation at 12000g for 30 min, the pellets (P₁) were collected and suspended in 0.5 ml of 0.1 M-Tris/HCl buffer, pH 8.0, and their glucosaminidase activity (O), and radioactivity (●), determined in 0.01 ml and 0.05 ml portions respectively.

Table 6. Dissociation of the concanavalin A- ^3H -labelled glycoprotein complex by α -methyl-D-glucoside and α -methyl D-mannoside: parallel behaviour of radioactivity and of glucosaminidase

Fractions S and Mic FT-S were prepared as described in the legends to Table 1 and Fig. 1. The conditions for the reaction of these fractions with concanavalin A and the subsequent interaction of the concanavalin A- ^3H -labelled glycoprotein complex with α -methyl D-glucoside and α -methyl D-mannoside are described in the text and in the legend to Fig. 7. Values in parentheses are percentages of the radioactivity (d.p.m.) or enzyme activity (units) in the concanavalin A precipitate.

Fraction	Radioactivity		Activity	
	(d.p.m.)	(%)	(units)	(%)
S (1.35 mg of protein)	6100	100	7900	100
Concanavalin A ppt.	3850	63	3845	48.6
α -Methyl D-glucoside supernatant	544	8.9 (14.1)	1152	14.6 (30.0)
α -Methyl D-mannoside supernatant	680	11.1 (17.6)	1411	17.7 (35.5)
Mic FT-S (1.27 mg of protein)	8940	100	10000	100
Concanavalin A ppt.	5060	56.6	4295	43.0
α -Methyl D-glucoside supernatant	656	7.3 (12.4)	1576	15.8 (36.5)
α -Methyl D-mannoside supernatant	1408	15.7 (27.8)	2147	21.6 (49.6)

glucosaminidase to its enzyme activity in units (Redman *et al.*, 1972), was highest in this fraction at 5 min, and hence that fraction Mic was the primary site of a very rapid process of nascent glucosaminidase assembly. As shown in Fig. 2(a) the quantitative association of radioactivity and of glucosaminidase activity in fraction Mic FT-S was maximal after barely 1 min of incubation.

As may be seen in Table 3, the polyribosome-rich fraction Mic FT-P retained throughout the first 5 min of incubation between 82 and 94% of the microsomal acid-insoluble radioactivity, together with 42–45% of the microsomal glucosaminidase. The sedimentation profile of the neuronal glucosaminidase with the polyribosomes is illustrated in Fig. 3(a), which demonstrates that virtually all polyribosomal classes associate with the enzyme. Two mutually compatible interpretations of this finding are possible: (a) a large number of polyribosomes is engaged in the synthesis of the glucosaminidase protein at any one time; and (b) virtually all of the partially completed glucosaminidase chains, still attached to polyribosomes, exhibit enzymic activity. The ubiquity of the glucosaminidase on the neuronal polyribosomes thus contrasts significantly with the relatively restricted presence of nascent catalase on hepatic polyribosomes (Uenoyama & Ono, 1972; Redman *et al.*, 1972; Sakamoto & Higashi, 1973).

Brief labelling of the neuronal homogenate (1 and 2 min; Figs. 3b and 3c), followed by the isolation of fraction FT-P, resulted in radioactivity profiles similar to those of glucosaminidase, particularly in the region of the heavy polyribosomes (tubes 1–7) and on the top of the gradient (tubes 13–16). A glucosaminidase peak was apparent on the top of the gradient even in the un-incubated control (Fig. 3a). Since this portion of a polyribosomal gradient contains the 'basal' (Morris, 1968) nascent polypeptides, i.e. finished chains released from the polyribosomes spontaneously, as against partially completed peptides or peptidyl-tRNA molecules which are released from polyribosomes by puromycin (Morris, 1968; Hamlin & Zabin, 1972), EDTA (Sakamoto & Higashi, 1973), ribonuclease (Uenoyama & Ono, 1972) or sodium dodecyl sulphate (Cioli & Lennox, 1973), we chromatographed the contents of tubes 13–16 on DEAE-cellulose and found that glucosaminidase A was by far the most abundant glucosaminidase component in this fraction (Fig. 4). We therefore conclude that glucosaminidase A constitutes the major portion of the glucosaminidase molecules synthesized at any one time. Fig. 4(c) shows a parallel increase with time of glucosaminidase A subcomponents and of spontaneously released radioactive polypeptides.

On discharge from the polyribosomes, completed polypeptide chains may enter the lumen of the endoplasmic reticulum, if synthesized on membrane-

bound polyribosomes, or the soluble cell sap (fraction S), if synthesized on the free polyribosomes. A further refinement of the former mode of intracellular disposition may also apply, i.e. newly formed chains, especially if glycoproteinaceous in nature, may remain temporarily attached to the inner surface of the endoplasmic reticulum, while further sugar residues are added to the nearly completed molecules (Redman & Cherian, 1972). A more recent examination of the finer details of the process of release of nascent hepatic microsomal glycoproteins (Kreibich & Sabatini, 1974) has revealed that some glycoproteins become very rapidly labelled after short pulses of [^{14}C]leucine and [^{14}C]glucosamine *in vivo*. Although we did not examine whether the neuronal microsomal proteins were synthesized on the free or the membrane-bound polyribosomes (Murthy, 1972), we did compare the rates of labelling of nascent polypeptide chains discharged into the cell sap (fraction S) or attached to microsomal structure before being released (fraction Mic FT-S). Fig. 5 shows that even though the proteins in both of the fractions quickly acquired similar specific radioactivities (Fig. 5b), the proportions represented by a given glucosaminidase component in each of them differed considerably (Table 4). Thus at 1 min, glucosaminidase B represented more than twice the amount of glucosaminidase A in fraction Mic FT-S, whereas the reverse was true in fraction S. At 5 min, the glucosaminidase B/A ratio in fraction Mic FT-S reached a value close to unity, while remaining unchanged in fraction S. We believe that these findings are consistent with the notion expressed above in the Results section, namely that at any one time, the different intracellular compartments contain different glucosaminidase components (Goldstone & Koenig, 1973, 1974). Thus our evidence suggests that glucosaminidase A, the preponderant component synthesized on the neuronal polyribosomes, resides preferentially in fraction S, whereas the less acidic component, glucosaminidase B, predominates in fraction Mic FT-S, where presumably it is en route to the lysosome.

The results of the experiments with concanavalin A (Table 5 and Fig. 7) confirm, for the glucosaminidase of rat brain neurons, the findings obtained with the sheep brain enzyme (Bishayee & Bachhawat, 1974a,b). They demonstrate in addition that only glucosaminidase A is precipitated with concanavalin A. Moreover, the presence of radioactivity in the methyl glucoside supernatants (Tables 5 and 6, and Figs. 7b and c) provides yet another quantitative element in support of the labelling of nascent glucosaminidase A.

Finally, the results on the convertibility of glucosaminidase A into glucosaminidase B (Figs. 6d, 7b and 7c), but not of glucosaminidase B into A (see also Beutler & Kuhl, 1975), although confirming our previous findings (Sellinger *et al.*, 1973), also provide

evidence in support of a mechanism mediating this process which does not depend on physical changes in the protein such as those brought about by freezing in 3M-NaCl (Beutler & Kuhl, 1975), or heating up to 50°C (Tallman *et al.*, 1974). Indeed, glucosaminidase B could be formed from glucosaminidase A in the absence of any physical stress, simply as a result of rechromatography on DEAE-cellulose at pH 7.0 (Fig. 6d) or through the reaction of the concanavalin A-glucosaminidase A complex with α -methyl D-glucoside. Since Seyama & Yamakawa (1974a) have shown that a highly purified preparation of horse kidney glucosaminidase A does not have sialidase as a contaminating activity, yet can be converted into glucosaminidase B merely by incubation at 37°C at pH 6.0, we tentatively conclude that the conversion of neuronal glucosaminidase A into glucosaminidase B is also not the result of a simple removal of sialic acid residues from the former component. Rather, at present, particularly because of the observed heterogeneity among glucosaminidase A subcomponents (Figs. 3, 4b and 4c), we opt for a mechanism based on the existence and the dissociation characteristics of a glucosaminidase A-glucosaminidase B complex such as depicted by Seyama & Yamakawa (1974b) for the glucosaminidase of horse kidney and similar to the hydrolase-acidic lipoglycoprotein complex proposed by Koenig & Patel (1975).

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