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N-Acetyl-β-Glucosaminidases in Human Spleen

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1. The *N*-acetyl-β-glucosaminidase of human spleen has been separated by gel electrophoresis into two components, an acidic form A and a basic form B. 2. The two forms are readily separated on DEAE-cellulose and have been concentrated 50-fold and sevenfold respectively. 3. They show similar K_m values towards 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, and have the same pH optima when compared in citrate, phosphate or acetate buffers. They are inhibited to a similar extent by acetate, heparin, *N*-acetylgalactosaminolactone, *N*-acetyl-β-D-galactosamine and *N*-acetyl-β-D-glucosamine. Specificity for C-4 orientation is not absolute and *p*-nitrophenyl β-galactosaminide is also hydrolysed but at a rate only 11·6% of that for the corresponding glucosaminide. 4. *N*-Acetyl-β-glucosaminidase B is stable over a wider pH range than is *N*-acetyl-β-glucosaminidase A, and is less easily denatured by heat. 5. Tissue fractionation indicates that both the A and B forms are present in the lysosomal fraction, whereas the supernatant contains the A form only. 6. Evidence is presented to indicate that the A form contains a number of sialic acid residues.

N-Acetyl-β-glucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) occurs in many tissues (Conchie, Findlay & Levvy, 1959), and is particularly abundant in organs where high rates of mucoid turnover might be expected, e.g. testis and spleen. The enzyme has been partially purified from pig epididymis (Findlay & Levvy, 1960) and from bovine spleen (Buddecke & Werries, 1964). Quantitative determinations of the activity in human tissues have been made on normal and pathological samples of serum (Furiya & Fukuda, 1963), plasma (Woollen & Turner, 1965), synovial fluid and leucocytes (Caygill & Jevons, 1966). In the present paper we describe our examination of this enzyme in human spleen and show that two distinct but possibly related forms are present.

EXPERIMENTAL

Materials. Human spleens were obtained at post mortem and stored in the deep freeze until required. No appreciable differences in the total activity or in the physical nature of the enzymes as described below were noted between spleens stored for different times from a few days to several months in these conditions.

4-Methylumbelliferyl *N*-acetyl-β-glucosaminide, *p*-nitrophenyl *N*-acetyl-β-glucosaminide, chondroitin sulphate and *N*-ethylmaleimide (Koch–Light Laboratories Ltd., Colnbrook, Bucks.), heparin (Boots Pure Drug Co. Ltd., Nottingham), dithiothreitol (Calbiochem, London, W. 1), salmine sulphate and A.R. salts for buffer constituents (British Drug Houses Ltd., Poole, Dorset) were purchased. *p*-Nitrophenyl *N*-acetyl-β-galactosaminide was a gift from Koch–Light Laboratories Ltd. The method of Findlay, Levvy & Marsh (1958) was used for the preparation of 2-acetamido-2-deoxygalactonolactone. In addition, the following commercial enzyme preparations were used: neuraminidase R.D.E. (Burroughs Wellcome, London, N.W. 1), lecithinase D and wheat-germ acid phosphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Enzyme assays. The methods used for β-glucuronidase and β-galactosidase were the same as those used by Robinson, Price & Dance (1967). Acid phosphatase was measured with β-glycerophosphate as substrate (Furth & Robinson, 1965), cytochrome oxidase by the method of Cooperstein & Lazarow (1951) and *N*-acetyl-β-glucosaminidase by the fluorimetric assay method of Leaback & Walker (1961). In inhibition studies the enzyme preparation (0·5ml.) was mixed with 0·5ml. of inhibitor solution in the appropriate buffer and warmed to 37° before the addition of 1ml. of buffer–substrate mixture, as described in the standard assay procedure. The results are expressed as percentage inhibition compared with controls in which buffer solution replaced the inhibitor solution.

The β-glucosaminidase and β-galactosaminidase activities were compared by using the *p*-nitrophenyl glycosides as described by Walker, Woollen & Heyworth (1961).

Cellular fractionation. A sample of fresh spleen was obtained at splenectomy on a female patient. It was immediately placed on ice and processed as follows within 90min. of removal. A 10g. sample was cut into small pieces and homogenized in 50ml. of ice-cold 0·25M-sucrose solution by two passes of a loose-fitting Teflon pestle in a Potter–Elvehjem-type homogenizer. The suspension was squeezed through two thicknesses of muslin and a 10ml. sample of the

filtrate kept for measurement of total activity. The remaining sample (40ml.) was centrifuged at 3000rev./min. for 2min. ($2 \times 10^3 g$-min.) in an MSE High Speed 17 refrigerated centrifuge at 4° with a type 69181 angle head. The sediment, containing unbroken cells and nuclei (fraction N in Table 2), was resuspended in 40ml. of 0·25M-sucrose and kept at 4°. The supernatant from fraction N was centrifuged at 10000rev./min. for 1min. at 4° ($13 \times 10^3 g$-min.). The supernatant was removed and the sediment resuspended in sucrose and centrifuged as before, the washings being added to the original supernatant from this stage. The sediment was resuspended in 40ml. of 0·25M-sucrose (fraction HM in Table 2). The pooled supernatants from the HM fraction (80ml.) were then centrifuged at 17000rev./min for 10min. ($313 \times 10^3 g$-min.) and resuspended and recentrifuged as before, and the sediment (fraction LM) was suspended in 40ml. of 0·25M-sucrose. Finally the supernatant and washings were centrifuged at 17000rev./min. for 90min. ($3 \times 10^6 g$-min.) to give a microsomal sediment (fraction Mic) and a soluble fraction (fraction S). All fractions were kept at 4° until required and assayed for acid phosphatase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, β-galactosidase, cytochrome oxidase and protein. When free activity was assayed all solutions contained 0·25M-sucrose and were adjusted to pH5·0 with the buffer used in the assay. Total activity was measured by incorporating 0·1% Triton X-100 to rupture the lysosome membranes.

DEAE-cellulose chromatography. The method of Furth & Robinson (1965) was used.

Gel-filtration and gel electrophoresis. The methods of separation and detection of protein and enzyme components were the same as those described by Robinson *et al.* (1967).

Protein assays. The extinction at 280mμ or the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used, with casein standards.

K_m values. These were calculated by the double-reciprocal plot method of Lineweaver & Burk (1934).

RESULTS

When the *N*-acetyl-β-glucosaminidase activities of 50mM-sodium citrate-buffer homogenates of five different human spleen samples were assayed at pH5·0 at 37°, as described by Leaback & Walker (1961), they were found to hydrolyse 48·5 ± 13·7mμmoles of the test substrate/hr./mg. of tissue.

Two distinct regions of enzyme activity of roughly equal intensity were found after electrophoresis of these homogenates at pH7·0 on starch gel. One component, which appeared to be negatively charged under these conditions, migrated rapidly towards the anode (component A) and the other (component B) moved slowly towards the cathode. When the gel buffer was changed to 5mM-citrate, pH5·0, component A remained at the origin and component B migrated more rapidly towards the cathode.

Separation of N-*acetyl-β-glucosaminidases A and B*

A 20% (w/v) homogenate of 5g. of human spleen in cold (4°) 10mM-sodium phosphate buffer, pH6·0, was centrifuged at 10000rev./min. for 20min. in an MSE High Speed 17 refrigerated centrifuge and the clear supernatant was applied to a DEAE-cellulose column (20cm. × 2cm. diam.) prepared in the same buffer. Assays of the column eluate fractions showed a clear separation of two enzyme components. About 33% of the original activity was not retained under these conditions and was washed rapidly through the column by the initial buffer mixture. Electrophoretic examination indicated this was component B. Component A was eluted after application of a chloride gradient and began to appear as a sharp peak of activity when the chloride concentration in the eluate reached 0·1M. About 40% of the original activity was obtained in this way in an elution volume of 21ml. This fraction showed a fivefold increase in specific activity over that of the original homogenate.

Further purification. A higher degree of purification of the two components was obtained when a preliminary protein fractionation was carried out (Table 1).

Step 1. A 20% (w/v) homogenate of spleen (330ml.) was centrifuged below 10° at 10000rev./min. for 10min. in an MSE High Speed 17 refrigerated centrifuge and the sediment discarded. The supernatant (300ml.) was fractionally precipitated with ammonium sulphate and that material precipitating between 40% and 70% saturation was collected by centrifugation, resuspended in 55ml. of 10mM-phosphate buffer, pH7·6, containing sodium chloride (0·4M) and

Table 1. *Partial purification of* N-*acetyl-β-glucosaminidases A and B from human spleen*

Activity is expressed as μmoles of substrate hydrolysed/hr. at pH4·5 and 37° in citrate buffer and specific activity as activity/mg. of protein. Details of the purification steps are given in the text.

Purification step	Total activity	Specific activity	Recovery of enzyme (%)
Spleen supernatant extract	2550	0·42	100
(1) 40%–70%-satd. $(NH_4)_2SO_4$ ppt.	1410	1·00	55
(2) Sephadex G-200 filtrate	490	7·00	19
(3) DEAE-cellulose peak A	257	21·50	10
peak B	104	3·0	4

EDTA (10mM), and recentrifuged to give a clear supernatant. This was concentrated by dialysis under vacuum at 4° against 1l. of 40mM-phosphate buffer, pH7·0.

Step 2. The concentrate from step 1 (13ml.) was filtered through a column of Sephadex G-200 (36cm. × 3cm. diam.), prepared in the buffer mixture used for step 1. The eluate was collected in 5ml. fractions at a rate of 15ml./hr. and assayed for enzyme activity, and the contents of the peak tubes were concentrated again by vacuum dialysis overnight against 40mM-phosphate buffer, pH7·0. Only one-third of the total activity applied to the column could be recovered, and this emerged as a single peak containing both forms.

Step 3. The *N*-acetyl-β-glucosaminidase concentrate (6·3ml.) was now separated into fractions A and B on DEAE-cellulose as described above and each sample was shown on gel electrophoretograms to be uncontaminated with the other.

The method resulted in an overall 50-fold purification of *N*-acetyl-β-glucosaminidase A and a sevenfold purification of *N*-acetyl-β-glucosaminidase B, although starch-gel electrophoresis showed that both preparations were still heterogeneous. The preparations were stored in the frozen state until required.

It was noted in step 2 that no separation of the two forms took place on gel filtration. Similarly, a mixture of the partially purified forms A and B (0·04ml.), when passed through a Sephadex G-200 column as described by Robinson *et al.* (1967), emerged as a single peak with an elution volume corresponding to mol.wt. approx. 100000.

Characterization of N-*acetyl-β-glucosaminidases A and B*

Ionic effects. Both enzymes were sensitive to the buffer constituents, showing symmetrical pH–activity curves with an optimum at 4·5–5·0 in sodium citrate buffer (50mM) or phosphate–citrate buffer (McIlvaine, 1921) whereas in sodium acetate buffer (50mM) the pH optimum was 5·5 and the curve was depressed on the acid side of the optimum. A similar situation was obtained with the *N*-acetyl-β-glucosaminidase of rat kidney (Robinson *et al.* 1967). Assays performed in citrate buffers in the concentration range 1·5–25mM at pH4·5 showed no differences in activity. The incorporation of phosphate produced slight inhibition, reaching 20% inhibition at 20mM. The effect of acetate was more pronounced and reached 80% inhibition at 20mM-acetate.

Stability to pH *changes.* Suitably diluted samples of the two enzymes were compared by maintaining the solution at 37° for 10min. in 5mM-phosphate buffer containing crystalline bovine serum albumin (0·1%) in the range pH2–8, and assaying them in 0·1M-citrate buffer, pH4·5. It was found that *N*-acetyl-β-glucosaminidase B was stable under these conditions between pH3 and pH7, whereas component A was rapidly denatured if the pH was varied by more than 1 unit from the optimum of 4·5.

K_m values. Both components had the same K_m value ($6{\cdot}7 \times 10^{-4}$M) when assayed with 4-methylumbelliferyl *N*-acetyl-β-glucosaminide.

Heat stability. Forms A and B could be distinguished by their different stability at 50° in citrate–phosphate buffer, pH4·5, containing crystalline bovine serum albumin (0·1%). When kept under these conditions for various time-intervals, followed by assay at 37° in the usual way, both showed a slow denaturation, the half-life of component A being 10min. and that of component B 27min. At pH5·0 and 50° component B was almost completely stable for 30min., whereas component A had decayed by 65% in that time.

Effect of inhibitors. It had previously been noted (Caygill, 1966) that heparin and other acidic mucopolysaccharides were potent inhibitors of *N*-acetyl-β-glucosaminidases from various sources. Both purified components A and B showed 50% inhibition at heparin concentrations of 1·25i.u./ml., but this inhibition was not increased by further addition of heparin and could be partially reversed by the addition of calcium chloride solution. Both enzymic forms were completely inhibited by *N*-acetylgalactosaminolactone concentrations of 0·1mM, which is consistent with the observation of Walker *et al.* (1961) that *N*-acetyl-β-glucosaminidase of rat kidney was not specific for the orientation of the hydroxyl group at C-4 and was able to bind the corresponding galactosamine substrates. Inhibition by the products of the reaction was also apparent. Enzymes A and B both reacted similarly to the presence of *N*-acetylglucosamine and *N*-acetylgalactosamine; the latter was particularly effective and produced 70% inhibition at a concentration of 1mM.

Under standard assay conditions the thiol reagent *N*-ethylmaleimide gave only 10% inhibition at 10mM, suggesting that reactive thiol groups were not essential components of the active site. The two forms showed a quantitative difference in their reaction to dithiothreitol. A maximum of 15% inhibition with enzyme A and one of 30% inhibition with enzyme B were noted when the inhibitor concentration reached 0·04M, increased concentrations up to 0·1mM having no further effect.

Ratio of N-*acetylglucosaminidase to* N-*acetylgalactosaminidase activities of components A and B*

The activities were compared at pH4·5 in citrate buffer by using the colorimetric method of Walker

et al. (1961) with 1·7mM-*p*-nitrophenyl glycosides as substrates. Both components A and B showed activity towards both substrates with a glucosaminidase/galactosaminidase ratio of 8·6.

Factors influencing the electrophoretic mobility of N-acetyl-β-glucosaminidases A and B

The ready association of heparin with these enzymes was further illustrated when heparin-inhibited enzyme samples (1·25i.u. of heparin/ml.) were examined by electrophoresis. The binding with component B was sufficiently strong to produce a negatively charged complex and reverse the direction of migration, although the mobility of the derivative was not increased to that of component A. The latter form was not visibly affected by this treatment, but the addition of salmine sulphate (2mg./ml.), on the other hand, destroyed its anodic mobility so that the detectable enzyme activity remained at the origin. When solutions of component A were allowed to denature slowly at room temperature it was possible to observe its apparent conversion into a form resembling component B before all activity disappeared. Treatment of components A and B by incubation with 1mg. of lecithinase D/ml. for 18hr. at room temperature in 0·1M-sodium citrate buffer, pH5·5, made no change in the electrophoretic mobility. Similarly treatment with wheat-germ acid phosphatase (1mg./ml. in 0·1M-citrate buffer, pH4·5) for 4hr. at room temperature did not produce any effect, nor did extraction by shaking enzyme solutions with 2-methylpropan-1-ol.

Action of neuraminidase on N-acetyl-β-glucosaminidase A. The acidic glycoprotein transferrin has been shown by Parker & Bearn (1963) to contain four sialic acid groups that can be successively removed by neuraminidase to give a sequence of progressively slower-moving components on electrophoresis, the rate of travel being dependent on the number of sialic acid groups remaining. A similar case is that of human serum alkaline phosphatase described by Robinson & Pierce (1964). The effect of neuraminidase on purified *N*-acetyl-β-glucosaminidase A was to produce a number of forms, each showing a stepwise decrease in anodic mobility. Neuraminidase R.D.E. was found to be free of glucosaminidase and thus suitable for this purpose. Samples (0·1ml.) of the enzyme solution obtained from the preparative procedure above were incubated with 0·1ml. of 0·1M-phosphate–citrate buffer, pH5·6, containing calcium chloride (20mM) and 0·2ml. of neuraminidase, either in the commercial form (100 units/ml.) or diluted four- or eight-fold. Control experiments in the absence of neuraminidase were also performed. Samples (0·05ml.) were removed immediately and after incubation at 37° for 3, 6 or 9hr. The samples were immediately frozen until required, and were subjected to electrophoresis side by side in starch gel as described above. The results (Fig. 1) showed that new bands of glucosaminidase activity were found even in samples kept frozen and that these were subsequently transformed into slower-moving components during the longer incubation experiments at 37°, the final result being dependent on time of incubation and neuraminidase concentration. Some non-enzymic decomposition took place and after 6hr. the control sample showed a new component similar in electrophoretic mobility to enzyme B, although no intermediate stages in its spontaneous formation could be detected.

If sufficient neuraminidase was added (0·5ml./0·1ml. of enzyme sample) the conversion of form A into form B took place without the intermediate stages being detectable. Quantitative scans of the fluorescent bands that developed on the gels, with an Aminco–Bowman fluoromicrophotometer fitted with a thin-layer scanning attachment, indicated that up to 45% of the original activity could be recovered in this form. Similar scans of control samples that had been spontaneously converted into form B before complete denaturation yielded not more than 18% of the original activity in this form. The mechanism of this spontaneous decomposition is not known, but the effect is in agreement with the fact that form A was less stable than form B or the intermediate forms.

Samples of component B were unaffected when treated as described above.

Intracellular distribution of N-acetyl-β-glucosaminidases A and B in human spleen

A fresh sample of human spleen was obtained at surgery and was fractionated by differential centrifugation. The distribution of *N*-acetyl-β-

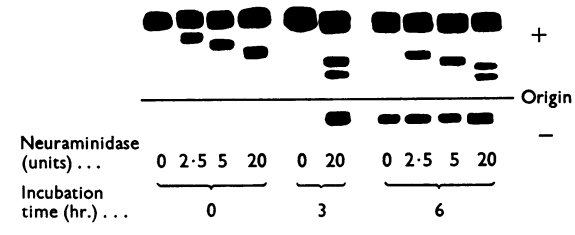

Fig. 1. Effect of neuraminidase on the electrophoretic mobility of *N*-acetyl-β-glucosaminidase A. Purified enzyme (0·1ml.) was incubated with neuraminidase R.D.E. (0·2ml.) and 0·1M-phosphate–citrate buffer, pH5·6, containing $CaCl_2$ (20mM). Samples (0·05ml.) were removed immediately and after 3 and 6hr. at 37° for electrophoresis as described in the text.

Table 2. *Distribution of* N-*acetyl-β-glucosaminidase and other hydrolases in subcellular fractions of human spleen*

Activities were measured as described in the text both in the absence and in the presence of 0·1% Triton X-100. Free activity is expressed as the percentage that is measurable in the absence of Triton X-100 of the total activity of the fraction.

	N-Acetyl-β-glucosaminidase		Acid phosphatase		β-Galactosidase		β-Glucuronidase	
Fraction	% of total activity of homogenate	Free activity in each fraction (% of total)	% of total activity of homogenate	Free activity in each fraction (% of total)	% of total activity of homogenate	Free activity in each fraction (% of total)	% of total activity of homogenate	Free activity in each fraction (% of total)
N	17	13	16	49	16	13	16	18
HM	15	8	33	14	16	9	15·5	17
LM	34	5	27	24	39	5	38	10·5
Mic	6	14	3	75	5	13·5	11	23
S	28	100	21	88	24	100	19·5	86

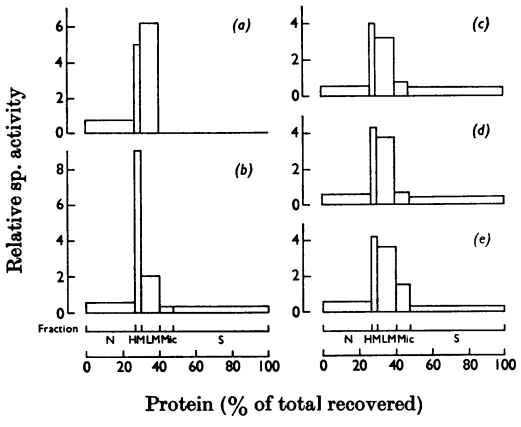

Fig. 2. Distribution pattern of *N*-acetyl-β-glucosaminidase and other enzymes in subcellular fractions of human spleen: (*a*) cytochrome oxidase; (*b*) acid phosphatase; (*c*) *N*-acetyl-β-glucosaminidase; (*d*) β-galactosidase; (*e*) β-glucuronidase. The conditions for isolation of the fractions and enzyme assays are described in the text. 'Relative specific activity' (% of total activity recovered/% of total protein recovered in that fraction) is represented on the ordinate and percentage recovery of protein on the abscissa.

glucosaminidase between the various fractions was assayed, and compared with those of β-galactosidase, β-glucuronidase and acid phosphatase, three typical lysosomal enzymes. In addition the degree to which each of the four enzymes existed as latent activities in the tissue fractions was measured by assaying them in the presence and absence of Triton X-100.

The results (Table 2) showed that 70–80% of the total activity of any of these enzymes was sedimentable and that a good deal of this was present in the 'nuclear' fraction, fraction N. All four enzymes showed a high degree of latency consistent with their occurrence within lysosome particles, and the highest specific activity was found in the heavy mitochondrial fraction (Fig. 2). The lysosomes of human spleen appeared to have a wider range of sizes than those of rat liver, and the different distribution of acid phosphatase compared with that of the other hydrolases made it possible that the lysosome population was heterogeneous with regard to enzyme content. An examination of rat spleen enzymes by Bowers & de Duve (1967) also emphasized the variations in distribution and the relatively large amount of activity in the N fraction when rat spleen is compared with rat liver.

The sucrose-washed sedimentable fractions were recentrifuged and then suspended in a small volume of water to produce a soluble enzyme concentrate by osmotic lysis. This was examined on gel electrophoresis as described above. *N*-Acetyl-β-glucosaminidases A and B both occurred in the N, HM and LM fractions, with component A predominating in the HM fraction. The 'ghost' membrane sediment from lysis of the LM fraction also contained both A and B forms. The soluble fraction, however, appeared to contain only the A form.

DISCUSSION

The *N*-acetyl-β-glucosaminidase of human spleen obtained by our extraction procedures is present in two distinct forms comprising roughly equal proportions of the total activity as assayed with 4-methylumbelliferyl *N*-acetyl-β-glucosaminide. The separation of the ram testicular enzyme on CM-cellulose reported by Caygill, Roston & Jevons (1966) appeared to show a similar situation in this tissue. The two forms reported here have similar

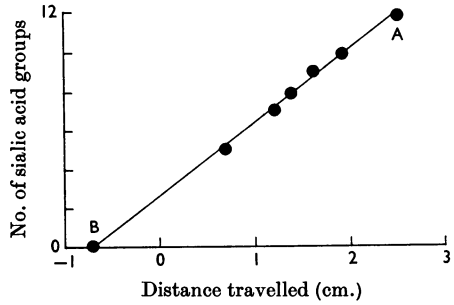

Fig. 3. Electrophoretic mobility of degradation products of *N*-acetyl-β-glucosaminidase A after treatment with neuraminidase. The positions of observed intermediates were plotted on a linear graph between the positions of forms A and B. The ordinate assumes that the minimum distance between two intermediates represents the removal of a single sialic acid group.

enzyme characteristics when assayed with synthetic substrates, but are of different stabilities to heat and pH changes, *N*-acetyl-β-glucosaminidase A being the less stable of the two.

It is generally assumed that the function of *N*-acetyl-β-glucosaminidase is to contribute to the hydrolytic degradation of glycoproteins, mucopolysaccharides or glycolipids. The spleen may be expected to play an active role in this respect and natural substrates may include the erythrocyte-membrane glycoproteins, blood-group substances and γ-globulins, all of which contain significant amounts of *N*-acetylhexosamine.

The existence of two enzymes in the same tissue having apparently identical actions is somewhat difficult to explain. One of these, *N*-acetyl-β-glucosaminidase A, could be itself a glycoprotein with sialic acid residues contributing to its overall acidity. This is supported by the changes in electrophoretic mobility that occur after attack by neuraminidase (Fig. 1), suggesting the stepwise removal of sialic acid residues. The change in electrophoretic mobility is roughly the same at each successive step (Fig. 3) and, on this basis, it is seen that it would require at least 12 molecules of sialic acid to be removed to explain the transformation of *N*-acetyl-β-glucosaminidase A into the basic form B. The intermediate forms would then represent the elimination of 2, 3, 4, 5 and 7 molecules of sialic acid in decreasing order of electrophoretic mobility. Since no evidence has been found to indicate any significant differences in molecular weight, as shown by molecular-sieving experiments, form A could be envisaged as a glycoprotein consisting of form B with up to 12 short carbohydrate side chains terminating with a sialic acid residue. Proof of this must await preparation of sufficiently pure samples of A and B for carbohydrate analysis to be meaningful.

The hypothesis on the biological role of glycoproteins expounded by Eylar (1965) would suggest that form A might represent an extracellular activity. Our preliminary experiments on the cellular fractionation of rat spleen indicated that two forms of the enzyme existed, that associated with the lysosomes (Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960) being the slower migrating form, whereas a more negatively charged species remains unsedimentable. The human spleen whose examination after subcellular fractionation is reported here was a pathological sample and grossly enlarged, but the condition had little effect on the integrity of the lysosome particles as judged by the high degree of latency they exhibited. In this case also the unsedimentable fraction contained only the type A *N*-acetyl-β-glucosaminidase, but it was clear that both forms were present in the sedimentable particles, and the two forms do not clearly indicate different cellular locations. The occurrence of form A in the debris could be due either to agglutination of microsomes or to the easily reversible adsorption of soluble activity on insoluble tissue components (Pugh, Leaback & Walker, 1957). An alternative explanation is that enzyme A represents a form present in membranes, and the specificity of such an enzyme would offer a possible 'recognition site' for transport of *N*-acetylglycosamine-containing substances or some other membrane reaction. A similar location and possible non-hydrolytic function has been suggested for β-glucuronidase (Fishman, Goldman & De Lellis, 1967). An examination of the enzymes present in macrophages from human peritoneal diffusate showed two forms of *N*-acetyl-β-glucosaminidase, which corresponded on electrophoresis with the two forms obtained from whole spleen. It is thus clear that both forms can occur in a single cell type.

A recent report of multiple forms of *N*-acetyl-β-glucosaminidase in calf brain reopens the question of specificity. Frohwein & Gatt (1966) found a particulate enzyme with both glucosaminidase and galactosaminidase activity and also two C-4-specific supernatant enzymes. The last two enzymes were obtained by having present either dithiothreitol or *N*-ethylmaleimide during the extraction, and it is possible that the substrate-binding activity of a single species may have been altered by the presence of one or other of these reagents to give the semblance of two distinct forms. It is not clear whether the presence of both these forms was ever demonstrated in the same preparation. Our inhibition studies with the same reagents gave no evidence that maintenance of thiol groups in the reduced state enhanced *N* - acetyl - β galactos - aminidase activity or that thiol-group blocking

favoured *N*-acetylglucosaminidase activity. The situation in brain tissue is more complex than in spleen and a preliminary examination of the human brain enzyme has shown a number of components on electrophoresis. The enzymes described in this paper both hydrolysed *N*-acetyl-β-glucosaminides and *N*-acetyl-β-galactosaminides and were inhibited by the corresponding glycosaminolactones and free sugars. There did not therefore appear to be complete C-4 specificity, although the preferred form for hydrolysis was the glucosaminide. The more potent inhibition by *N*-acetylgalactosamine compared with *N*-acetylglucosamine might indicate that the rate of hydrolysis of *N*-acetylgalactosaminides is limited by the slower dissociation of an enzyme–*N*-acetylgalactosamine complex.

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