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SIALYLATION IN VITRO OF PURIFIED HUMAN LIVER β-D-*N*-ACETYLHEXOSAMINIDASE *

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In order to study structure-function relationships of lysosomal enzymes, human liver β-*N*-acetylhexosaminidase (2-acetamido-2-deoxy-β-D-hexoside acetamidodeoxyhexohydrolase, EC 3 2 1 52) has been purified by an extraction/affinity chromatography/ion-exchange procedure The isoenzymes A and B, native as well as neuraminidase-treated, were incubated with a partially purified preparation of bovine colostrum sialyltransferase (CMP-*N*-acetylneuraminate D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2 4 99 1) Native β-*N*-acetylhexosaminidases were found to be poor acceptors for the sialyltransferase used However, incorporation of sialic acid into neuraminidase-treated β-*N*-acetylhexosaminidase A and B amounted to a 58 and 72% saturation of the theoretical acceptor sites, respectively The acceptor specificity of the sialyltransferase suggests that Galβ(1 → 4)-GlcNAc units may be present on at least part of the β-*N*-acetylhexosaminidase A and B molecules However, oligomannosidic-type chains may also occur on the lysosomal enzyme, as shown by sugar composition of the enzyme The presence and/or amount of sialic acid residues does not appear to affect the kinetic properties of β-*N*-acetylhexosaminidase A and B towards 4-methylumbelliferyl glycoside substrate

Introduction

The carbohydrate part of glycoproteins can play an important role in molecule-membrane interactions It was shown that the information for receptor-mediated uptake of glycoproteins by liver cells in vivo is contained in the structure of their oligosaccharide chains [1] Similarly, the recognition of an 'uptake marker' on the carbohydrate portion of lysosomal enzymes by lectin-like cell membrane receptors is a prerequisite for adsorption and specific endocytosis of these enzymes by various cell types [2] A defect in the formation of the marker structure could prevent the uptake of lysosomal enzymes by cells such as fibroblasts [3,4] Such a defect might be the cause of at least one severe disorder, the I-cell disease The lysosomal enzymes isolated from the culture medium of I-cell disease fibroblasts, unlike those from normal cell culture medium, are not specifically taken up by fibroblasts in vitro [4] Moreover, an elevation of lysosomal enzyme activity is observed in the extracellular fluids of I-cell disease patients [5], as well as in the culture medium from I-cell disease fibroblasts [6]

Several lysosomal enzymes have been purified and their carbohydrate compositions determined [7–12] All purified enzymes were shown to contain mannose, galactose and *N*-acetylglucosamine, whereas fucose, sialic acid and glucose were found in a few instances only With the exception of human urine

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Abbreviations CMP-AcNeu, cytidine 5'monophospho-*N*-acetylneuraminic acid, AcNeu, *N*-acetylneuraminic acid, GlcNAc, *N*-acetyl-D-glucosamine, GalNAc-Sepharose, *p*-aminophenyl-2-acetamido-2-deoxy-β-D-galactopyranoside derivatized CH-Sepharose, All sugars are of the D-configuration, unless otherwise noted SDS, sodium dodecyl sulfate

α-*N*-acetylglucosaminidase (EC 3 2 1 50) [13] no *N*-acetylgalactosamine could be detected in the enzymes These carbohydrate compositions are consistent with the view that *N*-glycosidically linked oligosaccharide chains prevail on the lysosomal enzymes

In view of our interest in the structure-function relationships of lysosomal enzymes we purified β-*N*-acetylhexosaminidase from human liver Specific modification of the carbohydrate portion of the lysosomal enzyme was accomplished by sialylation in vitro using a partially purified sialyltransferase from bovine colostrum [14] and the sialylated products were characterized

Materials and Methods

Materials

Human liver was kindly supplied by Dr R Donner from the AZVU hospital in Amsterdam and stored at −40°C until use Bovine serum albumin, *N*-acetylglucosamine, CDP-ethanolamine and ethanolamine were purchased from Sigma (St Louis, U S A) and 4-methylumbelliferyl glycosides, naphthol AS-BI-β-*N*-acetylglucosaminide and α-methylmannoside from Koch-Light Laboratories (Colnbrook, U K) CH-Sepharose 4B, DEAE-Sephadex A-50, CNBr-activated Sepharose 4B and Con A-Sepharose 4B were obtained from Pharmacia (Sweden) *Vibrio cholerae* neuraminidase was from Behringwerke (Marburg, F R G) En^{3}Hance solution and CMP-[^{14}C]AcNeu (specific activity 276 Ci/mol) were obtained from New England Nuclear (Boston, U S A) The sugar nucleotide was diluted with unlabeled CMP-AcNeu [15] to the desired specific radioactivity α_1-Acid glycoprotein used in these studies was provided by the American Red Cross National Fractionation Center with the partial support of the National Institutes of Health Grant No HL 13881 Desialylation of the α_1-acid glycoprotein was carried out as previously described [16] All other reagents were of analytical grade or the best grade available

Methods

Purification of β-N-acetylhexosaminidase All operations were carried out at 0–4°C unless otherwise stated Human liver (650 g) was homogenized in 4 vol (v/w) distilled water in a Sorvall Omnimixer at 16 000 rev /min for 3 × 20 s After gentle stirring for 1 h the homogenate was centrifuged for 60 min at 100 000 × *g* The pellet was resuspended in 0 1 M NaCl and then sonicated for 20 s After 1 h of stirring the suspension was centrifuged as before The supernatant solutions were combined and were brought to 0 05 M sodium phosphate buffer, pH 7 0, and 0 5 M NaCl

The resulting solution was applied to a column (5 × 5 cm) of Con A-Sepharose equilibrated in 0 05 M sodium phosphate buffer, pH 7 0/0 5 M NaCl, at a flow rate of 200 ml/h After washing with 5 bed vol of the equilibration buffer, bound glycoproteins were eluted at 20°C with a gradient of α-methylmannoside from 0–0 75 M in the same buffer at a flow rate of 100 ml/h The β-*N*-acetylhexosaminidase-containing fractions were pooled, concentrated 20-fold by ultrafiltration in an Amicon cell fitted with a PM-10 membrane and dialyzed for 16 h against 100 vol 0 05 M sodium phosphate buffer, pH 7 4/0 15 M NaCl

Affinity chromatography on a GalNAc-Sepharose column (5 0 × 2 5 cm) was carried out as previously described [17] After elution of the β-*N*-acetylhexosaminidase the enzyme-containing fractions were dialyzed for 16 h against 30 vol 0 05 M Tris-HCl buffer, pH 7 0/0 05 M KCl The retentate was concentrated by ultrafiltration

The isoenzymes A and B were separated by ion-exchange chromatography on a column (1 5 × 15 cm) of DEAE-Sephadex A-50 equilibrated in 0 05 M Tris-HCl buffer, pH 7 0/0 05 M KCl β-*N*-Acetylhexosaminidase B did not bind to the column, whereas β-*N*-acetylhexosaminidase A was eluted with a KCl-gradient from 0 05 to 0 55 M in the same Tris buffer Enzyme-containing fractions were pooled, dialyzed for 72 h against 30 vol distilled water (with changes at 24 and 48 h) and lyophilized The dry residue was taken up in a small volume of water and stored at −40°C

Characterization and analysis of purified β-N-acetylhexosaminidase The sugar compositions of the purified isoenzymes A and B were determined by gas-liquid chromatography as described by Reinhold [18], whereby the de-*O*-acetylation was carried out in 1 M NH_4OH in methanol for 30 min at 65°C

Polyacrylamide gel electrophoresis was carried out on 12% slab gels (0 1 × 9 0 cm) in the presence of

0 1% SDS, according to methods described in Ref 19 The samples, containing 5–50 μg protein in a maximal volume of 30 μl, were applied to the gel after treatment with 1% SDS and 0 1 M dithiothreitol at 100°C for 10 min, followed by addition of bromophenol blue and glycerol to a final concentration of 0 001 and 10%, respectively Electrophoresis was started with a current of 4 mA per gel, which was increased to 20 mA per gel when the samples had reached the separation gel Protein bands were visualized with 0 1% Coomassie brillant blue R-250 in H_2O/acetic acid/methanol (4 1 5, v/v) The molecular weight of the subunits was determined from a plot of the logarithms of the molecular weight of marker proteins vs their migration distance [20]

Gel electrophoresis on 6 5% slab gels was carried out in a similar way, with omission of SDS from the sample and from all buffers The gels were stained for enzymatic activity using 0 5 mM 2-naphthyl-AS-BI-β-*N*-acetylglucosaminide in 0 1 M sodium citrate buffer pH 4 5, to which shortly before use Fast Black (Serva, Heidelberg, F R G) had been added to a concentration of 1 0 mg/ml according to the procedure described in Ref 21

Isoelectric focusing on thin-layer polyacrylamide gels was performed on PAG-plates No 1804-101 (LKB) in the pH range from 3 5–9 5, using Multiphor equipment in accordance with Ref 22 The pH gradient was determined with a surface electrode at 4°C Protein bands were stained with Coomassie blue and enzyme activity was visualized as described under gel electrophoresis

Purification of bovine colostrum β-galactoside α(2 → 6)sialyltransferase Defatted bovine colostrum was dialyzed against 10 mM sodium cacodylate buffer at pH 6 5 and applied to a column of CDP-ethanolamine-Sepharose Bound sialyltransferase activity was eluted with 0 5 M NaCl as described [14] After this purification step the sialyltransferase was concentrated and dialyzed against 0 2 M Tris-maleate buffer, pH 6 8 The purified enzyme was assayed for neuraminidase, β-galactosidase, β-galactoside α(2 → 3)sialyltransferase and α-*N*-acetylgalactosaminide α(2 → 6)-sialyltransferase activities [23,24]

Sialylation of β-N-acetylhexosaminidase Prior to incubation with sialyltransferase, purified β-*N*-acetylhexosaminidase A (2 500 μg protein) and B (2 500 μg protein) were each treated with 0 5 U *V cholerae* neuraminidase in a total volume of 700 μl containing 35 μmol sodium acetate buffer, pH 5 5/105 μmol NaCl/6 3 μmol $CaCl_2$ The mixture was incubated for 3 5 h at 37°C in a dialysis bag immersed in 50 ml 0 050 M sodium acetate buffer, pH 5 5/0 15 M NaCl/ 9 mM $CaCl_2$ Controls contained neuraminidase which had been heated at 100°C for 10 min The desialylated enzyme was separated from neuraminidase activity by affinity chromatography on GalNAc-Sepharose as described [17] Released sialic acid was measured in the dialysis buffer with the periodate-resorcinol assay [25]

Sialylation was carried out as follows To suppress incorporation of ^{14}C-label into endogenous acceptors, the sialyltransferase preparation (45 mU) was preincubated with 1 0 μmol unlabeled CMP-AcNeu and 0 5 mmol of Tris-maleate buffer, pH 6 8, in a total volume of 2 5 ml, for 1 h at 37°C Desialylated β-*N*-acetylhexosaminidase A (230 μg protein, corresponding to 5 3 nmol theoretical acceptor sites as calculated from the number of galactosyl residues assayed by gas-liquid chromatography) or desialylated β-*N*-acetylhexosaminidase B (180 μg protein, corresponding to 4 9 nmol galactosyl residues) was added to a mixture containing 1 8 mU preincubated sialyltransferase/220 nmol CMP-[^{14}C]AcNeu (0 90 Ci/mol)/20 μmol Tris-maleate buffer, pH 6 8, in a total volume of 100 μl, and was incubated at 37°C After 2 h, additional amounts of sialyltransferase (1 8 mU), CMP-[^{14}C]AcNeu (220 nmol) and Tris-maleate (20 μmol) were added in a volume of 100 μl and incubation was continued for 22 h Sialylation of native β-*N*-acetylhexosaminidase A (200 μg protein, 2 2 nmol theoretical acceptor sites as calculated from the number of galactosyl residues minus the number of sialic acid residues determined by gas-liquid chromatography) and native β-*N*-acetylhexosaminidase B (200 μg protein, 2 4 nmol of theoretical acceptor sites) was performed in an analogous way The incubation volume was such that the concentrations of theoretical acceptor sites, sialyltransferase and nucleotide sugar were the same as in the incubation mixtures of the desialylated enzymes The time course of the incorporation of sialic acid into β-*N*-acetylhexosaminidase was followed by acid-precipitation of aliquots of the reaction mixture at different times The amount of radioactivity incorporated into acid-precipitable material was assayed as

described in Ref 16 with omission of the chloroform/methanol extraction step Control incubations lacking β-*N*-acetylhexosaminidase were run to assay the incorporation of [^{14}C]AcNeu into endogenous acceptors

Isolation and characterization of the sialylated products After incubation the reaction mixture was subjected to gel filtration on a column (1 5 × 47 cm) of Bio-Gel P-4, 200–400 mesh, equilibrated in 0 05 M sodium phosphate buffer, pH 7 4/0 15 M NaCl The high-molecular weight fractions were pooled and applied to a column (1 × 4 cm) of GalNAc-Sepharose After washing the column with 0 05 M sodium phosphate buffer, pH 7 4/0 15 M NaCl the retained β-*N*-acetylhexosaminidase was eluted with 100 mg *N*-acetylglucosamine/ml, in the same buffer Fractions of 5-ml were collected and assayed for β-*N*-acetylhexosaminidase activity and radioactivity Those containing enzyme activity were pooled, dialyzed against distilled water and lyophilized The dry residue was taken up in a small volume of water and stored at −20°C until use The sialylated endogenous acceptor did not adsorb to the affinity column This product was isolated similarly by pooling of the appropriate fractions followed by dialysis and lyophilization

The isolated radioactive materials were characterized by isoelectric focusing on polyacrylamide gels (pH 3 5–9 5) The gels were stained for β-*N*-acetylhexosaminidase activity and the radioactivity was counted in 3-mm slices which had been solubilized in H_2O_2 in screw-capped vials at 60°C for 8 h Alternatively, gels were soaked in En^{3}Hance solution, dried and subjected to fluorography [26] using RP Royal X-Omat X-Ray film (Kodak) Further characterization of the [^{14}C]sialylated materials was accomplished by incubation with *V cholerae* neuraminidase The digests were analyzed by gel filtration on a column (1 5 × 47 cm) of Bio-Gel P-4, 200–400 mesh, in 0 05 M sodium phosphate buffer, pH 7 4/0 15 M NaCl

Enzyme and protein assays Lysosomal glycosidase activities were assayed with 4-methylumbelliferyl glycosides dissolved to a concentration of 1 2 mM in 0 1 M citric acid/0 2 M Na_2HPO_4 buffer, pH 4 5, in the presence of 0 1 mg bovine serum albumin/ml buffer The incubation mixture was composed of 20 μl enzyme solution/100 μl substrate solution Incubations were carried out at 37°C for 15 min. The reaction was stopped with 100 μl 5% trichloroacetic acid and the liberated 4-methylumbelliferone was measured in 200 μl of the mixture after addition of 2 ml of a buffer containing 0 5 M Na_2CO_3 adjusted to pH 10 5 with glycine Enzymes assayed were α-galactosidase (EC 3 2 1 22), β-galactosidase (EC 3 2 1 23), α-mannosidase (EC 3 2 1 24), β-*N*-acetylglucosaminidase (EC 3 2 1 30), β-*N*-acetylgalactosaminidase, β-xylosidase (EC 3 2 1 37) and α-glucosidase (EC 3 2 1.20) β-Glucuronidase (EC 3 2 1 31) was assayed in 0 1 M sodium acetate buffer at pH 4 2 in an analogous way, and α-*N*-acetylglucosaminidase (EC 3 2 1 50) was assayed with *p*-nitrophenyl-α-*N*-acetylglucosaminide [27] Neuraminidase (EC 3 2 1 18) activity was determined using sialyllactit-[^{3}H]ol as a substrate at pH 4 2, as described in Ref 28 1 unit enzyme activity is defined as the amount of enzyme hydrolyzing 1 μmol substrate/min under the assay conditions β-Galactoside α(2 → 6)sialyltransferase was assayed as described in Ref 35 β-Galactoside α(2 → 3)sialyltransferase and α-*N*-acetylgalactosaminide α(2 → 6)sialyltransferase activities were assayed in an analogous way, using desialylated/defucosylated porcine submaxillary mucin as an acceptor 1 unit transferase activity is defined as the amount of enzyme transferring 1 μmol AcNeu/min to the acceptor using the assay system Protein determinations were carried out by the method of Lowry with bovine serum albumin as a standard [29]

Kinetic studies Apparent Michaelis constants (K_m values) were determined for the native, the neuraminidase-treated and the resialylated forms of β-*N*-acetylhexosaminidase A and B from an Eady-Hofstee plot Incubations were carried out in triplicate for 15 min at 37°C with varying concentrations of 4-methylumbelliferyl β-*N*-acetylglucosaminide in 0 1 M sodium citrate-phosphate buffer, pH 4 5

The pH vs activity curves of native, neuraminidase-treated and resialylated β-*N*-acetylhexosaminidase A and B were determined as follows To 20 μl of enzyme solution 100 μl 0 1 M sodium citrate-phosphate buffer of varying pH (3 6–6 4) containing 2 4 mM 4-methylumbelliferyl β-*N*-acetylglucosaminide were added The incubations were carried out in duplicate for 15 min at 37°C

Results

Purification of human liver β-N-acetylhexosaminidase

Human liver β-*N*-acetylhexosaminidase A and B were conveniently purified by the described procedure in an overall yield of 34% and a 4 000–8 000-fold enrichment (Table I) Differential assay of the isoenzymes A and B, based upon their separation on DEAE-Sephadex, showed that approx 55% of the total β-*N*-acetylhexosaminidase activity in liver is due to the isoenzyme A Therefore the yield of β-*N*-acetylhexosaminidase B appeared to be higher than that of form A However, calculation of separate yields of the isoenzymes A and B may be influenced by the spontaneous conversion of β-*N*-acetylhexosaminidase A to a B-like form during purification and storage [30] The affinity chromatography steps on Con A-Sepharose and GalNAc-Sepharose were essential in the purification procedure (Table I) Approx 10% of the applied enzyme activity did not adsorb to the GalNAc-Sepharose column This non-adsorbing material appeared to consist predominantly of isoenzyme B Only 50–60% of the applied activity was eluted with *N*-acetylglucosamine It seems unlikely that the lost activity represents a specific fraction of the total enzyme, since isoelectric focusing patterns did not indicate a gross change in the charge heterogeneity of the enzyme at this purification step

The β-*N*-acetylgalactosaminidase activity in the final enzyme preparations amounted to 13% of the β-*N*-acetylglucosaminidase activity of isoenzyme A as well as of isoenzyme B This is in agreement with what could be expected from earlier work [31] for an enzyme specific for both substrates The contamination by other lysosomal enzyme activities appeared to be minimal in both isoenzyme preparations β-Glucuronidase activity in isoenzyme B was 0 4% and β-galactosidase activity in isoenzyme A was 0 3%, whereas all other glycosidase activities were less than 0 02% of the β-*N*-acetylglucosaminidase activity of the isoenzymes

Characterization and analysis of the purified enzyme

Polyacrylamide gel electrophoresis in the presence of SDS and after reduction with dithiothreitol showed a protein band with a molecular weight of 27 000 for β-*N*-acetylhexosaminidase A as well as B Isoenzyme A contained an additional band at 52 000, and in some preparations a minor band at 35 000 Both isoenzymes contained minor protein bands between 23 000 and 26 000 (Fig 1 1) The minor bands are thought to be degradation products of β-*N*-acetylhexosaminidase (cf Ref 43) Without

TABLE I

PURIFICATION OF β-*N*-ACETYLHEXOSAMINIDASE FROM HUMAN LIVER

Human liver β-*N*-acetylhexosaminidase was purified by the extraction/affinity chromatography/ion-exchange procedure which is described in Materials and Methods

Steps	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification factor
Crude homogenate	106 600	3 806	0 036	100	1
Extract	51 750	3 514	0 068	92	1 9
Con A-Sepharose	1 029	3 253	3 2	85	89
GalNAc-Sepharose	16 8	1 851	110	49	3 056
DEAE-Sephadex					
β-*N*-Acetylhexosaminidase A	8 0	610	76	29 *	3 800 *
β-*N*-Acetylhexosaminidase B	5 1	685	134	40 *	8 375 *

* Final yields and purification factors were calculated for a ratio of activities of β-*N*-acetylhexosaminidase A and B in the crude homogenate of 55 45 This ratio was determined from a differential assay of the isoenzymes after separation on a DEAE ion-exchanger, as recommended in Ref 29

reduction of the sample, isoenzyme A and B showed a major band with a molecular weight of 52 000 and 54 000, respectively

In the absence of SDS β-*N*-acetylhexosaminidase B showed one protein band, which was enzymatically active The pattern of isoenzyme A consisted of five enzymatically-active protein bands (Fig 1, gels 2 and 3)

The purified isoenzymes both showed a considerable heterogeneity on isoelectric focusing, with major enzyme activity bands at pH 4 8 for isoenzyme A and at pH 7 0, 7 1 and 7 2 for isoenzyme B (Fig 2, a, e) Traces of B-like activity are found in the

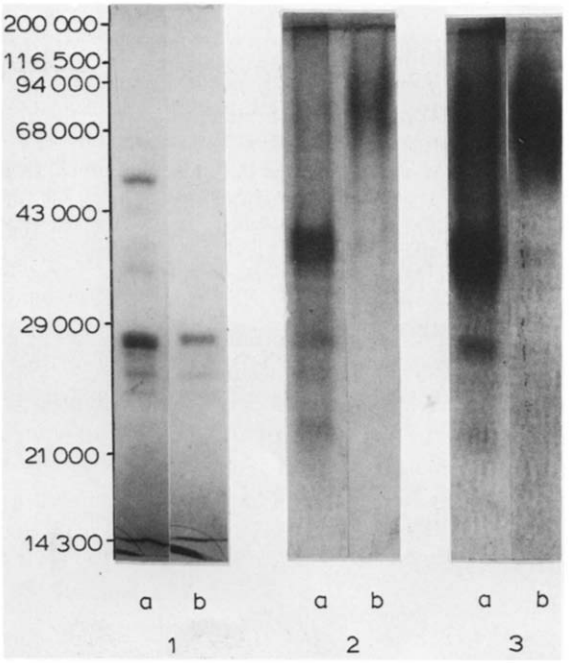

Fig 1 Polyacrylamide gel electrophoresis of human liver β-*N*-acetylhexosaminidase Electrophoresis was carried out according to the method described in Ref 19 using 12% polyacrylamide gels in the presence of SDS (panel 1) or 6 5% gels in the absence of SDS (panels 2 and 3) Gels were stained for protein with Coomassie blue (1, 2), or for enzyme activity with naphthol AS-BI-β-GlcNAc and Fast Black (3) Panel 1 lane a, β-*N*-acetylhexosaminidase A (8 μg protein), lane b, isoenzyme B (5 μg protein) Both samples were reduced with dithiothreitol prior to electrophoresis Migration distances of marker proteins are indicated Panel 2 lane a, isoenzyme A (32 μg protein), lane b, isoenzyme B (12 μg protein) Panel 3 lane a, isoenzyme A, lane b, isoenzyme B, each corresponding to 1 2 U of enzyme activity

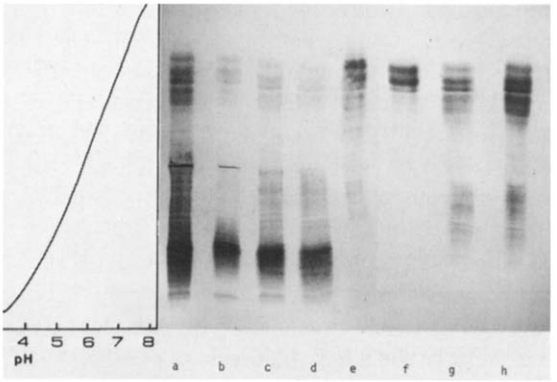

Fig 2 Isoelectric focusing patterns of β-*N*-acetylhexosaminidase A and B before and after desialylation and resialylation Isoelectric focusing of β-*N*-acetylhexosaminidase A and B was performed on polyacrylamide gel PAG-plates, at a pH gradient from 3 5–9 5 Enzyme activity was visualized with naphthol AS-BI-β-GlcNAc and Fast Black The patterns correspond to native (a), neuraminidase-treated (b), neuraminidase-treated, then resialylated (c) and native β-*N*-acetylhexosaminidase A incubated with sialyltransferase (d) Corresponding patterns for the B-enzyme are shown in e–h The pH gradient in the gel is indicated

purified A enzyme, whereas the preparation of β-*N*-acetylhexosaminidase B contains additional enzyme activity of intermediate isoelectric point This enzyme possibly corresponds to the previously

TABLE II

SUGAR COMPOSITION OF β-*N*-ACETYLHEXOSAMINIDASE PURIFIED FROM HUMAN LIVER

Carbohydrate compositions were determined by gas-liquid chromatography according to the method of Reinhold, the de-*O*-acetylation being carried out in 1 M NH_4OH in methanol [18] Results obtained with different batches of purified enzyme were generally consistent within 10%

Sugar	β-*N*-Acetylhexosaminidase A (nmol/mg protein)	β-*N*-Acetylhexosaminidase B (nmol/mg protein)
Fucose	⩽5	<5
Mannose	214	220
Galactose	26	34
N-Acetylglucosamine	62	65
N-Acetylgalactosamine	<5	<5
N-Acetylneuraminic acid	15	22

described I-form [32] All protein bands which were stained with Coomassie blue showed enzymic activity Due to neuraminidase treatment there is a shift in the activity of β-*N*-acetylhexosaminidase A and B from acidic to less acidic forms (Fig 2, b, f) Thus, it appears that at least part of the charge heterogeneity of both isoenzymes might be due to a different degree of sialylation

The purified β-*N*-acetylhexosaminidases A and B both contained mannose, galactose, *N*-acetylglucosamine and sialic acid (Table II) No *N*-acetylgalactosamine could be detected Although glucose was found in both isoenzymes, most of it appeared to be derived from dialysis tubing However, the possibility could not be excluded that some of it is a structural component of the oligosaccharide part of the enzyme Native β-*N*-acetylhexosaminidase A contained 26 nmol galactose and 15 nmol sialic acid/mg protein, enzyme B contained 34 nmol galactose and 22 nmol sialic acid/mg protein Upon neuraminidase treatment sialic acid was virtually completely removed from the isoenzymes A and B

Partial purification of bovine colostrum β-galactoside α(2 → 6)sialyltransferase

The purification step resulted routinely in a 1 000–2 000-fold purification of the sialyltransferase The purified enzyme contained no detectable neuraminidase, β-*N*-acetylhexosaminidase and β-galactosidase activity The activity of the β-galactoside α(2 → 3)sialyltransferase and the α-*N*-acetylgalactosaminide α(2 → 6)sialyltransferase together was less than 0 5% of the β-galactoside α(2 → 6)sialyltransferase activity The specific activity of the purified colostrum enzyme was between 25 and 30 mU/mg protein

Sialylation of β-N-acetylhexosaminidase

The native enzymes A and B, having 11 and 12 nmol theoretical acceptor sites/mg protein, respectively, appeared to be poor acceptors for the sialyltransferase from bovine colostrum (Fig 3) Incorporation of sialic acid proceeded slowly and even after prolonged incubation times the saturation of the acceptor sites increased only from 58 to 62% (isoenzyme A) and from 65 to 70% (isoenzyme B) However, incorporation into the neuraminidase-treated enzymes A and B, containing 23 and 27 nmol theoretical acceptor sites/mg protein, was much higher (Fig 3) After a rapid initial incorporation of sialic acid into desialylated β-*N*-acetylhexosaminidase A and B, further saturation of the acceptor sites took place at a much decreased rate, especially for the A enzyme The isoenzymes A and B were saturated up to 58 and 72%, respectively, in a 19 h incubation period (Fig 3)

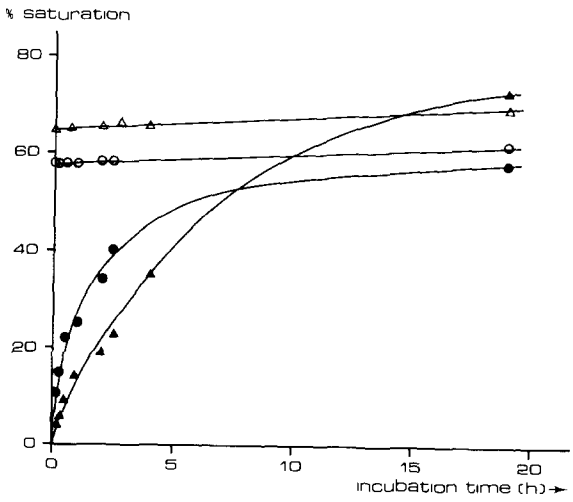

Fig 3 Incorporation of [^{14}C]AcNeu into β-*N*-acetylhexosaminidase A and B Native (○) and neuraminidase-treated (●) β-*N*-acetylhexosaminidase A, and native (△) and neuraminidase-treated (▲) β-*N*-acetylhexosaminidase B were incubated with sialyltransferase (18 mU/ml) and CMP-[^{14}C]AcNeu (2 20 mM) At different times the incorporation of sialic acid into acid precipitable material was assayed and the saturation of the theoretical acceptor sites calculated After 2 h more CMP-[^{14}C]AcNeu and sialyltransferase were added in amounts equal to that present initially

Characterization of the [^{14}C]sialylated products

The isolation of [^{14}C]sialylated β-*N*-acetylhexosaminidase by affinity chromatography on GalNAc-Sepharose led to complete removal of sialylated endogenous acceptor material but also to a small, inevitable loss of enzyme not binding to the affinity gel (Fig 4, a–e) At least 90% of the recovered enzyme activity and 50–90% of the recovered radioactivity were present in the fractions eluted with *N*-acetylglucosamine The radioactive product was not dialyzable, upon treatment with *V cholerae* neuraminidase, however, more than 85% of the label could be removed as a low-molecular weight compound

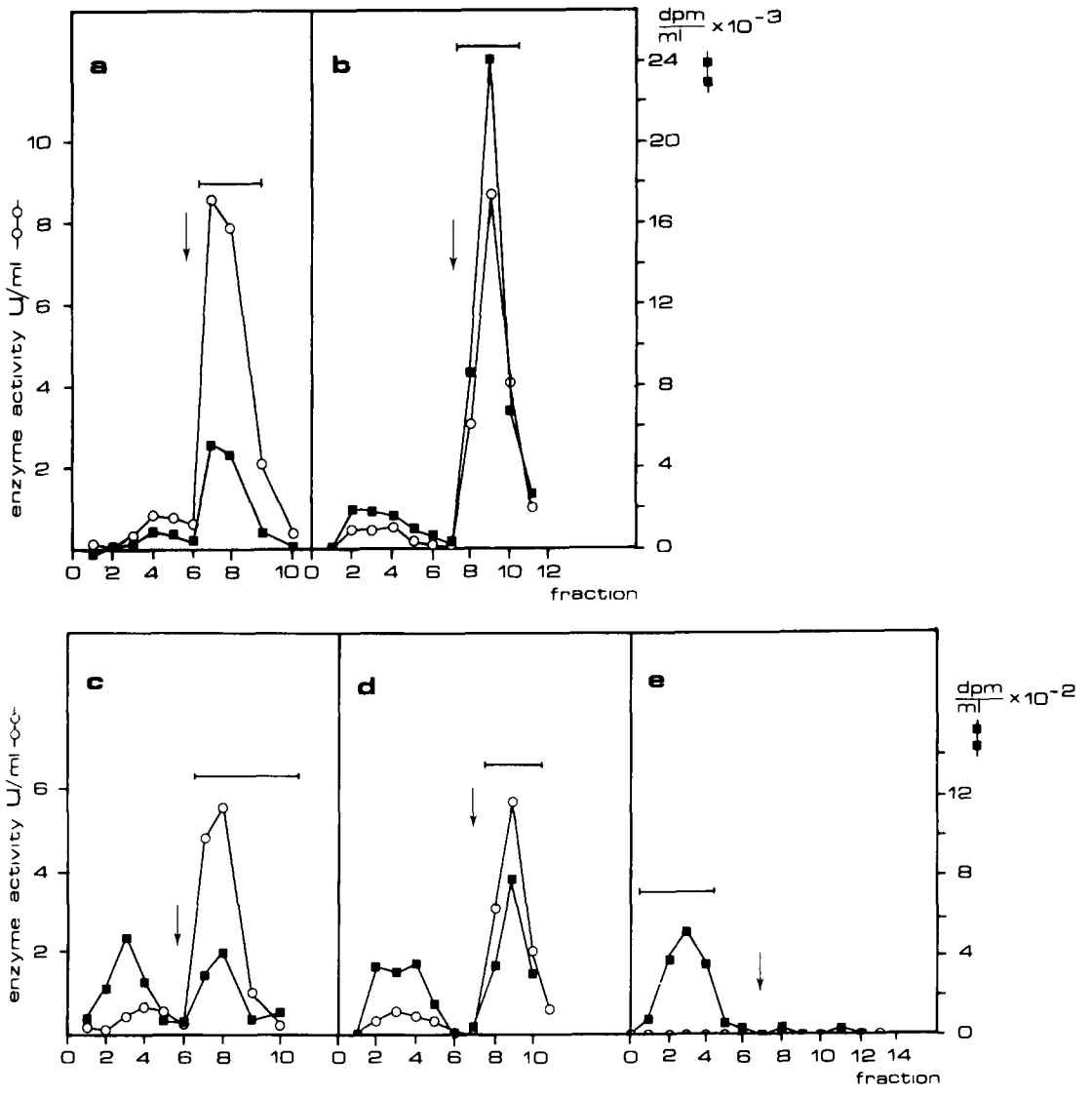

Fig 4 Affinity chromatography of in vitro [^{14}C]sialylated β-*N*-acetylhexosaminidase A and B The fractions containing material of high molecular weight, which had been obtained by gel filtration of the sialylation mixtures, were pooled and applied to a column (1 X 4 cm) of GalNAc-Sepharose The column was washed with equilibration buffer and eluted with 100 mg GlcNAc/ml in 0 05 M sodium phosphate buffer, pH 7 4, containing 0 15 M NaCl (arrow) Elution profiles correspond to the [^{14}C]sialylated products of neuraminidase-treated (a) and native (c) β-*N*-acetylhexosaminidase A, of neuraminidase-treated (b) and native (d) β-*N*-acetylhexosaminidase B, and of the endogenous acceptor (e) Fractions indicated by the bars were pooled for further studies ○———○, β-*N*-acetylhexosaminidase activity, ■———■, radioactivity

From the patterns obtained by polyacrylamide gel isoelectric focusing of the [^{14}C]sialylated β-*N*-acetylhexosaminidases, the native and the neuraminidase-treated enzymes (Fig 2), it is evident that the heterogeneity which was reduced upon neuraminidase treatment was restored after incubation with sialyltransferase Comparison of these enzyme-activity patterns with the densitometric recordings and the distribution of radioactivity in the gel for the [^{14}C]-sialylated products of neuraminidase-treated β-*N*-acetylhexosaminidase A and B (Fig 5) shows that the reappeared bands of enzyme activity were coinciding

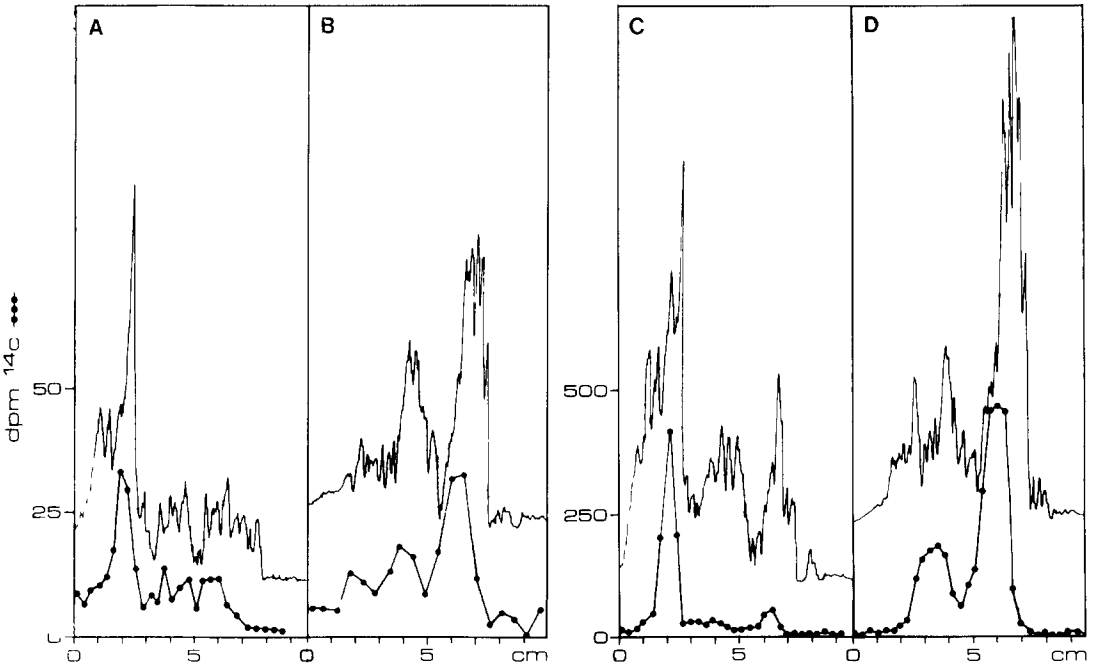

Fig 5 Incorporation of [^{14}C]AcNeu into β-*N*-acetylhexosaminidase A and B Distribution of radioactivity after isoelectric focusing After isoelectric focusing of in vitro [^{14}C]sialylated β-*N*-acetylhexosaminidase on polyacrylamide gel, enzyme activity was visualized with naphthol-AS-BI-β-GlcNAc and Fast Black and recorded using a densitometer Subsequently gels were cut into 3-mm slices, solubilized and radioactivity was counted Densitometric recordings (———) and distributions of radioactivity (•———•) are given for the [^{14}C]sialylated product of native β-*N*-acetylhexosaminidase A (A) and B (B) and of neuraminidase-treated β-*N*-acetylhexosaminidase A (C) and B (D)

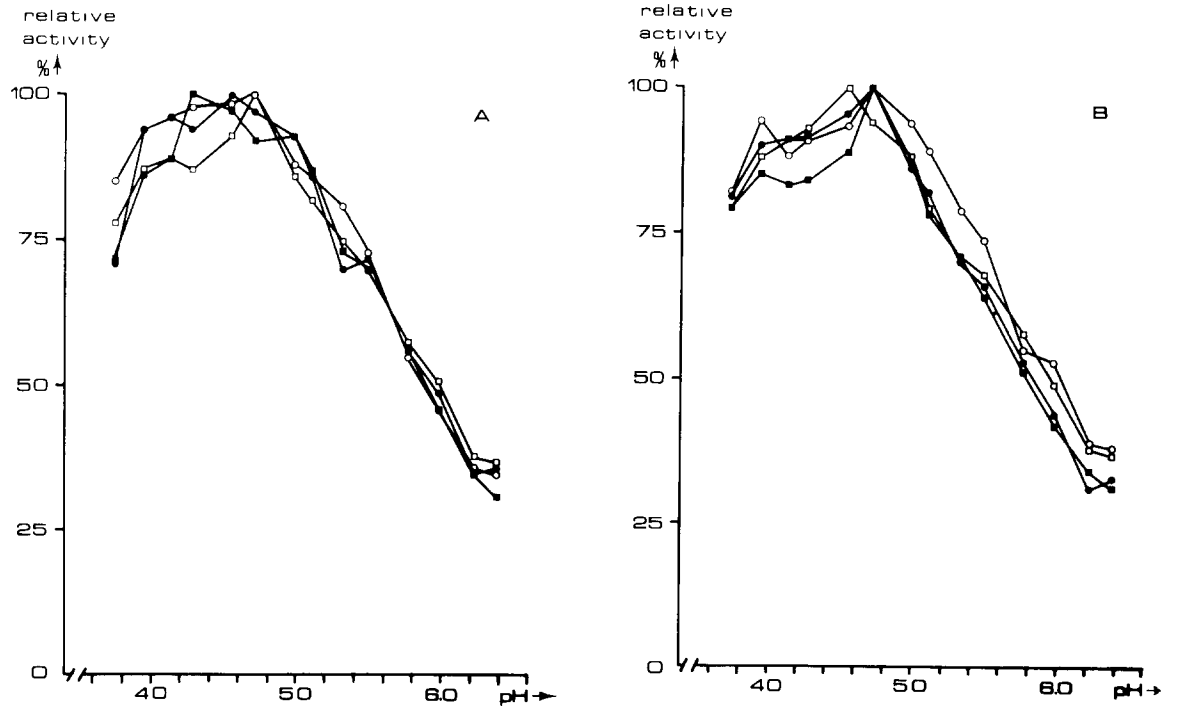

Fig 6 pH-Dependence of the activity of β-*N*-acetylhexosaminidase A and B The pH-activity curves were determined in citrate-phosphate buffer with 4-methylumbelliferyl-β-GlcNAc as a substrate, as described in Methods Activity is expressed as percentage of the maximal activity for each curve Panel A β-*N*-acetylhexosaminidase A, native, ▪, neuraminidase-treated, □, neuraminidase-treated, then resialylated, ○, native enzyme incubated with sialyltransferase, • Panel B β-*N*-acetylhexosaminidase B, native, □, neuraminidase-treated, ▪, neuraminidase-treated, then resialylated, ○, native enzyme incubated with sialyltransferase, •

TABLE III

APPARENT K_m VALUES FOR β-*N*-ACETYLHEXOSAMINIDASE A AND B

K_m values of β-*N*-acetylhexosaminidase A and B after desialylation and/or incubation with sialyltransferase were determined with 4-methylumbelliferyl β-*N*-acetylglucosaminide as a substrate The values given represent the means of two separate experiments performed in triplicate Deviation from the mean values was less than 10%

Treatment	β-*N*-Acetylhexosaminidase A (K_m (mM))	β-*N*-Acetylhexosaminidase B (K_m (mM))
None	0 76	0 78
Neuraminidase	1 04	0 83
Neuraminidase, then sialyltransferase	0 98	0 87
Sialyltransferase	0 83	0 70

with the radioactive bands This is consistent with the radioactivity pattern obtained by fluorography The radioactivity profile obtained from the [^{14}C]-sialylated endogenous acceptor was different from those of β-*N*-acetylhexosaminidase A and B The former product focused at a pH value of 5 5, whereas the radioactivity of [^{14}C]sialylated β-*N*-acetylhexosaminidase A focused at pH values between 4 2 and 4 7 and that of isoenzyme B between pH 6 7 and 6 9

Kinetic studies

Apparent Michaelis constants for the various neuraminidase and/or sialyltransferase-treated enzyme forms were close to those obtained for the native enzymes (Table III) The pH-activity curves for the native enzymes in phosphate-citrate buffer showed a broad optimum at pH 4 2–5 0 for both β-*N*-acetylhexosaminidase A and B The pH-activity curves for the desialylated and the resialylated enzyme forms were virtually identical to those of the native enzymes (Fig 6, A and B)

Discussion

In recent years several sialyltransferases have been purified and their specificities studied [14,23, 24,33,34] The β-galactoside α(2 → 6)sialyltransferase of bovine colostrum, which was used in this study in a partially purified form, has been demonstrated to link sialic acid α(2 → 6) specifically to Galβ(1 → 4)-GlcNAc structures on glycopeptides [35] and low molecular weight acceptors [33] Since our preparation was essentially free of activity towards mucin acceptors, incorporation of sialic acid into human liver β-*N*-acetylhexosaminidase A and B provided strong evidence that Galβ(1 → 4)GlcNAc units are present on the heteroglycan parts of both the A and B form of the lysosomal enzyme From the saturation percentages (Fig 3) it can be seen that at least 60–70% of the galactosyl residues in the enzymes are found in this type of structure

This conclusion is consistent with the carbohydrate analysis of the enzymes (Table II) The sugar compositions suggest that *O*-glycosidically linked oligosaccharide chains are absent and that at least part of the carbohydrate chains are of the *N*-acetyllactosaminic type Similar oligosaccharide chains containing sialic acid linked to galactose seem to be present on human urine α-*N*-acetylglucosaminidase [36], as suggested by uptake experiments However, oligomannosidic-type chains may also occur on the liver β-*N*-acetylhexosaminidase, as indicated by the preponderance of mannose in the enzyme (Table II) Such carbohydrate chains, in which part of the mannosine residues were phosphorylated, have been reported to occur on human urine α-*N*-acetylglucosaminidase [37], on bovine testicular β-galactosidase [38] and on a glycoprotein fraction inhibiting the assimilation of β-galactosidase by fibroblasts [39] The possibility cannot be excluded that the purified isoenzymes are each constituted of two sub-populations, in one of which the molecules exclusively bear oligomannosidic-type chains, the other consisting of molecules with *N*-acetyllactosaminic-type chains only The carbohydrate composition of the purified β-*N*-acetylhexosaminidase from human liver resembles that reported for the enzyme from human placenta [12], with the exception of the sialic acid content of the B-form, which is higher in the liver enzyme. It is unclear whether this difference depends on the tissue source of the enzyme or is caused by differences in the purification procedures

The charge heterogeneity observed upon isoelectric focusing of the purified β-*N*-acetylhexosaminidases A and B might be related to the existence of sub-populations of enzyme molecules bearing different types of carbohydrate structures, micro-

heterogeneity of the carbohydrate chains and differences in the protein parts of the enzymes At least part of these variations may have to be ascribed to the fact that the enzyme has been isolated from whole liver tissue, which contains several cell types and different subcellular structures In addition, some heterogeneity may arise as a result of postmortem changes and breakdown during isolation and purification of the enzyme In particular, part of the charge heterogeneity of β-*N*-acetylhexosaminidase A and B depends on differences in sialic acid content, since the complexity of isoelectric focusing patterns was reduced upon neuraminidase treatment, and could be restored by incubation with sialyltransferase from bovine colostrum A similar restoration of the original heterogeneity of a neuraminidase-treated glycoprotein enzyme after resialylation with sialyltransferase was previously reported for human plasma ceramide trihexosidase [40] and α-L-fucosidase from human liver [41]

The effect of the degree of sialylation on the pH-activity curves and K_m values of β-*N*-acetylhexosaminidase A and B appeared to be minimal So it seems likely that the presence and/or amount of sialic acid residues does not affect the kinetic properties of β-*N*-acetylhexosaminidase A and B Similar results were found for alkaline phosphatase from sheep brain [42] However, K_m values of human liver α-L-fucosidase were reported to be influenced by the sialic acid content of the enzyme [41]

Although native β-*N*-acetylhexosaminidase A and B were not fully sialylated, as shown by gas-liquid chromatography data (Table II), both isoenzymes appeared to be poor acceptors for the bovine colostrum sialyltransferase The absence of a significant transfer of sialic acid to acceptor structures on the native enzyme might be due to the substrate specificity of the sialyltransferase used This specificity was found to extend to structural features beyond the terminal *N*-acetyllactosamine units on the oligosaccharide chains of serum glycoproteins [35] and conceivably the branches most preferred by the sialyltransferase could already carry a terminal sialic acid residue Alternatively, sialic acid present on any branch of the heteroglycan part of the native β-*N*-acetylhexosaminidases A and B might have an inhibitory effect on a further sialylation of these enzymes

It remains to be investigated in which way variation in the sialic acid content of human liver β-*N*-acetylhexosaminidase could influence the uptake of the enzyme by various cell types Uptake experiments in vitro using specifically sialylated enzymes may provide more insight into the role of the carbohydrate moiety in receptor-mediated endocytosis and may contribute to the study of structure-function relationships of the lysosomal enzyme

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