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INTRANEURAL GLYCOSIDASES

II. PURIFICATION AND PROPERTIES OF α-FUCOSIDASE, β-FUCOSIDASE, α-MANNOSIDASE AND β-XYLOSIDASE OF RAT CEREBRAL CORTEX*

H. BRUCE BOSMANN AND BRIAN A. HEMSWORTH

Department of Pharmacology and Toxicology, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y. 14620 (U.S.A.)

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SUMMARY

α- and β-fucosidase (EC 3.2.1.–), α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) were purified 980-, 720-, 1900- and 2630-fold from rat cerebral cortex by solubilization with a non-ionic detergent, centrifugation, $(NH_4)_2SO_4$ precipitation, and Sephadex G-100 and DEAE-cellulose column chromatography. The purified enzymes hydrolyzed both *p*-nitrophenyl glycosides and glycoproteins at rapid rates. The purified β-fucosidase hydrolyzed *p*-nitrophenyl-β-fucopyranoside ($K_m = 2.66 \cdot 10^{-4}$ M) but not *p*-nitrophenyl-α-fucopyranoside. The purified α-fucosidase hydrolyzed *p*-nitrophenyl-α-fucopyranoside ($K_m = 1.33 \cdot 10^{-3}$ M), porcine submaxillary mucin ($K_m = 1.0 \cdot 10^{-5}$ M), porcine submaxillary mucin minus *N*-acetylneuraminic acid ($K_m = 2.7 \cdot 10^{-6}$ M) but not *p*-nitrophenyl-β-fucopyranoside or the α-1,3-fucose of orosomucoid. This enzyme appears to be specific for the α-1,2-linkage but not for the moiety to which fucose is bonded. The purified α-mannosidase catalyzed the hydrolysis of *p*-nitrophenyl-α-mannopyranoside ($K_m = 4.0 \cdot 10^{-4}$ M), fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine ($K_m = 2.7 \cdot 10^{-5}$ M), ovalbumin ($K_m = 8.2 \cdot 10^{-4}$ M), and ovalbumin *minus* *N*-acetylglucosamine ($K_m = 2.1 \cdot 10^{-6}$ M). β-Xylosidase hydrolyzed *p*-nitrophenyl-β-xylopyranoside ($K_m = 2.7 \cdot 10^{-3}$ M) and chondroitin sulfate *minus* $(N\text{-acetylgalactosamine, glucuronic acid})_n$, galactose, and galactose ($K_m = 3.2 \cdot 10^{-5}$ M). For hydrolysis of *p*-nitrophenylglycosides the following optimal pH's were found: α-fucosidase, 4.3; α-mannosidase, 4.1; β-fucosidase, 3.1; and β-xylosidase, 4.2. At a final concentration of 0.01 M, $HgCl_2$ inhibited the activity of the purified enzymes by 42 to 85%, with *p*-nitrophenylglycosides as substrates. The enzymes were rapidly inactivated by incubations for 1 h at temperatures above 40°.

* Address for reprints: Dr. H. B. Bosmann, Department of Pharmacology and Toxicology, University of Rochester, School of Medicine and Dentistry, 260 Crittenden Boulevard, Rochester, N. Y. 14620, U.S.A.

INTRODUCTION

The glycosidase enzymes have been studied in a variety of tissues but have not been studied in depth in the brain with respect to purification and properties[1-8]. In particular the less ubiquitous enzymes such as the fucosidases have not been studied with respect to purification and properties in the brain[8]. A previous study investigated the levels of glycosidases in rat and guinea pig cerebral cortex[9], and this paper reports the purification and properties of four of these enzymes from rat cerebral cortex, namely α-L-fucosidase (EC 3.2.1.–), β-L-fucosidase (EC 3.2.1.–), β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), and α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24).

The enzyme 1,2-α-L-fucosidase has been purified 500-fold from *Aspergillus niger*[10], and α-L-fucosidase has been purified from *Clostridium perfringens*[11] and abalone livers[12]. α-Mannosidase has been purified 3000-fold from *Phaseolus vulgaris*[13] and 90-fold from jack bean meal[2]. β-Xylosidase has been identified in a number of mammalian connective tissues and other sources[14]. β-D-Fucosidase has been identified histochemically in rat intestine[15]. In general, little work on the purification or characterization of these enzymes in nervous tissue has been published. This communication describes the procedure for the purification of α- and β-fucosidases, α-mannosidase and β-xylosidase from rat cerebral cortex and the kinetic, substrate specificity, and enzyme characteristic properties for these glycosidases.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl-α- and -β-L-fucopyranosides, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-xylopyranoside and other *p*-nitrophenyl-glycosides were purchased from Pierce Chemical Co., Rockford, Illinois.

Orosomucoid[16], fetuin[17], porcine submaxillary mucin[18,19], ovalbumin[20], and chondroitin sulfate[21] were prepared by published procedures as previously described[22,23]. All chemicals and biochemicals were of the highest purity commercially available. All solutions were made with water that was first distilled, then deionized, and finally glass distilled. The 0.1% Triton X-100 was made in 0.1 M Tris, pH 7.6, at all times.

Assay for glycosidase activity using p-nitrophenyl glycosides. The amount of a given glycosidase activity at pH 4.3 in any of the various enzyme preparations was determined using the *p*-nitrophenyl derivative in the following manner[5]: 25 μl (in some instances of high activity, 10 μl) of the enzyme extract were incubated with 6.0 μmoles of the *p*-nitrophenyl derivative (the final volume was 1.025 ml, 0.05 M in citrate, adjusted to pH 4.3) for 1 h at 37°. The reaction was terminated by the addition of 2 ml of 0.4 M glycine: NaOH buffer (pH 10.5), the reaction mixtures were centrifuged at 5000 × *g* for 10 min, and the absorbance of the released *p*-nitrophenol in the supernatant measured at 420 nm. From these data and a standard *p*-nitrophenol curve run simultaneously the nmoles hydrolyzed per hour were calculated. In each instance reactions were terminated at 10 min intervals to insure the linearity of the reactions up to 4 h. The substrates used were *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-galacto-pyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosamin-

ide, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-L-fucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside. *p*-Nitrophenol was used as a standard. In each experiment, assays and controls were run in duplicate. Controls consisted of assays in which glass distilled water was substituted for either the *p*-nitrophenyl glycoside substrate or the enzyme in the reaction mixture. These control values were averaged, added together and subtracted from the appropriate assays. Thus, light scattering contributions from the enzyme preparations or absorbance contributions from nonspecific hydrolysis of the *p*-nitrophenyl glycoside were subtracted from the results presented herein. Each experiment was performed at least 4 times. All solutions, suspensions, and buffers were made up in glass distilled water.

Protein. Total protein in any of the various enzyme preparations was determined by the method of LOWRY *et al.*[24]. Crystalline bovine serum albumin was used as a standard. In each instance of protein determination, the sample for analysis was precipitated with 30% trichloroacetic acid, washed twice with 10% trichloroacetic acid and once with ethanol–diethylether (2:1, v/v), and the resultant insoluble material dissolved in NaOH for analysis. Protein in fractions from the chromatography was determined by measuring the absorbance at 280 nm.

Fucose. Fucose was determined by the method of DISCHE AND SHETTLES[25].

Mannose. Mannose was determined by the anthrone procedure or the reducing sugar method of PARK AND JOHNSON[26] using mannose as a standard after ascertaining mannose was the only monosaccharide present by paper or gas–liquid chromatography[27].

Xylose. Xylose was determined by the method of TSIGANOS AND MUIR[28].

Glucuronic acid. Glucuronic acid was measured by the method of DISCHE[29].

Paper chromatography. Monosaccharides were determined by paper chromatography in *n*-butanol–pyridine–0.1 M HCl (5:3:2, by vol.) or pyridine–water–ethyl acetate–glacial acetic acid (5:3:5:1, by vol.).

Gas–liquid chromatography. In order to determine absolute amounts of monosaccharides present in the glycoproteins used, to identify and quantitate sugars released and to identify products of the enzymatic and hydrolytic preparations of substrates the methods of KIM *et al.*[30] were used to identify hexosamines by use of an amino acid analyzer and monosaccharides by gas liquid chromatography of the alditol acetate derivatives as previously described[31].

Assay for glycosidase activity with glycoprotein substrates. The assay for α- and β-fucosidase, α-mannosidase, and β-xylosidase with glycoprotein substrates was based on the release of fucose, mannose or xylose, respectively, from the designated substrate by gas–liquid chromatography or chemical determination. In all instances it was absolutely determined that the appropriate monosaccharide was the only sugar released by the enzyme. Absolute amounts of each monosaccharide present in each glycoprotein were determined by hydrolysis and gas–liquid chromatography as described above. Data are calculated based on the moles of monosaccharide in the substrate present and not on moles of the individual glycoprotein. The assay system contained 0.05 M citrate at pH 4.3 for α-fucosidase, pH 3.3 for β-fucosidase, pH 4.1 for α-mannosidase and pH 4.1 for β-xylosidase, a 100 μl sample of a 15 mM solution of substrate, and 10 to 100 μl of enzyme solution to a final volume of 0.7 ml. After incubation of the reaction mixture for 1 h at 37°, the reaction was terminated with 1 ml of 10% trichloroacetic acid. The mixture was centrifuged and analyzed for

released monosaccharide as given above by gas–liquid chromatography and chemical determination. In each instance substrate and enzyme controls run in duplicate were subtracted from the assays. Since the glycosidases studied herein are in all likelihood glycoproteins, it was especially important to run enzyme controls and subtract these values from the substrate containing reactions. These enzyme controls never were greater than 2% of the complete reaction, *i.e.* the enzymes did not function as efficient substrates for themselves. The substrate controls were never more than 4% of the reacted value in each instance. All analyses were run in duplicate. The substrates tested were as follows: for α-fucosidase (and β-fucosidase): orosomucoid, porcine submaxillary mucin, and porcine submaxillary mucin *minus* *N*-acetylneuraminic acid; for α-mannosidase: ovalbumin, ovalbumin *minus* *N*-acetylglucosamine and fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine; for β-xylosidase: chrondroitin sulfate *minus* (*N*-acetylgalactosamine, glucuronic acid)$_n$, galactose and galactose. Preparation of these substrates is given below and a structural presentation is given in Fig. 1.

Porcine submaxillary mucin minus N-acetylneuraminic acid. The porcine submaxillary mucin had the following percent composition of carbohydrate as determined by gas chromatography: galactose, 9; sialic acid, 10; fucose, 8; *N*-acetylgalactosamine, 20; mannose, 0; xylose, 0; glucose, 0, and *N*-acetylglucosamine, 0. Porcine submaxillary mucin *minus* *N*-acetylneuraminic acid was prepared by treatment of porcine submaxillary mucin[18,19] with a highly purified sialidase from *Clostridium perfringens*[32]. In a typical preparation 1 g of porcine submaxillary mucin was incubated with 5 mg of *C. perfringens* sialidase (purified 460-fold, free of proteolytic and other glycosidic enzymes) in 0.1 M acetate buffer at pH 5.0 for 40 h. The release of *N*-acetylneuraminic acid was monitored by the WARREN method[33] and total sialic acid was determined by the method of SVENNERHOLM[34]. Application of these procedures showed that 92.5% that of the sialic acid was removed. The sialidase was removed by precipitation with 1 *N* $HClO_4$ (the procedure removed 87% of the sialidase activity and 93% of the 5 mg added as protein). No fucose or galactose was detected by gas–liquid chromatography as being released during the enzymatic hydrolysis. This material with a fucose terminal residue linked $\alpha^{1,2}$ to galactose was used as substrate for the α- and β-fucosidase.

Fetuin minus N-acetylneuraminic acid, galactose and N-acetylglucosamine

The fetuin used in these studies had the following percent composition of carbohydrate as determined by gas chromatography: glucose, 0; galactose, 4; mannose, 3; *N*-acetylglucosamine, 6; sialic acid, 4; *N*-acetylgalactosamine, 0; fucose, 0; and xylose, 0. Fetuin minus *N*-acetylneuraminic acid, galactose, and *N*-acetylglucosamine was made from fetuin prepared by the method of SPIRO[17] as previously described[35]. The acceptor was desialized by hydrolysis in 0.05 M H_2SO_4 (approximately 10 mg fetuin per ml acid) at 80° for 1 h. Release of *N*-acetylneuraminic acid was monitored by the method of WARREN[33] and total sialic acid by the method of SVENNERHOLM[34]. The analytic procedures indicated that the hydrolysis removed 98% of the sialic acid residues. The acid was neutralized with 0.1 M NaOH and the hydrolysis mixture was exhaustively dialyzed against glass distilled water at 4°. The desialized fetuin (fetuin *minus* sialic acid) was lyophilized, dissolved in 0.02 M citrate buffer, pH 4.3, and reacted with β-galactosidase (0.1 mg enzyme protein per

7.0 mg fetuin) under toluene for 200 h at 37°[22]. The β-galactosidase, obtained by extraction from snail liver, did not contain measurable proteolytic activity or other glycosidase activity such as *N*-acetylglucosaminidase. The procedure removed 60–70% of the expected galactose as determined by the galactose oxidase (Galactostat, Worthington) or Anthrone procedures. After the incubation the β-galactosidase was precipitated with 0.34 *N* $HClO_4$ (this procedure removed 99% of the β-galactosidase activity and 92% of the added enzyme protein), and the product was neutralized with 1 M KOH, centrifuged, dialyzed, and lyophilized. This material was called fetuin *minus* sialic acid and galactose. *N*-Acetylglucosamine was then removed by either periodate and acid hydrolysis or enzymatic hydrolysis. The fetuin *minus* sialic acid and galactose was treated with sheep epididymal or jack bean meal hexosaminidase in order to remove hexosamine. In a typical preparation, fetuin *minus* sialic acid and galactose (400 mg in a total volume of 10 ml) was treated with 5 mg of epididymal or jack bean meal hexosaminidase in 0.02 M citrate buffer, pH 4.3, and incubated at 37° under toluene for 200 h. The sheep epididymal hexosaminidase preparation contains a highly active *N*-acetylglucosaminidase which was purified over 40-fold; the hexosaminidase from jack bean meal was purified over 80-fold by the method of LI AND LI[36]. Both enzyme preparations had no proteolytic activity when tested in large quantities (10–20 mg) against Azocoll (Calbiochem). Aliquots were tested at intervals by the REISSIG *et al.*[37] procedure for free *N*-acetylhexosamine. The hexosamine released from the fetuin by the hexosaminidase was examined by paper chromatography on borate paper[38] in butanol–pyridine–water (6:4:3, by vol.). The hexosaminidase-treated fetuin glycoprotein was obtained in the supernatant fluid after precipitation of the enzyme upon addition of ethanol to a concentration of 60%. (This precipitation removed 91% of the hexosaminidase activity and 91% of the added enzyme protein.) The precipitate was removed, and the supernatant solution was exhaustively dialyzed and lyophilized. This procedure released about 70–80% of the hexosamine. In each instance over 95% of the hexosaminidase-treated fetuin was found in the supernatant.

The fetuin *minus* sialic acid and galactose was also treated with periodate followed by acid hydrolysis in order to remove the *N*-acetylglucosamine residues. In a typical preparation, 300 mg of the fetuin *minus* sialic acid and galactose in 13.5 ml water was mixed with 12.5 ml 0.1 M sodium acetate, pH 4.6. After adding 100 mg of 0.1 M $NaIO_4$, the solution was placed in the dark at 4°. Aliquots were removed at specific times, dialyzed, and hydrolyzed in 4 M HCl for 3 h and analyzed for glucosamine. The reaction was stopped after 16 h by addition of 10 ml of 1.0 M glycerol. The pH was adjusted to 9.0 after 10 min followed by 40 ml of 1.0 M $NaBH_4$. After standing for 1 h, the solution was adjusted to pH 7 with acetic acid, and dialyzed against distilled water for 20 h. The material oxidized with periodate and subsequently reduced with $NaBH_4$ was next submitted to acid hydrolysis with 25 mM H_2SO_4 for 2 h at 90°. After hydrolysis the solution was neutralized with 1 M NaOH, dialyzed and lyophilized. Generally, from 70 to 80% of the material treated by acid in this manner was recovered as measured by protein analysis and 80–90% of the *N*-acetylglucosamine was removed. Little difference was noted between the material prepared by either the acid or enzymatic hydrolysis conditions described above. This material with a terminal mannose residue was used as substrate for α-mannosidase activity. Fetuin *minus* *N*-acetylneuraminic acid, galactose, and *N*-

acetylglucosamine had the following percent composition as determined by gas chromatography: glucose, 0; galactose, 0; mannose, 3; *N*-acetylglucosamine, 2; sialic acid, 0; *N*-acetylgalactosamine, 0; fucose, 0; xylose, 0.

Ovalbumin minus N-acetylglucosamine. The ovalbumin used in these studies had the following percent carbohydrate as determined by gas chromatography: glucose, 0; galactose, 0; mannose, 1.3; *N*-acetylglucosamine, 0.9; sialic acid, 0; fucose, 0; *N*-acetylgalactosamine, 0 and xylose, 0. *N*-acetylglucosamine was removed from ovalbumin by the procedures described above, using either sheep epididymal or jack bean meal hexosaminidase. The hexosaminidase-treated ovalbumin was obtained in the supernatant fluid after precipitation of the enzyme by addition of absolute ethanol to a final concentration of 62% (this procedure removed 94% of the hexosaminidase activity and 97% of the added enzyme protein). Application of the above analytic procedures indicated that 93.7% of the terminal *N*-acetylglucosamine residues were removed by these procedures. This material, which now has an additional terminal mannose residue, was used as a substrate for α-mannosidase. Ovalbumin *minus N*-acetylglucosamine had the following percent composition as determined by gas–liquid chromatography: glucose, 0; galactose, 0; mannose, 1.3; *N*-acetylglucosamine, 0.6; sialic acid, 0; *N*-acetylgalactosamine, 0; and xylose, 0.

Chondroitin sulfate minus glucuronic acid, galactose and galactose. Chondroitin sulphate was hydrolyzed for 20 h at 37° in 100 vol. of 0.18 M NaOH, to remove $(N\text{-acetylgalactosamine-glucuronic acid})_n$. Dialysis, lyophilization and analysis indicated that the hydrolyzed chondroitin sulphate contained the residue glucuronic acid–galactose–galactose–xylose. Glucuronic acid was removed from hydrolyzed chrondroitin sulfate by incubation of 1.5 g of hydrolyzed chondroitin sulfate with 12.5 mg of highly purified β-glucuronidase from bovine liver[39], for 30 h in 0.1 M sodium acetate at pH 4.5. Analysis indicated that 86.8% of the glucuronic acid was removed by this procedure. Galactose was removed using the β-galactosidase from snail liver[22] exactly as described above; 81% of the galactose was removed by this procedure. This material which has a terminal xylose residue linked β to a serine residue was used as a substrate for β-xylosidase activity.

Proteolytic activity. Proteolytic activity in the cortex preparations was determined in the following manner: 25 mg of Azocol (Calbiochem) were incubated at 37° with 250 μg of each of the purified enzyme preparations in 2 ml of 0.05 M citrate buffer, pH 7.5, for 30 min. Pronase controls were run simultaneously and the results are expressed as the amount of pronase giving the same activity as the indicated enzyme preparation per milligram of protein. It was possible to analyze for 0.01 μg of pronase by this method. Proteolytic activity in the various analytic enzymes was determined in the same manner except the incubation time was increased (*e.g.* 8 days).

Purification of enzymes. All steps of purification were carried out at 4° or the temperature of melting ice unless otherwise specified. Fractions from column chromatography were monitored for protein by measuring absorbance at 280 nm against a blank consisting of 0.1% Triton X-100 in 0.1 M Tris, pH 7.6. For the specific activity measurements the protein was determined by the method of LOWRY *et al.*[24].

Step 1. Homogenization. Rat cerebral cortices were removed and extracted with 0.1% Triton X-100 as previously described[9]. Routinely 6 cortices were minced in 0.1% Triton X-100 (4 vol.) and homogenized for 30 strokes in a Ten Broeck homo-

genizer. This material was used as crude enzyme source. In certain experiments 0.1 M Tris, pH 7.6, distilled water, or 0.25 M sucrose was substituted for the 0.1% Triton X-100 to evaluate the extraction procedure and enzyme stability. Furthermore, at certain steps in the procedure, fractions from separate groups of 6 cortices were combined in order to have enough material for column chromatography.

Step 2. Centrifugation. The extract was centrifuged for 1 h at 4000 × *g* in a Sorvall centrifuge and the pellet discarded.

Step 3. $(NH_4)_2SO_4$ precipitation. To the 4000 × *g* supernatant enzyme grade $(NH_4)_2SO_4$ (Mann) was added at 20 g per 100 ml by slow addition of the $(NH_4)_2SO_4$ with constant stirring for 4 h. The precipitate was allowed to settle for an additional 4 h and the suspension was then centrifuged at 10 000 × *g* for 1 h. To the supernatant was then added an addition of 30 g/100 ml $(NH_4)_2SO_4$ and the above procedure was repeated. The pellet was then resuspended in 0.1% Triton X-100 and dialyzed exhaustively against 0.1% Triton X-100. This material was used for column chromatography.

Step 4. Gel filtration on Sephadex G-100. The above solution (approximately 2.0 g of protein) was applied to a column of Sephadex G-100 (2.5 cm × 45 cm) packed in 0.1% Triton X-100 as recommended by the manufacturer. The column was eluted

TABLE I

PURIFICATION OF RAT CEREBRAL CORTEX α-FUCOSIDASE, β-FUCOSIDASE, α-MANNOSIDASE, AND β-XYLOSIDASE

Appropriate amounts of enzyme samples were incubated at 37° for 1 h with the indicated *p*-nitrophenylglycoside in 0.05 M citrate buffer, pH 4.3. The details of the purification procedures and assays are described in MATERIALS AND METHODS. Specific activity is presented for the highest fraction of the column chromatography, whereas total activity is given for the entire peak. Specific activity of the entire peak is given in parentheses. All numbers are rounded to the nearest tenth.

Purification step	*α-Fucosidase*				*β-Fucosidase*		
	Specific activity (nmoles/h per mg protein)	*Yield (%)*	*Purification factor*	*Total activity (μmoles/h)*	*Specific activity (nmoles/h per mg protein)*	*Yield (%)*	*Purification factor*
1. Homogenate	9.3	100	1	37.2	0.5	100	1
2. 4000 × *g*							
Supernatant	45.2	78	5	29.0	2.7	86	5
Pellet	1.5	14	0.2	5.1	0	0	0
3. $(NH_4)_2SO_4$ precipitation							
0–20%	12.0	13	1.3	4.8	0	0	0
20–50%	200.8	71	22	26.5	10.7	70	21.4
4. Sephadex G-100 eluate							
Tubes 32–34	1.6	0	0.2	0	360 (111)	46	720
Tubes 36–39	1520 (329)	50	163	18.6	0	0	0
Tubes 67–69	0	0	0	0	0	0	0
5. DEAE-cellulose eluate							
Tubes 62–68	9142 (2197)	13	980	4.8	0	0	0
Tubes 108–113	0	0	0	0	0	0	0

with 0.1% Triton X-100. 3 ml-fractions were collected with a flow rate of 10 ml/h.

Step 5. Column chromatography on DEAE-cellulose. A column (2.5 cm × 45 cm) was packed with DEAE-cellulose in 0.1% Triton X-100. An 8-ml sample (240 mg protein) of Tubes 36, 37, 38, and 39 from the above column eluate was applied to the column, and the column was eluted with a continuous salt gradient between 200 ml of 0.1% Triton X-100, 200 ml of 0.4 M NaCl in 0.1% Triton X-100, and 200 ml of 0.8 M NaCl in 0.1% Triton X-100. Fractions of 2.8 ml were collected with a flow rate of 8 ml/h.

Hexosamine. Hexosamine was determined by the method of BOAS[40], as previously described[41].

RESULTS

Substrates. The formulae of the glycoprotein substrates used in these experiments are given in Fig. 1.

Purification of enzymes. The purification scheme described above yielded preparations of enzymes which were essentially free of contaminating glycosidase or protease activity. The data given in Table I indicated that in general the enzymes

	α-Mannosidase				*β-Xylosidase*			
otal ctivity µmoles/h)	*Specific activity (nmoles/ h per mg protein)*	*Yield (%)*	*Purification factor*	*Total activity (µmoles/h)*	*Specific activity (nmoles/ h per mg protein)*	*Yield (%)*	*Purification factor*	*Total activity (µmoles/h)*
	2.0	100	1	8	3.7	100	1	14.8
.7	10.4	84	5	6.7	19.6	84	5	12.5
	0.2	6	0.1	0.5	0.6	12	0.2	1.8
	0.8	4	0.4	0.3	3.7	10	1	1.5
.4	40.6	68	20.3	5.4	71.6	62	19.4	9.3
.9	0	0	0	0	0	0	0	0
	800 (360)	21	400	1.7	0	0	0	0
	0	0	0	0	9762 (3212)	50	2630	7.4
	0	0	0	0	0	0	0	0
	3800 (1247)	11	1900	0.9	0	0	0	0

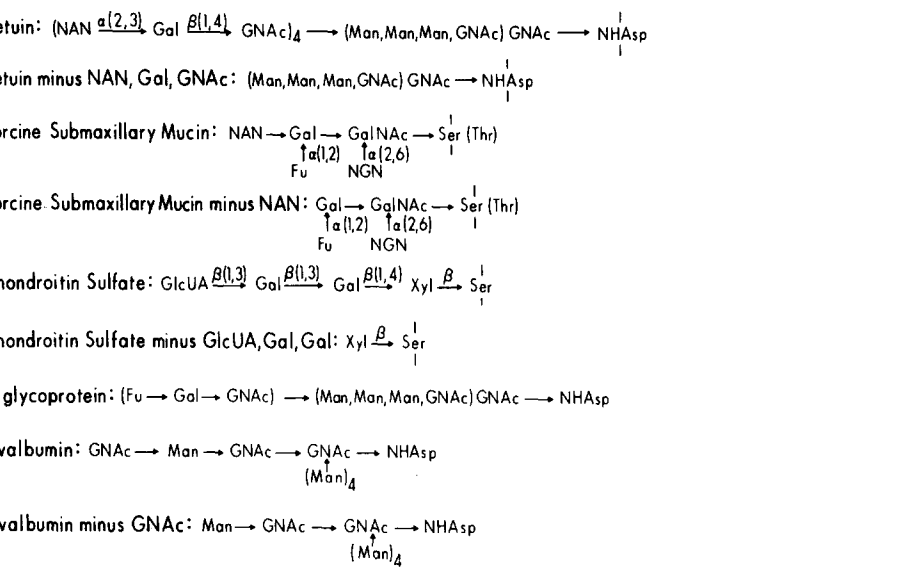

Fig. 1. Structural formulations for representative sugar sequences and sugar amino acid linkages of glycoproteins and glycoprotein fragments used as substrates. The original formulations are from the review of SPIRO[4] except for porcine submaxillary mucin[18,19]. Abbreviations: Thr, threonine; Ser, serine; NHAsp, asparagine; GlcUA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; GNAc, *N*-acetylglucosamine; NAN, *N*-acetylneuraminic acid; NGN, *N*-glycoylneuraminic acid; Gal, galactose; Man, mannose; Fu, fucose; Xyl, xylose. The structure for chondroitin is that of the linkage region of this polymer.

studied were solubilized by the 0.1% Triton X-100 and were associated with the 4000 × *g* supernatant. Extraction with 0.1 M Tris, pH 7.6, and distilled water did not prove to be as effective solubilizing agents as the non-ionic detergent. 0.25 M sucrose was a better solubilizing agent for α-fucosidase and α-mannosidase than the Triton; however, the 0.1% Triton X-100 had a stronger stabilizing effect on all the enzymes than the other extracting media (Table II). Surprisingly, the enzymes were more stable in the purified form from the Sephadex G-100 column than in the crude homogenate form (Table II).

The highest activity of α- and β-fucosidase, α-mannosidase, and β-xylosidase was found in the 20–50% $(NH_4)_2SO_4$ precipitate; α-fucosidase was purified 22-fold; β-fucosidase 21.4-fold; α-mannosidase, 20.3-fold and β-xylosidase, 19.4-fold. Sephadex G-100 column chromatography resulted in a further purification of the glycosidases (Fig. 2) and a separation of β-fucosidase and β-xylosidase; α-fucosidase and α-mannosidase were not entirely separated. Application of this α-mannosidase and α-fucosidase peak from the Sephadex G-100 column to a DEAE-cellulose column according to the methods described above and elution with a continuous NaCl gradient resulted in separation of the α-fucosidase and α-mannosidase (Fig. 3). The results in Table I indicate that for these procedures α-fucosidase was purified 980-fold; β-fucosidase, 720-fold; α-mannosidase 1900-fold, and β-xylosidase 2630-fold.

The data in Table III demonstrate that the β-fucosidase had very slight α-fucosidase contamination while the β-xylosidase was free of contaminating glycosidase or proteolytic activity. The α-fucosidase had slight β-*N*-acetylglucosaminidase activity

TABLE II

STABILITY AND EFFECTS OF EXTRACTION METHOD ON α-FUCOSIDASE, β-FUCOSIDASE, α-MANNOSIDASE AND β-XYLOSIDASE ACTIVITY OF RAT CEREBRAL CORTEX AND STABILITY OF PURIFIED RAT CEREBRAL CORTEX ENZYMES

All assays were performed on rat cerebral cortex which was homogenized in the indicated solution by 30 strokes with a Ten Broeck homogenizer. Sephadex G-100 enzymes refer to peaks of activity from the Sephadex G-100 column chromatography, *i.e.* for α-fucosidase, Tubes 36–39, for β-fucosidase, Tubes 32–34, for α-mannosidase, Tubes 36–39, and for β-xylosidase, Tubes 67–69. After 24 h at −26° the assays were again performed on the same material. Data are given as nmoles/h per mg protein. Numbers in parentheses are the percent of the initial rate.

Extraction	*α-Fucosidase*		*β-Fucosidase*		*α-Mannosidase*		*β-Xylosidase*	
	Initial	*24 h*	*Initial*	*24 h*	*Initial*	*24 h*	*Initial*	*24 h*
0.1 M Tris, pH 7.6	8.7	5.6 (64)	0.3	0.12 (40)	3.1	1.7 (54)	3.1	1.7 (54)
0.1% Triton X-100	9.3	8.0 (86)	0.5	0.25 (50)	2.0	1.3 (66)	3.7	3.0 (81)
Distilled water	7.2	5.8 (31)	0.2	0 (0)	1.7	1.4 (83)	3.0	2.0 (67)
0.25 M sucrose	11.3	7.6 (67)	0.4	0.03 (8)	2.6	1.4 (53)	2.6	0.9 (35)
Sephadex G-100 enzymes	1520	1537 (101)	360	357 (100)	800	809 (101)	9762	9410 (97)

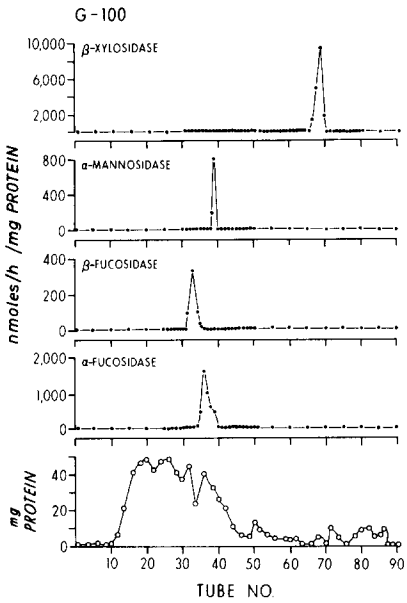

Fig. 2. Chromatography on Sephadex G-100. A sample of protein, 2.0 g in 5 ml of 0.1% Triton X-100 was applied to a Sephadex G-100 column (2.5 cm × 45 cm) previously equilibrated with the 0.1% Triton X-100. A maximum hydrostatic head of 20 cm was maintained during elution of the column with the 0.1% Triton X-100. Fractions of 3.0 ml were collected. Assays were conducted with *p*-nitrophenylglycosides as described in MATERIALS AND METHODS.

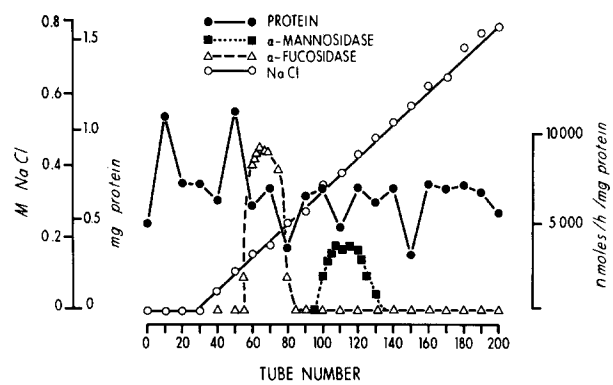

Fig. 3. Chromatography of partially purified α-mannosidase and α-fucosidase on DEAE-cellulose. To a column of DEAE-cellulose (2.5 cm × 45 cm) equilibrated with 0.1% Triton X-100 a sample of 240 mg of protein in 8 ml of 0.1% Triton X-100 was applied. The column was eluted in a continuous manner with 200 ml of 0.1% Triton X-100, 200 ml of 0.4 M NaCl in 0.1% Triton X-100 and 200 ml of 0.8 M NaCl in 0.1% Triton X-100. Fractions of 2.8 ml were collected. There was no α-mannosidase activity above Tube 136 or below Tube 95. There was no α-fucosidase activity below Tube 57 or above Tube 83. All assays were performed with the *p*-nitrophenylglycosides as given in MATERIALS ADN METHODS.

TABLE III

ENZYME SPECIFIC ACTIVITIES OF PURIFIED GLYCOSIDASE FRACTIONS

Activity was measured with *p*-nitrophenyl derivatives or azocoll as given in MATERIALS AND METHODS. Data are given as nmoles/h per mg protein and as means ± 1 S.D. (0 = no measurable activity).

Enzyme	*Sephadex G-100*		*DEAE-cellulose*	
	Tubes 32–34	*Tubes 67–69*	*Tubes 62–68*	*Tubes 108–113*
α-Glucosidase	0	0	0	1 ± 0.3
β-Glucosidase	0	0	0	0
α-Galactosidase	0	0	0	0
β-Galactosidase	0	0	0	0
β-*N*-Acetylgalactosaminidase	0	0	0	9 ± 2
β-*N*-Acetylglucosaminidase	0	0	3 ± 1	0
α-Mannosidase	0	0	0	3800 ± 16
β-Xylosidase	0	9762 ± 41	0	0
α-Fucosidase	1.6 ± 0.2	0	9142 ± 32	0
β-Fucosidase	360 ± 2	0	0	0
Pronase equivalents*	0	0	0	0

* Pronase equivalents were measured with azocoll. There was no activity when 10 mg of the purified enzyme preparations were incubated with azocoll as given in MATERIALS AND METHODS; it was possible to detect 0.01 μg of pronase by this method.

and the α-mannosidase had slight α-glucosidase and β-*N*-acetylgalactosaminidase activity when tested with *p*-nitrophenylglycosides or Azocoll for glycosidase or proteolytic activity, respectively.

pH optimum of enzymes. The effect of pH on the catalytic activity of the enzymes was studied using the *p*-nitrophenylglycosides in 0.05 M citrate or 0.05 M

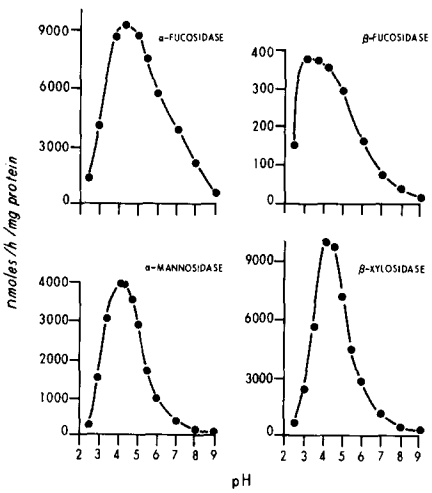

Fig. 4. pH–activity profile of purified rat cerebral cortex α-fucosidase, β-fucosidase, α-mannosidase, and β-xylosidase. The activity of each enzyme at pH 2.4 to 9.0 using 0.05 M citrate or 0.05 M citrate phosphate buffers was determined. The *p*-nitrophenylglycoside was employed as a substrate in each instance.

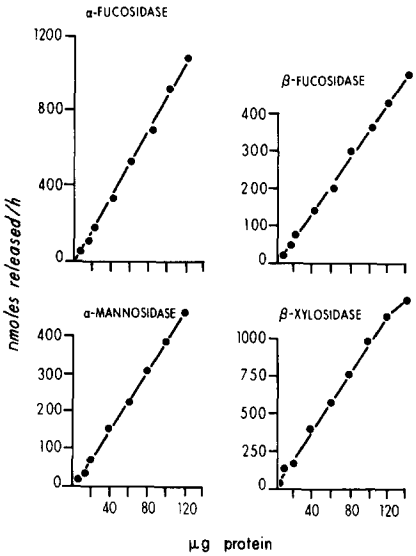

Fig. 5. Effect of purified enzyme concentration on hydrolysis of *p*-nitrophenylglycosides. Incubations were carried out at 37° for 1 h with the purified rat cerebral cortex glycosidase. The details of the assay are described in MATERIALS AND METHODS.

citrate phosphate buffers ranging from pH 2.5 to 9.0. The pH activity profiles shown in Fig. 4 indicate a pH optimum for α-fucosidase of 4.3, for α-mannosidase of 4.1, for β-fucosidase of 3.1, for β-xylosidase of 4.2. β-Xylosidase exhibited a sharp

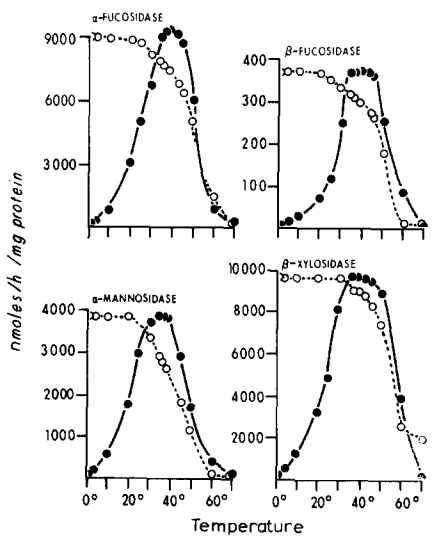

Fig. 6. Effect of temperature of incubation and temperature stability curves for purified α-mannosidase, β-xylosidase, and α- and β-fucosidase of rat cerebral cortex. Solid circles indicate assays incubated for 1 h at the indicated temperature. Open circles refer to incubation of the enzyme alone in the 0.05 M citrate buffer for 1 h prior to a normal assay at 37° for 1 h. All assays were performed with the purified enzymes and the appropriate *p*-nitrophenylglycosides as given in MATERIALS AND METHODS.

TABLE IV

EFFECT OF INHIBITORS AND ACTIVATORS ON PURIFIED α-FUCOSIDASE, β-FUCOSIDASE, α-MANNOSIDASE AND β-XYLOSIDASE

Purified α-fucosidase (DEAE-cellulose Tubes 62–68), β-fucosidase (Sephadex G-100 Tubes 32–34), α-mannosidase (DEAE-cellulose Tubes 108–113) and β-xylosidase (Sephadex G-100 Tubes 67–69) appropriate buffer substrate and indicated compound (in 50 μl buffer) were incubated for 1 h at 37° as described under MATERIALS AND METHODS. Inhibition or activations are expressed as percentage of control activity without addition, but with 50 μl of buffer substrate substituted for the addition. Molar concentration of protamine sulfate was calculated using a molecular weight of 10 000.

Compound	*Final conc.* × 10^2 *(M)*	*Control activity (%)*			
		α-Fucosidase	*β-Fucosidase*	*α-Mannosidase*	*β-Xylosidase*
$MnCl_2$	1	100	100	100	100
$PbCl_2$	1	106	78	96	85
$HgCl_2$	1	15	46	36	58
$CoCl_2$	1	103	106	90	102
$CuCl_2$	1	90	68	79	89
$FeCl_2$	1	100	100	100	100
$MgCl_2$	1	100	112	83	68
$CaCl_2$	1	98	124	100	83
EDTA	1	103	188	89	97
Eserine salicylate	0.1	146	150	130	147
Protamine sulfate	0.1	82	85	80	90
Iodoacetate	0.1	83	90	82	103
p-Hydroxymercuribenzoate	0.1	111	120	90	80
N-Ethylmaleimide	0.1	106	130	126	120
Bovine serum albumin	7.5	111	120	116	101

pH profile whereas the other glycosidases showed rather broad curves (Fig. 4).

Effect of enzyme concentration on purified rat cerebral cortex glycosidases. The results of varying enzyme concentration on the hydrolysis of the appropriate *p*-nitrophenylglycosides is presented in Fig. 5. A linear increase in hydrolysis was obtained up to 120 μg of protein in each instance.

Effect of temperature of reaction and temperature stability. The results of carrying out the reactions with the purified enzymes and the *p*-nitrophenylglycosides at various temperatures and of incubating the enzyme at a given temperature for 1 h before carrying out the reaction in the usual manner for 1 h at 37° are presented in Fig. 6. Each of the enzymes had its highest activity around 37° with little activity below 10° or above 60° incubation temperature. Incubation of the enzymes below about 25° for 1 h prior to assay did not adversely affect any of the glycosidases. Above 30° the activity fell off rapidly as the temperature of precincubation was increased; this was especially true with β-fucosidase (Fig. 6).

Effect of inhibitors and activators on purified rat cerebral cortex α-mannosidase, α- and β-fucosidase and β-xylosidase. The results of activation and inhibition studies are shown in Table IV. The most striking effect was that of 0.01 M $HgCl_2$ on each of the enzymes. Purified α-fucosidase activity was inhibited to 15% of the control activity by this cation. $MgCl_2$ (0.01 M) inhibited β-xylosidase activity to 68%; $CuCl_2$ (0.01 M) inhibited β-fucosidase activity to 68%. EDTA at 0.01 M activated the purified β-fucosidase activity to 188% of the control activity presumably by chelation of endogenous inhibitor (perhaps Cu^{2+}). The results with sulfhydryl reagents were variable. *p*-Hydroxymercuribenzoate inhibited α-mannosidase and β-xylosidase

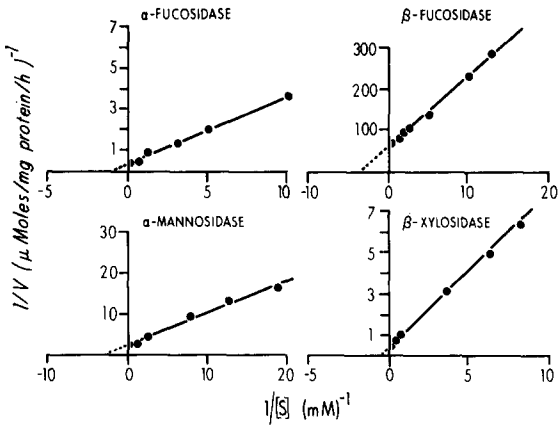

Fig. 7. Lineweaver–Burk plots of purified α-mannosidase, β-xylosidase and α- and β-fucosidase of rat cerebral cortex. The effect of substrate concentration was studied by using the *p*-nitrophenyl-glycosides as described in MATERIALS AND METHODS.

slightly and activated the α- and β-fucosidase slightly; *N*-ethylmaleimide also activated all the enzymes while iodoacetate inhibited all the glycosidases slightly. This may mean that the enzymes do not possess sulfhydryl groups or that the enzyme has sulfhydryl groups but these groups may be adjacent to, but not in, the active site[42]. Protamine sulfate inhibited all the purified glycosidases, whereas eserine (physostigmine) had a rather striking activating effect (Table IV).

TABLE V

v_{max} AND K_m VALUES FOR RAT CEREBRAL CORTEX PURIFIED α-FUCOSIDASE, β-FUCOSIDASE, α-MANNOSIDASE, AND α-XYLOSIDASE WITH SYNTHETIC, GLYCOPROTEIN, AND GLYCOPROTEIN FRAGMENT SUBSTRATES

Substrate concentration in all instances is based on molar amount of indicated sugar present and not on molar amount of glycoprotein.

Enzyme : substrate	v_{max} *(μmoles/mg protein per h)*	K_m *apparent (M)*
α-Fucosidase : *p*-nitrophenyl-α-fucopyranoside	3.33	$1.33 \cdot 10^{-3}$
α-Fucosidase : orosomucoid	No activity	No activity
α-Fucosidase : porcine submaxillary mucin	1.0	$1.0 \cdot 10^{-5}$
α-Fucosidase : porcine submaxillary mucin *minus* *N*-acetylneuraminic acid	0.33	$2.7 \cdot 10^{-6}$
β-Fucosidase : *p*-nitrophenyl-β-fucopyranoside	0.0167	$2.66 \cdot 10^{-4}$
α-Mannosidase : *p*-nitrophenyl-α-mannopyranoside	0.4	$4.0 \cdot 10^{-4}$
α-Mannosidase : fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine	0.4	$2.7 \cdot 10^{-5}$
α-Mannosidase : ovalbumin	1.6	$8.2 \cdot 10^{-4}$
α-Mannosidase : ovalbumin *minus* *N*-acetylglucosamine	0.3	$2.1 \cdot 10^{-6}$
β-Xylosidase : *p*-nitrophenyl-β-xylopyranoside	2.5	$2.7 \cdot 10^{-3}$
β-Xylosidase : chondroitin sulfate *minus* glucuronic acid, galactose and galactose	0.9	$3.2 \cdot 10^{-5}$

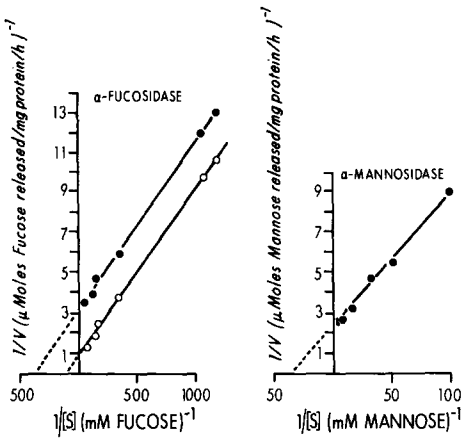

Fig. 8. Lineweaver–Burk plots of purified α-mannosidase and α-fucosidase of rat cerebral cortex. The effect of substrate concentration was studied using glycoproteins or degraded glycoproteins as given in MATERIALS AND METHODS. Substrate concentration in all instances is based on molar amount of indicated sugar present, not on molar amount of glycoprotein. The curve on the right is for α-mannosidase with fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetyl glucosamine as substrate. The open circle curve on the left is for α-fucosidase with porcine submaxillary mucin as substrate. The closed circle curve on the left is for α-fucosidase with porcine submaxillary mucin *minus* *N*-acetylneuraminic acid.

Effect of substrate. By performing experiments in which the *p*-nitrophenylglycoside concentration and the purified enzyme preparations were varied, data were obtained from which Lineweaver–Burk plots were determined. The plot of $1/v$ *versus* $1/[S]$ showed straight line relationships for each of the purified rat cerebral cortex glycosidases (Fig. 7). The values for apparent K_m and v_{max} as computed from the plots are given in Table V.

Substrate specificity. The enzymes described herein were purified and characterized with respect to their ability to release monosaccharide from *p*-nitrophenylglycosides. These purified enzymes were then reacted with glycoproteins and degraded glycoproteins as substrates. The data in Fig. 8 indicate Lineweaver–Burk plots obtained for three such experiments. Apparent K_m and v_{max} values for all the reactions studied are given in Table V. In each case the plot of $1/v$ *versus* $1/[S]$ showed straight line relationships for each of the purified enzymes studied with the glycoprotein substrates. Since in general, for the same enzyme the substrate with the lower K_m value is generally more reactive, the data in Table V are of interest. They demonstrate that in general the glycoprotein substrates were more reactive than the *p*-nitrophenylglycosides. The data in Table V also indicate that the desialyzed porcine submaxillary mucin was a more reactive substrate than the native porcine submaxillary mucin with respect to release of fucose by α-fucosidase from rat cerebral cortex. Presumably the *N*-acetylneuraminic acid causes steric hindrance for the action of the α-fucosidase. Similarly the α-mannosidase has a lower K_m with ovalbumin *minus* *N*-acetylglucosamine than with native ovalbumin presumably since another site has been exposed. In no instance was there any release of fucose from the glycoprotein substrates tested when β-fucosidase was substituted for α-fucosidase in the reaction.

TABLE VI

pH OPTIMUM AND EFFECT OF INHIBITORS ON PURIFIED α-FUCOSIDASE, α-MANNOSIDASE AND β-XYLOSIDASE IN REACTIONS INVOLVING GLYCOPROTEINS OR DEGRADED GLYCOPROTEINS AS SUBSTRATES

Purified enzymes were incubated with the indicated substrate at 37° for 1 h and assays performed as given in MATERIALS AND METHODS. pH optima were determined using 0.05 M citrate or 0.05 M citrate phosphate buffers. Inhibitor concentrations are final concentrations and results are expressed as % control activity.

Enzyme:substrate	*pH optimum*	*Inhibitor:*			
		$HgCl_2$ (0.01 M)	*$PbCl_2$ (0.01 M)*	*Iodoacetate (1 mM)*	*Protamine sulfate (1 mM)*
α-Fucosidase:orosomucoid	—	—	—	—	—
α-Fucosidase:porcine submaxillary mucin	4.2	2	42	70	74
α-Fucosidase:porcine submaxillary mucin *minus* *N*-acetylneuraminic acid	4.2	0	43	70	68
α-Mannosidase:fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine	4.1	14	48	54	72
α-Mannosidase:ovalbumin	4.1	12	56	72	72
α-Mannosidase:ovalbumin *minus* *N*-acetylglucosamine	4.1	6	49	73	73
β-Xylosidase:chondroitin sulfate *minus* glucuronic acid, galactose and galactose	3.7	36	94	90	86

Characteristics of glycoprotein substrate reactions. The results in Table VI give the optimum pH and inhibitor effects on each of the purified enzymes with the glycoprotein or glycoprotein fragment substrates. In each instance chromatography showed the indicated monosaccharide to be the only monosaccharide released during the experiment. The data indicate that the optimum pH of the enzymes remains fairly constant whether *p*-nitrophenylglycosides or glycoproteins were used as substrates (see Table VI and Fig. 4). The optimal pH for α-mannosidase and β-xylosidase was exactly the same for all substrates examined. The enzyme reactions involving the glycoprotein substrates were more sensitive to inhibition by cations and sulfhydryl group inhibitors than those involving the *p*-nitrophenylglycosides. $HgCl_2$ at a final concentration of 0.01 M completely inhibited fucose release from porcine submaxillary mucin and porcine submaxillary mucin *minus* *N*-acetylneuraminic acid. $PbCl_2$ was a much more potent inhibitor of glycosidase activity when the glycoprotein substrates were used in the reaction (See Tables IV and VI).

Percentage of carbohydrate released. The data in Table VII indicate the per-

TABLE VII

PERCENTAGE OF AVAILABLE CARBOHYDRATE MOIETIES RELEASED FROM GLYCOPROTEIN SUBSTRATES BY 40 μg OF PURIFIED ENZYMES PER h

Conditions of assay were as described in the text. The sugar released was the only carbohydrate moiety present.

Enzyme:substrate	*Sugar released*	*% Released*
α-Fucosidase:orosomucoid	None	0
α-Fucosidase:porcine submaxillary mucin	Fucose	1.7
α-Fucosidase:porcine submaxillary mucin *minus* *N*-acetylneuraminic acid	Fucose	4.3
α-Mannosidase:fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine	Mannose	1.3
α-Mannosidase:ovalbumin	Mannose	2.0
α-Mannosidase:ovalbumin *minus* *N*-acetylglucosamine	Mannose	6.7
α-Xylosidase:chondroitin sulfate *minus* glucuronic acid, galactose and galactose	Xylose	2.1

centage of available carbohydrate released by 40 μg of purified enzyme per h. The α-mannosidase released the lowest percentage of sugar, 1.3%, from fetuin *minus* *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine and the highest percentage, 6.7%, from ovalbumin *minus* *N*-acetylglucosamine.

DISCUSSION

Specificity of glycosidases which are capable of hydrolyzing monosaccharide residues from the nonreducing end of carbohydrate chains in polysaccharides, oligosaccharides, glycosaminoglycans, glycoproteins and glycolipids has been studied to a large extent recently. In particular the substrate specificity of glycosidases seems to fall into one of two classes: (1) enzymes specific for the sugar and a specific linkage or (2) enzymes specific for a terminal sugar, a specific linkage, and a specific adjacent residue (sugar, amino acid, *etc.*). BAHL[10] recently described an 1,2-α-L-fucosidase from *Aspergillus niger* which belongs to the second class, namely, an α-L-fucosidase specific for the α-1,2-linkage and an adjacent galactose. Data on nonmammalian fucosidases reported by AMINOFF AND FURUKAWA[11] and WATKINS[43] seem also to follow this pattern, *i.e.* they will hydrolyze only specific fucosidic linkages in oligo- and polysaccharides. Mammalian fucosidases[14] seem to be in the first class. The α-fucosidase reported herein seems also to fall in the first class; it hydrolyzes the α-1,2-linkage in porcine submaxillary gland mucin, does not hydrolyze the presumed α-1,3-linkage in orosomucoid[44] but does hydrolyze *p*-nitrophenyl-α-L-fucopyranoside. The β-xylosidase reported herein also does not require great substrate specificity since it will hydrolyze both *p*-nitrophenylxylopyranoside and an amino acid sugar linkage, namely, the xylose–serine β-linkage of chondroitin sulfate *minus* glucuronic acid, galactose and galactose.

The purified glycosidases are very sensitive to inhibition with $HgCl_2$ and to some extent $PbCl_2$; the inhibition is much greater for the glycoprotein substrates than with the *p*-nitrophenylglycosidases. α-Mannosidase, α-glucosidase, and β-galactosidase in guinea pig cerebellum[8] and β-*N*-acetylglucosaminidase in rat liver[45] have all been found to fractionate into multiple enzyme peaks, whereas in the present

experiments the glycosidases separated into single peaks on Sephadex G-100 and DEAE-cellulose. It is surprising that the mammalian glycosidases do not exhibit specificity with respect to the macromolecular or small molecule nature of the substrate since the enzymes which synthesize glycoproteins, namely the glycosyl transferases, show an extremely marked acceptor specificity and, in general, a requirement for macromolecular acceptors[46,47]. The glycosidases reported herein seem to require no metal ions or other cofactors for activity. The presence of 0.1% Triton X-100 stabilizes the enzymes especially in the purified form.

LI[48] has described the characteristics of purified α-mannosidase from jack bean meal and has reported that the enzyme hydrolyzes α-1,6'-, α-1,2'-, and α-1,3'-linked oligomannosides. The enzyme showed little specificity for the nature of the moiety to which the mannose was bonded[48]. AGRAWAL AND BAHL[13] have described an α-mannosidase from *Phaseolus vulgaris* which hydrolyzed either *p*-nitrophenyl-α-D-mannopyranoside or methyl-α-D-mannopyranoside and therefore it may be that the susceptibility of a glycoside bond to hydrolysis by a glycosidase does not always depend on either the nature of aglycon or its linkage to the glycosyl group. TANAKA *et al.*[12] have found that there are two α-fucosidases from abalone liver, one which acts on only *p*-nitrophenyl-α-L-fucoside and one which acts on *p*-nitrophenyl-α-L-fucoside and porcine submaxillary mucin. FUKADA *et al.*[49] have described a β-xylosidase from *Charonica lampas* which hydrolyzed both *O*-β-xylosyl-L-serine and stem bromelain glycopeptide. Each of the above instances documents glycosidase activity with certain specificities. Since all combinations of substrates were not utilized in the present experiments it is impossible to draw many conclusions about specificity. However, the following may be said about the specificity for the glycosidases of rat cerebral cortex studied: (1) the α-fucosidase of rat cerebral cortex was specific for α-1,2-linkages and quantitatively (*i.e.* K_m differences) but not qualitatively specific for the moiety to which the fucose was bonded. (2) The β-fucosidase of rat cerebral cortex was specific for β-fucose linkage, *i.e.* *p*-nitrophenyl-α-fucoside was not a substrate. (3) The α-mannosidase of rat cerebral cortex was not specific for the moiety to which the mannose was bonded and quantitative differences in substrate specificity were observed. (4) The β-xylosidase of rat cerebral cortex was similar to α-mannosidase in that no substrate specificity was observed. (5) In general, the enzymes tend to function only on terminal residues since other sugars were not released into the reaction mixture. It should be emphasized that these enzymes were purified (and therefore selected) on the basis of their ability to hydrolyze *p*-nitrophenylglycoside substrates. Other enzymes not catalyzing *p*-nitrophenylglycoside hydrolysis or hydrolyzing these substrates only to a limited extent but actively and specifically catalyzing hydrolysis of glycoprotein substrates may have been overlooked in the described purification scheme.

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