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ISOLATION AND CHARACTERIZATION OF β-GLUCOSIDASE* FROM THE CYTOSOL OF RAT KIDNEY CORTEX

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Summary

A procedure is described for the preparation of extensively purified β-D-glucosidase (EC 3.2.1.21) from the cytosol fraction of rat kidney. The specific activity of the β-glucosidase in the high speed supernatant (100 000 × *g*, 90 min) fraction of rat kidney homogenate is 700-fold greater than that in the same fraction from heart, skeletal muscle, lung, spleen, brain or liver. β-Glucosidase activity co-chromatographs with β-D-galactosidase, β-D-fucosidase, α-L-arabinosidase and β-D-xylosidase activities through the last four column steps of the purification and their specific activities are 0.26, 0.39, 0.028 and 0.017 relative to that of β-glucosidase, respectively. The specific activity of the apparently homogeneous β-glucosidase is 115 000 nmol of glucose released from 4-methylumbelliferyl-β-D-glucopyranoside per mg protein per h. All five glycosidase activities possess similar pH dependency (pH optimum, 6—7) and heat lability, and co-migrate on polyacrylamide disc gels at pH 8.9 (R_F, 0.67). β-Glucosidase activity is inhibited competitively by glucono-(1 → 5)-lactone (K_I, 0.61 mM) and non-competitively by a variety of sulfhydryl reagents including *N*-ethylmaleimide, *p*-chloromercuribenzoate, 5,5'-dithio-bis(2-nitrobenzoic acid), and iodoacetic acid. Although the enzyme will release glucose from *p*-nitrophenyl and 4-methylumbelliferyl derivatives of β-D-glucose, it will not hydrolyze xylosyl-*O*-serine, β-D-glucocerebroside, lactose, galactosylovalbumin or trehalose. The enzyme consists of a single polypeptide chain with a molecular weight of 50 000—58 000, has a sedimentation coefficient of 4.41 S and contains a relatively large number of acidic amino acids. A study of the

* IUPAC-IUB nomenclature: β-D-glucoside:glucohydrolase (EC 3.2.1.21).

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distribution of β-glucosidase activity in various regions of the dissected rat kidney indicates that the enzyme is probably contained in cells of the proximal convoluted tubule. The enzyme is also present in relatively large amounts in the villus cells, but not crypt cells, of the intestine. The physiological substrate and function of the enzyme are unknown.

Introduction

A number of investigators have described a β-D-glucosidase found in the kidney of a variety of species including the rat [1—13], pig [4] and human [5] which differs from the particulate, acid pH optimum lysosomal hydrolase of the same tissue in that the former: (1) has a pH optimum near neutrality, (2) exists in the cytosol of the cell, and (3) appears to be a single enzyme with rather broad substrate specificity. Although the soluble β-glucosidase of rat kidney will hydrolyze β-D-glucoside, β-D-galactoside, β-D-fucoside and α-L-arabinoside derivatives of *p*-nitrophenol and 4-methylumbelliferone, physiologic or natural substrates have not yet been identified; thus, the apparent function of the enzyme remains obscure. The finding, by histochemical methods, that this β-glucosidase is associated with cells of the proximal convoluted tubule of the kidney [6] that carry out monosaccharide readsorption, together with the fact that the inhibition constant for phloridzin of both porcine β-glucosidase activity and renal glucose transport are similar [4], has prompted some to suggest that the enzyme may play a role in the active transport of glucose in the kidney [7].

Clinical interest in this particular β-glucosidase derives from the observation that its concentration in the urine of laboratory animals with experimentally induced renal disease is elevated long before proteinuria is apparent [8].

Although a number of laboratories have described the properties of partially purified preparations of this kidney β-glucosidase from pig and rat, no report concerning the properties of this purified mammalian enzyme has appeared. We describe a relatively rapid procedure, employing conventional column chromatographic methods, which yields milligram quantities of an extensively purified preparation of β-D-glucosidase from the cytosol fraction of rat kidney cortex. The availability of the purified enzyme should facilitate studies concerning its physiological substrate and biological function.

Materials and Methods

Materials

Adult Sprague-Dawley rats were obtained from Zivic Miller Laboratories, Inc., Allison Park, Pa. The 4-methylumbelliferyl and *p*-nitrophenyl α- and β-D-glycopyranosides of glucose, galactose, mannose, *N*-acetylglucosamine and *N*-acetylgalactosamine were from Pierce Chemical Co., Rockford, Ill. Xylosyl-*O*-serine was the generous gift of Dr. Lennart Roden. $(NH_4)_2SO_4$ was special enzyme grade from Schwarz-Mann, DEAE-cellulose (Whatman type DE-52 processed before use according to Whatman information leaflet IL-2) was obtained from H. Reeve-Angel, Inc., Clifton, N.J. Hydroxyapatite gel (Bio-Gel HTP) and

DEAE-agarose (DEAE Bio-Gel A) were from Bio-Rad, Inc., Richmond, Calif. Sephadex G-200 and blue dextran were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. β-Mercaptoethanol was obtained from Eastman Organic Chemicals, Inc., Rochester, N.Y. *p*-Chloromercuribenzoate, 5,5′-dithio-bis(2-nitrobenzoic acid), *N*-ethylmaleimide, iodoacetic acid, equine cytochrome *c*, bovine liver catalase and serum albumin were all obtained from Sigma Biochemicals, Inc., St. Louis, Mo. All other chemicals were reagent grade.

Methods

Purification of rat kidney β-glucosidase: extraction of tissues. 150 pairs of freshly excised rat kidneys were trimmed of adhering fatty tissue and the capsules and adrenals were removed. The kidneys were minced and homogenized in five volumes of a medium containing sucrose (0.25 M), ethylenediamine tetraacetate (1 mM) and Tris · HCl (0.01 M, pH 7.2) buffer using 10 passes of a serrated Teflon pestle in a Potter-Elvehjem homogenizer. Unless otherwise stated, all procedures were carried out to 1—4°C. The homogenate was passed through four layers of cheesecloth and centrifuged (600 × *g*, 10 min). The supernatant fraction was centrifuged (100 000 × *g*, 90 min) to yield soluble and membrane fractions. Approx. 95% of the β-glucosidase activity was recovered in the soluble fraction (Table I). The purification of β-glucosidase was monitored using the substrates indicated in Table I.

$(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ fractionation was carried out by dissolving 16.4 g of solid $(NH_4)_2SO_4$ per 100 ml of soluble fraction and allowing it to stand for 15 min. An additional 18.1 g per 100 ml $(NH_4)_2SO_4$ was dissolved in the supernatant fraction obtained after centrifugation (10 000 × *g*, 10 min). After 15 min, centrifugation was repeated and the resultant pellet was dissolved in a minimal volume of distilled water.

Acid precipitation step. The redissolved $(NH_4)_2SO_4$ precipitate was dialyzed for 16 h against 200 volumes of 0.01 M sodium acetate buffer, pH 4.63. A voluminous precipitate was discarded after centrifugation as described above. β-Glucosidase activity was recovered in the supernatant fraction.

DEAE-cellulose column chromatography. The supernatant fraction from the acid precipitation step was applied to a 2 × 12 cm column of DEAE-cellulose previously equilibrated with 0.01 M sodium acetate buffer, pH 4.6. The column was washed with the same buffer until the absorbance of the eluate at 280 nm was below 0.01. A 600 ml linear, continuous gradient of KCl (0—0.2 M) in the same buffer was then applied to the column and 11-ml fractions were collected. A single, sharp peak of protein and glycosidase activity was eluted reproducibly at 0.06 M KCl (Fig. 1).

Hydroxyapatite gel column chromatography. The pooled fractions from the DEAE-cellulose column were applied directly, without dialysis, to a 1.2 × 10 cm column of hydroxyapatite gel previously equilibrated with 0.01 M sodium acetate buffer, pH 4.6. The column was washed with five bed volumes of 0.005 M Tris · HCl buffer, pH 7.0. A 200 ml linear, continuous gradient of sodium phosphate buffer, pH 7.0 (0.0—0.1 M) was then applied to the column adand 8-ml fractions were collected. β-Glucosidase activity was eluted from the column with approx. 0.025 M sodium phosphate (Fig. 2).

DEAE-agarose column chromatography. The pooled fractions from the

TABLE I

PURIFICATION OF β-D-GLUCOSIDASE FROM RAT KIDNEY

(1) Details of the purification procedure are described in the text. The overall yield of β-glucosidase activity was 24%. β-D-Glucosidase activity was determined using 4-methylumbelliferyl-β-D-glucopyranoside (4-MUGlc) as substrate; β-D-galactosidase with 4-methylumbelliferyl-β-D-galactopyranoside (4-MUGal); β-D-fucosidase with *p*-nitrophenyl-β-D-fucopyranoside (PNPFuc); α-L-arabinosidase with 4-methylumbelliferyl-α-L-arabinoside (4-MUAra); β-D-xylosidase with *p*-nitrophenyl-β-D-xylopyranoside (PNPXyl). (2) Glucocerebrosidase was assayed by a modification of the method of Erickson and Radin [26]. The incubation mixture contained in a final volume of 0.10 ml: 0.2 M sodium acetate, pH 6.0, 1.2% (w/v) sodium taurocholate, 11 000 cpm of ^{14}C-labeled *N*-stearoyl-glucocerebroside (700 cpm/nmol) and appropriate amounts of enzyme protein. After incubation for 1 h at 37°C, the incubation was terminated by the addition of 0.5 ml 1% KCl and 3 ml chloroform/methanol (2 : 1, v/v). After vortexing and centrifugation at maximum speed in a clinical centrifuge for 7 min to separate the mixture into two phases, the upper phase was discarded. The lower phase was evaporated, and the residue dissolved in 0.8 ml of 2% methanol in chloroform and applied to a 1.5 ml column of silicic acid (Unisil, Clarkson Chemical Co.) which had been equilibrated in the same solvent. The column was washed with two volumes of 1.5 ml and one volume of 2 ml of 2% methanol in chloroform in order to elute product (^{14}C-labeled *N*-stearoyl-ceramide) from the column. The 2% methanol elute containing the product was evaporated, and the residue was counted in 4.5 ml of toluene scintillation fluid containing 0.5 ml ethanol in a Packard tricarb scintillation counter. (3) The number in brackets indicates the overall purification achieved. (4) The number in parentheses indicates the ratio of the specific activity of a particular hydrolase activity to that of β-D-glucosidase.

Fraction	Volume (ml)	Protein (mg)	Total units ($\times 10^{-7}$)	Specific activity (nmol/h/mg protein)					
				β-D--Glucosidase (1) with 4-MUGlc	β-D--Galactosidase with 4-MUGal	β-D--Fucosidase with PNPFuc	α-L--Arabinosidase with 4-MUAra	β-D--Xylosidase with PNPXyl	β-D-Gluco-cerebrosidase (2)
Crude homogenate (600 × *g* supernatant)	1360	32600	8.94	273 [1](3)	210 (0.22)(4)	164(0.60)	43.7(0.16)	11.2(0.041)	12.5 (0.044)
High speed (100000 × *g*) supernatant	1360	7400	8.52	1150[4. 2]	287(0.25)	460(0.40)	51.7(0.045)	27.6(0.024)	7.26 (0.025)
Acid (pH 4. 6) dialysis	350	3260	5.79	1780[6. 5]	445(0.25)	605(0.34)	80.0(0.045)	32.0(0.018)	10.2 (0.0057)
DEAE--cellulose	72	405	3.89	9590[35]	2300(0.24)	3460(0.36)	364 (0.038)	230 (0.024)	7.26 (0.00076)
Hydroxy-apatite	80	80.9	2.52	31100[114]	6830(0.22)	6840(0.39)	870 (0.028)	560 (0.018)	1.22 (0.000039)
DEAE--agarose	140	33.0	1.99	60000[220]	11400(0.19)	24600(0.41)	1980 (0.033)	600 (0.010)	16.4 (0.00027)
Sephadex G-200	6.6	18.2	2.10	115000[420]	29900(0.26)	44900(0.39)	3220 (0.028)	1960 (0.017)	5.35 (0.000047)

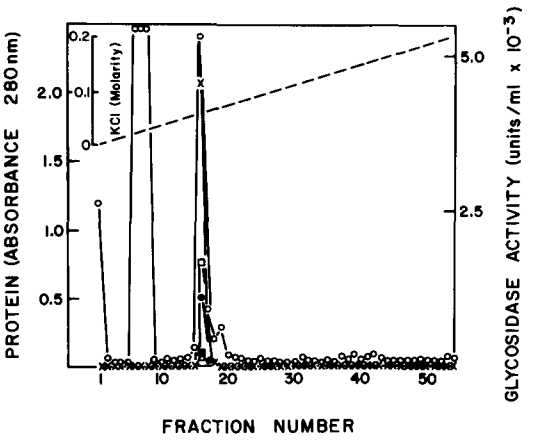

Fig. 1. Chromatography of rat kidney β-glucosidase on DEAE-cellulose. Determinations of protein (○) and various glycosidase activities were performed as described in the text and Table I. Fractions 16 and 17 were pooled. X, β-D-glucosidase; □, β-D-fucosidase; ●, β-D-galactosidase; ■, α-L-arabinosidase; △, β-D-xylosidase.

hydroxyapatite gel column were diluted with an equal volume of distilled water and applied directly to a 2 × 80 cm column of DEAE-agarose previously equilibrated with 0.005 M Tris · HCl buffer, pH 7.0. The column was washed with one bed volume of the same buffer and then developed with 300 ml of 0.05 M KCl in the same buffer. This resulted in the elution of several peaks of protein, but no β-glucosidase activity was eluted from the column. The column was then washed with 0.075 M KCl in the same buffer and a major peak of protein was eluted which contained greater than 95% of the β-glucosidase activity that had been applied to the column (Fig. 3). Fractions containing hydrolase activity were pooled and concentrated to 1.5 ml by pressure dialysis (Amicon Corp.) over N_2 gas using a UM-10 filter.

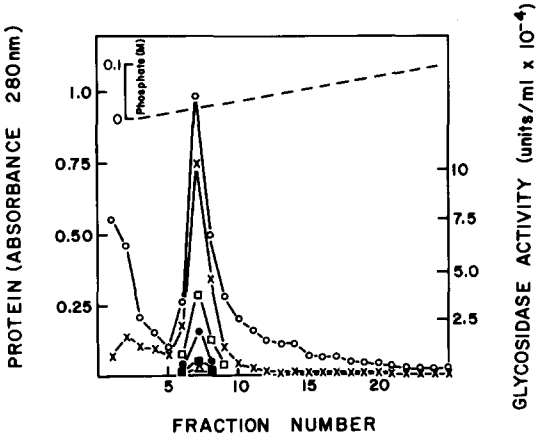

Fig. 2. Chromatography of rat kidney β-glucosidase on hydroxyapatite gel. Determinations of protein (○) and various glycosidase activities are described in the text and Table I. Fractions 6—9 were pooled. X, β-D-glucosidase; □, β-D-fucosidase; ●, β-D-galactosidase; ■, α-L-arabinosidase; △, β-D-xylosidase.

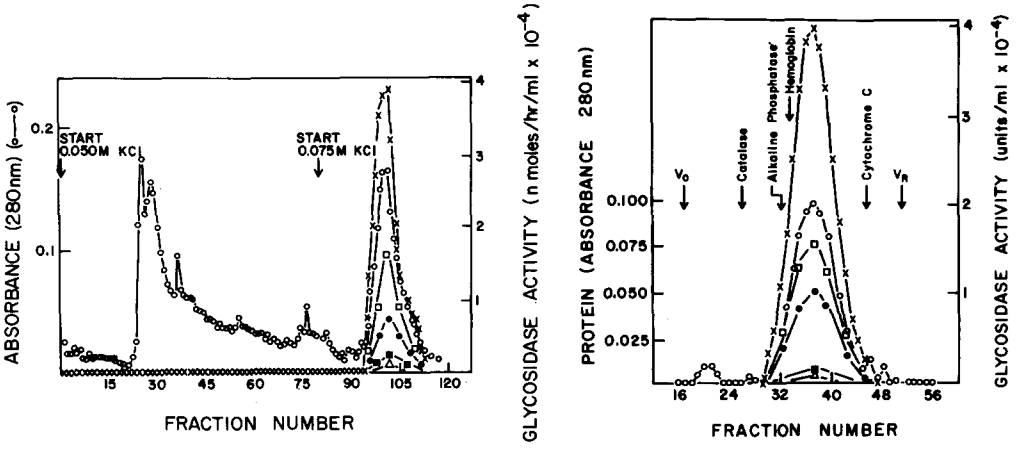

Fig. 3. Chromatography of rat kidney β-glucosidase on DEAE-agarose. Arrows indicate the beginning of each increase in the molarity of the KCl wash. Determinations of protein (○) and glycosidase activity were performed as described in the text and Table I. Fractions 96—109 were pooled. X, β-D-glucosidase; □, β-D-fucosidase; ●, β-D-galactosidase; ■, α-L-arabinosidase; △, β-D-xylosidase.

Fig. 4. Molecular sieve column chromatography of rat kidney β-glucosidase on Sephadex G-200. The source of β-glucosidase was the enzyme preparation from the first Sephadex G-200 column described in the text. Determinations of protein concentration (○) and glycosidase activity were performed as described in the text and Table I, X, β-D-glucosidase; □, β-D-fucosidase; ●, β-D-galactosidase; ■, α-L-arabinosidase; △, β-D-xylosidase. The column was calibrated with bovine catalase (mol. wt 232 000), human hemoglobin (mol. wt 64 500), bacterial alkaline phosphatase (mol. wt 86 000) and cytochrome *c* (mol. wt 11 700). The void (V_O) and retention (V_T) volumes of the column were determined using blue dextran and tritiated leucine, respectively.

Molecular sieve chromatography on Sephadex G-200. The concentrated β-glucosidase preparation from the DEAE-agarose column was applied to a 1.5 X 84 cm column of Sephadex G-200 (fine) that had been previously equilibrated with 0.005 M Tris · HCl buffer, pH 7.0. The column was eluted with the same buffer, employing a 20 cm constant pressure head from a Merriot bottle. Fractions containing β-glucosidase specific activities greater than 90 000 units per mg were concentrated by pressure dialysis to approximately 1 ml and rechromatographed on the same Sephadex G-200 column which was also calibrated for molecular weight estimation by the method of DeVincenzi and Hedrick [9]. The column was calibrated with the proteins indicated in Fig. 4. Purified rat kidney β-glucosidase eluted from this column as a single symmetrical peak of activity and protein at a position consistent with a molecular weight of 58 500. The specific activity of each of the various β-glycosidase activities tested was essentially constant (±8%) over the entire activity peak (Fig. 4).

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was performed at pH 8.9 according to the method of Ornstein [10]. Gels were stained for protein with Buffalo Blue-Black reagent for 2 h, destained electrophoretically, and scanned for absorbance at 600 nm using a Gilford linear transporter. Various glycosidase activity determinations were performed on replicate, unstained gels sliced into 2-mm sections. Slices were minced with a glass rod and extracted for 18 h at 20°C using 0.5 ml of 0.3 M sodium acetate buffer, pH 5.9,

and 50-μl aliquots were assayed for various glycosidase activities; the recovery of enzyme activity was approx. 23%.

Electrophoresis was also performed in a sodium dodecylsulfate-mercaptoethanol-containing buffer according to the method of Weber and Osborn [11] for evaluation of purity and estimation of subunit molecular weight. Bovine serum albumin, human immunoglobin (IgG) and equine cytochrome *c* were used as molecular weight standards. Gels were stained for protein with Coomassie blue, destained and scanned as described above.

Analytical ultracentrifugation. Sedimentation analyses were carried out using the Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control unit, a monochromator, a high intensity light source, and Rayleigh interference optics.

Molecular weight determinations were carried out using the short (3 mm) column approach-to-equilibrium method of Yphantis [12]. An AN-D rotor with a double-sector duraluminum-filled epoxy centerpiece and plane quartz windows was used. The final preparation of enzyme was dialyzed to equilibrium against 0.01 M Tris · HCl buffer, pH 7.0, and run against a buffer blank at 17 000 rev./min at 20°C for 36 h. The initial protein concentration was 0.405 mg per ml. The plates (Kodak spectrographic plates, emulsion type II-G) were measured using a Nikon profile projector.

Enzyme determinations. Unless otherwise stated, all determinations of glycosidase activity were carried out at 37°C under the following standard conditions of incubation: 0.10 M sodium acetate buffer (pH 6.0), 2.5 mM substrate (either 4-methylumbelliferyl or *p*-nitrophenyl glycoside) and 5 mM L-cysteine · HCl in a final volume of 0.10 ml. Release of *p*-nitrophenol ($A_{410\,nm} = 14.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) was monitored spectrophotometrically at 410 nm after stopping reactions by addition of 1.0 ml of glycine/NH_4OH buffer (0.3 M, pH 10.5). In the case of fluorogenic substrates, 4-methylumbelliferone release was determined fluorimetrically as described elsewhere [13]. Assays were linear with time and amount of enzyme protein. One unit of activity represents 1 nmol of product produced per h under the defined assay conditions.

Protein determinations. Determination of protein was performed by the method of Lowry et al. [14] using crystalline bovine serum albumin as standard or by ultraviolet absorption [15]. The latter method was used only to monitor the elution of protein from columns.

Amino acid analysis. Protein hydrolysis was carried out for 24 h at 108°C in evacuated tubes using 6 M HCl. Amino acid analysis was performed using a Beckman 120C amino acid analyzer according to the method of Moore and Stein [16]. Performic acid oxidation was performed according to the method of Moore [17]. Tryptophan was measured by the spectrophotometric method of Bencze and Schmidt [18].

Results

Comments on the isolation and purification

The purification can be accomplished in approx. 10 days with an overall yield of activity of 20—30% or about 10—20 mg of purified enzyme protein

TABLE II

EFFECT OF VARIOUS DETERGENTS, THIOL COMPOUNDS AND SULFHYDRYL REAGENTS ON RAT KIDNEY β-GLUCOSIDASE ACTIVITY

Addition	Final concentration	Percent of control activity
None	—	100*
Detergents (percent (w/v))		
Lubrol-WX	1	128
Tween-20	1	153
Cutscum	1	112
Digitonin	1	30
Lecithin	1	64
Deoxycholic acid	1	11
Taurocholic acid	1	26
Thiol compounds (mM)		
L-Cysteine	10	240
β-Mercaptoethanol	10	200
Dithiothreitol	10	72
Glutathione (reduced)	7	50
Glutathione (reduced	15	18
Glutathione (reduced)	25	16
Glutathione (oxidized)	7	100
Sulfhydryl reagents** (mM)		
N-Ethylmaleimide	5	2
p-Chloromercuribenzoate	5	1
5,5′-dithio-bis(2-nitrobenzoic acid)	5	5
Iodoacetic acid	5	19

* All activities are normalized to β-D-glucosidase activity (100%) assayed with 4-methylumbelliferyl-β-D-glucopyranoside in the standard assay described in the text.

** The enzyme was incubated with β-mercaptoethanol (1 mM final concentration) for 5 min at 20°C prior to the addition of sulfhydryl reagent.

from 150 pairs of rat kidneys (Table I). β-Glucosidase activity is stable in the frozen state (—70°C) and in saturated solutions of $(NH_4)_2SO_4$ at 4°C for at least 1 month. Although β-glucosidase activity is stimulated 1.5—2.0-fold by the inclusion of mercaptoethanol (10 mM) in the assay medium (Table II), its presence is not required during the actual purification steps. Cysteine, as well as mercaptoethanol, stimulates β-glucosidase activity; dithiothreitol is not stimulatory and is in fact slightly inhibitory; glutathione is a relatively potent inhibitor of activity (50% inhibition, 7 mM). At the same concentration, oxidized glutathione did not affect activity. The same enzyme from human kidney cortex is not inhibited by reduced glutathione (Glew, R.H., unpublished). The significance of, and basis for, this inhibition by reduced glutathione is unknown. The enzyme appears to contain at least one accessible and functionally essential sulfhydryl group since β-glucosidase activity is inhibited 81, 95, 99 and 98% by the sulfhydryl group reagents, iodoacetic acid, 5,5′-dithio-bis(2-nitrobenzoic acid), *p*-chloromercuribenzoate, *N*-ethylmaleimide, respectively (Table II). Several non-ionic detergents including Lubrol-WX, Tween 20, and Cutscum stimulate β-glucosidase activity by 12—53% whereas ionic detergents such as deoxycholate and taurocholate are relatively potent inhibitors.

TABLE III

VARIOUS GLYCOSIDASE ACTIVITIES IN DIFFERENT ORGANS OF THE RAT

Various rat organs were homogenized and centrifuged as described in the text to yield high speed (100 000 × *g*, 90 min) supernatant fractions which were assayed for the indicated glycosidases as described in Materials and Methods and Table I. The numbers in parentheses indicate the ratio of soluble to particulate β-D-glucosidase activity.

Enzyme	Specific activity (nmol/h/per mg)						
	Kidney	Brain	Lung	Heart	Liver	Spleen	Muscle
β-D-Glucosidase	761.1 (13.1)	1.14 (0.31)	0.77 (0.59)	0.16 (0.59)	0.67 (0.34)	1.06 (1.93)	0.308 (0.65)
β-D-Galactosidase	266.	8.69	13.2	4.12	6.47	59.8	4.04
α-L-Arabinosidase	16.9	<0.1	0.67	<0.1	0.363	3.21	<0.1
β-D-Fucosidase	172	<0.1	<0.1	<0.1	2.65	<0.1	<0.1
β-D-Xylosi-	7.64	<0.1	0.75	<0.40	0.48	<0.1	<0.1

With respect to subcellular distribution in the kidney, β-glucosidase activity appears to occur primarily in a soluble, non-particulate fraction. When homogenized in the presence or absence of sucrose (0.25 M), greater than 90% of the β-glucosidase activity is contained in the high speed supernatant fraction (Tables I and III). Price and Dance [2] also reported that the majority (86%) of the β-glucosidase activity of rat kidney occurs in a non-sedimentable fraction. In comparison with other rat organs, the β-glucosidase of rat kidney appears unique in several respects. First, the specific activity of the soluble enzyme is 700-fold greater in the kidney than in the high speed supernatant, cytosol fraction of the other tissues included in Table III. Secondly, under our assay conditions, the fraction of total β-glucosidase activity that is soluble rather than particulate is much higher in the kidney than in any other organ examined (Table III).

The specific activity of this soluble, near-neutral pH optimum β-glucosidase of rat kidney cytosol was not influenced by long term exposure (10 days) of the animals to high protein diets or by the inclusion of acidosis-inducing NH_4Cl (1.5%, w/v) in their drinking water.

Distribution of β-glucosidase activity in the kidney

In an effort to obtain information concerning the cellular localization of soluble β-glucosidase in the rat kidney, we employed a dissection procedure involving the preparation of serial slices from the cortex to the papilla regions as described by Curthoys and Lowry [19]. As an internal enzyme marker to assist us in the assignment of kidney regions, we assayed homogenates for γ-glutamyl transpeptidase activity which has been shown to occur almost exclusively in the proximal straight tubule cells of rat kidney [19]. All five of the

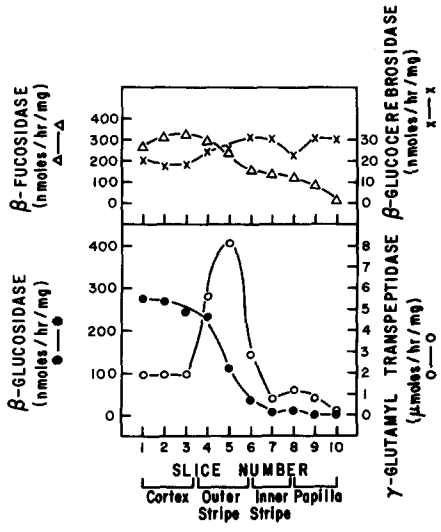

Fig. 5. Gross distribution of β-glucosidase activity in rat kidney. A cone from freshly excised rat kidney was sliced into 10 equivalent sections (1—2 mg) which were homogenized with 0.25 ml of the sucrose-Tris-EDTA medium described in Materials and Methods. Aliquots (5—20 μl) were assayed for various enzyme activities indicated as described in the text. γ-Glutamyl transpeptidase activity was determined according to Curthoys and Kuhlenschmidt [35]. Specific activities are expressed on the basis of protein which was determined by the method of Lowry et al. [14]. The gross appearance of the tissue together with the distribution of the γ-glutamyl transpeptidase marker [19] permitted us to define the various regions of the kidney. Although not shown, α-L-arabinosidase and β-D-galactosidase activities were distributed in a manner essentially identical to the β-D-fucosidase profile.

various hydrolase activities associated with the purified enzyme, namely β-D-glucosidase, β-D-galactosidase, β-D-fucosidase, β-D-xylosidase and α-L-arabinosidase, were enriched in the cortex region (Fig. 5). However, although the activities of the last four of the previously mentioned enzyme activities were still significant in the inner stripe region, β-D-glucosidase activity was essential restricted to the cortex region and dropped to very low levels in the 5th and 6th slices. β-Glucocerebrosidase, a lysosomal hydrolase [20], was distributed broadly throughout the various regions of the kidney and did not display a prominent peak. These results are consistent with the histochemical observation [6] that the major soluble, neutral pH optimum β-glucosidase that we have isolated from rat kidney is contained in convoluted proximal tubule cells comprising the cortex region.

Criteria of purity

Attempts to further increase the specific activity of the β-glucosidase preparation by chromatography on QAE-Sephadex (pH 7), DEAE-cellulose (pH 7) and carboxymethyl-cellulose (pH 6) were unsuccessful. The enzyme chromatographed as a single component on Sephadex G-200 (Fig. 4) and the specific activity of the glycosidase on each of the various substrates tested was constant over the entire profile of the major protein peak. Electrophoresis at pH 8.9 on ordinary disc gel yielded a single protein component (R_F, 0.67) that was coincident with β-glucosidase activity (Fig. 6). Staining of replicate gels with periodic acid-Schiff's base reagents according to Zacharius et al. [21] did not reveal

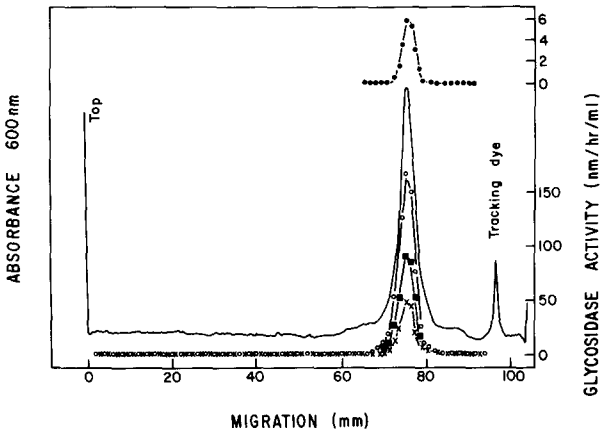

Fig. 6. Polyacrylamide disc gel electrophoresis of purified rat kidney β-glucosidase. Electrophoresis was performed at pH 8.9 as described in the text. The gels were first sliced (2-mm sections) and then extracted with 0.5 ml of 0.3 M sodium acetate buffer for 18 h at 20°C. Identical gels were stained for protein with Buffalo Blue-Black and scanned for absorbance at 600 nm (———) using a Gilford linear transport. ○, β-D-glucosidase; ■, β-D-fucosidase; X, β-D-galactosidase; ●, α-L-arabinosidase. The limited quantity of β-D-xylosidase activity on the gels prevented the measurement of this activity. Similar gels containing 60 μg of enzyme protein did not reveal detectable carbohydrate when subjected to the periodic acid-Schiff's base staining procedure of Zacharius et al. [21].

the presence of carbohydrate. Similarly, disc gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol indicated a single protein component that migrated very near the heavy subunit of human γ-globulin (Fig. 7). Additional evidence indicating the absence of significant contamination were the results of equilibrium centrifugation (Fig. 8); a plot of ln distance versus the square of the fringe displacement yielded a straight line. Under our stan-

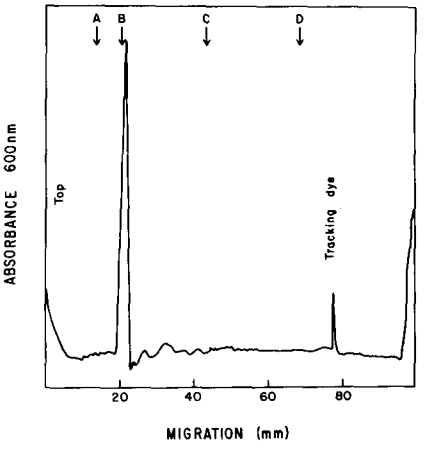

Fig. 7. Polyacrylamide gel electrophoresis of purified rat kidney β-glucosidase in a buffer containing sodium dodecyl sulfate and β-mercaptoethanol. Gels were stained for protein with Coomassie blue, and scanned for absorbance at 600 nm using a Gilford linear transport. The arrows indicate the migration positions of the following proteins used as molecular weight standards: A, human serum albumin (mol. wt 68 000); B, human immunoglobin (heavy chain, mol. wt 50 000); C, human immunoglobin (light chain, mol. wt 25 000); D, equine cytochrome *c* (mol. wt 11 700). These standards were run in replicate gels in the presence and absence of purified rat kidney β-glucosidase.

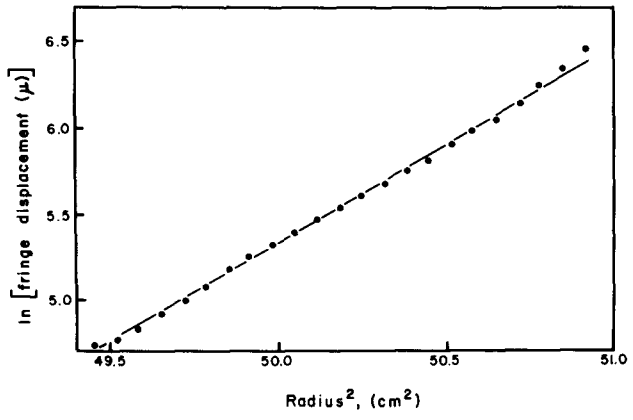

Fig. 8. Analytical ultracentrifugation molecular weight estimation of purified rat kidney β-glucosidase by the approach-to-equilibrium method of Yphantis [12]. Plot of the square of radial position in the rotor (cm) vs the ln of fringe displacement (μ). Details of method are described in the text. The slope of the plot is 1.17 and is consistent with a molecular weight of 57 700, assuming a partial specific volume of 0.727 ml per g which was calculated from the amino acid composition (Table VI) according to the method of Cohn and Edsall as described by Schachman [22].

dard conditions of assay, the specific activity of the apparently homogeneous β-glucosidase is 115 000 nmol of glucose released from 4-methylumbelliferyl-β-D-glucopyranoside per mg protein per h. Although the purification factor of 420 may seem low when compared with other glycosidases, it should be remembered that this glycosidase is found in the cytosol, while most other glycosidases are lysosomal or microsomal. Furthermore, the specific activity of this enzyme is relatively high in the rat kidney, the organ from which the enzyme was isolated, as compared to other organs.

Evidence for a single protein catalyzing the hydrolysis of several glycosides

The fact that purification of the β-D-glucosidase to constant specific activity and apparent homogeneity yielded a preparation capable of hydrolyzing β-D-galactosides, β-D-fucosides, α-L-arabinosides and β-D-xylosides suggested that a single enzyme was responsible for the hydrolysis of each of the substrates. In fact, on the last four column chromatographic steps of the purification procedure, the ratio of the specific activity of β-fucosidase, β-galactosidase, β-xylosidase or α-arabinosidase to that of β-glucosidase was nearly constant (Table I). In addition, when the purified enzyme preparation was subjected to disc gel electrophoresis in polyacrylamide gels, β-glucosidase, β-galactosidase, β-fucosidase, and α-arabinosidase activities co-electrophoresed (Fig. 6). The results of heat inactivation studies also indicate that a single protein is responsible for the hydrolysis of these various substrates (Table IV). For a given aglycone-containing substrate, regardless of the glycoside, the half-life ($t_{1/2}$) of the various glycosidase activities were the same; at 54°C, the $t_{1/2}$ of the enzyme was found to be 12.0, 12.0 and 11.8 min when assayed for activity using the 4-methylumbelliferyl derivatives of β-D-glucose, β-D-galactose, and α-L-arabinoside, respectively. Under the same conditions, the half-life of the purified glycosidase preparation was 19.0 min (±2.0 min) when determined with the *p*-

TABLE IV

HEAT INACTIVATION CHARACTERISTICS OF RAT KIDNEY GLYCOSIDASE DETERMINED WITH VARIOUS SUBSTRATES

200 μl of purified β-glucosidase (0.20 mg/ml) in 2 mM sodium phosphate buffer, pH 7.5 was heated at 54° C for various times (0—21 min at 3-min intervals) and rapidly cooled in an ice bath. Various glycosidase activities were determined using the standard assay described in the text and substrates indicated above. Plots of the logarithm of activity versus the time of heating yielded straight lines from which the half-life ($t_{1/2}$) was determined.

Substrate	$t_{1/2}$ (min)
p-Nitrophenyl-β-D-glucopyranoside	18.0
p-Nitrophenyl-β-D-fucopyranoside	20.4
p-Nitrophenyl-β-D-xylopyranoside	18.0
4-Methylumbelliferyl-β-D-glucopyranoside	12.0
4-Methylumbelliferyl-β-D-galactopyranoside	12.0
4-Methylumbelliferyl-α-L-arabinopyranoside	11.8

nitrophenyl derivatives of β-D-glucose, β-D-fucose and β-D-xylose. This apparent discrepancy in the half-life of the β-D-glucosidase when determined with the *p*-nitrophenyl and 4-methylumbelliferyl derivatives of β-D-glucose could be explained if different regions of the enzyme bind the *p*-nitrophenyl and 4-

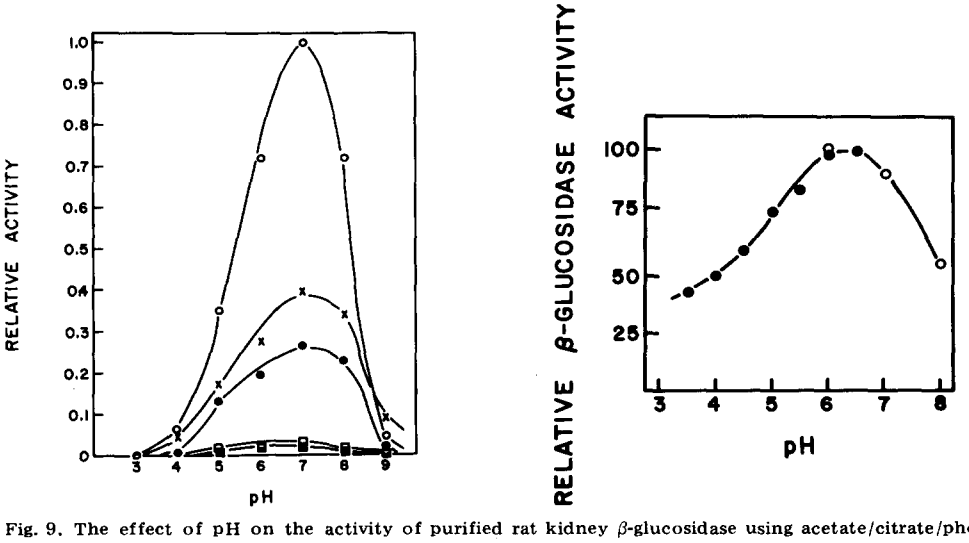

Fig. 9. The effect of pH on the activity of purified rat kidney β-glucosidase using acetate/citrate/phosphate buffer. Comparison of β-D-glucosidase (○), β-D-fucosidase (X), β-D-galactosidase (●), α-L-arabinosidase (□) and β-D-xylosidase (■) activities as a function of pH. The purified preparation of β-glucosidase was assayed with the substrates indicated in Table I using the standard assay conditions except that a single buffer containing sodium acetate, sodium citrate, and sodium phosphate, each at a final concentration of 0.15 M, was employed.

Fig. 10. Effect of pH on rat kidney β-glucosidase activity using sodium acetate and sodium phosphate buffer. Purified β-glucosidase was assayed at various pH in 0.10 M sodium acetate (●) and sodium phosphate (○) buffers using 4-methylumbelliferyl-β-D-glucopyranoside as substrate according to conditions described in the text.

methylumbelliferyl aglycones, and if these two regions of the enzyme are differentially sensitive to heat inactivation.

Finally, the observation that all five enzyme activities (β-D-glucosidase, β-D-galactosidase, β-D-fucosidase, β-D-xylosidase and α-L-arabinosidase) have very similar dependency on pH is consistent with the concept that one enzyme is capable of hydrolyzing all five substrates (Fig. 9). β-Glucosidase activity displays a relatively broad pH optimum with maximum activity between pH 6 and 7 when assayed in acetate or phosphate buffers and no specific buffer effects were observed (Fig. 10). Varying the concentration of either buffer between 0.01 and 0.25 M concentration had no effect on catalytic activity at pH 6.5.

Molecular weight-related parameters

Using partially purified preparations of soluble kidney β-glucosidase and based upon gel filtration chromatography data, various investigators [3,4] have reported the molecular weight of the enzyme to be approx. 50 000. The molecular weight of the current rat kidney enzyme was determined using several independent methods. When analyzed in the ultracentrifuge by the approach-to-equilibrium sedimentation procedure of Yphantis [12], a molecular weight of 57 700 was obtained (Table V, Fig. 8). This value was calculated using a partial specific value of 0.727 ml per g which was derived from the amino acid composition (Table VI) by the method of Cohn and Edsall as described by Schachman [22]. The empirical method of Weber and Osborn [11] involving polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulfate and mercaptoethanol yielded a lower value for the molecular weight, 49 500 (Fig. 7). Estimation of the molecular weight of rat kidney β-glucosidase using a gel filtration method (Fig. 4) according to DeVincenzi and Hedrick [9] yielded a value of 58 500. The sedimentation coefficient of the enzyme, determined by the empirical sucrose density gradient procedure of Martin and Ames [23], was 4.41 S.

TABLE V

SUMMARY OF MOLECULAR WEIGHT-RELATED PARAMETERS OF RAT KIDNEY β-GLUCOSIDASE

Molecular weight determination via	Molecular weight
(a) Ultracentrifuge	57770 (±1500)
(b) Sephadex G-200 chromatography	58500 (±1500)
(c) Sodium dodecyl sulfate disc gel electrophoresis	49500 (±2000)
Sedimentation coefficient *	4.41 (±0.25) S
Partial specific volume**	0.727 ml per g

* Determined by the sucrose density gradient centrifugation procedure of Martin and Ames [23] using bovine catalase as a standard with an assumed $S_{20,w}$ of 11.3.

** Calculated from amino acid composition by the method of Cohn and Edsall as modified by Schachman [22].

TABLE VI

AMINO ACID COMPOSITION OF SOLUBLE RAT KIDNEY GLYCOSIDASE

Amino acid	Residues per 58 000 g	
	Experiments value	Integer value
Lysine	25.5	26
Histidine	10.1	10
Arginine	24.5	25
Aspartic acid	51.7	52
Threonine	22.1	22
Serine	23.0	23
Glutamic acid	63.5	64
Proline	37.2	37
Glycine	35.3	35
Alanine	33.0	33
Half-cystine*	10.4	10
Valine	23.7	24
Methionine	7.2	7
Isoleucine	24.5	25
Leucine	35.4	35
Tyrosine	19.3	19
Phenylalanine	33.3	33
Tryptophan**	15.1	15

* Half-cystine was determined as cysteic acid after performic acid oxidation.

** Tryptophan was determined spectrophotometrically [18]. Glucosamine and galactosamine were not detected.

Amino acid composition

Rat kidney β-glucosidase contains a relatively large number of acidic amino acid residues and few basic amino acids (Table VI). Although not determined, the number of amide residues in the protein must be relatively small. This conclusion is supported by the observations that: (1) the enzyme has rapid mobility on polyacrylamide disc gels at pH 8.9 (Fig. 6), and (2) β-glucosidase activity binds firmly to DEAE-cellulose at pH values as low as 4.5 whereas most contaminating proteins do not. Since β-glucosidase does not contain a limiting amino acid residue, it was not possible to derive a minimum residue molecular weight from amino acid composition. The enzyme also contains a relatively large number of half-cystine residues (10 per molecule). The susceptibility of the enzyme to inhibition by a variety of sulfhydryl reagents as well as the stimulation of activity by various thiols (Table II) suggest that at least one free sulfhydryl group is present and that it is essential for activity. The absence of amino sugars indicates that the rat kidney β-glucosidase is not a glycoprotein.

Substrate specificity

A variety of glycosides were tested for their ability to serve as substrates for the purified enzyme and the results are summarized in Table VII. Of the non-physiologic, artificial substrates tested, only β-D-glucoside, β-D-galactoside, β-D-fucoside, β-D-xyloside and α-L-arabinoside derivatives of *p*-nitrophenyl or 4-methylumbelliferone were effectively hydrolyzed by the purified enzyme. Each of these substrates share the common structural feature of containing

TABLE VII

ANALYSIS OF THE SUBSTRATE SPECIFICITY OF PURIFIED RAT KIDNEY GLYCOSIDASE

Unless otherwise indicated, all substrates were tested under conditions described in the text for the standard assay. K_m values were obtained by varying substrate concentration over the range indicated; double reciprocal plots from reaction rates observed at 15 different substrate concentrations were linear.

Compound	Final concentration (mM)	V (nmol/h per mg)	K_m (mM)
4-Methylumbelliferyl-β-D-glucopyranoside	0.01— 1.0	115000	0.26
4-Methylumbelliferyl-β-D-galactopyranoside	0.01— 1.0	29900	0.18
4-Methylumbelliferyl-α-L-arabinoside	0.04— 4.0	3220	0.049
p-Nitrophenyl-β-D-glucopyranoside	0.2 —12.0	82000	2.06
p-Nitrophenyl-β-D-galactopyranoside	0.2 —12.0	21400	2.37
p-Nitrophenyl-β-D-fucopyranoside	0.04— 4.0	44900	3.22
p-Nitrophenyl-β-D-xylopyranoside	0.04— 4.0	1960	3.32
p-Nitrophenyl-β-D-mannopyranoside	2.5	<100	—
4-Methylumbelliferyl-β-D-*N*-acetylglucosaminide	1.0	<100	—
4-Methylumbelliferyl-β-D-acetylgalactosaminide	2.0	<100	—
p-Nitrophenyl-β-D-*N*-acetylglucosaminide	1.0	<100	—
4-Methylumbelliferyl-α-D-glucopyranoside	1.0	<100	—
4-Methylumbelliferyl-α-D-galactopyranoside	1.0	<100	—
4-Methylumbelliferyl-β-D-glucoronic acid	1.0	<100	—
p-Nitrophenyl-α-D-glucopyranoside	1.0	<100	—
p-Nitrophenyl-α-D-galactopyranoside	1.0	<100	—
p-Nitrophenyl-α-D-mannopyranoside	1.0	<100	—
Lactose*	0.1 — 5.0	<50	—
N-Stearoyl-β-D-glucosylceramide**	0.18	5.5	N.D.
Galactosyl-β-D-ovalbumin***	0.00043	<100	—
Xylosyl-*O*-serine†	1.0	<100	—
Trehalose*	1.0 —10	<50	—

* Glucose release was determined spectrophotometrically using the method of Lowry and Passonneau [38].

** ^{14}C-labeled *N*-stearoyl-β-D-glucosylceramide was prepared according to Erickson and Radin [26]. The assay conditions and procedure for ^{14}C-labeled *N*-stearoylceramide determination are described in Table I.

*** [^{14}C]galactosylovalbumin was prepared using UDP-[U-^{14}C]galactose, ovalbumin, and bovine serum galactosyltransferase purified 300-fold through the UDP-Sepharose column step as described by Barker et al. [29]. [^{14}C]Galactose release was determined by measuring the loss of trichloroacetic acid (5%)/phosphotungstic acid (1%)-precipitable radioactivity.

† Xylosyl-*O*-serine hydrolysis was determined by measuring serine release using the amino acid analyzer [16] and by measuring xylose release by the reducing sugar procedure of Park and Johnson [37].

trans, equitorial oxygen atoms at positions 1, 2 and 3 of the monosaccharide moiety. Since the *p*-nitrophenyl and 4-methylumbelliferyl derivatives of β-D-glucose are both hydrolyzed, the enzyme seems to be less specific for the aglycone moiety of the glycoside than the carbohydrate moiety. β-Glucuronyl, β-*N*-acetylglucosaminyl and β-*N*-acetylgalactosaminyl derivatives were not hydrolyzed by the purified enzyme. As reported by Patel and Tappel [1], lactose is not hydrolyzed by the soluble, broad specificity rat kidney hydrolase. Furthermore, the presence of high concentrations of lactose (100 mM) in the assay medium did not inhibit the hydrolysis of any of the effective substrates. This observation is consistent with the report of Swaminathan and Radhakrishnan [24] that kidney lactase occurs exclusively as a particulate enzyme.

Patrick [25] reported that tissues of patients with Gaucher's disease, which are markedly deficient in β-glucocerebrosidase activity [20], were also deficient in β-glucosidase activity when assayed with *p*-nitrophenyl β-D-glucose at pH 5.6 in a citrate/phosphate buffer. We therefore monitored β-glucocerebrosidase activity throughout the entire purification procedure using the authentic substrate, ^{14}C-labeled *N*-stearoyl-β-D-glucocerebroside [26]. Although the specific activity of the soluble β-glucosidase increased 420-fold during purification, the specific activity of the final purified hydrolase preparation on the β-glucocerebroside substrate actually decreased to less than half that of the initial kidney extract. Furthermore, β-glucocerebrosidase activity was predominantly (85%) particulate (100 000 × *g*, 90 min pellet) and the residual β-glucocerebrosidase activity that was contained in the corresponding supernatant fraction was not inactivated but could be recovered from the DEAE-cellulose column in fractions distinct from those that contained β-glucosidase activity. Thus, it appears that the enzyme we have purified is not involved in glycolipid catabolism.

Although the purified rat kidney enzyme will hydrolyze *p*-nitrophenyl-β-D-xylose, it will not cleave the glycosidic linkage in xylosyl-*O*-serine which occurs in mucopolysaccharides [27]. The possibility remains that such β-xylosyl residues may be cleaved by the enzyme we have isolated if they exist as part of a larger glycopeptide.

Recognizing that many glycoproteins contain β-linked galactosyl residues in the terminal regions of their oligosaccharide side chains [28], we prepared [^{14}C]galactosylovalbumin using extensively purified galactosyltransferase from fetal calf serum. It was shown by Barker et al. [29] that this particular galactosyltransferase synthesizes β-linked, terminal, non-reducing galactosyl residues. As shown in Table VII, the purified glycosidase was incapable of removing detectable quantities of terminal galactose residues from labeled galactosylovalbumin. Trehalose was not hydrolyzed by the purified β-glucosidase.

Inhibitors

As is true for the soluble β-glucosidase in the kidney of a variety of animals [1—3], glucono (1 → 5) lactone is a potent inhibitor of the current rat kidney β-glucosidase. The K_I values for this inhibitor were 0.61, 0.60, and 0.51 mM when the purified glycosidase preparation was assayed for β-D-glucosidase, β-D-galactosidase, and α-L-arabinosidase activities, respectively, using their 4-methylumbelliferone derivatives. In contrast to the porcine kidney enzyme which is phloridzin sensitive [4], the β-glucosidase of rat kidney was not inhibited when phloridzin was included in the assay at a final concentration of 2 mM. However, phloridzin was hydrolyzed by the β-glucosidase. Similarly, glucose (50 mM) or 2-deoxyglucose (50 mM) had no effect on rat kidney β-glucosidase activity.

Discussion

This present study has demonstrated that an extensively purified preparation of the major β-glucosidase in rat kidney cytosol is also capable of hydrolyzing a variety of substrates including β-linked galactosides, fucosides and

xylosides and α-linked arabinoside derivatives of 4-methylumbelliferone and *p*-nitrophenol. These results with the extensively purified enzyme are in agreement with the observations of a number of investigators who, using partially purified preparations of the cytoplasmic β-glucosidase from rat, pig and human kidney, concluded that a single enzyme catalyzes the hydrolysis of these various artificial glycosides [1—4]. A common structural feature shared by these five glycoside substrates is that each possesses the all *trans*-equitorial configuration with respect to the oxygen atoms at positions 1, 2 and 3 of the monosaccharide moiety. Furthermore, the 2-hydroxyl group might be essential for activity since 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide is not a substrate for the enzyme (Table VII).

As pointed out by Price and Dance [2], it is unwise to assume that the relative activity of the enzyme towards artificial, non-physiological substrates reflects it specificity towards natural substrates which are obscure at the present time. Although the enzyme is active on a broad range of artificial glycosides, it has not been possible to ascribe a function to the enzyme. In the case of the pig, the sensitivity of the kidney enzyme to inhibition by phloridzin as well as its location in the proximal convoluted cells of the kidney, has led to the suggestion that soluble hydrolases may be involved in renal reabsorption of sugars [7]. However, no direct evidence to support this concept exists. Indirect evidence which is consistent with the idea that the soluble β-glucosidase could play a role in glucose transport is the observation that the two rat tissues with the greatest capacity for glucose transport, the kidney and the intestine, are the tissues which contain β-glucosidase activity with the highest specific activities in cytosol fraction (Table III, Fig. 11). Furthermore, when the rat intestine was fractionated into a gradient of cells ranging from the crypt to the villus regions by the procedure of Weiser [30], the outermost villi cells were found to contain the greatest quantity of soluble, β-glucosidase activity (Fig. 11). It is the brush border region of mucosal cells that is thought to be the site where

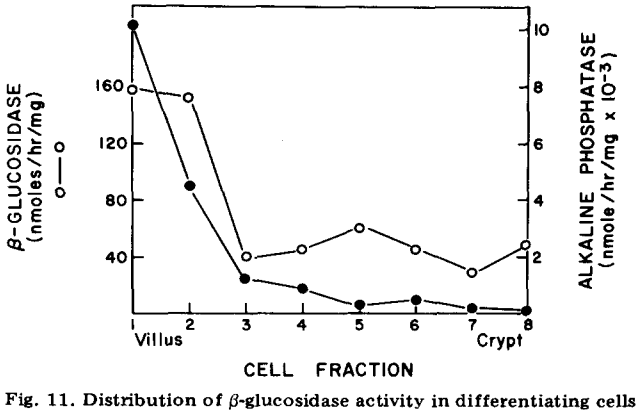

Fig. 11. Distribution of β-glucosidase activity in differentiating cells of the rat intestine. A gradient of cells (eight fractions) from the crypt to villus regions of the rat intestine were prepared as described elsewhere [30] and homogenized as described in Fig. 5. Aliquots (25 μl) were assayed for β-glucosidase activity (o) and alkaline phosphatase activity (•) as described elsewhere [36], the latter serving as a marker for villus cells. Specific activities are expressed on the basis of protein which was determined by the procedure of Lowry et al. [14].

monosaccharides are actively transported in the small intestine [31]. Therefore, in tissues with a high capacity for glucose transport, β-glucosidase activity is enriched in those cells which specifically carry out this transport function; specifically, soluble β-glucosidase activity is highest in the proximal convoluted tubule cells of the kidney (ref. 6, Fig. 5) and villus cells of the intestine (Fig. 11). It is noteworthy that a common system seems to be involved in the transport of glucose, galactose and xylose in the kidney [32] and that derivatives of these same monosaccharides are appropriate substrates for the hydrolase that we have described in the present report. Although molecular details of the events involved in sugar transport are lacking, such a hydrolase could function to release glucose intracellularly from some glycoside that is itself an intermediate in transport.

The finding that the purified enzyme will not hydrolyze the terminal, β-linked galactose residue from galactosylovalbumin (Table VII) indicates that it is probably not involved in the degradation of glycoproteins in the kidney. Considerable quantities of the serum glycoproteins contained in the glomerular filtrate are taken up and extensively degraded by epithelial cells of the proximal convoluted tubule. However, the observation that this process appears to involve the fusion of pinocytotic, glycoprotein-containing vesicles with lysosomes [33], makes it unlikely that the soluble cytoplasmic hydrolase that we have described with its near neutral pH optimum would participate in the degradation of the oligosaccharide side chains of these glycoproteins. The glycosidases responsible for the catabolism of glycolipids and glycoproteins are associated primarily with lysosomes and have relatively acidic pH optima [34].

The availability of pure preparations of the rat kidney glycosidase should facilitate the search for physiological and relevant substrates for the enzymes as well as studies directed at defining its function in the kidney. The list of possible candidates for authentic substrates should be expanded to include additional galactose-terminated glycoproteins and glycopeptides as well as various galactosyl and glucosyl derivatives of mammalian sterols.

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