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β-D-Glucosidases and Related Enzymic Activities in Pig Kidney

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1. The β-glucosidase activity of pig kidney is located in the unsedimentable fraction of the cell and is not associated with the lysosomes. 2. The enzyme is active towards β-D-glucosides, β-D-galactosides, β-D-xylosides and α-L-arabinosides. 3. These activities could not be separated by gel electrophoresis, gel filtration or DEAE-cellulose chromatography. 4. Response to inhibitors, heat-denaturation and competitive substrates suggests that a single active site is responsible for all four activities. 5. Two forms of the enzyme were found to occur either separately or together in kidneys of pigs from several different breeds. 6. Electro-focusing experiments show these to have a small difference in isoelectric point (4·9 and 5·1), and gel filtration gives an approximate molecular weight of 50000 for both forms. 7. The characteristics of these two enzymes are compared.

The occurrence of β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) in the kidneys of horses and cattle was reported by Neuberg & Hofmann (1935), and in pig intestinal mucosa by Steensholt & Viebel (1943). Robinson (1956), using the fluorigenic substrate used in the present paper, demonstrated its presence in liver, kidney, spleen and duodenum of rat, guinea pig, ferret, rabbit and mouse, and noted wide variations in the activity of the same organ from different species. Histochemical examination (Cohen, Tsou, Rutenburg & Seligman, 1952; Pearson, Andrews & Grose, 1961) also demonstrated a widespread occurrence, and the latter authors found that in the kidney only the cytoplasm of the proximal convoluted tubules was involved. The enzyme of rat kidney was characterized by Robinson, Price & Dance (1967) and shown to be different from a specific β-D-galactosidase that was also present. This rat kidney enzyme bore a close resemblance in its physical properties to that described by Chytil (1965) from ox liver.

After our preliminary report of β-D-xylosidase activity in pig kidney (Robinson & Abrahams, 1967) we have made a further study of renal β-D-glucosidase in this species.

EXPERIMENTAL

Materials. The following glycosides of 4-methylumbelliferone were prepared for use as fluorigenic substrates: β-D-glucoside (Robinson, 1956), β-D-galactoside, α-L-arabinoside (Robinson, 1964) and β-D-xyloside (de Bruyne & Loontiens, 1965). Arbutin, phlorrhizin, salicin, cellobiose, galactono-(1→4)-lactone and glucono-(1→5)-lactone were supplied by Koch–Light Laboratories, Colnbrook, Bucks. Lactose, xylose, sucrose, serine, buffer constituents and other salts were obtained from British Drug Houses Ltd., Poole, Dorset. Xylosyl-*O*-serine was the gift of Dr B. Lindberg, Karolinska Institutet, Stockholm, Sweden, and xylan (ex *Rhodymenia palmata*) was kindly supplied by Professor D. J. Manners, Heriot-Watt University, Edinburgh. The markers used for the estimation of molecular weights by gel filtration (Andrews, 1964, 1965) were horseradish peroxidase and cytochrome *c* (British Drug Houses Ltd.), *Escherichia coli* alkaline phosphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.) and Blue dextran 2000 (Pharmacia, Uppsala, Sweden).

Tissue preparations. Kidneys from pigs of known breed and sex were obtained immediately after slaughter (T. Wall and Sons Ltd., Willesden, London, N.W. 10) and kept frozen at −15° until required. Samples used for tissue-fractionation studies were not frozen and were processed within 1hr. of removal from the carcass in the following way. A 10% (w/v) homogenate was prepared in ice-cold 0·25M-sucrose containing 0·68mM-EDTA and adjusted to pH 7·0 with 0·25N-NaOH. After gentle homogenization in a smooth-glass Potter–Elvehjem-type homogenizer by two passes of a loose-fitting Teflon pestle operated by hand, the suspension was filtered through two thicknesses of muslin and then subjected to differential centrifugation in an MSE High Speed 17 refrigerated centrifuge. The following five successive fractions were obtained with force–time integrals corresponding to the fractionation described by Shibko & Tappel (1965): nuclear (N), 1600*g*-min. sediment; lysosomal–mitochondrial (LM), 10000*g*-min.; mitochondrial–microsomal (MM), 240000*g*-min.; microsomal (M), 6×10^6*g*-min.; an unsedimentable (S) fraction. The sediments were resuspended in 0·25M-sucrose for assay.

Enzyme assays. The fluorimetric assays have already been described for β-D-glucosidase and β-D-galactosidase (Price & Robinson, 1966), β-D-xylosidase (Robinson & Abrahams, 1967) and α-L-arabinosidase (Robinson, 1964). Acid phosphatase was measured as described by Furth & Robinson (1965), glucose 6-phosphatase by the method of Dallner (1963) with the liberated phosphate measured as

described by Allen (1940), succinate–neotetrazolium reductase as described by Slater & Planterose (1960), alkaline phosphatase by the method of Garen & Levinthal (1960) and peroxidase as described by Polis & Shmukler (1953).

Other assays. The following methods were used: cytochrome *c* (extinction at 412nm.), Blue dextran (extinction at 625nm.), protein (Lowry, Rosebrough, Farr & Randall, 1951), glucose (Dahlqvist, 1960), and xylose (Roe & Rice, 1948).

DEAE-cellulose chromatography and gel electrophoresis. The methods described by Robinson *et al.* (1967) were used.

Gel filtration. Sephadex G-200 was used as described by Robinson *et al.* (1967). Bio-Gel P-150 (100–200 mesh; BioRad Laboratories, Richmond, Calif., U.S.A.) was prepared by allowing the beads to swell overnight in 0·01M-sodium phosphate buffer, pH 7·6, containing 0·04M-NaCl and 0·01M-EDTA. The hydrated gel was then passed through a 20-mesh screen and packed under gravity in a 36cm. × 3cm. diam. column coated with dichlorodimethylsilane to minimize wall effects. Enzyme samples were applied in 2ml. of the same buffer and eluted at a flow rate of 10ml./hr.

Electro-focusing. Samples containing not more than 15mg. of protein were separated in an Ampholine column, capacity 110ml. (L.K.B. Instruments Ltd., London, S.E. 20). A pH gradient 3–6 was used with a 1% (w/v) carrier electrolyte solution in a sucrose density gradient [45–0% (w/v) sucrose]. The gradient was formed in 24 equal steps over a lower anode solution containing 12g. of sucrose dissolved in 14ml. of 0·5N-H_2SO_4.

The sample was distributed evenly through steps 9–16 and the gradient overlaid with 10ml. of 2% (w/v) ethanolamine as cathode solution. A potential of 800v at 2mA was applied for 48hr., the apparatus being maintained at 4°. After the focusing, the column was drained and collected in 1ml. fractions, which were assayed for β-D-glucosidase, their pH values measured and the peak-tube contents examined by gel electrophoresis.

Thin-layer chromatography. Glycosides, free sugars and serine were separated on 0·25mm. layers of Whatman CC 41 cellulose powder, in ethyl acetate–pyridine–water (12:5:4, by vol.) and detected by the method of Weiss & Smith (1967).

K_m, K_i and $V_{max.}$ values. The double-reciprocal plot method of Lineweaver & Burk (1934) was used.

RESULTS AND DISCUSSION

The mean activity of 53 kidneys was equivalent to 70 ± 23nmoles of substrate hydrolysed/hr. by 1mg. wet wt. of tissue. There was no appreciable loss of activity when the kidneys were stored for several months at −15°.

Tissue fractionation. Almost all the β-D-glucosidase activity that could be estimated in pig kidney was found to be localized in the supernatant when sucrose homogenates were fractionated as described (Table 1). This same fraction also contained the β-D-xylosidase and α-L-arabinosidase, the activity of all three enzymes was not further increased after the suspension had been frozen-and-thawed rapidly ten times and it was concluded that they did not occur in a latent form. On the other hand some β-D-galactosidase activity was present in the sedimentable fractions, where it exhibited the latency typical of lysosomally bound enzymes, and in the supernatant, where it was fully active without further treatment. The distribution of succinate–neotetrazolium reductase, glucose 6-phosphatase and acid phosphatase was as expected for mitochondrial, microsomal and lysosomal enzymes respectively. There is thus no evidence that the β-D-glucosidase is bound in or on the lysosomes, although in other organs this may be so (Beck & Tappel, 1968; Fisher, Whitehouse & Kent, 1967).

Gel electrophoresis. The β-D-glucosidase was detected after gel electrophoresis of homogenates at pH 7·0 as one or two sharply defined zones of

Table 1. *Intracellular distribution of pig kidney β-D-galactosidase, β-D-glucosidase, α-L-arabinosidase and β-D-xylosidase*

Distribution of total activity (%) after differential centrifugation of a 0·25M-sucrose homogenate of pig kidney was followed by enzyme assay as described in the text. Latency (in parenthesis) is the percentage of the activity in a given fraction that can only be estimated after alternately freezing-and-thawing the homogenate ten times.

	Total recovered activity in each fraction (%)				
Enzyme	Nuclear	Lysosomal–mitochrondrial	Mitochrondrial–microsomal	Microsomal	Soluble
β-D-Glucosidase	2·5 (0)	2·8 (0)	2·1 (0)	3·0 (0)	89·6 (0)
β-D-Galactosidase	4·4 (40)	16·2 (67·0)	8·1 (50)	2·3 (0)	69·1 (0)
α-L-Arabinosidase	2·5 (0)	2·8 (0)	3·8 (0)	3·1 (0)	87·8 (0)
β-D-Xylosidase	3·9 (0)	3·0 (0)	4·7 (0)	3·5 (0)	84·9 (0)
Acid phosphatase	31·9	33·6	14·7	2·7	16·9
Glucose 6-phosphatase	14·0	17·0	26·0	43·0	0
Succinate–neotetrazolium reductase	8·4	33·0	51·0	7·6	0

activity migrating rapidly towards the anode. These bands also reacted towards 4-methylumbelliferyl β-D-xyloside, β-D-galactoside and α-L-arabinoside and were present whether homogenates of cortex or medulla were used.

Of 54 animals examined, the kidneys of 23 had two bands of the broad specificity described, 29 had the faster of the two bands only and in two cases only the slower band was evident. There was no obvious correlation between the electrophoretic pattern and the sex or breed of the pig. In addition, electrophoresis of the lysosomal sediment from the tissue fractionation described revealed a slow-moving zone of β-D-galactosidase activity with no reaction towards the other substrates mentioned above. Similar patterns were described for the enzymes of the rat kidney by Robinson *et al.* (1967). The intensity of the fluorescent zones on the gel after it had been treated with substrate were compared on an Aminco–Bowman microspectrophotofluorimeter fitted with a thin-layer-scanning attachment. Preliminary experiments with serial dilutions of enzyme had shown that the plots obtained were proportional to the amount of enzyme present. As shown in Fig. 1, when two bands occurred in the same sample they were present in roughly equal amounts. The two forms have been named β-D-glucosidase 1 and 2 in order of decreasing electrophoretic mobility (cf. lactate dehydrogenase isoenzymes).

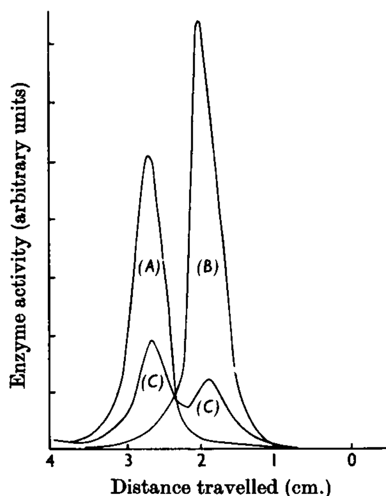

Fig. 1. Fluorimetric scan of starch-gel electrophoretograms of pig kidney β-D-glucosidase. The gels were kept in contact with filter paper soaked with 1mM-4-methylumbelliferyl-β-D-glucoside in 0·15M-sodium phosphate–citrate buffer, pH 5·5, for 10min. at 37° and the fluorescent bands that developed were scanned at 450nm. on the thin-layer attachment of the Aminco–Bowman microspectrophotofluorimeter. (*A*) Kidney with β-D-glucosidase 1 only; (*B*) kidney with β-D-glucosidase 2 only; (*C*) kidney with both forms.

Partial purification. A 20% homogenate in 0·25M-sucrose and 0·68mM-EDTA, pH 7·0, was centrifuged for 20min. at 10000rev./min. ($2.4\times 10^5 g$-min.) to sediment the lysosomal fraction and remove the associated β-D-galactosidase. The solution was then adjusted to pH 5 with 0·2M-citric acid, and heated to 37° and held at this temperature for 20min. The agglutinated protein was centrifuged off and the supernatant was dialysed under vacuum overnight at 4° against 0·01M-sodium phosphate buffer, pH 7. The concentrate was then subjected to a second agglutination as before and a further small amount of inactive protein centrifuged off. The supernatant solution contained over 50% of the original activity with a fivefold increase in specific activity. The preparation was conveniently stored as a freeze-dried powder, but the freeze-drying resulted in some loss of activity (Table 2).

DEAE-cellulose separation. The two forms of β-D-glucosidase were separated from each other and from the lysosomal β-D-galactosidase by stepwise elution with sodium chloride (0·02–0·1M at 0·02M intervals) from a DEAE-cellulose column at pH 7·0 in 0·01M-phosphate buffer. Each step consisted of 60ml. of buffer–salt solution. Under these conditions the lysosomal enzyme was not retained on the column when applied in buffer alone and β-D-glucosidases 1 and 2 were eluted in that order by 0·02M- and 0·06M-sodium chloride solutions respectively. Samples from kidneys having only

Table 2. *Partial purification of β-D-glucosidase from pig kidney*

Activity is expressed as μmoles of substrate hydrolysed/hr. at pH 5·5 and 37° in 0·2M-citrate–phosphate buffer and specific activity as activity/mg. of protein. Details of purification steps are given in the text.

Purification step	Total activity	Specific activity	Recovery (%)
Homogenate	24525	0·1858	100
Concentrate after first agglutination	17471	0·929	71·2
Concentrate after second agglutination	13824	1·0	56·3
Freeze-dried extract	8744	0·726	35·6

one of these enzyme components were also obtained in this way and appeared to be electrophoretically identical with the corresponding enzyme from kidneys having both forms.

Gel filtration. All attempts to separate the two forms of β-D-glucosidase by gel filtration have failed and it has not been possible to detect any difference in the molecular weight of the two isoenzymes. The calculated molecular weight by comparison with marker enzymes as described by Andrews (1964, 1965) is approx. 40000. There is, however, a possibility that the enzyme, being a carbohydrase, may show some affinity for the dextran and is thus unduly retarded by these columns, as has been shown for amylase (Flodin, 1962). The experiment was therefore repeated with Bio-Gel P-150, when a somewhat higher molecular weight (approx. 50000) was indicated. In the former case the enzyme was eluted after the peroxidase peak, whereas on the latter support it preceded this marker (Fig. 2).

Isoelectric points. The fact that the distinction between the two forms is one of charge rather than molecular size was demonstrated by electrofocusing. Fig. 3 shows that complete resolution was not obtained and the apparent isoelectric points may be somewhat displaced towards each other because of overlap of the two peaks. Nevertheless this did not result in gross contamination of one form by the other and it was possible to concentrate samples from each peak by vacuum dialysis. Each sample when run on gel electrophoresis showed only one of the two bands, the faster form being that with apparent isoelectric point 4·9 and the slower form that with isoelectric point 5·1.

Comparison of β-D-glucosidases 1 and 2. Some characteristics of β-D-glucosidases 1 and 2 are shown in Table 3. The two enzymes are identical in their broad specificity and relative affinity for substrates and inhibitors. They have the same pH optima and both are progressively denatured if the solution is maintained at 37° for short periods of time at less than pH 4·5. Between pH 5 and 7 both forms are stable at 37° for at least 30min. However, they can be differentiated by heat stability under suitable conditions. When the two enzymes were separately maintained at 53° in 0·15M-sodium phosphate–citrate buffer, pH 5·5, in the presence of 0·1% crystalline serum albumin half the total activity of β-D-glucosidase 2 was lost in 20min., whereas β-D-glucosidase 1 had lost only 2% of its original activity in that time.

(*a*) Substrate specificity. As already noted in the electrophoresis experiments, the enzymes appear to be active towards a number of structurally related glycosides and may have a common active site for all these substrates. This was examined in experiments in which the total enzymic hydrolysis of equimolar mixtures of pairs of these substrates was measured and the results were compared with the theoretical values expected for a single site or for two independent sites as calculated from V_{max}.

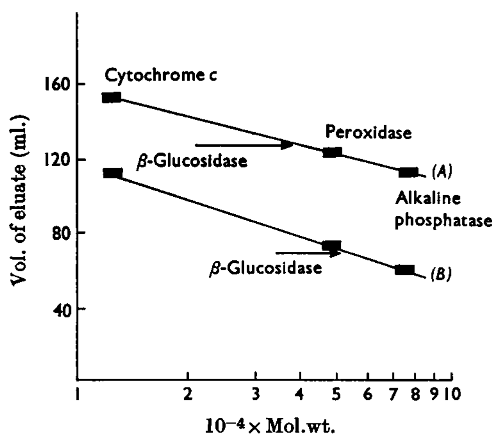

Fig. 2. Gel filtration of pig kidney β-D-glucosidase on Sephadex B-200 and Bio-Gel P-150. The elution volume of the kidney enzyme is compared with those of marker proteins of known molecular weight (Andrews, 1964). Conditions of separation and assay are as described in text. (*A*) Sephadex G-200; (*B*) Bio-Gel P-150.

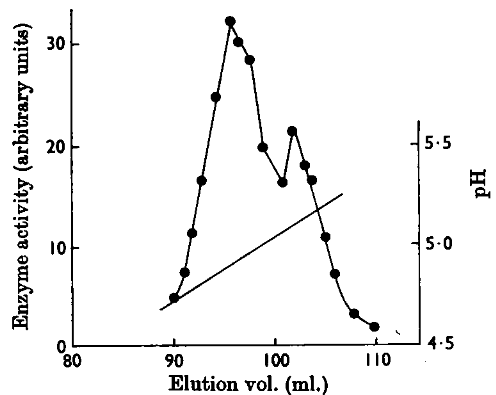

Fig. 3. Electro-focusing of pig kidney β-D-glucosidases 1 and 2 on an L.K.B. type 8100 Ampholine column (capacity 110ml.). Kidney homogenate (10mg. of protein) was focused for 48hr. at 4° as described in the Experimental section and 1ml. fractions were collected. Suitably diluted samples of these were assayed for β-D-glucosidase as described in the text, the pH values measured and forms 1 and 2 identified by gel electrophoresis of concentrates from peak tubes.

Table 3. *Comparison of some characteristics of β-D-glucosidases 1 and 2 from pig kidney*

	β-D-Glucosidase 1	β-D-Glucosidase 2
pH optimum*	5–6	5–6
K_m values		
β-D-Glucosidase	3.3×10^{-5}M	3.3×10^{-5}M
β-D-Galactosidase	11.1×10^{-5}M	11.7×10^{-5}M
α-L-Arabinosidase	1.75×10^{-5}M	1.75×10^{-5}M
β-D-Xylosidase	6.6×10^{-5}M	6.45×10^{-5}M
K_i (phlorrhizin)	2.9×10^{-6}M	2.9×10^{-6}M
Inhibition (%) by:		
Gluconolactone (14mM)	99	99
Galactonolactone (14mM)	70	66
Relative $V_{max.}$ values†		
β-D-Glucosidase	1	1
β-D-Galactosidase	1·72	2·24
α-L-Arabinosidase	0·25	0·40
β-D-Xylosidase	0·21	0·26

* The same results were obtained with all four substrates.

† Calculated from K_m values and assays under standard conditions.

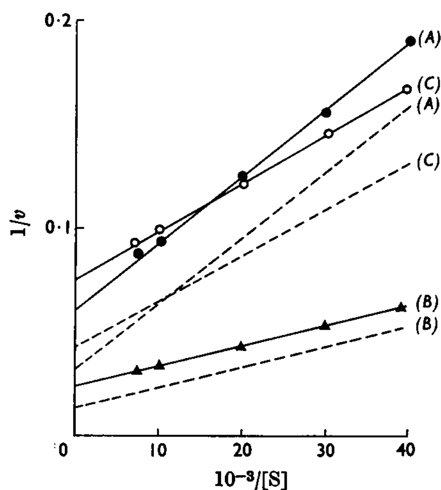

Fig. 4. Enzymic hydrolysis of equimolar mixtures of two competing substrates. A 1ml. sample of suitably diluted enzyme was incubated with 1ml. of an equimolar mixture of two substrates over the final total concentration range 0·125–0·025mM in 0·15M-sodium phosphate–citrate buffer, pH5·5. After 15min. incubation at 37° the liberated 4-methylumbelliferone was estimated by the standard assay procedure. Initial velocity, v, is expressed as μmoles of 4-methylumbelliferone liberated under these conditions; [S] is the final total substrate concentration. K_m and $V_{max.}$ for the individual substrates were determined at the same time. The lines show the hydrolysis expected on the assumption of competition of substrates at one site (——) or of two independent sites (----). (*A*) β-D-Glucoside–β-D-galactoside mixtures; (*B*) β-D-glucoside–β-D-xyloside mixtures; (*C*) β-D-xyloside–α-L-arabinoside mixtures.

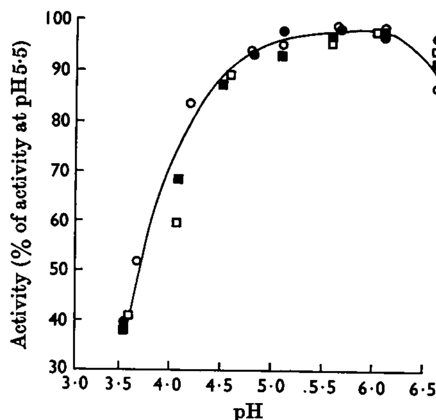

Fig. 5. Common pH–activity curves of pig kidney glycosidases in 0·2M-sodium phosphate–citrate buffer assayed as described in the text. ○, β-D-Glucosidase; ●, β-D-xylosidase; □, α-L-arabinosidase; ■, β-D-galactosidase.

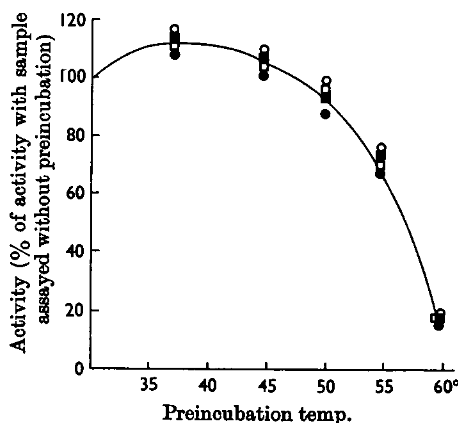

Fig. 6. Heat-inactivation of pig kidney glycosidases. Suitably diluted samples were preincubated at various temperatures in 0·2M-sodium phosphate–citrate buffer, pH5·5, for 15min. followed by assay at 37° as described in the text. ○, β-D-Glucosidase; ●, β-D-xylosidase; □, α-L-arabinosidase; ■, β-D-galactosidase.

and K_m values obtained with the same enzyme sample (Thorn, 1949). Fig. 4 shows that for β-D-glucosidase 1 the results with the substrate pairs glucoside–galactoside, glucoside–xyloside and xyloside–arabinoside all agree closely with the concept of a single site and by inference the remaining combinations of substrate pairs would give the same result. There was similar close agreement when the experiments were repeated with β-D-glucosidase 2. Further evidence for a common site was obtained from pH profiles. Plots

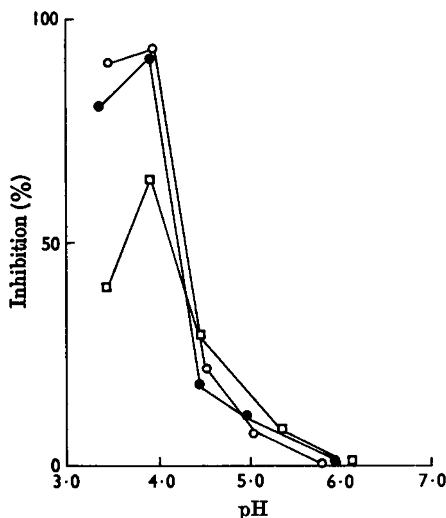

Fig. 7. pH-dependence of inhibition by NaCl. Results are expressed as percentage inhibition in the presence of 0·25M-NaCl in 0·15M-sodium phosphate–citrate buffer compared with assay in buffer alone at the same pH over the range 3·5–6·0. ●, β-D-Xylosidase; ○, β-D-glucosidase; □, α-L-arabinosidase.

of pH versus activity for β-D-glucosidase 1 acting on all four substrates were identical when normalized to the same maximum value to correct for different affinities (Fig. 5). In each case the enzyme showed a broad optimum at pH 5–6. Heat-inactivation at various fixed temperatures between 37° and 60° caused the same proportionate loss of activity towards all four substrates (Fig. 6).

(*b*) Inhibitors. It was noted in histochemical experiments (Rutenburg, Rutenburg, Monis, Teague & Seligman, 1958) that high concentrations of salt inhibited kidney β-D-glucosidase. In our experiments this was found to happen only when the assays were carried out on the acid side of the pH optimum. At pH 4·0 about 90% of the total activity measured in the absence of sodium chloride was inhibited by the presence of 0·25M-sodium chloride, but the same salt concentration had no effect at pH 6·0. Again the results were identical when β-D-xylosidase or α-L-arabinosidase was measured instead of β-D-glucosidase (Fig. 7). Gluconolactone and galactonolactone and a number of glycosides were also found to inhibit. The fact that both aldonolactones were effective again confirms a lack of specificity for the C-4 of the sugar, since these are well-known specific glycosidase inhibitors (Levvy, McAllan & Hay, 1962). It is noteworthy that the affinity of the glucoside phlorrhizin (K_i $2·9\times10^{-6}$M) is of the same order as that noted for the inhibition of sugar transport in the kidney (Diedrich, 1966). The possible role of glycosidases in the mechanism of active transport has been discussed by Semenza, Tosi, Vallotton-Delachaux & Mülhaupt (1964). Salicin (K_i $6·1\times10^{-4}$M) and arbutin (K_i 1×10^{-3}M) have less effect and are similarly weaker inhibitors of sugar transport in hamster and rat intestine (Alvarado & Crane, 1964).

(*c*) Action on natural glycosides. Since for the convenience of assay all the foregoing observations have been made on synthetic glycosides it is pertinent to ask whether the enzyme can function under similar conditions on some naturally occurring substrates. Such an enzyme could play an important role in the catabolism of mucopolysaccharides and glycolipids, and in particular the β-D-xylosidase activity could function in the cleavage of the xylosyl-*O*-serine links in mucopolysaccharide–protein complexes (Roden & Lindahl, 1965).

The partially purified enzyme prepared by agglutination of tissue homogenates was tested against some disaccharides and other possible substrates. The freeze-dried powder (100mg.), sufficient to liberate 70μmoles of glucose or 15μmoles of xylose in 1hr. from the fluorigenic substrates under normal assay conditions, was incubated with lactose, cellobiose or *R. palmata* xylan (2% solution in buffer, pH 5·5, for 24hr. at 37°) and the incubation mixture then tested for monosaccharides by thin-layer chromatography and assayed by the appropriate quantitative method. No significant hydrolysis could be detected under these conditions. The limits of detection of the thin-layer-chromatographic method would require 1% hydrolysis to give a 2μg./10μl. concentration of hydrolysis product, and the quantitative assay would detect down to 10μg./ml. Thus the degree of hydrolysis must be less than 0·05%. Neither serine nor free xylose could be detected on thin-layer chromatography after a similar incubation with xylosyl-*O*-serine. It is therefore not possible at present to ascribe a significant hydrolytic function to these enzymes with regard to these natural glycosidic links, and it is noteworthy that other authors have found some enzymes acting on disaccharides to be different from those detected by synthetic aryl glycosides (Doell & Kretchmer, 1962).

The possibility that the same enzyme may be responsible for the hydrolysis of both hexosides and pentosides of the same homomorphous series was recognized by Helferich, Winkler, Gootz, Peters & Günther (1932) and has been discussed by Baumann & Pigman (1957). Subsequently Manners & Mitchell (1967) and Conchie, Gelman & Levvy (1968) have confirmed that β-D-xylosidase and α-L-arabinosidase respectively may be associated

with the same active site on almond emulsin that hydrolyses β-D-glucosides, and the latter authors have compared this specificity with that of β-D-glucosidases and β-D-galactosidases of a number of other sources. In this respect the pig kidney enzyme more closely resembles almond emulsin than the mammalian enzyme studied (from rat epididymis).

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