Mammalian Glycosidases

2. PROPERTIES OF α -MANNOSIDASE AND β -GALACTOSIDASE FROM RAT EPIDIDYMISS*

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In a study of the occurrence of various glycosidases in mammalian tissues, the enzymes α -mannosidase and β -galactosidase were found to be widely distributed throughout the tissues of the mouse and the rat, particularly rich sources being the epididymis of these animals (Conchie, Findlay & Levvy, 1959a). Investigations have since been extended to the epididymis of other animals (Conchie, Findlay & Levvy, 1959b), but rat epididymis remains the best source of these two enzymes.

The present paper is concerned with some properties of α -mannosidase and β -galactosidase from this source, the chromogenic glycosides *p*-nitrophenyl α -mannoside, phenyl α -mannoside, *o*-nitrophenyl β -galactoside and phenyl β -galactoside being used as substrates. Some observations on mammalian β -galactosidase, in particular the enzyme from rat kidney, have been made by Cohen, Tsou, Rutenburg & Seligman (1952), using the chromogenic substrate 6-bromo-2-naphthyl β -galactoside.

EXPERIMENTAL

Materials

p-Nitrophenyl α -D-mannoside and D-mannono-1 $\rightarrow$ 4-lactone were prepared as described by Conchie *et al.* (1959a).

Phenyl α -D-mannoside, prepared as described by Helferich & Winkler (1933), had m.p. 131–132° and $[\alpha]_D^{17} + 113^\circ$ in water (c, 1.0).

o-Nitrophenyl β -D-galactoside was prepared by the method of Seidman & Link (1950), and had m.p. 193° and $[\alpha]_D^{18} - 51^\circ$ in water (c, 1.0).

Phenyl β -D-galactoside was prepared as described by Helferich (1944), and had m.p. 155–156° and $[\alpha]_D^{18} - 42^\circ$ in water (c, 1.0).

Galactono-1 $\rightarrow$ 4-lactone was obtained from L. Light and Co. Ltd.

Enzyme preparations

Adult rat epididymis was the source of all the enzyme preparations used in the experiments described below. Complete epididymides were homogenized in water (0.6 g. in 10 ml.) with a glass homogenizer (Jencons Ltd.), and incubated for 1 hr. at 37° in 0.1 M-acetic acid–NaOH buffer,

pH 5.0. The suspension was centrifuged at 1500 g for 15 min. and the resulting supernatant fractionated with $(\text{NH}_4)_2\text{SO}_4$, pH 5.0, the precipitate obtained between 20 and 80% saturation limits being collected at 10 000 g and dissolved in water (10 ml.). Such a stock solution could be kept for only a few days at 0° without an appreciable loss in α -mannosidase activity. On the other hand, even after several weeks at 0° there was little loss in β -galactosidase activity. For normal assay purposes the solution had to be diluted before use to liberate suitable amounts of aglycone (60–120 μg . of *p*-nitrophenol, 80–140 μg . of *o*-nitrophenol and 30–50 μg . of phenol). Typical dilutions for α -mannosidase were 1:25 and 1:10, and for β -galactosidase 1:10 and 1:5, for the nitrophenyl and phenyl substrates respectively.

Enzyme assays

Liberation of phenol. Liberation of phenol from phenyl α -mannoside and phenyl β -galactoside was estimated as by the procedure of Kerr, Graham & Levvy (1948), the colour intensity being measured on the Spekker photoelectric absorptiometer with Ilford 608 red filters (peak transmission 680 m μ). Incubation of enzyme with substrate was for 1 hr. at 37° in 0.1 M-acetic acid–NaOH buffer, pH 5.0 for α -mannosidase assays and pH 2.9 for β -galactosidase assays. The incubation mixture contained 0.5 ml. of enzyme preparation in a final volume of 1 ml. Except where otherwise stated, the substrate concentration was 10 mM for phenyl α -mannoside and 5 mM for phenyl β -galactoside.

Liberation of *p*-nitrophenol. Liberation of *p*-nitrophenol from *p*-nitrophenyl α -mannoside was measured as described by Conchie *et al.* (1959a). Except where otherwise stated, incubation of enzyme with substrate was for 1 hr. at 37° in 0.125 M-acetic acid–NaOH buffer, pH 5.0, the substrate concentration being 6 mM. The incubation mixture contained 0.5 ml. of enzyme preparation in a final volume of 4 ml.

Liberation of *o*-nitrophenol. Liberation of *o*-nitrophenol from *o*-nitrophenyl β -galactoside was measured as described by Conchie *et al.* (1959a). Except where otherwise stated, incubations were for 1 hr. at 37° in 0.125 M-acetic acid–NaOH buffer, pH 2.9, the substrate concentration being 2.5 mM.

RESULTS

Effect of pH

α -Mannosidase. The variation in relative activity with pH of an enzyme preparation [20–80% $(\text{NH}_4)_2\text{SO}_4$ fraction] over the range pH 2.5–7.5 is shown for the hydrolysis of 6 mM-*p*-nitrophenyl

* Part 1: Conchie, Findlay & Levvy (1959a).

α -mannoside in 0.125M-acetic acid-NaOH buffer (Fig. 1), and 10 mM-phenyl α -mannoside in 0.1M-acetic acid-NaOH buffer (Fig. 2). In each a smooth curve with a sharp peak at pH 5.0 resulted. Further determinations, with 6 mM-*p*-nitrophenyl α -mannoside as substrate, were made in 0.125M-citric acid-NaOH buffer and phosphate-citrate buffer (McIlvaine, 1921), as well as in 0.125M-acetic acid-NaOH buffer with substrate concentrations of 2 and 12 mM. In all cases, curves similar to that in Fig. 1 were obtained. Measurements in all the above-mentioned buffers with unfractionated buffer extracts of crude homogenates also gave similar curves. Enzyme preparations assayed at pH 5.0 in 0.125M-acetic acid-NaOH, 0.125M-citric acid-NaOH and phosphate-citrate buffers showed very little variation in their total activity with the nature of the buffer.

β -Galactosidase. Figs. 1 and 2 also show the pH-activity curves of the same enzyme preparation for the hydrolysis of 2.5 mM-*o*-nitrophenyl β -galactoside and 5 mM-phenyl β -galactoside respectively. In acetic acid-NaOH buffer, the pH optimum for the hydrolysis of the nitrophenyl substrate was at 2.9, whereas for the phenyl substrate two optima, at 2.9 and 3.5, were obtained. In phosphate-citrate and in citric acid-NaOH buffers, however, the same

two optima occurred with the nitrophenyl substrate. Results identical with the above were obtained when the supernatant from a buffer extract of a crude homogenate was used as the enzyme source. Determinations with 7.5 mM-*o*-nitrophenyl β -galactoside in 0.125M-acetic acid-NaOH buffer gave a curve similar to that obtained with a substrate concentration of 2.5 mM. Comparisons of enzyme activity in 0.125M-acetic acid-NaOH buffer, pH 2.9, 0.125M-citric acid-NaOH buffer, pH 3.6, and McIlvaine's phosphate-citrate buffer, pH 3.6, with the *o*-nitrophenyl substrate, showed the total activities to be within 8% of each other. Acetic acid-NaOH buffers, pH 2.9, were employed for all the remaining experiments with β -galactosidase described in this paper.

Cohen *et al.* (1952), in their study on rat-kidney β -galactosidase with 6-bromo-2-naphthyl β -galactoside in phosphate-citrate buffer, found the optimum of the enzyme to be at pH 5.0, and found very little activity displayed at pH 3.0.

Effect of substrate concentration

α -Mannosidase. The effect of variation in the substrate concentration on the reaction velocity at the pH optimum with *p*-nitrophenyl α -mannoside over the range 0.8–24 mM and phenyl α -mannoside over the range 2.5–40 mM is shown in Fig. 3. In neither case was a maximum velocity reached, but

Fig. 1. Hydrolysis at varying pH values of 6 mM-*p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer (■), 2.5 mM-*o*-nitrophenyl β -galactoside in 0.125M-acetic acid-NaOH buffer (●) and 2.5 mM-*o*-nitrophenyl β -galactoside in phosphate-citrate buffer (○), by enzymes from rat epididymis. For other assay conditions see text. An activity of 100 is equivalent to 110 μ g. of *p*-nitrophenol and 130 μ g. of *o*-nitrophenol liberated/hr.

Fig. 2. Hydrolysis at varying pH values of 10 mM-phenyl α -mannoside (○), and 5 mM-phenyl β -galactoside (●), in 0.1M-acetic acid-NaOH buffer by enzymes from rat epididymis. For other assay conditions see text. An activity of 100 is equivalent to 40 μ g. (○) and 33 μ g. (●) of phenol liberated/hr.

estimations at higher substrate concentrations could not easily be made owing to the limited solubility of the substrates in the incubation mixture. The results were analysed by the method of Lineweaver & Burk (1934), as in Fig. 8, plotting $1/S$ against $1/v$ (S being the substrate concentration and v the reaction velocity). Values obtained for K_m , the dissociation constants of the enzyme-substrate complexes, were 13 mM for *p*-nitrophenyl α -mannoside (mean of seven experiments) and 57 mM for phenyl α -mannoside (mean of three experiments). For routine assays all further estimations were made with the nitrophenyl substrate at a concentration of 6 mM, and the phenyl substrate at a concentration of 10 mM. Most of the experiments described below were carried out with the nitrophenyl substrate only.

β -Galactosidase. Fig. 3 also shows the variation in β -galactosidase activity with concentrations of *o*-nitrophenyl β -galactoside over the range 0.25–12.5 mM and phenyl β -galactoside over the range 0.5–40 mM. Maximal activity was reached with concentrations of 5 mM *o*-nitrophenyl β -galactoside and 15 mM-phenyl β -galactoside. In each case there was only slight inhibition by excess of substrate. Analysis by the method of Lineweaver & Burk (1934) gave values for K_m of 0.38 mM for *o*-nitrophenyl β -galactoside (mean of 12 experiments), and 1.6 mM for phenyl β -galactoside (mean of three experiments). For routine purposes, the substrate concentrations used were 2.5 mM for the

nitrophenyl substrate and 5 mM for the phenyl substrate. Both concentrations lie very near the respective optima for the substrates.

Effect of duration of incubation

Fig. 4 shows the amounts of *p*- and *o*-nitrophenol liberated from 6 mM-*p*-nitrophenyl α -mannoside and 2.5 mM-*o*-nitrophenyl β -galactoside after incubation with enzyme for varying periods. With α -mannosidase, the curve is approximately linear up to a period of 1 hr., after which there appears to be slow enzyme inactivation. The amount of *p*-nitrophenol liberated after 4 hr. represents about 6% of the total quantity present as glycoside. The rate of hydrolysis of *o*-nitrophenyl β -galactoside was linear with time up to a period of about 3 hr. After 4 hr. about 23% of the total amount of substrate present was hydrolysed.

Effect of enzyme concentration

The rate of liberation of *p*-nitrophenol from 6 mM-*p*-nitrophenyl α -mannoside was shown to be linear over an eightfold range of enzyme concentration. From this it may be noted that the substrate concentration was sufficiently high to prevent any change in the initial reaction velocity. At the highest concentration of enzyme about 6% of the total amount of substrate present was hydrolysed.

The rate of hydrolysis of 2.5 mM-*o*-nitrophenyl β -galactoside was linear over a 12-fold range of enzyme concentration. The higher values of liberated *o*-nitrophenol were read after dilution with a buffer mixture corresponding to the final

Fig. 3. Effect of varying the substrate concentration on the rate of hydrolysis of *p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer, pH 5.0 (○), phenyl α -mannoside in 0.1M-acetic acid-NaOH buffer, pH 5.0 (□), *o*-nitrophenyl β -galactoside in 0.125M-acetic acid-NaOH buffer, pH 2.9 (●), and phenyl β -galactoside in 0.1M-acetic acid-NaOH buffer, pH 2.9 (■), by rat-epididymal enzymes. For other conditions of assay see text.

Fig. 4. Effect of incubation at 37° on the hydrolysis of 6 mM-*p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer, pH 5.0 (○), and 2.5 mM-*o*-nitrophenyl β -galactoside in 0.125M-acetic acid-NaOH buffer, pH 2.9 (●), by rat-epididymal enzymes.

colorimetric medium. The highest value corresponded to hydrolysis of about 26% of the original amount of substrate present.

The linear relationship obtained in both cases indicated that there were no natural substrates, activators or inhibitors present in the enzyme preparations.

Effect of temperature

The variation in α -mannosidase and β -galactosidase activities after heating for 5 min. at various temperatures is shown in Fig. 5. The unbuffered enzyme preparation [20–80% $(\text{NH}_4)_2\text{SO}_4$ fraction], pH 5.5, was heated at the required temperature for 5 min., cooled in ice and then assayed. Results are expressed as percentage of residual activity, the control being an unheated portion. Under the conditions of the experiment β -galactosidase activity was almost completely destroyed at 70° and α -mannosidase activity at 75°.

Variation in stability with pH

Fig. 6 shows the residual α -mannosidase and β -galactosidase activities in an unbuffered enzyme preparation [20–80% $(\text{NH}_4)_2\text{SO}_4$ fraction] after incubation at various pH values for 1 hr. at 37°. Portions of the preparation were adjusted to the required pH with dilute HCl or NaOH, incubated at 37° for 1 hr. and, after a pH check, readjusted to the original pH of the preparation (5.5). All

portions were made up to a fixed volume and assayed as usual, the results being expressed as a percentage of a control which was not given a preliminary incubation. Both enzymes were stable over the pH range 5.0–6.5, but below pH 5.0 their stabilities were markedly different. α -Mannosidase became very unstable below pH 5.0, and the steep slope of the pH-activity curves in this region (Figs. 1, 2) would appear to be due to the fact that there is considerable inactivation of the enzyme. β -Galactosidase, on the other hand, remained completely stable as far as pH 3.0; below this value the enzyme was very unstable.

Inhibition by the corresponding aldonolactones

The aldonolactones mannono-1 $\rightarrow$ 4-lactone and galactono-1 $\rightarrow$ 4-lactone are strong inhibitors of α -mannosidase and β -galactosidase activities respectively (Conchie & Levvy, 1957a). Inhibition by these lactones is specific, no cross-inhibitory effects being obtained (Conchie *et al.* 1959a).

The effect on α -mannosidase activity of varying concentrations of mannono-1 $\rightarrow$ 4-lactone over the range 0.5–375 mM is shown in Fig. 7, the substrate used being 6 mM-*p*-nitrophenyl α -mannoside; 50% inhibition of α -mannosidase activity was obtained with 20 mM-lactone. Determinations of K_i , the dissociation constant of the enzyme-inhibitor complex, were carried out, assays being done in the

Fig. 5. Effect of heating for 5 min. at varying temperatures on the α -mannosidase (○) and β -galactosidase (●) activities of an unbuffered enzyme preparation from rat epididymis. For conditions of assay see text. Results are expressed as percentage of residual activity, the initial value being that of an unheated control.

Fig. 6. Effect of incubation for 1 hr. at 37° on the α -mannosidase (○) and β -galactosidase (●) activities of a rat-epididymal enzyme preparation adjusted to varying pH values with HCl or NaOH. For conditions of assay see text. Results are expressed as percentage of residual activity, the initial value being that of an untreated control.

presence and absence of 10 mM-lactone over a substrate concentration range of 0.8–20 mM for *p*-nitrophenyl α -mannoside and 2.5–40 mM for phenyl α -mannoside. Calculation of K_i was made by the method of Lineweaver & Burk (1934), and the analysis of a typical experiment with the nitrophenyl substrate is shown in Fig. 8*a*. Inhibition is competitive, and values for K_m and K_i were 13 and 11 mM respectively. The mean result of six determinations of K_i with the nitrophenyl substrate was 11 mM, and the mean of three determinations with the phenyl substrate was 19 mM. Experiments with the lactone at a concentration of 5 mM with each substrate gave results identical with those already obtained. Thus the inhibitor had an affinity for the enzyme equal to that of *p*-nitrophenyl α -mannoside, and about three times that of phenyl α -mannoside.

Fig. 7 also shows the percentage inhibition of the hydrolysis of 2.5 mM-*o*-nitrophenyl β -galactoside obtained with concentrations of lactones over the range 0.04–40 mM; 50% inhibition was obtained with 2.5 mM-lactone. Determinations of K_i , with

o-nitrophenyl β -galactoside concentrations over the range 0.5–7.5 mM and lactone at 2 mM, gave an average result of 0.51 mM, the mean of nine experiments. The analysis of a typical experiment by the Lineweaver & Burk plot, giving values of 0.4 and 0.64 mM for K_m and K_i respectively, is given in Fig. 8*b*, which also shows inhibition to be competitive. Measurements made with mM-lactone gave K_i values similar to those already obtained. Determinations of K_i for galactonolactone, with phenyl β -galactoside concentrations over the range 0.5–20 mM and lactone concentrations of 2 and 1 mM, resulted in a mean value of 0.21 mM. The lactone thus had an affinity for the enzyme equal to that of *o*-nitrophenyl β -galactoside and about eight times that of phenyl β -galactoside.

Table 1. *Effects of copper sulphate, silver nitrate and mercuric chloride on α -mannosidase activity after varying periods of pre-incubation with the enzyme*

The substrate was 6 mM-*p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer, pH 5.0. For 0 hr. values the enzyme was added last to the assay tubes. Results are expressed as percentage inhibition of controls similarly pre-incubated.

Metal ion	Final concn. (μ M)	Inhibition (%)			
		0 hr.	Period of pre-incubation (hr.)		
			0.5	1	2
Cu ²⁺	250	25	47	65	84
	500	36	75	84	97
Ag ⁺	10	12	37	48	73
Hg ²⁺	5	45	44	52	64
	10	62	67	77	88

Table 2. *Effects of copper sulphate, silver nitrate and mercuric chloride on β -galactosidase activity after varying periods of pre-incubation with the enzyme*

The substrate was 2.5 mM-*o*-nitrophenyl β -galactoside in 0.125M-acetic acid-NaOH buffer, pH 2.9. For 0 hr. values the enzyme was added last to the assay tubes. Results are expressed as percentage inhibition of controls similarly pre-incubated.

Metal ion	Final concn. (μ M)	Inhibition (%)			
		0 hr.	Period of pre-incubation (hr.)		
			0.5	1	2
Cu ²⁺	500	0	0	7	11
	4 000	5	36	61	86
	10 000	9	82	98	99
Ag ⁺	10	46	47	48	48
Hg ²⁺	0.1	0	1	0	0
	0.2	1	16	7	3
	0.4	23	100	100	100

Fig. 7. Inhibition of rat-epididymal α -mannosidase and β -galactosidase activities by varying concentrations of aldonolactones. Mannono-1 $\rightarrow$ 4-lactone as inhibitor, substrate 6 mM-*p*-nitrophenyl α -mannoside (O); galactono-1 $\rightarrow$ 4-lactone as inhibitor, substrate 2.5 mM-*o*-nitrophenyl β -galactoside (●). Other assay conditions were as described in the text.

Fig. 8. Relation between S , the substrate concentration (mM), and v , the rate of hydrolysis (μ g. of nitrophenol liberated/hr.), by rat-epididymal enzymes in presence (○) and absence (●) of lactone inhibitors, analysed by the method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. For conditions of assay see text. (a) Substrate, *p*-nitrophenyl α -mannoside; inhibitor, 10 mM-mannono-1 $\rightarrow$ 4-lactone. (b) Substrate, *o*-nitrophenyl β -galactoside; inhibitor, 2 mM-galactono-1 $\rightarrow$ 4-lactone.

Inhibition by metal ions

The metal ions Ag^+ , Hg^{2+} and, in certain cases, Cu^{2+} , have been shown to be powerful inhibitors of β -glucuronidase activity (Doyle, Katzman & Doisy, 1955; Levvy, Hay & Marsh, 1957; Levvy & Marsh, 1957). In view of this, the effects of these ions on the α -mannosidase and β -galactosidase activity of a 20–80% $(\text{NH}_4)_2\text{SO}_4$ fraction was studied. Tables 1 and 2 show some of the results obtained.

All three ions were strong inhibitors of α -mannosidase activity. The inhibitory effects of Cu^{2+} and Ag^+ ions were considerably increased when the enzyme preparation was pre-incubated at 37° with these ions, the amount of inhibition obtained increasing with the period of pre-incubation. Inhibition by Hg^{2+} ions, on the other hand, was only slightly increased by pre-incubation. Pre-incubations for longer periods were not attempted owing to the considerable inactivation of the enzyme that occurred when it was heated over these periods.

Both Ag^+ and Hg^{2+} ions were powerful inhibitors of β -galactosidase activity. Much higher concentrations of Cu^{2+} ions were required before inhibition was obtained, and this ion was not as effective an inhibitor of β -galactosidase as of α -mannosidase activity. Pre-incubation increased the amount of inhibition obtained with Cu^{2+} and Hg^{2+} ions, but only with Cu^{2+} ions did the length of time of pre-incubation have any effect.

Both enzymes, when incubated alone in their assay buffers over the periods given in Tables 1 and 2, showed much larger falls in activity than were observed when studying the effect of time of incubation on the reaction velocity (Fig. 4). Evidently the presence of substrate stabilizes the enzymes to a considerable extent.

Competing substrate experiments

α -Mannosidase. Investigations were made of the effect of 20 mM-phenyl α -mannoside on the hydrolysis of *p*-nitrophenyl α -mannoside in concentrations over the range 0.2–6 mM. In no case was inhibition of hydrolysis observed. A 40 mM-solution of methyl α -mannoside caused 14% inhibition of the hydrolysis of 6 mM-*p*-nitrophenyl α -mannoside and 19% inhibition of the hydrolysis of 10 mM-phenyl α -mannoside.

β -Galactosidase. Assays were done with *o*-nitrophenyl β -galactoside concentrations over the range 0.5–5 mM in the presence and in the absence of 10 mM-phenyl β -galactoside. Analysis of the results by the method of Lineweaver & Burk (1934) showed inhibition to be competitive, and gave a mean value for K_i for phenyl β -galactoside of 1.9 mM. A similar value was obtained with 5 mM-phenyl β -galactoside. The agreement between this value and the K_m for phenyl β -galactoside already determined, namely 1.6 mM, suggests that the same enzyme is responsible for the hydrolysis of both substrates.

DISCUSSION

The main purpose of the study of the two enzymes was to provide a basis for further investigations, and to compare their properties with each other and with those of the closely associated glycosidases β -glucuronidase and β -*N*-acetylglucosaminidase.

With α -mannosidase, neither of the substrates used could be employed at its optimum concentration owing to the relatively low affinity of the enzyme for the substrates, and limitations of solubility in the incubation mixture. Since the limiting velocity has not been reached, it is particularly important to keep within the limits of aglycone liberation specified (to ensure keeping within the same region of the first-order reaction curve), if results are to have any quantitative value in comparative tissue assays. If the optimum substrate concentrations could conveniently be employed, it is clear that the ultimate activity of α -mannosidase would be much higher relative to other glycosidases than is given in published papers (Conchie & Levvy, 1957*b*; Conchie & Mann, 1957; Conchie *et al.* 1959*a, b*; Conchie & Findlay, 1959). The necessary correction factors can be calculated from Fig. 3. Of the two substrates, *p*-nitrophenyl α -mannoside had a higher affinity for the enzyme and has been used for routine assays. The kinetics of β -galactosidase action on the other hand, are fairly simple. Both the chromogenic substrates employed have relatively high affinities for the enzyme, and can be used without difficulty at their optimum concentrations.

Both enzymes are specifically and competitively inhibited by the appropriate aldonolactones. Whereas K_i values for each lactone with the nitrophenyl substrates were not identical with those obtained with the phenyl substrates, they were sufficiently similar to suggest that in each case one enzyme was responsible for the hydrolysis of both nitrophenyl and phenyl substrates. Confirmation was obtained for β -galactosidase by the use of phenyl β -galactoside as a competing substrate in the hydrolysis of *o*-nitrophenyl β -galactoside. The K_i thus obtained for the phenyl substrate was very similar to its K_m . Similar experiments could not be carried out with α -mannosidase, however, since phenyl α -mannoside caused no inhibition of the hydrolysis of *p*-nitrophenyl α -mannoside. No satisfactory explanation of this behaviour can be given, but it seems unlikely that there are present in rat epididymis two separate α -mannosidases with identical pH-activity curves, and which are affected so similarly by mannonolactone and methyl α -mannoside.

Although the glycosidases α -mannosidase, β -galactosidase, β -*N*-acetylglucosaminidase and, to a

lesser extent, β -glucuronidase are all closely associated in rat epididymis, it is easy, by use of the appropriate inhibitors, to inhibit one or more of the enzymes as required (Conchie *et al.* 1959*a*). Moreover, concentrations of Cu^{2+} ions can be employed which would inhibit all α -mannosidase activity and leave β -galactosidase activity almost unaffected. From a study of the properties of α -mannosidase and β -galactosidase, it should be possible to obtain a preparation of one enzyme free from any activity of the other. If an enzyme preparation which has been fractionated with ammonium sulphate is incubated for 1 hr. at 37° and pH 3.5, all α -mannosidase activity will be removed whereas β -galactosidase is completely unaffected. On the other hand, heating an unbuffered preparation, pH 5.5, at 70° for 5 min. destroys almost all β -galactosidase activity but only about 33 % of the original α -mannosidase activity.

SUMMARY

1. Some properties of α -mannosidase and β -galactosidase from rat epididymis have been studied, with *p*-nitrophenyl α -mannoside and phenyl α -mannoside as substrates for α -mannosidase and *o*-nitrophenyl β -galactoside and phenyl β -galactoside as substrates for β -galactosidase.

2. The pH optimum of α -mannosidase in various buffers and for both substrates was at 5.0. The pH optimum of β -galactosidase in acetic acid-sodium hydroxide buffer with the nitrophenyl substrate was at 2.9; in citric acid-sodium hydroxide or in phosphate-citrate buffers with the nitrophenyl substrate, and in acetic acid-sodium hydroxide buffer with the phenyl substrate, two optima were obtained, at pH 2.9 and 3.5.

3. Mean K_m values obtained were: *p*-nitrophenyl α -mannoside 13 mM, phenyl α -mannoside 57 mM, *o*-nitrophenyl β -galactoside 0.38 mM and phenyl β -galactoside 1.6 mM.

4. When kept at 37° for 1 hr., α -mannosidase was stable over the pH range 5.0-6.5 and β -galactosidase over the pH range 3.0-7.0.

5. Heating at 70° for 5 min. destroyed β -galactosidase activity, and similar treatment at 75° destroyed α -mannosidase activity. α -Mannosidase was stable at 55-60° for 5 min.

6. Mean K_i values for mannono-1 $\rightarrow$ 4-lactone, a specific competitive inhibitor of α -mannosidase, were 11 mM with *p*-nitrophenyl α -mannoside as substrate and 19 mM with phenyl α -mannoside as substrate.

7. Mean K_i values for galactono-1 $\rightarrow$ 4-lactone, a competitive inhibitor of β -galactosidase, were 0.51 mM with *o*-nitrophenyl β -galactoside as substrate and 0.21 mM with phenyl β -galactoside as substrate.

8. Both enzymes are strongly inhibited by Ag^+ and Hg^{2+} ions in low concentrations; Cu^{2+} ions are powerful inhibitors of α -mannosidase activity and moderate inhibitors of β -galactosidase activity.

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The Biological Action of Substances Related to Thyroxine

11. ACETIC ACID ANALOGUES OF THYROXINE AND TRI-IODOTHYRONINE*

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The acetic acid analogues of thyroxine [3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)phenylacetic acid (tetraiodothyroacetic acid)] and tri-iodothyronine [3:5-di-iodo-4-(4-hydroxy-3-iodophenoxy)phenylacetic acid (tri-iodothyroacetic acid)] have been shown by Pitt-Rivers (1953) to possess thyroxine-like activity in rats. Thibault & Pitt-Rivers (1955) subsequently demonstrated that both compounds produced an immediate rise in the oxygen consumption of rat-kidney slices and in the basal metabolic rates of thyroidectomized rats. Immediate effects upon the glycolysis and respiration of cells have been reported (Heimberg, Park, Isaacs & Pitt-Rivers, 1955; Alexander & Bisset, 1958), but the immediate response of whole animals has not yet been confirmed by other workers. The possible physiological importance of tetra- and tri-iodothyroacetic acid, however, has been indicated by the chromatographic demonstration of their formation from thyroxine and tri-iodothyronine respectively in rat-kidney preparations (Roche, Michel, Jouan & Wolf, 1955; Albright, Larson, Tomita & Lardy, 1956). In clinical trials both compounds were shown to relieve the symptoms of myxoedema (Lerman & Pitt-Rivers, 1955; Trotter, 1955; Goolden, 1956) and there was some indication that they had a relatively greater effect upon the serum cholesterol than upon the basal metabolic rate.

Studies of the distribution of tri-iodothyroacetic acid in the tissues and excreta of the rat have been made by Roche, Michel, Jouan & Wolf (1956a) and by Larson & Albright (1958), who found that after the administration of small physiological doses the amount excreted as urinary iodide is less than when tri-iodothyronine was similarly used. In view of the possible clinical use of tri- and tetra-iodothyroacetic acid it seemed desirable to extend these studies by comparing the rates of excretion of both compounds with the corresponding iodothyronines. As deiodination processes have been shown to be important factors in the metabolism of the iodothyronines (Wilkinson, Sprott, Bowden & Maclagan, 1954; Wilkinson & Maclagan, 1954), the effect of the deiodination inhibitor, butyl 4-hydroxy-3:5-di-iodobenzoate (Sheahan, Wilkinson & Maclagan, 1951), has also been investigated in rats treated with the acetic acid compounds.

A preliminary report of some of the results has already appeared (Wilkinson, 1958).

MATERIALS AND METHODS

¹³¹I-labelled tri-iodothyroacetic acid. 3:5-Di-iodo-4-(4-hydroxyphenoxy)phenylacetic acid (11.1 mg.) (prepared by the method of Wilkinson, 1956) was dissolved at 0° in 2 ml. of methanol-aq. NH_3 soln. (sp.gr. 0.88) (1:1, v/v). A solution of I_2 (5.7 mg.) and Na^{131}I (5 mc) in aq. 50% methanol (4 ml.) was added gradually over 30 min. After setting

* Part 10: Wilkinson & Feetham (1955).