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The Preparation and Properties of β -Glucuronidase

5. SPECIFICITY

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Knowledge of the specificity of the enzyme β -glucuronidase present in animal tissues is far from complete. Masamune (1934) found that the enzyme would hydrolyse neither *laevomenthyl* α -D-glucuronide nor a variety of α - and β -D-glucosides. Phenyl β -D-glucoside was slowly hydrolysed by Masamune's enzyme preparation, but Graham (1946) obtained no hydrolysis of this compound with highly purified ox-spleen β -glucuronidase. Karunairatnam (1950) found that phenyl β -D-glucoside was hydrolysed by mouse-liver homogenates, but that this property was lost in the early stages of purification of β -glucuronidase. Further information on the specificity of this enzyme, not recognized as such by the original authors, is provided by the work of Karunairatnam & Levy (1949) who, in their search for an efficient inhibitor, found that the rate of hydrolysis of phenyl β -D-glucuronide was unchanged in the presence of methyl α -D-glucoside, methyl α -D-mannoside, methyl β -D-glucoside, methyl β -D-xyloside and methyl α -D-galactoside. Had any of these compounds been substrates for the enzyme, a fall in the rate of liberation of phenol might have been expected.

Meyer, Linker & Rapport (1951) found that β -glucuronidase hydrolysed the oligosaccharides which are the end products of the action of purified cesticular hyaluronidase on hyaluronic acid. In the absence of adequate knowledge of the specificity of β -glucuronidase, this finding could not be put forward as additional proof of the structure of hyaluronic acid. Implicit in the use of β -glucuronidase as a hydrolytic agent in the study of urinary steroids is the assumption that all the steroid set free in this way is initially conjugated with glucuronic acid. Further information on its specificity would increase the value of β -glucuronidase as a biochemical reagent. The difficulty of preparing suitable test compounds has been largely overcome

by the development of a method for the oxidation of hexosides to hexuronides with gaseous oxygen in the presence of platinum catalyst (Marsh, 1952: cf. Fernandez-Garcia *et al.* 1950; Mehlretter, Alexander, Mellies & Rist, 1951; Barker, Bourne & Stacey, 1951). The opportunity has been taken to study the action of mouse-liver β -glucuronidase on α - and β -glucuronides, galacturonides and mannuronides, and on a variety of other compounds.

EXPERIMENTAL

Materials. The following compounds were synthesized by methods given in the literature and were converted into the corresponding conjugated hexuronic acids (Marsh, 1952): (-)-menthyl α -D-glucopyranoside, methyl α - and β -D-galactopyranosides, methyl α - and β -D-mannopyranosides, and β -D-glucopyranose-1-phosphate. Biosynthetic α -D-glucopyranose-1-phosphate, presented by Dr R. J. Whelan, was likewise put through the oxidation procedure. It should be noted that β -D-glucuronic acid-1-phosphate has not been obtained pure. The glucuronic acid content of the specimen used here was 60% of theory for the tripotassium salt.

Methyl α -D-glucopyranoside, phenyl β -D-glucopyranoside and (-)-menthyl β -D-glucopyranoside were also prepared by standard methods. Methyl α - and β -D-glucopyranuronides were obtained in solution by treating the corresponding 3:6-lactones with dilute NaOH. Methyl 4-methyl-D-glucopyranuronide (mixture of α and β forms) was obtained in solution from the amide by heating the latter with NaOH (Smith, 1951). This amide and the two glucopyranuronolactones were presented by Prof. F. Smith. Oxycellulose (polyanhydroglucuronic acid, Yaackel & Kenyon 1942) was presented by Imperial Chemical Industries, Ltd. (Explosives Group). It had a COOH content of 22% (theoretical value for polyanhydroglucuronic acid, 26%), and was brought into solution by careful addition of dilute NaOH, so that the pH never exceeded 7.

The standard substrates, phenolphthalein, phenyl and (-)-menthyl β -D-glucuronides, were biosynthetic.

Enzyme preparation. Unless stated to the contrary, the enzyme was prepared as follows. Mouse liver (1-2 g.) was homogenized in water and made up to a final volume of 9 ml.

After adding 1 ml. *m*-acetate buffer, pH 5.2, the homogenate was incubated at 37° for 30–60 min., and centrifuged at 1500 *g* for 15 min. The supernatant was decanted and to it was added an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Precipitated protein was separated by centrifuging at 1500 *g* for 15 min., and taken up in a convenient volume of water.

For measuring the liberation of reducing sugar, and for certain other experiments, the preparation of the enzyme was modified as follows. The homogenate was incubated at pH 5.2 for 4 hr., and the limits of precipitation with $(\text{NH}_4)_2\text{SO}_4$ were narrowed from 0 and 50% to 31 and 50% saturation. The final enzyme solution was dialysed for 48 hr. at 4° before use.

Measurement of enzyme activity. Three methods were used. (1) The liberation of phenolphthalein from 0.00063 *m*-phenolphthalein glucuronide in acetate buffer, pH 5.2, in 1 hr. was measured by a modification (Levy, 1952) of the method of Talalay, Fishman & Huggins (1946). (2) The liberation of phenol from 0.015 *m*-phenyl glucuronide in citrate buffer, pH 5.2, in 1 hr. was measured by the method of Kerr, Graham & Levy (1948), but using Ilford no. 608 red filter in the Spekker absorptiometer instead of the blue filter recommended in the original procedure. (3) Liberation of reducing sugar from various possible substrates during 2 hr. incubation at 37° in acetate buffer, pH 5.2, was measured by the method of Levy (1946).

RESULTS

Effects on the hydrolysis of known substrates. All the compounds were examined for their effects on the hydrolysis of phenolphthalein glucuronide by

glucuronidase, and the results are shown in Table 1. As shown by the action of phenyl β -glucuronide, the release of phenolphthalein should be slowed in the presence of an alternative substrate for the enzyme. This test for possible substrates has the advantage of being less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis, but it does not discriminate between 'alternative substrate' and 'enzyme inhibitor'.

The galacturonides, mannanuronides, glucofuranuronides and the 4-methylglucopyranuronide had no effects of any kind on the release of phenolphthalein and were ruled out as possible substrates, as were also the galactosides, mannosides, glucose-1-phosphates and two of the glucosides.

Of the compounds which changed the rate of hydrolysis of phenolphthalein glucuronide, oxycellulose caused a fall which, on the basis of the glucuronic content, was equal to that produced by glucuronic acid under identical conditions of test (see Levy, 1952). On dialysis, the solutions of oxycellulose lost most of their depressant effect, but only 20% of the solid present in solution was found to be dialysable. The oxycellulose solutions were made up either to pH 5, in which case they were buffered with acetate and dialysed against the same buffer, or to pH 7, in which case the unbuffered solution was dialysed against water. Similar results were obtained in the two cases.

Table 1. *Effects of possible alternative substrates on the hydrolysis of 0.00063 m-phenolphthalein β -D-glucuronide by mouse-liver glucuronidase*

Compound	Final concentration (M)	Change in rate of phenolphthalein liberation (% control)
(-)-Menthyl α -D-glucuronide	0.01	+22*
(-)-Menthyl β -D-glucuronide	0.01	+5*
Phenyl β -D-glucuronide	0.01	-16
Methyl 4-methyl-D-glucuronide	0.001	0
Methyl α -D-glucofuranuronide	0.0005	0
Methyl β -D-glucofuranuronide	0.001	0
α -Glucuronic acid-1-phosphate	0.01	-48*
β -Glucuronic acid-1-phosphate	0.001†	-26*
Methyl α -D-galacturonide	0.01	0
Methyl β -D-galacturonide	0.01	0
Methyl α -D-mannuronide	0.01	0
Methyl β -D-mannuronide	0.01	0
(-)-Menthyl α -D-glucoside	0.001	+4
Methyl α -D-glucoside	0.01	0
(-)-Menthyl β -D-glucoside	0.0025	+10
Phenyl β -D-glucoside	0.01	0
α -Glucose-1-phosphate	0.01	0
β -Glucose-1-phosphate	0.01	0
Methyl α -D-galactoside	0.01	0
Methyl β -D-galactoside	0.01	0
Methyl α -D-mannoside	0.01	0
Methyl β -D-mannoside	0.01	0
Oxycellulose (freshly prepared solution)	0.01‡	-49
Oxycellulose (solution dialysed for 48 hr.)	0.01‡	-6
Oxycellulose (48 hr. old solution)	0.01‡	-42

* Enzyme precipitated between 31 and 50% saturation with ammonium sulphate and final solution dialysed.

† Calculated on glucuronic acid content.

‡ Calculated as glucurone (mol.wt. 176) before dialysis.

The depressant effects of the two glucuronic acid-1-phosphates on the hydrolysis of phenolphthalein glucuronide were consistent with the possibility that these compounds were substrates for the enzyme, but a number of other possibilities are considered later.

Activation of the hydrolysis of phenolphthalein glucuronide was observed in every experiment with menthyl α -glucuronide, varying somewhat in degree with different enzyme preparations. Activation was obtained with both menthyl glucosides and with menthyl β -glucuronide. With the β -glucuronide, activation of the enzyme apparently overcame the expected effect of substrate competition on the rate of liberation of phenolphthalein. Control experiments showed that neither of the menthyl glucuronides catalysed the decomposition of phenolphthalein glucuronide in absence of enzyme. Activation of the enzyme by menthol derivatives did not appear to be a surface effect since the surface-active agent, Triton X-100, did not activate the preparations used in the present experiments (Walker & Levvy, 1951). Mouse-liver glucuronidase can be separated into two fractions with pH optima at 4.5 and 5.2 respectively (Kerr, Campbell & Levvy, 1949). The activity of the mixture at a given pH is the sum of the activities of the two fractions at that pH. With a given enzyme preparation, activation by menthyl α -glucuronide was as great at pH 4.5 as at 5.2, which appeared to exclude the possibility of a change in the pH-activity curve.

Release of phenol from phenyl β -glucuronide was likewise accelerated by menthol derivatives, as shown in Table 2. On increasing the concentration of menthyl β -glucuronide, however, the effect of substrate competition became obvious. The affinities of phenyl and *laevomenthyl* β -glucuronides for the active enzyme centre, it should be noted, are

similar, whilst that of phenolphthalein glucuronide is much greater (Karunairatnam & Levvy, 1949).

Table 2. *Effects of menthol derivatives on the hydrolysis of 0.015M-phenyl β -D-glucuronide by mouse-liver glucuronidase*

Compound	Final concentration (M)	Change in rate of phenol liberation (% control)
(-)-Menthyl α -D-glucuronide	0.005	+19
(-)-Menthyl β -D-glucuronide	0.01	-20
(-)-Menthyl β -D-glucuronide	0.001	+3
(-)-Menthyl β -D-glucoside	0.0005	+6

The liberation of reducing sugar. Measurements were made of the liberation of reducing sugar from menthyl α -glucuronide and from α - and β -glucuronic acid-1-phosphate. For purposes of comparison, hydrolysis of menthyl β -glucuronide by the various enzyme preparations was also followed. Results of representative experiments are shown in Table 3. Menthyl α -glucuronide was neither a substrate for the enzyme, nor did it have any significant effect on the rate of hydrolysis of the β -anomer. Activation of the enzyme was presumably already at a maximum in the presence of the latter alone.

Neither α - nor β -glucuronic acid-1-phosphate yielded any reducing sugar after 2 hr. incubation at pH 5.2 in absence of enzyme, but both were hydrolysed in its presence. The glucuronic acid formed was insufficient to have accounted for the effects of the phosphates on the hydrolysis of phenolphthalein glucuronide (Table 1). Hydrolysis of the phosphates could have been due to β -glucuronidase or to a phosphatase in the preparation. Discrimination between these possibilities was made by the use of

Table 3. *Liberation of reducing sugar from various possible substrates by mouse-liver glucuronidase*

(Enzyme precipitated between 31 and 50% saturation with ammonium sulphate and final solution dialysed. Results expressed as glucuronic acid liberated in 0.8 ml. incubation mixture.)

Exp. no.	Substrate	Inhibitor	Glucuronic acid liberated (μ g.)	Inhibition (%)
1	0.01M-Menthyl β -glucuronide	None	69	—
	0.005M-Menthyl α -glucuronide	None	0	—
	0.01M-Menthyl β -glucuronide	0.005M-Menthyl α -glucuronide	67	3
2	0.01M-Menthyl β -glucuronide	None	129	—
	0.01M-Menthyl β -glucuronide	0.0001M-Saccharo-1:4-lactone	8	94
	0.01M- α -Glucuronic acid-1-phosphate	None	27	—
	0.01M- α -Glucuronic acid-1-phosphate	0.01M-NaF	20	26
	0.01M- α -Glucuronic acid-1-phosphate	0.0001M-Saccharo-1:4-lactone	26	4
3	0.01M-Menthyl β -glucuronide	None	113	—
	0.01M-Menthyl β -glucuronide	0.0001M-Saccharo-1:4-lactone	4	96
	0.002M- β -Glucuronic acid-1-phosphate*	None	37	—
	0.002M- β -Glucuronic acid-1-phosphate*	0.01M-NaF	27	27
	0.002M- β -Glucuronic acid-1-phosphate*	0.0001M-Saccharo-1:4-lactone	6	84

* Concentration calculated on glucuronic acid content.

saccharo-1:4-lactone, a powerful inhibitor for β -glucuronidase (Levy, 1952). As shown in Table 3, the concentration of the lactone chosen caused almost complete inhibition of the hydrolysis of menthyl β -glucuronide. Inhibition of the hydrolysis of β -glucuronic acid-1-phosphate was also marked, and this compound must therefore be regarded as a substrate for β -glucuronidase. The lactone had no significant effect on the rate of hydrolysis of α -glucuronic acid-1-phosphate. Hydrolysis in this case cannot therefore have been due to β -glucuronidase. The α -phosphate must have acted as an enzyme inhibitor, in the ordinary sense of the word, in the experiments with phenolphthalein glucuronide (Table 1).

Hydrolysis of both glucuronic acid-1-phosphates was slightly inhibited by fluoride, which does not inhibit β -glucuronidase (Karunairatnam & Levy, 1949), suggesting that a phosphatase was present in the enzyme preparations.

Resynthesis of glucuronides. Storey (1950) has suggested that the synthesis of a glucuronide in the animal body may occur by condensation of the aglycone with glucuronic acid-1-phosphate (anomer unspecified) under the influence of a phosphorylase. It could also conceivably occur under the influence of β -glucuronidase, acting as a transferase. In the mouse, glucuronide synthesis occurs predominantly in the liver (Karunairatnam, Kerr & Levy, 1949). The possibility had to be considered that resynthesis of phenolphthalein glucuronide might have occurred in the experiments with the glucuronic acid-1-phosphates shown in Table 1. The effects of the phosphates on the synthesis of *o*-aminophenyl glucuronide by mouse-liver preparations were therefore studied by the method of Levy & Storey (1949).

Mouse-liver homogenates were variously prepared in water, 0.9% (w/v) sodium chloride, 1.1% (w/v) potassium chloride and bicarbonate Ringer solution. In no instance did incubation of the homogenate with α - or β -glucuronic acid-1-phosphate (0.001 and 0.005M) and *o*-aminophenol (0.0025%) in bicarbonate Ringer solution give detectable synthesis of *o*-aminophenyl glucuronide. Addition of 0.01M-sodium fluoride or 0.0002M-saccharo-1:4-lactone, or both, to the incubation mixture did not lead to synthesis. In the synthesis of *o*-aminophenyl glucuronide by mouse-liver slices, addition of α - or β -glucuronic acid-1-phosphate (0.001 and 0.005M) caused no stimulation, nor did these compounds overcome the inhibition produced by anaerobiosis (Storey, 1950).

DISCUSSION

The synthesis of *laevomenthyl* β -D-glucuronide, identical with the biosynthetic compound (Marsh, 1952), has afforded final proof that this compound

and presumably other urinary glucuronides are β -D-glucopyranuronides. On the basis of the present work it would appear that mouse-liver glucuronidase is specific for this group of compounds, including β -D-glucopyranuronic acid-1-phosphate. It is remarkable that methyl β -D-glucofuranuronide was neither a substrate nor an inhibitor for the enzyme, since saccharo-1:4-lactone with a similar ring structure is a powerful competitive inhibitor (Levy, 1952).

Oxycellulose on a weight basis was about as effective as free glucuronic acid in slowing the hydrolysis of phenolphthalein glucuronide by the enzyme, but in the former case the effect was largely associated with dialysable material in the solution, which amounted to only 20% of the total solid. The net effect was too great to be accounted for in terms of free glucuronic acid present as impurity in the oxycellulose solution. It is considered likely that the action of oxycellulose was due to a mixture of oligosaccharides composed largely of β -linked glucuronic acid residues. Since oxycellulose is unstable to alkali it was found impossible to apply the measurement of reducing sugar, to decide whether any hydrolysis occurred in the presence of the enzyme.

Depression of the enzymic hydrolysis of phenolphthalein glucuronide by α - or β -glucuronic acid-1-phosphate was open to a variety of explanations. The phosphate might have been an alternative substrate for the enzyme, or it might have been an enzyme inhibitor in the usual sense of the word. Hydrolysis, either spontaneous at the pH of enzyme assay, or under the action of some other enzyme than β -glucuronidase, might have yielded sufficient glucuronic acid to have caused marked inhibition. Lastly, resynthesis of phenolphthalein glucuronide might have occurred, in accordance with the theory of Storey (1950). Eventually it was found that β -glucuronic acid-1-phosphate was a substrate for β -glucuronidase and that the α -anomer inhibited the enzyme, although both compounds were slowly hydrolysed at pH 5.2 by a phosphatase in the glucuronidase preparations.

It should not be concluded that the experiments dealing with glucuronide synthesis disprove Storey's theory that glucuronic acid-1-phosphate is concerned in this process in the animal body. Some unknown factor may have been missing or deficient in the experiments with liver homogenates (Dutton & Storey, 1951). In the experiments with liver slices, on the other hand, the glucuronic acid-1-phosphates may not have penetrated the cell membrane. The results of our experiments do, however, make it very unlikely that any synthesis of phenolphthalein glucuronide from phenolphthalein can have occurred in the presence of β -glucuronidase preparations and α - or β -glucuronic acid-1-phosphate.

SUMMARY

1. On the basis of its action on a number of conjugated hexuronic acids and other compounds, it is concluded that mouse-liver glucuronidase is specific for β -D-glucopyranuronides, including β -D-glucopyranuronic acid-1-phosphate.

2. Derivatives of *laevomenthol* caused slight activation of glucuronidase.

3. Mouse-liver preparations did not catalyse the condensation of *o*-aminophenol with α - or β -glucuronic acid-1-phosphate.

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The Mechanism of the Action of Uricase

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The enzyme uricase was considered formerly to oxidize uric acid with quantitative formation of allantoin, carbon dioxide and hydrogen peroxide (Wiechowski, 1907; Batelli & Stern, 1909; Keilin & Hartree, 1936a; Holmberg, 1939; Davidson, 1942), as shown in the following equation:

However, it was shown by Felix, Scheel & Schuler (1929) that under some conditions oxygen uptake was more rapid than carbon dioxide production, and Schuler (1932) found that at pH 8.9 the yield of carbon dioxide was only 17.8% of that expected from the oxygen consumption. These findings suggested that the first product of the

enzymic oxidation was not allantoin, and the pH changes during oxidation indicated that the primary product was a substance of greater acid strength than uric acid. Indications of the structure of this first product were obtained in experiments in which uric acid was oxidized with permanganate. Fischer &

Ach (1899) had found that oxidation of 1-methyl- and of 7-methyl-uric acid yields the same product, 3-methylallantoin, whilst 3-methyl- and 9-methyl-uric acid give 1-methylallantoin. Behrend (1904) pointed out that these findings can be explained by assuming that the first product of oxidation of uric acid by permanganate is a symmetrical compound, and he suggested that it possessed the structure I. This substance which Behrend was not able to isolate was named hydroxyacetylene diurein

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