The Kinetics of Hydrolysis of Synthetic Glucuronic Esters and Glucuronic Ethers by Bovine Liver and *Escherichia coli* β -Glucuronidase

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1. The relative rates of hydrolysis of synthetically prepared β -D-glucuronic esters [aglycone: benzoic acid, veratroyic (3,4-dimethoxybenzoic) acid, indol-3-ylacetic acid and ethylbutyric acid], and β -D-glucuronic ethers (aglycone: phenolphthalein, *p*-nitrophenol, 3,4-dimethoxyphenol, 3,4-dimethoxybenzyl alcohol) by commercial preparations of β -glucuronidase from bovine liver and *Escherichia coli* were investigated. The rates of hydrolysis of all compounds tested were followed by measuring the formation of glucuronic acid under conditions which do not affect the glycosidic ester bond.
2. The pH profiles of the substrates in reaction with the enzyme from both sources were determined, and substrate-saturation curves at the optimal pH for each substrate were constructed; double-reciprocal plots of activity against concentration were linear.
3. Comparison of kinetic data indicates that neither the type of sugar–aglycone linkage, nor the aglycone structure alone can explain the observed K_m and V_{max} values.
4. α -D-Glucuronic esters of benzoic and veratroyic acid resisted hydrolysis by β -glucuronidase from both sources.

Enzymic hydrolysis of glucuronic ethers by β -glucuronidase (EC 3.2.1.31) has been the subject of many investigations. However, only a few enzymic assays were performed with glucuronic esters, conjugates containing the β -ester linkage between the sugar moiety and the aglycone. This scarcity of information is partly due to the difficulties in obtaining pure samples of β -D-glucuronic esters and partly to the lack of adequate analytical methods that would allow measurement of free glucuronic acid in the presence of labile ester conjugates.

Levy & Worgan (1955) studied the activity of mouse liver β -glucuronidase toward biosynthetically prepared glucuronic esters of veratroyic (3,4-dimethoxybenzoic) acid (Sammons & Williams, 1946), 2-ethylbutyric acid and 2-ethylhexanoic acid (Kamil *et al.*, 1953) by measuring their effects in decreasing the enzyme-catalysed hydrolysis of phenolphthalein glucuronide. On the basis of the results obtained they concluded that glucuronic esters are substrates for β -glucuronidase. Experiments of this type in which glucuronic esters, isolated from biological material, were tested as competing substrates in the hydrolysis of a chromogenic substrate of known K_m value were also performed with β -glucuronidase from other sources (Levy, 1954; Levy & Conchie, 1966). Dutton (1956) presented evidence that UDP-glucuronic acid acts as the glucuronyl donor to a wide range of aglycones including carboxylic acids; the formed ester-type conjugates were treated with β -glucuronidase, the released aglycones were extracted with ether, and the extracts were compared with

known standards by paper chromatography. Eisenberg (1959) studied the β -glucuronidase-catalysed hydrolysis of biosynthetically prepared glucuronic ester of benzoic acid (Pryde & Williams, 1933) by using sodium acetate buffer to which ^{18}O -labelled water was added.

In this laboratory, an analytical procedure for direct measurement of glucuronic acid in mixtures containing glucuronic acid and labile glucosiduronic acids has been developed (Tomašić & Keglević, 1972a). The procedure is based on the colorimetric reaction of glucuronic acid with benzidine in the presence of acetic acid (Jones & Pridham, 1954), and the assay is carried out in buffered media suitable for enzymic reaction.

In the present study a comparative investigation of kinetic properties of β -glucuronidase from bovine liver and *Escherichia coli* in reaction with synthetically prepared glucuronic esters and structurally related glucuronic ethers was undertaken. The study was primarily designed to find out whether the rate of hydrolysis of the compounds examined was dependent on: (a) the type of sugar–aglycone linkage; and (b) the biological origin of the enzyme used. Some of the work described here has already been briefly reported (Keglević & Tomašić, 1972).

Experimental

Materials

Enzyme. β -Glucuronidase from bovine liver (type B-1, 600000 Fishman units/g) and of bacterial origin

(type II, approx. 1000 Sigma units/vial) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; just before use, the enzyme was dissolved in the appropriate buffer to the concentration of 100 Fishman units/ml. The enzyme activities were determined by incubating the bovine liver and the *E. coli* enzyme at 38°C in 0.1M-sodium acetate buffer, pH 5.0, and 0.06M-sodium-potassium phosphate buffer, pH 6.8, respectively, with phenolphthalein glucuronide as the substrate. One unit of enzyme activity is defined as the amount required to catalyze the liberation of 1 μ mol of phenolphthalein/min under the reaction conditions described below; the final substrate concentration was 0.2 and 0.1 mM with the bovine liver and *E. coli* enzyme, respectively.

Substrates. Phenolphthalein β -D-glucuronide (0.01M solution, pH 7.0) and *p*-nitrophenyl β -D-glucuronide were obtained from Sigma Chemical Co.

Other substrates used in this study were prepared in this laboratory and were analytically and anomerically pure. The synthesis of the following compounds has been described elsewhere: 1-O-benzoyl- β -D-glucopyranuronic acid, 1-O-(3,4-dimethoxybenzoyl)- β -D-glucopyranuronic acid and 1-O-(indol-3-ylacetyl)- β -D-glucopyranuronic acid (Keglević *et al.*, 1968); 1-O-(2-ethylbutyryl)- β -D-glucopyranuronic acid, 3,4-dimethoxyphenyl β -D-glucuronide and 3,4-dimethoxybenzyl β -D-glucuronide (Tomašić & Keglević, 1972b); 1-O-benzoyl- α -D-glucopyranuronic acid and 1-O-(3,4-dimethoxybenzoyl)- α -D-glucopyranuronic acid (Keglević *et al.*, 1968).

Other compounds. Benzidine (E. Merck, Darmstadt, Germany) was recrystallized from freshly redistilled aq. 96% (v/v) ethanol and stored for up to 2 months in the dark; 0.6% and 0.3% (w/v) solutions in glacial acetic acid, used as the colorimetric reagent, were made fresh daily. Great care should be taken in handling benzidine in view of its carcinogenicity. All other chemicals used as reagents or buffers were of analytical-reagent grade.

Methods

Conditions of the enzyme assays. All kinetic experiments were performed at 38°C. The assay mixture in a final volume of 1.90 ml was composed of the appropriate buffer, the substrate solution (0.005M in water) and the enzyme. After preincubation of the tube with buffer and substrate at 38°C for 10 min, the reaction was initiated by the addition of enzyme solution. The reaction was terminated at required times by adding 0.1 ml of acetic acid.

For experiments in which the pH was varied, sodium acetate buffer (0.1M) of various pH values was used for bovine liver β -glucuronidase and sodium acetate and sodium-potassium phosphate buffers (0.1M and 0.06M), respectively, for bacterial β -glucuronidase.

The rates of hydrolysis of the substrates were determined under conditions of linearity with respect to enzyme concentration and time and are expressed in terms of μ mol of glucuronic acid released/min per unit of enzyme. In all experiments, blanks (containing all components except substrate), appropriate controls (with the substrate added to the system after the incubation had been stopped) and standards (containing enzyme, buffer and known amounts of equimolar mixtures of glucuronic acid and the aglycone) were run simultaneously. All experiments were performed in duplicate.

Determination of glucuronic acid. Glucuronic acid liberated by the enzyme was determined by the colorimetric reaction with benzidine in the presence of acetic acid (Tomašić & Keglević, 1972a). Solutions of benzidine should be handled with great care since it is highly carcinogenic. To the acidified acetate digests, 1.5 ml of 0.6% benzidine reagent was added and, after heating at 100°C for 15 min, the absorbance was read against a blank with a Beckman DU spectrophotometer at 410 nm. In experiments in which the phosphate buffer was used, the acidified incubation mixture was diluted with 1 ml of water, 1.5 ml of 0.3% benzidine reagent was added and the mixture was further treated as described above. Sample reading, after subtraction of the control reading, was then compared with the values given by standards.

Determination of phenolphthalein. Phenolphthalein liberated was determined by the method of Talalay *et al.* (1946) as modified by Hirata & Teshima (1952). After incubation, the mixture was adjusted with 1M- Na_2CO_3 to pH 10.4 and diluted with water to 10.0 ml.

Polyacrylamide-gel electrophoresis. This was performed in the Ortec apparatus (Ortec, Oak Ridge, Tenn., U.S.A.) by using the system of Allen & Moore (1966) in a flat-bed vertical discontinuous polyacrylamide-gel system. The electrolyte was 65 mM-Tris-borate buffer, pH 9.0, and a constant current of 5 mA/sample was applied. Staining of the protein was carried out with Coomassie Brilliant Blue R 250 by the method of Chrambach *et al.* (1967). The β -glucuronidase activity was localized by incubating 2 mm portions of unstained duplicate gel with phenolphthalein glucuronide under standard conditions. The migration rate (R_F) was measured against that of the tracing dye Bromophenol Blue; the band associated with enzyme activity for bovine liver and *E. coli* enzyme showed R_F 0.253 and 0.426 respectively.

Results and Discussion

In the ester-type series, β -D-glucuronides of benzoic acid, veratric acid, indol-3-ylacetic acid and 2-ethylbutyric acid were tested as substrates for β -glucuronidase from bovine liver and *E. coli* origin. In the ether-type series, the comparative experiments were carried out with phenolphthalein glucuronide

Fig. 1. pH-response curves for bovine liver β -glucuronidase-catalysed hydrolysis of (a) glucuronic esters and (b) glucuronic ethers

Incubation mixtures contained in a total volume of 1.90 ml: 0.25 μmol of substrate, enzyme and 0.1 M-sodium acetate buffer at the indicated pH; the range of the enzyme added was 0.3–6.3 munits. At each pH the rates were obtained as a function of various enzyme concentrations, and the release of glucuronic acid was linearly related to time for at least 30 min up to 2 h. Other conditions were as described in the Experimental section. (a) β -D-Glucuronide of: $\circ$, benzoic acid; Δ , veratronic acid; ∇ , 2-ethylbutyric acid. (b) β -D-Glucuronide of: $\bullet$, phenolphthalein; $\blacksquare$, 3,4-dimethoxyphenol; $\blacktriangledown$, 3,4-dimethoxybenzyl alcohol. Each point represents the mean value of two experiments run in duplicate.

Fig. 2. pH-response curves for *E. coli* β -glucuronidase-catalysed hydrolysis of (a) glucuronic esters and (b) glucuronic ethers

Incubation conditions were as described in Fig. 1 with 0.1 M-sodium acetate (pH 5.0–5.6) or 0.06 M-sodium-potassium phosphate (pH 6.2–7.4) as buffer; the range of the enzyme added was 0.3–4.6 munits. Under conditions of the assay the release of glucuronic acid was linearly related to time for at least 7 up to 30 min. (a) β -D-Glucuronide of: $\circ$, benzoic acid; Δ , veratronic acid; ∇ , 2-ethylbutyric acid. (b) β -D-Glucuronide of: $\bullet$, phenolphthalein; $\blacksquare$, 3,4-dimethoxyphenol; $\blacktriangledown$, 3,4-dimethoxybenzyl alcohol. Each point represents the mean value of two experiments run in duplicate.

and *p*-nitrophenyl glucuronide (the two most commonly used substrates), and with 3,4-dimethoxyphenyl β -D-glucuronide and 3,4-dimethoxybenzyl β -D-glucuronide, both containing aglycones structurally related to that of the glucuronic ester of veratroid acid.

The activities of commercial preparations of β -glucuronidase from bovine liver and *E. coli*, used throughout the work, were defined with respect to the hydrolysis of phenolphthalein glucuronide by measuring the amount of the aglycone released. The reaction rates in comparative experiments were followed by measuring the formation of glucuronic acid; by using the benzidine procedure for direct quantitation of glucuronic acid (Tomašić & Keglević, 1972a) it was possible to employ a single analytical method for measuring the degree of hydrolysis of eight different substrates.

Since optimum conditions for enzymic hydrolysis of glucuronic esters were completely unknown, initial incubations were performed with 0.13 mM substrate during a fixed time (1 h) at pH values and with the enzyme concentrations affording maximum hydrolysis of phenolphthalein glucuronide. By these experiments it was established that all the β -D-glucuronic esters are substrates for bovine liver and *E. coli* β -glucuronidase, thus confirming the finding of Levvy & Worgan (1955) with biosynthetically prepared substrates. On the contrary, under identical experimental conditions, 1-O-benzoyl- α -D-glucopyranuronic acid and 1-O-veratroyl- α -D-glucopyranuronic acid were affected neither by the mammalian nor by the bacterial enzyme.

pH-activity curves

The measurements of initial velocities were made with equivalent amounts of the substrates at different pH values under conditions in which the enzyme activity was proportional to the enzyme concentration up to 10–20% conversion of the substrate. Because of limited availability, assays with glucuronic ester of indol-3-ylacetic acid were performed over the whole pH range with a fixed concentration of the enzyme to accomplish up to 50% hydrolysis.

The pH profiles of glucuronic esters and glucuronic ethers in reaction with bovine liver β -glucuronidase are presented in Fig. 1. For both types of substrates one-peak activity curves with pH optima between 4 and 5 were obtained. The pH optima for the hydrolysis of glucuronic ester of benzoic acid and glucuronic ethers of 3,4-dimethoxyphenol and 3,4-dimethoxybenzyl alcohol were practically identical. For phenolphthalein glucuronide, the shape of the curve and the rate of hydrolysis were comparable with the known data (Sarkar & Sumner, 1950; Plapp & Cole, 1966).

In Fig. 2 the pH-activity curves for β -glucuronidase from *E. coli* are given. In comparison with the bovine liver enzyme, the maximal activity was shifted to more basic values, and the pH optima for all substrates, except phenolphthalein glucuronide, were very close to 5.6.

Effects of substrate concentration

The effects of various substrate concentrations on the reaction rate were studied at the optimal pH of

Fig. 3. Substrate-saturation curves for (a) glucuronic esters and (b) glucuronic ethers in the bovine liver β -glucuronidase-catalysed hydrolysis.

The assay medium contained in a total volume of 1.90 ml: substrate at the concentration given, enzyme and 0.1 M-sodium acetate buffer at the optimal pH for each substrate. The range of the enzyme added was 0.3–4.0 units and the release of glucuronic acid was linearly related to time for at least 30 min up to 3 h. (a) β -D-Glucuronide of: $\circ$, benzoic acid; Δ , veratroid acid; $\square$, indol-3-ylacetic acid; ∇ , 2-ethylbutyric acid. (b) β -D-Glucuronide of: $\bullet$, phenolphthalein; $\blacktriangle$, *p*-nitrophenol; $\blacksquare$, 3,4-dimethoxyphenol; $\blacktriangledown$, 3,4-dimethoxybenzyl alcohol. Each point represents the mean value of three experiments run in duplicate.

each substrate at 38°C. The rates of glucuronic acid formation in the reaction with bovine liver β -glucuronidase are given in Fig. 3. The three glucuronic esters with aglycones containing an aromatic ring were hydrolysed at about the same rate and considerably faster than the conjugate of 2-ethylbutyric acid; the hydrolysis rates of the former compounds were similar to those observed for phenolphthalein glucuronide and *p*-nitrophenyl glucuronide. In the case of phenolphthalein glucuronide, considerable substrate inhibition occurred at high concentrations (Becker, 1965), whereas with the other substrates no inhibition was observed up to 0.5 mM. Hydrolysis of 3,4-dimethoxyphenyl glucuronide and particularly of 3,4-dimethoxybenzyl glucuronide proceeded at a considerably slower rate than that of the cognate glucuronic ester of veratroyic acid.

In the reaction with β -glucuronidase from *E. coli* (Fig. 4), the rates of glucuronic acid released, except for phenolphthalein glucuronide, were higher by one order of magnitude, or even more, than those obtained with the bovine liver enzyme. From Figs. 3 and 4 it is apparent that the relative $V_{\max}$ values for the bovine liver enzyme with glucuronic esters were very similar to those found for the *E. coli* enzyme; on the contrary, the relative $V_{\max}$ values for glucuronic ethers differed depending on the source of β -glucuronidase used.

For all compounds studied, plots of $1/v$ against $1/[S]$ were straight lines: the Michaelis constants and maximum-velocity values were derived from the Lineweaver-Burk plots and are summarized in Table 1.

The K_m and $V_{\max}$ values obtained with bovine liver β -glucuronidase for the three glucuronic esters containing aglycones with an aromatic ring, as well as for the glucuronic ethers of phenolphthalein and *p*-nitrophenol, were within a narrow range. The K_m value we obtained for *p*-nitrophenyl glucuronide was very close to the reported value of 0.13 mM obtained with rat liver microsomal β -glucuronidase (Lucier & McDaniel, 1972), and 0.118 mM with calf liver preparation (Fishman *et al.*, 1964). In the ester-type series neither replacement of the benzene hydrogen atoms by electron-releasing methoxy substituents, nor the alteration of the benzene ring to the indole nucleus were of any practical effect on K_m and $V_{\max}$ values. On the other hand, the K_m value for the glucuronic ester of 2-ethylbutyric acid, the only compound among the substrates tested possessing the aliphatic aglycone structure, was much higher.

In the ether-type series, the presence of two electron-releasing substituents in the benzene ring led to an increase in K_m and a decrease in $V_{\max}$ values. However, insertion of a methylene group between the *O*-glycosidic bond and the 3,4-disubstituted benzene

Fig. 4. Substrate-saturation curves for (a) glucuronic esters and (b) glucuronic ethers in the *E. coli* β -glucuronidase-catalysed hydrolysis

The assay medium contained in a total volume of 1.90 ml: substrate at the concentration given, enzyme and 0.1 M-sodium acetate buffer (except for phenolphthalein glucuronide where 0.06 M-sodium-potassium phosphate was used) at the optimal pH for each substrate. The range of the enzyme added was 0.3–4.0 munits and the release of glucuronic acid was linearly related to time for at least 5 min up to 15 min. (a) β -D-Glucuronide of: $\circ$, benzoic acid; Δ , veratroyic acid; $\square$, indol-3-ylacetic acid; ∇ , 2-ethylbutyric acid. (b) β -D-Glucuronide of: $\bullet$, phenolphthalein; $\blacktriangle$, *p*-nitrophenol; $\blacksquare$, 3,4-dimethoxyphenol; $\blacktriangledown$, 3,4-dimethoxybenzyl alcohol. Each point represents the mean value of three experiments run in duplicate.

Table 1. Kinetic parameters for the β -glucuronidase-catalysed hydrolysis of glucuronic esters and glucuronic ethers

All the kinetic constants were determined at optimal pH for each substrate, and K_m and V_{max} values were derived from double-reciprocal plots. Details of experimental procedures are given in Figs. 3 and 4. In V_{max}/K_m ratios, V_{max} is expressed as mmol of glucuronic acid/min per litre per enzyme unit and K_m is expressed in mmol/litre.

Substrate	Bovine liver β -glucuronidase			<i>E. coli</i> β -glucuronidase		
	K_m (mM)	Relative V_{max} (μ mol/min per enzyme unit)	V_{max}/K_m (min ⁻¹ ·enzyme unit ⁻¹)	K_m (mM)	Relative V_{max} (μ mol/min per enzyme unit)	V_{max}/K_m (min ⁻¹ ·enzyme unit ⁻¹)
β -D-glucuronide of:						
Benzoic acid	0.085	1.52	9.4	0.110	35.2	168.1
Veratroc acid	0.100	1.99	10.5	0.100	31.2	164.0
Indol-3-ylacetic acid	0.077	1.27	8.7	0.080	25.7	168.7
2-Ethylbutyric acid	1.000	2.37	1.25	0.290	15.2	27.5
Phenolphthalein*	0.110	1.73	8.3	0.030	1.44	2.5
<i>p</i> -Nitrophenol	0.120	2.28	10.0	0.100	48.6	256.0
3,4-Dimethoxyphenol	0.200	0.80	2.10	0.159	55.1	182.3
3,4-Dimethoxybenzyl alcohol	0.500	0.21	0.22	0.200	10.8	28.5

* Obtained by extrapolation of the linear portion of the double-reciprocal plots.

ring appeared to have a much stronger effect on the enzyme; the V_{max}/K_m ratio for 3,4-dimethoxyphenyl glucuronide was ten times as great as that for 3,4-dimethoxybenzyl glucuronide.

The K_m values for the action of *E. coli* β -glucuronidase were within a narrower range and, in general, lower than those obtained with the bovine liver enzyme, thus indicating a broader specificity of the bacterial enzyme in terms of K_m values. The K_m value we found for phenolphthalein glucuronide (0.03 mM) was comparable with the value of 0.04 mM reported by Didier-Fichet & Stoeber (1968), who used the same enzyme source.

However, as can be seen from Table 1, the relationships of the V_{max}/K_m ratio for the three glucuronic esters containing an aromatic ring versus the V_{max}/K_m ratio for the glucuronic ester of 2-ethylbutyric acid were essentially identical for the two enzymes. Very high relative V_{max} values obtained for *p*-nitrophenyl glucuronide and 3,4-dimethoxyphenyl glucuronide with the bacterial enzyme were in agreement with the results of Wang & Touster (1972), who reported that both electron-attracting and electron-releasing substituents facilitate the rate of hydrolysis by β -glucuronidase. However, the markedly lower V_{max}/K_m ratio calculated for 3,4-dimethoxybenzyl glucuronide points again to the importance of the relative positions of the *O*-glycosidic bond and the aromatic ring involved.

Conclusions

It appears that neither the type of sugar-aglycone linkage, nor the aglycone structure alone can explain the observed K_m values. It has already been observed (Krotz Kirk *et al.*, 1962) that the comparative activities of bacterial and mammalian β -glucuronidase preparations toward phenolphthalein glucuronide do not parallel their activities toward other substrates. Gel electrophoresis of commercial preparations of β -glucuronidase used in this study showed several protein bands; however, in all cases the enzyme activity was associated with only one band revealing different electrophoretic mobility with respect to the biological origin of the enzyme.

The present work has confirmed the validity of the benzidine procedure for direct spectrophotometric assay of glucuronic acid and thus provided the basis for further studies of enzyme-catalysed hydrolysis of ester-type glucuronides.

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