The Use of Phenol Glucuronide in the Assay of β -Glucuronidase

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Our present knowledge of the distribution in the body and the physical properties of β -glucuronidase is derived almost entirely from experiments, in which free glucuronic acid was determined by reduction of ferricyanide after enzymic hydrolysis of a conjugated glucuronide. Whilst it is possible to determine very small amounts of glucuronic acid by means of its reducing power (Levy, 1946), the lack of specificity in the reaction is a serious disadvantage in the routine assay of glucuronidase. Unless the enzyme preparation has been considerably purified, it may contain large amounts of materials other than glucuronic acid which reduce ferricyanide (Levy, 1948). Other impurities may interfere during the actual titration, if this is cerimetric, obscuring the end point and making it unstable. In studying the enzymic hydrolysis of oestriol glucuronide, Fishman (1939) determined free oestriol by the Kober colour reaction. Its scarcity, however, renders this substrate unsuitable for routine assays. Talalay, Fishman & Huggins (1946) have recently described a method whereby the hydrolysis of phenolphthalein glucuronide can be followed colorimetrically. This method appears to avoid the difficulties mentioned above in the determination of glucuronidase activities. It was found possible in this laboratory to apply the method of King & Armstrong (1934) for phosphatase determination to the assay of glucuronidase, using biosynthetic phenol glucuronide (Williams, private communication) as substrate. (We are greatly indebted to Dr Williams for details of the technique for obtaining this compound from rabbit urine.) Results obtained in studying the kinetics of hydrolysis of phenol glucuronide with enzyme obtained from two different organs, and a description of the procedure for the routine assay of glucuronidase in mouse tissues, are given below.

EXPERIMENTAL

Preparation of phenol β -D-glucuronide. The glucuronide was obtained from the urine of rabbits fed with phenol by the general procedure described by Williams (1943) for the isolation of aminophenol glucuronides. Before use, it was dried over P_2O_5 *in vacuo* for 10 hr. at 80°. Analysis showed it to be free from solvent of crystallization (m.p. 162°, corr.).

The colour reaction for free phenol. Phenol was determined with the reagent of Folin & Ciocalteu (1927). King & Armstrong (1934) give full details of the technique for this estimation. The colour intensity was measured with a Spekker photoelectric absorptiometer, using Ilford no. 602 blue filter. Free glucuronic acid did not interfere in the reaction. Phenol glucuronide in high concentration gave a faint colour, probably due to traces of free phenol (less than 0.2% of the total weight), since it varied from one sample to another. Contrary to the findings of Folley & Kay (1935), the reaction was not particularly sensitive to the Na_2CO_3 concentration and was complete in less than 20 min. at 37°. In constructing the calibration curve (Fig. 1),

Fig. 1. Calibration curve for estimation of phenol.

0.6 ml. standard phenol solution (Hawk & Bergeim, 1931) was added to 0.2 ml. 0.1 M-citrate buffer (pH 5.2), followed by 2 ml. of a 1:5 dilution of the Folin-Ciocalteu reagent. After mixing, 2 ml. of the liquid were taken for colour development as described below for enzyme extracts. Citrate, acetate and formate buffers had no effect on the colour reaction.

Preparation of the enzyme. Preparations from mouse liver and spleen were studied separately in these experiments. The organs were broken up in glass homogenizers (Umbreit, Burris & Stauffer, 1945). Unless stated to the contrary, the enzyme was partially purified following the method described by Talalay *et al.* (1946). The homogenate was brought to pH 5.0 or 5.2 with acetic or citric acid. Protein which separated after 30 min. at 37° was removed by centrifuging, and the enzyme in the supernatant liquid precipitated by adding an equal volume of saturated

(NH_4)₂SO₄ solution (SAS). The precipitate was dissolved in a convenient volume of water. No change in the amount of enzyme present was noted after purification in this way.

The effect of pH. The pH for optimum hydrolysis was determined over the range 3–6.5 at a substrate concentration of 0.01 M in citrate buffer. To 0.2 ml. of 0.1 M-buffer and 0.2 ml. of 0.04 M-substrate warmed to 37° was added 0.4 ml. enzyme, and the mixture was incubated at 37° for 1 hr. The initial effect of substrate and enzyme on the pH of the buffer was determined electrometrically. No further change in the pH occurred during the incubation.

With crude homogenates of liver and spleen, considerable amounts of substances giving the colour reaction were liberated in absence of substrate. It would appear that an enzymic process was responsible for the production of these compounds since the amount formed varied with the pH, with an optimum at 3.6–4.6. Partial purification of the

sponding to about 200 μg . phenol/g. for spleen and about 100 μg .g. for liver) before plotting the points. From Fig. 2 it can be seen that for enzyme from both sources the rate of hydrolysis rose to a maximum at pH 5.2 as the pH was lowered. After a drop at pH 4.75, the velocity again rose slightly at pH 4.5 and then fell off rapidly. The subsidiary peak at pH 4.5 was seen in other experiments when the pH increment was made small enough. A possible explanation of the shape of the curves in Fig. 2 is given by the work of Mills (1947), who found that β -glucuronidase from beef spleen could be separated into two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide. In acetate buffer, some results were obtained which suggested the pH optimum to be lower than in citrate, but this point was not further investigated. It was decided that for the routine assay of the enzyme the hydrolysis of phenol glucuronide should be done at pH 5.2 in citrate buffer.

Fig. 2. The variation in hydrolysis rate of phenol β -D-glucuronide with pH. Results expressed in terms of phenol liberated/g. moist tissue. $\times$ — $\times$ spleen enzyme; $\bullet$ — $\bullet$ liver enzyme.

homogenates as described above made the blank much smaller and very nearly independent of the pH. With purified enzyme from liver and spleen, the curves for variation in hydrolysis with pH assumed the shapes shown in Fig. 2. Homogenates from five mice were pooled for each organ before purifying, and all points on the curve were determined simultaneously. Corrections were applied for the enzyme blank at the appropriate pH (corre-

Fig. 3. The variation in initial hydrolysis rate with substrate concentration. Results expressed as fraction of maximum observed activity. $\times$ — $\times$ spleen enzyme; $\bullet$ — $\bullet$ liver enzyme.

The effect of substrate concentration. The effect of varying the substrate concentration at constant pH is shown in Fig. 3. The citrate buffer and substrate solutions were adjusted to pH 5.2. Addition of enzyme caused no appreciable change in pH. Three experiments were done with enzyme from each source and the averaged results are shown in the figure. It will be noted that inhibition by excess

substrate was marked in both cases. The substrate optimum for spleen (0.025M) was greater than for liver (0.01M). The results did not lend themselves to the accurate calculation of the Michaelis-Menten constant, K_m , by the method of Lineweaver & Burk (1934), but the value obtained graphically from Fig. 3 by finding the substrate concentration at

nide was directly proportional to the enzyme concentration. Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 3 hr. (Fig. 5).

Fig. 4. The relation between enzyme concentration and phenol liberated under standard conditions. Enzyme concentration in arbitrary units. $\times$ — $\times$ spleen enzyme; $\bullet$ — $\bullet$ liver enzyme.

Fig. 5. Phenol liberated after varying incubation periods. $\times$ — $\times$ spleen enzyme; $\bullet$ — $\bullet$ liver enzyme.

which half the observed maximum velocity of hydrolysis was developed was approximately the same (0.0035M) for enzyme from either source. The difference in shape between the curves for liver and spleen may be due to the presence of interfering substances in the tissue extracts rather than a qualitative difference in the enzyme system. A substrate concentration of 0.015M was adopted for further work with enzyme from both sources.

The effect of varying the enzyme concentration and the duration of hydrolysis. As shown in Fig. 4, the amount of phenol liberated in 1 hr. at 37° and pH 5.2 (citrate buffer) from 0.015M-phenol glucuro-

Reagents *The assay of β -glucuronidase*

Citrate buffer. (a) 0.3M, pH 5.2 (glass electrode); (b) 0.1M, prepared from 0.3M.

Substrate solution. 0.06M-Phenol glucuronide adjusted to pH 5.2; stored in the refrigerator.

Folin-Ciocalteu reagent. (a) Stock solution (British Drug Houses Ltd.); (b) Stock solution diluted 1 to 5 and prepared daily.

N-Sodium carbonate solution.

Saturated ammonium sulphate solution. Adjusted to pH 5.2.

Procedure

Spleen. The dissected organ was weighed in a chilled homogenizer tube and homogenized with 3 ml. water. The cell-free homogenate was transferred to a graduated centrifuge tube, using 1 ml. wash water. After addition of 0.5 ml. 0.3M-buffer, the tube was maintained at 37° for 30 min. Coagulated protein was separated by centrifuging for 15 min. The volumes of precipitate and supernatant liquid were noted and the latter was transferred to another graduated centrifuge tube. An equal volume of SAS was added and the tube centrifuged for 30 min. The liquid was discarded, and the residue dissolved in water and made up to a volume of 2 ml. The concentration of enzyme was then such that, in the case of normal animals, 0.4 ml. of the solution gave readings of 20–40 μ g. phenol after corrections had been applied for enzyme and substrate blanks. This volume of the enzyme solution was added to 0.2 ml. of 0.1M-buffer and 0.2 ml. substrate in a stoppered 10 ml. centrifuge tube. Prior to addition of the enzyme, the substrate and buffer were warmed to 37°. After incubation of the hydrolysis mixture for 1 hr. at 37°, 2 ml. of the diluted Folin-Ciocalteu reagent were measured into the tube. Protein was removed by centrifuging for 3 min. and 2 ml. of the supernatant transferred to a 10 ml. stoppered centrifuge tube containing 4 ml. Na_2CO_3 solution. The contents of the tube were mixed and the colour developed by incubating for 20 min. at 37°. Assays were carried out in duplicate.

Liver. The procedure was exactly as described above except that at certain stages the volumes used differed. Homogenization was done with 5 ml. water and 3 ml. of water were added in transferring the homogenate to the first graduated tube. In adjusting the pH, 1 ml. 0.3M-buffer was used. The solution of the enzyme after SAS precipitation was

made up to 4 ml., this volume giving readings of 20–40 μg . phenol in the assay after correction for blanks with livers from normal animals.

Controls. Every assay was controlled by incubating mixtures of enzyme and buffer containing 0.2 ml. water in place of substrate. The purified enzyme shows a small rise in the blank during incubation. Controls for phenol in the substrate were also done at frequent intervals, 0.2 ml. of the solution being incubated with 0.2 ml. buffer and 0.4 ml. water. Since there appeared to be a very small but perceptible increase in the reading when the substrate was incubated at pH 5.2, this procedure was considered preferable to that in which substrate is added to the enzyme after incubation (cf. Talalay *et al.* 1946).

RESULTS

The recoveries obtained in a series of experiments in which 0.2 ml. standard phenol solution was added to 0.2 ml. 0.1 M-buffer and 0.4 ml. enzyme (spleen and liver mixed) are shown in Table 1. In some experiments the mixture was incubated whilst in others the Folin-Ciocalteu reagent was added at once. Each figure shown in the table is the result of a determination carried out in duplicate and the results have been corrected for the enzyme blank (5.2 μg . unincubated, 7.7 μg . incubated).

Table 1. *Recovery of phenol added to buffered enzyme*

Phenol added (μg .)	Phenol recovered before incubation		Phenol recovered after incubation	
	(μg .)	(%)	(μg .)	(%)
3.9	3.7	95	4.1	105
7.9	8.0	101	8.0	101
15.7	15.6	99	15.8	101
31.4	31.1	99	31.2	99
62.9	63.1	100	63.0	100
105.0	105.0	100	104.9	100

In a series of 30 determinations in which 31.4 μg . phenol were added to enzyme and buffer as above,

and determined at once, the mean recovery was 31.5 μg . (100.3 %), and the standard deviation of a single determination from the mean was 0.19 μg . (0.6 %).

DISCUSSION

From the results given above it can be seen that phenol glucuronide is a convenient substrate for the assay of β -glucuronidase, using the reagent of Folin & Ciocalteu for estimation of phenol liberated by the enzyme. As compared with phenolphthalein glucuronide (Talalay *et al.* 1946), a much higher concentration of phenol glucuronide is required for optimum hydrolysis, and inhibition of the enzyme by excess substrate is more pronounced.

It has been generally assumed without any experimental justification that the conditions for optimum hydrolysis by β -glucuronidase are always the same no matter the source of the enzyme. The data obtained in this work for mouse liver and spleen glucuronidase support the assumption. Masamune (1934), in his pioneer work on glucuronidase, found hydrolysis of phenol glucuronide by beef kidney extracts, determined by estimation of glucuronic acid, to be at a maximum at pH 5.3–5.6 in citrate buffer. This is in good agreement with our own figure of 5.2 for mouse liver and spleen extracts.

SUMMARY

1. Phenol glucuronide is a convenient substrate for the assay of β -glucuronidase, using the Folin & Ciocalteu (1927) reagent for the estimation of free phenol.

2. In the procedure adopted for assay of glucuronidase in mouse liver and spleen, hydrolysis of phenol glucuronide was carried out in citrate buffer at pH 5.2 and a substrate concentration of 0.015 M.

3. The mean recovery of added phenol in the assay procedure was 100 %, and the standard deviation of a single observation from the mean was 0.6 %.

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