Purification of β -Glucuronidase from the Preputial Gland of the Female Rat

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Various methods have been reported for the purification of mammalian β -glucuronidase. To compare the different products, it is most convenient to express the specific activities as glucuronidase units/mg. of protein, where 1 unit liberates 1 μ g. of phenolphthalein from phenolphthalein glucuronide in 1 hr. at 38°. Smith & Mills (1953) applied metallo-protein reactions to the purification of the enzyme from ox liver and obtained a product with a specific activity of 32 000. This represented 800-fold purification, and the recovery was 5%. Starting with calf liver, Bernfeld, Nisselbaum & Fishman (1953) reached specific activities of the order of 60 000 (1500-fold purification and 10% recovery) after alkaline ammonium sulphate fractionation, followed by anion exchange and methanol fractionation. A specific activity of 107 000 was observed with the best individual preparation from calf liver, which was regarded as 85% pure on the basis of electrophoresis data and a specific property solubility test. The same procedure was less successful when applied to a commercial spleen powder (Bernfeld & Fishman, 1953), the final product having a specific activity of 7900 (1400-fold purification and 1% recovery). It seems possible that the fractionation procedure did not separate denatured enzyme. Sarkar & Sumner (1950) achieved approximately 1000-fold purification of ox-liver β -glucuronidase by fractionation with dioxan, but these authors did not follow the customary method for measuring the activity of the enzyme. However, from data given by Bernfeld *et al.* (1953), the specific activity as defined above would appear to have been 31 000. [The figures actually quoted by Sarkar & Sumner (1950) and by Bernfeld *et al.* (1953) for the degree of purification in their liver preparations were six times greater than those shown above, having been calculated by comparing the initial activity/mg. of moist tissue with the final activity/mg. of protein.]

Fishman and his co-workers (Bernfeld & Fishman, 1950; Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954) found that β -glucuronidase in an advanced state of purity displayed a fall in net activity on dilution. This effect could be reversed by adding a variety of substances, such as deoxyribonucleic acid (DNA), albumin or heat-inacti-

vated enzyme. In spite of the wide difference in specific activities, the calf-liver and spleen preparations described above were apparently equally prone to the dilution phenomenon, and the activities quoted for these two preparations were in fact obtained in the presence of DNA. Smith & Mills (1953) could observe no variation with concentration in the specific activity of their ox-liver preparation, but DNA and albumin did activate the enzyme at the pH of assay.

As shown by Beyler & Szego (1954), the preputial (clitoral) gland of the female rat has a β -glucuronidase activity of the order of 1500 phenolphthalein units/mg. of moist tissue (measured in the absence of DNA or albumin), making it the richest known source of the enzyme. Many other mammalian species lack this gland: it is present in the female mouse, but with negligible β -glucuronidase activity (Dr J. Conchie, personal communication). On the assumption that female-rat preputial gland has the same protein content as liver (16.5% of the moist weight), an assumption that was subsequently found to be nearly correct (see below), this gland would appear to have a specific β -glucuronidase activity of 9000. From the results of Bernfeld *et al.* (1953), completely pure calf-liver β -glucuronidase should have a specific activity of 120 000. On the further assumption that the specific activity of the pure enzyme is the same in calf liver and in female-rat preputial gland, one arrives at the rather startling conclusion that 7.5% of the protein in the preputial gland is β -glucuronidase.

From calculations such as these, it was decided that purification of the enzyme from the preputial gland would be profitable, and after a simple four-fold fractionation a specific activity of 455 000 was attained on assay in the presence of albumin.

EXPERIMENTAL AND RESULTS

Enzyme assay

Liberation of phenolphthalein. Biosynthetic phenolphthalein β -glucuronide (Talalay, Fishman & Huggins, 1946), final concn. 0.00063 M, was incubated with the enzyme for 1 hr. at 38° in 0.05 M-acetic acid-NaOH buffer, pH 4.5. The total volume of the incubation mixture was 4 ml., and

the remainder of the assay followed the procedure previously employed (Levy, 1952), with the slight modification described by Levy, Hay & Marsh (1957) for determining optimum pH values.

Liberation of *o*-nitrophenol. Incubation of *o*-nitrophenyl β -galacturonide (Marsh & Levy, 1958b) with the enzyme was carried out as described for phenolphthalein β -glucuronide. After 1 hr. at 38°, the reaction was terminated by adding 4 ml. of 0.4 M-glycine-NaOH buffer, pH 10, and the mixture was centrifuged at 1500 *g* for 15 min. The intensity of the *o*-nitrophenol colour in the supernatant was read with a Spekker photoelectric absorptiometer, Ilford no. 601 violet filters being used. Under these conditions the substrate was stable to alkali.

Liberation of phenol. Hydrolysis of phenyl β -glucuronide and phenyl β -galacturonide (Marsh & Levy, 1958b) in 0.05 M-acetic acid-NaOH buffer, pH 4.5, was followed by the procedure of Kerr, Graham & Levy (1948), the colour intensity being measured in the Spekker absorptiometer with Ilford no. 608 red filters.

Protein determination

Protein was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951), with the Spekker absorptiometer with Ilford no. 608 red filters. Since the colour intensity varies with different proteins, the choice of a standard is important with this method (Fig. 1). Albumin was selected because it has approximately the same intensity as mixed tissue proteins (Lowry *et al.* 1951). The choice of this standard was confirmed by the results of micro-Kjeldahl determinations of N (Karunairatnam & Levy, 1951) in preputial-gland homogenates and in the enzyme at all stages of purification: the N figures ranged from 15 to 17% of the protein, estimated as albumin. Fishman and his co-workers (Bernfeld & Fishman, 1953; Bernfeld *et al.* 1953, 1954) employed chymotrypsin as standard in determining the protein content of purified

β -glucuronidase by the colorimetric method. Chymotrypsin is 1.5 times as chromogenic as albumin (Lowry *et al.* 1951; Fig. 1).

Purification procedure

General comments. Results obtained by the final procedure are illustrated in Table 1. Since it tended to give higher and more reproducible figures, crystalline bovine albumin (L. Light and Co. Ltd.) was employed to activate the enzyme instead of DNA. Parallel assays were done without activator, but are not shown since the only factor that governed the extent of activation by albumin in this work was the enzyme concentration in β -glucuronidase units/ml. of incubation mixture (cf. Fig. 2). In other words, there was no change in the degree of activation (about 100% at the usual level of assay) from beginning to end of the purification.

Preliminary experiments having shown that consecutive fractionations with ammonium sulphate or ethanol led to little purification after the first precipitation, and often to extensive inactivation, alternate fractionation with these two reagents was adopted. It can be seen that only at stage 5 was there much inactivation in the final procedure, most of the other losses resulting from the discard of side-fractions.

Precipitates were allowed to settle for 30 min. at 0° before centrifuging, which was done at the same temperature for 10 min. at 20 000 *g*. The high speed and low temperature were not essential before stage 4. Solutions were dialysed at 0° against 0.01 M-acetic acid-NaOH buffer, pH 5.2, at which pH the enzyme is stable (Levy *et al.* 1957). General manipulation of the preparation was done at ordinary room temperature. For successful fractionation it was essential to increase the initial buffer concentration to 0.1 M. The final product (obtained in about 1 week) was a clear colourless solution, in which the enzyme was stable at 0° for at least several weeks, provided that the buffer was not more dilute than 0.01 M. There was 25-fold purification and 26% recovery.

Stage 1 (homogenate). A mixture of white and hooded adult female rats, usually discards from our breeding stock, was employed. From 25 rats a total of 2.72 g. of preputial gland was obtained. This was suspended in portions in water by means of a chilled glass homogenizer [Jencons (Scientific) Ltd., Hemel Hempstead, Herts]. The homogenate was made 0.1 M with respect to acetic acid-NaOH buffer, pH 5.2, in a final volume of 27.2 ml., and incubated for 4 hr. at 38°. It was stored overnight at -20°. The figure given in Table 1 for the total enzyme activity of the homogenate and the derived figure for the protein content (12% of the fresh tissue) are close to the means for several preparations.

Stage 2 (supernatant). After thawing the homogenate, insoluble material was centrifuged off and washed on the centrifuge with an equal volume of 0.1 M-buffer which was added to the supernatant.

Stage 3 (first ammonium sulphate fractionation). Saturated ammonium sulphate solution (at 0°) was added to the supernatant to give a final saturation of 20% and the precipitate was discarded. The preparation was brought to 80% saturation with ammonium sulphate and this precipitate was retained. (Water was added where necessary in all stages of fractionation to correct for volume changes after mixing the reagents.) The precipitate was dissolved in

Fig. 1. Calibration curves for different proteins determined by the method of Lowry *et al.* (1951) with the Spekker photoelectric absorptiometer (6.5 ml. scale): O, crystalline bovine-pancreas chymotrypsin (L. Light and Co. Ltd.); x, crystalline salt-free chymotrypsinogen (L. Light and Co. Ltd.); ●, crystalline bovine zinc-insulin (British Drug Houses Ltd.); Δ, crystalline bovine albumin (L. Light and Co. Ltd.); ▲, salmon-roe protamine sulphate (L. Light and Co. Ltd.). Nitrogen contents were checked by micro-Kjeldahl.

the minimal volume of 0.01 M-acetate buffer, pH 5.2, and dialysed against the same buffer for 2 days. Material that separated from solution during the dialysis was discarded, and the buffer concentration was increased to 0.1 M.

Stage 4 (first ethanol fractionation). Cold ethanol was added stepwise to give concentrations of 20, 45 and 65% (v/v). All three precipitates were retained and dissolved in minimal volumes of 0.1 M-buffer. Traces of insoluble material were discarded.

Stage 5 (second ammonium sulphate fractionation). Fraction 4b was made 33, 60 and 75% saturated with ammonium sulphate. All three precipitates were brought into solution and dialysed as in stage 3.

Stage 6 (second ethanol fractionation). After removing insoluble material and increasing the buffer concentration to 0.1 M, fraction 5b was treated with cold ethanol in final concentrations of 40, 45 and 65%. All three precipitates were dissolved in 0.1 M-buffer, in which they were completely soluble. Only the first fraction was pigmented. There should be no precipitate when the supernatant from fraction 6 is brought to an ethanol concentration of 50%.

Properties of the enzyme

Effects of varying the enzyme concentration and the duration of hydrolysis. The specific activity of the purified enzyme (and of crude preputial-gland homogenates) fell progressively on dilution as described by Bernfeld *et al.* (1954). This effect was abolished by adding albumin, DNA (from herring sperm; L. Light and Co. Ltd.) or concentrated heat-inactivated enzyme. The rate of hydrolysis of the substrate declined as the period of incubation with the enzyme was prolonged (cf. Bernfeld *et al.* 1954), but remained constant up to 2 hr. in the presence of albumin. These experiments were done with 0.00063 M-phenolphthalein β -glucuronide in 0.05 M-acetate buffer, pH 4.5 or 5.2.

Variation in activity with pH. To establish the best conditions for enzyme assay during the purification, the experiments shown in Fig. 2 were done.

The pH-activity curves for the hydrolysis of phenolphthalein β -glucuronide by crude homogenates and by the purified enzyme displayed two peaks, one at pH 4.5 and the other at pH 5.0–5.2. Activation by albumin caused little change in the shape of the curve. In agreement with Smith & Mills (1953), however, DNA altered the character of the curve by inhibiting at low and activating at high pH. The exact effect produced was a function of enzyme purity as well as dilution.

It was recently reported (Marsh & Levy, 1958a) that partially purified β -glucuronidase from the preputial gland (stage 3 above) hydrolysed 0.01 M-phenyl β -glucuronide in 0.125 M-acetate buffer with a single optimum at pH 4.5, and that the same enzyme hydrolysed 0.01 M-phenyl β -galacturonide with a single optimum at pH 3.9. As shown in Fig. 3, repetition of these experiments with our purest enzyme preparations in 0.05 M-acetate buffer gave similar results. Fig. 3 also shows that DNA acted as an inhibitor in the hydrolysis of the galacturonide below pH 6. The effect of DNA on the hydrolysis of phenyl β -glucuronide was similar to that shown in Fig. 2 where the substrate was phenolphthalein β -glucuronide. Albumin acted purely as an activator in the hydrolysis of both phenyl β -glucuronides.

From Fig. 3 it can be seen that the ratio of β -glucuronidase to β -galacturonidase activity at pH 4.5 was 1.4 in the purified enzyme preparation. The figure previously reported for the partially purified preparation (Marsh & Levy, 1958a) was 3.1 at this pH, due no doubt to tissue constituents producing a differential effect like DNA. Specific activities determined for the purified enzyme as in Table 1, but with 10 mM-phenyl β -glucuronide as substrate, were only 25% of the figures obtained with phenolphthalein β -glucuronide. (When the

Table 1. *Purification of β -glucuronidase from female-rat preputial gland*

One glucuronidase unit (G.U.) liberates 1 μ g. of phenolphthalein from 0.00063 M-phenolphthalein β -glucuronide in 1 hr. at pH 4.5 in the presence of 0.01% of albumin. Percentages of saturated ammonium sulphate solution (SAS) and ethanol are by volume, and indicate limits for precipitation. At stages 4 and 5, fraction b was taken for further purification.

Stage	Description	Specific activity (G.U./mg. of protein)	Recovery (G.U./g. of moist tissue)
1	Homogenate	18 400	2 210 000
2	Supernatant	21 800	2 010 000
3	20–80% SAS	44 600	2 000 000
4 a	0–20% Ethanol	11 000	16 500
b	20–45% Ethanol	81 500	1 830 000
c	45–65% Ethanol	417	4 570
5 a	0–33% SAS	54 300	26 500
b	33–60% SAS	217 000	930 000
c	60–75% SAS	26 900	185 000
6 a	0–40% Ethanol	180 000	133 000
b	40–45% Ethanol	455 000	584 000
c	45–65% Ethanol	106 000	117 000

enzyme activities are expressed as moles of aglycone liberated instead of $\mu\text{g.}$, it can be seen that the two β -glucuronides are hydrolysed at nearly the same rate.)

Effect of varying the substrate concentration in the

Fig. 2. Hydrolysis of 0.00063M-phenolphthalein β -glucuronide at varying pH values in 0.05M-acetate buffer by (a) a crude homogenate of preputial gland and (b) a purified preparation with a specific activity of 341 000 (as defined in Table 1); $\times$, no addition; O , 0.03% of DNA present; $\bullet$, 0.01% of albumin present.

presence and the absence of inhibitors. In previous work (Marsh & Levvy, 1958a), it was concluded that the β -glucuronidase and β -galacturonidase activity of a preputial-gland preparation are due to a single enzyme, because figures for K_i for various competitive inhibitors were practically identical with either phenyl β -glucuronide or phenyl β -galacturonide as substrate. This conclusion was borne out by the results of similar experiments with purified β -glucuronidase (Table 2), except when

Fig. 3. Hydrolysis of 0.01M-phenyl β -glucuronide ($\bullet$) and phenyl β -galacturonide (O) at varying pH values in 0.05M-acetate buffer by purified β -glucuronidase (specific activity 455 000, see Table 1) in the absence of activator, and the effect of 0.03% of DNA on the hydrolysis of phenyl β -glucuronide (Δ) and phenyl β -galacturonide ($\times$).

Table 2. Dissociation constants (mean values) for the complexes formed by purified β -glucuronidase with various substrates (K_m) and competitive inhibitors (K_i)

Experiments were done in 0.05M-acetate buffer, pH 4.5, and unless otherwise stated 0.01% of albumin was present. Saccharate and mucate solutions were boiled before use (Levy, 1952). The specific activity (see Table 1) of the enzyme preparation was 434 000.

Substrate	K_m (μM)	K_i		
		Saccharo- 1:4-lactone (μM)	Saccharate* (μM)	Mucate† (μM)
Phenolphthalein β -glucuronide in the absence of albumin	50	0.17	—	26
Phenolphthalein β -glucuronide	57	0.11	—	14
Phenyl β -glucuronide	510	—	0.31	14
Phenyl β -galacturonide	6500	—	0.32	15
o-Nitrophenyl β -galacturonide	840	—	1.1	70

* In a boiled saccharate solution, one-third of the anion is present in the form of the inhibitory saccharo-1:4-lactone (Levy, 1952).

† Inhibition due to lactone formation in solution.

o-nitrophenyl β -galacturonide was employed as substrate. Both inhibitors acted competitively, and the results were analysed by the method of Lineweaver & Burk (1934).

One possible explanation of the anomalous results obtained with *o*-nitrophenyl β -galacturonide is that a different enzyme was responsible for the hydrolysis of this substrate. It seems most unlikely, however, that a β -glucuronidase preparation with a β -glucuronidase activity of 434 000 units/mg. of protein should also contain a highly active and specific *o*-nitrophenyl β -galacturonidase; under the conditions specified in Table 2, the preparation liberated 350 000 μ g. of *o*-nitrophenol/mg. of protein from 0.01 M β -galacturonide in 1 hr. at 38°.

A more probable explanation of this phenomenon is that the nitro group modifies the combination of substrate with enzyme. The same phenomenon was encountered with a partially purified limpet β -glucuronidase preparation (Marsh & Levy, 1958*a*), which gave the following values for the dissociation constants of the complexes formed with the enzyme: *o*-nitrophenyl β -galacturonide 1.2 mM, boiled saccharate solution 2.9 μ M, boiled mucate solution 61 μ M. The last two figures are ten times greater than those obtained with phenyl β -glucuronide or phenyl β -galacturonide as substrate (cf. Marsh & Levy, 1958*a*).

Inhibition by excess of substrate was not observed with any of the substrates except phenolphthalein β -glucuronide, with which it became apparent above 0.00063 M, particularly in the absence of albumin, as illustrated by a typical experiment in Fig. 4.

Fig. 4. Effect of varying the substrate concentration (S) on the rate of hydrolysis of phenolphthalein β -glucuronide at pH 4.5 in 0.05 M-acetate buffer by purified β -glucuronidase (specific activity 434 000, see Table 1) in the presence (O) and the absence (x) of 0.01 % of albumin. Rates are expressed as a percentage of the optimum in each case.

DISCUSSION

We consider that our figures for specific β -glucuronidase activity are directly comparable with those obtained by Bernfeld *et al.* (1953), despite their use of the more chromogenic chymotrypsin instead of albumin as standard protein (cf. Fig. 1), because their assays were done at pH 5 in the presence of DNA instead of pH 4.5 in the presence of albumin. Their stated optimum was at pH 4.5, and it is therefore probable that only two-thirds of the maximum enzyme activity was displayed under their conditions of assay (cf. Fig. 2). It appears that β -glucuronidase at high dilution does require the presence of other proteins to display its full activity, but DNA cannot be regarded as a satisfactory substitute since its effect is influenced by other tissue constituents and by pH.

Although we have obtained a specific activity of 455 000, as compared with the theoretical figure of 120 000 put forward by Bernfeld *et al.* (1953) for the pure enzyme from calf liver, we do not feel that we have necessarily approached the ultimate figure for the enzyme from preputial gland. Paucity of material precluded systematic application of the usual physical tests of homogeneity. On paper electrophoresis at pH 6.9 in phosphoric acid-NaOH buffer, ionic strength 0.05, protein migrated almost entirely as a single band when the specific activity was only of the order of 50 000, and this band stained uniformly for β -glucuronidase when sprayed with phenolphthalein β -glucuronide in the usual acetate buffer, and then with alkaline buffer after a short period of incubation.

Where more than one peak is observed in the pH-activity curve for β -glucuronidase, Smith & Mills (1953) consider on the basis of earlier ammonium sulphate fractionations that each peak is due to a distinct protein. An alternative explanation (Levy, 1953) is that the shape of the pH-activity curve, even when there is only one peak, is a function of the environment (including the nature and concentration of tissue constituents, buffer and substrate). In view of the different curves obtained for different substrates with the purified preputial-gland enzyme in the presence or the absence of DNA, we favour the latter explanation.

The differential action of DNA on the β -glucuronidase and β -galacturonidase activities of the enzyme suggests that the multiplicity of enzymes in the mammalian cell may sometimes perhaps be more apparent than real. Complex formation between an active protein and other tissue constituents could feasibly yield products of differing specificity in a simple fractionation procedure, in particular one based on centrifuging the enzyme in aqueous tissue suspensions.

β -Glucuronidase finds its major application at the present time in the hydrolysis of urinary steroid glucuronides, and where rats are freely available the female preputial gland would appear to be a useful source of the enzyme in a concentrated form. It has an activity, in phenolphthalein units/g. of fresh tissue, 100 times greater than rat liver and 20 times greater than the visceral hump of the limpet (*Patella vulgata*). The product obtained at stage 3 above is probably sufficiently pure for most purposes.

SUMMARY

1. β -Glucuronidase from the female-rat preputial gland has been purified 25-fold to give a final activity of 455 000 phenolphthalein units/mg. of protein on assay in the presence of albumin.

2. In agreement with Bernfeld *et al.* (1954), the specific activity fell progressively on dilution, and this effect was abolished by adding albumin.

3. The purified enzyme also hydrolysed β -galacturonides.

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The Thiol Groups of Normal Adult Human Haemoglobin

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The analysis of the thiol groups in normal adult human haemoglobin has been the subject of a number of recent papers (Ingram, 1955; Benesch, Lardy & Benesch, 1955; Hommes, Santema-Drinkwaard & Huisman, 1956; Murayama, 1956). These workers all used the method of amperometric titration with silver nitrate (Kolthoff & Harris, 1946; Benesch & Benesch, 1948). They found that, in general, there were four reactive thiol groups/molecule of native protein and eight in the denatured protein. On the other hand, Ingbar & Kass (1951), using the same method but with sodium chloride in the supporting electrolyte, found two thiol groups/molecule of native protein. The same result was obtained by Hughes (1950) with methylmercuric iodide.

The reason for the discrepancy between the different findings is suggested by some of Ingram's (1955) results. As well as titration with silver

nitrate, he carried out amperometric titrations with mercuric chloride and a rotating platinum indicator electrode (Kolthoff, Stricks & Morren, 1954). Kolthoff and his colleagues found that, under the conditions specified, the endpoint of the mercuric chloride titration occurs when the ratio of mercury to thiol is 1:1. With simple thiols such as cysteine and glutathione this corresponds to the compound $(RS)_2Hg_2$, where one mercury atom is firmly bound as the dimercaptide $(RS)_2Hg$ and the other is less firmly bound, probably by carboxyl. With proteins steric considerations may make it difficult for two thiols to react with one mercury atom and a compound of the type $Prot \cdot S \cdot HgCl$ is formed. Thus Edelbach, Katchalsky, Maybury, Hughes & Edsall (1953) showed that mercaptalbumin ($Alb \cdot SH$) reacts rapidly with mercuric chloride:

A slow reaction follows with excess of protein:

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