The β-Glucuronidase of the Roman Snail (*Helix pomatia*)

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β-Glucuronidase has been found in the crop fluid of locusts (Robinson, Smith & Williams, 1953) and in the digestive gland of certain marine molluscs (Dodgson, Lewis & Spencer, 1953). The enzyme has also been reported in the digestive gland of *Helix laeda* Gould by Utusi, Huzi, Matumoto & Nagaoka (1949), but its properties were not given in detail. The enzyme used by these authors was prepared from a 'stock porridge' 3 years old.

The work reported in the present paper shows that the digestive gland of the Roman snail possesses a high β-glucuronidase activity; the other species of snails examined showed less activity. The Roman snail is a convenient object for study because of its large size, and the digestive gland, which weighs about 1·5 g., can be rapidly and easily dissected. As far as present observations go, only the digestive gland and the crop contain appreciable amounts of the enzyme. This work has been carried out in conjunction with histochemical studies on the digestive gland (Billett & McGee-Russell, unpublished observations), in an attempt to establish the localization of the enzyme.

Materials

Animals. Adult snails weighing about 40 g. were used. The snails had been kept in captivity for several months; under these conditions their food consisted mainly of cabbage. The snails were originally obtained from a colony which lives on the Cotswold Hills in the vicinity of Cheltenham.

Digestive gland. After the snail had been killed by decapitation, the digestive gland was carefully dissected out of the visceral mass. Care was taken to ensure that the gland was not contaminated with intestine and ovo-testis which are closely apposed to it.

Crop fluid. A ligature was tied round the crop behind the buccal mass. The crop was severed in front of the ligature and dissected out back towards the digestive gland. Another ligature was tied near the gland and the crop severed behind this. The crop with its contained fluid was removed to a watch glass. Puncture of the isolated crop gave 1–2 ml. of fluid. The fluid varied in appearance, but was usually a brown, rather viscous liquid. Sometimes the fluid contained undigested pieces of food; these were removed by centrifuging. Microscopic examination of the fluid failed to reveal the presence of micro-organisms. The fluid was diluted 100-fold with distilled water and assayed directly for glucuronidase.

Analytical methods

Robinson *et al.* (1953) used quinolyl-8-glucuronide as the substrate in their method for assaying the β-glucuronidase activity of locust-crop fluid, and the amount of 8-hydroxyquinoline liberated was estimated colorimetrically after coupling with diazotized di-*o*-anisidine. In the present work the same substrate was used but the 8-hydroxyquinoline formed was coupled with diazotized sulphanilic acid for purpose of estimation.

Quinolyl-8-glucuronide. This was prepared according to the method of Robinson *et al.* (1953).

Standardization of method. A standard solution of 8-hydroxyquinoline was prepared by dissolving a known weight in water made slightly alkaline with NaOH. Samples (5 ml.) of standard solution, containing 100–500 μg. of 8-hydroxyquinoline, were measured out and to each was added 1 ml. of freshly prepared diazotized sulphanilic acid in the form of Ehrlich's diazo reagent (Hawk, Oser & Summerson, 1947), followed by 2 ml. of 5N-NaOH. The diazotization was carried out at approximately 5°. After addition of the alkali the mixtures were diluted to 100 ml. with water and kept for 30 min. at room temperature to allow maximum colour development (the colour was stable for at least 24 hr. if kept in the dark). The extinction coefficients were measured at 465 mμ. in a Unicam SP. 350 Spectrophotometer (Unicam Instruments (Cambridge) Ltd.). There was a linear relationship between the extinction coefficient and amount of 8-hydroxyquinoline.

Measurement of enzyme activity. The solutions to be assayed were warmed to 25° and 1 ml. samples mixed with 1 ml. of 0·005M quinolyl-8-glucuronide in M acetate buffer, pH 4·2, and 1 ml. water. Control tubes were set up in which 1 ml. 0·001M potassium hydrogen saccharate (prepared according to Levvy, 1952) replaced the water; the saccharate solution completely inhibited the enzyme. The mixtures were incubated at 25°. After a suitable time interval, which varied between 10 min. and 2 hr., the tubes were plunged into ice-water and 1 ml. 20% (w/v) trichloroacetic acid was added to each. The mixtures were centrifuged at 1000 *g* for 10 min. and the supernatants decanted off. The residues were suspended in 3 ml. distilled water and centrifuged as above. The 8-hydroxyquinoline was estimated in the combined supernatants and washings. The colours produced required diluting twice to tenfold before a suitable reading could be obtained on the spectrophotometer. Negligible amounts of colour were produced in the controls.

Preparation of the digestive-gland extract. After being weighed, the gland was homogenized in distilled water using an all-glass homogenizer (Potter & Elvehjem, 1936). The preparation so obtained was diluted to a 1% (w/v) suspension of the tissue and sufficient toluene was added to

saturate the mixture, which was placed in a refrigerator overnight (12–16 hr.) and then centrifuged at about 1000 *g* for 10 min. After the supernatant had been decanted off, the residue was suspended in the original volume of distilled water and centrifuged under the same conditions as before. The combined extracts appeared to contain all the enzyme. The residue showed no appreciable activity. Most of the enzyme appears to be extracted within 2 hr. of homogenizing. Extractions carried out for longer than 24 hr. showed a diminished activity.

No reaction was apparent in the absence of substrate. A slight hydrolysis of the substrate occurred when it was incubated under the conditions of the assay in the absence of the enzyme preparation. Glucuronic acid in amounts equivalent to those formed by the enzymic hydrolysis of the substrate gave no colour with the diazo reagent. Complete recovery of added 8-hydroxyquinoline was obtained under the conditions of the assay.

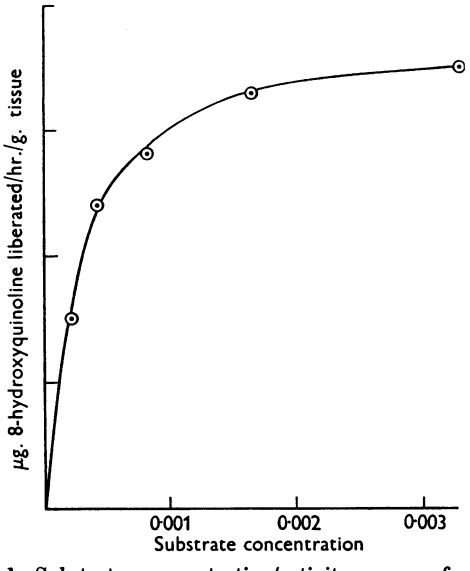

Fig. 1. Substrate concentration/activity curve for β-glucuronidase of *H. pomatia*. The substrate was quinolyl-8-glucuronide in 0·33 M acetate buffer, pH 4·2; temp. 25°.

After it had been established that the reaction rate was approximately linear for the first hour a series of determinations were made using an incubation period of 40 min.

RESULTS

Optimum substrate concentration. The activity of the digestive-gland extracts was measured at substrate concentrations ranging from 4×10^{-4} to 3×10^{-3} M, at pH 4·2. The result is shown in Fig. 1 and indicates an optimum substrate concentration in the region of 0·002 to 0·003 M. The solubility of quinolyl-8-glucuronide in M acetate buffer, pH 4·2, is between 0·005 and 0·01 M at about 18°. Because of this low solubility a slightly sub-optimum substrate concentration was used in the main determinations.

Optimum pH. Determinations were carried out as already described except that the pH of the acetate buffer was varied from 3·7 to 5·5. The pH/activity curve is shown in Fig. 2. The optimum pH appears to be in the region of 4·2.

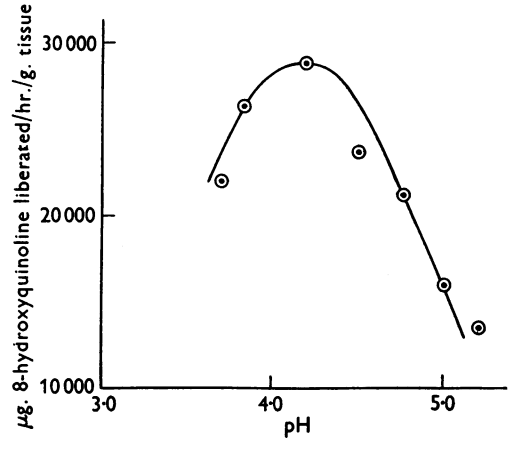

Fig. 2. pH/activity curve for *H. pomatia* β-glucuronidase. The substrate was 0·0017 M quinolyl-8-glucuronide in 0·33 M acetate buffer; temp. 25°.

Table 1. *β-Glucuronidase activity of mollusc extracts at* 25°

The substrate was 0·0018 M quinolyl-8-glucuronide in M acetate buffer, pH 4·2. The activity is expressed in terms of μg. of 8-hydroxyquinoline/hr./g. or ml.

Species	Tissue	Activity		No. of determinations
		Mean	Range	
Helix pomatia	Digestive gland	30 900	18 900–45 000	14
	Crop fluid	12 200	5 400–16 500	6
	Kidney	400	0–900	4
	Albumen gland	1 600	1 170–2 100	4
	Salivary gland	700	0–1 200	4
	Mantle ridge	50	0–200	4
	Foot	400	0–1 200	4
	Ovo-testis	2 500	700–7 200	4
H. aspersa	Digestive gland	13 500	8 600–17 000	6
Limnea stagnalis	Digestive gland	500	0–1 800	6
Anodonta cygnea	Digestive gland	4 800	4 400–5 600	6
Planorbis corneus	Digestive gland	4 300	2 800–5 400	6

Activity of the digestive-gland extracts and crop fluid. After optimum conditions of substrate concentration and pH had been established, a series of consecutive determinations were made on the enzyme preparations. Each preparation was obtained from a separate snail. The results are given in Table 1. The activities are expressed as μg. of 8-hydroxyquinoline liberated/g. of tissue/hr. in the case of the digestive gland and as μg. of 8-hydroxyquinoline/ml./hr. in the case of the crop fluid.

Distribution of β-glucuronidase in organs of H. pomatia *and other molluscs.* Included in Table 1 are the results of determinations on a number of organs of *H. pomatia* besides the digestive gland. The activities of the digestive glands of the garden snail, two fresh-water gastropods and the swan mussel were also determined in the same way and under the same conditions as those described for the digestive gland of *H. pomatia*.

DISCUSSION

The results show that the digestive glands of *H. pomatia* and *H. aspersa* contain appreciable amounts of β-glucuronidase. The glucuronidase activity of these terrestrial species is significantly higher than that of certain fresh-water species. In the Roman snail, high enzyme activity is confined to the digestive gland. The albumen gland appears to possess a slight activity. No significance can be attached to the activity associated with the ovotestis because it is difficult to dissect this organ free of the investing digestive gland.

The digestive gland of the Roman snail appears to be one of the richest sources of glucuronidase so far discovered. The activity is comparable with that of locust-crop fluid (Robinson *et al.* 1953) and of the digestive gland of marine molluscs (Dodgson *et al.* 1953); it is probably greater. A direct comparison is not possible because of the different conditions of assay used by the various authors. In particular it must be noted that, in the experiments described in this paper, incubation was at 25°, which is nearer to that normally obtaining in the animal than is 37°; at the latter temperature the activity of the extracts is more than doubled.

The pH optimum of 4·2 found for *H. pomatia* glucuronidase is to be compared with the value 4·5, found by Robinson *et al.* (1953) for locust-crop fluid using the same substrate. Dodgson *et al.* (1953) found an optimum pH of 4·0 for the glucuronidase of marine molluscs using *p*-chlorophenylglucuronide as substrate. These pH data suggest a fairly close relationship for the enzymes from invertebrate sources. The present work has not revealed a second optimum pH which appears to be a characteristic of the enzyme isolated from certain vertebrate tissues (Mills, 1948; Kerr, Campbell & Levvy, 1949). Utusi *et al.* (1949) investigated the action of glucuronidase from the digestive gland of *H. laeda* on 0·025M β-menthol glucuronide in citrate buffer, pH 4·5. About 30% of the substrate was hydrolysed after incubating for 1 hr. at 37°. Optimum conditions do not appear to have been determined.

The function of the enzyme in molluscs is probably similar to that in locusts and in the micro-organisms which exist in the gut of ruminants (Marsh, Alexander & Levvy, 1952). As suggested by the various authors concerned, the enzyme probably acts on a substance containing a β-glucuronide link which normally exists in the food of these herbivorous animals. This substance may be hemicellulose (Marsh *et al.* 1952). The glucuronidase of the snail may be considered to be a component of 'cytase', a mixture of enzymes concerned with the breakdown of cellulose and related substances (Faberge, 1945; Holden, Pirie & Tracy, 1950; Holden & Tracy, 1950).

Histochemical investigations (Billett & McGee-Russell, unpublished observations) indicate that the enzyme is localized in particular cells in the digestive gland of the snail. These investigations suggest that the tissue itself, and not micro-organisms which may reside in the crop and digestive gland, is responsible for the production of the enzyme which is found in the crop fluid.

SUMMARY

1. The digestive gland and the crop of the Roman snail, *Helix pomatia*, appear to contain large amounts of β-glucuronidase. This enzyme has an optimum pH of 4·2 when acting on quinolyl-8-glucuronide as a substrate.

2. The 8-hydroxyquinoline formed by the enzymic hydrolysis of the substrate was estimated by coupling it with diazotized sulphanilic acid. The concentration of the azo dye produced was measured spectrophotometrically.

3. The β-glucuronidase of the Roman snail is related to, and probably identical with, the enzyme known to exist in the digestive gland of certain marine molluscs and the crop fluid of locusts.

4. The digestive glands of *Limnea stagnalis*, *Planorbis corneus* and *Anodonta cygnea* contain only small amounts of glucuronidase compared with *Helix pomatia* and *H. aspersa*.

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β-Amylolysis: Union of Enzyme and Substrate

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It is well known that β-amylase liberates maltose from starch and its components in the β form (Kuhn, 1925; Freeman & Hopkins, 1936). This was established by observing the mutarotation of the fission products and establishing that it was the maltose itself which was undergoing this change. In one type of experiment the optical rotation of the β-amylase/starch reaction mixture is read continuously during the reaction and samples are withdrawn from time to time and treated with alkali to stop enzyme action and hasten mutarotation. The rotations of the former series are lower than the corresponding ones of the latter (corrected for dilution) because β-maltose ($[\alpha]_D + 118°$) has yet to change into αβ-maltose ($[\alpha]_D + 137°$).

In repeating some experiments of this kind and taking direct readings of optical rotation of the reaction mixture very soon after mixing the enzyme and substrate it was observed that the rotation was higher, not lower, than that of the corresponding mutarotated solution. After the reaction had proceeded further this position was reversed and subsequent readings conformed to those previously reported by the above authors. The phenomenon was confirmed with starch, amylose and a short-chain amylose dextrin and was considered to be well outside the range of experimental error. The simplest explanation is that an enzyme-substrate compound is formed, the optical rotation of which (dextrorotatory) is greater than that of the substrate.

MATERIALS AND METHODS

Substrates. Soluble starch was prepared from potato starch by the method of Hanes & Cattle (1938), $[\alpha]_D^{20} + 198°$ in water; amylose from potato starch by precipitation with thymol followed by repeated recrystallizations with butanol as described by Hopkins & Jelinek (1948). Achroic amylose dextrin was prepared by hydrolysing amylose with bacterial α-amylase (prepared from *B. subtilis*) to the achroic stage and its separation from the lower-molecular hydrolytic products in a charcoal-Celite column as described by Bailey, Whelan & Peat (1950). The lower fractions eluted by ethanol up to 25% (v/v) were separated and the remainder eluted with ethanol (30%, v/v, and higher) but not further fractionated, $[\alpha]_D^{20} + 186°$ in water.

Enzymes. We are indebted to the Wallerstein Laboratories, New York, for a preparation of β-amylase of high purity from ungerminated barley, and to Dr A. K. Balls for crystalline sweet potato β-amylase (4 times recrystallized).

Reactions were performed as follows. The substrate and enzyme were prepared in solution, and after standing for a period of 1 hr. at the temperature at which the reaction was to be carried out, the optical rotation of each was measured in the polarimeter at that temperature. Our soluble starch fell in $[\alpha]_D$ by about 0·6 angular degree for each degree (° C) rise in temperature. In the case of amylose special precautions were necessary, such as reading immediately after filling the polarimeter tube (see Results). The reaction mixtures contained no added buffers, the pH being about 6.

On mixing suitable volumes of amylase (5 ml.) and substrate (100 ml.), after a short vigorous shaking a portion was transferred to a jacketed polarimeter tube and the first reading obtained as quickly as possible, and later readings at intervals. These are represented graphically in Figs. 1–3 and are described there as 'continuous' curves. The remainder of the reaction mixture was kept in a flask in a water bath at the same temperature. Samples (10 ml.) were withdrawn at intervals and rapidly run into 1 ml. of 0·2 N-NaOH. After about 30 min. the optical rotation was measured at the reaction temperature. The results (corrected for dilution) are shown in the figures as 'withdrawals' curves. Such small fall in α_D of starch or other substrate or maltose as was caused by the alkali (after correcting for dilution) was allowed for, control experiments having been performed. Portions of the final reaction mixture, after amylolysis had ceased, treated with equal volumes of NaOH and