Studies in Detoxication

45. β-GLUCURONIDASE AND ARYLSULPHATASE IN THE CROP FLUID OF LOCUSTS

BY D. ROBINSON, J. N. SMITH AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received* 7 *May* 1952)

Studies in progress in this laboratory on the detoxication mechanisms of locusts involved an investigation of the occurrence of the enzymes β-glucuronidase and arylsulphatase in these insects. It was soon discovered that a β-glucuronide-decomposing enzyme and an arylsulphatase occurred in the crop liquor of these insects to an appreciable extent.

For the estimation of the β-glucuronidase activity in the crop liquor the easily prepared quinolyl-8-glucuronide was found to be a convenient substrate, whilst arylsulphatase could readily be estimated by means of potassium 2-hydroxy-5-nitrophenylsulphate as suggested by Robinson, Smith, Spencer & Williams (1952).

EXPERIMENTAL

Preparation of locust crop fluid and the enzyme solution

Adult locusts of two species (*Locusta migratoria migratorioides* and *Schistocerca gregaria*) were provided by the Anti-Locust Research Centre, British Museum (Natural History) and were kept on a diet of grass at 35–40° and relative humidity of 70%. The crop fluid of the insects was obtained by picking them up by the thorax and placing the end of a blood pipette to their mouths. On handling in this way, the insects become excited and expel a large drop of clear brownish fluid which then runs into the pipette (for discussion of the production of this drop of fluid see Chauvin, 1941). Volumes up to 0·04 ml. (average 0·02 ml.) could be obtained from each insect during a single manipulation. After a meal of grass the crop liquor is often greenish in colour, cloudy and more dilute than the brown liquor.

For estimation on individual insects the crop fluid was used without dilution, whereas with the pooled fluid from several insects it was diluted with water as needed. Three specimens of pooled crop fluid, on drying at 110°, were shown to contain 10·26, 10·57 and 10·3% of solid matter. The nitrogen content (Kjeldahl) of pooled material was 8·75 mg. N/ml. (two estimations).

Determination of β-glucuronidase activity using p*-chlorophenylglucuronide as substrate*

In the initial stages of the investigation β-glucuronidase activity was assayed by the method of Spencer & Williams (1951). The crop fluid was diluted with water, usually about 200 times. It was found that the enzyme solutions contained material which absorbed ultraviolet light of wavelength less than 280 mμ., so that it was not always possible to use the 245 mμ. band of the *p*-chlorophenol anion. Estimations were therefore carried out using the 298 mμ. band of this anion. The use of this wavelength was also advantageous because of the high glucuronidase activity of the crop fluid.

Table 1. *β-Glucuronidase activity of brown crop fluid of individual locusts*

(Activity estimated at pH 5·2 with *p*-chlorophenylglucuronide.)

Locust species	Phenol liberated (μg./hr./ml. crop fluid)
Locusta migratoria	3600, 11350, 14000, 14500, 18150
Schistocerca gregaria	9650, 18400, 23500, 30500

All measurements were made at pH 5·2 which, as we found later, was not the optimum pH of the locust enzyme. The results obtained with five specimens of *Locusta migratoria* and four of *Schistocerca gregaria* are given in Table 1 which shows that the crop fluid of these insects may contain from 10 000 to 30 000 chlorophenyl units/ml. of β-glucuronidase at pH 5·2, or about 200–300 units from each drop (0·02 ml.) of fluid obtained by a single manipulation of one of the insects.

Determination of β-glucuronidase with quinolyl-8-glucuronide

Preparation of quinolyl-8-glucuronide. This compound, and some of its metallic salts, was prepared by Brahm (1899), but no rotation or full analysis was quoted for the glucuronide itself. Our method of preparation, however, is new and relatively simple. A total of 18 g. of 8-hydroxyquinoline were fed by stomach tube, in water, to six rabbits. The 24 hr. urine (750 ml.), after filtration through glass wool, was brought to pH 4 with glacial acetic acid (about 3 ml.) and left at 0° for 2 days. The β-8-quinolylglucuronide separated slowly as large greenish crystals (12 g.) which were filtered and then recrystallized several times (charcoal) from hot water. The second 24 hr. urine, by the same procedure, yielded a further 1 g. of crude glucuronide. The *trihydrate* formed pale yellow-green prisms of m.p. 153° (decomp.); $[\alpha]_D^{20°} - 75° \pm 2·5°$ in water (*c*, 0·1). (Found: C, 47·4; H, 5·6; N, 3·7. $C_{15}H_{15}O_7N.3H_2O$ requires C, 48·0; H, 5·6; N, 3·7%.) It was sparingly soluble in water, ethanol and ether, but more soluble in boiling water.

*The colour reaction between 8-hydroxyquinoline and diazotized di-*o*-anisidine*

Conditions of colour development. When a solution of Brentamine Fast Blue B salt (referred to later as BFB) (Imperial Chemical Industries, Ltd.) is added to 8-hydroxy-

quinoline a cherry-red colour develops which is proportional to the amount of phenol used. Quinolyl-8-glucuronide gives no colour with BFB. The colour obtained is stable for 20 min. after reaching its maximum, between the pH limits of 4 and 6·5. Above pH 6·5 the colour tends to precipitate, whereas below pH 4 it develops extremely slowly. The limits of stability of the colour are therefore within the pH range of maximum activity of β-glucuronidase. The solution of BFB was therefore made up in a solution of 0·01 M-potassium acid saccharate in 0·3 M-acetate buffer at pH 4·5, the saccharate serving to inhibit the β-glucuronidase, and the acetate to provide a pH at which the colour has a maximum stability. A 0·1 % solution of BFB was found to be the most suitable and economical. Using these solutions the colour attained its maximum in 5 min. at 37°, or in 15 min. at room temperature.

The standard curve was prepared as follows. A solution of 8-hydroxyquinoline (3 ml., 4–30 μg.) in 0·3 M-acetate buffer at pH 4·5 was treated with 1 ml. of 0·1 % BFB. The solution was allowed to stand 15 min. at room temperature and then the colour measured in the Spekker absorptiometer using an Ilford no. 603 blue-green filter and 0·5 ml. cells. The standard curve obtained was a straight line.

Method of assay

Enzyme solution. In most cases, 0·1 ml. of crop fluid diluted to 200 ml. with distilled water contained enough enzyme for incubation periods up to 1 hr.

Substrate solution. Quinolyl-8-glucuronide is very sparingly soluble in water (about 0·003 M), but fortunately the optimum substrate concentration (see below) is about 0·0003 M and a 0·001 M solution of the glucuronide is easily obtained. The stock substrate solution was therefore a 0·001 M solution of the glucuronide in water. This solution does not deteriorate in 4–5 days.

BFB solution. This consisted of a 0·1 % solution of BFB in a solution of 0·01 M-potassium acid saccharate in 0·3 M-acetate buffer of pH 4·5. This solution has to be freshly made just before use since it deteriorates in a few hours.

The final method of assay of β-glucuronidase in the crop fluid was therefore as follows. To each of two tubes was added 1 ml. of 0·001 M-substrate solution, 1 ml. M-acetate buffer of pH 4·5 and 1 ml. of enzyme solution. These tubes were then incubated at 37°, together with a control tube containing 1 ml. substrate and 1 ml. acetate buffer. After the desired period of incubation (usually 1 hr.), 1 ml. of the BFB solution was added to all three tubes and 1 ml. of the enzyme solution to the control tube. After 5 min. (at 37°) the colour of the test solutions was measured in the Spekker absorptiometer (filter, Ilford no. 603 and 0·5 ml. cells) with the control solution in the blank cell of the instrument. The amount of 8-hydroxyquinoline liberated was then read off from the standard curve (see Tables 2 and 3 for results). This method was not applicable to the assay of β-glucuronidase of animal tissues (e.g. rat liver) since the tissue extracts interfered considerably with the development of colour.

Table 2. *β-Glucuronidase content of fluids of* Locusta migratoria

(Activity estimated with quinolyl-8-glucuronide at pH 4·5.)

Fluid	8-Hydroxyquinoline liberated (μg./hr./ml.)
A pooled sample of brown crop fluid	64000
Green crop fluid from individual insects	3850, 5500, 8000, 9700, 14000
Pooled crop fluid from 20 hoppers (5th instar)	4200
Pooled haemolymph* from five adults	300
Ecdysial fluid†	<200 (5 specimens)

* To obtain haemolymph, the insect was punctured in the thorax and gently squeezed, and the drop of pale yellow-green fluid (about 0·05 ml.) which appeared was collected in a blood pipette.

† When the insect is about to moult it hangs upside down and moves its abdomen. When found in this position, one section of the abdomen was removed with a sharp scalpel and then the old skin came away easily. The unbroken new skin could be seen underneath. The ecdysial fluid is found between the old and new skins and resembles haemolymph. It was collected by means of a blood pipette and as much as 0·2 ml. could be obtained from one locust.

Optimum pH

For the pooled enzyme solution this was determined as above, except that the pH of the acetate buffer was varied from pH 3·8 to 5·7. A single optimum was found at pH 4·5 (see Fig. 1). Under standard conditions the amount of 8-hydroxyquinoline liberated by a constant amount of a given specimen of crop liquor was found to be proportional to the time of incubation, up to 4 hr. The optimum substrate concentration was determined at pH 4·5 and 37° as above, using

Table 3. *β-Glucuronidase activity of locust* (Locusta migratoria) *crop fluid at different stages of development*

Age of locust (days)	Stage of development (instar)	Activity of crop fluid of individual insects at pH 4·5 (μg. 8-hydroxyquinoline liberated/hr./ml.) Average	Range	No. of individual insects tested
6	1st or 2nd?	5 200	590–12 200	9
8		18 200	9 700–24 000	6
13	3rd	6 890	3 150–10 600	5
17		11 400	8 300–14 800	6
21		14 300	9 700–16 000	6
23	4th	7 020	2 500–12 000	5
26		16 300	14 800–18 000	6
28	5th	16 300	13 000–17 750	8
34		20 560	16 000–24 000	5

substrate concentrations varying from 0·1 to 10 × 10^{-4} M and incubating for 1 hr. Maximum activity under these conditions was attained at a concentration of 3 × 10^{-4} M. Beyond this value there was no increase in activity and no inhibition by excess substrate.

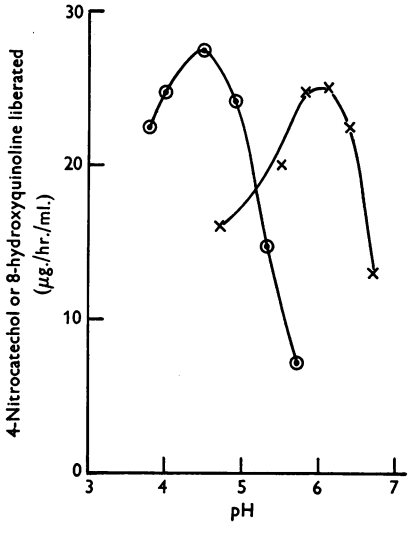

Fig. 1. pH-Activity curves for the β-glucuronidase (⊙—⊙) and arylsulphatase (×—×) of crude locust crop fluid (*Locusta migratoria*).

Inhibition by saccharate

D-Glucosaccharic acid has been shown to be a specific inhibitor of β-glucuronidase from mammalian tissues (Karunairatnam & Levvy, 1949). The enzyme of locust crop fluid is also inhibited by this substance. To study this effect potassium acid saccharate was incorporated in the acetate buffer. The procedure described above was used. A 50% inhibition of the enzyme acting on 0·0003 M-quinolyl-8-glucuronide at pH 4·5 and 37° was noted when the saccharate concentration reached 0·0004 M, whilst complete inhibition was observed at 0·01 M. These results are similar to those found by Karunairatnam & Levvy (1951) for mouse-liver β-glucuronidase which is completely inhibited by 0·01 M-saccharate when acting on 0·00125 M-phenolphthalein glucuronide at pH 4·5 in acetate buffer. This suggests that the locust enzyme is a β-glucuronidase. (Recent work by Levvy (1952) suggests that the inhibition by saccharate solutions is probably due to the presence of saccharo-1:4-lactone. To ensure the presence of this lactone in saccharate solutions they should be boiled before use.)

Detection of glucuronic acid. Karunairatnam & Levvy (1951) have pointed out that the detection of a phenol as a result of the action of an enzyme preparation supposed to contain β-glucuronidase on a phenolic glucuronide is not sufficient to characterize the enzyme as a β-glucuronidase. The detection of the glucuronic acid is also necessary (cf. Levvy, 1948). Saturated aqueous solutions (1 ml.) of several glucuronides were therefore incubated at 37° for 18 hr. with 0·2 ml. of pooled locust crop liquor, and 0·2 ml. of each solution was then separated chromatographically on paper in *n*-butanol:acetic acid:water (cf. Partridge, 1948), along with authentic samples of D-xylose, glucuronic acid and glucurone. Quinolyl-8-glucuronide, *o*- and *m*-aminophenylglucuronides and *p*-iodophenylglucuronide were used and in each case glucuronic acid, but not glucurone or xylose, was demonstrated using a naphthoresorcinol spray (Partridge, 1948). Free *o*- and *m*-aminophenols were revealed by the salicylaldehyde reagent (Robinson, Smith & Williams, 1951*a*) and 8-hydroxyquinoline by its fluorescence in ultraviolet light. Crop liquor, at the concentration used, gave no interfering spots.

These results, therefore, show that locust crop liquor has an enzyme with the properties of a true β-glucuronidase.

Arylsulphatase activity

The arylsulphatase activity of locust crop liquor was determined by one of the methods of Robinson *et al.* (1952). This method depends on the release from potassium 2-hydroxy-5-nitrophenylsulphate of 4-nitrocatechol which gives a red colour with alkali.

It was assumed that the optimum substrate concentration of the crop liquor sulphatase would be similar to that of takadiastase, i.e. 0·0025 M (Robinson *et al.* 1952), and the enzyme solution used was prepared by diluting locust crop liquor roughly 25 to 50 times.

With individual locusts a measured volume (approx. 0·02 ml.) of the crop fluid was added to 2 ml. of buffer-substrate solution (0·0025 M-potassium 2-hydroxy-5-nitrophenylsulphate in 0·5 M-acetate buffer of pH 5·9). This solution and a control buffer-substrate solution (2 ml.) were incubated at 37° for 1 hr.

The results (Table 4) show that locust crop liquor contains appreciable amounts of arylsulphatase (approx. 1000 4-nitrocatechol units/ml. or 100–120 units/mg. N) comparable with the takadiastase (800–900 units/g. or 200 units/mg. N) used in earlier experiments (Robinson *et al.* 1952).

Table 4. *The arylsulphatase activity of the crop fluid and haemolymph of locusts*

Animal	Source	4-Nitrocatechol liberated (μg./ml./hr.)
Locusts		
Locusta migratoria	Brown crop fluid	850, 900, 960, 1000, 1035*
	Haemolymph	67·5, 137, 187, 165*
Schistocerca gregaria	Brown crop fluid	1300, 1130*
Humans	Saliva (5 specimens)	0
Goat	Saliva (1 specimen)	0
Frog hoppers	'Cuckoo spit'	0

* Specimens from different insects.

The haemolymph of these insects was also tested and was found to possess about one-tenth of the activity of the crop liquor. Human and goat saliva contained no arylsulphatase activity nor did the froth ('cuckoo spit') of frog hoppers (see Table 4).

Optimum pH. For the locust enzyme this was determined essentially as above, varying the pH of the buffer. It was found to be in the region of pH 6 (see Fig. 1), and is thus similar to that of takadiastase (Robinson *et al.* 1952).

Inhibitors of locust arylsulphatase

Some of the inhibitors which inhibited the arylsulphatase activity of takadiastase (Robinson *et al.* 1952) were also tested against the locust enzyme. For this purpose 2 ml. of 0·04 M inhibitor solution in M-acetate buffer at pH 5·9 were incubated with 1 ml. of 0·1 M substrate solution in M-acetate buffer at pH 5·9 and 1 ml. of the diluted crop fluid, together with the appropriate control without the enzyme. The colour was developed by adding 1 ml. of 10% (w/v) NaOH to the test solutions and 1 ml. each of NaOH and of enzyme solution to the control. The inhibition found was very similar in extent to that of takadiastase (see Robinson *et al.* 1952). With 0·02 M-K_2SO_3, NH_2OH, HCl and KCN, the inhibition was 87, 80 and 100% respectively. Dzialoszynski (1947) has reported that urea inhibits the arylsulphatase of 'Clarase'. We also tested urea against the locust enzyme and found that a 0·02 M solution caused a 24% inhibition of the enzyme.

An attempt has been made to apply the above method to partly purified extracts of animal tissues (Robinson, Smith & Williams, 1951*b*). Dr Roy of Edinburgh University (in a private communication) has carefully applied the method to tissues and informs us that the method will probably not be of any value for the assay of animal sulphatase, as fairly highly purified preparations must be used to ensure quantitative recovery of the liberated nitrocatechol, and also the kinetics of the reaction appear to be complex. He has come to the conclusion that the animal and mould enzymes have very different properties (see Roy, 1953).

DISCUSSION

The crop liquor of locusts has been shown to contain a number of enzymes which are obviously related to digestion, for Chauvin (1941) reports that it contains a dextrinase, an amylase and a sucrase (see also Uvarov, 1948). It is not clear whether the sulphatase and glucuronidase we have found in crop liquor is secreted by the locust or is of microbiological origin. Dr A. E. Oxford has examined the crop fluid for us and found no large Protozoa, but some medium-sized micro-organisms more or less similar to those found, by Karunairatnam & Levvy (1951), to carry most of the glucuronide-decomposing activity of sheep rumen contents. This may be associated with the digestion of polysaccharides containing β-glucuronoside links as has been suggested by Marsh, Alexander & Levvy (1952) for a number of domestic animals, and the relatively high activity may be correlated with the reported high rate of digestion in locusts (Uvarov, 1948). A digestive role for these enzymes seems to be supported by the slight activity of haemolymph and ecdysial fluid (Table 2) and by the observation that the glucuronidase activity of crop fluid tends to be less at the beginning of an instar (Table 3) when the insects have not been feeding. Normally, the locust stops feeding on the day before moulting and does not feed readily again till the day after.

It is interesting to note that the locust enzyme has the same optimum pH (4·5) with quinolyl-8-glucuronide as Sarkar & Sumner (1950) and Talalay, Fishman & Huggins (1946) have found for glucuronidase preparations of animal origin acting on phenolphthalein glucuronide.

The pH optimum of locust arylsulphatase, about pH 6, was also similar to that found in takadiastase (Robinson *et al.* 1952) and rat liver (Robinson *et al.* 1951*b*), but there appears at the moment to be no obvious function for this enzyme in locusts.

SUMMARY

1. The crop fluid of two species of locusts has been proved to contain appreciable amounts of β-glucuronidase and an arylsulphatase.

2. The β-glucuronidase was estimated with quinolyl-8-glucuronide as substrate, the liberated 8-hydroxyquinoline being estimated by coupling under specified conditions with Brentamine Fast Blue B salt (diazotized *o*-anisidine).

3. The β-glucuronidase of the crude crop liquor showed a single pH optimum at 4·5 and was present in all stages of development from the first instar to the full adult.

4. The formation of glucuronic acid by the action of the crop liquor on a number of β-glucuronides was proved by paper chromatography.

5. Arylsulphatase was detected by the release of 4-nitrocatechol from potassium 2-hydroxy-5-nitrophenylsulphate. With this substrate the enzyme showed an optimum at about pH 6.

6. Arylsulphatase activity was not found in human or goat salivas.

7. The results are discussed and the suggestion made that β-glucuronidase may be connected with digestion in locusts.

We are grateful to the Anti-Locust Research Centre (British Museum) for supplies of locusts, to Imperial Chemical Industries, Ltd., for a gift of Brentamine Fast Blue B salt, to Prof. E. C. Amoroso for a sample of goat saliva, to Dr A. E. Oxford for microscopic examination of locust crop liquor and to Dr G. A. Levvy for information prior to publication.

REFERENCES

Brahm, C. (1899). *Hoppe-Seyl. Z.* **28**, 439.
Chauvin, R. (1941). *Ann. Soc. ent. Fr.* **110**, 147.
Dzialoszynski, L. M. (1947). *Nature, Lond.*, **160**, 464.
Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
Karunairatnam, M. C. & Levvy, G. A. (1951). *Biochem. J.* **49**, 210.
Levvy, G. A. (1948). *Biochem. J.* **42**, 2.
Levvy, G. A. (1952). *Biochem. J.* **50**, xv.
Marsh, C. A., Alexander, F. & Levvy, G. A. (1952). *Nature, Lond.*, **170**, 163.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Robinson, D., Smith, J. N., Spencer, B. & Williams, R. T. (1952). *Biochem. J.* **51**, 202.
Robinson, D., Smith, J. N. & Williams, R. T. (1951*a*). *Biochem. J.* **50**, 228.
Robinson, D., Smith, J. N. & Williams, R. T. (1951*b*). *Biochem. J.* **49**, lxxiv.
Roy, A. B. (1953). *Biochem. J.* **53**, 12.
Sarkar, N. K. & Sumner, J. B. (1950). *Arch. Biochem.* **27**, 453.
Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
Uvarov, B. P. (1948). *Anti-Locust Bull.* **1**, 11.

Studies in Detoxication

46. THE METABOLISM OF ALIPHATIC ALCOHOLS. THE GLUCURONIC ACID CONJUGATION OF ACYCLIC ALIPHATIC ALCOHOLS

BY I. A. KAMIL, J. N. SMITH AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received* 7 *May* 1952)

A large number of aliphatic alcohols are used industrially as solvents and as starting materials for the manufacture of other chemicals such as esters and ethers. Those most commonly used are the monohydric and dihydric alcohols, and glycerol. According to von Oettingen (1943) those containing more than three hydroxyl groups are of no industrial toxicological importance. Aliphatic alcohols, other than ethanol, also occur in small amounts in wines and liquors, but their nature and amounts are not accurately known. In analysis, these alcohols are lumped together as 'higher alcohols' or fusel oil. Rum, brandy and whisky may contain 100–400 mg./100 ml. of higher alcohols, the amounts varying according to the 'mash' used, the yeast culture employed and the conditions of fermentation. *n*-Propanol, *iso*propanol, *n*-butanol, *iso*butanol, *n*-, active and *iso*-amyl alcohols have been reported in fusel oil (Kirk & Othmer, 1947). In addition to these alcohols, heptyl alcohol has been reported in a 25-year-old brandy (Ordinneau, 1886; cf. Herstein & Jacobs, 1948), and Hewitt (1928) states that alcohols as high as octyl and nonyl occur in some wines and spirits. The bouquet or flavour of wines is in part due to the presence of esters of these alcohols. The amyl alcohols appear to arise by fermentation of leucine and isoleucine (Herstein & Jacobs, 1948). Appreciable quantities of higher alcohols are therefore being constantly consumed by human beings. Methanol up to 0·36 % may occur in the cheap brandies (marc brandy); this usually arises as the result of the fermentation of pectin.

Most of the known data on the toxicity and metabolism of the aliphatic alcohols have been assembled by von Oettingen (1943), and it is clear from his publication that our knowledge of the metabolism of these compounds is but fragmentary. In general, however, it is known that aliphatic alcohols are oxidized *in vivo*, the primary alcohols being initially oxidized to aldehydes and the secondary to ketones (see Williams, 1947). Some of the more volatile alcohols are undoubtedly eliminated to some extent in the unchanged state by the lungs. The tertiary alcohols, *tert.*-butyl and amyl are known to be partly excreted in the urine as glucuronides. Our knowledge of the fate of methanol and ethanol is fairly extensive, and a valuable study of the metabolism of seven out of the eight possible isomers of amyl alcohol has been made by Haggard, Miller & Greenberg (1945). The latter workers showed quite clearly that the rate of the metabolism of the amyl alcohols in the rat was in the order primary > secondary > tertiary.

In most of the studies on alcohols few workers have paid any attention to whether these alcohols give rise to conjugated glucuronic acids. Neubauer (1901), from qualitative observations on dogs and rabbits, concluded that a number of alcohols were excreted as glucuronides, including ethanol but not methanol. Deichmann & Thomas (1943), however,