- Earle, D. P., Smull, K. & Victor, J. (1942). *J. exp. Med.* **76**, 317.
 Himsworth, H. P. (1943). *Lancet*, **2**, 465.
 Himsworth, H. P. (1947). *Lectures on the Liver and its Diseases*, 1st ed. Oxford: Blackwell.
 Lichtman, S. S. (1949). *Diseases of the Liver, Gall-bladder and Bile Ducts*, 2nd ed. London: Kimpton.
 Pohl, F. (1904). *Arch. exp. Path. Pharmacol.* **51**, 341.
 Roberts, E. & Eckstein, H. C. (1944). *J. biol. Chem.* **154**, 367.
 Roth, J. S. & Allison, J. B. (1950). *J. biol. Chem.* **183**, 173.

- Schiff, L. (1946). *The Differential Diagnosis of Jaundice*, 1st ed. Chicago: The Year Book Publishers.
 Seligson, D., McCormick, G. J. & Sborov, V. (1952). *J. clin. Invest.* **31**, 661.
 Sherlock, S., Summerskill, W. H. J., White, L. P. & Phear, E. A. (1954). *Lancet*, **2**, 453.
 Vigneaud, V. du (1952). *A Trail of Research*, pp. 137-42. Ithaca: Cornell University Press.
 Walshe, J. M. (1953). *Quart. J. Med.* **22**, 483.
 Watson, C. J. (1949). *Ann. intern. Med.* **31**, 405.

A Glucuronic-decomposing Enzyme from Rumen Micro-organisms

3. MODE OF ACTION, SPECIFICITY AND INHIBITION BY ACIDIC SUGAR DERIVATIVES*

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Other papers in this series (Karunairatnam & Levvy, 1951; Marsh, 1954) have described the discovery of a glucuronide-decomposing enzyme in micro-organisms normally present in the sheep rumen, and the partial purification of the enzyme and the kinetics of its action upon some biosynthetic glucosiduronic acids. No attempts have hitherto been made to demonstrate the hydrolytic action of the so-called β -glucuronidases of *Escherichia coli* and other micro-organisms, and while in the previous work on the rumen enzyme there was no evidence of any alternative mode of action, classification of it has been delayed until the sugar residue after enzyme action could be identified with certainty. The action upon simple α - and β -glycosides and glycosiduronic acids of the three common hexose series was examined. For this specificity study the general method, adopted previously for the study of tissue β -glucuronidase (Levy & Marsh, 1952), whereby possible alternative substrates were tested as competitive inhibitors for the enzymic decomposition of a known standard substrate, was used throughout.

Inhibition of the glucuronide-decomposing rumen enzyme by D-glucosaccharo-1 $\rightarrow$ 4-lactone was noted by Levvy (1952); the negligible lactonization at the nearly neutral pH of optimum activity of the enzyme explained the negative results previously obtained by Karunairatnam & Levvy (1951) with saccharate solutions. The inhibitory effects of this and other hexaric acids and derivatives, and of the common hexuronic acids, were investigated.

The results presented in this paper show that the enzyme isolated from the sheep rumen has the same *in vitro* action and specificity as tissue β -glucuronidase, and may therefore be classified as a true β -glucuronidase.

* Part 2, Marsh (1954).

MATERIALS AND METHODS

Materials. The following compounds were synthesized: phenyl α -D-glucopyranoside (Fischer & v. Mechel, 1916), phenyl β -D-glucopyranoside (Fischer & Armstrong, 1901), (-)-menthyl α -D-glucopyranoside (Fischer & Bergmann, 1917), (-)-menthyl β -D-glucopyranoside (Treibs & Franke, 1950), methyl α -D-glucopyranoside (Helferich & Schäfer, 1948), methyl α - and β -D-galactopyranosides (Dale & Hudson, 1930), methyl α -D-mannopyranoside (Fischer, 1895), methyl β -D-mannopyranoside isopropanol solvate (Isbell & Frush, 1940).

From the above glycosides the following glycosiduronic acids were prepared by catalytic oxidation (Marsh, 1952): (-)-menthyl α -D-glucosiduronic acid, methyl α - and β -D-galactosiduronic acids, methyl α - and β -D-mannosiduronic acids; 3-O-methyl D-glucuronic acid was similarly prepared from 1:2-O-isopropylidene 3-O-methyl D-glucose (Vischer & Reichstein, 1944). Hydrolysis of methyl α -D-mannosiduronic acid by the method of Ault, Haworth & Hirst (1935) yielded D-mannuronic acid, which was converted into mannuronate by treatment with dil. NaOH.

Methyl α -D-xyloside was donated by Professor E. L. Hirst to Dr G. A. Levvy, and the di-O-isopropylidene derivative of 2-keto L-gulonic acid by Dr F. Bergel; to remove the acetone groups of the latter compound, a 10% (w/v) aqueous soln. was boiled for 30 min. (Reichstein & Grüssner, 1934). The following compounds were presented by Professor F. Smith to Dr G. A. Levvy: D-glucosaccharo-1 $\rightarrow$ 4- and 6 $\rightarrow$ 3-monolactones and 1 $\rightarrow$ 5-6 $\rightarrow$ 3-dilactone (Smith, 1944), D-mannosaccharo-1 $\rightarrow$ 4-6 $\rightarrow$ 3-dilactone (Haworth, Heslop, Salt & Smith, 1944), methyl β -D-glucofuranosiduronate 6 $\rightarrow$ 3-lactone (Owen, Peat & Jones, 1941) and methyl 4-O-methyl D-glucosiduronic acid amide (mixture of α - and β -forms) (Smith, 1951). By treating the corresponding lactones with dil. NaOH, solutions of D-mannosaccharate and methyl β -D-glucofuranosiduronate were prepared; the latter compound unlike phenolic glucofuranosiduronate solutions (see Tsou & Seligman, 1952), is stable at neutral pH (Owen *et al.* 1941). Methyl 4-O-methyl D-glucosiduronate was prepared by heating an aqueous solution of the amide with dil. NaOH (Smith, 1951), and the glycosidic linkage

was completely hydrolysed by subsequent boiling with $N\text{-HCl}$ for 3 hr.; on neutralizing the hydrolysate with dil. NaOH the product behaved as a lactone rather than as a free acid, and it was assumed that 4-methyl D -glucurone was formed in the initial acid hydrolysis.

The following biosynthetic ester type glucosiduronic acids were presented by Professor R. T. Williams to Dr G. A. Levvy; veratroyl glucuronide (Sammons & Williams, 1946), and the potassium salts of diethyl acetyl and α -ethylhexanoyl glucuronide (Kamil, Smith & Williams, 1953).

Oxycellulose (polyanhydroglucuronic acid) (Yackel & Kenyon, 1942) was donated by Imperial Chemical Industries (Explosives) Ltd. This product had a stated carboxyl content of 22% (w/w) (theoretical COOH for polyanhydroglucuronic acid, 26%). It was brought into solution by the careful addition of dil. NaOH so that the pH did not exceed 7; the titration to neutrality gave a COOH value of 19% (w/w). D -Glucurone was donated by Corn Products Refining Co. Ltd. and was converted into glucuronate by treatment with dil. NaOH .

The following products were obtained commercially: α - D -galacturonic acid (Roche Products Ltd.), salicin (Hopkin and Williams Ltd.); L -ascorbic acid (L. Light and Co. Ltd.); potassium hydrogen saccharate, mucic acid, and D -glucono-1 $\rightarrow$ 5-lactone (British Drug Houses Ltd.). This last compound, m.p. 151–153° after recrystallization from ethanol, was converted into gluconate by treatment with dil. NaOH .

In general, solutions of alternative substrates or inhibitors were adjusted to pH 6.1 before addition to the assay solution, but this was omitted when testing solutions of neutral lactones. The preparation of free acids from the lactones by treatment with dil. NaOH was controlled in each case by the glass electrode, small quantities of alkali being added until equilibrium at pH 9 was reached, followed by readjustment to pH 6.1. In experiments designed to compare the effects of different treatments of the alternative substrate or inhibitor solutions, all solutions were tested simultaneously on a single enzyme preparation. All melting points are corrected.

Enzyme preparation and measurement of enzyme activity. The source of the enzyme and its preparation have already been fully described (Marsh, 1954). For experiments using possible alternative substrates or inhibitors, enzyme C preparations (Marsh, 1954) were employed, i.e. after two stages of $(\text{NH}_4)_2\text{SO}_4$ fractionation, followed by dialysis for 48 hr. The specific activity was then about 1000 enzyme activity units/mg. protein N , an enzyme activity unit being defined as that which liberated 1 μg . phenolphthalein/hr. at pH 6.1 from 0.0005 M phenolphthalein glucuronide under the conditions described by Karunairatnam & Levvy (1951). The liberation of phenolphthalein from 0.0005 M phenolphthalein glucuronide in 1 hr. at 38° in the presence and absence of alternative substrates or inhibitors was measured using as incubation mixture 1.0 ml. 1.5 N - H_3PO_4 - NaOH buffer pH 6.1, 0.5 ml. 0.004 M phenolphthalein glucuronide pH 6.1, 2.0 ml. aqueous soln. of competing substrate or inhibitor and 0.5 ml. enzyme soln. Appropriate controls were set up using water in place of the substrate, inhibitor or enzyme. In experiments using oxycellulose or ascorbic acid, which gave a background colour after development at an alkaline pH, further controls, omitting both substrate and enzyme, were included. The assays and controls were performed in duplicate, with colour development by addition

of glycine- Na_2CO_3 buffer, pH 10.7, as described by Karunairatnam & Levvy (1951).

For investigations of the products after rumen enzyme action, enzyme C preparations were again fractionally precipitated between the limits of 42–60% $(\text{NH}_4)_2\text{SO}_4$ saturation, followed by dialysis of the concentrated enzyme solution for 48 hr. at 15°.

Mouse-liver β -glucuronidase was prepared according to the procedure of Levvy & Marsh (1952) for use in the reducing sugar method.

EXPERIMENTAL AND RESULTS

Comparison of free phenolphthalein and glucuronic acid in enzyme digests of phenolphthalein glucuronide

A series of experiments was made to determine the relative proportions of phenolphthalein and reducing material formed in incubates of the enzyme with phenolphthalein glucuronide, a substrate which has a much higher affinity for the enzyme than others examined (Marsh, 1954).

Trial experiments showed that glucuronic acid could not be estimated by cerimetric methods in the presence of phenolphthalein or phenolphthalein glucuronide; the former interfered with the end point of the cerimetric titration (see Levvy, 1946), and phenolphthalein glucuronide gave high and variable reducing blanks. Mixtures containing up to 300 μg . phenolphthalein and 700 μg . phenolphthalein glucuronide were acidified with 5% (w/v) trichloroacetic acid (TCA), followed by continuous extraction in a micro extractor (Levy, 1948) with ethyl acetate (A.R.) previously washed with water. After 30 min. all traces of free or conjugated phenolphthalein were extracted; all subsequent extractions with ethyl acetate were carried out for 1 hr., at the end of which the pH of the acid solution had risen to about 5.0, indicating the simultaneous removal of TCA. When standard quantities of glucuronic acid were included, cerimetric determination after ethyl acetate extraction indicated a negligible fall in reducing value.

To test the efficiency of the method, mouse-liver β -glucuronidase was initially used for the enzymic hydrolysis, using as incubation mixture 2.0 ml. 0.1 N acetic acid- NaOH buffer pH 5.2, 1.0 ml. 0.004 M phenolphthalein glucuronide pH 5.2 and 1.0 ml. enzyme soln. After incubation for 1 hr. at 38°, the solutions were rapidly cooled to 15° and 0.25 ml. withdrawn and added to 3.75 ml. 0.1 N acetic acid- NaOH buffer, pH 5.2, followed by 4 ml. glycine- Na_2CO_3 buffer, pH 10.7. After centrifuging at 1500 g for 15 min., free phenolphthalein was estimated. For the determination of free glucuronic acid, 2.0 ml. of the remaining incubate mixture were withdrawn and 2.0 ml. 10% (w/v) TCA added. After centrifuging at 1500 g for 15 min., 2.5 ml. of the supernatant were withdrawn and extracted continuously with ethyl acetate to remove phenolphthalein and unchanged substrate. Traces of ethyl acetate in the aqueous layer were removed by a current of air over the warmed solution, and the volume was made up to 5 ml.; 1.0 ml. samples were then taken for the measurement of reducing value by the procedure of Levvy (1946). The results of a typical experiment (Table 1) show that the amounts of liberated phenolphthalein and glucuronic acid were equivalent.

This experiment was then repeated using preparations of the rumen enzyme. The final substrate concentration was

Table 1. *Estimations of free phenolphthalein and reducing uronic acid (calculated as glucuronic acid) in incubates of phenolphthalein glucuronide with mouse-liver β -glucuronidase and rumen enzyme*

Enzyme	Substrate concentration (M)	Period incubation at 38°	Total liberated phenolphthalein (μ g.)	Total liberated glucuronic acid		Theoretical free glucuronic acid (μ g.)
				Reducing method (μ g.)	Naphthoresorcinol method (μ g.)	
Mouse-liver β -glucuronidase	0.004	1 hr. at pH 5.2	1020	626	—	622
Rumen enzyme	0.002	24 hr. at pH 6.5	1840	1102	1137	1123

raised to 0.002 M, and 0.1 N citric acid–NaOH buffer, pH 6.5, was substituted for the acetate buffer used above. Employing the procedure described above, there was an apparent loss of about 60% free glucuronic acid, assuming this to be the liberated reducing material. It has previously been shown that free glucuronic acid present in rumen enzyme preparations is precipitated together with protein prior to the estimation of reducing value (Marsh, 1954). The following modifications were finally made: after 24 hr. incubation at 38°, followed by addition of TCA, 2.5 ml. of the mixture was immediately removed and extracted with ethyl acetate without prior removal of precipitated protein. Further material, probably mucopolysaccharide, was observed to coagulate at the water/ethyl acetate interface. After removal of ethyl acetate, the aqueous suspension was allowed to stand for 12 hr. at 15°, then diluted to 5 ml. and centrifuged at 1500 *g* for 15 min. Reducing value estimations on the supernatant were carried out as before, together with estimation of non-extractable uronic acid by the Tollens reaction with naphthoresorcinol, using the procedure of Hanson, Mills & Williams (1944). The uronic acid was calculated as D-glucuronic acid from a standard curve. As shown in Table 1, similar values for liberated glucuronic acid were given by the reducing method and by the naphthoresorcinol reaction, and these were also equivalent to the amount of aglycone liberated.

Identification of free D-glucuronic acid from enzyme digests of phenolphthalein glucuronide

By paper chromatography. After incubating the rumen enzyme solution (1.0 ml.) with 0.005 M phenolphthalein glucuronide (1.0 ml.), pH 6.5, for 24 hr. at 38°, followed by dialysis into water for 24 hr. at 0°, the dialysate was found to contain 450 μ g. free glucuronic acid, estimated cerimetrically. The dialysate was evaporated to dryness *in vacuo*, and extracted with 0.2 ml. water. Chromatographic separation was then carried out on paper in an amyl alcohol–pyridine–water mixture (7:7:6, by vol.) (Jeanes, Wise & Dimler, 1951), together with authentic samples of D-glucurone, D-glucuronic acid, D-galacturonic acid and D-xylose. There was no interference in the separation of the reducing sugars in the presence of phenolphthalein or its glucuronide nor from dialysable products in the enzyme preparation. After development, the papers were sprayed with *o*-phenetidine and benzidine to demonstrate the presence of uronic acids and xylose respectively. Only one spot from the dialysed enzyme digest was observed, and this corresponded to D-glucuronic acid.

By isolation of the dibenziminazoyl derivative of D-glucosaccharic acid. An authentic specimen of the di-(benziminazol-2-yl) derivative of D-glucosaccharic acid was prepared

from 180 mg. D-glucurone by oxidation and coupling with *o*-phenylenediamine (British Drug Houses Ltd.) according to the procedure of Lohmar, Dimler, Moore & Link (1942) as modified by Levvy (1948). This yielded 110 mg. (30% of theory) of the dibenziminazoyl derivative, m.p. 240° (decomp.). The dihydrochloride and dipicrate were prepared from this as described by Levvy (1948). The dihydrochloride had m.p. 267–268° (decomp.), $[\alpha]_D^{25} + 52.0 \pm 0.5^\circ$ in water (c, 2); m.p. of dipicrate 212° (decomp.) with change of form at 144°.

For the enzymic hydrolysis, 25 ml. of the rumen enzyme preparation, containing 160 enzyme units/ml., were incubated with 25 ml. 0.03 M phenolphthalein glucuronide, pH 6.4, for 60 hr. at 38°; the pH was checked at intervals of 12 hr. The solution was then dialysed into 500 ml. water at 0° for 48 hr. The dialysate was acidified to pH 1.3 with 5 N-H₂SO₄, and extracted 10 times with ethyl acetate (total 1 l.). After testing for the absence of free or conjugated phenolphthalein, the aqueous residue was concentrated *in vacuo* to 100 ml. and shaken with BaCO₃ to remove sulphate. The aqueous soln. was filtered and estimated by the method of Hanson *et al.* (1944) to contain 75 mg. glucuronic acid (including volume of solution in dialysis sac); this would correspond to 52% hydrolysis, but this value is low due to retention of free glucuronic acid by the undialysable residue.

This free uronic acid solution was then oxidized and condensed with *o*-phenylenediamine as described above. Before condensation, a small sample was boiled for 1 hr. at pH 3 and tested as an inhibitor for mouse-liver β -glucuronidase as described by Levvy (1952), from which it was calculated that about 21 mg. total D-glucosaccharic acid was present (29% of theory on oxidation). This yielded 28 mg. of the dibenziminazole derivative (23% of theory from the glucuronic acid content), m.p. 238–240° (decomp.), from which were prepared the dihydrochloride m.p. 266° (decomp.) and the dipicrate m.p. 211–213° (decomp.) with change of form at 142°. None of these derivatives, when mixed with the corresponding authentic specimens, produced any depression of the melting points.

Effects of possible alternative substrates upon the enzymic hydrolysis of phenolphthalein glucuronide

Possible alternative substrates which had no appreciable effect upon the liberation of phenolphthalein from 0.0005 M phenolphthalein glucuronide by the rumen enzyme are shown in Table 2. The remaining substances, which caused a noticeable change in the liberation of phenolphthalein when present in the incubation mixture, are shown in Table 3.

Table 2. *Possible alternative substrates having negligible effect on rate of liberation of phenolphthalein from 0.0005 M phenolphthalein glucuronide at pH 6.1 by rumen enzyme*

Compound	Final concentration (M)	Change in rate of phenolphthalein liberation (% control)
Methyl α -D-glucopyranoside	0.01	0
Phenyl α -D-glucopyranoside	0.01	0
Phenyl β -D-glucopyranoside	0.01	0
(-)-Menthyl α -D-glucopyranoside	0.001	0
Methyl α -D-galactopyranoside	0.01	0
Methyl β -D-galactopyranoside	0.01	0
Methyl α -D-mannopyranoside	0.01	0
Methyl β -D-mannopyranoside	0.01	-2
Salicin	0.01	-2
Cellobiose	0.01	0
Methyl β -D-xylopyranoside	0.01	0
Methyl α -D-galactosiduronic acid	0.01	0
Methyl β -D-galactosiduronic acid	0.01	0
Methyl α -D-mannosiduronic acid	0.01	0
Methyl β -D-mannosiduronic acid	0.01	-2
Methyl 4-O-methyl D-glucosiduronic acid*	0.002	0
Methyl β -D-glucosiduronic acid	0.001	-2

* Mixture of α - and β - anomers.Table 3. *Possible alternative substrates affecting the rate of liberation of phenolphthalein from phenolphthalein glucuronide at pH 6.1 by rumen enzyme*

Compound	Final concentration (M)	Standard substrate concentration (M)	Change in rate of phenolphthalein liberation (% control)
(-)-Menthyl β -D-glucopyranoside	0.001	0.0005	+4
(-)-Menthyl α -D-glucosiduronic acid	0.01	0.0005	+8
Oxycellulose (freshly prepared)	0.01*	0.0005	-15
Veratroyl glucosiduronic acid	0.0005	0.0002	-29
Diethylacetyl glucosiduronic acid	0.004	0.0002	-21
α -Ethylhexanoyl glucosiduronic acid	0.001	0.0002	-22

* Calculated as glucurone.

Table 4. *Inhibitions of oxycellulose solutions on the liberation of phenolphthalein from 0.0005 M phenolphthalein glucuronide by rumen enzyme*

For preparation of solutions see text. Concentrations are calculated as glucurone.

Preparation	Concentration (M)	Inhibition of rate of phenolphthalein liberated (%)
Freshly prepared soln.	0.01	15
Soln. kept 48 hr.	0.01	11
Residue after complete dialysis	0.008*	6-9
Dialysate	0.001*	7

* Calculated from results obtained by dialysing a soln. at pH 7 into water for 48 hr. at 15°.

Definite inhibitory effects were produced only by oxycellulose and the ester glucuronides of veratric, diethylacetic and α -ethylhexanoic acids. To conserve material, the concentrations of the last three compounds were considerably reduced, and the

standard substrate concentration was accordingly decreased to 0.0002 M.

Direct measurements of the liberation of reducing material after incubating oxycellulose with the enzyme could not be made, since this material is very unstable at an alkaline pH. Its inhibitory power after dialysis was investigated, however. A solution of oxycellulose (0.02 M, calculated as D-glucurone) in 0.3 N-H₃PO₄-NaOH buffer, pH 6.1, was dialysed for 48 hr. at 15° into an equal volume of the same buffer. A similar solution was dialysed for the same period into several changes of fresh buffer to remove all dialysable material; approximately 20% was lost in this time. The inhibitory powers of the first dialysate, the residue from complete dialysis, and a non-dialysed solution allowed to stand at room temperature were then compared (Table 4). The inhibitory power decreased after dialysis, and the inhibition caused by the dialysate at the lower concentration was as high as that of the undialysable residue.

Effects of possible inhibitors on the enzymic hydrolysis of phenolphthalein glucuronide

Results of preliminary tests for the inhibition of the liberation of phenolphthalein from 0.0005M phenolphthalein glucuronide by the rumen enzyme, in the presence of acidic sugar derivatives, are shown in Table 5.

Table 5. *Effects of acidic sugar derivatives and their lactones on rate of liberation of phenolphthalein from 0.0005M phenolphthalein glucuronide by rumen enzyme*

Compound	Final concentration (M)	Inhibition of rate of phenolphthalein liberation (%)
D-Gluconate	0.01	2
D-Gluconate (after 1 hr. at 100° and pH 2)	0.01	4
D-Glucono-1→5-lactone	0.01	2
D-Glucuronate	0.01	21
D-Glucuronate	0.002	11
3-O-Methyl D-glucuronate	0.002	5
4-O-Methyl D-glucuronate	0.002	3
D-Glucurone	0.01	3
4-O-Methyl D-glucurone*	0.002	0
D-Galacturonate	0.01	0
D-Mannuronate	0.01	2
D-Mannurone	0.01	2
D-Glucosaccharate	0.01	6
D-Glucosaccharate (after 6 hr. at 20° and pH 3)	0.01	12
D-Glucosaccharate (after 1 hr. at 100° and pH 3)	0.001	49
D-Glucosaccharo-1→4-lactone	0.00025	49
D-Glucosaccharo-6→3-lactone	0.01	18
D-Glucosaccharo-1→5.6→3-dilactone	0.001	6
Mucate (D-galactosaccharate)	0.005	-7
Mucate (after 2 hr. at 100° and pH 2)	0.005	0
D-Mannosaccharate	0.001	2
D-Mannosaccharo-1→4.6→3-dilactone	0.001	3
L-Ascorbate	0.01	73†
L-Ascorbate	0.001	7
2-Keto-L-gulonate	0.002	4

* Initial product from methyl 4-O-methyl-D-glucosiduronic acid after acid hydrolysis.

† This value is probably high since 0.01M ascorbic acid interferes with the phenolphthalein colour reaction.

Table 6. *Inhibition by 0.00025M D-glucosaccharo-1→4-lactone of liberation of phenolphthalein from 0.0005M phenolphthalein glucuronide by suspensions of rumen micro-organisms and by rumen enzyme preparations*

For details of enzyme purification, see Marsh (1954).

Stage of enzyme purification	Inhibition of rate of phenolphthalein liberation (%)
Suspension of active micro-organisms	44
Enzyme A (crude cell-free extract)	46
Enzyme B (after 1st (NH ₄) ₂ SO ₄ fractionation)	49
Enzyme C (after 2nd (NH ₄) ₂ SO ₄ fractionation)	49

Of particular interest were the inhibitory effects caused by the hexaric acids and their lactones. Whereas freshly prepared D-glucosaccharic acid solutions had little inhibitory power, after a period of ageing at an acid pH, and particularly on boiling for a short time, marked inhibition was caused. The inhibition by the 1→4-lactone, of D-glucosaccharic acid was relatively so powerful that the inhibitions caused by the 6→3-lactone and 1→5.6→3-dilactone could well be explicable as due to a trace (about 0.5%) of the 1→4-lactone in these preparations. The inhibition of the rumen enzyme by D-glucosaccharo-1→4-lactone was quantitatively independent of the period of incubation at 38° and pH 6.1, indicating that the inhibitor is stable at this pH.

A solution of D-glucosaccharo-1→4-lactone had a similar inhibitory power for enzyme preparations of varying purity, and inhibited the liberation of phenolphthalein from its glucuronide by the intact micro-organisms. Table 6 shows the effect upon enzyme preparations at stages A, B and C, and a suspension of active micro-organisms, prepared according to the procedure of Marsh (1954). Microscopic examination of the suspension before incubation showed the organisms to be then still viable.

The kinetics of the inhibition of the enzymic hydrolysis of phenolphthalein glucuronide by 0.01M D-glucuronic acid and 0.00025M D-glucosaccharo-1→4-lactone at varying substrate concentrations were investigated. In both cases percentage inhibition decreased with increasing substrate concentration, showing that inhibition was competitive. Using the procedure previously adopted (Marsh, 1954) for the determination of the Michaelis constant K_s for alternative substrates for the rumen enzyme, mean values of 3.6×10^{-3} M for D-glucuronic acid, and 1.9×10^{-5} M for D-glucosaccharo-1→4-lactone, were obtained for K_s , the apparent dissociation constant of the enzyme-inhibitor complex.

DISCUSSION

Detection and assay of the rumen enzyme have hitherto always been made by colorimetric measurement of the liberated aglycone. It is clear from the

experimental evidence, however, that the enzyme has a hydrolytic *in vitro* action; it is thus a β -glucuronidase. The direct chemical isolation of glucuronic acid liberated in enzyme digests is difficult, although Miwa (1932) has isolated the potassium salt from incubates of flavone glucosiduronic acids with baicalinase, a plant enzyme which has now also been identified as a β -glucuronidase (Levy, 1954). The oxidation method of Lohmar *et al.* (1942), whilst giving low yields, is more convenient for the identification of small quantities of glucuronic acid such as were obtainable from digests with the rumen enzyme. The equivalence of liberated reducing uronic acid, calculated as D-glucuronic acid, with the amount of free aglycone in enzyme digests is further strong evidence of a hydrolytic action.

The substrate specificity of the rumen enzyme seems to be very similar to that of animal tissue β -glucuronidase, as described by Levy & Marsh (1952), in that the rumen enzyme is specific for the hydrolysis of β -D-glucosidopyranuronic acids. The inhibition of the enzymic hydrolysis of the standard substrate by ester glucosiduronic acids may be due to their effect as alternative substrates or as true inhibitors. The lability of these compounds in alkaline solution (Pryde & Williams, 1933) has made the direct investigation of their hydrolysis difficult, but Levy (1954) has stated that these compounds are substrates for mouse-liver β -glucuronidase, and this is therefore probably also true for the rumen enzyme.

Oxycellulose has a slightly lower inhibitory effect than D-glucuronic acid on the rumen enzyme, but the inhibition was apparently associated mostly with the dialysable material in the preparation. Since the latter formed only 20% of the total weight, the inhibition could not be accounted for by glucuronic acid formed by complete hydrolysis. The inhibitory material in oxycellulose would thus seem to be polysaccharides of low molecular weight, and these may be substrates for the enzyme. It is therefore interesting to note that there was no experimental evidence of an action of the rumen enzyme upon methyl 4-O-methyl β -D-glucosiduronic acid. This suggests that if the function of this and other similar alimentary enzymes in herbivores is to break down hemicellulose material, as has been previously suggested (Marsh, Alexander & Levy, 1952; Robinson, Smith & Williams, 1953; Billett, 1954), the site of such enzyme action is probably only at the linkage of a terminal glucuronic acid residue at the non-reducing end of the polysaccharide. This may also be true of animal tissue β -glucuronidase, on which the effects of oxycellulose and substituted glycosiduronic acids are similar to those on the rumen enzyme (Levy & Marsh, 1952).

The slight activation of rumen β -glucuronidase

activity by (-)-menthyl α -D-glucosiduronic acid and (-)-menthyl β -D-glucoside resembles the effects of (-)-menthol derivatives on the tissue enzyme (Levy & Marsh, 1952), but the rumen enzyme differs from the latter in that no activation was observed in the presence of the alternative substrate (-)-menthyl β -D-glucosiduronic acid (Marsh, 1954). A saturated solution of (-)-menthol had no appreciable effect.

Of the compounds examined as possible true inhibitors of rumen β -glucuronidase, D-glucosaccharo-1 $\rightarrow$ 4-lactone was by far the most powerful, causing 50% inhibition of enzyme activity at a concentration of 0.00025 M with substrate concentration 0.0005 M. This confirms the results previously obtained by Levy (1952). The inhibitory power was appreciably lower for the rumen enzyme than for mouse-liver β -glucuronidase, for at the same inhibitor concentration over 90% inhibition of animal tissue β -glucuronidase activity was noted with a substrate concentration of 0.00063 M, and a 0.000004 M lactone solution caused 50% inhibition under the same conditions (Levy, 1952). The inhibition of the rumen β -glucuronidase by acidified D-glucosaccharic acid preparations was correlated with formation of the 1 $\rightarrow$ 4-lactone in these solutions as found by Levy (1952). Since this inhibitor was found to be stable at the normal pH (5.9–6.2) of the sheep rumen, and appeared to inhibit effectively the glucosiduronic acid-decomposing ability of the whole organisms, it may prove of value in attempting to elucidate the physiological function of the enzyme.

There is a marked difference between rumen and tissue β -glucuronidases in their susceptibilities to inhibition by mucic acid preparations. The boiling of a mucic acid solution at an acid pH is said to result in rapid lactone formation (Levene & Simms, 1925), although the evidence, largely titrimetric, for this is circumstantial, for no lactones of mucic acid have been isolated. Levy (1952) found that boiled mucic acid solutions (0.0007 M) caused strong inhibition of mouse-liver β -glucuronidase activity, whereas unboiled mucic acid (0.002 M) had little effect. In the case of rumen β -glucuronidase, boiled mucic acid at the latter concentration had no effect, whereas unboiled preparations caused slight activation of enzyme activity (Table 5). This difference was not due to changes in composition of mucate solutions at pH 5.2 and 6.1, for by experiment there was found to be equally strong inhibition of mouse-liver β -glucuronidase at pH 5.2 (acetate buffer) and pH 6.1 (phosphate buffer).

D-Glucuronic acid, a product of the enzyme action, caused inhibition of rumen β -glucuronidase, but the monomethyl derivatives had less effect, and D-glucurone and (hypothetical) 4-O-methyl D-glucurone were apparently inactive. In this respect

the rumen enzyme closely resembles tissue β -glucuronidase (see Karunairatnam & Levvy, 1949; Spencer & Williams, 1951; Levvy, 1952), but inhibition of the latter by D-glucuronic acid is more marked.

Quantitative measurement of the enzyme activity in the presence of high concentrations of L-ascorbic acid was difficult, but some inhibition was apparent. Becker & Friedenwald (1949) found that this compound strongly inhibited rat-liver β -glucuronidase (see also Mills, Paul & Smith, 1953). Levvy (1952) found no inhibition of mouse-liver β -glucuronidase by 0.001 M ascorbic acid, and at 0.01 M concentration Wong & Rossiter (1951) found negligible inhibition of rabbit polymorphonuclear leucocyte β -glucuronidase. No satisfactory explanation has been given for these varying results with animal tissue β -glucuronidase preparations.

In the case of D-gluconic acid, any inhibitory power for β -glucuronidase seems to be associated with an impurity. In earlier experiments it was noted that solutions of 0.01 M gluconic acid after boiling at pH 2 for 1 hr. caused up to 10 % inhibition of rumen enzyme activity (substrate concentration 0.0005 M), but the inhibitory effect became negligible after several recrystallizations of the D-glucono-1 $\rightarrow$ 5-lactone. Some authors, e.g. Karunairatnam & Levvy (1949), and Mills *et al.* (1953) have described slight inhibition by gluconate of tissue β -glucuronidase acting on phenyl glucuronide, whereas Wong & Rossiter (1951) and Levvy (1952) found no effect using phenolphthalein glucuronide as the substrate. These differing results might partly be due to the higher affinity of phenolphthalein glucuronide for the enzyme compared with phenyl glucuronide, but a positive effect with gluconate or gluconolactone might well be due to traces of saccharic acid formed during their manufacture.

From this survey of the inhibitory effects of acidic sugar derivatives and their lactones upon rumen β -glucuronidase, it seems that the essential requirements for an effective specific inhibitor, as for the β -glucuronidase from animal tissues, are that the configuration of the secondary hydroxyl groups of the D-glucose molecule must be preserved and unsubstituted in a cyclic structure containing a carboxylic acid grouping at the C₍₆₎ position, i.e. there must be a close resemblance to a β -D-glucopyranuronide structure.

SUMMARY

1. The glucuronide-decomposing enzyme in the sheep rumen was found to be a true β -glucuronidase. Phenolphthalein and glucuronic acid were liberated in equivalent amounts by enzymic action on the conjugated glucosiduronic acid.

2. Liberated glucuronic acid was identified by oxidation to D-glucosaccharic acid, from which derivatives were prepared.

3. Rumen β -glucuronidase was found to be specific for β -D-glucosidpyranuronic acids, and had no effect upon glycosides or on glycosiduronic acids of other sugars, nor upon methyl 4-O-methyl glucosiduronic acid.

4. Acylglucuronides and dialysable components of oxycellulose inhibited enzyme action upon phenolphthalein glucuronide. These compounds may be substrates for the enzyme.

5. D-Glucosaccharo-1 $\rightarrow$ 4-lactone was a strong competitive inhibitor of rumen β -glucuronidase, although its affinity for this enzyme is lower than for tissue β -glucuronidase.

6. Boiled mucic acid preparations had no effect upon the rumen enzyme, in which respect the latter differs from tissue β -glucuronidase.

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REFERENCES

- Ault, R. G., Haworth, W. N. & Hirst, E. L. (1935). *J. chem. Soc.* p. 517.
- Becker, B. & Friedenwald, J. S. (1949). *Arch. Biochem.* **22**, 101.
- Billett, F. (1954). *Biochem. J.* **57**, 159.
- Dale, J. K. & Hudson, C. S. (1930). *J. Amer. chem. Soc.* **52**, 2534.
- Fischer, E. (1895). *Ber. dtsch. chem. Ges.* **28**, 1429.
- Fischer, E. & Armstrong, E. F. (1901). *Ber. dtsch. chem. Ges.* **34**, 2898.
- Fischer, E. & Bergmann, M. (1917). *Ber. dtsch. chem. Ges.* **50**, 711.
- Fischer, E. & v. Mechel, L. (1916). *Ber. dtsch. chem. Ges.* **49**, 2813.
- Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.
- Haworth, W. N., Heslop, D., Salt, E. & Smith, F. (1944). *J. chem. Soc.* p. 217.
- Helferich, B. & Schäfer, W. (1948). *Org. Synth. Coll.* **1**, 2nd edn, p. 364.
- Isbell, H. S. & Frush, H. L. (1940). *J. Res. nat. Bur. Stand.* **24**, 125.
- Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
- Kamil, I. A., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 137.
- Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
- Karunairatnam, M. C. & Levvy, G. A. (1951). *Biochem. J.* **49**, 210.
- Levene, P. A. & Simms, H. S. (1925). *J. biol. Chem.* **65**, 31.
- Levy, G. A. (1946). *Biochem. J.* **40**, 396.

- Levy, G. A. (1948). *Biochem. J.* **42**, 2.
 Levy, G. A. (1952). *Biochem. J.* **52**, 464.
 Levy, G. A. (1954). *Biochem. J.* **53**, 462.
 Levy, G. A. & Marsh, C. A. (1952). *Biochem. J.* **52**, 690.
 Lohmar, R., Dimler, R. J., Moore, S. & Link, K. P. (1942). *J. biol. Chem.* **143**, 551.
 Marsh, C. A. (1952). *J. chem. Soc.* p. 1578.
 Marsh, C. A. (1954). *Biochem. J.* **53**, 609.
 Marsh, C. A., Alexander, F. & Levy, G. A. (1952). *Nature, Lond.*, **170**, 163.
 Mills, G. T., Paul, J. & Smith, E. E. B. (1953). *Biochem. J.* **53**, 232.
 Miwa, T. (1932). *Acta phytochim.*, Tokyo, **6**, 154.
 Owen, L. N., Peat, S. & Jones, W. J. G. (1941). *J. chem. Soc.* p. 339.
 Pryde, J. & Williams, R. T. (1933). *Biochem. J.* **27**, 1210.
 Reichstein, T. & Grüssner, A. (1934). *Helv. chim. acta*, **17**, 311.
 Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 125.
 Sammons, H. G. & Williams, R. T. (1946). *Biochem. J.* **40**, 223.
 Smith, F. (1944). *J. chem. Soc.* p. 633.
 Smith, F. (1951). *J. chem. Soc.* p. 2646.
 Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
 Treibs, W. & Franke, I. (1950). *Ann. Chim. (Phys.)*, **570**, 76.
 Tsou, K.-C. & Seligman, A. M. (1952). *J. Amer. chem. Soc.* **74**, 5605.
 Vischer, E. & Reichstein, T. (1944). *Helv. chim. acta*, **27**, 1332.
 Wong, E. & Rossiter, R. J. (1951). *Canad. J. med. Sci.* **29**, 195.
 Yackel, E. C. & Kenyon, W. O. (1942). *J. Amer. chem. Soc.* **64**, 121.

The N-Terminal Amino Acid Residues of Gelatin

3. ENZYMIC DEGRADATION

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The specific action of proteolytic enzymes on small peptides is well known (for reviews see Neurath & Schwert, 1950; Smith, 1951). The work reported in the present paper describes the hydrolytic effect of pepsin, trypsin, chymotrypsin and papain on gelatin using the *N*-terminal residue technique to follow the extent of degradation and to examine enzyme specificity. The *N*-terminal residue technique was employed in Part 2 of this series (Courts, 1954*b*) to examine thermal degradation of gelatin catalysed by hydrogen and hydroxyl ions. The technique does not permit the bonds broken to be specified, but indicates the amino acid residues carrying the amino groups which are released during hydrolysis.

MATERIALS AND METHODS

Gelatin. An alkali-processed ox-bone gelatin of isoionic point pH 5.1 was used, being identical with the material used in Part 2 of this series. The substrate was 2.5 g. in 50 ml. water adjusted to a suitable pH value.

Trypsin. Crystallized trypsin (Armour Laboratories, Chicago, U.S.A.) (0.02 g.) was used at pH 8.5. The reaction was allowed to proceed for 2.5 hr. at 21, 35, 44, 56 and 65°. The author is most grateful to Dr Watson of the Courtauld Laboratories for a gift of this enzyme.

Pepsin. Crystallized pepsin (Armour Laboratories) (0.02 g.) was used at pH 1.87 and 37°. The reaction was carried out for 6 and 19 hr. Controls were run without enzyme to allow for thermal degradation at this pH value.

Chymotrypsin. Crystallized chymotrypsin (Armour Laboratories) (0.02 g.) was used at pH 8.0 and 37°. Reaction times were 2, 4 and 6 hr. A second series was carried out to observe the effect of enzyme concentration and of variation of pH; thus 0.002 g. of chymotrypsin was incubated at 37° for 4 hr. with solutions of substrate at pH 5.1 (isoionic point of the gelatin) and at pH 8.0 (reputed optimum for this enzyme).

Papain. Commercial papain (Norman Evans and Rais, Manchester) (0.002 g.) was used at 37°. Hydrolysis was allowed to continue for 2 and 5 days at (a) pH 5.05, the pH optimum of papain for partially hydrolysed protein and (b) pH 6.95, the pH optimum for intact protein substrates. The extent of thermal degradation under these conditions was also examined.

N-Terminal residue determinations

The enzymically degraded gelatins were adjusted to pH 8.5 and allowed to react with 1:2:4-fluorodinitrobenzene (FDNB). The preparation of dinitrophenyl (DNP) derivatives, their subsequent hydrolysis and the evaluation of *N*-terminal residues were all carried out using the methods previously described by Courts (1954*a, b*).

Considerations of proteinase inactivation

No attempts were made to remove or inactivate the proteinase before allowing its digest to react with FDNB. Pepsin would be automatically inactivated at pH 8.5. It is likely that chymotrypsin would be inactivated by FDNB through substitution at its tyrosyl phenolic groups (cf. Sizer, 1945). Papain may likewise be inactivated by blocking of sulphhydryl groupings (Smith, 1951), although for both