A Glucuronide-decomposing Enzyme from Rumen Micro-organisms

2. PURIFICATION AND KINETICS

By C. A. MARSH

Rowett Research Institute, Bucksburn, Aberdeenshire

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The first paper in this series (Karunairatnam & Levvy, 1951) reported the discovery of an enzyme, normally present in micro-organisms in the rumen of the sheep, which decomposed β -D-glucosiduronic acids. Other enzymes, apparently having a similar action, have since been reported in the contents of the alimentary tracts of several species of mammals (Marsh, Alexander & Levvy, 1952), in the crop liquor of locusts (Robinson, Smith & Williams, 1953) and in the digestive tracts of some marine molluscs (Dodgson, Lewis & Spencer, 1953). These enzymes have provisionally been classified as β -glucuronidases, until their modes of action and specificities have been more thoroughly studied. A further examination of the enzyme occurring in the sheep rumen has been made, and in this paper a method of extraction and purification of the enzyme and the kinetics of enzyme action are described.

It has not yet been ascertained with which organism, or group of organisms, this enzyme is associated. The method of Karunairatnam & Levvy (1951), using as starting material a centrifuged fraction of the mixed rumen population, has therefore been adopted. These workers prepared cell-free extracts by mechanical disintegration of the organisms; for larger preparations it was found more convenient to prepare aqueous extracts of acetone-treated powders. The later stages of purification of the enzyme were controlled by the use of the specific-property solubility test of Falconer & Taylor (1946), who used it as a criterion of absolute purity of a liver esterase preparation. For the multi-stage purification of an enzyme by salt precipitation it has been found very convenient in defining at each stage the limits of salt saturation to be employed at the following stage, in order to achieve a known degree of purification with economical recovery of enzyme activity.

EXPERIMENTAL AND RESULTS

Materials and general methods

Substrates. Biosynthetic phenolphthalein β -D-glucuronide (Fishman, Springer & Brunetti, 1948), phenyl β -D-glucuronide (Garton, Robinson & Williams, 1949) and (-)-menthyl β -D-glucuronide (Williams, 1938) were used.

Measurement of enzyme activity. The liberation of phenolphthalein from phenolphthalein glucuronide (5×10^{-4} M) in 0.3 N- H_3PO_4 : NaOH buffer, in 1 hr. at 38°, was measured by the procedure of Karunairatnam & Levvy (1951); one unit of enzyme activity was defined as that which liberated 1 μ g. phenolphthalein/hr. at pH 6.1 from this substrate. The relative purity of an enzyme preparation was measured in terms of its specific activity, i.e. activity units/mg. N.

In experiments using phenyl glucuronide as the substrate, the liberation of phenol by the enzyme in 0.05 N citric acid-NaOH buffer was measured by a modification (Levy & Marsh, 1952) of the procedure of Kerr, Graham & Levvy (1948). Preliminary experiments showed that, as in the case of animal β -glucuronidase (Kerr *et al.* 1948), there was quantitative recovery of added phenol after incubation with the enzyme preparation.

Attempts to measure the enzymic liberation of reducing sugar from (-)-menthyl glucuronide in 0.05 N citric acid-NaOH or 0.2 N phthalic acid-NaOH buffers were made using the cerimetric method as described by Levvy (1946). The ceric sulphate solution (approx. 0.012 N) was standardized by arsenious oxide (A.R.) (Vogel, 1943). In the determination of the glucuronic acid equivalent of ceric sulphate according to the procedure of Levvy (1946) it was found that D-glucurone and D-glucuronic acid (prepared by titrating a D-glucurone solution with diluted alkali to a stable, slightly alkaline pH) had equivalent reducing powers. It was calculated that 1 μ g. glucuronic acid was equivalent to 0.002 ml. 0.0127 N ceric sulphate; Levvy (1946) gives the equivalent of 1 μ g. glucuronic acid = 0.002 ml. 0.0124 N ceric sulphate. Protein precipitation was carried out using copper tungstate (Levy, 1946) or 5% (w/v) trichloroacetic acid (TCA) (Fishman, 1939a). The former method necessitated the use of phthalate buffer; when TCA was used the concentration of Na_2CO_3 in the alkaline ferri cyanide was increased accordingly to neutralize the free acid present.

Nitrogen determinations. For stages before the preparation of cell-free extracts of the enzyme, determination of total N was performed. For cell-free extracts, protein N was determined after precipitation with 5% TCA. After standing at 0° for 12 hr., the precipitate was sedimented by centrifuging at 1500 g for 15 min. and washed thrice on the centrifuge with 5% TCA. Control experiments showed that this removed all traces of $(NH_4)_2SO_4$ introduced in previous operations. The precipitate was finally resuspended in water and samples removed for duplicate determinations of protein N.

Kjeldahl digestion and measurement of total or protein N were as described by Karunairatnam & Levvy (1951).

Ammonium sulphate precipitation. Since the solubility of $(NH_4)_2SO_4$ varies appreciably with the temperature, an aqueous solution (ca. 3.5 M) saturated at 0° was used for

protein precipitation. A calculated quantity was added to the enzyme solution, buffered with $0.3N-H_2PO_4-NaOH$, pH 6.1, at 0° , to give the desired $(NH_4)_2SO_4$ concentration, expressed as percentage saturation. The slight error thereby introduced owing to the volume contraction on dilution of the saturated $(NH_4)_2SO_4$ solution was neglected. After precipitation, the mixture was allowed to equilibrate at 0° for 30 min. before centrifuging at 1500 g for 15 min. at 15° . The required precipitates after $(NH_4)_2SO_4$ fractionation were redissolved in water and dialysed against running tap water for 48 hr. before further fractionation or use of the specific-property solubility test.

Specific-property solubility tests. The enzyme solution, buffered with $0.3N-H_2PO_4-NaOH$, pH 6.1, was added to suitable mixtures of saturated $(NH_4)_2SO_4$ and water to give systems of equal volumes and enzyme content with differing $(NH_4)_2SO_4$ concentrations. After standing at 0° for 30 min., and centrifuging at 1500 g for 15 min., the precipitates were redissolved in water and samples removed for enzyme assay and for determination of protein N. The total supernatant activity and protein N were then calculated by difference. Direct enzyme assay of the supernatant activity in the presence of high concentrations of $(NH_4)_2SO_4$ was found to be unreliable.

pH Measurement. This was made with the glass electrode on the Cambridge pH meter.

Source of the enzyme

Rumen liquor was withdrawn from adult male castrated Cheviot sheep, with permanent rumen fistulae. Initially the animals' diet consisted of hay and concentrate (two parts linseed oil cake and one part crushed oats), with free access to water and salt lick. Later the concentrate was excluded. Much cleaner preparations were thereby obtained, without deterioration of the health of the animals or noticeable alteration of the glucuronide-decomposing activity of the whole rumen liquor. The daily diet then consisted of 750 g. hay fed at 7 a.m., and a further 750 g. hay fed at 4 p.m. The rumen liquor was withdrawn at 9.30 a.m., and for large-scale preparations the samples from several sheep were pooled, not more than 1500 ml. being withdrawn from a single sheep each time.

Preparation of cell-free extracts

The rumen liquor was first strained through six layers of surgical gauze to remove coarse food particles, and the suspension of micro-organisms was then fractionated by centrifuging to obtain the fraction which sedimented between the limits of 15 g for 2 min. and 1500 g for 30 min. This greenish sediment was washed with water on the centrifuge, and then contained 45–60% of the apparent total enzyme activity of the strained rumen liquor. It was resuspended in 5 vol. water, and poured into 20 vol. of rapidly stirred acetone at 15° , and mechanical stirring was continued for a further 30 min. After being centrifuged at 1500 g for 6 min., the brown precipitate was washed on the centrifuge with a further 3 vol. acetone. (Omission of this washing yielded a dark brown mass which proved difficult to resuspend in water.) The product, after being dried *in vacuo* over $CaCl_2$, was a friable, almost white powder (yield 1 g./500–800 ml. strained rumen liquor).

The acetone-treated powder was suspended in water by homogenizing in a glass homogenizer (Umbreit, Burris &

Stauffer, 1949) for 1 min. at 20° . The aqueous suspension was diluted to the original volume of the aqueous suspension of micro-organisms before acetone treatment, and stirred slowly at 20° for 4 hr.; it was then centrifuged at 8300 g for 30 min., and the supernatant re-centrifuged under the same conditions. The final turbid supernatant was almost free of visible particles under the high-power light-microscope, and contained about 40% of the total enzyme activity of the suspension of acetone-treated powder. Soluble-enzyme activity was defined as that which remained in this supernatant. In large-scale preparations a further quantity of soluble-enzyme activity (10–15% of that already extracted) could be recovered by resuspending the cell debris in half the original quantity of fresh water and repeating the extraction procedure.

Preliminary extractions with $0.6N-Na_2HPO_4-0.3N$ citric acid mixed buffer (McIlvaine, 1921) over the pH range 4.3–8.0 indicated that whereas the ratio supernatant activity/suspension activity remained almost constant, there was appreciable loss of total enzyme activity in buffered solutions during the extraction period, particularly outside the pH range 6–7. Maximum supernatant activity resulted from extraction with water alone. Extractions with aqueous solutions of the non-ionic surface active agents Triton A 20 and Triton X 100 (Rohm & Haas Co., Philadelphia), at a concentration (0.003%, w/v), which did not affect the subsequent enzyme assay, gave results similar to extraction with water. Maximum extraction with water was reached in approximately 2 hr. at 20° ; subsequently the slow increase in enzyme extraction was almost nullified by the loss of total activity. Although the rate of enzyme inactivation in the aqueous suspension at 3° was less than at 20° , the extracted enzyme activity at the lower temperature was less, even after 72 hr. extraction. Attempts to render soluble a further part of the enzyme activity associated with the residual cell debris after aqueous extraction, by further treatment with acetone as described above, led to loss of more than 50% of the residual activity of the debris.

Purification of the enzyme

Stage A. Precipitation of mucilaginous material. Precipitation of non-active mucilaginous material in the crude cell-free extract at pH 4.5, according to the procedure used by Karunairatnam & Levvy (1951), caused a slight increase in the specific activity (see Table 1), although little solid was precipitated. The product (enzyme A) contained 85–90% of the enzyme activity of the crude extract.

Stage B. 1st fractionation with $(NH_4)_2SO_4$. An initial fractionation of enzyme A, between the limits of 20–80% $(NH_4)_2SO_4$ saturation, precipitated all the enzyme activity (enzyme B). The solubility-test curves of the variation of enzyme B activity and protein N at varying $(NH_4)_2SO_4$ concentrations are

shown in Figs. 1a and 1b respectively. Precipitation of enzyme *B* activity and of protein did not commence below 30% $(\text{NH}_4)_2\text{SO}_4$ saturation, and were both completed at 75% saturation. Thus there was little non-overlapping non-active protein at this stage. The steeper gradient of Fig. 1b at commencement of precipitation, as compared with that of Fig. 1a, indicated the presence of less soluble non-active protein, with a similar effect above 65% $(\text{NH}_4)_2\text{SO}_4$ saturation due to more soluble non-active protein. These conclusions are more easily seen in the specific-property solubility test (Fig. 1c). In this curve the constant gradient between points A and B, at about 42 and 62% $(\text{NH}_4)_2\text{SO}_4$ saturation respectively, indicated the maximum purification which could be achieved by subsequent $(\text{NH}_4)_2\text{SO}_4$ fractionation of enzyme *B*. From Fig. 1a it can be seen that ca. 80% of the total enzyme *B* activity should be precipitated between these limits.

Stage C. 2nd fractionation with $(\text{NH}_4)_2\text{SO}_4$. The enzyme *B* preparation was fractionated between the limits of 42–60% $(\text{NH}_4)_2\text{SO}_4$ saturation, and after redissolving the precipitate in water and dialysing, solubility tests of the resulting enzyme (enzyme *C*) activity and protein N between the limits of 35–70% $(\text{NH}_4)_2\text{SO}_4$ saturation were performed. The specific-property solubility test for enzyme *C* is shown in Fig. 2. Much impurity is shown to be precipitated below 50% $(\text{NH}_4)_2\text{SO}_4$ saturation, with a linear range of maximum specific activity between the limits of 50–60% $(\text{NH}_4)_2\text{SO}_4$ saturation, which should yield a theoretical recovery of 60% enzyme activity.

Stage D. 3rd fractionation with $(\text{NH}_4)_2\text{SO}_4$. Enzyme *D* was obtained after fractionation of the enzyme *C* preparation between the limits 50–60% $(\text{NH}_4)_2\text{SO}_4$ saturation. The specific-property solubility test for enzyme *D* is shown in Fig. 3. It was found that neither enzyme activity nor protein N was precipitated below 45% $(\text{NH}_4)_2\text{SO}_4$ saturation. Overlapping impurity was still obvious at both ends of the precipitation range, and the curve shows a maximum specific activity gradient between the limits of 50–60% $(\text{NH}_4)_2\text{SO}_4$ saturation. Fractionation of enzyme *D* between these limits should result in 90% recovery of enzyme activity.

Stage E. 4th fractionation with $(\text{NH}_4)_2\text{SO}_4$. Since attempts to decrease the limits of fractionation to less than a difference of 10% $(\text{NH}_4)_2\text{SO}_4$ saturation were not very successful, the enzyme *D* preparation was again fractionated between the limits of 50–60% $(\text{NH}_4)_2\text{SO}_4$ saturation. In practice ca. 60% of the enzyme activity was recovered. The specific-property solubility test on the final enzyme solution (enzyme *E*) is shown in Fig. 4. This is an almost linear curve, extrapolating through the origin, indicating the apparent absence of more soluble

Fig. 1. a, b: precipitation of enzyme activity and protein N at varying concentrations of $(\text{NH}_4)_2\text{SO}_4$. Enzyme preparation *B* (Table 1). c: first specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *B*. $(\text{NH}_4)_2\text{SO}_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points A and B = 900 enzyme units/mg. protein N.

impurity. The experimental point corresponding to the original solution (i.e. 0% salt saturation) does not lie on the linear curve, indicating the continued presence of less soluble inactive protein. The difficulty experienced in removing this impurity suggests that it may be denatured enzyme.

The final product after stage *E* was a colourless liquid with a very slight opacity.

In Table 1 is shown the apparent recoveries of enzyme activity obtained in a single large-scale preparation, together with the specific activities of the solutions or suspensions at each stage of the purification. For the cell-free solutions the specific activities agreed closely with the estimates given by the previously conducted specific-property solubility tests. In the preparation illustrated the loss

of enzyme activity during the initial centrifugal fractionation of the rumen liquor was higher than was normally found. The recovery of enzyme activity in the initial cell-free extract was 17.5% of that in the strained rumen liquor, with a ninefold enzyme purification (calculated in total N content). The recovery of enzyme *E* activity was 10.5% of that in the initial crude cell-free extract, with a 42-fold purification (calculated on protein N). The apparent overall enzyme purification from the strained rumen liquor to enzyme *E* was thus 378-fold.

Other preparations, in which $(\text{NH}_4)_2\text{SO}_4$ fractionations were carried out between the same limits as in Table 1, but not as far as stage *E*, gave values of the specific activities at the various stages similar to those given in Table 1.

Fig. 2. Second specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *C* (Table 1). $(\text{NH}_4)_2\text{SO}_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points *A* and *B* = 9000 enzyme units/mg. protein N.

Fig. 3. Third specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *D* (Table 1). $(\text{NH}_4)_2\text{SO}_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points *A* and *B* = 12000 enzyme units/mg. protein N.

Test for glucuronide-decomposing enzymes in the diet

Appreciable amounts of pigmented food particles present in normal rumen liquor are sedimented during the fractional centrifuging of the liquor, and these cannot readily be separated from the micro-organisms present in the various fractions (Davidson, 1954). There was, however, no liberation of phenolphthalein when phenolphthalein glucuronide (0.00125M) was incubated at 38° and pH 6.1 with suspensions of ground hay of variable particle size up to ca. 0.5 mm. diameter, before or after acetone treatment as described above. It was concluded that none of the glucuronide-decomposing enzyme activity occurring in the rumen could have originated in the feed.

Fig. 4. Fourth specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *E* (Table 1). $(\text{NH}_4)_2\text{SO}_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between the origin and point *A* = 13600 enzyme units/mg. protein N.

Table 1. *Stages in the purification of the rumen glucuronide-decomposing enzyme*

Substrate 0.0005M phenolphthalein β -D-glucuronide. Unit of enzyme activity as specified in Methods section.

Stage	Vol. (ml.)	Enzyme activity		Nitrogen content (total or protein N) (μ g. N/ml.)	Specific activity (units/mg. N)	Theoretical specific activity from Figs. 1c, 2, 3 (units/mg. N)
		Units/ml.	Total units			
Strained rumen liquor	7 600	33.4	257 000	1440*	23	—
Active fraction of micro-organisms	424	220	93 400	2530*	87	—
Cell-free extract of acetone-dried powder	1 120	40	44 800	202* 139†	198 288	—
Enzyme A (after pptn. at pH 4.5)	1 140	36	41 200	113†	318	—
Enzyme B (after 1st $(\text{NH}_4)_2\text{SO}_4$ fractionation)	635	42	26 700	128†	325	—
Enzyme C (after 2nd $(\text{NH}_4)_2\text{SO}_4$ fractionation)	101	163	16 500	173†	950	900
Enzyme D (after 3rd $(\text{NH}_4)_2\text{SO}_4$ fractionation)	60	150	9 000	18.2†	8 300	9 000
Enzyme E (after 4th $(\text{NH}_4)_2\text{SO}_4$ fractionation)	11.5	408	4 700	33.8†	12 000	12 000

* Total N. † Protein N.

Effects of varying the buffer, enzyme concentration and period of assay

Free inorganic phosphate was rigorously excluded from an enzyme C preparation after extraction of the acetone-treated powder; 0.1N citric acid-NaOH buffer, pH 6.1, was employed before each $(\text{NH}_4)_2\text{SO}_4$ fractionation. After the final dialysis, determination by the procedure of Fiske & Subbarow (1925) gave a free phosphate content of less than 0.2 μ g. P/ml. Assays of enzyme activity of this preparation at pH 6.1 in 0.3N- H_3PO_4 -NaOH, 0.1N citric acid-NaOH and 0.2N phthalic acid-NaOH buffers gave identical values.

The amount of phenolphthalein liberated in 1 hr. at 38° and pH 6.1 from 0.0005M phenolphthalein glucuronide, using the usual enzyme preparation in phosphate buffer, was directly proportional to the concentration of enzyme C. The reaction velocity, with constant enzyme concentration, remained unchanged after 4 hr. incubation at 38° in phosphate buffer, pH 6.1.

These results confirm the findings of Karunairatnam & Levvy (1951); their significance is discussed later.

Variation of enzyme activity with pH

The variation of enzyme activity with pH, using 0.00125M phenolphthalein glucuronide as substrate, in 0.6N- Na_2HPO_4 -0.3N citric acid mixed buffer, pH 4.5-8.5, was studied using the procedure of Karunairatnam & Levvy (1951). It was found (Fig. 5) that the enzyme showed only a single optimum, but this varied slightly with the purity of the enzyme preparation. After the first $(\text{NH}_4)_2\text{SO}_4$ fractionation (enzyme B), optimum enzyme activity was at pH 6.0-6.1, as found by Karunairatnam & Levvy (1951), whereas after a further $(\text{NH}_4)_2\text{SO}_4$ fractionation (enzyme C) the optimum shifted to pH 6.5-6.6.

Fig. 5. Variation of rumen enzyme activity with pH. Substrate 0.00125M phenolphthalein glucuronide; assays in 0.3N- H_3PO_4 -NaOH buffer. ●—●, enzyme preparation B; ○—○, enzyme preparation C (Table 1).

The liberation of phenol from 0.015M-phenyl glucuronide in 0.05N citric acid-NaOH buffer in 1 hr. at 38° by enzyme C preparations showed a single optimum at pH 6.6, i.e. almost identical with that obtained with phenolphthalein glucuronide as substrate and enzyme C.

Variation of enzyme activity with substrate concentration

Fig. 6a shows the variation of enzyme activity with the concentrations of the substrates phenolphthalein and phenyl β -D-glucuronides in typical experiments. For phenolphthalein glucuronide,

inhibition by excess substrate was evident at concentrations above $0.005M$, whereas there was no evidence of the attainment of maximum enzyme activity even at the highest concentration ($0.02M$) of phenyl glucuronide used. Enzyme *C* preparations were employed in each case.

Analysis of these results by the graphical methods of Lineweaver & Burk (1934) and of Hofstee (1952) showed some anomalies in the kinetics. The plots of $1/v$ against $1/S$, S/v against S and v against v/S (Fig. 6*b*), where v represents the initial reaction velocity (enzyme activity) and S the substrate concentration, gave non-linear curves at all substrate levels in each case, whereas according to Michaelis-Menten theory these curves should be linear in the absence of substrate inhibition. The effect obtained was one of enzyme activation at increasing substrate concentration in the lower range; thus in Fig. 6*b* there is an increase in the gradient of the curve as the substrate concentration increases (i.e., v/S decreases) with a sudden reversal of gradient in the case of phenolphthalein glucuronide due to the subsequent effect of substrate inhibition. Statistical advice (from Mr A. W. Boyne of this Institute) was that no true straight line could be fitted to the experimental points at concentrations below the inhibition level.

This made impossible the determination of K_s or K_i , the respective dissociation constants of the active binary enzyme-substrate complex ES and the inactive complex ES^n formed in the region of substrate inhibition, or of n , the number of substrate molecules in the latter complex, by the graphical method of Lineweaver & Burk (1934). The apparent value of K_s for phenolphthalein glucuronide was therefore determined from Fig. 6*a*, for from the Michaelis-Menten theory, K_s is equal to the substrate concentration required to give a reaction velocity of $V_m/2$, where V_m is the theoretical maximum reaction velocity. Values of K_s for phenolphthalein glucuronide obtained in different experiments are shown in Table 2, the mean values being $7.7 \times 10^{-5}M$ at pH 6.5, and $6.0 \times 10^{-5}M$ at pH 6.1.

Since the maximum reaction velocity for the substrate phenyl glucuronide was not reached, the value of K_s for this compound was measured by the indirect method described below.

a

b

Fig. 6. *a*: effect of varying the substrate concentration (S) on the rate of decomposition (v) of phenolphthalein and phenyl glucuronides by enzyme preparation *C* (Table 1). $\bigcirc-\bigcirc$, Decomposition of phenolphthalein glucuronide in $0.3N-H_3PO_4-NaOH$ buffer, pH 6.5; $\bullet-\bullet$, decomposition of phenyl glucuronide in $0.05N$ citric acid- $NaOH$, pH 6.6. *b*: results plotted according to Hofstee (1952). For decomposition of phenolphthalein glucuronide ($\bigcirc-\bigcirc$), v/S in units of $10^5 \mu g./hr.$ For decomposition of phenyl glucuronide ($\bullet-\bullet$), v/S in units of $10^4 \mu g./hr.$

Table 2. Apparent Michaelis constants of substrates for the rumen enzyme

Substrate	Constant	Values obtained $M \cdot 10^{-5}$	pH	Apparent relative affinity (mean)
Phenolphthalein	K_s	6.3, 9.1	6.5	1
β -D-glucuronide		5.0, 3.7, 9.5, 6.0	6.1	
Phenyl β -D-glucuronide	$K_i (=K_s)$	220, 320	6.1	0.022
(-)-Menthyl β -D-glucuronide	$K_i (=K_s)$	260, 320	6.1	0.021

Table 3. Recovery of glucuronic acid after addition to enzyme C preparations, with and without incubation at 38° for 5 hr. in 0.05N citric acid-NaOH buffer, pH 6.5. Protein pptn. with 5% trichloroacetic acid

Glucuronic acid added (μg.)	Glucuronic acid recovered (μg.)			
	Non-incubated		After incubation	
	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme
250	238	182	253	185
300	306	249	306	239

Measurements of reducing sugar

Attempts were made to measure the liberation of reducing sugar from the enzymic decomposition of 0.01M (-)-menthyl β-D-glucuronide, using concentrated enzyme preparations (enzyme C, after 48 hr. dialysis). Although there was evidence of the liberation of reducing sugar at pH 5.5-7.0, the amounts measured, estimated as glucuronic acid and using periods of incubation at 38° of up to 4 hr., were invariably very low. Measurement of glucuronic acid recovery after addition of this compound to enzyme preparations showed, however, an apparent loss (ca. 30%) as shown in Table 3. There was no further change in the recovery of glucuronic acid after incubation at 38° for 5 hr., suggesting that the apparent loss was due to adsorption by and co-precipitation with protein or polysaccharide, and not to enzymic destruction or to the liberation from the substrate of a free sugar with a reducing power widely different from that of glucuronic acid.

It was concluded that (-)-menthyl β-D-glucuronide was a substrate for the rumen enzyme, but the kinetics of enzyme action on this substrate could not be measured directly. The indirect determination of K_s is described below.

Inhibition of phenolphthalein glucuronide decomposition by alternative substrates

The effects of 0.01M phenyl and (-)-menthyl glucuronides on the enzymic decomposition of phenolphthalein glucuronide were studied. In such experiments these alternative substrates acted as competitive inhibitors of the enzyme, and it was assumed that their concentrations remained virtually unchanged throughout the incubation period. Assays were conducted in phosphate buffer at pH 6.1, using enzyme C preparations; Fig. 7 shows the variation of enzyme activity (liberation of free phenolphthalein) with phenolphthalein glucuronide concentration, in the absence and presence of each alternative substrate.

By considering these alternative substrates as competitive inhibitors, their affinities for the enzyme can be determined from the Michaelis equation for a competitive system.

$$v = \frac{V_m S}{K_s (1 + I/K_i) + S},$$

Fig. 7. Effect of standard substrate concentration on the liberation of phenolphthalein from phenolphthalein glucuronide in absence and presence of (a) 0.01M phenyl β-D-glucuronide, (b) 0.01M (-)-menthyl β-D-glucuronide, in 0.3N phosphate buffer, pH 6.1. ○—○, No alternative substrate present; ●—●, phenyl β-D-glucuronide present; ×—×, (-)-menthyl β-D-glucuronide present.

where v is the initial reaction velocity (enzyme activity) with substrate concentration S and inhibitor concentration I , and V_m the theoretical maximum reaction velocity (see Levy & Marsh, 1954). The apparent values of K_s and $(1 + I/K_i)$ were obtained directly from Fig. 7, being equal to the substrate (phenolphthalein glucuronide) concentration required for half maximum reaction velocity of the respective non-inhibited and inhibited reactions, V_m being unchanged. The values of K_i , the Michaelis constant of the competitive inhibitor, for phenyl and (-)-menthyl glucuronides, were then calculated, and the values obtained from different experiments are shown in Table 2. The value of K_i should be identical with that of K_s for the second substrate, for both in this case represent the dissociation constant of the enzyme-alternative substrate complex. Lederberg (1950) has used this method of indirect determination of K_s of various substrates for the β-galactosidase of *Escherichia coli*.

It is seen from Table 2 that the values of K_i (or K_s) for phenyl and (-)-menthyl glucuronides were approximately equal (ca. 3×10^{-3} M). The affinity of the rumen enzyme for phenolphthalein glucuronide is thus considerably higher than for the other substrates examined.

DISCUSSION

The degree of purification achieved with the rumen enzyme compares unfavourably with that of animal tissue β -glucuronidase obtained recently. Thus using different techniques, Sarkar & Sumner (1950), Bernfeld & Fishman (1950) and Smith & Mills (1953) have each obtained an approximately 1000-fold purification of the tissue enzyme, whereas after preparation of the cell-free extracts, a 40-fold purification of the rumen enzyme was obtained, mostly in the ammonium sulphate fractionations. The method adopted would seem valuable in the earlier stages of purification before the use of a more specific technique, however, by indicating the limiting purity which may be achieved by a multi-stage salt fractionation.

In the preparation given in Table 1, the protein N recovered after the final ammonium sulphate fractionation was about 2% of that present in the original cell-free extract. The specific activity gradient of 13 600 units/mg. protein N obtained for the purified rumen enzyme in the final specific-property solubility test may be compared with the values for highly purified tissue β -glucuronidase, e.g. 200 000 units/mg. protein N for ox-liver β -glucuronidase (Smith & Mills, 1953); the rumen enzyme has a higher affinity for phenolphthalein glucuronide than has animal β -glucuronidase. It seems unlikely that the final product represents the rumen enzyme in a high state of purity. Falconer & Taylor (1946) have pointed out that in circumstances where protein impurities may have identical solubilities with those of the enzyme studied, a linear specific-property solubility test is not a criterion of absolute purity; it also appears possible that a homogenous solid solution of enzyme and inactive protein is precipitated.

Since the rumen enzyme is wholly intracellular, assay of the enzyme activity of a suspension of the intact micro-organisms cannot be compared quantitatively with that of a suspension of the acetone-treated powder or with subsequent stages in which the enzyme is fully soluble, and the recoveries given for the earlier stages of purification are probably inaccurate. Strains of haemolytic streptococcus containing a glucuronide-decomposing enzyme have shown a 5- to 10-fold increase in activity after sonic disintegration (Jacox, 1953), and with the rumen enzyme a slightly higher total activity was noted after acetone treatment. Assays of enzyme activity in the original rumen liquor are even less reliable, since decomposition of the standard substrate may well be inhibited competitively by natural substrates present in the liquor.

Endogenous inhibitors appear to be absent from partially purified cell-free preparations, however, since there was found a linear variation of enzyme

activity with enzyme content (see Levvy & Marsh, 1954); Karunairatnam & Levvy (1951) found no evidence of a thermostable coenzyme in cell-free extracts of the rumen enzyme, and their results may also be interpreted as indicating the absence of a thermostable inhibitor, e.g. one similar to that found in rat-liver β -glucuronidase preparations (Walker & Levvy, 1953).

There is a fraction of the enzyme activity closely associated with the insoluble disrupted cell contents which, although fully active, cannot easily be brought into solution. Thus aqueous extraction after acetone treatment removed only 45% of the total enzyme activity; Karunairatnam & Levvy (1951) obtained an extraction of 57% of the activity from cells disintegrated by mechanical treatment.

The single pH optimum obtained with different preparations of the rumen enzyme at 6.1 or 6.5 may be compared with values given for similar enzymes of microbial origin, e.g. those from *Escherichia coli* (Buehler, Katzman & Doisy, 1949; Smith & Mills, 1950), pH-optimum 6.2, and from *Streptococcus haemolyticus* (Jacox, 1953), pH-optimum 5.5-7.0. Animal tissue β -glucuronidases have a pH optimum at 4.5 and some an additional optimum at pH 5.0-5.2; baicalinase, the only plant β -glucuronidase which has been investigated, has a single pH optimum varying between pH 3.8 and pH 4.5, dependent upon the buffer (Levy, 1954). This supports the experimental evidence that the rumen enzyme is of microbial origin and does not originate from plant particles in the diet or secretion of tissue β -glucuronidase into the rumen by the host animal.

The phenolphthalein glucuronide concentration (0.005M at pH 6.5) for maximum enzyme activity was higher than the value of 0.00062M at pH 6.1 obtained by Karunairatnam & Levvy (1951). This, and also the slight shift in the pH optimum in enzyme preparations of different stages of purity, may be due to the removal of interfering substances by ammonium sulphate fractionation; Walker & Levvy (1953) observed that in the absence of its endogenous inhibitor the optimum substrate concentration for rat-liver β -glucuronidase preparations was almost doubled. The difficulties found here in applying the graphical methods of Lineweaver & Burk (1934) to the experimental results were not experienced by Karunairatnam & Levvy (1951), or generally by other workers with animal tissue β -glucuronidase, but Fishman (1939b) has obtained anomalous curves of enzyme activity and substrate concentration for purified ox-spleen β -glucuronidase somewhat similar to those obtained by the author. A possible explanation of the activating effect of the enzyme at moderate substrate concentrations may be that, apart from an active binary complex ES, other active complexes ESⁿ may also be formed. Hofstee (1952) has shown that the same effect may

be caused by the simultaneous action of two similar enzymes of differing affinities for the same substrate. In view of the complications of both apparent activation and inhibition by excess substrate, the apparent values of K_s and of $K_i (=K_s)$ for the alternative substrates, obtained by direct graphical methods, represent the most accurate values obtainable from the data.

The affinity ($1/K_s$) of phenolphthalein glucuronide for the rumen enzyme was found to be somewhat higher than for animal β -glucuronidase (e.g. see Levvy, 1952); the much lower affinities of phenyl and (-)-menthyl glucuronides were approximately equal, and of the same order, viz. $1/K_s = 300 \text{ M}^{-1}$ for both enzymes. The rumen enzyme differs from mouse-liver β -glucuronidase (Levy & Marsh, 1952) in that there was no apparent activation of the decomposition of phenolphthalein glucuronide by (-)-menthyl β -D-glucuronide.

The mode of action of the rumen enzyme, e.g. whether or not it is a true β -glucuronidase, has still to be determined. The fact that an enzyme preparation free from inorganic phosphate gave identical assays of enzyme activity in different buffers makes it unlikely, however, that the enzymic liberation of phenolphthalein from its glycosiduronic acid takes place by a mechanism of phosphorolysis. An investigation of the reaction products of enzyme action, and of the specificity and inhibition of the rumen enzyme, will be published later.

SUMMARY

1. A partial purification of the glucuronide-decomposing enzyme associated with the medium-sized micro-organisms in the sheep rumen is described. This was achieved by aqueous extraction of an acetone-treated powder and acid precipitation of inactive material, followed by repeated fractional precipitation with ammonium sulphate. The fractionation limits were controlled by use of the specific-property solubility test of Falconer & Taylor (1946).

2. The overall purification (in terms of enzyme activity/mg. N) which was achieved, commencing with strained rumen liquor, was approximately 400-fold.

3. The single pH optimum of enzyme activity varied slightly with the state of purity of the preparation between the limits 6.1 to 6.6 and was the same for phenyl and phenolphthalein glucuronides.

4. The effect of varying substrate concentration was studied and certain anomalies are discussed.

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