A Glucuronide-Decomposing Enzyme from Rumen Micro-organisms

1. PREPARATION AND ASSAY

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The most characteristic feature of ruminant digestion lies in the use of cellulose and the associated ill-defined polysaccharides which are collectively known as hemicellulose. Digestion of these substances is brought about by the mixed microbial population of the rumen, and considerable study has been made of the end products of this process, in particular of the volatile fatty acids which are absorbed through the rumen wall into the blood stream of the host (Elsden & Phillipson, 1948). Such studies, however, cast little light on the decomposition of cellulose or hemicellulose in its early stages, since qualitatively similar end products result from the digestion of starch and simple sugars in the rumen. The scattered and inconclusive evidence available from studies of cellulose degradation by bacterial cultures (for reviews see Norman & Fuller, 1942; Fähræus, 1947; Hungate, 1950) suggests that glucose and cellobiose may be intermediates in this process. Caution is, however, necessary in attempting to apply conclusions arrived at with individual organisms to changes brought about by a complex population such as that of the rumen (Norman & Fuller, 1942; Elsdén & Phillipson, 1948). If reducing sugars are formed in the rumen from cellulose or hemicellulose, it would appear that the insolubility of the substrate makes the process a slow one, followed by rapid destruction of the sugars by the micro-organisms. Such considerations make a direct approach to the study of the enzymic mechanism of cellulose and hemicellulose fission in the rumen a matter of very great difficulty. It has been suggested (e.g. Boswell, 1941), although on evidence which is open to criticism (Fähræus, 1947), that microbial fission of the cellulose chain involves preliminary oxidation to a polyglucuronide. Be that as it may, glucuronic acid residues enter into the composition of certain hemicellulose polysaccharides in the plant (Norman, 1937).

It was considered that the identification and characterization of β -glycosidases present in the total rumen contents in significant amounts would constitute a useful contribution to knowledge of the special features of ruminant digestion. For the initial identification of such enzymes, it was proposed to make use of soluble β -glycosides containing aglycones which, in the free state, are stable and

readily detected in the presence of intact rumen micro-organisms. This approach permits treatment of the rumen liquor as though it were one tissue.

Biosynthetic phenolphthalein β -D-glucuronide, which has been recommended for use in the assay of animal β -glucuronidase by Talalay, Fishman & Huggins (1946), appeared to be a suitable substrate. As described below, a fraction of the micro-organisms in the sheep rumen has been found to contain an enzyme or system of enzymes which liberates phenolphthalein from the glucuronide. The present paper deals with the preparation of the enzyme in cell-free extract and the determination of optimum conditions for assay.

EXPERIMENTAL AND RESULTS

General methods

Enzyme assay. On the basis of results obtained in early experiments, the following general procedure was adopted. To 3 ml. 0.1M-phosphate buffer, pH 6.1, were added 0.5 ml. 0.004M-phenolphthalein glucuronide solution (adjusted to pH 6.1) and 0.5 ml. enzyme preparation (rumen liquor, cell suspension or cell-free extract). After incubating the mixture at 37° for 1 hr., the reaction was arrested and the colour of the free phenolphthalein developed by addition of 4 ml. of a buffer solution, pH 10.7 (0.4M-glycine, 0.2M-NaCl and 0.25M-Na₂CO₃). The mixture was centrifuged at 1500 g for 15 min., and the intensity of the red colour in the supernatant was read with a Spekker photoelectric absorptiometer, using Ilford no. 605 yellow-green filter. Results were read from a calibration curve constructed by replacing substrate and enzyme in the mixture by a phenolphthalein solution containing 10–80 μ g., and are expressed as μ g. phenolphthalein liberated in 1 hr. at 37°.

The addition of Na₂CO₃ was found to increase the efficiency of the glycine buffer recommended by Talalay *et al.* (1946) for colour development. Increasing the final Na₂CO₃ concentration beyond 0.125M, however, led to rapid colour fading.

When phenolphthalein was added to suspensions of rumen micro-organisms and incubated as described above, the recovery was quantitative.

Nitrogen determination. Kjeldahl digestion was carried out according to the procedure of Chibnall, Rees & Williams (1943). NH₃ was evolved in the Markham still (Markham, 1942), absorbed in 2% boric acid containing methyl red and bromocresol green (see Conway, 1947), and titrated with 0.01N-HCl.

Measurement of pH was done with the glass electrode.

Table 1. *Fractionation of rumen contents*

Fraction no.	Relative centrifugal force (g)	Period of centrifuging (min.)	Enzyme activity (μ g. phenolphthalein)		Microscopic observations
			per mg. total N	Total in fraction	
1	15	2	80	6,000	Large protozoa, food debris and a few organisms from 2
2	1500	30	240	21,000	Quin's organism, small protozoa and large bacteria
3	5000	30	60	800	Small bacteria and a few organisms from 2
4	Supernatant from 3		0	0	Almost cell-free

Rumen liquor. Cheviot sheep with permanent rumen fistulae were fed daily with 1 lb. hay at 8 a.m., 0.5 lb. concentrate (2 parts linseed oil cake and 1 part crushed oats) at 11 a.m. and 1 lb. hay at 8 p.m. The animals had free access to water and salt lick. Rumen liquor was withdrawn at 10.30 a.m. and strained through eight layers of surgical gauze. Eight animals have been used in the course of this work. Under the conditions stated, no marked variation in the composition of the rumen microbial population was observed from animal to animal or from day to day. The pH was 6.0-6.2.

Centrifugal fractionation of rumen contents

On the basis of preliminary experiments in which the strained rumen liquor was centrifuged at different speeds for varying times, the procedure shown in Table 1 was drawn up for fractionating the micro-organisms according to their enzyme activity. The precipitate obtained at each stage was resuspended in water and samples were taken for measurement of enzyme activity and total N. Whilst no systematic attempt was made to assess the relative importance of the different rumen micro-organisms in this connexion, Table 1 suggests that the enzyme activity was associated with the medium-sized species which sediment in fraction 2. These comprise Quin's yeast-like organism (Quin, 1943), small protozoa and large bacteria. The activities of fractions 1 and 3, consisting mainly of large protozoa and small bacteria respectively; were probably due to contamination with fraction 2. Large protozoa (*Holotrich* and *Oligotrich* ciliates) in bacteria-free preparations, and enriched cultures of bacteria fermenting xylose and glucose (small cocci and cocco-bacilli), obtained from the sheep rumen by Dr A. E. Oxford in this Institute, were unable to decompose phenolphthalein glucuronide. The fact that the final supernatant in Table 1 had no activity shows that the enzyme was neither extracellular nor derived from body fluids.

Preparation of the enzyme

Stage A. Fraction 2 of the rumen micro-organisms (see Table 1) was washed on the centrifuge four times with thrice its volume of water to remove pigmented material, and resuspended in water.

Stage B. To obtain a cell-free extract, the suspension was shaken in small batches with half volumes of Ballotini, Grade 12 (Chance Bros., Ltd.) in the Mickle Tissue Disintegrator (Mickle, 1948) for 1.5 hr. The aqueous layer was centrifuged at 8300 g for 30 min. and the supernatant separated. Precipitated material was suspended in water and put through the extraction procedure once more. The combined

extracts were again centrifuged at 8300 g for 30 min., to give a supernatant which was almost free from microscopic particles.

Stage C. Before proceeding with $(\text{NH}_4)_2\text{SO}_4$ fractionation, it was found necessary to remove mucilaginous material. Since the enzyme is unstable at acid pH (see below), the following procedure was adopted. To the cell-free solution was added M-acetate buffer, pH 4.3, till the pH of the mixture was 4.5. After centrifuging in chilled cups at 1500 g for 15 min., the supernatant was separated and brought to pH 6.1 with 2N-NaOH.

Stage D. To the enzyme solution was added one-tenth of its volume of M-phosphate buffer, pH 6.1, followed by sufficient saturated $(\text{NH}_4)_2\text{SO}_4$ solution to give a final saturation of 30%. The bulky precipitate obtained on centrifuging at 1500 g for 15 min. was discarded. The supernatant was brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ and again centrifuged. The supernatant was discarded, and the precipitate dissolved in water and dialysed overnight against running tap water. Attempts to narrow the limits for $(\text{NH}_4)_2\text{SO}_4$ fractionation led to considerable loss of enzyme.

Table 2 shows the recoveries obtained at various stages in the above procedure.

Table 2. *Recovery of enzyme at various stages of preparation*

Stage	Vol. (ml.)	Total enzyme activity (μ g. phenolphthalein)
A, washed micro-organisms	250	51,000
B, cell-free extract	350	29,300
C, solution after adjusting B to pH 4.5	400	21,500
D, ppt. at 30-70% saturation with $(\text{NH}_4)_2\text{SO}_4$	150	15,000

Tests for the existence of a coenzyme

No suggestion was obtained in the experiments described above of the existence of a coenzyme. Nevertheless, experiments were carried out to test this point directly. Dialysis of the cell-free extract at stage B for 20 hr. against running tap water caused no significant fall in enzyme activity. Immersion of the enzyme preparation in boiling water for 3 min. caused complete loss of activity. This preparation when added to an unboiled enzyme extract produced no increase in the activity of the latter.

Variation in activity with pH

Fig. 1 shows the effect of varying the pH on the rate of decomposition of phenolphthalein glucuronide by rumen preparations. To 3 ml. phosphate-citrate buffer (mixtures of 0.2M- Na_2HPO_4 and 0.1M-citric acid) were added 0.5 ml. enzyme preparation (stage B) and 0.05 ml. 0.01M-phenolphthalein glucuronide solution. The mixture was incubated at 37° for 1 hr. There was little change in the pH during incubation. After addition of 4 ml. Na_2CO_3 -glycine buffer, the contents of all tubes were brought to pH 10.3–10.5 with NaOH and diluted to 10 ml. As can be seen from Fig. 1, the activity showed a sharp optimum at pH 6.1.

Fig. 1. Effect of pH on the rate of decomposition of phenolphthalein glucuronide by rumen enzyme in phosphate-citrate buffer.

The activity of an enzyme preparation at pH 6.1 was the same in phosphate-citrate, phosphate, citrate or acetate buffer.

Variation in stability with pH

The stability of the enzyme at varying pH is illustrated in Fig. 2. About 15 ml. enzyme preparation (stage B) adjusted to the required pH with HCl or NaOH, was incubated at 37° for 15 min. or 1 hr. The preparation was then rapidly cooled to room temperature and brought to pH 6.1, and the activity was assayed in the usual way. Comparison of Figs. 1 and 2 makes it evident that the peak in the pH-activity curve (Fig. 1) was largely determined by irreversible inactivation of the enzyme outside the pH range 6–7.

Variation in activity with substrate concentration

The effect of varying the substrate concentration on the rate of decomposition of phenolphthalein glucuronide by a rumen enzyme preparation (stage D) in phosphate buffer, pH 6.1, is shown in Fig. 3. Similar relationships were found in other experiments. It can be seen that inhibition by excess substrate was pronounced. The experimental results were analysed by the graphical method of Lineweaver & Burk (1934), as shown in Fig. 4. A value of $3.05 \times 10^{-5}\text{M}$ was obtained for K_s , the dissociation constant of the active enzyme-substrate complex, whilst n , the number of substrate molecules

Fig. 2. Inactivation of the enzyme after 15 min. (x -- x) and after 1 hr. (o — o) at 37° and varying pH. Final assay at pH 6.1. Results expressed as fraction of the activity before incubation.

per active enzyme centre in the inactive complex was 2 (the actual value given by Fig. 4 is 1.97), and K_2 , the dissociation constant of the inactive complex, was 0.0126M. The optimum activity of the enzyme was reached at a substrate concentration of $6.2 \times 10^{-4}\text{M}$ ($= \sqrt{[K_s K_2]}$, cf. Lineweaver & Burk, 1934). A substrate concentration of 0.0005M was selected for routine enzyme assay.

Fig. 4 also gives a value of 38.2 μg . phenolphthalein liberated for V_m , the theoretical maximum velocity of reaction in the absence of inhibition for the particular enzyme preparation employed. This contrasts with a figure of 34.8 μg . phenolphthalein for the observed optimum velocity of hydrolysis by the preparation. The line shown in Fig. 3 was drawn

Fig. 3. The effect of varying the substrate concentration (S) on the rate of decomposition (v) of phenolphthalein glucuronide. The points were obtained experimentally whilst the line was drawn through values calculated by substituting for K_s , K_2 , n and V_m (see Fig. 4) in the equation

$$v = \frac{V_m S}{S + K_s + S^n/K_2}$$

Fig. 4. Derivation of K_s , K_2 , n and V_m by the method of Lineweaver & Burk (1934). (a) When S/v is plotted against S at low substrate concentrations, the slope is $1/V_m$ and the intercept at $S=0$ is K_s/V_m . (b) When $\log (S/v - K_s/V_m - S/V_m)$ is plotted against $\log S$ at high substrate concentrations, the slope is n and the intercept at $S=1$ is $-\log K_2 V_m$.

Fig. 5. Phenolphthalein liberated by varying enzyme concentrations under standard conditions.

Fig. 6. Phenolphthalein liberated after varying periods of incubation.

through values of v calculated by substituting for K_s , K_2 , n and V_m in the equation

$$v = \frac{V_m S}{S + K_s + S^n/K_2}$$

where v is the reaction velocity at different values of S , the substrate concentration. There was excellent

agreement between the experimentally derived points and the line given by the equation.

While the mechanism of this reaction remains a matter of conjecture the simplicity of the kinetics suggests that they are derived from a single reaction, either a simple hydrolysis of the glucuronide or the rate-determining reaction in a more complex process.

Effect of varying the enzyme concentration and the duration of hydrolysis

As shown in Fig. 5, the amount of phenolphthalein liberated in 1 hr. at 37° and pH 6.1 (phosphate buffer) from 0.0005M-phenolphthalein glucuronide was directly proportional to the concentration of rumen enzyme (stage D). Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 6 hr. (Fig. 6). Since the amounts of phenolphthalein liberated after incubation for more than 2 hr. were too great for direct reading with the Spekker absorptiometer, suitable samples of the reaction mixture were diluted to 4 ml. with phosphate buffer prior to colour development.

Action of saccharic acid

Karunairatnam & Levvy (1949) found that hydrolysis of phenylglucuronide by preparations of animal β -glucuronidase was inhibited by saccharic acid. The inhibition was 50% with 2×10^{-4} M-saccharate, and practically complete with less than 0.01M (substrate concentration 0.015M). Hydrolysis of 0.00125M-phenolphthalein glucuronide in phosphate buffer, pH 6.1, by preparations of the rumen enzyme (stage D) showed only 8% inhibition in presence of 0.01M-saccharate and 44% with 0.1M-saccharate. With mouse-liver β -glucuronidase, however, hydrolysis of 0.00125M-phenolphthalein glucuronide in acetate buffer, pH 4.5, was completely inhibited by 0.01M-saccharate. It is evident that saccharic acid causes little inhibition of the rumen enzyme compared with its effect on animal glucuronidase.

DISCUSSION

Classification of this rumen glucuronide-decomposing enzyme awaits studies of its specificity and of its possible action on polysaccharides in the diet of the sheep. These in turn require preparation of the enzyme in a comparatively pure state. It must also be shown that the sole reaction product from phenolphthalein glucuronide, apart from free phenolphthalein, is glucuronic acid. It should be borne in mind that the liberation of phenolphthalein may result, not from simple hydrolysis of the glucosidic link, but from the combined actions of a

decarboxylase and xylosidase, or some other such complex process. Whilst direct investigation of this point is contemplated, the simplicity of the kinetic data suggests that in the present instance we are dealing with a hexuronidase. Moreover, the loss in enzyme activity in passing from the whole cell suspension to the partially purified cell-free extract was smaller than might be expected for a complex system involving steps such as decarboxylation.

Since the source of this enzyme is not a pure culture, but a mixed suspension of micro-organisms, it is possible that the final activity is influenced by factors contributed by more than one type of organism. This state of affairs is, however, that obtaining in the rumen, and the present approach avoids the difficulties of interpretation which attend pure culture work in this field.

It is considered possible that the natural substrate for this enzyme is not a conjugated glucuronide such as is found in urine, but a plant polysaccharide containing uronic acid residues, or a fission product thereof. It is interesting to note that preliminary observations have been made in this Institute (Moir & Marsh, 1950) in which variations in the number of Quin's organisms (Quin, 1943) present in rumen liquor *in vivo* appeared to be correlated with variations in the activity of the glucuronide-decomposing enzyme. No evidence, however, exists to suggest that this micro-organism is particularly concerned in the early stages of hemicellulose degradation.

Whilst enriched cultures of xylose-fermenting bacteria from the rumen were unable to liberate phenolphthalein from its glucuronide, our colleagues Dr A. E. Oxford and Mr C. A. Marsh have found that they develop this property after growth on a medium containing menthyl glucuronide. The adaptive response to the presence of a conjugated glucuronide appears analogous to that shown by other micro-organisms, for example *Escherichia coli* (Buehler, Katzman & Doisy, 1949). Since there is no evidence to suggest that small bacteria of the xylose-fermenting type with the ability to decompose glucuronides normally occur in the rumen, it is not proposed to study the adaptive enzyme in question any further at present.

Consideration of the composition of skeletal polysaccharides in the plant suggests other compounds worthy of use as 'model' substrates in studying digestive enzymes in the rumen along the lines described in the Introduction. Use of one of these, phenyl β -D-glucoside, is contemplated in the immediate future.

SUMMARY

1. An intracellular glucuronide-decomposing enzyme has been prepared in cell-free state from medium-sized micro-organisms in the rumen of the sheep.

2. The optimum pH for the decomposition of phenolphthalein glucuronide by the enzyme was 6.1.

3. Irreversible inactivation of the enzyme occurred on incubation for a short period at a pH lower than 6 or higher than 7.

4. On the assumption that the rate of liberation of phenolphthalein from the glucuronide was governed by a single reaction a value of 3.05×10^{-5} M was obtained for the dissociation constant of the active enzyme-substrate complex. Excess substrate

caused pronounced inhibition of the enzyme. The inactive complex contained two substrate molecules per active enzyme centre, and the second dissociation constant was 0.0126 M.

5. Saccharate caused only slight inhibition of the enzyme in comparison with its effect on animal β -glucuronidase.

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REFERENCES

- Boswell, J. G. (1941). *New Phytol.* **40**, 20.
 Buehler, H. J., Katzman, P. A. & Doisy, E. A. (1949). *Fed. Proc.* **8**, 189.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Conway, E. J. (1947). *Micro-Diffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood.
 Elsdon, S. R. & Phillipson, A. T. (1948). *Ann. Rev. Biochem.* **17**, 705.
 Fähræus, G. (1947). *Symb. bot. upsaliens.* **9**, no. 2.
 Hungate, R. E. (1950). *Bact. Rev.* **14**, 1.
 Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
 Moir, R. J. & Marsh, C. A. (1950). Unpublished observations.
 Norman, A. G. (1937). *The Biochemistry of Cellulose, the Polyuronides, Lignin etc.* Oxford: University Press.
 Norman, A. G. & Fuller, W. H. (1942). *Advanc. Enzymol.* **2**, 239.
 Quin, J. I. (1943). *Onderstepoort J. vet. Sci.* **18**, 91.
 Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.

The Chromatographic Fractionation of Ribonuclease

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During a study of the partial hydrolysis of ribonuclease (Porter, 1950), it became necessary to separate enzymically active products from the original enzyme and other substances. In some cases the degradation appeared to be slight and little change in solubility or other physical properties seemed to have occurred. Conventional methods of protein purification, such as precipitation with salts or organic solvents proved unsuccessful, and attention was therefore turned to partition between two liquid phases. The system ammonium sulphate-water-ethanol was used very successfully by Herbert & Pinsent (1948) in the purification of bacterial catalase. In some preliminary experiments with haemoglobin (Martin, 1949), the system ammonium sulphate-water-ethylene glycol monoethyl ether ('cellosolve') was found to be less likely than most to cause denaturation. This system was therefore chosen for investigation, though ribonuclease is so stable a protein that many systems may well be satisfactory. In one case, however, the ratio of the partition coefficient of ribonuclease to

that of the aggregate of its hydrolysis products was about 3:2. This system could yield a satisfactory separation only with the aid of a counter-current extraction train of very high efficiency, or of a partition chromatogram. The latter was chosen, using kieselguhr to support the stationary phase, since cellulose, starch or precipitated silica would have probably too fine a structure to admit a protein molecule.

These columns proved very successful, and revealed the presence of two enzymically active components in the original crystalline ribonuclease.

MATERIALS

Ribonuclease was prepared from ox pancreas by the method of Kunitz (1940) and also by the modified procedure of McDonald (1948). It was recrystallized several times and was homogeneous in the Tiselius electrophoresis apparatus at pH 7.4 and 8.5.

The kieselguhr was Hyflo Supercel obtained from the Johns Manville Co. Ltd., London. It was used without pre-treatment.