there were increased amounts of oxidation products estimated by the Roe & Kuether method. Much of the injected material was not accounted for.

2. In guinea pigs, injected dehydroascorbic acid produced toxic symptoms which include atrophic changes in the fur, fatty liver and death.

3. In human subjects oral dehydroascorbic acid behaved quantitatively like ascorbic acid.

4. Dehydroascorbic acid methanolate was safely injected into human subjects. It was largely reduced to ascorbic acid. The plasma level and urinary excretion of ascorbic acid increased. Approximately 3 mg. of dehydroascorbic acid were equivalent to 1 mg. of ascorbic acid in this respect.

5. Dehydroascorbic acid injected into human subjects led in plasma and urine to a greater rise in oxidation products than in ascorbic acid. Dehydroascorbic acid did not appear in the urine.

6. Cortisone and ACTH did not modify the tissue metabolism of dehydroascorbic acid injected into guinea pigs. In particular, oxidation products of ascorbic acid did not appear in the adrenals.

7. In one human subject the amount of ascorbic acid in the urine increased with ACTH. In one subject the oxidation products increased and in another there was a small rise in dehydroascorbic acid excretion.

Our thanks are due to Dr W. A. R. Thompson who arranged a supply of dehydroascorbic acid by Messrs Boots Ltd.; to Dr A. L. Morrison of Roche Products Ltd. for supply of dehydroascorbic acid methanolate. ACTH and cortisone were supplied by the Medical Research Council. We are indebted to Dr J. M. Tanner for considerable help with the statistical analyses; to Mr A. L. Wooding for photographic assistance.

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β-Glucosidase from Rumen Liquor

PREPARATION, ASSAY AND KINETICS OF ACTION

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(*Received* 10 *March* 1954)

The most important feature of ruminant digestion is the utilization of cellulose and other fibrous plant substances. There is as yet no clear evidence of the steps occurring during the initial breakdown of these materials, but it appears probable that some form of enzymic depolymerization takes place, followed by further splitting of glycosidic linkages. This would finally result in the production of simple sugars, which could easily be transformed into the volatile fatty acids known to be the main end-products of polysaccharide digestion that are absorbed from the rumen. It seems likely that, in the case of cellulose at least, β-glucosidase plays an important part in such a scheme, and it was with this in mind that a study of this enzyme in the rumen of the sheep was undertaken.

For preliminary studies on rumen β-glucosidase, it was decided to use a chromogenic substrate, to enable the rumen liquor to be treated as one whole tissue, and thus ensure that no important active fractions are overlooked. Such a procedure was adopted successfully by Karunairatnam & Levvy (1951) in their investigation of a glucuronide-decomposing enzyme in sheep rumen. After synthesizing and testing several chromogenic β-D-glucosides, *o*-nitrophenyl β-D-glucoside was finally

selected for the work described in this paper. *p*-Nitrophenyl β-D-glucoside, which was first used by Aizawa (1939), has been employed extensively in β-glucosidase studies, mainly by Japanese workers, and *o*-nitrophenyl β-D-galactoside has been used by Lederberg (1950), who investigated β-galactosidase from *Escherichia coli*.

EXPERIMENTAL

Materials. *o*-Nitrophenyl β-D-glucoside was prepared using the method described by Seidman & Link (1950) for *o*-nitrophenyl β-D-galactoside. The product, recrystallized from ethanol, had m.p. 152° and $[\alpha]_D^{16} - 106{\cdot}6°$ in water (*c*, 1·2), properties identical with those of the compound described by Montgomery, Richtmyer & Hudson (1942).

Sodium gluconate and glucono-1:5-lactone were both obtained from British Drug Houses Ltd.; the glucono-1:5-lactone was twice recrystallized from ethanol before use: m.p. 153°; $[\alpha]_D^{18} + 63{\cdot}1°$ in water (*c*, 1·8). Glucono-1:4-lactone was prepared from calcium gluconate as described by Hedenburg (1915), and recrystallized from ethanol: m.p. 135°; $[\alpha]_D^{18} + 67{\cdot}0$ in water (*c*, 1·8). Rumen liquor, obtained as described below, was strained through nine layers of surgical gauze before use.

Enzyme assay. As a result of preliminary experiments, the following procedure was adopted. To 3 ml. of 0·2M-Na_2HPO_4–0·1M citric acid buffer (McIlvaine, 1921), pH 5·4, was added 0·5 ml. of 0·04M *o*-nitrophenyl β-D-glucoside solution (giving a final concentration in the incubation mixture of 5 mM) and 0·5 ml. of the enzyme preparation. The mixture was incubated for 1 hr. at 37°, and the reaction then stopped by the addition of 2 ml. 5% (w/v) trichloroacetic acid solution. After centrifuging at 1500 *g* for 15 min., 4 ml. of the supernatant were measured into another tube, and 1·5 ml. of 0·5N-NaOH added, followed by 2·5 ml. of 0·25M glycine–Na_2CO_3 buffer, pH 10·0. The intensity of the yellow-orange colour developed was measured in a Spekker photoelectric absorptiometer (Hilger & Watts Ltd., London, N.W. 1), using Ilford no. 601 (425 mμ.) violet filters, and the weight of liberated *o*-nitrophenol was measured from a calibration curve. Blanks were carried out for enzyme and substrate incubated separately.

Inhibition experiments. For inhibition experiments, the following modification of the above procedure was adopted. To 2·5 ml. of 0·2M-Na_2HPO_4–0·1M citric acid buffer, pH 5·4, were added 0·5 ml. of 0·04M *o*-nitrophenyl β-D-glucoside, 0·5 ml. inhibitor solution (or 0·5 ml. of water in the case of the control) and 0·5 ml. enzyme preparation. After incubation the normal assay procedure was followed. Where necessary the pH of the inhibitor solution was adjusted to 5·4 at the glass electrode before use, but this was unnecessary in the case of the lactone solutions used.

Sampling regime and diet. The animals used were Cheviot sheep fitted with permanent rumen fistulae. Their daily diet consisted of 500 g. hay at 7 a.m. and a similar quantity at 4 p.m., with free access to water and salt lick. Rumen liquor was always withdrawn at 9.30 a.m., not more than 1500 ml. being removed from each sheep at any time.

Preparation of cell-free extracts of the enzyme. The required fraction of the rumen contents was obtained by centrifuging (see below), washed 4 times on the centrifuge with NaCl (1%, w/v; 10 ml./ml. of deposit) and suspended in about 5 times its own volume of water. Washing with 1% NaCl removed no enzyme activity. The suspension was then poured, with stirring, into 4 times its volume of acetone, stirring being continued at room temperature for 40 min. After centrifuging at 1500 *g* for 6 min., the deposit was well washed with half the previous volume of acetone, and centrifuged for a further 6 min., after which it was dried overnight *in vacuo* at room temperature. The dried powder was suspended in an 0·05% (w/v) solution of the non-ionic surface-active agent Triton X-100 (Rohm and Haas Co., Philadelphia, U.S.A.) using 60 ml./g. acetone powder and, after grinding in a glass homogenizer, was extracted at room temperature, with stirring, for 4 hr. After centrifuging at 10000 *g* for 30 min. in an M.S.E. High Speed Angle-13 Centrifuge, a second extraction of the debris was carried out using half the previous volume of Triton. The supernatants in each case were recentrifuged and then combined. Fifty per cent of the total activity was extracted. It was found that Triton X-100 gave a better extraction (about 20% more) than either water alone, or buffers of varying composition and pH. Control experiments showed that the above concentration of Triton had no effect on the activity of an aqueous extract of acetone-dried powder, and did not interfere with the colour reaction for *o*-nitrophenol determination. Except where stated otherwise, all experiments described in this paper were carried out using enzyme solution prepared by this method from combined Fractions I and II (see below).

Nitrogen estimations. Digestion was carried out according to the method of Chibnall, Rees & Williams (1943). The ammonia was removed by steam distillation using the apparatus of Markham (Markham, 1942), absorbed in 2% boric acid containing methyl red and bromocresol green (Conway, 1947) and titrated with 0·01N-HCl.

RESULTS

Fractionation of rumen liquor and estimation of enzyme activity in the whole-cell fractions. To ascertain in which size of particle the main β-glucosidase activity lay, fractionation of the rumen liquor was carried out by centrifuging at various speeds for predetermined periods, and washing and assaying the fractions so obtained (Table 1). Centifuging in this manner gave a rough separation of the rumen liquor micro-organisms into protozoa (fraction I), the larger bacteria with some small protozoa (fraction II), and the smaller bacteria (fraction III). There were probably also plant fragments in all fractions (see below). The optically clear supernatant from fraction III had no activity, indicating that there was no β-glucosidase present in solution in rumen liquor. By far the greatest total activity lay in fraction II. Since further experiments, described below, showed fractions I and II to give enzyme preparations of similar properties, these fractions were in later experiments combined and used in the preparation of extracts of β-glucosidase.

Variation in activity with pH. The variation in relative activity with pH of an extract of an acetone-dried powder from fractions I and II combined is

Table 1. *Distribution of β-glucosidase activity in fractions obtained by centrifuging rumen liquor*

Strained rumen liquor (100 ml.) was separated, by centrifuging, into the fractions detailed below, each fraction being washed four times with NaCl (100 ml.; 1%, w/v) and finally suspended in water. Samples of each fraction (0·5 ml.) were assayed using the standard conditions described in the text. Fractions I and II were obtained using M.S.E. centrifuges with swing-out heads, while fraction III was obtained using an M.S.E. High Speed Angle-13 Centrifuge.

Fraction	Relative centrifugal force (*g*)	Period of centrifuging (min.)	Final vol. of fraction (ml.)	Enzyme activity: μg. *o*-nitrophenol/mg. N	Enzyme activity: Total *o*-nitrophenol liberated (μg.)
I	15	2	20	2032	5 880
II	1 500	30	100	1676	31 800
III	10 000	30	70	1323	5 040
IV	Supernatant (cell-free)				0
Strained rumen liquor (uncentrifuged)			100	608	41 100

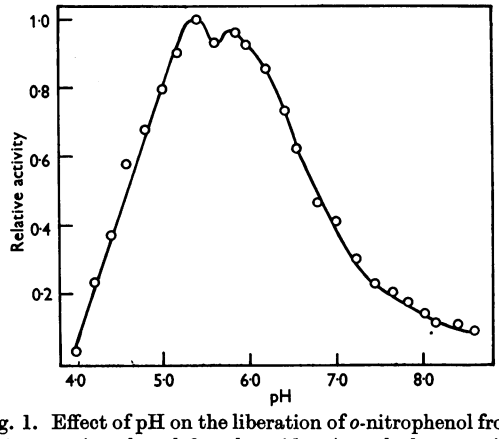


Fig. 1. Effect of pH on the liberation of *o*-nitrophenol from 5 mM *o*-nitrophenyl β-D-glucoside. A washed suspension of fractions I and II combined (see text) was used in 0·2 M-Na_2HPO_4–0·1 M citric acid buffer of appropriate pH, and the normal assay procedure described in the text carried out. The final assay sample was brought to pH 10·0 with 0·5 N-NaOH, and adjusted to a final volume of 8 ml. with 0·25 M glycine–Na_2CO_3 buffer pH 10·0. An activity of 1·0 is equivalent to 309 μg. *o*-nitrophenol liberated/hr.

shown in Fig. 1. The pH of the buffers was varied by increments of 0·2 unit within the range 3·6–8·4, the pH of each hydrolysis mixture being measured before and after incubation, and the average value taken. It was found that the pH altered only slightly during incubation. Determinations of the variation in activity with pH were also carried out on the original suspensions of all the fractions separated as described above, as well as on extracts of acetone-dried powders of each of these separate fractions. In every case the same pattern of curve was obtained, as shown in Fig. 1, with peaks of about the same relative activity at pH 5·4 and 5·8.

Most of the later experiments described were carried out at pH 5·4, though in certain cases (mentioned below) a duplicate experiment was performed at pH 5·8.

The activity of an extract of an acetone-treated powder of combined fractions I and II in H_3PO_4–NaOH, phthalic acid–KOH, citric acid–NaOH, and sodium acetate–acetic acid buffers of 0·15 M concentration at pH 5·4 was the same as that in 0·2 M-Na_2HPO_4–0·1 M citric acid buffer. A determination of the pH optimum in sodium acetate–acetic acid buffer gave the same pattern of curve as shown in Fig. 1 for Na_2HPO_4–citric acid buffer.

Variation in activity with substrate concentration. Fig. 2*a* shows the effect on the activity of the enzyme at pH 5·4 of varying the substrate concentration. The maximum activity of the preparation was reached at a substrate concentration of 5 mM. In no experiment was inhibition by excess substrate noted. The results were analysed by the graphical method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. The results in Fig. 2*a* gave a value of 0·88 mM for K_m, the dissociation constant of the enzyme–substrate complex (Fig. 2*b*). A mean value of 0·86 mM was obtained for K_m in six experiments. A determination at pH 5·8 gave a value of 0·80 mM. A substrate concentration of 5 mM was selected for routine assay purposes.

Effect of varying the duration of hydrolysis. The rate of hydrolysis of *o*-nitrophenyl β-D-glucoside is linear with time for 3–4 hr. (Fig. 3), thus showing that the preparation is stable at 37° and saturated with substrate for that period. Where necessary, the pigment was diluted with glycine buffer before measuring its colour intensity.

Effect of varying the enzyme concentration. The effect on the activity of varying the enzyme concentration over a tenfold range is shown in Fig. 4. Since the reaction velocity is proportional to the concentration, it would appear that there is no natural inhibitor or substrate present in the preparation. The presence of either would have caused a change in the slope of the activity/concentration curve, since their concentration would have varied as the enzyme concentration was altered (see Levvy & Marsh, 1954). If necessary, the pigment was diluted as described in the preceding section.

Variation in stability with pH. The variation in stability of the enzyme with pH is shown in Fig. 5. Similar curves were obtained with incubation periods of 15 min., 1 hr. and 2 hr., indicating that the variation in stability was an instantaneous effect of extremes of pH, rather than of incubation at these pH values. The enzyme was quite stable at the pH of optimum activity.

It is also clear from a comparison with the pH optimum curve (Fig. 1), that the shape of the latter is, in the lower range of pH, dependent on the fact that there is considerable inactivation there.

β-Glucosidase in the hay diet. Davidson (1954), investigating plant pigments in the rumen of sheep, found that all microbial fractions obtained by centrifuging were to a greater or lesser extent contaminated by pigmented plant particles from the diet. In view of this, the hay fed to the sheep was examined for β-glucosidase activity. A sample of the hay was ground in a hammer mill to pass a sieve of mesh 0·55 mm. The ground hay (1 g.) was suspended in water (40 ml.) and incubated for 4 hr. at 37°, after which an assay was carried out. This suspension was then put through the treatment described for rumen liquor, assays being performed

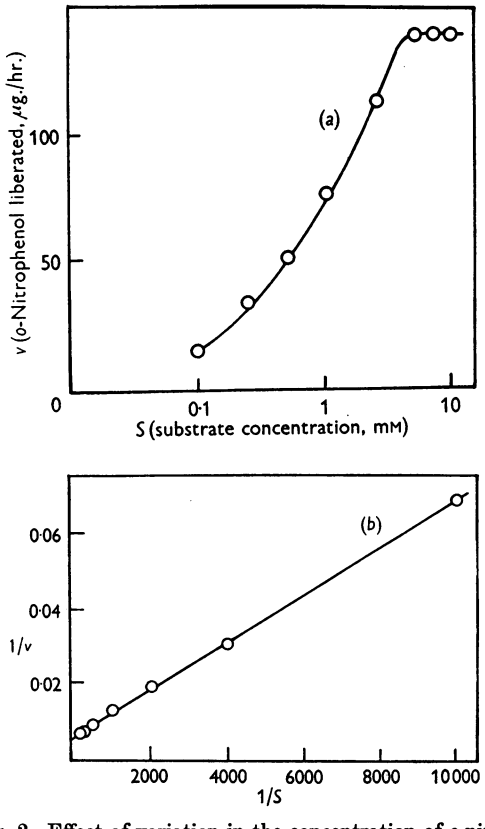


Fig. 2. Effect of variation in the concentration of *o*-nitrophenyl β-D-glucoside on the liberation of *o*-nitrophenol by rumen β-glucosidase (a cell-free extract prepared as described in text) in 0·2M-Na_2HPO_4–0·1M citric acid buffer, pH 5·4, after incubation for 1 hr. at 37°. (*a*) Plotting the substrate concentration (*S*) against the rate of decomposition (*v*). (*b*) Plotting $1/S$ against $1/v$ (Lineweaver & Burk, 1934).

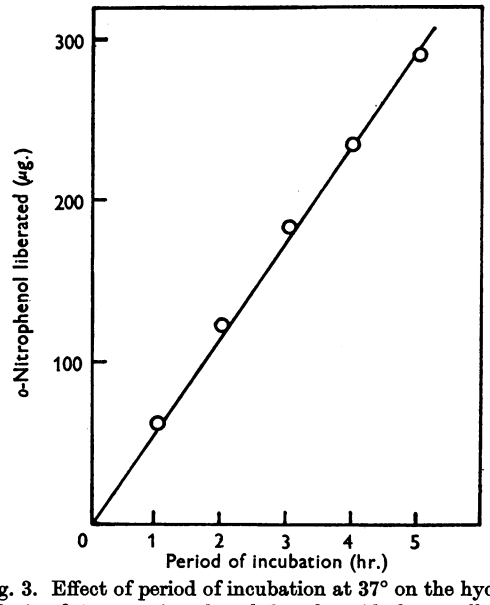


Fig. 3. Effect of period of incubation at 37° on the hydrolysis of 5 mM *o*-nitrophenyl β-D-glucoside by a cell-free extract of the enzyme in 0·2M-Na_2HPO_4–0·1M citric acid, pH 5·4. Other conditions as for standard assay procedure (see text).

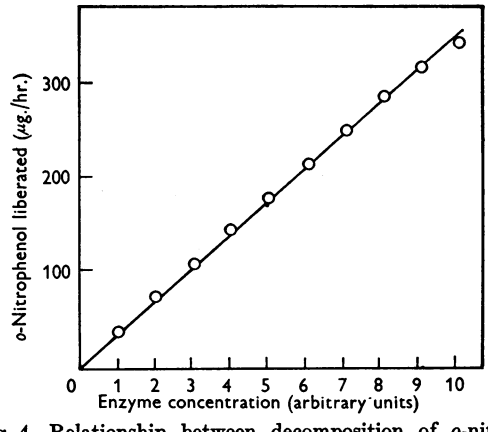


Fig. 4. Relationship between decomposition of *o*-nitrophenyl β-D-glucoside (5 mM) and concentration of the enzyme over a tenfold range. Standard conditions of assay as described in text. The activity value at concentration '10' is that of a cell-free extract obtained from an acetone-dried powder of fractions I and II combined; 0·5 ml. of this extract liberated 350 μg. *o*-nitrophenol/hr. The other concentrations were obtained by dilution of this extract, 0·5 ml. of the diluted solution being used for each assay.

at various intermediate stages (see Table 2). On washing the suspension of hay 4 times with 1% NaCl (80 ml. each time), suspending the washed deposit in 50 ml. of water, and carrying out an assay, it was found that 63% of the total activity in the original suspension had been removed. An acetone-treated powder of the suspension was prepared as for rumen liquor fractions, and the dried powder extracted with Triton X-100 (0·05%; 30 ml.). Assays were carried out on both the suspension of the whole acetone-treated powder, and on the centrifuged supernatant. The activity in this supernatant was 9·5% of that in the suspension of the whole acetone-treated powder, and 3·9% of the activity in the original hay suspension. This is in contrast to the enzyme prepared from rumen liquor, which did not pass into solution on washing the centrifugal fractions, and 50% of which could be extracted from the acetone-treated powder with Triton X-100. It would thus appear that the enzyme dealt with in the rumen liquor fractions was essentially microbial in origin.

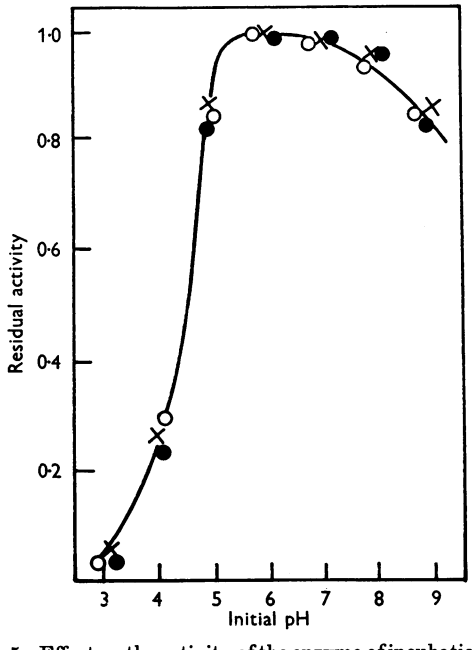


Fig. 5. Effect on the activity of the enzyme of incubation at varying pH at 37° for 15 min. (○—○), 1 hr. (×—×), and 2 hr. (●—●). A cell-free extract (4 ml.), was adjusted with HCl or NaOH to the required pH and incubated at 37° for the above periods of time, after which the solution was adjusted to pH 5·4, the volume made up to 10 ml., and the standard assay procedure carried out (see text) using 0·5 ml. of the solution. The results are expressed as fractions of the activity of a similarly diluted solution of a cell-free extract which was not incubated. An activity of 1·0 corresponds to 292 μg. *o*-nitrophenol liberated/hr.

Inhibition by gluconolactones

Both glucono-1:4-lactone and glucono-1:5-lactone were found to be strong inhibitors of rumen β-glucosidase activity. Measurements of enzyme activity were carried out in the presence and absence of 0·1 mM solutions of glucono-1:4-lactone and glucono-1:5-lactone, using concentrations of substrate varying from 0·1 to 7·5 mM. The results were analysed by the graphical method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$ (Fig. 6). In both cases, the lines obtained in presence and absence of inhibitor had a common intercept $1/V$ (V = maximum velocity), indicating that inhibition was competitive. Determination of K_i, the dissociation constant of the enzyme–inhibitor

Table 2. *Measurement of β-glucosidase activity in hay*

Suspension of milled hay (1 g.) in water (40 ml.) incubated for 4 hr. at 37°, and then centrifuged and sediment washed with 1% NaCl (4 × 80 ml.). Resuspended in water (50 ml.) and precipitated with acetone (200 ml.). Acetone-treated powder suspended in Triton X-100 solution (30 ml.; 0·05%) and solid centrifuged off. Assays carried out on various fractions under standard conditions described in text.

Treatment	Total activity in 1 g. hay (μg. *o*-nitrophenol liberated/hr.)
Whole hay suspension in water (after 4 hr. incubation at 37°)	6160
Washed suspension (resuspended in water)	2300
Acetone-treated powder in suspension	2520
Supernatant from suspension of acetone-treated powder	240

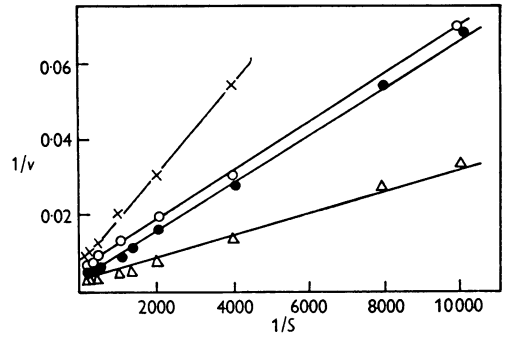


Fig. 6. Relationship between concentration of *o*-nitrophenyl β-D-glucoside and activity of the enzyme (*a*) in presence (●—●) and in absence (△—△) of 0·1 mM glucono-1:4-lactone; (*b*) in presence (×—×) and in absence (○—○) of 0·1 mM glucono-1:5-lactone, analysed by the method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. Different enzyme preparations (cell-free extracts) were used in *a* and *b*. The standard conditions for inhibition experiments (see text) were used. For units of v and S see Fig. 2.

complex gave, in the experiments shown, values of 0·08 mM for the 1:4-lactone, and 0·072 mM for the 1:5-lactone.

Mean values obtained for K_i were 0·094 mM for glucono-1:4-lactone from three experiments and 0·091 mM for glucono-1:5-lactone from three experiments. Similar values were obtained with 0·2 mM solutions of the lactones. The lactones thus each had an affinity for the enzyme about ten times that of *o*-nitrophenyl β-D-glucoside. Gluconic acid, in the form of sodium gluconate, showed only very slight inhibitory power, about 4·9% inhibition at millimolar concentration. Of the other compounds tested as possible inhibitors at millimolar concentration, saccharate, boiled and unboiled (see Levvy, 1952), showed no inhibitory power, while thiophenyl β-glucoside caused 5·4% inhibition.

Variation in inhibition with concentration of lactones. The effect on the activity of the preparation of varying the concentration of the lactones is shown in Fig. 7 which gives curves for glucono-1:4-lactone and glucono-1:5-lactone over the range 0·01 to 10 mM. Fifty per cent inhibition was obtained with concentrations of 0·25 mM glucono-1:4-lactone and 0·20 mM glucono-1:5-lactone. Similar curves were obtained when the experiments were repeated in 0·2 M-Na_2HPO_4–0·1 M citric acid buffer, pH 5·8.

Effect of time and temperature on the inhibitory power of aqueous solutions of lactones. Both glucono-1:4-lactone and glucono-1:5-lactone mutarotate when in aqueous solution, though at different rates (Hedenburg, 1915; Nef, 1914), and it was observed that the changes in rotation were accompanied by corresponding changes in inhibitory power. Fig. 8 shows the results of a series of such experiments, carried out at room temperature (19°). The solutions used for observing the changes in rotation of the lactones were, after suitable dilution, also used for the inhibition experiments.

It should be noted that although final equilibrium has not been reached in either case, the 1:5-lactone is closely approaching it, while the 1:4-lactone would require a much longer period (about 14 days, according to Hedenburg). Nevertheless, the fall in rotation, due to formation of gluconic acid, which has $[\alpha]_D - 6·7°$ (Rehorst, 1928), is followed in every case by a comparable drop in inhibitory power. The amount of lactone present at each time interval can be calculated from the percentage inhibition observed (using Fig. 7); the amounts so obtained agree well with those calculated from the observed rotations, assuming that gluconic acid is being produced, and that it has no inhibitory power. The percentage of glucono-1:4-lactone remaining after 119 hr. at room temperature was 59% (calculated from the percentage inhibition data of Fig. 7), and 63% (calculated from the rotation measurements). In the case of the glucono-1:5-lactone solution the values obtained are 18% from inhibition, and 19% from rotation measurements.

On heating a solution of either lactone to 100°, equilibrium is fairly rapidly reached, with a preponderance of 1:4-lactone and gluconic acid. Table 3 shows the changes in inhibition and rotation of solutions of both lactones over a period of 24 hr. at 100°. Again, the amounts of lactone calculated from the percentage inhibition using Fig. 7 (assuming

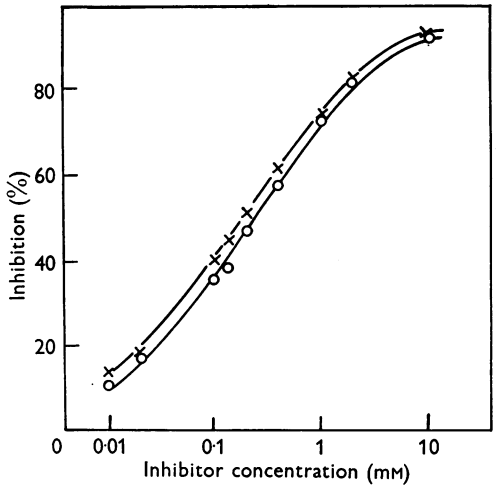


Fig. 7. Relationship between the percentage inhibition of the decomposition of 5 mM *o*-nitrophenyl β-D-glucoside and the concentration of glucono-1:4-lactone (O—O) and glucono-1:5-lactone (×—×). The enzyme preparation was a cell-free extract, and the standard conditions for inhibition experiments were used (see text).

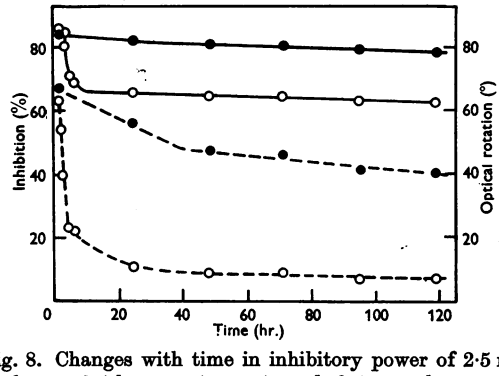


Fig. 8. Changes with time in inhibitory power of 2·5 mM glucono-1:4-lactone (●—●) and 2·5 mM glucono-1:5-lactone (O—O) and rotation of glucono-1:4-lactone (●---●) (*c*, 1·8 in water) and glucono-1:5-lactone (O---O) (*c*, 1·8 in water) at room temperature (19°). The enzyme preparation was a cell-free extract prepared as described in the text. In the inhibition experiments the solutions used in measuring optical rotation of the lactones were diluted to give a final incubation concentration of 2·5 mM. The standard conditions for inhibition experiments (see text) were used.

Table 3. *Changes with time in inhibitory power and rotation of aqueous solutions of glucono-*1:4*-lactone and glucono-*1:5*-lactone at* 100°

For inhibitory power tests the solutions used in measuring optical rotation were finally diluted to millimolar concentration and the standard procedure for inhibition experiments (see text) was used. The enzyme preparation was a cell-free extract of Fractions I and II combined. Calculations from the inhibition determinations (using Fig. 7) of the percentage of lactone remaining after 24 hr. at 100°, give values of 66% for both the glucono-1:4-lactone and glucono-1:5-lactone solutions. From the rotation measurements values of 65% for the 1:4-lactone solution and 61% for the 1:5-lactone solution are obtained. These figures are based on the assumption that the main lactone present after 24 hr. at 100° is the 1:4-lactone (Hedenburg, 1915).

	Glucono-1:4-lactone		Glucono-1:5-lactone	
Time (hr.)	Inhibition (%)	$[\alpha]_D^{18}$ (*c*, 1·8)	Inhibition (%)	$[\alpha]_D^{18}$ (*c*, 1·8)
0	71·6	+67·0°	77·0	+63·1°
1	67·2	—	66·9	—
2	67·3	—	66·8	—
3	66·7	—	66·2	—
4	66·9	—	66·7	—
5	67·0	—	66·8	—
17	66·9	—	67·0	—
24	66·8	+40·9°	66·7	+38·0°

inhibition to be due to the 1:4-lactone) are in good agreement with those calculated from the observed rotation. Gluconic acid on heating at 100° would be expected to attain the same equilibrium, and though on heating a solution of sodium gluconate with an equivalent of HCl at 100° for 24 hr. neither the percentage inhibition nor the rotation was as high as that obtained on heating the lactones, there was, nevertheless, a notable increase in inhibitory power, and agreement with the rotation. A millimolar solution of sodium gluconate, which before heating with HCl caused 4·9% inhibition, after 24 hr. heating at 100° caused 60·8% inhibition and had $[\alpha]_D^{16} + 23·1°$ in water (*c*, 1·8, calculated as lactone). Presumably equilibrium had not yet been finally established. Early attempts to obtain solutions of the 1:4-lactone by heating sodium gluconate solutions with HCl showed little or no increase in inhibition, and led to a false conclusion regarding the inhibitory power of the 1:4-lactone published in a preliminary communication (Conchie, 1953).

DISCUSSION

Of the total β-glucosidase activity demonstrated to be present in the rumen by the use of *o*-nitrophenyl β-D-glucoside, only the enzyme in the larger particles has been studied in detail, though preliminary observations on the remaining fraction indicate that it has similar properties. By neglecting this fraction for the time being, larger quantities of rumen liquor could be dealt with. Moreover, the fractions examined contain by far the greater proportion of the total activity.

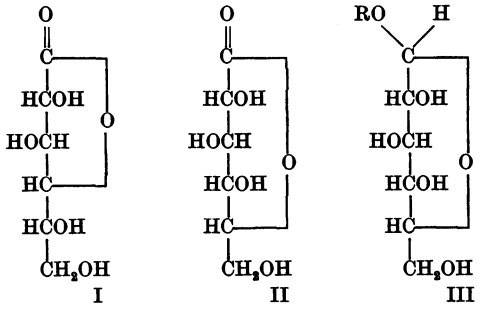
Criticism of the use of *p*-nitrophenyl β-D-glucoside as a means of estimating total enzyme activity has come from Jermyn (1952). After applying the techniques of filter-paper electrophoresis and paper chromatography to test the homogeneity of β-glucosidases from *Aspergillus oryzae* he found that the preparation consisted of a number of components, none of which had absolute specificity for any one substrate. On the other hand, only one of these components would hydrolyse *p*-nitrophenyl β-D-glucoside, and this appeared to be part of a separate system, not necessarily related to the other β-glucosidases. Niwa (1943), investigating the specificity of β-glucosidase preparations from animal viscera, studied the activities of preparations obtained under varying conditions from ox liver, and found that while the activity on salicin always varied parallel to that on phenyl β-glucoside, the activities on *p*-nitrophenyl and β-naphthyl β-glucosides varied irregularly. In the case of horse-kidney β-glucosidase, he obtained a different pH optimum using *p*-nitrophenyl β-glucoside compared with that obtained using salicin and phenyl β-glucoside.

While it is true that, in some cases at least, the nitrophenol colorimetric method does not seem to indicate all the β-glucosidase activity present in a preparation, it is nevertheless useful in a preliminary survey. The above evidence merely emphasizes that it would be unwise to carry out too extensive a study of the enzyme relying solely on this method of assay. An indication that the preparation discussed in this paper is not solely an aryl β-glucosidase, but also capable of hydrolysing cellobiose, is given by the fact that a 10 mM solution of cellobiose causes about 30% inhibition of the hydrolysis of *o*-nitrophenyl β-D-glucoside. It was considered inadvisable to carry out measurements of the liberation of reducing sugar until some further purification of the preparation had been done. This is in progress.

Until recently it was considered that particles in rumen liquor brought down at relatively high centrifuge speeds consisted essentially of micro-organisms of various types. However, Davidson (1954) has shown by measurement of their plant pigment content, that all centrifugal fractions in rumen liquor, even those composed of the smallest particles, contain plant fragments derived from the diet. In the present connexion, this introduced the possibility that plant enzyme might contribute to the total β-glucosidase activity in the rumen fractions. Accordingly, investigations on the β-glucosidase activity of the hay fed to the sheep were carried out.

A whole suspension of hay showed β-glucosidase activity, a large proportion (63%) of which could be removed by washing with 1% NaCl solution. Only a very small proportion of the enzyme activity in an acetone-treated powder prepared from the hay suspension could be extracted with an 0·05% (w/v) solution of Triton X-100. Since the enzyme in the fractions prepared from rumen liquor by centrifuging was not extracted by 1% NaCl solution, but was largely extractable, after acetone treatment, by 0·05% Triton X-100, it would appear to be of microbial origin.

In view of the fact that the β-glucosidase activity of hay could be extracted with 1% NaCl solution, it is remarkable that the supernatant obtained after freeing rumen liquor from all particulate matter showed no β-glucosidase activity, since the β-glucosidase present in the rumen is stable at the pH of rumen liquor. This absence of activity in the supernatant must mean that any β-glucosidase present in the hay eaten by the sheep is rapidly adsorbed, absorbed or metabolized by the rumen micro-organisms as soon as it goes into solution.



Levvy (1952), studying inhibitors of β-glucuronidase, found that while saccharo-1:4-lactone was an inhibitor, saccharic acid was not, and suggested that this was due to the fact that the general formula for a β-D-glucuronide more closely resembled that for saccharo-1:4-lactone than saccharic acid, having a ring system involving $C_{(1)}$ and a carboxyl group on $C_{(5)}$. A similar comparison might be made in the case of β-D-glucosides (III) and the lactones of gluconic acid (I and II). Since both lactones have about the same degree of affinity for the enzyme, it would appear that, while a ring system is necessary, it can involve $C_{(4)}$ or $C_{(5)}$ without appreciable difference in effect. Saccharo-1:4-lactone had no inhibitory effect on rumen β-glucosidase, so a primary alcohol group on $C_{(6)}$ is probably a requisite for inhibition of this enzyme.

Ezaki (1940), investigating the β-glucosidases of takadiastase and almond emulsin, found that gluconic acid, and its 1:4-lactone, were inhibitors of β-glucosidase activity in takadiastase, but that gluconic acid did not inhibit the β-glucosidase activity of emulsin. This work was further extended by Horikoshi (1942), who found that the β-glucosidase of emulsin was inhibited by glucono-1:4-lactone. On the basis of inhibitor studies these authors found that β-glucosidase preparations from various sources were of two types—the 'emulsin' type or the 'taka' type. Neither author appears to have tested the 1:5-lactone.

The most interesting feature of the inhibition experiments described in this paper is the prospect that not only β-glucosidases, but β-glycosidases and possibly glycosidases are in general inhibited by the aldonolactones of configuration corresponding in the secondary hydroxyl groups to the sugar residues and with identical groups at $C_{(6)}$.

SUMMARY

1. A β-glucosidase preparation, essentially microbial in origin, has been obtained as a cell-free extract from sheep rumen contents, and its properties have been investigated using *o*-nitrophenyl β-D-glucoside as substrate.

2. The preparation has two pH optima, at pH 5·4 and pH 5·8 respectively.

3. A value of 0·86 mM was obtained for K_m, the dissociation constant of the enzyme–substrate complex, for *o*-nitrophenyl β-D-glucoside.

4. The enzyme is stable between pH 5 and 7, and is stable at the temperature of assay, 37°, for 4 hr.

5. Both glucono-1:4-lactone and glucono-1:5-lactone are strong competitive inhibitors of rumen β-glucosidase activity, having an affinity for the enzyme about 10 times that of *o*-nitrophenyl β-D-glucoside. Gluconic acid has no appreciable inhibitory effect.

6. The changes in inhibitory power of the two lactones in aqueous solutions at room temperature and 100° can be correlated with the structural changes occurring in the lactones.

The author wishes to express his thanks to Dr G. A. Levvy for his interest and encouragement, and for his suggestion of gluconolactone as a possible inhibitor, to Dr C. A. Marsh for the sample of thiophenyl β-glucoside, and to Miss E. F. Wallace and Mr I. Strachan for technical assistance.

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The Action Pattern of Potato Phosphorylase

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(*Received* 15 *April* 1954)

The action of phosphorylase is expressed by the equation

$$n \text{ (glucose 1-phosphate)} + \text{primer} \rightleftharpoons \text{amylose} + n \text{ (orthophosphate)}.$$

The enzyme belongs to the group of carbohydrate-transferring enzymes, the function of which is the transfer of sugar residues from the donor substrate (in this case, glucose 1-phosphate or amylose), either singly or severally to an acceptor substrate containing a hydroxyl group (primer or orthophosphate).

The present investigation is concerned with two problems which are common to these transferring enzymes. The main problem is the elucidation of the mode of action of the enzyme, but this is bound up with the problem of the function of the maltodextrins (maltosaccharides) in the forward (synthetic) reaction. Confining the attention to this synthetic reaction two patterns of action may be envisaged. These are described as 'single-chain' and 'multi-chain'. In the former, a molecule of phosphorylase continuously increases the length of a single primer chain until the molecule becomes immune to further enzyme action (e.g. by precipitating from solution), before transferring its activity to a second molecule of primer. In the multi-chain type of action random synthesis occurs and all the primer chains grow at approximately equal rates. This seems the more probable mechanism and has generally been assumed in the past (e.g. see Swanson, 1948), Bernfeld & Meutémédian (1948), however, favoured single-chain action. If it could be shown that multi-chain synthesis occurred, then by the use of primer molecules of known size, the synthesis of linear chain molecules of any predetermined average length could be accomplished.

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These substances would provide the material for studies of the variation in physical and chemical properties in a homologous series of polymers and of the action patterns of other enzymes. In this paper it is established that synthesis by phosphorylase follows the multi-chain pattern, and synthetic amyloses have been prepared for use in studies of variation in iodine stain with chain length (Whelan & Bailey, to be published) and of the action patterns of Q-enzyme (Peat, Whelan & Bailey, 1953) and β-amylase (Whelan & Bailey, to be published). Through the initiative of Mould & Synge (1954) they have also been used in developing a new method of fractionating large molecules.

The second problem required a study of the behaviour of maltodextrins as primers in the synthetic reaction. Hanes (1940) first showed that the synthetic reaction did not proceed in the absence of starch, dextrins or maltose. Green & Stumpf (1942) failed to detect priming action with maltose but confirmed Hanes's other observations. Weibull & Tiselius (1945) hydrolysed a starch dextrin (produced amylolytically) with acid and fractionated the products on charcoal using aqueous ephedrine as eluting agent. They found that the product of lowest molecular weight to exhibit priming activity was the trisaccharide, maltotriose. We have re-investigated the priming activity of the maltodextrins using material prepared as described by Whelan, Bailey & Roberts (1953). It will be shown that the choice of primer can exercise a profound influence on the polydispersity of the synthetic amylose; if maltotriose had been the only primer used in the study of action pattern the conclusion might well have been that single-chain synthesis was taking place. Maltotriose is unique among the maltodextrins in respect of the action on it of starch-metabolizing enzymes. It is very slowly attacked by β-amylase (Whelan *et al.* 1953)