Cellulolytic preparations from Micro-organisms of the Rumen and from *Myrothecium verrucaria*

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SUMMARY: A study has been made of the degradation of different forms of cellulose ('cellulolysis'), comprising soluble substituted derivatives (carboxymethyl-cellulose), insoluble cellulose powder, swollen cellulose, hydrocellulose and native cotton fibres, by concentrated suspensions of mixed rumen micro-organisms and by the aerobic fungus *Myrothecium verrucaria*. Mixed rumen micro-organisms are shown to be one of the most powerful sources of cellulolytic enzyme, in that they produce almost complete solubilization of all the above forms of cellulose in a relatively short period (3 days). Enrichment cultures of rumen micro-organisms were prepared by growing concentrates of mixed micro-organisms on cellulose powder. Cellulolysis was followed by determining the cellulose disappearance, formation of cellulolytic activity and gas evolution. Freeze-dried powders and their derived acetone powders obtained from washed concentrated suspensions (non-enrichment cultures) of mixed rumen micro-organisms solubilized up to 80 % of insoluble cellulose powder. Cell-free extracts were isolated: (a) from concentrated suspensions and from freeze-dried powders of rumen micro-organisms by extraction with butanol; (b) from concentrated suspensions of enrichment cultures by grinding with alumina at low temperature. The butanol extracts solubilized cellulose powder to a small extent (10 % solubilization) and were more effective against the soluble carboxymethyl-cellulose. In contrast, the alumina extracts were more active against insoluble cellulose (30 % solubilization) but were only weakly active against carboxymethyl-cellulose. Cell-free filtrates from *Myrothecium verrucaria*, an aerobic fungus much used in work on cellulose metabolism, were shown to possess cellulolytic properties very similar to those of alumina extracts from enrichment cultures of rumen micro-organisms. The significance of the results is discussed in relation to the mode of breakdown of cellulose.

Despite much work, little appears to be known about the conditions governing the biological breakdown of cellulose. Many workers have obtained extracts, from animal, vegetable and microbial sources, with only feeble activity on insoluble forms of cellulose, and have turned to using as substrates extensively degraded cellulose or even the soluble substituted cellulose derivatives. Whilst such substrates usually prove to be more susceptible to hydrolysis, they leave the primary question unanswered, namely, how is true or insoluble cellulose hydrolysed for utilization by a select group of micro-organisms? Numerous examples of 'cellulase' preparations which fail to attack insoluble cellulose with any significance are given in an excellent monograph by Siu (1951). More recent cases are quoted by Halliwell (1957) where calculations show that hydrolysis of cellulose powder or cotton never exceeds more than about 6 % of the substrate.

Despite the feeble action demonstrated by these extracts, the parent organism, whether fungus or bacterium, is often capable of producing complete

solubilization of most forms of cellulose. This difference has been attributed to the presence of at least two enzymes, the first of which attacks more complex and insoluble molecules and thus renders them sensitive to degradation by a further enzyme (Reese, Siu & Levinson, 1950). Hence, truly cellulolytic micro-organisms containing the two (or more) enzymes are capable of hydrolysing native cellulose as well as degraded celluloses and the soluble cellulose derivatives, e.g. carboxymethylcellulose. Non-cellulolytic organisms are said to lack this primary enzyme and hence fail to attack insoluble cellulose, although soluble cellulose derivatives are often readily hydrolysed. On the other hand, Whitaker (1953) believed that a single enzyme is responsible for hydrolysis of native cellulose and carboxymethylcellulose. However, his results with a purified concentrate of cellulase from *Myrothecium verrucaria* indicate not more than 2% hydrolysis of cotton linters no matter how much enzyme was added.

In rumen cellulolysis the problem appears to be further complicated by the necessity for anaerobiosis. No one has yet obtained extracts or even pure strains of micro-organisms which exhibit significant cellulolysis of undegraded cellulose. Invariably, workers have employed hydrolysed and degraded forms of cellulose.

In work on cellulose degradation in the rumen it is preferable to employ mixed micro-organisms rather than isolated pure strains because (1) there is less divergence from *in vivo* cellulolytic activity, and (2) our mixed cultures, unlike pure strains of other authors, act relatively rapidly and completely on all forms of insoluble and undegraded cellulose tested by us (possibly because more than one strain of micro-organism is essential for complete cellulolysis).

In the work presented here an attempt has been made to determine the conditions controlling the hydrolysis of cellulose and the production of 'cellulase'. To this end we have used two sources of enzyme: (1) the anaerobic system of mixed rumen micro-organisms; (2) the aerobic fungus, *Myrothecium verrucaria*. Cellulolysis has been determined gravimetrically from the loss in weight of the residual cellulose rather than by determination of the increase in reducing sugar value. The latter procedure, as noted in the Discussion, can lead to false and high values for hydrolysis.

METHODS

Substrates. These were identical to those reported earlier (Halliwell, 1957). Sodium carboxymethylcellulose, a soluble cellulose derivative with a degree of substitution of about 0.5, was generously supplied as Cellofas B by Imperial Chemical Industries Ltd.

Source of cellulase. Rumen liquor (R.L.), rumen liquor supernatant fluid (R.L.S.) obtained by high speed centrifugation and rumen liquor concentrates (R.P.) were prepared as described by Halliwell (1957).

Preparation of enrichment cultures of rumen micro-organisms. A bicarbonate + salts medium (see Halliwell, 1957), with cellulose powder (Whatman),

sodium sulphide for anaerobiosis, R.L.S. and yeast extract to supply additional nutrients, and an inoculum of an eight-fold concentrate (R.P.) of rumen micro-organisms, were incubated at 37° under CO₂ for the times stated in the text. The fermentation vessel consisted of a conical flask fitted with a Bunsen valve. For gas analysis experiments, the exit tube of the valve was replaced by a direct glass connexion to an inverted measuring cylinder over dilute hydrochloric acid. The composition of the medium is tabulated below:

Composition of enrichment medium (% w/v): NaHCO₃, 1.47; KCl, 0.04; KH₂PO₄, 0.018; MgSO₄·7H₂O, 0.03; (NH₄)₂HPO₄, 0.09; CaCl₂·6H₂O, 0.06; Cellulose powder (Whatman) 0.4, 1.2, 2 or 3.0; Yeast extract, Difco Bacto, 0.03; Na₂S, 9H₂O, 0.02; R.L.S., 20 (v/v); Inoculum, eight-fold concentrate of R.L. micro-organisms, 5 (v/v); pH value with CO₂, adjusted to 6.8.

Preparation of enzyme extracts from enrichment cultures of rumen micro-organisms using alumina. Extracts capable of producing appreciable hydrolysis of insoluble cellulose were obtained by centrifuging enrichment cultures, after a suitable period of fermentation, at 20,000 g for 20 min. at 1° and washing the precipitated organisms with 0.02 % Na₂S, 9H₂O (pH 7.2). The rumen liquor concentrate thus obtained was cooled to 0° for 1 hr. and then ground in portions at that temperature in a previously cooled mortar with a total of 3 times its weight of dried washed alumina for 15 min. (McIlwain, 1948). The ground powder was then extracted with 3 vol. of sulphide + phosphate buffer (0.02 % w/v, Na₂S, 9H₂O in 0.067 M-Na₂HPO₄ + 0.067 M-KH₂PO₄, pH 7.2) centrifuged at 25,000 g for 30 min. at 1°, the supernatant fluid removed and centrifuged for 20 min. The final optically clear yellow supernatant fluid (pH 7.2) possessed appreciable activity with insoluble cellulose powder (Whatman, C.P.W.), up to 30 % being solubilized, but the preparation had only feeble activity (compared with the butanol preparation described below) on carboxymethylcellulose.

Preparation of enzyme extracts from rumen micro-organisms with butanol. Morton's technique (1950) was adapted to prepare enzyme extracts from rumen liquor concentrates (R.P.). The latter, obtained by centrifugation as in the previous paper, were cooled to 1° and stirred at that temperature with about 0.4 vol. of *n*-butanol (A.R.) for 15 min., after which the whole suspension was centrifuged at 20,000 g for 20 min. at 1°. The aqueous phase containing butanol was carefully separated from over- and under-lying layers and re-centrifuged to provide a clear orange-coloured supernatant fluid (pH 7.6). This enzyme preparation exhibited appreciable activity on carboxymethylcellulose (C.M.C), but did not cause more than 10 % hydrolysis of cellulose powder (Whatman) and did not attack cotton fibres at all.

Preparation of freeze-dried powders from rumen micro-organisms and of derived acetone powders. These powders retained much of the true cellulolytic capacity of the parent micro-organisms, and were more effective than either butanol extracts or alumina extracts, in that they produced almost complete solubilization (up to 80 %) of cellulose powder (Whatman). The preparation of this freeze-dried powder is outlined below, where 'recovery of activity' refers to the total 'cellulase' value (proportional to the weight of cellulose solubilized

by unit original volume) as determined by the standard assay on cellulose powder in bicarbonate buffer (see below):

Centrifuge Rumen liquor at 1800 g, 1°, 30 min.	
Precipitate 1 (17 % recovery of activity): suspend in 0.02 % Na ₂ S, 9H ₂ O (pH 7.1) stir gently to wash, centrifuge as above for 55 min.	Supernatant fluid of large volume but of low acti- vity; rejected
Precipitate 2 (60 % recovery of activity of precipitate 1): suspend in 0.02 % Na ₂ S, 9H ₂ O (pH 7.1) adjust to pH 6.9 and freeze dry	
Freeze-dried powder (100 % recovery of activity of precipitate 2 or 11 % recovery of activity of rumen liquor); Suspend in redistilled acetone at 1° (30 vol.), stir at 1° for 20 min.; centrifuge at 1800 g. for 15 min. at 1°	
Precipitate of acetone powder: suspend in 0.02 % Na ₂ S, 9H ₂ O (pH 7.1) to assay cel- lulase (50 % recovery of activity of freeze dried powder)	Supernatant fluid rejected

Myrothecium verrucaria cultures (I.M.I. 45541, Commonwealth Mycological Institute, Kew, Surrey) were grown on the salt medium of Saunders, Siu & Genest (1948) with cellulose powder (Whatman) as substrate. The cell-free culture fluid itself, unlike that of rumen liquor, possessed true cellulolytic activity against cellulose powder, without recourse to extraction of the organism. The fluid was first filtered through a grade 3 sintered glass filter to remove undissolved cellulose and mycelium, then centrifuged at 20,000 g for 30 min. at 1°, and subsequently sterilized by filtration through a sintered glass (5/3) bacteriological filter.

Determination of the cellulase activity of micro-organisms and of enzyme extracts. (a) Rumen micro-organisms and extracts were examined for 'cellulase' by the assay method described earlier (Halliwell, 1957), except that lyophilized powders, acetone powders and enzyme extracts were incubated for 72 hr. instead of 40 hr. as with organisms. (b) *Myrothecium verrucaria* cultures and enzyme filtrates were assayed in Sørensen phosphate buffer (pH 6.0; 0.067 M-Na₂HPO₄ + 0.067 M-KH₂PO₄) with 30 or 50 mg. cellulose at 30° or at 50° in test tubes, 15 mm. × 150 mm., or in 25 ml. conical flasks. The total volume was made up to 6 ml. including 1 ml. or 2 ml. of microbial suspension or filtrate.

'Cellulase' unit. For comparative purposes in the text a 'cellulase unit' may be defined as the minimum amount which in an extract or suspension produces *c.* 70 % cellulolysis under standard conditions. One ml. of rumen liquor from hay-fed sheep, which hydrolyses about 70 % of the standard amount of cellulose powder in 40 hr. (Halliwell, 1957; Table 1), is the minimum volume with this activity. Concentrate-fed sheep may show much less activity in this period.

Method of determining degree of cellulose hydrolysis. This was done by gravimetric estimation of the residual cellulose (Halliwell, 1957). Control cellulose determinations containing insoluble cellulose and all reagents without micro-organisms or extracts therefrom, showed no loss of cellulose in periods up to 7 days at 37°. Assay tubes containing extracts of organisms and incubated overnight or longer were examined for the growth contamination of bacteria as films treated by Gram's method and by inoculation of samples into fresh cellulose + buffer mixture. The results were always negative. Further evidence of freedom from bacteria was shown by the constancy of pH value of the buffers used. Rumen micro-organisms produce a rapid change in pH value of the buffer in less than 15 hr. (see Results).

Cellulose weights. In the text initial cellulose weights are referred to as ' % concentration'; this indicates g. insoluble cellulose/100 ml.

Reducing sugar determinations. Hydrolysis of carboxymethylcellulose (C.M.C.) to sugars was followed by a modification of the colorimetric procedure of Folin & Malmros (1929) adapted to the Spekker photoelectric absorptiometer (Hilger).

RESULTS

Properties of concentrates of rumen micro-organisms

In order to obtain concentrated preparations for enzymic investigations, rumen micro-organisms were centrifuged at 20,000 *g* for 20 min. (preferably at 1°) the supernatant fluid rejected and the precipitated organisms (R.P.) preserved at 1° until used. No loss of 'cellulase' occurred in R.P. stored at this temperature up to 3 days. Some of the general properties of R.P., particularly in relation to the instability of its true cellulase, have been described earlier (Halliwell, 1957, Table 1). Several washing methods were examined by the cellulase assay procedure in order to determine the most suitable way to obtain relatively clean preparations which were active against cellulose. As Table 1 shows, the most suitable process for obtaining concentrated washed suspensions of organisms involved the use of 0.02 % (w/v) sodium sulphide in 0.16 *M*-sodium chloride (pH 6.9). The consistently low value obtained by washing with cysteine + phosphate buffer is possibly an effect associated with potassium phosphate in absence of ammonium phosphate (which supports growth) at this pH value. A similar effect was observed with phosphate as a washing medium before freeze-drying of concentrated suspensions of rumen micro-organisms. The effect was not examined further; it may be noted that Sijpesteijn & Elsdon (1952) found that dipotassium hydrogen phosphate exerted an inhibitory action on the muscle succinic dehydrogenase system.

The 'cellulase' of rumen micro-organisms appears to be extremely unstable, as observed in attempts at concentration which involved exposure of large surfaces to the air or to elevated temperatures. Thus, representative samples of rumen liquor were treated as follows: (1) separate samples were centrifuged successively in a single centrifuge tube at 15000 *g* for 30 min. (uncooled; initial temperature of 18°); the supernatant fluid was rejected and the precipitated micro-organisms covered with fresh rumen liquor and re-centrifuged; (2) the

samples were treated in separate centrifuge tubes by centrifuging as above, after which the supernatant fluid was rejected and the precipitate stored at 1°; (3) samples were treated exactly as in (2), but at 1° throughout and with 0.02 % sulphide added to the rumen liquor. Each treatment was repeated and the precipitated organisms pooled until a three-fold concentration of the original rumen micro-organisms had been achieved. The pooled concentrates from each treatment, when examined by the standard cellulase assay, gave 4, 60 and 90 % hydrolysis of cellulose respectively, and thus indicated the advantages to be gained by maintaining a low temperature and reducing conditions.

Table 1. *The effect of washing procedures on recovery of cellulase activity from micro-organisms deposited (R.P.) by centrifugation of rumen liquor*

R.L.S. = supernatant fluid after centrifugation of rumen liquor

Treatment	(NH ₄) ₂ HPO ₄ absent	(NH ₄) ₂ HPO ₄ present
	Cellulolysis* (by standard assay method) (%)	
(1) Organisms resuspended in R.L.S.	Not done	85
(2) Organisms resuspended in NaHCO ₃ + salts buffer containing 0.02 % Na ₂ S (pH 6.8)	Not done	70
(3) Organisms resuspended in NaHCO ₃ + salts buffer containing 0.02 % Na ₂ S (pH 6.8) after:		
(a) Washing with Sørensen phosphate buffer (pH 6.9) and recentrifuging	44	44
(b) After washing with 0.004 M- cysteine in Sørensen phosphate buffer (pH 6.4)	19	71
(c) After washing with 0.004 M- cysteine + 0.16 M-NaCl (pH 6.4)	43	61
(d) After washing with 0.02 % Na ₂ S + 0.16 M-NaCl (pH 6.9, 6.4)	66	81

* Cellulolysis corrected for endogenous blank of deposited organisms.

*Action of concentrated preparations of micro-organisms
on various forms of cellulose*

Fig. 1 illustrates the capacity of concentrated preparations of micro-organisms from rumen liquor (R.P.) to hydrolyse most forms of cellulose, from the degraded forms such as hydrocellulose to chemically untreated cotton fibres. Hydrocellulose and cellulose powder (Whatman) were hydrolysed to about the same degree (90 %), with a higher initial rate of attack on cellulose powder. Swollen cellulose powder was attacked even more rapidly, but ultimately left a resistant residue amounting to c. 20 % of the initial cellulose. In contrast, cotton fibres underwent only about 70 % solubilization in the same period (70 hr.). Dewaxing of the fibres by ethanol-ether-NaOH extraction gave no significant increase in the rate of cellulolysis. In all cases the initial rapid cellulolysis was followed by a much slower phase approaching complete degradation of substrate. The pH changes during the fermentation

are shown in Fig. 2. Hydrolysis of the soluble cellulose derivative carboxymethylcellulose to sugars by R.P. was difficult to follow except in presence of bacterial growth inhibitors to prevent conversion to fatty acids. However, as shown below, cell-free extracts of R.P., obtained by butanol treatment, readily attacked carboxymethylcellulose (Fig. 3).

Fig. 1

Fig. 2

Fig. 1. Action of rumen liquor concentrates (R.P.) on different forms of cellulose. Standard cellulase assay. Cellulose solubilized as % of initial weight; cotton fibres Δ , cellulose powder (Whatman) $\square$, swollen cellulose powder (Whatman) $\blacksquare$, hydrocellulose $\blacktriangle$.

Fig. 2. Changes in pH value during fermentation of different forms of cellulose by rumen liquor concentrates (R.P.). Conditions as in Fig. 1. Cotton fibres $\blacksquare$, cellulose powder (Whatman) Δ , swollen cellulose powder (Whatman) $\square$, hydrocellulose $+$.

Cellulolytic extracts from concentrated suspensions of rumen micro-organisms (non-enriched)

Several attempts were made to obtain active cellulolytic extracts from concentrated suspensions (R.P.), after carefully adjusting conditions to pH 7.0 and 1° in presence of 0.02 % (w/v) sulphide. Grinding (for 15 min.) of such R.P. preparations in presence of R.L.S. in a glass homogenizer diminished the total cellulase value to 70 % of the value of an unhomogenized preparation, whereas repeated freezing and thawing of R.P. from -30° to $+30^\circ$, over a period of 2 days, produced no change in the total cellulolytic activity. However, both these treatments did not release enzyme to centrifuged supernatant fluid fractions. R.P. suspensions were also treated in the Mickle disintegrator, (Mickle, 1948) with ballotini beads (grade 12; Chance Bros. Ltd.,) at pH 6.2 for 30 min. at 1° , and provided material with up to four-fold the activity of the initial R.P. suspension, whereas similar treatments at pH 8.2 retained only 30 % of the total original cellulase activity. Acetone powders from R.P.,

prepared in the usual manner, resulted in almost complete loss of cellulolytic activity when assayed on cellulose powder (Whatman) or on hydrocellulose. The supernatants centrifuged at 20,000g from the Mickle disintegrates never gave more than 5% hydrolysis of cellulose powder (Whatman), whilst an aqueous extract from the acetone powder was completely inactive. Grinding of R.P. with alumina at 1° and extraction with Sørensen phosphate buffer containing 0.02% (w/v) sodium sulphide over the pH range 6.7–7.5 provided optically clear yellow extracts which were devoid of cellulase activity. Butanol preparations of R.P. (see Methods) were more successful, but such extracts never gave more than about 10% hydrolysis of cellulose powder (Whatman), although they acted quite rapidly on the soluble cellulose derivative carboxymethylcellulose (25% hydrolysis of susceptible links in 18 hr.; Fig. 3).

Preparation and properties of freeze-dried powders from rumen micro-organisms and of derived acetone powders

By careful control of conditions it was possible to obtain a 'standard' freeze-dried powder, and from this an acetone powder, in both of which the 'cellulase' was sufficiently potent to solubilize up to 83% of the initial amount of cellulose powder (Whatman) (see 'Methods' for preparation). To obtain active preparations it was necessary to complete the separations at 1° and to avoid undue exposure to air (0.02%, w/v, Na₂S, 9H₂O was used in washing procedures). On the large scale (4 l.) described under Methods, much cellulase activity was lost in the primary low speed (1800g) centrifugation. Some of this activity was recovered by centrifugation of the rejected supernatant fraction at high speed, but the minor improvement in specific activity (cellulase activity/g. dry weight freeze dried powders) of the ultimate freeze-dried product did not warrant the effort involved. The presence of sulphide was most critical at the actual freeze-drying stage because its absence caused marked losses on drying. Direct lyophilization of rumen liquor, without the preliminary concentration and washing, yielded dried powders with an over-all recovery of cellulase similar to that of the standard powder; however, the specific activity was only about one-twelfth of that of the standard dried product. The pH value at which lyophilization is done must likewise be carefully chosen and as Fig. 4 indicates the optimal value required in order to obtain high 'cellulase: dry-weight ratios' is about pH 7.4. Preparations were adjusted with HCl or NaOH to different pH values and the effect of the final pH value of the preparation after drying was determined (Fig. 4).

Another kind of freeze-dried powder was prepared by centrifuging rumen liquor for a short period (800g for 30 sec. at 1°) to remove larger particles. The cloudy supernatant fluid was then fractionated by centrifugation and washing exactly as in the standard procedure described above. Freeze-dried powders obtained by this modified procedure looked more homogeneous and gave more uniform suspensions, but never possessed more than half of the total cellulase activity of the standard dried powder. The latter appeared to be reasonably stable in that it still retained up to one-third of its total initial cellulase activity after 7 weeks at 1° under vacuum over calcium chloride.

It was possible that the freeze-dried powders contained viable micro-organisms and in order to eliminate this possibility acetone powders were prepared from lyophilized preparations by the usual techniques (see 'Methods' under 'Preparation of freeze-dried powders'). Approximately half of the total cellulase content of the parent freeze-dried product was recovered in the acetone powder which itself was capable of hydrolysing up to 80 % of cellulose powder (Whatman) in the standard cellulase assay. The freeze-dried powders appeared to be much more stable in aqueous suspension than did acetone

Fig. 3

Fig. 3. Hydrolysis of carboxymethylcellulose by cell-free butanol extract of rumen liquor concentrates (R.P.). Conditions, 0.2 ml. butanol extract on carboxymethylcellulose (10 mg.) in Sørensen phosphate buffer (pH 5.5). Total assay volume 4 ml.; 37°. Susceptible links calculated from Spurlin (1939).

Fig. 4

Fig. 4. Effect of pH value at freeze-drying on recovery of cellulase activity. Activity given as specific activity/g. freeze-dried powder.

powders prepared from freeze-dried powders. Thus, suspensions of a freeze-dried powder (final natural pH *c.* 6.7) in: (1) de-aerated water; (2) 0.01 % (w/v) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, pH 7.0 (adjusted with HCl); (3) 0.01 % (w/v) sulphide, pH 10.4, followed by a standard cellulase assay after each treatment, gave 80, 80, and 72 % hydrolysis of cellulose powder (Whatman), respectively. Even after incubation at pH 9.7 for 3 hr. at 30° in an 0.01 % (w/v) sulphide suspension, freeze-dried powders were still capable of producing 74 % hydrolysis of cellulose powder (Whatman). In contrast, acetone powders (prepared from freeze-dried powders) appeared to be far more sensitive to alkali. Thus a suspension of an acetone powder in 0.01 % (w/v) sulphide at pH 10.4, gave only half the cellulolytic activity of the same powder suspended in sulphide at pH 7.0, although, due to buffering action of the acetone powder, the final pH value was 6.7 in both cases.

*Stimulatory action of rumen liquor supernatant fluid
on cellulolysis by lyophilized powders*

The cellulase assays of the dried powders described above, were completed in presence of the high speed supernatant fluid (R.L.S.) from rumen liquor. R.L.S., which itself was devoid of cellulase, when added to the assay system increased the activity of freeze-dried preparations eight-fold in the standard cellulase assay. When R.L.S. was heated (by immersion of its container in boiling water for 1 min.) before addition to the assay system, the activity of lyophilized powders was raised only two-fold.

Certain fatty acids were found to stimulate the growth of a rumen isolate on ball-milled filter paper and on glucose (Bryant & Doetsch, 1955), and also to stimulate cellulolysis by mixed rumen micro-organisms (Bentley, Lehmkuhl, Johnson, Herschberger & Moxon, 1954). In order to determine whether such acids accounted for the activating effect of R.L.S. on our freeze-dried powders, *n*-valeric and *isovaleric* acids were used, singly and together, at 10, 100 and 500 % of the concentrations suggested by Bentley *et al.* (1954). These acids had no stimulating effect on the cellulase activity of our freeze-dried preparations. This result was not unexpected if the principal action of the fatty acids were supposed to be on growth of micro-organisms, rather than on cellulolysis.

*Preparation of cellulolytic extracts from lyophilized powders
of rumen micro-organisms and derived acetone powders*

Autolysis of a freeze-dried powder suspension in 0.01 % (w/v) sulphide at pH 7.0 or at pH 9.7, in evacuated vessels for 3 hr. at 30°, produced no serious loss in cellulase activity. Likewise, hand grinding of freeze-dried powders with ballotini beads in a mortar, or grinding followed by autolysis in 0.01 % sulphide (pH 7.0) for 1 hr. at 30°, did not decrease cellulase activity. On the other hand, 0.05 % (w/v) Triton X-100 (Rohm and Haas Co., Philadelphia, U.S.A.), 0.1 % (w/v) Teepol XL (B.D.H.; Walker & Levvy, 1951) or 0.2–2 % (w/v) sodium cholate when used as solubilizing agents, all produced complete loss of cellulolytic activity. The aqueous supernatant fractions obtained by high-speed centrifugation from any of the above treatments, did not hydrolyse cellulose powder (Whatman). Likewise, acetone powders prepared from lyophilized powders and subsequently extracted with water, with 0.01 % (w/v) sulphide (pH 7.0) or with Sørensen phosphate buffer containing 0.01 % sulphide (pH 7.0), also did not give an active cellulase in the centrifuged supernatant fluid. However, some cellulolytic activity was obtained from the lyophilized powder by suspension in 0.01 % sulphide, (pH 7.0) and separation of the supernatant fluid before or after treatment with butanol, in a manner similar to that described for butanol extracts of R.P. (Methods). The aqueous butanol extract hydrolysed up to 10 % of the cellulose powder (Whatman) under the standard conditions. A sample of the aqueous supernatant fluid which was separated before treatment with butanol, was compared with the butanol extract for β -glucosidase activity on *o*-nitrophenyl- β -glucoside (by Dr J. Conchie). Approximately twice as much activity was present in the

butanol extract as in the aqueous supernatant fluid. This suggests that glucosidase activity, like cellulase, is relatively closely associated with insoluble material, in agreement with Conchie (1954).

Enrichment cultures of rumen micro-organisms

In order to increase the cellulase content and to decrease the number of non-cellulolytic organisms in the concentrated suspensions of rumen liquor micro-organisms (R.P.), cultures were prepared grown on cellulose powder (Whatman). At first the standard assay conditions (see Methods) were used unmodified on the large scale. With rumen liquor itself as an inoculum (12 %, v/v) a much slower rate of cellulolysis was obtained than on the smaller scale of the standard assay. This difficulty was eventually overcome by the use of an eight-fold concentrate of rumen micro-organisms in presence of yeast extract and the clear high-speed supernatant (R.L.S.) from rumen liquor (see final medium in Methods). Where samples were required for analysis at successive periods of the fermentation, it was found advisable to use replicate vessels rather than to take successive samples from a single vessel after mixing by gentle shaking. The latter treatment invariably decreased the degree of cellulolysis to less than one-third of the value obtained by replicate sampling (Fig. 5). In some experiments, retardation of fermentation by shaking was so severe that cellulose utilization did not exceed 12 % of the available cellulose in 4 days. Such findings were in agreement with the work reported earlier on the inhibitory action of shaking on the cellulolysis of cellulose powder (Whatman) by rumen micro-organisms (Halliwell, 1957). Cellulose utilization was examined in enrichment cultures on 0.4 % (w/v), 1.2 % (w/v) and 3 % (w/v) cellulose powder (Whatman), and cellulase formation was examined on the 1.2 and 3 % cellulose powder (Whatman) suspensions (Fig. 5). Samples of fermented medium were removed at intervals from replicate vessels and analysed for cellulose and for cellulase by the standard techniques. As the results indicate, cellulase production (as determined by the standard cellulase assay) reached an optimum well before complete utilization of cellulose. When approaching the end of fermentation, as determined from cellulose utilization, some fermentations did not show any trace of cellulase when samples were removed to fresh cellulose powder (Whatman), suspensions, although residual cellulose was still being solubilized in the parent fermentation.

Three basic types of buffer salts media were used in the present investigation with enrichment cultures in order to compare their effectiveness in supporting cellulase formation: (1) the acetate salts medium of Sugden (1953) as used previously for protozoal cellulolysis (Halliwell, 1957); (2) the bicarbonate buffer medium of Bryant & Doetsch (1954) who used this medium to isolate rumen cellulolytic organisms; (3) the standard bicarbonate buffer medium of the present investigation (see preparation of enrichment cultures under 'Methods'). Fig. 6 illustrates the superiority of our standard bicarbonate medium, based on that of Elsden (1945), over the other two. Whereas the former produced rapid and active cellulolysis and cellulase formation, the

alternative media gave die-away curves for cellulose utilization and for enzyme synthesis. The main difference between the three media is in the higher NaCl concentration in medium 1 and the lower bicarbonate and magnesium concentrations of media 1 and 2. As shown previously (Halliwell, 1957; Table 1) NaCl at 0.1 M (0.6%), a concentration near that (0.5%), of medium 1, diminished cellulolysis by rumen micro-organisms to about one-half. The same table also indicates that decrease of the magnesium concentration probably accounted for some of the inferiority of media 1 and 2 when used

Fig. 5

Fig. 6

Fig. 5. Enrichment cultures of rumen liquor; effect of sampling procedure on cellulolysis and effect of cellulose concentration on cellulose solubilized and cellulase produced. Cellulase determinations by standard procedure. Substrate: cellulose powder (Whatman). On 0.4% initial cellulose: cellulose solubilized by sampling from separate vessels ▲, by sampling repeatedly from single vessel □. Results on 0.4% cellulose for 3 days fermentation only. On 3% initial cellulose: cellulose solubilized ■, cellulase activity +. On 1.2% initial cellulose: cellulose solubilized △, cellulase activity ×.

Fig. 6. Enrichment cultures. Effect of different media on cellulose solubilized and cellulase activity on 1.2% (w/v) cellulose powder (Whatman). Acetate medium (Sugden, 1953) cellulose solubilized ▲, cellulase activity +; Bryant & Doetsch medium (1954) cellulose solubilized △, cellulase activity □; bicarbonate medium (present author) cellulose solubilized ■, cellulase activity ×.

for cellulolysis in enrichment cultures. The main factor supporting maximum cellulolytic activity in the standard bicarbonate salts medium appeared to be its higher bicarbonate content. In this respect it was found that rumen liquor lost none of its cellulase when adjusted to pH 5.8 for 5 min. at 18° with 0.01 M-HCl and then returned to pH 6.8 for the standard cellulase assay. When, however, rumen liquor at pH 5.8 was assayed as usual, but in standard bicarbonate buffer at pH 5.8 (by using more dilute bicarbonate) instead of pH 6.8, no cellulose was hydrolysed. Whether the effect on cellulolysis is due to lowered concentration of bicarbonate or to the pH value was not

determined. However, the effect appears to be associated with bicarbonate concentration because all three media of Fig. 6 possessed approximately the same initial pH value (see Fig. 7), whilst cellulolytic activity increased as the added bicarbonate increased (from 0% in medium 1 to 0.2% in medium 2 and 1.5% in medium 3). The buffer capacity of medium 3 (standard bicarbonate buffer) was much greater than the other media and was better able to resist the acidifying effect due to the metabolic products of cellulolysis (Fig. 7). Bryant & Doetsch (1954) observed a decrease in acid production by a cellulolytic organism growing on glucose when the bicarbonate of the medium was decreased from 0.2 to 0%; they attributed this action to the bicarbonate without reporting pH changes.

Fig. 7

Fig. 8

Fig. 7. Enrichment cultures. Effect of different media on pH value of fermentation on 1.2% (w/v) cellulose powder (Whatman). Acetate medium (Sugden, 1953) ×; Bryant & Doetsch medium (1954) +; bicarbonate medium (present author) □.

Fig. 8. Enrichment cultures. Gas evolution, cellulose solubilization and cellulase activity on cellulose powder (Whatman). Gas evolution on 1.2% (w/v) cellulose +, on 2% cellulose Δ, on 3% cellulose ×. On 1.2% cellulose: cellulose residual □, cellulase activity ■. Cellulose residual given, not cellulose solubilized, to avoid confusion of the curves.

Determination of the course of fermentation from gas evolution

As shown above, cellulase formation appeared greatest in enrichment cultures at the early stages of fermentation, only to fall rapidly later when cellulose utilization was approaching a maximum. To facilitate the identification of the optimal time for harvesting organisms with maximum cellulase content, the fermentation vessels were connected so that issuing gases were collected and measured (see Methods). Thus, the state of metabolism (recorded in volumes of gas) was determined immediately, and after calibration against cellulose utilization and cellulase formation, provided a rapid method of following cellulolysis to the harvesting period.

Fig. 8 indicates the gas evolution over the complete fermentation cycle on cellulose powder (Whatman) at 1.2, 2 and 3 % concentration, together with the cellulose utilization and cellulase production on 1.2 % cellulose powder (Whatman). This concentration of cellulose was finally adopted for the preparation of organism extracts; the results should also be compared with those of Fig. 5. Gas evolution on 0.4 % cellulose powder (Whatman), approximately the concentration used on the small scale of the standard cellulase assay, was also examined but the curves obtained were always flatter and less decisive. Cultures growing on 1 % initial cellulose appeared most useful for obtaining concentrated preparations of cellulase-containing organisms in that residual cellulose was less troublesome when making aqueous extracts of enzyme. Maximal cellulase synthesis was found in the early stages of cellulose utilization, at about the 40 % level, just before the rate of gas evolution began to fall off.

*Preparation of cellulolytic extracts from enrichment cultures
of rumen micro-organisms*

A concentrated precipitate of cellulase-containing micro-organisms obtained from the above enrichment cultures by high-speed centrifugation was used to prepare alumina-ground extracts (see Methods). Under optimal conditions the enzyme preparation so obtained was capable of solubilizing up to 30 % of the cellulose powder (Whatman) supplied, but did not hydrolyse de-waxed cotton fibres. Maximum cellulolytic activity was obtained by grinding and extracting the organisms with alumina at pH 7.2 rather than at pH 6.5; cellulase action appeared to be associated with yellow-coloured extracts which were more readily obtained at higher pH values. Corresponding alumina extracts from concentrated precipitates of non-enrichment cultures did not yield cellulase in the extracts. The orange coloured butanol extracts prepared from enrichment cultures like those directly from concentrates of rumen micro-organisms (non-enriched), never hydrolysed more than c. 10 % of the cellulose powder (Whatman) in 2 days at pH 6.8 (standard bicarbonate assay buffer) or at pH 6.0 (in Sørensen phosphate buffer). As shown above (Fig. 3), rumen butanol extracts were more effective against the soluble cellulose derivative carboxymethylcellulose.

Comparison of the cellulolytic activity of the various preparations

It was also found that the cell-free filtrate (see Methods) obtained from *Myrothecium verrucaria* cultures growing on cellulose powder (Whatman) had properties very similar to those of the alumina-ground extracts of rumen micro-organisms from enrichment cultures. Thus, the extracts from concentrates of rumen micro-organisms solubilized about 30 % of cellulose powder (Whatman) whilst our best filtrates (unconcentrated) from *M. verrucaria* were equally active in the same period (72 hr). Likewise, the rumen alumina-extract and the *M. verrucaria* cell-free filtrates had only feeble activity (in contrast to the butanol preparations from rumen micro-organisms) against carboxymethylcellulose. Thus the enzyme activity ratios of a butanol extract (from

a concentrated preparation of micro-organisms) and of a cell-free filtrate (unconcentrated) from *M. verrucaria*, were approx. 5:1 at 37° with carboxymethylcellulose as substrate (assays by courtesy of Dr G. N. Festenstein). On the other hand, with insoluble cellulose as substrate, the butanol extract (rumen) possessed only one-half the activity on cellulose powder (Whatman) and one-fifth the activity on swollen cellulose powder, of the *M. verrucaria* filtrate. Table 2 shows some of the properties of *M. verrucaria* filtrates on these substrates. The table also indicates that the active principle in *M. verrucaria* filtrates towards cellulose powder was unstable at higher temperatures.

Table 2. *Action of Myrothecium verrucaria cell-free filtrate at (pH 6.0) in Sørensen phosphate buffer*

Standard *M. verrucaria* cellulase assay (see Methods). C.P.W.=cellulose powder (Whatman); S.C.P.W.=swollen C.P.W.

		30°		50°
		Static tubes	Shaken flasks	Static tubes
Period of incubation (hr.)		Cellulose used as % of initial		
Substrate				
C.P.W.	48	19	20	8
S.C.P.W.	18	—	75	88

DISCUSSION

In a previous paper (Halliwell, 1957) it was emphasised that the results of work on cellulolysis by micro-organisms or extracts thereof, depend not only on the substrate used but also on the method of cellulase assay employed. This theme was then developed to analyse the validity of results obtained by different methods of assay, particularly those which depend on formation of reducing sugars from insoluble forms of cellulose. Different forms of cellulose contain chains of very different length, ranging from the complex native cotton fibre down to the much-degraded cellodextrins and the soluble but substituted cellulose derivative carboxymethylcellulose. Certain micro-organisms can produce complete solubilization of all these forms of cellulose and, as shown, rumen micro-organisms are one of the most active sources known. Many workers have prepared extracts, from animal and plant sources and from cellulolytic and non-cellulolytic micro-organisms, which rapidly hydrolyse soluble, but substituted, cellulose derivatives, and have little or no action on insoluble cellulose, particularly cotton fibres. Recent examples of such preparations have been collected (Halliwell, 1957) and calculation shows that the degree of hydrolysis ranged from 0.2 % to about 6 % of the available cellulose powder or cotton. Reese, Siu & Levinson (1950) explained this difference by suggesting that action on insoluble cellulose involves a preliminary cellulase (C_1), sparsely distributed in nature, followed by the action of a more universal enzyme (C_x). The latter is also believed to hydrolyse the much degraded soluble forms of cellulose or cellulose derivatives. The low degrees of hydrolysis of insoluble cellulose obtained by these workers (0.2–6 % solubilization) may well represent enzymic breakdown of non-reducing, non-

true cellulosic fractions (xylans, glucosans) associated with relatively undegraded cellulose. Hydrolysis of such 'impurities' by a 'cellulase' preparation, when analysed by a reductimetric method would yield false evidence of cellulolysis. The error assumes major importance when the observed degree of 'cellulolysis' represents only a fraction of the available cellulose. Support for this belief of 'impurities' being present is to be found in the identification of xylose from a fermentation of cellulose powder (Alphacel) by rumen micro-organisms (Kitts & Underkofler, 1954) and of the short-chained polysaccharides obtained by aqueous extraction from filter-paper, wood cellulose and cotton linters (Huffman, Rabers, Spriesterbach & Smith, 1955). An example of this low degree of cellulolysis of insoluble cellulose found by a reductimetric method of cellulase assay is given by the work of Kitts & Underkofler (1954), who obtained a preparation from rumen bacteria which produced only 3% cellulolysis of cellulose powder (Alphacel) in 49 hr.; further example appears to be the preparation from *Myrothecium verrucaria* obtained by Whitaker (1953). After concentration and purification of *M. verrucaria* filtrates, the latter hydrolysed no more than 2% of the weight of cotton linters and no more than 7% of a 'cellulose' precipitated from cotton dissolved in concentrated sulphuric acid. Thus, hydrolysis of the cotton linters was low, possibly due to the few susceptible short chains, whilst hydrolysis of the acid-degraded cellulose, with more of the shorter chains, was correspondingly greater. Whitaker's cellulase may have been exhibiting uni-enzymic action, but only towards the shorter molecular species of cellulose chains present in most forms of insoluble cellulose.

Our own findings tend to support the views expressed by Reese *et al.* (1950) that more than one enzyme (C_1 and C_x) or set of conditions may be necessary for significant hydrolysis of insoluble cellulose. Thus, our rumen butanol preparations acted much more effectively against the soluble carboxymethylcellulose, but had only slight hydrolytic action (maximum 10% hydrolysis) on insoluble cellulose powder. On the other hand, alumina extracts from rumen micro-organisms and cell-free filtrates from *Myrothecium verrucaria* were relatively feeble in attacking carboxymethylcellulose as compared with the rumen butanol extract, but produced up to 30% solubilization of insoluble cellulose powder. Such a reversal of enzyme activity on a soluble cellulose derivative and on insoluble cellulose suggests multi-enzymic action (or conditions). A similar variation of activity in filtrates of other organisms was reported by Gilligan & Reese (1954). Further evidence for the dissimilarity of the enzyme which hydrolyses carboxymethylcellulose from that which hydrolyses insoluble cellulose appears in the work by Reese *et al.* (1950) who showed that *M. verrucaria* filtrates acting on soluble carboxymethylcellulose were about twice as active at 50° as compared with 30°. In contrast, with an insoluble cellulose powder (Whatman) our own work indicates that *M. verrucaria* filtrates exhibited less than half their activity when incubated at 50°, as compared with their effect at 30°.

The nature of the initial action on insoluble cellulose is still uncertain. If it be enzymic in nature, our results suggest 'the enzyme' may be highly unstable

or requires a special combination of conditions for (1) no true cellulase was detected in the medium external to rumen micro-organisms growing on insoluble cellulose, (2) true cellulase activity of rumen micro-organisms fermenting insoluble cellulose powder was severely repressed by the presence of antiseptics (Halliwell, 1957) as compared with their action on the soluble carboxymethylcellulose, (3) exposure of cellulase-active rumen micro-organisms, of freeze-dried powders or of acetone powders of rumen organisms, to excessive aeration or elevated temperatures produced heavy losses of cellulase and (4) no one has yet succeeded in preparing cellulolytic extracts which attack undegraded cotton fibres with anything remotely approaching the vigour of micro-organisms.

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