The Cellulolytic Activity of Pure Strains of Bacteria from the Rumen of Cattle

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(Received 18 March 1963)

SUMMARY

The *in vitro* breakdown of degraded and undegraded varieties of cellulose was examined by using pure strains of bacteria isolated from the rumen of cattle. One strain of *Bacteroides succinogenes*, two strains of *Ruminococcus albus* and two strains of *Ruminococcus flavefaciens* were allowed to ferment ground cellulose powder (prepared from filter paper), cellulose powder (Whatman) and undegraded cotton fibres, the extent of breakdown being followed by loss of weight of the insoluble substrate. All five organisms were highly active on degraded ground cellulose powder and dissolved 72-90 %, but only one organism, *B. succinogenes* strain s-85, was equally effective on cellulose powder (Whatman) or on undegraded cotton fibres. *R. flavefaciens* strain FD-1 was somewhat less potent on the latter substrates, achieving 40 and 60 % dissolution, respectively, of cellulose powder (Whatman) and cotton fibres. *R. albus* strain 7 and *R. flavefaciens* strain c-94 had negligible effects on cotton fibres (10 and 0 % solubilization, respectively). *R. albus* strain D-89, producing 40 % solubilization of cotton fibres, was intermediate in activity between *R. albus* strain 7 and *R. flavefaciens* strain FD-1. Cell-free preparations from culture filtrates of *B. succinogenes* strain s-85 gave only 4 % breakdown of ground cellulose powder and up to 9 % breakdown of cellulose powder (Whatman) in 17 days. Cell-free filtrates from the metabolism fluid of *R. flavefaciens* strain FD-1 or from the disintegrated organisms brought about 46 and 36 % solubilization, respectively, of ground cellulose powder, but failed to attack cotton fibres. The results support the view that the capacity of an organism or cell-free enzyme to attack any one particular form of cellulose is no criterion of its ability to attack less degraded or undegraded types of cellulose.

INTRODUCTION

Several attempts have been made to isolate and describe the activity of rumen cellulolytic micro-organisms by using cellulosic substrates previously degraded by chemical or physical treatments and hence rendered more susceptible to biological attack. Thus, acid-treated and ball-milled cotton has been used by Hungate (1950*a, b*), a ball-milled filter paper by Bryant & Burkey (1953*a*) and soluble cellulose derivatives, such as carboxymethylcellulose, by Kitts, Carr & Underkofler (1954) and Underkofler, Kitts & Smith (1953). The micro-organisms in question have not, however, been examined on native undegraded cellulosic substrates such as

cotton fibres, although it is known that this form of cellulose can be readily and completely dissolved *in vitro* by a mixed population from the rumen of sheep (Halliwell, 1957*b*, 1961*a*). The present work compares the *in vitro* bacterial solubilization of different forms of cellulose, extending from degraded ball-milled filter paper through cellulose powder (Whatman) to undegraded, purified cotton fibres. The bacteria used were pure strains, isolated from bovine rumen, of *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*.

METHODS

General procedures for the preparation of media, isolation of bacteria and descriptions of strains (Bryant & Burkey, 1953*a*; Bryant & Doetsch, 1954; Bryant, Small, Bouma & Robinson, 1958*a*), the nature of the three cellulosic substrates, namely, ground cellulose powder prepared by grinding filter paper (Whatman no. 1) in a ball-mill (Bryant & Burkey, 1953*a*), cellulose powder (Whatman) and dewaxed Texas cotton fibres (Halliwell, 1957*a*) and the determination of residual cellulose (Halliwell, 1957*a*), are described in the references cited. Additional details or modifications are given below.

Bovine bacteria. *Bacteroides succinogenes* strain s-85, *Ruminococcus albus* strains 7 and D-89 and *Ruminococcus flavefaciens* strains C-94 and RD-1 were isolated from bovine rumen and then maintained as stab cultures in agar at -50 to -70° . Two or three loopfuls of the stock cultures were transferred to tubes containing 5 ml. cellulose broth and grown at 37° for 24–48 hr. to provide sufficient actively growing inoculum for up to 200 ml. of cellulose broth.

Culture media. Cellulose broth was used to grow the organisms and also as a basal medium to examine the cell-free enzymic breakdown of the different forms of cellulose. Broth (100 ml.) contained: 30 ml. whole rumen fluid (prepared by expressing rumen fluid through surgical gauze, allowing to stand overnight at 1° and separating off the middle phase from the lower sediment and upper floating material); 3.75 ml. mineral solution 1 (0.6%, w/v, K_2HPO_4); 3.75 ml. mineral solution 2 (w/v: 0.6%, KH_2PO_4 ; 1.2, $(NH_4)_2SO_4$; 1.2, NaCl; 0.25, $MgSO_4 \cdot 7H_2O$; 0.12, $CaCl_2$); 0.1 ml. 0.1%, w/v, resazurin solution; 5 ml. 8%, w/v, Na_2CO_3 ; 2 ml. 2.5%, w/v, cysteine hydrochloride; water to 100 ml. The broth was equilibrated with, and maintained under, O_2 -free CO_2 . Ground cellulose powder, cellulose powder (Whatman) or dewaxed Texas cotton fibres were incorporated in the medium at a final concentration of about 0.2%, w/v.

Cellulolysis or solubilization of cellulose. In the standard cellulase assay for whole bacteria, the strains were inoculated under CO_2 into test tubes (16×150 mm.) of cellulose broth (10 ml.) and incubated anaerobically at 37° . In the standard cellulase assay for cell-free preparations an aqueous suspension of cellulose was added to different amounts of culture filtrate as indicated in the text; the assays were made at 37° under sterile and anaerobic conditions in presence of CO_2 and with additional cysteine at the concentration found in the culture medium. Reagents and tubes were sterilized by autoclaving at 120° and enzyme solutions by filtration.

Solubilization of cellulose was followed by gravimetric estimation of residual cellulose by using sintered-glass filter crucibles, porosity M, medium grade (nominal maximum pore size 10–15 μ) for cellulose powders, or porosity C, coarse grade (40–60 μ) for cotton fibres. With the pure cultures used here the washing procedure

with HCl, NH_4OH , Teepol and ethanol (Halliwell, 1957*a*), for the removal of contaminating non-cellulosic material from mixed rumen bacterial fermentations, was not required. Residual cellulose was washed copiously with distilled water, dried overnight and weighed: the 'enzyme blank' (bacteria incubated in cellulose-free media) gave negligible values after washing and filtration.

Cell-free enzyme preparations. Large-scale cultures with about 400 ml. cellulose broth containing 0.2 % ground cellulose powder, were inoculated from actively growing cultures of *Bacteroides succinogenes* strain s-85 or *Ruminococcus flavefaciens* strain FD-1, grown for the preceding 24–48 hr. in an identical medium. At suitable intervals the large-scale cultures were agitated temporarily with a magnetic stirrer to provide homogeneous suspensions for sampling. The cultures were regassed with CO_2 during and after sampling. After the desired stage of fermentation had been reached the cultures were centrifuged at 27 000 *g* for 20 min. at 3° (Servall Machine, Model RC2, Rotor SS-34, Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.) and the cell-free supernatant phase removed and maintained under CO_2 for assay. In some experiments the precipitated bacteria were also disintegrated in a Mini-Mill (Gifford-Wood Co., Hudson, New York) under CO_2 and cooled in ice. Extensive breakage of organisms required 30–50 min. treatment in the Mill, and was judged by safranin stained smears.

Alkali solubility of cellulosic substrates. Dewaxed Texas cotton fibres, cellulose powder (Whatman), and a hydrocellulose (prepared by soaking absorbent cotton wool in 11 *N*-HCl; Halliwell, 1957*a*), were air-dried materials. Ground cellulose powder and ground hydrocellulose, which were prepared in the usual manner in a ball mill (Bryant & Burkey, 1953*a*; Hungate, 1950*a*), were stored and used in aqueous suspension. The hydrocellulose and its ground counterpart had been used as cellulosic substrates in earlier work with rumen micro-organisms, but in the present work they were used only for comparative purposes in the alkaline solubility estimations.

About 50 mg. of each form of cellulose in water was mixed in a test tube with an equal volume (6 ml.) of 2 *N*-NaOH, the suspension heated in a boiling water bath for 15 min. under a glass pear-bulb, cooled for 5 min. and transferred quantitatively to a sintered glass crucible (porosity M) by washing with 20 ml. *N*-NaOH. The cellulose was sucked almost dry, washed with 40 ml. *N*-NaOH, again sucked almost dry, and washed with water, 0.1 *N*- H_2SO_4 and water until neutral. The crucible + cellulose were dried overnight at 105° and weighed.

RESULTS

Bacterial solubilization of ground cellulose powder, cellulose powder and dewaxed cotton fibres

Bacteroides succinogenes strain s-85, *Ruminococcus albus* strains 7 and D-89, *R. flavefaciens* strains C-94 and FD-1 were grown in cellulose broth containing about 0.2 % ground cellulose powder (Whatman) or dewaxed cotton fibres. It is evident from Table 1 that the five organisms attacked ground cellulose powder and, with the exception of *B. succinogenes* strain s-85, attacked this powder, a degraded substrate, more effectively than they did fibres. Only *B. succinogenes* strain s-85, *R. flavefaciens* strain FD-1 and possibly *R. albus* strain D-89 gave significant breakdown of undegraded cellulose as found in cotton fibres.

In an attempt to promote more rapid and extensive attack on cotton fibres we re-examined some of the organisms recorded in Table 1 after incorporating cellobiose at 0.02 % final concentration in the medium. The results (Table 2) confirmed

Table 1. *Solubilization of ground cellulose powder and dewaxed cotton fibres by rumen bacteria*

Conditions: standard cellulase assay (see Methods). Initial weight of ground cellulose powder was 23 mg. (average) and of cotton fibres 20 mg. Period of incubation for ground cellulose powder was 5 days and for cotton fibres 7 days.

Organisms	Substrate	
	Ground cellulose powder	Cotton fibres
	Cellulose solubilized as % of initial weight	
<i>Bacteroides succinogenes</i> strain s-85	88	97
<i>Ruminococcus albus</i> strain 7	88	10
strain D-89	88	40
<i>R. flavefaciens</i> strain C-94	72	0
strain FD-1	90	55

Table 2. *Solubilization of dewaxed cotton fibres in presence of cellobiose*

Conditions: as in Table 1 but with cellobiose in the medium at 0.02 % final concentration. Initial weight of cotton fibres was 21 mg. (average).

Organisms	Time of action of culture	
	4 days	8 days
	Cotton fibres solubilized as % of initial weight	
<i>Bacteroides succinogenes</i> strain s-85	86	86
<i>Ruminococcus albus</i> strain 7	0	10
<i>R. flavefaciens</i> strain FD-1	30	60

the cellulolytic nature of *B. succinogenes* strain s-85 and of *R. flavefaciens* strain FD-1, whereas *R. albus* strain 7 once again showed little inclination to solubilize undegraded cellulose of cotton fibres. This suggests that the cellulolytic activity of *R. albus* strain 7 is restricted to simpler forms of cellulose (Table 1).

Cellulose powder (Whatman) appears to retain some of the properties of native cellulose more than do certain other powdery forms of cellulose; it is more soluble in N-alkali than cotton fibres (see Discussion) but resists solubilization by cell-free cellulolytic filtrates from fungi although not to the same degree as do cotton fibres (Halliwell, 1961*b*). If particle size were the only obstacle to microbial breakdown of fibrous cellulose we should expect the powdery nature of cellulose powder

(Whatman) to encourage attack by those organisms which find difficulty in metabolizing fibres. Three amounts of cellulose powder (Whatman), about 10, 20 and 50 mg., were supplied to *Bacteroides succinogenes* strain s-85, *Ruminococcus flavefaciens* strain FD-1 and *R. albus* strain 7. At all substrate concentrations the first two strains showed rapid breakdown of cellulose up to the fourth day of fermentation, whilst *R. albus* displayed only feeble activity extending to the second day (Figs. 1, 2).

Fig. 1. The effect of time of incubation on bacterial cellulolysis of cellulose powder (Whatman). Conditions: standard cellulase assay with *Bacteroides succinogenes* strain s-85; initial weight of cellulose 13 mg., $\circ$; 22 mg., $\triangle$; 54 mg., $\square$. *Ruminococcus albus* strain 7; initial cellulose 11 mg., $\bullet$; 20 mg., $\blacktriangle$; 51 mg., $\blacksquare$. Cellulose incubated in absence of bacteria for 13 days lost no weight. Percentages near curves indicate the final extent of solubilization of the initial cellulose.

Fig. 2. The effect of time of incubation on bacterial cellulolysis of different amounts of cellulose powder (Whatman). Conditions: standard cellulase assay with *Ruminococcus flavefaciens* strain FD-1; initial weight of cellulose 11 mg., $\circ$; 22 mg., $\bullet$; 53 mg., $\square$. Other details as in Fig. 1.

After the fourth day the rate of solubilization of cellulose decreased with *B. succinogenes* strain s-85 and *R. flavefaciens* strain FD-1 and complete hydrolysis was achieved only by the former organism in 7 days. With *R. flavefaciens* strain FD-1, increasing the fermentation period by 9 days produced little change in the figure of about 40 % breakdown attained after the first 4 days at the lower cellulose concentrations. At all three cellulose concentrations *R. albus* strain 7 dissolved no more than 10 % of the substrate in 2 or 13 days fermentation.

Cellulolytic enzymes from *Bacteroides succinogenes* strain s-85 and from *Ruminococcus flavefaciens* strain FD-1

The action of cell-free enzyme preparations from *Bacteroides succinogenes* strain s-85 on ground cellulose powder and on cellulose powder (Whatman). Strain s-85 was grown in 400 ml. broth containing 0.22 % ground cellulose powder, as described in Methods. After 29 hr. the organisms had decreased the amount of cellulose to 37 % of its initial value and to 21 % in a further 12 hr. The culture was allowed a further 23 hr. (total 64 hr.) to attack most of the remaining cellulose and thus to encourage release of any adsorbed enzyme from its cellulosic substrate (see Halliwell, 1961b). The

culture was centrifuged to obtain the aqueous phase which was sterilized by filtration; 10 ml. of this cell-free enzyme solution were incubated with 20 mg. ground cellulose powder + cysteine at 37° for 22 days. This produced only a 4 % loss of weight of cellulose. Other cultures of *B. succinogenes* strain s-85, grown under identical conditions to those described, metabolized 86 % of ground cellulose powder in 44 hr. and 88 % in 72 hr. Cell-free culture filtrates were obtained at both stages of attack by the standard procedures and their activities measured by using 20 mg. cellulose powder (Whatman) and 20 ml. of each of the enzymic filtrates. These dissolved only 9 and 3 %, respectively, of the available cellulose powder (Whatman) in 17 days at 37°. The small extent of breakdown of ground cellulose powder and cellulose powder (Whatman) is not considered significant in view of the nature of these substrates (see Discussion).

The action of cell-free enzyme preparations from Ruminococcus flavefaciens strain FD-1 on ground cellulose powder and on dewaxed cotton fibres. Tables 1 and 2 and Figs. 1 and 2 indicate that *R. flavefaciens* strain FD-1 was as active as *Bacteroides succinogenes* strain s-85 on ground cellulose powder, but was not as effective in solubilizing less-degraded substrates such as cellulose powder (Whatman) and cotton fibres. Thus *R. flavefaciens* strain FD-1, like *B. succinogenes* strain s-85, dissolved ground cellulose powder to within 90 % of completion, but gave only 40–60 % loss of weight of cellulose powder (Whatman) and cotton fibres, as compared with the 100 % loss effected by *B. succinogenes* strain s-85. The latter organism did not provide a cell-free cellulase preparation producing marked breakdown of either of the substrates tested and the results indicate that *R. flavefaciens* strain FD-1 was the only remaining bacteria likely to possess this enzyme system.

Ruminococcus flavefaciens strain FD-1 was grown by the standard procedure in 400 ml. broth + 0.2 % ground cellulose. Of the cellulose 36 and 80 % was catabolized after 18 and 40 hr., respectively. The older culture supplied two enzyme preparations: the first was the supernatant phase obtained by centrifuging the culture medium; the second was prepared by resuspending the sediment in the minimum necessary volume of some of the above supernatant phase followed by treatment in the Mini-mill disintegrator whereby the enzyme was released into the aqueous phase. This was collected by centrifugation as described earlier. The two enzyme preparations, sterilized by filtration, were incubated in 15 and 5 ml. samples each with 20 mg. ground cellulose powder for 20 days during which period they solubilized 46 and 36 %, respectively, of the substrate. Samples (20 ml.) of the first enzyme preparation were also incubated for 20 days with 10 mg. dewaxed cotton fibres; no loss of weight of cellulose was observed.

DISCUSSION

In the field of cellulose metabolism by micro-organisms or cell-free enzyme preparations, difficulties of definition are more important than real experimental discrepancies. There is much to be said for adhering only to the terms β -polyglucosidase or 1,4- β -glucanase for the enzymes which attack polymers of the cellulose type, and for reserving the term cellulase until such time as the mechanism that accomplishes the initial degradation or sensitization of undegraded cellulose or native cotton fibres has been elucidated. The same difficulties of interpretation arise with the term 'cellulolytic organism' because not all organisms thus styled can

attack cotton fibres. Whilst one is entitled to use the term cellulolytic for an organism that attacks any 1,4- β -glucan, such organisms are undoubtedly divided into two groups in accordance with their ability or inability to act on cotton fibres.

Soluble cellulose derivatives or similar biologically susceptible forms of insoluble but degraded cellulose are often used as substrates for enzymes in whole organisms or in the cell-free state. The results obtained, however, are difficult to assess in absence of simultaneous studies with an undegraded substrate such as cotton fibre. Whilst all forms of cellulose are believed to be chemically identical in consisting solely of 1,4- β -linked anhydroglucose units, there are marked differences between them in physical properties such as chain length and the presence of crystalline and amorphous regions. A distinction between various types of cellulose is shown by their relative susceptibilities to alkali. Five cellulosic substrates (dewaxed cotton fibres, cellulose powder (Whatman), a hydrocellulose powder prepared from absorbent cotton wool by soaking in concentrated HCl, the same hydrocellulose after grinding in a ball-mill, ground cellulose powder prepared from filter-paper) were digested with N-NaOH for 15 min. as described in Methods, and lost 0, 17, 16, 17 and 21 % of their weight, respectively (corrected for moisture content). A more comprehensive estimate of the smaller molecular chains of cellulose is given by the β - and γ -cellulose contents. These two fractions, arbitrarily defined as the material soluble in 17.5 % (w/w) NaOH at 20°, contain molecules with a degree of polymerization from about 10 to 200. α -Cellulose is the fraction insoluble in the same concentration of alkali and has chains with a degree of polymerization greater than 200. The β - + γ -cellulose contents of dewaxed Texas cotton fibres and of cellulose powder (Whatman) were 2 and 30 %, respectively, as determined by loss of weight on 50 mg. of each substrate and corrected for moisture contents. This difference in physical properties between celluloses is reflected in the relative activities of the rumen bacteria examined in this report. Five organisms, *Bacteroides succinogenes* strain s-85, both strains of *Ruminococcus albus* and both strains of *R. flavefaciens*, were most effective in solubilizing a large proportion (72–90 %) of a degraded ground cellulose powder (Table 1); but only *B. succinogenes* strain s-85 produced a comparable degree of cellulolysis of undegraded cotton fibres and of cellulose powder (Whatman). In this respect it is similar to mixed rumen micro-organisms (Halliwell, 1957*b*, 1961*a*). *R. flavefaciens* strain FD-1 and *R. albus* strain D-89 were less effective in rate and extent of cellulolysis and were the only other strains to achieve marked breakdown of cotton fibres, 55 and 40 %, respectively (Tables 1, 2). On cellulose powder (Whatman) the action of *R. flavefaciens* strain FD-1, the most effective of the ruminococci in Table 1, was similarly restricted to 40 % solubilization of the substrate, even with prolonged incubation (Fig. 2). *R. albus* strain 7 and *R. flavefaciens* strain C-94 produced extensive solubilization (88 and 72 %) of ground cellulose powder but only 10 and 0 % breakdown of cotton fibres (Table 1). Other strains of *R. albus* which did not attack even the ground cellulose powder have been described (Bryant *et al.* 1958*b*). As only one strain of *B. succinogenes* was available for the present study, it is not known whether this species would, in general, more extensively solubilize fibrous forms of cellulose as compared to ruminococci. It is of interest in this respect that *B. succinogenes* was found in much greater numbers than ruminococci in the rumen of a cow fed wheat-straw, but ruminococci were more numerous when good quality alfalfa hay was fed (Bryant & Burkey, 1953*b*).

It has frequently been suggested that fibrous forms of cellulose are unsuitable substrates for rumen micro-organisms because of the relatively small surface area exposed to attack, and recourse has been made to finely ground varieties of cellulose. An increased susceptibility to biological attack that accompanies the transition from fibrous to powdery cellulose is liable to be associated not only with increased surface area, but also with degradative changes in the cellulose molecules. If the surface area of the cellulose were of paramount importance in favouring bacterial attack then cellulose powder (Whatman) should be broken down to a greater extent than cotton fibres. The results shown in Figs. 1 and 2 do not support this view. *R. albus* strain 7 and *R. flavefaciens* strain FD-1 found cellulose powder (Whatman) just as difficult to solubilize as cotton fibres, suggesting that this powdered cellulose retained some of the characteristics of the fibrous material.

The small degree of cellulolysis of ground cellulose powder (4 %) and cellulose powder (Whatman) (3–9 %) shown by cell-free enzyme preparations from *Bacteroides succinogenes* strain s-85 is probably insignificant as far as undegraded cellulose is concerned, since the effect might be confined to the smaller cellulosic molecules produced in the manufacture. This lack of cellulolytic activity shown by cell-free systems from *B. succinogenes* strain s-85 is in marked contrast to the pronounced activity of the intact organism on all three forms of cellulose (Tables 1, 2; Figs. 1, 2). *R. flavefaciens* strain FD-1, although as active on ground cellulose powder as *B. succinogenes* strain s-85, was only about half as effective as *B. succinogenes* strain s-85 on cellulose powder (Whatman) and on cotton fibres, but yielded cell-free enzyme preparations which solubilized up to 46 % of ground cellulose powder. The same preparations did not attack cotton fibres, suggesting that different mechanisms are responsible for the breakdown of degraded and undegraded cellulose.

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