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The Degradation of Cotton Cellulose by the Extracellular Cellulase of *Myrothecium verrucaria*

2. THE EXISTENCE OF AN 'EXHAUSTIBLE' CELLULASE*

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A cellulolytic micro-organism almost certainly attacks cotton fibre by means of an extracellular enzyme, but filtrates derived from actively growing cultures have been reported to have little action on cotton that has not been made more accessible by swelling or partial degradation (Reese, 1956); this fact has not been explained. It has been suggested that there exist several 'cellulases', some of which preferentially attack substrates of short chain length whereas others are more active towards materials of higher degrees of polymerization. In none of this work, which has been reviewed by Gascoigne & Gascoigne (1960) and Selby (1963), has extensive degradation of unswollen cotton by cell-free extracts of the micro-organisms been demonstrated. It is the purpose of the present paper to describe how such degradation has been achieved and to provide additional evidence that more than one enzyme species is involved in the hydrolytic breakdown of unswollen cotton by cell-free filtrates from *Myrothecium verrucaria*.

The mode of enzymic attack on a soluble substrate can readily be determined by comparing the resulting changes in suitably chosen physical and chemical properties. With an insoluble substrate, however, the location of chemically identical linkages will affect both their ease of scission and the contribution the scission makes to the measured changes in the properties of the substrate. In fibrous cotton certain parts of each cellulose molecule are so arranged relative to parts of others that a region of high lateral order and strong interchain bonding occurs; such regions are termed 'crystalline'. Between them the chains are disordered to various extents and the interchain bonding is weaker; these regions, which have been termed 'amorphous', are more accessible to chemical attack because they are more easily swollen and penetrated (Howsmon & Sisson, 1954). On a larger scale the bundles of cellulose chains are grouped together in a microfibrillar structure (Preston, 1962). Such a structure will contain holes between the fibrils and these may be the smallest cavities into which cellulase can penetrate, whereas chemical reagents of relatively small molecular bulk can also attack the regions of high lateral disorder and the surface of the crystalline regions. The resistance to penetration by cellulase will be one of the chief factors regulating enzymic hydrolysis (Walseth, 1952; Reese, Segal & Tripp, 1957), and the consequent immobility of the enzyme within the substrate is presumably the cause of the localized attack observed (Selby, 1961).

Halliwell (1962) has stated that the lack of solubilization of undegraded cotton by culture filtrate from *M. verrucaria* 'may be due to the enzymic production of physical changes in cellulose which are not reflected in the formation of soluble products'. The nature of these physical changes is not made clear but as an example he cites his demonstration that hydrocelluloses lose more weight when heated with N-sodium hydroxide at 100° for 15 min. than does an undegraded cotton. It is well established, however, that treatment with hot dilute alkali causes chemical degradation of β-(1→4)-glucans containing free reducing groups, with consequent loss of weight (Clibbens, Geake & Ridge, 1927; Corbett, 1959). Under the conditions of alkali treatment used the free reducing groups produced by the exposure of cotton to culture filtrate

* Part 1: Selby (1961).

would, by the same mechanism, give rise to the losses of weight observed by Halliwell (cf. Selby, 1961).

The resistance of fibrous cotton to attack by enzymes is understandable. In seeking to explain why the whole organism should be able to degrade cotton rapidly whereas the derived filtrate cannot, it seems necessary to suggest that some enzyme species or cofactor, present in the vicinity of the growing organism and necessary for the breakdown of unswollen cotton, must be in short supply in the cell-free extract as normally prepared. Reese, Siu & Levinson (1950) found that certain fungi could grow on accessible or solubilized forms of cellulose but not on native cotton fibre. These organisms were said to produce a 'cellulase' termed 'C_x', active only towards the more accessible forms of cellulose. It was suggested that organisms capable of growth on unswollen cotton must produce in addition a cellulase, termed 'C_1', capable of splitting, from fibrous cellulose, individual anhydroglucose chains that are then depolymerized by 'C_x', but no positive demonstration has been given of 'C_1' activity in cell-free preparations.

The subsequent controversy as to the multiplicity of 'cellulases' has centred round these 'C_x' factors, since it has concerned cellulase action on substrates swollen or in solution. In the work described below attention has been concentrated on the cellulolytic degradation of unswollen cotton fibre.

MATERIALS

The carboxymethylcellulose was Cellofas B. 50 from Imperial Chemical Industries Ltd. The methylcellulose was Celacol M.M.100 (degree of substitution 1·99) from British Celanese Ltd. Celite 535 was supplied by Johns–Manville and Co. Ltd. Sephadex G-25 was supplied by AB Pharmacia, Uppsala, Sweden. The organism was *Myrothecium verrucaria* (strain 45 541) from the Commonwealth Mycological Institute.

The cotton flock on which the organism was grown was supplied as C/8 flock by Hutchinson Ltd., Ramsey Mill, Chadderton, Oldham, Lancs. It was a scoured mechanically-powdered flock with a viscosity-average degree of polymerization ($\bar{P}_v$) of about 2900, calculated from the fluidity (8·3 P^{-1}) of a standard (0·5%, w/v) solution in standard cuprammonium (cf. Selby, 1961).

The cotton yarn used for measurements of cellulase activity was a Sudan Sakel of count 20 (0·295 mg./cm.) that had been given two successive 3 hr. treatments with 1% (w/v) NaOH at 140° in an autoclave. It had a $\bar{P}_v$ of about 4100, calculated from the fluidity (5·2 P^{-1}) of a standard (0·5%, w/v) solution in cuprammonium.

METHODS

Preparation of the culture filtrate. The methods of Whitaker (1953) were employed, but cotton flock was used in place of linters as the sole carbon source in the shake-culture medium. The organism was grown in 300 ml. batches of culture medium by incubation at 30° for the times stated. Each culture was chilled, centrifuged (35 000 *g*) at 2° and filtered through glass paper (Whatman GF/B). The filtrate was checked for the absence of bacterial contamination by microscopical examination, and was then saturated with washed chloroform as a preservative. The pH was readjusted to 6·5 and the solution stored at 2° until required.

Filtrates derived from cultures grown under apparently identical conditions for the same time often differed in activity towards both carboxymethylcellulose and cotton. The effect of these variations was minimized by making comparative experiments and using for each a single batch of culture filtrate.

Measurement of activity towards carboxymethylcellulose. Carboxymethylcellulase activity was measured viscometrically by a modified version of a method described by Selby (1961). Carboxymethylcellulose was dissolved in water and filtered with the aid of Celite 535; the solids content was measured, and the solution was diluted and phosphate buffer added to give 0·25% (w/v) of carboxymethylcellulose, a final concentration of 0·1 M of total phosphate and pH 6·5. This stock solution was preserved by saturation with washed chloroform; its viscosity remained unchanged during storage for several months at room temperature. Plasma albumin, previously included, was omitted because these cellulase preparations were unpurified. Since enzymic action causes the viscosity to fall at a rapidly decreasing rate it is necessary to make the initial measurements soon after the addition of the enzyme. Suspended-level (Ubbelohde) viscometers with a flow-time for water of about 50 sec. were convenient and sufficiently accurate. The kinetic-energy correction was kept below a tolerable limit (0·4 sec.) by making the flow-volume small (1·2 ml.). Carboxymethylcellulose solution (5 ml.) in the viscometer was brought to temperature in a thermostat at 30°. The enzyme solution (0·125 ml.) was added and mixed by applying alternate suction and pressure to one of the tubes of the viscometer. The reaction was timed from the moment of addition and the first measurement of viscosity was started within 1 min. The activity of the enzyme solution in carboxymethylcellulase units was defined (Selby, 1961) as the rate of change (min.$^{-1}$) of $1/\eta_{sp.}$; since the rate fell with time when the modified technique was used, its average value over the range from $\eta_{sp.} = 1$ to $\eta_{sp.} = 0{\cdot}5$ was taken.

Measurement of the strength of cotton yarns. Because of the uncertainty of any correction for variation in the linear density of cotton yarn that might be applied to measured breaking-loads, and the inconvenience of distinguishing between yarn samples of different linear densities in one experiment, samples (19 cm., measured under a tension of 25 g.) were weighed to the nearest 0·5 mg. and collected in batches of equal weight. After the yarns in a batch had been incubated with cellulase, they were washed, dried in the air and 'conditioned' at 65% relative humidity. The strength was then measured on a pendulum-type single-thread testing machine with a jaw separation of 1 cm. and a rate of loading adjusted so that the sample broke after about 6 sec. Ten breaking-loads were measured along the length of each sample and the mean, $\overline{BL}$, was calculated. The strengths of untreated samples of a range of weights were measured; from a graph of $\overline{BL}$ against weight the initial strength of a weighed sample could be predicted to within ±3% (S.E.). In any one experiment samples of equal

weight were used and, if the measured losses of strength were small, the accuracy was increased by making further measurements of the initial strength of samples in the same batch.

Enzymic degradation of cellulose. The samples of cotton yarn were wound into coils of about 1 cm. diameter and, unless otherwise stated, were incubated for 24 hr. at 30° with a cell-free enzyme preparation adjusted to pH 6·5 and saturated with washed chloroform. A liquor:cotton ratio of 300:1 (v/w) was employed unless otherwise stated. When repeated 24 hr. treatments were to be given the liquor was decanted and immediately replaced by fresh enzyme solution; only after the final treatment was the cotton sample washed and dried. Samples described as 'pretreated' were given three such successive treatments with fresh culture filtrate and were not washed or dried before incubation with the solution whose activity was to be measured. As a control pretreated samples were simultaneously incubated in the appropriate buffer solution to confirm that there was no further loss of strength when no more enzyme was added.

RESULTS AND DISCUSSION

Degradation of cotton by repeated treatments with fresh culture filtrate. In trying to discover why the fungus should be so much more effective than the derived filtrates in degrading unswollen cotton, it was reasoned that for extensive degradation to occur it might be necessary for the supply of a labile enzyme or cofactor to be maintained, or for an inhibitory by-product of the degradation to be removed. Repeated 24 hr. treatments of cotton with fresh portions of a cell-free culture filtrate from *M. verrucaria* were therefore given; by this means a culture filtrate has, for the first time, been made to produce degradation comparable with that caused by a cellulolytic organism. Complete loss of strength with little change in $\bar{P}_v$ [4100 to 3250; see Selby (1961) for comment on the significance of these determinations] and large losses of weight were obtained (Figs. 1 and 2). In contrast, when hydrolysis with hydrochloric acid is used to decrease the strength of unswollen cotton by 75 % the $\bar{P}_v$ falls to about 260 (calculated from the results of Clibbens & Ridge, 1928) and there is little loss of weight. The weakening of cotton with little accompanying change in $\bar{P}_v$ has long been known to be a characteristic of microbial attack (Searle, 1929; Fargher, 1953). This effect was attributed to the highly localized growth of the organism, causing whole regions of the substrate to be digested away and leaving others untouched, but the present results confirm that this is also characteristic of the enzymic attack, a view put forward by Selby (1961) on indirect evidence.

Loss of activity during the reaction. After incubation for 5 days a culture filtrate was unable to degrade cotton further (Fig. 1) but could still weaken a series of fresh cotton samples treated in succession, each suffering a similar loss of strength. The extent of this loss in 24 hr. was 15 %, which is distinctly less than that produced by fresh culture filtrate (cf. Fig. 1). The loss of activity was also evident in a culture filtrate that had been exposed for 24 hr. to each of three fresh cotton samples in sequence and was then unable to weaken further a

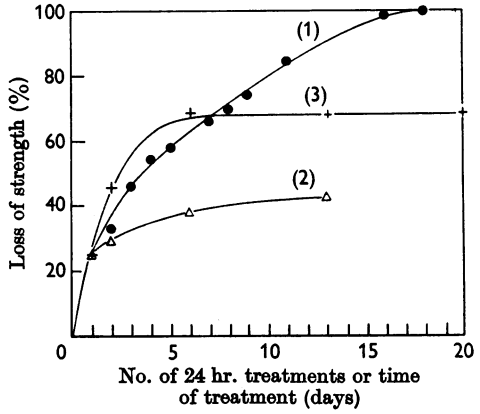

Fig. 1. Weakening of cotton yarn by: ●, repeated daily treatments with culture filtrate (300:1, v/w); △, a single treatment for 13 days at the same liquor:cotton ratio; +, a single treatment for 20 days at 16 times the liquor:cotton ratio, to establish that the increased degradation in curve (1) was not due only to the quantity of enzyme used. Experimental details are given in the text.

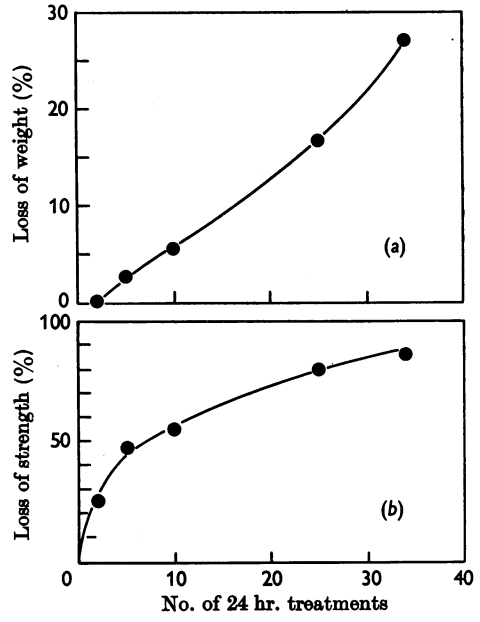

Fig. 2. Loss of (*a*) weight and (*b*) strength by cotton yarn after repeated daily treatments with fresh culture filtrate (300:1, v/w). Experimental details are given in the text.

cotton sample that had already received three pretreatments with fresh culture filtrate (Fig. 3).

The contrast between the complete loss of activity towards pretreated cotton and the persistence of a limited activity towards fresh cotton suggested that two factors must be present. The loss of activity could have been due to the loss of a cofactor or to the accumulation of an inhibitor, but evidence is presented below that diffusible or thermostable cofactors or inhibitors are probably not involved. We therefore postulate the existence of two enzymes attacking cotton cellulose, and ascribe the loss of activity to 'exhaustion' of one of them without making assumptions about the mechanism. This exhaustible cellulase, without which pretreated cotton cannot be further weakened, will be called 'A-enzyme', and the other, which accounts for the residual activity against fresh yarns, 'B-enzyme'. A-enzyme has the ability to attack those linkages in fibrous cotton that have to be broken to produce losses of strength of more than 15–20 %. B-enzyme, on the other hand, cannot attack these linkages and hence on its own cannot decrease the strength of fibrous cotton by much more than 15 %. A-enzyme is rapidly exhausted in its attack on cotton and must be replaced if extensive degradation is to occur; it may be detected and assayed by using as a substrate pretreated cotton, which is resistant to B-enzyme. B-enzyme appears to include those 'cellulases' that attack swollen or substituted celluloses. This working hypothesis was tested in a number of ways.

It seemed possible that the strength of the cotton was lost so much more easily in the earlier stages of the attack that a very small residual quantity of A-enzyme in exhausted culture filtrate, and not B-enzyme, was responsible for the continued ability to attack fresh samples. This alternative explanation was supported by the demonstration that the initial weakening of a fresh sample in a 24 hr. incubation could be caused by considerably diluted culture filtrate (Fig. 4). To determine conclusively whether the remaining activity in exhausted culture filtrate towards fresh cotton samples was due not to A-enzyme but to another enzyme (B-enzyme) it was therefore essential to exhaust the culture filtrate very thoroughly of A-enzyme. This was done by successive 24 hr. treatments with samples of cotton; a portion of the filtrate was retained after four, eight and twelve treatments and its activities towards carboxymethylcellulose and both fresh and pretreated cotton samples were measured. Consideration of the relation between enzyme concentration and the strength lost by a fresh cotton sample (Fig. 4) showed that cellulase concentration could most accurately be measured when the loss of strength was about 8 %. Each exhausted culture filtrate was therefore diluted to produce this loss of strength in fresh cotton, and the diluted filtrate tested for its activities towards carboxymethylcellulose and towards cotton already weakened by 45 % by three pretreatments with fresh culture filtrate. The carboxymethylcellulase activities of these diluted solutions (Table 1) varied by only a small amount, supporting the view that the initial loss of strength by cotton and the decrease of viscosity of carboxymethylcellulose solution are caused by the same enzyme (B-enzyme). None of these diluted exhausted culture filtrates had any measurable activity towards pretreated cotton, confirming that there must be more than one enzyme

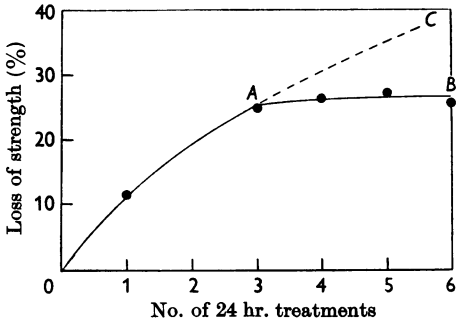

Fig. 3. Contrast between the losses of strength produced by fresh and by exhausted culture filtrates during repeated treatment of cotton yarns. Origin to *A*: repeated daily treatments with fresh filtrate; *A* to *B*: repeated daily treatments with filtrate exhausted by three successive exposures to fresh cotton; *A* to *C*: expected curve for further repeated daily treatments with fresh filtrate [cf. curve (1) of Fig. 1]. Experimental details are given in the text.

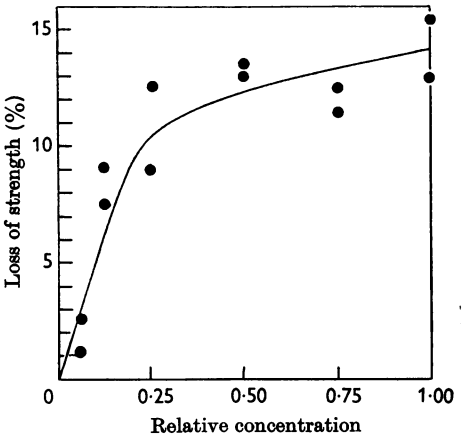

Fig. 4. Influence of dilution on the loss of strength produced during a single 24 hr. treatment of cotton yarn with culture filtrate. The concentration of culture filtrate is given on the abscissa as a fraction of the original (1·00). Experimental details are given in the text.

species active towards cotton; the A-enzyme was rapidly exhausted during four exposures to fresh cotton, but the activity of B-enzyme both towards fresh cotton and towards carboxymethylcellulose was lost, during many more exposures, at a much lower rate (Table 1); some loss would in any case be expected from adsorption of the enzyme on successive samples.

Attempts to detect cofactors or inhibitors. The possibility that the loss of A-enzyme activity is caused by loss of a cofactor or accumulation of an inhibitor was next considered. Such a cofactor or inhibitor might be diffusible, and to test this possibility fresh and exhausted culture filtrates were each dialysed against 0·01 M-phosphate buffer solution, pH 6·5, by gel-filtration through a column of Sephadex G-25. In the gel-filtration procedure no attempt was made to attain high resolution in terms of molecular weight. The columns were characterized with haemoglobin and nitrophenylaspartic acid (convenient coloured substances of high and low molecular weight respectively) and used under conditions that would just permit their complete separation. The eluate was collected in two fractions, fast and slow, that would contain all the haemoglobin and nitrophenylaspartic acid respectively. When culture filtrate was applied to the column all the carboxymethylcellulase emerged in the fast fraction. Since this fraction was twice the volume of the culture filtrate put on the column its activity against cotton was compared with that of the culture filtrate diluted with an equal volume of the phosphate buffer. This comparison revealed no decrease in the activity of a fresh culture filtrate or increase in the activity of an exhausted culture filtrate.

It was still possible that, if a cofactor or inhibitor were present but too firmly bound to the cellulase to be separated by dialysis, it might be freed by denaturing the enzyme by heat. A cofactor in heated fresh culture filtrate should then be capable of reactivating exhausted culture filtrate, or an inhibitor in heated exhausted culture filtrate of deactivating fresh culture filtrate.

Culture filtrate was adjusted to pH 6·5 with dilute acetic acid and its buffer capacity increased, at the same pH, by adding M-sodium phosphate buffer solution (1:100, v/v). Some of this solution was exhausted of its activity towards cotton by three successive 24 hr. exposures to fresh cotton samples. Portions of the exhausted filtrate and of the fresh buffered filtrate were heated in a water bath at 100° for 10 min. Samples of cotton were pretreated by repeated exposure for 24 hr. to three quantities of fresh buffered filtrate. To test for the presence of a heat-stable inhibitor in exhausted filtrate, exhausted heated filtrate was mixed with an equal volume of fresh filtrate and, for comparison, samples of exhausted heated filtrate and of fresh filtrate were each diluted with an equal volume of 0·01 M-phosphate buffer solution, pH 6·5. Similarly, to test for the presence of a cofactor in fresh filtrate, fresh heated filtrate was mixed with an equal volume of exhausted filtrate and again, for comparison, each component of the mixture was diluted with the phosphate buffer solution. The activities of these six solutions were compared by incubating each in three successive portions with a pretreated cotton sample and measuring the resulting loss of strength. Control measurements were made on three similarly pretreated samples incubated with 0·01 M-phosphate buffer solution, pH 6·5, for the same times. The results (Table 2) showed that if there was any reactivation it was not enough to restore the activity to that of the fresh culture filtrate, and that if there was any deactivation it did not lower the activity to that of the exhausted filtrate. This is

Table 1. *Effect of repeated exposure to fresh cotton samples on the activity of culture filtrate towards cotton and towards carboxymethylcellulose*

A culture filtrate was repeatedly exposed for periods of 24 hr. to fresh cotton samples. At intervals the activity of the filtrate, and of dilutions made from it, were tested against fresh cotton to determine the dilution that caused an 8% loss of strength in 24 hr. This dilution was then tested for its action on carboxymethylcellulose. Other details are given in the text.

No. of 24 hr. exposures	Dilution necessary to produce an 8% loss of strength	$10^2 \times$ Carboxymethylcellulase activity, after dilution (units)
0	6·0-fold	1·83
4	3·0-fold	1·72
8	1·7-fold	1·70
12	1·2-fold	1·54

Table 2. *Test for a thermostable cofactor in fresh culture filtrate and for an inhibitor in used culture filtrate*

Fresh culture filtrate, heated or unheated, and exhausted culture filtrate, heated or unheated, were mixed in equal volumes with one another or with 0·01 M-phosphate buffer. The activities of these six mixtures were compared by incubating each in three successive portions with pretreated cotton that had already been weakened by 42%. Other details are given in the text.

Composition of incubation fluid			Average further loss of strength (%)
Unheated component	Heated component	Buffer	
Fresh	—	+	14
Fresh	Exhausted	−	11_5
—	Exhausted	+	−3
Exhausted	—	+	2
Exhausted	Fresh	−	5
—	Fresh	+	−1
—	—	+	0

negative evidence and the possibility remains that an inhibitor might be present which is not desorbed and readsorbed on the enzyme under our conditions.

Comparison of loss of weight with loss of strength. During the later stages of experiments involving repeated treatment of cotton samples with culture filtrate small fragments of cotton hairs always separated, and when loss of weight was measured it was necessary to retain these within the reaction vessel. The cotton and culture filtrate (40 mg. in 12 ml.) were placed in a sintered-glass crucible (porosity no. 3) contained in a weighing bottle, and sufficient chloroform was placed outside the crucible to prevent any significant quantity of either fluid flowing through the sintered disk into the other. After each treatment the spent liquor was withdrawn by suction and immediately replaced by fresh culture filtrate; in this way no insoluble material was lost during the experiment. This was confirmed by refiltering all the spent liquor and washings from each experiment through a small disk of fine glass filter paper (Whatman GF/C), which was subsequently shown to be free from carbohydrate by treatment for 1 hr. with boiling 0·2 N-potassium dichromate solution in 30 % (v/v) sulphuric acid. The excess of dichromate was determined, after dilution, by titration with 0·1 N-ferrous ammonium sulphate in 2 % (v/v) sulphuric acid; phenylanthranilic acid was used as an internal indicator. It was also confirmed that the final thorough washing of the cotton sample before weighing removed almost all the residual nitrogenous material (nitrogen in residual solids approx. 0·025 %). All the cotton samples were weighed after washing and drying to constant weight over phosphorus pentoxide in a vacuum oven at 60°.

The considerable solubilization of fibrous cotton subjected to repeated applications of culture filtrate (Fig. 2*a*) illustrates the effectiveness of this technique in imitating the action of the fungus. Comparison of Fig. 2 (*b*) with curve (1) of Fig. 1 shows a difference in the A-enzyme activity of the culture filtrate used. Although such differences were commonly found, the relation between loss of strength and loss of weight was little changed. For example, in other experiments losses of strength of 67 and 80 % were accompanied by losses of weight of 10·5 and 17 % respectively. No loss of weight occurred during the first 24 hr. treatment with a fresh culture filtrate, during which time the sample was weakened by about 20 %. Whether this reflects differences in the modes of action of A-enzyme and B-enzyme cannot be established until A-enzyme has been isolated. Previously recorded losses of weight have been low, usually 4 % or less (Halliwell, 1957).

Recovery of enzyme from the culture solids. The strong adsorption of cellulases on cellulose is probably one of the main reasons for the low activity towards fibrous cotton of cell-free culture filtrates of cellulolytic organisms, and it was thought that a quantity of A-enzyme might be recovered from the culture solids that are usually rejected. Van Haga (1958) has demonstrated the splitting of complexes of cellulases and carbohydrate material by washing them through a column of cellulose powder with dilute alkali, which suggests that the cellulase–substrate complex is less stable in alkaline conditions. We therefore attempted to elute cellulase from culture solids of *M. verrucaria* with solutions of borax. The mycelium and cellulose remaining after filtration through glass paper (Whatman GF/B) of a chilled 5-day culture of *M. verrucaria* on cotton flock was mixed with an equal bulk of Celite 535 and dispersed in sufficient phosphate buffer solution (0·02 M with respect to total phosphate), pH 6·5, at 2° to form a slurry. This was formed into a column 2·5 cm. in diameter and 20 cm. long; the subsequent elutions were done in a cold room at 2°. The column was first washed with the same buffer solution (2 l.; rate of flow 25 ml./hr.) and then with 0·01 M-sodium tetraborate buffer, pH 9·0. The eluate was collected automatically in 10 ml. fractions in test tubes that already contained 0·08 M-sodium dihydrogen phosphate (5 ml.) to return the pH to 6·0–6·5 and thus minimize exposure of the enzyme to high pH. Each fraction was then stirred for about 30 min. by a very slow stream of nitrogen bubbles from a glass tube automatically introduced into the test tube. The activities of the eluted fractions towards carboxymethylcellulose and towards cotton were measured. For the latter determination coiled cotton samples were placed in the phosphate solution in alternate test tubes, which after collection of the fractions were incubated at 30° for 5 days. The eluates were active towards both carboxymethylcellulose and cotton (Fig. 5). Peak activities were comparable with those of culture filtrates (e.g. carboxymethylcellulase activity = $1{\cdot}8 \times 10^{-2}$ unit; loss of strength by cotton after incubation for 5 days = 35 %). Later fractions (at about 2900 ml.) had activities towards cotton as great as earlier fractions (at about 2600 ml.), but with only half of the carboxymethylcellulase activity, showing a partial separation of A- and B-enzymes. The second peak of activity at 2800 ml. was not explained.

The elution technique was subsequently improved by using a competing soluble substrate. Column techniques proved unsatisfactory as reasonable rates of flow could not be obtained unless excessive quantities of Celite 535 were added; batchwise extraction at 30° was therefore employed. A 5-day culture of *M. verrucaria* was centrifuged at 35 000 *g* for 10 min., and 50 g. of the wet solids remaining was stirred with 120 ml. of 0·02 M-phosphate buffer solution, pH 6·5, for 30 min. Again the solids were separated by centrifuging, and the

extraction was repeated once with the same buffer solution, then twice with 0·1 M-phosphate buffer, pH 5·5. One half of the solids was again extracted with the phosphate buffer, pH 5·5, and the other with the same solution containing 1 % (w/v) of carboxymethylcellulose. The extracts were diluted twofold in such a way that they finally contained the same amount of carboxymethylcellulose and had the same pH and ionic strength. The activities of these extracts were measured towards carboxymethylcellulose and towards a cotton sample already weakened by 35 % by pretreatment with culture filtrate (Table 3). In a comparative experiment the extraction was made with 1 % (w/v) methylcellulose solution (Table 4). The inclusion of carboxymethylcellulose or methylcellulose in the eluting buffers yielded products with enhanced activity towards cotton.

Effect of growing the organism in the presence of carboxymethylcellulose. The inclusion of carboxymethylcellulose (1 %) in cultures of *M. verrucaria* on cotton flock in a mineral medium caused an increase in the relative A-enzyme activity of the culture filtrate with no corresponding increase in carboxymethylcellulase activity. The filtrate from the culture containing carboxymethylcellulose weakened a pretreated cotton sample, that had already lost 35 % of its initial strength, by a further 34 % during incubation for 7 days. In the absence of carboxymethylcellulose the corresponding further loss of strength was only 24 %; both filtrates had a carboxymethylcellulase activity of 17×10^{-2} unit. Presumably a continuous transfer of the A-enzyme from the cotton to the carboxymethylcellulose occurred. By contrast, the addition of carboxymethylcellulose to a culture filtrate immediately before application to cotton diminished the resulting loss of strength (7-day treatment) from 37 to 17 %. Apparently the carboxymethyl-

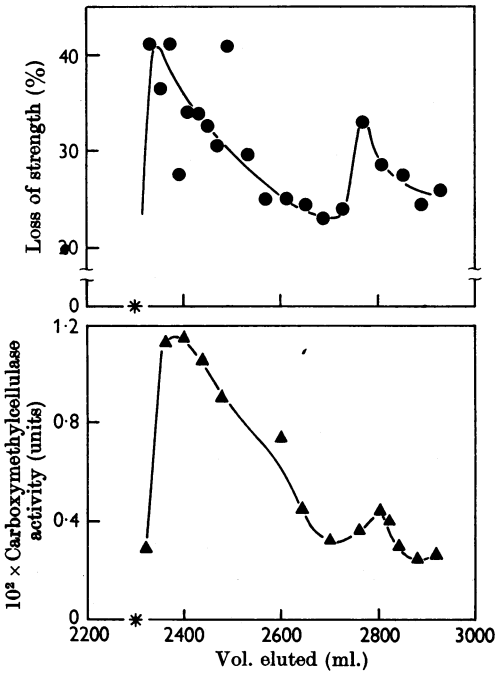

Fig. 5. Elution of cellulase from residual culture solids with tetraborate buffer. The mycelium and cellulose from a 5-day culture of *M. verrucaria* were mixed with Celite 535 and packed to form a column of 2·5 cm. diam. and 20 cm. long, which was washed first with 2 l. of 0·02 M-phosphate buffer, pH 6·5, then with 0·01 M-sodium tetraborate, pH 9·0. Tetraborate emerged from the column at the point marked with an asterisk. Incubations with cotton were for 5 days at 30°. Other details are given in the text.

Table 3. *Elution of cellulase from residual culture solids by carboxymethylcellulose solution*

Solids from a 5-day culture were twice extracted (30 min.) with phosphate buffer, pH 6·5, twice with phosphate buffer, pH 5·5, and divided into two parts. These were again extracted, one with phosphate buffer, pH 5·5, the other with the same buffer containing carboxymethylcellulose. After additions of buffer and carboxymethylcellulose solution to make pH, ionic strength and carboxymethylcellulose concentration the same, the extracts were tested on carboxymethylcellulose and on pretreated cotton. Other details are given in the text.

	10^2 × Carboxymethylcellulase activity (units)	Further loss of strength by pretreated cotton (%)
Culture filtrate	2·3	5
Third extract, with buffer (pH 5·5)	0·2	3
Fifth extract, with buffer (pH 5·5)	0·1	4
Fifth extract, with buffer (pH 5·5) + 1 % of carboxymethylcellulose	$0{\cdot}4_5$	19

Table 4. *Elution of cellulase from residual culture solids by solutions of carboxymethylcellulose and of methylcellulose*

The experimental procedure is described in Table 3; the final extractions were made with phosphate buffer, pH 5·5, 1 % carboxymethylcellulose and 1 % methylcellulose solutions.

Extraction solution	Loss of strength by a fresh cotton sample after incubation for 5 days at 30° (%)
Buffer (pH 5·5)	5
Buffer (pH 5·5) + 1 % carboxymethylcellulose	18_5
Buffer (pH 5·5) + 1 % methylcellulose	18

cellulose again competes for the cellulase, this time at the expense of the degradation reaction.

When the organism was grown on carboxymethylcellulose alone the filtrate had much less activity towards pretreated cotton than when fibrous cellulose was included; the respective losses of strength were 4 and 24 %, both filtrates having first been diluted to the same carboxymethylcellulase activity ($3{\cdot}5 \times 10^{-2}$ unit). The inoculum was of spores and mycelium from an actively growing culture on cellulose and therefore adapted to cellulase production; it thus appears that the presence of fibrous cellulose in the growth medium is necessary for the production of A-enzyme by the organism. The same conclusion was reached from an experiment in which cultures of *M. verrucaria* were grown in mineral media containing different quantities of cellulose.

Effects of varying the amount of cellulose present in the growth medium. It was hoped that, when the organism was grown in the presence of less cellulose, the more complete digestion of the substrate might release into solution cellulase otherwise trapped in the cellulose or adsorbed on it. Gilligan & Reese (1954) measured the ratio of the activities of culture filtrates towards a swollen partially-degraded cellulose and towards carboxymethylcellulose. The ratio increased as the amount of cellulose in the culture medium was decreased; the results of our experiment (Table 5) show that, although A-enzyme activity was enhanced relative to carboxymethylcellulase activity, the effect was small and both activities were diminished.

A possible mechanism for the exhaustion of A-enzyme. The reason for the exhaustion of the A-enzyme in a culture filtrate on exposure to cotton is not understood. The strong adsorption by cellulose of cellulase, and the ability of carboxymethylcellulose to compete for the enzyme, suggest the formation during cellulolysis of an enzyme–carbohydrate complex that persists either on the cellulose substrate, and thus immobilizes the enzyme, or in solution. In the latter case the deactivation might be only partial, depriving the enzyme of A-enzyme activity but not of B-enzyme activity; the B-enzyme found in culture filtrates may be formed from A-enzyme in this way. There is, however, as yet no direct evidence to support this idea.

Table 5. *Effect of the amount of cellulose in the culture medium on the production of cellulase by* Myrothecium verrucaria

Experimental details are given in the text.

Concn. of cellulose in culture (g./l.)		30	3·3	0·33
$10^2 \times$ Carboxymethylcellulase activity of filtrate (units)		18	3·5	0·8
Further loss of strength by a cotton sample, pretreated to a loss of strength of 35 %, on incubation for 7 days (%)	In filtrate	25	27	19
	In filtrate diluted to a carboxymethylcellulase activity of $0{\cdot}8 \times 10^{-2}$ unit	13	16	19

SUMMARY

1. The extensive degradation of cotton by cellulolytic micro-organisms has been imitated by the use of cell-free culture filtrates from *Myrothecium verrucaria*. Complete loss of strength with little change in degree of polymerization, and large losses of weight, were obtained.

2. The culture filtrate loses its activity towards cotton during attack and must be repeatedly renewed for extensive degradation to occur. A filtrate that has ceased to attack a particular cotton sample is, however, still capable of weakening a fresh sample by about 15 %. This limited activity is ascribed to a stable cellulase (B-enzyme) that may be identifiable with the 'cellulases' active towards swollen or soluble cellulosic substrates and that is distinct from the labile enzyme (A-enzyme) necessary for extensive degradation of cotton.

3. A-enzyme is adsorbed by cotton and can be desorbed by alkali and by solutions of cellulose derivatives. Cotton on which the organism has grown thus provides a source of cellulase.

4. The production of A-enzyme requires the presence of fibrous cellulose in the culture medium and is increased if carboxymethylcellulose is added.

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The Catalysis of the Auto-oxidation of 2-Mercaptoethanol and other Thiols by Vitamin B_{12} Derivatives

POLAROGRAPHIC AND OTHER INVESTIGATIONS

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During a study of the exchange reaction between CO_2 and the carboxyl group of pyruvic acid catalysed by preparations from *Peptostreptococcus elsdenii* (previously referred to as rumen organism LC), it was found that the auto-oxidation of 2-mercaptoethanol (monothioethylene glycol) is greatly accelerated by catalytic amounts of vitamin B_{12} derivatives at pH 7 (Peel, 1962*a*). Such an effect does not appear to have been reported previously at physiological pH, except for an incidental statement by Dubnoff (1950) that vitamin B_{12} catalysed the oxidation of certain thiols in a non-enzymic system. In view of the current interest in vitamin B_{12} derivatives, the increasing use of 2-mercaptoethanol as a reducing agent in incubation mixtures and the possibility that this catalytic property might be of use for the detection and assay of vitamin B_{12} derivatives, the reaction was investigated further. The present paper reports the results, some of which have appeared in brief (Peel, 1962*b*).

MATERIALS AND METHODS

Chemical reagents

The thiols used were as follows: 2-mercaptoethanol, Eastman grade (from Kodak Ltd., Liverpool); 2,3-dimercaptopropanol (BAL) and L-cysteine hydrochloride (from L. Light and Co. Ltd.); thioglycollic acid, laboratory-reagent grade (from British Drug Houses Ltd.); glutathione [from The Distillers Co. (Biochemicals) Ltd., Liverpool]; DL-dihydro-6-thioctic acid, assay 98%, and DL-dihydro-6-thioctic amide, assay 100% (from Farmochimica Cutolo-Calosi S.p.A., Naples; gifts from Dr V. Massey). The 2-mercaptoethanol, 2,3-dimercaptopropanol and thioglycollic acid were distilled under reduced pressure before use and then assayed to be 100, 100 and 95% pure respectively by iodometric determination. The 2-mercaptoethanol was subsequently used over an extended period during which it was stored at room temperature. After 6 months the purity had fallen to 92% and the material was distilled again. In experiments where the amount of thiol was critical, solutions were standardized iodometrically before use. Where necessary thiol solutions were neutralized to pH 7·1 with NaOH, immediately before use. Aqueous 50% (v/v) methanol was used to dissolve the dihydrothioctic acid amide. Controls showed that this solvent had no effect on the polarographic measurements.

The vitamin B_{12} derivatives were obtained as gifts and, apart from the exceptions mentioned below, were from Dr E. Lester Smith. Their structural relationships to cyanocobalamin are indicated in Table 3. The 5,6-dimethylbenzimidazolylcobamide coenzyme (Barker *et al.* 1960*b*) was obtained from Dr H. A. Barker. As the cobamide coenzymes are very labile to light, experiments with this compound were done in near darkness with minimum artificial illumination. Factors A and B (Ford & Porter, 1953) and Factor D (Brown, Cain, Gant, Parker & Smith, 1955) were obtained as solutions in aqueous 70% (v/v) ethanol from Dr J. W. G. Porter and Dr H. R. V. Arnstein. These solutions were standardized by measurement of the extinction at 367 mμ in 0·1 M-KCN and assuming ϵ to be $30{\cdot}4 \times 10^3$ l. mole^{-1} cm.$^{-1}$ as for dicyanocobalamin (Barker *et al.* 1960*a*). The term Factor B is usually used to denote the monocyano derivative of cobinamide, derived from cyanocobalamin by removal of the nucleotide, but as this compound readily takes up an additional molecule of cyanide, the scope of the term has sometimes been extended to include the dicyano form as well. The spectrum of the Factor B sample used in all the experiments reported below corresponded to a mixture of the mono- and di-cyano forms. On adding excess of cyanide, the spectrum changed to one closely resembling that for dicyanocobinamide given by Ford & Porter (1953). Factor B has not yet been crystallized; as far as the author is aware, reliable extinction coefficients, which would permit an evaluation of the proportions of the two forms in the mixture, are not available. The