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The Breakdown of Cellulose and its Derivatives by Enzymes from *Myrothecium verrucaria*

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(*Received* 29 *March* 1962)

Halliwell (1961) examined some of the circumstances controlling the slow solubilization of native cellulose and the complete solubilization of swollen cellulose powder and swollen fibres by filtrates from *Myrothecium verrucaria*. The organism itself experiences no difficulty in rapidly decomposing undegraded cellulose of the type associated with cotton fibres. However, the purified 'cellulases' described so far show marked activity on carboxymethylcellulose, but are less effective than culture filtrates against undegraded cellulose (cf. Youatt & Jermyn, 1959).

Cell-free filtrates were used in the present work, which extends my previous findings (Halliwell, 1961) and presents further properties of the cellulolytic enzymes together with an analysis of the requirements for the quantitative determination of carboxymethylcellulase.

MATERIALS AND METHODS

Source of enzymes

Preparations of cell-free culture filtrates from *M. verrucaria* and from rumen bacteria were used as sources of cellulolytic enzymes and were as described by Halliwell (1961, 1957*b*). In the present work, rumen fluid was strained through surgical gauze and centrifuged at 300*g* for 1 min. to remove most of the protozoa. The supernatant liquor was subsequently centrifuged at 20 000*g* for 30 min. at 1° (Spinco model L centrifuge, rotor 30). The precipitate was resuspended in the minimal volume of supernatant, decanted to a conical flask at 1°, and 0·4 vol. of butan-1-ol (AnalaR) was added slowly with stirring. After the addition of butanol had been completed, stirring was continued for a further 15 min. The mixture was centrifuged, at 20 000*g* as above, and the aqueous phase was carefully separated from over- and under-lying layers and recentrifuged to give a clear orange phase. An occasional opacity in some preparations was removed by the dropwise addition of water and mixing. Crystalline pepsin (porcine mucosa) and crystalline trypsin (bovine pancreas) were commercial preparations (Armour Pharmaceutical Co. Ltd.).

Substrates

Dewaxed cotton fibres, cellulose powder (Whatman), swollen cellulose powder and hydrocellulose were as described by Halliwell (1961, 1957*a*).

Carboxymethylcellulose (Cellofas B, ref. no. MH.Y.2, degree of substitution about 0·5) solutions (1%, w/v) were prepared by mixing 0·3 g. in 30 ml. of boiling water and heating at 100° for 30 min. with occasional stirring to dissolve particulate matter. After cooling and making up to 30 ml. with water the 'solution' was centrifuged at 1800*g* for 15 min. to remove fibrillar matter. The viscous but clear supernatant phase was removed with a pipette and served as the stock solution for carboxymethylcellulase assays. It could be stored at room temperature (20°) or 3° for about 3 days (see the Results section).

Analytical procedure

Determination of the activities of cellulolytic enzymes. The methods of assay with the soluble substrate, carboxymethylcellulose, and with insoluble forms of cellulose were as described by Halliwell (1961). In the carboxymethylcellulase assay a point of particular importance was the need for care in pipetting small volumes of reagents into a compact reaction volume; in this respect conical Quickfit tubes of the type described by Levvy & McAllan (1959) were useful. With cellulose, hydrolysis was measured by two procedures, namely the indirect procedure, based on the amount of soluble carbohydrates transferred to the aqueous phase, and the direct procedure (Halliwell, 1958). The latter was only used with fibres. It was modified to include a preliminary alkaline digestion of the fibres and the volumes of reagents were increased 4·5-fold to deal with the greater weight of fibres present (5 mg.). In addition the heating period at 100° was extended to 1½ hr. After

enzymic digestion the aqueous phase was sucked off from the fibres with a filter-stick and the fibres were washed with water which was removed in the same way. The filter-stick was transferred to a clean tube. The fibres, in their original tube, received 7 ml. of N-NaOH and were heated at 100° for 15 min. under a pear-bulb. The tube was cooled for 5 min. The filter-stick was re-introduced to remove the alkaline digest from the fibres which were washed rapidly with four lots of 5 ml. of N-NaOH, four lots of 5 ml. of water and 4 ml. of 1·6N-H_2SO_4. The filtrates were discarded. Finally, any contents of the tube which had held the filter-stick during the alkaline digestion of the fibres were washed with water into the tube containing the fibres, which were now sucked as dry as possible. Water (4 ml.), followed by 9 ml. of 0·5% $K_2Cr_2O_7$–H_2SO_4, was added to the fibres plus filter-stick and the mixture heated at 100° for 1½ hr. under a pear-bulb. The solution was cooled, carefully diluted with 13 ml. of water, mixed and sucked rapidly through the filter-stick into a Quickfit boiling tube. There was no unfilterable residue. This was followed by further water washings of the tube until a total of 45 ml. of water had been added to the original 13 ml. of water–chromic acid oxidizing mixture. A 'blank' tube containing 4 ml. of water and 9 ml. of 0·5% $K_2Cr_2O_7$–H_2SO_4 was heated as above, but without a filter-stick, and, after dilution, was treated with 0·9 ml. of 20% (w/v) Na_2SO_3 to give the colorimetric 'blank'.

Determination of pepsin and trypsin. These activities were measured as described by Herriott & Laskowski (1955) and Northrop (1939), with bovine haemoglobin, enzyme–substrate powder (Armour Pharmaceutical Co. Ltd.) and Folin's phenol reagent. Colorimetric readings were made on the Spekker absorptiometer (Hilger) with Ilford red filters (no. 608, peak transmission 700 mμ) and tyrosine for the standard reference curve.

Alkali solubility of cotton fibres, cellulose powder and hydrocellulose. Each form of cellulose (30 mg.) was boiled at 100° in 7 ml. of N-NaOH for 15 min. under a pear-bulb, cooled for 5 min. and transferred to a sintered-glass crucible (porosity 3). The contents of the test tube were washed out with 10 ml. of N-NaOH on to the cellulose, which was then sucked almost dry, washed with 20 ml. of N-NaOH, sucked dry and washed with water (about five lots of 20 ml.) until neutral. The crucible with its cellulose was dried overnight at 105° and weighed.

Estimation of protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall as modified by Halliwell (1961). The cell-free rumen bacterial preparations contained 3 mg. of protein (as albumin)/ml. and the cell-free fungal filtrates 0·18 mg. of protein/ml.

Solutions used for measuring the effect of pH *on the stability of carboxymethylcellulase and cellulase.* The following buffer solutions were employed: for pH 2, 3, 4 and 5, 0·2N-sodium acetate–HCl (readjusted, after incubation with enzyme, to pH 5·5 with M-sodium acetate or 0·18M-KH_2PO_4); for pH 6 and 7, 0·05M-KH_2PO_4–NaOH (readjusted, after incubation with enzyme, to pH 5·5 with 0·2M-KH_2PO_4 or 0·02N-acetic acid); for pH 8 and 9, 0·05M-boric acid–KCl–NaOH (readjusted, after incubation with enzyme, to pH 5·5 with 0·02N-acetic acid).

The experimental tubes, containing 0·6 ml. of buffer of the required pH, 0·6 ml. of water and 0·3 ml. of cell-free filtrate from *M. verrucaria*, were incubated for 15 min. at 37°, followed by readjustment of the pH to 5·5 (the normal assay reaction) as described above. A known volume of the mixture was removed into a second tube containing swollen cellulose powder (final vol. 4 ml.), pH 5·5, and assayed for cellulase in the standard procedure by measuring the production of soluble carbohydrates. A second portion was transferred into a third tube and diluted with water to give a suitable dilution of carboxymethylcellulase for removal of 0·1 ml. to tubes containing carboxymethylcellulose and the buffer for this assay.

Control tubes containing untreated enzyme were incubated at pH 5·5 and 37°, with appropriate buffer mixtures.

Peptic and tryptic digests of carboxymethylcellulase and cellulase. To estimate possible inactivation of cellulolytic enzymes merely by a pH effect, control tubes were prepared containing all reagents, except the proteinase, present in the experimental tubes. Briefly, 0·6 ml. of buffer solution (pH 5 for pepsin, pH 6·3 for trypsin) plus 0·6 ml. of water plus 0·3 ml. of cell-free filtrate, pH 5·9, from *M. verrucaria* (0·1 mg. of protein) were incubated at 37°, adjusted to pH 5·5 and subsequently divided into portions for assay on swollen cellulose powder and on carboxymethylcellulose as described above. The composition and treatment of the experimental tubes were identical with those of the control tubes except for replacement of the 0·6 ml. of water by 0·6 ml. of pepsin or trypsin.

In the usual 10 min. assay of pepsin at 37° and pH 1·8 on haemoglobin the maximum value on the standard tyrosine calibration curve was attained with 7×10^{-4} pepsin units [units calculated from Northrop (1939)]. In the peptic digests of cellulase and carboxymethylcellulase at pH 5 where peptic activity is much reduced (see text) I used 10 times this amount of pepsin, and in addition increased the incubation period to 60 min.

In the usual 10 min. assay of trypsin at 37° and pH 7·6 on haemoglobin the maximum value on the standard tyrosine calibration curve was obtained with 8×10^{-4} trypsin units (units calculated as with pepsin). In the tryptic digests of the cellulolytic enzymes at pH 6·3 where tryptic activity is reduced (see text) I employed the same amount of trypsin as above but increased the incubation period to 35 min.

RESULTS

Action of cell-free filtrates from rumen bacteria and from Myrothecium verrucaria *on carboxymethylcellulose.* There are difficulties in the estimation of carboxymethylcellulase activity. With cell-free filtrates from *Aspergillus oryzae* there is a variable susceptibility of carboxymethylcellulose to enzymic attack, depending on the method of preparation of the substrate (Jermyn, 1952); 'aging' of the substrate was also reported, together with failure to achieve saturation of the enzyme from rumen bacteria with carboxymethylcellulose (Festenstein, 1959); and with filtrates from *M. verrucaria* there is a lack of proportionality between enzyme concentration and activity (Miller, Blum, Glennon & Burton, 1960).

For quantitative work these variables must be eliminated and this I have attempted as follows, using cell-free filtrates from rumen bacteria and

from *M. verrucaria*. Solutions of carboxymethylcellulose, prepared as described in the Materials and Methods section, may be stored at 3° for up to 3 days and give essentially the same assay values with rumen carboxymethylcellulase. With samples stored for 3 days at room temperature (20°) the 'blank' value of carboxymethylcellulose increases significantly on the third day giving an apparent net 4–8 % decrease in enzymic activity, since the experimental-tube reading remains at the normal value.

Provided that at no substrate concentration is the glucose production allowed to exceed 11 μg., it is possible to achieve saturation. The substrate concentration (0·3–0·35 ml. of 1 %, w/v, carboxymethylcellulose/0·6 ml. of total assay volume) used in the normal fungal carboxymethylcellulase assay (Halliwell, 1961) proved to be sufficient for saturation of the rumen bacterial preparation, and glucose production was restricted to less than 11 μg. since this was the limit of the method.

The Michaelis constant, K_m, of the enzyme–substrate complex for the bacterial carboxymethylcellulase was 0·8 g. of carboxymethylcellulose/l., compared with the fungal enzyme value of 0·5 g./l. (Halliwell, 1961). The reaction velocity is proportional to the concentration of carboxymethylcellulase from rumen bacteria. A similar linearity was obtained with the fungal enzyme.

Action of cellulolytic enzymes on carboxymethylcellulose and on cellulose. The bacterial preparation described above was more active on the soluble carboxymethylcellulose than was the fungal filtrate, whereas the reverse situation occurred with insoluble swollen cellulose as substrate, irrespective of the higher protein concentration of the dialysed bacterial preparation (Table 1). The breakdown of swollen cellulose by the fungal preparation after 1 hr. corresponds to about 6 % hydrolysis.

Neither preparation, however, was particularly effective in solubilizing undegraded cellulose of dewaxed cotton fibres. This was true even with successive additions of fungal enzyme over a period of 4 days. The effect did not exceed 4 %. This may be due to enzymic production of physical changes in cellulose which are not reflected in the formation of soluble products. Thus dewaxed Texas cotton fibres, cellulose powder (Whatman) and a hydrocellulose (prepared by treatment with hydrochloric acid; Halliwell, 1957*a*) are all insoluble in water, but after digestion with N-sodium hydroxide for 15 min. at 100° followed by filtration, washing and drying at 105°, the losses in weight (corrected for moisture contents of 8, 7 and 6 % respectively) were 3, 17 and 14 % respectively, indicating physical differences between undegraded cellulose in cotton fibres and the more degraded celluloses. I have used this alkaline treatment in the determination of the microbial breakdown of cotton fibres by *M. verrucaria* and find that it gives weights for residual cellulose which are 20 % less than those given by previous washing techniques (Halliwell, 1957*a*), presumably owing to a more efficient removal of fungal mycelium, but possibly also because of solubilization of microbially degraded cellulose.

When the alkaline procedure was applied to cotton fibres, previously incubated with daily additions of *M. verrucaria* filtrates, the extent of solubilization was greater than formerly, but simultaneously was more variable, sometimes providing maximum breakdown of cellulose in 1 day, sometimes needing several days to achieve the same stage with other preparations (Fig. 1). In no case has solubilization been extensive, the values usually being restricted to a maximum of 10–13 % of the weight of fibres.

Activity of cellulolytic enzymes on carboxymethylcellulose and on swollen cellulose powder at different temperatures. Cell-free filtrates from *M. verrucaria* are active on both soluble carboxymethylcellulose and on cellulose over the range 17–80° with maximum effect around 50° (Fig. 2). The butanol-extracted enzyme from rumen bacteria has no action on insoluble cellulose (Table 1), but with carboxymethylcellulose it provided temperature–

Table 1. *Comparison of the activity of rumen bacterial filtrates and* Myrothecium verrucaria *filtrates on carboxymethylcellulose and swollen cellulose powder*

Enzymic activities were assayed by the standard procedures: chromate oxidation of the soluble products (in 0·9 ml. of filtrate) for cellulase and reducing-sugar estimations for carboxymethylcellulase (see text).

Source of cell-free enzyme	Protein content (mg./ml. of undiluted enzyme)	Activity at pH 5·5: On swollen cellulose powder with undiluted enzyme (mg. of 'glucose' formed)	Activity at pH 5·5: On carboxymethylcellulose with enzyme diluted 1:100 (μg. of 'glucose' formed)
Rumen bacteria	3	0	6·3
M. verrucaria	0·18	0·29	3·3

activity curves similar to those displayed by the fungal enzyme for that substrate in Fig. 2. Compared with the standard assays at 37° the activity of *M. verrucaria* carboxymethylcellulase increased by 70% at 50° whereas cellulase was 100% more efficient at 50°.

Stability to heating of the carboxymethylcellulase and cellulase activities of Myrothecium verrucaria *filtrates.* The retention of considerable activity in the presence of substrate at the elevated temperatures described above led me to investigate the effect of duration of heating in the absence of substrate at 100° (Fig. 3): 80% of the enzymic activity on the two substrates is lost in the first 10 min. and this is followed by a slow but progressive loss of a further 10% of activity over the next 20 min. After being boiled for 30 min., however, the cell-free filtrates still apparently possessed a small fraction (10%) of activity, which, with cellulase, represents less than 0·5% hydrolysis of cellulose and is not therefore truly representative of attack on structures other than the shorter, more degraded, chains in this substrate. This may represent non-enzymic extraction of short-chain molecules by the boiled tissue preparation. Cellulose in the absence of boiled enzyme preparation showed no loss in weight from the substrate. Alternatively a small residual fraction of enzymic activity may be acting on readily-hydrolysable short-chain molecules.

Variation in stability with pH. Fig. 4 illustrates the effect of pH adjustment on the stability of fungal carboxymethylcellulase and cellulase. Cell-free filtrates were mixed with the appropriate buffers to give the required final pH, incubated at 37° for 15 min., and then readjusted to pH 5·5; this was followed by the addition of cellulose or carboxymethylcellulose for the usual determination of activity. The pH–stability curve for cellulase resembles its pH–activity curve described by Halliwell (1961) in which maximum values were obtained at pH 5.

Action of proteolytic enzymes on carboxymethylcellulase and cellulase. The pH–stability curve (Fig. 4) indicates a rapid loss of enzymic activity below pH 5, whereas at pH 3·0 and 5·0 respectively peptic activity on haemoglobin was 50 and 0·4% of that exhibited at pH 1·8 in 10 min. at 37°. Hence, in the attempt to prepare peptic digests of cellulase and carboxymethylcellulase, pH 5 was chosen to obtain both activity of pepsin and stability of the cellulolytic enzymes. In these diges-

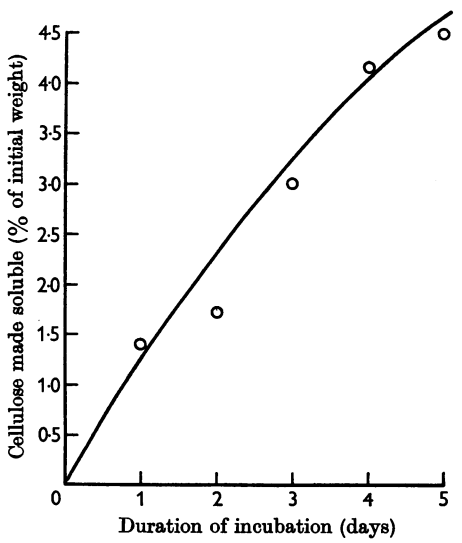

Fig. 1. Solubilization of dewaxed Texas cotton fibres by cell-free filtrates of *Myrothecium verrucaria.* Fibres (5 mg.) were incubated with 1 ml. of acetate buffer, pH 5·5, and 1 ml. of cell-free filtrate at 37° for 5 days. The same amount of filtrate was added daily over 4 days. Residual fibres were separated, digested with NaOH and estimated with dichromate as described in the Analytical procedure section.

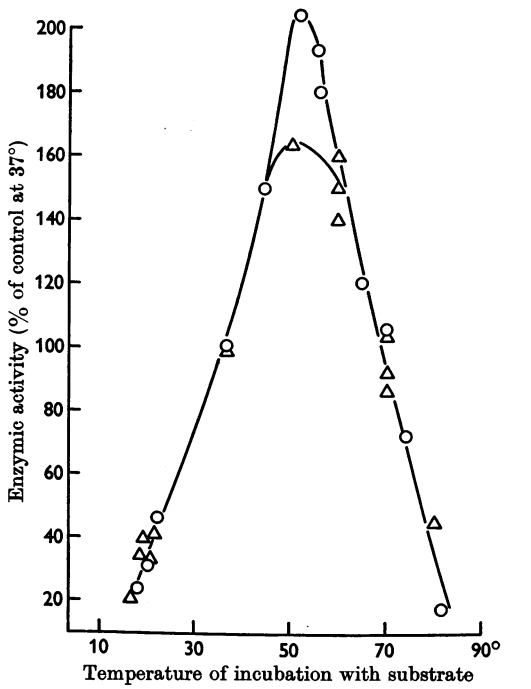

Fig. 2. Variation in the activity of carboxymethylcellulase and cellulase with temperature. Cell-free filtrates from *M. verrucaria* were assayed on carboxymethylcellulose or swollen cellulose powder under the standard conditions described in the Analytical procedure section but at the temperatures shown. ○, Cellulase; △, carboxymethylcellulase.

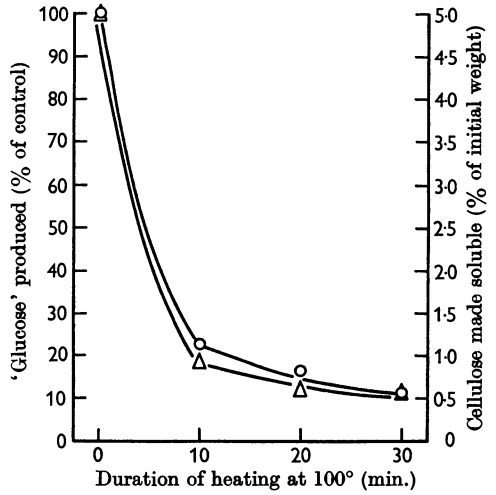

Fig. 3. Effect of duration of heating at 100° on the enzymic activity of cellulase and carboxymethylcellulase of *Myrothecium verrucaria*. Cell-free filtrates (undiluted for cellulase, or diluted approx. 1:70 for carboxymethylcellulase as in the standard assay procedure) were heated in a water bath at 100° for the periods shown. Samples were removed at intervals and transferred to the standard assay systems containing swollen cellulose powder, or carboxymethylcellulose, pH 5·5, and estimated as described in the Analytical procedure section. ○, Cellulase; △, carboxymethylcellulase.

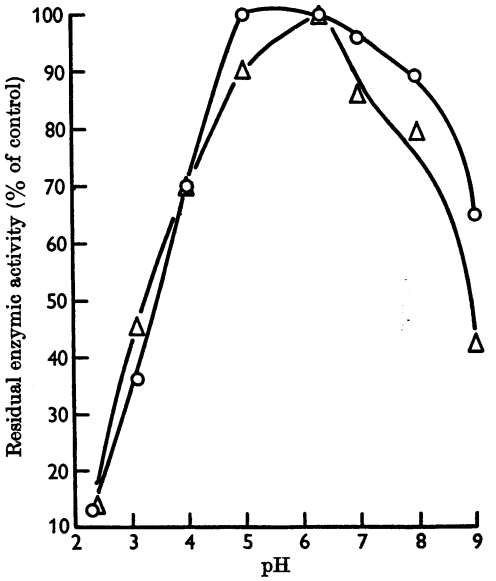

Fig. 4. Effect of pH on the stability of cellulase and carboxymethylcellulase of *Myrothecium verrucaria*. Cell-free filtrates, natural pH 5·9, were mixed with acetate, phosphate or borate buffers (see the Materials and Methods section) to give the final pH shown, incubated for 15 min. at 37°, readjusted to pH 5·5 and then assayed directly on swollen cellulose powder, or, after dilution, on carboxymethylcellulose by the standard procedures described in the Analytical procedure section. ○, Cellulase; △, carboxymethylcellulase.

tion experiments the reduced activity of pepsin at pH 5 was compensated to some extent by using 10 times the amount of pepsin employed in the standard 10 min. assay of this enzyme at pH 1·8, and by prolonging the incubation to 1 hr. Such treatment, however, failed to produce any loss in carboxymethylcellulase or cellulase activities.

In measuring the effect of trypsin on the cellulolytic enzymes, I chose the region of maximum stability, pH 6·3 (Fig. 4), at which value trypsin had 40 % of the activity shown in its usual 10 min. assay reaction at pH 7·6. Cell-free filtrates from *M. verrucaria*, treated with trypsin under these conditions but in an extended incubation lasting 35 min., showed no loss in activity of carboxymethylcellulase and cellulase. These more convincing experiments, compared with pepsin, demonstrate the stability of the cellulolytic enzymes to proteolytic attack. 'Controls' showed no cellulolytic activity of the pepsin or trypsin preparations.

DISCUSSION

The cellulase problem is made more complex by the wide range of substrates, from the highly complex and insoluble cellulose of cotton fibres to the other extreme of the relatively simple molecules corresponding in degree of polymerization to carboxymethylcellulose or even simpler cellodextrins. Even with carboxymethylcellulose, variations in enzymic susceptibility are encountered which result from differences in the degree of polymerization and of substitution. Jermyn (1952), working with a carboxymethycellulase from *Aspergillus oryzae*, found that solutions prepared by one method were hydrolysed twice as fast as other preparations, but were unstable, and after 3 days the susceptibility fell to the lower level, with the separation of gel-like material. The use of carboxymethylcellulose prepared under the described conditions, together with the recommended analytical procedure, has enabled me to obtain reproducible and quantitative responses with carboxymethylcellulase, including the determination of the K_m of a system (rumen bacterial carboxymethylcellulase) which formerly defied analysis.

The results achieved with cell-free enzymes from rumen micro-organisms and from *M. verrucaria* provide a comparison between powerfully cellulolytic bacteria and a somewhat less active fungus. Enzyme preparations from the latter organism are very active on both the soluble carboxymethyl-

cellulose and on insoluble swollen cellulose, but the bacterial extracts, which are even more effective on carboxymethylcellulose than the fungal enzyme preparation, are inert towards swollen cellulose. Neither preparation is particularly effective against undegraded cellulose of cotton fibres, although cell-free filtrates from *M. verrucaria* produced about 10% solubilization of this substrate, as measured by the alkaline-digestion procedure.

Carboxymethylcellulase appears to be relatively stable to heat, as is evident from the increased reactivity at about 50°, thus confirming work by Reese, Siu & Levinson (1950). In addition, Fig. 2 illustrates that cellulase not only has an optimum temperature similar to that of carboxymethylcellulase, but also a relatively greater hydrolytic effect at 50° than has that enzyme. This can be attributed to the corresponding difference in their susceptibilities to heat treatment at 50° (cf. 'treatment A' of Fig. 6, Halliwell, 1961).

Both enzymes are relatively stable to the pH of the medium during short periods of exposure at pH 5–7 but outside this region activities drop sharply in a manner resembling the pH–activity curve described above.

Existing cellulase preparations fail to solubilize undegraded cellulose found in, for example, cotton fibres, and it was thought that this might be due to proteolytic attack on cellulolytic systems. However, digestion experiments indicated that both cellulase and carboxymethylcellulase lost no more activity in the presence of pepsin or trypsin than could be ascribed to the acidity of the medium.

This report extends the findings of Halliwell (1961) on this subject by defining the conditions required for the quantitative determination of cellulase and carboxymethylcellulase, as well as enumerating more fully the situations which govern the reaction of cellulase on its insoluble substrate.

SUMMARY

1. The action of cell-free filtrates from *Myrothecium verrucaria* and from rumen bacteria was examined on soluble carboxymethylcellulose, insoluble swollen cellulose powder and on cotton fibres.

2. Conditions are defined for reproducible and quantitative determinations of carboxymethylcellulase. The Michaelis constant of rumen bacterial carboxymethylcellulase is 0·8 g. of carboxymethylcellulose/l.

3. Maximum activity of carboxymethylcellulase and cellulase is evident at 50°.

4. Carboxymethylcellulase and cellulase exhibit maximum stability between pH 5 and 7.

5. Neither enzymic system is inactivated by digestion with pepsin or trypsin.

I sincerely thank Dr G. A. Levvy for his advice and encouragement and Mr G. Pratt for valuable technical assistance.

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The Decomposition and Toxicity of Dialkylnitrosamines in Rats

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(*Received* 26 *March* 1962)

Dimethylnitrosamine inhibits protein synthesis (Magee, 1958; Brouwers & Emmelot, 1960; Emmelot & Mizrahi, 1961), causes centrilobular necrosis of the liver (Barnes & Magee, 1954; Magee, 1958) and is a potent carcinogen, producing cancers in the liver, kidney and lung (Magee & Barnes, 1956, 1959, 1962; Schmähl & Preussmann, 1959; Zak, Holzner, Singer & Popper, 1960; Argus & Hoch-Ligeti, 1961; Dontenwill & Mohr, 1961). Several other dialkylnitrosamines have been