metabolism of these injured rats is altered at two points, and the help they give in planning further experiments to elucidate the altered mechanisms.

SUMMARY

1. The early effects of bilateral hind-limb ischaemia on the concentrations of phosphorylated glycolytic intermediates, pyruvate, lactate and α -oxoglutarate, in muscle and liver have been investigated in intact, intact cold-acclimatized, adrenal-medullectomized and adrenalectomized rats at air temperatures between 18° and 22° and also in intact rats at an air temperature of 30°.

2. The changes in the phosphorylated glycolytic intermediates in the injured muscle during ischaemia were the same as those reported for anoxic muscle *in vitro*.

3. In the uninjured fore-limb muscle the fructose 1:6-diphosphate and pyruvate concentrations rose after hind-limb ischaemia. These changes were attributed to increased glycogenolysis and to decreased utilization of fructose 1:6-diphosphate and possibly also of pyruvate.

4. The most constant change in the liver after limb ischaemia was an increase in the pyruvate concentration and also in that of fructose 1:6-diphosphate when conditions were such that the liver temperature fell. These changes are attributed to a decrease in the utilization of fructose 1:6-diphosphate and in the oxidation of pyruvate.

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

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Many papers on the action of extracts from cellulolytic fungi on various cellulosic substrates have been published over the last two decades. It is now well established that any pretreatment of cellulose involving either swelling or dissolution increases its accessibility both to attack by cellulase and to other reagents. This aspect of the subject has been

adequately reviewed elsewhere (Pigman, 1951; Cumberbirch, 1955). It is still uncertain, however, whether the attack of the enzyme on the accessible glucoside linkages in the cellulose is random or endwise, as it is known to be with certain other glucosidases. An answer can only be obtained by examining the relation between such factors as the

change in degree of polymerization, the loss of weight and the number of end groups produced during hydrolysis.

Whitaker (1957) has investigated the hydrolytic action of the cellulase of *Myrothecium verrucaria* on a cellulose that had previously been swollen and partially degraded by treatment with 15N-phosphoric acid and has shown that the changes of both weight- and number-average degree of polymerization in relation to the loss of weight during the hydrolysis are consistent with a theory of random attack on all the β -glucoside linkages. It is well known, however, that the degradation of cotton cellulose by fungi produces very much smaller changes in degree of polymerization (as measured by the fluidity of a standard solution of the cellulose in cuprammonium), for a given loss of strength, than does acid degradation (Searle, 1929; Fargher, 1953). Although this difference has been attributed to the highly localized growth of the fungus, which causes whole regions of the substrate to be digested away and leaves others untouched, Walseth (1952), working with a cellulase from *Aspergillus niger*, has suggested that it is due to the limited accessibility of the substrate to attack by the enzyme. The theory of random attack proposed by Whitaker will then apply only to a cellulose substrate sufficiently swollen to be more than usually accessible to large molecules. The experiments described in this paper have shown that the degradation of cotton cellulose, even when it has been swollen by pretreatment with sodium hydroxide solution (mercerized), by the extracellular extract of *M. verrucaria* is neither random nor end-wise, and an alternative mechanism is suggested.

EXPERIMENTAL

Organism. The organism was *Myrothecium verrucaria*, obtained as strain 45,541 from the Commonwealth Mycological Institute (originally Q. M. 460 isolated by M. H. Downing from a cotton bale, Washington, D.C., U.S.A.).

Enzyme preparations. The methods of Whitaker (1953) were followed as far as the fractionation with ethanol to provide a partially purified cellulase preparation. This was preserved by freeze-drying and storage at 0°. No loss of activity towards carboxymethylcellulose could be detected after several months. The organism was also grown on a smaller scale in the same medium (300 ml. batches in 1 l. shake flasks) to provide simple culture filtrates which were used without purification.

Cellulose substrates. (a) Cotton yarn. A Sudan Sakel yarn of count 20 (0.295 mg./cm.) was given two successive scouring treatments by heating in an autoclave with 1% sodium hydroxide solution at 140° for 3 hr. It had a viscosity-average degree of polymerization ($\bar{P}_v$) of approximately 6000, calculated from the fluidity (3.5 P⁻¹) of a standard (0.5%, w/v) solution in standard cuprammonium.

(b) Cotton sliver. A scoured Texas sliver with approx.

$\bar{P}_v$ 6500 (fluidity of 0.5% solution in cuprammonium, 2.6 P⁻¹).

(c) Cotton flock (supplied as C/8 flock by Hutchinson Ltd., Ramsey Mill, Chadderton, Oldham, Lancs.). This was scoured, mechanically powdered flock having approx. $\bar{P}_v$ 2900 (fluidity of 0.5% solution in cuprammonium, 8.3 P⁻¹).

Mercerization. The cotton was swollen by immersion without tension in 25% sodium hydroxide solution at 2° for 4 hr. After removal from the alkali the sample was washed until the wash-water remained neutral and stored at 2° under water to keep it swollen.

Assay of the activity of the enzyme towards carboxymethylcellulose. To a solution of carboxymethylcellulose (Cellofas B, Imperial Chemical Industries Ltd.; 200 mg. in 10 ml. of 0.1 M-acetate buffer, pH 5.6) were added water (9 ml.) and a solution of crystalline bovine plasma albumin (300 μ g. in 1 ml. of water) (Whitaker, 1953). The solution was filtered through a sintered-glass disk and brought to 30° in a thermostat. An appropriate volume of the cellulase preparation was measured from a micrometer pipette into a small glass vessel. The volume was made up where necessary to approximately 0.5 ml. with water and the vessel was dropped into the carboxymethylcellulose solution. The two solutions were mixed and quickly introduced into an Ostwald viscometer, which was already in position in the thermostat. Measurements of viscosity were made after known degradation times, zero time being taken as the time at which the enzyme was added to the substrate. Values of specific viscosity ($\eta_{sp.} = \eta_{rel.} - 1$) were calculated. The reciprocals of the specific viscosities were plotted against the times of degradation in minutes (cf. Thomas, 1956). The slope of the line so generated was found to be proportional to the enzyme concentration over the experimentally convenient range of concentrations. The numerical value of this slope (min.⁻¹), calculated for a concentration of original enzyme preparation of 0.500 ml./20 ml. of substrate, was chosen as a measure of the activity of the preparation (carboxymethylcellulase units). Since the enzymic degradation was continuing during the measurement, the viscosity of the solution was falling continuously. It was assumed that the fluidity of the solution varied linearly with time over the small range involved during the making of any one measurement, and hence the time of degradation was taken as half-way between the start and finish of the flow. This was shown not to involve any serious error when the rate of change of fluidity was of the order normally found. The specific viscosity of a solution of carboxymethylcellulose, prepared as described above, but with the addition of the same quantity of enzyme inhibited with mercuric acetate, was 4.05.

Enzymic degradation of cellulose. The cotton sample (1.5 g.) was placed in a 250 ml. conical flask, to which was then added sodium acetate buffer (0.1 M; pH 5.6; 60 ml.) containing an antiseptic (sodium pentachlorophenate, 0.05%), crystalline bovine plasma albumin (5 ml. of a solution containing 300 μ g./ml.), water (25 ml.) and finally the appropriate enzyme solution, contained in 10 ml. of water. The samples were placed on a rotary shaking machine at 30° for the required time. To check the sterility of the preparation a sample of the liquor was then used to inoculate a slope of oatmeal-agar. The loss of weight was determined by filtering off the remaining insoluble material, which was washed and dried over phosphorus pentoxide to

constant weight in a vacuum oven at 60°. The fluidity of a standard solution in cuprammonium was measured on a sample (300 mg.) taken from each residue and a further sample (approx. 1 g.) was used for the determination of the loss of weight on heating with alkali.

Degradation of cellulose by hydrochloric acid. The cotton yarn [substrate (a) above; 1 g.] was immersed in 6N-hydrochloric acid (30 ml.) at 30° for from 2 to 240 hr. with occasional mechanical shaking. The acid was removed by filtration, the residual cellulosic material was washed with water at room temperature until the washings were acid-free, and dried to constant weight in a vacuum oven at 60° over phosphorus pentoxide and the loss of weight was calculated. The fluidity of a standard solution of the residue in cuprammonium, and the loss of weight on heating with alkali, were measured.

Determination of the viscosity-average degree of polymerization ($\bar{P}_v$) of cellulose. The fluidity (P^{-1}) of a standard (0.5%, w/v) solution of the cellulose in standard cuprammonium (containing 15 g. of copper/l., 200 g. of ammonia/l.) at 20° was measured by the method of Calvert & Clibbens (1951). The intrinsic viscosity (i) was obtained from table VIII given by these authors. $\bar{P}_v$ (as defined by Flory, 1953) was calculated from the intrinsic viscosity by means of the relation:

$$i = 0.0319 \cdot \bar{P}_v^{0.657}$$

derived by Cumberbirch & Harland (1958). This relation is based on experimental data for a range of $\bar{P}_v$, 190–2000, and therefore the values above 2000 are only approximate. This approximation is justified, since lack of accuracy in these figures does not affect the argument presented here.

Determination of loss of weight on heating with alkali. The loss of weight on heating with alkali (Clibbens, Geake & Ridge, 1927) was used as an empirical measure of the terminal aldehyde groups in the cellulose samples. An accurately weighed amount of the dried sample of cellulose or modified cellulose (approx. 1 g.) was placed in a 150 ml. conical flask of Pyrex glass, which was then nearly filled with cold 1% sodium hydroxide solution. The flask was closed with a pear-bulb stopper to exclude air and heated in a boiling-water bath for 6 hr. The undissolved residue was collected in a sintered-glass crucible of porosity 3, washed, dried in a vacuum over phosphorus pentoxide at 60° and weighed. The percentage weight loss was calculated and is referred to below as the loss of weight on heating with alkali.

RESULTS

Degradation of cellulose. Degradation of the various substrates already described at enzyme activities ranging from 0.4×10^{-2} to 50×10^{-2} carboxy-

Fig. 1

Fig. 1. Degradation of cotton cellulose. Relations between the loss of weight during hydrolysis and the subsequent loss of weight with alkali. (1) Action of *M. verrucaria* cellulase on unmercerized cellulose; (2) action of *M. verrucaria* cellulase on mercerized cellulose; (3) action of 6N-hydrochloric acid at 30° on unmercerized cellulose.

Fig. 2

Fig. 2. Degradation of cotton cellulose. Relations between loss of weight during hydrolysis and $\bar{P}_v$ of the insoluble residue. (1) Action of *M. verrucaria* cellulase on unmercerized cellulose; (2) action of *M. verrucaria* cellulase on mercerized cellulose; (3) action of *M. verrucaria* cellulase on cellulose swollen with phosphoric acid [interpolated from results given by Whitaker (1957)]; (4) action of 6N-hydrochloric acid at 30° on unmercerized cellulose. The broken line at the top of the graph is the theoretical curve for endwise attack.

methylcellulase units, and for various times, yielded data which are presented in Figs. 1 and 2. The relation between the loss of weight during enzymolysis and the subsequent loss on heating with alkali is shown in Fig. 1. Fig. 2 relates the loss during enzymolysis to the $\bar{P}_v$ of the insoluble residue. It was not possible to cause more than 2.5% loss of weight by enzymic hydrolysis of the unswollen celluloses and the range of the experiments was extended by using cellulose substrates which had been made more accessible by mercerization. With these materials weight losses of up to 45% were obtained. The results obtained at the different times and different enzyme activities (with both crude and partially purified preparations) lie on the same curves, as do those with the different substrates, except for those from the mercerized samples, which lie on curves that can be distinguished from those from the unmercerized samples in both Figures.

Results for the acid degradation of unmercerized cotton cellulose and those obtained by Whitaker (1957) for the enzymic degradation of his swollen cellulose are given for comparison. Whitaker used the relation $\bar{P}_w = 107i$ (where $\bar{P}_w$ = weight-average degree of polymerization) to calculate, from the viscosity of the derived nitrate in ethyl acetate solution, values of degree of polymerization which are plotted here as values of $\bar{P}_v$.

DISCUSSION

Since there is no satisfactory absolute method for the determination of terminal aldehyde groups in modified celluloses the loss of weight on heating with alkali under standardized conditions, which has been shown to have the same analytical significance as copper reducing power (Clibbens *et al.* 1927), has been used as an empirical measure of the content of these groups. If, as is deduced below, enzymic attack occurs only in the most accessible regions of cellulose it seems probable that the aldehyde groups released will be at least as accessible to degradation by alkali as those from acid attack. If a difference in accessibility to alkali exists it will therefore cause underestimation of the content of terminal aldehyde groups in the acid-hydrolysed celluloses. The contrast between acid and enzymic attack may therefore be greater than the results (Fig. 1) show. It is clear from Fig. 1 that, for the production of a given number of terminal aldehyde groups in the insoluble residue, considerably more substrate is removed during enzymic than during acid attack. Hence, whether or not the acid hydrolysis of cellulose is a random process with respect to all the β -glucoside linkages, the enzymic hydrolysis cannot be. This evidence, taken alone, would suggest predominantly endwise attack. However,

reference to Fig. 2 shows that the attack is not endwise. For endwise attack the relation between the amount of weight lost on hydrolysis and the $\bar{P}_v$ of the residue is represented approximately by the straight line, which was calculated on the assumption of a uniform initial chain length. For a given loss of weight the effect on $\bar{P}_v$ produced by the enzymic degradation is clearly much greater than that corresponding to endwise attack. The relation between the weight loss during hydrolysis and the $\bar{P}_v$ of the insoluble residue found by Whitaker (curve 3; Fig. 2) is very similar to that found for material degraded by acid (curve 4; Fig. 2) and is markedly different from that for either unmercerized or even mercerized cellulose after attack by cellulase.

The mode of action of cellulase on an unswollen cellulose suggested by these experiments is one of initial attack at relatively few sites along the length of a molecule. This is then followed by cleavage of a number of linkages in the immediate vicinity of these sites. The material removed at this stage is converted into soluble carbohydrates. The reason for this restricted attack is not apparent until one considers the cellulose molecule as a component of the cotton hair. Cotton cellulose is known to contain regions of varying accessibility and it is suggested that since *M. verrucaria* cellulase has a large molecule (Whitaker, Colvin & Cook, 1954) it is probably incapable of penetrating any but the regions of highest accessibility, and will also have little mobility within the cellulose structure. When the cotton has been swollen by treatment with alkali it is more generally accessible, but the attack of the enzyme, although much more extensive, is still far from random, presumably because the enzyme molecules still hydrolyse preferentially linkages in the immediate vicinity of their initial points of attack. If, however, the cellulose is swollen in phosphoric acid, or if the molecular size of the catalyst is smaller (for example, if an acid is used in place of an enzyme), attack of a more random nature is observed. This is presumably due to the increased mobility of the catalyst molecule within the substrate.

SUMMARY

1. Extracellular extracts of *Myrothecium verrucaria* have been used to study the mechanism of cellulase action on unmercerized and mercerized cotton cellulose.

2. Relations between loss of weight during hydrolysis and (1) decrease of viscosity-average degree of polymerization and (2) the further loss of weight on heating with alkali have been determined and compared with results similarly obtained by (a) acid degradation of unmercerized cellulose and (b) enzymic degradation of swollen cellulose.

3. These comparisons suggest that the enzyme, being composed of large molecules with restricted mobility in the substrate, removes a number of adjacent glucose residues from each of relatively few sites of attack.

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Folic Acid and the Synthesis of Methionine by Extracts of *Escherichia coli*

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The synthesis of methionine from homocysteine and serine by enzymic extracts of *Escherichia coli* has been found in earlier work from this Laboratory to require a cofactor of the folic acid group. In experiments with dialysed and Dowex 1-treated enzymic extracts, the folic acid cofactor can be supplied either by an extract of heated *E. coli*, or under certain conditions by tetrahydropteroylglutamate (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). These two sources of cofactor differ in their properties, and in the conditions under which they show activity with extracts of *E. coli* PA15, a glycine or serine auxotroph. First, the cofactor activity of tetrahydropteroylglutamate is rapidly lost on exposure to air, whereas that of the extract of heated *E. coli* is stable under these conditions. Secondly, the utilization of tetrahydropteroylglutamate by extracts of strain PA15 is dependent upon cobalamin. With ultrasonic extracts the addition of cobalamin to the reaction mixtures is sufficient to allow tetrahydropteroylglutamate to act as cofactor (Guest *et al.* 1960), whereas with extracts of acetone-dried organisms it is necessary that the organisms should have been grown in the presence of cobalamin for tetrahydropteroylglut-

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amate to be effective (Kisliuk & Woods, 1960). On the other hand, when extract of heated *E. coli* is used as the source of cofactor, cobalamin is not needed with either type of enzymic extract. Thirdly, the methionine synthesis promoted by extract of heated *E. coli* is inhibited by tetrahydropteroylglutamate in the absence of cobalamin (Kisliuk & Woods, 1960; Guest *et al.* 1960).

E. coli 121/176 is an auxotroph requiring cobalamin or methionine for growth. Ultrasonic extracts prepared from organisms grown in the presence of methionine differ from those of strain PA15 in that cobalamin is needed for methionine synthesis promoted by either extract of heated *E. coli* or by tetrahydropteroylglutamate.

The observed differences between extract of heated *E. coli* and tetrahydropteroylglutamate in this reaction suggest that the latter is not identical with the factor or factors present in the extract of heated *E. coli*. Although most work with reactions requiring folic acid cofactors has been done with tetrahydropteroylglutamate, experiments with two other such reactions have also shown that this compound is not identical with the active substance or substances in extracts of heated organisms.

Wright (1958) has found seven compounds, all pteridine derivatives, in heated extracts of *Clostridium cylindrosporium* which act as cofactors for the