Induction of Cellulase (C_x) in *Verticillium albo-atrum*

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SUMMARY

Of a large number of sugars and polysaccharides, only cellulose substrates or cellobiose induced C_x (B-1,4-glucan glucano hydrolase; Enzyme Commission no. 3.2.1.4) in *Verticillium albo-atrum*. Many of the sugars tested inhibited the induction of C_x in the presence of carboxymethyl-cellulose (CM cellulose); this is explained on the basis of a balance between growth and enzyme production such that a low growth rate is usually coupled with high C_x levels and vice versa. One per cent glucose completely repressed C_x induction in 0.1 % cellobiose medium. Cellobiose caused slight inactivation of C_x by end-product inhibition. If the mycelium has no physical contact with insoluble cellulose in liquid cultures, virtually no growth occurs and C_x values are insignificant. These results are discussed in relation to the postulated C_1 enzyme involved in the initial stages of cellulose degradation.

INTRODUCTION

The breakdown of naturally occurring cellulose in plant cell walls by fungal cellulases is not yet well understood (Wood, 1960; Mandels & Reese, 1965) but it is generally believed that a C_1 enzyme acts upon insoluble cellulose to produce linear chains which are broken down by one or more of a second type of enzyme termed C_x (B-1,4-glucan glucano hydrolase; Enzyme Commission no. 3.2.1.4). The activity of the latter can conveniently be studied using various forms of soluble cellulose, including the Na^+ salt of carboxymethyl-cellulose (CM cellulose), as substrates.

It is known that in many fungi the production of cellulases is adaptive so that in the absence of cellulose the enzyme is not formed in detectable quantities. Whether or not actual contact between the fungus and cellulose is necessary for the initiation of this process is conjectural. Since the substrate is highly insoluble it is difficult to ascertain how the fungus 'recognizes' its presence, but Mandels & Reese (1960), working with *Trichoderma viride* and other fungi, have suggested that sufficient traces of enzyme are formed to start cellulose hydrolysis. Once begun, the soluble products are thought to induce the production of larger amounts of enzyme. Cellobiose, one of the common products of hydrolysis, has been shown by these workers to induce C_x ; other disaccharides which also contain a β -glycosidic linkage such as lactose and sophorose act in a similar way (Mandels, Parrish & Reese, 1962). In an investigation with *Verticillium albo-atrum*, a vascular wilt organism, Talboys (1958) reported that cellobiose or cellulose would induce C_x whereas glucose, sucrose, lactose and starch all inhibited the formation of this enzyme. In a further study of this fungus, Whitney, Chapman & Heale (1969) showed that three main C_x components were produced when cellulose or

cellobiose was present in the culture medium. The approximate molecular weights of these components were 75,000, 32,000 and 16,000. They also found that incubation of C_x with CM cellulose produced only cellobiose and some medium length polymers. Glucose was absent in the culture filtrates and cellobiase (β -glucosidase) activity was not detectable.

In the present paper, we report on an investigation into the following aspects of the C_x system in *Verticillium albo-atrum*: the nature of the inducing molecule, inhibitory substances, inducer/inhibitor reactions and substrate/fungus contact. An accompanying paper (Heale & Gupta, 1970) deals with the utilization of cellobiose as the soluble product of cellulose degradation.

METHODS

Cultural procedures and carbon sources

A strain of *Verticillium albo-atrum*, isolated from wilted lucerne (*Medicago sativa*) in Norfolk during 1964, was used throughout this investigation. Modified Dox medium contained (g./l.): NaNO_3 , 2; KCl , 0.5; KH_2PO_4 , 1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5. The following carbon sources were used: D (—) fructose (glucose-free B.D.H.), D (+) galactose (B.D.H.), D (—) glucose (Analar), α -Me-D-glucoside (Sigma Chemical Co.), β -Me-D-glucoside (Sigma Chemical Co.), cellobiose (B.D.H.), gentiobiose (B.D.H.), lactose (B.D.H.), maltose (B.D.H.), melibiose (B.D.H.), sucrose (Analar), trehalose (B.D.H.), cellulose powder (Whatman standard grade, Balston Ltd), CM cellulose (Na^+ salt, D.S. 0.45 to 0.55, B.D.H.), pectin (Sigma Chemical Co.) and starch (soluble, B.D.H.).

The culture media were adjusted with phosphate buffer (0.1 M) to pH 5.5 before autoclaving. Sixteen oz. medicine bottles with cotton-wool plugs were employed as culture vessels containing 100 ml. liquid medium. Five replicate bottles were used for each treatment and were sterilized by autoclaving at 121° for 15 min. except in the case of pectin and those saccharides with a low melting point which were autoclaved at 115° for 10 min. Cultures were inoculated with a 6 mm. disc, cut with a sterile cork borer, from the growing margin of a 3 week parent culture of the fungus grown on potato-glucose agar. The bottles were incubated at $24 \pm 1^\circ$ in the dark for 14 days, except where stated otherwise, and the mycelial mats were separated by centrifugation at 4000 rev./min. for 20 min. in a MSE Super Medium centrifuge equipped with a universal swing-out head. Toluene (1 ml./l.) was added to the supernatant fluid. Growth was estimated by weighing mats that had been dried to constant weight in an oven at 60° .

In the substrate-contact experiments two different methods involving agar and liquid cultures were used. In the agar tests, modified Dox medium was mixed with 1 % agar (Davis), autoclaved, and 15 ml. samples poured into 10 replicate sterile Petri plates for each carbon source. When the medium had solidified, a well was cut on one side of the dish with a sterile no. 6 cork-borer and filled with warm modified Dox medium containing either cellobiose, CM cellulose or cellulose powder (all at 1 %); unsupplemented medium served as a control. The cellulose powder had previously been washed by centrifugation three times in sterile distilled water to remove traces of sugars. The agar was inoculated 40 mm. from the well and the cultures were incubated at $24 \pm 1^\circ$. Growth towards the substrate was measured after 3, 6, 9, 12 and 14 days, after which an agar block was removed from about halfway between the well and the leading edge of the hyphae, and homogenized in about 10 ml. acetate buffer (0.2 M, pH 5.2). The

homogenate was centrifuged at 4500 *g* for 30 min. at 0° in a high speed 18 (MSE). The supernatant fluid was tested for C_x activity and for protein levels in the normal way. In the liquid culture tests a special apparatus (Fig. 1) was used; it consisted of two flat flange joints with side arms (Quick Fit, FG 25) bent at right angles and held together with a metal clip. The mouth of each of the vertically positioned side arms was stoppered tightly with a rubber bung joined by glass tubing to a sterile filtration column filled with nonabsorbent cotton wool. The arms were separated centrally at the flange by a membrane filter (size 5 cm., standard grade, Oxoid). One arm was filled with 50 ml. of modified Dox medium and the other with Dox medium supplemented with either 1 % CM cellulose or 1 % cellulose powder (washed carefully as before) and the whole apparatus autoclaved.

Fig. 1. Apparatus used for the substrate contact experiment.

Protein determination. Extracellular protein production in all supernatant fluids was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). A 5 % final concentration of trichloroacetic acid was used to precipitate protein which was then dissolved in 0.1 % (w/v) NaOH solution. Casein standards were used.

CM cellulase (C_x) assay. Ability of the culture supernatant fluid to degrade CM cellulose was tested by using an Ostwald type viscometer (BS/IP/U-BS.188, size D). The enzyme reaction mixture contained 25 ml. of 1 % (w/v) CM cellulose (dissolved by mechanical stirring at 20° for 10 min. in 0.2 M-sodium acetate buffer (pH 5.2); toluene added at 1 ml./l.) and 1 ml. of supernatant fluid. After incubation for 1 h. at 30°, the flow time (sec.) was measured in the viscometer. Enzyme activity was initially calculated as the decrease of viscosity in CM cellulose equivalents. Arbitrary enzyme units (e.u.) were used to standardize the results and allow a quantitative comparison between treatments (Whitney *et al.* 1969).

Sugar determinations. Reducing sugars present in the culture medium before inoculation and in the supernatant fluid after growth were estimated quantitatively as glucose equivalents by a procedure utilizing the potassium ferricyanide–potassium ferrocyanide reduction reaction. The reduction in intensity of the yellow ferrocyanide was measured in an autoanalyser (Technicon) at 420 nm. (Hoffman, 1937). Sucrose was estimated by a modification of the resorcinol method of Roe (Dutton, Carruthers & Oldfield, 1961). Trehalose, α -Me-glucoside and β -Me-glucoside were estimated by the anthrone method (Winder, Brennan & McDonnell, 1967).

RESULTS

The effect of a variety of sugars and polysaccharides on C_x induction

In initial experiments, the fungus was grown for 14 days in modified Dox medium containing the following single carbon sources at 1 %: galactose, glucose, fructose, α -Me-glucose, β -Me-glucose, cellobiose, gentiobiose, lactose, melibiose, maltose, sucrose, trehalose, CM cellulose, cellulose powder, pectin, starch.

After centrifugation, the culture filtrates were assayed viscometrically for C_x activity; sugar and protein in the filtrates were determined and dry weights of mycelial mats calculated.

Table 1. C_x activity, extracellular protein and dry wt for *Verticillium albo-atrum* grown on various carbon sources at 1 %

	C _x activity e.u./ml. of supernatant fluid	μ g./ml. Extra- cellular protein supernatant fluid	Dry wt of mycelium mg./flask
Monosaccharides			
Galactose	0	4.8	270
Glucose	0	2.8	230
Fructose	0	2.0	206
α -Me-glucoside	0	2.3	105
β -Me-glucoside	0	2.3	95
Disaccharides			
Cellobiose	34.0	11.0	550
Gentiobiose	3.0	6.0	338
Lactose	0	6.8	260
Melibiose	0	3.6	258
Maltose	0	5.0	220
Sucrose	0	3.6	228
Trehalose	0	2.5	112
Polysaccharides			
CM cellulose	44.0	21.5	135
Cellulose powder	33.5	10.8	105
Pectin	0	10.2	230
Starch	0	3.5	190

The results in Table 1 showed that *only* cellulosic substrates or cellobiose induced C_x; gentiobiose, however, led to barely detectable quantities of enzyme and so might be considered as having some slight inducing activity. Where C_x was induced, there was a corresponding increase in the levels of extracellular protein. Cellobiose was clearly the best source of carbon in terms of dry weight production. Polysaccharides such as pectin or starch promoted more growth than CM cellulose. It was not possible to measure accurately the yield of cultures grown with cellulose powder because of the residual cellulose, but since the combined weights here were less than the mycelial yields in CM cellulose-grown cultures, it is possible to conclude that cellulose powder was attacked more slowly than soluble CM cellulose. Measurements of sugar uptake showed that cellobiose was completely utilized by the end of the 14 days growth period, i.e. a total uptake of 1.8 mg. of cellobiose/mg. dry wt of mycelium (i.e. dry wt after 14 days). Cultures containing the other disaccharides showed lower levels of sugar uptake (on a dry wt basis) and lower dry weights as compared with cellobiose

media, but sugar uptake was consistently higher than in monosaccharide cultures, the uptake of glucose being 1.3 mg./mg. dry wt. This suggested some mechanism that facilitated the utilization of cellobiose (and other disaccharide molecules to a lesser extent) relative to glucose and other monosaccharides.

Inhibition of C_x by glucose

The fungus was grown for 2 weeks on modified Dox medium containing 1 % CM cellulose and either 0.1, 0.5, 1, 1.5 or 2 % glucose. Control cultures were grown in 1 % cellulose medium without glucose. Fig. 2 shows the inhibitory effect of glucose on C_x induction; even 0.1 % glucose caused a marked decrease in enzyme values as compared with those for 1 % CM cellulose alone. The opposite effect was observed for growth, which increased correspondingly with higher amounts of glucose; at the same time,

Fig 2. Effect on C_x induction of various glucose concentrations (0.1 to 2 %) in the presence of 1 % CM cellulose. ■, C_x activity (e.u./ml.); □, extracellular protein (μ g./ml.); ▨, dry wt of mycelium (mg./flask).

extracellular protein amounts were decreased, reflecting the decrease in C_x . To test whether this effect involved the inhibition of C_x production or some form of enzyme inactivation, glucose was added at various concentrations (0.1 to 2 %) to a series of enzyme preparations and the mixtures assayed, with appropriate controls. In all cases the activity in the presence of glucose was unaltered, showing that its effect was on enzyme production.

The effect of small concentrations (0.1 %) of sugars and polysaccharides on C_x induction in the presence of CM cellulose

The fungus was grown in modified Dox medium containing 1 % cellulose and one of a variety of saccharides at 0.1 %; control cultures contained 1 % CM cellulose.

Glucose had the strongest inhibitory effect of the monosaccharides (Table 2); of the disaccharides, sucrose was very inhibitory and more so than either glucose or fructose, alone. Compared with control cultures C_x values/ml. were greater with cellobiose, with gentiobiose, and to a lesser extent with lactose. Relating enzyme production to mycelial dry wt, however, showed that C_x production was slightly less with cellobiose than in unsupplemented CM cellulose cultures. Of the polysaccharides, starch was strongly inhibitory, pectin slightly so. All the sugars were tested for their possible effects on the activity of C_x . With the exception of cellobiose, which at 2 % caused slight inactivation of C_x , the results were entirely negative.

Table 2. C_x activity, extracellular protein and mycelial dry wt for *Verticillium albo-atrum* in 1 % CM cellulose medium in the presence of various sugars and polysaccharides at 0.1 %

Cellulose supplement	C_x activity e.u./ml. of supernatant fluid	μ g./ml. Extra-cellular protein of supernatant fluid	Dry wt of mycelium mg./flask
Monosaccharides			
Galactose	42.5	27.0	196
Glucose	24.0	17.2	135
Fructose	34.0	25.0	265
α -Me-glucoside	43.4	24.7	185
β -Me-glucoside	45.0	25.3	164
Disaccharides			
Cellobiose	59.2	31.5	253
Gentiobiose	50.0	28.0	305
Lactose	45.9	26.8	434
Melibiose	39.8	25.3	396
Maltose	22.0	12.0	152
Sucrose	10.0	5.9	230
Trehalose	22.9	12.4	200
Polysaccharides			
Pectin	32.0	24.0	159
Starch	15.0	11.5	124
1 % CM cellulose alone (control)	44.0	21.0	133

C_x induction by cellobiose, and interactions with glucose

To study the time course of cellobiose-induced C_x production, the fungus was grown in modified Dox medium containing 0.1, 1 or 2 % cellobiose and harvested after 3, 6, 9, 12 and 14 days. Similar changes in C_x activity per unit dry wt of mycelium were found (Fig. 3) but the higher concentrations led to increased C_x values. The curves at all concentrations increased steeply to maxima at 4 to 6 days, after which there was a decline. This resulted from a slightly increasing rate of growth accompanied by a decrease in the rate of C_x formation, particularly from the 9th day onwards. A close correlation was found between the rates of hexose utilization per unit of mycelial dry wt at 1 and 2 % cellobiose. In 0.1 % cellobiose medium, all of the cellobiose was utilized by the 6th day when C_x values slowly declined until by the 14th day C_x was only just detectable.

To investigate glucose/cellobiose interaction the fungus was cultured in modified

Dox medium containing 0.1 % cellobiose + 1 % glucose, and the cultures harvested at regular intervals. C_x induction was completely inhibited by the glucose and the enzyme was not detected at any time during the growth period.

Fig. 3. C_x induction in 0.1, 1 and 2 % cellobiose media. +—+, 2 %; ●—●, 1 %; ○—○, 0.1 %.

The effect of substrate contact on the breakdown of cellulose

In the first series of tests to show whether physical contact between the fungus and its insoluble cellulose substrate was essential for degradation to occur, the substrate (cellobiose, CM cellulose, or washed cellulose powder) and inocula were placed 40 mm. apart on plates containing modified Dox agar medium. The fastest growth rate was towards cellobiose (Fig. 4), a somewhat slower rate towards CM cellulose, and there was no difference between the control (unsupplemented agar medium) and washed cellulose powder. Agar blocks from between fungus and substrate showed no C_x activity, even with CM cellulose agar. The C_x in the latter case may have been irreversibly bound to the agar, or alternatively may have been inactivated during the extraction procedure.

In the second series of tests, liquid cultures were used in the apparatus shown in Fig. 1. The fungus was separated from its substrate by a membrane filter. The cellulose was inoculated *in situ* in one treatment, and in a second the modified Dox medium was inoculated in the other arm of the apparatus; CM cellulose as a soluble cellulose and unsupplemented Dox medium were included as controls (Table 3). At intervals of 2 days, liquid was sucked from one arm to the other (in both directions) to ensure that diffusible substances passed across the filter. After 9 days the supernatant fluid from both arms was assayed for C_x and the usual tests made. Growth was negligible and

C_x activity hardly detectable when the fungus was separated from cellulose powder by the membrane filter; when grown in contact with cellulose powder, however, moderate growth and enzyme activity were recorded. As expected, inoculation of Dox medium in the opposite arm to the one containing CM cellulose resulted in similar growth and enzyme activity in both arms.

Fig 4. Growth rates in substrate-contact experiment carried out using agar plates (inoculum 40 mm. from substrate). ●—●, Cellobiose; ○—○ CM cellulose; △—△, unwashed cellulose powder; ▲—▲, washed cellulose powder; □—□, control water agar.

Table 3. Effect on C_x induction of physical contact of *Verticillium albo-atrum* with cellulose substrate. A membrane filter separated the two arms of the apparatus (Fig. 1)

Arm of apparatus	Modified Dox medium	Whether inoculated	Dry wt of mycelium (mg.)	Extra-cellular protein (μ g./ml.)	C_x activity (e.u./ml.)
Left	Unsupplemented	Inoculated	34	< 1	< 1
Right	Supplemented with cellulose powder	Uninoculated	0.0	< 1	< 1
Left	Unsupplemented	Uninoculated	0.0	8.5	21.0
Right	Supplemented with cellulose powder	Inoculated	98.0	12.0	24.0
Left	Unsupplemented	Inoculated	105.0	18.0	42.0
Right	Supplemented with CM cellulose	Uninoculated	0.0	18.0	38.0
Left	Unsupplemented	Inoculated	14.0	< 1	0.0
Right	Unsupplemented	Uninoculated	0.0	< 1	0.0

DISCUSSION

The data show that the induction of extracellular C_x activity in *Verticillium albo-atrum* depended upon the presence of cellobiose (or cellulose which was degraded to liberate cellobiose units) in the medium. This means that the molecular configuration of the inducing substance is limited to a disaccharide having a β -glycosidic link between C1 and C4 of the two glucose units. Any departure from these characteristics is critical; thus gentiobiose, which has only negligible inducing activity, is very similar to cellobiose except that it has a β -1,6 linkage. Since cellobiose is the main soluble product of cellulose degradation by this fungus (Whitney *et al.* 1969), it follows that the process is a self-maintaining one. Mandels & Reese (1960) reported that cellobiose induced C_x in *Trichoderma viride* and several other fungi but little in comparison with cellulose. They explained this on the basis of the very high growth-promoting characteristics of cellobiose. Under natural conditions (simulated by the use of cellobiose octa-acetate), slow rates of degradation of the insoluble substrate resulted in small amounts of cellobiose being released which were rapidly consumed, accompanied by high values of C_x production. However, with *V. albo-atrum* cellobiose is both an excellent growth source and a potent inducer of C_x . Amongst other disaccharides having inducing activity in *T. viride* are lactose, characterized by a β -1,4 link, and sophorose, a β -1,2 sugar, which is a very powerful inducer, producing 200 times the value of C_x as compared with cellobiose (Mandels *et al.* 1962). When *V. albo-atrum* is grown on 0.1 % cellobiose, the sugar is rapidly utilized and none is detectable after 6 days. Coinciding with the exhaustion of cellobiose, the value of C_x reaches a maximum at about the 6th day and falls thereafter. In *T. viride*, C_x does not appear until after the cellobiose has been consumed, which is why Mandels & Reese (1960) have suggested that the actual inducer is a product of cellobiose metabolism rather than cellobiose itself. We cannot at this stage rule out the possibility that an oligosaccharide (Heale & Gupta, 1970) could act as the internal inducer in *V. albo-atrum*, but it does seem quite clear that the induction mechanism in these two fungi is different in several important respects. These differences might be attributable to the distinct ecological roles involved. *T. viride* being a soil saprophyte, *V. albo-atrum* a vascular wilt pathogen which probably does not begin to degrade cellulose until the death of the host (Whitney *et al.* 1969) and which shows a rapidly declining phase when incorporated into soil along with the host residues (Heale & Isaac, 1963).

Slight end-product inhibition of C_x was demonstrated by adding cellobiose to reaction mixtures during viscometric assays. Similarly, Reese, Gilligan & Norkrans (1952) found that cellobiose inhibited most of the 36 cellulases tested. It is possible, therefore, that the rate of CM cellulose degradation by *Verticillium albo-atrum* could be somewhat limited by inactivation of C_x . It is doubtful, however, whether such an effect could operate as a significant controlling mechanism during cell-wall degradation, when the rate of cellobiose production is probably well below the potential utilization rate.

Cellobiose and some of the other disaccharide sugars such as gentiobiose were much better carbon sources for growth in *Verticillium albo-atrum* than glucose, and measurements of the rates of uptake strongly suggest that some mechanism facilitates the utilization of these disaccharides as compared with glucose.

Monosaccharides such as glucose and fructose, disaccharides such as sucrose,

trehalose and maltose, and the polysaccharide starch all inhibited C_x induction in the presence of CM cellulose. Again, 1 % glucose completely repressed the formation of C_x in the presence of 0.1 % cellobiose. Similar results were reported by Talboys (1958) for glucose, sucrose and starch using cellulose as C_x inducer. The mechanism of such generalized repression is clearly related to the balance between growth and C_x formation. In general, a low growth rate is coupled with high enzyme levels and vice versa; this effect is best seen over a range of glucose concentrations in the presence of 1 % CM cellulose (Fig. 2), but it is also observed during growth on different sugars in the presence of CM cellulose. A similar phenomenon has been reported by Mandels & Reese (1960) when, using *Trichoderma viride*, they showed that rapidly consumed metabolites reduced the levels of C_x in the medium. These findings are generally consistent also with an earlier investigation (Whitney *et al.* 1969) which showed that C_x levels in the diseased host were only three times that of the healthy uninoculated control plants until the terminal stages of the disease are reached. At this time when the host dies, the level of C_x rises to 20 times that of the controls. This sudden increase in C_x was attributed to a derepression effect resulting from the exhaustion of the more easily assimilable sugars present in the cells, and it is only at this stage that generalized cell-wall degradation occurs.

The results of the substrate-contact experiment must be considered in relation to the widely held view that the initiation of the induction mechanism is due to very small amounts of C_x produced 'constitutively' which liberate some cellobiose from cellulose and so induce the enzyme in detectable quantities. Since growth was negligible in the Dox medium (minus carbon source) separated by a porous membrane from the cellulose powder on which fungus grows if inoculated *in situ*, it seems clear that actual contact is necessary for the initiation of the cellulose degrading system. According to Selby (1963), evidence from different sources shows that some enzyme system (C_1) present in the vicinity of the growing micro-organism is necessary for the breakdown of natural cellulose and is missing from extracellular extracts as normally prepared. Until more evidence is forthcoming on the nature and role of the C_1 enzyme in this and other fungi it is difficult to decide whether separation of the mycelium from the insoluble substrate affects the induction of both C_1 and C_x or only C_1 . The absence of C_1 would mean that the initial step in cellulose degradation would not occur and following from this the lack of cellobiose liberation would be reflected in insignificant C_x values.

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REFERENCES

- DUTTON, J. V., CARRUTHERS, A. & OLDFIELD, J. F. T. (1961). The synthesis of sucrose by extract of the root of sugar beet. *Biochemical Journal* **81**, 266-272.
- HEALE, J. B. & ISAAC, I. (1963). Wilt of lucerne caused by species of *Verticillium*. IV. Pathogenicity of *V. albo-atrum* and *V. dahliae* to lucerne and other crops; spread and survival of *V. albo-atrum* in soil and in weeds; effect upon lucerne production. *Annals of Applied Biology* **52**, 439-451.
- HEALE, J. B. & GUPTA, D. P. (1970). The utilization of cellobiose by *Verticillium albo-atrum*. *Journal of General Microbiology* **63**, 175-181.
- HOFFMAN, W. S. (1937). A rapid photoelectric method for the determination of glucose in blood and urine. *Journal of Biological Chemistry* **120**, 51-55.

- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, A. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MANDELS, M. & REESE, E. T. (1960). Induction of cellulase in fungi by cellobiose. *Journal of Bacteriology* **79**, 816-826.
- MANDELS, M. & REESE, E. T. (1965). Inhibition of cellulases. *Annual Review of Phytopathology* **3**, 85-102.
- MANDELS, M., PARRISH, F. W. & REESE, E. T. (1962). Sophorose as an inducer of cellulase in *Trichoderma viride*. *Journal of Bacteriology* **83**, 400-408.
- REESE, E. T., GILLIGAN, W. & NORKRANS, B. (1952). Effect of cellobiose on the enzymatic hydrolysis of cellulose and its derivatives. *Physiologia plantarum* **5**, 379-390.
- SELBY, K. (1963). The effect of cellulolytic enzymes on some properties of cotton fibres. In *Advances in Enzymatic Hydrolysis of Cellulose and Related Materials*. Edited by E. T. Reese. Oxford: Pergamon Press.
- TALBOYS, P. W. (1958). Degradation of cellulose by *Verticillium albo-atrum*. *Transactions of the British Mycological Society* **41**, 242-248.
- WHITNEY, P., CHAPMAN, J. M. & HEALE, J. B. (1969). Carboxymethylcellulase production by *Verticillium albo-atrum*. *Journal of General Microbiology* **56**, 215-225.
- WINDER, F. G., BRENNAN, P. J. & McDONNELL, I. (1967). The effects of isoniazid on the composition of mycobacteria with particular reference to soluble carbohydrates and related substances. *Biochemical Journal* **104**, 385-393.
- WOOD, R. K. S. (1960). Pectic and cellulolytic enzymes in plant disease. *Annual Review of Plant Physiology* **2**, 299-322.