INDUCTION OF CELLULASE IN FUNGI BY CELLOBIOSE

MARY MANDELS AND ELWYN T. REESE

Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Massachusetts

Received for publication October 19, 1959

Cellulase is an adaptive enzyme in fungi. The inducing substrate, cellulose, is insoluble. How then does the induction occur?

It is possible that soluble products of enzyme action are the natural inducers of the enzymes that attack insoluble substrates. This theory assumes that small amounts of the inducible enzymes are produced even in the absence of inducer. When substrate is present it is hydrolyzed, the soluble products then enter the cell and induce more enzyme. Examples of products acting as inducers of polysaccharases have been cited in a previous paper (Mandels and Reese, 1957), and cellobiose can act as an inducer of cellulase (Mandels and Reese, 1957; Talboys, 1958). However, the amount of cellulase produced when *Trichoderma viride* was grown on cellobiose was less than 5 per cent of the yield on cellulose. If products are the natural inducers, why do they induce so poorly? This paper attempts to show that cellobiose is the natural inducer of cellulase, and that the low enzyme yields obtained in cellobiose cultures are due to inhibitory and inactivating effects resulting from rapid growth on sugar.

MATERIALS AND METHODS

The methods used in this study, including cultural conditions and determination of cellulase (Cx) activity, have been described in an earlier paper (Mandels and Reese, 1957). For most fungi other than *T. viride*, the pH of the culture medium was adjusted to 6.0, and for maximal cellulase production, proteose peptone (Difco) at 1 g per L was added to the basal medium.

For induction studies with washed mycelium, *T. viride* was grown for 4 days in shake flasks on the basal medium with 0.6 per cent glycerol. The mycelium was filtered off and washed with water. For each induction test, 20 mg (dry weight) of mycelium were suspended in 20 ml of a solution containing: (a) 0.05 M phosphate buffer (pH 2.8), (b) nutrient salts of the basal medium, at one fourth the concentration used in growth tests, and (c) 10 mg of the compound to be tested as an inducer. In tests employing the Basidiomycete QM 806, the mycelium was grown for 4 days on the basal medium with 1 per cent starch, and 20 mg (dry weight) of washed mycelial pellets were suspended in 20 ml of pH 4.0 buffer (0.025 M citrate + 0.025 M phosphate) containing 20 mg of proteose peptone and 10 mg of the compound to be tested as inducer. These tests were conducted at 29 C on the shaker, and cellulase determined on the extracellular solution at 48 and 72 hr as Cx units per ml, or Cx units per mg inducer.

Laminaribiose and laminaritriose were prepared by hydrolysis of laminarin by filtrates of *Rhizopus arrhizus* QM 1032 (Reese and Mandels, 1959) and isolated on a carbon column (Darco G60, 1 part; celite 545, 2 parts) by elution with increasing concentrations of ethanol. Enzymatically produced cellobiose and cellotriose were similarly prepared from phosphoric acid swollen cellulose by the action of filtrates of *Streptomyces* species QM B814 (Reese *et al.*, 1959).

Cellobiitol was prepared by reduction of cellobiose with sodium borohydride. Cellotriose, cellotetraose, cellopentaose, and cellohexaose were obtained from G. L. Miller of this laboratory. Isomaltose was obtained from Allene Jeanes of the U. S. Department of Agriculture, Peoria, Illinois.

Chromatograms were made using Whatman no. 1 paper and developed with isopropyl alcohol-glacial acetic acid-water (54:8:18). Carbohydrates were located by spraying with sodium periodate (3 per cent) followed by benzidine. Reducing sugars were detected with benzidine (Horrocks, 1949).

Cellulase is an extracellular enzyme complex produced by all cellulolytic fungi. It degrades cellulose chains by hydrolysis of the β-1,4-glucosidic bonds. The activity (Cx) is measured by production of reducing sugar from carboxymethyl cellulose.

TABLE 1

Cellulase production by fungi grown on various substrates

QM No.	Organism	Cellulose	Cellobiose Octa-acetate	Cellobiose	Lactose	Glucose	Glycerol
		Cx u/ml	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*
B814	*Streptomyces* sp.	8.0	0.1	0.9	0.8	0	0
806	Basidiomycete	100+	0	3.8	29.0	0	0
812	*Schizophyllum commune*	2.0	4.4	1.2	0.5	0	0
1013	*Polyporus versicolor*	3.0	0.1	0	0	0	0
826	*Sporotrichum pruinosum*	35.0	0	0.6	0.9	0	0
381	*Pestalotiopsis westerdijkii*	80.0	39.0	0.3	0	0	0
34e	*Humicola fuscoatra*	19.0	3.5	0.2		0	0
94d	*Stachybotrys atra*	9.0	10.0	0.3		0	0
527	*Fusarium moniliforme*	5.6		0	0	0	0
6a	*Trichoderma viride*	97.0	0	16.0	24.0	12.0	0
460	*Myrothecium verrucaria*	26.0	1.6	3.4	0	0	0
873	*Aspergillus luchuensis*	2.0	0	1.7	0.9	0.4	0
1852	*Penicillium helicum*	32.0	28.0	0.1		0	0
137g	*Penicillium pusillum*	8.0	0	4.1	1.2	0	0
474	*Penicillium funiculosum*	6.5	8.0	0	0.9	0	0
4e	*Penicillium lilacinum*	0.3	0.4	4.1	0.3	0	0

Basal medium plus carbon source (0.4 per cent cellobiose octaacetate, 0.5 to 1.0 per cent other substrates), in shake flasks at 29 C.

RESULTS

A. Cellobiose as an inducer of cellulase. The cellulase content of the culture filtrate of a number of organisms was determined (table 1). Cellulolytic fungi produce up to 100 or more Cx units per ml when grown on cellulose. Cellulase is not produced on glycerol (or on mannitol or starch) despite good growth. Most fungi that produce cellulase when grown on cellulose also produce cellulase on cellobiose, but the yields are relatively low. Of 49 cultures that we have tested, 36 produced measurable cellulase when grown on cellobiose. Many fungi produce Cx on lactose, often in higher yield than on cellobiose. In connection with differences in yields of cellulase on cellobiose compared to lactose, it is interesting to note that cellobiose is an excellent growth substrate for these fungi and is rapidly consumed, whereas lactose, which differs from cellobiose only in the configuration around the number 4 carbon in the glycoside ring, is a poor growth substrate for fungi and is slowly consumed.

Some fungi grow on cellobiose octaacetate. This insoluble substrate is attacked by organisms capable of producing an esterase that can split off the acetate groups (Reese, 1957). Such organisms produce Cx when grown on cellobiose octaacetate, often in higher yield than when grown on cellobiose, and sometimes in higher yield than when grown on cellulose. *Pestalotiopsis westerdijkii*, *Penicillium funiculosum*, *Humicola fuscoatra* (and other *Humicola* species and *Pestalotia* species not listed in table 1) are examples of such organisms. Cellobiose octaacetate is not an inducer of Cx for fungi unable to solubilize it. On the other hand, some noncellulolytic organisms solubilize cellobiose octaacetate, but do not produce Cx when grown on it.

In this survey two organisms, *T. viride* and the Basidiomycete QM 806, were outstanding producers of cellulase. Filtrates of *T. viride* have not only high Cx activity, but are the most effective in breaking down native undegraded celluloses (Reese, 1956). QM 806, the conidial stage of an unidentified Basidiomycete (Reese and Mandels, 1959), produces the highest Cx yields of any organism we have tested. Both fungi grow and produce cellulase on all celluloses tested, including soluble derivatives of low degrees of substitution (table 2) but the yield varies markedly.

In general, maximal yields were obtained on the finely divided, relatively undegraded celluloses, which are readily consumed by the fungi. Somewhat lower yields were obtained when the fungi were grown on the partially degraded celluloses. The soluble cellulose derivatives give low cellulase yields. Soluble derivatives of high degree of substitution are not consumed by the organisms and do not induce Cx, even when another carbon source is provided to support growth of the fungi. The efficacy of cellulosic materials as inducers seems to depend on their susceptibility to attack by the fungus. This indicates that the induction is not a contact phenomenon, but that it is more likely that the soluble products of cellulase action are the inducers.

TABLE 2

Effect of growth substrate on cellulase production

Substrate*	Basidiomycete QM 806	*Trichoderma viride* QM 6a
	Cx u/ml	*Cx u/ml*
Cellulose		
Cotton		
Unwashed (40 mesh)	82	
Gray duck (40 mesh)	82	
Sheeting (40 mesh)	84	21
Sliver (ball milled)	90	30
Phosphoric acid swollen (Walseth)	55	8
Wood (Solka floc)	48	29
Fungal (Saprolegnia)	10	4
Bacterial (Acetobacter)	3	5
Animal (Tunicate)	28	7
Derivatives (soluble)		
Sulfate, D.S.† 0.4	6	3
Carboxymethyl D.S. 0.5	14	4
Methyl, D.S. 1.8	2	0
Sugars and glycosides		
Lactose	13	22
Salicin	8	0
Cellobiose	4	1
Glucose (reagent)	0	4

* Basal medium plus 0.5 per cent substrate, shake flasks at 29 C.

† D.S. = degree of substitution.

Cellobiose is usually the first, detectable, soluble product of cellulase action. The oligoglucosides, cellotriose to cellohexaose, are hydrolyzed very rapidly and have only a transient existence (Whitaker, 1954; Reese, *et al.*, 1959). Longer chains are insoluble. Cellobiose does induce, but the yields are very low. The only other inducers found are the β-1,4-glucoside, salicin, for the Basidiomycete, and glucose (reagent grade) for *T. viride*. Other materials that supported growth of the Basidiomycete at 0.5 per cent, but did not induce Cx include: xylose, arabinose, galactose, fructose, sucrose, maltose, glycerol, mannitol, gluconolactone, methyl β-glucoside, calcium cellobionate, calcium lactobionate, starch, chitin of *Aspergillus luchuensis*, succinate, peptone, and coconut oil. No growth or Cx production occurred on shrimp chitin, acetate, or pyruvate. Similar information has been published for *T. viride* (Mandels and Reese, 1957).

The concentration of inducer has a marked

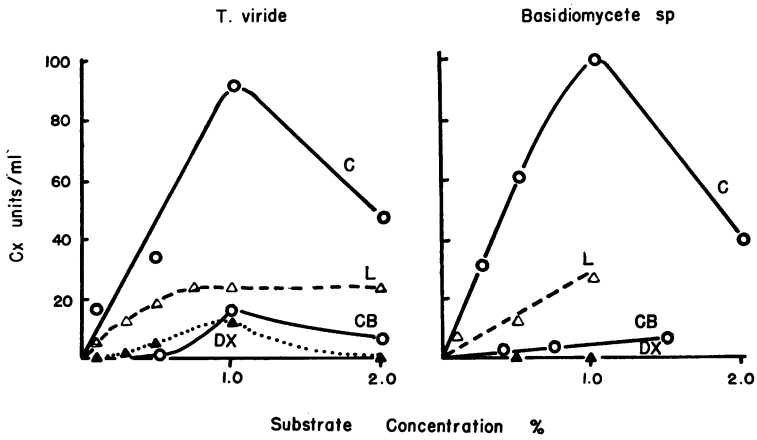

Figure 1. Effect of substrate concentration on cellulase production. Cultures grown on basal medium plus substrate in shake flasks at 29 C. *C* = cellulose (solka floc); *L* = lactose; *CB* = cellobiose; and *DX* = glucose.

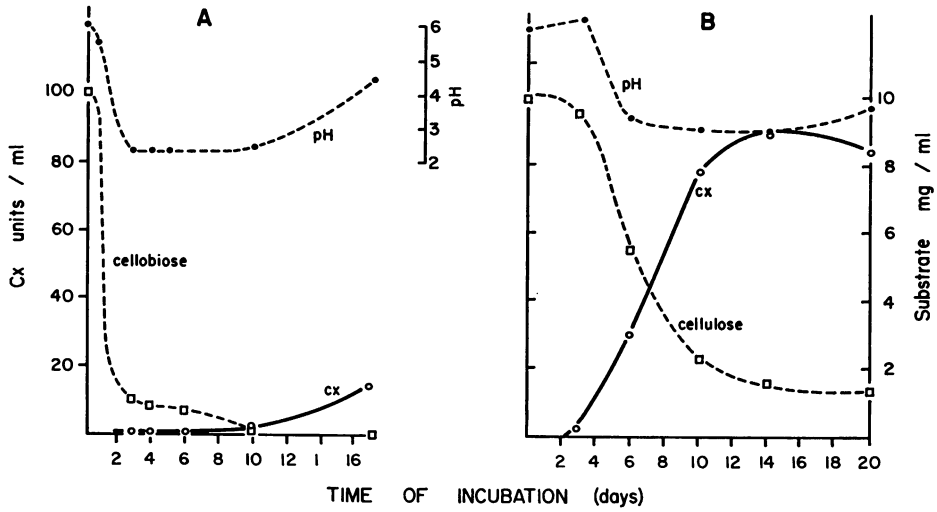

Figure 2. Production of cellulase by *Trichoderma viride* grown on *A*, cellobiose, and *B*, cellulose. Basal medium + 1 per cent substrate. Cellulose weight includes weight of residual mycelium.

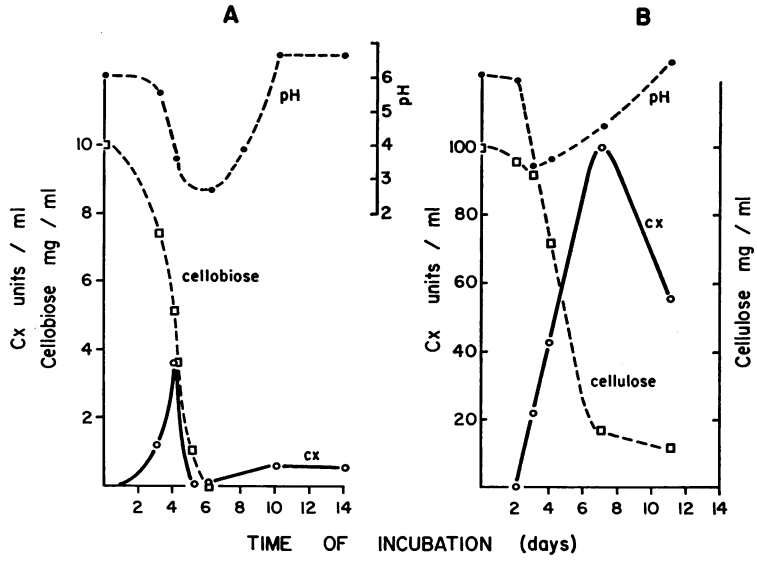

Figure 3. Production of cellulase by Basidiomycete QM 806 grown on *A*, cellobiose, and *B*, cellulose. Basal medium + 1 per cent substrate. Cellulose weight includes weight of residual mycelium.

effect on Cx production. Maximal yields were obtained on 1 per cent substrate (figure 1). On cellulose (figures 2 and 3) (and this is also true of lactose) Cx is produced during growth on, and consumption of, the substrate, and Cx production ceases when the cellulose has been consumed. When *T. viride* (figure 2) grows on cellobiose, cellulase does not appear until after the sugar has been consumed. This is not just a matter of release for we are unable to detect Cx in the mycelium before it appears in the filtrate. *T. viride* consumes 0.5 per cent cellobiose rapidly and then produces 1 Cx μ per ml (figure 1). On 1.0 per cent cellobiose *T. viride* consumes about 90 per cent of the cellobiose rapidly, and then, perhaps because of the low pH that develops, consumes the last 10 per cent quite slowly. Under these conditions 16 Cx u per ml are produced (figure 1). When the Basidiomycete 806 grows on cellobiose Cx is produced in the presence of the

TABLE 3
Inducers of cellulase (washed mycelium tests)

Substrate	*Trichoderma viride*	Basidiomycete QM 806
	Cx u/ml	*Cx u/ml*
Wood cellulose (Solka floc)	5.2	19.2
Carboxymethyl cellulose (D.S. 0.5)	1.6	2.9
Cellulose SO_4 (D.S. 0.4)	1.0	1.7
Methyl cellulose (D.S. 1.8)	0	0.5
Glucose	0.3	0
Cellobiose	0.6	2.1
Cellobiose (enzymatic)	0.6	
Cellotriose	0.4	
Cellotriose (enzymatic)	0.6	
Cellotetraose	0.4	
Cellopentaose	0.4	
Cellohexaose	0.4	
Salicin	0	2.1
Lactose	7.2	2.7
Inducer* present in reagent glucose	200.0	0
Cellobiose transfer product†	14.0	

* Impurity from reagent glucose separated on carbon and eluted with 15 per cent ethanol, tested at 0.01 to 0.05 mg carbohydrate per ml.

† R_G 0.4 material eluted from carbon column with 15 per cent ethanol (figure 6).

cellobiose, but diminishes as the cellobiose is consumed (figure 3).

Washed pellets of the Basidiomycete, or washed mycelium of *T. viride*, readily produce Cx in the presence of a suitable inducer. No other materials need be added, but maximal production occurs if peptone or nutrient salts are included and the suspension buffered to pH 4.0 for the Basidiomycete, or to 2.7 for *T. viride*. The same compounds that induce in growth experiments induce with washed mycelium (table 3). Cx begins to appear in 18 hr and reaches a maximum in 48 hr. In addition to the compounds tested in growth experiments the following have been found to be noninducers with washed mycelium of *T. viride*: laminaribiose, laminaritriose, gentiobiose, phloridzin, cellobiitol, cellobionic acid, butyl β-glucoside, *p*-nitrophenyl β-glucoside, *o*-nitrophenyl β-galactoside, phenyl β-thioglucoside, sinigrin, sinalbin, progoitrin, α,α-trehalose, isomaltose, melibiose, sorbitol, Δ-gluconolactone, γ-gluconolactone, 2-deoxyglucose, *N*-acetylglucosamine, glucosamine-HCl. Acetate strongly inhibits Cx formation with washed mycelium (10^{-2} M for the Basidiomycete, 10^{-3} M for *T. viride*). Formate, propionate, and butyrate act similarly.

Thus, cellulase is an adaptive enzyme in fungi. The inducers are, in approximate order of efficiency, cellulose ≧ cellobiose octaacetate > lactose > cellobiose (table 1). All of these inducers contain the β-1,4-glycosidic linkage.

Glucose (reagent grade) is an inducer only for *T. viride*. We have tested many fungi on other media (those of Reese and Levinson, 1952, and of Jermyn, 1953) but Cx was produced only by *T. viride*. When *T. viride* grows on glucose, the glucose is consumed before Cx appears. Filtrates of glucose cultures examined after the consumption of glucose, but before the appearance of Cx, contain an alcohol-soluble, heat-stable material that induces Cx with washed glycerol-grown mycelium. This material moves on paper, or on a carbon column like a disaccharide. Although we thought at first that this was a reversion product formed by the fungus from glucose, further investigation showed that it is an impurity present in the glucose itself.

The inducer is present in glucose produced by acid hydrolysis of corn starch (Clinton; Corn Products; Pfanstiehl, CP; Merck USP; J. T. Baker, reagent grade; and Eastern). We separated it from the glucose on a carbon column by gradient elution with ethanol. The crude inducer carbohydrate equals about 0.15 per cent of the weight of original glucose (Pfanstiehl) and is highly active (table 3). Corn starch, glucose which has been separated from the inducing carbohydrate by passage through a carbon column, or glucose (Staley) produced by enzymatic hydrolysis of corn starch do not induce cellulase in *T. viride*.

If cellobiose is the true inducer of Cx in cellulose cultures, we should be able to get yields on cellobiose equal to those on cellulose. Actually, the yields are very low. In *T. viride*, however, cellobiose appears to inhibit the production of Cx (figure 2) and in the Basidiomycete, the metabolism of cellobiose leads to the inactivation of cellulase (figure 3). Perhaps these effects will explain the low yields of cellobiose.

B. Inhibition of cellulase production by cellobiose. When *T. viride* grows on 0.5 per cent glucose, the sugar is consumed and Cx has appeared at 40 hr. The addition of glucose or cellobiose at 24 hr delays the appearance of Cx for several days

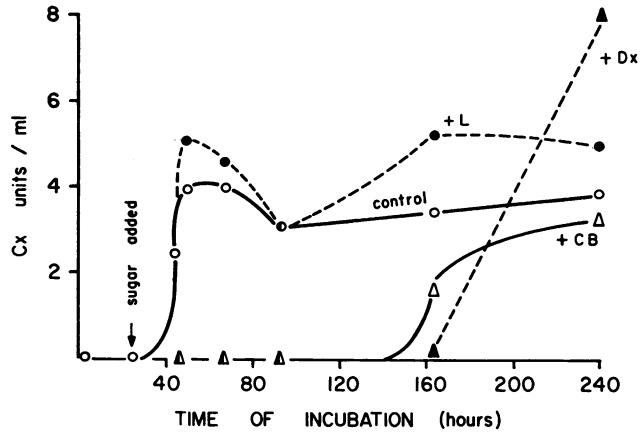

Figure 4. Effect of addition of sugars on cellulase production by *Trichoderma viride* grown on glucose. ○——○ = Control, 0.5 per cent glucose, no addition; ●- - -● = 0.5 per cent lactose (*L*) added at 24 hr; ▲- - -▲ = 0.5 per cent glucose (*Dx*) added at 24 hr; and △——△ = 0.5 per cent cellobiose (*CB*) added at 24 hr.

(figure 4). Amylase formation is also inhibited during this period. The added sugar is rapidly consumed and the pH falls to 2.1 for several days. Lactose added at 24 hr is also consumed with a sharp fall in pH, but Cx production is not delayed. The added lactose, however, does not give increased induction. The glucose- or cellobiose-induced lag can be prolonged by increasing the concentration of sugar, by lowering phosphate or trace metals in the basal medium, or by addition of 0.1 per cent gluconate or oxalate to the culture. The lag can be shortened or eliminated by decreasing the concentration of sugar, or by raising the phosphate or trace metal concentration in the medium.

C. Inactivation of cellulase during cellobiose metabolism. Cellulase separated from the mycelium is a stable enzyme. Culture filtrates can be stored indefinitely at refrigerator temperatures. They can be concentrated in a film evaporator at 40 C, precipitated with acetone, or lyophilized without loss of activity. Therefore, the loss of Cx that follows consumption of cellobiose in the Basidiomycete culture (figure 3) is a puzzling phenomenon. We have made attempts to stabilize this Cx by addition of phosphate, metals, gelatin, peptone, Versene, or sugars to the culture, and by varying nitrogen level or sources in the medium. None have succeeded. In all cases, the Cx decreased when cellobiose was finally consumed.

When cellobiose is added to a cellulose culture of the Basidiomycete that has produced Cx, the cellobiose is quickly consumed and the cellulase activity falls markedly (figure 5). After consumption of the cellobiose the Cx rises again sharply.

The effect is not specific for cellobiose. Other rapidly consumed metabolites such as glycerol or glucose cause a similar inactivation. Cellobiose, however, is the most active compound. The same sharp fall in Cx occurs when these sugars are added to cellulose cultures of *T. viride*, *Sporotrichum pruinosum*, *Myrothecium verrucaria*, *P. westerdijkii*, or *Stachybotrys atra*; and for glucose and lactose cultures of *T. viride*. In all cases, the fall parallels the consumption of the sugar, and is accompanied by a pH fall. The fall in Cx is often accompanied by a marked rise in other extracellular enzymes, e. g., amylase and β-1,3-glucanase.

The loss of Cx in these cultures can be prevented or decreased by adding extra phosphate with the added sugar or by the addition of merthiolate which prevents the consumption of the added sugar. It can also be prevented by removal to unshaken conditions or by addition of peptone or of protein with the sugar. Cellobiose does not inactivate Cx in culture filtrates from which the mycelium has been removed.

Recovery from inactivation occurs only in young cellulose cultures. This sharp recovery to the original level makes the phenomenon look like a reversible inactivation, but, despite considerable effort, we have never been able to reactivate the enzyme in the culture filtrate (which still contains residual cellulose) in the absence of

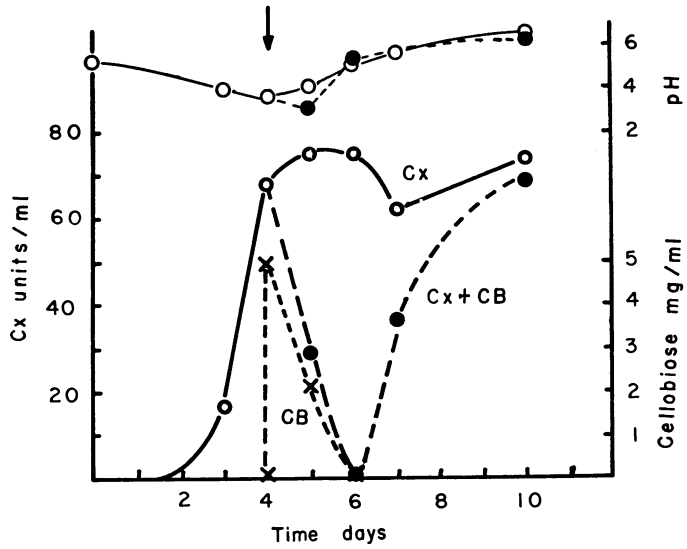

Figure 5. Inactivation of cellulase by cellobiose. Basidiomycete QM 806 grown on basal medium + 1.0 per cent cellulose. Cellobiose added at 4 days (↓). *Upper curves:* pH, O——O, control; ●——● cellobiose added. *Lower curves:* O——O, *Cx* in control, 1.0 per cent cellulose (*Cx*); ●——●, Cx after addition of 0.5 per cent cellobiose (*Cx* + *CB*); X- - -X, addition and consumption of cellobiose.

living mycelium. Protein decreases in the culture filtrate as the Cx falls, and reappears before the Cx rises again. If there is a reversible inactivation the enzyme must be precipitated or adsorbed on the mycelial complex. However, we have not been able to extract the enzyme from the mycelial residue. It is released only under conditions that could also allow synthesis of new enzyme.

The loss of Cx appears to be related to low pH. However, in older cellulose cultures the fall is not accompanied by low pH, the addition of PO_4 prevents fall with little effect on pH, and under some conditions Cx is produced at pH values as low as those under which the inactivation occurs.

D. High cellulase yields on cellobiose by inhibition of growth. The inhibitory effects of cellobiose on Cx production seem to be related to rapid metabolism of the cellobiose. In cellulose cultures, cellobiose is slowly released, and slowly consumed. Lactose and cellobiose octaacetate are slowly consumed, and they are better Cx inducers than is cellobiose.

We therefore attempted to slow down the consumption of cellobiose in various ways. One of these was by adding it slowly to cultures. Adding 0.5 per cent cellobiose to a culture of the Basidiomycete slowly over a 3-day period raised Cx production to 8 units per ml as compared to 2.5 units per ml in the control grown on 0.5 per cent cellobiose given as one dose. In general our attempts along this line did not result in any marked increases.

We had more success by using deficient media

TABLE 4

Effect of mineral deficiency on growth and cellulase production by Basidiomycete QM 806 grown on cellobiose

Medium*	Sugar Remaining at 4 Days	Maximum Enzyme Produced
	mg/ml	*Cx u/ml*
Full (control)	0.8	3
−Minor elements	2.8	7
−Mg − minor elements	2.8	8
−Mg	3.0	8
−Ca	4.2	9
−Ca − minor elements	5.0†	22
−Ca − Mg	NG, 22 days‡	
−Ca − Mg − minor elements	NG, 22 days	

* Basal medium + 0.5 per cent cellobiose. Ca = 0.03 per cent $CaCl_2$; Mg = 0.03 per cent $MgSO_4 \cdot 7H_2O$; (minor elements = Fe, 1.0; Zn, 0.8; Co, 0.5; Mn, 0.5 ppm).

† Visible growth in 10 days.

‡ NG = no growth.

(table 4). Growth rate is inversely related to the sugar remaining at 4 days. The Basidiomycete QM 806 requires calcium and trace metals for good growth in our medium. When calcium and magnesium are both omitted no growth occurs. If only one is omitted, or if the trace metals are omitted, growth is slow and cellulase yields are increased.

T. viride, on the other hand, does not require added trace metals for good growth, but no Cx is produced on glucose or cellobiose unless they are added. The trace metal requirement can be met by cobalt alone (Mandels and Reese, 1957). In the absence of cobalt, growth on cellobiose is slowed a trifle and there is no Cx (table 5). At 0.1 ppm of cobalt, growth is good, but there is is only a trace of Cx. As cobalt is increased to 10 ppm, growth (as indicated by sugar consumption) is delayed, and Cx production reaches a maximum. At higher cobalt concentrations, both growth and Cx produced are inhibited. Possibly the role of cobalt in Cx production may be related to the inhibition of sugar metabolism.

TABLE 5

Effect of cobalt on growth and cellulase production by Trichoderma viride grown on cellobiose

Addition	Sugar Remaining at 2 Days	Maximum Enzyme Produced
	mg/ml	*Cx u/ml*
None	1.1	0.1
Full minor elements*	0	1.0
Cobalt		
0.1 ppm	0	0.2
0.5 ppm	0.5	0.5
1.0 ppm	1.8	0.7
10.0 ppm	4.1	5.5
50.0 ppm	5.0	0.8
100.0 ppm	5.0	0.2

Basal medium with trace metals omitted, plus 0.5 per cent cellobiose.

* Minor elements = Fe, 1.0; Co, 0.5; Zn, 0.8; Mn, 0.5 ppm.

Another means of adding cellobiose slowly is through the use of cellobiose octaacetate. We selected three organisms that do not grow on cellobiose octaacetate and so do not produce Cx on it (table 6). When the esterase of *H. fuscoatra* is added aseptically to cellobiose octaacetate cultures, the cellobiose octaacetate is solubilized, the fungi grow on the products of hydrolysis, and Cx is produced. In another test cellobiose octaacetate was hydrolyzed by the esterase of *H. fuscoatra*, and about 10 per cent was solubilized. Chromatograms of these soluble products showed a series of carbohydrate spots, presumably various acetates of cellobiose. Such soluble products probably can enter the cell, but are slowly consumed. In our tests these soluble products induced Cx at a low concentration. Cellobiose itself did not induce at the same concentration (table 6).

Finally, we tried slowing down consumption of cellobiose by growing the fungi without shaking and at less favorable temperatures. Most organisms grew too slowly in stationary culture and produced little Cx. *T. viride*, however, when shaken for 24 hr at 29 C to allow germination and then removed from the shaker, grew fairly well and completely consumed 1 per cent cellobiose at 18 to 40 C. Growth was most rapid at 34 C, but Cx production was poor. At 29 C, 33

TABLE 6

Induction of cellulase by cellobiose octaacetate

Organism	Substrate				
	Cellobiose octaacetate (0.4%) plus:			Soluble products† (0.04%)	Cellobiose (0.04%)
	Water	Boiled esterase*	Active esterase		
	Cx u/ml	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*
Trichoderma viride QM 6a	0	0	6.6	5.8	0
Penicillium pusillum QM 137g	0	0	6.5		
Basidiomycete QM 806	0	0.2	28.0	6.5	0

Cultures grown on basal medium plus indicated substrate.

* Esterase = culture filtrate of *Humicola fuscoatra* grown on cellobiose octaacetate, added at 1:10.

† Soluble products produced by action of esterase on cellobiose octaacetate.

units per ml were produced, double the yield obtained in shake culture. At 24 C growth was delayed, but 94 Cx units per ml were produced, a yield equal to that obtained on cellulose (table 7).

TABLE 7

Effect of temperature on cellulase production by Trichoderma viride grown on cellobiose

Temp	Sugar Remaining at 6 Days	Maximum Enzyme Production
C	*mg/ml*	*Cx u/ml*
18	2.2	27
24	0.75	94
29	0	33
34	0	2.2
40	2.1	0.1

Cultures grown on basal medium plus 1.0 per cent cellobiose for 24 hr at 29 C shaken, 48 hr at 29 C unshaken, then to indicated temperatures unshaken.

E. Induction of cellulase by a transfer product from a cellobiose culture of T. viride. Why should slow consumption of cellobiose lead to higher cellulase yields? With cultures like the Basidiomycete 806 where Cx is produced in the presence of cellobiose, the cellobiose seems to be a direct inducer, but the rapid metabolism of cellobiose leads somehow to destruction of the enzyme. Slowing down the metabolism decreases this destructive effect. In cultures of *T. viride*, however, cellobiose inhibits the appearance of Cx. Since the Cx appears only after the cellobiose has been consumed the direct inducer may be a product of cellobiose metabolism. The oligosaccharases of fungi are transferases. In ordinary hydrolysis the glucosyl group is transferred to water, but it can also be transferred to an alcohol or to another carbohydrate molecule. Thus, Buston and Jabbar (1954) showed that cell-free extracts of *Chaetomium globosum* acting on cellobiose formed glucose, gentiobiose, sophorose, and laminaribiose

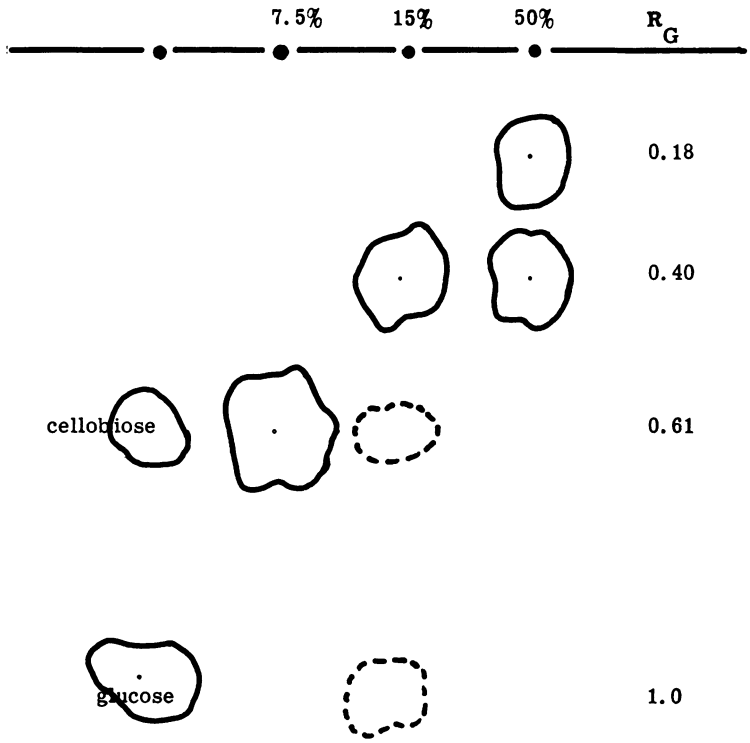

Figure 6. Chromatogram of carbohydrates separated from a culture of *Trichoderma viride* grown on cellobiose. *T. viride* was grown for 5 days on 2 per cent cellobiose, then 420 ml of culture filtrate poured on a carbon column and eluted with water and ethyl alcohol of the concentrations noted. R_G = ratio of movement of unknown/movement of glucose. *Products:* 900 mg cellobiose (7.5 per cent ethanol); 500 mg R_G 0.4 carbohydrate (15 per cent ethanol); 140 mg R_G 0.4 + R_G 0.18 carbohydrate (50 per cent ethanol).

TABLE 8

Cellulase induction by a product isolated from a cellobiose culture of Trichoderma viride. (Growth experiment)

Organism	Cellobiose	R_G 0.4	Cellotriose	Lactose	Cellohexaose
	Cx u/ml	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*	*Cx u/ml*
Basidiomycete QM 806	1.0	2.3	2.0	2.1	
Pestalotiopsis westerdijkii	1.0	4.2	0.5	0	1.1
Myrothecium verrucaria	0.4	1.1	0	0	
Trichoderma viride	0	1.0	0.1	1.0	0
Sporotrichum pruinosum	1.2	2.4	0.8	0	
Penicillium helicum	0.2	0.7			

Basal medium plus carbohydrate at 0.2 per cent.

R_G 0.4 material from cellobiose culture, eluted from carbon column with 15 per cent ethanol (figure 6). Other inducers included for comparison.

as well as β-linked trisaccharides. When we grow *T. viride* on cellobiose and chromatogram the culture filtrate when the reducing value has decreased about 50 per cent, we find beside cellobiose a series of carbohydrate spots of which the most prominent have R_G values of 0.4 and 0.18. Cellobiose in this system has an R_G value of 0.6 and cellotriose of 0.18. These materials were adsorbed on a charcoal column and separated by elution with water and ethanol (figure 6). The R_G 0.4 material, eluted with 15 per cent ethyl alcohol, was tested as a cellulase inducer. This transfer product does induce cellulase in several fungi and is generally more active than cellobiose, cellotriose, or lactose (table 8). It is also an active inducer with washed mycelium (table 3).

DISCUSSION

To serve as an inducer, a substance must reach the site of enzyme production. The relatively poor inducing ability of the soluble cellulose derivatives leads us to believe that contact with the outer cell surface is not enough, since these offer so much better contact than does insoluble cellulose. We believe that the natural inducers of cellulase are soluble products of cellulose hydrolysis. Since cellobiose is the only soluble product commonly found during enzymatic hydrolysis of cellulose, our efforts have been directed to it.

We have now shown that cellobiose does, indeed, act as an inducer of cellulase in many fungi. The problem that concerns us is why the enzyme yields are usually so low compared to those obtained on cellulose. Our results indicate that inhibitory and inactivating factors are responsible, and that when these are overcome, as by slowing down the growth rate, the yields of cellulase on cellobiose can be as great as those on cellulose.

The inhibitory effects of sugars, particularly glucose, on enzyme induction have been frequently noted (Cohn, 1956; Neidhardt and Magasanik, 1956) but poorly understood. In cellulase induction, the inhibition may be related to mineral metabolism as suggested in our experiments; or the inhibition may be caused by certain metabolic products, particularly organic acids. Inactivation of enzymes in the absence of substrate, or during consumption of a new substrate, has been attributed to proteolytic enzymes (Spiegelman and Dunn, 1947; Jeuniaux, 1958). Whether the cellulase inactivation is of this sort, we do not know.

Cellulase inducers other than cellobiose and cellulose have been found: (a) lactose (β-1,4-galactosido-D-glucose), (b) salicin (a β-glucoside), (c) hydrolysis products of cellobiose octaacetate, (d) a compound present in reagent glucose, and (e) a transfer product from cellobiose cultures of *T. viride*. It is to be anticipated that compounds resembling the natural inducer will have inducing ability and that they may even exceed the natural inducer in this capacity. The activity of the transfer product from cellobiose suggests that it may be an intermediate in the induction reaction, and that in *T. viride* cellobiose is not the direct inducer. The impurity in reagent glucose may be 200 times as active as cellobiose for *T. viride*. Although we have not identified this we expect it will be found chemically related to cellobiose.

Pure glucose is not an inducer of cellulase. This is a retraction of a conclusion published previously before we knew of the impurity in reagent grade glucose that acts as an inducer.

We believe that cellobiose and related oligosaccharides are the true inducers of cellulase in cellulose cultures. The cellulose is an inducer only by virtue of its being hydrolyzed to these by cellulase. Other active carbohydrates probably owe their inducing ability to their similarity to cellobiose.

SUMMARY

Cellulase is an adaptive enzyme in fungi and is produced when they are grown on cellulose or on cellobiose. Other inducers include lactose, cellobiose octaacetate, and salicin. Glucose is not an inducer. All known inducers contain a β-glycosidic linkage, but noninducing compounds may also contain this linkage.

The poor cellulase yields when fungi are grown on cellobiose under optimal conditions are attributable to inhibitory or inactivating influences. The cellulase yields on cellobiose can be markedly increased by slowing the growth rate.

REFERENCES

BUSTON, H. W. AND JABBAR, A. 1954 Synthesis of β-linked glucosaccharides by extracts of *Chaetomium globosum*. Biochim. et Biophys. Acta. **15,** 543.

COHN, M. 1956 On the inhibition of glucose of the induced synthesis of β-galactosidase in *E. coli*. In *Enzymes, units of biological structure and function*, pp. 41–48. Academic Press, Inc., New York.

HORROCKS, R. H. 1949 Paper partition chromatography of reducing sugars with benzidine as a spraying reagent. Nature, **162,** 164–444.

JERMYN, M. A. 1953 Fungal cellulases. III. *Stachybotrys atra* growth and enzyme production on non-cellulosic substrates. Australian J. Biol. Sci., **6,** 48–69.

JEUNIAUX, C. 1958 Researches on chitinases. Arch. intern. physiol. biochem., **66,** 408–427.

MANDELS, M. AND REESE, E. T. 1957 Induction of cellulase in *Trichoderma viride* as influenced by carbon source and metals. J. Bacteriol., **73,** 269–278.

NEIDHARDT, F. AND MAGASANIK, B. 1957 Effect of mixtures of substrates on the biosynthesis of inducible enzymes in *Aerobacter aerogenes*. J. Bacteriol., **73,** 260–263.

REESE, E. T. 1956 A microbiological process report. Enzymatic hydrolysis of cellulose. Appl. Microbiol., **4,** 39–45.

REESE, E. T. 1957 Biological degradation of cellulose derivatives. Ind. Eng. Chem., **49,** 89–93.

REESE, E. T. AND LEVINSON, H. 1952 A comparative study of the breakdown of cellulose by microorganisms. Physiol. Plantarum, **5,** 345–366.

REESE, E. T. AND MANDELS, M. 1959 β-D-1,3-Glucanases in fungi. Can. J. Microbiol., **5,** 173–185.

REESE, E. T., SMAKULA, E., AND PERLIN, A. S. 1959 Enzymatic production of cellotriose from cellulose. Arch. Biochem. Biophys., **85,** 171–175.

SPIEGELMAN, S. AND DUNN, D. 1947 Interactions between enzyme forming systems during adaptation. J. Gen. Physiol., **31,** 153.

TALBOYS, P. W. 1958 Degradation of cellulose by *Verticillium albo-atrum*. Trans. Brit. Mycol. Soc., **41,** 242–248.

WHITAKER, D. R. 1954 Hydrolysis of a series of β-1,4-oligoglucosides by *Myrothecium verrucaria* cellulase. Arch. Biochem. Biophys., **53,** 439–449.