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Effect of Cellobiose on the Enzymatic Hydrolysis of Cellulose and its Derivatives

By

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In a previous study of the products of cellulose hydrolysis by microbial filtrates, cellobiose was found to be the end-product of the reaction mediated by the 1,4 β-polyglucosidase, Cx (Levinson *et al.* 1951). In actively growing cultures the further metabolism of cellobiose occurs *within* the organism. It has been shown that the cleavage products of enzymic hydrolysis of carbohydrates often are inhibitors of the enzyme concerned, e.g. glucose and fructose inhibit the activity of invertase, maltose that of amylase. If cellobiose is indeed the product of Cx activity, it might be possible to inhibit the reaction by the addition of cellobiose. Karrer *et al.* (1924) have shown that cellobiose (0.075 per cent) inhibits lichenin hydrolysis by snail lichenase, but glucose, fructose and galactose had an almost equal effect. Norkrans (1950) has shown that cellobiose (0.03 per cent) inhibits hydrolysis of cellulose (reprecipitated from cuprammonium solution) by extracellular, cellulolytic enzymes of the fungus, *Tricholoma nudum*. No other sugars were used for comparison.

This paper is restricted to a consideration of the effect of cellobiose on the hydrolysis of several cellulosic substrata by cell-free filtrates of various microorganisms. Other sugars and methyl cellulose have been used to determine whether the effects observed are peculiar to cellobiose.

Methods

Three different cellulosic substrata were used: a cellulose derivative (carboxymethylcellulose), a swollen linter cellulose, a native cellulose (ground,

[1] Part of this work was done during a visit by Dr. Norkrans to our laboratory.

dewaxed cotton fiber 40—60 mesh). Most of the experiments were performed with carboxymethylcellulose (CMC) and the hydrolysis was followed in a Hoeppler viscosimeter (Levinson *et al.* 1951). The results were based on a conversion of the fall time of the ball to specific viscosity (η_{sp}):

$$\eta_{sp} = \frac{\eta_{solution}}{\eta_{solvent}} - 1$$

$$\eta_{sp} = c\,F - 1$$

and

$$\phi = \frac{1}{\eta_{sp}} = \frac{1}{c\,F - 1}$$

where F = fall time of ball
c is a constant that includes characteristics of ball and solution — under the test conditions
and ϕ = specific fluidity

The reaction mixture contained 1 per cent of CMC in 0.05 *M* citrate buffer. Most routine measurements were made at pH 5.4—5.6, and at a temperature of 35° C. The rate of change in fluidity is a function of the enzyme activity.

The swollen cellulose was prepared by treatment of linter cellulose with 85 per cent phosphoric acid as described by Walseth (1948). The washed material remains well suspended. The extent of hydrolysis of the native and swollen cotton cellulose was measured gravimeterically. Three to four hours enzymic treatment at 50° C was sufficient to give a measurable loss in weight of the swollen cellulose. The native cellulose was incubated 18—24 hours at 40° C.

In the CMC viscosity test, very low enzyme concentrations are required (0.3—2.0 per cent, based on volume of enzyme filtrate). In the swollen cellulose tests, the filtrate represented 30 per cent, and in the cotton tests 90 per cent of the total volume of suspension. The substrate concentration varied with the tests, being one per cent for CMC and for ground cotton, and about 0.75 per cent for the swollen cellulose.

The enzymes used were prepared by growing the organism in shake flasks on a cellulosic substratum (Reese *et al.* 1950). The residue was removed by filtration, and the cell-free filtrates preserved with 0.01 per cent merthiolate in a refrigerator.

Only commercially available materials were used. Two sources of cellobiose were employed (Pfanstiehl and Difco). The CMC was obtained from Hercules Powder Company as grade 70 M, and is of medium viscosity. The methocel is a product of Dow Chemical Company and has a degree of substitution (DS) of about 1.8.

Results

(1) *Hydrolysis of CMC (viscosity data)*

We first observed cellobiose inhibition in our laboratory by the viscosity method. Filtrates of cultures of several organisms were tested (Table 1), and all but two showed inhibition of Cx activity by 2 per cent cellobiose. The two exceptions, filtrates of *Streptomyces sp.* and of *Myrothecium verrucaria,* were

Table 1. *Effect of cellobiose and of glucose on hydrolysis of CMC by the Cx's of various organisms* (Viscosimetric Method).

Filtrate of	QM No.	Cellobiose pH 5.0	Cellobiose 7.0	Glucose 5.0	Glucose 7.0
Aspergillus flavus	10e	I	I	0	
Aspergillus fumigatus	45h	I			
Aspergillus niger	458	I			
Memnoniella echinata	1c	I			
Myrothecium verrucaria	460	S	0		
Penicillium pusillum	137g	I	I	0	0
Pestalotia palmarum	381	I		0	
Streptomyces sp.	B814	S	I	0	0
Trichoderma viride	6a	I			
Torula sp.	986	I		S	

I = Inhibition; S = Stimulation; 0 = No effect.

stimulated by the addition of cellobiose. It was this peculiar behavior that led us to look further into the problem.

Greatest stimulation was observed in the case of the *Streptomyces sp.* filtrate. To determine whether stimulation was general among members of this genus, five other cellulolytic isolates were tested. Three of these were stimulated by 1 per cent cellobiose (95—169 per cent stimulation); and two were inhibited (33—62 per cent inhibition). The isolate most studied (QM B 814) produced a filtrate stimulated under the same conditions to an extent of 260 per cent.

In all, filtrates of 36 organisms were tested for the effect of 2 per cent cellobiose on the hydrolysis of CMC at pH 5.4. These include representatives of all major groups of fungi, several actinomycetes and one bacterium (*Sporocytophaga myxococcoides*). Of these 26 were inhibited, 3 were unaffected, and 7 were stimulated. Glucose had a stimulatory effect in 2 cases where cellobiose inhibited (*Trichoderma viride, Torula sp.*); in all other cases glucose was without effect. Those filtrates that were stimulated by cellobiose include the 4 strains of Streptomyces (above), *Myrothecium verrucaria, Chaetomium elatum* QM 606, and *Fusarium roseum* QM 38 g. The range of activity covered by cellobiose is from over 60 per cent inhibition (*Streptomyces sp.* JTB 2604 and unidentified Basidiomycete cultures) to over 200 per cent stimulation (*Streptomyces sp.* QM B 814).

For further tests, two organisms (*Streptomyces sp.* QM B 814; *Myrothecium verrucaria* QM 460) were selected to represent those whose filtrates were stimulated by cellobiose, and two organisms (*Trichoderma viride* QM 6 a; *Penicillium pusillum* QM 137 g) to represent those whose filtrates were inhibited. Increasing the cellobiose concentration (Figure 3) in the range

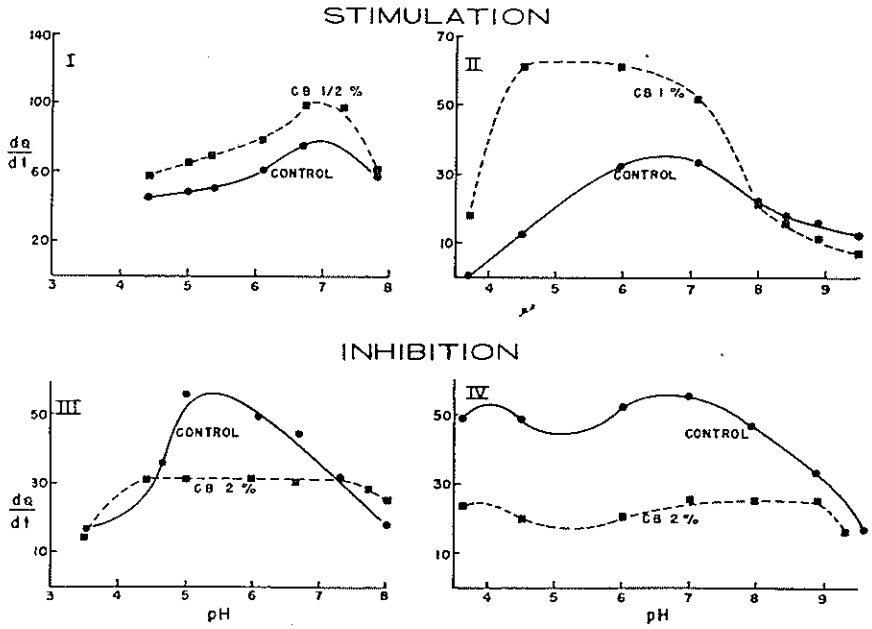

Figure 1. *Effect of pH on stimulation and inhibition by cellobiose as measured by changes in fluidity* (CMC).

I *Myrothecium verrucaria* ----- with cellobiose
II *Streptomyces sp.* ——— without cellobiose
III *Trichoderma viride*
IV *Penicillium pusillum*

$d\phi/dt$ is the rate of change of fluidity; $\phi = \frac{1}{\eta_{sp}}$

1—4 per cent, increased the inhibition. Where stimulation occurred, the optimum concentration varied with the source being 1 per cent for *Streptomyces sp.* and 0.5 per cent for *Myrothecium verrucaria* under the conditions of this test.

Hydrogen ion concentration has a marked effect on the amount of inhibition or of stimulation produced by addition of cellobiose. These effects on the shape of the pH-activity curves are shown in Figure 1. For *Streptomyces sp.* filtrate, the addition of cellobiose increases the rate of hydrolysis and broadens the zone of optimum activity. As a result, at pH 4.5 where the control activity is about $^1/_3$ maximal, the test solution containing cellobiose remains at its maximum value. Such differences in the shapes of the curves lead to different amounts of stimulation or of inhibition at the various pH levels.

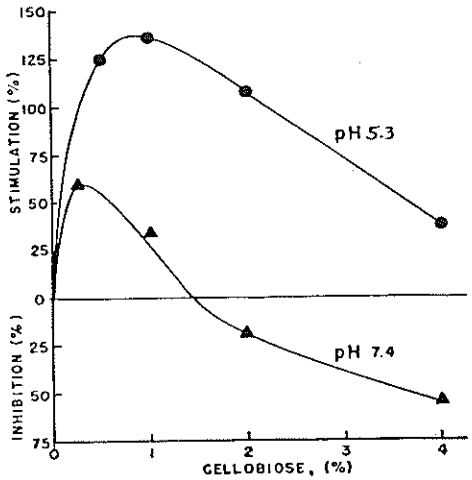

Figure 2. *Effect of cellobiose concentration on stimulation of Streptomyces sp. filtrate at various pH's* (CMC).

A flattening out of the pH-activity curve by cellobiose similar to that observed above is found with *Trichoderma viride* filtrates. In this case, however, the effect of the cellobiose is to inhibit. For the other two filtrates the control and cellobiose curves remain more or less parallel over most of the range. It is obvious that we have drawn the pH-activity curve for *Penicillium pusillum* with little regard for the experimental points. The curves have been so drawn on the basis of additional data showing a bimodal nature. A similar curve for pH vs cellulase activity has been reported for the enzymes of the larvae of *Stromatium fulvum* (Mansour and Mansour-Bek 1937). Such bimodal pH-activity curves appear to be rare. We suppose they may indicate the presence of two cellulases of different pH optima. Except for *Streptomyces sp.*, the region of maximum cellobiose effect appears to be near the pH range of optimal activity. Most interesting is the decrease in stimulation (and in inhibition) as the pH rises, and the change from stimulation to inhibition (and vice versa).

There is an interaction between cellobiose concentration and pH. This was suggested by a comparison of some of the earlier data with later results. In Table 1, it is indicated that for *Streptomyces sp.* B 814, stimulation was observed at pH 5.0 and inhibition at pH 7.0. This is true for all cellobiose concentrations above 1.5 per cent (Figure 2). Below that value, only stimulation is apparent (in the range pH 5.3—7.4). As the pH rises, the value for maximum stimulation decreases and shifts toward the region of lower cellobiose concentrations.

Other sugars have been tested for their effect on hydrolysis of CMC in the viscosimeter. The conditions chosen were those which proved optimal for

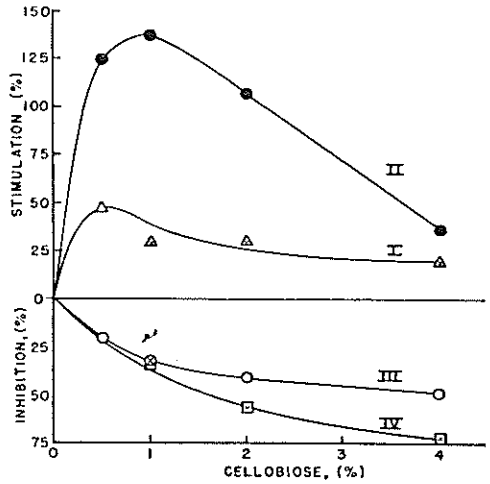

Figure 3. *Effect of cellobiose concentration on stimulation and inhibition at pH 5.3* (CMC — Fluidity tests).

I *Myrothecium verrucaria*
II *Streptomyces sp.*
III *Trichoderma viride*
IV *Penicillium pusillum*

cellobiose. Sucrose, glucose and maltose were tested with each of the four filtrates under comparison. In only one case, was there any significant effect, — and here the effect was opposite to that observed with cellobiose. The *Trichoderma viride* filtrate, normally inhibited by cellobiose, was stimulated by glucose (maltose and sucrose had no effect). The effect is slight (15—20 per cent), greatest at a glucose concentration of 2 percent, and is relatively independent of pH.

Methocel (Methyl cellulose, DS 1.8) is a water soluble cellulose derivative of high degree of substitution. As a result, it is resistant to hydrolysis by cellulolytic enzymes. Preliminary tests had shown methocel to be very effective in inhibiting Cx. It was now retested with the four filtrates under consideration (Figure 4). All four filtrates were inhibited by methocel, but to greatly varying degrees. Most sensitive was that of *Trichoderma viride,* followed by *Myrothecium verrucaria, Penicillium pusillum* and *Streptomyces sp.* This is exactly the same order previously observed for adsorption of the enzyme Cx of these filtrates on a purified wood cellulose (unpublished). In the latter tests, the Cx was found to be adsorbed much more firmly than was β-glucosidase, from which it is inferred that enzyme-substrate attraction was involved. It has been shown, however, (Teragama *et al.* 1950) that cellulose sulfate inhibits catalase activity. The methocel in our experiments may be acting in a similar non-discriminatory manner.

Since carboxymethyl cellulose is a moderately strong acid, there is a tendency to explain any differences observed in behavior between it and cellulose on the basis of the characteristics of the acidic group. This would be particularly so in explaining the effect of pH on cellobiose stimulation (or

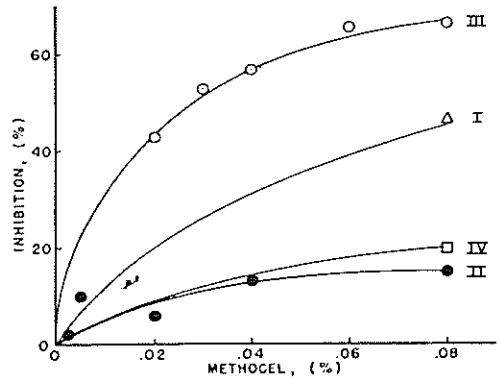

Figure 4. *Inhibition of CMC hydrolysis by methocel* (Viscosimetric Method).

I *Myrothecium verrucaria*
II *Streptomyces sp.*
III *Trichoderma viride*
IV *Penicillium pusillum*

inhibition) of the hydrolysis rate. We have, therefore, repeated some of the above experiments using non-acidic water soluble cellulose derivatives:

Cellulose acetate (Eastman Kodak Co.) DS 0.76

Hydroxyethyl cellulose (Carbide and Carbon Chem. Co.)

With the cellulose acetate, hydrolysis by the *Streptomyces sp.* filtrate was stimulated by cellobiose; hydrolysis by *Penicillium pusillum* filtrate was inhibited by cellobiose. With the hydroxyethyl cellulose, cellobiose stimulation of hydrolysis by *Streptomyces sp.* filtrate was greatest at the lower pH values and decreased as the pH became higher. At pH 7.5, maximum stimulation was found at 0.7 per cent cellobiose; inhibition occurred above 2.5 per cent cellobiose. All of these data are in agreement with those obtained using carboxymethyl cellulose as substrate. The effects reported would seem to be independent of the nature of the substituent present.

(2) *Hydrolysis of unsubstituted cellulose*

Hydrolyses of both swollen cellulose (Figure 5), and of ground cotton fiber (Table 2), are inhibited by the addition of cellobiose. All filtrates behave in the same manner. No stimulation was observed at any time.

In the swollen cellulose tests, glucose, maltose, and sucrose were compared with cellobiose at the 1 per cent level. The results, for the sugars other than cellobiose, are somewhat erratic because the weights are so near those of the controls. The maximum values for inhibitions by a 1 per cent concentration of glucose, maltose, or sucrose are of the magnitude obtained for the action of 0.1 per cent cellobiose (Figure 5). Six other filtrates (in addition to those shown in Figure 5) were tested on swollen cellulose. Three of these, *Aspergillus flavus, Torula sp.* and *Scopulariopsis brevicaulis,* were inhibited by cellobiose but not by glucose. Of the others, that of *Sporotrichum pruinosum*

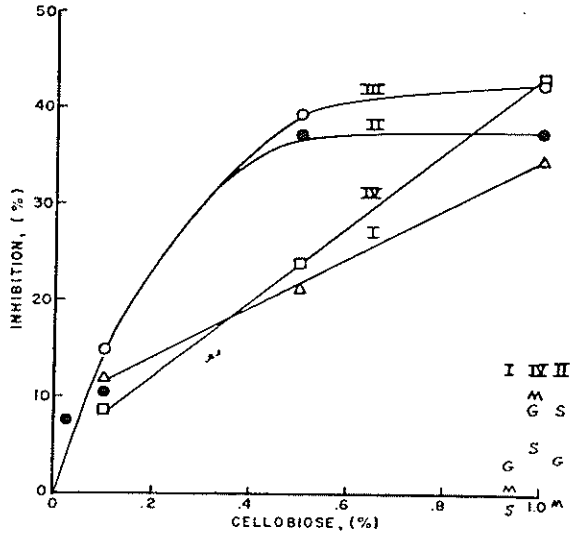

Figure 5. *Inhibition of cellulolytic activity by cellobiose* (loss in weight of swollen cellulose).

I *Myrothecium verrucaria*
II *Streptomyces sp.*
III *Trichoderma viride*
IV *Penicillium pusillum*

Effects of other sugars at the one percent level.
M = Maltose
G = Glucose
S = Sucrose

produced very little loss in weight, while those of *Pestalotia palmarum* and of *Botrytis cinerea* were inhibited to a slight but equal extent by both cellobiose and glucose. Similar, but more limited data were obtained with cotton cellulose used as substratum. The inhibition, thus, appears to be specific for cellobiose.

Methocel was employed in but one test with cotton fiber as substratum. The filtrate selected (*Trichoderma viride*) was that whose Cx was inhibited most in the CMC experiments. For this set of conditions, 0.01 per cent metho-

Tab. 2. *Effect of cellobiose on hydrolysis of cotton cellulose* by cell-free filtrates.

Filtrate of	QM No.	Cellobiose Conc. %	pH	Loss in Wt. % [2]		Inhibition, % [1]
				No CB	+CB	
Myrothecium verrucaria	460	4.	5.2	7.6	3.8	50
		4.	6.4	8.3	2.9	65
Penicillium pusillum	137g	0.4	5.2	8.8	5.0	43
		1.0	5.2	8.8	3.0	66
		5.0	5.2	11.5	3.7	68
Streptomyces sp.	B814	1.	5.7	6.9	2.7	61
		4.	5.7	6.9	1.45	79
Trichoderma viride	6a	1.0	5.2	7.4	2.2	70
		5.0	5.2	4.6	0.5	89

[1] Hydrolysis measured as loss in weight of cotton cellulose.

$$\% \text{ Inhibition} = \frac{(\text{loss in absence of CB}) - (\text{loss in presence of CB})}{\text{loss in absence of cellobiose}} \times 100.$$

[2] Four replicates for each condition.

cel gave a 55 per cent inhibition of hydrolysis as compared to a 57 per cent inhibition by 1 per cent cellobiose. Methocel is, then, about 100 times as active as cellobiose in inhibiting this particular enzyme.

Discussion

For purposes of convenience, the stimulatory effect of cellobiose will first be considered, followed later by a discussion of the inhibitory effect. The *stimulation* by cellobiose of the Cx activity of *Streptomyces spp.* filtrates takes place only when the substrate is a form of cellulose solubilized by the introduction of various substituents (CMC, hydroxyethyl cellulose, cellulose acetate). When unsubstituted cellulose is used the stimulation is absent. The effect is of significance here only as it may lead to some understanding of the action of enzymes on modified substrates. The facts regarding the Streptomyces filtrate are:

(1) Cellobiose stimulation of Cx at low pH
(2) Cellobiose inhibition of Cx at high pH
(3) Methocel inhibition of Cx

Because of the similarity of cellobiose and methocel to the natural substrate (i.e. cellulose), we assume that they compete with the CMC for the active sites on the enzyme Cx, i.e. they all have an affinity for the same loci. When CMC occupies the active position, it remains there until hydrolysis occurs, and the products are then removed. Cellobiose occupies the site for the least time, the natural tendency being to thrust off the end products. Methocel, however, is retained longer at the locus since it resists hydrolysis. Now it is assumed that the configuration of the Cx of *Streptomyces sp.* is a bit different from the Cx of most other organisms studied in that the hydrolysis products of the CMC are not as readily removed from the active position. The result is that the enzyme is unable to combine with another molecule of substrate. It is then possible that cellobiose, competing for the same areas, aids in the removal of the refractory products. This explanation does not consider the very marked effect of pH on cellobiose stimulation of *Streptomyces sp.* filtrates. For a constant cellobiose concentration the stimulation is greatest at the lower part of the pH range. It may be that in this region the binding force of the refractory-CMC-products to the enzyme is greatest, since other tests have shown that adsorption of Cx on cellulose increases with increasing hydrogen ion concentration. On the other hand, the fact that stimulation is greatest at low pH may also be explained on the basis of cellobiose protection of Cx by preventing enzyme inactivation. Similar protective actions have been reported for

(1) yeast invertase by protein at low (but not at the optimum) pH

(2) invertase by sucrose

(3) α amylase by starch and its hydrolysis products.

The objection to this »protective» supposition is that the stimulation is not apparent when *un*substituted celluloses (cotton, swollen cellulose) are used as substrates.

We will now consider the *inhibitory* action of cellobiose. Cellobiose inhibits the Cx of most of the organisms studied. Even in those cases where stimulation is observed at low cellobiose concentrations (i.e. with CMC), the inhibitory effect becomes evident as the cellobiose concentration is increased. Since other sugars have little or no effect, the specificity of the reaction lends support to the belief that cellobiose is the end product of the enzyme (Cx) hydrolysis. This is confirmation of earlier work reporting the presence of cellobiose in hydrolysates of soluble cellulose derivatives (Levinson *et al.* 1951).

The inhibition of a reaction by its end products may lead to a better understanding of the nature of the enzyme action. For purposes of comparison, the paucity of data on the effect of maltose on β-amylase and on α-amylase is unfortunate. It appears (Schwimmer 1950) that maltose inhibits α-amylase in a non-competitive manner, but that hydrolyzable dextrins act competitively. This is consistent with the theory of random action coupled with the greater affinity of this enzyme for the longer chains. No data of this kind have come to our attention regarding the effect of maltose on β-amylase, though it has long been known (Kühn 1925) that maltose does inhibit β-amylase. It might be argued (in the absence of data) that the inhibition should be competitive since maltose results from each hydrolytic act of the β-amylase. Our data show that cellobiose specifically inhibits the Cx activity of most, but not of all the microorganisms that we have examined. Based on meager CMC results (unreported), it appears that cellobiose usually acts non-competitively, similar in effect to maltose on α-amylase. This is in agreement with the earlier data supporting random splitting of the cellulose chain:

(1) Fåhraeus (1946): Cellophane strips lost rapidly in tensile strength before any glucose could be demonstrated.

(2) Norkrans (1950): Rapid depolymerization of regenerated cellulose occurred without a corresponding increase in sugar formation.

(3) Levinson and Reese (1950): A rapid loss in viscosity of CMC per unit increment in reducing value was found in the early stages of hydrolysis.

Cellobiose inhibits cellulolytic action as measured by loss in weight of native cotton, as well as that measured by loss in viscosity of CMC. This

observation must be examined for its effect on the two step mechanism postulated for the breakdown of cellulose.

$$\text{Native Celluose} \xrightarrow{C_1} \text{Chains} \xrightarrow{Cx} \text{Cellobiose}$$

We believe that it is the action of Cx on *insoluble* fragments (resulting from C_1 activity) that leads to the losses in weight which have been measured.

We have considered following farther the comparison of Cx with the amylases. β-amylase acts on starch to produce β-maltose, its action proceeding from the non-reducing end of the molecule. Why the configuration is β rather than α is as yet unknown. α-amylase splits the chains in a random fashion, the final product being α-maltose. Where the hydrolysis reaction proceeds rapidly, it is possible to detect the α or β form of maltose, and thus to determine whether the amylase is of the α or β type. To date, we have been unable to find a substrate of suitable nature to permit of Cx activity of the magnitude required. An activity resulting in the production of 0.3 per cent cellobiose within 30 minutes would probably be sufficient to permit detection of the α or the β form of cellobiose, before mutarotation resulted in the establishment of the equilibrium mixture.

Further comparison of Cx with amylases forces us to recognize that the characterization of fungal amylases as α-type may no longer hold. It now appears (Okazaki 1950) that both α and β amylases are present in the same fungus. The possibility that there are similarly two (or more) Cx types in each organism must not be overlooked. The bimodal pH-activity curves support such a view. While the evidence for such mixtures of Cx types is scanty, this may be due entirely to insufficient experimentation. Most of our present data, however, support the view that the filtrates of a fungus contain one Cx type acting in a random fashion similar to α-amylase.

Summary

Cellobiose inhibits the hydrolysis of cellulose by filtrates of most of the organisms tested. Glucose does not inhibit the hydrolysis. The action of cellobiose is believed to be that of an end product inhibiting an enzymatic hydrolysis, in much the same manner that maltose inhibits hydrolysis of starch by α-amylase.

Filtrates of seven organisms (of 36 tested) have been found where the hydrolysis of carboxymethyl cellulose is *stimulated* by low cellobiose concentrations. As the cellobiose concentration was increased, the stimulation decreased, and finally inhibition occurred. The hydrolysis of *unsubstituted* cellulose by these same filtrates is inhibited by cellobiose.

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