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Products of Enzymatic Hydrolysis of Cellulose and Its Derivatives

H. S. Levinson, G. R. Mandels and E. T. Reese

From the Quartermaster General Laboratories, Philadelphia, Pennsylvania

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INTRODUCTION

In previous papers (Reese, Siu, and Levinson, 1950; Levinson and Reese, 1950) the mechanism of cellulose degradation has been investigated through the use of soluble cellulose derivatives. On the basis of data obtained with cellulolytic and non-cellulolytic organisms, it was postulated that at least two steps were involved. The first, characteristic of all cellulolytic fungi involved an enzyme C_1 , whose specific action was not known but which presumably resulted in the formation of linear chains of anhydroglucose units. The second step was mediated by an enzyme, C_x , present in some non-cellulolytic as well as in all cellulolytic microorganisms. This enzyme is capable of attacking cellulose derivatives. Measurements of viscosity changes and the formation of reducing substances during hydrolysis of carboxymethylcellulose (CMC) by C_x led to the postulate of a random splitting of the chains. In the present paper, the recently available tools of filter paper chromatographic analysis and of the specific glucose aerodehydrogenase (glucose oxidase, notatin) were applied to the determination of the end products formed by the action of C_x on cellulose and its derivatives. Specifically, it is attempted to resolve the problem as to whether glucose or cellobiose is the end product of cellulose degradation by microorganisms. In the preceding paper in this series (Reese, Siu, Levinson, 1950), it was hypothesized that glucose was formed directly by the action of C_x on CMC. The present data negate this conclusion and show that cellobiose is the main end product of hydrolyses effected by C_x .

METHODS

1. Filtrates

Filtrates having cellulolytic activity were prepared by culturing the organisms in flasks on a reciprocating shaker at 30°C. for from 3 to 14 days at which time they were filtered through sintered glass. Where necessary the filtrates were preserved with merthiolate (0.01%).

2. Substrates

Several soluble cellulose derivatives have been employed in the present work. In our previous work (Reese *et al.*, 1950; Levinson and Reese, 1950) we used carboxymethylcellulose, Na salt (CMC). Actually, hydroxyethylcellulose and cellulose sulfate give essentially the same type of results. We have used cellulose sulfate¹ for the present work because of its solubility, its low viscosity, its low degree of substitution, and its rapid hydrolysis by filtrates containing Cx. In our experiments, dialyzed solutions have been used, since this treatment has been found to remove some of the sulfate ion.

3. Analysis of Hydrolysates for Reducing Substances

The enzymatic hydrolysates were autoclaved, prior to assay, for 15–20 min. at 15 lb. in order to inactivate the filtrates. This treatment was found to have no significant effect on the analyses. The dinitrosalicylic acid (DNS) method of Sumner (1944) was used for determination of the total reducing value (in terms of glucose) and for other purposes as indicated below.

(a) *Determination of glucose.* Keilin and Hartree (1948) have proposed the use of glucose oxidase (notatin) for the specific determination of glucose in mixtures of sugars or other substances. Instead of following the reaction manometrically as proposed by these authors, we have determined the decrease in reducing value of the sample, as measured by the DNS method, after incubation with the enzyme. Foster (1949) has pointed out that glucose aerodehydrogenase is a more correct name for this enzyme.

Glucose aerodehydrogenase was produced by growing *Penicillium notatum* [Merck MF-70 (50 R 333)]² on a glucose-yeast extract, mineral salts solution in shake flasks for 5–7 days, filtering, and using the filtrate without further treatment. More than 1 mg./ml. glucose is oxidized in the reaction mixture (8 ml. 0.2% glucose, 1 ml. 0.5M citrate pH 5.0, 1 ml. filtrate containing glucose aerodehydrogenase) when the contents are agitated on a shaker at 40°C. for 1 hr. Under these conditions no oxidation of cellobiose has been detectable.

(b) *Detection and estimation of cellobiose* was accomplished by hydrolysis of cellobiose to glucose with β -glucosidase followed by determination of the glucose formed with glucose aerodehydrogenase. It is not practical to measure cellobiose by the increase in reducing value after hydrolysis, because the increase over the high initial

¹ Cellulose sodium sulfate (Eastman Kodak # 103205 of D.S. 0.4) was kindly supplied by Dr. C. J. Malm.

² We are indebted to Dr. H. B. Woodruff for this culture.

value is very slight when measured with DNS. The β -glucosidase preparation was obtained by growing *Aspergillus luchuensis* (QM 873) on cellobiose in shake flasks for 17-33 days at 30°. After filtering through fritted glass funnels the filtrates were used without treatment except to add merthiolate as preservative. This filtrate had no *Cx* activity.

(c) *Measurement of cellobiose and glucose* in hydrolysates was accomplished by simultaneous determination of total reducing values, glucose and cellobiose, according to the following schedule: 8 ml. of hydrolysate was added to each of four test tubes which were then treated as shown in Table I.

Total reducing substances are shown in Table I by tube No. 1, glucose by the difference between tube No. 1 and No. 2, and cellobiose is represented by the difference between the total glucose after hydrolysis with β -glucosidase and the original glucose, or

$$\text{Cellobiose} = [(3-4) - (1-2)] 0.95$$

The value thus obtained is multiplied by 0.95 to correct for the addition of water occurring during hydrolysis.

TABLE I

Scheme for Simultaneous Determination of Glucose and Cellobiose

Step number	Tube number			
	1	2	3	4
1	1 ml. H ₂ O	1 ml. H ₂ O	1 ml. β -Glucosidase	1 ml. β -Glucosidase
2	----- Incubate 2 hr. at 50° -----			
3	1 ml. H ₂ O	1 ml. Glucose oxidase	1 ml. H ₂ O	1 ml. Glucose oxidase
4	----- Incubate 2 hr. at 40° on shaker -----			
5	----- Determine reducing sugars by DNS -----			

The validity of this method rests on the purity of the enzyme solutions used. Tests show the notatin preparation to have no *Cx* or β -glucosidase activity, i.e. no action on CMC 50T or salicin, and the β -glucosidase preparation to have no *Cx* or glucose aerodehydrogenase activity. Additional tests showed nearly quantitative recovery of added cellobiose (92, 92, and 96%); glucose shows no "cellobiose."

One limitation of the procedure is the assumption that cellobiose is the primary sugar acting as substratum for β -glucosidase. Actually, Grassmann, Zechmeister, Toth, and Stadler (1933) have shown that chains of 3, 4, and 6 glucose units are also hydrolyzed by this enzyme. Our "cellobiose" values are probably also subject to this limitation and should thus be considered as maximum values. This method is superior to acid hydrolysis in its greater specificity but leaves much to be desired.

4. Analysis of Hydrolysates by Paper Chromatography

Confirmation of the presence of glucose and of cellobiose in the hydrolysates has been obtained by means of paper-partition chromatography. The apparatus used was essentially that described by Consden, Gordon, and Martin (1944). Strips of Whatman No. 1 filter paper 11.5 × 45 cm. were employed. Small quantities (3-4 μ l.) of

the hydrolysates and of control sugars (1%) were applied with a capillary pipet along a penciled line approximately 6 cm. from an end, and at intervals of approximately 1.5 cm. This end of the paper was secured by glass slides in a trough containing the solvent.³ After 18–24 hr. incubation in a closed vessel, the solvent had irrigated 30–35 cm. of the paper. The paper was removed, dried in a circulating hot-air oven at 100°, sprayed with the developer⁴ and developed at 100° for 5–7 min. With the benzidine developer the migrating solute appeared as brown spots, varying in intensity with the concentration of sugars present. After developing the chromatogram, the R_F of each spot produced by the unknown was compared with those of known sugars.

It was found in a number of cases, that the concentration of reducing sugars in the hydrolysates was too low for the spots to be detected with certainty. Consequently, it has been our practice to concentrate the hydrolysate, either by lyophilization or by evaporation at 100° to dryness, and to reconstitute the solids to 1/10 the original volume. Tests show that neither procedure changes the amount of glucose or cellobiose found. Often, in examining CMC or its hydrolysates by the method of paper-partition chromatography, the solute did not migrate readily, with the result that an undesirable trail was formed. In such cases, removal of the longer molecules was accomplished by precipitation with 50% ethyl alcohol, the smaller molecules remaining in solution. This procedure does not significantly affect the amounts of glucose or cellobiose present.

5. Determination of β -Glucosidase

The activity of β -glucosidase is customarily evaluated by determining the rate at which the enzyme hydrolyzes some commercially available material such as β -methylglucoside or salicin. Pigman (1944) has clearly demonstrated that β -glucosidases of various origins hydrolyze different β -glucosides at different rates. For estimating the activity of the β -glucosidase, cellobiase, cellobiose is naturally the preferred substratum, but it is objectionable because of its high initial reducing value. Of the available substitutes, salicin is a better substratum than β -methylglucoside for the filtrates tested because it is hydrolyzed more rapidly and has a lower initial reducing value. At 0.052 M in the reaction mixture, the zero value for salicin is 0.00–0.01 mg./ml. and for β -methylglucoside $0.13 \pm .01$ mg./ml., as measured by the DNS method. The detailed procedure is to mix 1 ml. 0.5 M citrate buffer (pH 5.0) with 8 ml. 0.925% salicin, and add 1 ml. of the filtrate to be tested. The mixture is incubated for 1 hr. at 50°C., and an increase in reducing value is determined by the DNS method.

6. Determination of Cx

Cx , which can be considered similar to the β -glucopolysaccharase of Grassmann *et al.* (1933), acts upon the same β -1,4-type linkage as does β -glucosidase, but in the longer chains. The end product as shown in this paper is at least primarily cellobiose, but other reducing compounds formed also react with the DNS reagent used. The

³ Solvent: 75% isopropyl alcohol, 90 ml.; glacial acetic acid, 10 ml.

⁴ Developer (Horrocks, 1949): benzidine, 0.5 g.; glacial acetic acid, 20 ml.; absolute ethyl alcohol, 80 ml.

reducing values obtained after hydrolysis with Cx are to be considered only as relative and not in any absolute manner. Since the optimum conditions for Cx are nearly the same as for β -glucosidase the same procedure is used except for the substitution of CMC 50T (in a final concentration of 0.5%) for salicin.

EXPERIMENTAL

Filtrates from cultures of several microorganisms grown on different substrata were tested for β -glucosidase and for Cx activity. The results (Table II) demonstrate, with the exception of *Aspergillus luchuensis*, very slow hydrolysis of salicin or cellobiose and rapid hydrolysis of

TABLE II
Hydrolysis of Salicin, Cellobiose and CMC by Culture Filtrates

Filtrate	Grown on	Rate of hydrolysis, mg./ml./hr.		
		Salicin	Cellobiose	CMC 50T
H 26 <i>Aspergillus fumigatus</i> QM 45h	Duck	0.01	0.04	0.61
H 11 <i>Aspergillus fumigatus</i> QM 45h	Duck	0.01	0.01	0.64
K 2 <i>Actinomyces</i> sp. QM-B814	HEC	0.00	0.00	0.98
A 15 <i>Myrothecium verrucaria</i> QM 460	Duck	0.02	0.03	0.91
A 13 <i>Myrothecium verrucaria</i> QM 460	CMC	0.01	0.01	0.43
D4e-5 <i>Penicillium lilacinum</i> QM 4e	Dextran A ^a	0.00	0.00	0.01
J 47 <i>Aspergillus luchuensis</i> QM 873	Duck	0.24	0.14	0.36
C 15 <i>Trichoderma viride</i> QM 6a	Duck	0.00	0.02	0.89
W Water, control	—	0.00	0.00	0.00

^a R. S. from dextran A: 0.56 mg./ml./hr.; i.e., this filtrate contains an α -1,6-polysaccharase.

CMC. The relatively low β -glucosidase activity and the high Cx activity of these filtrates suggested that if such filtrates were allowed to act upon cellulose derivatives an analysis of the hydrolysates should indicate whether cellobiose or glucose units were split off by the enzyme Cx .

The course of one such hydrolysis of cellulose sulfate (Fig. 1A) by a *Trichoderma viride* filtrate showed that the total reducing value increased rapidly during the first hour. There is a corresponding increase in cellobiose. Both curves flatten out abruptly after reaching maximum values. Glucose does not appear until later and it, too, reached a constant value. The amount of glucose present at the end of 24 hr. was much less than the amount of cellobiose. The *T. viride* filtrate used in

this experiment was known to have a high *Cx* activity and a low β -glucosidase activity.

If the β -glucosidase activity of the above reaction mixture is increased by addition of a filtrate of *A. luchuensis* with a high β -glucosidase and low *Cx* activity, the cellobiose is rapidly hydrolyzed with the result that the final hydrolysate contains much glucose but no cellobiose (Fig. 1B). The total reducing value is also slightly increased

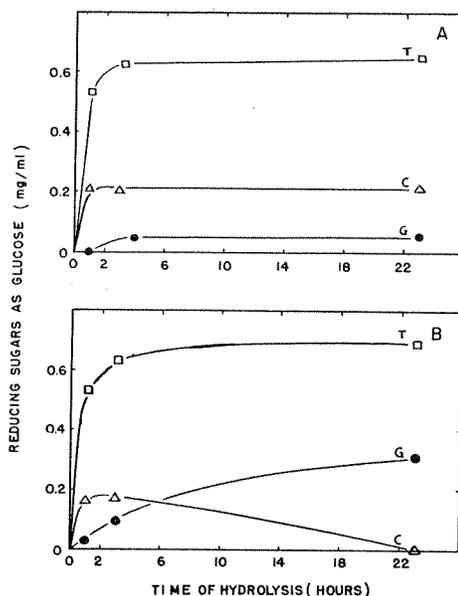


FIG. 1. Appearance of glucose and cellobiose during hydrolysis of cellulose sulfate by filtrate of *Trichoderma viride*. A. No β -glucosidase added. B. β -glucosidase added at the beginning of the hydrolysis. T = Total reducing value in mg./ml. as glucose, C = Cellobiose in mg./ml., G = Glucose in mg./ml.

since the reducing value of the glucose obtained from the cellobiose is greater than the reducing value of the cellobiose that has been hydrolyzed.

The above solutions were also subjected to paper-partition chromatography (Fig. 2). It will be observed that the reaction with *T. viride* filtrate in the absence of β -glucosidase gave a very light glucose spot at 3.5 hr. This spot became darker in the later stages of hydrolysis, i.e.,

the glucose concentration increased. When additional β -glucosidase was present, glucose appeared earlier (60 min.), and cellobiose disappeared completely by the end of 23 hr. These findings confirm the results obtained by the method described in the preceding paragraph. Similar results have been obtained with filtrates of *Aspergillus fumigatus*, *Actinomyces* sp., *Sporocytophaga myxococcoides* (QM-B482), and *Myrothecium verrucaria*. All these solutions have high *Cx* and low β -glucosidase activities.

A better idea of the relationship of the appearance of glucose to the appearance of cellobiose is obtained if the amount of each sugar is

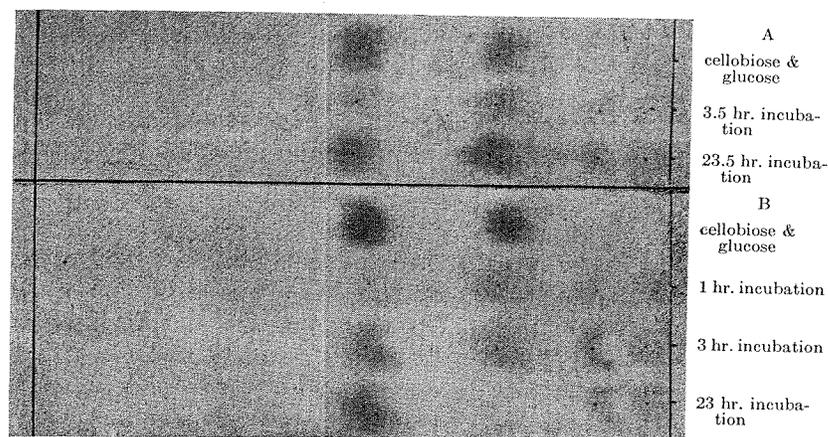


FIG. 2. Products of hydrolysis of cellulose sulfate by a filtrate of *Trichoderma viride*. A. In the absence of added β -glucosidase. B. In the presence of added β -glucosidase.

plotted against the total reducing value of the hydrolysate. This procedure eliminates "rate of hydrolysis," and permits comparison of filtrates of different *Cx* activities. The data (Fig. 3) from experiments with filtrates of four different organisms show (1) that glucose is not produced until late in the reaction, and (2) that the subsequent increase in glucose is accompanied by a decrease in cellobiose. It must be concluded that glucose arises from cellobiose, and that even though the amount of β -glucosidase in the filtrates is very low, it is the enzyme responsible for the hydrolysis of cellobiose.

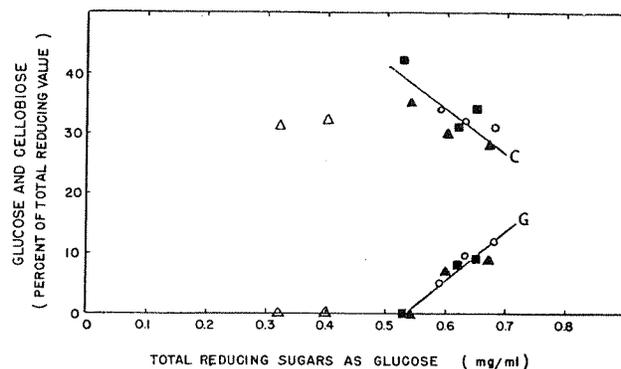


FIG. 3. Appearance of glucose and cellobiose during hydrolysis of cellulose sulfate by filtrates of

Sporocytophaga myxococcoides Δ
Trichoderma viride \blacksquare
Myrothecium verrucaria \circ
Aspergillus fumigatus \blacktriangle

C = cellobiose, G = glucose.

The data (Fig. 3) do not show the relationship between the amount of "cellobiose" and the total reducing value in the early part of the hydrolysis. From other experiments dealing with this point, it has been found that, prior to the appearance of glucose, the ratio, cellobiose: total reducing value, remains constant. For the hydrolysis of cellulose sulfate (Table III) a value of 40% was found, i.e., the cellobiose in mg./ml.

TABLE III

Comparison of Relative Amounts of Cellobiose + Glucose Found in Hydrolysates of Cellulose Dextrin and of Cellulose Sulfate

Cellobiose in mg./ml. as cellobiose; glucose in mg./ml. as glucose;
 but total reducing value as mg./ml. glucose

Organism	Per cent of total reducing value due to cellobiose + glucose in	
	Cellulose dextrin hydrolysate	Cellulose sulfate hydrolysate
<i>Myrothecium verrucaria</i>	77 (5 hr.)	41 (3 hr.)
	78 (16 hr.)	43 (23 hr.)
<i>Trichoderma viride</i>	75 (4 hr.)	39 (3 hr.)
	82 (24 hr.)	43 (23 hr.)
<i>Sporocytophaga myxococcoides</i>	72, 72, 62 (23 hr.)	32, 33 (23 hr.)
	—	38 (3 hr.)
<i>Aspergillus fumigatus</i>	—	37 (23 hr.)

was 40% of the total reducing value calculated as glucose. Though the absolute value of the ratio is of no significance, the fact that it appears to remain constant may be important in the final analysis of the mechanism of hydrolysis. Similar results have been obtained using cellulose dextrin prepared by treatment of linter cellulose with 72% sulfuric acid. The cellulose dextrin hydrolysate has a relatively larger per cent of cellobiose and glucose. In fact, 75-80% of the total reducing value is due to these two sugars. This is about twice the amount obtained from cellulose sulfate.

Since the DNS method is not specific for end group determination, an analysis of a cellulose sulfate hydrolysate by the iodine oxidation method of Caldwell, Doebbeling, and Manian (1936) was carried out, as in Fig. 1A. The initial reducing group concentration was 1.35 mM, and the increase in 22 hr. was 2.40 mM. Of this increase, 0.55 mM was found as glucose, and 1.0 mM as cellobiose.

Analysis of the hydrolysates for products other than glucose and cellobiose was made by the method of paper-partition chromatography. Several celluloses and substituted celluloses were hydrolyzed by the filtrates of two different fungi for 3 days at 50° (Fig. 4). This incubation

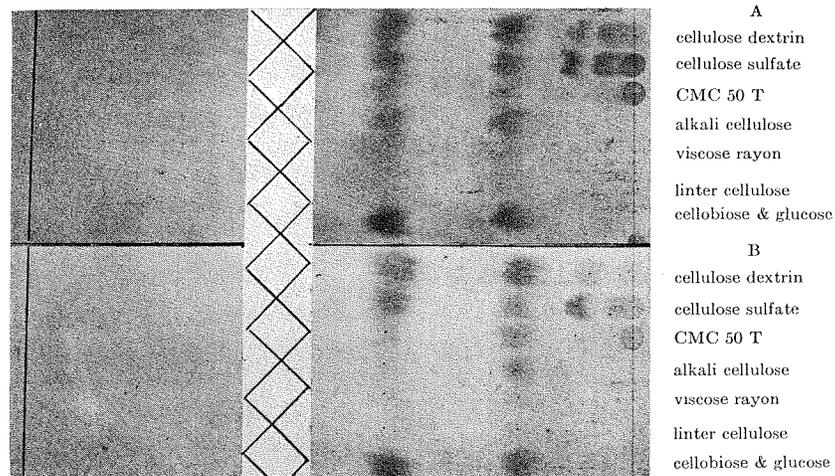


FIG. 4. Products of the hydrolysis of cellulose and some of its derivatives by filtrates of
 A. *Aspergillus fumigatus*,
 B. *Trichoderma viride*.

time was considerably longer than required for hydrolysis of the soluble cellulose derivatives but was employed so that measurable amounts of reducing compounds could be produced from the insoluble substrata. All substrata tested yielded both glucose and cellobiose. The amount of each produced was to some extent a function of the solubility or degree of dispersion of that substance. Least was obtained from linter cellulose, more from viscose rayon, and still more from alkali cellulose. From these three substrata no other reducing substance was found. The hydrolysates of the substituted celluloses, CMC 50T and cellulose sulfate, contained at least three reducing substances in addition to glucose and cellobiose. One of these did not move from the original spot during irrigation. Cellulose dextrin behaves more like the substituted celluloses than it does the unmodified cellulose in having additional reducing substances in the final hydrolysate.

Since modified cellulose has been used as the substratum in the series of experiments employed, the question arises as to what action the filtrates may exert on the linkage between cellulose and the side group. It is known (Eyler, Klug, Diephuis, 1947) that strong mineral acid splits off glycolic acid from CMC. Strong acid similarly splits off the sulfate from cellulose sodium sulfate. Do the hydrolytic enzymes found in our filtrates do likewise? Dialyzed cellulose sulfate was hydrolyzed with a filtrate of *T. viride* for a time far in excess of that required to reach the maximum reducing value. No precipitate formed on the addition of BaCl_2 . From the results of several experiments, it was estimated that enzyme hydrolysis split off less than 5% of the sulfate (in 3 days at 50°). Indeed, we are inclined to believe that no sulfate is split off by our enzyme filtrates.

While the ester type linkage of cellulose sulfate is not hydrolyzed by the filtrates, there remains the possibility that an ether type linkage as found in CMC might be attacked. CMC 70M (D.S. 0.89) was hydrolyzed with *T. viride* filtrate. The hydrolysate was acidified and extracted with ether in a continuous extractor for 18 hr. A trace of glycolic acid was obtained from the unhydrolyzed CMC, and there was no increase in glycolic acid due to enzyme hydrolysis. The evidence appears to support the contention that the cellulolytic filtrates here employed do not split off the side groups attached to the cellulose chains. Thus, the action of the enzyme on the substituted cellulose derivatives tested is distinct from that of strong mineral acids. While acids can hydrolyze

both the side-group linkages and the β -1,4-glucosidic linkages, enzymatic action is confined to the latter.

Since the β -glucosidase activity of the cellulolytic filtrates is very low, and cellobiose actually accumulates in the medium on enzyme hydrolysis of cellulose derivatives, it appears that cellobiose may be the sugar that enters the fungus cell. A few experiments were set up to observe the comparative rates of disappearance of cellobiose and of glucose from the medium by cultures of organisms actively growing on cellulose (cotton duck). The sugars were added after good growth had taken place. The cell-free filtrates were simultaneously tested for their ability to hydrolyze cellobiose, under the same conditions as in the growing culture.

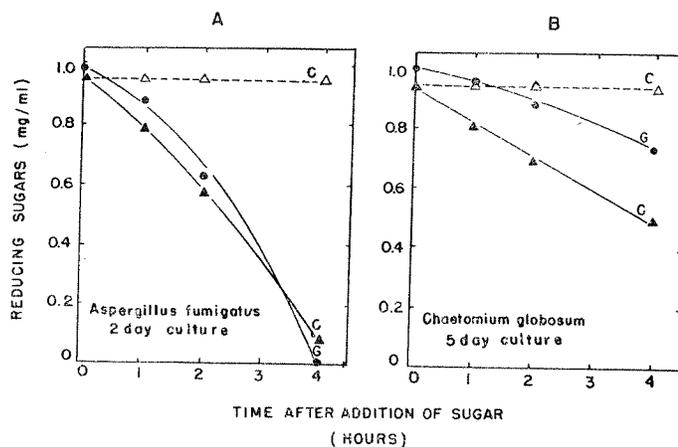


Fig. 5. Absorption of sugars by fungi in shake flasks. C = cellobiose, G = glucose, solid line = growing culture, broken line = filtrate.

In general, the rates of disappearance of cellobiose and of glucose from the medium were similar. The cellobiase activity of the filtrates was in most cases much too low to explain the disappearance of cellobiose by assuming that it was first hydrolyzed to glucose and then absorbed by the organism. Details of the results are shown for two of the organisms (Fig. 5). The *Aspergillus fumigatus* culture (2 days old) consumed glucose and cellobiose at nearly the same rate. The filtrate, without mycelium, was able to hydrolyze only 3% of the cellobiose under the same conditions. Obviously cellobiose was not hydrolyzed

prior to coming in contact with the growing mycelium. A test of the sugar remaining at the end of the 4 hr., showed only cellobiose in the cellobiose-pellets flasks. The *Chaetomium globosum* culture appears to consume cellobiose slightly more rapidly than it does glucose, and the filtrate alone was unable to hydrolyze cellobiose under the same conditions. In these respects, the results are similar to those for *A. fumigatus*. The cellobiose-pellets cultures, however, show an appreciable amount of glucose at the end of 4 hr. Since this could not have been due to action of β -glucosidase in the outer solution, it is interesting to speculate on its origin. If β -glucosidase is located at the surface of the cell, then the product of hydrolysis, i.e. glucose, might diffuse out into the medium as well as into the cell. This appears to be the likely explanation. An alternate hypothesis is that glucose is liberated from the old cells undergoing autolysis. *Myrothecium verrucaria* and *Actinomyces sp.* cultures also show nearly equal consumption rates for glucose and cellobiose. The filtrates alone hydrolyze some cellobiose, but insufficient to account for the amount of cellobiose consumed. Glucose appears in the cellobiose-pellets flasks at the end of 4 hr., as in *C. globosum*. Finally, with *Sporocytophaga myxococcoides*, the hydrolysis of cellobiose in the cell-free filtrate was of a magnitude sufficient to explain consumption of the cellobiose by the cells on the basis of hydrolysis to glucose, with subsequent absorption of glucose.

DISCUSSION

In a recent report, we concluded that the enzyme *Cx*, capable of hydrolyzing a straight chain of anhydroglucose units in β -1,4-glucosidic linkage, acts on the chain to produce glucose directly without the formation of the classical cellobiose intermediate. The data on which we based this conclusion are:

- (a) Glucose was detected as the osazone in hydrolysates of cellulose by enzyme filtrates. No specific determination of cellobiose was made.
- (b) No β -glucosidase was detectable in the cellulolytic filtrates used.

We reasoned that the absence of the enzyme β -glucosidase and the presence of glucose favored the hypothesis made. The above facts are not altered by our present data, but their interpretation must fit the additional information. The hydrolysis of cellulose is slow, requiring days to obtain amounts of end product suitable for detection. Under these conditions, amounts of β -glucosidase too slight to be detectable

in a 1- or 2-hr. assay may still be sufficient to account for the hydrolysis of the cellobiose to glucose in the long incubation period used for cellulose hydrolysis. In the present work, employing new techniques and short hydrolysis periods, we have detected cellobiose in the hydrolysates, appearing earlier than glucose. This cellobiose is rapidly converted to glucose when additional β -glucosidase is added to the reaction mixture. Filtrates of most cellulolytic microorganisms are deficient in β -glucosidase.

The definite occurrence of cellobiose in hydrolysates and the high rate of disappearance of cellobiose from the culture medium show that cellobiose can be metabolized by fungus cells. While we have not proved that the cell membrane is permeable to cellobiose, we have shown that in some cases the rate of disappearance greatly exceeds the rate of hydrolysis in the outer medium. This need not be explained on a permeability basis, but, as such, these data are in accordance with the results of Höfler (1926) who has reported that the rate of penetration of disaccharides may equal that of monosaccharides. In some cases, it has been shown that a cellulolytic organism can use cellobiose, but cannot use glucose (McBee, 1948). These observations support the direct utilization of cellobiose. Possibly the inability to utilize glucose hinges upon the production by these organisms of the enzyme, glucose oxidase, which would lead to self-destruction by the gluconic acid or H_2O_2 produced. In the cases examined by us, glucose is readily absorbed. Variation in the permeability of the protoplasmic membranes of different organisms to cellobiose and to glucose probably does exist. The point we emphasize is that cellulolytic activity does not necessarily hinge on the ability of the organism to absorb glucose.

Hydrolysis of both cellulose and its derivatives led, in all cases, to the formation of cellobiose and of glucose. From cellulose and alkali cellulose, no other intermediates are detectable on our chromatograms. The reaction thus appears to pass rapidly over the intermediate stages, probably because the soluble intermediates are much more available to the enzymes than are the molecules of the insoluble substratum. From the modified celluloses, other end products are detectable. These are reducing substances moving more slowly on the chromatogram than cellobiose. They might be either cellotriose, cellotetraose, etc., or substituted glucose, substituted cellobiose, etc. We are inclined toward the latter view. If cellotriose accumulates from cellulose derivatives, it might also be expected in the hydrolysates of alkali cellulose

Glucose 100% alkali cellulose 100% 1954

cellotriose, cellotetraose, etc.
substituted glucose, substituted cellobiose, etc.

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and of linter cellulose which it is not. Furthermore, cellotriose should be hydrolyzed by β -glucosidase (Grassmann *et al.*, 1933) and addition of this enzyme to the hydrolysates of substituted cellulose derivatives should result in conversion of this compound to glucose with elimination of the spot from the chromatogram. This does not happen—addition of β -glucosidase does not eliminate the unidentified spots.

Our data show that neither *Cx* nor any other enzyme in cellulolytic filtrates can split off the side chains, either of the ether or ester type, which are present in the substituted derivatives. Should such a splitting off of the substituents occur, one could expect (a) precipitation of the longer insoluble cellulose dextrin-type chain as a result of the loss of the solubilizing group, and (b) the complete hydrolysis of the resulting chains to cellobiose and glucose. Actually neither of these expectations is realized. In addition, if the enzymes were capable of splitting off the substituents it would be expected that cellulolytic organisms and filtrates from their culture media would be able to do so and thus be in a position to utilize the highly substituted compounds (i.e. of D.S. = 1.0–3.0). Previous data (Reese *et al.*, 1950) have shown that this is not the case—the highly substituted soluble cellulose derivatives being almost completely immune to enzymatic hydrolysis.

SUMMARY

Cellobiose and glucose were determined in a mixture of the two carbohydrates by methods involving the use of glucose oxidase and of β -glucosidase.

Paper-partition chromatography is used as a confirmatory method in the identification of the hydrolysis products and in the detection of the various constituents.

The cellulolytic organisms studied produce large amounts of the enzyme *Cx*, which diffuses into the medium. Only small amounts of β -glucosidase are found outside the cell. Cellobiose resulting from *Cx* activity can enter the cells as rapidly as can glucose.

The role of cellobiose as a principal product in the hydrolysis of cellulose is confirmed. It is hypothesized that the principal final product of *Cx* activity is cellobiose, and that the presence of cellobiase in the medium is not a prerequisite to utilization of cellobiose by the organism. This is a correction of the hypothesis previously published stating that glucose appeared to be the final product of *Cx* activity.

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