ABSORPTION AND UTILIZATION OF OLIGOSACCHARIDES
BY *CELLVIBRIO GILVUS*

by

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INTRODUCTION

The microbial degradation of cellulose to cellobiose and glucose and the subsequent metabolism of these sugars is a complex process. In an attempt to understand the process, studies on the enzymology and physiology of a single cellulolytic organism were initiated some years ago (8, 9). *Cellvibrio gilvus* is of physiological interest because it elaborates an enzyme system which seems to degrade cellulose to cellobiose, and it prefers cellobiose to glucose as an energy source for growth both of these characteristics being typical of bacterial attack on cellulose as contrasted to the more thoroughly studied fungal attack.

To date extracellular enzymes of three substrate specificities have been identified in culture filtrates of *C. gilvus* hydrolyzing cellulose to cellobiose, an enzyme system which attacks crystalline cellulose which was identified by Flora (7), an enzyme which hydrolyzes aryl-β-glucosides which was observed incidentally in the course of the present research, and a group of exo-β-(1,4)-glucan hydrolases capable of liberating

reducing sugar groups from carboxymethylcellulose or cellulodextrins which was isolated by Storvick and King (14) and described in further detail by Cole and King (3) and Storvick, Cole, and King (15). This latter enzyme system was resolved into four electrophoretically distinguishable components which have been partially purified (14). The mode of action of each of these components was determined by analysis of intermediates and final products of hydrolysis of cellotriose, cellotetraose, cellopentaose, reduced cellopentaose, and reduced cellohexaose (3, 14). Each of these components cleaves cellobiosyl and cellotriosyl units from the non-reducing end of the substrate and fails to hydrolyze these products further at an appreciable rate. The frequency of attack at bonds yielding cellobiose and cellotriose is characteristic for each component and depends upon the substrate. Component IV also catalyzed the hydrolysis of cellobiose but only to a limited extent. The hydrolysis of cellotriose, when incubated in pure form with the four components, proceeded at a rate which is 5-10% of the rate of hydrolysis of the higher members of the series. For reasons not yet

understood, the trisaccharide has never been identified in either culture media or in hydrolysates of cellulosic substrates by crude enzyme preparations.

The metabolism of cellobiose and glucose was investigated in an attempt to explain the physiological basis for the disaccharide preference (8, 9). Differences in the nature of the respiratory end products, the rate of assimilation, the pH optima for growth on the two sugars, and the observation of non-additive respiratory rates of cultures growing on the two sugars indicated that cellobiose and glucose might not be metabolically equivalent. An intracellular cellobiose phosphorylase was demonstrated which catalyzes the cleavage of cellobiose to glucose and α-D-glucose-1-phosphate. Experiments using cell free systems suggested that these two products are not equivalently oxidized. The data indicated that glucose may follow two pathways, one leading to gluconate which is not further metabolized and one involving a conversion to glucose-6-phosphate and passing through fructose-1,6-diphosphate to pyruvate. These data also indicated that the glucose-1-phosphate is converted

to pyruvate via fructose-1,6-diphosphate by a pathway which has not yet been identified. The end products of metabolism have been identified as CO_2, volatile organic acids, volatile neutral products, non-volatile organic acids, and non-volatile neutral products. By measuring the relative contributions of the reducing and non-reducing glucose groups of cellobiose to respiratory CO_2 Swisher, Storvick and King (16) have presented additional evidence in support of the hypothesis that the two glucosyl moieties of cellobiose are not metabolized equivalently. Using cellobiose uniformly labeled in the reducing glucosyl moiety 74% of the respiratory CO_2 was found to arise from the reducing glucose portion of the disaccharide.

There are many voids in the current knowledge of the degradation and utilization of cellulose by C. gilvus. The nature of the enzyme which initially attacks cellulose, the importance of the aryl-β glucosidase, and the properties of the cellobiose phosphorylase are still not understood. The question, can oligosaccharides larger than cellobiose be absorbed by the cell and utilized as energy sources,

developed from the observation that cellotriose failed to accumulate in the culture medium and the report that intracellular enzymes of a mold (18) and rumen bacteria (11) could hydrolyze oligosaccharides larger than cellobiose.

In the present investigation studies measuring the disappearance of oligosaccharide from resting cell suspensions and growth of cultures on oligosaccharides were designed to test the hypothesis that _C. gilvus_ is capable of actively removing oligosaccharides larger than cellobiose from the medium and to elucidate in general terms the mechanism of the process.

MATERIALS AND METHODS

The bacterial culture and media used were those described by Hulcher and King (8) except that the vitamins were omitted and cellobiose was included at 0.2%. The culture was stored at 4 C on slants consisting of the same medium plus 1.5% agar. These were transferred every 2-4 weeks. Routinely and prior to each experiment the culture was tested for purity by examining gram-stained smears.

In preparing resting cell suspensions for kinetic studies, 24-hr cultures were washed once by 5-min centrifugation at 25 C and 13,300 x G in 0.067 M potassium phosphate buffer at pH 7.0. The washed culture was resuspended in the same buffer and stirred for 10 min with a magnetic stirrer to break up clumps. The suspension was diluted with the phosphate buffer to give a final Klett Summerson turbidity value of 250 corresponding to 2.6×10^9 cells per ml, and aliquots of the diluted suspension were placed in 250-ml Erlenmeyer flasks fitted with cotton stoppers. After a 30-min starvation period at 25 C, cellodextrins were added at a final

concentration of 0.5 μM per ml in a total volume of 10 ml. The flasks were then incubated at 25 C on a mechanical shaker. Immediately after addition of oligosaccharide and at intervals thereafter samples were removed from the flasks and filtered through a 0.65-μ Millipore filter. The filtrates were analyzed for sugar by the phenol-sulfuric acid procedure described by Dubois et al. (4) and modified by Timell (17), and for degree of polymerization (D.P.) of the sugars by the borohydride reduction procedure described by Timell (17). Absorbancy readings for both analyses were obtained using the Spectronic 20 Spectrophotometer at 480 mμ.

Growth experiments were carried out in the basal medium described by Hulcher and King (8) with the following modifications: casein hydrolysate was added as 0.05% of the medium (w/v), and no yeast extract was included. Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose were added at a final concentration of 0.03 mM per ml on a hexose basis. The basal medium was sterilized by autoclaving for 15 min at 121 C. The vitamin supplement and cellodextrins were

sterilized by filtration through a 0.45- μ Millipore filter and added aseptically to the basal medium. Tubes containing 5 ml of the broth were inoculated with 0.1 ml of a 1:50 dilution of a 24-hr culture and incubated at 25 C on a mechanical shaker.

Turbidity was always measured with a Klett Summerson colorimeter using the green filter. To correlate cell concentration with turbidity measurements a 24-hr culture was diluted 1:100 with a 0.1% crystal violet solution. The stained cells were counted in a Levy-Hauser heamocytometer. Dilutions of the same culture were made with potassium phosphate buffer, and the turbidity was measured. A standard curve was plotted and used throughout the study.

The chemicals used were reagent grade where possible. The cellodextrins were prepared and purified by the method of Miller (12) as modified by Storvick, Cole, and King (15). The glucose used was obtained from the National Bureau of Standards.

RESULTS

Absorption of oligosaccharides

Figure 1 illustrates the disappearance of cellodextrins from the medium of a resting cell suspension. These data show that the rate of disappearance of each oligosaccharide was linear with respect to time and decreased as the number of anhydroglucose units per molecule increased. To determine the effect of the filtrate on the oligosaccharides, a control experiment was carried out in which the cells were omitted. Filtrates taken from resting cell suspensions after the 30 min starvation period were incubated with the oligosaccharides in 250 ml Erlenmeyer flasks for 1 hr on a mechanical shaker and then boiled 5 min to terminate any enzymatic action. Analyses of these filtrates indicated that no change in either the concentration of cellodextrin or in the D.P. had occurred during the incubation (Table 1). From these data it was concluded that the disappearance of sugar depended upon the presence of the cells and that no β-(1,4)-glucan hydrolase was present in the medium prior to the

Fig. 1 - Disappearance of oligosaccharide from the supernatant of resting cell suspensions. G_1, G_2, G_3, G_4, G_5, and G_6 represent glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose, respectively.

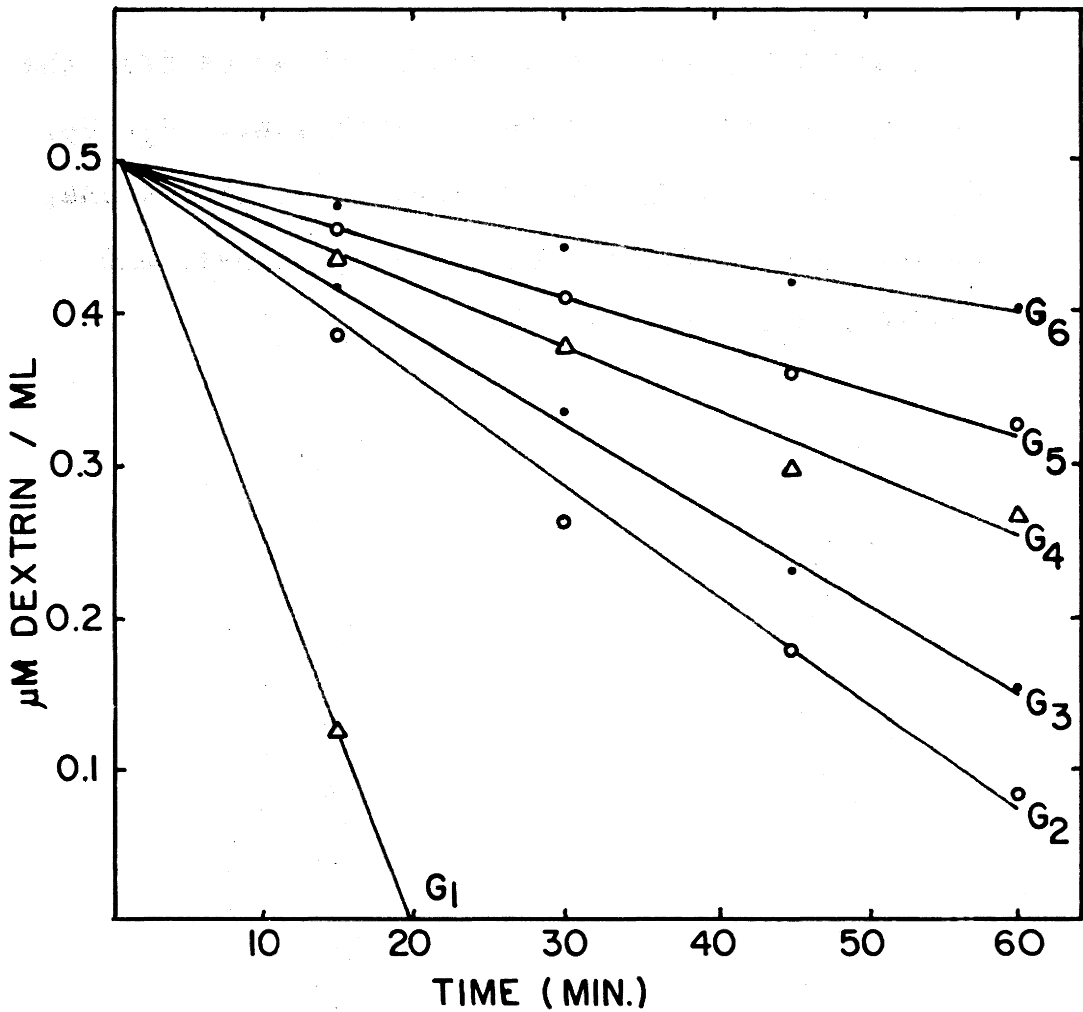

Table 1

Effect of resting cell suspensions and cell filtrates on the degree of polymerization of oligosaccharide during 1 hr incubation

Theoretical D.P.	Experimental D.P.			
	With Cells		Without Cells	
	0	1 hr	0	1 hr
2	2.11	2.00	1.87	2.00
3	3.22	3.08	3.09	2.92
4	3.94	4.23	3.78	4.22
5	5.70	4.85	5.35	5.55
6	5.49	5.35	--	--

addition of oligosaccharide to the resting cell suspensions.

The data from the experiment summarized in Figure 1 permit calculation of the number of glucosyl moieties metabolized per cell per unit time as shown in Table 2. On a hexose basis approximately equivalent amounts of oligosaccharide were absorbed per cell per min suggesting that the rate of removal of oligosaccharides was controlled by a glucose requirement of the cell.

<u>Growth response to oligosaccharides</u>

The results of growth studies compiled in Table 3 show that there was a significant increase in growth on the oligosaccharides when compared with a non-carbohydrate control. During logarithmic growth, the cultures grew at approximately the same rate on all the carbohydrates; however, a marked variation was observed in final cell densities.

Maximum cell yield occurred at 30 hours on the hexasaccharide. On glucose the maximum cell yield over that obtained on the basal medium was 68% as great, and on cellobiose, cellotriose, cellotetraose, and

Table 2

Absorption rates of oligosaccharides

D.P. Glucose Units/ Molecule	Molecules Absorbed/ Cell/Min (millions)	Molecules of Glucose Obtained/ Cell/Min (millions)
1	39	39
2	20	41
3	14	43
4	11	42
5	9	45
6	6	37

Table 3

Growth response on glucose and oligosaccharide as compared with a non-carbohydrate control

Incubation Time (hrs)	Turbidity*						
	Control	Glucose	Cello-biose	Cello-triose	Cello-tetraose	Cello-pentaose	Cello-hexaose
0	1	1	1	1	1	1	1
3	4	4	4	4	6	5	7
6	9	16	14	15	13	5	13
9	12	59	47	45	45	41	41
12	30	163	151	152	153	155	155
15	70	263	251	251	241	243	252
18	80	318	328	324	318	333	366
21	83	355	388	385	383	390	465
24	83	377	427	426	431	433	518
30	79	384	458	460	463	493	523

* Expressed in Klett-Summerson units.

cellopentaose the corresponding values were 85%, 86%, 87%, and 93%, respectively.

DISCUSSION

These data indicate that oligosaccharides larger than cellobiose are actively removed from a culture medium at a rate which appears to be controlled by the respiration of the cell. Furthermore an intracellular phosphorolytic cleavage of the cellulodextrins is suggested.

The linearity of oligosaccharide uptake (Figure 1) indicates that the removal is by an active absorption mechanism. If simple diffusion accounted for the disappearance, an exponential curve would be expected. Secondly, the results from the control experiment show that the cells are necessary for removal of oligosaccharide and that there is no hydrolase in the medium when the cells are presented with oligosaccharide. If extracellular hydrolysis were to take place prior to uptake, a lag would be predicted.

That the oligosaccharides were removed intact is suggested by the constancy of the D.P. of the oligosaccharides seen in Table 2. Smaller fragments present in the medium resulting from extracellular hydrolysis would cause a decrease in the apparent D.P.

The conclusion that the rate of uptake is controlled by the rate of respiration of the cell is dictated by the results in Table 1. These data show that independent of the D.P. of the carbohydrate, the same number of glucose molecules was removed from the medium per cell per unit time. This behavior is strongly reminiscent of a system using Streptococcus faecalis with which Abrams (1) obtained data indicating that the absorption of oligosaccharide was dependent upon the rate of glycolysis.

It is evident from Table 3 that C. gilvus is capable of utilizing the oligosaccharides as a source of energy. Moreover, although supplemented with an equivalent amount of carbohydrate on a hexose basis, the cells appear to metabolize larger dextrins with greater efficiency suggesting that the degradation of oligosaccharide once absorbed involves a phosphorolytic breakdown of the molecules. In a phosphorolytic reaction inorganic phosphorus becomes esterified without the expenditure of energy. It has been demonstrated that phosphorylases in both plant and animal systems initiate the formation of glucose-1-phosphate from polysaccharides and inorganic phosphate. Among bacteria the breakdown

of disaccharides by phosphorolytic reactions has repeatedly been demonstrated (5, 6, 10), and the preference for cellobiose over glucose exhibited by some cellulolytic bacteria has been attributed to this reaction (2, 9, 13).

Extensive studies of the respiratory machinery of C. gilvus have given evidence that the initial step in cellobiose metabolism involves the esterification of the non-reducing glucosyl moiety with inorganic phosphate through the action of a cellobiose phosphorylase (9). The initial steps in the oxidation of glucose appear to involve ATP dependent conversions to gluconate and glucose-6-phosphate. It would seem that the formation of sugar phosphates from a molecule of cellobiose would entail the expenditure of only one-half the amount of ATP as the phosphorylation of an equivalent of glucose. Assuming that the oligosaccharides are absorbed by the cells intact and that these cells elaborate a phosphorylase capable of handling oligosaccharides larger than cellobiose, it can be postulated that the greater the number of anhydroglucosyl residues per molecule of carbohydrate, the greater the number of

sugar phosphates formed without the expenditure of ATP. The ATP conserved would then be free to participate in other biochemical processes requiring energy for chemical work and may in part account for the variation in final cell densities observed on the oligosaccharides.

It should be emphasized, however, that there is no evidence in this report from which it can be concluded that the oligosaccharides actually enter the cell. It is possible that once absorbed, the oligosaccharides are hydrolyzed at the site of absorption.

SUMMARY

To test the hypothesis that oligosaccharides larger than cellobiose could be absorbed and utilized by a cellulolytic bacterium, Cellvibrio gilvus, the disappearance of oligosaccharides from a resting cell suspension was measured and the growth response to the oligosaccharides was observed.

Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose were incubated with resting cell suspensions. At various times samples were removed and filtered. The filtrates were analyzed for sugar concentration and D.P. The oligosaccharides appeared to be removed from the medium at a linear rate with respect to time indicating an active absorption system. The data also gave evidence that the rate of uptake was controlled by the respiration rate of the cell since the number of glucosyl units removed per cell per minute was a constant value independent of the D.P. of the oligosaccharide.

That the D.P. of the oligosaccharides remained constant after an incubation of one hour with the

cells was taken as an indication that the oligosaccharides were absorbed intact. Results of a control experiment in which oligosaccharide was incubated with cell filtrates confirmed these data.

To determine the efficiency of utilization of oligosaccharide the turbidity of cultures growing on the sugars was measured. After a 30 hr incubation period maximum cell density was observed on the hexasaccharide. In comparison maximum cell yield above the non-carbohydrate control on glucose and the di-, tri-, tetra-, and pentasaccharides were 68, 85, 86, 87, and 93%, respectively. This suggested that the degradation and phosphate esterification of the larger dextrins might be in part the result of a phosphorolytic cleavage of the absorbed oligosaccharides.

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ABSTRACT

The ability of a cellulolytic bacterium *Cellvibrio gilvus*, to absorb and utilize members of the cellulose oligosaccharide series was investigated.

Resting cell suspensions prepared from 24-hour cultures were incubated with the cellulodextrins. At various times samples were removed from the incubation flasks and filtered. The filtrates were analyzed for sugar concentration by a modified phenol-sulfuric acid procedure and degree of polymerization (D.P.) by a borohydride reduction phenol-surfuric acid method. The rate of disappearance of the oligosaccharides from the supernatant of the resting cell suspensions was linear with respect to time indicating an active absorption mechanism. The conclusion that the rate of absorption was controlled by the respiration of the cell was based on the observation that independent of the D.P. of the cellulodextrin, the number of glucose molecules removed per cell per minute was approximately the same. The D.P. of the substrates remained constant over the experimental period with the cells suggesting that the molecules were removed intact. No effect on

sugar concentration or D.P. was observed when the oligosaccharides were incubated with the filtrates which confirm these data. It was not possible from these results to determine if the oligosaccharides entered the cell or were metabolized at the cell wall.