METABOLIC BASIS FOR DISACCHARIDE PREFERENCE IN A CELLVIBRIO[1]

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A previous publication described a new species of *Cellvibrio* for which cellobiose seems to be superior to glucose as an energy source (Hulcher and King, 1958). The present report describes experiments designed to elucidate the basis for this anomalous carbohydrate preference.

MATERIALS AND METHODS

Reagent grade chemicals were used where available. Adenosine triphosphate and coenzyme A were obtained from the Pabst Laboratories; riboflavin-5′-phosphate was obtained from the California Foundation for Biochemical Research; sugar phosphates were obtained from Nutritional Biochemicals Corporation. "Levigated Alumina," A-542, from Fisher Scientific Company, was used in preparing ground-cell suspensions and cell-free extracts. All chromatographic solvents were freshly distilled.

The analytical procedures used were those described by Somogyi (1945) and Nelson (1944) for reducing sugars, Pan *et al.* (1953) modified for differential analysis of mixtures of cellobiose and glucose, Johnson (1941) for total nitrogen, Fiske and SubbaRow (1925) for total and inorganic phosphorus, and Lowry *et al.* (1951) for protein.

Separation of sugar phosphates by paper chromatography was conducted using a modification of the procedures described by Mortimer (1952). A solvent consisting of methyl ethyl ketone, methyl cellosolve, ammonia, and water in the volume ratio of 7:2:3:1 was found to yield higher R_f values and greater resolution than Mortimer's solvent. The R_p values in the modified solvent were as follows: inorganic phosphate, 100; glucose-1-phosphate, 173; glucose-6-phosphate, 154; fructose-1,6-diphosphate, 4.6; and fructose-6-phosphate, 242. The R_p value is 100 times the ratio of the distance which a compound moves to the distance moved by inorganic phosphate, the measurements being made to the leading edge of all spots.

Sugar phosphates were detected on paper chromatograms by using the procedure of Hanes and Isherwood (1949). Glucose-6-phosphate was distinguished from glucose-1-phosphate by spraying the papers first with aniline hydrogen phthalate (Partridge, 1949) and then with the sugar phosphate spray. Sugar phosphates were recovered from experimental systems as their barium salts (Umbreit *et al.*, 1951) and then converted to their ammonium salts according to the method of Wade and Morgan (1955) prior to separation by paper chromatography. In isolating enzymatically synthesized glucose-1-phosphate from cell-free enzyme systems, the ion-exchange procedure of McCready and Hassid (1944) was used.

Active cell suspensions were obtained from 3- or 4-L broth cultures grown for 18 hr under forced aeration at room temperature. The medium was that used previously (Hulcher and King, 1958) supplemented with 0.025 g of enzymatic casein hydrolyzate per L and with 1 ppm Antifoam-A (Dow Chemical Company). Cell yields were 2 to 4 g of packed, wet cells per L. All cultures were tested for contamination by examining Gram stains and nigrosine stains before harvesting the cells by high speed centrifugation in the cold.

Cell-free extracts were obtained by washing the packed cells twice in tris(hydroxymethyl)-aminomethane (Tris) buffer (0.01 M or 0.03 M, pH 7.0) in the cold. The washed cells were then ground with twice their weight of alumina in a chilled mortar for 10 to 20 min. "Soluble enzyme" preparations consisted of the clear supernatants obtained by centrifugation at 20,000 × G for 30 min at 0 to 4 C. Such extracts contained several of the enzymes participating in the initial reactions of carbohydrate metabolism, but con-

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tained only a fragmentary electron transport system. In experiments involving measurement of oxygen uptake, washed cells were ground by the same procedure, but the centrifugation was carried out at 3500 rpm for 10 min, thereby removing the alumina and intact cells. The resulting supernatants, containing soluble enzymes and particulate debris from crushed cells, consumed oxygen actively. Differential staining of these suspensions using both nigrosine and methylene blue indicated that the preparation contained almost no intact cells.

RESULTS

Metabolism of intact cells. The results of a comparison of the ability of intact cells grown on cellobiose to oxidize glucose and cellobiose separately and in mixture are shown in figure 1. The total carbohydrate concentration on a hexose basis was the same in all flasks. The rates of oxidation of both sugars were essentially linear indicating that adaptive processes were not involved in either the absorption or the oxidation of glucose. In repeated experiments of this type the rate of oxidation of cellobiose was consistently about 10 per cent higher than the rate of oxidation of glucose. In addition oxidation of the mixed sugars proceeded distinctly more rapidly than that of either alone, suggesting that independent pathways of metabolism might be involved prior to the participation of the terminal oxidase. Control experiments established that neither sugar had a catalytic effect on the oxidation of the other.

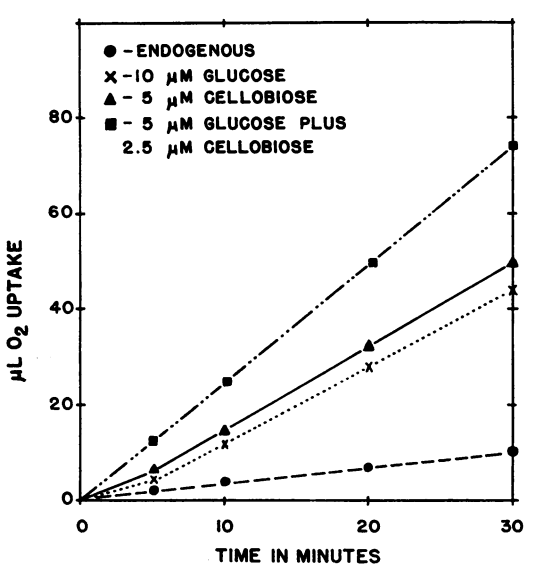

Figure 1. Oxidation of glucose and cellobiose by intact cells of *Cellvibrio gilvus*. Cellobiose-grown cells washed twice in 0.067 M potassium phosphate buffer at pH 7.0 in the cold. Flasks contained 1.0 ml of cell suspension, 0.8 ml of 0.067 M potassium phosphate (pH 7.0), 0.2 ml of 10 per cent KOH in the center well, and 1.0 ml substrate.

TABLE 1

Effect of glucose and cellobiose on the esterification of inorganic phosphate by resting cells

Substrate	Increase in Organic Phosphate
	μg P/100 μg cell N
None	0.23
0.005 M cellobiose	1.63
0.01 M glucose	9.67
0.005 M glucose + 0.0025 M cellobiose.	9.56

Reaction mixtures consisted of 15.0 ml cell suspension in 0.005 M potassium phthalate buffer at pH 6.5 and 5.0 ml of substrate. Flasks were shaken on a rotary shaker at 25 C for 30 min. At zero time and after 30 min, samples were analyzed for total and inorganic phosphate and total nitrogen. Phosphate esterified was then calculated by difference.

The effect of glucose and cellobiose on phosphate esterification by suspensions of resting cells is shown in table 1. These suspensions consisted of cellobiose-grown cells, washed four times in potassium phthalate (0.005 M, pH 6.5) and then in 0.85 per cent NaCl until the washings contained no phosphate. It can be seen that both sugars stimulated esterification of phosphate although the response to glucose was greater than that to cellobiose. The effect of the two sugars in the presence and absence of exogenous phosphate on the respiration of intact cells washed free of extractable phosphate is illustrated in table 2. Here again the synergistic effect of the two sugars on respiration was seen. The response of cells respiring the two sugars to exogenous inorganic phosphate was conspicuously different.

Analysis of the intracellular sugar phosphate composition of cells grown in glucose or cellobiose broth showed 1.05 and 3.25 mg phosphate per g of wet cells, respectively. The only phosphate compounds detectable in either type of cells were traces of inorganic phosphate and large amounts of fructose-6-phosphate (R_p of authentic

TABLE 2

Effect of exogenous inorganic phosphate on oxidation of glucose and cellobiose by washed intact cells

Substrate	Oxygen Consumed
	μL/hr/flask
None	51
Cellobiose	64
Glucose	92
Cellobiose + inorganic phosphate	94
Glucose + inorganic phosphate	93
Glucose + cellobiose + inorganic phosphate	109

Flask contents: 1.8 ml cell suspension in 0.067 M potassium phthalate at pH 6.5, 1.0 ml substrate (0.01 M glucose or 0.005 M cellobiose) in the same buffer, 1.0 ml of 0.01 M potassium phosphate at pH 6.5, plus water to a total volume of 4 ml. Center wells contained 0.2 ml of 10 per cent KOH. The temperature was 30 C.

fructose-6-phosphate, 241; R_p of compound extracted, 239).

Metabolism of soluble enzyme extracts. The data obtained using intact cells indicated that the basis for the difference in growth response to the two sugars lay somewhere in intracellular metabolism rather than in permeability differences or adaptive utilization of glucose. Cell-free preparations were found to exhibit preferential utilization of cellobiose and distinctive responses to added phosphate resembling those manifested by intact cells as shown in table 3.

From these data it can be seen that cellobiose utilization was distinctly more rapid than glucose utilization regardless of phosphate supplementation; that adenosine triphosphate (but not inorganic phosphate) stimulated glucose utilization; that inorganic phosphate (but not adenosine triphosphate) stimulated cellobiose utilization, and that cellobiose utilization (but not that of glucose) was accompanied by rapid esterification of inorganic phosphate. These characteristics resemble strongly the behavior of intact cells described earlier.

Carbohydrate conversions in soluble enzyme preparations. Similar cell-free enzyme preparations under identical conditions converted cellobiose and inorganic phosphate to fructose-6-phosphate and glucose, as detected by paper chromatography of the reaction products. On addition of adenosine triphosphate and triphosphopyridine nucleotide, however, the glucose failed to accumulate, being further metabolized with formation of a compound with the chromatographic characteristics of gluconate, together with traces of fructose-1,6-diphosphate.

Using paper chromatographic procedures, the soluble enzyme preparations were shown to catalyze also each of the step reactions of glycolysis between glucose and fructose-1,6-diphosphate. When cellobiose was the substrate, however, conversion of the glucose-1-phosphate (produced by phosphorolysis of the disaccharide) to glucose-6-phosphate could not be demonstrated although fructose-6-phosphate was detected as a product of either glucose-1-phosphate or cellobiose metabolism. This apparent absence of phosphoglucomutase has not been explored further.

Demonstration of a cellobiose phosphorylase. The inorganic phosphate-dependence of cellobiose metabolism and the observation that glucose depressed the conversion of cellobiose to sugar phosphates even in the presence of inorganic phosphate suggested that the major route of cellobiose dissimilation was through the action of

TABLE 3

Sugar utilization and phosphate esterification by cell-free preparations

Addenda				Utilization of Sugars	Phosphate Esterification
Glucose	Cellobiose	Inorganic phosphate	Adenosine triphosphate		
1×	*2×*			*μmoles/hr/flask*	*μg/flask*
0	0	0	0	0.00	0.0
+	0	0	0	1.00	0.0
+	0	+	0	0.33	—
+	0	0	+	2.84	10.0
+	0	+	+	2.84	—
0	+	0	0	4.20	80.0
0	+	+	0	7.25	160.0
0	+	0	+	3.10	—
0	+	+	+	3.86	—

The reaction mixtures contained 1 ml of soluble enzyme extract, 1.2 mg of carbohydrate, 3.3 μmoles of adenosine triphosphate and 3.3 μmoles of inorganic phosphate in a total volume of 2 ml. All reagents were adjusted to pH 7.0 prior to mixing. Incubation was at 30 C without shaking. Dashes indicate that analyses were not made.

a cellobiose phosphorylase (Sih *et al.*, 1957). The presence of the phosphorylase was established by characterization of the sugar phosphate produced from phosphorolysis of cellobiose and by demonstration of net synthesis of cellobiose from glucose and glucose-1-phosphate using soluble enzyme extracts.

To 21 ml of enzyme extract containing 1.6 mg protein per ml were added 14.6 mmoles of cellobiose, 7.3 mmoles of potassium phosphate at pH 7.0, and sodium fluoride and $MgSO_4$ at final concentrations of 6×10^{-3} M and 9×10^{-6} M, respectively. After incubation for 12 hr at 30 C the inorganic phosphate was removed as the magnesium ammonium phosphate complex at pH 10. The sugar phosphate was isolated by the ion-exchange procedure of McCready and Hassid (1944) and crystallized from a mixture of methanol and acetone at −16 C. The isolated crystals were not distinguishable from α-D-glucose-1-phosphate on paper chromatograms. Hydrolysis of the nonreducing phosphate in N HCl for 10 min yielded reducing sugar and *o*-phosphate in the ratio of 1.03. The sugar which was released behaved chromatographically like glucose. The specific optical rotation of the crystalline sugar phosphate was $[\alpha]_D^{23} = +67°$ (C = 1 per cent in water). An authentic sample of α-D-glucose-1-phosphate had an $[\alpha]_D^{23}$ of + 69°. Reithel (1945) has reported that β-D-glucose-1-phosphate had an $[\alpha]_D^{20}$ of +12°.

When the reverse reaction was carried out, using equivalent concentrations of glucose plus α-D-glucose-1-phosphate as substrates, a reducing sugar was isolated by paper strip chromatography which, like authentic cellobiose, showed a 2.2-fold increase in reducing power when hydrolyzed in N HCl for 45 min. The compound had the same R_f as authentic cellobiose (1 solvent only) and gave the color reaction typical of reducing 1,4-β-disaccharides when sprayed with the differential indicator of Buchan and Savage (1952); the color reaction was identical to that of authentic cellobiose and readily distinguishable from maltose. After acid hydrolysis, only glucose could be detected as a product.

When 20 μmoles of fructose-1,6-diphosphate, 5 μmoles of diphosphopyridine nucleotide, 5 μmoles of triphosphopyridine nucleotide, and 0.5 μmoles of methylene blue were incubated in 0.4 ml of 0.1 M potassium phosphate buffer of pH 7.0 with 0.5 ml of soluble enzyme extract for 45 min at 30 C, pyruvate was detected among the products, using the procedures of Friedman and Haugen (1943) and Cavallini and Frontali (1954). The paper chromatograms actually revealed two acidic 2,4-dinitrophenylhydrazones, one of which had the same R_f as the 2,4-dinitrophenylhydrazone of authentic pyruvate. After elution from paper chromatograms the absorption spectra of the isolated dinitrophenylhydrazone and the dinitrophenylhydrazone of pyruvate were indistinguishable.

Oxidation of glucose and cellobiose by ground-cell suspensions saturated with fructose-1,6-diphosphate. The data obtained with both intact cells and soluble enzyme extracts indicated that the paths of metabolism of glucose and cellobiose were in part independent. A common pathway through fructose-6-phosphate and fructose-1,6-diphosphate to pyruvate was indicated, and therefore differences in metabolism could be presumed to occur prior to the formation of fructose-6-phosphate. An adenosine triphosphate-dependent conversion of glucose to gluconate was specifically implicated. The following experiment

TABLE 4

Effect of cofactors on the oxidation of fructose-1,6-diphosphate by ground-cell suspensions

Flask Contents				Oxygen Consumed
Cofactors		Fructose-1,6-diphosphate	Deletions	
1×	2×			μL/hr/flask
—	—	—	—	75
+	—	—	—	73
+	—	+	—	85
—	+	+	—	91
—	+	—	—	71
+	—	+	DPN	68
+	—	+	TPN	83
+	—	+	CoA	73
+	—	+	Meth.-B	27
+	—	+	—	83

Contents of Warburg flasks were 10 μmoles of fructose-1,6-diphosphate, 1.0 μmole of diphosphopyridine nucleotide (DPN), 1.0 μmole of triphosphopyridine nucleotide (TPN), 0.1 μmoles of coenzyme A (CoA), and 0.5 μmoles of methylene blue (Meth.-B) all in 2 ml of 0.1 Tris buffer at pH 7.0 plus 1.0 ml of ground-cell suspension. Substrate was placed in side arms and added to main reaction mixture after 20 min of endogenous respiration at 30 C. "2×" indicates cofactors added at double the stated concentrations.

was designed to establish whether metabolic processes other than those involving fructose-1,6-diphosphate were significant in over-all cell respiration of carbohydrate as indicated by oxygen consumption.

A variety of procedures for preparation of soluble extracts failed to yield extracts capable of appreciable oxygen uptake even in the presence of substrate and added cofactors, although such preparations had previously been shown to convert both sugars to pyruvate. Active oxygen uptake was obtained, however, in ground-cell suspensions containing cell debris but essentially no intact cells. After three successive grindings with alumina for 20 min at −4 to 0 C, 89 per cent of the cells were ruptured. Most of the remaining intact cells were removed with the alumina by low speed centrifugation. The resulting suspensions, containing 2.17 mg of N per ml, oxidized fructose-1,6-diphosphate at the rates shown in table 4. These data indicated dependence of the oxygen uptake on diphosphopyridine nucleotide, coenzyme A, and methylene blue. Doubling of the cofactor concentration stimulated oxidation of fructose-1,6-diphosphate only 7 per cent and had no effect on the endogenous oxidations.

Immediately after completion of the experiment above, the same enzyme preparation was used to establish the concentration of fructose-1,6-diphosphate required to saturate the capacity of the preparation for oxygen uptake when fortified with diphosphopyridine nucleotide, coenzyme A, and methylene blue. The results shown in figure 2 indicate that the system was essentially saturated at a fructose-1,6-diphosphate concentration of 200 μmoles per flask. Table 5 summarizes the results of examining the effect of glucose, cellobiose, and adenosine triphosphate on oxygen uptake by ground-cell suspensions saturated with fructose-1,6-diphosphate and supplemented with diphosphopyridine nucleotide, methylene blue, and coenzyme A. Since further addition of fructose-1,6-diphosphate failed to increase oxygen uptake under these conditions, the increase resulting from either glucose or cellobiose must result from oxidations by routes other than that involving fructose-1,6-diphosphate. The inhibitory effect of adenosine triphosphate supplementation was consistent but unexplained.

Evidence of the nature of the oxidative shunt was obtained by incubation at 30 C for 60 min of 1 ml of a ground-cell suspension with 200 μmoles of fructose-1,6-diphosphate, 0.5 μmole of methylene blue, 5.0 μmoles of adenosine tri-

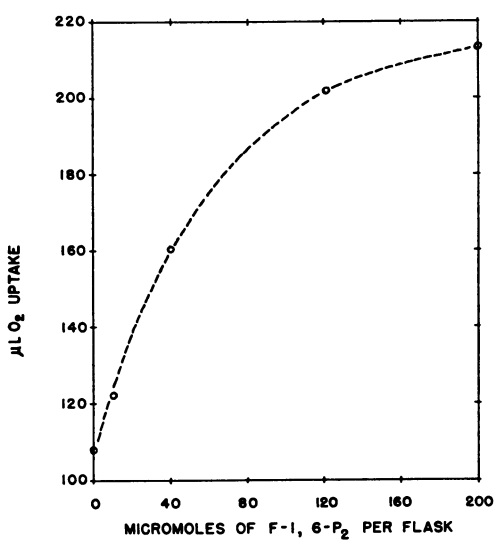

Figure 2. Determination of concentration of fructose-1,6-diphosphate (F-1,6-P_2) required for saturation of ground-cell metabolism. Experimental conditions were the same as those described in table 4.

TABLE 5

Oxidation of glucose and cellobiose by ground-cell suspensions saturated with fructose-1,6-diphosphate

Flask Contents					Oxygen Consumed
Fructose-1,6-diphosphate	Cofactors	Adenosine triphosphate	Glucose	Cellobiose	
					μL/hr/flask
—	—	—	—	—	17
+	—	—	—	—	53
+	+	+	—	—	71
+	+	+	+	—	100
+	+	—	+	—	123
+	+	+	—	+	101
+	+	—	—	+	122
+	—	—	+	—	108
+	—	+	+	—	94
+	—	—	—	+	106
+	—	+	—	+	45

Conditions were the same as those in table 4 with "1×" cofactors but with 200 μmoles of fructose-1,6-diphosphate per flask. Glucose was added as 10 μmoles per flask, cellobiose as 5 μmoles per flask, and adenosine triphosphate as 5 μmoles per flask.

phosphate, 1.0 μmole of triphosphopyridine nucleotide, 1.0 μmole of diphosphopyridine nucleotide, 0.1 μmole of coenzyme A, 40 μmoles of potassium phosphate, and either 10 μmoles of glucose or 5 μmoles of cellobiose. A third vessel containing glucose, 6×10^{-3} M NaF, and 9×10^{-6} M $MgCl_2$ was included. After removal of protein with trichloracetic acid and adjustment of the pH to 4.0 with NH_4OH, 50-μL aliquots were chromatographed in the isopropanol–pyridine–water–acetic acid (volume ratio 8:8:4:1) of Gordon *et al.* (1956). Gluconate was detected by spraying with ferrocyanide, drying, and then spraying with ferric ammonium sulfate according to the procedure of Martin (1955). Known samples of gluconate, glucuronate, glucose, and cellobiose were also chromatographed. A spot having the specific R_f and color characteristics of gluconate was observed from systems with either glucose or cellobiose as substrate, including the system containing fluoride.

DISCUSSION

The data presented serve to establish modestly the physiological basis of the superior growth response to cellobiose, but do not allow an explicit description of the key events at the enzyme level. The approximately equivalent respiration rates on glucose and cellobiose using intact, cellobiose-grown cells (figure 1) indicate that there is no marked deficiency in the capacity of the cells to absorb or to oxidize glucose. In addition, intact cells were found to esterify phosphate during oxidation of either sugar (table 1). Several independent observations indicated, however, that the metabolism of the two sugars involved at least partially distinct pathways; among these observations were (a) the difference in the yield of acid obtained with the two sugars (Hulcher and King, 1958), (b) the synergistic effect of the two sugars on oxygen uptake (figure 1), and (c) the distinctly different effect of inorganic phosphate on oxidation of the two sugars by washed, intact cells (table 2).

Demonstration that cell-free preparations resembled intact cells in their relative rates of oxidation of the two sugars and in the response of these oxidations to inorganic phosphate suggests that the difference in growth rates on the two sugars resulted from events occurring within the cell.

Both sugars were found to be convertible in part to fructose-1,6-diphosphate, but the data in table 5 indicate that the oxidations subsequent to fructose-1,6-diphosphate are not the sole respiratory reactions of the cell. These same data indicate that the divergences in glucose and cellobiose metabolism occur prior to the formation of fructose-1,6-disphosphate. Since both sugars were found to be converted to fructose-6-phosphate, it appears likely that the divergences occur prior to the appearance of fructose-6-phosphate.

Qualitative evidence indicated that glucose was metabolized by two pathways; one leading through glucose-6-phosphate to fructose-1,6-diphosphate, the other leading to gluconate. Cellobiose was found to be cleaved phosphorolytically yielding glucose plus glucose-1-phosphate. The latter is convertible to fructose-6-phosphate and fructose-1,6-diphosphate, but the pathway is obscure, since phosphoglucomutase is apparently absent. On a molar basis, then, less cellobiose would presumably be shunted into the gluconate pathway. If it is assumed that the molar energy yield from the gluconate shunt is less than that from the pathway through fructose-1,6-diphosphate to pyruvate, a reasonable hypothesis for the enhanced growth on cellobiose may be advanced: the cell derives from cellobiose slightly more energy per mole of hexose metabolized than is obtained from glucose. Substantiation of this hypothesis must await more detailed identification of the specific pathways involved and quantitative determination of the relative rates at which the two sugars participate in the alternate reaction sequences.

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SUMMARY

The metabolic basis of preferential utilization of cellobiose over glucose has been sought using both intact cells and cell-free preparations of *Cellvibrio gilvus*. A cellobiose phosphorylase has been demonstrated to initiate metabolism of the disaccharide. Convergence in the metabolism of glucose and cellobiose has been shown to occur at two points: by the direct formation of glucose from half of the cellobiose as a result of the phosphorolytic cleavage; and later, by the formation of fructose-6-phosphate from both

sugars. The data suggest the hypothesis that the difference between the sugars as energy sources for growth stems from the direct oxidation of glucose to gluconic acid; cellobiose is cleaved phosphorolytically and further metabolized by reactions leading to more complete oxidation and therefore a greater yield of energy per mole of hexose consumed.

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