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METABOLIC NONEQUIVALENCE OF THE TWO GLUCOSE MOIETIES OF CELLOBIOSE IN *CELLVIBRIO GILVUS*

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ABSTRACT

SWISHER, ELIZABETH J. (Virginia Polytechnic Institute, Blacksburg), WALDEMAR O. STORVICK, AND KENDALL W. KING. Metabolic nonequivalence of the two glucose moieties of cellobiose in *Cellvibrio gilvus*. J. Bacteriol. 88:817-820. 1964.—Cellobiose was synthesized in 40% yield with uniform C^{14} labeling in the reducing glucose moiety and no label in the nonreducing glucosyl. Resting-cell suspensions of *Cellvibrio gilvus* respiring the labeled cellobiose derived approximately 80% of their respiratory CO_2 from the reducing glucosyl and 20% from the nonreducing glucose. Analysis of isotope content in CO_2 from cells respiring a mixture of labeled cellobiose and unlabeled glucose confirmed that the glucose-1-phosphate produced from phosphorolysis of cellobiose is less extensively converted to CO_2 than is either the glucose released by phosphorolysis of cellobiose or glucose absorbed from the medium. In crude cell extracts, release of glucose from cellobiose was shown to be P_i-dependent, the pH optimum of cellobiose phosphorylase being 6.2.

Among cellulolytic organisms, a number of instances were described in which growth on cellobiose is superior to that on glucose (Hutchinson and Clayton, 1918; McBee, 1948; Enebo, 1949; Hungate, 1950; Sijpesteijn, 1951; Hall, 1952; Hulcher and King, 1958*a*). In the three instances where the metabolic basis for cellobiose preference was sought most thoroughly, the primary extracellular product of cellulose hydrolysis was found to be cellobiose. The three organisms, *Clostridium thermocellum* (Sih and McBee, 1955; Sih, Nelson, and McBee, 1957; Alexander, 1961), *Ruminococcus flavefaciens* (Ayers, 1958, 1959), and *Cellvibrio gilvus* (Hulcher and King, 1958*a*, *b*), also have in common a high intracellular level of cellobiose phosphorylase, which appears to initiate the reaction sequences leading to oxidation of cellobiose by producing free glucose and α-D-glucose-1-phosphate.

In contrast to *C. thermocellum* and most strains of *R. flavefaciens*, *C. gilvus* does grow on glucose, although the lag phase is prolonged and the maximal growth rate is lower than on cellobiose (Hulcher and King, 1958*a*). Several lines of evidence indicated that cellobiose and glucose followed at least partially independent metabolic pathways in *C. gilvus*, among them the respiratory end products and pH optima for growth on the two sugars, nonadditive respiration rates for the two sugars, distinct differences in the rates of phosphate esterification in the presence of the two sugars, and demonstration in a cell-free system of a respiratory shunt bypassing fructose-1,6-diphosphate through which free glucose passed readily but apparently not glucose-1-phosphate (Hulcher and King, 1958*b*).

Because glucose and glucose-1-phosphate are the initial products of cellobiose metabolism in *C. gilvus*, these data suggested that the two glucosyl moieties of cellobiose must not be metabolically equivalent, but previous data did not prove the point. The present research tested this hypothesis directly by measuring the relative contributions of the reducing and nonreducing glucose groups of cellobiose to respiratory CO_2 when intact cells oxidized cellobiose in which the reducing glucose was labeled with C^{14} while the nonreducing glucose moiety was unlabeled.

MATERIALS AND METHODS

The culture and the medium used were those described by Hulcher and King (1958*a*), except that the vitamins were omitted and cellobiose was included at 0.2%. Before harvesting, all cultures were tested for purity by examining both Gram- and nigrosine-stained smears.

In preparing cell-free preparations, the cells were washed twice by centrifugation at 0 C in 0.1 N sodium citrate buffer at pH 6.2. The cell paste was then resuspended in 2 volumes of the

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cold buffer and disrupted in a French pressure cell at 12,000 psi. Intact cells and cell debris were removed by centrifugation for 30 min at 0 C and 17,500 × *g*. The clear supernatant fluid was used as the crude source of cellobiose phosphorylase.

For manometric experiments, the cells were washed three times in 0.067 M potassium phosphate buffer at pH 7.0 in a centrifuge at 0 C, resuspended in the same buffer, and filtered through glass wool to remove clumps. The filtered suspension was then diluted with the same buffer so that a 1:10 dilution gave 25% transmittance in a Bausch & Lomb Spectronic-20 colorimeter at 430 mμ. Carbon dioxide was determined by the indirect method of Umbreit, Burris, and Stauffer (1957).

C^{14} analyses of CO_2 were carried out in a Tri-Carb liquid scintillation spectrometer by use of 0.1 ml of aqueous sample dispersed in 15 ml of liquid scintillator composed of 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(5-biphenyloxazolyl)] benzene in a solvent consisting of 20% ethanol in toluene. For counting glucose and cellobiose, 3 ml of aqueous sample were mixed into 15 ml of a scintillator consisting of 10% naphthalene, 1% 2,5-diphenyloxazole, and 0.025% 1,4-bis-[2-(5-biphenyloxazolyl)] benzene in dioxane. Internal standards consisting of C^{14}-benzoic acid of known absolute disintegration rate were used to determine counting efficiency.

Reducing sugars were determined as described by Nelson (1944) and modified by Somogyi (1952). Total sugars were determined by use of the procedure of Dubois et al. (1956). The ethyl acetate-pyridine-water solvent of Walker and Whelan (1960) was used for paper chromatography. Ionophoretic separations of glucose and sorbitol followed the procedure of Bourne, Hutson, and Weigel (1959). Protein analyses were conducted as described by Lowry et al. (1951). The notatin procedure of Saifer and Gerstenfield (1958) was used to determine glucose in the presence of cellobiose.

RESULTS AND DISCUSSION

Observations on the cellobiose phosphorylase from C. gilvus. Crude cell-free extracts prepared as described above yield cellobiose phosphorylase having a fivefold increase in specific activity, with a 63% recovery when the protein is precipitated between 20 and 40% saturation with $(NH_4)_2SO_4$. At 40% saturation, precipitation of the enzyme was complete, in contrast with the analogous enzyme from *R. flavefaciens* which Ayers (1959) reported to require 70% saturation for complete precipitation. With this enzyme fraction, production of glucose from cellobiose as substrate was completely dependent on orthophosphate, indicating that β-glucosidase levels were negligible. The pH optimum was approximately 6.2. In previous investigations of cellobiose phosphorylase, pH values of 6.9 to 7.6 have been used (Ayers, 1958, 1959; Alexander, 1961· Sih et al., 1957; Hulcher and King, 1958*b*).

Synthesis of asymmetrically labeled cellobiose Preparative scale synthesis of cellobiose labeled in the reducing glucosyl employed a reaction system consisting of 20 μmoles of glucose-*U*-C^{14} (10 μc per mg), 100 μmoles of α-D-glucose-1-phosphate, 4.5×10^{-3} μmoles of $MgCl_2$, 3 μmoles of NaF, and 0.7 ml of crude cell extract in a total volume of 1.2 ml of 0.1 N sodium citrate at pH 6.2. After incubation at 37 C for 2 hr, the cellobiose was isolated by the procedure of Miller (1960). The yield was approximately 2 mg of cellobiose having a specific activity of approximately 5 μc per mg, a 40% recovery of C^{14} in the final product. Infrared spectra between 2 and 16 μ of the synthetic compound were indistinguishable from those of authentic cellobiose, and were markedly different from maltose spectra. The differential spray reagent of Buchan and Savage (1952) was reported by Buston and Khan (1956), and was confirmed by us, to distinguish between cellobiose, maltose, and all of the isomeric β-diglucosides. In both R_F and color reaction, the synthetic compound was indistinguishable from authentic cellobiose. Cellobiitol, gentiobiitol, laminaribiitol, and sophoritol were prepared from authentic samples of the parent disaccharides and from the C^{14}-disaccharide by reduction with borohydride. Ionophoretic separation according to Bourne et al. (1959) showed the reduced synthetic compound to migrate identically with reduced cellobiose and to be distinct from the other β-disaccharides. When 25 μg of the labeled synthetic product were dissolved with 1.0 g of authentic cellobiose in water and carried through three successive crystallizations, the specific activity held constant. The initial mixed solution had a specific activity of 355 counts per min per mg, and the three successive crops of crystals

gave values of 328, 362, and 379 counts per min per mg. To be certain that the label was exclusively in the reducing glucosyl, a portion of the synthetic product was reduced with a sevenfold molar excess of sodium borohydride. Completeness of reduction was verified by the absence of reducing sugars. After acid hydrolysis, glucose and sorbitol were separated by paper electrophoresis in the system described by Bourne et al. (1959). All radioactivity moved with the sorbitol, indicating that the starting disaccharide had been labeled solely in the reducing glucose moiety.

Respiration of asymmetrically labeled cellobiose. Four Warburg flasks, two for the measurement of O_2 consumption and two for the indirect determination of CO_2, were set up for each substrate system and for endogenous controls. The main compartment contained 1.0 ml of turbidimetrically standarized cell suspension and 0.5 ml of 0.067 M potassium phosphate at pH 7.0. In the first side arm was 1.0 ml of buffered substrate or buffer. The second side arm contained 0.3 ml of 2 N HCl, and the center wells contained paper wicks with 0.2 ml of 10% KOH or water. After a 20-min thermal equilibration at 37 C, the contents of the first side arm were dumped. After O_2 uptake had proceeded to approximately 70% of completion, the reaction was stopped by dumping the acid from the second side arm, and shaking was continued for 10 min to allow complete absorption of CO_2. The center well contents, including the wicks, were transferred to 5.0-ml volumetric flasks and were extracted with water for 24 hr before determining C^{14} in the scintillation counter. From the manometric and the $C^{14}O_2$ data, the percentages of CO_2 derived from each glucose moiety of cellobiose were calculated (Table 1).

If the two halves of the cellobiose molecule were metabolically equivalent for *C. gilvus*, one would expect CO_2 evolved by cells respiring cellobiose to be derived equally from the two halves of the molecule. Therefore, the expected specific activity of CO_2 evolved from asymmetrically labeled cellobiose would be the same as the specific activity of the substrate as a whole or 1,455 counts per min per μmole of carbon. The observed specific activity of CO_2 evolved by cells respiring asymmetrically labeled cellobiose was 2,331 counts per min per μmole of carbon, a value distinctly higher than that expected if the two halves of the cellobiose molecule contributed equal amounts of CO_2 (Table 1). Results of this type may be explained only if the reducing glucose moiety of cellobiose contributed more than one-half of the total CO_2 evolved from the C^{14}-cellobiose substrate. The percentage of the CO_2 which was contributed by the reducing glucosyl moiety of cellobiose was calculated, and was found to be in the neighborhood of 80% in several experiments.

TABLE 1. *Respiration of glucose and asymmetrically labeled cellobiose by resting cells*

Substrate per flask	CO_2 evolved	Specific activity*		Percentage of CO_2 from reducing glucose of cellobiose
		Substrate	CO_2	
	μmoles			
C^{14}-cellobiose (12.5 μM)	36†	1,455	2,331	80
C^{14}-cellobiose (6.25 μM) plus glucose (12.5 μM)	35	728	1,207	41
C^{14}-glucose (25 μM)	33	7,125	7,507	100

* Expressed as counts per minute per micromole.

† Corrected for endogenous CO_2 of 2.2 μmoles.

Since the reducing glucosyl moiety of cellobiose is released as glucose after phosphorolysis, it was anticipated that exogenous glucose might behave similarly to the reducing glucosyl moiety of cellobiose. If this were true, and if the two halves of the cellobiose molecule were equivalent for the organism, one would expect the specific activity of CO_2 evolved from the C^{14}-cellobiose-glucose mixture to approach the specific activity of the whole substrate or 728 counts per min per μmole of carbon. Again, the observed specific activity of CO_2 evolved was higher than predicted (Table 1), indicating either a difference in the metabolism of the two halves of the cellobiose molecule or a difference in the metabolism of the reducing glucosyl moiety of cellobiose and free glucose. The percentage of CO_2 contributed by the reducing glucose moiety of cellobiose in this case was 41%, although the reducing half of the cellobiose molecule represented only 25% of the carbon source. These data also indicate that the two halves of the cellobiose molecule are metabolized differently.

The C^{14}-glucose substrate served as an experimental control, since the specific activity of all CO_2 evolved by cells respiring this substrate must theoretically be the same as the specific activity of the C^{14}-glucose. Deviation from this theoretical specific activity reflects the sum of (i) experimental error, (ii) nonuniformity of the labeling pattern in the C^{14}-glucose used as substrate (as well as in the synthesis of C^{14}-cellobiose), and (iii) a difference in endogenous respiration in the absence and presence of added substrate. The observed specific activity of CO_2 evolved by cells respiring this substrate was approximately 6% higher than the expected value (Table 1).

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