Pathways of Glucose Metabolism by Rough and Smooth Variants of *Bacillus stearothermophilus*  

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The radiorespirometric method was used to study the pathways of glucose metabolism in the rough and smooth variants of *Bacillus stearothermophilus* NCA 1518. The Embden-Meyerhof (EM) pathway was more active in the smooth variant than in the rough variant. The participation of the hexose monophosphate shunt (HMP) and EM pathways in the smooth variant was calculated as 4.2 and 95.8%, respectively. The rough variant utilized glucose via the EM pathway exclusively or in combination with a pathway other than the HMP pathway. The estimated efficiency of the tricarboxylic acid system in the rough and smooth variants was 81.3 and 4.9%, respectively.

The rough and smooth variants of many bacteria differ in their metabolism and nutritive requirements. The rough variant is generally less active biochemically (6, 7, 11) and, in the case of *Bacillus stearothermophilus* NCA 1518, is nutritionally less demanding than the smooth form (W. M. Hill, Ph.D. Thesis, Univ. of Missouri, 1966). Since the rough and smooth transitions are thought to be in the nature of a biochemical mutation (3), it would be expected that differences in metabolism would exist between the two variants.

Three pathways of glucose catabolism in microorganisms are: the Embden-Meyerhof (EM) pathway, the hexose monophosphate cycle (HMP), and the Entner-Doudoroff (ED) pathway. The EM pathway results in the formation of two molecules of pyruvic acid. The carboxyl carbons of the pyruvate are derived from C-3 and C-4 of glucose. Decarboxylation of the pyruvate molecules liberates two CO₂ molecules and usually two molecules of acetate. The HMP cycle results in the preferential oxidation of C-1 of the glucose molecule, resulting in the formation of a pentose phosphate sugar. Recycling of the pentose sugar forms hexose phosphate, with the C-2 atom of the pentose becoming the C-1 of the hexose. The third cycle, discovered by Entner and Doudoroff (4) with their studies on *Pseudomonas saccharophila*, forms pyruvate and glyceraldehyde phosphate. The carboxyl carbon of the pyruvate is derived from C-1 of the glucose molecule, and the aldehyde carbon of the glyceraldehyde phosphate is derived from C-4 of glucose. There is another pathway for glucose metabolism which exists in mammalian cells and may function in some microorganisms (2). This pathway was first suspected by Hollmann and Touster (9) and is described as the glucuronic acid-xylulose cycle. In this pathway, C-6 of glucose is preferentially oxidized to CO₂. The pentose d-xylulose-5-phosphate is eventually formed, linking this pathway with the HMP cycle.

The study of carbohydrate metabolism has been greatly advanced over the past decade with the advent of differentially labeled glucose isotopes. With these radioactive compounds and other isotopic substrates, investigators have demonstrated functional EM, HMP, and other glycolytic pathways in microorganisms.

Wang et al. (13) used a radiorespirometric method to study glucose catabolism by several microorganisms. For the bacilli tested, they found the following pathway participation: *Escherichia coli*, 72% EM and 28% HMP; *B. subtilis*, 65% EM and 35% HMP; *Pseudomonas saccharophila*, 100% ED; *Pseudomonas reptilivora*, 72% ED and 28% HMP. Blumenthal (2) studied the changes in glucose metabolism of *B. cereus* spores during germination and outgrowth. The EM pathway always accounted for at least 70% of the glucose utilized by the germinated spores. Glucose C-6 was preferentially oxidized by spores grown in dialyzed Casitone medium to the elongated stage. Hollman (8) also lists several microorganisms and animal tissues and the percentage of participation of the respective pathways.

Research conducted in our laboratory on the metabolic and growth characteristics of a flat-sour bacterium, *B. stearothermophilus* NCA 1518,
demonstrated significant differences between the two variants of this organism. This paper presents the results of an investigation of the pathways of glucose catabolism by this organism as determined by a radiorespirometric technique.

**Materials and Methods**

*Preparation of vegetative cell suspensions.* The rough and smooth variants of *Bacillus stearothermophilus* NCA 1518 were obtained from pure cultures originally isolated by the single-cell method (12). The cells were maintained on Trypticase Soy Agar (TSA) slants at 4 C. Prior to being used, streaks of the cultures were made on a medium containing TSA, 0.5% maltose, and 0.4% bromoresol purple. After incubation for 20 to 22 hr at 55 C, the colonies were examined under a dissecting microscope to determine the homogeneity of the populations. The smooth variant readily utilizes the maltose and produces enough acid to cause a purple to yellow color change in the medium within 12 to 14 hr at 55 C. The rough variant produces little or no detectable acid in this medium after incubation for 48 hr at 55 C.

Typical colonies of the variants (5) were picked and streaked onto the surface of TSA plates (10 plates for the rough and 15 plates for the smooth). These plates were held at 55 C for 8 hr, and the cells were removed by pouring about 5 ml of 0.85% NaCl into the plates and gently dislodging the cells from the agar surface with the tip of a pipette.

The resulting suspensions of each variant were combined in 50-ml polyethylene centrifuge tubes. The cells were centrifuged twice for 15 min at 4 C at 12,000 × g in a refrigerated centrifuge. The washed cells of each variant were resuspended in 20 ml of 0.85% NaCl. Phosphate buffer was not used, because it restricts cell division in the rough variant of this organism, causing elongated cells (*unpublished data*), and apparently can interfere with glucose metabolism (10). Also, there was not enough acid produced through respiration by either variant under the conditions of this test to be detected with bromoresol purple.

The final suspension of the rough variant contained approximately 6 × 10⁹ viable cells per ml, which is equivalent to 1.1 mg of nitrogen and 8.4 mg (dry weight). The smooth-variant suspension contained about 2 × 10⁹ viable cells per ml or 0.44 mg of nitrogen or 1.6 mg (dry weight). Figure 1 shows the standard curves used to relate dry weight, nitrogen content, and viable-cell count.

*Radioactive glucose.* Glucose C-1, C-2, and C-6 were obtained from Baird Atomic Inc., Cambridge, Mass. The glucose C-3, 4 and sodium acetate C-2 were obtained from Volk Radiochemical Co., Burbank, Calif.

*Scintillation counting.* The scintillation liquid was prepared by combining 4.0 g of 2,5-diphenyloxazole with 40 mg of dimethyl 1, 4-bis-(2-5-phenyloxazolyl)benzene and diluting to 1 liter with toluene. The scintillation compounds were purchased from Packard Instrument Co., Inc., Downers Grove, Ill. The samples were counted for 10 min on a Packard Tri-Carb scintillation spectrometer (model 314X).

The radiorepirometer. The apparatus for radiorespirometric measurements is a modification of the instrument designed by Wang et al. (13). It consists of gas-flow control Nupro valves (Fig. 2A) and a number of sintered-glass filter CO₂ traps (Fig. 2C) and Warburg reaction flasks.

The flasks and traps are mounted on holders, which are mounted on a shaking device. The gas flow can be adjusted independently for each of the eight sets by turning the Nupro valves (Nuclear Products Co., Cleveland, Ohio). The gas was routed through the reaction flasks which were held in a constant-temperature water bath. The CO₂ resulting from respiration was carried by the air flow to a three-way stopcock (Fig. 2B) and then was routed to one of the sintered-glass filters containing 10 ml of ethanolamine-ethyl alcohol (2:1, v/v). The CO₂ was collected in the trapping solution for a desired length of time, and then the stopcock was turned to direct the gas flow to the second trap. The trapping solution-CO₂ mixture was removed from the trap by releasing the rubber hose clamp and applying pressure at the top of the sintered-glass filter. As the liquid passed through the sintered glass and rubber tubing, it was caught in a 50-ml graduate cylinder. The trap was washed with 5 ml of absolute ethyl alcohol which was also collected in the graduate cylinder. The graduate cylinder was capped and the contents were vigorously shaken. From the graduate cylinder, 5 ml was removed and pipetted into a 20-ml scintillation vial containing 10 ml of scintillation liquid.

*Conditions of the experiment.* The variant cell suspension (4 ml) was pipetted into the center well of a reaction flask. The side arm of the flask contained 1 ml of carbon-labeled glucose (1 µc/20 µmoles). In the mixed population and sodium acetate utilization

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**Figure 1.** Standard curve relating dry weight, nitrogen content, and viable cell count of rough and smooth cell suspensions.
experiments, 0.5 µc and 10 µmoles of substrate were employed. The cell suspensions were allowed to reach thermal equilibrium at a water bath temperature of 50 °C for 10 min, at which time the substrate was tipped into the reaction vessel. Shaking was continuous except for the time required to remove the contents of the CO₂ traps. Gas flow was adjusted to 40 to 45 ml/min.

Upon termination of the CO₂ collection, 4 ml of the suspension was removed from the flasks and centrifuged. The supernatant fluid was analyzed for carbohydrate content by the dinitrosalicylic acid test (1).

RESULTS

The per cent recovery of radioactive CO₂ from labeled glucose utilized by the rough variant is shown in Fig. 3B and 4B. The cumulative recovery of radioactive CO₂ was greatest from the C-3,4-labeled glucose (88.9%), followed by the C-2-labeled substrate (64.7%). The recovery of CO₂ from C-6 glucose (36.1%) was only slightly higher than that from C-1 glucose (35.8%).

The per cent recovery from the breakdown of labeled glucose by the smooth variant is shown in Fig. 3A and 4A. The cumulative recovery was 5.5, 5.4, 57.9, and 1.35% from glucose C-1, C-2, C-3,4, and C-6, respectively. The utilization of glucose C-3,4 by the smooth variant was much greater than the utilization of glucose C-1, C-2, and C-6 by either the smooth or rough variant. The breakdown of glucose C-1, C-2, and C-6 by the smooth variant was less than that of the rough variant.

Sodium acetate C-2 oxidation by the rough and smooth variants is shown in Fig. 5. The rough variant utilized 66.7% of the acetate after 2 hr of incubation, whereas the smooth utilized only 4.5%.

The interaction between mixed populations of rough and smooth cells in the breakdown of glucose C-3,4 is shown in Fig. 6. The rough variant utilized 25.4% of the glucose C-3,4, the smooth variant utilized 36.3% of the glucose, and the mixed population (30% rough, 70% smooth) utilized 62.0% of the glucose after 60 min of CO₂ collection. The mixed population contained one-half as many smooth and rough cells as the pure variant populations.

DISCUSSION

The data obtained from the utilization of different glucose isotopes by one variant can be compared with each other without consideration of cell numbers.
or dry weight. However, a problem arises when attempts are made to compare data obtained from the two variants. As shown in Figure 1, suspensions of the rough and smooth variants which had the same number of viable cells by plate count did not have the same dry weight or nitrogen content. When the data are corrected and expressed on a per milligram of dry weight or milligram of nitrogen basis, the smooth variant will be favored because it contains more individual cells per milligram of dry weight or milligram of nitrogen than the rough variant. Conversely, when the data are expressed on a per cell basis, the rough variant will be favored because of its greater weight per cell (5). Further complications arise from the characteristic of the rough variant to form chains of three or four individual cells, which would result in plate counts being lower than when the chains did not occur. The data obtained in this research were not corrected to a common dry weight or cell count basis. The smooth suspension had about three times more cells, by plate count, than the rough. However, when the individual rough cells in chains are taken into account, the data for the numbers of cells in the two suspensions will become more equal. Also, correcting the data to a dry weight basis will change the magnitude of the values but not the observed relationships between the two variants.

The two variants differ in their utilization of glucose by the EM pathway and the tricarboxylic acid cycle. As indicated by the data on glucose C-3,4 utilization, the smooth variant has a very active EM pathway, whereas the rough demonstrates a moderate EM pathway activity. Conversely, the data on glucose C-6 and C-2 oxidation indicate that the rough variant has an active tricarboxylic acid cycle, whereas the smooth has a relatively inactive cycle. This comparison of oxidative systems in the variants is supported by the results of acetate C-2 breakdown. Since the major means of acetate utilization is via the tricarboxylic

![Figure 4](https://example.com/figure4.png)  
**FIG. 4.** Per cent cumulative recovery of $^{14}$CO$_2$ from labeled glucose utilized by the smooth (A) and rough (B) variants. Substrate: 1 µc and 20 µmoles.

![Figure 5](https://example.com/figure5.png)  
**FIG. 5.** Oxidation of sodium acetate C-2 by rough and smooth variants. Substrate: 0.5 µc and 10 µmoles.

![Figure 6](https://example.com/figure6.png)  
**FIG. 6.** Utilization of glucose C-3,4 by pure and mixed (30% rough and 70% smooth) populations of the variants. Substrate: 0.5 µc and 10 µmoles.
acid cycle (11), labeled acetate is a reliable means of estimating the tricarboxylic acid activity. Also, the fact that growing cultures of the smooth variant accumulate appreciable quantities of acids (6; W. M. Hill, Ph.D. Thesis, Univ. of Missouri, 1966) indicates that this variant has a reduced tricarboxylic acid cycle activity. However, it is also possible that the smooth variant utilizes much of the two-carbon material derived from pyruvate decarboxylation in fatty acid synthesis.

There is a possibility that the rough variant is capable of utilizing glucose by a system similar to the gluconic acid-xylulose pathway in which glucose C-6 is preferentially oxidized. The ratio of CO₂ formed from glucose C-6 to CO₂ formed from glucose C-1, from five separate experiments with the rough variant, ranged from 0.95 to 1.22 with an average of 1.02. The average ratio suggests that the rough variant utilizes glucose exclusively by the EM pathway, since a ratio of 1.0 would be expected in such a circumstance (8).

If there were no CO₂ formed from glucose C-1 other than that derived from EM activity, the recovery of CO₂ from glucose C-1 and C-6 would be theoretically identical. This is the case with the rough variant, but the smooth variant produces more CO₂ from glucose C-1 than glucose C-6. Therefore, some CO₂ derived from glucose C-1 is being used under the action of a second pathway in the smooth variant, presumably the HMP shunt. By use of the formulas developed by Wang et al. (13), values of 4.2 and 95.8% are obtained for the per cent participation of the HMP and EM pathways, respectively, in the smooth variant.

The increased utilization of glucose C-3,4 in mixed populations of the variants indicates a definite interaction between the variants. It is possible that combining the two variants results in a complementary enzyme system which has the high EM activity of the smooth variant and the high tricarboxylic acid activity of the rough variant. Thus, as the two-carbon compounds are formed by the smooth variant, they are quickly oxidized by the rough variant. This would be a more balanced system of glucose utilization than is found in either of the variants alone.

An estimation of the efficiency of the tricarboxylic acid cycle in using the two-carbon fragments formed via the EM pathway may be calculated from data in this research. Efficiency values of 81.3 and 4.8% were obtained for the rough and smooth variants, respectively, by use of the following formula:

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\text{per cent recovery of CO}_2 \text{ from glucose C-6} = \left( \frac{\text{per cent recovery of CO}_2 \text{ from glucose C-3,4}}{2} \right) \times 100
\]

More research is required to determine whether a gluconic acid-xylulose pathway is operative in the rough variant of this organism. Experiments using 0.02 m NaF as an enzyme inhibitor have been initiated. The results thus far obtained showed that C-1 oxidation by the rough variant was decreased 15% and that by the smooth variant was increased 48%. Also, C-6 oxidation by the rough was reduced 30 to 40%, whereas that by the smooth variant was decreased 90 to 95%. Unless other factors such as cell permeability and transport of the NaF are evolved, the inhibition results also show that these variants differ in their pathways of glucose metabolism.

**Literature Cited**


