AN ENZYMATIC STUDY OF THE UTILIZATION OF GLUCONIC ACID BY *PROPIONIBACTERIUM PENTOSACEUM*

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Generally the so-called Meyerhof-Embden scheme is considered to be the major pathway of hexose dissimilation by members of the genus *Propionibacterium*. Two lines of evidence supporting this premise are the isolation, coupled with a demonstration of their utilization, of several of the intermediates of glycolysis (Wood, Stone, and Werkman, 1937; Werkman, Stone, and Wood, 1937; Barker and Lipmann, 1949) and the distribution of a major portion of the isotopic labeling of various carbohydrates in the product commensurate with such a pathway (Wood, Stjernholm, and Leaver, 1955). There is additional evidence, however, for the existence of simultaneous alternate pathways of carbohydrate dissimilation by this microbe. Indicative of such alternate pathways is the relative insensitivity of glucose dissimilation and oxidation by *Propionibacterium* to the inhibitors of normal glycolysis (Werkman, Stone, and Wood, 1937; Fukui, 1952) and isotopic data best explained by additional pathways (Wood, Stjernholm, and Leaver, 1955). However, studies of carbohydrate utilization on an enzyme level with this organism have been lacking.

Fukui (1952) while studying the ability of various species of the genus *Propionibacterium* to adapt to aerobic growth, noted that various members of this group were capable of utilizing sodium gluconate as an energy source. Since the utilization of gluconate is generally considered to be indicative of a pathway or pathways alternate to conventional glycolysis (Gunsalus, Horecker, and Wood, 1955), an enzymatic study of gluconate dissimilation by *Propionibacterium pentosaceum* strain E214 seemed significant. Growth of this organism on sodium gluconate presumably has the advantage of inducing greater activity of the pathways alternate to conventional glycolysis and thus their enzymatic study is quantitatively facilitated.

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METHODS AND MATERIALS

The culture of *P. pentosaceum* strain E214 used in this study was grown on a medium composed of 0.5 per cent each of tryptone, yeast extract, K_2HPO_4, and sodium gluconate or glucose. The culture was grown in 3 L of this medium, contained in a 4-L Erlenmeyer flask, from a 1 per cent inoculum at 30 C for 18 hr. The resultant cell crop was centrifuged from the growth medium, washed once with distilled water, recentrifuged, and resuspended in distilled water to make a thin paste (approximately 1 per cent of the original growth volume). For experiments using whole cells this paste was diluted 10-fold before use. For the preparation of sonic extracts the paste was exposed in a Raytheon Magnetostriction oscillator using a frequency of 9 kc per sec for 45 min. The exposed suspension was then centrifuged at 12,000 rpm for 15 min. The resulting supernatant containing approximately 10 mg protein per ml was used as a crude enzyme preparation.

The fructose-1,6-diphosphate (F-1,6-P) was a commercial preparation, being further purified as its strychnine salt according to the method of Sable (1952). The substrates fructose-6-phosphate (F-6-P), glucose-6-phosphate (G-6-P), and 6-phosphogluconate (6-PG) were prepared from F-1,6-P as described by Newberg *et al.*, (1943) and Wood and Horecker (1953). The ribose-5-phosphate (R-5-P), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), and adenosine triphosphate (ATP) were commercial preparations.

Metabolic gas exchange was measured at 37 C using conventional manometric methods (Umbreit *et al.*, 1949) in the Warburg respirometer. Protein was estimated using the Folin-phenol method of Lowry *et al.* (1951). Pentose and sedoheptulose were determined using the method of Horecker *et al.* (1953). Identification of phosphate esters was obtained by dephosphorylation and paper chromatography of the nonphosphorylated

carbohydrates as described previously (VanDemark and Wood, 1956).

Enzymatic dehydrogenase activity was determined spectrophotometrically by the reduction of DPN or TPN at 340 mμ according to the method of Adler *et al.* (1939). Aldolase activity was determined by the method of Sibley and Lehninger (1949).

RESULTS AND DISCUSSION

As shown in figure 1 gluconate-grown cells of *P. pentosaceum* readily oxidize both sodium gluconate and glucose, while glucose-grown cells oxidize glucose somewhat slower. Although initially, glucose-grown cells do not significantly utilize sodium gluconate, after several hours' incubation (not shown in figure 1) gluconate oxidation is induced. Under the same conditions neither type cell utilizes sodium 2-ketogluconate.

Crude sonic preparations of gluconate-grown *P. pentosaceum* strain E214 readily oxidized those phosphorylated intermediates which were considered as the most likely intermediates of gluconate dissimilation (figure 2). This ready oxidation of F-1,6-P, G-6-P, 6-PG, and R-5-P by this crude enzyme preparation is presumptive evidence that these compounds may be intermediates in the enzymatic breakdown of gluconate.

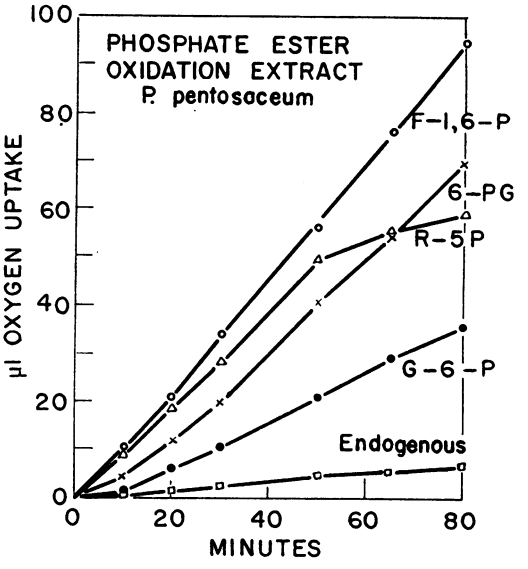

Figure 2. The oxidation of phosphorylated intermediates by a sonic extract of *Propionibacterium pentosaceum.* The Warburg cups contained approximately 6 mg of protein, 10 μmoles of the substrates indicated, 120 μg of DPN, 0.5 ml of M Tris buffer, pH 7.4, and water to 3 ml.

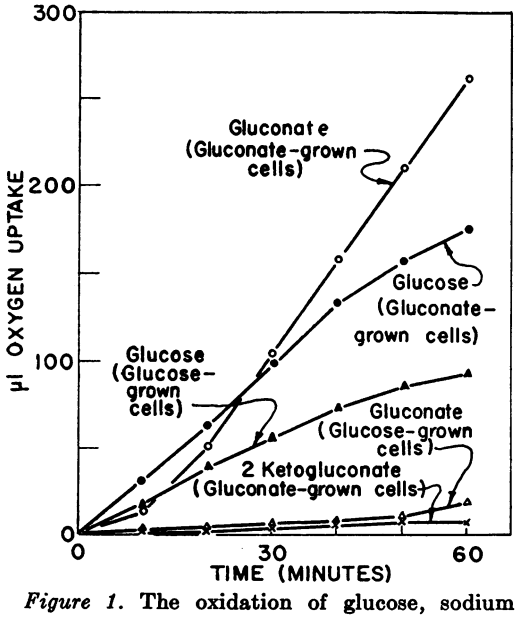

Figure 1. The oxidation of glucose, sodium gluconate, and sodium-2-ketogluconate by whole cells of *Propionibacterium pentosaceum.* The Warburg cups contained 1 ml of cell suspension (approximately 20 mg protein), 25 μmoles of the substrates indicated, 1.0 ml of 0.1 M phosphate buffer, pH 7.4, and water to 3 ml. The endogenous respiration was subtracted from the data shown.

Spectrophotometric studies of the reduction of TPN by G-6-P and 6-PG with the crude enzyme (figure 3) demonstrated the existence of G-6-P and 6-PG dehydrogenases respectively. Both dehydrogenases were TPN-specific, failing to reduce DPN, and in this regard similar to the G-6-P and 6-PG dehydrogenases in yeast and *Escherichia coli.* Since the crude sonic preparation contains G-6-P dehydrogenase, the reduction of TPN with F-6-P by this enzyme preparation (figure 3) is indicative of the additional presence of phosphohexoisomerase.

Having established the presence of a TPN-specific 6-PG dehydrogenase in the crude enzyme, reduction of TPN with sodium gluconate in the presence of ATP is evidence of gluconokinase activity in the sonic preparation (figure 4). This kinase activity required ATP and magnesium ions for maximum activity. As further shown in figure 4, glucose also serves as a substrate to reduce TPN, indicating the presence of glucokinase in the crude enzyme.

Since these data would seem to establish the

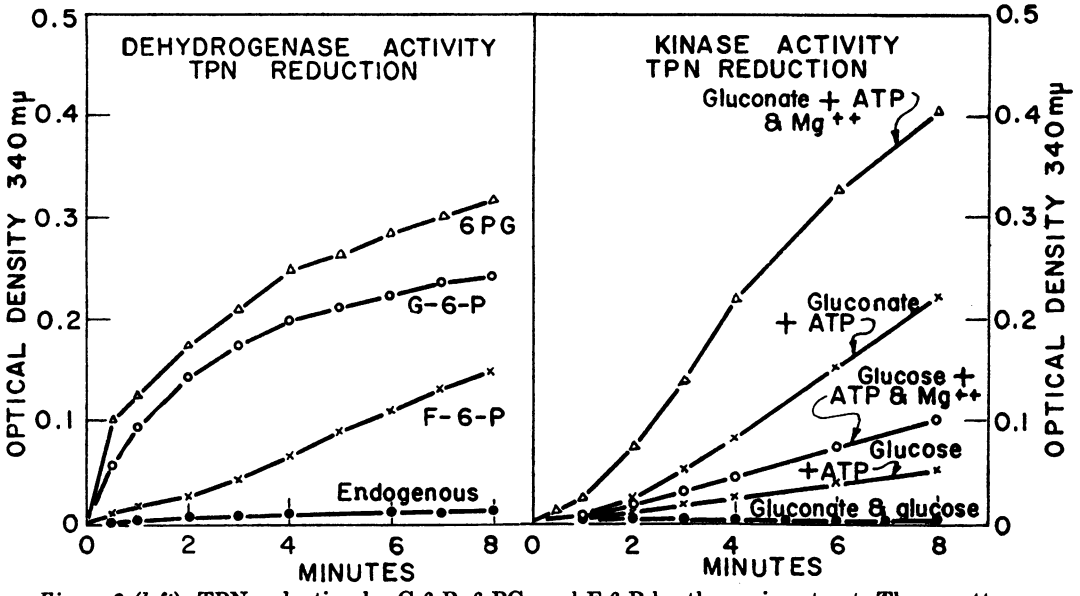

Figure 3 (left). TPN reduction by G-6-P, 6-PG, and F-6-P by the sonic extract. The cuvettes contained approximately 4 mg of protein, 25 μmoles of the substrates indicated, 0.5 μmoles of TPN, 0.5 ml M Tris buffer, pH 7.4, and water to 3 ml volume.

Figure 4 (right). TPN reduction by gluconate and glucose, with and without ATP and magnesium. The cuvettes contained 1.0 ml sonic extract (approximately 7 mg protein), 25 μmoles of the substrates indicated, 0.5 μmoles of TPN, 0.5 ml M Tris buffer, pH 7.4, and where indicated, 20 μmoles of $MgCl_2$ or 20 μmoles of ATP, with water added to bring the total volume to 3.0 ml.

ability of the *P. pentosaceum* strain E214 extract to phosphorylate gluconate and then oxidize the resulting 6-PG, it seemed essential to determine the products of the latter dissimilation. On the manometric oxidation of 6-PG, initial R. Q.'s approaching 1 were obtained, suggesting an oxidative decarboxylation of 6-PG to form pentose phosphate. However, on analysis of the reaction mixture with orcinol, only low levels of pentose could be detected. On chromatographic analysis of the dephosphorylated reaction mixture a variety of products, including ribulose, ribose, sedoheptulose, fructose and pyruvate were detected.

Anaerobic incubation of 6-PG with the crude enzyme under the conditions outlined by Kovachevich and Wood (1955) to detect the presence of the 6-PG splitting system first reported in *Pseudomonas saccharophilia* by Entner and Doudoroff (1952), resulted in negligible pyruvate formation. Similar negative evidence for this 6-PG splitting system has been reported by Kovachevich and Wood (1955) with *Propionibacterium shermanii*. Thus it would appear the 6-PG degradation in this microorganism is via a yeast-type 6-PG dehydrogenase forming pentose phosphate, presumably riboluse-5-phosphate.

In an attempt to determine the pathway of pentose phosphate degradation by *P. pentosaceum* strain E214 an enzymatic study of R-5-P utilization was undertaken. The anaerobic breakdown of R-5-P resulted in the accumulation of compounds giving an orcinol reaction characteristic of sedoheptulose. A chromatographic analysis of this reaction mixture detected the formation of ribulose, fructose, and pyruvic acid, in addition to sedoheptulose. These data would appear to indicate that the ribulose-5-phosphate, formed on the oxidative decarboxylation of 6-PG, in isomeric equilibrium with R-5-P is transformed to sedoheptulose-7-phosphate (S-7-P) by transketolase, the heptulose being then converted to F-6-P by transaldolase. Thus, these chromatographic data serve as qualitative evidence for the presence of those enzymes unique to the "Horecker cycle" in *P. pentosaceum*.

However, the dissimilation of pentose phosphate apparently does not occur solely via this pathway, for on incubation of R-5-P with the crude sonic preparation under the conditions essential for the demonstration of triose phosphate dehydrogenase, i. e., in the presence of glutathione and arsenate, reduction of DPN could be demonstrated spectrophotometrically

(figure 5). Since the G-6-P and 6-PG dehydrogenases have been shown to be TPN-specific, DPN reduction under these conditions is indicative of the formation of triose phosphate and a 2-carbon fragment from pentose phosphate with the subsequent stepwise oxidation of the triose phosphate to pyruvic acid. Thus, it would appear that under the appropriate conditions a substantial portion of R-5-P degradation occurs by way of the latter route. Similar results have been demonstrated with the closely related bacterium *Microbacterium lacticum* (VanDemark and Wood, 1956).

The crude sonic preparation of gluconate-grown *P. pentosaceum* strain E214 also appeared to contain those enzymes characteristically found in conventional glycolysis. The presence of aldolase and phosphofructokinase were demonstrated by the methods of Sibley and Lehninger (1949), by which enzymatically formed triose phosphates are fixed as hydrazones and later estimated as chromogen formed by adding 2,4-dinitrophenylhydrazine and alkali. As shown in figure 6, triose phosphates are rapidly formed from F-1,6-P or

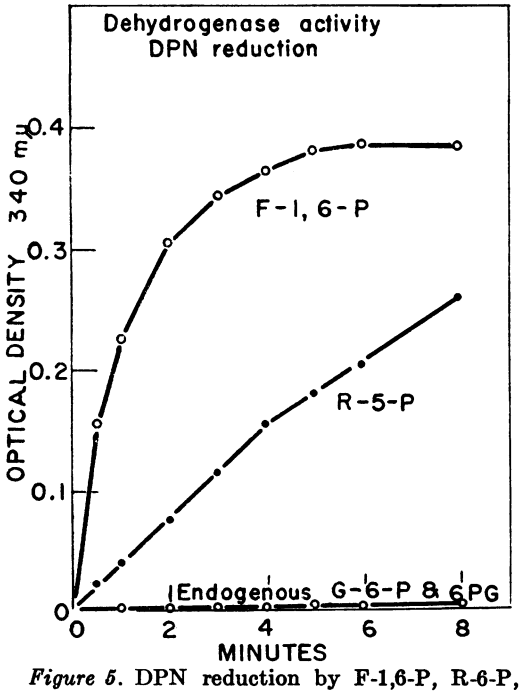

Figure 5. DPN reduction by F-1,6-P, R-6-P, and 6-PG. The cuvettes contained 1.5 ml 0.1 M veronal buffer, pH 7.8, 0.3 ml cell extract (approximately 3 mg protein), 0.5 μmoles DPN, 25 μmoles substrate, 12 μmoles sodium glutathione, 18 μmoles sodium arsenate, and water to 3 ml volume.

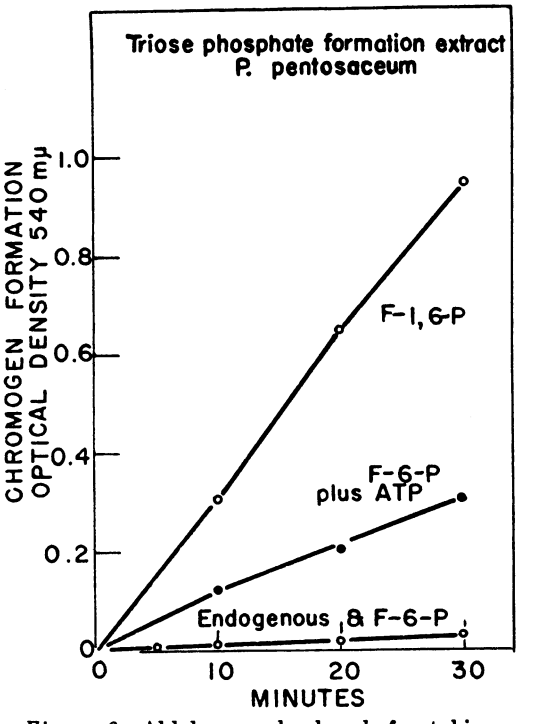

Figure 6. Aldolase and phosphofructokinase activity of a *Propionibacterium pentosaceum* extract. The incubation mixture contained 15 μmoles of substrate, 1.0 ml of enzyme (approximately 7 mg protein), 0.5 ml M Tris buffer, pH 8.6, 0.65 ml of 0.56 M hydrazine and water to a total volume of 7.5 ml. 6.0 μmoles ATP was added as indicated. One ml aliquots were added to 2 ml of 10 per cent trichloroacetic acid at the time intervals indicated and analyzed for the formation of triose phosphate ("chromogen").

F-6-P plus ATP. DPN reduction occurred on incubation of F-1,6-P with the crude enzyme (figure 5), indicating the presence of the enzymes aldolase, triose phosphate isomerase, and triose phosphate dehydrogenase.

The high activity of the enzymes of conventional glycolysis in the sonic preparation would seem to indicate that this pathway is participating in this dissimilation. A possible site of the entrance of the gluconate dissimilation into conventional glycolysis is through the "Horecker cycle" and the formation of the hexose phosphates.

However, the utilization of gluconate and the functioning of a Horecker cycle under anaerobic conditions must require a coupled dismutation with a hydrogen acceptor. Since the major end products of gluconate fermentation are propio-

nate and acetate, this dismutation may be in the reduction of various dicarboxylic acids leading to formation of propionate via succinate.

Considering the results of this enzymatic study, one notes the diversity of pathways operating in the degradation of gluconate. Considered in conjunction with the isotope data with the propionic acid bacteria (Wood *et al.*, 1955) this enzymatic diversity may well account for the considerable isotopic activity in all positions of the products resulting from the fermentation of labeled hexoses. Noting the high activity of those enzymes common to conventional glycolysis in the present study, this confirms the conclusions of Wood *et al.* from isotopic data that the major route of glucose dissimilation is via an Embden-Meyerhof type cleavage. Furthermore, the ready adaptation to gluconate and the presence of those enzymes which constitute the hexose monophosphate pathway, in addition to the presence of transketolase and transaldolase, supports the isotopic evidence for cleavages alternate to conventional glycolysis. For in the breakdown of glucose any portion of the substrate dissimilated by these alternate pathways results in a randomization of the isotopic labeling and a resultant complex isotope distribution pattern.

SUMMARY

Resting cell suspensions of gluconate-grown *Propionibacterium pentosaceum* strain E214 readily oxidize both glucose and sodium gluconate but not sodium 2-ketogluconate.

Sonic extracts of these cells oxidize glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-phosphate (F-1,6-P), 6-phosphogluconate (6-PG) and ribose-5-phosphate (R-5-P). Evidence for the existence in this preparation of gluconokinase, phosphohexose isomerase, phosphofructokinase, aldolase, and triose phosphate dehydrogenase were obtained.

In addition, TPN-linked G-6-P and 6-PG dehydrogenases were demonstrated. 6-Phosphogluconate was degraded to pentose phosphate, S-7-P, and F-6-P, and pyruvate. Anaerobically R-5-P is also degraded to the same products. Ribose-5-phosphate will also serve as a substrate for the reduction of DPN, thus indicating the occurrence of a more direct breakdown of this substrate.

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