PATHWAYS OF CARBOHYDRATE DEGRADATION IN *PSEUDOMONAS FLUORESCENS*[1]

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The unique ability of heterotrophic bacteria to degrade carbohydrates rapidly for the production of energy and intermediates for biosynthesis, often in preference to other natural organic compounds, has aroused the curiosity of all microbiologists. The wide variety of fermentation types described in the last 25 years has suggested the existence of many dissimilatory routes and has emphasized the differences among organisms. With the elucidation of the glycolytic mechanism of energy generation in muscle and in yeasts by Embden, Meyerhof and others, however, the concept evolved that this glycolytic pattern is the basic mechanism in all bacteria capable of carbohydrate utilization. The variation in ability to attack different carbohydrates and to form an array of products was not considered to be incompatible with this concept but rather to be due to diverse pathways to and from the glycolytic scheme at both the hexose and pyruvate stages. By an extension of these ideas even facultative and obligate aerobes could utilize the glycolytic route as part of the pathway for oxidation of glucose to pyruvic acid, which in turn is oxidized to carbon dioxide and water via an oxidative cycle such as the tricarboxylic acid cycle.

The presence of reactions converting carbohydrates to glycolytic intermediates and the presence of diverse routes from pyruvate to fermentation products have been amply documented. In addition, there is evidence that the glycolytic mechanism operates in lactobacilli, streptococci, members of the coli-aerogenes group, and in many other genera. While facultative aerobes have been reported to utilize the glycolytic and other pathways for glucose oxidation, evidence is not available for the participation of a glycolytic mechanism in carbohydrate oxidation by obligate aerobes. In fact, several observations, particularly in the pseudomonads and acetobacter species suggest that carbohydrate oxidation involves completely different reactions. Among these are the observations of Bernhauer and Knobloch (2), Lockwood *et al.* (21), and Koepsell (17) that large amounts of gluconic and ketogluconic acids are formed during growth on glucose. Another is the observation of Barron and Friedemann (1) that glucose oxidation by certain pseudomonads is insensitive to fluoride.

Thus, during the late 1940's when these and similar unrationalized observations with other microorganisms and tissues renewed a widespread interest in the mechanism of carbohydrate degradation, the lack of concepts applicable to bacterial carbohydrate oxidation prompted our investigation of this process in *Pseudomonas fluorescens*. This research was undertaken with the aim of elucidating the pathways from glucose to carbon dioxide which furnish biologically useful energy and intermediates for biosynthesis. Implicit in this aim was the necessity of studying individual enzymatic reactions and the effect of growth conditions upon enzyme activity. The rapid advances in knowledge and in concepts generated by the study of many tissues and cells have greatly aided this research. Particularly valuable have been the experiments of Horecker, Racker, Doudoroff and their collaborators. Since the approach to this problem required a measurement of each transformation or step in a metabolic chain, experiments were performed with crude extracts, obtained by sonic disruption, or with partially purified enzyme preparations.

The oxidative capacities of a crude extract of glucose-grown cells are shown in figure 1. These preparations, in the absence of accessory hydrogen acceptors, such as methylene blue, rapidly oxidize glucose, gluconate, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), 6-phosphogluconate (6-PG) and ribose-5-phosphate (R-5-P). Fructose-1,6-diphosphate (F-1,6-P) is oxidized very slowly, whereas 2-ketogluconate and pentoses are not

[1] Based upon the Eli Lilly and Company Award address in Bacteriology and Immunology. Delivered at the Fifty-fifth Annual Meeting of the Society of American Bacteriologists in New York, New York, May 12, 1955.

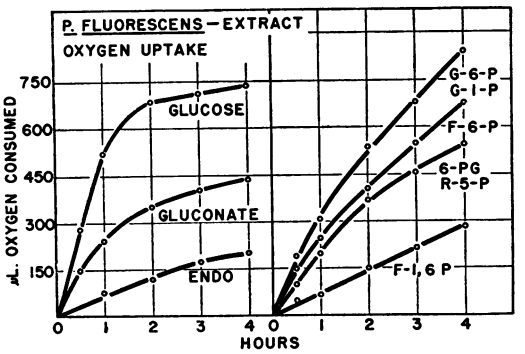

Figure 1. Oxidation of various substrates by a sonic extract of *Pseudomonas fluorescens*. The Warburg cups contained approximately 35 mg of protein, 20 μM of substrate, 100 μg of DPN (80 per cent purity), and Veronal buffer pH 7, in a fluid volume of 3 ml. Temperature, 37 C (35).

attacked. The oxidation of phosphate esters continues essentially to completion. For instance, as much as 5 μM of oxygen were consumed and 5 μM of CO_2 evolved per μM of G-6-P added. With glucose and gluconate, however, carbon dioxide was not produced. Thus, crude sonic extracts are excellent preparations for study since intact pathways for hexose phosphate degradation and the associated electron transport systems are present.

OXIDATION PRIOR TO PHOSPHORYLATION

Although hexose monophosphate was initially considered to be an intermediate in glucose oxidation, the fact that glucose-6-phosphate and 6-phosphogluconate oxidation yielded carbon dioxide whereas that of glucose and gluconate did not, suggested that independent systems mediated in the oxidation of glucose and G-6-P. Additional evidence supporting this conclusion (34) was obtained by adding phosphate buffer in manometric experiments with crude preparations or by employing enzyme preparations precipitated with ammonium sulfate. These treatments abolished G-6-P and 6-PG oxidation but left glucose and gluconate oxidation intact (figure 2). Thus it is clear that G-6-P and 6-PG are not intermediates in glucose and gluconate oxidation. Stokes and Campbell (31) also eliminated a role of phosphate esters in glucose oxidation by dried *Pseudomonas aeruginosa* by showing the ATP did not stimulate and that fluoride did not decrease the oxidation rate.

With 10 μM each of glucose and gluconate, 10 and 5 μM of oxygen were consumed respectively as would be expected from the conversion of these substrates to ketogluconate. Analysis of the cup contents by paper chromatography revealed ketogluconate to be the sole product arising from glucose and gluconate oxidation. The product was considered to be 2-ketogluconate rather than 5-ketogluconate since Entner and Stanier (10) have identified 2-ketogluconate as the product of glucose oxidation by this strain of *P. fluorescens*.

The complete systems for glucose, gluconate and G-6-P were associated with a red particulate fraction which was completely sedimented at 100,000 to 150,000 times gravity, or precipitated from one-third saturated ammonium sulfate. These particles, whose chemical composition and size distribution have been studied by Schachman, Pardee and Stanier (27), are intact respiratory units, which can be freed of soluble con-

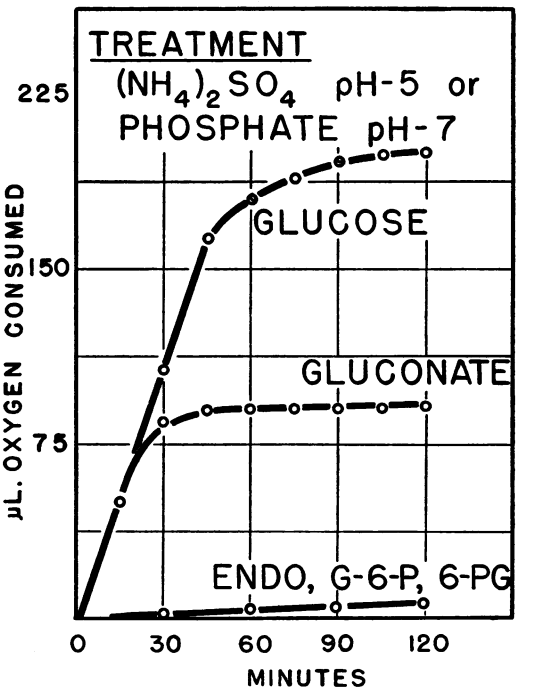

Figure 2. Glucose and gluconate oxidation in the absence of glucose-6-phosphate and 6-phosphogluconate oxidation. A sonic extract was either precipitated at 75 per cent ammonium sulfate saturation, pH 5, and tested in Veronal buffer, pH 7, or tested without ammonium sulfate treatment in 0.1 M phosphate buffer, pH 7. Other conditions were as in figure 1 except that 10 μM of substrate were used. (34)

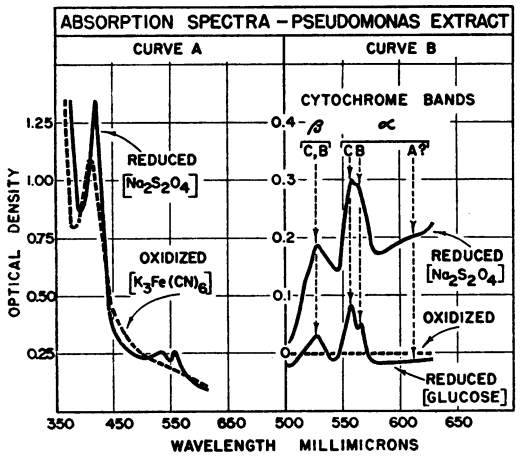

Figure 3. The cytochrome absorption spectra of crude extracts and particles from *Pseudomonas fluorescens*. Curve A, the cuvettes contained 0.5 ml of crude extract, 1.5 ml of 4 per cent sodium desoxycholate, 1 ml of water, 0.05 ml of one per cent potassium ferricyanide, and a few crystals of sodium hydrosulfite or glucose as indicated. The absorption was measured with a Beckman model DU spectrophotometer against a water blank. Curve B was the same as Curve A, except that the cuvette contained 1 ml of 4 per cent sodium desoxycholate and 2 ml of a particle suspension. A cuvette containing oxidized particles was used as the blank for setting the instrument. (34)

stituents by sedimentation or precipitation and dried *in vacuo* without loss of activity.

Several lines of evidence indicate that glucose and gluconate are oxidized by enzymes which differ from those heretofore described. In contrast to liver glucose dehydrogenase which utilizes diphosphopyridine nucleotide (DPN) as the hydrogen acceptor, DPN and triphosphopyridine nucleotide (TPN) neither stimulate the oxidation rate nor act as hydrogen acceptors for glucose oxidation. Similarly, the pseudomonas system does not reduce tetrazolium compounds as is characteristic of flavoproteins including glucose oxidase. Examination of the absorption spectrum in the presence of either glucose, gluconate, or hydrosulfite as reductants, revealed the formation of absorption bands which disappeared upon aeration or upon the addition of ferricyanide. The absorption spectra are shown in figure 3. The addition of hydrosulfite or glucose produced two small peaks at approximately 520 and 560 mμ and a more intense absorption in the Soret region (curve A). This spectrum is essentially identical with that reported by Stanier *et al.* (30) for particles from a mandelate-oxidizing preparation of this organism. To obtain more detailed spectra, the particles were separated from the soluble proteins and resuspended in a desoxycholate solution to improve the light transmission. The effect of light scattering was eliminated by using the oxidized cytochromes as a blank and measuring the difference spectrum due to reduction. As shown on the right hand side of figure 3, with glucose as the electron donor, a clearly defined absorption peak at 558 mμ and another peak in the region of 565 mμ were obtained. These correspond approximately to the α absorption peaks of mammalian cytochrome c and cytochrome b, respectively. The β absorption of these components occurred at about 530 mμ. With hydrosulfite the peak at 565 mμ is partly obscured and is visible only as a shoulder of the 558 peak. It is noteworthy that a peak was not observed in the region of 600 to 620 mμ. This fact together with the lack of appreciable cyanide inhibition at 10^{-3} M concentration suggests that a typical cytochrome oxidase is not present.

Thus, particles from *P. fluorescens* oxidize glucose and gluconate to 2-ketogluconate as observed in living cells. Since 2-ketogluconate was not degraded further, this system, for the time being, was considered to function in energy generation via oxidative phosphorylation but not to furnish carbon structures for biosynthesis.

OXIDATION OF PHOSPHATE ESTERS

As shown in figure 1, sonic preparations rapidly oxidize hexose monophosphate, 6-phosphogluconate and ribose-5-phosphate (34). In contrast to glucose and gluconate oxidations which result in 2-ketogluconate accumulation, phosphate ester oxidation continues essentially to completion. Further, the initial oxidations of G-6-P and 6-PG are catalyzed by pyridine nucleotide-linked dehydrogenases. These were studied in more detail by spectrophotometric measurement of pyridine nucleotide reduction. Typical reduction curves are shown in figure 4. In contrast to the yeast and mammalian G-6-P and 6-PG dehydrogenases, both DPN and TPN serve as electron acceptors. DPN reduction differs markedly from that of TPN in that following the initial reduction, a lag or stationary period was observed; after 1 to 15 minutes there was a return to the maximal rate.

Subsequently, it was found that the concentration of DPN·H could be rapidly decreased even in the presence of excess glucose-6-phosphate, by stirring the cuvette contents. Hence the lag was attributed to equal rates of DPN·H formation (at the expense of G-6-P) and DPN·H oxidation by dissolved oxygen. Since tetrazolium compounds serve as hydrogen acceptors for G-6-P oxidation in the presence of DPN, and for the oxidation of DPN·H alone, a flavoprotein DPN·H dehydrogenase appears to mediate in electron transport from DPN·H to the cytochromes.

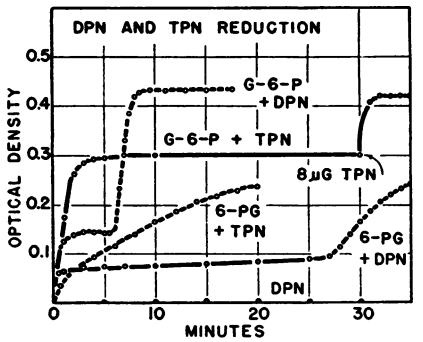

Figure 4. DPN and TPN reduction by glucose-6-phosphate and 6-phosphogluconate. The quartz micro cuvettes (l = 1 cm) contained 0.015 ml of sonic extract, 0.05 ml of Veronal buffer, pH 7, 0.04 μM of DPN or 0.03 μM of TPN, 3.2 μM of G-6-P or 6-PG, and water to 0.5 ml. (35)

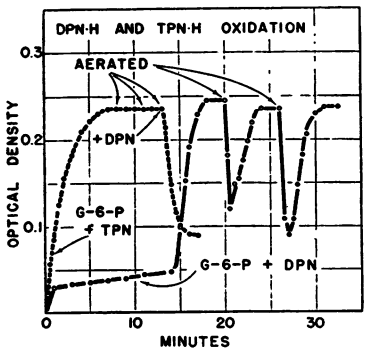

Figure 5. DPNH and TPNH oxidation by a sonic extract of *Pseudomonas fluorescens*. The cuvettes contained 0.15 ml of sonic extract, 5 μM of $MgCl_2$, 12 μM of glycylglycine buffer, pH 7.4, and 0.05 μM of TPN or DPN. 0.02 μM (solid circles) or 1.6 μM (open circles) of G-6-P were added. The volume was 0.5 ml. The reaction mixture was aerated by vigorous stirring. DPN was added as indicated. (35)

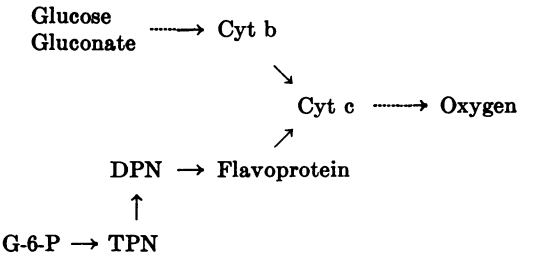

Figure 6. Electron transport in particles from *Pseudomonas fluorescens*.

TPN reduction occurs without a lag and TPN·H oxidation even in absence of G-6-P is not initiated by aeration at the points indicated (figure 5). Upon the addition of DPN, however, a rapid oxidation of TPN·H occurred. This activation of TPN·H oxidation is considered evidence for the presence of pyridine nucleotide transhydrogenase, an enzyme discovered in *P. fluorescens* by Colowick *et al.* (4), which reduces DPN with TPN·H as the donor. The DPN·H is then oxidized as has been described.

These data suggest that electrons follow different routes in glucose and G-6-P oxidation. The pathways may be similar to those postulated by Slater (29) for the oxidation of succinate and G-6-P by a beef heart preparation (figure 6) as follows: G-6-P dehydrogenase oxidizes G-6-P with the reduction of TPN, which is not reoxidizable except via DPN. In the presence of DPN, pyridine nucleotide transhydrogenase forms DPN·H, which in turn is oxidized by a diaphorase presumably with the reduction of cytochrome c. Glucose oxidation like that of succinate is on another leg of this system, not involving pyridine nucleotide carriers but reducing cytochromes b and c instead. Unidentified carriers may operate in the region of the dotted lines. Aside from glyceraldehyde-3-phosphate dehydrogenase which also utilizes DPN as an acceptor, these are the only oxidations thus far encountered between glucose and pyruvate.

ALTERNATE ROUTES OF 6-PHOSPHOGLUCONATE UTILIZATION

During the foregoing studies several observations were made which could not be explained. Among these were the disappearance of 6-PG without significant pentose formation, the oxidation of 6-PG by the soluble protein fraction with DPN but not TPN as the electron acceptor (33),

and an apparently unrelated observation made in collaboration with Doctor Martin Gibbs that resting cells converted carbon atom 3 or 4 of glucose to carbon dioxide at the same rate as carbon one. These observations were clarified by the discovery of Entner and Doudoroff (9) that enzymes from *Pseudomonas saccharophila* cleaved 6-phosphogluconate between carbon atoms 3 and 4 to form pyruvate and a triose phosphate, presumably glyceraldehyde-3-phosphate (G-3-P).

Subsequently, it was shown that two routes of 6-PG degradation were present, the oxidation presumably to pentose phosphate and anaerobic conversion to pyruvate and triose phosphate. The velocity of the latter reaction, however, was at least 10-fold the rate of 6-phosphogluconate dehydrogenase (35). Thus, this alternate route from 6-phosphogluconate to pyruvate and by-passing pentose phosphate, accounted for the lack of pentose production accompanying 6-PG utilization. Similarly, the observed reduction of DPN by the soluble proteins can now be attributed to the production of triose phosphate from 6-PG via 2-keto-3-deoxy-6-phosphogluconate and the oxidation of G-3-P by G-3-P dehydrogenase. Entner and Doudoroff (9) postulated a two-step reaction mechanism involving the dehydration of 6-phosphogluconate between carbon atoms 2 and 3, ketonization of the enol produced to form 2-keto-3-deoxy-6-phosphogluconate (KDPG), and cleavage of KDPG between carbons 3 and 4 to yield pyruvate and G-3-P.

In order to test this postulate and to elucidate the mechanism of this new and quantitatively important pathway in *P. fluorescens*, purification and separation of the enzymes were undertaken. Since the identity of the intermediates in this pathway had not been established, the rates of 6-PG disappearance and of pyruvate formation were utilized for the enzyme assays. A fractionation procedure which completely resolved the system into two activities with approximately 30-fold purification of each fraction is shown in figure 7 (18, 19). Fraction A, precipitated between 25 per cent and 40 per cent ammonium sulfate saturation, catalyzes the disappearance of 6-PG and the formation of an unidentified phosphate ester which serves as a substrate for fraction B. Fraction A was completely inactive unless both ferrous ions and glutathione (GSH) were added.

In the presence of Fe^{++} and GSH, large amounts of the intermediate were produced and isolated as the barium salt essentially free of 6-PG. The ester was distinguished from other compounds by its Rf value and by reaction with carbonyl spray reagents such as *m*-phenylenediamine, *p*-anisidine and alkaline copper reagent. The presence of organic phosphate was revealed by the arsenomolybdate spray reagent. Evidence for the location of the carbonyl group was obtained by using 6-phosphogluconate-1-C^{14} as the substrate. Quantitative decarboxylation of the intermediate by ceric sulfate gave a complete recovery of the radioactivity as carbon dioxide. Although no evidence was obtained for the deoxy group, these data are consistent with the intermediate being the postulated 2-keto-3-deoxy-6-phosphogluconate.

Fraction B, in the presence of crystalline rat liver lactic dehydrogenase and DPN·H, completely converts KDPG to lactate with a corresponding decrease in optical density at 340 mμ.

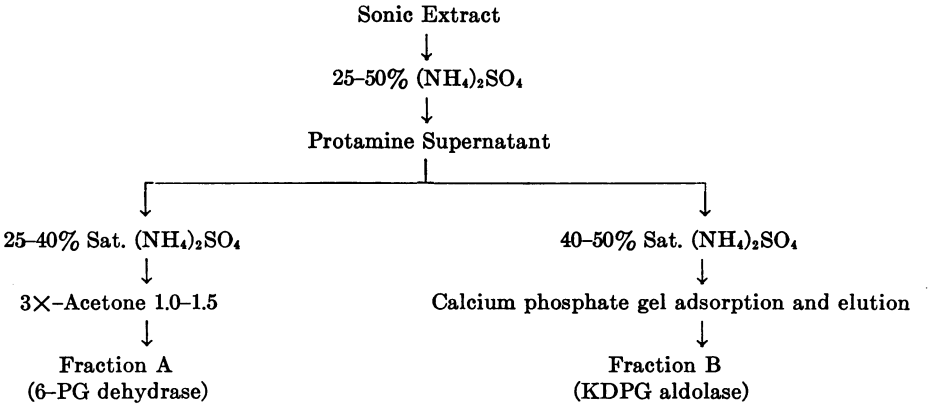

Figure 7. Fractionation of 6-PG cleaving system from *Pseudomonas fluorescens*.

This coupled reaction affords a means of KDPG determination which was used to measure the stoichiometry of 6-PG utilization (figure 8). Within the limits of measurement of 6-PG disappearance and KDPG formation, 6-PG is completely converted to KDPG. The broken line indicates the amount of 6-PG added.

The products of KDPG cleavage by fraction B were identified and the stoichiometry determined spectrophotometrically with crystalline enzymes (figure 9). In a cuvette containing DPN·H, crystalline lactic dehydrogenase and KDPG, the addition of fraction B or KDPG aldolase caused a rapid decrease in optical density equivalent to the amount of substrate added, thereby indicating the complete conversion of KDPG to pyruvate. After the reaction had ceased, the addition of crystalline rabbit muscle G-3-P dehydrogenase regenerated essentially the original amount of DPN·H. Thus an equivalent amount of G-3-P also was present. The center portion of the graph illustrates the same stoichiometry except that G-3-P was determined first and pyruvate second. The right-hand part of the graph shows a test for the presence of triose phosphate isomerase in KDPG aldolase, or fraction B. In the presence of crystalline rabbit muscle fructose-1,6-phosphate aldolase and G-3-P dehydrogenase, added F-1,6-P yields one equivalent of DPN·H rather than the two expected if isomerase were present. After DPN reduction had ceased, the addition of KDPG aldolase did not produce more DPN·H, whereas the addition of a partially purified rabbit muscle triose phosphate isomerase resulted in the formation of another increment of DPN·H. Since isomerase is not present in KDPG aldolase, it can be concluded that 2-keto-3-deoxy-6-phosphogluconate cleavage yields equimolar amounts of pyruvate and D-glyceraldehyde-3-phosphate.

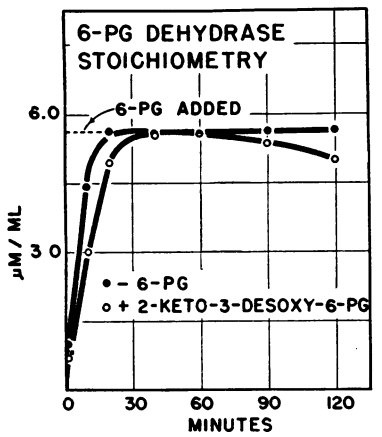

Figure 8. The conversion of 6-PG to KDPG by 6-PG dehydrase. The reaction mixture contained 200 μM of tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.65, 2 units of KDPG aldolase (40 to 50 per cent $(NH_4)_2SO_4$ fraction), 3 μM of NaGSH, 6 μM of $FeSO_4$, 5.6 μM of Na-6-PG, 3 units of 6-PG dehydrase, and water to 1 ml. Aliquots were removed at the intervals indicated, heated in boiling water for 2 minutes, and a suitable dilution assayed for 6-PG and KDPG. (18)

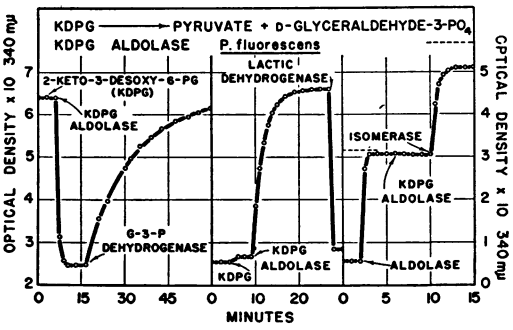

Figure 9. The identification of the end-products of KDPG aldolase action. In the left-hand section, the 3 ml reaction mixture contained 500 μg of lactic dehydrogenase, 300 μM of Tris buffer, pH 7.65, 0.14 μM of DPNH, 0.14 μM of DPN, 6 μM of sodium arsenate, 12 μM of NaGSH, 0.17 μM of KDPG, 3 units of KDPG aldolase, and 34 μg of crystalline G-3-P dehydrogenase. The KDPG, KDPG aldolase, and G-3-P dehydrogenase were added at the times indicated. The conditions were the same for the center section, except that DPNH was not added, and KDPG, KDPG aldolase, and lactic dehydrogenase were added at the times indicated. In the right-hand section the test conditions were those of the center section, except that the 3 ml reaction mixture contained 3.4 mg of G-3-P dehydrogenase, 500 μg of crystalline aldolase, 0.127 μM of fructose-1,6-diphosphate, and 0.03 ml of partially purified triose phosphate isomerase. The aldolase, KDPG aldolase, and triose phosphate isomerase were added as indicated. (19)

The approximate equilibrium of KDPG cleavage can be seen in figure 10. On the right, with β-glycerolphosphate buffer, which does not react with G-3-P, the utilization of KDPG and the formation of pyruvate and G-3-P, ceased after 30 minutes. At this point the reaction had proceeded about 70 per cent in the direction of

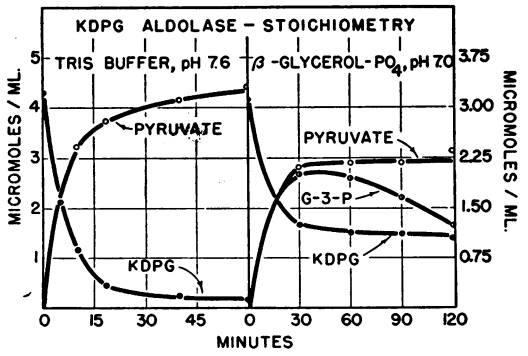

Figure 10. Stoichiometry of KDPG aldolase action. In the left-hand section the 1 ml reaction mixture contained 200 μM of Tris buffer, pH 7.65, 4.3 μM of KDPG, and 2 units of KDPG aldolase. Aliquots of the reaction mixture were removed at the times indicated, the reaction was stopped by boiling for 2 minutes, and aliquots were removed for pyruvate and KDPG determinations. In the right-hand section the conditions were the same, except that the reaction mixture contained 100 μM of β-glycerol phosphate hydrochloride buffer, pH 7, and 3.15 μM of KDPG. The reaction was stopped by adding trichloroacetic acid. (19)

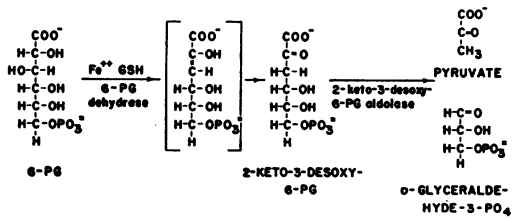

Figure 11. Mechanism of 6-phosphogluconate cleavage.

cleavage. Beyond this time G-3-P began to disappear. An approximate equilibrium constant calculated from the 30-minute values is

$$\frac{(\text{Pyr})(\text{G-3-P})}{(\text{KDPG})} = 3.4 \times 10^{-3} \text{ m/l.}$$

With trishydroxymethylamino methane buffer, the cleavage reaction proceeds essentially to completion. The shift in equilibrium is attributed to a reaction between the buffer and G-3-P as reported by Segal and Boyer (28).

While this investigation was in progress, MacGee and Doudoroff (22) using enzyme preparations from *P. saccharophila*, isolated the crystalline trisodium salt of the intermediate, established its structure as 2-keto-3-deoxy-6-phosphogluconate and demonstrated its conversion to pyruvate and G-3-P. A sample of this material donated by them was cleaved by KDPG aldolase from *P. fluorescens* to yield an equivalent amount of pyruvate.

Thus the results obtained with enzymes from *P. fluorescens* are in agreement with the mechanism postulated by Entner and Doudoroff, and are summarized in figure 11. 6-phosphogluconate is converted to 2-keto-3-deoxy-6-phosphogluconate by an enzyme requiring ferrous ions and glutathione. A dehydration between carbon atoms 3 and 4 and a presumably non-enzymatic ketonization of the enol in brackets would accomplish this conversion. Hence the enzyme provisionally is termed 6-phosphogluconate dehydrase. The second enzyme termed KDPG aldolase catalyzes an aldolase-type cleavage of 2-keto-3-deoxy-6-phosphogluconate. A combination of these purified enzymes effects the over-all conversion originally observed. Since glyceraldehyde-3-phosphate is converted to pyruvate by crude extracts, 2 moles of pyruvate are formed as in glycolysis. In contrast to the glycolytic scheme, however, one pyruvate carboxyl arises from carbon 1 and one from carbon 4. By this mechanism plus decarboxylation of pyruvate, carbon dioxide can be formed equally well from carbon atoms 1 and 4 of glucose as was previously observed.

REACTIONS OF RIBOSE-5-PHOSPHATE

Although 6-phosphogluconate oxidation by sonic preparations, presumably to pentose phosphate, occurs slowly, the rapid oxidation of ribose-5-phosphate, and its utilization under anaerobic conditions indicates an important role of this intermediate in carbohydrate oxidation. Analysis of reaction mixtures by paper chromatography revealed that ribose-5-phosphate is degraded by reactions similar to those already described by Horecker *et al.* (16) and de la Haba *et al.* (5) for rat liver, spinach and yeast preparations in that heptulose phosphate is a reaction product. The disappearance of ribose was accompanied by a sequential appearance and disappearance of ribulose, sedoheptulose, fructose and finally glucose. Quantitative determination of this reaction sequence is shown in figure 12. The rapid disappearance of approximately 17 μM of pentose was accompanied by an initially rapid accumulation of heptulose. After 10 minutes, however, heptulose began to disappear and hexose

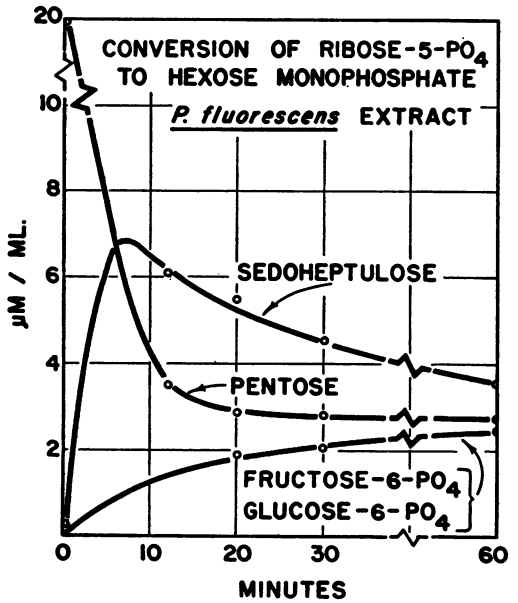

Figure 12. The conversion of ribose-5-phosphate to hexose monophosphate by crude extracts. The reaction mixture contained 3.5 ml crude extract (approximately 20 mg per ml) 20 μM of R-5-P, 0.7 ml Tris buffer, pH 7.65, and water to 7 ml. Aliquots were removed at the intervals indicated for analysis.

monophosphate formation was evident. These data indicate that *P. fluorescens* contains transaldolase and transketolase, two transferases discovered by Horecker (13–15), which were shown by Racker *et al.* (26) and Horecker *et al.* (12, 15) to convert pentose phosphate to fructose-6-phosphate. Thus, as in yeast and mammalian tissue, the oxidation of hexose monophosphate via pentose phosphate and heptulose phosphate can regenerate fructose-6-phosphate and glucose-6-phosphate. This cyclic mechanism was proposed by Horecker (11) and Racker (25) as a pathway for hexose monophosphate oxidation. The products would be carbon dioxide and glyceraldehyde-3-phosphate. Whether this cycle actually operates under oxidative conditions where G-3-P dehydrogenase can compete for the G-3-P required as an acceptor for transaldolase has not been established.

THE GLYCOLYTIC PATHWAY

Several lines of evidence indicate that *P. fluorescens* does not possess a complete Embden-Meyerhof glycolytic system. Firstly, resting cells do not ferment glucose, and secondly, enzyme preparations are unable to ferment G-6-P or F-1,6-P, or oxidize F-1,6-P. An inventory of glycolytic enzymes revealed that aldolase and phosphohexose isomerase were present in low concentration compared to the amount of glyceraldehyde-3-phosphate dehydrogenase and enzymes for conversion of 3-phosphoglycerate to pyruvate. Furthermore, hexokinase and phosphohexokinase could not be detected. The lack of an intact glycolytic scheme is in agreement with the conclusions reached by Lewis and collaborators (20) who found that in resting *P. fluorescens*, C^{14} from position-labeled glucose reached acetate and pyruvate by non-glycolytic pathways. In the light of the foregoing data on the hexose monophosphate pathways, the presence of those glycolytic enzymes which are present is best explained by their functioning in the hexose monophosphate systems.

2-KETOGLUCONATE TO PYRUVATE

With the presence of an active glucose-oxidizing system and of hexose monophosphate routes amply demonstrated, a serious gap in our knowledge was evident. The oxidation of glucose could furnish energy for biosynthesis, presumably via oxidative phosphorylation, and the hexose monophosphate routes could form intermediates such as pyruvate and pentose phosphate for biosynthesis. No means had been discovered, however, for converting glucose to intermediates —that is, a connecting link between the pathways of hexose or hexonic acid oxidation and of phosphate ester degradation had not been found. Repeated attempts to demonstrate hexokinase, using the sensitive spectrophotometric determination of glucose-6-phosphate, were negative. In addition, there had been no success in demonstrating reactions for glucose dissimilation beyond 2-ketogluconate.

A solution to this problem was ultimately found in the observations that sonic extracts phosphorylate both gluconate and 2-ketogluconate and that this process is accompanied by pyruvate formation (23, 24). These reactions are catalyzed by typical magnesium requiring kinases similar to the inducible gluconokinase discovered by Cohen (3) in *Escherichia coli* and the inducible gluconokinase and 2-ketogluconokinases found in *Aerobacter cloacae* by DeLey (6). As shown in figure 13, the formation of both gluconokinase and 2-ketogluconokinase in *P.*

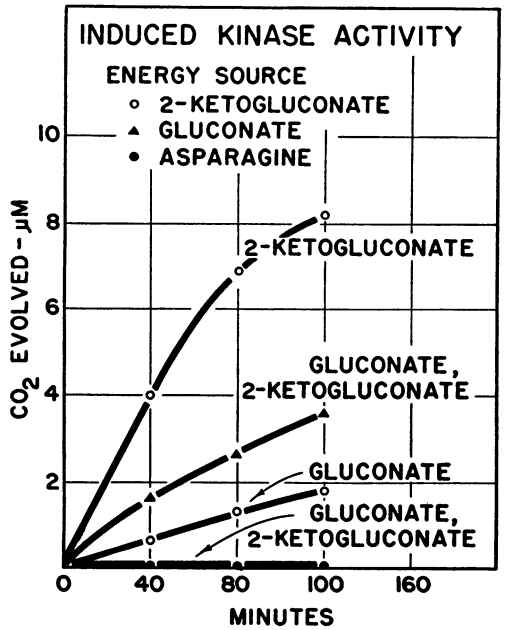

Figure 13. Gluconokinase and 2-ketogluconokinase content of cells grown on gluconate (▲), 2-ketogluconate (○), and asparagine (●). Kinase assays were performed in Warburg vessels containing 0.3 ml of 0.43 M sodium bicarbonate, 0.3 ml of 0.1 M magnesium chloride, 0.3 ml of 0.1 M ATP (30 µM), 1.0 ml of enzyme extract (approximately 20 mg per ml), 0.3 ml of 0.1 M gluconate or 2-ketogluconate (30 µM) and 1.1 ml of water. The gas phase was 95 per cent N_2-5 per cent CO_2. The incubation temperature was 30 C. The dried *Pseudomonas fluorescens* used as a kinase source was initially grown in an asparagine medium and then transferred aseptically to a secondary energy source of either 0.5 per cent 2-ketogluconate, gluconate or asparagine before harvesting. Extracts were prepared by sonic vibration and tested for kinase activity as indicated.

fluorescens is induced by growth on their respective substrates. Glucose and gluconate-grown cells contained both gluconokinase and 2-ketogluconokinase as would be expected, since both gluconate and 2-ketogluconate are formed by glucose oxidation. 2-ketogluconate-grown cells contain largely 2-ketogluconokinase whereas asparagine-grown cells are devoid of both kinases. This experiment revealed that 2-ketogluconokinase formation can be induced without concomitant gluconokinase formation. Hence different kinases are involved in gluconate and 2-ketogluconate phosphorylation.

The product of gluconate phosphorylation serves as the substrate for purified yeast 6-phosphogluconate dehydrogenase, and migrates on paper chromatograms identically with known 6-PG. The product of 2-ketogluconate phosphorylation by crude extracts is readily converted to pyruvate and hence does not accumulate. After partial purification of 2-ketogluconokinase by ammonium sulfate fractionation and dialysis, however, the phosphate ester accumulates. Large quantities of this compound were prepared and recovered as the barium salt, purified as the ammonium salt by chromatography on powdered cellulose and finally obtained as the barium salt essentially free of inorganic phosphate, nucleotides and 2-ketogluconate. The ester contains one acid-stable phosphate group per reducing group, or per molecule. After treatment with phosphatase, 2-ketogluconate is regenerated and the expected amount of inorganic phosphate appears. The properties of the phosphate ester, 2-ketogluconate and the product of dephosphorylation are compared in table 1. The ultraviolet absorption spectra of 2-ketogluconate semicarbazone and that of the phosphate ester are identical with a maximum at 350 mµ. Similarly, the spectra of the quinoxalines are identical in the ultraviolet region with a maximum at 335 mµ; the 330 to 360 ratio is 1.5 in both cases.

Paper chromatography in methanol-ammonia-water (60:10:30) and in water-saturated phenol gives identical Rf values for 2-ketogluconate and for the dephosphorylation product. With both, the Rf value is considerably different from that of the phosphate ester. All three compounds reduce copper reagent, and give a brown spot with *p*-anisidine. The phosphate ester is differentiated from 2-ketogluconate by the blue color produced with the phosphate spray. Similarly, with *o*-phenylenediamine, a green spot is obtained with the phosphate ester, whereas a purple spot is obtained with both 2-ketogluconate and the dephosphorylation product. These data are all consistent with the phosphate ester being 2-keto-6-phosphogluconate (23, 24) and are in agreement with DeLey's (7, 8) characterization by similar tests of the product of 2-ketogluconate phosphorylation by *A. cloacae*.

Both 2-keto-6-phosphogluconate and 2-ketogluconate plus ATP yield approximately 2 moles of pyruvate per mole of substrate added. The pyruvate was identified by column and paper chromatography as the sole keto acid produced. The melting point of its 2,4-dinitrophenylhydrazone agreed with that of the authentic pyruvate 2,4-dinitrophenylhydrazone.

TABLE 1

Properties of the product of 2-ketogluconate phosphorylation

Test	Phosphate Ester	2-Ketogluconate	Dephospho Compound
Derivatives			
Semicarbazone	Max. 350 mμ	Max. 350 mμ	—
Quinoxaline	Max. 335 mμ 320/360 = 1.5	Max. 335 mμ 320/360 = 1.5	—
Chromatography			
Rf			
MeOH-NH_4OH-H_2O (60:10:30)	0.46	0.60	0.60
Phenol-H_2O	0.00	0.40	0.40
Acetone-25% TCA (35:65)	0.43	0.68	—
Spray reaction			
Cu-arsenomolybdate	Blue	Blue	Blue
Semicarbazide	UV absorb.	UV absorb.	UV absorb.
NH_4 molybdate-$HClO_3$	Blue	Colorless	Colorless
p-anisidine	Brown	Brown	Brown
o-phenylenediamine	Purple	Yellow-green	Yellow-green

The pathway from 2-ketogluconate to pyruvate has been investigated with 2-ketogluconate-1-C^{14} and 2-ketogluconate-6-C^{14} as substrates (32). These were prepared by quantitative oxidation of the corresponding glucoses to 2-ketogluconate with the bacterial particles already described. In the presence of ATP and the sonic extract, these labeled substrates were converted to pyruvate. The pyruvate was isolated on celite columns and degraded to locate the isotope. With 2-ketogluconate-1-C^{14}, the carboxyl group of pyruvate contained 56 per cent the specific activity of carbon 1 of 2-ketogluconate-1-C^{14}, whereas with 2-ketogluconate-6-C^{14}, the methyl group of pyruvate contained approximately 20 per cent the specific activity of carbon atom 6. The other positions of pyruvate were unlabeled in each case. These data indicate that pyruvate is formed by a pathway in which the carboxyl groups arise from carbon 1 and an unlabeled carbon presumably 4, and the methyl group from carbon atom 6 and an unlabeled carbon presumably carbon atom 4. The difference in dilution of isotope with 2-ketogluconate-1-C^{14} as compared with 2-ketogluconate-6-C^{14} may be due to the slower conversion of carbon atoms 4, 5 and 6 to pyruvate. The data can be explained by the operation of dehydration and cleavage reactions for 2-keto-6-phosphogluconate similar to those in the Entner-Doudoroff cleavage of 6-phosphogluconate. In this case, however, 2,4-diketo-3-deoxy-6-phosphogluconate would be the intermediate which is formed and then cleaved to pyruvate and 3-phosphoglycerate. The 3-phosphoglycerate can then be converted to pyruvate. These data do not eliminate another possibility suggested by DeLey (private communication); namely, that a mixed dismutation between glyceraldehyde-3-phosphate and 2-keto-6-phosphogluconate to form phosphoglycerate and 6-phosphogluconate is functioning. The 6-phosphogluconate so formed is then degradated via the Entner and Doudoroff mechanism.

Thus our current concepts of the pathways of glucose oxidation have been expanded to include this new pathway from 2-ketogluconate to pyruvate. Further, these hexonic acid phosphorylations provide an insight as to the means of entering the pathways of degradation involving phosphate esters.

SUMMARY

The pattern of carbohydrate dissimilation in *Pseudomonas fluorescens* is summarized in figure 14. Glucose is oxidized by a particulate respiratory unit to gluconate and 2-ketogluconate without substrate phosphorylation. There is evidence for cytochromes b and c but not for pyridine nucleotides and flavoproteins functioning in these oxidations. 2-ketogluconate is phosphorylated to 2-keto-6-phosphogluconate. Approximately 2 moles of pyruvate are formed from 2-ketogluconate or from 2-keto-6-phosphogluconate by an undefined and presumably new pathway. Thus one route from glucose to pyruvate proceeds across the top and down the right-hand side of figure 14.

Two alternate routes begin with the phosphorylation of gluconate to 6-phosphogluconate. One involves the ferrous ion and thiol-dependent

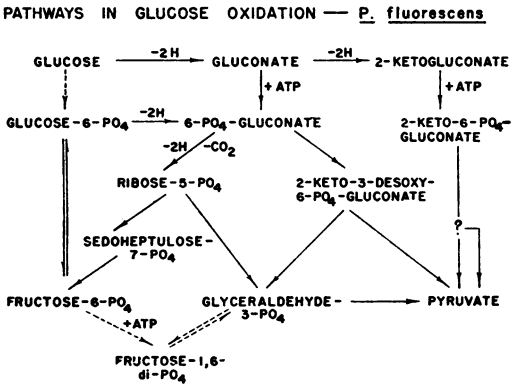

Figure 14. Pathways of glucose oxidation in *Pseudomonas fluorescens.*

dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate and cleavage of this intermediate to pyruvate and D-glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is then oxidized to pyruvate. The other route involves 6-phosphogluconate oxidation presumably to a mixture of ribose-5-phosphate and ribulose-5-phosphate which are then converted to sedoheptulose-7-phosphate, fructose-6-phosphate and glucose-6-phosphate. Glyceraldehyde-3-phosphate is produced as a result of hexose phosphate synthesis. This also can yield pyruvate. Glucose-6-phosphate is oxidized to 6-phosphogluconate and part of the 6-phosphogluconate may then form ribulose-5-phosphate and proceed around the cycle again.

The glycolytic reactions are shown at the left of figure 14. Evidence has not been obtained for hexokinase, and phosphohexokinase and aldolase activity is weak. Phosphohexose isomerase, glyceraldehyde-3-phosphate dehydrogenase and the enzymes for the conversion of 3-phosphoglycerate to pyruvate have been found. As emphasized by this graphic portrayal of the reactions, these glycolytic enzymes may function equally well in the hexose monophosphate systems. Fructose-1,6-diphosphate is the only glycolytic intermediate not involved in the pathways thus far demonstrated; hence its function has not been established in *P. fluorescens.*

Just as our concepts of glucose oxidation at the initiation of this research now seem naive, so undoubtedly will be these ideas in the near future. However, one point has been amply substantiated namely that *P. fluorescens* truly illustrates the concept of alternate or multiple pathways of carbohydrate degradation.

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Figures 1, 2, 3, 4, 5, 8, 9, and 10 were originally published in *The Journal of Biological Chemistry* in the cited references. We thank the copyright owner for permission to reproduce these.

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