DISSIMILATION OF GLUCOSE AND GLUCONIC ACID BY *PSEUDOMONAS NATRIEGENS*[1]

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Received for publication November 10, 1961

ABSTRACT

EAGON, R. G. (University of Georgia, Athens) AND C. H. WANG. Dissimilation of glucose and gluconic acid by *Pseudomonas natriegens*. J. Bacteriol. **83**:879-886. 1962—When glucose dissimilation of a marine pseudomonad, *Pseudomonas natriegens*, was studied, enzymes of both the glycolytic pathway and of the hexose monophosphate pathway were detected in extracts of glucose-grown cells. Enzymes of the Entner-Doudoroff pathway and phosphoketolase were not detected. Data from radiorespirometric experiments indicated that approximately 92 and 8% of glucose actually catabolized were routed via the glycolytic and the hexose monophosphate pathways, respectively.

When *P. natriegens* was induced to utilize gluconate, it was demonstrated that gluconokinase and enzymes of the Entner-Doudoroff pathway were induced. Radiorespirometric experiments with cells under growing conditions revealed that gluconate was dissimilated predominantly (80%) via the Entner-Doudoroff pathway. This observation was in contrast to the observation that the glycolytic pathway is practically the exclusive catabolic pathway for glucose dissimilation. A minor portion of substrate gluconate was also catabolized by this organism via the hexosemonophosphate pathway. However, the pentose phosphate derived from substrate gluconate is believed not to be catabolized extensively.

The important facet uncovered by these experiments was the extensive operation of the glycolytic route of glucose dissimilation. This is in contrast to other pseudomonads studied to date, which have been reported to dissimilate glucose predominantly via the Entner-Doudoroff pathway and which do not utilize the glycolytic pathway.

[1] Contribution no. 37 from the University of Georgia Marine Institute at Sapelo Island.

The bacterium used in this investigation, *Pseudomonas natriegens*, was first isolated from salt-marsh mud, and its morphology and physiology were described by Payne (1958, 1960) and by McRorie, Williams, and Payne (1959). It was observed from these studies that this bacterium had the interesting property of producing enough acid in carbohydrate broth under aerobic conditions to give a positive methyl red test. These acids, resulting from glucose dissimilation, were identified by Payne, Eagon, and Williams (1961) as acetic, lactic, and pyruvic acids. CO_2 was also produced. It was also reported that this microorganism contained a constitutive enzyme system(s) for the dissimilation of glucose and that it was predominately an aerobe with both the rate and extent of growth being greatly reduced under anaerobic conditions (Lobley and Eagon, 1961; Payne, 1958). At the same time, there was preliminary evidence for the participation of the Krebs' tricarboxylic acid cycle.

Additional studies were carried out on its morphology and physiology, and the specific epithet, *P. natriegens*, was proposed by Payne et al. (1961).

The observation of the accumulation of acetic, lactic, and pyruvic acids and the liberation of CO_2 from aerobic cultivation in glucose offered interesting speculations regarding probable pathways of carbohydrate dissimilation. Thus, experiments described in this paper were undertaken to elucidate the pathways involved in glucose and in gluconic acid dissimilation by *P. natriegens*.

MATERIALS AND METHODS

Growth of organism. P. natriegens was grown in 500-ml Erlenmeyer flasks containing 100 ml of medium of the following composition: glucose or potassium gluconate, 2 g; dehydrated nutrient broth, 8 g; sea salt, 15 g; Na_2HPO_4, 12.5 g; KH_2PO_4, 5 g; $(NH_4)_2SO_4$, 0.5 g; NH_4Cl, 0.5 g; and distilled water to 1 liter. Additional Na_2HPO_4

was added to pH 7.0. Each flask was inoculated with 1 ml of a fully grown culture and incubated on a rotary shaker for 12 to 16 hr at 30 C.

Preparation of cell-free extracts. The cells were harvested by centrifugation, washed with a 0.052 molar solution of $MgCl_2$, suspended in two volumes of 0.1 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.5), and disintegrated with a Raytheon 200-w, 10-kc magnetostrictive oscillator for 10 min. The solution was then centrifuged at 12,000 $\times$ *g* for 30 min at 0 C to remove cell debris. This extract was dialyzed against 0.1 M tris buffer (pH 7.5) for 24 hr. Extracts prepared in this manner could be frozen for several weeks with no appreciable loss of activity.

Radiotracer experiments. Radiorespirometric experiments, which measure the rate of $C^{14}O_2$ production from individual substrate carbon atoms, were carried out in a manner essentially the same as that described by Wang et al. (1958). C^{14} specifically labeled glucose samples were obtained from the National Bureau of Standards through the kind cooperation of H. S. Isbel, except [3,4-C^{14}] glucose, which was prepared in this laboratory according to the method of Wood, Lifson, and Lorber (1945). C^{14} specifically labeled gluconate samples were prepared from correspondingly labeled glucose specimens according to the method of Moore and Link (1940). The radioactivity in CO_2 samples (in the form of hyamine carbonate), cells, and incubation media was determined by means of a liquid scintillation counter in the manner described by Wang and Krackov (*in press*).

RESULTS

Enzymological studies on extracts of glucose-grown cells. Table 1 indicates those enzymes detected, as well as those not detected, in extracts of glucose-grown cells. Glucokinase was demonstrated manometrically by the phosphorylation of glucose by adenosine triphosphate (ATP) and cell-free extract. Phosphorylation of fructose and mannose was not observed.

Aldolase activity was determined by using hydrazine to trap triose phosphates formed from cleavage of fructose-1,6-diphosphate, followed by development of a colored product with 2,4-dinitrophenyl-hydrazine according to the Sibley and Lehninger method as modified by Beck (1955).

Glyceraldehyde-3-phosphate dehydrogenase was detected by coupling with aldolase to generate glyceraldehyde-3-phosphate from fructose-1,6-diphosphate. This enzyme had a specific requirement for diphosphopyridine nucleotide (DPN) cofactor; no activity was observed when triphosphopyridine nucleotide (TPN) cofactor was used.

TABLE 1. *Enzymes detected and not detected in extracts of glucose-grown Pseudomonas natriegens*

Enzymes detected	
Glucokinase	6-Phosphogluconate dehydrogenase
Aldolase	Transaldolase
Glyceraldehyde-3-phosphate dehydrogenase	Phosphoriboisomerase
Phosphofructokinase	Phosphoketopentoepimerase
Glucose-6-phosphate dehydrogenase	

Enzymes not detected

6-Phosphogluconate dehydrase
2-Keto-3-deoxy-6-phosphogluconate aldolase
Phosphoketolase

Phosphofructokinase was detected by using fructose-6-phosphate as substrate and ATP as cofactor. This system was coupled with aldolase and glyceraldehyde-3-phosphate dehydrogenase and the reduction of DPN observed.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were detected by using glucose-6-phosphate and 6-phosphogluconate as substrates and observing the reduction of TPN. No activity was observed when DPN cofactor was used.

The presence of transaldolase, transketolase, phosphoriboisomerase, and phosphoketopentoepimerase was indicated by the formation of phosphates of sedoheptulose, fructose, and ketopentose from ribose-5-phosphate by cell-free extract. Fructose-phosphate and sedoheptulose-phosphate were determined by Dische's sulfuric acid-cysteine reaction as modified by McDonald, Cheldelin, and King (1960). Ketopentose-phosphate was determined as cysteine carbazole-reactive ketopentose according to Axelrod and Jang (1954).

6-Phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase were not detected when assays were carried out according to

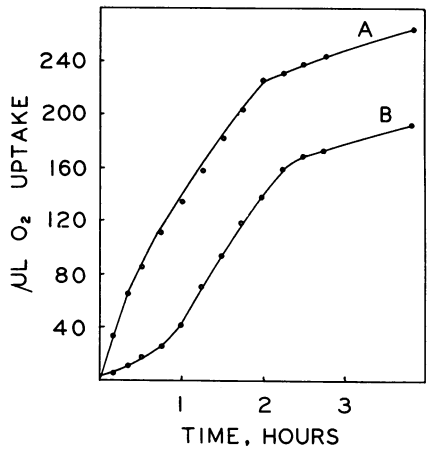

FIG. 1. *Rate and extent of oxidation of gluconate by gluconate-grown cells of Pseudomonas natriegens (curve A) and glucose-grown cells (curve B). Four μmoles of gluconate per Warburg vessel were used.*

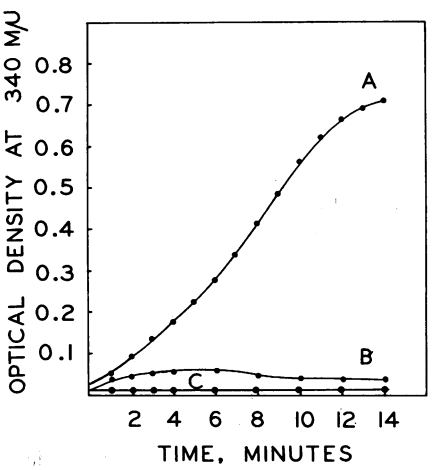

FIG. 2. *Demonstration of gluconokinase. Beckman spectrophotometer, 30 C; 100 μmoles tris (pH 8); 0.5 μmole TPN; 20 μmoles ATP; 20 μmoles $MgCl_2$; 10 μmoles gluconate; 0.1 ml dialyzed extract (2.1 mg protein); total volume, 3.0 ml. Curve A, extract from gluconate-grown cells; curve B, extract from glucose-grown cells; curve C, ATP omitted from reaction mixtures.*

the technique of Kovachevich and Wood (1955*a*, *b*). Phosphoketolase was not detected when assayed by the technique of Ciferri and Blakley (1959).

Induction to gluconate utilization. The results in Fig. 1 show the rate and extent of oxidation of gluconate with whole resting cells of *P. natriegens*

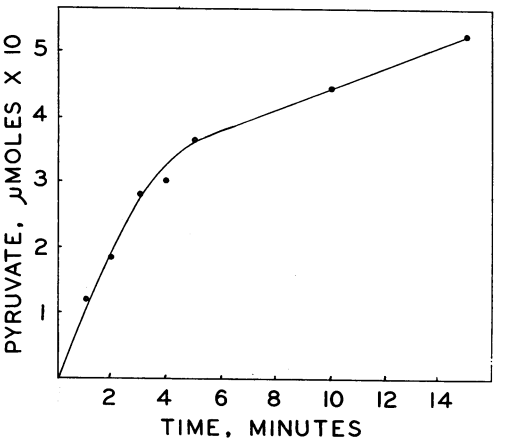

FIG. 3. *Demonstration of the Entner-Doudoroff pathway. Klett-Summerson colorimeter, 30 C; 200 μmoles tris (pH 7.65); 3 μmoles reduced glutathione; 6 μmoles $FeSO_4$; 7 μmoles 6-phosphogluconate; 0.1 ml extract (2.4 mg protein); total volume, 1.0 ml. At the time intervals indicated, pyruvate was assayed according to the method of Friedemann and Haugen (1943), and the resulting "chromogen" was measured using Klett filter no. 42.*

grown on gluconate (curve A) and glucose (curve B). These data indicate that enzyme systems for the dissimilation of gluconate are readily inducible.

The presence of gluconokinase was established by coupling this enzyme with 6-phosphogluconate dehydrogenase present in the extracts and measuring formation of reduced TPN (TPNH) at 340 mμ. The data in Fig. 2 indicate that cultivation of *P. natriegens* on gluconate induced the formation of gluconokinase (curve A) and that gluconokinase was not detected in extracts from glucose-grown cells (curve B). As indicated by curve C, there was no activity in the absence of ATP.

The presence of 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase was demonstrated according to the technique of Kovachevich and Wood (1955*a*,*b*). The results (Fig. 3) indicate that initially there was a rapid production of pyruvate, followed by a diminished rate. These enzymes were not detected in extracts of glucose-grown cells and their formation was induced by cultivation of *P. natriegens* on gluconate.

Participation of pathways during glucose dissimilation. Since key enzymes of both the glycolytic pathway and the pentose cycle have

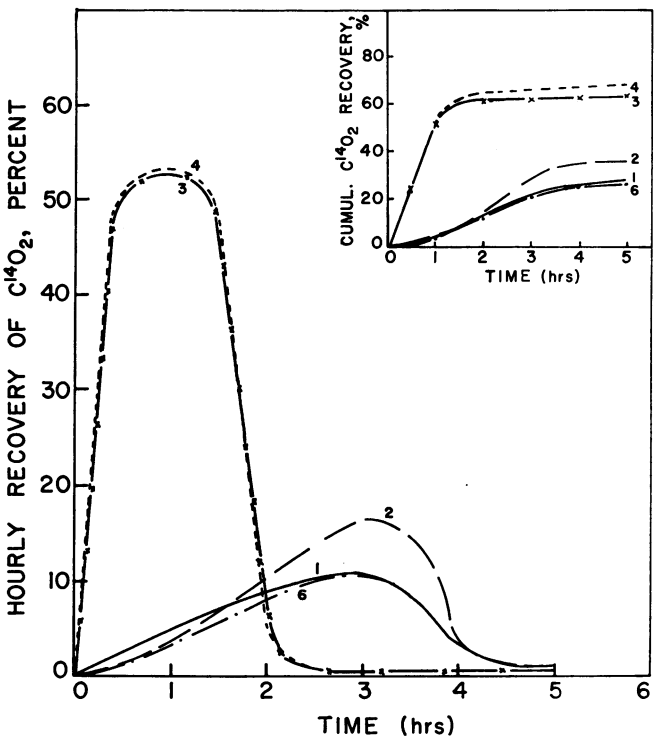

FIG. 4. *Radiorespirometric patterns for the utilization of C^{14} specifically labeled glucose samples by Pseudomonas natriegens cells under aerobic growing conditions.* [*1-C^{14}*] *glucose,* ————; [*2-C^{14}*] ***glucose,*** — — — —; [*3-C^{14}*] *glucose,* —X—X—X; [*4-C^{14}*] *glucose,* ----------; [*6-C^{14}*] *glucose,* —·—·—. ***The data for C-4 of glucose was obtained indirectly from results observed in the*** [*3-C^{14}*] *glucose and* [*3,4-C^{14}*] ***glucose experiments. The incubation suspension consisted of 2.5 mg cells in 10 ml of a medium containing basal salts, 0.01% yeast extract, and 12 mg of the specifically labeled glucose.***

been demonstrated in extracts of glucose-grown cells, it appeared desirable to attempt to estimate the relative participation of the pentose phosphate pathway and the glycolytic Krebs cycle route in the over-all catabolism of hexose in this organism.

Radiorespirometric experiments were carried out with washed cells under aerobic growing conditions. Two distinct phases can be recognized during the incubation period (Fig. 4). First, during the active assimilation phase while substrate glucose was rapidly utilized via the primary breakdown processes, the extensive operation of the glycolytic pathway was observed, as evidenced by the high yields of $C^{14}O_2$ from C-3 and C-4 of glucose. Meanwhile, the preferential conversion of C-1 of glucose to CO_2, as compared to C-6, points to the occurrence of the hexose monophosphate pathway. The assimilation phase terminated at approximately 3 hr after substrate administration, presumably reflecting the exhaustion of glucose from the medium.

The assimilation phase was immediately followed by the depletion phase, during which time the degradation products of substrate glucose previously assimilated were routed back to the respiratory mechanism, resulting in the burst of $C^{14}O_2$ production from several carbon atoms of substrate glucose. The order of $C^{14}O_2$ yield observed during the depletion phase, i.e., C-2 > C-1 > C-6, indicated that the glycolytic Krebs cycle sequence was playing an important role in the biosynthesis of cellular constituents. This is true in view of the fact that C-2 and C-5 of glucose, upon conversion to carboxyl carbon atoms of acetate via the glycolytic pathway, can be oxidized preferentially in comparison with the methyl carbon atom of acetate derived from C-1 and C-6 of glucose via the glycolytic scheme.

The incorporation of specifically labeled carbon

TABLE 2. *Dissimilation of C^{14} specifically labeled glucose samples by Pseudomonas natriegens**

Substrate	Radiochemical inventory†			
	Resp. CO_2	Medium	Cells	Total
[1-C^{14}] Glucose	29	8	65	102
[2-C^{14}] Glucose	34	8	61	103
[3-C^{14}] Glucose	63	6	32	101
[4-C^{14}] Glucose‡	66	7	24	97
[6-C^{14}] Glucose	23	9	69	100

* Experimental conditions: 2.5 mg of cells in 10 ml of medium containing basal salts, 0.01% yeast extract, and 12 mg of the specifically labeled glucose samples having 0.25 μc of radioactivity.

† Data taken at the end of the incubation experiments and expressed as the percentage recovery of substrate radioactivity in each category of the metabolic products.

‡ Calculated value from date observed in the [3-C^{14}] glucose and [3,4-C^{14}] glucose experiments.

atoms of substrate glucose into cellular constituents and fermentation products in the incubation medium is given in Table 2. Data presented are those observed at the end of each of the incubation experiments. The preferential incorporation of C-6 and C-1 of glucose into cellular constituents, as compared to other carbon atoms, is in accordance with the conclusion drawn from $C^{14}O_2$ data that the glycolytic Krebs cycle sequence constitutes the predominant catabolic route in this organism. Repetition of these experiments provided radiorespirometric data with closely parallel results between the experiments.

Participation of pathways during gluconate dissimilation. Washed cells (grown on glucose) were allowed to catabolize C^{14} specifically labeled gluconate samples under aerobic growing conditions. The time course plot of $C^{14}O_2$ production from individual carbon atoms of gluconate is given in Fig. 5.

An examination of existing information on

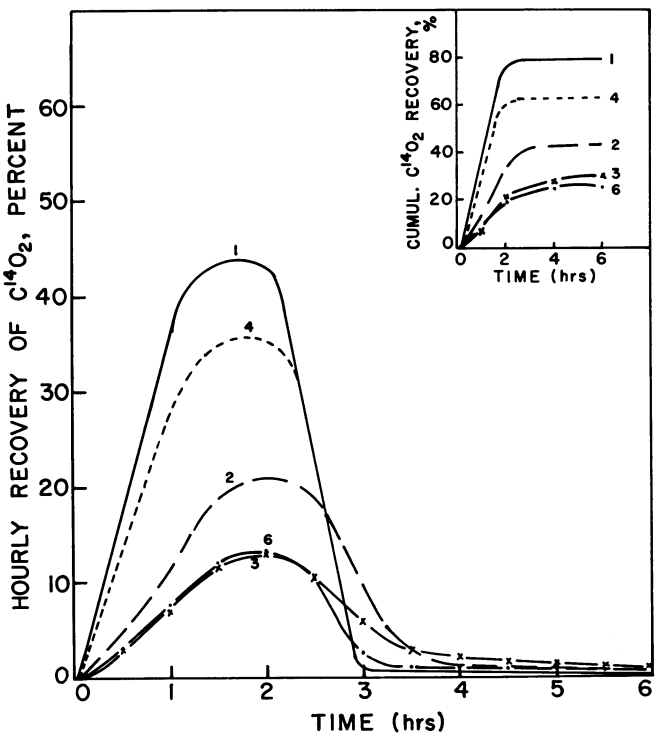

FIG. 5. *Radiorespirometric patterns for the utilization of C^{14} specifically labeled gluconate samples by Pseudomonas natriegens cells under aerobic growing conditions.* [1-C^{14}] *gluconate,* ————; [2-C^{14}] *gluconate,* — — — —; [3-C^{14}] *gluconate,* —×—×—×; [4-C^{14}] *gluconate,* ----------; [6-C^{14}] *gluconate,* —·—·—. *The data for C-4 of gluconate were obtained indirectly from results observed in the* [3-C^{14}] *gluconate and* [3,4-C^{14}] *gluconate experiments. The incubation suspension consisted of 2.5 mg cells in 10 ml of a medium containing basal salts, 0.01% yeast extract, and 12 mg of the specifically labeled gluconate.*

gluconate catabolism indicated that in microorganisms gluconate can be catabolized via one or more of the three major catabolic sequences.

(i) Via the Entner-Doudoroff scheme, gluconate, upon phosphorylation, can be degraded to 1 mole of pyruvate and 1 mole of triosphosphate. The latter can be in turn converted to pyruvate. The mechanism of the cleavage reaction is such that complete metabolic equivalence between C-1 and C-4, C-2 and C-5, or C-3 and C-6 can be realized if the Entner-Doudoroff pathway constituted an exclusive route for gluconate catabolism (Wang et al., 1958).

(ii) Gluconate, upon phosphorylation, can be catabolized via the pentose cycle reactions, giving rise to fructose-6-phosphate. The latter compound, when isomerized to glucose-6-phosphate, will then be in turn converted to 6-phosphogluconate, thus making one complete turn of the pentose cycle. Repeated operation in the nature of this mechanism would convert the top three carbon atoms of the gluconate skeleton to CO_2 with rates following the order of C-1, C-2, and C-3. Such a mechanism has been observed in *Acetobacter suboxydans* (Kitos et al., 1958).

(iii) Similar to the foregoing mechanism, it is also possible that fructose-6-phosphate, derived from gluconate via the pentose cycle reactions, can be converted to fructose-1,6-diphosphate and, hence, catabolized via the glycolytic scheme.

In view of the foregoing understandings and the observed radiorespirometric pattern, it appears that gluconate is catabolized in *P. natriegens*, primarily by way of the Entner-Doudoroff pathway, with the hexose monophosphate pathway playing concurrently a minor role. This conclusion is drawn from the fact that, although C-1 and C-4 of gluconate were extensively recovered in the respiratory CO_2, complete catabolic equivalence of these two carbon atoms was not realized, and the CO_2 yield from C-1 was slightly greater in magnitude. Since the CO_2 yield from C-3 of gluconate was approximately the same as that from C-6, it is concluded that pentose phosphate derived from substrate gluconate via phosphogluconate decarboxylation was not involved extensively in respiratory activities; otherwise one would expect a greater recovery of C-3 of gluconate in CO_2 over that of C-6. Repetition of these experiments provided radiorespirometric data with closely parallel results between the experiments.

TABLE 3. *Dissimilation of C^{14} specifically labeled gluconate samples by Pseudomonas natriegens**

Substrate	Radiochemical inventory†			
	Resp. CO_2	Medium	Cells	Total
[1-C^{14}] Gluconate	79	11	9	99
[2-C^{14}] Gluconate	43	5	55	103
[3-C^{14}] Gluconate	30	7	62	99
[4-C^{14}] Gluconate‡	63	7	24	94
[6-C^{14}] Gluconate	26	6	68	100

* Experimental conditions: 2.5 mg of cells in 10 ml of medium containing basal salts, 0.01% yeast extract, and 12 mg of the specifically labeled gluconate samples having 0.25 μc of radioactivity.

† Data taken at the end of the incubation experiments and expressed as the percentage recovery of substrate radioactivity in each category of the metabolic products.

‡ Calculated value from date observed in the [3-C^{14}] gluconate and [3,4-C^{14}] gluconate experiments.

The estimation of pathway participation with respect to gluconate catabolism has also been carried out according to the method of Wang et al. (1958). From the substrate inventory data, particularly in the 1-C^{14} gluconate experiment (Table 3), it was found that 11% of the substrate gluconate was left in the incubation medium, presumably in the form of products of gluconate metabolism. This fact implies that 89% of the substrate gluconate has entered the cells and hence engaged in active metabolic processes. Use is then made of the cumulative $C^{14}O_2$ yield data from C-1 and C-4 of gluconate at 2 hr after substrate administration (i.e., 78 and 62%, respectively) for pathway estimation. It appears that, insofar as the portion of substrate gluconate that engaged in catabolism is concerned, approximately 80% is catabolized via the Entner-Doudoroff pathway and the remaining 20% is presumably utilized via the hexose monophosphate pathway.

DISCUSSION

P. natriegens appears capable of oxidizing glucose through the known routes of carbohydrate metabolism. This bacterium was demonstrated to possess enzymes of the glycolytic pathway of glucose dissimilation and also enzymes of the pentose phosphate pathway. A Krebs tricar-

boxylic acid cycle has been indicated also. Estimation of the participation of pathways in the catabolism of glucose has been carried out according to the method of Wang et al. (1958), employing cumulative $C^{14}O_2$ yield data observed in the radiorespirometric experiment. In the present case, estimation was made on the basis that the pentose phosphate derived from substrate glucose was not involved in further respiratory activities. Judging from the amount of C-3 and C-4 of glucose incorporated into the cellular constituents, it was concluded that approximately 72% of the substrate glucose was engaged in catabolic activities. The calculation indicated that, for the portion of substrate glucose actually catabolized, approximately 92 and 8% were routed via the glycolytic and the hexose monophosphate pathways, respectively.

These results are in accordance with previous findings reported by Payne et al. (1961) that this organism under growing conditions aerobically converted over 90% of the catabolized glucose to acetate, lactate, pyruvate, and carbon dioxide. The exact nature of the hexose monophosphate pathway functioning in this organism cannot be defined in the present study. However, the pentose phosphate derived from substrate glucose via the hexose monophosphate pathway did not appear to be further catabolized extensively. The conclusion is drawn from the fact that the CO_2 yield from C-3 of glucose is practically the same as that from C-4 of glucose and that the CO_2 yield from C-6 of glucose was indeed very low during the assimilation phase. In fact, the catabolic structure observed with this organism remarkably resembles previous findings with baker's yeast (Wang et al., 1958). In both cases, the glycolytic pathway plays a predominant role in glucose catabolism accompanied by the limited operation of the hexose monophosphate pathway. The pentose phosphate derived from substrate glucose does not appear to be extensively catabolized, despite the fact that the pentose-cycle enzymes have been detected in both of these organisms.

It was also demonstrated that *P. natriegens* could be induced to grow on gluconate. Gluconokinase, 6-phosphogluconate dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase have been demonstrated to be present in the extracts of gluconate-grown cells but not in extracts of glucose-grown cells.

Results from a series of radiorespirometric experiments revealed that gluconate can be utilized by glucose-grown cells at a moderate rate via predominantly the Entner-Doudoroff pathway. Although there also exists evidence that a portion of the substrate gluconate was assimilated via the hexose monophosphate pathway, the pentose-cycle sequence does not appear to play an important role in the catabolism of pentose phosphate derived from substrate gluconate. The slow rate of utilization of gluconate by glucose-grown cells used in the radiorespirometric experiments reflects the necessity of enzyme induction when the cells are exposed to gluconate.

An important facet uncovered by the present study is that the catabolic mechanism for glucose recognized in *P. natriegens* is basically different from that operative in several other species of pseudomonads so far studied (Stern, Wang, and Gilmour, 1960), in which the Entner-Doudoroff pathway plays a predominant role in glucose catabolism. It should be further emphasized that *P. natriegens* is the only marine pseudomonad studied thus far and that enzymes of the Entner-Doudoroff pathway were not detected in this organism grown on glucose as the carbon source.

The results reported from this experiment do not offer an explanation as to why the glycolytic pathway is operated almost exclusively when enzymes of the pentose-cycle pathway can be detected in cell-free extracts. Possible explanations include a barrier in the conversion of glucose-6-phosphate to 6-phosphogluconate in the intact cell, a deficiency of TPN, a block in the oxidation of TPNH, or ratios and concentrations of ADP, ATP, and Pi unfavorable to the operation of the hexose monophosphate pathway.

ACKNOWLEDGMENTS

We are indebted to Conrad Lobley and Julia Krackov for their very capable technical assistance.

This investigation was supported in part by research grants (G-9865 and G-19997) from the National Science Foundation and a research grant (E-1144) from the National Institutes of Health, U. S. Public Health Service.

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