Arch. Microbiol. 99, 15–23 (1974)

The Utilization of 2-Ketogluconate by *Hydrogenomonas eutropha H 16*

H. G. Nandadasa, Marion Andreesen, and H. G. Schlegel

Institut für Mikrobiologie der Gesellschaft
für Strahlen- und Umweltforschung mbH, München, in Göttingen

Received April 23, 1974

Abstract. During growth of *Hydrogenomonas eutropha H 16* on 2-ketogluconate, 2-ketogluconate kinase and 2-keto-6-phosphogluconate reductase were formed. These enzymes were absent from cells grown on fructose, gluconate, acetate, succinate or autotrophically. There was no evidence for extracellular oxidation of glucose, fructose or gluconate with the formation of ketogluconic acids.

The utilization of 2-ketogluconate is neither subject to catabolite inhibition by hydrogen nor is 2-keto-6-phosphogluconate reductase inhibited by ATP, ADP or phosphoenolpyruvate. The enzyme is characterized by a high affinity for its substrates.

Key words: *Hydrogenomonas eutropha H 16* — Growth on 2-Ketogluconate — 2-Ketogluconate Kinase — 2-Keto-6-phosphogluconate Reductase — Low Cooperative — Homotropic Effects — Hydrogen Bacterium.

Besides fructose and gluconate, 2-ketogluconate is one of the few hexose derivatives utilized by *Hydrogenomonas eutropha* (Davis *et al.*, 1970). By the wild type strains glucose is neither utilized (Gottschalk *et al.*, 1964; Kuehn and McFadden, 1968) nor oxidized to gluconic acid; only a rare mutant (PHB$^-$5) defective in PHB synthesis oxidized glucose to gluconic acid (Schlegel *et al.*, 1970). With respect to these properties *H. eutropha* strain *H 16* differs markedly from some pseudomonads which have recently been investigated for the metabolic route of 2-ketogluconate degradation. In *Pseudomonas aeruginosa* a glucose dehydrogenase and a gluconate dehydrogenase act extracellularly, therefore, glucose is metabolized by parallel pathways (Roberts *et al.*, 1973). In *Pseudomonas putida* the non-phosphorylative pathway to 2-ketogluconate followed by phosphorylation, reduction and further degradation via the Entner-Doudoroff system is even the main pathway of glucose utilization (Vicente and Cánovas, 1973a).

Although *H. eutropha H16* is able to grow on 2-ketogluconate the degradative pathway had not been studied. We have, therefore, investigated the pattern of induction of the enzymes involved, *i.e.* 2-ketogluconokinase (E.C. 2.7.1.63), 2-keto-6-phosphogluconate reductase (E.C.

1.1.1.69), 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase as well as glucose-6-phosphate dehydrogenase and gluconokinase, using extracts of cells grown on various carbon sources including autotrophically grown cells. Furthermore, the mutant strain *H16*—PHB$^-$5 was considered which oxidizes glucose to gluconic acid by an extracellularly acting enzyme (Schlegel *et al.*, 1970). Although this ability had been clearly shown, a possible further oxidation to 2-ketogluconate had not been tested.

Methods

Organisms and Growth. Hydrogenomonas eutropha H16 (= *Alcaligenes eutrophus H16*; ATCC 17699) and the mutants *H16*-PHB$^-$4 and *H16*-PHB$^-$5 derived therefrom (Schlegel *et al.*, 1970) were taken as lyophylized samples from the culture collection of this institute.

For preparing the enzyme extracts the cells were grown in the usual media (Schlegel *et al.*, 1961; Bowien and Schlegel, 1972) containing either 0.5% fructose or 0.5% gluconate or 0.2% 2-ketogluconate or 0.2% acetate under air. Autotrophically the cells were grown under a gas mixture of 10% oxygen, 10% carbon dioxide and 80% hydrogen. 6 l flat-bottomed round flasks connected to gas reservoirs or an air supply were used as culture vessels. The suspension was magnetically stirred (400 rpm). The growth temperature was 30°C.

Preparation of Bacterial Extracts. The cells harvested were washed once using 0.067 M phosphate buffer pH 7.0 and were either frozen and stored at −20°C or used immediately. The cell suspension was sonicated (1 min/ml) applying a Branson sonifier (20 kcycles, 600 W). Intact cells and cell debris were removed by centrifuging for 20 min at 10000 *g*. For removing particles including NADH oxidase centrifugation at 120000 *g* for 60—90 min in an Omikron-centrifuge (Heraeus-Christ, Osterode) was applied. The supernatant was used for enzyme determinations and for the preparation of 2-keto-6-phosphogluconate. The determinations were repeated using extracts from which low molecular weight compounds had been removed by filtration through Sephadex G-25.

Preparation of 2-Keto-6-phosphogluconate. 5 ml of freshly prepared extract of 2-ketogluconate grown cells (approx. 20 mg protein) was added to 50 ml 0.05 M TEA buffer pH 7.6 containing 2 g 2-ketogluconate, 2 mmole ATP and 0.25 mmole $MgCl_2$. During incubation at 30°C the progress of the 2-ketogluconokinase reaction was followed by measuring the decrease of ATP. After overnight incubation the reaction mixture was centrifuged at 30000 rpm for 1 h, and the supernatant was passed through a diaflo-cell for deproteinization. 10 g of active charcoal (norite) was added to the filtrate, then filtered through a Büchner funnel and washed with water. 0.5 mmol barium acetate followed by 500 ml ice cold ethanol were added to the filtrate (100 ml). The precipitate was separated by centrifugation and dissolved in 10 ml 0.1 N HCl; some 2 N HCl was necessary for dissolution. The solution was passed through a Dowex 50 column and the pH was adjusted to pH 7.0 with NaOH. The concentration was determined by an optical test with a partly purified preparation of 2-keto-6-phosphogluconate reductase to be 22 mM. The yield was 616 μmole. For the removal of non-phosphorylated 2-ketogluconate a small aliquot of the solution was chromatographed on cellulose thin layer plates using a mixture of n-propanol—ammonia—water = 6:3:1 as a solvent system.

Enzyme Assays. All enzyme activities were measured by optical tests. The reaction mixtures for glucose-6-phosphate dehydrogenase, 6-phosphogluconate

dehydratase, 2-keto-3-deoxy-6-phosphogluconate aldolase and for the latter both enzymes together [Entner-Doudoroff (ED)-system] were essentially those used by Gottschalk *et al.* (1964) and Blackkolb and Schlegel (1968a).

For 2-keto-6-phosphogluconate reductase the reaction mixture contained 0.84 ml 50 mM TEA-buffer pH 7.6, 0.03 ml 100 mM $MgCl_2$, 0.03 ml 12.5 mM $NADH_2$, 0.05 ml 22 mM 2-keto-6-phosphogluconate and 0.05 ml extract in 1.00 ml volume.

2-Ketogluconate kinase was measured in a test coupled with 2-keto-6-phosphogluconate reductase; the reaction mixture contained 0.74 ml 50 mM TEA buffer pH 7.6, 0.03 ml 100 mM $MgCl_2$, 0.3 ml 12.5 mM $NADH_2$, 0.1 ml 15 mMATP, 0.05 ml 25 mM 2-ketogluconate in 1.00 ml total volume.

For measuring the activities of fructose-, gluconate- and 2-ketogluconate kinases the coupled test with pyruvate kinase and lactate dehydrogenase as auxiliary enzymes was used. Extracts filtered through Sephadex G-25 had to be used to keep inspecific reactions minimal. The reaction mixture contained in 3.00 ml: 2.55 ml 50 mM TEA buffer pH 7.6, 0.1 ml 15 mM ATP, 0.1 ml 100 mM $MgCl_2$, 0.1 ml 15 mM PEP, 0.05 ml 25 mM $NADH_2$, 0.01 ml pyruvate kinase (5 mg/ml), 0.01 ml lactate dehydrogenase (5 mg/ml), 0.1 ml extract, 0.1 ml 25 mM fructose (gluconate or 2-ketogluconate, respectively).

An Eppendorf photometer equipped with a recorder was used for all spectrophotometric assays. One unit of activity was defined as 1 μmol of NAD(H) reduced (or oxidized) per minute. All values were corrected for a low but measurable NADH oxidation which occurred in the absence of any of the substrates. K_m values were determined by conventional methods. Protein was determined by the method of Beisenherz *et al.* (1953) applying a modul of 19 mg per sample for calculating protein from extinction at 546 nm.

Materials. Enzymes and biochemicals were obtained from Boehringer GmbH, Mannheim. Conventional chemicals for nutrient solutions were purchased from E. Merck, Darmstadt, W.-Germany. 2-Ketogluconate was obtained as the calcium salt from E. Merck (Art.-Nr. 5190); the cation was removed by treatment with a column of Dowex 50 (H^+-form).

Results

Growth

Growth of the wild type strain *Hydrogenomonas eutropha H 16* occurred with a doubling time of 110 min at 30°C with 2-ketogluconate as a substrate compared *e.g.* to fructose or gluconate with doubling times of 120 and 90 min, respectively.

The respiration of fully adapted starved cells responded to the addition of 2-ketogluconate more slowly than to the addition of fructose or gluconate (Fig. 1). This lag of respiratory oxygen uptake is regarded as a consequence of the early reduction step in the degradation pathway of 2-ketogluconate. In starved cells the generation of reducing power necessary for reduction of 2-keto-6-phosphogluconate depends on the dehydrogenation of glyceraldehyde phosphate and pyruvate as well as the TCC intermediates; these reactions are late steps in the degradative pathway; the function of an early step is dependent on a product of late steps. Therefore, the respiratory rate increased slowly. The lag was

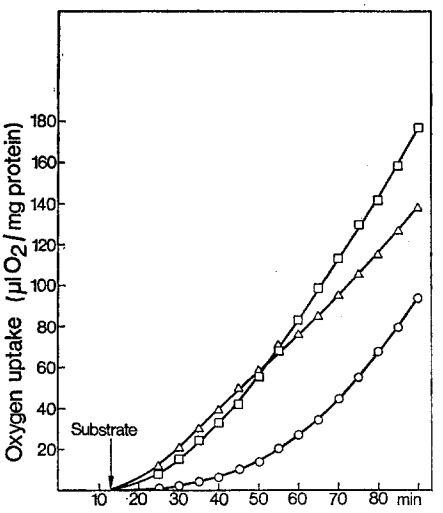

Fig. 1

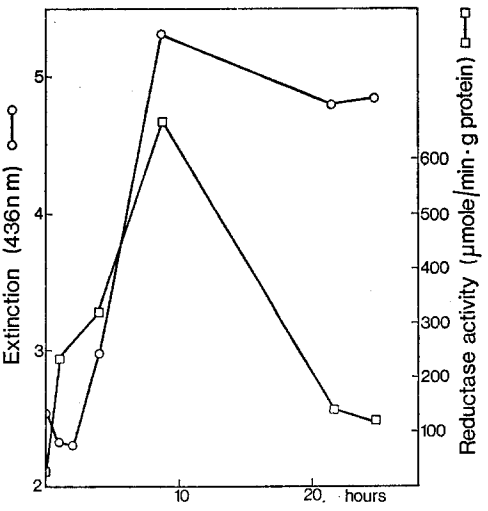

Fig. 2

Fig. 1. Time course of oxidation of 2-ketogluconate compared to fructose and gluconate by fully adapted, starved cells of *Hydrogenomonas eutropha H16* mutant PHB$^-$4. The cells of this mutant, different from the wild type by the incapability to synthesize and accumulate poly-β-hydroxybutyrate, had been grown on fructose, gluconate or 2-ketogluconate and after harvesting had been starved (shaken in mineral medium under air) for 5 h. The course of oxygen uptake was measured manometrically. The substrate was added after 13 min. Since the densities of the three suspensions differed slightly, the rates were recalculated based on an equal cell protein content (determined after Schmidt *et al.*, 1963). Symbols: o——o 2-ketogluconate, □——□ gluconate, △——△ fructose

Fig. 2. Synthesis of 2-keto-6-phosphogluconate reductase after transfer of gluconate grown cells to a 2-ketogluconate medium. o——o turbidity (E_{436}), □——□ specific activity of reductase

especially pronounced when cells devoid of storage materials were used; for the experiment shown in Fig. 1 the mutant PHB$^-$4 was used which lacks the ability to synthesize and accumulate poly-β-hydroxybutyrate.

While growth on fructose is repressed (Gottschalk, 1965) as well as inhibited (Blackkolb and Schlegel, 1968a) by hydrogen, the utilization of gluconate and 2-ketogluconate is not impaired by hydrogen. These observations already indicated that the enzymes involved in the degradation of 2-ketogluconate are not subject to such a strict control by catabolite inhibition as is glucose 6-phosphate dehydrogenase (Blackkolb and Schlegel, 1968b; Bowien *et al.*, 1974). If gluconate grown cells were transferred to a medium containing 2-ketogluconate as the sole carbon source the enzymes of 2-ketogluconate metabolism were quickly induced. The specific activity of 2-keto-6-phosphogluconate reductase even reach-

Table 1
Assay of enzymes of 2-ketogluconate metabolism of *Hydrogenomonas eutropha H 16* grown heterotrophically on various carbon sources and autotrophically

Enzyme determined	Specific activity of enzymes (μmoles/min · g protein) Cells grown on				
	2-Keto-gluconate	Gluconate	Fructose	Acetate	Autotrophically
Glucose 6-phosphate dehydrogenase	31	16	201	4	3
6-phosphogluconate dehydrogenase	149	123	138	24	7
KDPG-Aldolase	230	178	107	46	10
ED-System	130	210	83	3	< 1
2-Keto-6-phosphogluconate reductase	488	< 1	13	< 1	5
2-Ketogluconokinase	65	< 1	< 1	< 1	< 1
Gluconokinase	6	14	< 1	< 1	< 1
$NADH_2$-oxidase	5	4	5	2	2

ed values higher than those measured in cells harvested at the end of the exponential growth phase (Fig. 2).

The specific enzyme activities listed in Table 1 are in accordance with the assumption that 2-ketogluconate is degraded via 2-keto-6-phosphogluconate and 6-phosphogluconate followed by the Entner-Doudoroff pathway. As has been tested by two kinds of optical assay, 2-ketogluconate is activated by a specific kinase. Using a test with 2-keto-6-phosphogluconate reductase as the auxiliary enzyme the specific activity was determined. From the results of the second test using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes the conclusion could be drawn, that 2-ketogluconate kinase is only synthesized during growth on 2-ketogluconate and neither on fructose or gluconate nor on acetate or during autotrophic growth.

With respect to the induction pattern the same results have been obtained for the second enzyme, 2-keto-6-phosphogluconate reductase. This enzyme is only present in 2-ketogluconate grown cells. Using a purified preparation of 2-keto-6-phosphogluconate and the crude extract or a partially purified 2-keto-6-phosphogluconate reductase of 2-ketogluconate grown cells the product of the reduction of 2-keto-6-phosphogluconate was identified as 6-phosphogluconate: after $NADH_2$ oxidation due to the reduction of 2-keto-6-phosphogluconate the reaction mixture was supplemented by NADP and 6-phosphogluconate dehydrogenase; the fast increase of optical density indicated the product to be 6-phosphogluconate.

2-Keto-6-phosphogluconate Reductase

The enzyme has a rather high specific activity in 2-ketogluconate grown cells (approx. 500 units/g protein). It was purified twenty fold by removing the nucleic acids by protamine sulfate precipitation, followed by precipitating the reductase by ammonium sulfate (40% saturation) and DEAE-cellulose chromatography; the enzyme was eluted by approx. 50 mM phosphate buffer pH 7.0. The preparation had a specific activity of 9600 units/g protein. The enzyme is characterized by high affinities for its substrates (Figs. 3 and 4). The $S_{0.5}$ for 2-keto-6-phosphogluconate amounted to 0.16 mM. The enzyme used both reduced nicotinamide dinucleotides, $NADH_2$ and $NADPH_2$; the $S_{0.5}$-values were 0.02 mM $NADH_2$ and 0.1 mM $NADPH_2$.

The substrate saturation curves indicate that the enzyme does not follow straight Michaelis-Menten kinetics. In the reciprocal plot the curve is bent slightly upwards indicating weak positive cooperativity. Hill plots confirmed this impression; n-values were calculated for the three enzyme substrates tested: $n = 1.3$ for 2-keto-6-phosphogluconate; $n = 1.4$ for $NADPH_2$ and $n = 1.5$ for $NADH_2$. The cooperativity is apparently limited to homotropic effects. ATP and PEP, which exert a pronounced inhibitory effect to the glucose 6-phosphate dehydrogenase of the same organism (Blackkolb and Schlegel, 1968b), do not impair

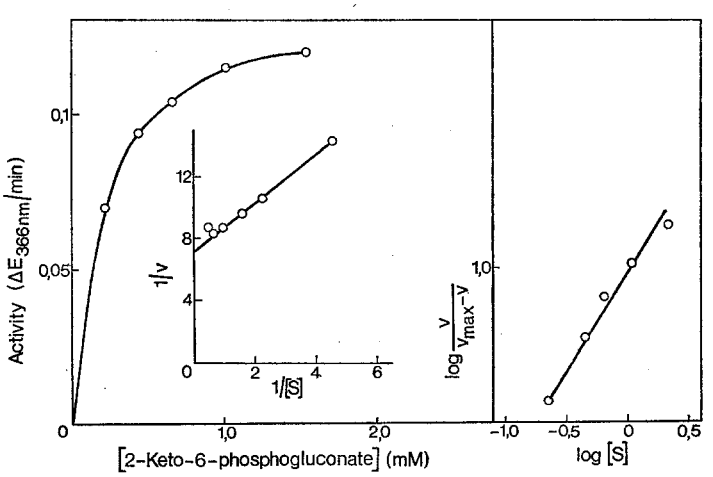

Fig. 3. Substrate (2-keto-6-phosphogluconate)saturation curve of a 10-fold purified preparation of 2-keto-6-phosphogluconate reductase from *Hydrogenomonas eutropha H16*. The reaction mixture contained 0.05 ml enzyme preparation containing 4.7 µg protein, otherwise as described under "Methods". The insertion shows the reciprocal (Lineweaver-Burk) plot from which $S_{0.5}$ was calculated. The Hill-plot resulted in $n = 1.3$

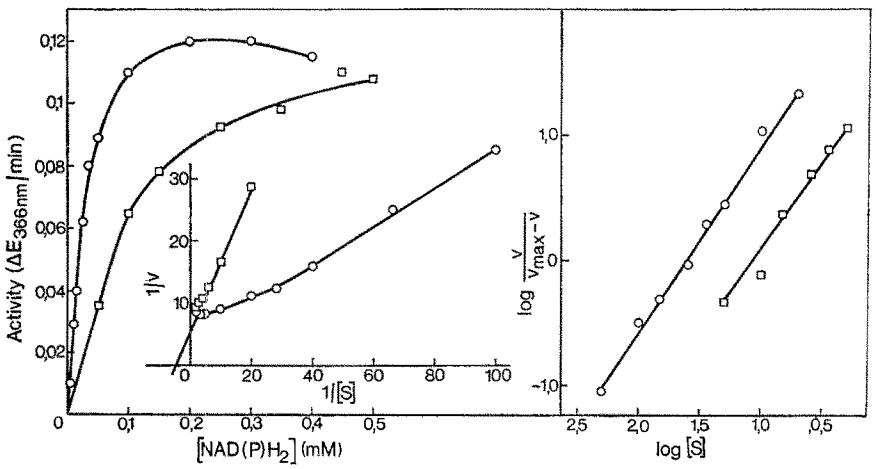

Fig. 4. Substrate [NAD(P)H_2]saturation curves of the purified preparation of 2-keto-6-phosphogluconate reductase. Conditions and insertion as in Fig. 1. The Hill-plot resulted in $n = 1.5$ for $NADH_2$ and $n = 1.4$ for $NADPH_2$. Symbols: o——o $NADH_2$-saturation curve, □——□ $NADPH_2$-saturation curve

the enzyme at all even at 1.5 mM concentration. Other metabolites possibly causing heterotropic effects at the enzyme have not been tested. The results obtained are in accordance with the in vivo observation that the utilization of 2-ketogluconate is not inhibited by hydrogen in the gas atmosphere.

The mutant *H 16*—PHB$^-$5, which oxidized glucose to gluconic acid (Schlegel *et al.*, 1970), was grown on succinate (0.5%) as well as on succinate supplemented by 0.5% fructose or glucose. Although glucose was converted to gluconate by the mutant cells, the cells did not exhibit any activity of 2-ketogluconokinase and 2-keto-6-phosphogluconate reductase; even the enzymes of the Entner-Doudoroff pathway and glucose 6-phosphate dehydrogenase were absent.

Discussion

The presence of the Entner-Doudoroff pathway in gluconate grown *Escherichia coli* (Eisenberg and Dobrogosz, 1967) as well as the utilization of glucose by *Pseudomonas putida* via gluconate and 2-ketogluconate (Narrod and Wood, 1956; Vicente and Cánovas, 1973a, b) and the utilization of glucose via gluconate and 2-ketogluconate by parallel pathways in *P. aeruginosa* were surprising and emphasize the diversity of glucolytic routes in bacteria. Compared to the mechanisms described for these organisms *Hydrogenomonas eutropha H 16* offers a trivial case. The enzymes for the utilization of gluconate or 2-ketogluconate are

induced by these substrates. The basal level of the substrate specific enzymes is rather low. Since after growth on 2-ketogluconate the specific activities of 2-ketogluconate kinase and 2-keto-6-phosphogluconate reductase are very high, the conclusion appears justified that the utilization of 2-ketogluconate in *H16* occurs via 2-keto-6-phosphogluconate and 6-phosphogluconate. With the wild type strains no observations were made indicating a direct oxidation of glucose or gluconic acid by extracellularly acting dehydrogenase.

2-Ketogluconate belongs to those substrates whose primary degradation steps depend on a metabolite which is produced in a later step of the degradative pathway. In the case of 2-ketogluconate and hydrocarbons (alkanes, aranes) this metabolite is reduced pyridine nucleotide. Carbon dioxide and ATP represent further examples.

The carboxylation of an early intermediate, β-methylcrotonyl coenzyme A to form β-methylglutaconyl-CoA is involved in the degradative pathway of leucine or isovaleric acid. This carboxylation reaction precedes steps in which carbon dioxide is generated. Respiration and growth of an *Achromobacter* strain on isovalerate proved to be highly dependent on the CO_2 partial pressure (Lafferty, 1963).

ATP is required to support the transport and initiate the degradation of hexoses. The short lag phase of hexose utilization by fully induced starved cells may be attributable to the lag of energy generation. A severe retardation of sulfate respiration was observed when cells of *Desulfovibrio desulfuricans* depleted from ATP were exposed to a non-fermentable substrate (hydrogen) as H-donor and sulfate as the H-acceptor. The lag could be overcome by the addition of pyruvate (which provides ATP generated by fermentation) or sulfite (which served as H-acceptor without having to be activated beforehand) (Peck, 1960).

Acknowledgements. H.G.N. would like to thank the Deutsche Akademische Austauschdienst for granting a post-doctoral fellowship.

References

Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., Pfleiderer, G.: Diphosphofructose-Aldolase, Phosphoglycerinaldehyd-Dehydrogenase, Glycerophosphat-Dehydrogenase und Pyruvat-Kinase aus Kaninchenmuskel in einem Arbeitsgang. Z. Naturforsch. 8b, 555–577 (1953)

Blackkolb, F., Schlegel, H. G.: Katabolische Repression und Enzymhemmung durch molekularen Wasserstoff bei *Hydrogenomonas*. Arch. Mikrobiol. **62**, 129–143 (1968a)

Blackkolb, F., Schlegel, H. G.: Regulation der Glucose-6-phosphat-Dehydrogenase aus *Hydrogenomonas H16* durch ATP und $NADH_2$. Arch. Mikrobiol. **63**, 177–196 (1968b)

Bowien, B., Cook, A. M., Schlegel, H .G.: Evidence for the *in vivo* regulation of glucose 6-phosphate dehydrogenase activity in *Hydrogenomonas eutropha H16*

from measurements of the intracellular concentrations of metabolic intermediates. Arch. Microbiol. **97**, 273–281 (1974)

Bowien, B., Schlegel, H. G.: Der Biosyntheseweg der RNS-Ribose in *Hydrogenomonas eutropha* Stamm *H16* und *Pseudomonas facilis*. Arch. Mikrobiol. **85**, 95–112 (1972)

Davis, D. H., Stanier, R. Y., Doudoroff, M., Mandel, M.: Taxonomic studies on some gram negative polarly flagellated "hydrogen bacteria" and related species. Arch. Mikrobiol. **70**, 1–13 (1970)

Eisenberg, R. C., Dobrogosz, W. J.: Gluconate metabolism in *Escherichia coli*. J. Bact. **93**, 941–949 (1967)

Gottschalk, G.: Die Verwertung organischer Substrate durch *Hydrogenomonas* in Gegenwart von molekularem Wasserstoff. Biochem. Z. **341**, 260–270 (1965)

Gottschalk, G., Eberhardt, U., Schlegel, H. G.: Verwertung von Fructose durch *Hydrogenomonas H16* (I). Arch. Mikrobiol. **48**, 95–108 (1964)

Kuehn, G. D., McFadden, B. A.: Enzymes of the Entner-Doudoroff path in fructose-grown *Hydrogenomonas eutropha*. Canad. J. Microbiol. **14**, 1259–1260 (1968)

Lafferty, R. M.: Kohlendioxyd-Fixierung durch organotrophe Bakterien. Arch. Mikrobiol. **44**, 373–405 (1963)

Narrod, S. A., Wood, W. A.: Carbohydrate oxidation by *Pseudomonas fluorescens*. V. Evidence of gluconokinase and 2-ketogluconokinase. J. biol. Chem. **220**, 45–55 (1956)

Peck, H. D., Jr.: Evidence for oxidative phosphorylation during the reduction of sulfate with hydrogen by *Desulfovibrio desulfuricans*. J. biol. Chem. **235**, 2734–2738 (1960)

Roberts, B. K., Midgley, M., Dawes, E. A.: The metabolism of 2-oxogluconate by *Pseudomonas aeruginosa*. J. gen. Microbiol. **78**, 319–329 (1973)

Schlegel, H. G., Kaltwasser, H., Gottschalk, G.: Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38**, 209–222 (1961)

Schlegel, H. G., Lafferty, R., Krauss, I.: The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. **71**, 283–294 (1970)

Schmidt, K., Liaaen-Jensen, S., Schlegel, H. G.: Die Carotinoide der Thiorhodaceae. I. Okenon als Hauptcarotinoid von *Chromatium okenii* Perty. Arch. Mikrobiol. **46**, 117–126 (1963)

Vicente, M., Cánovas, J. L.: Glucolysis in *Pseudomonas putida:* Physiological role of alternative routes from the analysis of defective mutants. J. Bact. **116**, 908–914 (1973a)

Vicente, M., Cánovas, J. L.: Regulation of the glucolytic enzymes in *Pseudomonas putida*. Arch. Mikrobiol. **93**, 53–64 (1973b)

Prof. Dr. H. G. Schlegel
Institut für Mikrobiologie
D-3400 Göttingen
Grisebachstraße 8
Federal Republic of Germany