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Carbohydrate Metabolism in *Rhodopseudomonas spheroides*

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SUMMARY: The wild-type strain of *Rhodopseudomonas spheroides* 2.4.1 does not utilize exogenously supplied gluconate and grows poorly with glucose, fructose or mannose as substrates, accumulating aldonic acids and 2-keto-3-deoxy gluconic acid in media containing these sugars. Mutants which are capable of growing well with glucose without the accumulation of acids acquire the enzyme 'phosphogluconic acid dehydrase' and oxidize hexoses mainly via the pathway described previously in *Pseudomonas saccharophila*. The Embden-Meyerhof pathway is constitutive in the wild-type and mutant strains of *R. spheroides* but appears to have a limited function as a result of very low aldolase activity. Phosphogluconic acid dehydrogenase is either absent or too low in activity to permit the utilization of the pentose-phosphate oxidative pathway.

A new type of particulate 'aldose dehydrogenase system', constitutive in all strains is involved in the oxidation of glucose and mannose to the corresponding aldonic acid lactones. Gluconic and mannonic acids are both converted to 2-keto-3-deoxy acid through the action of two distinct dehydrating enzymes. A mutation leading to the utilization of gluconic acid is associated with the acquisition of a high level of '2-keto-3-deoxy gluconic acid kinase', but not of phosphogluconic acid kinase. A permeability factor may also be involved in the adaptation of gluconate.

In his studies on the non-sulphur purple bacteria, van Niel (1944) recorded that the species of *Rhodopseudomonas spheroides* is capable of utilizing glucose, fructose, mannose and gluconate as substrates for photosynthetic growth and that acidic products accumulate in media containing hexose sugars. These observations were confirmed by us with *R. spheroides* strain 2.4.1 obtained from van Niel's collection. It was shown further that the pattern of substrate utilization and of acid formation by this strain is similar in cultures grown aerobically in the dark to that found in anaerobic cultures in the light. The utilization of gluconate, however, for both photosynthetic and oxidative metabolism required a period of adaptation or 'training' of the cultures by their serial transfer in media containing this compound. It was further observed that after several passages in glucose or fructose media, the cultures grew more rapidly and profusely than they did on initial transfer and ceased to produce acid. The adaptation of the cultures to gluconate, glucose, and fructose media was shown to be due to the selection of mutant strains appearing in the original population. The mutant colonies could be recognized by their rapid growth and large size on agar media containing the appropriate compound as the principle carbon source. The altered properties of the mutant were retained after many transfers in media which did not contain the substrate to which the clones had originally been adapted. Two types of 'glucose' mutant

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strains were isolated, one which grows well with either glucose or fructose and produces no acid from either sugar, and another which grows well with glucose but not with fructose, and produces acid from the latter but not from the former.

The studies to be reported here were conducted with growing culture, intact resting cells and cell-free preparations of the wild type strain and of selected mutant strains derived from it in an attempt to elucidate the biochemical pathway of sugar metabolism in *Rhodopseudomonas spheroides* and the nature of the enzymic differences in the various strains.

METHODS

Five strains of *Rhodopseudomonas spheroides* were studied in the course of this work. Two of these designated as 'parent strains' were indistinguishable in their behaviour in sugar media and in their enzymic composition with respect to carbohydrate metabolism.

Parent strain 1. A single colony isolate (2.4.1C) from the original or 'wild-type' strain 2.4.1 from the collection of Professor C. E. van Niel.

Parent strain 2. Spontaneous 'green' pigment mutant derived from parent-strain 1 by Cohen-Bazire, Sistrom & Stanier (1957) and kept in the collection of the Department of Bacteriology, University of California, under the designation 2.4.1Ga.

Glucose strain 1. Mutant strain isolated from Parent-strain 1 on glucose medium. This strain grows well with glucose or fructose as carbon source and less profusely with mannose. No acid accumulates in media with glucose or fructose and relatively little acid with mannose.

Glucose strain 2. Mutant strain isolated after many passages of parent-strain 2 in glucose medium. This strain grew well with glucose but not with fructose or mannose. Large amounts of acid were produced from the two latter sugars, but none from glucose. Fructose-utilizing mutants were obtained from this strain by passage on fructose media.

Gluconate strain. Mutant strain derived from glucose-strain 2 by serial transfers in liquid media containing glucose + gluconate, with subsequent isolation on gluconate medium.

Stock cultures and liquid cultures in which sugars were not used as principal sources of carbon were grown in 0.02M-KH₂PO₄Na₂HPO₄ (pH 6.8) buffer containing per litre: 4 g. amino acid mixture (Difco Casamino acids), 3 g. yeast extract (Difco); 20 ml. Hutner's basal mixture of minerals and vitamins (described as 'concentrated base' by Cohen-Bazire *et al.* 1957). For the study of growth with carbohydrates as principal sources of carbon, the concentration of nitrogenous organic nutrients was reduced. The media contained per litre of 0.02M-phosphate buffer: 2 g. (NH₄)₂SO₄, 1 g. amino acid mixture, 20 ml. Hutner's base and between 2.0 and 3.8 g. of the carbohydrate substrate (autoclaved separately). Aerobic cultures were incubated in the dark in flasks on a high-speed rotary shaker, while anerobic cultures were grown in special flat containers continuously illuminated with tungsten lamps (at 1000 foot-candles) and constantly aerated with a gas mixture consisting of

95% nitrogen and 5% (v/v) CO₂. All cultures were incubated at 30°. Growth was measured by the estimation of the optical density of the suspensions at 680 mμ, which was calibrated against dry-weight determinations. Organisms used for manometric experiments and for the extraction of enzymes were harvested in the early stationary phase of growth. Enzyme preparations were generally made by sonic disintegration of 10% (wet weight/volume) washed bacterial suspensions for 8–10 min. in a 9 KC Raytheon magnetostrictor oscillator. The suspending medium was usually 0.75 M-Tris (pH 7.5) buffer (trishydroxymethyl aminomethane) prepared with either phosphoric or hydrochloric acid, and containing a small quantity of deoxyribonuclease and, in some cases, glutathione. Unbroken organisms and coarse debris were removed by low-speed centrifugation. For the separation of the 'soluble' and 'particulate' enzyme fractions, the extracts were centrifuged for 90 min. at 100,000 g in a Spinco ultra-centrifuge. Good preparations of the soluble enzymes were also obtained by grinding the organisms with levigated alumina (method of McIlwain, 1948). For the detection of certain enzymes which are relatively unstable, such as fructokinase and phosphohexokinase, freeze-dried organisms were used.

Protein was determined by the method of Lowry, Rosenbrugh, Farr & Randall (1951) and phosphate according to Fiske & SubaRow (1925). Hydrolysis for 10 min. at 100° with N-H₂SO₄, or for 20 min. at room temperature with 1.5 N-KOH, was used for determining acid-labile and alkali-labile phosphate, respectively. Reducing sugars were estimated by the procedure of Schales & Schales (1945) and glucose by the use of a colorimetric method employing glucose oxidase ('Glucostat', Worthington Biochemical Corp. Freehold, N.J., U.S.A.).

Fructose and fructose phosphates were determined by the reagents of Roe (1934). Crystalline glyceraldehyde phosphate dehydrogenase and partially purified glucose-6-phosphate dehydrogenase from yeast were used for the detection of the substrates of these enzymes. Sugar acid lactones were determined by the method of Hestrin (1949) and the sugar acids by the same method after lactonization by boiling for 5 min. with N-HCl. Pyruvic acid was estimated by the toluene extraction method of Friedmann & Haugen (1948). Other keto acids were estimated by the method of Lanning & Cohen (1951). 2-Keto-3-deoxy-6-phosphogluconic acid (KDPG), as well as the dephosphorylated analogue (KDG) and a highly purified KDPG aldolase, were obtained from previous studies with *Pseudomonas saccharophila* (MacGee & Doudoroff, 1954). The crystalline calcium salt of KDG was also obtained from Dr Joseph Merrick. Both ascending and descending paper chromatography were used for the identification and purification of various compounds. Glucose, gluconolactone, gluconic acid, KDG and 6-phosphogluconic acid were separated with acetone + pyridine + water (2:1:1). With this solvent, the *R_f* values for the above compounds were 0.75, 0.89, 0.36, 0.49, and 0.27, respectively. Fructose-6-phosphate, fructose diphosphate and dihydroxyacetone phosphate were separated with *n*-butanol + propionic acid + water (10:5:7), while gluconohydroxamic acid was identified by chromatography with *n*-propanol + distilled formic acid + water (6:3:1) and *n*-butanol + pyridine + water (6:4:3).

A mixture of *n*-butanol, ethanol and water (5:1:4) was used for the chromatography of 2,4-dinitrophenyl hydrazone of pyruvic acid. Alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) was used for the detection of reducing compounds on chromatograms and *o*-phenylenediamine spray (Lanning & Cohen, 1951) for 2-keto-3-deoxy hexonic acids. Fructose and its phosphate esters were located by the use of resorcinol reagent and lactones with hydroxylamine (Block, Durrum & Zweig, 1958). Phosphate esters were developed according to Hanes & Isherwood (1949).

RESULTS

The wild-type strain 2.4.1.C of *Rhodopseudomonas spheroides* did not grow aerobically in Hutner's basal medium with glucose unless this medium was supplemented with amino acids. In a medium containing 0.1% (w/v) cas-amino acids, the addition of glucose at 0.2% (w/v) did not affect the initial growth rate (0.125 generations/hr.) and the growth curves for cultures grown with and without glucose were virtually identical to the point at which the maximum stationary phase was reached in the sugar-free controls (0.31 mg. dry wt./ml.). Thereafter, the cultures in glucose medium continued to grow at an extremely low rate (0.03 generation/hr.) so that the ultimate yield of organism was approximately three times greater than that found in the control cultures. A portion of the growth curves is shown in Fig. 1A. Glucose disappeared rapidly throughout the initial period of growth and was converted in large part to a mixture of gluconic and 2-keto-3-deoxygluconic acids. Gluconic acid was identified by its chromatographic behaviour and by the following reactions of the chromatographically-isolated compound. It could be lactonized by boiling with *N*-HCl and converted with hydroxylamine to a compound indistinguishable by chromatography from gluconohydroxamic acid. With the highly specific particulate gluconic oxidase of *Pseudomonas fluorescens*, it was oxidized to 2-ketogluconic acid, which was identified chromatographically. 2-Keto-3-deoxygluconate was identified by its *R_f* value and specific colour reactions on chromatograms and, subsequently, by its phosphorylative decomposition to yield pyruvic acid in the presence of enzymes obtained from the gluconate-oxidizing strain of *R. spheroides*. In all respects, the compound behaved identically with authentic samples of 2-keto-3-deoxy gluconic acid.

From 11.0 μ mole glucose, a maximal yield of 8.3 μ mole of sugar acids was obtained after 42 hr. of cultivation. This comprised 5.0 μ mole gluconic acid and 3.3 μ mole of KDG. On subsequent incubation the gluconic acid content of the medium remained constant, while KDG disappeared slowly, so that at 70 hr. its concentration decreased to about one third of its maximal value.

The behaviour of the wild-type strain under anaerobic conditions in the light resembled that found under aerobic conditions except that the rate of growth and the total yield of organism were greater (Fig. 1B). The maximal accumulation of sugar acids was almost the same as in aerated cultures (4.8 μ mole gluconic acid and 2.4 μ mole of KDS after 42 hr. of cultivation).

The negligible contribution of glucose to the development of cultures during the early phase of growth suggested that little if any glucose carbon would be incorporated into cell material. This, however, was not found to be the case. In an experiment in which uniformly-labelled glucose- ^{14}C was supplied in the medium, more than half of the carbon of the newly formed cells was found to be derived from this compound during the first 23 hr. of development, both under aerobic and anaerobic conditions. Most of the glucose carbon could be accounted for as sugar acids and cell material. Even after 70 hr. of anaerobic growth, over 91 % of the glucose carbon was recovered in the medium and in the organisms, while in an aerobic culture, about 86 % was found in the soluble products and cell materials. Hence, only a small fraction of the sugar was converted to CO_2 in either photosynthetic or respiratory metabolism.

Fig. 1. Growth of *Rhodospseudomonas spheroides* parent-strain 1 and glucose-strain 1 in media with and without glucose. The glucose-free medium was prepared with 1 g. Casamino acids/l. as described in the text. The glucose medium contained, in addition, 2 g. glucose/l. A = aerobic cultures in the dark (atmosphere, air); B = anaerobic cultures in the light (atmosphere, 95 % (v/v) N_2 , 5 % (v/v) CO_2).

Like glucose, fructose, mannose and ribose were oxidized by the wild-strain with the formation of sugar acids. None of these sugars, however, could be used for growth in Hutner's basal medium, and neither mannose nor ribose contributed appreciably to growth in media supplemented with amino acids. Gluconic acid and KDG were identified as the principal excreted products in the metabolism of fructose and of ribose. During growth with mannose,

mannonic acid, KDG and traces of gluconic acid were found to accumulate. Mannonic acid was identified by chromatography, lactonization, and conversion to mannono-hydroxamic acid in the same manner as was gluconic acid. It was differentiated from gluconic acid by the fact that it was not oxidized by the gluconic acid oxidase of *Pseudomonas fluorescens*. Subsequently, the compound was further identified by its conversion to KDG with a mannonic acid dehydrase preparation obtained from *Rhodospseudomonas spheroides*.

Resting cell suspensions of the wild-type strain grown on yeast-extract medium oxidized glucose and fructose very slowly in phosphate buffer at pH 6.8, even in the presence of 5×10^{-3} Mg^{++} , which was found to stimulate oxygen uptake with these substrates. With glucose as substrate, a lag of 15–60 min. was generally observed before the maximal rate of O_2 consumption was established. The maximal Q_{O_2} varied in different batches of organisms, being generally lower than 10. The total amount of oxygen consumed by such suspensions usually exceeded the amount required for the formation of sugar acids and the chromatographic analysis of the reaction mixtures showed the presence of only traces of gluconic acid and of KDG after the glucose had disappeared. In one experiment, however, in which organisms grown in a medium containing amino acids and glucose were allowed to oxidize 10 μmoles glucose, 5.03 μmole oxygen were consumed and 9.6 μmole sugar acids were found (6.1 μmole gluconate; 3.5 μmole KDG). Gluconate was neither oxidized nor converted to KDG by resting cell suspensions.

Unlike the parent strains, the mutant 'glucose strains' could grow to some extent in Hutner's basal medium with glucose as carbon source; and they grew much more rapidly in media supplemented with amino acids in the presence of glucose than in its absence. For example, glucose-strain 1 grew almost exactly like the wild-type strain with 0.1% Casamino acids as carbon source (0.125 generations/hr., 0.34 mg. maximal dry wt. cells/ml. under aerobic conditions). When the same medium was supplemented with 0.2% glucose, the growth rate was increased to 0.29 generation/hr. and remained almost constant until a maximum crop equiv. 1.2 mg. dry wt. cells/ml. was attained. The differences in the growth of the two strains with glucose is clearly shown in Fig. 1A. Also in contrast to the wild-type strains, the glucose-strains did not accumulate appreciable amounts of gluconic acid or KDG during growth with glucose. The photosynthetic growth of the glucose-strains was also more rapid and more profuse in the presence than in the absence of glucose, and no sugar acids were found to accumulate (see Fig. 1B). An experiment in which glucose-strain 1 was grown with Casamino acids and glucose- ^{14}C showed that approximately 50% of the cell carbon was derived from glucose during aerobic and anaerobic development. In both aerobic and anaerobic cultures, a portion of the glucose carbon (22 and 24% respectively) appeared in extracellular products of metabolism. No sugar acids were detected in the medium and a significant part of the labelled extracellular material was accounted for as viscous complex polysaccharide of unknown composition which is produced by *Rhodospseudomonas spheroides* in a variety of media.

About 80 % of the labelled glucose carbon was lost as CO_2 in the aerobic culture and about 10 % in the anaerobic culture.

Glucose-strain 2 was similar to glucose-strain 1 in its growth with glucose, but differed from the latter in its inability to grow more rapidly when fructose or mannose was added to an amino acid medium. Both of these sugars were oxidized almost quantitatively to a mixture of mannonic acid and KDG, which were identified as described earlier. No gluconic acid was detected in the cultures.

The difference between the glucose-strains on the one hand and the parent strains on the other was reflected not only in growth rates and products of metabolism in glucose media but also in the rates of oxygen uptake by resting suspensions provided with glucose. Washed suspensions of glucose-strain 2, for example, oxidized the sugar without an appreciable initial lag and with a Q_{O_2} of 84.

The following well-known enzymic activities were found in cell-free extracts of the parent strains grown in yeast extract amino acid medium, and may therefore be said to be constitutive in these strains: glucose kinase, fructose kinase, phosphoglucokinase, phosphoglucose isomerase, phosphoglucomutase, fructose di-phosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and an active KDGP aldolase or 'splitting enzyme' which converts 2-keto-3-deoxy-6-phosphogluconic acid to pyruvic acid and D-glyceraldehyde-8-phosphate (Kovachevich & Wood 1955*b*). An extract of Parent-strain 1 decomposed 16 μmole KDPG/hr./mg. protein at 80° in 12.5 mM.-Tris buffer (pH 7.5) with 7.5 mM substrate. Other constitutive enzymes of the parent strains discussed later included a new type of particulate 'aldose dehydrogenase', δ -gluconolactonase, gluconic acid dehydrase, mannonic acid dehydrase, two types of phosphatases, and a KDG kinase which was present only in trace amounts. An inducible mannose isomerase (Palleroni & Doudoroff, 1956) was formed rapidly when the wild-type cells were transferred to fructose or mannose media.

Both of the glucose strains and the gluconate strain differed from the parent strains in that they produced an active constitutive phosphogluconic acid dehydrase (Kovachevich & Wood, 1955*a*) which was never formed, even in sugar-containing media, by wild-type organisms. A cell-free extract of glucose-strain 1 dehydrated 12.5 μM -phosphogluconate at the rate of 3.6 $\mu\text{mole/hr./mg.}$ protein at 80° in 25 mM-imidazole buffer (pH 7.0). The gluconate-strain differed from all other strains in its ability to produce very large amounts of KDG kinase, a constitutive enzyme formed only in trace amounts by other strains even in media containing gluconate.

Evidence for the occurrence of transaldolase, transketolase, ribose kinase and the pentose phosphate epimerases in *Rhodopseudomonas spheroides* was first obtained from growth experiments in which Parent-strain 1 was found to accumulate gluconic acid and KDG in ribose media. Later it was shown that cell-free extracts of the glucose strains produced fructose-6-phosphate (identified by chromatography and the Roe reaction) and glucose-6-phosphate (identified by the glucose dehydrogenase reaction) in the presence of ribose-5-phosphate or ribose and ATP but not with ribose alone as substrate.

No dehydrogenases capable of reducing DPN or TPN with glucose, mannose or mannose-6-phosphate were found in any of the strains under any conditions, nor was there any evidence for the occurrence of a glucose-fructose isomerase, phosphomannose isomerase, glucose-mannose waldenase, or gluconic acid kinase under any circumstances. Although the presence of trace amounts of a phosphogluconic acid dehydrogenase could not be rigorously ruled out, most attempts to demonstrate such an enzyme were unsuccessful. Many experiments were conducted with different cultures, using DPN, TPN, ferricyanide and oxygen as possible hydrogen acceptors for the oxidative decarboxylation of phosphogluconic acid. Only with DPN was any reaction ever observed and the reduction of this coenzyme might have been due to side-reactions catalysed by the crude enzyme preparations. The highest rates of DPN reduction ever observed were only of the order of $0.5 \mu\text{mole/hr./mg. protein}$.

Fructose di-phosphate aldolase was detected as a constitutive enzyme in all of the strains, but the activity was extremely small in every case, in spite of many efforts to find optimal conditions for the enzyme. With various modifications of the spectrophotometric and chemical methods of assay for this enzyme (Taylor, 1955) the maximal activities observed in extracts of parent and mutant strains were of the order of $0.1\text{--}0.2 \mu\text{mole of fructose di-phosphate split/hr./mg. protein}$. Additional evidence for low aldolase activity was obtained in experiments with an extract of glucose-strain 2 acting on 6-phosphogluconate. In this preparation a constant supply of both triose phosphates was ensured by the consecutive action of phosphogluconic acid dehydrase, KDPG aldolase and triose phosphate isomerase. After incubation for 4 hr. when essentially all of the substrate had disappeared, less than 5 % of the triose phosphate was found to have been converted to fructose diphosphate, most of it being recovered as dihydroxyacetone phosphate.

No direct evidence was obtained for the absence of a kinase capable of phosphorylating both glucose and fructose in the parent strains or in glucose-strain 1. The occurrence of two distinct kinases was evident, however, from the observation that glucose-strain 2 could phosphorylate glucose but not fructose. The reduction of TPN could be demonstrated spectrophotometrically when crude enzyme preparations of the parent strains were incubated with fructose, ATP and magnesium in the presence of the coenzyme. This reduction was the result of the combined action of kinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase present in the extracts. Under these conditions the rate of TPN reduction was found to be between ten and twenty times greater than when fructose-1-phosphate was substituted for fructose and ATP. It may therefore be concluded that the product of fructose phosphorylation is fructose-6-phosphate rather than fructose-1-phosphate.

The phosphorylation of 2-keto-3-deoxygluconic acid by a 'KDG kinase' (Cynkin & Ashwell, 1959) was shown indirectly with extracts of the gluconate-strain. Since such preparations contained KDPG aldolase but no similar enzyme capable of splitting the unphosphorylated keto acid, the production of pyruvic acid from KDG in the presence of ATP was used as an assay for the kinase reaction. In 50 mM-Tris buffer (pH 8) with 3 mM-KDG, 10 mM-ATP

and 5 mM-MgCl₂, the rate of phosphorylation was found to be about 2.0 μ mole/hr./mg. protein at 30°. When gluconate was substituted for KDG at the same concentration in the reaction mixture, pyruvate formation did not begin immediately, but reached a maximal rate only after about 10 min. of incubation. This maximal rate was relatively low (less than 0.3 μ mole/hr./mg. protein) and was identical with the rate of conversion of gluconate to KDG by the same preparation in the absence of ATP. This was taken as evidence that no direct phosphorylation of gluconate took place and that the formation of pyruvate from gluconate was the result of the consecutive action of gluconic acid dehydrase that will be described below, and KDG kinase. KDG kinase activity was detected in all of the strains studied but in never more than trace amounts (less than 10% of the activity found in the gluconate strain). This enzyme is probably responsible for the slow disappearance of KDG in old cultures of the parent strains grown with glucose.

All the strains were found to possess constitutive enzymes capable of dehydrating gluconic and mannonic acids. The product of both reactions, 2-keto-3-deoxygluconic acid, was assayed by the method of Lanning & Cohen (1951). Since phosphogluconic acid dehydrase is not present in the wild-type strain, it cannot be identical with either of the enzymes which dehydrate the unphosphorylated sugars. That at least two different enzymes can attack gluconic acids was evident from the observation that the relative rates of dehydration of the two substrates could be changed by incubating the organisms with fructose or mannose. An extract of wild-type organisms grown in yeast-extract medium, incubated with 50 mM-Tris buffer (pH 8.0) and 8.3 mM-gluconate produced 0.8 mole of KDG/mg. protein/hr. at 30°. Under the same conditions, mannonate was dehydrated at the rate of 0.5 μ mole/hr. An extract made from similar organisms, but after these had been incubated with aeration in a mannose medium for several hours, had the same activity with gluconate, but dehydrated mannonate at a rate of 1.0 μ mole/hr./mg. protein. Although the above observations do establish that two enzymes can be produced, they do not exclude the possibility that either or both enzymes may have some affinity for both substrates. It seems appropriate, however, to refer to them as gluconic acid dehydrase and mannonic acid dehydrase respectively, on the basis of the relatively greater activity with one or the other substrate. Dehydrase activity for galactonic acid was also present in the extracts (De Ley & Doudoroff, 1957).

A constitutive enzyme system capable of oxidizing a variety of aldose sugars was found in the particulate enzyme fraction of both wild-type and mutant strains. Either oxygen or ferricyanide can act as a hydrogen acceptor for the oxidation of glucose by this system, while DPN, TPN and methylene blue are not reduced. Carbon dioxide or organic hydrogen acceptors can presumably be used as ultimate hydrogen acceptors in photosynthetic metabolism, since gluconic and mannonic acids accumulate in cultures grown anaerobically in the light with glucose and mannose, respectively. Under these conditions, the oxidizing agent may be generated by photochemical reactions. An assay for this 'aldose dehydrogenase' system was developed, based on the

estimation of ferrocyanide formed from ferricyanide, essentially according to the method of Folin & Malmros (1929). With this procedure it was shown that magnesium ion and an unidentified co-factor present in the soluble enzyme fraction are required for the activity of the particulate enzyme (see Table 1). The unknown co-factor was found to be resistant to boiling in the presence of acid, could be removed by charcoal or anion exchange resins and could not be replaced by any one of a variety of inorganic ions or of the better known coenzymes that have been tested. The 'aldose dehydrogenase system' did not oxidize D-xylose or D-gluconate, and had only a slight activity with D-arabinose as substrate. On the other hand, D-ribose, L-arabinose, D-mannose, D-galactose and D-glucosamine were oxidized at rates ranging between 70 and 100 % of that observed with glucose. The product of glucose oxidation appeared to be a gluconolactone, since gluconohydroxamic acid accumulated when the reaction was carried out in the presence of hydroxylamine.

Table 1. *Aldose dehydrogenase system in Rhodopseudomonas spheroides*

Glucose oxidation as determined by ferricyanide reduction at 30°. Complete system contained/ml. reaction mixture: washed particulate enzyme (9.5 mg. protein); tris-phosphoric acid buffer (pH 7.8) 63 μ mole; $MgCl_2$, 6.7 μ mole; $K_3Fe(CN)_6$, 10 μ mole; glucose, 18 μ mole; Na_2CO_3 , 10 μ mole; soluble fraction of 10 % (wet weight/vol.) extract of organisms heated for 15 min. at 100° and centrifuged 0.33 ml.

	Rate of ferri- cyanide reduction (as μ mole glucose oxidized/hr./ml.)
Complete system	41
Complete system without glucose	1
Complete system without particulate enzyme	0
Complete system without boiled extract	4
Complete system without $MgCl_2$	18

The soluble fraction of the extracts of the wild-type strain possessed gluconolactonase activity. In 0.03M-phosphate buffer (pH 6.8) with 3M- Mg^{++} and 22 mM-glucono- δ -lactone, the rate of hydrolysis of the lactone was approximately four times greater in the presence of the crude enzyme (about 7 mg. total protein/ml.) than in its absence. This observation suggests that the product of glucose oxidation by the aldose dehydrogenase system may be glucono- δ -lactone, as is the case with the particulate enzyme of *Pseudomonas fluorescens* (Jermyn, M., unpublished).

At least two types of phosphatases were found in the soluble enzyme fraction of the extracts of wild-type organisms. An acid phosphatase hydrolysed 6-phosphogluconate at a rate of 0.7 μ mole/hr./mg. protein in 25 mM-maleate buffer (pH 6.2) with 12.5 mM-6-phosphogluconate and 12.5 mM- Mg^{++} at 30°. Under similar conditions, phosphate was liberated at the rate of 0.4 μ mole/hr./mg. protein from 12.5 μ M-fructose diphosphate. Under alkaline conditions, on the other hand, 6-phosphogluconate was practically unattacked, while fructose diphosphate was rapidly hydrolysed. In Tris buffer (pH 8.5) with

12.5 $\mu\text{M-Mg}^{++}$ and substrate the rate of phosphate liberation was 0.1 and 2.2 $\mu\text{mole/hr./mg. protein}$ at 30° for the two substrates, respectively. Fructose-6-phosphate and fructose-1-phosphate were hydrolysed very slowly at pH 8.5, the rate of phosphate production with these substrates being less than one-tenth of that found with fructose diphosphate. Although the alkaline hydrolysis of fructose diphosphate by crude enzyme preparations is undoubtedly a complex phenomenon, it seems probable that a specific fructose-1-6-diphosphatase is in large part responsible for phosphate liberation under these conditions. Two lines of evidence support this conclusion. In the first place, the products of enzymic hydrolysis were more resistant to acid hydrolysis than the initial substrate, suggesting the removal of phosphate in the 1-position. Furthermore, TPN, which was reduced at a very low rate when added to the enzyme together with fructose diphosphate, was reduced rapidly when added after the enzyme and substrate had been incubated together for some time. This was taken as evidence that glucose-6-phosphate had been formed as a consequence of phosphatase and phosphoglucose isomerase activities. Somewhat surprising was an almost complete absence of phosphatase activity in the particulate fraction of the extracts at both pH 6.2 and 8.5 with either phosphogluconate or fructose-diphosphate as substrate.

DISCUSSION

Rhodopseudomonas spheroides can utilize at least three parallel biochemical pathways for the aerobic and photosynthetic oxidation of glucose, two of which involve dehydration reactions in which either gluconic or 6-phosphogluconic acid is converted to its 2-keto-3-deoxy analogue. The constitutive glycolytic or Embden-Meyerhof pathway appears to be of very limited significance because of low degree of aldolase activity. No good evidence for the occurrence of a 'monophosphate shunt mechanism' requiring the oxidative decarboxylation of phosphogluconic acid could be found. If a phosphogluconic dehydrogenase is present at all, its activity is very low. The rapid and efficient utilization of glucose and fructose without acid production is dependent on the mutational acquisition of the enzyme phosphogluconic acid dehydrase. This enzyme is absent from the wild-type strain studied and probably in most isolates of the species, since the production of acids is a species characteristic. It must be pointed out, however, that the enrichment procedures used for the isolation of *R. spheroides* from nature may be responsible for the selection of strains of such 'wild-type' rather than of the mutant types, because fermentable sugars cannot be used advantageously in the enrichment media.

The discovery of the 'dehydrative' pathways of carbohydrate metabolism in the photosynthetic *Rhodopseudomonas*, was not unexpected, this type of dissimilation, with various modifications, having previously been found to be characteristic of other pseudomonads, including the chemo-organotrophic *Pseudomonas fluorescens* (Kovachevich & Wood, 1955*a, b*), and *Xanthomonas phaseoli* (Hochster & Katznelson, 1958), and the facultatively chemolitho-

Table 2. Practically all of the phenomena which have been observed *in vivo* can be explained on the basis of *in vitro* studies of the enzymes found in the different strains. Although the observed rates of enzymic activity were in some cases (e.g. for kinases) somewhat lower than would be required for the observed metabolic rates, no great effort was made to determine optimal conditions for the assay of each enzyme and some of the enzymes were found to be extremely labile.

Table 2. Comparison of parent and mutant strains of *Rhodopseudomonas spheroides*

	Wild-type parent- strains 1 and 2	Glucose- strain 1	Glucose- strain 2	Gluconate- strain
In cultures				
Utilization of glucose	Poor	Good	Good	Good
Utilization of fructose	Poor	Good	Poor	Poor
Utilization of gluconate	None	None	None	Good
Acids formed with glucose	{ Gluconic KDG	None	None	None
Acids formed with mannose	{ Gluconic manno- nic KDG	Traces	{ Mannonic KDG	Not tested
In cell-free extracts				
Fructose kinase	Present	Present	Absent	Not tested
Phosphogluconic acid dehydrase	Absent	Present	Present	Present
KDG kinase	Trace	Trace	Trace	Present

A dual origin of the gluconic and 2-keto-3-deoxy gluconic acids that accumulate in cultures of the wild-type strains grown with glucose, fructose or mannose is supported by the following evidence. Glucose-strain 2, which does not possess fructose kinase, accumulates only mannonic acid and KDG when growing with fructose or mannose. This can be explained by the presence of the enzymes mannose, isomerase, 'aldose dehydrogenase' and mannonic acid dehydrase. On the other hand, the parent strains which possess fructose kinase, do produce gluconic acid from fructose and to a lesser extent from mannose. These strains possess phosphoglucose isomerase but no glucose isomerase, phosphomannose isomerase or mannose-6-phosphate dehydrogenase. Hence, in the metabolism of fructose and mannose, gluconic acid must arise from the hydrolysis of phosphogluconic acid, which can be derived indirectly from fructose-6-phosphate. A similar origin of gluconic acid must be postulated in the metabolism of ribose by *Rhodopseudomonas spheroides*, the enzymes necessary for the conversion of pentose phosphates to glucose phosphate having been demonstrated. It therefore seems likely that the wild-type strain produces gluconic acid from glucose both by direct oxidation and by the consecutive action of glucose kinase, glucose-6-phosphate dehydrogenase and the acid phosphatase that has been found to attack 6-phosphogluconate. Since the wild-type strain has no phosphogluconic acid dehydrase, and probably no phosphogluconic acid dehydrogenase, phosphatase must play an

important role in preventing the accumulation of phosphorylated metabolic intermediates in the cells. The soluble alkaline phosphatase would aid this process by converting any fructose diphosphate that accumulates, as a result of low aldolase activity, to fructose-6-phosphate.

The relatively low content of aldolase was somewhat surprising in view of the constitutive presence of other enzymes of the Embden-Meyerhof pathway and of the presumed importance of aldolase in photosynthetic CO_2 fixation. The inability of the wild-type strain to grow well with glucose as substrate, however, was entirely compatible with the *in vitro* observations.

The 'aldose dehydrogenase' system found in *Rhodospseudomonas spheroides* is of interest for several reasons. Unlike other particulate aldose-oxidizing enzymes that have been studied, it requires a soluble heat-stable co-factor, which has not yet been identified. Furthermore, the specificity of this system for different aldose sugars cannot easily be explained on the basis of the stereochemical configuration of the substrates. The simplest explanation requires the assumption that the system is a complex of two or more dehydrogenases with different specificities closely associated with an electron-transport system. Further studies on this system are in progress.

2-Keto-3-deoxy gluconate accumulates during the growth of the parent strains with glucose, fructose or mannose, and of the glucose-strain 2 with fructose or mannose. This can be ascribed to the action of gluconic and mannonic acid dehydrases, which have been demonstrated in cell-free extracts of both types. It has been shown that while KDG disappears slowly in cultures of the parent strains after the exhaustion of glucose, the gluconate which had accumulated does not disappear. Furthermore, gluconate is not oxidized or converted to KDG when supplied to such cultures during growth. It must be concluded that the dehydrases can act only on intracellularly formed aldonic acids. Once excreted, gluconic acid apparently becomes unavailable to the dehydrase, possibly due to its ionization. The gluconate-strain utilizes gluconic acid by converting it to KDG and phosphorylating the latter compound. The slow disappearance of KDG in the course of glucose metabolism by the wild-type strain may be attributed to the presence of a low level of KDG kinase activity that has been demonstrated in the cells. The high content of the kinase in the gluconate-strain does not, by itself, explain the ability of this strain to use exogenously supplied gluconate. It seems possible, therefore, that the mutation leading to gluconate utilization involves a change in permeability to the substrate as well as an increase in KDG kinase. The extreme difficulty of obtaining gluconate-utilizing strains that has been encountered in the present studies may, indeed, have been due to the necessity for selecting double mutants, possessing both a gluconate permease and a high level of KDG kinase.

The isolation of glucose-strain 2 was a fortunate accident, because the absence of fructose kinase in this strain made it possible to intercept the role of other enzymes in the metabolism of the species. It is curious that the loss of this enzyme accompanied the acquisition of KDPG dehydrase in this strain. It is quite possible, however, that the two distinguishing characteristics were

not acquired as a result of a single mutational event, since the adapted type was isolated after a long period of selection on glucose medium. A large number of other isolates of 'glucose'-strains had the culture characteristics resembling those of glucose-strain 1.

The present discussion would not be complete without a consideration of the apparently anomalous utilization of glucose carbon as a source of cell material by the wild-type strain. Tracer experiments have shown that the sugar, although very poorly utilized as a hydrogen donor in both respiratory and photosynthetic metabolism, provides a major fraction of cell carbon, in spite of the fact that the medium contains a mixture of amino acids and vitamins. The growth curves suggest that the amino acids are the main substrates for the energy metabolism of this strain. Hence, the situation appears to be the reverse of that generally accepted for such nutritionally exigent organisms as the lactic acid bacteria, which require glucose as a source of energy, but are presumed to synthesize cell substance mainly from preformed organic 'building blocks'.

Assuming that a rate-limiting reaction in sugar degradation is the splitting of fructose diphosphate, the explanation may in part invoke the accumulation of reserve polysaccharide and capsular materials. Although a complete study of reserve substances had not yet been undertaken with *Rhodopseudomonas spheroides*, another photosynthetic bacterium, *Rhodospirillum rubrum*, has been shown to produce a glycogen-like polysaccharide as the principal reserve material from certain organic substrates during photosynthesis. The occurrence of a constitutive phosphoglucomutase in *R. spheroides* is at least suggestive of a similar role of polysaccharides in this species. A supporting observation was that actively growing cultures had a higher content of glucose carbon than old cultures. The likelihood that polysaccharide capsular material may also contribute to the glucose carbon of the cells was suggested by the accumulation of ethanol-precipitable viscous slime in cultures of the mutant strains; this slime had a high content of glucose carbon. The formation of nucleotides through the transaldolase-transketolase reactions is another mechanism for the incorporation of glucose carbon into cell constituents. Finally, it must be remembered that even though the aldolase reaction may be too slow to permit a high rate of sugar dissimilation, the triose phosphates produced in this reaction may be rapidly assimilated while other constituents of the medium are more readily oxidized.

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