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Different Degradation Pathways for Glucose and Fructose in *Rhodopseudomonas capsulata*

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Abstract. In *Rhodopseudomonas capsulata* the enzymes of the Entner-Doudoroff pathway and the Embden-Meyerhof pathway have been examined. Fructose-grown cells contained inducible activities of phosphoenolpyruvate-fructosephospho-transferase and 1-phosphofructokinase and only low levels of fructokinase and 6-phosphofructokinase. Although fructose-grown cells contained, in addition, all the enzymes of the Entner-Doudoroff pathway together with fructose-1,6-diphosphatase and phosphoglucose isomerase, the Entner-Doudoroff pathway was not operative in fructose catabolism and served only the degradation of glucose. The functional separation of glucose and fructose catabolism via the Entner-Doudoroff and a modified Embden-Meyerhof pathway, respectively, was confirmed by different approaches:

1. Radiorespirometric experiments with glucose and fructose labelled in positions 1, 2, 3, 3+4 and 6 have been carried out. The pattern of $^{14}CO_2$-evolution from position-labelled glucose was characteristic for the Entner-Doudoroff pathway, that from position-labelled fructose for the Embden-Meyerhof pathway.

2. In the presence of arsenite up to 50% of glucose- and fructose-carbon was excreted as pyruvate. Using 1-^{14}C-glucose, 86% of the pyruvate was labelled in the carboxyl group, whereas using 1-^{14}C-fructose only 19% of the pyruvate was labelled in the carboxyl group.

3. A glucose-6-phosphate dehydrogenase-deficient mutant was isolated which lacked a functional Entner-Doudoroff pathway but which was unaltered in its ability to grow on fructose.

Key words: Rhodopseudomonas capsulata – Hexose catabolism – Entner-Doudoroff pathway – Embden-Meyerhof pathway – PEP-fructose-phosphotransferase – Mutant isolation – Radiometric techniques.

Non-Standard Abbreviations. EMP = Embden-Meyerhof pathway; EDP = Entner-Doudoroff pathway; PPP = pentose-phosphate pathway; PEP-fructose-PTS = phosphoenolpyruvate-fructose-phosphotransferase; 1-PFK = 1-phosphofructokinase; 6-PFK = 6-phosphofructokinase; PGI = phosphoglucose isomerase; F-6-P = fructose-6-phosphate; F-1-P = fructose-1-phosphate; G-6-P = glucose-6-phosphate; 6-PG = 6-phosphogluconate; FDP = fructose-1,6-diphosphate; KDPG = 2-keto-3-deoxy-6-phosphogluconate; DHAP = dihydroxyacetonephosphate; GAP = glyceraldehyde-3-phosphate; Ru-5-P = ribulose-5-phosphate; PEP = phosphoenolpyruvate

In bacteria, hexoses are usually degraded by one of the three major pathways, the Embden-Meyerhof pathway (EMP), the Entner-Doudoroff pathway (EDP) and the pentose-phosphate pathway (PPP) or by the concerted action of the EMP and PPP or the EDP and PPP (Wang et al., 1958).

A modified EMP was discovered in *Enterobacter aerogenes* (Hanson and Anderson, 1966, 1968):

$$\text{fructose} \xrightarrow{\text{PEP-fructose-PTS}} \text{F-1-P} \xrightarrow{\text{1-PFK}} \text{FDP} \xrightarrow{\text{aldolase}} \text{DHAP} + \text{GAP}$$

The first phosphorylated sugar derivative is F-1-P instead of F-6-P; F-1-P is formed by the action of a PEP-fructose-PTS and is further converted to FDP by 1-PFK. This pathway is only operative with fructose; glucose on the contrary is catabolized by the normal type of EMP via F-6-P:

$$\text{glucose} \xrightarrow{\text{PEP-glucose-PTS}} \text{G-6-P} \xrightarrow{\text{PGI}} \text{F-6-P} \xrightarrow{\text{6-PFK}} \text{FDP} \xrightarrow{\text{aldolase}} \text{DHAP} + \text{GAP}$$

The same pattern of glucose and fructose metabolism was shown to occur in *Escherichia coli* (Fraenkel, 1968; Ferenci and Kornberg, 1971, 1973), *Bacillus*

subtilis (Gay and Rapoport, 1970), and *Clostridium thermocellum* (Patni and Alexander, 1971). Although it has been known for some bacteria, that glucose and fructose were degraded by different modifications of the EMP, there was so far no evidence that these sugars might be degraded by the EDP and the EMP.

Among the Rhodospirillaceae, only few species are known to utilize hexoses as substrates, and until now, only three species, *Rhodospirillum rubrum*, *Rhodopseudomonas capsulata*, and *Rhodopseudomonas sphaeroides*, have been investigated with respect to their hexose catabolic pathways. *R. capsulata* and *R. sphaeroides* are able to utilize both glucose and fructose, while *R. rubrum* utilizes only fructose. *R. rubrum* was shown by radiorespirometric methods to catabolize fructose via EMP (Gibson and Wang, 1968). In *R. capsulata* grown on glucose, only the enzymes of the EDP were found. These results and radiorespirometric experiments indicated that glucose is broken down by the EDP (Eidels and Preiss, 1970). In *R. sphaeroides*, all the enzymes of the EDP have been demonstrated in glucose and fructose grown cells (Szymona and Doudoroff, 1960), suggesting that for the breakdown of these hexoses the EDP is operative. Fructose-grown cells, however, contain in addition a PEP-fructose-PTS (Saier et al., 1971) and, recently, we demonstrated 1-PFK in fructose-grown *R. capsulata*, *R. sphaeroides* and other species of phototrophic bacteria (Conrad and Schlegel, 1974). These observations suggested that in both phototrophic bacteria, fructose might be degraded via F-1-P and the EMP, while glucose is apparently degraded via EDP.

In the present paper, conclusive evidence for the operation of these pathways in *R. capsulata* has been obtained by enzymatic analysis, by isolation and study of a mutant, by tracing the labelled carbon in a degradation product of ^{14}C-labelled sugars and by radiorespirometry.

MATERIALS AND METHODS

Organism and Growth Conditions

Rhodopseudomonas capsulata strain Kb1 (Klemme, 1968), DSM Göttingen 155, was grown in a mineral medium containing 12 mmoles KH_2PO_4, 10 mmoles NH_4Cl, 2 mmoles $MgSO_4$, 7 mmoles NaCl, 0.35 mmoles $CaCl_2$ and 10 ml trace element solution SL4 (Pfennig and Lippert, 1966) in 1000 ml destilled water, adjusted to pH 6.9. Unless otherwise stated, the medium was supplemented for growth with 10–20 mmoles of either Na-DL-malate, D-glucose or D-fructose and 0.1 mg thiamine. Glucose and fructose were filtersterilized and added aseptically. For phototrophic cultures glass bottles of 100 or 500 ml volume were completely filled, closed with tightly fitting screw caps and incubated at 30° C with illumination of approximately 1000–2000 Lux. For aerobic cultures, fluted Erlenmeyer flasks were used and agitated on a rotatory shaker at 150 rev./min and 30° C. Inocula were prepared in the same manner and grown on the same substrate as the main culture. The extinction was measured at 650 nm wavelength using a Bausch & Lomb Spectronic 70 photometer. Growth of phototrophic cultures was followed by direct measurement of the extinction using screw capped tubes of 20 ml volume as culture vessels, which fitted into the sample compartment of the photometer. Purity of the cultures was checked by use of Difco AC medium and agar plates containing 0.3% peptone + 0.3% yeast extract.

Isolation of Mutants

Mutants failing to grow on glucose were isolated according to the method of Marrs et al. (1972). Cells were grown aerobically on glucose and harvested during the exponential growth phase. They were then washed and resuspended in mineral medium to a density of 10^9 cells/ml. N-methyl-N'-nitro-N-nitrosoguanidine (0.1 mg/ml) was added and the suspension incubated at 30° C in a water bath. After 30 min the cells were washed free of the mutagen and resuspended in the double volume of mineral medium containing 0.1% yeast extract and 20 mM Na-pyruvate. This suspension was dispensed into 5 flasks, which were aerated for 60 h on a rotary shaker at 30° C to allow the surviving cells to grow to stationary phase. The suspensions were then centrifuged, the cells washed and resuspended in mineral medium containing thiamine to a density of about 2×10^8 cells/ml. These suspensions were shaken for 7.5 h, adding then 25 mM glucose. After 5 h incubation Penicillin G (160 units/ml) was added and the incubation continued for 20 h. The surviving cells (about 10^4 cells/ml) were washed free of penicillin and spread on agar plates containing 25 mM glucose and 0.2 mM of either fructose or Na-pyruvate. The agar plates were incubated aerobically in the dark for 9 days. Small pinpoint colonies were picked from the plates and transferred to agar plates containing 0.3% peptone + 0.3% yeast extract. After 3 days aerobic incubation, the Lederberg technique was used to transfer cells from the colonies to agar plates containing either glucose, fructose or pyruvate. The mutants, which formed colonies on fructose- and pyruvate- but not on glucose-agar, were picked from the master plate and purified by streaking them on peptone-yeast extract agar. The mutant strains, which lost the ability to grow on glucose, were verified by incubation on glucose, fructose and pyruvate plates which were kept aerobically in the dark as well as anaerobically in the light.

Preparation of Cell-Free Extracts

The cells were harvested in the late exponential growth phase, washed twice in 50 mM phosphate buffer (pH 7.6) and resuspended to an extinction (650 nm) of 100–200. Cell extracts were prepared from these suspensions by sonication (2 min/5 ml) with a Braun Sonic 300 (Quigley, Rochester, U.S.A.) and centrifugating at 20000 × g for 30 min and 120000 × g for 90 min. The supernatant was filtered through Sephadex G-25 (Pharmacia, Uppsala, Sweden) and used immediately for enzyme assays. Protein was measured after the method of Lowry et al. (1951). Crude cell-free extracts for the assay of the PEP-fructose-PTS were prepared by suspending the cells in 2 mM phosphate buffer (pH 7) containing 140 mM KCl and 1 mM dithioerythritol, followed by sonication and centrifugating at 2000 × g for 15 min. The crude extract was fractionated into a pellet fraction and a soluble fraction by centrifugating at 200000 × g for 2 h.

Spectrophotometric Enzyme Assays

All enzyme assays were done at 25° C. Specific activity was defined as nmoles of substrate utilized per min and per mg protein.

The enzyme activites were determined in a photometer (PM6, Zeiss, Oberkochen, Germany) connected to a Servogor S recorder, measuring NADH oxidation or NAD(P) reduction at 366 nm. The rates of the enzyme reactions were linear with time and were proportional to the protein concentration. Controls were done by omitting the individual substrates or the cell extract in the test assay. Saturating substrate concentrations were used. The assay conditions of the individual enzymes were essentially those used by Probst and Schlegel (1973) and are described in detail below:

Glucokinase (EC 2.7.1.2): 1.79 ml 50 mM TEA buffer (pH 7.6), 0.60 ml glucose solution (200 mg/ml), 0.20 ml 100 mM $MgCl_2$, 0.20 ml 15 mM NADP, 0.10 ml 15 mM ATP, 10 µl G-6-P dehydrogenase (3.5 units), 0.10 ml cell extract.

Fructokinase (EC 2.7.1.4): 1.79 ml 50 mM TEA buffer (pH 7.6), 0.60 ml fructose solution (200 mg/ml), 0.20 ml 100 mM $MgCl_2$, 0.20 ml 15 mM NADP, 0.10 ml 15 mM ATP, 10 µl G-6-P dehydrogenase (3.5 units), 5 µl PGI (3.5 units), 0.10 ml cell extract.

Phosphoglucose isomerase (EC 5.3.1.9): 2.54 ml 50 mM TEA buffer (pH 7.6), 0.20 ml 100 mM $MgCl_2$, 0.10 ml 15 mM NADP, 0.10 ml 100 mM F-6-P, 10 µl G-6-P dehydrogenase (3.5 units), 0.05 ml cell extract.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49): 2.60 ml 50 mM TEA buffer (pH 7.6), 0.10 ml 100 mM $MgCl_2$, 0.10 ml 15 mM NADP, 0.10 ml 120 mM G-6-P, 0.10 ml cell extract.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44): 2.55 ml 50 mM TEA buffer (pH 7.6), 0.20 ml 100 mM $MgCl_2$, 0.10 ml 15 mM NADP or NAD, 0.10 ml 25 mM 6-PG, 0.05 ml cell extract.

6-Phosphogluconate dehydratase (EC 4.2.1.12): 0.855 ml TEA buffer (pH 7.6), 30 µl 100 mM $MgCl_2$, 20 µl 15 mM NADH, 15 µl KDPG aldolase (0.9 units), 10 µl L-lactate dehydrogenase (20 units), 50 µl 25 mM 6-PG, 20 µl cell extract.

KDPG aldolase (EC 4.1.2.14): 0.825 ml TEA buffer (pH 7.6), 30 µl 100 mM $MgCl_2$, 20 µl 15 mM NADH, 5 µl L-lactate dehydrogenase (10 units), 100 µl 4 mM Na-KDPG, 20 µl cell extract.

6-Phosphofructokinase (EC 2.7.1.11): 2.34 ml 50 mM TEA buffer (pH 8), 0.09 ml 100 mM $MgCl_2$, 0.20 ml 100 mM glutathion-reduced, 0.05 ml 15 mM NADH, 10 µl aldolase (0.18 units), 20 µl glycerolphosphate dehydrogenase (2.2 units)/triosephosphate isomerase (26 units), 0.10 ml 30 mM ATP, 0.10 ml 100 mM F-6-P, 0.10 ml cell extract.

1-Phosphofructokinase (EC 2.7.1.56): identical with the test conditions of 6-PFK, except using 10 mM F-1-P instead of 100 mM F-6-P.

FDP aldolase (EC 4.1.2.13): 2.73 ml 50 mM TEA buffer (pH 7.6), 0.05 ml 15 mM NADH, 20 µl glycerolphosphate dehydrogenase (2.2 units)/triosephosphate isomerase (26 units), 0.10 ml 20 mM FDP, 0.10 ml cell extract.

Fructose-1,6-diphosphatase (EC 3.1.3.11): 2.47 ml 100 mM Tirs buffer (pH 8.8), 0.10 ml 20 mM EDTA, 0.10 ml 100 mM $MgCl_2$, 0.10 ml 15 mM NADP, 20 µl G-6-P dehydrogenase (7 units), 5 µl PGI (3.5 units), 0.10 ml 100 mM FDP, 0.10 ml cell extract.

Phosphofructomutase (activity converting F-1-P to F-6-P): 2.58 ml 50 mM TEA buffer (pH 7.6), 5 µl 20 mM FDP, 0.20 ml 20 mM EDTA, 0.05 ml 100 mM $MgCl_2$, 0.05 ml 15 mM NADP, 0.10 ml 100 mM F-1-P, 10 µl G-6-P dehydrogenase (3.5 units), 5 µl PGI (3.5 units), 0.10 ml cell extract.

Radioactivity Measurement

Radioactivity was measured in a liquid scintillation counter (Tri-Carb 3375, Packard, U.S.A.). Toluene-ethanol-scintillation cocktail (Wilkerson and Eagon, 1972) and Aquasol (New England Nuclear, Dreieichenhain, Germany) were used as scintillation fluids. For determining the radioactivity of $^{14}CO_2$, which has been absorbed in KOH solution, a mixture of 9 ml Aquasol and 1 ml water was used as scintillation cocktail. In that case the scintillation flasks were allowed to stand for at least 5 h, so that the chemoluminescence, which was caused by the alkaline sample, had disappeared. An external standard was used to correct for quenching. The radioactivity was then calculated as disintegrations per min (dpm).

PEP-Fructose-Phosphotransferase

The method of Newsholme et al. (1967), which has been applied to the determination of the PEP-fructose-PTS by Sobel and Krulwich (1973) was used. The assay conditions were those of Saier et al. (1971). The reaction was carried out at 25° C using a concentration of 10 mM U-^{14}C-fructose (1.36 µCi/µmol). The reaction was stopped after 30 min by addition of one volume ethanol. Radioactivity measurement was carried out in 15 ml toluene-ethanol-scintillation cocktail.

Incorporation of Labelled Hexose Carbon into Pyruvate

Cells were grown aerobically on either glucose or fructose and harvested during the exponential growth phase. The cells were washed twice in 12 mM phosphate buffer (pH 6.9) and resuspended to an extinction (650 nm) of 6. Depending on the experiment, 1 ml cell suspension of glucose- or fructose-grown cells was placed together with 5 µmoles Na-arsenite into the main compartment of a Warburg flask. The sidearm contained 1 µmol of either ^{14}C-glucose or ^{14}C-fructose (3 µCi/µmol). The open Warburg flask was then attached to a reciprocal shaker and shaken at 30° C for 30 min at 100 rev./min. Then the ^{14}C-hexose was added from the sidearm, and shaking was continued for 60 min. The cells were then harvested by centrifugation and washed once with distilled water. The ^{14}C-pyruvate, which was excreted during the experiment, was isolated from the combined supernatants by means of ion exchange chromatography (Thauer et al., 1970) adding 5 µmoles unlabelled Na-pyruvate as carrier. The isolated pyruvate was then decarboxylated according to the method of Fromageot and Desnuelle (1935). The decarboxylation was carried out in Warburg flasks with double sidearms. The main compartment contained 1 ml ^{14}C-pyruvate solution, the first sidearm 0.1 ml 10 N H_2SO_4 and the second sidearm 0.1 ml $Ce(SO_4)_2$ saturated 1 N H_2SO_4. For the absorption of evolving $^{14}CO_2$, 0.2 ml 10% KOH-solution and a small piece of filter paper were placed into the centre well. The Warburg flasks were then closed with serum stoppers and the reaction was started by adding the contents from the two sidearms into the main compartment. The flasks were shaken at 30° C for 3 h. Then the flasks were opened and the KOH-solution and the filter paper transferred into Aquasol, rinsing the centre well three times with the scintillation cocktail. The radioactivity of an aliquot from the main compartment was also determined. The efficiency of the decarboxylation procedure was checked by decarboxylating 1-^{14}C-pyruvate: 98% of the radioactivity was recovered as $^{14}CO_2$ under the conditions described.

Radiorespirometry

Cells were grown aerobically in a glucose or fructose salts medium and harvested in the middle of the exponential growth phase. The cells were washed free of nutrients and resuspended in mineral medium with thiamine to an extinction (650 nm) of 2. The radiorespirometric experiments were carried out in Warburg flasks. Cell suspension (0.8 ml) was pipetted into the main compartment and

0.2 ml 10% KOH-solution and a small piece of filter paper were placed into the centre well. The sidearm contained 0.2 ml 10 mM ^{14}C-glucose or ^{14}C-fructose labelled in different positions. The Warburg flasks were closed with serum stoppers and attached to a reciprocal shaker. They were shaken with sidearms open for 10 min at 30°C and 100 rev./min. The sidearms were then closed with the insert plug and the ^{14}C-hexose added from the sidearm into the main compartment. The reaction was subsequently stopped by injecting 0.1 ml 4 N HCl into the main compartment. The flasks were shaken for a further 30 min to allow quantitative absorption of CO_2. Then the flasks were opened and the content of the centre well was transferred into Aquasol, the centre well being further rinsed three times with scintillation fluid. The cells were harvested by centrifugation and aliquots of the resuspended cells and of the supernatant medium were added to Aquasol. The total radioactivity recovered was in the range of 84–106%.

Preparation of Position-Labelled Fructose

Fructose labelled in position 2, 3 or 3+4, was prepared from the correspondingly labelled glucose by the method of Muntz and Carroll (1960), which was modified and described in detail by Avigad et al. (1966). The following modifications were applied: During the course of our preparation, only 50 µmoles glucose were inserted and correspondingly less $NaBH_4$-solution was added. For desalting the reaction mixture, a mixed resin bed of Dowex 50W X8H^+ and Dowex 1X1OH^- (1:1 mixture, 50–100 mesh, Serva, Heidelberg, Germany) was used. The enzymatic reaction converting sorbitol to fructose was carried out in 200 mM phosphate buffer (pH 8) and at 90 min incubation time. The fructose was separated from unreacted sorbitol by thinlayer chromatography using Cellulose G 1440 glass plates (20 × 20 cm, 0.1 mm thickness, Schleicher & Schüll, Dassel, Germany). The chromatograms were developed ascending 3 times for 3 h in n-butanol/ethanol/H_2O (52:33:15). Rechromatography of the eluted fructose showed that it was free from contaminating sorbitol. The yield of ^{14}C-fructose was 50–60%.

Chemicals

Analytical grade chemicals, D-glucose (biochemical grade) and D-fructose (purissimum, DAB 7) were purchased from Merck, Darmstadt, Germany. N-Methyl-N′-nitro-N-nitrosoguanidine was obtained from Serva, Heidelberg, Germany, Penicillin G sodium salt from Dauelsberg, Göttingen, Germany, dithioerythritol from Sigma, U.S.A., $Ce(SO_4)_2$ from the British Drug House, Poole, England and Na-arsenite from Riedel de Haën, Hannover, Germany. Biochemicals and enzymes were supplied from Boehringer, Mannheim, Germany. Dr. H. von Hugo supplied purified 1-PFK from *Clostridium pasteurianum*. KDPG aldolase was purified from cell extracts of *Alcaligenes eutrophus* H 16 according to the description of Robra (1971). KDPG was prepared from 6-phosphogluconate using a crude cell-free extract of a KDPG aldolase-deficient mutant of *A. eutrophus* H 16 (Robra, 1971). The radiochemicals were purchased from Amersham-Buchler, Braunschweig, Germany. 3-^{14}C-glucose and 3,4-^{14}C-glucose were obtained from New England Nuclear, Dreieichenhain, Germany and 6-^{14}C-fructose was from Mallinckrodt, St. Louis, U.S.A.

Table 1. Specific activities of the PEP-fructose-phosphotransferase in crude extracts of *Rhodopseudomonas capsulata*[a]

Substrate supporting growth	Growth conditions	
	Phototrophic	Aerobic-dark
Malate	0.7	0.1
Glucose	0.7	0.7
Fructose	5.2	5.5

[a] Specific activity expressed as nmoles substrate utilized per mg protein and per min

RESULTS

Demonstration of Phosphoenolpyruvate-Fructose-Phosphotransferase

The induction of 1-PFK by fructose (Conrad and Schlegel, 1974) suggested that fructose is degraded by the EMP via F-1-P. This required the presence of an enzyme activity forming F-1-P from fructose. *Rhodopseudomonas capsulata* possessed PEP-fructose-PTS activity which was about 8 times higher in fructose-grown than in glucose- or malate-grown cells (Table 1). The activity was induced under both aerobic and phototrophic growth conditions. In the crude extract the reaction of the PEP-fructose-PTS was linear for more than 30 min and the reaction rate was proportional to the extract protein concentration up to 10 mg/ml assay mixture.

To characterize the phosphotransferase, the crude extract was fractionated by centrifuging for 2 h at 200000 × g, and the activity was tested in the crude extract, the pellet and the soluble fraction without and with ATP or PEP as phosphate donor. In the crude extract and the pellet fraction, the specific activity of the phosphotransferase was higher with PEP than with ATP (Table 2). Significant ATP-dependent activity was found only in the soluble fraction. This activity was in the range of that of fructokinase found in spectrophotometric enzyme assays of Sephadex G-25-treated cell extracts (Table 4). Without any exogenous phosphate donors only a low specific phosphotransferase activity was detected. 98% of the PEP dependent phosphotransferase activity was localized in the pellet fraction (Table 3). The fractionation resulted in a relatively high loss of PEP-dependent phosphotransferase activity, while the loss of protein and of ATP-dependent activity was rather low (Table 3). A similar high loss of activity was observed in the absence of exogenous phosphate donor. The loss of activity may be explained by the loss of a necessary factor from the membranes during the fractionation step. In *Rhodospirillum rubrum*, a factor with a molecular weight of 200000, was removed by washing the membrane fraction with water resulting in inactivation of the PEP-fructose-PTS. In *R. sphaeroides* the factor was removed even by washing with KCl-solution (Saier et al., 1971).

Table 2. Specific activities of the PEP-fructose phosphotransferase in fractionated extracts of cells grown phototrophically on fructose

Phosphate donor	Specific activity (nmoles/mg protein per min) in		
	Crude extract	Pellet fraction	Soluble fraction
PEP	4.4	2.6	0.2
ATP	1.7	0.6	4.6
No exogenous	1.1	0.4	0.5

Table 3. Localisation of the PEP-fructose-phosphotransferase activity in fractionated extracts of cells grown phototrophically on fructose

Phosphate donor	Loss of phosphotransferase activity during fractionation of the crude extract (%)[a]	Distribution of the recovered phosphotransferase activity (%)	
		Pellet fraction	Soluble fraction
PEP	47	98	2
ATP	24	41	59
No exogenous	63	81	19
Protein	15	85	15

[a] The absolute activities corresponding to 100% activity of the crude extract were those listed in column 1 of Table 2

In order to determine the position in which fructose was phosphorylated by the PEP-fructose-PTS, the reaction was carried out with the pellet fraction. After being deproteinized, the reaction mixture was assayed for F-1-P and F-6-P formed. The assays of F-1-P and F-6-P were those used for the determination of 1-PFK and 6-PFK, respectively: F-1-P and F-6-P were omitted and replaced by the enzymes 1-PFK and 6-PFK. After 10 min reaction time, only F-1-P but not F-6-P could be detected in the reaction mixture (about 80 nmoles F-1-P per mg protein), indicating that fructose was phosphorylated by the PEP-fructose-PTS in 1-position.

Possible Fate of Fructose-1-Phosphate

R. capsulata was known to use the EDP for the catabolism of glucose (Eidels and Preiss, 1970). Cells grown on fructose, however, contained both EDP and EMP enzymes (Conrad and Schlegel, 1974) and it was uncertain whether one or the other or both systems were operative during fructose catabolism. Table 4

Table 4. Specific activities of some enzymes of carbohydrate metabolism in cell extracts of *R. capsulata*

Enzyme	Specific activity (nmoles/mg protein per min) after phototrophic growth in mineral medium[a] containing		
	Malate	Glucose	Fructose
Glucokinase	107	101	117
Fructokinase	7	7	8
Phosphoglucose isomerase	322	542	474
Phosphofructomutase (converting F-1-P to F-6-P)			n.d.[b]
6-PG dehydrogenase			
NADP	n.d.	n.d.	n.d.
NAD	n.d.	n.d.	n.d.

[a] The mineral medium contained 0.05% yeast extract
[b] n.d. = not detectable

shows that cell extracts contained only a relatively low fructokinase activity, while the glucokinase activity was high. This indicated that fructose catabolism proceeded via F-1-P rather than F-6-P. As 1-PFK and FDP aldolase were present in fructose-grown cells, the degradation of F-1-P via EMP appeared to be possible. Therefore the question of the metabolic fate of F-1-P focused attention on whether F-1-P could be additionally channelled into the EDP. To rule out that F-6-P was formed from F-1-P by a phosphofructomutase activity—an enzyme yet to be found—, this activity was tested by using the phosphoglucomutase assay (Bergmeyer et al., 1974). G-1-P and GDP were replaced by F-1-P and FDP and PGI was also added. No activity could be detected using TEA-buffer pH 7.6 and 8.0 and Tris-buffer pH 8.8 with or without EDTA. The activity was also absent in cells grown aerobically on fructose. Therefore, it was concluded that F-6-P could originate from F-1-P only via FDP. As only a low 6-PFK activity was present, degradation of F-6-P via EMP was unlikely. F-6-P should be converted into G-6-P by the high constitutive activity of PGI allowing further degradation via EDP. Consequently, fructose catabolism via EDP could so far not be excluded.

Mutants Defective in the EDP

To decide whether the EDP is necessary for fructose metabolism in *R. capsulata*, we isolated mutants which were defective in one of the enzymes of the EDP, but were still able to utilize fructose. Out of 510 isolated colonies 4 mutant strains were isolated, which were not able to grow aerobically or photo-

trophically on agar plates containing glucose as sole substrate, but were unaltered in their ability to utilize fructose and pyruvate. Figure 1 shows the growth of a representative mutant GP33 on fructose and glucose under phototrophic conditions in liquid culture. Growth on fructose occurred in both wildtype and mutant, with a similar generation time of about 3–4 h, whereas glucose was utilized only by the wildtype but not by the mutant. To determine the enzymic defect of the mutant, the enzymes of hexose metabolism were measured in cell extracts of wildtype and mutant cells grown phototrophically in peptone-yeast extract medium containing glucose. To avoid possible non-induction of glucose catabolic enzymes by exogenous glucose, the enzymes were also measured in fructose grown cells. Table 5 shows the specific activities of enzymes in the wildtype and the mutant GP33. Under no growth conditions was G-6-P-dehydrogenase activity detectable in the mutant, while it was easily measurable in the wildtype. In addition, the mutant had a low specific activity of 6-PG dehydratase. Possibly the enzyme is induced by 6-PG, which is not formed when G-6-P-dehydrogenase is defective. All the other enzymes tested were present in comparable specific activities in the mutant and the wildtype. The properties of the mutant GP33 were shared by the other 3 mutants which have been isolated.

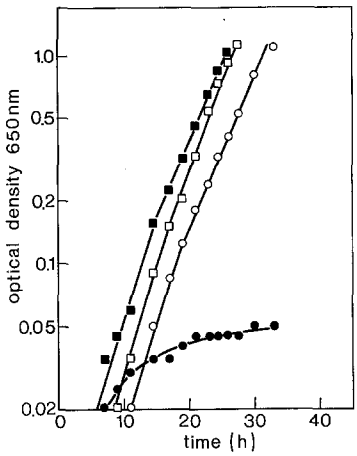

Fig. 1
Phototrophic growth of wildtype and mutant GP33 of *R. capsulata* on glucose and fructose. The cultures were incubated with malate-grown cells. Wildtype growing on fructose (□——□) and glucose (○——○); mutant GP33 growing on fructose (■——■) and glucose (●——●)

Incorporation of 1-^{14}C-Hexose into Pyruvate

To decide which role the EDP plays in the catabolism of fructose in *R. capsulata* wildtype, a radiolabelling technique was applied. The catabolism of 1-^{14}C-hexose via the EDP gives rise to 1-^{14}C-pyruvate, whereas catabolism via the EMP yields 3-^{14}C-pyruvate. *R. capsulata* did not excrete pyruvate or lactate when grown on glucose or fructose under phototrophic or aerobic conditions. Resting cells, however, incubated in the presence of arsenite, excreted up to 50% of the supplied hexose-carbon as lactate under phototrophic conditions and as pyruvate under aerobic conditions. Incorporation of U-^{14}C- and 1-^{14}C-glucose and -fructose into pyruvate was studied under aerobic conditions. The yield of radioactivity in the isolated pyruvate was the same with U- and 1-^{14}C-hexose, indicating that both C_3-halves of the hexose were excreted in equal amounts.

Table 6 shows the distribution of radioactivity in the pyruvate derived from labelled glucose and fructose. The carboxyl-group of pyruvate originating from U-^{14}C-hexose was labelled less than 33.3% of the total radioactivity. The deviation from this theoretical value was similar to the results obtained with *Micrococcus luteus* (*Sarcina lutea*) reported by Dawes and Holms (1958) and may be explained by exchange

Table 5. Specific activities of some enzymes of carbohydrate metabolism in cell free extracts of wild type and mutant GP33 of *R. capsulata*[a]

Enzyme	Wild-type growth conditions			Mutant GP33 growth conditions		
	Fructose phototrophic	Fructose aerobic	Glucose phototrophic[b]	Fructose phototrophic	Fructose aerobic	Glucose phototrophic[b]
Glucokinase	117[c]			146		128
Fructokinase	8[c]			15		14
G-6-P dehydrogenase	48	115	52	n.d.[d]	n.d.	n.d.
6-PG dehydratase	96	161	101	6	14	5
KDPG aldolase	103	164	110	81	52	82
1-Phosphofructokinase	45	71	1	65	86	3
6-Phosphofructokinase	6			1		2
FDP aldolase	90	73	73	127	78	94
FDPase	39	37	25	72	30	16

[a] Specific activity expressed as nmoles substrate utilized per mg protein and per min
[b] The mineral medium contained 0.1% peptone and 0.1% yeast extract
[c] The mineral medium contained 0.05% yeast extract
[d] n.d. = not detectable

Table 6. Decarboxylation of pyruvate excreted by *R. capsulata* on 1-^{14}C-glucose or 1-^{14}C-fructose

Substrate for pyruvate excretion	Pyruvate used for decarboxylation (dpm)	Recovery of radioactivity (%) after decarboxylation of the pyruvate[a] in		
		CO_2	Acetate	Total recovery
U-^{14}C-glucose	290284	29.1	72.1	101.2
1-^{14}C-glucose	228923	74.6	25.3	99.9
1-^{14}C-glucose[b] (corrected values)		86	14	100
U-^{14}C-fructose	258204	26.9	74.0	100.9
1-^{14}C-fructose	225823	15.6	85.7	101.3
1-^{14}C-fructose[b] (corrected values)		19	81	100

[a] The values are mean values from three estimates; the maximal deviation from the mean value was 1%

[b] The procedure used for correcting the data is explained in the text, the total recovery of radioactivity was taken into account

reactions with unlabelled CO_2 via Wood-Werkmann reaction. The loss of $^{14}CO_2$ from the carboxyl group of pyruvate which is derived from U-^{14}C-hexose, was used for correcting the data obtained with pyruvate from 1-^{14}C-hexose. The assumption was made, that the relative loss of radioactivity in the carboxyl-group was the same with U- and 1-^{14}C-hexose. After correction of the labelling data for pyruvate derived from 1-^{14}C-fructose, only 19% of the total radioactivity was present in the carboxyl-group. On the other hand, 1-^{14}C-glucose gave rise to pyruvate, which contained 86% of the total radioactivity in the carboxyl-group. These results show that fructose was degraded mainly via EMP and glucose mainly via EDP.

Radiorespirometry

Radiorespirometry is a useful tool in the analysis of hexose degradative pathways under aerobic growth conditions (Wang et al., 1958). Radiorespirometric experiments were carried out with glucose and fructose labelled in 1-, 2-, 3-, 3,4-, and 6-position. The $^{14}CO_2$, which was generated from a position-labelled hexose in a certain time period, was trapped in KOH and counted. The percentage yield of $^{14}CO_2$ originating from the radioactive hexose was determined. The rate of $^{14}CO_2$ evolution from 4-^{14}C-hexose was calculated by difference from the rates obtained from 3,4-^{14}C-hexose and 3-^{14}C-hexose.

The results show a completely different pattern of radiorespirometry with fructose and glucose as substrates (Fig. 2). With glucose as substrate, the rates of $^{14}CO_2$ evolution from 1-^{14}C- and 4-^{14}C-glucose were nearly the same and exceeded those from 3-^{14}C- and 6-^{14}C-glucose. With fructose as substrate, on the other hand, the rates of $^{14}CO_2$ evolution from 3-^{14}C- and 4-^{14}C-fructose exceeded those from 1-^{14}C- and 6-^{14}C- fructose. These results are in accordance with glucose being catabolized via EDP and fructose via EMP. The results did not indicate any participation of the PPP in glucose or fructose degradation, which is consistent with the fact that 6-PG dehydrogenase was not detectable in cell extracts of *R. capsulata* (Table 4). An additional radiorespirometric experiment on fructose was carried out with the G-6-P dehydrogenase$^-$-mutant GP33 (Fig. 3). The results were comparable to those obtained with the wildtype, suggesting that fructose catabolism proceeded exclusively via EMP in *R. capsulata*.

DISCUSSION

Our radiorespirometric experiments have shown that in *Rhodopseudomonas capsulata* fructose was catabolized by means of the EMP while glucose was degraded by the EDP (Fig. 4). This finding agrees with the data of Eidels and Preiss (1970) on glucose metabolism and confirms our previous conclusions (Conrad and Schlegel, 1974). No attempt was made to calculate the relative contribution of the EMP and EDP to hexose breakdown (see Katz and Wood, 1963), as we did not follow the respiratory CO_2-evolution to exhaustion of the carbon source. A comparison of fructose radiorespiration by the wildtype and by the G-6-P dehydrogenase$^-$-mutant, however, indicated only an insignificant contribution of the EDP to fructose metabolism.

The results of the radiorespirometric experiments were confirmed by pyruvate-labelling experiments. Only a small fraction of fructose or glucose was catabolized via the converse pathway (EDP and EMP, respectively). An estimation of the exact proportion of hexose carbon being catabolized by one or the other pathway is not possible as the extent of triose-phosphate conversion to the pyruvate pool is not known. It cannot be excluded, that the unphysiologi-

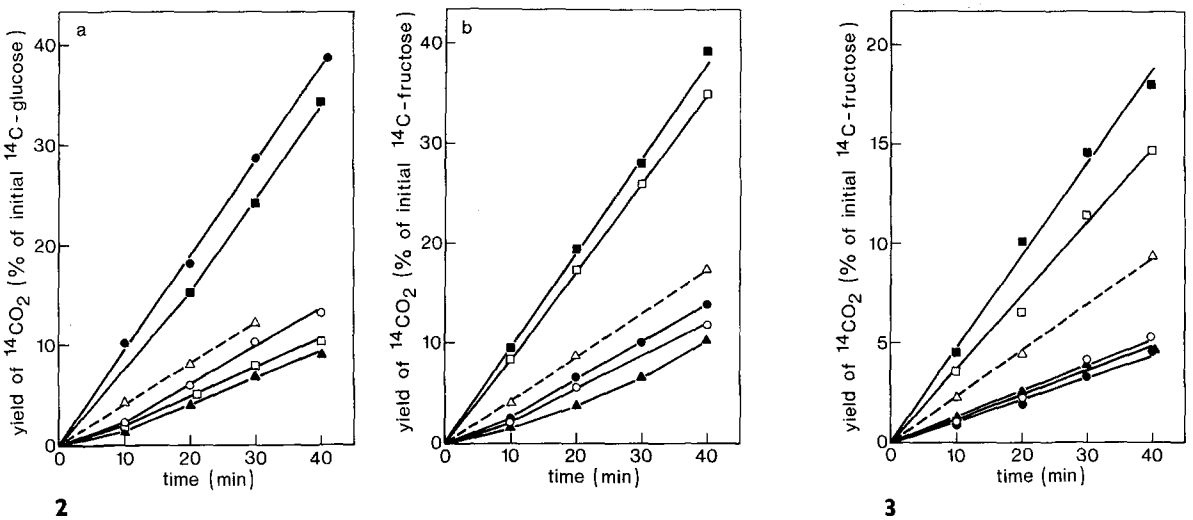

Fig. 2a and b. Radiorespirometry of position-labelled glucose and fructose by *R. capsulata*. Each incubation mixture contained: 0.8 ml resuspended bacteria (E_{650} = 2) aerobically grown on glucose or fructose and 0.2 ml of glucose or fructose (10 µmoles/ml) labelled in different carbon atoms. The radioactivity used was: (a) 1-^{14}C-glucose, 1.83×10^5 dpm; 2-^{14}C-glucose, 2.07×10^5 dpm; 3-^{14}C-glucose, 1.83×10^5 dpm; 3,4-^{14}C-glucose, 2.05×10^5 dpm; 6-^{14}C-glucose, 1.88×10^5 dpm; U-^{14}C-glucose, 1.66×10^5 dpm; (b) 1-^{14}C-fructose, 1.52×10^5 dpm; 2-^{14}C-fructose, 1.47×10^5 dpm; 3-^{14}C-fructose, 1.56×10^5 dpm; 3,4-^{14}C-fructose, 1.72×10^5 dpm; 6-^{14}C-fructose, 6.51×10^4 dpm; U-^{14}C-fructose, 1.71×10^5 dpm. ●——● 1-^{14}C-hexose; □——□ 3-^{14}C-hexose; ▲——▲ 6-^{14}C-hexose; ○——○ 2-^{14}C-hexose; ■——■ 4-^{14}C-hexose; △----△ U-^{14}C-hexose

Fig. 3. Radiorespirometry of position-labelled fructose by G-6-P dehydrogenase$^-$-mutant of *R. capsulata*. The experimental conditions were the same as for Figure 2. The radioactivity used was: 1-^{14}C-fructose, 1.47×10^5 dpm; 2-^{14}C-fructose, 1.46×10^5 dpm; 3-^{14}C-fructose, 1.57×10^5 dpm; 3,4-^{14}C-fructose, 1.69×10^5 dpm; 6-^{14}C-fructose 6.72×10^4 dpm; U-^{14}C-fructose, 1.64×10^5 dpm

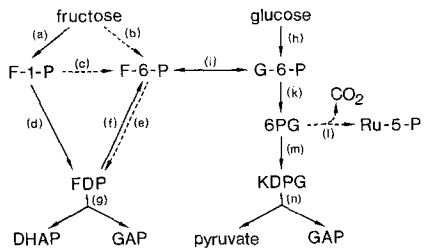

Fig. 4. Pathways of glucose and fructose catabolism in *R. capsulata*. A solid line indicates that the enzyme activity was present; a broken line indicates that the enzyme activity was only low or not detectable. Enzymes: (*a*) PEP-fructose-PTS, (*b*) fructokinase, (*c*) phosphofructomutase, (*d*) 1-PFK, (*e*) 6-PFK, (*f*) FDPase, (*g*) FDP aldolase, (*h*) glucokinase, (*i*) PGI, (*k*) G-6-P dehydrogenase, (*l*) 6-PG dehydrogenase, (*m*) 6-PG dehydratase, (*n*) KDPG aldolase

cal conditions applied for achieving pyruvate excretion influenced the amount of hexose carbon distributed to the EDP or the EMP.

The radiometric techniques have only been applied to aerobically growing cells. Since, however, the mutant deficient in G-6-P dehydrogenase was unaltered in its ability to grow phototrophically on fructose and did not grow on glucose, it is assumed that the pattern of sugar metabolism is the same under phototrophic as well as aerobic conditions.

The presence of an inducible PEP-fructose-PTS and 1-PFK together with a low fructokinase activity and the absence of phosphofructomutase activity suggested that fructose was degraded via F-1-P (Fig. 4). Bacteria, which catabolize fructose exclusively via F-1-P, require the activity of FDPase and PGI for the synthesis of carbohydrates originating from G-6-P; FDPase$^-$-mutants of *Enterobacter aerogenes* for example were not able to grow on fructose (Sapico et al., 1968). As G-6-P, which is formed during the carbohydrate synthesis, can also be used for catabolism via EDP, a pathway leading from fructose via F-1-P, FDP, F-6-P and G-6-P into the EDP is conceivable. This was recently shown by Sawyer et al. (1977), who demonstrated that *Pseudomonas aeruginosa*, *P. putida*, *P. stutzeri* and *P. acidovorans* catabolized fructose mainly via EDP although PEP-fructose-PTS and 1-PFK have been induced under these conditions. Only a 6-PG dehydratase$^-$-mutant of *P. putida*, which was still able to grow on fructose (Vincente and Cánovas, 1973) catabolized fructose via EMP. The results of our study on *R. capsulata* show, on the other hand, that fructose was mainly catabolized via EMP and not via EDP.

In glucose-grown cells, the PEP-fructose-PTS and the 1-PFK were not induced. Only the enzymes of the

EDP were present. 6-PFK activity was very low in cell extracts, suggesting that the EMP was not used for glucose catabolism. This was confirmed by the pattern of glucose radiorespirometry and by the inability of a G-6-P dehydrogenase$^-$-mutant to grow on glucose, although the data of the pyruvate labelling experiment suggested a low contribution of the EMP to glucose catabolism.

The most astonishing result of this study was that in one bacterium two sugars, which are as similar to each other as glucose and fructose, were degraded via two very different pathways, the EDP and the EMP. The reason for the differences in the degradation of both sugars is seen in the phosphorylation of fructose to F-1-P instead of F-6-P (Fig. 4). If fructose would be catabolized via F-6-P, it must be assumed that F-6-P and G-6-P are equilibrated by the PGI-reaction efficiently enough to allow degradation of both fructose and glucose by the same pathway. PGI occupies a key position in distributing the hexose molecules into the different pathways, which either start with F-6-P, like the EMP, or with G-6-P, like the EDP and PPP. It was shown that the mammalian PGI-activity can be effected by 6-PG as competitive inhibitor (Kahana et al., 1960). In vivo, however, the PGI-reaction is apparently working fast enough to allow considerable equilibration between F-6-P and G-6-P (Landau and Katz, 1964). Therefore, it is obvious that a functional compartmentation of glucose and fructose catabolism is achieved by the initial formation of F-1-P rather than F-6-P. Several bacteria are already known to possess the enzymes necessary for fructose breakdown via F-1-P. The PEP-fructose-PTS and/or 1-PFK, have been demonstrated in *Arthrobacter pyridinolis* (Sobel and Krulwich, 1973), species of *Clostridium* (Patni and Alexander, 1971; du Toit et al., 1972; von Hugo and Gottschalk, 1974), *Bacteroides symbiosus* (Hsu and Reeves, 1970), *Butyrivibrio fibrisolvens* (Kistner and Kotzé, 1973), phototrophic bacteria (Conrad and Schlegel, 1974), species of *Beneckea* and *Photobacterium* (Gee et al., 1975), *Pseudomonas doudoroffii* (Baumann and Baumann, 1975) and other species of *Pseudomonas* (Sawyer et al., 1977). In addition, some bacteria have been shown to catabolize fructose via F-1-P and glucose via F-6-P, both by means of the EMP (for references see introduction). After applying several experimental methods, we have now obtained evidence that in *R. capsulata* the conversion of fructose to F-1-P results in a functional separation of the EMP from the EDP.

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