An Alternative Pathway for the Degradation of Endogenous Fructose during the Catabolism of Sucrose in *Rhodopseudomonas capsulata*

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Sucrose catabolism was studied in *Rhodopseudomonas capsulata*. Sucrose was hydrolysed by the action of a constitutive cytoplasmic sucrose. The use of a glucose-6-phosphate dehydrogenase-deficient mutant and radiorespirometric experiments demonstrated that both the glucose and the fructose moieties of sucrose were catabolized via the Entner–Doudoroff pathway. This result was confirmed by enzyme analysis and studies on sugar assimilation. All the enzymes of the Entner–Doudoroff pathway were present in bacteria grown on sucrose but fructokinase (EC 2.7.1.4) activity was relatively low. In contrast, phosphoenolpyruvate:fructose phosphotransferase and 1-phosphofructokinase, the key enzymes for the catabolism of exogenous fructose, were only partially induced. Bacteria grown on sucrose and treated with chloramphenicol were, therefore, not able to assimilate exogenous fructose. We conclude that under these conditions endogenous fructose is catabolized via the Entner–Doudoroff pathway, while exogenous fructose is degraded via fructose 1-phosphate and the Embden–Meyerhof pathway.

INTRODUCTION

Recently, we have shown that in *Rhodopseudomonas capsulata* fructose is degraded via fructose 1-phosphate and the Embden–Meyerhof pathway (EMP) (Conrad & Schlegel, 1977a). Glucose, on the other hand, is catabolized via the Entner–Doudoroff pathway (EDP) (Conrad & Schlegel, 1977a; Eidels & Preiss, 1970). *Rhodopseudomonas capsulata* is able to grow on sucrose as well as fructose and glucose. The question therefore arose as to whether glucose and fructose originating from sucrose hydrolysis were catabolized through the same pathways as when supplied exogenously as the free hexoses. In the present paper we provide evidence that both the glucose and the fructose moieties of sucrose were degraded via the EDP; the catabolic pathway of endogenously formed fructose was thus different from that of exogenous fructose.

METHODS

Bacteria and growth conditions. *Rhodopseudomonas capsulata* strain Kb1, DSM 155, was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G. The mutant strain GP33 was isolated from the wild type as described earlier (Conrad & Schlegel, 1977a). This mutant was deficient in glucose-6-phosphate dehydrogenase activity and was unable to grow on glucose. The growth conditions were the same as used previously (Conrad & Schlegel, 1977a).

Preparation of protoplasts. Protoplasts from bacteria grown aerobically on sucrose were prepared by treatment with lysozyme/EDTA (Karunairatnam, Spizizen & Gest, 1958). The protoplasts were stable in 50 mM-phosphate buffer (pH 7.6) containing 1 M-mannitol and 50 mM-MgSO₄. The protoplast suspension was centrifuged for 30 min at 15000 *g* and 4 °C; this supernatant was designated the periplasmic fraction. The pellet was resuspended in 50 mM-phosphate buffer (pH 7.6), incubated in the presence of DNAase for

5 min at 25 °C and then freed from membranes by centrifugation for 30 min at 20000 g and 4 °C; this supernatant was designated the cytoplasmic fraction.

Enzyme assays. The preparation of bacterial extracts and the enzyme assays were done as described by Conrad & Schlegel (1977a). Sucrase activity was measured using a mixture similar to that for the fructokinase (EC 2.7.1.4) assay, with fructose replaced by sucrose.

Determination of glucose and fructose. Glucose was measured by means of a glucose oxidase-coupled colour reaction (Biochemica blood sugar test combination, Boehringer). Fructose was determined by the resorcinol-thiourea test (Dische, 1962).

Assimilation of radioactive sugars. Bacteria were grown aerobically in mineral medium containing glucose, fructose or sucrose and harvested during the exponential growth phase. The bacteria were washed free of nutrients and resuspended in mineral medium, supplemented with thiamin ($0.1 \mu\text{g ml}^{-1}$) and chloramphenicol ($50 \mu\text{g ml}^{-1}$), to an absorbance (650 nm) of 1.5. The bacterial suspension (2 ml) was distributed into Erlenmeyer flasks (25 ml) and shaken at 25 °C and 150 rev. min^{-1} . The reaction was started by adding 20 μl of [^{14}C]glucose, [^{14}C]fructose or [^{14}C]sucrose (final concentration, 1 mM; $0.3 \mu\text{Ci } \mu\text{mol}^{-1}$). At intervals, samples (100 μl) were withdrawn with an automatic pipette and rapidly filtered through Sartorius cellulose nitrate membrane filters (pore size $0.45 \mu\text{m}$). The filters were washed with 10 ml ice-cold mineral medium and placed in 15 ml toluene/ethanol scintillation cocktail. The radioactivity was measured as described by Conrad & Schlegel (1977a). Cell protein was determined by the method of Schmidt, Liaaen-Jensen & Schlegel (1963).

Preparation of [fructose- ^{14}C]sucrose and radiorespirometry. Samples of [^{14}C]sucrose labelled in the fructose moiety in positions 1, 1,6 and uniformly were prepared from the correspondingly labelled fructose specimens by the method of Chassy & Krichevsky (1972), except that the last step of the purification of sucrose phosphorylase (chromatography on Sephadex G-200) was omitted. Despite this omission, the enzyme preparation contained no hydrolytic activity. [^{14}C]Fructose (2 μmol , 1 μCi) was incubated overnight with 160 μmol glucose 1-phosphate and approximately 0.09 units of sucrose phosphorylase at room temperature. The solution was then desalted on a mixed resin bed of Dowex 50W-X8 (H^+ form) and Dowex 1-X1 (OH^- form) (1:1 mixture, 50 to 100 mesh; Serva, Heidelberg, F.R.G.) and concentrated at 40 °C in an evaporator (Rotavapor R, Büchi, Switzerland). The radioactive sucrose was separated from unreacted fructose by thin-layer chromatography using Cellulose G 1440 glass plates ($20 \times 20 \text{ cm}$, 0.1 mm thick; Schleicher & Schüll, Dassel, F.R.G.). The chromatograms were developed with ascending solvent twice for 2 h in 1-butanol/ethanol/water (52:33:15, by vol.). The radioactive sucrose was located by means of a radiochromatogram spark chamber (Birchover Isotopen Kamera 450, Zinsser, Frankfurt, F.R.G.). The radiorespirometric experiments were done as described by Conrad & Schlegel (1977a). The total radioactivity recovered at the end of the experiments was in the range of 88 to 98 %.

Chemicals. Chemicals were from the same sources as described earlier (Conrad & Schlegel, 1977a). Sucrose, analytical grade, was from J. T. Baker, Deventer, The Netherlands. Chloramphenicol was purchased from Serva, Heidelberg, F.R.G. The radiochemicals were supplied by Amersham-Buchler, Braunschweig, F.R.G., except [$1,6\text{-}^{14}\text{C}_2$]fructose, which was from Calbiochem.

RESULTS

Essential enzymes of sucrose catabolism

The enzymes of *Rhodopseudomonas capsulata* strain kbl were analysed in bacteria grown phototrophically (doubling time 5 to 6 h) or aerobically (doubling time 6 to 7 h) on sucrose. Sucrase was formed constitutively; its specific activity after growth on malate, fructose, glucose and sucrose under phototrophic or aerobic conditions ranged between 35 and 68 nmol sucrose cleaved $\text{min}^{-1} (\text{mg protein})^{-1}$. Sucrose was hydrolysed to glucose and fructose above pH 6.8 with maximum activity; no reaction occurred with raffinose as substrate. All the enzymes necessary for the breakdown of intracellular glucose via the EDP were present in the extracts (Table 1), i.e. glucokinase (EC 2.7.1.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14). Although phosphoglucose isomerase (EC 5.3.1.9) and fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) were also active, degradation of glucose via fructose 6-phosphate and the EMP appeared unlikely, since 6-phosphofructokinase (EC 2.7.1.11) activity was very low. The absence of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activity indicated that the pentose phosphate pathway was not involved in sucrose catabolism. In contrast to the high glucokinase activity, fructokinase

Table 1. *Specific activities of some enzymes of sucrose metabolism in R. capsulata*

Specific activities are expressed as nmol substrate utilized min⁻¹ (mg protein)⁻¹ at 25 °C.

Enzyme	Specific activity after growth on sucrose	
	Phototrophic conditions	Aerobic conditions
Sucrase	55	68
Glucokinase	197	238
Fructokinase	6	6
Phosphoglucose isomerase	443	886
Glucose-6-phosphate dehydrogenase (NADP)	148	268
6-Phosphogluconate dehydrogenase (NADP and NAD)	ND	ND
6-Phosphogluconate dehydratase	140	228
2-Keto-3-deoxy-6-phosphogluconate aldolase	134	179
Phosphoenolpyruvate:fructose phosphotransferase	1.5	2.1
1-Phosphofructokinase	10	15
6-Phosphofructokinase	4	3
Fructose-1,6-bisphosphate aldolase	42	40
Fructose-1,6-bisphosphatase	21	10

ND, Not detected.

(EC 2.7.1.4) activity was low irrespective of the growth substrate (Tables 1 and 2; Conrad & Schlegel, 1977a).

Exogenous fructose was catabolized via fructose 1-phosphate and the EMP (Conrad & Schlegel, 1977a). In sucrose-grown bacteria the activities of the key enzymes of this pathway, phosphoenolpyruvate: fructose phosphotransferase and 1-phosphofructokinase (EC 2.7.1.56), were only 15 to 40 % of those present in fructose-grown bacteria; however, the enzyme levels were two to five times higher than in glucose-grown bacteria (Table 1; Conrad & Schlegel, 1974, 1977a). On the basis of these results and the low fructokinase activity, it was uncertain whether the fructose moiety of sucrose was catabolized like exogenous fructose via the EMP or like glucose and the glucose moiety of sucrose via the EDP.

Localization of the sucrose-splitting enzyme

To test the possibility that sucrose was hydrolysed outside the cytoplasmic membrane and the resulting fructose was catabolized like an exogenous substrate, protoplasts were prepared and sucrase activity was determined in the periplasmic and cytoplasmic cell fractions (Table 2). Although the periplasmic fraction contained more protein (75 %) than the cytoplasmic fraction (25 %), only 30 % of the total sucrase activity was found in the periplasmic fraction; this percentage was comparable with that of three cytoplasmic indicator enzymes and was, therefore, probably the result of leakage from the protoplasts. The specific activity of the sucrase in the cytoplasmic fraction was seven times higher than in the periplasmic fraction. No activity was found in the membrane fraction. These results indicate that sucrase was a cytoplasmic enzyme and sucrose was cleaved inside and not outside the cytoplasmic membrane.

Possible excretion of fructose or glucose and the capacity of the cells to assimilate sugars

To exclude the possibility that glucose or fructose were excreted after hydrolysis of sucrose, bacteria were grown on sucrose under phototrophic as well as under aerobic conditions. Throughout the growth cycle neither fructose nor glucose was detected in the medium.

If fructose or glucose had been excreted into the medium and then immediately taken up and catabolized as an exogenous substrate, sucrose-grown cells should be able to assimilate these sugars as exogenous substrates. However, chloramphenicol-treated sucrose-grown

Table 2. *Localization of sucrase activity in R. capsulata*Specific activities are expressed as nmol substrate utilized min⁻¹ (mg protein)⁻¹ at 25 °C.

Enzyme	Distribution of activity (%)		Specific activity	
	Cytoplasmic fraction	Periplasmic fraction	Cytoplasmic fraction	Periplasmic fraction
Sucrase	70	30	43	6
Glucokinase	81	19	145	12
Fructokinase	77	23	11	1
Glucose-6-phosphate dehydrogenase	89	11	297	13

Fig. 1. Assimilation of $[U-^{14}\text{C}]$ fructose, $[U-^{14}\text{C}]$ glucose and $[U-^{14}\text{C}]$ sucrose by chloramphenicol-treated bacteria grown aerobically on fructose, glucose or sucrose. Bacteria grown on fructose ($\circ$), glucose ($\square$) or sucrose ($\bullet$) were incubated in 2 ml mineral medium containing thiamin ($0.1 \mu\text{g ml}^{-1}$), chloramphenicol ($50 \mu\text{g ml}^{-1}$) and $2 \mu\text{mol}$ ($0.6 \mu\text{Ci}$) of $[U-^{14}\text{C}]$ fructose (a), $[U-^{14}\text{C}]$ glucose (b) or $[U-^{14}\text{C}]$ sucrose (c).

cells did not assimilate fructose at a higher rate than glucose-grown cells, while fructose-grown cells incorporated fructose rapidly (Fig. 1a). In addition, sucrose-grown cells assimilated sucrose carbon three times faster than fructose carbon (Fig. 1a, c). Identical results were obtained when phototrophically grown cells were examined under phototrophic conditions (not shown). Hence we concluded that the low activity of the phosphoenolpyruvate:fructose phosphotransferase/1-phosphofructokinase system measured in sucrose-grown cells could not allow the degradation of the fructose moiety of sucrose.

Glucose carbon, on the other hand, was assimilated by sucrose-grown cells at a significantly higher rate than by fructose-grown cells (Fig. 1b) and at approximately the same rate as sucrose carbon was assimilated (Fig. 1b, c). The cells were, therefore, able to assimilate any glucose eventually excreted after sucrose hydrolysis. Sucrose carbon, however, was only assimilated by sucrose-grown cells, and not by cells grown on glucose or fructose (Fig. 1c) indicating that an inducible sucrose-transport system was involved in sucrose utilization.

Radiorespirometric and mutant experiments

To determine the degradative pathway of the fructose moiety of sucrose, radiorespirometric experiments were done with cells grown aerobically on sucrose. The cells were incubated with $[U-^{14}\text{C}]$ sucrose or with sucrose which was labelled in the fructose moiety in

Fig. 2. Radiorespirometry of specifically-labelled sucrose. Each incubation mixture contained 0.8 ml resuspended bacteria ($A_{650} = 2$) grown aerobically on sucrose and 0.2 ml sucrose ($5 \mu\text{mol ml}^{-1}$) labelled in different carbon atoms. The yield of $^{14}\text{CO}_2$ from [fructose-6- ^{14}C]sucrose was calculated as $2 \times (\text{yield from [fructose-1,6-}^{14}\text{C}_2\text{]sucrose}) - (\text{yield from [fructose-1-}^{14}\text{C]sucrose})$. Substrate radioactivities were: [fructose-1- ^{14}C]sucrose, 0.81×10^5 d.p.m.; [fructose-1,6- $^{14}\text{C}_2$]sucrose, 0.82×10^5 d.p.m.; [fructose-U- ^{14}C]sucrose, 0.67×10^5 d.p.m.; [U- ^{14}C]sucrose, 1.03×10^5 d.p.m. ●, [fructose-1- ^{14}C]sucrose; ■, [fructose-6- ^{14}C]sucrose; ○, [fructose-U- ^{14}C]sucrose; △, [U- ^{14}C]sucrose.

positions 1, 1,6 or uniformly. The $^{14}\text{CO}_2$ released from radioactive sucrose in a specified time period was trapped in alkali and counted. The rate of $^{14}\text{CO}_2$ evolution from [fructose-6- ^{14}C]sucrose was calculated by difference from the rates obtained with [fructose-1,6- $^{14}\text{C}_2$]sucrose and [fructose-1- ^{14}C]sucrose.

$^{14}\text{CO}_2$ was generated at almost equal rates from [U- ^{14}C]sucrose and from [fructose-U- ^{14}C]sucrose (Fig. 2), the difference being within the limits of experimental error. This result shows that the glucose and fructose parts of the sucrose were catabolized at similar rates. The rate of $^{14}\text{CO}_2$ release from [fructose-1- ^{14}C]sucrose was much higher than that from [fructose-6- ^{14}C]sucrose. The rate of $^{14}\text{CO}_2$ release from [fructose-U- ^{14}C]sucrose was intermediate (Fig. 2). This radiorespirometric pattern was characteristic for the operation of the EDP during the breakdown of the fructose moiety of sucrose.

The need for a functional EDP for the breakdown of sucrose was confirmed by a growth experiment with the glucose-6-phosphate dehydrogenase-deficient mutant strain GP33 described previously (Conrad & Schlegel, 1977a). This mutant was unable to grow on sucrose under either phototrophic or aerobic conditions, but retained the ability to grow on fructose with the same doubling time (3 to 4 h) as the wild type. When the mutant was grown in peptone/yeast extract medium supplemented with sucrose as the inducing substrate, glucose-6-phosphate dehydrogenase activity was not detectable and 6-phosphogluconate dehydratase activity was low. All the other enzyme activities, however, were similar to those of the wild type, except fructokinase, which was three times higher in the mutant than in the wild type.

DISCUSSION

Our radiorespirometric data show that the fructose moiety of sucrose was degraded via the EDP, while previous experiments have shown that exogenous fructose is catabolized via the EMP; both exogenous and endogenous glucose was degraded via the EDP (Conrad &

Fig. 3. Catabolic pathways of fructose, glucose and sucrose. F-1-P, Fructose 1-phosphate; F-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G-6-P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate.

Schlegel, 1977*a*; Fig. 3). Additional evidence for the degradation of both hexose moieties of sucrose via the EDP was obtained from the inability of a glucose-6-phosphate dehydrogenase-deficient mutant to grow on sucrose. Our conclusion that both hexose moieties of sucrose were catabolized via the EDP is also in accordance with the enzyme data; in sucrose-grown cells all the enzymes of the EDP were present, but 6-phosphogluconate dehydrogenase and 6-phosphofructokinase were absent or low. Only a partial induction of phosphoenolpyruvate:fructose phosphotransferase and 1-phosphofructokinase was observed. These activities, however, were apparently too low to enable the vectorial phosphorylation of exogenous fructose and its assimilation. A partial induction of these enzymes has also been observed in mannitol-grown *Pseudomonas aeruginosa*, provided the cells contained the enzymes for the conversion of mannitol to endogenous fructose (Phibbs *et al.*, 1978). In a fructokinase-deficient mutant of *Enterobacter (Aerobacter) aerogenes*, phosphoenolpyruvate:fructose phosphotransferase and 1-phosphofructokinase were induced in the presence of sucrose (Kelker, Hanson & Anderson, 1970). The authors concluded that in this mutant the accumulating endogenous fructose derived from the cleavage of sucrose was catabolized like exogenous fructose via fructose 1-phosphate and the EMP. In *Rhodospseudomonas capsulata*, the activity of fructokinase was low compared with that of glucokinase, but it was evident that endogenous fructose had to be converted to a hexose 6-phosphate ultimately, otherwise its catabolism via the EDP could not have occurred. We did not attempt to find out which enzyme activity was actually responsible for the conversion of endogenous fructose to fructose-6-phosphate. It is conceivable that the fructokinase activity was higher *in vivo* than *in vitro*, or that the bacteria contained a fructokinase activity which is more active with a phosphate donor other than ATP or phosphoenolpyruvate, like the acylphosphate:hexose phosphotransferase of *E. aerogenes* (Anderson & Kamel, 1966).

The first step of sucrose degradation in *R. capsulata* was the hydrolysis to glucose and fructose by a sucrase. Although the properties of this enzyme were not investigated, it was probably not a β -fructosidase, since raffinose could not serve as a substrate. The sucrase activity was confined to the cytoplasm: sucrase was neither located outside the cell membrane, as in some yeasts and fungi (Sutton & Lampen, 1962; Metzenberg, 1963; Kidby & Davies, 1970; Janda & Hedenström, 1974), nor excreted into the medium, as in *Streptococcus mutans* (Fukui, Fukui & Moriyama, 1974) and *Bacillus subtilis* (Pascal *et al.*, 1971).

The sucrase activity of *R. capsulata* was constitutive and was not subject to catabolite repression by glucose or by fructose as in *B. subtilis* (Prestidge & Spizizen, 1969), *S. mutans* (Tanzer, Brown & McInerney, 1973) and *Clostridium pasteurianum* (Laishley, 1975).

Although sucrase and all the other enzymes necessary for sucrose breakdown were also present in cells grown on glucose or fructose (Conrad & Schlegel, 1974, 1977*a*), sucrose was only taken up by sucrose-grown bacteria. In *Staphylococcus aureus* (Hengstenberg, Egan & Morse, 1968) and *B. subtilis* (Lepesant & Dedonder, 1968) the transport of sucrose is mediated by a phosphoenolpyruvate phosphotransferase system, which phosphorylates the glucose moiety in the 6-position. In *R. capsulata*, however, we were unable to detect a phosphoenolpyruvate:sucrose phosphorylating activity (unpublished results), suggesting that sucrose was transported in the unphosphorylated state. The ability of sucrose-grown cells to assimilate glucose to some extent suggests either that glucose was transported by the same transport system as sucrose or that a glucose transport system was partially induced by sucrose or by endogenously formed glucose.

The differences in the degradative pathways of exogenous and endogenous fructose are considered to be due to the phosphorylation of exogenous fructose to fructose 1-phosphate and of endogenous fructose to fructose 6-phosphate. This difference results in a functional compartmentation of the degradation of fructose 1-phosphate and fructose 6-phosphate, respectively, and has already been discussed (Conrad & Schlegel, 1977*a*). A similar separation of the catabolic pathways for exogenous fructose on the one hand, and for endogenous fructose and glucose derived from sucrose on the other, has been demonstrated in *E. aerogenes* (Kelker *et al.*, 1970) and *B. subtilis* (Lepesant *et al.*, 1972; Gay & Rapoport, 1970). In these bacteria, however, the difference in the catabolic pathways is in the utilization of 1-phosphofructokinase for exogenous fructose and of 6-phosphofructokinase for endogenous fructose; in both cases fructose is catabolized via the EMP. Similar results were obtained with respect to the degradation of fructose and glucose in *Escherichia coli* (Fraenkel, 1968; Ferenci & Kornberg, 1971, 1973) and *Clostridium thermocellum* (Patni & Alexander, 1971), to the degradation of fructose and ribose in *Pseudomonas doudoroffii* (Baumann & Baumann, 1975) and to the degradation of fructose and sorbitol in *B. subtilis* (Delobbe, Chalumeau & Gay, 1975). However, the presence of two pathways, one via fructose 1-phosphate and the other via fructose 6-phosphate, glucose 6-phosphate and the EDP, does not necessarily imply that each of these pathways is used for the breakdown of its specific substrate exclusively: it has been demonstrated for *P. aeruginosa* and other *Pseudomonas* species (Sawyer *et al.*, 1977*a*), for marine species of *Alcaligenes* (Sawyer, Baumann & Baumann, 1977*b*) and for *Rhodopseudomonas sphaeroides* (Conrad & Schlegel, 1977*b*), that exogenous fructose is mainly catabolized via the EDP, although the enzymes of fructose catabolism via fructose 1-phosphate and the EMP have been induced. The role of fructose 1,6-bisphosphate aldolase, fructose-1,6-bisphosphatase and pyrophosphate dependent 6-phosphofructokinase for the distribution of fructose carbon into the EMP and the EDP has been discussed by these authors. Recently it has been demonstrated that mutants of *P. aeruginosa* deficient in either phosphoglucose isomerase or glucose 6-phosphate dehydrogenase failed to grow on mannitol, which is catabolized via endogenous fructose and the EDP, whereas they retained the ability to grow on exogenous fructose (Phibbs *et al.*, 1978). However, the low growth rate on fructose confirmed that a functional EDP is necessary in addition to the enzymes of the EMP for the catabolism of exogenous fructose in *P. aeruginosa*. In *R. capsulata*, however, both catabolic pathways are apparently well separated (Fig. 3): the pathway via fructose 1-phosphate and the EMP for exogenous fructose, and the pathway via fructose 6-phosphate, glucose 6-phosphate and the EDP for endogenous fructose as well as both exogenous and endogenous glucose.

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