METABOLISM OF CARBOHYDRATES BY *PSEUDOMONAS SACCHAROPHILA*[1]

I. Oxidation of Fructose by Intact Cells and Crude Cell-Free Preparations

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Pseudomonas saccharophila was originally described because of its rather striking ability to use certain complex carbohydrates more readily than the constituent monosaccharides (Doudoroff, 1940). The subsequent discoveries of a "sucrose phosphorylase," trehalase, and amylases in this organism have not given an answer to the problem posed by the original observations (Doudoroff 1945, 1951). The recent elucidation of a new pathway for glucose metabolism in this bacterium (Entner and Doudoroff, 1952; MacGee and Doudoroff, 1954) has merely underlined the lack of understanding of its behavior *in vivo*. The studies to be reported do not explain the anomalous inability of the wild-type strain to use glucose and fructose for growth and respiration, but do present some new information which, it is hoped, will eventually be of use to the understanding of the enzymatic basis of cellular permeability to exogenous substrates. The first report deals mainly with the behavior of intact cells in the presence of sucrose, glucose, and fructose with a special emphasis on the utilization of fructose.

MATERIALS AND METHODS

The culture of *Pseudomonas saccharophila* used in these studies has been carried for 16 years by mass transfers in a liquid sucrose medium. This culture, although apparently heterogeneous with respect to genetic variants, has remained remarkably constant with respect to most of its physiological characteristics. It can, of course, be expected that mutations have occurred and mutants with high survival value have been selected in the course of years. Some slight changes in behavior, particularly with regard to maltose utilization, have indeed been observed. In the following discussion, the term "wild type" will be used to designate re-isolates of the mass culture which resemble the culture originally described in being able to grow rapidly on sucrose and starch media but requiring a period of adaptation to grow on glucose or fructose. The synthetic media used contained KH_2PO_4-Na_2HPO_4 buffer at pH 6.8, 0.033 M; NH_4Cl, 0.1 per cent; $MgSO_4$, 0.05 per cent; ferric ammonium citrate, 0.005 per cent; calcium chloride, 0.0005 per cent; and usually 0.25 per cent of the sugar supplied as source of carbon and energy. The cultures were grown on a rotary shaker at 30 C.

The methods used for the studies with cell-free preparations have been described previously (Entner and Doudoroff, 1952).

RESULTS

When mass cultures of *P. saccharophila* were transferred from synthetic medium with sucrose as carbon source to media containing either glucose or fructose as the organic substrate, a more or less prolonged lag period usually preceded the further development of cultures. This was especially true of cultures transferred to fructose media, which often required several days of incubation before growth was observed. Once the culture had developed with glucose or fructose, the organisms were found to be capable of growing rapidly on further transfer in the homologous medium. This suggested that a certain small number of cells capable of growing in the monosaccharide media were always present in the parent culture. Further evidence that a selection of a few clones from the parent strain was involved in the adaptation was obtained by plating sucrose-grown cultures on agar media with either glucose or fructose as substrate. In such experiments almost all of the plated cells produced tiny

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translucent colonies which stopped growing after three or four days. A few colonies, however, which soon showed a more opaque appearance, continued to grow and attained a considerable size. Under proper conditions, opaque papillae or sectors could be observed in some of the translucent colonies, and these gave rise to large colonies. Organisms isolated from such large colonies were capable of forming large colonies on the homologous agar media, and of growing rapidly in liquid media with the sugar on which they had been isolated. Clones obtained by such isolation retained their ability to grow rapidly with the monosaccharide even after many transfers on sucrose medium and were reminiscent of the "glucose mutants" described in *P. putrefaciens* (Klein and Doudoroff, 1950).

While freshly isolated colonies of the parent strain grown on sucrose medium gave very few rapidly growing colonies on glucose or fructose media, cultures which had been transferred many times with sucrose as substrate contained from a fraction of 1 per cent to several per cent of such colonies. This indication that a dynamic equilibrium is soon established between the parent and mutant stocks in each culture was supported further by the observation that different clones of the parent strain showed characteristically different patterns with regard to the heterogeneity of the population, and that these patterns persisted through many subcultures of the particular clones when mass transfers were used. Thus, most of the parent culture clones showed very few large colonies on fructose medium and a somewhat greater number on glucose medium. Occasional clones, however, showed a different distribution, and one, which has been studied in further detail, was repeatedly found to contain from 3 to 7 per cent of cells capable of growing with fructose and a very much smaller fraction of cells capable of growing with glucose.

This culture was transferred to sucrose and to fructose media, and its growth followed nephelometrically. At various times, differential counts were made by spreading appropriate dilutions on the surface of plates containing sucrose, glucose and fructose, respectively. Growth on sucrose medium was immediate and rapid, and the fraction of cells capable of producing large colonies on fructose and glucose media remained more or less constant throughout the exponential phase of growth and during 10 hours in the maximum stationary phase. Between 2 and 7 per cent of the cells gave rise to large colonies on fructose, and approximately 0.1 per cent produced large colonies on glucose. When the culture was transferred to fructose medium, a long lag period was observed. No growth of the major portion of the initial population could be detected, but the number of cells capable of producing large colonies on fructose increased exponentially until such cells predominated in the population. The further growth of the culture could also be accounted for by the exponential growth of these cells. A particularly convincing argument for the fact that growth of this culture was due to the initial presence and development of a particular clone of mutant cells may be found in the fact that the "fructose adapted" colonies derived from this culture were of a rarely encountered type to be described later as an "F" strain. In this experiment the fraction of cells capable of producing large colonies on glucose medium again remained small and approximately constant at between 0.01 and 0.1 per cent.

A simple method based on Newcombe's technique (Newcombe, 1949) was used to demonstrate that the mutational event conferring on the cultures the ability to grow with monosaccharides occurs spontaneously in sucrose media. A young, freshly isolated colony of the parent strain was streaked heavily on a sucrose-agar plate and incubated until a single confluent band of growth had developed. The strip of agar containing the streak was then cut out, inverted on a fructose-agar plate and smeared across the surface of the plate at right angles to the original axis of the streak. The fructose plate was then incubated until large colonies capable of growing with fructose appeared on a background of confluent "marginal growth" of the parent strain. Many of these colonies occurred in regular rows parallel to the direction of the smear. This was interpreted to indicate that the original streak on the sucrose plate already contained microcolonies of cells capable of growing on fructose medium and that the mutations to the ability to use the monosaccharide had occurred at various times prior to the transfer to fructose agar. Analogous experiments done with glucose agar gave similar results.

It became apparent early that the mutant types isolated on glucose and fructose media fell

TABLE 1

Growth and respiration of Pseudomonas saccharophila strains with different sugars

P. saccharophila	Growth Medium Containing 0.25% of Following Substrates	Mean Division Time (min.)	Q_{O_2}* with 0.02 M Substrates		
			Sucrose	Glucose	Fructose
Wild type	Sucrose	150	126	9	0
G type (strain G-1)	Sucrose	195	115	84	0
	Glucose	370	17	166	6
F type (strain F-1)	Sucrose	145	102	4	107
	Fructose	160	13	2	109
GF type (strain GF-1)	Sucrose	150	139	25	61
	Glucose	290	1	93	87
	Fructose	430	2	3	99

* Q_{O_2} determined at 30 C with cells harvested from early stationary phase cultures. Endogenous respiration rate (Q_{O_2} 20–28) was substracted.

into three main categories which will be designated as follows:

G type was capable of growing with sucrose or glucose but not with fructose. This type was isolated fairly frequently from glucose plates.

F type was capable of growing with sucrose or fructose but not with glucose. This type occurred very rarely and was isolated only on three occasions from fructose cultures.

GF type. This was the commonest of the types isolated from fructose plates and occasionally from glucose plates. The organisms were characterized by their ability to grow with sucrose, glucose or fructose. Development with fructose, however, was usually slow and markedly dependent on the concentration of substrate. Thus, one strain had division times of 2,000, 750, 580, and 520 minutes, respectively, soon after transfer from sucrose medium to media with fructose concentrations of 0.125, 0.25, 0.5, and 1.0 per cent. The growth rate increased slightly after several divisions in fructose media. Strains utilizing either glucose or fructose could also be obtained from either G or F types by isolating large colonies on the nonhomologous monosaccharide media.

All of the above types retained their characteristics even after prolonged cultivation on sucrose medium.

Studies on the metabolism of sugars by selected strains of the various types showed further interesting differences in their behavior (table 1). While the parent strain grown with sucrose does not oxidize free glucose or fructose at an appreciable rate, the mutant strains retain varying abilities to oxidize the monosaccharides. In all cases, the rate of utilization is increased by growing the culture with the homologous sugar. A striking effect of ageing or starvation was observed with the F strain grown with sucrose. Cells harvested in the exponential phase of growth oxidized fructose slowly, but the rate increased with the establishment of the stationary phase. An increase in the rate of fructose oxidation could also be induced by harvesting the cells and aerating them for several hours either in a sugar-free but otherwise complete medium or simply phosphate buffer (table 2).

An unexpected and strong inhibition of fructose oxidation was observed in fructose-adapted cells of both F and GF types when glucose was present. Even in very low concentration, glucose depressed or completely abolished the excess oxygen uptake due to the oxidation of added fructose. In the cases where very small amounts of glucose were used, this sugar was apparently slowly oxidized and the inhibitory effect disappeared after a

TABLE 2

Effect of starvation on oxidation of fructose by F strain

Exper. No.	Conditions	Q_{O_2} (Minus Endogenous) with 0.02 M Substrates	
		Sucrose	Fructose
1	12-hr sucrose culture (late exponential)	88	37
	Same at 24 hr (stationary)	103	106
2	12-hr. sucrose culture (late exponential)	76	23
	Same washed, aerated 12 hr in sugar-free medium	96	87
3	10-hr sucrose culture (exponential)	80	25
	Same washed, aerated 14 hr in pH 6.8 buffer	101	96

TABLE 3

Inhibition of fructose utilization by F strain of Pseudomonas saccharophila in the presence of various sugars

Exper. No.	Growth Substrate	Additions (in μM) per 2 ml of Cell Suspension	Q_{O_2} (Minus Endogenous)*
1	Fructose	40 fructose	84
		40 D-glucose	2
		40 fructose + 40 D-glucose	6
2	Fructose	40 fructose	112
		0.1 D-glucose	0
		40 fructose + 0.1 D-glucose	21
3	Fructose	40 fructose	128
		40 fructose + 5 D-glucose	10
		40 fructose + 5 D-mannose	49
		40 fructose + 5 D-galactose	22
		40 fructose + 5 maltose	57
		40 fructose + 5 cellobiose	36
		40 fructose + 40 meso-inositol	33
		40 fructose + 40 L-glucose	112
4	Fructose	40 fructose	146
		40 fructose + 40 D-xylose	10
		40 fructose + 40 L-xylose	146
		40 fructose + 40 D-arabinose	117
		40 fructose + 40 L-arabinose	11
		40 fructose + 40 α-methyl-glucoside	75
		40 fructose + 0.2 D-xylose	81
		40 fructose + 0.1 D-glucose	54
		40 fructose + 0.1 D-glucose + 0.2 D-xylose	39
5	Sucrose (one division)	10 fructose	30
		10 fructose + 10 D-glucose	4
		10 sucrose	83
		10 sucrose + 10 D-glucose	87

* Endogenous Q_{O_2} varied from 20 to 25 in different experiments.

period of time proportional to the amount of glucose initially added. No similar inhibitory effect of glucose could be found on the oxidation of sucrose. Several other sugars, notably D-xylose, D-mannose, D-galactose, maltose and cellobiose were also found to have an inhibitory effect on fructose utilization. Since these sugars were not themselves oxidized at a high rate, their effect persisted for many hours (see table 3).

In experiments reported earlier, it has been shown that the mechanism of glucose utilization in *P. saccharophila* appears to involve the phosphorylation of glucose to glucose-6-phosphate by a specific glucokinase, the further oxidation of the ester to 6-phosphogluconic acid, the rearrangement of this compound to 2-keto, 3-deoxy, 6-phosphogluconic acid by an enzyme referred to as 6-PGA[2] dehydrase and the aldolase-like split of the keto-acid by a "PGA aldolase" to yield pyruvic acid and glyceraldehyde-3-phosphate. A triose phosphate dehydrogenase is also present, and, under proper conditions, two moles of pyruvic acid can be shown to be formed from one mole of glucose.

Strong evidence that a similar mechanism is used in fructose oxidation was obtained in experiments in which fructose-adapted F and GF mutant strains were allowed to oxidize fructose in the presence of metabolic poisons. As is the case

[2] The following abbreviations are used in the present paper: PGA: phosphogluconic acid; DPN: diphosphopyridine nucleotide; TPN: triphosphopyridine nucleotide; ATP: adenosinetriphosphate.

with glucose oxidation, the addition of arsenite or dinitrophenol caused the accumulation of pyruvic acid, while in the presence of iodoacetate, pyruvic acid and methyl glyoxal were produced in approximately equimolar amounts (Entner and Doudoroff, 1952). Phosphohexoisomerase was shown to be present in mutant cells adapted to fructose as well as in sucrose-grown parent culture cells and in G-type cells grown with glucose. A glucose-6-phosphate dehydrogenase capable of reducing either DPN or TPN and requiring Mg ions was also found in all types of cells tested, as were 6-PGA dehydrase, 6-PGA aldolase and triose phosphate dehydrogenase.

Further evidence that an initial phosphorylation is required for the oxidative metabolism of fructose was obtained with cell-free alumina-ground extracts to which methylene blue had been added as hydrogen carrier. Such preparations from glucose-adapted cells had previously been shown to produce pyruvic acid in the presence of ATP, glucose, and arsenite (Entner and Doudoroff, 1952).

A 20 per cent wet weight alumina-ground preparation of GF cells grown with fructose was made in 0.033 M pH 6.8 phosphate buffer. To 2-ml aliquots of this preparation were added sodium arsenite, 2 μM; DPN, 0.2 μM; TPN, 0.2 μM; methylene blue, 0.5 μM; and various substrates. The volume was in each case made up to 3 ml and the reaction mixtures were shaken with air for 90 minutes at 30 C. Pyruvic acid was determined in each reaction vessel. The following amounts of pyruvic acid were found in excess of that produced in the absence of substrates:

a. With 10 μM ATP added; none.
b. With 20 μM fructose added; 0.3 μM.
c. With 20 μM fructose and 10 μM ATP; 6.8 μM.
d. With 20 μM glucose and 10 μM ATP; 0.6 μM.

This experiment demonstrates convincingly the specificity of the preparation for fructose and the requirement for ATP, but the observed rate of pyruvate formation was extremely low as compared with the rate of fructose metabolism by intact cells. This phenomenon had been noted in previous studies on glucose metabolism.

The relatively poor activity of crude cell-free preparations in the oxidation of sugars is attributable to several factors. The enzymes involved in the phosphorylation, particularly that of fructose, have been found to be extremely unstable and the kinase activities, at best, rather low. The hydrogen transport system between reduced DPN or TPN and oxygen is almost entirely lacking in such preparations and even methylene blue is a very poor mediator in the oxidation of the reduced coenzymes. In addition, the coenzymes appear to be destroyed in the course of the experiment. This destruction can be partially prevented by the addition of ATP but not of nicotinamide. Finally, the ATP and the phosphate esters are subject to enzymatic hydrolysis.

The preparations were capable of producing pyruvate from glucose-6-phosphate and fructose-6-phosphate in the absence of ATP, and from 6-phosphogluconate in the absence of either ATP or oxygen. In some cases pyruvate was produced from fructose-1-phosphate, but in no instance at a rate equal to that observed with fructose-6-phosphate or glucose-6-phosphate.

DISCUSSION

The evidence presented above indicates that the ability of *P. saccharophila* to utilize a given monosaccharide, such as fructose, is independent of the capacity of the organism to use a disaccharide (sucrose) containing the hexose moiety and may depend on three types of factors. These include the genetic make-up of the cells, the inductive effect of the substrate, and, in some cases, the physiological state of the cells with respect to growth phase and availability of endogenous reserves. Experiments with cell-free preparations strongly suggest that an essential step in the utilization of fructose is the phosphorylation of the sugar with ATP by a specific kinase. Studies with both poisoned intact cells and cell extracts support the view that the further metabolism of fructose follows the pathway which has been described previously for glucose oxidation (Entner and Doudoroff, 1952; MacGee and Doudoroff, 1954). The special aspects of the initial reactions involving fructose will be treated in a separate paper, in which an attempt will be made to correlate the *in vivo* behavior of various cultures of *P. saccharophila* with their enzymatic composition.

SUMMARY

Three types of mutants of *Pseudomonas saccharophila* can be differentiated from the parent strain by their ability to grow with glucose, fructose, or either hexose as substrate.

The ability to oxidize fructose by fructose-utilizing strains is conditioned by the growth substrate and by the physiological state of the cells.

Glucose and certain other carbohydrates have a strong inhibitory effect on fructose oxidation.

Evidence is presented for the phosphorylation of fructose and its subsequent oxidation via the 2-keto, 3-deoxy, 6-phosphogluconic acid pathway.

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