METABOLISM OF CARBOHYDRATES BY *PSEUDOMONAS SACCHAROPHILA*[1]

II. Nature of the Kinase Reaction Involving Fructose

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In studies reported previously (Doudoroff Palleroni, MacGee, and Ohara, 1956) it has been shown that cell-free preparations of a mutant strain of *Pseudomonas saccharophila* adapted to grow with fructose as a substrate could oxidize fructose only if ATP[2] was added. The products of the oxidation of fructose by such preparations and by intact cells inhibited with metabolic poisons were identical with the products of glucose oxidation by glucose-adapted cultures. Glucose, however, was not oxidized by fructose-adapted cells of some strains or by cell-free extracts of such cells even in the presence of ATP.

These observations made it appear likely that a specific kinase is involved in the phosphorylation of fructose. A glucokinase incapable of phosphorylating fructose has been described previously in glucose-adapted mutants (Entner and Doudoroff, 1952). The kinases of *P. saccharophila* have been found to be extremely labile and have not yielded to purification procedures. A complicating factor for the study of kinase specificity was the presence of a "mannose isomerase" which was discovered in the course of the present studies. In the following report, which deals with the phosphorylation reactions involving fructose, all of the experiments were carried out with crude preparations and some of the interpretations are based on indirect evidence and "tracer" techniques.

METHODS

The properties of the various strains of *P. saccharophila* used in the present studies and methods for their cultivation were described in the previous paper (Doudoroff, Palleroni, MacGee and Ohara, 1956). Cell-free extracts were prepared as before, except that M/100 glutathione was added to the cell suspension before grinding with alumina or sonerating in a 9-kc Raytheon oscillator. Ordinarily 20 per cent wet weight suspensions were sonerated for 20 minutes and the debris removed by centrifugation at 22,000 × G for 30 minutes. The dry weight of the original suspension before soneration (22 per cent of the wet weight) was used as a basis for calculations of enzymatic activity of the extracts.

Transphosphorylation between ATP and hexoses was usually measured by the disappearance of the free sugar from the medium. Reducing sugars were measured by the procedure described by Schales and Schales (1945). Fructose was estimated by the method of Roe (1934). Radioactivity measurements were done by the use of a thin window Geiger-Muller counter.

RESULTS

Activity of kinase with fructose could be demonstrated with cell-free extracts of F or GF cells grown with fructose, as well as with wild type *P. saccharophila* grown with sucrose. Transphosphorylation between ATP and fructose could be shown readily using the disappearance of fructose, of ATP (Entner and Doudoroff, 1952), or the production of CO_2 from bicarbonate buffer as criteria (Colowick and Kalckar, 1943).

In the course of these experiments, it became apparent that a fraction of added fructose always disappeared rapidly even in the absence of ATP. This was shown to be due to the presence of a new enzyme, named "mannose isomerase," which catalyzes the interconversion of free fructose and mannose (Doudoroff, Palleroni and MacGee, 1955). At equilibrium, about 29 per cent of the fructose initially present is isomerized. No glucose is produced in the reaction, and this sugar has only a slight inhibitory action on the isomerization of fructose. A complete account of the

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[2] The following abbreviations are used in the present paper: ATP: adenosinetriphosphate; ADP: adenosinediphosphate; DPN: diphosphopyridine nucleotide; TPN: triphosphopyridine nucleotide; Tris: tris(hydroxymethyl) aminomethane. Molarity of tris buffers is expressed as tris.

partial purification and properties of this enzyme will be published elsewhere (Palleroni and Doudoroff, 1956).

The specific nature of the fructokinase was best demonstrated with fructose-grown cells of a GF mutant. Activity of kinase was measured as previously described (Entner and Doudoroff, 1952). The rate of transphosphorylation was found to be 0.4 μM per hour per mg of dry weight with 0.05 M fructose as substrate. The same preparation phosphorylated glucose at a rate of 0.05 μM per hour per mg.

It has been previously reported that certain sugars, including glucose, are very inhibitory to the utilization of fructose by intact cells, while L-xylose has no inhibitory effect (Doudoroff, Palleroni, MacGee and Ohara, 1956). Therefore, the effect of glucose and of D- and L-xylose on the activity of kinase was tested. A preparation of GF cells grown with fructose which had no demonstrable glucokinase activity was used. This preparation did not phosphorylate L-xylose and showed a very slight and doubtful kinase activity with D-xylose (0.08 $\pm$ 0.05 μM per hour per mg dry weight of bacteria). The rate of fructose phosphorylation was measured in the presence and absence of the other sugars by determining the disappearance of total free sugar in the presence of ATP. Each reaction was carried out for 60 minutes at 30 C in a total volume of 0.8 ml containing enzyme, 0.2 ml (extract of 8.8 mg dry weight bacteria); tris HCl buffer pH 7.4, 120 μM; $MgCl_2$, 10 μM; KCl, 20 μM; glutathione, 2 μM; ATP, 20 μM; KF, 4 μM; fructose, 10 μM; and 10 μM of each of the other sugars.

Fructose alone was phosphorylated at a rate of 0.62 ($\pm$0.05) μM per hour per mg dry bacteria. In the presence of D-glucose, D-xylose and L-xylose in addition to fructose, the rate of sugar disappearance was found to be 0.69 ($\pm$0.05), 0.58 ($\pm$0.05), and 0.67 ($\pm$0.05) μM, respectively. This experiment demonstrates conclusively that glucose and L-xylose in concentrations equivalent to that of fructose do not inhibit the phosphorylation of fructose. D-Xylose, which may have been phosphorylated to a slight extent during the incubation, may have had at most a slight inhibitory effect on the phosphorylation of fructose.

An experiment conducted with fructose-grown F strain, in which the disappearance of free fructose over and above that due to the isomerase was used as a measure of kinase activity, also showed that glucose does not have any inhibitory or competitive effect with the fructokinase and that the observed low rates of transphosphorylation are not due to the accumulation of ADP. The rate of phosphorylation was found to be 0.65 μM per hour per mg dry weight with 0.01 M fructose and 0.02 M ATP. Fructose disappeared at a rate of 0.68 μM per hour under similar conditions, but with 0.01 M glucose in addition to fructose. In a parallel experiment, in which only a catalytic amount of ATP was added (0.002 M) and creatine phosphate and creatine phosphate transferase were used as a source of high energy phosphate, the rate of fructose disappearance was found to be 0.61 μM per hour per mg dry weight.

In order to identify the products of the phosphorylation, an enzyme preparation from fructose-grown GF strain was allowed to react with ATP and fructose in 0.07 M tris buffer at pH 7.4 and 0.025 M NaF for 60 minutes at 30 C. The reaction mixture was deproteinized with trichloracetic acid and fractionated according to Umbreit *et al.* (1949). The barium-soluble, alcohol-insoluble fraction was found to be composed of a mixture of glucose and fructose esters. The presence of both glucose-6-phosphate and fructose-6-phosphate was shown by paper chromatographic analysis of the mixture before and after hydrolysis with alkaline phosphatase, and the glucose-6-phosphate was further identified enzymatically by the reduction of TPN in the presence of purified yeast glucose-6-phosphate dehydrogenase.

Attempts to purify the fructokinase were unsuccessful. Since fructose-1-phosphate is known to be the product of the initial phosphorylation with some fructose kinases, and since this compound did occasionally give rise to pyruvic acid with cell-free preparations, a tracer experiment was carried out designed to rule out this compound as a primary product of phosphorylation. One ml of alumina-ground cell-free extract of fructose-grown cells of a GF mutant (20 per cent wet weight) was incubated for 20 minutes at 30 C in a total volume of 2.5 ml of reaction mixture containing glucose-6-phosphate, 20 μM; fructose-6-phosphate, 20 μM; fructose-1-phosphate, 20 μM; fructose, 10 μM; ATP, 10 μM; labeled in the pyrophosphate moieties with P^{32} (1×10^5 cpm per μM), $MgCl_2$, 10 μM; KF, 10 μM; and tris buffer at pH 7.5, 60 μM. The reaction was stopped with trichloracetic acid, and the

phosphate esters were fractionated with barium according to the method of Umbreit. Inactive inorganic phosphate and ATP were added to the barium-soluble, alcohol-insoluble fraction and the mixture was refractionated. The barium-soluble, alcohol-insoluble fraction was then hydrolyzed for 30 minutes at 100 C with N HCl. The inorganic phosphate produced on hydrolysis was precipitated as barium phosphate and its specific activity was determined and compared with the specific activity of the total phosphorus of the barium-soluble fraction. Assuming all of the inorganic phosphate to have been derived from fructose-1-phosphate, a maximum value of 1,530 cpm per μM could be computed for this compound. The mixture of glucose-6-phosphate and fructose-6-phosphate was found to have a minimum specific activity of 2,500 cpm per μM. Since a considerable fraction of fructose-6-phosphate would have been hydrolyzed in 30 minutes, the true specific activity of the fructose-1-phosphate was undoubtedly much lower than the value calculated, while the specific activity of the glucose-6- and fructose-6-phosphates must have been higher. While the experiment does not rule out fructose-1-phosphate as one of the reaction products, it does exclude the possibility that it is the precursor of the other esters.

In view of the fact that an active mannose isomerase was present in fructose-grown cells, it was essential to determine whether the substrate for kinase activity in such cells might not actually be mannose rather than fructose. An indirect method was used to clarify this point. Duplicate samples of a crude cell-free preparation of fructose-grown F cells were incubated with ATP, fructose and mannose. Each sample contained 1.5 ml of a 20 per cent sonerated cell extract; ATP, 90 μM; $MgCl_2$, 100 μM; KF, 40 μM; fructose, 84 μM; and mannose, 36 μM; in a total volume of 3.55 ml of 0.1 M tris-maleic buffer at pH 7.4. After 20 minutes at 30 C, a small amount (4 μM) of fructose totally labeled with C^{14} was added to one tube and about 1 μM of totally labeled mannose to the other. Ten μM of ATP were introduced at the same time into each tube. One minute later, the reaction was stopped by the addition of trichloracetic acid, and the phosphate esters were precipitated by the addition of barium acetate, NaOH to pH 8.5 and four volumes of alcohol. The barium precipitates were redissolved and reprecipitated several times, with the addition of inactive fructose and mannose to remove the labeled free hexoses. The radioactivity of the initial alcoholic supernatants and of the precipitates was then determined, as well as the total amounts of each free sugar present at the end of the experiment. From these figures, it was possible to determine the specific activities of the mannose and fructose at the time of labeling (table 1, line *b*). Using the maximum values for rates of isomerase activity found in

TABLE 1

Phosphorylation of labeled fructose and mannose

	Sample 1		Sample 2	
	Fructose	Mannose	Fructose	Mannose
a. Total activity in barium precipitate (cpm)....	39,000		11,000	
b. Initial specific activity on addition of labeled sugar (cpm/μM).....	35,000	0	0	30,000
c. Minimum specific activity of initially labeled sugar after 1 minute of isomerase action (cpm/μM).....	30,000	—	—	17,500
d. Maximum specific activity of initially unlabeled sugar after 1 minute (cpm/μM).....	—	11,600	4,000	—
e. Minimum amount of initially labeled sugar phosphorylated in 1 minute, assuming only kinase for that sugar to be present (*a*/*b*)....	1.1 μM	—	—	0.37 μM
f. Maximum amount of initially labeled sugar phosphorylated in 1 minute assuming only kinase for that sugar to be present (*a*/*c*)....	1.3 μM	—	—	0.63 μM
g. Minimum amount of initially unlabeled sugar phosphorylated in 1 minute assuming only kinase for that sugar to be present (*a*/*d*)....	—	3.4 μM	2.8 μM	—

such preparations, it was possible to compute the minimum specific activity of each of the labeled sugars added and the maximum specific activity of the unlabeled sugar after 1 minute of equilibration. These values are given in lines *c* and *d* of table 1. From the total activity of the precipitates (line *a*, table 1), a minimum and a maximum value were then computed for the amount of each sugar phosphorylated, on the assumption that only fructose or only mannose served as substrates for the kinase (table 1, lines *e* and *f*). On the same assumption, the minimum amount of phosphorylation of each of the initially unlabeled sugars was computed on the basis of the maximum possible activity of that sugar after equilibration (table 1, line *g*). The assumption that either fructose alone or mannose alone is phosphorylated can be ruled out by the comparison of the values in lines *f* and *g*. The results clearly indicate that mannose cannot be the obligate intermediate in the phosphorylation of fructose, since a maximum of 0.63 μM of mannose could have been phosphorylated in one tube and a minimum of 3.4 μM would have to be phosphorylated in the other. Applying similar reasoning, one is led to the conclusion that fructose can not be an obligate intermediate in the phosphorylation of mannose, since in one tube a maximum of 1.3 μM of fructose could have been phosphorylated, while in the other the phosphorylation of a minimum of 2.8 μM would have been required.

It may be pointed out that the actual values for the specific activities of the phosphorylated sugars would be much closer to the initial values than those in lines *c* and *d*. Thus, the figures in line *f* would be actually lower, and those in line *g* higher, making the discrepancies even greater in both cases.

While the above experiment does not give any indication as to whether a single kinase acts on both fructose and mannose or two kinases are involved, it does prove that either sugar can be phosphorylated independently without previous isomerization to the other. It is interesting to note that the sum of the two minimum (and most probable) values obtained for the two sugars (line *e*) is 1.47 μM. This would approximate the total amount of esterification if both hexoses were independently phosphorylated. This figure agrees closely with the observed average rate of free sugar disappearance over the 21-minute experimental period. The latter, calculated from chemical analyses, was 1.3 μM per minute, and represents a minimum figure for the 1-minute period after labeling, since additional ATP was introduced.

Evidence that entirely different kinases are involved in the phosphorylation of mannose and fructose was obtained from experiments with the wild type strain grown on maltose as substrate. The cells grown with maltose did not oxidize exogenously supplied glucose or fructose, but could oxidize maltose with a Q_{O_2} of 196 (above endogenous) in the presence of 0.001 M maltose. Extracts of such cells did not contain mannose isomerase and were virtually devoid of fructokinase. They did, however, possess strong glucokinase and mannokinase activities. In order to identify the product of phosphorylation of mannose, an extract of maltose-grown cells was incubated for 50 minutes at 30 C with mannose and ATP. The reaction mixture contained mannose totally labeled with C^{14} (total counts, 43,000 cpm), 200 μM; ATP (Na), 250 μM; KF, 56 μM; $MgCl_2$, 200 μM; glutathione, 40 μM; and 4.2 ml of 20 per cent (wet weight) sonerated cell extract in a total volume of 11.9 ml 0.1 M tris-HCl buffer at pH 7.4. The reaction was stopped by the addition of trichloracetic acid and the phosphate esters were fractionated according to Umbreit *et al.* (1949). The barium-insoluble fraction, after reprecipitation, contained no measurable radioactivity, while the barium-soluble, alcohol-insoluble fraction contained 13,000 cmp, representing approximately 60 μM of phosphorylated mannose. The total organic phosphorus content in this fraction was 73 μM. This fraction was reprecipitated and assayed for glucose-6-phosphate and fructose-6-phosphate with glucose-6-phosphate dehydrogenase and the Roe reaction, respectively. Approximately 8 per cent of the total ester was found to be glucose-6-phosphate and 5 per cent fructose-6-phosphate. Hydrolysis with N HCl at 100 C for varying periods of time showed that only a small amount of easily hydrolyzable esters was present in the preparation (4 per cent was hydrolyzed in 10 minutes and 7.5 per cent in 30 minutes). The above observations indicated that the bulk of the ester was mannose-6-phosphate. This was supported by a comparison of the rates of acid hydrolysis of the ester with that of a known sample of mannose-6-phosphate. In the interval between 30 and 180 minutes of hydrolysis with N HCl, 8.3 per cent of the ester obtained in the reaction was hydrolyzed at 100 C. Under identical conditions, 7.2 per cent of mannose-6-

phosphate was decomposed. The slightly higher value can be attributed to the presence of fructose-6-phosphate in the preparation.

Although a small amount of glucose and fructose esters was found in the preparations, the extracts did not convert either known mannose-6-phosphate or their own product of mannose phosphorylation to other esters at an appreciable rate, nor could any reduction of DPN or TPN be observed when either compound was used as substrate with the cell-free extracts.

Since the level of the fructokinase activity of most cell-free preparations of fructose-grown cells was lower than that necessary to account for the utilization of fructose by intact cells, it seemed possible that the enzyme had actually little function *in vivo*. An experiment was therefore designed to show that phosphate esters of both glucose and fructose accumulate in intact cells during the oxidation of fructose. Two 25-ml aliquots of a heavy suspension of cells of the GF strain containing 1.73 g (dry weight) of cells each in 0.03 M tris-HCl buffer at pH 7.2 were vigorously aerated with pure oxygen. Two ml of 0.4 M fructose were added to one aliquot and 2 ml of water to the control. After 10 minutes of aeration, 3 ml of 40 per cent trichloracetic acid were added rapidly to each suspension and the extracts were cooled to 0 C. The precipitates were removed by centrifugation and reextracted for 10 minutes at 0 C with 10 ml of 4 per cent trichloracetic acid. After centrifugation, the first and second extracts were pooled, neutralized, and treated with barium and alcohol to precipitate the phosphate esters. The precipitates were dried, redissolved in 5 ml of dilute HCl, and fractionated according to the method of Umbreit *et al.* (1949). The "barium-soluble, alcohol-insoluble" fractions were reprecipitated three times and analyzed for glucose and fructose phosphates. The fructose ester was measured as fructose-6-phosphate with the reaction of Roe, while glucose-6-phosphate was estimated quantitatively from the reduction of TPN in the presence of glucose-6-phosphate dehydrogenase.

The suspension aerated in the absence of sugar was found to contain no fructose phosphate and about 0.4 ($\pm$ 0.2) μM of glucose-6-phosphate per g (dry weight) of cells. In the presence of fructose, the cells contained 1.0 ($\pm$ 0.2) μM of glucose-6-phosphate and 1.9 ($\pm$ 0.2) μM fructose phosphate per g. The organic phosphorus present in the cold trichloracetic acid extracts of cells incubated with and without fructose was 21.6 and 9.6 μM per g (dry weight), respectively. The rather high accumulation of both fructose and glucose esters in the "steady state" of fructose oxidation supports the view that fructokinase and phosphoglucose isomerase are functionally active in the intact organisms.

DISCUSSION

The experiments described in the present studies indicate that the utilization of fructose by fructose-adapted mutant strains involves the phosphorylation of fructose to fructose-6-phosphate and the conversion of this ester to glucose-6-phosphate by phosphoglucose isomerase which has been found in all strains of *P. saccharophila* grown on a variety of substrates. The identity of the subsequent pathways for the metabolism of glucose and fructose in different mutant strains was suggested by the similarity of the products of glucose and fructose oxidation by intact cells poisoned with iodoacetate and arsenite. It was further supported by the demonstration that the enzymes required for the transformation of glucose-6-phosphate to pyruvate are present in fructose-adapted cultures (Duodoroff, Palleroni, MacGee and Ohara, 1956).

Mannose, which is produced from fructose by the "mannose isomerase," is phosphorylated by a different enzyme, the product being mannose-6-phosphate. The fate of mannose-6-phosphate is unknown at present. It is not attacked by the glucose-6-phosphate dehydrogenase present in the cells and no dehydrogenase for the ester has been demonstrated. Mannose phosphate does not, however, appear to be of a major importance as an intermediate in fructose utilization. Not only was the rate of phosphorylation of mannose by an extract of fructose-adapted cells shown to be relatively low as compared with that of fructose, but the isomerization of fructose would have to precede the mannokinase action. Although the activity of mannose isomerase in extracts is high compared with fructokinase activity, the equilibrium constant of the reaction is unfavorable to the accumulation of mannose. In intact cells oxidizing fructose it is quite possible that virtually no mannose-6-phosphate is formed. Alternatively, the mannose-6-phosphate may be hydrolyzed by phosphatase and the resulting mannose reepimerized to fructose by the isomerase.

Of special interest is the lack of inhibition of the

kinase reactions involving fructose by glucose, since glucose is a strong inhibitor of fructose oxidation in intact cells. The mannose isomerase has also been shown to be almost insensitive to glucose inhibition. Neither phosphoglucose isomerase nor glucose-6-phosphate dehydrogenase is inhibited by free glucose.

The distribution of the mannose isomerase and of the enzymes involved in the phosphorylation of glucose and fructose in different strains of *P. saccharophila* grown with different substrates will be discussed in a subsequent report.

SUMMARY

Fructose-adapted mutant strains of *Pseudomonas saccharophila* contain a "mannose isomerase" which interconverts fructose and mannose. Fructose is phosphorylated with adenosine triphosphate to fructose-6-phosphate. The kinase is specific and distinct from the kinase(s) involved in the phosphorylation of glucose and mannose. Fructose-1-phosphate is not the initial product of phosphorylation and neither adenosine diphosphate nor glucose have a significant inhibitory effect on the reaction. Mannose can be phosphorylated slowly by fructose-adapted strains. The wild-type strain grown on maltose, which contained neither mannose isomerase nor fructokinase was shown to phosphorylate mannose to mannose-6-phosphate.

Both glucose-6-phosphate and fructose phosphate were shown to accumulate in intact cells of a fructose-adapted culture during the oxidation of fructose.

REFERENCES

COLOWICK, S. P. AND KALCKAR, H. M. 1943 The role of myokinase in transphosphorylations. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphates. J. Biol. Chem., **148**, 117–126.

DOUDOROFF, M., PALLERONI, N. J., AND MACGEE, J. 1955 Utilization of fructose by *Pseudomonas saccharophila*. Bacteriol. Proc., **1955**, 127.

DOUDOROFF, M., PALLERONI, N. J., MACGEE, J., AND OHARA, M. 1956 The metabolism of carbohydrates by *Pseudomonas saccharophila*. I. Oxidation of fructose by intact cells and crude cell-free preparations. J. Bacteriol., **71**, 196–201.

ENTNER, N. AND DOUDOROFF, M. 1952 Glucose and gluconic acid oxidation by *Pseudomonas saccharophila*. J. Biol. Chem., **196**, 853–862.

PALLERONI, N. J. AND DOUDOROFF, M. 1956 Mannose isomerase of *Pseudomonas saccharophila*. J. Biol. Chem., **218**, 535–548.

ROE, J. H. 1934 A colorimetric method for the determination of fructose in blood and urine. J. Biol. Chem., **107**, 15–22.

SCHALES, O. AND SCHALES, S. S. 1945 A simple method for the determination of glucose in blood. Arch. Biochem., **8**, 285–292.

UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F. 1949 *Manometric techniques and tissue metabolism*, pp. 185–197. Burgess Publishing Co., Minneapolis, Minn.