# SOME PROPERTIES OF THE HEXOKINASE OF PSEUDOMONAS PUTREFACIENS[1]

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Received for publication June 11, 1953

In a recent paper (Klein and Doudoroff, 1950) the mutation of *Pseudomonas putrefaciens* to glucose utilization was reported. The data presented at that time indicated that the biochemical basis of this mutation might be the production of the enzyme hexokinase. Mutant cells, in contrast to wild type cells, could be induced to form hexokinase in the presence of glucose. Furthermore, the kinase appeared to catalyze the phosphorylation of glucose but not of fructose.

It was the purpose of this study to investigate some of the properties of the kinase of *P. putrefaciens*, particularly in view of the fact that preliminary studies had shown it to differ from yeast, brain, and plant hexokinases with respect to substrate specificity. The latter enzymes have been studied extensively and have been shown to be capable of phosphorylating glucose, fructose, and mannose (Berger *et al.*, 1946; Kunitz and MacDonald, 1946; Slein *et al.*, 1950; Wiebelhaus and Lardy, 1949; Saltman, 1953).

## MATERIALS AND METHODS

The organism, *P. putrefaciens*, and the conditions for its cultivation have been described previously (Klein and Doudoroff, 1950).

Oxygen uptake by resting cellular suspensions was determined in air at 30 C using conventional Warburg techniques.

For all enzyme determinations, cells were harvested after 18 to 24 hours of incubation, washed twice in M/30 phosphate buffer (pH 6.8), and ground with alumina in the cold as described by Hayaishi and Stanier (1951). The protein content of these preparations was determined by the method of Stadtman *et al.* (1951).

The standard assay for hexokinase was performed by placing together, in a total volume of 1.5 ml: 5 micromoles of sugar, 10 micromoles of adenosine triphosphate, 10 micromoles of $MgCl_2$, 25 micromoles of phosphate buffer (pH 6.8), and the enzyme preparation containing 1.0 to 1.5 mg protein. The mixtures were kept in an ice bath, and immediately after the addition of the enzyme an initial sample of 0.5 ml was removed and treated with $Ba(OH)_2$ and $ZnSO_4$ to precipitate phosphate esters according to the method of Somogyi (1945). The tubes were incubated then in a 30 C water bath for 30 minutes after which a final 0.5 ml sample was removed and treated to remove phosphate esters. Subsequently, both the initial and final samples were analyzed for free reducing sugars, using the method of Saifer *et al.* (1941). A decrease in reducing sugar under these conditions constitutes a measure of hexokinase activity (*cf* Saltman, 1953).

A second method for the determination of hexokinase was used in certain experiments, described below, in which high concentrations of sugar were employed. In these experiments, glucose-6-phosphate was determined directly in the reaction mixture at the end of the incubation period by measuring the reduction of triphosphopyridine nucleotide in the presence of zwischenferment (Slein *et al.*, 1950). For these determinations the reaction was stopped with 5 per cent trichloroacetic acid, and the proteins were removed by centrifugation. The centrifugate was heated at 100 C for 7 minutes in 1 N HCl in order to destroy any remaining adenosine triphosphate,[2] and following neutralization an aliquot was added to a quartz cell containing, in addition, 0.1 ml of triphosphopyridine nucleotide (200 $\mu$g), 0.15 ml of zwischenferment, and enough M/20 tris (hydroxymethyl) amino-methane buffer (pH 8.0) to bring the total volume to 3.0 ml. Reduction of triphosphopyridine nucleotide was followed at 340 m$\mu$ using a Beckman spectrophotometer.

Finally, a third assay procedure was employed in experiments in which radioactive glucose was used. After incubation under "standard assay"

[1] Supported in part by State of Washington funds for medical and biological research.

[2] This step was necessary because the zwischenferment preparation was found to contain hexokinase.

conditions, the proteins were removed by trichloroacetic acid precipitation, and the supernatant neutralized. At this point 45 micromoles of unlabeled potassium glucose-6-phosphate were added as a carrier, and the phosphate esters were fractionated according to conventional methods (Umbreit *et al.*, 1945). The "alcohol-insoluble" fraction was redissolved in 1 N HCl and reprecipitated twice. After the final precipitation, the residue was washed with 95 per cent alcohol and evenly suspended in absolute

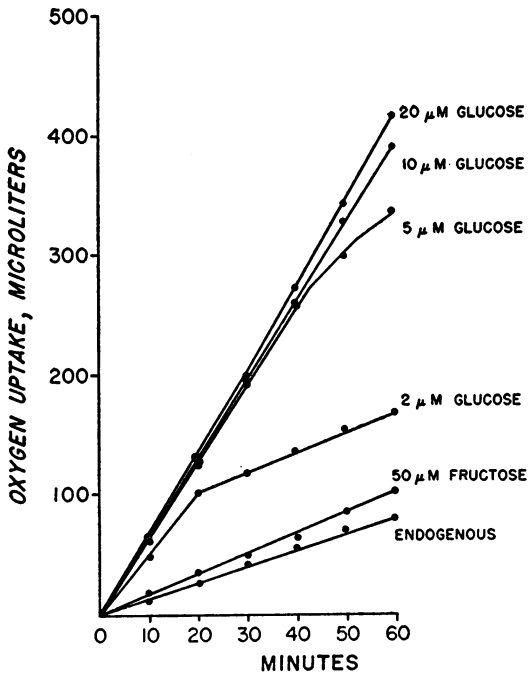


*Figure 1.* Oxidation of glucose and fructose by glucose grown cells of *Pseudomonas putrefaciens*. Approximately 10 mg glucose grown cells (18 hours old) in M/10 phosphate buffer, pH 7.2; volume = 2.0 ml. Vessel side arms contained 0.2 ml substrate dissolved in distilled water.

alcohol. Aliquots of this suspension were plated on copper discs for radioactivity determinations. The latter were performed using a Tracerlab Autoscaler equipped with a windowless gas-flow counter. All radioactivity measurements were corrected subsequently for the usual errors.

The methods of measurement of phosphoglucomutase and phosphohexose isomerase activity, of fructose, and of phosphate have been described previously (Klein and Doudoroff, 1950).

All sugars used in this study were of cp grade. Radioactive glucose was purchased from Nuclear Chemical Co.; adenosine triphosphate was obtained as the dipotassium salt from Pabst Co.; barium glucose-6-phosphate, triphosphopyridine nucleotide (80 per cent purity), and zwischenferment were obtained from Sigma Chemical Co.

## RESULTS

*Oxidation of glucose and fructose by glucose grown cells.* Cells of *P. putrefaciens* grown in glucose oxidize this substrate rapidly but exhibit a very feeble respiration of fructose. As is seen in figure 1, the glucose oxidizing system appears to be fully saturated in the presence of 5 micromoles of glucose, whereas the oxidation of 50 micromoles of fructose proceeds very slowly. These results, of course, might be attributed to a difference in the permeability of these cells toward glucose and fructose, a contention which implies that the phosphorylation of these substrates by cell-free extracts should proceed at roughly equivalent rates. However, upon testing for hexokinase activity with crude enzyme preparations, the specificity for glucose still held.

TABLE 1

*Phosphorylation of glucose, fructose, and mannose by extracts of Pseudomonas putrefaciens*

| ENZYME PREPARATION | PER CENT SUGAR DISAPPEARANCE* | | |
|---|---|---|---|
| | Glucose | Fructose | Mannose |
| A | 57 | 1 | — |
| B | 49 | 1 | — |
| C | 77 | 2 | 1 |
| D | 54 | 1 | 1 |

* Standard assay conditions.

*Phosphorylation of glucose, fructose, and mannose by extracts.* Extracts prepared from several different batches of glucose grown cells all evidenced marked specificity towards glucose (table 1). These results are similar to those obtained by Slein *et al.* (1950), who showed that rat muscle hexokinase phosphorylates glucose but not fructose. Colowick (1951) confirmed the findings of the above investigators but made the additional significant observation that this difference was apparent only at low concentrations of sugar. Indeed, at high concentrations the enzyme was reported to phosphorylate fructose at a higher rate than glucose. It is clear, therefore, that al-

though the rat muscle preparation has a low affinity for fructose, the latter can serve as an excellent substrate when present in high concentrations. The possibility existed, therefore, that fructose might function as a phosphate acceptor at higher concentrations than those employed in the standard assay. Accordingly, to test this, the enzyme was incubated with increasing concentrations of fructose, and analyses were made for glucose-6-phosphate. This method is entirely satisfactory even with fructose as the initial substrate since the enzyme preparations contain a potent hexose isomerase, and fructose-6-phosphate rapidly comes to equilibrium with glucose-6-phosphate. The findings in one such experiment are given in table 2. It is apparent that at the lowest concentration of sugars used approximately ten times more glucose is phosphorylated than fructose, whereas at the highest concentrations less than a third as much glucose-6-phosphate was formed from fructose as from glucose. Thus, it is clear that this kinase resembles the rat muscle enzyme described above with respect to its low affinity for fructose. But for the bacterial enzyme, fructose remains a poor substrate even at high concentrations.

TABLE 2

*The influence of increasing concentrations of fructose on the formation of glucose-6-phosphate by extracts of Pseudomonas putrefaciens**

| CONCENTRATION (μM PER 1.5 ML) | | MICROMOLES GLUCOSE-6-PHOSPHATE FORMED |
|---|---|---|
| Glucose | Fructose | |
| 10 | 0 | 5.03 |
| 0 | 10 | 0.52 |
| 0 | 40 | 1.78 |
| 0 | 80 | 1.72 |
| 0 | 100 | 1.76 |
| 100 | 0 | 5.56 |

* Incubation time in this experiment, 90 minutes.

The different affinities for glucose and fructose are, perhaps, better illustrated in experiments in which both substrates are present in competition for the same enzyme site. If a small amount of radioactive glucose is added to a series of tubes which contain graded amounts of unlabeled fructose, the effect of high concentrations of fructose on glucose phosphorylation can be determined. Table 3 contains data on one such experiment, from which it is clear that the enzyme preferentially phosphorylates glucose.

*Phosphorylation of various sugars by extracts of P. putrefaciens.* Since the experiments recorded above demonstrated a restricted substrate specificity, it was of some interest to determine whether sugars other than glucose could serve as substrates in the standard assay. Of a number of carbohydrates tested, only glucose and glucosamine appear to be significantly phosphorylated (table 4). The latter compound has been shown to be phosphorylated by animal (Harpur and Quastel, 1949) and plant (Saltman, 1953) hexo-

TABLE 3

*Competition between glucose and fructose for the hexokinase of Pseudomonas putrefaciens**

| SUBSTRATES | | TOTAL RADIOACTIVITY OF PHOSPHATE ESTERS | PER CENT INHIBITION OF GLUCOSE PHOSPHORYLATION |
|---|---|---|---|
| Glucose | Fructose | | |
| μM | μM | cpm | |
| 5 | — | 2,730 | 0 |
| 5 | 5 | 2,470 | 9.5 |
| 5 | 12.5 | 1,880 | 31.0 |
| 5 | 25 | 1,770 | 35.1 |
| 5 | 50 | 1,550 | 43.1 |
| 5† | — | 540 | — |

* Standard assay conditions.

† Control; no adenosine triphosphate was added to this tube.

TABLE 4

*Phosphorylation of carbohydrates by extracts of Pseudomonas putrefaciens**

| SUBSTRATE | PER CENT OF GLUCOSE ACTIVITY† | SUBSTRATE | PER CENT OF GLUCOSE ACTIVITY† |
|---|---|---|---|
| D-Glucose..... | 100 | D-Glucosamine. | 50–70 |
| D-Fructose.... | 0–10 | D-Xylose..... | 0 |
| D-Mannose.... | 0–5 | D-Lyxose..... | 0 |
| L-Sorbose..... | 0 | D-Ribose..... | 0–10 |
| D-Galactose... | 0 | L-Fucose..... | 0 |
| D-Arabinose... | 0–5 | Maltose..... | 0 |
| L-Arabinose... | 0 | | |

* Standard assay conditions. Data represent a summary of several experiments.

† $\frac{\text{Micromoles substrate disappearance}}{\text{Micromoles glucose disappearance}} \times 100.$

kinases, enzymes that also utilize fructose and mannose as substrates. Since these hexoses are poor substrates for the enzyme of *P. putrefaciens*, one might expect the configuration around the second carbon atom of glucose to be critical in determining substrate specificity. It is somewhat surprising, therefore, that glucosamine is phosphorylated by extracts made from this organism.

*Effect of inhibitors on glucose phosphorylation.* Several groups of investigators have presented evidence to support the contention that yeast and mammalian hexokinases contain essential sulfhydryl groups (Bailey and Webb, 1948; Griffiths, 1949; Colowick, 1951). On the other hand, in their study of the properties of crystalline yeast hexokinase, Berger *et al.* (1946) found no basis for this claim. To test for the presence of sulfhydryl groups essential to the activity of the hexokinase of *P. putrefaciens*, the phosphorylation of glucose was carried out in the presence of sulfhydryl poisons. Table 5 provides data illustrating the inhibition of glucose esterification by low concentrations of iodoacetate and phenylmercuric acetate and the reversal of this inhibition by excess cysteine.

TABLE 5

*Inhibition of hexokinase activity by sulfhydryl poisons, and reversibility with cysteine**

| ADDITIONS (FINAL MOLAR CONC × $10^3$) | | | PER CENT INHIBITION |
|---|---|---|---|
| Iodoacetate | Phenyl mercuric acetate | Cysteine | |
| 0.1 | — | — | 0 |
| 1.0 | — | — | 26 |
| 3.3 | — | — | 100 |
| — | 0.01 | — | 33 |
| — | 0.1 | — | 100 |
| — | 1.0 | — | 100 |
| — | 0.1 | — | 100 |
| — | 0.1 | 0.33 | 8 |
| — | 0.1 | 1.0 | 0 |

* Standard assay conditions.

While these results appear to be presumptive evidence for essential sulfhydryl groups in the enzyme preparation, it should be noted that the criteria of inhibition and reversal are not always sufficient in designating an enzyme as a sulfhydryl enzyme (Olcott and Fraenkel-Conrat, 1947).

Glucose-6-phosphate, one of the products of the hexokinase reaction, has been found to inhibit brain hexokinase (Weil-Malherbe and Bone, 1951; Crane and Sols, 1952) but not the yeast enzyme (Slein *et al.*, 1950). The effect of this substance on the enzyme of *P. putrefaciens* was tested; when added to the standard assay system, glucose-6-phosphate was not inhibitory up to the highest concentration used (0.012 M).[3] By contrast, a 72 per cent inhibition of brain hexokinase was reported by Weil-Malherbe and Bone (1951) at a concentration of 0.0018 M glucose-6-phosphate.

*The product of glucose phosphorylation.* In crude enzyme mixtures, the initial product of glucose phosphorylation could not be determined since these extracts contained phosphoglucomutase. It seemed possible that the initial product of glucose phosphorylation might be glucose-1-phosphate, which subsequently could be converted to the stable glucose-6-phosphate. (Pertinent to this argument are the studies on yeast galactokinase; Wilkinson (1949) demonstrated that galactose phosphorylation results in the formation of galactose-1-phosphate rather than galactose-6-phosphate.) In order, therefore, to test for the product of glucose phosphorylation, phosphoglucomutase had to be removed or inhibited.

TABLE 6

*Product of glucose phosphorylation**

| GLUCOSE DISAPPEARANCE | PHOSPHATE IN ALC-INSOL FRACTION | | GLUCOSE-6-PHOSPHATE FORMED‡ |
|---|---|---|---|
| | $P_{tot}$† | $P_o + P_7$† | |
| μM | μM | μM | μM |
| 5.8 | 5.6 | 0 | 4.7 |

* Standard assay conditions, except glucose increased to 10 μM.

† $P_{tot}$, total phosphate; $P_o$, inorganic phosphate; $P_7$, phosphate hydrolyzable in 1 N HCl in seven minutes.

‡ Determined spectrophotometrically.

Removal of phosphoglucomutase activity by fractionation of the crude enzyme preparation of *P. putrefaciens* proved unnecessary when it was found that certain batches of crude extract were

[3] Other compounds noninhibitory to glucose phosphorylation were glucose-1-phosphate (0.0067 M), fructose-6-phosphate (0.0067 M), and sodium fluoride (0.05 M).

active only in the presence of cysteine. These were, therefore, completely devoid of phosphoglucomutase activity in the standard assay system. Using such preparations the immediate product of glucose phosphorylation could be identified. After incubating such extracts with glucose and adenosine triphosphate, the hexose-monophosphate fraction was collected. Phosphate analyses revealed that all the phosphate in the fraction was resistant to seven minute hydrolysis by 1 N HCl, thus suggesting that glucose-6-phosphate was the glucose ester formed. Further specific identification was achieved using the spectrophotometric method described above. Table 6 contains data summarizing the results of these determinations.

## DISCUSSION

Plants and yeast appear to contain a single protein capable of catalyzing the phosphorylation of glucose and fructose, as well as of mannose. Similarly, brain tissue has been shown to contain a hexokinase having comparable specificity. It might at first seem reasonable, therefore, to regard the enzyme from *P. putrefaciens* as a distinctly different type of kinase. It should be emphasized, however, that studies on the kinetics of yeast and brain hexokinases have shown these enzymes to have a greater affinity for glucose than for fructose (Dixon and Needham, 1946; Meyerhof and Wilson, 1948). These differences in affinity are apparent only at extremely low concentrations because both yeast and brain preparations become saturated with substrate at low concentrations. On the other hand, rat muscle hexokinase has a considerably higher affinity for glucose than for fructose and does not become saturated with fructose until high levels of this sugar are used. All these enzymes therefore may be considered as varieties of hexokinase, differing mainly in their affinities for fructose. On this basis, the enzyme of *P. putrefaciens* might be classed with the other members of this group. Indeed, except for the fact that fructose, even at high concentrations, is not readily phosphorylated by this enzyme, there exists a similarity between the hexokinase of *P. putrefaciens* and of rat skeletal muscle.

Whether a similar type of hexokinase is present in other bacteria is not known at present; further investigations are needed in order to clarify the point. In this connection, it is of interest to note the findings of T. J. Starr of this department, who recently isolated eight strains of marine myxobacteria, two of which were capable of oxidizing glucose but not fructose (unpublished experiments). In cases such as these, it would seem that a specific kinase might be operative. With microorganisms that utilize glucose and fructose at comparable rates, it is generally assumed that a hexokinase similar to the yeast enzyme is present. This assumption may, however, be erroneous in some cases. For example, extracts of cells of *P. putrefaciens*, grown in sucrose, readily phosphorylate both hexoses. However, when such extracts are treated with acetone, the fraction precipitating between 25 to 40 per cent acetone is unable to phosphorylate fructose while retaining its activity towards glucose (unpublished experiments). It is clear that in this case the cells must have contained at least two hexokinases. In a recent study on another bacterium, *Escherichia coli*, Cardini (1951) found conditions under which a hexokinase active on glucose could be separated from a crude mixture that had originally been active also on the related hexoses, fructose and mannose.

The properties of this hexokinase may explain previous observations (Klein, 1951) on the growth of this organism with fructose as the carbon source. At that time, it was reported that *P. putrefaciens* could be grown with fructose provided that this sugar was present at high concentrations. Even under these conditions, however, growth of the organisms was slow, and the final cell yield was low, thus indicating an inefficient utilization of the sugar. These effects can be interpreted now in the light of knowledge gained from the present studies. The presence in these cells of a hexokinase, which has a low affinity for fructose, and which catalyzes a relatively feeble phosphorylation of this sugar at high concentrations, could explain the earlier observations. Evidence supporting this view came from an experiment in which cells, unadapted to glucose, were inoculated into a synthetic medium containing fructose. After the cells were harvested and ground with alumina, the resulting extract exhibited properties identical with those described for glucose grown cells.

## SUMMARY

It has been shown that the hexokinase of *Pseudomonas putrefaciens* phosphorylates glucose

and glucosamine at low substrate concentrations. Fructose, mannose, and a number of other carbohydrates do not serve as substrates under these conditions.

The enzyme has a low affinity for fructose, the latter serving as a poor substrate at high concentrations.

Using preparations devoid of phosphoglucomutase activity, the initial product of glucose phosphorylation has been identified as glucose-6-phosphate.

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