GLUCOSAMINE-6-PHOSPHATE DEGRADATION BY PROTEUS VULGARIS AND ISOLATION OF PHOSPHOGLUCOSAMINISOMERASE

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Abstract

Aron, H. (University of California, Santa Barbara), H. H. Yonenaka, and H. I. Nakada. Glucosamine-6-phosphate degradation by Proteus vulgaris and isolation of phosphoglucomaminisomerase. J. Bacteriol. 87:1123–1128. 1964.—The presence of the enzyme, phosphoglucomaminisomerase, was demonstrated in a strain of Proteus vulgaris. Fructose-6-phosphate and ammonia were shown to be the primary reaction products. The optimal pH was 7.2 with a broad peak. This differs from previously reported bacterial phosphoglucomaminisomerasers which had a pH optimum of about 5.8. Other properties of the enzyme are presented.

An earlier report described an enzyme isolated from Proteus vulgaris 31 M that catalyzed the direct oxidation of free N-acetylgalactosamine to the corresponding N-acetylhexosaminic acids (Hochstein, Wolfe, and Nakada, 1959). Because of the requirement for oxygen and the formation of peroxide, this enzyme was presumed to be a flavoprotein dehydrogenase. Crude extracts of P. vulgaris 31 M were also capable of converting glucosamine to glucosic acid (Wolfe and Nakada, unpublished data). Furthermore, this strain of P. vulgaris showed no indication of a phosphoglucomaminisomerase such as that described in Escherichia coli, Aerobacter cloacae, or pig kidney (Wolfe, Morita, and Nakada, 1956; Wolfe and Nakada, 1956; Wolfe, Britton, and Nakada, 1957; Comb and Roseman, 1958; Imanaga, 1957; and Leloir and Cardini, 1956). Deterioration of this oxidative ability led to a search for other P. vulgaris strains containing N-acetylhexasamine or hexosamine dehydrogenase. P. vulgaris 13315 GL, which caused the rapid disappearance of added hexosamines or N-acetylhexasamines, was provided by G. Litwack. Preliminary studies with crude extracts of this bacterium showed the absence of a flavoprotein oxidase, but indicated a metabolic pathway that involved phosphorylation and the formation of ammonia and a phosphorylated sugar. The latter product could be assayed by ketose formation or by coupling the reaction to the glucose-6-phosphate dehydrogenase system.

Thus, the reaction could either catalyze the conversion of glucosamine-6-phosphate to fructose-6-phosphate and ammonia in a manner similar to the previously mentioned phosphoglucomaminisomerase, or catalyze the deamination of glucosamine-6-phosphate directly to glucose-6-phosphate and ammonia. In either case, the mechanism of this reaction was of interest because of the lack of such a system in a previously studied strain of P. vulgaris.

In this paper, we wish to briefly report on the partial purification and properties of a phosphoglucomaminisomerase from P. vulgaris 13315 GL which catalyzes the conversion of glucosamine-6-phosphate to fructose-6-phosphate and ammonia.

Materials and Methods

Organism. P. vulgaris 13315 GL was obtained through the kindness of Gerald Litwack, University of Pennsylvania, Philadelphia.

Culture. All cultures were grown in a liquid medium of the following final composition: 1% Proteose Peptone (Difco), 0.5% sodium chloride, 0.5% glucose, and 0.5% glucosamine. Aeration and agitation during growth were accomplished either by shaking or by the passage of acid- and alkali-washed air.

Chemicals. Glucosamine-6-phosphate was synthesized both enzymatically (Brown, 1951) and chemically, according to the procedure used by Seegmiller and Horecker (1951) for the synthesis of glucose-6-phosphate. All other materials were obtained from commercial sources.

Analytical methods. Total and inorganic phos-
phorus were determined by the method of Fiske and SubbaRow (1925); ketose sugars by the carbazole method of Dische and Borenfreund (1951); hexosamine by the method of Levy and McAllan (1959); the concentration of protein and nucleic acids spectrophotometrically according to formulas proposed by Warburg and Christian (1941); and ammonia by the method of Branganca, Quastel, and Schucker (1954). A mixture of acetic acid-ethyl acetate-water (3:3:1) was used as the developing system for chromatography (Mortimer, 1952). The chromatograms were sprayed with p-anisidine phosphate (Cerbulis, 1955), ninhydrin (Payne and Kieber, 1954; Klenk and Lauenstein, 1952), or aniline diphenylamine (Saroja, Venkataraman, and Giri, 1955; Schwimmer and Bevenue, 1956). The glucosamine-6-phosphate deaminating system was assayed spectrophotometrically by coupling the reaction to the glucose-6-phosphate dehydrogenase system (added to the reaction vessels to insure adequate levels of this enzyme), or by following either the formation of ketose and ammonia or the disappearance of hexosamine, or both.

FIG. 1. Spectrophotometric assay of phosphoglucoamminosomerase and phosphoglucoisomerase. Assays were recorded with a Beckman model DB double-beam spectrophotometer. Each cuvette contained: potassium phosphate buffer (pH 7.2), 12.5 μmoles; MgSO₄, 5 μmoles; cysteine, 10 μmoles; nicotinamide adenine dinucleotide phosphate, 0.5 μmoles; glucose-6-phosphate dehydrogenase, excess; enzyme, 0.1 ml; glucosamine-6-phosphate or fructose-6-phosphate, 1 μ mole; and water to a total volume of 1.4 ml.

Results and Discussion

Figure 1 shows a typical assay resulting from coupling the glucosamine-6-phosphate deaminating system to glucose-6-phosphate dehydrogenase with the use of a relatively crude enzyme preparation that also contained an active phosphoglucoisomerase. This indicated that the principal product of the deamination was either glucose-6-phosphate or fructose-6-phosphate. Because of the presence of phosphoglucoisomerase which rapidly catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate, it was impossible to define clearly which of the two hexose phosphates was the primary product of enzyme action on phosphoglucoisomerase. Studies on the E. coli phosphoglucoamminosomerase (Wolfe et al., 1956, 1957; Wolfe and Nakada, 1956) showed that the mechanism of action followed the Lobry de Bruyn-Van Ekenstein rearrangement, as does phosphoglucoisomerase. Thus, there were three possible schemes by which glucosamine-6-phosphate could have been converted to a hexose phosphate: (i) this strain of P. vulgaris could have a phosphoglucoaminosomerase similar to that found in E. coli; (ii) there could be a deaminase that converts glucosamine-6-phosphate to glucose-6-phosphate; or (iii) the isomeric deamination could have been catalyzed by phosphoglucoisomerase with specificity characteristics that include the hexosamine phosphate.

We felt that the best way to distinguish among these possibilities was to separate, if possible, phosphoglucoisomerase from the enzyme responsible for glucosamine-6-P deamination.

Enzyme preparation. A crude extract was prepared by manually grinding approximately 10 g of frozen P. vulgaris 13315 GL in a frozen mortar with 10 g of powdered glass, and extracting with 50 ml of potassium phosphate buffer (0.05 M; pH 7.2). After centrifugation, the precipitate was discarded, and finely ground ammonium sulfate was added to the supernatant fluid to give a 0 to 70% saturated fraction. The precipitate obtained after centrifugation was dissolved in 50 ml of water; 0.05 volume of 1 M manganous chloride was added to precipitate nucleic acids, and the resulting suspension was dialyzed overnight against potassium phosphate buffer (0.005 M; pH 8.0). After centrifugation, the supernatant fluid containing the crude enzyme extract was either fractionated further
TABLE 1. Preliminary purification of enzyme*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Nucleic acids (%)</th>
<th>Vol (ml)</th>
<th>Δ A0 per min at 340 μμ with GA-6-P</th>
<th>Specific activity</th>
<th>Δ A0 per min at 340 μμ with F-6-P</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 70</td>
<td>2.00</td>
<td>4.5</td>
<td>110</td>
<td>0.015</td>
<td>0.075</td>
<td>0.32</td>
<td>1.60</td>
</tr>
<tr>
<td>0 to 40</td>
<td>0.54</td>
<td>2.0</td>
<td>10</td>
<td>0.005</td>
<td>0.063</td>
<td>0.16</td>
<td>2.96</td>
</tr>
<tr>
<td>40 to 50</td>
<td>2.12</td>
<td>2.2</td>
<td>10</td>
<td>0.05</td>
<td>0.236</td>
<td>0.38</td>
<td>1.79</td>
</tr>
<tr>
<td>50 to 60</td>
<td>3.54</td>
<td>3.0</td>
<td>10</td>
<td>0.17</td>
<td>0.480</td>
<td>0.70</td>
<td>1.97</td>
</tr>
<tr>
<td>60 to 80</td>
<td>3.90</td>
<td>3.5</td>
<td>10</td>
<td>0.02</td>
<td>0.051</td>
<td>0.58</td>
<td>1.47</td>
</tr>
</tbody>
</table>

* Abbreviations: GA-6-P, glucosamine-6-phosphate; F-6-P, fructose-6-phosphate.
† Defined as the amount of substrate converted per min per mg of protein. (See Fig. 1 for assay procedure.)

with an ammonium sulfate to give 0 to 40, 40 to 50, 50 to 60, and 60 to 80% saturated fractions, or passed through a diethylaminoethyl (DEAE)-cellulose column, previously equilibrated with 0.005 M potassium phosphate buffer (pH 8.0), followed by elution with 100 ml of each of the following potassium phosphate buffers: (I) 0.005 M, pH 8.0; (II) 0.05 M, pH 7.6; (III) 0.1 M, pH 7.2; (IV) 0.15 M, pH 7.0; (V) 0.2 M, pH 6.8; and (VI) 0.2 M, pH 6.5 (sodium chloride added to a concentration of 0.2 M).

The results of fractionation with ammonium sulfate are given in Table 1. No separation of the two enzymes was achieved in this manner. Although contaminated with phosphoglucoisomerase, the 50 to 60% ammonium sulfate fraction was used for many of the studies of the enzyme properties because of higher yields.

The second procedure resulted in the separation of the phosphoglucoisomerase from the phosphoglucominiosomerase. Each of the eluted fractions was analyzed for protein content from optical density readings at 280 μμ (Fig. 2). Fractions showing the highest protein content were concentrated by dialysis against polyvinylpyrrolidone. (The different peaks are indicated by Roman numerals in Fig. 2.)

Because both phosphoglucoisomerase and phosphoglucominiosomerase catalyze the formation of fructose-6-phosphate from their respective substrates, glucose-6-phosphate or glucosamine-6-phosphate, we could assay for both enzymes by following the formation of ketose from the above substrates. Each of the concentrated fractions was checked for enzyme activity with the use of both glucose-6-phosphate and glucosamine-6-phosphate.

The results of two studies are given in Table 2, in which the protein concentration (mg/ml) of fraction IV was 0.045 and 0.153, and that of fraction VI, 0.081 and 0.093. These results clearly showed that separation of the two enzymes was achieved by chromatography on DEAE-cellulose, with no cross contamination of activities in either of these fractions.

Identification of the product of deamination as fructose-6-phosphate was obtained by paper chromatography. The unidimensional ascending or descending method was employed. Incubation of fraction IV with glucosamine-6-phosphate yielded two spots; one was identified as glu-
TABLE 2. Separation of phosphoglucoisomerase from phosphoglucoisomerase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glucosamine-6-phosphate</th>
<th>Glucose-6-phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>7.2</td>
<td>10.3</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each reaction vessel contained the following: substrate, 2 µmoles; potassium phosphate buffer (pH 7.2), 10 µmoles; and enzyme, 0.1 ml, in a total volume of 0.5 ml. (See text and Fig. 2 for fractions).

From these results it can be concluded that the probable metabolic pathway of glucosamine-6-phosphate degradation by P. vulgaris 13315 GL was similar to that found in E. coli (Wolfe et al., 1956, 1957; Wolfe and Nakada, 1956; Comb and Roseman, 1958).

**Studies of properties of phosphoglucoisomerase.** To demonstrate further the presence of phosphoglucoisomerase, a balance study correlating the disappearance of glucosamine and the appearance of ketose and ammonia was run (Fig. 4). As shown, an approximately stoichiometric relationship was obtained between the disappearance of hexosamine and the appearance of the products.

The effects of different concentrations of glucosamine-6-phosphate on reaction rate are shown in Fig. 5. When these results were plotted according to the double reciprocal method of Lineweaver and Burk (1934), the Michaelis-Menten (1913) constant of $1.25 \times 10^{-2} \text{ M}$ was obtained. The Michaelis-Menten constant from E. coli phosphoglucoisomerase was reported to be $1.5 \times 10^{-2} \text{ M}$ (Comb and Roseman, 1958).

According to Leloir and Cardini (1956), phosphoglucoisomerase obtained from animal tissues required N-acetylglucosamine-6-phosphate for activation. The enzyme from P. vulgaris 13315 GL was not influenced by this compound.

**FIG. 3.** Paper chromatography of the reaction mixtures from assay of the purified enzyme fractions.

cosamine-6-phosphate, and the other as fructose-6-phosphate. No glucose-6-phosphate was detectable. If this fraction were contaminated by phosphoglucoisomerase, part of the fructose-6-P should have been converted to glucose-6-P. Incubation of fraction VI with glucose-6-phosphate produced spots for glucose-6-phosphate and fructose-6-phosphate, indicating the presence of phosphoglucoisomerase (Fig. 3).

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**FIG. 4.** Balance study comparing glucosamine-6-phosphate disappearance and product formation. The reaction vessel contained: glucosamine-6-phosphate, 4 µmoles; potassium phosphate buffer (pH 7.2), 20 µmoles; enzyme, 0.2 ml, in a total volume of 1.0 ml.
No effect was shown with 2-deoxyglucose-6-phosphate, a substance that had been reported to inhibit phosphoglucomutase (Wick et al., 1956).

Both in the course of purification and in studies of the properties of phosphoglucomutase, it was found that the *P. vulgaris* enzyme was labile to heat and low pH, being easily destroyed at a pH of 5.0 and inactivated when heated to 55°C for 5 min.

The phosphoglucomutase showed an optimal pH of about 7.2 with approximately 50% of optimal values from 6.8 to 7.4 (Fig. 6). This broad optimal pH range differed from that found with the *E. coli* phosphoglucomutase which had greatest activity at pH 5.8. In the studies presented here, three buffer systems were used: acetate, phosphate, and tris(hydroxymethyl)aminomethane chloride. The plotted hydrogen ion concentrations were based on the pH of the reaction mixture. The different buffers had no effect on the reaction rate.

The relationship of enzyme concentration to reaction rate was examined. The rate of ketose formation was followed by incubating glucosamine-6-phosphate with varying concentrations of enzyme. The expected kinetics were not observed (Fig. 7). No obvious explanation for this nonlinearity was apparent. Similar results were obtained with different enzyme preparations by following the course of the reaction spectrophotometrically or by following hexosamine disappearance or ketose formation.

![Graph showing effect of pH on phosphoglucomutase reaction](image1)

**Fig. 5.** Effect of pH on the phosphoglucomutase reaction. Each reaction vessel contained: buffers as indicated, 10 μmoles; glucosamine-6-phosphate, 2 μmoles; and enzyme, 0.1 ml. Total volume was 0.5 ml. The pH values plotted are the values obtained from the reaction mixtures at the end of the incubation period.

![Graph showing effect of substrate concentration](image2)

**Fig. 6.** Effect of substrate concentration. Each flask contained: glucosamine-6-phosphate in amounts indicated; enzyme, 0.1 ml; potassium phosphate buffer (pH 7.2), 10 μmoles; and water to a total volume of 0.5 ml.

![Graph showing effect of enzyme concentration](image3)

**Fig. 7.** Effect of enzyme concentration. Each reaction vessel contained enzyme in the amounts indicated; glucosamine-6-phosphate, 2 μmoles; potassium phosphate buffer (pH 7.2), 10 μmoles; and water to a total volume of 0.5 ml.
Studied for reversibility, in which enzyme was incubated with fructose-6-phosphate and ammonia under a variety of conditions, gave no indication of glucosamine-6-phosphate formation.

In contrast with studies on _P. vulgaris_ 31 M, a flavoprotein glucosamine oxidase could not be detected in _P. vulgaris_ 13315 GL. It is interesting that two strains of the same bacteria should have such differing mechanisms for the metabolic breakdown of hexosamines.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**


