ENZYMATIC FORMATION OF PENTOSE PHOSPHATE FROM 6-PHOSPHOGLUCONATE*

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In 1933 (1) Warburg and Christian reported the formation of 6-phosphogluconate from glucose-6-phosphate and later the further oxidation of phosphogluconate (2). Triphosphopyridine nucleotide (TPN), the coenzyme for both reactions, was isolated from horse red blood cells. Further investigation of the oxidation of 6-phosphogluconate revealed subsequent decarboxylation, without participation of phosphate, and Lipmann proposed the formation of 2-keto-6-phosphogluconate which gave rise to D-arabinose-5-phosphate (3). The bacterial oxidation of gluconate to 2-ketogluconate had been described earlier (4).

Dickens (5) found a system oxidizing phosphogluconate in brain homogenates and partially purified an enzyme from yeast extracts. These preparations also were active with glucose-6-phosphate as the substrate in contrast to Negelein and Gerischer's (6) Zwischenferment, which acts only on glucose-6-phosphate. Dickens and McIlwain (7) found that phenazine dyes were efficient as hydrogen acceptors in this system.

Dickens (8) described another enzyme preparation from yeast which was able to oxidize ribose-5-phosphate. Yeast extract also fermented both ribose-5-phosphate and D-arabinose-5-phosphate, the former at a much faster rate (9). It was suggested that ribose-5-phosphate was the pentose formed in the breakdown of phosphogluconate by way of 2-keto-6-phosphogluconate.

Warburg and Christian (10) and Dickens (8) attempted to isolate and identify the end-products of phosphogluconate oxidation. Some fractions gave strong pentose reactions and compounds with 3 to 6 carbon atoms were obtained. Because of the difficulty of preparation of the components of the system, the amounts of reaction products which were isolated were too small to permit further analysis.

Our decision to reinvestigate this system arose from an interest in the origin of ribose and desoxyribose. In working with bacteriophage, Cohen

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(11, 12) had found that uninfected Escherichia coli under certain conditions synthesized ribose nucleic acid (RNA) and desoxyribose nucleic acid (DNA) in a ratio of about 3:1, but that on infection the same amount of P was used to synthesize only the virus nucleic acid, DNA. He proposed ribose-5-phosphate as a common precursor for the synthesis of the ribose and desoxyribose phosphates and RNA and DNA respectively. He suggested that virus infection then shunted all the P passing through the ribose-5-phosphate step into the DNA path.

Micromethods have been devised for the separation and characterization of the reaction products of this system (13, 14). Their application to analysis of the reaction products of this system will be described, as well as some attributes of an enzyme preparation from yeast which oxidizes and degrades 6-phosphogluconate to form pentose phosphate. A brief summary of the results of these investigations has appeared (13).

Materials

Components of System—The system we have used was essentially that described by Dickens. It is composed of the enzyme (7), TPN, glucose-6-phosphate or phosphoglucconate as the substrate, and phenazine as the hydrogen carrier. The reaction was carried out in 0.003 M phosphate buffer, keeping the pH between 6.5 and 7.4.

Brewers' bottom yeast from Cooper's Brewery in Philadelphia was washed with water and dried slowly on paper at room temperature. Autolysis of a suspension of the dried yeast in 3 parts of water was continued until fermentative activity ceased. This Lebedev extract was diluted with 10 volumes of water and the enzyme was precipitated with 0.02 N acetic acid at pH 4.6. This precipitate was washed with 0.02 N acetic acid and dried by lyophilization. The dried enzyme was brought into solution by the careful addition of 0.05 N NaOH to a pH value no higher than 7.4.

Although the activity of a preparation remained constant for a long time when kept in the dry state, the activities of different preparations of enzyme made in the same way from the same yeast varied widely. The ratio of activity on phosphoglucconate and activity on glucose-6-phosphate varied with the preparation. With constant amounts of enzyme and the same concentration of TPN and phosphate, five different enzyme preparations had ratios of O₂ uptake on phosphoglucconate to that on glucose-6-phosphate, which ranged from 0.39 to 0.63. The preparations used in most of the experiments to be described below were tested in reaction mixtures containing 10 mg. of enzyme, 1 mg. of TPN, 9.4 μM of substrate, and 0.06 mg. of phenazine methochloride, in 2 cc. of 0.01 M phosphate buffer at pH 7.0. On incubation in air at 38° they consumed 6.8 to 7.3
and 3.6 to 3.7 \(\mu M\) of \(O_2\) in 60 minutes, with glucose-6-phosphate and 6-phosphogluconate respectively. The maximal initial rate obtained was 0.22 mole of \(O_2\) per minute with 1.74 \(\mu M\) of phosphogluconate per mg. of enzyme.

TPN was prepared from horse liver (15). Purification was not carried beyond Warburg's Step III and the purity of TPN in our earliest preparations was about 4 per cent or below, as determined by the method either of Warburg (10), of Haas (16), or of LePage (17). The new method of preparation of TPN by LePage and Mueller (18) has not yet been tried in its entirety. One step, the passage of a solution of 4 per cent TPN through a charcoal (Nuchar C) column, effected an increase in purity to 16 per cent. This preparation and a preparation of 55 per cent purity, kindly given to us by Dr. B. Horecker and Dr. A. Kornberg of the National Institutes of Health, have been used in recent experiments.

Phenazine methochloride (7) has been used as the hydrogen carrier. Some commercial preparations were not pure. Solutions of this compound were not stable and the solid deteriorated even when kept in the cold \textit{in vacuo}. Consequently, solutions of the carrier were freshly prepared and an excess was added to the reaction mixture.

Glucose-6-phosphate was prepared by the method of LePage and Umbreit (19) from Embden's ester, which had been isolated by the method of DuBois and Potter (20). 6-Phosphogluconate was prepared by bromine oxidation of glucose-6-phosphate (21). The isolated barium salt contained 1 to 3 per cent of a mixture of glucose-6-phosphate and a reducing acid, identified as the 2-keto-6-phosphogluconic acid. Fructose-6-phosphate has been prepared from the Ca fructose-1,6-diphosphate obtained from the Schwarz Laboratories. The barium salt of ATP was isolated from rabbit muscle and was hydrolyzed to yield ribose-5-phosphate, which was isolated as the Ba salt. Synthetic d-arabinose-5-phosphate and xylose-5-phosphate were kindly supplied by the Levene Collection of the Rockefeller Institute. Glyceraldehyde-3-phosphate was obtained by hydrolysis of the synthetic dimeric phosphoglyceraldehyde generously given us by Dr. O. Meyerhof, who also gave us samples of glucose-1-phosphate. We are grateful to Dr. H. Green of this University for very active preparations of alkaline phosphatase (22).

\textbf{Analytical Methods}—Three methods were employed to follow the progress of the reaction. The method of Burris (23), involving the time of decolorization of methylene blue, was used in initial experiments to compare the activities of enzyme preparations, to determine optimal concentrations of the compounds, to give an estimate of amounts of TPN activity at various steps during preparation of the TPN, and to test the activity of the system with different substrates. Oxygen uptake and \(CO_2\) pro-
duction were measured in Warburg manometers in an atmosphere of air at 38°. The Beckman spectrophotometer was used for the determination of TPN by the Haas or Warburg method, with measurement at 340 μμ.

Pentose was determined in the Bial reaction by the method of Miller et al. (24), with ribose as the standard. Reducing values were determined by either the Hagedorn-Jensen titrimetric method (25) or the Nelson colorimetric method (26), with glucose as the standard. Trichloroacetic acid inhibited color formation in the Nelson method. The procedure of King was used for the estimation of phosphorus (27). Ketose was estimated by the method of Roe (28).

Paper Chromatography—The squares No. 597 (Schleicher and Schüll) have proved satisfactory. Ascending and descending chromatograms of carbohydrates in sealed aquaria were run in various solvent mixtures, as described by Partridge (29).

Our Rₚ values obtained in s-collidine are different from those of Partridge and are given at the appropriate places below. The presence of the carbohydrates on the papers was detected by heating with m-phenylenediamine dihydrochloride (30), or Benedict’s reagent; they were subsequently viewed under an ultraviolet beam. The heating of these papers was carefully controlled in a glass-walled oven which had a maximal variability of 1° in all parts of the oven. Acids were indicated by spraying with 0.04 per cent brom phenol blue in 95 per cent alcohol.

The carbohydrates were freed from phosphate by incubating isolated esters in 0.3 per cent MgCl₂ and 0.1 unit of purified alkaline phosphatase (22) per 0.065 mg. of organic P. The incubation mixtures were maintained at pH 8.8 for 3 hours to complete hydrolysis. It was readily seen on chromatograms that fructose-6-phosphate gave rise to fructose, glucose-6-phosphate and glucose 1-phosphate liberated glucose, and ribose-3-phosphate and ribose-5-phosphate yielded ribose. No other sugars were detected in these tests.

The chromatography of phosphate esters has been summarized elsewhere (13). In addition to the separation of ribose-5-phosphate and D-arabinose-5-phosphate by means of ethanol-boric acid therein described, the ready detection of glyceraldehyde-3-phosphate and 6-phosphogluconic acid may be noted as a function of the Rₚ values, fluorescence, and reactivities with m-phenylenediamine dihydrochloride, the former being reactive before heating, the latter being unreactive.

Elution of carbohydrates and the phosphorylated derivatives with water from cut strips of the dried chromatograms was accomplished with good recoveries. These eluates were centrifuged and analyzed.

Ion Exchange Separations—These techniques were employed to effect a separation of neutral and acidic carbohydrates and to eliminate disturbing
salt effects on paper chromatograms. The cation exchange resin Amberlite IR-100 and the anion exchange resin Amberlite IR-400A were converted to the hydrogen and chloride forms respectively and washed until filtrates rose to pH 5. A column of the appropriate resin 14 cm. high and 1 cm. in diameter was used in the apparatus described by Tompkins et al. (31). With a flow rate of 0.5 cc. per minute a filtration of 98 per cent of neutral sugar, e.g. ribose, was effected with a 2-to 3-fold dilution. This flow rate was also efficacious in the separate elution of the acids.

In a typical experiment 10 cc. of a mixture of 8.8 mg. of ribose, 9.05 mg. of gluconic acid, and 10.0 mg. of 2-ketogluconic acid as calcium salts at pH 7.0 were added to the anion column and washed through with water. The neutral filtrate, followed by water, was passed through the cation exchange column until the effluent rose to pH 5. Over-all recoveries of pentose of 96 per cent were found at this point. Ag₂O was added cautiously to the acid filtrate with shaking until the pH approached 7. The AgCl was filtered or centrifuged after standing overnight at 4°, since the low salt concentration inhibited rapid complete precipitation.

The anion column was eluted with 0.01 N HCl. Gluconate was estimated as described elsewhere (14), and 2-ketogluconate by the Nelson procedure. The elution patterns are presented in Fig. 1. A considerable separation of these acids was effected since the pK of an α-keto acid is
lower than that of an α-hydroxy acid. The recoveries of gluconate and 2-ketogluconate were 102 and 99 per cent respectively. In this experiment 94 per cent of the gluconate was obtained containing 4 per cent of ketogluconate, and in turn 96 per cent of the ketogluconate contained 6.6 per cent of gluconate. Further purification could be effected by recycling, as was necessary when gluconate was present in excess of 2-ketogluconate.

![Graph](image)

**Fig. 2.** The substrate specificity of the enzyme system oxidizing 6-phosphogluconate. The reaction mixture contained 20 mg. of enzyme, 0.1 mg. of TPN, 55 per cent purity, and the substrate in 2 cc. of 0.0035 M phosphate, pH 6.6. One side bulb contained 0.6 mg. of phenazine methochloride in 0.2 cc., tipped at 0 time. The other side bulb contained 0.2 cc. of 0.5 N NaOH. The center well contained 0.3 cc. of TCA (50 per cent). Incubation was in air at 38° for 60 minutes. Substrates were glucose-6-phosphate 5.4 μM, fructose-6-phosphate 7.5 μM, phosphogluconate 11.3 μM, or 10 μM of glucose-1-phosphate, glucose-4-phosphate, glucose, ribose-3-phosphate, ribose-5-phosphate, xylose-5-phosphate, or glyceraldehyde-3-phosphate.

**Results**

*Properties of Enzyme System*—Only glucose-6-phosphate, fructose-6-phosphate, and phosphogluconate showed a significant oxygen consumption, as shown in Fig. 2. The end-points of the reaction for glucose-6-phosphate, fructose-6-phosphate, and 6-phosphogluconate were 1, 1, and 0.5 moles of O₂ per mole of substrate. The system had a very slight activity on ribose-5-phosphate and on xylose-5-phosphate. It was unable to oxidize glucose-1-phosphate, indicating the absence of active phospho-
glucomutase. Glyceraldehyde-3-phosphate, glucose-4-phosphate, ribose-3-phosphate, and glucose were also not oxidized.

It was shown that the enzyme preparation contained phosphohexoisomerase. Thus the oxidation of fructose-6-PO₄ is probably explained by its conversion to glucose-6-PO₄. On incubation of 928 γ of fructose-6-phosphate with 5 mg. of enzyme in 1 cc. of 0.003 M phosphate buffer at pH 7.0 in the absence of TPN and phenazine, 522 γ, or 44 per cent, disappeared in 17 minutes. Conversely, on incubation of 1500 γ of glucose-6-phosphate with 10 mg. of enzyme in 2 cc. of buffer, 443 γ of fructose-6-phosphate equivalent (28) appeared in that time.

The reaction did not proceed in the absence of TPN. When TPN of low purity was used, sodium cyanide increased the rate of oxygen consumption and, therefore, was usually added to the reaction mixture. Na-CN was added to a concentration of 0.01 M, but since no cyanide was added to the alkali for absorption of CO₂, the effective concentration of cyanide ion in the reaction mixture was much lower. When the effective concentration was brought to 0.01 M by addition of cyanide to the alkali, there was some inhibition of the reaction.

CO₂ was determined by acidification of the reaction mixtures in the Warburg vessels with trichloroacetic acid. The CO₂:O₂ ratios usually remained in the range of 0.90 to 1.40. The amount of CO₂ produced varied with the enzyme preparation, the concentration of phosphate, and the purity of the TPN.

Higher phosphate concentrations caused inhibition of activity, as shown in Table I. Dickens (8) found that the velocity of oxidation varied inversely with the phosphate concentration with phosphate concentrations greater than 0.05 M. Our results show a decrease in pentose production at phosphate concentrations of 0.01 M, although the oxygen consumption was not decreased. It may also be seen that pentose formation closely paralleled CO₂ production in this experiment. The TPN in this experiment was 55 per cent pure. In recent experiments with TPN of this purity, pentose formation has usually approached about 0.4 mole of pentose per mole of phosphogluconate.

Fig. 3 shows a typical experiment with our system. It can be seen that O₂ consumption, CO₂ production, and the appearance of pentose are not simply interrelated in this system. Also, the dependence of these functions on the presence of phenazine is demonstrated. It had not previously been shown that the increase in pentose in the system arose from the degradation of phosphogluconate. By using the Schneider procedure (32), the acid-soluble pentose was determined in the cold trichloroacetic acid filtrate, and the nucleic acid pentose was determined after extraction of the cold trichloroacetic acid precipitate with hot trichloroacetic
acid. As can be seen in Table II, the increased acid-soluble pentose did not arise from the nucleic acid fraction of the enzyme. It was also found

<table>
<thead>
<tr>
<th>Phosphate concentration</th>
<th>Oxygen uptake</th>
<th>CO₂ produced</th>
<th>Pentose produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00015</td>
<td>5.84</td>
<td>4.9</td>
<td>4.6</td>
</tr>
<tr>
<td>0.00015</td>
<td>6.45</td>
<td>4.5</td>
<td>5.3</td>
</tr>
<tr>
<td>0.0011</td>
<td>6.48</td>
<td>4.4</td>
<td>4.9</td>
</tr>
<tr>
<td>0.01</td>
<td>7.73</td>
<td>2.6</td>
<td>2.7</td>
</tr>
<tr>
<td>0.1</td>
<td>2.20</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>0.2</td>
<td>1.80</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>0.3</td>
<td>0.85</td>
<td>1.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The reaction mixture contained 17 mg. of enzyme, 12.8 μM of phosphogluconate, 0.05 mg. of TPN (55% per cent), and 0.6 mg. of phenazine methochloride in 2.4 cc. The well contained 0.3 cc. of TCA (50% per cent) and the side bulb 0.2 cc. of 0.5 N NaOH. The vessels were incubated in air at 38° for 90 minutes.

Fig. 3. Accumulation of pentose in phosphogluconate degradation. In each vessel were 2 cc. of enzyme mixture containing 10 mg. of enzyme, 2.34 mg. of TPN (3% per cent), 23.2 μM of phosphogluconate, and 0.06 cc. of 0.2 M NaCN. Phosphate buffer of pH was 0.003 M. Side arm 1 contained 0.6 mg. of phenazine methochloride in 0.2 cc. and was tipped at 0 time. Side arm 2 contained 0.2 cc. of 0.5 N NaOH. The well contained 0.2 cc. of 5% per cent trichloroacetic acid which was tipped at the time indicated.

that, when ribose 5-phosphate was incubated for 2 hours with an enzyme reaction mixture, there was no significant change in the pentose content of the mixture.
There was no increase in inorganic phosphate during the reaction. There was a shift of organic phosphate from the barium-insoluble phosphogluconate to barium-soluble compounds. All the increase in reducing sugars and pentose was found in the barium-soluble fractions.

E. coli As Source of Dehydrogenases—We have confirmed Racker’s (33) findings that E. coli contains enzymes active in this system. E. coli, strain B, was cultivated in a synthetic medium (34) containing glucose until the concentration was about 2 to 3 × 10⁹ bacteria per cc. The bacteria in 3 liters of the suspension were harvested by centrifugation and were washed. The wet bacterial pellet weighing 4.7 gm. was then ground for 3 minutes in a mortar with 2½ times its weight of alumina No. 301, kindly supplied by the Aluminum Corporation of America. The paste was extracted three times with 10 cc. of 0.01 m phosphate, pH 7.4, and centrifuged. The combined extracts were brought to a volume of 30 cc. and tested for dehydrogenases by the method of Burris. There was decolorization of methylene blue with phosphogluconate, ribose-5-phosphate, and glucose-6-phosphate. There was no activity with glucose, gluconic acid, or ribose, but addition of TPN caused decolorization with glucose. With 10-fold dilution of the extract, TPN was necessary for the reduction of methylene blue by glucose-6-phosphate and phosphogluconate. There was no diminution of activity of the extract after standing 5 days in the cold.

Fractionation and Analyses of Reaction Products—Preliminary character-

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Free pentose</th>
<th>Increase in mixture</th>
<th>Change in protein-bound pentose</th>
<th>Change in protein-bound pentose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - TPN</td>
<td>39 γ per cc.</td>
<td>59 γ per cc.</td>
<td>131 γ per cc.</td>
<td>-15 γ per cc.</td>
</tr>
<tr>
<td>0 + “</td>
<td>139 γ per cc.</td>
<td></td>
<td>116 γ per cc.</td>
<td>-18 γ per cc.</td>
</tr>
<tr>
<td>10</td>
<td>178 γ per cc.</td>
<td>39 γ per cc.</td>
<td>112 γ per cc.</td>
<td>-21 γ per cc.</td>
</tr>
<tr>
<td>20</td>
<td>181 γ per cc.</td>
<td>42 γ per cc.</td>
<td>110 γ per cc.</td>
<td>-4 γ per cc.</td>
</tr>
<tr>
<td>30</td>
<td>235 γ per cc.</td>
<td>96 γ per cc.</td>
<td>127 γ per cc.</td>
<td>-7 γ per cc.</td>
</tr>
<tr>
<td>60</td>
<td>288 γ per cc.</td>
<td>149 γ per cc.</td>
<td>124 γ per cc.</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>310 γ per cc.</td>
<td>171 γ per cc.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2 cc. of the enzyme mixture contained 10 mg. of enzyme D, 2.0 mg. of TPN (4 per cent purity), 3.6 mg. of phosphogluconate, 0.06 cc. of NaCN (1 per cent), 0.3 cc. of phosphate buffer, 0.02 m, pH 7.4, and 0.2 cc. of phenazine (0.3 per cent). The vessels were incubated in air at 38°. 0.2 cc. samples were removed and Bial determinations were run on cold and hot TCA extracts (32).
PHOSPHOGLUCONATE DEGRADATION

ization was first attempted by means of paper chromatography of the sugars freed with alkaline phosphatase. In a typical experiment a reaction mixture consisting of 8 mg. of enzyme, 0.5 mg. of TPN (about 4 per cent purity), 0.6 mg. of phenazine, and 5.5 mg. of phosphogluconate in 2 cc. of 0.003 M phosphate buffer at pH 7.0 was incubated at 37°. Aliquots were removed and treated with phosphatase as described previously. After centrifugation of the magnesium phosphate, the supernatant fluids were concentrated to 0.1 cc. and chromatographed on paper in collidine-water and phenol-water mixtures. The papers were sprayed with ammioniacal silver nitrate, silver nitrate containing 0.2 N sodium hydroxide, or m-phenylenediamine. Spots having $R_F$ values and colors characteristic of ribose, arabinose, and 2-ketogluconate were found.

It should be noted that 2-ketogluconic acid does not reduce ammoniacal AgNO$_3$ readily, nor does it react with m-phenylenediamine. However, it reacts on heating at 100° with Benedict's reagent or ammoniacal AgNO$_3$ containing 0.2 N NaOH. The latter reaction on paper is slow, and in air continues long after heating and gives rise to a purple spot. The detection of the 2-keto acid in the hydrolyzed reaction products appeared quite distinctive, since a compound appeared having these reactivities as well as the correct $R_F$ value.

In another type of procedure, the mixtures which had been incubated for various times were treated with barium hydroxide and barium acetate which precipitated all the barium-insoluble phosphates and the protein. The barium-soluble phosphates were precipitated with 4 volumes of alcohol. The alcohol precipitates were dissolved in water and the barium was removed with sodium sulfate. These solutions were dephosphorylated and chromatographed in a butanol-water mixture. The results are shown in Table III. In addition, spots were seen after irrigation with butanol-water at $R_F$ 0.39 to 0.41, $R_F$ values not ascribable to any compounds which we have investigated. The barium-insoluble precipitates were essentially free of reducing sugar.

The chromatograms of the reaction products treated in these various ways gave definite evidence that as the reaction proceeded the phosphogluconate disappeared, and that substances appeared having the chromatographic characteristics before and after hydrolysis of ribose phosphate, arabinose phosphate, sometimes 2-keto-6-phosphogluconate, sometimes glyceraldehyde phosphate, and also unidentified compounds. However, in order to characterize these products more definitely and to avoid salt effects on the chromatograms, more elaborate fractionations of larger amounts of material were desirable.

Large Scale Experiments—In Fig. 4 is presented a flow sheet describing the operations and analyses to which the reaction products were system-
D. B. M. SCOTT AND S. S. COHEN

519

The enzymatic reactions were run in Warburg vessels and the O2 consumption of the separate vessels was followed. Each vessel contained 15 mg. of enzyme, approximately 1 mg. of TPN, 3.6 per cent purity, 6 to 7 mg. of phosphogluconate, and 0.9 mg. of phenazine metho-

**TABLE III**

*Paper Chromatography with Butanol-Water Mixture*

<table>
<thead>
<tr>
<th>Standards</th>
<th>RF values</th>
<th>Hydrolyzed reaction products</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Ketogluconate</td>
<td>0.094</td>
<td>0.05-0.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.07-0.13</td>
<td>0.036-0.08</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.145</td>
<td>0.18-0.15</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.22</td>
<td>0.18- 0.25</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>0.49</td>
<td>0.50- 0.56</td>
</tr>
<tr>
<td>2-Ketogluconate</td>
<td>0.094</td>
<td>0.05-0.12</td>
</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Glyceraldehyde</td>
<td>0.49</td>
<td>0.50- 0.56</td>
</tr>
</tbody>
</table>

FRACTIONATION AND ANALYSIS OF PRODUCTS OF PHOSPHOGLUCONATE DEGRADATION

**FIG. 4.** Flow sheet.

chloride in 3.3 cc. of 0.003 M phosphate buffer at pH 7.0 or 7.4, and 0.003 M sodium cyanide. CO2 was absorbed by 0.3 cc. of 0.5 M sodium hydroxide in a side arm. The center well contained 0.3 cc. of 50 per cent trichloroacetic acid (TCA). At the desired level of O2 consumption, the reactions
PHOSPHOGLUCONATE DEGRADATION

were stopped with TCA and the CO₂ production was measured. The contents of nine to twelve vessels were pooled, chilled, centrifuged at 2000 r.p.m. for 10 minutes, and the sediment washed once with cold 5 per cent TCA. The extracts were analyzed for pentose, total P, and inorganic P, and, in some cases, P hydrolyzable in N HCl at 100° for 10 minutes.

To the TCA extracts were added NaOH to bring the pH to 8.8 and enough Ba⁺⁺ to precipitate the P if it had all been inorganic. 4 volumes of 95 per cent alcohol were added and the mixture permitted to stand overnight at 4°. The precipitate was removed by centrifugation, washed with 95 per cent ethanol and ether, and dried in vacuo over P₂O₅. The supernatant fluids were analyzed for P and pentose.

The dried Ba salts were worked into solution with dilute HCl, the pH not being permitted to fall below 2. A very small insoluble sediment was removed by centrifugation. Aliquots of the extract were analyzed for organic P, reducing sugar, and pentose, and on paper chromatograms in ethanol mixtures. The dried chromatograms were examined by spot tests or were cut into strips. The strips were eluted at 100° for 5 minutes with H₂O and the centrifuged eluates were analyzed for pentose, reducing sugar, P, and their ultraviolet absorption spectra in a Beckman spectrophotometer.

The bulk of the material was dephosphorylated with alkaline phosphatase and the sediment, containing only slight traces of reducing sugar, was discarded after centrifugation. The neutral supernatant fluid was analyzed for reducing sugar and pentose and slowly passed through the anion exchange resin. The acids were eluted from the column by 0.01 N HCl and the fractions analyzed for gluconate with gluconate-adapted bacteria (14) and reducing sugar, with 2-ketogluconate as a standard. The filtrate was analyzed for pentose and reducing sugar, and passed through the cation exchange resin. This acid filtrate was freed of chloride with Ag₂O, and finally the neutral sugar solution was concentrated in vacuo to a small volume. This neutral fraction was analyzed on paper chromatograms by spot tests and studies on eluates of cuts. It was also analyzed by pentose-adapted bacteria for ribose and D-arabinose (14).

In Tables IV and V are presented the analyses of three sets of reaction mixtures stopped at different levels of oxidation and subjected to the complete fractionation procedure in the flow sheet in Fig. 4. It may be noted that a large amount of pentose is derived from the TPN. The recovery of this TPN pentose was only about 75 per cent complete, due to its partial combination with the first TCA precipitate. Also, the recovery of the total P was only about 85 to 90 per cent complete in alcohol-precipitable Ba salts, the remainder being in the main recoverable in the
alcoholic supernatant fluid. Although this P was organic P, only about 10 per cent was found to be pentose phosphate. Inorganic phosphate was essentially unchanged, at least after TCA precipitation. Clearly, however, alcohol supernatant fluid. Although this P was organic P, only about 10 per cent was found to be pentose phosphate. Inorganic phosphate was essentially unchanged, at least after TCA precipitation. Clearly, however,

TABLE IV

Analyses at Levels of Phosphogluconate Degradation

<table>
<thead>
<tr>
<th></th>
<th>Before reaction</th>
<th>Intermediate oxidation, 40 min. at 37°</th>
<th>Complete oxidation, 120 min. at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated input</td>
<td>Found in barium ppt.</td>
<td>Calculated input</td>
</tr>
<tr>
<td>Inorganic P</td>
<td>2.87 mg.</td>
<td>2.67 mg.</td>
<td>3.40 mg.</td>
</tr>
<tr>
<td>Phosphogluconate P</td>
<td>7.04 mg.</td>
<td>8.44 mg.</td>
<td>7.30 mg.</td>
</tr>
<tr>
<td>TPN P</td>
<td>0.54 mg.</td>
<td>0.65 mg.</td>
<td>12.09 mg.</td>
</tr>
<tr>
<td>Organic P</td>
<td>7.58 mg.</td>
<td>5.32 mg.</td>
<td>12.45 mg.</td>
</tr>
<tr>
<td>Total P</td>
<td>10.44 mg.</td>
<td>7.99 mg.</td>
<td>12.19 mg.</td>
</tr>
<tr>
<td>Alcohol-soluble P*</td>
<td>3.69 mg.</td>
<td>2.74 mg.</td>
<td>4.43 mg.</td>
</tr>
</tbody>
</table>

* Found in alcohol supernatant.

TABLE V

Molar Relationships at Levels of Phosphogluconate Degradation

<table>
<thead>
<tr>
<th></th>
<th>Intermediate oxidation</th>
<th>Complete oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphogluconate input, μM</td>
<td>272</td>
<td>218</td>
</tr>
<tr>
<td>Oxygen consumption, μM</td>
<td>90</td>
<td>150</td>
</tr>
<tr>
<td>μM O₂</td>
<td>0.33</td>
<td>0.68</td>
</tr>
<tr>
<td>μM PG</td>
<td>1.0-1.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Increase in pentose, μM</td>
<td>32</td>
<td>51</td>
</tr>
<tr>
<td>&quot; reducing sugar, μM</td>
<td>78</td>
<td>113</td>
</tr>
<tr>
<td>μM pentose</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>μM O₂</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pentose was formed in the experiments as oxidation progressed and recovered in the main in Ba salts precipitable by 80 per cent ethanol. The yield of the total reducing substance isolated in Ba salts was about 0.5
mole per mole of substrate under conditions of complete oxidation. Pentose accounted for about half of this.

The three solutions of Ba salts were analyzed as follows: Three 0.5 cc. aliquots of each solution were applied to paper and irrigated in descending chromatograms with ethanol-acetate. One strip was sprayed with brom phenol blue, another with m-phenylenediamine, and the last was cut into parallel strips of 1 cm. width, eluted with water, and analyzed for pentose.

**Table VI**

*Chromatography in Ethanol-Acetate of Phosphates Isolated at Various Levels of Oxidation*

<table>
<thead>
<tr>
<th>Standards</th>
<th>Initial mixture</th>
<th>Intermediate</th>
<th>Complete oxidation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_f )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acids</td>
<td>PD*</td>
<td>Acids</td>
<td>PD*</td>
</tr>
<tr>
<td>Ribose-5-phosphate</td>
<td>0.50</td>
<td>0.50</td>
<td>0.52</td>
<td>0.44-0.60</td>
</tr>
<tr>
<td></td>
<td>0.31-47</td>
<td></td>
<td>0.31-47</td>
<td></td>
</tr>
<tr>
<td>Arabinose-5-phosphate</td>
<td>0.56</td>
<td>0.56</td>
<td>0.58</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>0.31-47</td>
<td></td>
<td>0.31-47</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>0.73</td>
<td>0.71</td>
<td>0.72</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>0.83</td>
<td></td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>0.31-47</td>
<td></td>
<td>0.31-47</td>
<td></td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>0.89</td>
<td>0.86</td>
<td>0.89</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>0.31-47</td>
<td></td>
<td>0.31-47</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction with m-phenylenediamine.

In Table VI are presented the results of these tests. By qualitative examination only two components could be identified. The substance of \( R_f \) 0.86 to 0.89 was an acid which did not react with m-phenylenediamine dihydrochloride (PD) and decreased in amount very markedly as the oxidation progressed. This \( R_f \) value, as well as other characteristics, was that of 6-phosphogluconate. The substance which reacted immediately with PD with a fluorescent front of \( R_f \) 0.84 appeared late in the reaction. These characteristics would appear to describe glyceraldehyde-3-phosphate. The reaction products also appeared to accumulate in the
pentose phosphate positions. An analysis of the pentose content of the strips showed that the pentose of the reaction products was indeed at the pentose phosphate positions, i.e. at $R_F$ 0.5 to 0.6 (13). This was quite marked in the completely oxidized material.

In Fig. 5 is presented a chromatogram of the intermediate oxidation product in ethanol-boric acid. 1 cc. of solution was applied containing 473 $\gamma$ of reducing sugar, 318 $\gamma$ of apparent pentose, and 475 $\gamma$ of phosphate. In the summated 5 cc. eluates of the cuts, 447 $\gamma$ of reducing sugar were recovered, 329 $\gamma$ of apparent pentose, and 439 $\gamma$ of phosphate. The distribution of pentose and reducing sugar was in the main slightly in advance of the arabinose-5-phosphate position. Although the reducing sugar to pentose ratio in the peak symmetrical between $R_F$ 0.26 and 0.39 was about 2:1, the ratio at $R_F$ 0.22 to 0.26 was 1:1, as would be given by arabinose-5-phosphate. The phosphate analyses of these eluates revealed a rough correspondence to the pentose curve with an additional sharp peak at $R_F$ 0.74 to 0.82, a position free of reducing groups or pentose.

The Bial-reactive material $R_F$ 0.26 to 0.39 was not a typical pentose, since the color developed in 40 minutes in the orcinol-FeCl$_3$ reaction showed an additional peak at 465 m$\mu$ as well as the usual peak at 660 m$\mu$. These spectra are presented in Fig. 6. The ultraviolet absorption spectrum of the eluate was not distinctive. This substance represented over one-third
of the apparent pentose and one-half of the total reducing sugar of these products.

Properties of Dephosphorylated Neutral Sugars—The remainder of the Ba salts was dephosphorylated and separated on the anion exchange column. The neutral fractions were desalted and evaporated. The material available for study contained 1.30 mg. of reducing sugar and 0.48 mg. of pentose in 4.4 cc. for the starting material, 7.6 mg. of reducing sugar and 3.6 mg. of pentose in 4.6 cc. for the intermediate oxidation product, and 6.0 mg. of pentose in 3.0 cc. for the complete oxidation product. These represented losses of pentose from the amounts present in the various reaction mixtures through the steps after isolation of Ba salts of 0.83, 0.84, and 0.75 mg. of pentose respectively. This suggested the loss of some common source of pentose present in the starting mixture, such as one of the TPN nucleosides freed after phosphatase treatment but absorbed by one of the resins.

Collidine chromatograms of some of these materials are presented in Fig. 7. Pentose was clearly found at the arabinose and ribose positions in the intermediate and complete oxidation products. Analysis of reducing sugar in the strips, as shown in Fig. 7, revealed reducing groups only at the ribose and arabinose positions in the intermediate oxidation product and at the glucose position in the starting material. This last was due to a small amount of glucose-6-phosphate in this phosphogluconate substrate.
The oxidation product was also chromatographed in a butanol-H$_2$O mixture. On treatment with $m$-phenylenediamine weak spots were revealed at $R_f$ 0.12 and 0.18, which are those of arabinose and ribose, respectively. Pentose analysis of the cut chromatograms confirmed these qualitative tests, pentose recovery in the eluates approaching 100 per cent.

Paper was treated with 0.3 cc. aliquots of the sugar solutions containing 234 $\gamma$ and 600 $\gamma$ of pentose from the intermediate and complete oxidation products, respectively. The papers were irrigated with collidine. The thoroughly dried papers were cut between $R_f$ 0.45 and 0.65 and eluted with 5 cc. aliquots of H$_2$O. These solutions were then analyzed by adapted bacteria for ribose and $d$-arabinose. It was found that 17 to 21 per cent of the original applied pentose recoverable in these eluates was fermented by ribose-adapted bacteria and 8.5 to 14 per cent was fermented by the arabinose-adapted bacteria.

Direct analyses of larger aliquots were then made by adapted bacteria. In 0.5 cc. aliquots of the neutral fraction of the complete oxidation product were found 251 $\gamma$ of ribose or 25 per cent of the total pentose. In 1.5 cc. aliquots of the corresponding fraction of the intermediate oxidation product were found 154 $\gamma$ of ribose and 130 $\gamma$ of arabinose or 13 and 11

![Diagram showing the distribution of reducing sugar in two of the paper chromatograms developed in $s$-collidine-H$_2$O. The products described in Table IV are herein analyzed. The papers were irrigated for about 25 cm., cut, and dried. 1 cm. strips were cut and eluted with 5 cc. of H$_2$O.]
per cent of the total pentose respectively. These figures are considered more reliable than those reported for the eluted chromatogram.

Distribution of Acids from Ion Exchange Studies—The acids of the various dephosphorylated fractions adsorbed on the anion exchange resins were eluted at pH 2 and collected in small separate fractions. The fractions were analyzed for gluconate with adapted bacteria and for reducing sugar. The distribution of these substances in the eluates is presented in Fig. 8. Three points deserve comment: (1) gluconic acid disappeared as oxidation progressed; (2) only a small amount of keto-onic acid was present as such in these materials and apparently existed in the phosphogluconate prepared by the Br₂ oxidation of glucose-6-phosphate; (3) a new, very weak acid or otherwise adsorbed material appeared among the degradation products. This material was not isoascorbate, as estimated by means of the 2:6-dichlorophenol-indophenol reaction, and indeed gave a strong Bial reaction comparable in the quality of color to that of the major unknown Bial-reactive phosphate described previously. The elution pattern of isoascorbate was found to be comparable to that of gluconate and 5-ketogluconate when absorbed in the presence of 2-keto-
DISCUSSION

It has been shown by paper chromatography of the intact phosphates and the isolation of gluconate freed by hydrolysis that, as oxidation progressed, 6-phosphogluconate disappeared; i.e., the O\textsubscript{2} consumption of about 0.5 mole of substrate did not merely reflect a series of oxidations of a portion of the phosphogluconate but oxidation of all of this substrate. This fact, taken with the R. Q. of this system of about 1, means that a single oxidative decarboxylation of 6-phosphogluconate to form pentose phosphate cannot account for the observed phenomena. Such an oxidative decarboxylation would yield an R. Q. of 2. Two other possibilities exist: (1) there is another oxidation of 6-phosphogluconate which does not lead to decarboxylation and pentose, or (2) there are two successive reactions, i.e. oxidation and decarboxylation leading to pentose. In the first instance one might conceive of the formation of 5-keto-6-phosphogluconate which would be expected to be cleaved by aldolase to 1 mole of triose phosphate and tartronic acid semialdehyde. In the second, the formation of 2-keto-6-phosphogluconate is a possibility. However, insufficient keto-hexonic acid has been obtained to account satisfactorily for the R. Q. and pentose formed. The apparent accumulation in our system of glyceraldehyde-3-phosphate may be related to these questions, since we have not observed a degradation of ribose-5-phosphate in our system.

Since ribose was produced in the system, an inversion has occurred at the C\textsubscript{2} hydroxyl of the pentose. A simple degradation should yield D-arabinose. It is possible that the inversion may occur either around the oxidation of the 6-carbon compound or after pentose phosphate formation. One mechanism would suggest the formation of a 2,3-enediol which would be decarboxylated to form a 1,2-enediol pentose phosphate. The parent enediol would probably not be a diphospho derivative since the oxidation proceeded in the absence of inorganic phosphate. This intermediate would be theoretically capable of forming ribose-5-phosphate, arabinose-5-phosphate, and araboketose-5-phosphate. Still another mechanism might suggest the direct formation of D-arabinose-5-phosphate or araboketose phosphate by oxidative decarboxylation. These substances might then be transformed by some series of epimerization steps to ribose-5-phosphate.

The compounds found suggest the formation of some of these possible intermediates. First, a Bial-reactive phosphate was obtained in high yield.
which moved in ethanol-boric acid. This compound had an $R_F$ value slightly greater than arabinose-5-phosphate and developed a marked peak at 465 μm in the Bial reaction unlike arabinose-5-phosphate. We have observed that ascorbic and D-isoascorbic acids give such a peak, in contrast to ribose, arabinose, xylose, and their phosphates. Derivatives of certain phosphorylated ascorbates or xyloketose might be expected to migrate in ethanol-boric acid, since they would not contain contiguous cis hydroxyls. Araboketose-5-phosphate would not be expected to move in this mixture.

Finally we have noted in dephosphorylated reaction products the presence of a weakly acidic Bial-reactive substance which was very rapidly eluted from the anion exchange column. This compound was less acidic than the isoascorbates and possibly might be a 1,2-enediol pentose. The quality of its Bial reaction further indicated similarity to the Bial-reactive phosphorylated constituent migrating in ethanol-boric acid. Although a 1,2-enediol pentose phosphate would have contiguous cis hydroxyls at the $C_3$ and $C_4$ positions, it is conceivable that the phosphate or the enediol might interfere with the formation of a boric acid complex.

Thus the study has raised many new problems and has pointed to the possible existence of a number of hitherto undescribed compounds and reactions involved in the degradation of 6-phosphogluconate. Their existence rests on several lines of evidence: (1) the finding of materials behaving like ribose-5-phosphate and (2) the finding that the amounts found of ribose phosphate or arabinose-phosphate accounted for only a small part of the Bial-reactive material which accumulated, or the pentose which could have accumulated as a result of the observed oxidation and decarboxylation. The purification of the enzymes involved should assist in the elucidation of some of the individual reaction steps.

**SUMMARY**

An enzyme preparation oxidizing and degrading 6-phosphogluconate comparable to the system described by Dickens has been isolated from yeast. A system oxidizing 6-phosphogluconate in the presence of TPN has also been demonstrated in *E. coli* extracts. The specificity of the yeast preparation and its dependence on TPN and the carrier phenazine methochloride have been studied. The $O_2$ consumption, $CO_2$ production, and pentose production in a complete system have been measured, and it has been demonstrated that the pentose formed is derived from the 6-phosphogluconate. In this system 0.5 mole of $O_2$ was consumed per mole of 6-phosphogluconate; the R. Q. was close to 1, and 0.25 to 0.40 mole of pentose was produced.

The products of the enzymatic degradation of 6-phosphogluconate have

1 Cohen, S. S., and Arbogast, R., unpublished data.
been isolated, fractionated, and analyzed. Analysis by paper chromatography of the dephosphorylated products has revealed substances behaving like ribose and D-arabinose. Analysis of the phosphates by this technique has revealed the disappearance of 6-phosphogluconate and the appearance of substances behaving like ribose-5-phosphate and arabinose-5-phosphate and a new unknown Bial-reactive phosphate. This latter substance had properties not ascribable to any known substance and comprised about one-half of the apparent pentose formed. A compound reacting like glyceraldehyde-3-phosphate was also observed to accumulate.

Adaptive enzymatic analysis of the isolated pentoses revealed D-ribose and D-arabinose in amounts of about 25 and 10 per cent respectively of the total apparent pentose present, in confirmation of the chromatographic analyses.

The ion exchange analysis of the acidic products after removal of the phosphate revealed the disappearance of 6-phosphogluconate as the reaction progressed, with slight, if any, accumulation of 2-ketogluconate. A small amount of slightly acidic Bial-reactive material was found only in the reaction products. It was not an ascorbate and it is thought that it might be a pentose enediol. The significance of these reaction products has been discussed.

BIBLIOGRAPHY

PHOSPHOGLUCONATE DEGRADATION

ENZYMATIC FORMATION OF PENTOSE PHOSPHATE FROM 6-PHOSPHOGLUCONATE

Dwight B. McNair Scott and Seymour S. Cohen