THE METABOLISM OF HEXOSE AND PENTOSE PHOSPHATES IN HIGHER PLANTS*

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(Received for publication, October 1, 1952)

The pathway of carbohydrate degradation, which begins with the action of G-6-P dehydrogenase¹ on G-6-P, has been studied by a number of workers (1–9). Their results have shown the operation of this scheme in a variety of tissues (yeast, red blood cells, and mammalian liver). The existence of G-6-P dehydrogenase in various seeds (10) suggests the occurrence of this system in higher plants. The present paper reports the results of an investigation of this system in extracts of spinach and pea leaves. The sequence of reactions beginning with G-6-P is as follows: The hexose phosphate is dehydrogenated by an apparently conventional TPN enzyme to 6-PG and then oxidatively decarboxylated to pentose phosphate by a TPN 6-PG dehydrogenase. The resulting pentose phosphate, a mixture of ribose and ribulose cells, is then converted, in part, to sedoheptulose phosphate. Balance experiments carried out with R-5-P indicate that 2 moles of R-5-P give rise to 1 mole of triose phosphate and 1 mole of heptulose phosphate. Time lags in attaining the proper ratio indicate the transient formation of a non-ribose, non-heptulose compound that may be a precursor to the heptulose phosphate; however, efforts to show the existence of glycolaldehyde were negative. The heptulose phosphate itself is in large part transformed to a mixture of G-6-P and F-6-P. In the presence of TPN (DPN is considerably less effective) the triose phosphate is transformed to pyruvic acid and inorganic phosphate. Unlike its better known counterpart in the Embden-Meyerhof scheme, this reaction does not require ADP, nor is its action accelerated by arsenate in the absence of ADP.

* Report of work supported in part by the Herman G. Frasch Foundation for Agricultural Chemistry.

¹ The following abbreviations will be used: ADP, adenosinediphosphate; DPN, diphosphopyridine nucleotide; FDP, fructose-1,6-diphosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; GSSG, oxidized glutathione; GSH, reduced glutathione; 6-PG, 6-phosphogluconic acid; R-5-P, ribose-5-phosphate; TCA, trichloroacetic acid; Tham, tris(hydroxymethyl)aminomethane; TPN, triphosphopyridine nucleotide.
Materials and Methods

All of the reagents were of commercial origin, unless otherwise noted. R-5-P was prepared by the acid hydrolysis of adenosine-5-phosphate (11). Ribulose was prepared according to Glatthaar and Reichstein (12). The contaminating aldose was oxidized to aldonic acid with bromine and removed with Permutit S.² Dr. N. K. Richtmyer of the National Institutes of Health kindly supplied us with the various heptuloses.

The spinach acetone powder was made by grinding frozen leaves with twice their weight of acetone in a blender at −20°. The residue was reextracted, freed of residual acetone, and stored at −20°. The enzyme extract was made by suspending the acetone powder in 8 times its weight of distilled water and extracting for 15 minutes. All operations were conducted at 2°. Insoluble material was removed by centrifugation at 19,000 × g for 20 minutes (Stage I). The resultant solution was made 0.7 saturated with respect to ammonium sulfate and the pH adjusted to 7 with strong KOH. The precipitate formed after 15 minutes was collected by centrifugation, dissolved in a small quantity of water, and dialyzed for 4 hours against two changes of distilled water. Such preparations have been stored at −20° for 7 months without loss of the activities studied (Stage II).

The pea leaf enzyme consisted of a water extract of an acetone powder of pea leaves, prepared as described above, and then dialyzed for 3 hours against two changes of distilled water. Pentose determinations were made by the method of Mejbaum (13). Pentose values are corrected for heptose interference. Heptulose was determined by a modification of the Dische, Shettles, and Osnos CyRI procedure for hexose (14). The density of the resultant solutions was measured at 505 mù after 18 hours. The determinations are possible in the presence of glucose, fructose, G-6-P, F-6-P, FDP, R-5-P, ribose, ribulose, and dihydroxyacetone phosphate. The method does not permit distinction between mannoheptulose, sedoheptulose, and glucoheptulose. FDP and triose phosphate were determined as alkali-hydrolyzable phosphorus after incubation of the solutions to be tested with muscle aldolase and cyanide (15).

Pyruvate was determined colorimetrically directly upon its isolation as the 2,4-dinitrophenylhydrazone from the reaction mixture (16). In some cases the determination of 2,4-dinitrophenylhydrazone was made after its chromatographic isolation on Whatman No. 1 paper, with an aqueous 1:1 mixture of 1.5 N NH₄OH and saturated (NH₄)₂CO₃ as solvent.³ Pyruvate was determined colorimetrically directly upon its isolation as the 2,4-dinitrophenylhydrazone from the reaction mixture (16). In some cases the determination of 2,4-dinitrophenylhydrazone was made after its chromatographic isolation on Whatman No. 1 paper, with an aqueous 1:1 mixture of 1.5 N NH₄OH and saturated (NH₄)₂CO₃ as solvent.³ Pyruvate was determined colorimetrically directly upon its isolation as the 2,4-dinitrophenylhydrazone from the reaction mixture (16). 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ruvic acid gives two spots with this solvent ($R_f$ 0.27 and 0.49), owing to the existence of the syn- and antihydrazones. The optical density of the solutions resulting from the elution of the hydrazones into ethanol was determined at 356 nm.

Crystalline DPN glyceraldehyde phosphate dehydrogenase was prepared by the method of Cori, Slein, and Cori (17). Chromatography of the phosphorylated compounds was carried out by the procedure of Bandurski and Axelrod (18). The free sugars were chromatographed by the method of Partridge (19), with the butanol-ethanol-water mixture (10:1:2) of Williams and Bevenue (20) as solvent. GSII was determined by the method of Kassell and Brand (21).

**Results**

*G-6-P Dehydrogenase and 6-PG Dehydrogenase—*These enzymes, both TPN-specific, were readily demonstrated in spinach and pea leaf preparations by measuring the reduction of TPN spectrophotometrically in the presence of the appropriate substrate. For all concentrations of substrates tested, the rate of reaction with 6-PG exceeded the rate with G-6-P. Since diaphorase was present in these preparations, these reactions could be followed manometrically by adding methylene blue or colorimetrically with 2,6-dichlorophenol indophenol (Fig. 1). The presence of GSSG reductase permitted the reactions to be followed by measuring the GSH formed.

Conn and Vennesland, studying G-6-P dehydrogenase in wheat germ (22), found that CO$_2$ was produced and concluded that this was due to phosphogluconate dehydrogenase activity. R-5-P was identified among the products resulting from the action of the pea leaf enzyme on 6-PG, by cochromatography of the C$^{14}$-labeled product with authentic R-5-P. The experiment was performed as follows: A solution of 19 $\mu$m of 6-PG and 19 $\mu$m of GSSG, made up to a volume of 1.6 ml. and adjusted to pH 7.5, was introduced into a Warburg vessel containing 0.3 ml. of Tham buffer, pH 7.1, 0.15 M, 0.1 ml. of solution containing 130 $\gamma$ of TPN, and 1.0 ml. of the pea leaf enzyme. The radioactive 6-PG was made by phosphorylating uniformly labeled glucose with wheat germ particulate hexokinase (23) and oxidizing the product with Br$_2$. The center well contained 0.2 ml. of 20 per cent NaOH. The side arm contained 0.5 ml. of 2 $\times$ H$_2$SO$_4$, which was tipped into the mixture after the reaction had run for 3000 seconds at 37$^\circ$. Following addition of the acid, the vessel was shaken an additional 15 minutes to permit absorption of all the CO$_2$ liberated. The contents of the center well were then transferred to a wet combustion apparatus and the

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4 A number of plant materials were found to contain 6-phosphogluconate dehydrogenase, which has been the subject of a more extensive investigation by Dr. Jan Links at the California Institute of Technology.
radioactive carbonate collected as BaCO₃. The counts removed from the center well, 8260, represented a 68 per cent recovery of one-sixth of the activity in the original 6-PG. The enzyme reaction mixture was fractionated to obtain the "barium-soluble alcohol-insoluble" phosphate esters (24) which were freed of barium with Dowex 50 and cochromatographed in two dimensions with authentic R-5-P. Juxtaposition of the main spot on the radioautograph with the blue spot shown by the authentic R-5-P after spraying established the probable identity of R-5-P as a reaction product.

![Graph](http://www.jbc.org/)

**Fig. 1.** Reduction of 2,6-dichlorophenol indophenol, coupled to TPN oxidation of G-6-P and 6-PG by pea leaf enzyme. Reaction conditions, 0.5 ml. of G-6-P (or 6-PG), 0.0025 M, 0.5 ml. of potassium phosphate buffer, 0.2 M, pH 6.5, 0.5 ml. of 2,6-dichlorophenol indophenol (40 μg), 1.0 ml. of TPN, (80 μg, 65 per cent purity), 0.5 ml. of enzyme, Stage I, and 3.0 ml. of H₂O. Reaction temperature 25°C. Absorption measured in an Evelyn calorimeter with 540 nm filter. Reduction in absence of substrate was negligible.

**Formation of Other Sugars from R-5-P**—When spinach enzyme was permitted to act on R-5-P, a decrease in Mejbaum-reactive material occurred. Chromatographic (Table I) and chemical analysis revealed the formation of a number of sugars as follows:

When spinach enzyme acted upon R-5-P in the presence of bisulfite, alkali-labile phosphate, indicative of triose phosphate, was formed (Fig. 2). Similar results were obtained in the absence of bisulfite. Dische (28) had shown in 1938 that erythrocyte hemolysates formed triose phosphate from ribose derivatives. The alkali-labile phosphate was determined as the total of alkali-labile phosphate and FDP phosphate. The molar ratio of pentose disappearing to alkali-labile phosphate appearing was somewhat higher than the expected value of 2.0. The high ratio may be, in part, explained by the observation that triose phosphate disappears on prolonged incubation.
The anomalous disappearance of the pentose discussed below also tends to make the ratio too high. However, when the triose phosphate was oxidized as it was formed (see section below, "Triose phosphate oxidation"), the calculation, on the basis of GSSG reduced, showed a ratio more nearly 2.0.

Hypoiode oxidation established that the triose was almost entirely dihydroxyacetone phosphate, but, since triose phosphate isomerase was present in the mixture, the triose would be expected to occur predominantly in the ketose form. The inclusion of bisulfite as a carbonyl-trapping agent did not alter the results. Since bisulfite is somewhat less than 80 per cent as efficient as cyanide as a trapping reagent, when used in the

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>( R_F - F ) = \frac{\text{mobility of carbohydrate}}{\text{mobility of fructose}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.72, 0.72</td>
</tr>
<tr>
<td>Sedoheptulose†</td>
<td>0.83</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.00</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.10, 1.04</td>
</tr>
<tr>
<td>Ribose</td>
<td>1.48</td>
</tr>
<tr>
<td>&quot;Ribose X&quot;</td>
<td>1.47, 1.47, 1.48</td>
</tr>
<tr>
<td>Ribulose</td>
<td>1.65</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>2.7‡</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>2.9‡</td>
</tr>
<tr>
<td>&quot;Fast spot&quot;</td>
<td>2.54</td>
</tr>
</tbody>
</table>

* The position of the "unknown" alongside the authentic sugars is compatible with their behavior with the aniline-trichloroacetate spray (25), the alkaline AgNO₃ spray (26), and the orcinol spray (27), as well as with their mobilities.

† The putative sedoheptulose had the same mobility as an authentic sample in a separate experiment, but was slightly faster than mannose and glucose-6-phosphate. No \( R_F - F \) values were obtained.

‡ These values are the unpublished data of A. Bevenue obtained in separate experiments and may not be strictly applicable.
aldolase assay, it is conceivable that ketotriose could be formed from aldotriose in the presence of bisulfite. Cyanide could not be used because it combined readily with the R-5-P. Further identification of the product as triose phosphate was provided by its oxidation by crystalline DPN glyceraldehyde phosphate dehydrogenase of rabbit muscle (Fig. 3) and its conversion to pyruvic acid by the TPN dehydrogenase of the spinach extract as discussed below. When the phosphate esters arising from the action of the spinach enzyme on R-5-P were dephosphorylated and chromatographed, a fast moving substance appeared which possibly corresponded to dihydroxyacetone (Table I).

Ribulose was located on the chromatogram by virtue of its unique behavior with the orcinol (heptulose) spray of Klevstrand and Nordal (27). After the chromatogram is sprayed and then heated for 1 to 2 minutes at 100°, this sugar forms a brightly white yellow fluorescent area as viewed under long ultraviolet light. On heating for another 15 to 20 minutes the area assumes a pink to violet color, which becomes deeper as the chromatogram is stored.

Heptulose, as its phosphate, was first detected by the use of the Klevstrand-Nordal orcinol spray on two-dimensional chromatograms of the reaction products. The chromatograms obtained with the dephosphorylated sugars also showed the presence of material giving the characteristic blue color of heptulose with the orcinol spray. The substance giving the
blue color moved with the same \( R_f \) as did authentic sedoheptulose. The anhydride of sedoheptulose, sedoheptulosan, does not respond to the orcinol spray. An equilibrium mixture consisting of 80 per cent of the anhydride and 20 per cent sedoheptulose results upon treating the sugar or anhydride with HCl (29). Chromatography of the unknown sugars before and after acid treatment showed a marked decrease in orcinol-

![Graph](attachment:graph.png)

**FIG. 3.** Reduction of DPN in the presence of rabbit muscle triose phosphate dehydrogenase by glyceraldehyde phosphate formed from R-5-P by the action of spinach enzyme. Reaction conditions, 1.0 ml. of pyrophosphate, 0.06 M, pH 8.5, 0.2 ml. of potassium arsenate, 0.1 M, pH 8.5, 0.1 ml. of NaF, 1 M, 0.5 ml. of DPN (500 \( \gamma \), 90 per cent purity), 0.1 ml. of a suspension of twice crystallized rabbit muscle triose phosphate dehydrogenase, 0.5 ml. of R-5-P, 0.008 M, the indicated volume of spinach enzyme (Stage II), and H\(_2\)O to make 3.3 ml. (On the lowest curve, the enzyme was withheld to the time indicated by the arrow.) Room temperature, 25\(^\circ\); temperature of the cuvette compartment not controlled. Wave-length, 340 \( \text{m}_{\mu}\). Optical path 1.00 cm. Rabbit muscle triose phosphate dehydrogenase was present in great excess over that required for the maximal rate of reduction.

reacting material and thus provided evidence that heptulose was present. During the course of our work, Horecker and Smyrniotis (6) announced the formation of sedoheptulose phosphate by the action of rat liver acetone powder extracts on pentose phosphate. The Dische cysteine-sulfuric acid test not only served for the quantitative measurement of heptulose, but the absorption spectrum helped to establish the identity of the sugar as a heptulose. Results obtained with this method applied directly to the reaction mixture agreed well with the heptulose values obtained after quantitative chromatography and elution of this heptulose, as the free
sugar, thus indicating the absence of interfering substances and the equivalence of the free sugar and the phosphate.

Fig. 4 shows the change in the absorption spectrum obtained in this test after increasing periods of enzyme action on R-5-P. It will be noted that heptulose decreases with prolonged incubation. For comparison the absorption spectra of G-6-P and sedoheptulosan are also shown. The effect of varying the concentration of R-5-P on the rate of ribose disappearance and heptulose formation is shown in Table II. The molar ratio of twice the increase in heptulose to the decrease in ribose approaches 1 as the reaction approaches completion. It is of course obvious that prolonged reaction times would cause the disappearance of heptulose and result in an apparent decrease in this ratio.

The formation of hexose was demonstrated by application of the modified Dische test (Fig. 4) as well as by the appearance of fructose and glucose
on the chromatograms of the dephosphorylated reaction products (Table I). G-6-P was also identified by its reaction with TPN and a purified preparation of G-6-P dehydrogenase.

**Stoichiometry of Reaction**—In the modified Dische test, G-6-P and F-6-P are equivalent on a molar basis. Calculations based on the data presented in Fig. 4 indicate a good recovery of the R-5-P consumed in the reaction (Table III) if one assumes that the ratio of R-5-P disappearance to triose phosphate formation is 2. Such a ratio is approached when the triose phosphate is oxidized as formed.

**Action of Various Inhibitors and Activators on Ribolytic Reaction**—The effect of Mg$$^{++}$$, Mn$$^{++}$$, and F$$^{-}$$ concentrations of 0.01, 0.01, and 0.1 M, respectively, on the rate of R-5-P transformation to heptulose was almost negligible. Each reaction tube contained in a total volume of 1.0 ml., 0.05 ml. of spinach enzyme Stage II, 2.89 μM of R-5-P, and 75 μM of Tham-HCl, pH 7.5. Arsenite at 5 × 10⁻³ M also was without effect. The reaction times were 30 and 60 minutes, at 37°. When the buffer was replaced by arsenate, pH 8.5, 0.01 M, or Veronal, pH 8.5, 0.014 M, there was

**TABLE II**

Molar Equivalence of Ribose Destruction and Heptulose Formation

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Ribose destroyed</th>
<th>Moles heptulose formed × 2 Moles ribose destroyed</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>0.57</td>
</tr>
<tr>
<td>16</td>
<td>41</td>
<td>0.85</td>
</tr>
<tr>
<td>64</td>
<td>78</td>
<td>0.93</td>
</tr>
</tbody>
</table>

In each 1.0 ml. of reaction mixture 0.2 ml. of Tham-HCl, 0.15 M, pH 7.5, 0.05 ml. of spinach enzyme, Stage II, and 2.53 μM of R-5-P were present. Temperature, 37°.

**TABLE III**

Relation of Heptulose and Hexose Formation to Pentose Disappearance

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Heptulose</th>
<th>Hexose</th>
<th>Triose*</th>
<th>Ribose</th>
<th>Heptulose + hexose</th>
<th>Heptulose + triose</th>
<th>7/10 × pentose</th>
<th>Heptulose + hexose</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>24.4</td>
<td>4.0</td>
<td>13.2</td>
<td>-44.0</td>
<td>41.6</td>
<td>-30.8</td>
<td>28.4</td>
<td></td>
</tr>
<tr>
<td>1275</td>
<td>15.5</td>
<td>21.8</td>
<td>15.8</td>
<td>-52.5</td>
<td>53.1</td>
<td>-36.8</td>
<td>37.3</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated as 3/10 × pentose (on the assumption that 0.5 mole of triose phosphate is formed per mole of pentose phosphate disappearing).
no significant effect, although phosphate, pH 8.5, 0.01 \text{ M}, caused a 20 to 30 per cent reduction in the rate of ribose loss and heptulose appearance. At 10^{-4} \text{ M}, p$\text{-}$chloromercuribenzoate inhibited the disappearance of ribose by 58 per cent under conditions approximately as described above. The inhibition of heptulose formation was even greater in similar experiments, with the result that the molar ratio (2 × heptulose)/(ribose) was less than 0.5. Ethylenediamine tetraacetate was completely without effect on the reaction at pH 7.5 (Tham buffer), whereas at pH 8.5 (Veronal buffer) it caused an 85 per cent decrease in heptulose formation and a 40 to 50 per cent decrease in ribulose formation. Borate buffer at pH 8.5 at a concentration of 0.05 \text{ M} and \text{NH}_2\text{OH} at pH 7.5, 0.2 \text{ M}, completely inhibited the disappearance of Mejbaum-reactive material when R-5-P was present in a concentration of 6 \times 10^{-3} \text{ M}. No heptulose was formed.

Utilization of Ribulose-5-phosphate—Thanks to the kindness of Dr. B. L. Horecker in supplying us with some ribulose-5-phosphate, it was possible to compare this substrate with an approximately equivalent concentration of R-5-P as a heptulose precursor. The initial rate of heptulose formation was significantly more rapid with the ribulose phosphate (Table IV).

Triose Phosphate Oxidation—Although TPN was not required for the operation of the R-5-P-destroying system, it was found that added TPN was reduced. Fig. 5 illustrates the reduction of 2,6-dichlorophenol indophenol when added to the TPN-containing system. The appreciable reduction of dye in the absence of added ribose phosphate is presumably due to some endogenous substrate, since this activity was lost during storage of the enzyme for a short time at room temperature. As indicated below, the observed reduction of TPN is probably not due to a pentose dehydrogenase but rather a dehydrogenase acting on triose phosphate formed from pentose phosphate.

To permit further investigation of the dehydrogenase it was necessary to supply some means of reoxidizing reduced TPN. This was accomplished by the addition of GSSG, since glutathione reductase was present. No \text{CO}_2 was formed by the action of the enzyme system on R-5-P in the presence of GSSG and TPN. 2.3 \mu M of acid were formed from 5.5 \mu M of R-5-P, as demonstrated manometrically by the liberation of \text{CO}_2 from \text{HCO}_3^- buffer.

An earlier observation (30) that TPN was reduced by leaf enzyme in the presence of FDP suggested that triose phosphate might be the dehydrogenase substrate. That this is probably the case is shown by the fact

\text{Dr. Martin Gibbs has recently reported the existence of both DPN and TPN triose phosphate dehydrogenase in the green plant (Thirty-sixth annual meeting of the Federation of American Societies for Experimental Biology, April, 1952, New York).}
TABLE IV

Production of Heptulose from Ribose-5-phosphate and Ribulose-5-phosphate

<table>
<thead>
<tr>
<th>Time</th>
<th>Ribose-5-phosphate*</th>
<th>Ribulose-5-phosphate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>20</td>
<td>0.29</td>
<td>0.48</td>
</tr>
<tr>
<td>40</td>
<td>0.56</td>
<td>0.67</td>
</tr>
<tr>
<td>80</td>
<td>0.92</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* The reaction mixture contained in 1.0 ml., 3.5 μM of R-5-P, 0.2 ml. of Tham-HCl, 0.15 M, pH 7.5, and 0.1 ml. of 1:4 spinach enzyme, Stage II. Temperature, 37°.
† Same as above but with 4.1 μM of ribulose-5-phosphate instead of R-5-P.

Fig. 5. Reduction of 2,6-dichlorophenol indophenol in the presence of TPN and R-5-P. Reaction conditions, 1 ml. of 200 γ per ml. of TPN, 80 per cent pure, 0.5 ml. of R-5-P, 0.027 M, 0.5 ml. of 2,6-dichlorophenol indophenol, 120 γ per ml., 0.5 ml. of 0.15 M Tham-HCl, and 0.2 ml. of enzyme, Stage I. Total volume, 6.0 ml., pH 7.5; room temperature, 25°. Density determined in the Evelyn colorimeter with 540 mpf filter.

that, in the presence of TPN and a means for its reoxidation, no triose phosphate accumulated when the enzyme acted on R-5-P (Fig. 6). As can be seen, there is instead a liberation of 0.57 μM of inorganic phosphate per micromole of pentose phosphate destroyed.
The system has not been sufficiently purified to permit detailed characterization of the enzyme catalyzing the oxidation of the triose phosphate. The observation, however, of the disappearance of triose phosphate, the formation of an acid group, the reduction of TPN, and the formation of pyruvic acid suggests the operation of a TPN-linked triose phosphate dehydrogenase, together with the glycolytic enzymes required to bring about the conversion of phosphoglyceric acid to pyruvic acid.

Several observations suggest that enzymes other than the conventional glycolytic enzymes are concerned in the production of pyruvic acid from triose phosphate. It is notable that the addition of AsO$_4^{3-}$ or PO$_4^{3-}$ did not particularly alter the rate of formation of GSH or disappearance of pentose in a test mixture containing GSSG, TPN, and R-5-P (Table V). It will be noted that approximately 1 mole of GSII is formed per mole of R-5-P utilized, as would be expected if the molar ratio of R-5-P disappearance to triose phosphate formation were 2.0.

To preclude the possibility that the rate-limiting steps were triose phosphate formation from pentose phosphate, or TPN reoxidation, an experi-
ment was performed in which the R-5-P and all constituents other than
TPN were preincubated 1/2 hour with the enzyme. Each cuvette contained
R-5-P 12.6 μM, Tham 72 μM, GSH 7.2 μM, AsO₄⁺ 50 μM, TPN 0.42 μM,
and spinach enzyme (Stage II) 0.1 ml. in a final volume of 3.2 ml. Under
these conditions, TPN reduction as measured at 340 mμ was inhibited 13
per cent by the AsO₄⁺. For this experiment the enzyme was dialyzed
an additional 20 hours against two changes of 2 liters of 10⁻³ M cysteine,
pH 7.0.

ADP, 5-AMP, and 3-AMP inhibited TPN reduction 43, 40, and 40
per cent, respectively, when present in concentrations of 1.4 × 10⁻³ M for
ADP and 5 × 10⁻³ M for 5- and 3-AMP. The observed reduction of TPN
could not be due simply to action of G-6-P dehydrogenase on G-6-P

| TABLE V |
| Effect of Phosphate, Arsenate, and Fluoride on Oxidative System |
|-------------------|-------------------|
|                    | R-5-P disappearing | GSH formed |
|                    | μM per tube        | μM per tube |
| Control            | 2.16               | 2.29        |
| Phosphate          | 1.98               | 2.41        |
| Arsenate           | 2.24               | 2.29        |
| Fluoride           | 2.29               | 2.36        |

Each tube contained in a total volume of 1.2 ml., 9.7 μM of GSSG, 6.17 μM of R-5-P,
17.9 μM of Tham-HCl, pH 7.5, 9.8 μM of MgSO₄, 0.13 μM of TPN, 0.1 ml. of enzyme,
Stage II, and 0.1 ml. of 0.05 M phosphate, 0.05 M arsenate, or 0.10 M NaF. Reaction
time, 2000 seconds. Temperature, 37°.

formed by the system, since the product of the reaction was pyruvic acid.
The other inhibitor tested, 0.02 m NaF, was without effect (Table V).
The enzyme utilized DPN at approximately one-half the rate of an equiva-
lent amount of TPN. Enzymatic assay with G-6-P dehydrogenase showed
no TPN contamination of the DPN.

DISCUSSION

Whatever the importance of this scheme to higher plants, the enzymes

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\text{Glucose-6-phosphate} \rightarrow \text{TPN} \rightarrow \text{6-phosphogluconate} \rightarrow \text{TPN} \rightarrow \text{ribulose phosphate} \\
\text{triose phosphate + sedoheptulose phosphate} \\
\text{TPN} \rightarrow \text{pyruvate} \\
(\text{glucose-6-phosphate} \leftrightarrow \text{fructose-6-phosphate})
\]
to actuate the scheme are present. It has been possible to find in a single tissue, the spinach leaf, the enzymes which catalyze the accompanying sequences. The G-6-P can reenter the scheme and the net result of the forward operation would be the ultimate conversion of G-6-P to pyruvic acid and CO₂.

With this enzyme system the conversion of hexose phosphate to pyruvate can be independent of adenosine diphosphate, phosphate, and di-phosphopyridine nucleotide. The formation of pyruvate appears to involve enolase, since it is inhibited by a mixture of F⁻, Mg⁺⁺, and PO₄³⁻; however, the subsequent formation of pyruvic acid and inorganic phosphate does not require ADP.

The probable identification of R-5-P as one of the reaction products resulting from phosphogluconate oxidation was established by radiochromatograms, but the possibility that this was not the primary product was suggested by the findings of Cohen and Scott (31) that other 5-carbon sugars could be formed in such reactions, and by the isolation of ribulose-5-phosphate as a first product by Horecker, Smyrniotis, and Seegmiller (32) from the products of yeast phosphogluconate dehydrogenase. The fact that ribulose appeared to be in our reaction mixture and the observation that ribulose-5-phosphate was somewhat better than R-5-P in forming heptulose are our basis for placing ribulose phosphate in the same place in our scheme that Horecker and coworkers have chosen in their scheme.

It may be noted from some of the data above (Table II) that the ratio \((2 \times \text{moles of heptulose formed})/(\text{moles of ribose destroyed})\), based on the Fe-orcinol test, approaches more closely a value of 1 the longer the reaction runs. This apparent anomaly is probably not due to the more rapid transformation of R-5-P to ribulose phosphate than the subsequent transformation of the ribulose phosphate to heptulose phosphate, since the data of Horecker et al. (32) show the chromogenic value of ribulose-5-phosphate to be only about 12 per cent less than that for aldopentose in the Mejbaum test. This difference is not sufficient to account for the discrepancies and the suggestion is therefore offered either that there is another compound which occurs between ribulose phosphate and heptulose phosphate in the scheme or that such a compound is in equilibrium, via a side reaction, with components in the main reaction sequence. However, no new spots were found on chromatographing sugars obtained from the products formed under conditions which gave rise to an anomalous ratio, with the possible exception of "ribose X" (Table I). At present the only

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\(^6\) An enzyme in spinach leaves which converts phospho-enol pyruvate to pyruvate and inorganic phosphate has been subjected to a 20-fold purification by two of us (R. S. B. and C. M. G.) and found to act independently of the presence of ADP,
basis for postulating this as a new component is the observation that there appears to be a substance with the mobility of ribose, which, unlike ribose, gives the same color as ribulose with the orcinol spray.

Although heptulose phosphate is shown as passing directly to G-6-P, it is obvious that stoichiometrical considerations could not permit the reaction as written. However, it appears that the partial disappearance of heptulose is largely accounted for by the appearance of G-6-P and F-6-P.

The conversion of two C₅ units to a C₄ and C₇ unit might be expected to involve a C₂ compound. Such a fragment was sought on the premise that it might be glycolaldehyde, but efforts to detect it in reaction mixtures directly, and in distillates therefrom, as the readily recognized dinitrophenylhydrazone or hydrazone were unrewarding. The enzyme preparation could oxidize glycolaldehyde at the expense of TPN.

The failure to show a metal requirement for the enzyme system, converting R-5-P to heptulose phosphate, may have been due to the limited purification of the preparation. However, the action of ethylenediamine tetraacetate in inhibiting the reaction at pH 8.5, but not at pH 7.5, indicates that an alkaline earth ion may be involved. The inhibitory action of p-chloromercuribenzoate implicates a sulfhydryl group in at least one of the enzymes in the system.

**SUMMARY**

1. It has been shown that an enzymatic system is present in spinach leaf extracts which oxidizes glucose-6-phosphate via triphosphopyridine nucleotide. The resulting 6-phosphogluconic acid is further oxidatively decarboxylated by another triphosphopyridine nucleotide-requiring enzyme to form a mixture of ribose and ribulose phosphates.

2. The pentose phosphates are further metabolized to sedoheptulose phosphate and triose phosphate.

3. Balance studies indicate the formation of 1 mole of heptulose phosphate and 1 mole of triose phosphate for each 2 moles of ribose phosphate which are utilized. However, in the early stages of the reaction less heptulose appears than is expected on the basis of pentose disappearance, thus suggesting the transient formation of an additional component.

4. The triose phosphate so formed is converted to pyruvic acid and inorganic phosphate in the presence of triphosphopyridine nucleotide.

5. The heptose phosphate is further metabolized to hexose phosphate.

6. The complete system results in the conversion of hexose phosphate to pyruvic acid and inorganic phosphate independent of the presence of adenyl phosphates and inorganic phosphate.
PHOSPHATE METABOLISM IN HIGHER PLANTS

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THE METABOLISM OF HEXOSE AND PENTOSE PHOSPHATES IN HIGHER PLANTS
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