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PREPARATION AND SOME PROPERTIES OF RIBULOSE-1, 5-DIPHOSPHATE

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PREPARATION AND SOME PROPERTIES OF RIBULOSE-1, 5-DIPHOSPHATE

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January 1956

INTRODUCTION

Recent work in various laboratories has confirmed the suggestion that ribulose-1, 5-diphosphate (RuDP) acts as the primary carbon dioxide acceptor of photosynthesis, the RuDP being carboxylated to yield two molecules of phosphoglyceric acid.

In view of the key function played by this compound in nature it became of great interest to prepare it in quantity and to examine some of its properties. Previous work in this laboratory had utilized an extraction of Scenedesmus followed by chromatographic purification as a source of RuDP; this method was tedious and gave small quantities admixed with other sugar diphosphates. A more attractive method, attributable to Weissbach et al., lay in the formation of RuDP from incubation of ribose-5-phosphate (RMP) and adenosine triphosphate (ATP) with an enzyme preparation from spinach, and attention was directed towards this procedure.

MATERIALS AND METHODS

A. Ribose-5-phosphate: Schwarz Ba salt used. Contains 5.7% P by weight (Ba RMP·5H₂O requires 6.8%).

Adenosine triphosphate: Pabst disodium salt used. Analysis of a solution gave the following figures:

Strength of adenylic acid, assayed spectrophotometrically at 260 mµ (€ at pH 7.0 = 15.4 x 10³) = 133 µM/ml. Inorganic P = 12.9 µM/ml. Acid-labile P = 223.1 µM/ml.

B. Analysis

1. Phosphorus. Total phosphorus was determined colorimetrically by the technique of Allen, which was suitable for aliquots containing 10 to 150 µg of phosphorus. The amount of phosphorus was read from a calibration curve obtained for the Klett colorimeter with a 660-mµ filter (Fig. 1). This method
Fig. 1. Calibration curve for Klett colorimeter for determination of phosphorus (aliquots containing 10 to 150 μg P).
was scaled down to one-fifth scale for the determination of aliquots containing
0 to 35 mg of phosphorus (Fig. 2).

Inorganic phosphorus in the presence of acid-labile phosphorus was determined
by the method of Lowry and Lopex, using ascorbic acid at pH 4.0 as reducing
agent.

2. Pentose. Pentose was determined by the Bial reaction with orcinol-HCl. It
was important to purify the orcinol by recrystallization from benzene. Then
2 ml of the pentose-containing solution was treated with a solution of orcinol in
ethanol (0.2 ml of 10%) and a solution of FeCl₃ in concentrated HCl (2.0 ml of
0.1%). The mixture was heated for 40 minutes at 100° and the spectrum deter-
mined. A calibration curve relating the concentration of aldopentose to the
optical density at 670 mμ was constructed, (Fig. 3) using solutions of both
arabinose and ribose. The spectrum obtained is given in Fig. 4; the ratio of
the optical density at 540 mμ to that at 670 mμ is constant at 0.19.

The data for ribulose were those of Horecker: the extinction coefficient given by ribulose at 670 mμ is 50% of that given by
aldopentoses. By use of these data, a mixture of ribulose and aldopentose could
be analyzed by adding a known amount of aldopentose, determining the resulting
orcinol spectrum, and solving simultaneous equations giving the concentrations
of the two components.

3. Protein. Total protein in solution was determined by the method of
Bücher, which involves a measurement of the turbidity produced by a standard
concentration of trichloroacetic acid and ammonium sulfate in a given time. An
aliquot of the protein solution (containing 1 to 4 mg protein) was made up to 6 ml
with water; (NH₄)₂SO₄ solution (2 ml; 0.24 M), and trichloroacetic acid (0.25 ml;
3 m), and water to 10 ml was added. The tube was shaken and the turbidity
measured after 1 minute in the Klett colorimeter, using a blue filter. A cali-
bration curve (Fig. 5) was constructed with a solution of blood albumen. This
method was found to be rather unsatisfactory in practice, and the values obtained
with it must be regarded as approximate.

4. Ammonium sulfate. When protein fractions obtained from ammonium
sulfate precipitations were re-dissolved in phosphate buffer, it was necessary to
determine the amount of (NH₄)₂SO₄ brought through with the precipitated protein.
This was done by taking 500 ml of the solution in question (at 0° C), diluting to 25 ml
with distilled water at room temperature, and measuring conductivity of the
resulting solution with a conductivity bridge (Industrial Instruments, Inc., Model
RC 16). By use of a calibration curve (Fig. 6) the concentration of (NH₄)₂SO₄
could be determined.

C. Ion-Exchange Chromatography

For the chromatography of the reaction product, Dowex-1 (200 to 400-mesh;
8% cross-linked) in the chloride form, freed from fines by decantation and cycled
Fig. 2. Calibration curve for Klett colorimeter for determination of phosphorus (aliquots containing 0 to 35 µg P).
Fig. 3. Calibration curve for Cary spectrophotometer for orcinol test.
Fig. 4. Spectrum of orcinol product from ribose.
Fig. 5. Calibration curve for Klett colorimeter for turbidimetric protein determination (blue filter).
Fig. 6. Conductimetric determination of \((NH_4)_2SO_4\) concentration in presence of phosphate buffer. The calibration solution consisted of phosphate buffer (0.05 M, pH 7.3, 0° C) plus an aliquot of \((NH_4)_2SO_4\), all made up to 25 ml at room temperature. Abscissa represents concentration of \((NH_4)_2SO_4\) in g/100 ml of the stock solution at 0° C.
twice between the hydroxide and chloride forms, was used. The columns were developed by using the graded elution technique of H. Busch et al. The presence of phosphates in the eluate fractions was detected by spotting out 15-μl aliquots onto filter paper and spraying with the Hanes-Isherwood phosphate spray. Adenine nucleotides were detected by their absorption at 260 μm, and ribulose diphosphate by the brownish color it gave in the orcinol test.

RESULTS

Elution characteristics of RMP and the adenine nucleotides

The separation of a mixture of adenylic acid (AMP, 261 μmoles), ATP (199 μmoles), and RMP (90 μmoles) on the Dowex-1 column under the conditions of graded elution was first studied. The column size was 2.2 by 21 cm; the developer (0.2 M KCl + 0.015 M HCl) was run into a mixing pot containing 750 ml water. The mixture of AMP, ATP, and RMP dissolved in 10 ml water was brought to pH 7.8 with ammonia, and poured onto the column; this was washed with 25 ml water before development at 2.1 ml/min was commenced. Fractions of 8 ml were collected. The results are shown in Fig. 7; the curve represents the results of a separate experiment to determine the rise in molarity of the developer solution issuing from the mixing flask. Here 0.2 M HCl was run into the flask containing 750 ml water, with stirring, at the same rate as solution was withdrawn the pH of which was measured at suitable intervals.

Preparation of Enzyme System

Six separate preparations of enzyme from spinach were made; the first three preparations gave extracts that showed very little activity. The reason for this is not known, but it may be because the protein fractions were centrifuged at 40,000 rpm in a Spinco centrifuge (No. 40 head). Milder centrifuging was used in the later preparations which showed reasonable activity. The procedures used in each of the last three preparations were very similar, hence only one is reported in detail.

Enzyme Preparation 4 (all at 0°C)

Sixty grams of spinach leaves (Lucky Stores' "American spinach") were homogenized in a Waring Blender for 3 min and the brei filtered through a coarse sintered glass filter. The filtrate was brought to pH 7.0 with ammonia, and centrifuged in a Spinco centrifuge (No. 20 head) 5 min at 20,000 rpm; 200 ml of the clear, pale green extract was brought to 45% saturation with ammonium sulfate (61.6 g) and the precipitate was centrifuged off and discarded. To the supernate was added 35.6 g (NH₄)₂SO₄, bringing it to 70% saturation, and the precipitate was centrifuged in the Spinco centrifuge (No. 20 head) 5 min at 5,000 rpm. The resulting precipitate was dissolved in phosphate buffer (ca. 20 ml; 0.05 M; pH 7.4) giving Fraction I of volume 22.6 ml. When 0.5 ml of I was made up to 25 ml with distilled water at room temperature it had a
Fig. 7. Separation of ATP, AMP, and RMP on Dawex-1.
resistance of 188 ohms (as measured in the conductivity cell); this is equivalent to 5.04 g \((NH_4)_2SO_4\) per 100 ml of Fraction I. \((NH_4)_2SO_4\) (4.78 g) was added to bring saturation to 41%, the resulting precipitate was centrifuged off and discarded, and \((NH_4)_2SO_4\) (1.29 g) was added to the filtrate to bring saturation to 50%. The resulting precipitate was collected by centrifuging at 5,000 rpm in Spinco No. 40 Head, and dissolved without stirring in phosphate buffer (3.5 ml of 0.05 M; pH 7.4) to give Fraction II. Protein concentration = 45 mg/ml.

Weissbach et al. further purified Fraction II by adsorption on calcium phosphate gel followed by elution with sodium pyrophosphate. This step was not carried out in any of the preparations described, however, as both the testing for activity and the actual large-scale incubation were carried out with Fraction II.

It may be noted that after each addition of \((NH_4)_2SO_4\) the pH was adjusted with ammonia; as the preparation proceeded the extract turned a dark brown color.

The activity of the extract was tested by the procedure of Colowick and Kalckar. This procedure utilizes the fact that phosphorylation of an alcoholic group with a molecule such as ATP produces one extra mole of acid at pH >7.5:

\[
\begin{align*}
\text{R} \cdot \text{OH} + \text{Ad} & \rightarrow \text{R} \cdot \text{P} \cdot \text{OH} + \text{Ad} \\
\text{R} \cdot \text{P} \cdot \text{OH} + \text{Ad} & \rightarrow \text{R} \cdot \text{P} \cdot \text{O} \cdot \text{OH} + \text{Ad} \\
\text{R} \cdot \text{P} \cdot \text{O} & \rightarrow \text{R} \cdot \text{P} \cdot \text{OH} + \text{Ad} \\
\end{align*}
\]

By working in \(NaHCO_3\) solution, one can measure this acid production manometrically as \(CO_2\) evolution. The following Warburg flasks (15 ml) were set up and flushed with 96% \(N_2\)-4% \(CO_2\) for 10 minutes at 37°. After equilibration, the contents of the sidearms were tipped in (Table I) and the gas evolved was measured at regular time intervals (Table II). It will be noted that a steady evolution of gas took place in Flask 2, which is a symptom of some enzymatic dephosphorylation of ATP; this would, of course, liberate \(CO_2\) for the same reason as kinase activity. Flask 3 showed a still larger volume change, which is a measure of the kinase activity of the enzyme extract. In this experiment the theoretical yield of \(CO_2\) = 290 \(\mu\)l, hence after 145 min, 45% of this value had been evolved.

Two large-scale incubations were then performed in large Warburg vessels (185 ml capacity) at 37°, with mercury as manometric fluid. The contents of the flasks were of the following compositions:

1 ml of RMP (200 \(\mu\)M/ml; pH 7.0),
3 ml of ATP (140 \(\mu\)M/ml; pH 7.0),
13 ml of \(NaHCO_3\) (0.032 M) + \(MgCl_2\) (0.01 M),
0.12 ml of versene (0.01 M, pH 7.4);
the sidearm contained 1.5 ml of Fraction II.
Table I

Compositions of reaction mixtures

<table>
<thead>
<tr>
<th>Substance</th>
<th>Contents (ml.)</th>
<th>Flask No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NaHCO₃ (0.032 M) + MgCl₂ (0.01 M)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Versene (0.01 M; pH 7.4)</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>RMP (135 µM/ml; pH 7.0)</td>
<td>0.10</td>
<td>--</td>
</tr>
<tr>
<td>ATP (150 µM/ml; pH 7.0)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Sidearms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ (0.032 M) + MgCl₂ (0.01 M)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Phosphate buffer (0.05 M; pH 7.4)</td>
<td>0.15</td>
<td>--</td>
</tr>
<tr>
<td>Fraction II</td>
<td>--</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Table II

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flask 1 (µl)</th>
<th>Flask 2 (µl)</th>
<th>Flask 3 (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10</td>
<td>12.8</td>
<td>147</td>
<td>145</td>
</tr>
<tr>
<td>20</td>
<td>14.5</td>
<td>170</td>
<td>233</td>
</tr>
<tr>
<td>30</td>
<td>19.6</td>
<td>173</td>
<td>261</td>
</tr>
<tr>
<td>45</td>
<td>14.5</td>
<td>176</td>
<td>282</td>
</tr>
<tr>
<td>60</td>
<td>18.0</td>
<td>185</td>
<td>298</td>
</tr>
<tr>
<td>145</td>
<td>19.6</td>
<td>206</td>
<td>336</td>
</tr>
<tr>
<td>235</td>
<td>19.6</td>
<td>218</td>
<td>341</td>
</tr>
</tbody>
</table>

The flask was flushed with 96% CO₂ - 4% N₂ for 10 min, and the enzyme was then tipped in. After 75 minutes the evolution of gas was virtually complete; the contents of the flasks were then poured straight onto the Dowex column and washed with 25 ml water. The column was developed at 1.85 ml/min, and 14-ml fractions were collected. The result of the separation is shown in Fig. 8.

Total recovery of phosphate = 2.13 millimoles (73%).

The performance of the column was impaired by a deposit of denatured protein forming at the top; in subsequent experiments the solution was de-proteinized before chromatography.

The tubes containing the RuDP were pooled (90 ml) and concentrated to 50 ml by distillation under a reduced pressure of nitrogen at 30°. Barium acetate (100 mg) and ethanol (200 ml) were added and the solution was left to stand at 0° for 1 hour. At the end of this time the white flocculent precipitate was centrifuged down, washed with ethanol (40 ml of 80%), and dried in vacuum over P₂O₅, giving 126 mg of white powder—RuDP-I.
Fig. 8. Separation of product from Enzyme Preparation 4.
Analysis of RuDP-I

45.8 mg of the dried Ba salt was suspended in water (1.8 ml) and H₂SO₄ (0.35 ml of 0.2 N) was added. Na₂SO₄ (0.2 M) was added until the precipitation of Ba was just complete; 0.27 ml was needed. The BaSO₄ was washed several times with water, and the combined washings (1.2 ml) were added to the solution of the phosphate. The resulting solution, pH 3.5, was brought to 6.5 with NaOH and the volume adjusted to 5 ml. A phosphatase solution was prepared by shaking 10 mg of "Phosphatase" (General Biochemicals, Inc.) with water (1 ml) for 20 min and centrifuging, and the supernate was added to 4.6 ml of the above solution; the mixture was then incubated at 37°. Samples were withdrawn periodically and their inorganic phosphate content was determined. After 7 hours all the organic phosphate had been released, and samples of the solution were chromatographed unidimensionally in water-saturated phenol and butanol-propionic acid-water. The former solvent was found to be more satisfactory and separated the dephosphorylated sample into two spots, of Rf 0.58 and 0.65, which were identified as ribose and ribulose on the basis of co-chromatography and colors given by orcinol-trichloroacetic acid and aniline-phthalic acid sprays. No other aldose sugar was revealed by spraying with aniline-trichloroacetic acid-acetic acid. The spectrum of the product from the orcinol reaction showed two peaks at 540 and 670 mμ; the ratio of the former to the latter is 0.46.

These experiments give the following analytical data for the RuDP-I solution:

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PO₄</td>
<td>18.1 µM/ml</td>
</tr>
<tr>
<td>Inorganic PO₄</td>
<td>0.9 µM/ml</td>
</tr>
<tr>
<td>PO₄ released by phosphatase preparation</td>
<td>17.9 µM/ml</td>
</tr>
<tr>
<td>Total pentose</td>
<td>7.5 µM/ml</td>
</tr>
<tr>
<td>Pentose total / PO₄</td>
<td>1.241</td>
</tr>
<tr>
<td>Pentose composition:</td>
<td>Ribulose = 56</td>
</tr>
<tr>
<td>Percentage of Ba salt as pentose diphosphate</td>
<td>31%</td>
</tr>
</tbody>
</table>

In view of the high proportion of ribose in the dephosphorylated product, the phosphatase preparation was tested for pentose phosphate isomerase activity.
Test of phosphatase (G. B. I.) for pentose phosphate isomerase activity

To 2.0 ml of a solution of RMP (135 μM/ml) at pH 6.6 was added the extract of "Phosphatase" (G. B. I., 5mg) in water (0.5 ml); the resulting solution was incubated at 36° for 9 hours, by which time all the organic phosphate had been liberated. The spectrum of the product from the orcinol reaction showed two peaks at 540 and 670 mμ,

$$\frac{\varepsilon_{540 \text{ mμ}}}{\varepsilon_{670 \text{ mμ}}} = 0.32.$$  

This corresponds to a ribulose:ribose ratio of 32:68, i.e. the equilibrium mixture of RMP and ribulose-5-phosphate at 37°. Chromatography with phenol-water showed the presence of ribose and ribulose.

Separation of acid phosphatase from Polidase-S

Cohen reported that the majority of the acid phosphatase activity in Polidase-S (Schwarz) was contained in the fraction precipitating between 83% and 100% ammonium sulfate saturation at pH 7.7. This procedure was applied to a sample of Polidase-S, and the precipitated protein was redissolved in water (1.5 ml).

This phosphatase preparation was tested for pentose phosphate isomerase activity in an acetate buffer solution at pH 5.0, as follows:

RMP (400λ; 34 μM/ml)

Acetate buffer (4 ml, 0.2 M + 0.01 M MgCl₂; pH 5.0)

Phosphatase solution (400λ)

After 2 hours all the organic phosphate had been liberated and the resulting solution was analyzed by the orcinol reaction and by chromatography with phenol-water. The spectrum of the orcinol product was that of a typical aldopentose, with no peak at 540 mμ,

$$\frac{\varepsilon_{540 \text{ mμ}}}{\varepsilon_{670 \text{ mμ}}} = 0.12$$

The only pentose detected after chromatography was ribose (R₇ 0.58); the aniline-phthalic acid spray showed a brownish streak running at R₇ ~ 0.30, the nature of which is not known.

The subsequent preparation of RuDP were analyzed with this purified acid phosphatase preparation. It may be noted that the purified Polidase preparation gave a distinct green color in the orcinol reaction which, beyond a certain level,
impaired the accuracy of the pentose determination. This impairment was shown by determining a known amount of arabinose in the presence of 25% of the enzyme solution. The blank solution turned quite green, and when this was used as blank in the Cary spectrophotometer, the resulting spectrum of the arabinose-containing solution gave a value for the pentose that was 17% low. For this reason, it was important that the amount of enzyme added to the incubation mixture be such that the orcinol reaction could be performed in the presence of a maximum of approximately 5% of enzyme solution. At such concentrations, the color from the blank did not significantly affect the pentose determination.

Enzyme Preparation 5

With the procedure outlined for Preparation 4, 40% of the theoretical yield of CO$_2$ was evolved in the manometric test of kinase activity with this preparation. The large-scale incubation was carried out with the use of an automatic titrating device, in which KOH (2 M) was automatically titrated into the mixture to keep the pH at 7.8; the reaction vessel was maintained at 38$^\circ$ under an atmosphere of nitrogen. This method possessed decided advantages over the manometric one: (a) the operation is easier; (b) in the absence of CO$_2$ the yield of RuDP would be unaffected by the presence of any carboxydismutase in the enzyme extract.

The incubation mixture consisted of

- RMP (3.7 ml; 135 $\mu$M/ml),
- ATP (6.7 ml; 150 $\mu$M/ml),
- MgCl$_2$ (0.85 ml; 0.2M),
- versene (0.15 ml; 0.01 M),
- water (5 ml).

The pH was brought up to 7.8 before addition of the enzyme extract (2.65 ml), which had been brought to pH 7.8 with KOH immediately beforehand. The uptake of alkali (0.151 ml, 2 N) substantially ceased after 30 min, and the pH was then lowered to 6.5 by the addition of HCl. The solution was transferred to a bath at 80$^\circ$ for 3 min and the precipitated protein centrifuged down and washed with two 1-ml portions of phosphate buffer (0.05 M; pH 7.3), these washings were added to the supernatant solution. The combined solution was transferred to a Dowex-1 column (2.2 x 25 cm) and washed with 25 ml water. The development was as before: flow rate = 1.9 ml/min. First fraction = 180 ml, subsequent fractions = 5 ml. The RuDP came through in fractions Nos. 119 through 131, which were combined (74 ml), brought to pH 7, filtered, and analyzed for total phosphorus (155 $\mu$M). Slight excesses of barium acetate solution (0.2 ml; 1 M) and ethanol (296 ml) were added, and the solution was left for 1 hour at 5$^\circ$. The precipitate was centrifuged down, washed with ethanol (80%), and dried in vacuo over P$_2$O$_5$. Weight = 59.2 mg RuDP-II.
Analysis of RuDP-II

7.27 mg was weighed out into water (300 \( \lambda \)) and \( \text{H}_2\text{SO}_4 \) (70\( \lambda \); 0.2 N) added, followed by just sufficient \( \text{Na}_2\text{SO}_4 \) (60 \( \lambda \); 0.2 M) to precipitate the barium. The barium precipitate was washed with water and the combined washings and original supernatant solution were made up to 1 ml.

A sample of this solution was incubated with purified Polidase preparation,

RuDP-II solution (0.4 ml),

acetate buffer (1 ml, 0.2 M + 0.01 M MgCl\(_2\); pH 5.0),

phosphatase solution (0.1 ml).

At the end of 8 hours at 37\( ^{\circ} \) no further organic phosphate was released and an aliquot (100\%\) of the solution was analyzed by the orcinol reaction. The spectrum was determined against a blank containing 7\% of the phosphatase solution plus acetate buffer (70\%), and showed two peaks at 540 and 670 m\( \mu \),

\[
\frac{\varepsilon_{540 \text{ m\( \mu \)}}}{\varepsilon_{670 \text{ m\( \mu \)}}} = 0.55.
\]

Chromatographic analysis in phenol-water showed ribulose, a trace of ribose, and a brownish streak (with aniline-phthalic acid spray) at \( R_f \sim 0.3 \) (cf. p 17).

From these data the following analytical figures are obtained: the phosphate from 7.27 mg RuDP-II in water (1 ml) gives a solution containing

\begin{align*}
\text{total } \text{PO}_4^2- & : \quad 14.8 \text{ \mu M/ml}, \\
\text{inorganic } \text{PO}_4^2- & : \quad \text{below limit of detection}, \\
\text{PO}_4^2- \text{ released by phosphatase solution} & : \quad 14.0 \text{ \mu M/ml}, \\
\text{total pentose} & : \quad 6.86 \text{ \mu M/ml}, \\
\text{pentose/total } \text{PO}_4^2- & : \quad 1:2.15, \\
\text{pentose composition:} & \quad \frac{\text{ribulose}}{\text{ribose}} = \frac{69}{31}; \\
\text{Percentage of Ba salt as pentose diphosphate:} & \quad 29.2.
\end{align*}
Enzyme Preparation 6

The scale of the previous two experiments was doubled, and an enzyme extract was made from 120 g of spinach. The extract, dissolved in phosphate buffer (5 ml, 0.05 M, pH 7.4), showed a very low activity in the Warburg assay, only 20% of the theoretical yield of CO₂ being evolved. In spite of this low activity two large-scale incubations were done, using the titration apparatus; composition of the incubation mixture in each case was

\[
\text{RMP (2.0 ml, 200 \mu M/ml),} \\
\text{ATP (5.0 ml, 150 \mu M/ml),} \\
\text{versene (0.15 ml; 0.01 M),} \\
\text{MgCl₂ (0.85 ml; 0.2 M),} \\
\text{glutathione (0.25 ml; 0.42 M),} \\
\text{water (5.0 ml).}
\]

The glutathione was added in view of Racker's finding that phosphopentokinase required the presence of -SH containing compounds such as cysteine and glutathione.

The two incubation batches consumed 180.6 and 202.4 \lambda of KOH (2 N) respectively, the reaction time being 35 minutes. They were combined and chromatographed on Dowex-1 (2.5 x 25 cm) as described previously; flow rate = 2.25 ml/min, fraction No. 1 = 190 ml, subsequent fractions = 5 ml. The RuDP came through in fractions Nos. 154 through 174, which were combined (122 ml) and analyzed for total phosphate; 462 \mu M was found. The barium salt was precipitated with barium acetate (0.70 ml, 1 M) and dried; weight = 136 mg RuDP-III.

Analysis of RuDP-III

A sample of Ba salt (32.23 mg) was weighed out into water (0.40 ml), and H₂SO₄ (0.36 ml, 0.2 N) was added. The precipitation of barium was completed with Na₂SO₄ (0.15 ml, 0.41 M), and the solution made up to 2 ml at pH 5.5. An aliquot of this solution was phosphatased with the purified Polidase preparation,

\[
\text{RuDP solution (125\lambda),} \\
\text{acetate buffer (600\lambda; 0.2 M + 0.01 M MgCl₂; pH 5.0),} \\
\text{phosphatase solution (25\lambda).}
\]

The organic phosphate was released after 30 hours at 37° and the solution was analyzed by the orcinol reaction, using a 100-\lambda aliquot and 3\lambda of phosphatase.
solution in the blank mixture. The spectrum showed two peaks at 540 and 670 m\(\mu\),

\[
\frac{\epsilon_{540 \, m\mu}}{\epsilon_{670 \, m\mu}} = 0.63.
\]

Thus, the phosphate from 32.23 mg RuDP-III in 2 ml water gives

- **total PO\(_4\):** 40 \(\mu\)M/ml,
- **inorganic PO\(_4\):** 4 \(\mu\)M/ml,
- **PO\(_4\) released by phosphatase enzyme:** 38.4 \(\mu\)M/ml,
- **total pentose:** 17.8 \(\mu\)M/ml,
- **pentose/total PO\(_4\):** 1: 2.25,
- **pentose/organic PO\(_4\):** 1: 2.16,
- **pentose composition:** \(\frac{\text{ribulose}}{\text{ribose}} = 78:22\)

**Percentage of RuDP-III as pentose diphosphate:** 36%.

**Chromatography of RuDP**

The paper chromatography of RuDP in several solvents was studied. Because of its instability under alkaline conditions it was not possible to use any basic solvent system; the following acidic solvents were investigated:

(a) methanol–formic acid (88%)-\(\text{H}_2\text{O}\) (80:15:5);\(^{21}\)
(b) acetone–trichloracetic acid (25%) (75:25);\(^{22}\)
(c) tertiary butanol–picric acid–water (78:2:22);\(^{14, 23}\)
(d) isopropyl alcohol (75 ml)–trichloracetic acid (5 g)–ammonia (0.25 ml, sp. gr. = 0.9)–water (25 ml).\(^{24}\)

Of these solvents, (d) was found to be the most satisfactory, giving the following \(R_f\) values (all phosphates put on paper at pH 5): fructose-1,6-diphosphate 0.48; RuDP, 0.49; RMP, 0.60; orthophosphate, 0.77. Although the diphosphates run as clearly defined spots, none of the solvent systems was capable of differentiating between individual diphosphate esters. It is possible that borate complexing may be used successfully in this respect.\(^{25}\) The effect of adding boric acid (0.5 g) to Solvent (d) was tried, but the \(R_f\) values of the above-mentioned phosphate esters remained substantially the same.
It is of interest that the only spots revealed by phosphate spray on the chromatographed RuDP-III were those of the diphosphate and a small quantity of orthophosphate.

**Acid hydrolysis of RuDP**

The hydrolysis of the crude (C\(^{14}\))-RuDP obtained from Scenedesmus, which had been treated with C\(^{14}\)O\(_2\), \(^1\) was examined in the early part of 1954. Samples of the (C\(^{14}\))-RuDP were heated in 0.1 N HCl and in 1 N HCl at 100\(^\circ\) and 35\(^\circ\) respectively. Aliquots were chromatographed two-dimensionally in phenol-water and in butanol-propionic acid-water. It was found in both cases that only a small accumulation of ribulose monophosphate(s) could be observed, free ribulose appearing instead. It seemed that both phosphate groups must be hydrolyzing at approximately the same rate, in contrast to fructose or glucose-1,6-diphosphates, in which the 1-phosphate hydrolyzes several times as rapidly as does the 6-phosphate. After 1 hr in 0.1 N HCl at 100\(^\circ\) about 35\% of unchanged RuDP remained, several regions of radioactivity appearing on the chromatogram were not identified, and about 40\% of the original count of RuDP could not be recovered. Treatment with N HCl at 35\(^\circ\) resulted in less destruction of the products.

Weissbach et al. \(^3\) also studied the hydrolysis of RuDP in 1 N HCl at 100\(^\circ\) and found both phosphate groups equally labile; the half time of hydrolysis was about 30 min.

The hydrolysis behavior of RuDP-I in 1 N HCl at 37\(^\circ\) was then investigated. A barium-free solution of RuDP-I in 1 N HCl (5 ml) was maintained at 37\(^\circ\), aliquots being withdrawn at intervals for inorganic phosphate determination (Table III). A similar experiment was performed with a solution of RMP in 1 N HCl at 37\(^\circ\) (Table IV). A sample of the RuDP hydrolyzate after 137 hours at 37\(^\circ\) was analyzed by the orcinol reaction. Two peaks at 540 and 670 m\(\mu\) were visible:

\[
\frac{\varepsilon_{540\ m\mu}}{\varepsilon_{670\ m\mu}} = 0.53;
\]

this corresponds to

\[
\frac{\text{ribulose}}{\text{ribose}} = \frac{68}{32}.
\]

Analysis of the RMP hydrolyzate by orcinol reaction gave a typical aldopentose spectrum

\[
\frac{\varepsilon_{540\ m\mu}}{\varepsilon_{670\ m\mu}} = 0.18.
\]
### Table III

Hydrolysis of RuDP-I in N HCl at 37° C.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Inorganic PO₄ (µM/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.34</td>
</tr>
<tr>
<td>18.5</td>
<td>1.16</td>
</tr>
<tr>
<td>44.5</td>
<td>2.22</td>
</tr>
<tr>
<td>64.5</td>
<td>2.74</td>
</tr>
<tr>
<td>95.5</td>
<td>3.37</td>
</tr>
<tr>
<td>121.0</td>
<td>3.35</td>
</tr>
<tr>
<td>137.0</td>
<td>3.52</td>
</tr>
</tbody>
</table>

Total PO₄ in solution 3.9

### Table IV

Hydrolysis of RMP in N HCl at 37° C.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Inorganic PO₄ (µM/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>20</td>
<td>0.03</td>
</tr>
<tr>
<td>40</td>
<td>0.03</td>
</tr>
<tr>
<td>71</td>
<td>0.09</td>
</tr>
<tr>
<td>96</td>
<td>0.11</td>
</tr>
<tr>
<td>113</td>
<td>0.14</td>
</tr>
<tr>
<td>137</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Total PO₄ in solution 4.2
A sample of the RuDP hydrolyzate was neutralized to pH 7 with NaOH and washed through a column of Dowex-1 (x2; formate form) containing 2 ml of resin. The resulting free-sugar solution was analyzed by the orcinol reaction and gave two peaks at 540 and 670 m\(\mu\),

\[
\frac{\varepsilon_{540 \text{ m}\mu}}{\varepsilon_{670 \text{ m}\mu}} = 0.62,
\]

corresponding to

\[
\begin{align*}
\text{ribulose} & = 75, \\
\text{ribose} & = 25
\end{align*}
\]

Chromatography in phenol-water of the two acid hydrolyzates and spraying with aniline-phthalic acid gave the picture shown in Fig. 9.

**Spectroscopic measurements**

Some spectroscopic measurements were made on RuDP and other model compounds to see if any information could be gained on the form of RuDP in solution.

The data available for compounds other than carbohydrates are given in Table V; those marked with an asterisk were measured in this laboratory (Fig. 10).

The spectra of certain classes of carbohydrates have been the subject of several investigations. The main findings of these can be summarized as follows:

The low-intensity absorption at 280 m\(\mu\) of glucose, as described by Niederhoff,\textsuperscript{26} and attributed to free -CHO, has since been found by Henri and Schou\textsuperscript{27} to be due to impurities; they concluded that the amount of free -CHO present in solution was below the limit of spectroscopic detection, viz., <0.3\%. This conclusion was confirmed by Cantor and Peniston,\textsuperscript{28} using polarographic methods, although pentoses were found to contain higher proportions of aldehydo-sugar; e.g., ribose contains 8.5\% at pH 7.0 (0.1 M). Work by several investigators\textsuperscript{29,30,31} has demonstrated that certain ketohexoses do show absorption at 278-280 m\(\mu\); in general \(\varepsilon\) is below 10. If glucose, galactose, or any of certain pentose solutions is raised to pH 12, a characteristic absorption spectrum forms (Fig. 11), which disappears on acidification.\textsuperscript{29}

No work on the uv spectra of phosphorylated sugars has been found in the literature. Table VI contains the spectroscopic measurements (in water) that have been made in this laboratory.
Fig. 9. Chromatogram of products from acid hydrolysis of RuDP and RMP.
Table V

Spectroscopic measurements on some noncarbohydrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ (m$\mu$)</th>
<th>$\xi_{\text{max}}$</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$-CO-CH$_3$</td>
<td>n-hexane</td>
<td>188</td>
<td>900</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>water*</td>
<td>265</td>
<td>~15</td>
<td>7</td>
</tr>
<tr>
<td>CH$_2$=CH$_2$</td>
<td></td>
<td>175</td>
<td>15,000</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(submerged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>max.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_2$=CH$_2$</td>
<td></td>
<td>190</td>
<td>10,000</td>
<td></td>
</tr>
<tr>
<td>CH$_2$=CH*</td>
<td>ethanol (95%)</td>
<td>225</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>OEt$\nearrow$C=O*</td>
<td>ethanol (95%)</td>
<td>Continuous absorption from 300 m$\mu$.</td>
<td>No peak</td>
<td></td>
</tr>
<tr>
<td>C=C=CH-C=CH$_2$OH</td>
<td>water</td>
<td>245</td>
<td>acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>265</td>
<td>alkali</td>
<td></td>
</tr>
<tr>
<td>C - C = C - C - C - CH$_2$OMe</td>
<td>water</td>
<td>229</td>
<td>6,000</td>
<td></td>
</tr>
</tbody>
</table>
1. Vinylene carbonate in 95% EtOH (15Å in 5 ml.)
2. Acetone in water
3. Ethyl carbonate in 95% EtOH (55Å in 5 ml.)

Fig. 10. Spectra of Vinyl carbonate in ethanol, acetone in water, and ethyl carbonate in ethanol.
Spectrum of mixture of 0.5 Mol Glucose solution and 0.5 Mol NaOH at 0 hr.

Fig. 11. Spectrum of mixture of 0.5 M glucose solution and 0.5 M NaOH at 0 hr. (From Gabryelski and Marchlewski, Biochem. 2, 250, 385 (1932).
Table VI

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>$\lambda_{\text{max}}$ (m$\mu$)</th>
<th>$\varepsilon_{\text{max}}$ (m$\mu$)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydroxyacetone</td>
<td>City Chemical Corp</td>
<td>270</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>From C. Ballou as</td>
<td>262</td>
<td>22.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>$\text{CH}_2\text{OH} \cdot \text{C-CH}_2\text{OP}=\text{O}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EtO</td>
<td>R  O  O</td>
<td>R  O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>where R = cyclo-hexylammonium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-diphosphate</td>
<td>Mg salt (G. B. I.)</td>
<td>258</td>
<td>56</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>Ba salt (Schwarz)</td>
<td>258</td>
<td>55.5</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Reprecipitated in ethanol (80%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RuDP</td>
<td>Sample of RuDP-III</td>
<td>260</td>
<td>342</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Reprecipitated Ba salt</td>
<td>260</td>
<td>494</td>
<td>6.0</td>
</tr>
</tbody>
</table>
The spectra of the sugar phosphates were measured in a micro Cary Cell (5 cm, 0.7 ml) made of Teflon; the value of the extinction coefficients is subject to an error of about ± 2.5% (Fig. 12).

The dihydroxyacetone phosphate derivative was dissolved in a known quantity of 0.01 N H₂SO₄ and left to stand at 37° for 12 hours in order to hydrolyze the acetal grouping. The spectrum was measured against a blank solution containing an equivalent amount of cyclo-hexylammonium sulfate at pH 2.

The Mg salt of fructose diphosphate was dissolved in water and the strength of the solution in terms of total phosphate was measured; the concentration of the hexose diphosphate used for calculating η is one-half of this phosphate concentration. The Ba salt (Schwarz) was brought into solution with H₂SO₄-Na₂SO₄ treatment; to the resulting Ba-free solution (at pH 7) was added a slight excess of barium acetate (1 M) and 4 volumes of ethanol. The precipitated Ba salt was washed with ethanol (80%) and dried in vacuum over P₂O₅. A Ba-free solution was made in the same way as above and its strength was determined from the phosphate content.

A sample of RuDP-III was used for the first ribulose diphosphate determination, and the concentration of the phosphate in solution was measured and divided by 2.25 to obtain the pentose diphosphate strength. The value of η is calculated on the assumption that all the pentose diphosphate is ribulose diphosphate. This value may well be too low if the sample actually contains 22% of ribose.

To the stock solution of RuDP-III used for the preparation of the spectroscopic solution was added a slight excess of barium acetate (at pH 7) and four volumes of ethanol. The precipitated salt was washed with ethanol (80%) and dried in vacuum over P₂O₅. A Ba-free solution of this salt was made by H₂SO₄-Na₂SO₄ treatment and assayed for total phosphate, and the concentration of RuDP was obtained as above.

**Weygand-Csendes test for Ene-diols**

The Weygand-Csendes test for ene-diols was applied to a solution of RuDP as follows: To 1 mg of the pentose diphosphate in methanol (3 ml, 95%) + pyridine (1 ml), was added one drop of TiCl₃ solution (5%). A colorless precipitate formed. A second test was carried out, similar to the first, except that the pyridine was omitted. The solution remained colorless.

Similar negative results were given by acetoin, dihydroxyacetone, and dihydroxyacetone phosphate. Color changes were observed on application of the test to ascorbic acid and salicylaldehyde.
A. Ribulose diphosphate x 1/5 reprecipitated Ba salt
B. Fructose-1,6-diphosphate reprecipitated Ba salt
C. Dihydroxyacetone phosphate as
   \[ \text{CH}_2\text{OH}-\text{C}-\text{CH}_2\text{OP} = \text{O} \]
   \[ \text{EtO} \text{OEt} \text{O} \text{O} \]
   where \( R = \text{Cyclohexylammonium} \)
   vs. equivalent Cyclohexylammonium sulfate
D. Dihydroxyacetone in H\(_2\)O

Fig. 12. Spectra of sugar phosphates.
DISCUSSION

The hydrolysis of RuDP

The interesting behavior of RuDP on acid hydrolysis, viz., the equal lability of each phosphate group, is most probably due to intramolecular phosphate group migration.

\[
\begin{align*}
\text{CHOH} & \quad \text{CHOH} \\
\text{CH}_2\text{O} & \quad \text{CH}_2\text{O} \\
\text{P}=\text{O} & \quad \text{P}=\text{O} \\
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{C}=\text{O} & \quad \text{C}=\text{O} \\
\text{OH} & \quad \text{OH} \\
\text{P(OH)}_2 & \quad \text{P(OH)}_2 \\
\text{acid} & \quad \text{acid} \\
\end{align*}
\]

A similar migration to C atom 3 could take place and it would be expected that a phosphate grouping in this position would be acid-labile. With greater quantities of RuDP it would be interesting to see if cyclic phosphates could be isolated after appropriate treatment.

The analysis of the acid hydrolyzates of RuDP and RMP showed two unexpected features:

(a) the appearance of significant amounts of a pentose other than ribulose in the RuDP hydrolyzate,

(b) the presence of arabinose in both the RuDP and RMP hydrolyzates.

The results of the treatment of RuDP with purified Polidase-S indicated that the pentose diphosphate contained some ribose-diphosphate; from the method of preparation it is to be expected that this would be ribose-1,5-diphosphate (RiDP) arising from the action of a phosphopentokinase on RMP. The 1-phosphate group in the RiDP is known to be much more acid-labile than the 5-phosphate, and hence it would be expected that a mixture of RuDP and RiDP, on acid hydrolysis at 37\(^\circ\) with 1 N HCl, should yield a mixture of ribulose and RMP. The parallel experiment with a sample of RMP under these conditions showed its extremely slow hydrolysis. Hence, on passing the diphosphate hydrolyzate through Dowex-1 in the formate form, we confidently expected that only ribulose would be present in the elute. The presence of 25% aldopentose is difficult to reconcile with any other behavior than some rearrangement at the 1,2-C atoms of RuDP on hydrolysis.

The fact that the above-mentioned aldopentose appears to be arabinose (and no ribose), is again unexpected, as is the appearance of arabinose together with ribose, in the RMP solution.
It is clear that before any conclusions can be drawn the experiment should be repeated under very carefully controlled conditions, and the relative amount of the sugars present in the hydrolyzate should be quantitatively determined rather than estimated by use of spray reagents.

SPECTROSCOPIC MEASUREMENTS

There is a surprising number of possible forms in which RuDP might exist in solution; this unusual property is due to the inability of the compound to form a furanose ring. The number is also multiplied by uncertainty as to the position of the phosphate group other than that on C₅. By analogy with other phosphopentokinase and phosphohexokinase systems, the second phosphate group could be expected to reside on C₁. The alkaline oxidation of RuDP to give phosphoglyceric and phosphoglycollic acids favors a 1,5-diphosphate, although the stoichiometry of this reaction has never been accurately determined. The possibility of phosphate migration through intermediate cyclic phosphate formation under both acid and alkaline conditions must also be borne in mind in any interpretation of degradative data. However, if a 1,5-diphosphate is adopted as a basis for argument, the following structures suggest themselves:

\[
\begin{align*}
\text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} \\
\text{C=O} & \quad \text{CHOH} & \quad \text{C-OH} & \quad \text{C-OH} & \quad \text{C-OH} & \quad \text{C-OH} & \quad \text{C-OH} \\
\text{CHOH} & \quad \text{C=O} & \quad \text{CH} & \quad \text{C-OH} & \quad \text{C-OH} & \quad \text{C-OH} & \quad \text{C-OH} \\
\text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} \\
\text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} & \quad \text{CH}_2\text{OP} \\
\end{align*}
\]

Structure II is rendered improbable by the isolation of ribulose after enzymatic dephosphorylation and the nonappearance of CO₂ on periodate treatment.

Structure III is attractive (a) because compounds such as CH₃-CO-CH₂OH are believed to exist partly in the acetol form,

\[
\text{CH}_3\text{-C(OH)-CH}_2
\]

(b) the attack of a bicarbonate ion could be visualized as an opening of the epoxide ring.
Structures V and VI are cyclic phosphate variants of the ene-diol formulation IV. The ene-diol formulations, although the most attractive as regards a mechanism for the carboxylation reaction, suffer from the lack of a suitable conjugating group which could stabilize the ene-diol.

It will be seen that structures I to III, and IV to VI, form two groups which should show different uv absorption, and so a survey was made of various model compounds, including certain sugar phosphates and RuDP (Tables V and VI). The most striking feature that emerges is the relatively high absorption of RuDP, at $\lambda_{\text{max}}$ 260 m$\mu$. The value of the molar extinction, 350 to 500, is too high for this to be due to the low-intensity peak of $-\text{C}=\text{O}$ at 260 to 270 m$\mu$. Hence it seems quite probable that the absorption peak could be due to the presence of an ene-diol type of chromophore; a form of RuDP that would give molar extinction coefficients higher than about 50 is difficult to envisage without invoking this type of chromophore. This seems the most definite statement that can be made at the moment. However, great caution is necessary when dealing with compounds of low-intensity absorption in the 200 to 300 m$\mu$ region, as their purity must be of a very high order before any absorption characteristics can be unequivocally assigned. The classical example of this difficulty is, of course, the alleged absorption of glucose at 260 m$\mu$; the purification of glucose to optical purity standard is a tedious process. Hence, the purification of RuDP up to analytical purity is most desirable. It is encouraging to note that the reprecipitation of the Ba salt of RuDP resulted in increased absorption.

This work was done under the auspices of the U. S. Atomic Energy Commission.
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35. Chemistry of Carbon Compounds (Elsevier) 1A, 709 (1951).