The Hydrolysis of Phosphate Esters of \( \alpha \)-Hydroxy Acids Catalyzed by Molybdate*

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SUMMARY

The hydrolysis of phosphate esters of 2-hydroxy acids is catalyzed by molybdate. Half-times were determined for the hydrolysis of 2,3-phosphoglyceric acid (PGA) (24 min), 2-PGA (34 min), phosphoglycolic acid (35 min), and phospholactic acid (46 min). Conditions are given for determining the distribution of \(^{32}\)P in 2,3-PGA which is hydrolyzed to 3-PGA and inorganic phosphate.

Frequent observations have been made of the increased rate of hydrolysis of relatively labile phosphate compounds in acid molybdate solution (e.g. phosphocreatine (1), acetyl phosphate (2), and 1,3-diphosphoglyceric acid (3)). A study by Weil-Malherbc and Green (4) indicated that ATP, pyrophosphate, phosphoenolpyruvate, and fructose 1,6-diphosphate were hydrolyzed appreciably in the pH range 3 to 5, while fructose 1,6-diphosphate, glucose 1-phosphate, \( \alpha \)-glycerophosphate, and glucose 6-phosphate were quite stable.

The fact that monophosphoglyceric acids form complexes with high optical rotation in the presence of molybdate led to the use of polarimetric procedures for the quantitative determination of these compounds (5). Molybdate forms complexes with many oxygen-containing organic compounds and the affinity for \( \alpha \)-hydroxy acids is particularly high (6). The present study began with the observation of Bartlett that the monophosphoglyceric acids are hydrolyzed at widely different rates in the presence of molybdate (7). The specificity, pH, and temperature dependences have been examined. In the pH range 3 to 5 there is a marked specificity of hydrolysis for phosphate esters of \( \alpha \)-hydroxy acids. A procedure has been worked out for the quantitative hydrolysis of the susceptible compounds. With it, it has been possible to analyze the distribution of isotope in the two phosphate groups of 2,3-diphosphoglyceric acid since only the phosphate on C-2 is liberated as inorganic phosphate which can then be extracted from the unhydrolyzed phosphate with organic solvents.

EXPERIMENTAL PROCEDURE

Materials and Methods

2-PGA\(^1\) (sodium salt), 3-PGA, and 2,3-PGA (cyclohexylamine salts) were purchased from Boehringer and Söhne. 3-PGA (barium salt) was purchased from Nutritional Biochemicals and Schwarz Laboratories. \( \alpha \)- and \( \beta \)-Glycerol-P were purchased from Sigma. Ammonium molybdate was from Merck. 2,3-PGA was also isolated from pig blood (8). Phosphoglycolic acid was obtained as a byproduct of the preparation of glyceraldehyde 3-phosphate from fructose 1,6-diphosphate (9). Phosphoglycolic acid, phospholactic acid, and phosphohydroxypropionic acid were synthesized by chemical methods. The procedures used were essentially those described by Ballou and Fischer for the synthesis of 2-PGA (10). Phosphorylation of the methyl esters with diphenylphosphorochloridate was followed by catalytic hydrogenation to remove the phenyl groups and saponification. The cyclohexylamine salts of phospholactic acid and phosphohydroxypropionic acid were recrystallized from ethanol and acetone. The phosphoglycolic acid was prepared as the barium salt from aqueous solution. Acetol phosphate was a gift from Dr. W. J. Rutter to Dr. I. A. Rose. Crystalline preparations of lactic dehydrogenase, pyruvate kinase, enolase, and phosphoglycerate mutase were purchased from Boehringer and Söhne. These enzymes were used to detect and measure pyruvate, phosphoenolpyruvate, 2-PGA, and 3-PGA (11).

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer with \( \text{H}_2\text{O}-\text{toluene-ethyl alcohol (1:20:10)} \) mixtures. The toluene contained 0.4\% 2,5-diphenyloxazole.

\(^1\)The abbreviations used are: 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; 2,3-PGA, 2,3-diphosphoglyceric acid.

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**Determination of Inorganic Phosphate**—Acid-washed tubes of heat-resistant plastic should be used for the hydrolysis since the prolonged heating in acid molybdate solution produced high blank values in the colorimetric procedures. The method usually followed in this study used Elon as the reducing agent for the phosphomolybdate acid (12). To a sample containing 0.02 to 0.20 μmole of inorganic phosphate in a volume of 0.76 ml, add sulfuric acid, 10 x (0.04 ml), 5% ammonium molybdate solution, 0.28 x (0.04 ml), and Elon reagent (0.16 ml). Mix well after each addition. Read in a spectrophotometer at 660 μm 10 min after the last addition. With each experiment blanks and standards should be subjected to the conditions of the hydrolysis. The extinction coefficient for phosphate was 3.6. Alternatively, the very sensitive procedure of Mozersky, Pettinati, and Kolman (13) may be used in which the absorption of the yellow phosphomolybdic acid is read after extraction into an organic solvent. When isobutanol (water-saturated) was used for the extraction (14) rather than the isobutanol-benzene mixture used by Mozersky et al., an extinction coefficient of 24.4 was found at 320 μm.

**Procedure for Molybdate-catalyzed Hydrolysis**

Satisfactory conditions for hydrolysis were developed with the three phosphoglyceric acids as test compounds. A complete dependence on molybdate and a pH optimum of 4 (Fig. 2) was observed. To apply this procedure to enzymatic studies it may be necessary to remove protein and P₂O₅ prior to hydrolysis.

**Removal of Protein**—If protein is present it must be removed before the addition of molybdate. Trichloroacetic acid added to a concentration of 5% is a satisfactory precipitant in most cases. If, however, it is desired to determine phosphate by the procedure of Mozersky et al. (13) their deproteinization procedure should be followed. The protein precipitate is removed by centrifugation and a measured portion of clear supernatant is used for subsequent steps.

**Preliminary Extraction of Inorganic Phosphate** Sulfuric acid is added to a concentration of 0.1 x. The molybdate solution is added in sufficient amount to extract the inorganic phosphate, 0.10 ml of 0.28 x being used routinely for the extraction of up to 2 μmoles of inorganic phosphate. The compound present under strongly acidic conditions is the heteropoly acid, H₄P₂Mo₇O₃₆ (15). A P₂O₅:Mo ratio of 1:12 was sufficient for the complete extraction of 25P₂ from the aqueous phase into isobutanol. If the amount of inorganic phosphate is large and not known, additional portions of molybdate may be added during successive extractions until the yellow color of phosphomolybdic acid no longer forms when molybdate is added. Water-saturated isobutanol (2 ml) is added to the strongly acidic aqueous phase, the contents of the tube are mixed vigorously, and the phases are separated by a brief low speed centrifugation. The isobutanol layer is removed as completely as possible with a capillary pipette. Since it is not possible to remove the organic phase quantitatively by this procedure, the extraction is repeated twice more with 2-ml portions of isobutanol and the organic phase is removed as completely as possible each time.

**Molybdate-catalyzed Hydrolysis**—In a test tube is placed a neutralized sample (0.5 ml) containing not more than 0.2 μmole of hydrolyzable compound. The following substances are added: 0.1 M sodium acetate buffer, pH 4.9 (0.06 ml), 1.0 M acetic acid (0.06 ml), and 0.28 x ammonium molybdate solution (0.04 ml).

![Fig. 1. Rates of hydrolysis of the phosphoglyceric acids. Samples were removed at various times from incubations containing 2-PGA (C), 3-PGA (△), and 2,3-PGA (X). The phosphate color was determined with Elon as reducing agent and the value was corrected to a standard volume with the aid of glucose-14C included in each incubation.](http://www.jbc.org/)

The test tube is covered and placed in a rack in a boiling water bath for at least five half-lives. After cooling, H₂SO₄ (10 x) (0.04 ml) is added. The inorganic phosphate released may be determined by addition of a reducing reagent. Alternatively, the reducing agent may be omitted and, after the acidification with sulfuric acid, the volume is brought to 1 ml with water. Two milliliters of water-saturated isobutanol are added and the phosphate is extracted. The color of the phosphomolybdic acid in the isobutanol layer may be read as described above. Portions of the aqueous phase, containing the unhidrolyzed phosphate compounds, and the organic phase, containing the hydrolyzed phosphate, may be used for counting 32P in a scintillation counter. Quenching will occur if excess molybdate is present. The amounts indicated in the method do not cause quenching.

**RESULTS**

This study has centered about the usefulness of molybdate in the preferential hydrolysis of phosphate bonds in the phosphoglyceric acids. It was found that when 2-PGA and 3-PGA were heated in molybdate solution near pH 4, the phosphate was released from 2-PGA but not from 3-PGA; 2,3-PGA released just 1 eq of phosphate under the same conditions (Fig. 1). The half-times for hydrolysis were 24 min for 2,3-PGA and 34 min for 2-PGA. Other compounds hydrolyzed under these conditions were phosphoglyceric acid (half-time 30 min) and phosphoäetic acid (half-time 46 min). Compounds in addition to 3-PGA that were not hydrolyzed in 2 hours were 3-phosphohydroxypropionic acid, α-glycerol-P, β-glycerol-P, and acetal phosphate (1-phospho-2-ketopropanol). The hydrolysis appears to be specific for a phosphate group adjacent to a carboxyl. No migration of phosphate between carbon atoms 2 and 3 occurs under these conditions of mild acidity (10, 16).

When only 1 eq of phosphate was released from 2,3-PGA, and in view of the specificity shown by the monophosphates, it was expected that the product of the hydrolysis would be 3-PGA. With enzymatic methods, 3-PGA was identified in the reaction mixture after molybdate hydrolysis. Although the amount of 3-PGA recovered was about 20% less than that expected from the phosphate release, a similar 20% loss of enzymatically assayable phosphoäetic acid was noted. The amount of 3-PGA released was 0.30 eq compared to the expected 0.38 eq. This loss was consistent for both 2-PGA and 3-PGA. It is possible that some decomposition of 3-PGA also occurred under these conditions.
Table I

Rates of hydrolysis of some sugar phosphates

Each incubation was done in a 0.5-ml volume and contained 0.2 μmole of the test compound. A duplicate set of incubations contained no molybdate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No molybdate</th>
<th>With molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole</td>
<td>μmole</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.023</td>
<td>0.055</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>0.003</td>
<td>0.144</td>
</tr>
<tr>
<td>2,3-PGA</td>
<td>0</td>
<td>0.114</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of pH on the rate of hydrolysis. Each sample contained in a volume of 0.9 ml: 0.4 μmole of phosphoglyceric acid and sodium molybdate solution (0.6 μmole) buffered with acetate (for pH 4 and above) or with acetate-HCl (below pH 4). The samples were heated 20 min at 100°C and then cooled. The pH of each sample was determined and found unchanged. Portions of each hydrolyzed sample were used to determine the phosphate released with Elson as the reducing agent.

Table II

Test for formation of acyl phosphate

In a 0.5-ml volume: 2,3-PGA (3 μmole), molybdate (0.08 ml), neutralized hydroxylamine (2 ml) (0.1 ml). Final pH was 6.5. Controls lacking hydroxylamine contained Tris buffer. Final pH 6.5. Heated 90 min at 100°C. Acylhydroxamic color had an extinction coefficient of 0.850 in the presence of heated samples containing molybdate.

<table>
<thead>
<tr>
<th>Inorganic phosphate formed</th>
<th>1.02 μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxamate</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

The work of Weil-Malherbe and Green (4) showed that the hydrolysis of sugar phosphates could be catalyzed by molybdate under certain conditions. Molybdate stimulated the hydrolysis of fructose-6-P and fructose-1,6-di-P while glucose-6-P was not hydrolyzed even after 120 min (Table I). The hydrolysis of fructose-1,6-di-P and especially of fructose-6-P was appreciable even in the absence of molybdate. Interference by these two compounds may be eliminated by borohydride reduction prior to hydrolysis.

The pH dependence of the hydrolysis was determined for phosphoglyceric acid. Fig. 2 shows that the rate is most rapid at pH 4. This is characteristic of reactions in which the phosphate monoanion is the reactive species and is the pH profile found for the hydrolysis of several classes of phosphate esters in the absence of molybdate (17).

The observation that a carboxyl group was required for hydrolysis, as illustrated by the lack of reactivity of α-glycerol-P and acetol phosphate, suggested that the reaction might occur with the intermediate formation of an acyl phosphate which would be hydrolyzed readily to free acid and phosphate in the presence of molybdate. This hypothesis was tested by having hydroxylamine present during the hydrolysis of 2-PGA or 2,3-PGA to trap any acyl phosphate formed and produce a hydroxamic acid. The reaction was carried out at pH 6.5 at which the hydrolysis was slow but the acyl phosphate would be more stable. Table II shows that no hydroxamate formation could be detected. It was found possible to trap added acetyl phosphate quantitatively as the hydroxamate when it was added to control incubations at any time during the heating period. The color of the hydroxamic acid in the Lipmann and Tuttle procedure (18) was diminished in the presence of molybdate but this was corrected for by the use of internal standards. Acyl phosphate at a level of 15% of the amount of the total reaction, as measured by phosphate release, could have been observed.

**Discussion**

In early attempts to analyze the 32P isotope distribution in 2,3-PGA, various enzymatic procedures were tried. It seemed unlikely that nonspecific phosphatases which were found to have high K₉ values for 2,3-PGA, and which hydrolyzed 3-PGA more rapidly than 2,3-PGA, would give unambiguous results. The pyrophosphate-activated 2,3-PGA phosphatase from rabbit muscle described by Zancan, Recondo, and Leloir (19) did remove only one phosphate and could be used to determine 2,3-PGA quantitatively but it contained monophosphoglycerate mutase. Attempts in this laboratory to remove the mutase by purification procedures were not successful (20). Hashimoto, Nakao, and Yoshikawa (21) and Hashimoto, Takahashi, and Yoshikawa (22) reported the preparation of a 2,3-PGA phosphatase, also from rabbit muscle, that produced only 3-PGA as product and could be used in the desired manner. When the enzyme was prepared according to their directions, the resulting preparation had the same ratio of 2,3-PGA phosphatase to phosphoglycerate mutase as the crystalline rabbit muscle phosphoglycerate mutase obtained commercially. Both preparations could be activated to the same extent by pyrophosphate. It was concluded that the 2,3-PGA phosphatase activity of rabbit muscle was not due to a separate enzyme but to the phosphoglycerate mutase itself (see also Grisolia and Tecson (23)).

An attempt to use the 2,3-PGA co-factor activity in the monophosphoglycerate mutase reaction as an analytical procedure was similarly unsuccessful. In the presence of a high concentration of 3-PGA, a rate-limiting amount of mutase was coupled to enolase, pyruvate kinase, and lactic dehydrogenase and the course of the reaction was observed spectrophotometrically. It was expected that 2,3-PGA which was labeled in the phosphate on C-2 would be converted to 2-PGA and trapped in the subsequent
reactions. It was found that, after the reaction had gone to 8 times
the amount of the 2,3-PGA present, the 2,3-PGA had lost only
42% of its radioactivity. It was concluded that 2,3-PGA does
not turn over rapidly in this system (see also Reference 24).

The molybdate method for the hydrolysis of phosphate esters
of α-hydroxy acids may be useful as a means of determining the
concentration of 2,3-PGA when the enzymatic methods usually
employed would be inapplicable because of the presence of inter-
facing substances. It differentiates between 2-PGA and 3-PGA
and can analyze their phosphate groups separately. Further
study of the mechanism and analytical applications would be of
interest, but are not planned at this time.

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