

References:

Fiske, C.H.; Subbarow, Y. *J. Biol. Chem.* **1925**, 66, 375.

Palmer, R.E. *J. Chem. Ed.* **1985**, 62(10), 898.

Introduction

The phosphomolybdate method is one of the more common methods for determining inorganic phosphate in samples. Molybdic acid (generated by addition of sulfuric acid to ammonium molybdate) is added to the solution containing inorganic phosphate, and the colorless phosphomolybdic acid complex is formed. A reducing agent is then added which converts the complex to the intensely blue phosphomolybdous acid complex. This complex is quite stable and color generation follows Beer's Law. The method will not detect organically bound phosphate, so it can be used to determine inorganic phosphate, even in the presence of organic phosphates. Organic phosphates may then be hydrolyzed to yield inorganic phosphate, and this method used to quantitate organic phosphate.

Organic phosphates have a central role as important intermediates in many metabolic pathways. Release of inorganic phosphate from these compounds under gentle hydrolysis conditions, allows one to characterize different forms of organic phosphate. Phosphate bonds with a free energy of hydrolysis more negative than -5.0 kcal/mol, such as phosphoanhydrides and acetal-phosphates, can be hydrolyzed at 100°C with dilute acid. The large free energy change indicates that these classes of bonds are relatively unstable. Conversely, bonds with small free energy changes, such as phosphoesters, will not hydrolyze under these conditions. Analysis of samples for inorganic phosphate, following gentle hydrolysis, and comparison with unhydrolyzed samples will allow one to quantitatively determine the presence of high energy organic phosphate linkages. Total phosphate determination will allow one to determine the other organic phosphate linkages.

Solutions:

- 1) Reducing Solution (200mL): 0.4g 1-amino-2naphthol-4-sulfonic acid, 2.4g sodium bisulfite, 2.4g sodium sulfite.
- 2) 2.5 M Sulfuric Acid
- 3) 1 M Hydrochloric Acid
- 4) 1 M Sodium Hydroxide
- 5) 2.5% Ammonium Molybdate Solution
- 6) 10% $\text{Mg}(\text{NO}_3)_2$ in EtOH
- 7) 10.0 mM Stock Phosphate Solution (potassium phosphate, monobasic)
- 8) 10.0 mM Stock Standard Glucose-6-Phosphate
- 9) 10.0 mM Stock Standard Glucose-1-Phosphate
- 10) 10.0 mM Stock Standard AMP (Adenosine 5'-triphosphate, disodium salt)
- 11) 10.0 mM Stock Standard ATP (Adenosine 5'-monophosphoric acid, sodium salt (assumed dihydrate))

Laboratory:

Phosphomolybdate Method – (For **Inorganic Phosphate Standards**, and for **Organic Phosphate Bond Analysis**)

To the sample (standard P_i or reacted organic phosphate) add 1 mL of 2.5 M H₂SO₄, and then 1 mL of 2.5% ammonium molybdate. After mixing, add 0.1 mL of the reducing solution and then add distilled water to bring the total volume up to 10.00 mL. Mix thoroughly. After 10 minutes read the absorbance at 650 nm.

Standard Curve – (**Inorganic Phosphate Standards**)

Do a **1:5 dilution of a stock standard phosphate** solution (10.0 mM) to yield a working standard phosphate solution (2.00 mM). Prepare 5 test tubes for color standards. Test tube #1 (blank) will contain no working standard phosphate solution. Pipet 0.20, 0.40, 0.60, and 0.80 mL of the working standard solution into the remaining tubes. Add the rest of the reagents in the order described above.

Gentle Hydrolysis – (**Of Your Organic Phosphate Molecule**)

Add 1 mL of 1 M HCl to 1 mL of the stock solution for your organic phosphate molecule, and heat in a boiling water bath for exactly 7 minutes. Immediately neutralize by adding 1 mL of 1 M NaOH and add 2 mL of distilled water to bring the total volume up to 5 mL. **Analyze 0.5 mL aliquots** for inorganic phosphate by the phosphomolybdate method as described above.

Total Phosphate – (**Of Your Organic Phosphate Molecule**)

Add 6 drops of 10% Mg(NO₃)₂ in ethanol(w/v) to 1 mL of the stock solution for your organic phosphate molecule. **Slowly and cautiously** heat the solutions until dry, where a white ash remains. Add 1 mL of 1 M HCl, and heat in a boiling water bath for 10 minutes. Next add 1 mL of 1 M NaOH and 3 mL of distilled water to bring the final volume up to 5 mL. **Analyze 0.5 mL aliquots** by the phosphomolybdate method. All phosphate from the samples should have been liberated.

Report:

- 1) In your notebook, include a brief summary of the experiment and describe what the gentle hydrolysis accomplishes along with the phosphomolybdate method when studying organic phosphate bonds.
- 2) In your notebook, include the values for the ΔG° , and write down the reactions, for hydrolysis of phosphoester, phosphoanhydride and acetal phosphate linkages.
- 3) Prepare a Table and a Figure, using a spreadsheet, for the phosphate standards with the regression results and best-fit line as usual.
- 4) Prepare a Table showing the concentrations of organic phosphates obtained from no hydrolysis, gentle hydrolysis and total phosphate for each of the sample compounds studied. (Spreadsheet provided by instructor.)
- 5) Write a brief abstract for the experiment with purpose, methods, results and conclusions as usual.