Phosphate Determination using the Varian DMS 90 Spectrophotometer

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Introduction
The ubiquity of phosphates, both organic and inorganic, is so well known that it requires no further comment. Hydrolysis or digestion of phosphates to yield orthophosphate followed by conversion to a phosphomolybdate complex forms the basis for quantitative analysis of these compounds. After digestion and formation of the phosphomolybdate complex, the complex is reduced to the blue chromophore that is determined spectrometrically.

The various methods in common use differ in the oxidizing agents employed in the digestion and in the reducing agents used for reduction of the phosphomolybdate complex. Sulphuric acid and 1-aminonaphthol-4-sulphonic acid (ANSA) are employed as oxidizing and reducing agents respectively in the methods of Fiske and Subbarow (1) and Bartlett (2); perchloric acid and ANSA in the methods of King (3) and variations on it (4), and magnesium nitrate and ascorbic acid in the method of Ames (5). The blue chromophore generated by reduction of the phosphomolybdate complex is measured at either 830 nm (5) or 820 nm (1-4).

Two procedures are described below; the methods employing perchloric acid, ANSA (4) and magnesium nitrate and ascorbic acid (5). In the illustrations presented here, the modification of the procedure of Bartlett (2) using perchloric acid in the sample digestion (4) was employed. Results from an analysis of a protein which was being examined to determine if an O-blocked tyrosyl residue was due to tyrosine-O-phosphate are given as an example of use of one phosphate determination method (6).

Equipment
1. Varian DMS 90 UV-Visible Spectrophotometer (Part No. 00-100222-00).
2. 1 cm pathlength semi-micro cuvettes (Part No. 00-998798-54).
3. Digestion heater (4) or microburner.
4. Boiling water bath or electrical heating block heater.
5. Lang-Levy constriction micropipettes or Eppendorf or Gilson pipettors.

Spectrophotometer Operating Parameters
Wavelength 830 nm (Method 1); 820 nm (Method 2)
Slit width 4 nm (based on a natural bandwidth of ca 75 nm)

Reagents
Method 1: (Modified Procedure of Bartlett)
Standard phosphate solution, 10 mM:
1.36 g of dried potassium dihydrogen phosphate (KH₂PO₄) is dissolved in 1000 mL of chloroform-saturated, deionized water. Store at 4°C. Dilute to 1.0 mM for use in preparing a standard curve.

Ammonium molybdate solution:
4.4 g of ammonium molybdate (NH₄MO₇O₂₄·4H₂O) is dissolved in 300-500 mL of water. 14 mL of H₂SO₄ (conc) are added and the volume adjusted with water to 1000 mL.

ANSA (1-aminonaphthol-4-sulphonic acid):
Stock solution 0.5 g ANSA, 30 g of sodium hydrogen sulphite (NaHSO₃), and 6 g of sodium sulphate (Na₂SO₄) are dissolved in 250 mL of water in the dark.
The reagent is allowed to stand at room temperature for 3-4 hours filtered into an amber bottle and stored at 4°C. The stock reagent is stable for more than one month.
ANSAL Working solution:
The stock solution is diluted in water to 1/12 of its initial concentration.
70-72% perchloric acid (analytical reagent).
Method 2: (Procedure of Ames)
Phosphate Standard: See Method 1.
Ammonium molybdate solution:
0.42% (w/v) 4.2 g is ammonium molybdate dissolved in 500 mL of water, 28.6 mL H2SO4 (conc) and water to 1000 mL.
Ascorbic acid:
10% (w/v) in H2O. The working reagent is prepared from 1 volume of ascorbic acid stock reagent plus 6 volumes of the ammonium molybdate solution. This reagent is stable for 8 hours in an ice bath.
Magnesium nitrate:
10% (w/v) Mg(NO3)2.6H2O in 95% ethanol.
0.5 N hydrochloric acid.

Procedure
Method 1
Phosphate containing solutions (10-200 μL; 0-150 nmol PO4) are placed in 13 x 100 mm Pyrex or Kimax test tubes and evaporated to dryness, taking care to avoid spattering. 0.4 mL of 70-72% perchloric acid (0.48 mL of 60% has been used without difficulty) is added and the samples digested on the heating rack until colorless or a light straw yellow. After cooling to room temperature, 2.4 mL of ammonium molybdate and 2.4 mL of the working ANSA solution are added and the samples mixed on a vortex mixer. Samples are heated for 10 minutes in a boiling water bath, cooled, and the absorbance read at 830 nm. Blanks (either deionized water, dialysate or perchloric acid alone), standards and unknown samples are run in duplicate. Absorbance at 830 nm for a sample that contains 100 nmol of PO4 is 0.43 ± 0.02. Linear regression to a straight line is used to produce the standard curve and the phosphate concentration in the unknown samples calculated from the regression equation.
Method 2
Phosphate containing solutions (10-100 μL, 0-70 nmol PO4) are placed in 13 x 100 mm Pyrex or Kimax test tubes, 30 μL of the magnesium nitrate solution is added and the sample taken to dryness over a Bunsen or Moeker burner. The samples are heated over the flame until the brown fumes cease to be evolved. After cooling, 300 μL of 0.5 N HCl are added and the sample heated in a boiling water bath or in an electric block heater for 15 minutes. (Tubes are "capped" with marbles to minimize evaporative losses.) The tubes are cooled to room temperature and 700 μL of the ascorbic acid/ammonium molybdate working solution are added. The chromophore is produced by heating the samples at 37°C for 1 hour or 45°C for 20 minutes. After cooling, the absorbance is read at 820 nm. Absorbance for a solution containing 10 nmol of PO4 is approximately 0.26. Blanks (either deionized water or dialysate), standards and unknown samples are run in duplicate. For protein and other samples that contain only small amounts of PO4 relative to the amount of hydrocarbon, the ashing procedure may need to be repeated to ensure complete digestion.

Results
Standard curves, one prepared using samples that contained 5 mg of bovine plasma albumin (Cohn Fraction V, Sigma Chemical Co., St. Louis, MO) per sample and the other only the usual PO4, are shown in Figures 1a and 1b, respectively. The albumin was added to ascertain if protein was adversely affecting the digestion procedure and that 0.1 mole of PO4 per mol protein could be satisfactorily determined. No blank (dialysate) correction was made to the data shown in the figure. Approximately 0.3 mol of PO4/mol of albumin was detected in the albumin sample. The slopes of these standard curves (4.43 x 10^-3 A/nmol Figure 1a and 4.06 x 10^-3 A/nmol Figure 1b) were not significantly different from the mean slope determined for 7 different standard curves with the same reagents, 4.31 x 10^-3 ± 0.19 x 10^-3 A/nmol. Bovine blood clotting factor X2 was examined for the presence of phosphate as tyrosine-O-phosphate because of the demonstration of a blocked tyrosine residue in this protein (6). Less than 0.1 mol of PO4 mol protein that could be associated with the blocked tyrosyl residue was found leading to the conclusion that the substituted tyrosyl residue was not tyrosine-O-phosphate but most probably tyrosine-S- sulphate.

Discussion
Method 1, the modified procedure of Bartlett is routinely used in the author's laboratory using devices such as Eppendorf or Pipetman micropipettors. This approach, a compromise that sacrifices precision and accuracy for convenience produces linear standard curves with slopes that are reproducible from day to day to within ±4% (1 std dev) and with an average correlation coefficient of 0.999. For greater precision and accuracy, classical procedures employing calibrated glassware and pipettes should obviously be employed. When necessary calibrated Lang-Levy micropipettes have been used.
The sensitivity of both procedures described above makes them highly susceptible to contaminating phosphate, particularly from detergents commonly used for cleaning glassware. Such contamination and losses due to spattering during digestion are most likely the principal sources of error in these assay procedures. Separate test tubes that are only cleaned with dichromate-sulphuric acid cleaning solution and rinsed with deionized water are maintained in the author's laboratory to minimize the phosphate contamination error.
Arsenate and, in some circumstances, silicates may react to form a blue chromophore that can contribute very significantly to the measured absorbance. The original references should be consulted for further information about these interferences and the conditions under which they are significant. Perchloric acid and perchlorates can be explosive and, thus, when large numbers of samples over an extended period of time are analyzed, the accumulation of perchlorates in the fume hood exhaust system should be prevented.

References