

A Micro Phosphate Method¹

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A spectrophotometric method for the determination of inorganic phosphate in tissue extracts has been developed by Griswold, Humoller, and McIntyre (1). It uses the reducing agent (aminonaphtholsulfonic acid) of the Fiske and SubbaRow method (2) and the strong acid conditions necessary for the development of the heteropoly blue complex described by Boltz and Mellon (3). The main advantage of this method over previous methods is that nonspecific molybdenum blue color production is prevented and the heteropoly blue color which is proportional to the phosphate concentration is stable for at least 24 hours.

To make this method applicable to the determination of organic phosphorus we have used the acid required for optimal color development to digest the organic compounds and perform the whole procedure (digestion and color development) in a micro Kjeldahl flask. The volumes of reagents have been reduced and their concentrations altered such that accumulated pipet deliveries bring the reaction solution to the correct final volume. We have also found it necessary to increase the acidity and to read at a slightly different wavelength than described originally by Griswold, Humoller, and McIntyre (1). Although the reaction is linear over the range of 0-10 μg phosphorus, the accuracy of reading the absorbance scale on the Beckman DU spectrophotometer limits the most accurate range between 0 and 4 μg phosphorus.

Apparatus and Reagents. All absorbance readings were made with a Beckman Model DU spectrophotometer, using 1 cm quartz cells and water in the reference cell.

Standard Phosphate Solution. Dissolve exactly 0.3510 gm of pure dry monopotassium phosphate in water. Add 10 ml of 10 N H_2SO_4 and dilute to 1 liter. This solution contains 80 μg phosphorus per milliliter and is stable indefinitely. Fresh working standards are made from suitable dilutions of the standard solution.

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Ammonium Molybdate Tetrahydrate. 0.28% (w/v) in water and stored in a polyethylene bottle.

7.2 N H_2SO_4 . Dilute 200 ml concentrated H_2SO_4 to 1 liter. Standardization is not necessary.

Aminonaphtholsulfonic Acid (ANS). Weigh into a 25 ml volumetric flask 50 mg of anhydrous sodium sulfite, 150 gm sodium bisulfite, and 25.0 mg of recrystallized ANS (recrystallized according to the method of Fiske and SubbaRow (2)). Grind the ANS into solution with a glass stirring rod using a minimal volume of water. When dissolved, dilute to volume. This solution will keep about 2 weeks if protected from light.

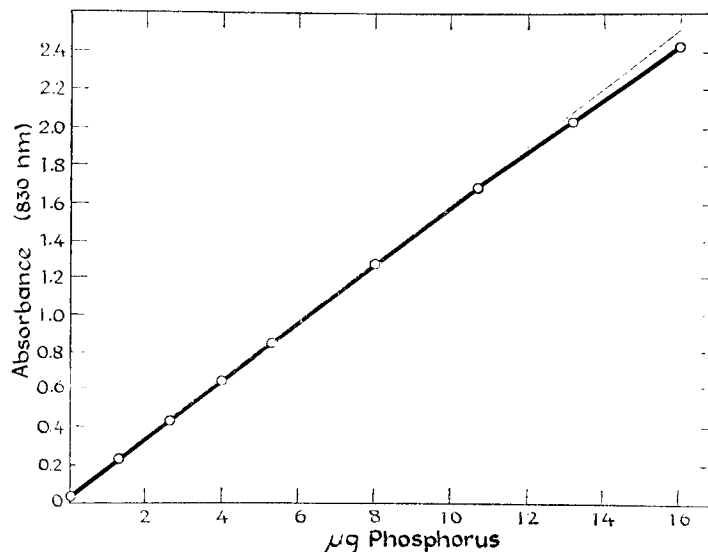


FIG. 1. Phosphorus standard curve.

Procedure for Organic Phosphorus Determination. The sample is pipetted into a 30 ml micro Kjeldahl flask (Microchemical Specialties, Berkeley, California) along with 1.0 ml of 7.2 N H_2SO_4 and a few acid-washed Carborundum boiling chips. The sample is evaporated on a micro Kjeldahl digestion rack (Microchemical Specialties) until constant-boiling sulfuric acid is obtained (white fumes of SO_3 begin to rise in the neck of the flask). The flame is reduced to prevent the loss of these SO_3 fumes and digestion is continued for 30 minutes. These hydrolysis conditions proved satisfactory for use with nucleic acids and their derivatives; however, for more resistant phosphate esters additional treatment may be necessary. One or two drops of 30% H_2O_2 may be cautiously added directly to the sample and digestion continued until the solution

clears. The addition of H_2O_2 may be repeated as needed but the blanks should be similarly treated.

Cool the flasks in tap water and add 4.5 ml of the molybdate solution and 0.5 ml of the ANS solution. Attach a test tube clamp to each of the flasks and hang them on the edge of a boiling water bath so that the bottom part of the flask floats on the water but most of the neck of the flask is not heated (i.e., not over the bath). Heat the sample for 10 minutes to develop the color. Cool in tap water ~ 3 minutes, mix any condensate on the neck of the flask, and read the absorbance at 830 nm.

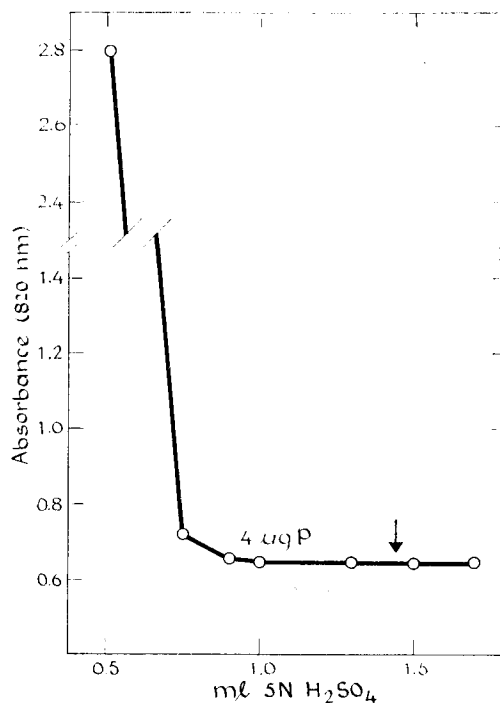


FIG. 2. Effect of sulfuric acid concentration on formation of heteropoly blue. Arrow indicates concentration recommended. All samples contained 4 μ g P.

A standard curve is generated by treating aliquots of the standard phosphate solution in the same manner as described above. A plot of the observed absorbance versus μ g phosphorus on linear paper provides a standard curve from which the phosphorus content of the unknown samples can be obtained (Fig. 1).

Discussion. Phosphate reacts with molybdic acid to form heteromolybdic acids. Boltz and Mellon (3) observed that, in the presence of 1 *N* sulfuric acid and heat, the heteropoly complexes produce a blue color

the intensity of which is proportional to the amount of phosphorus present. Molybdic acid is not affected under these conditions. When color development is carried out at lower acidity, as in the method of Fiske and SubbaRow (2), the molybdic acid itself (even in the absence of phosphorus) is readily reduced to a blue complex ("molybdenum blue") that has a maximum around 650 nm rather than at 830 nm as observed for the heteropoly blue complex. Furthermore, the heteropoly blue color is stable for at least 24 hours.

Griswold, Humoller, and McIntyre (1) used the reductant of the Fiske and SubbaRow method (2) and the acidity of the Boltz and Mellon method (3) and developed a very sensitive method for determining inorganic phosphate in tissues.

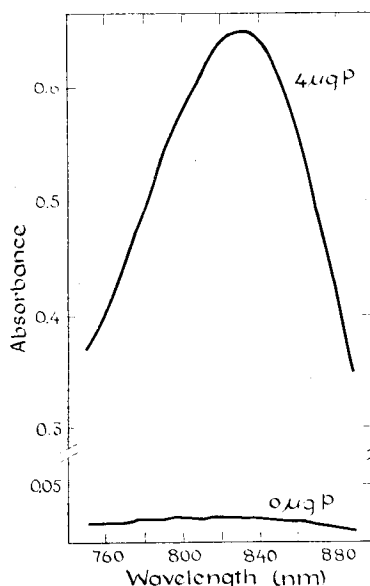


Fig. 3. Absorption spectra for reagent blank and a sample containing 4 μ g P.

We have further modified this method so that it can be used for the determination of organic phosphorus by (1) reducing the volume of reagents used, (2) conducting all steps of the procedure (digestion and color development) in a micro Kjeldahl flask, and (3) using the pipetted addition of reagents to bring the reaction mixture to the correct volume.

More recently we observed that the acidity used by Griswold, Humoller, and McIntyre (1) is barely the minimum required to give a stable color (Fig. 2). For this reason we increased the final acidity from 1 to 1.44 *N*. The necessity for this increase was first brought to light by the fact that

duplicate standards sometimes did not agree. This was traced to the fact that during the evaporation and digestion steps some of the acid had been lost as SO_3 by using too hot a flame. By increasing the acidity a slight loss of SO_3 is not as critical but is still an important step to control if reproducible results are to be obtained.

The heating time in the boiling water bath for the development of the heteropoly blue color is not critical in the range of 7 to 13 minutes. At shorter times full color is not developed but can be brought up to full color with further heating. This means that the time interval between adding the reagents and the heating step is also not critical. Heating as long as 20 minutes resulted in an absorbance increase of 0.010 compared to the sample heated for 10 minutes. This increase is probably due to evaporation.

Griswold, Humoller, and McIntyre (1) read their solutions at 820 nm. We find the peak to be at 830 nm as did Boltz and Mellon (3) and now read our samples at this wavelength, although it is granted that due to the flatness of the curve in this area the differences in the readings between 820 and 830 nm are slight (Fig. 3).

The facts that the reductant solution is stable for at least a week, that no nonspecific color is produced at the higher acidity used, that the heteropoly color specific for phosphorus is stable for at least 24 hours, and that the spectrophotometric sensitivity of the method is excellent between 0 and 4 μg phosphorus, combined with the modification for handling organic phosphorus samples, have made this method particularly effective for the determination of the phosphorus content of the nucleic acids and their derivatives (4). It is also of particular value for the determination of inorganic phosphorus in samples that might otherwise be too dilute for assay by other methods.

REFERENCES

1. GRISWOLD, B. L., HUMOLLER, F. L., AND MCINTYRE, A. R., *Anal. Chem.* **23**, 192 (1951).
2. FISKE, C. H., AND SUBBAROW, Y., *J. Biol. Chem.* **66**, 375 (1925).
3. BOLTZ, D. F., AND MELLON, M. G., *Anal. Chem.* **19**, 873 (1947).
4. SMITH, K. C., AND MEUN, D. H. C., *Biochemistry* **7**, 1033 (1968).