Assay of Inorganic and Organic Phosphorus in the 0.1–5 Nanomole Range

For microchemical studies of retina in small animals, we needed a highly sensitive and reliable assay for nanomole amounts of organically combined P such as in phospholipids and phosphoproteins, and of picomole amounts of inorganic P liberated in enzymic reactions. The most sensitive method of P detection is based on the principle that at a low pH Malachite Green forms a complex with phosphomolybdate (1) with a marked shift in absorption maximum and a high molar absorption coefficient (112,000). To stabilize the dye–phosphomolybdate complex, Tween 20 has been employed in a method described for assay of phospholipid P (2). Another method, described only for assay of inorganic P, has been published (3,4) that uses a different detergent (Sterox) and a lower concentration of dye. We found Sterox to be a better detergent, giving improved clarity of color, stabilization, and reproducibility, while the lower dye concentration led to lower blank values. We have used these and other factors to develop a Malachite Green method for analysis of organic P in the 1–8 nmole range and of inorganic P in the 0.1–8 nmole range.

MATERIALS

Chemicals

(a) Aqueous solution of 0.045% Malachite Green hydrochloride indicator (Matheson, Coleman & Bell), mixed with a Teflon-coated stirring bar and magnetic stirring plate; the solution was stored at room temperature in a plastic-stoppered brown glass bottle; when new solution was required the bottle was rinsed thoroughly with distilled water and drained, but no further cleaning with acid was used and detergent was avoided at all times. (b) Ammonium molybdate (4.2%) in 4 n HCl. (c) Ammonium molybdate–Malachite Green solution: to a polyethylene bottle 3 vol of the 0.045% Malachite Green and 1 vol of the 4.2% ammonium molybdate in 4 n HCl were added and mixed by magnetic stirring for 30 min; the solution was filtered through Whatman nos. 5 or 6 filter paper. (d) Ammonium molybdate–Malachite Green–Sterox color reagent: to 5 ml of the ammonium molybdate–Malachite Green solution,
100 μl of 2% Sterox were added and mixed. This solution was stable for about a week at 4°C, with some increase in blank values. Sterox (2% solution) was from Coleman Instruments (flame photometer standard). Some Sterox may contain objectionable amounts of P, such as Harleco Sterox, recommended by Hohenwallner and Wimmer (4). (e) Perchloric acid, 60%, A. R. (f) Inorganic phosphorus standard consisted of KH$_2$PO$_4$ (dried 4 hr at 110°C) as a stock solution in 0.01 N H$_2$SO$_4$; dilutions were made so that 20 μl portions contained 0.1–5 nmoles P. (g) Organic P standard consisted of sphingomyelin, one of the most difficult phospholipids to hydrolyze. A 10 mg sphingomyelin standard (Supelco) was diluted to 10 ml with chloroform:methanol (2:1, v/v). From this dilutions were made so that 20 μl portions contained 0.5–5 nmoles P. The volume of standard was restricted to 40 μl or less, in conformity with the 40 μl vol of acid digestion mixture added in the routine test.

**Apparatus**

For acid digestion of organic P compounds at 190°C in the routine method, a heating base (Hallikainen Instruments, Richmond, Calif., max temp 300°C) was used, to which a heating block (Temp-Blok) with 20 × 10 mm holes was adapted by packing aluminum foil around it. Disposable culture tubes (or Pyrex tubes) 75 mm long and 10 mm in diameter were cut uniformly to the same height (50 mm) with an electric cutting wheel, so as to project out of the heating block only a few millimeters. This allowed uniform, rapid loss of perchloric acid during the digestion step and enabled grasping of the tubes with forceps. Tubes for the ultramicro inorganic P method were 6 mm diam Kimax microtubes.

**Precautions**

All glassware was washed with 50–75% sulfuric acid and rinsed in distilled water; reaction tubes and fused silica microcuvettes were washed with the acid immediately after use. Safety precautions for perchloric acid have been described in detail (5). The use of microvolumes reduces but does not eliminate the possibility of an explosive incident when the acid is heated with organic material, and the presence of sulfuric acid increases the danger. The experiment should be conducted in a hood with strong draft. In addition, safe elimination of the perchloric acid fumes can best be achieved by exhausting them through a large inverted funnel mounted over the heating block and attached to a water aspirator or pump. This prevents contamination of the fume hood and ducts and discharges the potentially explosive vapors to the water of the drain.
PROCEDURE

**Routine Organic and Inorganic P Method**

For analysis of organic P samples (which require digestion to release the P) standards of organic P and inorganic P, and water blanks were pipetted into microtubes and the water or organic solvent was removed either by heating block or vacuum pump. Volumes were preferably kept within 40 μl, the volume of digestion mixture to be added. To the dry samples, standards, and blanks, 10 μl of 10 N H₂SO₄ and 30 μl of 60% perchloric acid were added and mixed. The tubes were heated 25 min at 190°C in the heating block in a hood with strong draft and the vapors were exhausted directly by an inverted funnel connected to a water pump. The perchloric acid completely evaporated under these conditions and, in the case of tissue lipid extracts, the residue was perfectly clear with a nearly invisible film of sulfuric acid left at the bottom of the tube. If in unusual conditions so large an amount of organic material was present that the sample was not clear after 25 min of digestion, an additional 30 μl of 60% perchloric acid was added and heating continued another 25 min; this redigestion was repeated if necessary to achieve clarity.

After the tubes had been removed from the heat and cooled, a 75 μl vol of water was added to all tubes and mixed. A 400 μl vol of ammonium molybdate-Malachite Green-Sterox reagent was then added to each tube during agitation with a vortex mixer. A brilliant green color developed immediately in the tubes containing P, while the blank was yellow. After 10 min, or just before reading, a group of samples was remixed and let stand briefly for bubbles to clear before transfer to a cuvette. The color was read at 660 nm in fused quartz microcuvettes (10 × 25 × 3 mm) in a Gilford 2400 spectrophotometer with microcell carrier and pinhole. The color was stable and if the complex settled out after many hours, it could be resuspended by vortex mixing. Blanks read 0.070–0.160 against air, depending on age of the color reagent (d) and the freedom of glassware and individual chemical ingredients from P contamination.

For analysis of inorganic P samples, the digestion step was omitted. To the dry samples and blanks, 10 μl of 10 N H₂SO₄ were added and mixed, followed by 75 μl of water and 400 μl of ammonium molybdate-Malachite Green-Sterox reagent, and the test was continued as described above.

**Ultramicro Inorganic P Method**

To accommodate the method to the 0.1–1 nmole range for inorganic P, the assay system consisted of 10 μl of water added with standard or
sample, 5 μl of 3 N H₂SO₄, and 75 μl of ammonium molybdate–Malachite Green–Sterox color reagent, mixed during addition.

RESULTS AND DISCUSSION

Digestion of Organic P Compounds

The digestion step was modified from that of Chalvardjian and Rudnicki (2), who heated with perchloric acid alone for 15 min at an unspecified temperature. We found inclusion of sulfuric acid in the digestion mixture essential for reproducible results. In 25 min at 190°C, comparable absorptions were obtained with equal amounts of inorganic P and of sphingomyelin standards (Fig. 1). Results for the inorganic P standard, but not for the phospholipid, tended to depart from linearity at the highest concentrations when carried through the digestion procedure. To

![Figure 1](image-url)

**FIG. 1.** Standard curves of KH₂PO₄ and sphingomyelin in routine organic P assay (480 μl final volume). Each point is a mean ± Standard Deviation (SD) for triplicate determinations. The molar absorption coefficient is 112,000.
test reproducibility of the method for tissue phospholipids, rat retinas were extracted with 19 vol of chloroform:methanol (2:1, v/v) and washed lower phase lipids were prepared as described previously for brain (6). The extract was diluted with 2:1 chloroform:methanol so that 10–30 μl vol would contain approximately 1–3 nmoles of phospholipids. Linearly greater optical densities were obtained with successively greater volumes of lipid extract (Fig. 2).

In the routine method for inorganic P (no digestion, 500 μl final volume) the curve was linear even at the higher concentrations. The possible formation of inorganic pyrophosphate at high concentrations of inorganic P during the digestion procedure may explain a decline in linearity when inorganic P was used as a standard in the organic P method.
Ultrasensitive assay of inorganic P

Picomole quantities of inorganic P could be analyzed by the reduction of the volume of the test to 90 μl (Fig. 3). We did not try to determine organic P on this scale, although with the appropriate sized heating block and matching tubes the digestion procedure probably could be carried out.

REFERENCES

Laboratory of Vision Research, National Eye Institute, National Institutes of Health, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland

Received May 6, 1974; accepted July 17, 1974