those reported for free bases and nucleosides chromatographed on normal paper with water as a developing solvent.[7] After migration of the bases and nucleosides with water as solvent, the papers may be dried and then developed with 0.3 *M* LiCl in order to move nucleoside diphosphate sugars and monophosphates; they may also be developed with 0.8 *M* LiCl in order to separate di- and triphosphates.

*Separation of Pyrophosphorylase Reaction Products.* In a typical experiment, 1 micromole each of the nucleoside triphosphate and the hexose 1-phosphate, 2 micromoles of $Mg^{++}$, and enzyme are added to Tris-acetate buffer (pH 7.8) to a total volume of 0.3 ml and incubated. The mixture is heated to 100° for 1–2 minutes to stop activity, centrifuged, and spotted directly on PEI paper.[8] Separation of the reactants and products may be achieved in about 50 minutes with 0.4 *M* LiCl, and the results are qualitatively similar to those illustrated in Fig. 2.

*Direct Characterization of Nucleoside Diphosphate Sugars on PEI Paper.* As chromatography with 0.3 *M* LiCl separates nucleoside diphosphate sugars from other nucleotides and from each other (with the exception of adenosine and inosine compounds), the determination of their positions relative to GMP ($R_{GMP}$) gives a preliminary qualitative identification. The base may often be characterized by directly scanning the papers at various wavelengths of light. When scanning at 250, 260, and 280 mμ, characteristic values of the absorbancy ratios may be calculated as follows[1]: $R_a\ \Delta = (250\text{–}260)/(250\text{–}280)$. This technique has proved very useful for the separation of inosine and adenosine compounds, and under some conditions it might be very helpful with other nucleosides.

[7] G. R. Wyatt, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 250. Academic Press, New York, 1955.

[8] H. Verachtert, S. T. Bass, and R. G. Hansen, *Biochem. Biophys. Res. Commun.* **15**, 158 (1964).

## [10] Assay of Inorganic Phosphate, Total Phosphate and Phosphatases

By Bruce N. Ames

### Assay of Inorganic Phosphate[1]

*Principle.* The phosphomolybdate complex is reduced by ascorbic acid. The method is about 7 times as sensitive as the Fiske-SubbaRow

[1] P. S. Chen, Jr., T. Y. Toribara, and H. Warner, *Anal. Chem.* **28**, 1756 (1956).

procedure and involves less pipetting. One can easily determine 0.01 micromole of phosphate.

*Reagents*

(a) Ascorbic acid, 10%. This should be kept in the refrigerator and is good for about a month.

(b) 0.42% Ammonium molybdate·4 $H_2O$ in 1 *N* $H_2SO_4$ (28.6 ml conc. $H_2SO_4$ and 4.2 g ammonium molybdate·4 $H_2O$ to 1000 ml $H_2O$). This solution is stable at room temperature.

Mix: 1 part of (a) to 6 parts of (b). This solution will keep in an ice bath for the day.

*Procedure.* Add 0.70 ml of the Mix to 0.30 ml of the phosphate solution in a small test tube (or to 0.30 ml of water for the blank) and incubate 20 minutes at 45° or 1 hour at 37°. Read at 820 mμ; 0.01 micromole of inorganic phosphate results in an absorbancy of 0.260. The color is stable for several hours. The readings are proportional to phosphate concentrations to an optical density of at least 1.8. (It is necessary that the proper phototube, sensitive to light at 820 mμ, be in position in the spectrophotometer; otherwise low readings will be obtained.)

*Comments.* All glassware should be scrupulously clean (free of phosphate) because of the sensitivity of the method. Water rinses often are not sufficient to remove phosphate adsorbed to cuvettes or test tubes so that detergents containing trisodium phosphate often cause difficulties. Cuvettes reserved for phosphate determinations are desirable.

Pyrophosphate breaks down about 5% in the method and compounds such as glucose 1-phosphate also break down somewhat, so that the method is not very satisfactory for determining inorganic phosphate if labile phosphate esters are present in large excess. However, the presence of 1 labile phosphate group (out of 2 phosphates determined by total phosphate analysis) can be demonstrated in compounds such as UDP-glucose by showing that 1 phosphate appears after 10 minutes' hydrolysis at 100° in 1 *N* HCl. Compounds such as AMP, glucose 6-phosphate, and ribose 5-phosphate are stable.

### Ashing Procedure for Total Phosphate[2]

(Suggested by R. Kielley)

*Principle.* The sample of organic phosphate and a drop of magnesium nitrate solution in a small test tube are taken to dryness by shaking the tube in flame. This procedure takes a few seconds and completely ashes

[2] B. N. Ames and D. T. Dubin, *J. Biol. Chem.* **235**, 769 (1960).

organic phosphates. This procedure is coupled with the very sensitive inorganic phosphate method just described.

*Reagents*

10% $Mg(NO_3)_2 \cdot 6\ H_2O$ in 95% alcohol
HCl, 0.5 *N*
Mix described in assay for inorganic phosphate

*Procedure.* To 0.01–0.10 ml of phosphate sample (containing up to 0.07 micromole of phosphate) in a Pyrex or Kimax 13 × 100-mm test tube is added 0.03 ml of the magnesium nitrate solution. The material is taken to dryness and ashed by shaking the tube over a strong flame until the brown fumes disappear. The tube is then allowed to cool and 0.30 ml of 0.5 *N* HCl is added; the tube is capped with a marble and heated in a boiling water bath for 15 minutes to hydrolyze to phosphate any pyrophosphate formed in the ashing. Cooling the tops of the tubes by a stream of air helps to minimize evaporation. After the tube has cooled, 0.7 ml of the Mix is added and the tubes are incubated as described in the assay for inorganic phosphate. Absorbancy: 0.01 micromole of organic phosphate results in an absorbancy of 0.240.

*Comments.* The ashing procedure is rapid and is good for various biological materials and phosphate esters such as nucleic acid, carbohydrate phosphate esters, viruses, phospholipids, etc. In material containing little phosphate relative to organic matter, e.g., a phosphoprotein, it may be necessary to ash twice with the $Mg(NO_3)_2$. The $Mg(NO_3)_2$ causes a small lowering of the extinction so that a phosphate standard should be run through the ashing procedure.

Some silicate is formed from the tubes during the ashing, and this gives a very small amount of color, but it cancels out when read against a blank with the same amount of silicate. Pyrex tubes give a different amount of silicate than Kimax tubes, so the tubes should not be mixed.

### Assay of Phosphatases[3]

*Principle.* The method for inorganic phosphate described can be used as an assay for phosphatases hydrolyzing stable phosphate esters, e.g., glucose-6-phosphate, ribose-5-phosphate, histidinol phosphate, AMP, etc. No deproteinization is usually necessary as the method is so sensitive that little enzyme is required. The enzyme incubation can be stopped with the one ascorbic-molybdate solution thus avoiding an extra pipetting.

[3] B. N. Ames, B. Garry, and L. H. Herzenberg, *J. Gen. Microbiol.* **22**, 369 (1960).

*Procedure.* To 0.3 ml of incubation mixture containing buffer and enzyme and substrate is added 0.7 ml of the Mix, and the tube is incubated as described under inorganic phosphate. It is usually convenient to run a blank without substrate, e.g., glucose 6-phosphate but containing the enzyme, buffer, etc. A separate blank for the substrate can be run occasionally, and if this contains a small amount of inorganic phosphate this value can be subtracted. Protein up to about 200 $\mu$g does not interfere with the assay if the blank also contains protein. If deproteinization is necessary, 0.3 ml of 5% trichloroacetic acid or perchloric acid does not interfere in the assay.