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A Simplified Colorimetry without Incineration of Phosphorus in Phosphatides

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In order to determine the total phosphorus content of a sample by a colorimetric method, the organically bound phosphate must be brought into inorganic form. In cases of glycerophosphatides which contain extremely stable linkages of glycerophosphate, this has usually been accomplished by wet incineration in the presence of a strong acid and an oxidizing agent, and the orthophosphate thus released has been assayed by one of the modified methods of the classical Fiske and Subbarow procedure¹⁾ It is, however, impractical to involve the step of incineration especially when many samples have to be routinely analyzed, since wet incineration requires relatively longer time and special facilities and often suffers from accidental loss of the content of a digestion flask.

This note describes a new and simple colorimetric method of phosphatide-P determination which does not employ incineration. It has been used in this laboratory for hundreds of determinations and found particularly suited for the routine estimation of total phosphorus contents of phosphatide samples. The method consists of periodic acid oxidation of the α -glycerophosphate produced by acid hydrolysis of glycerophosphatides to give glycolaldehyde phosphate which in turn is readily hydrolyzed to yield

orthophosphate. The hydrolysis and oxidation are designed to take place simultaneously. The resulting orthophosphate is then determined colorimetrically by the method of Griswold et al.²⁾ with a slight modification.

Procedure for Glycerophosphatide-Phosphorus Determination

Reagent A. Three volumes of 10 N sulfuric acid and 1 volume of 0.1 M periodic acid are mixed and ethanol is added, on the day of use, to make 10 volumes. This reagent is stable for at least twenty-four hours.

Reagent B. Three volumes of 2.5% ammonium molybdate, 5 volumes of aminonaphthol-sulfonic acid solution and 40 volumes of water are mixed on the day of use. This reagent has been found to give the same color development after the storage for twenty-four hours at room temperature. The aminonaphtholsulfonic acid solution consists of 0.1% 1-amino-2-naphthol-4-sulfonic acid, 0.2% anhydrous sodium sulfite and 6% sodium bisulfite.

A glycerophosphatide sample in 0.2 ml chloroform is mixed with 1.0 ml of Reagent A and heated in a test tube without stopper in a vigorously boiling water bath for forty-five minutes. During this heating the volume of the sample solution is reduced to approximately 0.3 ml, being due to the evaporation of organic

1) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

2) B. L. Griswold, F. L. Humoller and A. R. McIntyre, *Anal. Chem.*, **23**, 192 (1951).

solvents. Then 4.8 ml of Reagent B is added to the mixture and heated again in a vigorously boiling water bath for exactly ten minutes. The test tube is quickly withdrawn from the bath and cooled in running water. After the solution is cooled to room temperature, optical density at 820 m μ is read in a spectrophotometer. The color is stable for at least twenty-four hours.

Justification and Limitation of the Method

The validity of the method proposed here will be discussed with particular reference to three major points of modification, namely the effect of periodate on the oxidation and on the color development, quantitativity of the hydrolytic reactions on phosphatides in aqueous acid suspension, and the effort made here to minimize the steps involved.

The oxidation of α -glycerophosphate with periodate to yield glycolaldehyde phosphate has been well known. Leva and Rapoport³⁾ and Burmaster⁴⁾ studied the conditions for the determination of phosphorus of α - and β -glycerophosphates by employing periodate oxidation, although their procedures were not simple enough to be applied for routine determinations. Direct estimation of phosphatide-phosphorus without previous hydrolysis and separation of the water-soluble phosphate from fatty acids has not been reported as yet. By using a soy-bean lecithin preparation (Tokyo Kasei Co.) and by comparing the results with those of ordinary wet incineration,⁵⁾ we have examined the conditions for direct production of orthophosphate from the lipid. It has been found that when periodate is used at the concentration of 2 mM or less as described above, any extra reducing reagents such as sulfite⁴⁾ or bisulfite³⁾ is not required in addition to Reagent B for the quantitative formation of molybdenum blue. The sulfite and bisulfite contents in our

Reagent B were sufficient enough to reduce the excess of periodate and iodate formed. When the periodate is used at higher concentrations, sulfite, bisulfite or compounds such as glycerol must be added to consume the excess periodate. Fig. 1 shows that the

FIG. 1. Time Required for the First-Step Heating with Reagent A

Soy-bean lecithin, 2×10^{-7} moles, dissolved in 0.2 ml chloroform was heated at 100°C with 1.0 ml of Reagent A for varying times, and then mixed with 4.8 ml of Reagent B, heated at 100°C for ten minutes and the blue color was estimated at 820 m μ .

time needed for the first-step heating of phosphatides with sulfuric acid and periodate is approximately twenty minutes. The high efficiency of this step is reasonable since three reactions, i.e., hydrolysis of phosphatides to glycerophosphate, periodate oxidation of glycerophosphate to glycolaldehyde phosphate, and hydrolysis of glycolaldehyde phosphate to form inorganic phosphate are carried out simultaneously in the present method so that the product at each step is removed from the field of action, thus shifting the equilibrium to the right. When the amount of phosphatides to be analyzed was higher, the heating of more than twenty minutes was necessary. We recommend, therefore, in the standard procedure to employ the first-step heating of forty-five minutes.

For estimation of the orthophosphate produced by the oxidation and hydrolysis, we have chosen, with a minor modification

3) E. Leva and S. Rapoport, *J. Biol. Chem.*, **149**, 47 (1943).

4) C. F. Burmaster, *ibid.*, **164**, 233 (1946).

5) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

as mentioned later, the method of Griswold et al.²⁾ which takes advantage of the desirable features of the Fiske and Subbarow method¹⁾ and of the Boltz and Mellon method⁶⁾. The sulfuric acid, bisulfite and sulfite used in their method are efficiently utilized here in the production of orthophosphate and the subsequent color development. The color of molybdenum blue has been found stable for at least twenty-four hours, so that it can be estimated at a convenient time. As Fig. 2

FIG. 2. Range of Linearity of the Method

Chloroform solutions of various lecithin concentrations were treated as described in the text. Up to 4 mM, or 8×10^{-7} moles in the total 5 ml final solution, O. D. readings at 820 mμ were proportional to the lecithin quantity. Beyond this concentration, the curve (—) turned aside from the straight line (----) which represented the results obtained by wet incineration⁵⁾. The O. D. readings of more than 1.5 were obtained with the solutions suitably diluted with water.

shows, the blue color follows Beer's law and gives exactly the same optical density (O. D.) readings as the ones by wet incineration, up to the lecithin concentration of 4 mM in the sample chloroform solution. Above this concentration, the O. D. readings are not directly proportional to the sample phosphatide concentrations. The relationships between the O. D. readings and the phosphatide concentrations have, however, been

found highly reproducible, and once a calibration curve is made, the determination can be carried out for samples with concentrations far beyond 4 mM.

To obtain α -glycerophosphate quantitatively, we have found that the sample phosphatide must be homogeneously dispersed in the aqueous acid solution. Various types of detergents which would "solubilize" lipids in water had been examined, but none of them

FIG. 3. DEAE-Cellulose Column Chromatography of Phosphatides of *Escherichia coli*

Cells of *E. coli* K12 uniformly labeled with ^{32}P were harvested at their logarithmic growth phase and killed by trichloroacetic acid. The lipids extracted with methanol and chloroform were fractionated by DEAE-cellulose column chromatography with chloroform-methanol (4:1) and a linear concentration gradient of ammonium hydroxide. Each eluted fraction was analyzed for its phosphorus content both by the colorimetric method as described in the text (—) and by the radioactivity measurement with a G. M.-automatic counter (----). The peaks correspond, from left to right, to phosphatidyl ethanolamine, phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidic acid and phosphatidyl serine, respectively.

6) D. F. Boltz and M. G. Mellon, *Anal. Chem.*, **19**, 873 (1947).

was found useful in the present work. Synthetic detergents commercially available such as sodium dodecyl sulfate gave blue color upon heating with Reagent B, probably because they contain phosphate and/or silicate, and other detergents such as dodecylamine or deoxycholate were insoluble in the sulfuric acid solution. We therefore employed ethanol to obtain a uniform emulsion of phosphatides which were originally dissolved in chloroform. The ratio of the amounts of chloroform, ethanol and water recommend by us as described above was not only suitable for lecithin but also for all glycerophosphatides of *Escherichia coli* including phosphatidyl serine. This has been proved by comparing the O. D. readings at $820\text{m}\mu$ and ^{32}P radioactivity of all fractions prepared by DEAE-cellulose column chromatography of *Escherichia coli* lipids which has been uniformly labeled with ^{32}P . Fig. 3 showed that the ratio was uniform throughout the entire range of the chromatographic elution.

The applicability of present colorimetric method has not been tested with phosphorus-containing lipids other than phosphatidyl compounds, but plasmalogens in which more acid-labile acetal linkages are substituted for the esters of phosphatidyl lipids can obviously be analyzed by this method. The phosphorus of phosphonolipids

with direct carbon-phosphorus bonds can not be freed by the present method. We have not tested sphingolipids but we assume that the phosphorus of this type of lipids may also be analyzed with only minor changes in procedure.

Griswold et al.²¹ prepared three reagents separately for color development and required twice of volume adjustment. We have examined and found that the previous mixing of all reagents to form Reagent B does not interfere the quantitative color development, in contrast with what has been said in textbooks, as we have therefore purposely designed the procedure to involve only two reagents, namely Reagent A for the hydrolysis and oxidation and Reagent B for the color development, and to make the final blue solution of a uniform volume without adjustment to have practically permissible deviations from 5.0 ml. These modifications have made the method extremely simple and useful in routine analysis of many phosphatide samples. The method also enables the use of ordinary tubes such as those used in fraction collectors for color development. When more accurate measurements are desired, the use of graduated test tubes and the adjustment of sample volumes before O. D. estimation are recommended.