Phosphorus determination in phosphoglycerides from thin-layer chromatograms

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SUMMARY Phosphoglyceride phosphorus is readily determined directly on silica gel removed from thin-layer chromatograms, without elution, by a nondigestive procedure with a sulfuric–periodic acid reagent. The method is specific for hydrolyzable phosphoglyceride containing two acid-hydrolyzable groups. Cariolipin reacts only partially, while sphingomyelin, diether lecithin, and phosphonate cephalin fail completely to react.

SUPPLEMENTARY KEY WORDS periodic–sulfuric acid · nondigestive procedure

RECENTLY Shibuya, Honda, and Maruo (1) showed that phosphorus can be quantitatively liberated from lecithin without digestion by heating with alcoholic sulfuric–periodic acid. We have found that this reaction can be used in determining the phosphorus of certain phospholipids separated by thin-layer chromatography. The phospholipids need not be eluted first from the silica gel zones scraped off the plate. Phosphorus is liberated only from phosphoglycerides containing hydrolyzable substituents, and not from diether lecithin, sphingomyelin, or phosphonate derivatives of diacyl glycerol.

Materials. Chromatographically homogeneous egg lecithin was prepared from egg yolks (2, 3). Chromatographically pure lysolecithin, phosphatidyl ethanolamine, phosphatidyl inositol, and cardiolipin were obtained from Supelco, Inc., Bellefonte, Pa. These lipids were obtained from natural sources; the last-named three lipids showed a number of minor impurities on TLC. Phosphatidyl serine (natural), sphingomyelin (natural), and *rac*-1,2-dioctadecyl glycerophosphorylcholine (diether lecithin, synthetic) were obtained from Mann Research Labs. Inc., New York. *sn*-Glycerol 1,2-dipalmitate 3-dihydrogen phosphate [dipalmitoyl L-α-phosphatidic acid (4) containing a small amount of lysophosphatidic acid] and chromatographically pure *sn*-glycerol 1,2-dipalmitate 3-(2′-aminoethylphosphonate) [phosphonate cephalin (5, 6)] were synthetic materials prepared according to published procedures.

Methods. A mixture of 10 N sulfuric acid–0.1 M periodic acid–95% ethanol 3:1:6, mixed on the day of use, was used to liberate phosphate (1).

After the Silica Gel G plate has been developed and dried, it was exposed briefly to iodine vapor. Each outlined phosphatide spot was carefully scraped off onto glassine paper and transferred to an 18 × 150 mm test tube. 1 ml of the above reagent was added and the unstoppered tubes were placed into a boiling water bath for 45 min. The volume of the liquid was reduced by the heating to 0.3 ml. To the residual liquid in each tube was added 4.8 ml of the mixture 2.5% ammonium molybdate–standard aminonaphtholsulfonic acid (ANSA) reagent (containing by weight 0.2% ANSA, 0.5% sodium sulfite, and 7.5% sodium bisulfite)–water 3:5:40, freshly mixed. (The mixed reagent is stable for at least 24 hr.) The tubes were returned to the boiling water bath for 10 min and cooled. A blank area of the silica gel on the plate about equal in size to the phosphatide-containing spots was treated similarly and used as the reagent blank. Inorganic phosphate solution was used as a standard; it was sufficiently concentrated (0.01 M) that its volume was negligibly small (< 0.1 ml).

After the silica had been centrifuged off, the molyb-

Abbreviation: TLC, thin-layer chromatography.

denum blue color was read in a spectrophotometer at any appropriate wavelength (e.g., 660 mμ).

In many experiments results obtained by this procedure were compared to those obtained by a method employing digestion with perchloric acid (7).

Results: Inorganic Phosphate. Phosphorus in standard inorganic phosphate solution was determined, without chromatography, both by the periodic–sulfuric acid method and by perchloric acid digestion. Rather surprisingly, the former was much more sensitive (Fig. 1), and yielded twice as much color as the perchloric acid procedure.

If the phosphate solution was first applied to Silica Gel G plates and the spots were then scraped off for phosphorus determination, the same results were obtained. Silica does not, therefore, inhibit the color reaction. Thus, phosphate solution, not applied to a plate but taken through the heating procedure, could be used as the standard for the phosphorus determinations after chromatography.

Lecithin. Phosphorus in various known concentrations of lecithin dissolved in trichloroethylene was determined by the sulfuric–periodic acid method applied directly to the solution, to the spot resulting from application of the solution to Silica Gel G without chromatography, and to the spot resulting from chromatography of the solution on Silica Gel G in chloroform–methanol–water 65:25:4. In the first two cases the optical densities obtained with equal quantities of lecithin were essentially identical (Fig. 2, part 1). The greatest variation found between duplicates of any point on the graphs was 1.4%. The slightly lower (about 3%) values obtained after chromatography probably reflect the presence in the original lecithin of unrecognized traces of phosphorus-containing contaminants, which had been removed during TLC.

The phosphorus content of the lecithin solution, determined without chromatography by the periodic acid method, appeared to be 5.0% lower than with the perchloric acid digestion method (Fig. 2, part 2).

Lysolecithin. Fig. 3, part 1 shows the determination of phosphorus in chromatographically pure lysolecithin by the periodic acid method with and without TLC. The virtually identical results found show that the method is inherently capable of quantitative recoveries after TLC. The chromatographic solvent was the same as that used for lecithin.

The periodic acid method here gave yields of phosphorus 98% as great as with the perchloric acid method (Fig. 3, part 2).

Sphingomyelin. Shibuya et al. suggested (1), without trial, that sulfuric–periodic acid would also be useful to determine sphingomyelin phosphorus. Rather unexpectedly, we observed that no phosphate at all was liberated under the conditions described. In fact, we have shown (8) that lecithin could even be determined in unchromatographed lecithin–sphingomyelin mixtures as long as the latter was not present in such large excess that sphingomyelin particles mechanically interfered by adsorbing molybdenum blue from the solution.

Other Phospholipids. The following phosphoglycerides (0–1.0 μmole) were readily determined by the periodic acid procedure before or after TLC, with phosphorus yields of 95–102% compared to the perchloric acid digestion method: phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and phosphatidic acid. Cardiolipin did not give quantitative recoveries (see Discussion).

Phosphonate cephalin released no phosphate under the conditions described; it did not interfere with lecithin determinations in mixtures of the two lipids. Diether lecithin also yielded no phosphate, but did interfere slightly with the determination of lecithin.

Discussion. The structural criterion for phosphorus determination by the periodic–sulfuric acid method seems to be the ability to be converted transiently to glycerol 3-phosphoric acid by brief acid hydrolysis. As

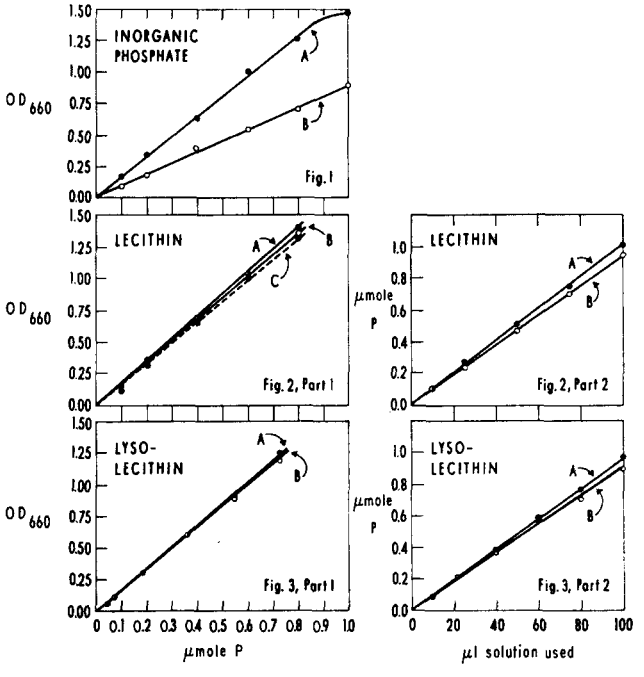

FIG. 1. Inorganic phosphate standard curve. *A*, by periodic acid method; *B*, by perchloric acid method.

FIG. 2. Part 1: determination of lecithin phosphorus by periodic acid method. *A*, without TLC; *B*, applied to TLC plate but not chromatographed; *C*, chromatographed. Part 2: determination of lecithin phosphorus without TLC. *A*, by perchloric acid method; *B*, by periodic acid method.

FIG. 3. Part 1. Determination of lysolecithin phosphorus by periodic acid method. *A*, without chromatography; *B*, after TLC. Part 2: determination of lysolecithin phosphorus without TLC. *A*, by perchloric acid method; *B*, by periodic acid method.

long as competing intermediates (e.g., glycerol 1,2- and 1,3-diphosphates, as would result with cardiolipin), which prevent quantitative glycerophosphate release, cannot be formed in the mixture being analyzed, quantitative analytical results may be expected.

The method is the only procedure so far described for direct analysis of phosphoglycerides in the presence of their phosphonate analogues (and vice versa, if a digestion procedure is used in conjunction). The method may also be used to indicate the presence of unreactive impurities (e.g. diether phosphoglycerides) in TLC spots consisting mainly of ester phosphoglyceride, and as a criterion of purity in synthetic or isolated pure phosphoglycerides.

The procedure may also be useful when various amounts of one phosphoglyceride are to be analyzed after TLC, since the method has several advantages over incinerative procedures: no digestion racks or other special apparatus are required, noxious fumes are not evolved, and the color reaction is more sensitive.

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