notes on methodology

Removal of the sphingolipid impurity from preparations of yeast phosphatidyl inositol

WILLIAM E. TREVELYAN

Distillers Company (Yeast) Ltd., Great Burgh, Epsom, Surrey, England

SUMMARY A complex sphingolipid containing inositol and mannose, present in lipid extracted from toluene-autolyzed baker's yeast, was eluted from silicic acid columns immediately after phosphatidyl inositol, and was the main nitrogenous impurity in crude preparations of this phospholipid. Nitrogen-free phosphatidyl inositol was obtained by rechromatography on alumina. Modifications to the chromatographic procedure also gave diphosphatidyl glycerol containing the theoretical 4.29% P.

KEY WORDS yeast · phosphatidyl inositol · sphingolipid · silicic acid chromatography · alumina chromatography · diphosphatidyl glycerol

RECENTLY (1) we described a simple method for the preparation from baker's yeast of the sodium salt of phosphatidyl inositol, in good yield, and of a purity now estimated to be about 90%. The presence of impurity was indicated by the formation of interfacial material when fatty acid esters were extracted by light petroleum after methanolysis (1), and, more directly, by the nitrogen content of the preparations. This amounted to 0.20%, in spite of the fact that the principal nitrogenous yeast phospholipids (lecithin, phosphatidyl ethanolamine, and phosphatidyl serine) had been largely destroyed, before lipid was extracted from the yeast, by autolysis in the presence of toluene (2). It was later found that little advantage was gained by extending the period of autolysis from 5 to 23 hr, even though this modification reduced still further the amount of lecithin and of phosphatidyl ethanolamine in the lipid extract.

Sphingolipids. More recent studies have shown that lipid extracted from autolyzed yeast contains an appreciable proportion of sphingolipid, and that this was the contaminant responsible for the nitrogen content of the original preparations of yeast phosphatidyl inositol. From that fraction of lipid insoluble in isopropanol (1) a crude sphingolipid preparation was obtained (after phosphoglycerides had been degraded by hydrolysis with alkali at 38°C) which amounted to 1.7 mg/g yeast (dry weight), about one-sixth of the quantity of phosphatidyl inositol found in this type of yeast.

When the isopropanol-insoluble phospholipids were fractionated by chromatography on Whatman SG 34 silicic acid (1), chloroform–methanol–water 86:14:1 was found to elute diphosphatidyl glycerol, and an 80:20:1 mixture, some phosphatidyl ethanolamine. Better resolution than before was obtained by the use of solvent systems that contained water. Chloroform–methanol–water 25:25:1 (4 bed volumes) eluted phosphatidyl inositol, and a further 3 bed volumes eluted phosphatidyl inositol together with a sphingolipid. Mild alkaline hydrolysis of this fraction yielded 0.4 mg/g yeast of a sphingolipid, which was recovered as a chalky-white solid by cooling its solution in warm chloroform–methanol–water 86:14:1, or by precipitation with methanol.

This and similar preparations were very resistant to acid hydrolysis, a two-stage procedure being most effective. The sphingolipid (20.5 mg) was first refluxed for 6 hr with 5 ml methanol and 1 ml of 12 N hydrochloric acid (3). Water (3 ml) was added to the cooled hydrolysate, and lipids then extracted with 3 × 10 ml of chloroform. The extracts were evaporated and the dry lipid was refluxed for 2 hr with 5% methanolic HCl. A methyl ester fraction (7.0 mg) and a long-chain base fraction (5.3 mg) were isolated and weighed, substantially as described by Kates (4). Thin-layer chromatography on Silica Gel G (Kieselgel G, E. Merck A.G., Darmstadt, Germany) with benzene–acetone 50:1 as solvent showed the ester fraction to be predominantly methyl esters of hydroxy fatty acids, R_f 0.29 (16.5 μmoles, calculated as ester of C_{26} acid). The base gave a predominant ninhydrin-positive spot, R_f 0.17, when the plate was developed in the chloroform–methanol–ammonia system of Sambasivarao and McCluer (5) (16.7 μmoles, calculated as C_{18} phytosphingosine).

The lipid-free methanol–water phase obtained after the first stage of the hydrolysis procedure yielded on evaporation 9.6 mg of water-soluble material that contained phosphorus (17.8 μmoles), inositol (17.7 μmoles), and mannose (9.8 μmoles). Inositol was estimated after anion exchange chromatography of its borate complex, as described below. Mannose was determined by an anthrone procedure, and was also reduced to mannitol by $NaBH_4$, the polyol being then estimated after isolation by chromatography on Dowex-1 borate.

Except for its low content of mannose, the preparation thus resembled the yeast sphingolipid described originally by Law and Fitz (6) and more recently, under the name of "mycoglycolipid," by Wagner and Zofcsik (7). This material, it was calculated, would have contributed 0.1% N to a preparation of phosphatidyl inositol made by the original procedure.

Purification of Phosphatidyl Inositol. Sodium phosphatidyl inositol which had less than 0.03% N was obtained

by rechromatography on an alumina column [cf. Long and Owens (8)], from which it was eluted with chloroform–methanol–water 2:3:1. The final product contained 3.61% P and 2.60% Na [calculated (1), 3.64% and 2.70% respectively]. The ratio of fatty acid to phosphorus was 1.99 moles/g-atom P (theory, 2.00), and no interfacial material was formed during the methanolysis procedure. Glycerol and inositol were estimated, with a precision of ±2%, by a procedure which involved hydrolysis in a sealed tube (under nitrogen) with 2 N HCl for 72 hr at 110°C, neutralization with Dowex-1 bicarbonate, and anion exchange chromatography of the borate complexes of the polyols on short Dowex columns. The molar ratio of glycerol:inositol:phosphorus was 0.98:0.96:1.00. The phospholipid gave a single spot, R_f 0.30, on thin-layer plates (Merck, Kieselgel G) with propanol–chloroform–water 4:1:1.

Diphosphatidyl glycerol. Diphosphatidyl glycerol, purified by rechromatography on silicic acid and by washing with citrate buffer (1), was finally precipitated from solution in ether by acetone, to give a colorless wax containing 4.29% P (calculated for the sodium salt, 4.29%). On thin-layer plates developed in propanol–chloroform–water a single spot with R_f 0.71 was seen.

The only polyol detected by anion exchange chromatography, after hydrolysis with acid as described above, was glycerol. All the phospholipid P was rendered water-soluble, but the resultant esters were not completely split, 0.86 mole of free phosphate and 1.27 moles of glycerol being obtained per g-atom of P. On the basis of paper chromatographic evidence it is thought that some glycerol diphosphate was formed in the initial stage of hydrolysis and was then only partly split to glycerol and phosphate.

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References

1. Trevelyan, W. E. 1966. *J. Lipid Res.* **7:** 445.
2. Harrison, J. S., and W. E. Trevelyan. 1963. *Nature.* **200:** 1189.
3. Sweeley, C. C., and E. A. Moscatelli. 1959. *J. Lipid Res.* **1:** 40.
4. Kates, M. 1964. *J. Lipid Res.* **5:** 132.
5. Sambasivarao, K., and R. H. McCluer. 1963. *J. Lipid Res.* **4:** 106.
6. Law, J. H., and F. Fitz. 1961. *Abstracts of Papers*, Division of Biological Chemistry. 139th Meeting of the American Chemical Society, St. Louis, Mo., March 1961. 68C.
7. Wagner, H., and W. Zofcsik. 1966. *Biochem. Z.* **344:** 314.
8. Long, C., and K. Owens. 1966. *Biochem. J.* **99:** 617.