Phosphatidic Acid and Phosphatidylinositol Metabolism in *Schizosaccharomyces Pombe*

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The phospholipid composition of *Schizosaccharomyces pombe* was not markedly affected by changes in the phosphate concentration of the medium or phase of growth. The major fatty acids in the total lipid extract and purified phosphatidylinositol were palmitic acid and oleic acid. Phosphatidic acid was synthesized by acylation of L-3-glycerophosphate in *Schiz. pombe* and phosphatidate phosphohydrolase was present. Phosphatidylinositol synthesis from inositol occurred in the absence of CDP-diglyceride. Even with dialysed cell-free preparations, the inositol lipid was synthesized by an apparently energy-independent route, at rates greater than would be required during cell growth. Phosphatidylinositol appeared to be broken down by a phospholipase D. All the enzymes examined were particulate; similar activities were found in *Saccharomyces cerevisiae*.

Schizosaccharomyces pombe is a fission yeast that has an absolute requirement for inositol and does not catabolize it (Posternak, 1966). This yeast is therefore a convenient micro-organism in which to study phosphatidylinositol metabolism. Posternak (1966) has reviewed the effect of inositol and its antagonists on *Schiz. pombe*. The enzymes responsible for phosphatidylcholine and phosphatidylethanolamine biosynthesis have been studied in yeast (Wittenberg & Kornberg, 1953; Kennedy & Weiss, 1956). Lysophosphatidic acid synthesis has also been investigated by Kuhn & Lynen (1965). More recently, the formation of CDP-diglyceride by cell-free extracts of yeast has been reported (Hutchison & Cronan, 1968).

In the present study, the phospholipid composition of *Schiz. pombe* has been determined and the metabolism of phosphatidic acid and phosphatidylinositol investigated in this species.

MATERIALS AND METHODS

Materials. Special reagents were obtained as follows: [32 P]orthophosphate (PBS 1), [U- 14 C]myo-inositol, (CFB 49) and [2- 14 C]ethanolamine hydrochloride (CFA 329) from The Radiochemical Centre, Amersham, Bucks., U.K.; 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase and glycerokinase from Boehringer und Soehne G.m.b.H. (through Courtin and Warner, Lewes, Sussex, U.K.); ATP, CTP, CDP, CMP, CoA, CDP-choline, DL-3-glycerophosphate, bovine serum albumin (fraction V), GSH, cysteine, tris (type 7-9) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium

chloride from Sigma Chemical Co., London S.W.6, U.K.; myo-inositol, nicotinic acid, thiamin hydrochloride, calcium D-pantothenate, pyridoxine hydrochloride and D-biotin from British Drug Houses, Poole, Dorset, U.K.; Cutscum (iso-octylphenoxy polyethoxyethanol) from Kodak Ltd., Kirkby, Lancs., U.K.; Helicase freeze-dried enzyme from the juice of the snail *Helix pomatia* from Industrie Biologique Française S.A., Gennevilliers, France. Dr R. O. Thomson of Wellcome Research Laboratories, Beckenham, Kent, U.K., kindly supplied a concentrate of *Clostridium perfringens* type A filtrate as a source of phospholipase C.

Analytical methods. All the methods used in the present work were as described previously (Atherton & Hawthorne 1968).

Yeasts. Cultures of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were obtained from Professor J. S. Hough of this Department. *Schiz. pombe* was maintained on agar slopes and grown in the medium of Norris & Darbre (1956) with added myo-inositol (100 mg/l) and at two concentrations of KH_2PO_4 (0.1 or 1.5 g/l). Three 50 ml flasks containing 10 ml of medium were inoculated with *Schiz. pombe* and the yeast was allowed to grow for 24 h. Three 1-litre flat-bottomed culture flasks were half-filled with basal medium and 10 ml of yeast suspension was added. *Schiz. pombe* was grown with stirring and continuous aeration for 24-30 h at 25°C (exponential phase) or for 48 h if the stationary phase was needed. For work on a larger scale, two or three 5-litre batches were grown.

Electron microscopy. Whole cells were stained with 2% KMnO_4 containing 6.7% KCl at 4°C for 2.5 h. After dehydration, the sample was embedded in Araldite, sectioned and over-stained with uranyl acetate and viewed on an Elmiskop 1 electron microscope. Cell fractions were

fixed in 6.25% glutaraldehyde, stained with 1% OsO₄ and treated as previously described.

Extraction and analysis of phospholipids. A large proportion of the *Schiz. pombe* phospholipid can be removed from whole cells by four extractions with chloroform-methanol (2:1, v/v). Three subsequent extractions with chloroform-methanol-conc. HCl (800:400:3, by vol.) extracted a further 18% of the recovered lipid phosphorus. Deacylation of the lipid extract and chromatography of the resulting phosphate esters followed the method described by Prottey & Hawthorne (1966). A complete hydrolysis of the alkali-labile phospholipids was obtained by using 15 mol of NaOH/mol of lipid phosphorus. The fatty acid composition of the lipids was determined by the method of Prottey & Hawthorne (1966).

Preparation of lipid substrates. Calcium phosphatidate was prepared from egg phosphatidylcholine by the procedure of Hübscher & Clarke (1960) with cabbage phospholipase D (stage 2 enzyme; Davidson & Long, 1958). After the extraction into light petroleum, phosphatidic acid was eluted with methanol-chloroform (3:47, v/v) from a silicic acid column containing no Celite. Calcium phosphatidate was converted into the sodium salt by re-emulsifying with 150 mM-sodium EDTA, pH 7.0, and dialysing against 1.5 mM-sodium EDTA. After six dialyses of several hours each against EDTA, three against 20 mM-NaCl and one against water, less than 0.5% of the lipid was in the calcium form. Sodium [¹⁴C]phosphatidate was produced by the method of Brindley, Smith, Sedgwick & Hübscher (1967) followed by dialysis as above with sodium EDTA.

CDP-diglyceride was made from sodium phosphatidate by the method of Agranoff & Suomi (1963) as modified by Prottey & Hawthorne (1967) with one further alteration: after dialysis of the sodium phosphatidate against 5 mM-HCl followed by water, the whole emulsion was freeze-dried.

Phosphatidylinositol was prepared from *Sacch. cerevisiae* by the method of Trevelyan (1966a) and from brain and *Schiz. pombe* by the method of Kai, White & Hawthorne (1966).

[³²P]Phosphatidylinositol and [³²P]phosphatidylethanolamine were prepared from the tissues of a guinea pig that had been injected intraperitoneally with 2 mCi of [³²P]-orthophosphate. Liver, lungs, heart, kidneys, spleen and brain were removed and extracted with chloroform-methanol (2:1, v/v), and the lipids were purified on successive alumina and silicic acid columns (Kai *et al.* 1966). The phospholipid extracts were washed with 0.2 vol. of 50 mM-sodium EDTA, pH 7.5, by the procedure of Folch, Lees & Sloane-Stanley (1957) before being loaded on the silicic acid column. [³²P]Phosphatidylethanolamine was eluted with chloroform-methanol (9:1, v/v) and had a specific radioactivity of 94 c.p.m./μg of P as determined in a Geiger-Müller counter (efficiency 6%). T.l.c. on silicic acid in chloroform-methanol-water (19:7:1, by vol.) showed no impurities when spots equivalent to 9 μg of P were applied. [³²P]Phosphatidylinositol was eluted from the column with chloroform-methanol (4:1, v/v), contained traces of lysophosphatidylethanolamine and had a specific radioactivity of 104 c.p.m./μg of P.

Phosphatidylglycerol was made from spinach leaves by the method of Haverkate & van Deenen (1965).

L-1,2-Diglyceride was prepared from egg phosphatidyl-

choline by using *Clostridium perfringens* phospholipase C; the product was purified as described by Gurr, Brindley & Hübscher (1965).

Terminally labelled [³²P]ATP was produced as described previously (Kai *et al.* 1966) and glycerol[³²P]phosphate was made from it by the method of Prottey & Hawthorne (1967).

Preparation of subcellular fractions. All centrifuging was done at 5°C, 0.3 M-sucrose was used to wash and suspend the fractions. *Schiz. pombe* was harvested by centrifuging at 12500g for 5 min. The cells were washed twice with 0.3 M-sucrose, the sedimentation being first at 25000g for 10 min and then at 17000g for 5 min. Yeast was crushed in an X-press of 5 ml capacity (AB Biox, Nacka 2, Sweden) pre-cooled at -20°C. After being allowed to thaw, the crushed yeast was homogenized with six strokes of a tightly fitting Potter-Elvehjem homogenizer (10 ml capacity, radial clearance approx. 0.05 mm) with a Teflon pestle rotating at 600 rev./min. The material was centrifuged at 2000g for 5 min and the resulting loose white pellet was washed twice with 0.3 M-sucrose by using the centrifuge and resuspending with three strokes of the same homogenizer. The final white sediment was reasonably firm and contained whole cells and large cell debris. Light microscopy showed that the pooled supernatant (fraction A) contained no whole cells. The cell-free homogenate was centrifuged at 104000g for 60 min. The red gelatinous sediment (fraction B) was resuspended by hand in the homogenizer. The supernatant was taken as fraction C.

Enzyme preparations. *Schiz. pombe* was usually harvested in the exponential phase of growth. Sometimes the yeast was washed and crushed in water instead of 0.3 M-sucrose and treated as above. Some preparations of the cell-free homogenate in water (10-20 mg of protein/ml) were dialysed for several hours against 4-6 litres of demineralized water at 4°C, with two changes. An acetone-dried powder was prepared at 4°C by mixing 5 ml of fraction A and 50 ml of acetone (pre-cooled to -20°C), shaking for 5 min and filtering off the precipitate. The powder, after being allowed to dry, was resuspended in the same volume of 0.3 M-sucrose.

Before fractionation *Sacch. cerevisiae* was treated by washing at 17500g for 10 min with water to remove impurities. After being crushed, whole cells were removed as described above.

Enzyme assays. Aqueous dispersions of all lipid substrates were prepared immediately before use, by treatment for 1 min in the MSE 60W Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.). Unless otherwise stated, the various enzyme assay systems had a total volume of 1 ml.

Succinate dehydrogenase (EC 1.3.99.1) was determined by the method of Pennington (1961) in the presence of 2 mM-EDTA (Porteous & Clark, 1965). Preincubation at 0°C for 30 min was necessary to obtain full activity.

Phosphatidate phosphohydrolase (EC 3.1.3.4) was measured by the method of Coleman & Hübscher (1962) under the following conditions: 40 mM-sodium succinate buffer, pH 5.3, 4 mM-sodium phosphatidate and enzyme protein not exceeding 2.0 mg. After preincubation for 2 min at 37°C, the assay was started by adding enzyme and the incubation was continued for 60 min.

Phospholipases B and C were measured in the following assay system: 40 mM-tris-HCl buffer, pH 7.4 (or 25 mM-

tris-HCl buffer, pH 8.3, or 40 mM-sodium succinate buffer, pH 5.3), 1–4 mM-phosphatidylinositol, 4–8 mM-MnCl₂ (sometimes omitted or replaced by CaCl₂) and 2 mg of enzyme protein. The reaction was started by the addition of enzyme and was allowed to continue for 60 min at 25°C or 37°C. The reaction was stopped as described by Coleman & Hübscher (1962) for phosphatidate phosphohydrolase and total phosphate was determined.

Phospholipase D was assayed in the following system: 25 mM-tris-HCl buffer, pH 8.3, 1 mM-[³²P]phosphatidylinositol (sodium salt), 4 mM-CaCl₂ and 1 mg of enzyme protein. After preincubation for 1 min at 25°C, the reaction was started by adding enzyme and incubation continued for 60 min at 25°C. The reaction was stopped and the mixture treated as described previously (extraction method A; Kai *et al.* 1966). The phosphatidic acid region of the paper chromatograms, as shown by markers, was cut out and digested with 3 ml of 60% (w/v) perchloric acid containing 1 drop of 1% ammonium molybdate and radioactivity was measured.

Synthesis of phosphatidic acid by acylation of L-3-glycero[³²P]phosphate was measured in a system containing: 50 mM-potassium phosphate buffer, pH 7.4, 2 mM-neutralized cysteine, 3 mg of bovine serum albumin (fraction V), 3 mM-DL-3-glycero[³²P]phosphate (approx. 4000 c.p.m./μmol in a counter of 6% efficiency), 0.1 mM-neutralized CoA, 1.6 mM-neutralized ATP, 8 mM-MgCl₂, 1.6 mM-potassium palmitate and not more than 1 mg of enzyme protein. After preincubation for 1 min, the enzyme was added to start the reaction and the tubes were incubated at 30°C for 30 min. Radioactivity of the labelled lipid was measured as described by Prottey & Hawthorne (1967).

Phosphatidic acid synthesis from L-1,2-diglyceride was measured by using the following assay system: 25 mM-tris-HCl buffer, pH 8.3, or 25 mM-potassium phosphate buffer, pH 7.4, 1 mM-neutralized [³²P]ATP (900 000 d.p.m./μmol), 10 mM-MgCl₂, 1 mM-L-1,2-diglyceride and 2.4 mg of enzyme protein. After preincubation for 1 min, the reaction was started by adding enzyme and the incubation continued for 60–120 min at 25°C. The lipids were extracted as described above and radioactivity was determined after chromatography.

Phosphatidylinositol synthesis was measured in an incubation mixture containing: 25 mM-tris-HCl buffer, pH 8.3, 4 mM-MnCl₂, 5 mM-[¹⁴C]myo-inositol (20 000 d.p.m./μmol) and up to 3 mg of enzyme protein. After preincubation for 1 min, the reaction was started by adding enzyme and the incubation continued for 60 min at 25°C. The rest of the method followed that described for the measurement of phosphatidylinositol synthesis in brain (Salway, Harwood, Kai, White & Hawthorne, 1968).

In an attempt to measure an exchange reaction between ethanolamine and inositol in phospholipids, the following assays were used: 25 mM-tris-HCl buffer, pH 8.3, 1 mM-[³²P]phosphatidylethanolamine (50 d.p.m./μmol), 4 mM-MnCl₂, 5 mM-myoinositol and 2–3 mg of enzyme protein. Tubes were incubated for 60 min at 25°C. Phosphatidylinositol radioactivity was measured after extraction and chromatography, by following method A of Kai *et al.* (1966). In the second method the concentrations were: 50 mM-tris-HCl buffer, pH 8.3 (or 40 mM-tris-HCl buffer, pH 7.4, or 50 mM-potassium phosphate buffer, pH 7.4, or 40 mM-sodium succinate buffer, pH 5.3), 4 mM-MnCl₂,

1 mM-phosphatidylinositol (sodium salt), 1 mM-[¹⁴C]ethanolamine (200 000 d.p.m./μmol) and 2–3 mg of enzyme protein. The contents were incubated as above but in stoppered tubes. Lipid radioactivity was measured by the method used for assay of phosphatidylinositol synthesis from [¹⁴C]myoinositol (Salway *et al.* 1968). In another approach, 1–2 mM-phosphatidylethanolamine was added to the normal assay for phosphatidylinositol synthesis in order to measure the increased incorporation of inositol.

Formation of phosphatidylinositol from labelled phosphatidic acid was measured in the following mixture: 25 mM-tris-HCl buffer, pH 8.3, 4 mM-MnCl₂, 5 mM-myoinositol, 1 mM-sodium [¹⁴C]phosphatidate (54 000 d.p.m./μmol) and 2 mg of enzyme protein. After preincubation for 1 min, enzyme was added and the incubation was continued for 60 min at 25°C. The resulting lipids were separated by chromatography on formaldehyde-treated paper. Phosphatidic acid and phosphatidylinositol were applied in separate lanes as markers. These lanes only were stained with Nile Blue (Kai *et al.* 1966), since the dye interfered with scintillation counting of ¹⁴C. The developed chromatograms were cut into 4 cm strips parallel to the solvent front and each strip was extracted with chloroform-methanol-conc. HCl (800:400:3, by vol.) The extracts were neutralized with saturated aqueous NaHCO₃ and evaporated to dryness. Radioactivity was determined by the method of Salway *et al.* (1968) for phosphatidylinositol.

RESULTS

Electron microscopy showed that the yeast had a similar appearance to the strains already described by Maclean (1964) and Havelková (1966). Vesicles appeared to develop during the exponential phase of growth in 2% glucose. The doubling time was 5 h at 25°C.

Table 1 shows the phospholipid pattern of *Schiz. pombe* under a variety of conditions. Four different cultures were analysed. Variation in the KH₂PO₄ content of the medium (0.1 or 1.5 g/l) did not affect the phospholipid composition, nor was there a detectable difference between stationary and exponential phases of growth. The fatty acid compositions of the total lipid fraction and purified phosphatidylinositol are shown in Table 2.

The pH of a preparation of crushed cells containing about 20 mg of protein/ml of water was 6.2 before dialysis and 5.4 after dialysis against water. This value for *Schiz. pombe* (pH 6.2) is similar to that determined by Polakis & Bartley (1965) for *Sacch. cerevisiae* (pH 6.45). The pH differs from that of the growth medium and also from the optimum pH for the enzymes studied: phosphatidic acid synthesis (overall system), pH 7.4; phosphatidate phosphohydrolase, pH 5.6; phosphatidylinositol synthesis, pH 8.0.

Biosynthesis of phosphatidic acid in Schiz. pombe. In the experiments with glycero[³²P]phosphate, the labelled lipid extracted from the incubation

Table 1. *Phospholipid composition of Schiz. pombe and Sacch. cerevisiae*

	% of total lipid P			
	Present work* <i>Schiz. pombe</i> (mean $\pm$ S.E.M.)	Letters (1966)† <i>Sacch. cerevisiae</i>	Longley <i>et al.</i> (1968)‡ <i>Sacch. cerevisiae</i> (mean $\pm$ S.E.M.)	Deierkauf & Booiij (1968)§ <i>Sacch. cerevisiae</i>
Phosphatidylcholine	44.9 $\pm$ 3.5	42.0	27 $\pm$ 0.7	45.2
Phosphatidylethanolamine	19.4 $\pm$ 1.0	22.4	29 $\pm$ 0.7	16.7
Phosphatidylinositol	17.6 $\pm$ 1.6	22.5	23 $\pm$ 1.0	{ 19.8 9.3
Phosphatidylserine	5.7 $\pm$ 1.0	7.3		
Methyl glycerophosphate	3.9 $\pm$ 0.4	—	—	—
Total alkali-labile P	91.7 $\pm$ 1.2	—	—	—

* Phospholipids were estimated by paper chromatography after deacylation (Dawson, 1960). Some methyl glycerophosphate is also produced as an artifact. The results are from four different cultures.

† Extraction with acidified solvent. The following values are also given in this paper: phosphatidylglycerol, 1.4; cardiolipin, 2.2; phosphatidylmethylethanolamine, 2.2.

‡ These authors also quote: phosphatidic acid, 12; lysophosphatidylcholine, 7. The yeast was N.C.Y.C. 366.

§ The phosphatidylinositol value includes a small amount of phosphatidylglycerophosphate. These authors also quote: phosphatidic acid + cardiolipin, 5.3; phosphatidylglycerol, 2.5. The yeast was a mutant g_1G_2 .

Table 2. *Fatty acid composition of lipids of Schiz. pombe*

Values are means of three analyses.

Fatty acid	Composition (mol/100 mol)	
	Total lipid	Purified phosphatidylinositol
C _{18:1}	89.3	61.0
C _{16:0}	8.6	37.6
C _{14:0}	0.3	1.0
C _{12:0}	1.0	0.7
C _{10:0}	0.8	0.2

mixture was shown to be phosphatidic acid by chromatography on formaldehyde-treated paper with phosphatidic acid as marker. Acylation of glycerophosphate by *Schiz. pombe* was found to require ATP, DL-3-glycerophosphate and CoA (Figs. 1 and 2). A marked inhibition of synthesis was seen with high concentrations of CoA. Albumin, cysteine and Mg^{2+} or Mn^{2+} ions all stimulated the synthesis. The rate of synthesis was constant for 30 min, after which it fell off sharply. The rate was proportional to enzyme protein concentration in the range 0–1 mg/ml. The optimum pH for phosphatidic acid synthesis was 7.4 (Fig. 2) and the rate was higher in phosphate buffer than in tris-HCl buffer.

The enzyme system required added fatty acid for full activity. The effect of various fatty acids is shown in Fig. 3. Comparison of maximum activities gave the following sequence: C_{14:0} > C_{18:1} > C_{16:0} > C_{18:0} > C_{12:0} = C_{10:0} > C_{8:0}. Acids C_{10:0}, C_{12:0} and C_{18:1} produced marked inhibition at high concentrations. The enzyme was stable at -20°C for

Fig. 1. Phosphatidic acid formation from glycerophosphate by *Schiz. pombe* homogenate. The curves were obtained by varying the concentrations of individual components as follows: (a) ATP; (b) glycerophosphate; (c) albumin; (d) cysteine; (e) bivalent ions. O, Mg^{2+} ; ●, Mn^{2+} . Results were calculated on the assumption that L-3-glycerophosphate was the substrate (cf. Kuhn & Lynen, 1965).

Fig. 2. Phosphatidic acid synthesis by *Schiz. pombe*. Details are as given in the legend to Fig. 1. (a) Effect of pH. Each point is the average of pH determinations before and after the 30 min incubation: $\circ$, 50 mM-potassium phosphate buffer, $\bullet$, 50 mM-tris-HCl buffer. (b) Effect of CoA concentration.

Fig. 3. Phosphatidic acid formation from glycerol[³²P]-phosphate by *Schiz. pombe* homogenate. Details are as given in the legend to Fig. 1. Since there was some activity without added fatty acid each curve is corrected for this. a: $\circ$, C_{8:0} acid; $\square$, C_{10:0} acid; b: $\circ$, C_{12:0} acid; $\square$, C_{14:0} acid; c: $\circ$, C_{18:0} acid; $\square$, C_{16:0} acid; d: $\square$, C_{18:1} acid.

Fig. 4. Activity of phosphatidate phosphohydrolase in *Schiz. pombe* (a) Substrate concentration-activity curves at pH 5.6 ($\bullet$) and pH 7.4 ($\circ$); (b) pH-activity curve with succinate buffers ($\circ$) and tris-HCl buffers ($\bullet$).

several weeks. *Sacch. cerevisiae* homogenates showed a similar order of activity in the synthesis of phosphatidic acid when measured under the same conditions.

When attempts were made to measure phosphatidic acid synthesis from L-1,2-diglyceride and labelled ATP, no activity could be detected under a wide variety of conditions. Diglyceride kinase therefore appears to be absent from *Schiz. pombe*.

Phosphatidate phosphohydrolase in *Schiz. pombe*. The properties of phosphatidate phosphohydrolase were first measured at pH 7.4 (optimum pH for phosphatidic acid synthesis), then at pH 5.6 (pH optimum for phosphatidic acid breakdown). No detectable activity could be measured with less than 2 mM-sodium phosphatidate at pH 7.4 (Fig. 4).

Table 3. Effect of bivalent cations on phosphatidate phosphohydrolase

Chlorides of the metal ions were used.

Concn. of metal ion (mM)	Metal ion ...	Phosphatidate hydrolysis ($\mu\text{mol/h}$ per mg of protein)		
		Mn ²⁺	Ca ²⁺	Mg ²⁺
0		0.91	—	—
2		1.32	1.28	1.27
4		0.17	0.79	1.30
8		0.05	0.17	0.24
12		0	0	0.22

Fig. 5. Phosphatidylinositol synthesis in *Schiz. pombe*. Chromatography of the product on silicic acid. ¹⁴C was determined on 1 ml samples from each 10 ml fraction. Abbreviations: NL, neutral lipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

The K_m was 0.91 mM and the maximum velocity was 1 $\mu\text{mol/h}$ per mg of protein at pH 5.6. At the pH optimum (5.6) the rate remained constant for 100 min and at pH 7.4 for 60 min. At each pH the rate was proportional to protein concentration over the range 0–2.5 mg of protein/ml. Particulate enzyme, prepared by centrifuging the cell-free homogenate at 104 000g for 60 min, was used to examine the effect of bivalent cations since it had a higher specific activity. At 2 mM concentration Ca²⁺ and Mn²⁺ stimulated, whereas at 8 mM and 12 mM they strongly inhibited (Table 3). *Sacch. cerevisiae* homogenate also contained phosphatidate phosphohydrolase.

Phosphatidylinositol synthesis from [¹⁴C]inositol in *Schiz. pombe*. To investigate the products of this reaction the chloroform phases from several incubations were filtered through glass-fibre paper to remove protein, and the material was loaded on to a silicic acid column (1.5 cm × 4 cm) in chloroform together with unlabelled yeast phosphatidylinositol (200 μg of P). The eluate was monitored by scintillation counting and t.l.c. The carrier phosphatidylinositol together with most of the radioactivity was eluted with chloroform-methanol (1:1, v/v) (Fig. 5).

Fig. 6. Effect of bivalent cations on phosphatidylinositol synthesis in *Schiz. pombe*. The assay system is described in the Materials and Methods section. ○, Mn²⁺; ●, Mg²⁺; □, Ca²⁺.Table 4. Effect of nucleotides on phosphatidylinositol synthesis in *Schiz. pombe*

Nucleotide	Phosphatidylinositol synthesis (nmol/h per mg of protein)	
	1.5 mM	3.0 mM
Expt. 1: dialysed homogenate		
Concn. of addition ...		
ATP	1.7	2.1
CTP	4.0	3.0
CDP	3.8	3.4
CMP	4.0	3.2
CDP-choline	3.9	3.5
CDP-diglyceride	2.3	3.8
No addition (dialysed enzyme)		1.9
No addition (undialysed enzyme)		4.0
Expt. 2: undialysed homogenate		
CTP	4.7	3.2
CTP with equimolar phosphatidate	3.1	2.1
CMP	6.2	5.7
CMP with equimolar phosphatidate	4.4	3.5
No addition		12.6

Fractions containing phosphatidylinositol were pooled, evaporated to dryness and chromatographed on formaldehyde-treated paper together with phosphatidic acid and phosphatidylinositol markers. After the markers and one lane had been stained with Nile Blue, the other lane was cut into strips (3 cm × 15.2 cm), eluted with chloroform-methanol-conc. HCl (800:400:3, by vol.) and its radioactivity measured. In the phosphatidylinositol region 90% of the radioactivity was recovered. The above evidence and the fact that phosphatidylinositol was the most effective phospholipid inhibitor indicate that it was the product of the reaction.

To examine in detail the effects of bivalent cations and nucleotides on phosphatidylinositol synthesis, the *Schiz. pombe* homogenate was usually dialysed against water. This resulted in a loss of about 50% of the activity whether the total dialysis

Table 5. *Effect of phospholipids on phosphatidylinositol synthesis in Schiz. pombe*

The basic assay system was used with dialysed cell-free homogenate. The values represent the percentage of the corresponding control rate with dialysed homogenate and no added phospholipid.

Phospholipid	Concn. of phospholipid ...	4 mM-GSH		No GSH 1 mM
		1 mM	2 mM	
Sodium phosphatidate		107	112	113
CDP-diglyceride (potassium salt)		18	17	21
Phosphatidylethanolamine		112	117	124
Phosphatidylcholine		46	47	76
Phosphatidylglycerol (sodium salt)		98	74	—
Phosphatidylinositol (sodium salt)		14	11	11
L-1,2-Diglyceride		—	—	104
Control rates (nmol of phosphatidylinositol/h per mg of protein)				
Undialysed homogenate		11.6	11.6	9.02
Dialysed homogenate		7.12	7.12	6.96

Fig. 7. Effect of CDP-diglyceride on phosphatidylinositol synthesis. (a) Tris-HCl buffer, pH 8.3, 4 mM-MnCl₂: O, undialysed *Schiz. pombe*; ●, undialysed *Sacch. cerevisiae*; □, dialysed *Schiz. pombe*. (b) *Schiz. pombe*, undialysed, 2 mM-MnCl₂: ▲, tris-HCl buffer, pH 7.4; △, tris-HCl buffer, pH 8.3.

time was 3 h or 21 h. The enzyme was stable at -20°C for 25 days, and GSH (4 mM) sometimes restored the activity of freshly dialysed preparations.

Of the bivalent cations investigated, only Mn²⁺ ions activated the synthesis to any degree (Fig. 6). The maximum reaction rate was obtained with concentrations of inositol between 4 mM and 8 mM. The rate of phosphatidylinositol synthesis was constant for 90 min and proportional to enzyme protein concentration in the range 0–3 mg/ml; the optimum pH was 8.0.

When nucleotides were added, ATP had little effect and CDP-diglyceride was less effective at 1.5 mM than at 3.0 mM. The highest activity was with 1.5 mM-CTP, but CMP was equally effective (Table 4). In a second experiment with undialysed enzyme, CMP and CTP both inhibited phosphatidylinositol synthesis, even in the presence of added sodium phosphatidate.

Phosphatidylethanolamine, and to a smaller extent sodium phosphatidate, produced a small but definite stimulation (Table 5). Phosphatidylinositol and CDP-diglyceride produced a marked inhibition. Fig. 7 shows the effect of CDP-diglyceride in more detail. An absolute requirement for this coenzyme was never found in the yeast systems, which thus differ from those mammalian tissues in which phosphatidylinositol synthesis has been studied.

The addition of 1% (w/v) Cutscum decreased the activity dramatically from 6.3 to 1.0 nmol of inositol incorporated/h per mg of protein. In brain, Cutscum stimulated phosphatidylinositol synthesis (J. G. Salway, G. L. White & J. N. Hawthorne, unpublished work).

Exchange enzymes. The absence of a CDP-diglyceride requirement and the stimulation of phosphatidylinositol synthesis by phosphatidate or

phosphatidylethanolamine suggested that the following reactions might be taking place:

However, when [^{32}P]phosphatidylethanolamine and inositol were incubated with cell-free homogenates of *Schiz. pombe*, no radioactive phosphatidylinositol was produced. If [^{14}C]ethanolamine and phosphatidylinositol were mixed with such homogenates, again there was no detectable production of labelled lipid. Sodium [^{14}C]phosphatidate was not converted into phosphatidylinositol in the presence of inositol and the same enzyme preparation. Hence pathways (1) and (2) could not be demonstrated. Phosphatidylinositol synthesis from [^{14}C]inositol in cell-free homogenates of *Schiz. pombe* was practically abolished when an acetone-dried powder was prepared and could not be restored by adding either the extracted lipids or sodium phosphatidate.

Phosphatidylinositol hydrolysis. No release of total water-soluble phosphate from phosphatidylinositol by yeast preparations could be detected under a variety of conditions. The procedure should measure directly phospholipases B and C together with other combinations of enzymes that would yield soluble organic or inorganic phosphate. However, [^{32}P]phosphatidylinositol was converted into phosphatidic acid, indicating the presence of a phospholipase D catalysing reaction (1) above. Ca^{2+} ions activated this enzyme, the optimum concentration being 4mM. Mn^{2+} ions were less effective and there was detectable activity in the absence of metal ions. Cutscum did not appear to stimulate. The assay system was limited by the amount of phospholipid that could be separated clearly on formaldehyde-treated paper and also by the difficulty in producing phosphatidylinositol of great purity and high specific radioactivity.

A cell-free homogenate of *Schiz. pombe* (exponential phase of growth) had an activity at pH 8.3 of 16.7nmol of phosphatidate released/h per mg of protein. The same preparation synthesized phosphatidylinositol at a rate of 13.6nmol/h per mg of protein.

Fractionation of activities. The sediment produced by spinning cell-free homogenate at 104000g for 60min contained a few membranes and numerous particles, probably ribosomes and most of the succinate dehydrogenase activity (Table 6): about 55% of the protein appeared in the supernatant. Phosphatidic acid synthesis was largely in the particulate fraction. On adding back supernatant in a 1:1 ratio (based on protein content), synthesis was inhibited by about 50%. Phosphatidate phosphohydrolase was also located in the particulate fraction. Adding back supernatant to the particles in an approx. 1:1 protein ratio had little effect (1100 compared with 1160nmol of phosphatidate hydrolysed/h per mg of protein after addition of supernatant).

Although the assays for phosphatidate synthesis and breakdown were conducted at different temperatures (30°C and 37°C respectively), the potential for phosphatidic acid breakdown was considerably greater than that for synthesis of the compound.

The system responsible for phosphatidylinositol synthesis appeared to be particulate; the presence of albumin improved the recoveries. Addition of supernatant to the particles caused a marked stimulation, resulting in complete recovery of the activity when the particle/supernatant protein ratio was 1:2. Heating at 95°C for 5min destroyed the effect. Cytidine nucleotides (1.5mM and 3mM) stimulated the particulate activity, although CMP was more effective than CTP. Preliminary work using snail-juice enzyme to digest the cell wall of *Schiz. pombe* indicated that phosphatidylinositol synthesis was not specifically located in mitochondria.

Table 6. *Distribution of enzymic activity in the subcellular fractions of Schiz. pombe homogenate*

Specific activities are expressed as nmol/h per mg of protein.

	Cell-free homogenate		Particulate		Supernatant		Recovery (%)	No. of expts.
	Activity (%)	Sp. activity	Activity (%)	Sp. activity	Activity (%)	Sp. activity		
Protein	100	—	58.0	—	54.3	—	112.3	4
Succinate dehydrogenase	100	382	90.9	594	5.7	42	96.6	3
Phosphatidate synthesis	100	13.8	98.8	21.3	0	0	98.8	1
Phosphatidate phosphohydrolase	100	827	92.4	1275	0	0	92.4	2
Phosphatidylinositol synthesis without albumin	100	7.7	50.2	4.2	9.8	1.4	60.0	3
with albumin	100	9.5	58.2	8.8	10.2	1.8	68.4	2

drial membranes, as identified by succinate dehydrogenase activity.

DISCUSSION

Biosynthesis of phosphatidylinositol. In animal tissues the biosynthesis of phosphatidylinositol takes place according to the following reaction (Paulus & Kennedy, 1960):

CDP-diglyceride can be synthesised by *Sacch. cerevisiae* (Hutchison & Cronan, 1968) and appears to be involved, directly or indirectly, in the synthesis of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylglycerol by micro-organisms (Kates, 1966). CDP-diglyceride is also involved in cardiolipin synthesis (Stanacev, Chang & Kennedy, 1967). Our failure to find evidence for reaction (3) in *Schiz. pombe* and *Sacch. cerevisiae* was therefore most unexpected. No requirement for CDP-diglyceride could be demonstrated, though this coenzyme inhibited the incorporation of labelled inositol into phosphatidylinositol with both dialysed and undialysed preparations. Reaction (3) cannot be excluded completely, since the enzyme preparations may have contained firmly bound CDP-diglyceride. Hutchison & Cronan (1968), for instance, failed to show a requirement for phosphatidate in the synthesis of CDP-diglyceride by extracts of *Sacch. cerevisiae*. However, the yeast system is strikingly different from that in pancreas (Prottey & Hawthorne, 1967), which shows a clear requirement for CDP-diglyceride. In mammalian tissues phosphatidylinositol can be synthesized from labelled inositol in a system that depends on added CTP and phosphatidate rather than CDP-diglyceride. This could not be demonstrated in the present study with yeast (Table 4).

The results suggest that phosphatidylinositol synthesis in yeast does not require high-energy cytidine nucleotides. Earlier studies have indicated that inositol may be incorporated into phosphatidylinositol by an exchange reaction dependent on Mn^{2+} ions (Paulus & Kennedy, 1960; Hübscher, 1962), although the reaction is still undefined:

The yeast reaction also requires Mn^{2+} ions and is stimulated by cytidine nucleotides at concentrations of Mn^{2+} above 2 mM in the absence of phosphate buffer. The exchange reaction described by Paulus & Kennedy (1960) was only stimulated by CMP when the concentration of Mn^{2+} ions was much lower, or in the presence of buffer.

Of the phospholipids tested as possible acceptors

in reaction (4), only phosphatidylethanolamine and phosphatidic acid showed activity. Phosphatidylinositol, phosphatidylcholine and phosphatidylglycerol were inhibitory. Yang, Freer & Benson (1967) also failed to demonstrate transphosphatidylation between phosphatidylcholine and inositol with cabbage phospholipase D and high concentrations of inositol. The present experiments with labelled phosphatidylethanolamine gave no evidence that it could take part in reaction (4). The specific radioactivity of this lipid, however, was only sufficient to give a count rate of about twice background in the product, assuming that the rate of phosphatidylinositol synthesis was 6 nmol/h per mg of protein. Labelled phosphatidate added to the yeast system did not appear to be converted into phosphatidylinositol by reaction (1). However, the reverse reaction (phospholipase D activity) could be demonstrated with labelled phosphatidylinositol. Bivalent cations activated this phospholipase D, Mn^{2+} being less effective than Ca^{2+} . The enzyme was active at pH 8.0 and the rate of hydrolysis in the presence of Ca^{2+} ions was of the same order as the rate of phosphatidylinositol synthesis in the presence of Mn^{2+} ions. The detergent Cutscum did not activate either synthesis or hydrolysis. In spite of certain similarities, it is not yet clear whether the phospholipase D activity is concerned with phosphatidylinositol synthesis. The equilibrium in reaction (1) is likely to favour hydrolysis rather than synthesis of phosphatidylinositol, unless energy can be provided in some way. The most obvious way would be by the formation of a nucleotide intermediate, but there is little evidence that cytidine derivatives are involved.

Assuming that *Schiz. pombe*, like *Sacch. cerevisiae*, has 15% of protein based on wet weight (Long, 1961), it can be calculated from the doubling time of 5 h at 25°C in exponential growth that 0.13 mg of lipid P is formed per 150 mg of protein in 5 h. Since 18% of the total lipid P represents phosphatidylinositol, the rate of synthesis under these growth conditions will be approx. 1 nmol/h per mg of protein. The cell-free system described in this work can form phosphatidylinositol at approx. 12 nmol/h per mg of homogenate protein (Table 5), a rate more than adequate for growth, turnover and synthesis of new membrane in the yeast cell.

Phosphatidic acid synthesis and hydrolysis. Kuhn & Lynen (1965) studied the acylation of glycerophosphate by *Sacch. cerevisiae*, but their product was largely lysophosphatidic acid. The transferase synthesizing lipid from palmitoyl-CoA (EC 2.3.1.15) had an optimum pH of about 7.0. The pH optimum for the *Schiz. pombe* enzyme system in the present work was 7.4 with palmitate and CoA. Calculation from the results of Kuhn & Lynen (1965), assuming that 43% of the yeast dry weight was protein (Long,

1961), gives a specific activity of about $1\mu\text{mol}$ of glycerophosphate incorporated/h per mg of protein with palmitoyl-CoA or oleoyl-CoA. The highest activity for palmitate incorporation in the present work was much less than this (43 and 35 nmol of glycerophosphate/h per mg of protein for *Sacch. cerevisiae* and *Schiz. pombe* respectively). This indicates that palmitoyl-CoA:L-glycerol-3-phosphate *O*-palmitoyltransferase was not the rate-limiting enzyme in the synthesis of phosphatidic acid from palmitate. As in *Escherichia coli* (Love, 1967; Kito & Pizer, 1969) acylation of glycerophosphate is the predominant pathway for phosphatidic acid synthesis. Comparison of the rates of incorporation of various fatty acids (Fig. 3) indicates some specificity in this enzyme system, since the results are not predictable simply on the basis of the dispersibility of the respective acids in an aqueous medium. For instance, laurate ($\text{C}_{12:0}$ acid) was much less effective than either the $\text{C}_{10:0}$ acid or the $\text{C}_{14:0}$ acid. Brindley & Hübscher (1966) found that myristate was most rapidly incorporated into glycerides synthesized via phosphatidic acid in cat intestinal mucosa. These authors found inhibition by higher concentrations of $\text{C}_{18:0}$ acid and $\text{C}_{12:0}$ acid, results that also resemble the present findings. Of the three fatty acids incorporated most readily into phosphatidate by *Schiz. pombe* ($\text{C}_{14:0}$ acid, $\text{C}_{18:1}$ acid and $\text{C}_{16:0}$ acid), two are major components of the yeast lipid ($\text{C}_{18:1}$ acid and $\text{C}_{16:0}$ acid; Table 2). Diglyceride kinase appears to be absent from *Schiz. pombe*. The phosphatidate phosphohydrolase of *Schiz. pombe* appears very similar in its properties to the enzyme occurring in animal tissues (Coleman & Hübscher, 1962; Sedgwick & Hübscher, 1965).

Yeast phospholipid composition. The phospholipid composition of *Schiz. pombe* is very similar to that of *Sacch. cerevisiae* as determined by Letters (1966) and Deierkauf & Booiij (1968) (Table 1). The results of Longley, Rose & Knights (1968) for *Sacch. cerevisiae* differ considerably from the present results and those of the other workers. This is presumably a reflection of the analytical method used, one-dimensional t.l.c., although Jollow, Kellerman & Linnane (1968) report that the content of phosphatidylcholine is decreased when yeast is cultured under aerobic rather than anaerobic conditions. All workers report a relatively high content of phosphatidylinositol in yeast. The value represents approx. 20% of the total lipid P and contrasts with values of about 5% for mammalian tissues (Ansell & Hawthorne, 1964). This difference makes our failure to demonstrate the CDP-diglyceride biosynthetic pathway in yeast even more noteworthy. Raising the phosphate concentration of the growth medium from 2mM to 4mM has no effect on the phospholipid pattern of *Sacch. cerevisiae*

(Trevelyan, 1966b). This also applies to *Schiz. pombe* over the range 0.7–11mM.

The fatty acid composition of phosphatidylinositol from *Schiz. pombe* differed from that of the total lipid extract (Table 2), the purified phospholipid containing more palmitic acid and less oleic acid. No palmitoleic acid was found in *Schiz. pombe* phosphatidylinositol, which thus differs from that of *Sacch. cerevisiae* (Trevelyan, 1966a,b).

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