Biogenesis of Mitochondria*

PHOSPHOLIPID SYNTHESIS *IN VITRO* BY YEAST MITOCHONDRIAL AND MICROSOMAL FRACTIONS

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The ability *in vitro* of yeast mitochondrial and microsomal fractions to synthesize lipid *de novo* was measured. The major phospholipids synthesized from *sn*-[2-³H]glycerol 3-phosphate by the two microsomal fractions were phosphatidylserine, phosphatidylinositol and phosphatidic acid. The mitochondrial fraction, which had a higher specific activity for total glycerolipid synthesis, synthesized phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid, together with smaller amounts of neutral lipids and diphosphatidylglycerol. Phosphatidylcholine synthesis from both *S*-adenosyl[*Me*-¹⁴C]methionine and CDP-[*Me*-¹⁴C]choline appeared to be localized in the microsomal fraction.

Investigations into the mechanism whereby mitochondria are assembled have shown that some mitochondrial components are derived from mitochondrial synthetic systems, but most of the mitochondrial components are coded for by nuclear DNA and are synthesized on cytoplasmic ribosomes. It is known that mitochondria have their own specific DNA, which in yeast and mammals has the coding capacity for at least mitochondrial rRNA and tRNA. Mitochondria can synthesize some protein components of the mitochondrial adenosine triphosphatase and cytochrome oxidase complexes, and can probably also synthesize components of the adenine nucleotide transporter (Ashwell & Work, 1970; Linnane *et al.*, 1972; Borst, 1972; Tzagoloff *et al.*, 1973; Haslam *et al.*, 1973; Perkins *et al.*, 1973).

The subcellular sites of synthesis of phospholipid components of the mitochondrial membranes are well characterized only in the mammal (McMurray & Dawson, 1969). Mammalian mitochondria synthesize only phosphatidic acid, lysophosphatidic acid, phosphatidylglycerol and diphosphatidylglycerol (Dennis & Kennedy, 1972), and a mechanism operates to transfer the other phospholipids between the endoplasmic reticulum and the mitochondria (McMurray & Dawson, 1969; Wirtz *et al.*, 1972). Although the main pathways of lipid synthesis in yeast have been described (Steiner & Lester, 1972) the distribution of lipid synthetic activities between the mitochondrial and microsomal fractions has not been determined in this organism. This distribution of activities has been investigated in the present study, since such knowledge is important to our under-

standing of the assembly process of mitochondrial membranes.

Experimental

Reagents and enzymes

Materials were purchased from the following sources: creatine phosphate, creatine kinase (EC 2.7.3.2), glycerol kinase (EC 2.7.1.30), cysteine, serine, glycerol 3-phosphate, *S*-adenosylmethionine, CDP-choline, CTP, ATP and CoA from Sigma Chemical Company (St. Louis, Mo., U.S.A.); *myo*-inositol and Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid] from Calbiochem Australia Pty. Ltd. (Sydney, N.S.W., Australia); silica gel 60 HR from E. Merck A.-G. (Darmstadt, Germany); Cab-O-sil from Packard Instrument Co. (Melbourne, Vic., Australia). All other chemicals were reagent grade. *S*-Adenosyl[*Me*-¹⁴C]methionine and [2-³H]glycerol were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and CDP-[*Me*-¹⁴C]choline from New England Nuclear, Boston, Mass., U.S.A. [³²P]Phosphate (carrier-free) was purchased from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. *sn*-[2-³H]Glycerol 3-phosphate was synthesized by the method of Chang & Kennedy (1967).

Growth conditions of the organism

Saccharomyces cerevisiae, strain L410 (α *ura his*) was grown at 30°C under forced aeration in an 11-l fermentor. The growth medium contained salts (Proudlock *et al.*, 1971), 0.5% (w/v) yeast extract, uracil (25 µg/ml), histidine (50 µg/ml) and 1% (w/v)

* This paper is no. 35 in the series. No. 34 is Howell *et al.* (1973).

Fig. 1. *Preparation of subcellular fractions*

A total cell-free supernatant fraction was prepared as described in the text and was further fractionated by centrifugation.

ethanol. The cells were harvested at the mid-exponential phase of growth (2–3 mg dry wt. of cells/ml). For the incorporation of ^{32}P cells were grown aerobically with shaking in a 2 litre fluted flask containing 600 ml of the above medium, with the addition of $[\text{P}^{32}]\text{P}_i$ (4 $\mu\text{Ci/ml}$). The cells were inoculated at 10^{-3} mg/ml and harvested at the mid-exponential phase of growth (after 11–12 generations).

Preparation of subcellular fractions

The cells were washed and protoplasts prepared by treatment with snail gut enzymes (Helicase, L'Industrie Biologique Francaise, Paris, France) as described by Watson *et al.* (1970). Protoplasts were disrupted by passage through a French pressure cell at 10000 kPa in a medium containing sorbitol (0.6 M), EDTA (1 mM) and Tris buffer (15 mM), the final pH being 7.2 (buffer A). Whole cells, nuclei and cell debris were removed by three successive centrifugations at 1000 $g_{av.}$ for 10 min. The supernatant was designated the total cell-free supernatant fraction. Mitochondria were collected by centrifugation at 12000 $g_{av.}$ for 10 min. Mitochondrial fragments were collected as an intermediate pellet which was discarded. Further centrifugations of this supernatant yielded microsomal fractions 1 and 2 (Fig. 1). The crude mitochondrial pellet was further purified

by layering on a continuous 40–80% (w/v) sorbitol gradient containing EDTA (2 mM), Hepes buffer (20 mM), final pH 7.2 and centrifuging for 2 h at 64700 $g_{av.}$ in a Spinco SW 25-1 rotor (Beckman Instruments Inc., Palo Alto 20, Calif., U.S.A.). All fractions were resuspended in buffer A to give a protein concentration of 5 mg/ml.

Assay techniques

NADPH-cytochrome *c* reductase activity was assayed spectrophotometrically as described by Masters *et al.* (1965). NADH oxidase activity was determined by the method of Pullman *et al.* (1960) and protein concentration was determined by the method of Gornall *et al.* (1949). The difference spectra of subcellular fractions were determined by using a Carey Model 14 spectrophotometer with sodium dithionite as the reductant and potassium ferricyanide as the oxidant as described by Chance (1957).

Incubation conditions

The incubation mixture used for the incorporation of *sn*-[2- ^3H]glycerol 3-phosphate into lipid was a modification of that used by Steiner & Lester (1972) and contained MgCl_2 (2.6 mM), MnCl_2 (5.3 mM),

CTP (1.6mM), ATP (5.0mM), creatine phosphate (8.0mM), creatine kinase (0.03mg), cysteine (20mM), CoA (0.09mM), sucrose (100mM), inositol (3mM), serine (3mM), *sn*-[2-³H]glycerol 3-phosphate (0.3mM; 3.1 μ Ci), KH₂PO₄ (1mM), NaF (2mM), Hepes buffer (20mM), all adjusted to pH7.2 with 2M-KOH, and the subcellular fraction (0.5mg of protein). The total volume was 1.0ml. For incubations at pH8.4, Tris buffer (20mM) was substituted for Hepes buffer. For the incorporation of CDP-[*Me*-¹⁴C]-choline CTP, inositol, serine and *sn*-[2-³H]glycerol 3-phosphate were omitted; *sn*-glycerol 3-phosphate (0.3mM) and CDP-[*Me*-¹⁴C]choline (0.05mM; 0.5 μ Ci) were added. The incubation mixture for the incorporation of S-adenosyl[*Me*-¹⁴C]methionine consisted of cysteine (20mM), sucrose (100mM), KH₂PO₄ (1mM), NaF (2mM), Tris buffer (20mM) adjusted to pH8.4 with 2M-KOH, and S-adenosyl[*Me*-¹⁴C]methionine (0.1mM; 0.5 μ Ci) in a total volume of 1.0ml. All incubations were at 30°C. Incubation mixtures were always checked for bacterial contamination by plating and direct scoring of colonies.

Extraction and isolation of lipids

The incubation was terminated and the lipids were extracted by a method based on that of Bligh & Dyer (1959). Methanol-chloroform (2:1, v/v; 3.75ml) was added to the incubation mixture (1.0ml). After 10min the precipitated protein was sedimented in a bench centrifuge and the supernatant withdrawn. The pellet was resuspended in water (1ml) and the extraction procedure repeated. Water (2.5ml) and chloroform (2.5ml) were added to the combined supernatants and the tubes shaken vigorously. The solvent phases were allowed to separate at 0-4°C overnight. The upper phase was discarded

and the lower chloroform phase evaporated to dryness under a stream of nitrogen. The lipid was dissolved in 1.0ml of chloroform and 0.1ml was withdrawn for the determination of radioactivity. The remainder was concentrated by evaporation and used for t.l.c.

Thin-layer chromatography

The lipids were separated by two-dimensional chromatography on silica gel HR plates. Solvent 1 consisted of chloroform-methanol-water (65:25:4, by vol.) and solvent 2 was chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5, by vol.). The chromatography plate was sprayed with Rhodamine 6G (0.2%, w/v) and areas of the gel containing lipids were outlined and scraped into scintillation vials. Cab-O-sil and scintillation fluid were added (Bruno & Christian, 1961). The radioactivity was determined in a Phillips liquid-scintillation analyser.

Results

Characterization of subcellular fractions

Yeast cells were ruptured in a French pressure cell after helicase enzyme digestion and a total cell-free supernatant fraction, a mitochondrial fraction and two microsomal fractions were prepared as described in the Experimental section and in Fig. 1.

Membrane-specific enzymes were assayed to assess the degree of cross-contamination of the fractions (Table 1). The microsomal fractions were less than 1% contaminated by the mitochondrial fraction as assessed by NADH oxidase activity. Measurements of cytochrome *aa*₃ concentration indicated a somewhat higher contamination of about 5%.

The microsomal contamination of the mitochondrial fraction is inherently more difficult to

Table 1. *Properties of subcellular fractions*

Subcellular fractions were prepared and the percentage of total cell-free supernatant protein in each fraction was determined. An intermediate mitochondrial fragment pellet (8.8% of total cell-free supernatant protein) was discarded. Membrane-specific enzymes were assayed as described in the Experimental section. Specific activities are expressed as: NADH oxidase, nmol of NADH oxidized/min per mg of protein; NADPH-cytochrome *c* reductase, nmol of cytochrome *c* reduced/min per mg of protein. Cytochrome *aa*₃ content is given as nmol of cytochrome *aa*₃/mg of protein; *E*_{mM} of cytochrome *c* = 16.0 (Estabrook & Holowsinsky, 1961). The results represent the average of four determinations $\pm$ S.D.

Fraction	% of the total cell-free supernatant protein	NADH oxidase activity	Cytochrome <i>aa</i> ₃ concentration	NADPH-cytochrome <i>c</i> reductase activity
Total cell-free supernatant	100	0.36 $\pm$ 0.13		13.7 $\pm$ 0.6
Mitochondrial fraction	4.6	6.1 $\pm$ 0.22	3.63 $\pm$ 0.28	4.5 $\pm$ 0.8
Microsomal fraction 1	4.1	$\sim$ 0.02	0.20 $\pm$ 0.15	25.0 $\pm$ 1.0
Microsomal fraction 2	15.9	$\sim$ 0.01	$\sim$ 0.01	10.0 $\pm$ 0.3
Soluble supernatant	61.9			

measure owing to the absence of a readily assayable microsomal marker in yeast. Measurements of NADPH-cytochrome *c* reductase activities suggest that the mitochondrial fraction could be contaminated by approximately 20% with the microsomal protein. However, owing to the high endogenous rate encountered in these assays, this estimate of contamination is probably a maximum value. Electron microscopic examination of the mitochondrial fraction showed the presence of some non-mitochondrial membranous material, which is consistent with a contamination of 10–20% by the microsomal fraction. The results suggest that the fractions used in the present study are not grossly contaminated and as shown below, the extent of contamination does not vitiate the results.

Phospholipids of subcellular fractions

Cells were grown for 11–12 generations in the presence of $^{32}\text{P}_i$ and subcellular fractions were then prepared. The lipids were then extracted and separated by t.l.c. The phospholipid composition of the total cell-free supernatant fraction was the same as that of the whole cells and is similar to that found for other strains of yeast (Letters, 1966).

The mitochondrial and microsomal fractions are quite different with respect to their phospholipid composition (Table 2). The mitochondrial fraction is characterized by a high diphosphatidylglycerol and phosphatidylethanolamine content and a low phosphatidylserine and phosphatidylinositol content compared with the microsomal fraction. These distributions are similar to those previously reported for yeast (Jakovcic *et al.*, 1971) and for mammalian mitochondria (Hostetler *et al.*, 1972). The microsomal fraction 1 is characterized by a high proportion of phosphatidylserine and has a diphosphatidylglycerol content of 5.8%. As shown above, contamination of the microsomal fraction by the mitochondrial fraction does not exceed 5%. Thus the diphosphatidylglycerol content of the microsomal fraction cannot be due to contamination with the mitochondrial fraction and it may be concluded that some diphosphatidylglycerol is localized in this fraction, as has been observed in other organisms (for review see White, 1973).

The unknown component, which has a low R_F value, is probably polyphosphoinositide (Lester & Steiner, 1968).

Synthesis of total lipid by sub-cellular fractions

The incorporation of *sn*-[2- ^3H]glycerol 3-phosphate into total lipids by each fraction *in vitro* was investigated. For all fractions, the reaction rate was proportional to protein concentrations up to

Table 2. Phospholipids of subcellular fractions

Fraction	^{32}P content (% of total lipid P_i)							
	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-choline	Phosphatidic acid	Phosphatidylethanolamine	Diphosphatidylglycerol	Phosphatidylglycerol	Unknown
Total cell-free supernatant	18.4 ± 0.2	8.9 ± 0.2	33.3 ± 2.2	0.8 ± 0.5	28.6 ± 2.6	8.4 ± 0.2	0.5 ± 0.1	1.1 ± 0.4
Mitochondrial fraction	8.2 ± 0.2	2.1 ± 0.2	32.8 ± 0.4	2.8 ± 0.7	37.4 ± 2.9	18.1 ± 0.3	0.5 ± 0.1	0.3 ± 0.1
Microsomal fraction 1	16.9 ± 1.1	13.5 ± 0.3	32.0 ± 1.6	2.2 ± 0.4	27.6 ± 2.8	5.8 ± 1.0	0.2 ± 0.0	1.8 ± 0.7
Microsomal fraction 2	19.8 ± 0.5	10.1 ± 0.6	37.7 ± 1.5	1.4 ± 0.2	26.5 ± 2.0	3.1 ± 0.5	0.3 ± 0.1	1.2 ± 0.2
Soluble supernatant	24.1 ± 1.2	7.6 ± 0.1	37.8 ± 3.8	1.3 ± 0.1	25.3 ± 1.6	1.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.0

Cells were grown to a constant specific radioactivity in $^{32}\text{P}_i$ as described in the Experimental section. Subcellular fractions were prepared; the lipids were extracted and then separated by t.l.c. The radioactivity in each lipid species, expressed as the % of total lipid P_i , is presented as the mean ± s.d. of four determinations.

Fig. 2. Synthesis of total lipid by subcellular fractions

The time-course for the incorporation of *sn*-[2-³H]-glycerol 3-phosphate into total lipid by subcellular fractions (0.5 mg of protein) was determined as described in the Experimental section. □, Total cell-free supernatant fraction; Δ, mitochondrial fraction; ●, microsomal fraction 1; ○, microsomal fraction 2. The result is a typical example from a series of five experiments.

1 mg/ml. The rate of lipid synthesis by the mitochondrial fraction (Fig. 2) remained constant for at least 30 min, whereas the rate of synthesis by both microsomal fractions and by the total cell-free supernatant fraction slowed after 2 min. As a result, after 30 min the mitochondrial fraction had synthesized the largest amount of lipid, although the initial rate of lipid synthesis by this fraction was lowest.

To obtain these rates NaF, which is a phosphatase inhibitor, was included in the incubation media. If NaF was omitted (results not shown) a high proportion of diacylglycerol accumulated, and lipid synthesis ceased completely after 10 min, owing to hydrolysis of phosphatidic acid and *sn*-[2-³H]-glycerol 3-phosphate. ATP and CoA were required for maximum rates of synthesis by all the fractions. The optimum concentrations were found to be 5 mM and 90 μM respectively. Some limited synthesis occurred even in the absence of these compounds, and was probably due to the utilization of endogenous fatty acyl-CoA esters.

Synthesis of individual phospholipids

The synthesis of lipid by the microsomal fractions 1 and 2 is shown in Fig. 3. A very rapid initial synthesis of phosphatidylserine and phosphatidic acid occurred but the concentration of phosphatidic acid in both fractions soon reached a steady-state concentration.

Fig. 3. Synthesis of individual phospholipids by microsomal fractions

The time-course for the incorporation of *sn*-[2-³H]glycerol 3-phosphate by (a) microsomal fraction 1 (0.5 mg of protein) and (b) microsomal fraction 2 (0.5 mg of protein) was determined as described in the Experimental section. ●, Phosphatidylserine; ▼, phosphatidic acid; ○, phosphatidylinositol; ■, phosphatidylethanolamine; □, neutral lipids; ▲, diphosphatidylglycerol; ▽, phosphatidylglycerol; △, phosphatidylcholine. The result is a typical example from a series of five experiments.

The synthesis of phosphatidylethanolamine by the microsomal fraction 1 was much greater than that by the microsomal fraction 2. With both fractions, there was little synthesis of phosphatidylglycerol, diphosphatidylglycerol and neutral lipid.

The pattern of lipid synthesis shown by the mitochondrial fraction was entirely different from that of the other fractions (Fig. 4). After an initial lag, there was a rapid synthesis of phosphatidylglycerol and

Fig. 4. Synthesis of individual phospholipids by the mitochondrial fraction

The time-course for the incorporation of *sn*-[2-³H]glycerol 3-phosphate by the mitochondrial fraction (0.5mg of protein) was determined as described in the Experimental section. The symbols used are as in Fig. 3. The result is a typical example from a series of five experiments.

Fig. 5. Synthesis of individual phospholipids by total cell-free supernatant fractions

The time-course for the incorporation of *sn*-[2-³H]glycerol 3-phosphate by total cell-free supernatant fraction (0.5mg of protein) was determined as described in the Experimental section. The symbols used are as in Fig. 3. The result is a typical example from a series of five experiments.

phosphatidylethanolamine. The concentrations of phosphatidylserine and phosphatidic acid soon reached a steady state but substantial synthesis of diphosphatidylglycerol and neutral lipids occurred.

The synthesis of individual phospholipids by the total cell-free supernatant fraction is shown in Fig. 5. The major phospholipid species synthesized were phosphatidic acid, phosphatidylserine and phosphatidylinositol. A very rapid initial synthesis of phosphatidic acid and phosphatidylserine occurred, but after 10min the initial rate of synthesis slowed down. Only small amounts of phosphatidylethanolamine, neutral lipid, phosphatidylglycerol and diphosphatidylglycerol were formed. The phospholipid species synthesized by the total cell-free supernatant fraction were similar to those synthesized by the microsomal fractions; this is a predictable result as the microsomal fraction constitutes a larger proportion of the cell-free supernatant protein compared with the mitochondrial fraction (Table 1).

The soluble supernatant fraction did not synthesize phospholipid. Moreover, when it was added to incubations containing other fractions, the soluble supernatant fraction did not alter the extent or the pattern of the lipids synthesized. The synthesis of phosphatidylcholine from *sn*-[2-³H]glycerol 3-phosphate was not observed in any fraction.

Confirmation of synthetic pathways

The incubation conditions were modified to confirm the presence of conventional synthetic pathways. All effects noted were reproducible. The omission of inositol from the incubation mixture resulted in a substantial decrease in the proportion of phosphatidylinositol synthesized by the microsomal fractions. However, the extent of phosphatidylinositol synthesis in the mitochondrial fraction was almost unaffected (Table 3). This could be due to a high endogenous concentration of inositol in the mitochondrial fraction or to generation of free inositol through turnover of phosphatidylinositol. The omission of CTP appreciably affected phosphatidylinositol synthesis, an observation which is consistent with this proposal. With all three particulate fractions, the omission of serine resulted in a decreased synthesis of phosphatidylserine and phosphatidylethanolamine. The precursor-product relationship between phosphatidylserine and phosphatidylethanolamine was confirmed by the addition of hydroxylamine, which is a potent inhibitor of phosphatidylserine decarboxylation (Kaback & Stadtman, 1968). Under these conditions, accumulation of phosphatidic acid and phosphatidylserine in both the mitochondrial fraction and microsomal fraction 1 occurred and no phosphatidylethanolamine synthesis was observed (Table 3). The omission of

Table 3. *Effect of alterations in the incubation conditions on phospholipid synthesis*

Subcellular fractions were prepared and incubated for 30 min as described in the Experimental section and under the conditions shown in the Table. Lipids were extracted and separated by t.l.c. The radioactivity in the total extract and in each lipid species was determined and results are presented as mol of product/100 mol of glycerolipid [^3H]glycerol in each species. The results represent the average of two experiments.

Incubation conditions	Total lipid synthesized (d.p.m./30 min per 0.5 mg of protein)	Amount of lipid synthesized (mol/100 mol of glycerolipid [³ H]glycerol)							
		Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-choline	Phosphatidic acid	Phosphatidyl-ethanolamine	Diphosphatidylglycerol	Phosphatidyl-glycerol	Neutral lipid
Mitochondrial fraction									
Normal	334290	14.7	14.5	0.2	9.0	25.9	4.6	22.9	7.8
-Serine, -inositol	315110	15.5	2.5	0.3	20.2	9.6	13.2	6.3	32.1
+Hydroxylamine	186340	11.2	39.2	0.2	36.7	0.4	1.1	8.5	8.2
-CTP	148610	1.4	0.6	0.2	80.6	0.9	0.9	0.7	12.1
-ATP	166261	0.9	1.2	0.4	0.2	0.1	11.0	85.3	0.4
pH8.4	40310	0.6	1.0	0.4	1.9	0.2	30.1	64.9	0.7
Microsomal fraction 1									
Normal	332805	24.6	40.5	0.2	17.0	12.1	0.5	3.2	1.0
-Serine, -inositol	321654	7.0	6.2	0.3	56.5	9.4	1.2	12.8	5.6
+Hydroxylamine	322805	17.1	56.1	0.3	24.9	0.6	0.1	0.1	1.2
-CTP	368174	2.4	3.0	0.1	88.6	1.9	0.1	0.1	3.8
pH8.4	158656	25.1	29.2	0.2	24.2	12.0	1.8	3.7	2.5
Microsomal fraction 2									
Normal	190907	27.7	38.9	0.2	27.4	3.3	0.1	1.3	1.3
-Serine, -inositol	171285	3.5	14.8	0.2	68.0	2.9	0.1	2.1	6.8
pH8.4	134290	29.6	41.6	0.4	24.1	2.9	0.2	0.4	2.2

CTP from both the mitochondrial fraction and the microsomal fraction 1 incubations resulted in the synthesis of phosphatidic acid and neutral lipid only.

Analysis of the lipids synthesized by the mitochondrial fraction in the absence of ATP showed that phosphatidylglycerol was the major product. An examination of the time-course of the reaction in the absence of ATP showed that all products were synthesized in the usual proportions for the first 5 min, after which only phosphatidylglycerol and diphosphatidylglycerol were synthesized. The biochemical basis for this observation requires further investigation.

The synthesis of diphosphatidylglycerol by the mitochondrial fraction was stimulated in the absence of serine and inositol, conditions under which CDP-diacylglycerol would accumulate. The increased synthesis is largely accounted for by the decrease in accumulation of phosphatidylglycerol, suggesting that diphosphatidylglycerol may be synthesized by the condensation of phosphatidylglycerol and CDP-diacylglycerol, as in mammals (Hostetler *et al.*, 1972) and not by the condensation of two phosphatidylglycerol molecules, as in *E. coli* (Hirschberg & Kennedy, 1972).

Synthesis of phosphatidylcholine

The occurrence in yeast of an *S*-adenosylmethionine methylation pathway for phosphatidylcholine synthesis is well established (Steiner & Lester, 1970; Waechter & Lester, 1971). It has also been postulated that the CDP-choline pathway for phosphatidylcholine synthesis occurs in yeast (Steiner & Lester, 1972). When *S*-adenosylmethionine was added to the

standard incubation mixture, no synthesis of phosphatidylcholine from *sn*-[2-³H]glycerol 3-phosphate was observed. The optimum pH value for the *S*-adenosylmethionine pathway *in vitro* is 8.4 (Steiner & Lester, 1970), but even at this pH, no phosphatidylcholine synthesis was observed (Table 3). At pH 8.4, the mitochondrial fraction synthesizes phosphatidylglycerol and diphosphatidylglycerol almost exclusively, whereas the products of the microsomal system are almost the same as those synthesized normally.

Similarly, the addition of CDP-choline to the incubation mixture did not result in detectable synthesis of phosphatidylcholine in any fraction (results not shown). This failure to synthesize phosphatidylcholine from *sn*-[2-³H]glycerol 3-phosphate is probably due to isotopic dilution of the newly synthesized radioactive phosphatidylethanolamine and diacylglycerol precursors by the large excess of these compounds already present in the membrane.

This problem was overcome by the use of radioactive *S*-adenosylmethionine and CDP-choline. The incubation of fractions with *S*-adenosyl[Me-¹⁴C]methionine at pH 8.4 resulted in the synthesis of phosphatidylcholine by all fractions (Fig. 6). The synthesis in combined microsomal fractions 1 and 2 was much greater than that in the mitochondrial fraction. The intermediate compound dimethylphosphatidylethanolamine was observed, but monomethylphosphatidylethanolamine was not. This agrees with the observations of Steiner & Lester (1970), who reported that a crude total particulate fraction from yeast synthesized more dimethylphosphatidylethanolamine than monomethylphosphatidylethanolamine.

Fig. 6. Synthesis of phosphatidylcholine via the *S*-adenosylmethionine pathway

The cell-free supernatant fraction, mitochondrial fraction and a combined microsomal fraction were prepared and incubated at pH 8.4 with *S*-adenosyl[Me-¹⁴C]methionine as described in the Experimental section. The time-course of incorporation by (a) the total cell-free supernatant, (b) the combined microsomal fractions and (c) the mitochondrial fraction (all 0.5 mg of protein) into dimethylphosphatidylethanolamine (▲) and phosphatidylcholine (●) was measured. The data are the average results of two experiments.

Fig. 7. Synthesis of phosphatidylcholine via the CDP-choline pathway

The time-course of the incorporation of CDP-[Me-¹⁴C]-choline into phosphatidylcholine was determined as described in the Experimental section. ▲, Total cell-free supernatant fraction; ■, mitochondrial fraction; ●, combined microsomal fractions. The data are the average results of two experiments.

The substrate for phosphatidylcholine synthesis by the CDP-choline pathway is diacylglycerol. The accumulation of diacylglycerol can be obtained by omitting CTP from the normal incubation mixture. Under these conditions phosphatidylcholine synthesis from CDP-[Me-¹⁴C]choline was observed in all fractions (Fig. 7). As in the incubations with *S*-adenosylmethionine, the synthesis of phosphatidylcholine via the CDP-choline pathway was far more extensive in the combined microsomal fraction than in the mitochondrial fraction.

The extent of phosphatidylcholine synthesis in the mitochondrial fraction is consistent with the degree of contamination of this fraction by the microsomal fraction. However, we cannot discount the possibility that some mitochondrial phosphatidylcholine synthesis does occur.

Discussion

In the present experiments, subcellular fractions of yeast have been shown to synthesize *in vitro* all of the major phospholipids which occur *in vivo*. The rate of synthesis of total phospholipids by a cell-free lysate fraction is similar to that reported by Steiner & Lester (1972). Of the fractions studied, the mitochondrial fraction had the highest specific activity (per mg of protein) for phospholipid synthesis, followed by the microsomal fraction 1. The microsomal fraction 2,

which sedimented at a higher centrifugal force, had the lowest specific activity, which is probably due to a greater amount of ribosomal material in this fraction.

The synthesis of diphosphatidylglycerol and phosphatidylglycerol was localized entirely in the mitochondrial fraction, a localization which is similar to that found in mammals (Kiyasu *et al.*, 1963; Davidson & Stanacev, 1971). However, unlike the mammalian mitochondrial fraction the yeast mitochondrial fraction can also synthesize considerable quantities of phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine. The different response of the mitochondrial and microsomal fractions to incubation at pH 8.4 shows that the synthesis of these phospholipids is not due to microsomal contamination. Under these conditions, the synthesis of phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine ceased in the mitochondrial fraction but continued in both microsomal fractions. Further, under normal incubation conditions, the specific activity of the mitochondrial fraction for the synthesis of these phospholipids is comparable with or greater than that of the microsomal fraction and is therefore too high to be accounted for by contamination.

Phosphatidylserine was the major product of phospholipid synthesis by both microsomal fractions, with smaller amounts of phosphatidic acid and phosphatidylinositol being synthesized. The major difference between the microsomal fractions with respect to lipid synthesis is that phosphatidylethanolamine is synthesized at an appreciably greater rate in microsomal fraction 1. However, the synthesis of phosphatidylethanolamine by decarboxylation of phosphatidylserine is largely localized in the mitochondrial fraction. The synthesis of phosphatidylglycerol, which is the major mitochondrial product, is negligible in microsomal fraction 1, which shows that the phosphatidylethanolamine synthesis in fraction 1 is not due to mitochondrial contamination.

The synthesis of phosphatidylcholine by both the CDP-choline and the *S*-adenosylmethionine pathways appears to be localized mainly in the microsomal fractions. Although not investigated in the present study, it is probable that the pathway for phosphatidylethanolamine synthesis via CDP-ethanolamine also occurs in yeast, as CDP-ethanolamine is found in yeast (Kennedy & Weiss, 1956) and [¹⁴C]ethanolamine added to yeast cultures is incorporated into phosphatidylethanolamine (G. S. Cobon, P. D. Crowfoot & A. W. Linnane, unpublished work).

The high capacity of yeast mitochondrial fractions to synthesize phospholipids *in vitro* indicates that they may be able to synthesize most of their own phospholipids *in vivo*. As the mitochondrial fraction contains a high proportion of phosphatidylcholine

Fig. 8. Pathways of phospholipid synthesis *de novo* in yeast and mammalian mitochondria (excluding base-exchange mechanisms)

The pathways indicated are the major ones found in yeast (—) and mammalian (----) mitochondrial fractions. It is possible that a limited amount of phosphatidylcholine synthesis also occurs in both cases. The mammalian data are taken from McMurray (1973) and from Thompson (1973).

Fig. 9. Pathways of phospholipid synthesis *de novo* in yeast and mammalian microsomal fractions (excluding base-exchange mechanisms)

The pathways indicated are the major ones found in yeast (—) and in mammalian (----) microsomal fractions. The microsomal localization of phosphatidylethanolamine synthesis from diacylglycerol in yeast is tentative.

(Table 2) it is probable that some mechanism, perhaps similar to that which is found in mammals (McMurray & Dawson, 1969; Wirtz *et al.*, 1972; Wirtz & Zilversmit, 1968; Kamp *et al.*, 1973) operates to exchange phospholipids between sub-cellular membrane systems in yeast.

The relative capacities of yeast and rat liver mitochondrial and microsomal fractions to synthesize phospholipids are compared in Figs. 8 and 9. Unlike yeast mitochondrial fractions, rat liver mitochondrial fractions are only capable of the synthesis of phosphatidic acid, phosphatidylglycerol and diphosphatidylglycerol *de novo*, but they can decarboxylate phosphatidylserine to form phosphatidylethanolamine and interconvert phospholipids by base-exchange mechanisms. The other component phospholipids are synthesized in the microsomal fractions and transported via a protein-mediated mechanism. Yeast and rat liver microsomal fractions have similar pathways of phospholipid synthesis except that yeast microsomal fractions contain CDP-diglyceride-L-serine *O*-phosphatidyltransferase (EC 2.7.8.8.) which is absent from rat liver microsomal fractions (Thompson, 1973).

The broader capacity of yeast mitochondrial fractions for lipid synthesis is evidence for a greater biosynthetic autonomy of the yeast mitochondrion as compared with the mammalian mitochondrion.

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