

# Regulation of phosphatidic acid biosynthetic enzymes in *Saccharomyces cerevisiae*

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**Abstract** Phosphatidic acid is the biosynthetic precursor of all glycerolipids. To understand how phosphatidic acid biosynthesis is controlled in *Saccharomyces cerevisiae*, we studied the regulation of three enzyme activities involved in the synthesis of this glycerolipid precursor, i.e., glycerophosphate acyltransferase (GPAT), dihydroxyacetone phosphate acyltransferase (DHAPAT), and acyl DHAP reductase. GPAT activity was increased 3-fold, while DHAPAT activity was increased up to 9-fold in wild type cells grown in a nonfermentable carbon source compared to that of glucose-grown cells. The ratio of GPAT/DHAPAT activity was 12 in glucose-grown cells but only 4 in cells grown in glycerol/ethanol. In the previously characterized *tpa1* mutant, (T. S. Tillman and R. M. Bell, 1986. *J. Biol. Chem.* 261: 9144–9149), GPAT was decreased 2-fold and DHAPAT 27-fold compared to activities in the wild type. Acyl DHAP reductase activity in both wild type and *tpa1* cells grown on a nonfermentable carbon source was increased approximately 2-fold over that of glucose-grown cells. All three enzymatic activities increased as wild type cells grown on glucose entered the stationary phase of growth. Therefore, GPAT, DHAPAT, and acyl DHAP reductase activities appear to be regulated by the respiratory state of the cell. None of the activities was affected to a great extent by inositol, which is a key regulator of many enzymes involved in the synthesis of PtdOH-derived phospholipids in *S. cerevisiae*, nor by deletion of the mitochondrial genome. ■ These data show that *i)* the PtdOH biosynthetic enzymes GPAT, DHAPAT, and acyl DHAP reductase are increased during respiratory growth but are not affected by inositol; *ii)* the extent of derepression differs for the acyltransferases GPAT and DHAPAT in the wild type cell; and *iii)* the extent of reduction of the two enzyme activities is strikingly different in the *tpa1* mutant. The results suggest that GPAT and DHAPAT are probably different enzymes, and that both the G3P and acyl DHAP pathways may be important for glycerolipid synthesis in yeast. — Minskoff, S. A., P. V. Racenis, J. Granger, L. Larkins, A. K. Hajra, and M. L. Greenberg. Regulation of phosphatidic acid biosynthetic enzymes in *Saccharomyces cerevisiae*. *J. Lipid Res.* 1994. 35: 2254–2262.

**Supplementary key words** glycerophosphate acyltransferase • dihydroxyacetone phosphate acyltransferase • acyl dihydroxyacetone phosphate reductase • carbon source • growth phase • inositol • *rho*<sup>0</sup> mutant • *tpa1* mutant

Phosphatidic acid (PtdOH) is the precursor for the major phospholipids present in cell membranes. While much is known regarding the biosynthesis of PtdOH-derived phospholipids, regulation of synthesis of PtdOH itself is not well understood. Two routes to PtdOH synthesis have been described in eukaryotic cells (**Fig. 1**). Acylation of glycerol 3-phosphate (G3P) by acyl-CoA can be catalyzed by glycerophosphate acyltransferase (GPAT) to form 1-acyl-glycerophosphate (lyso-PtdOH). Alternatively, dihydroxyacetone phosphate (DHAP) acylation by acyl-CoA's can be catalyzed by DHAP acyltransferase (DHAPAT) to produce acyl DHAP, which can be enzymatically (acyl DHAP reductase) reduced by NADPH to lyso-PtdOH. Acylation of lyso-PtdOH yields PtdOH. In animals, the acyl DHAP pathway is utilized in the synthesis of ether lipids (1). While ether lipids have not been detected in yeast (2, 3), both GPAT and DHAPAT activities have been observed and characterized in *Saccharomyces cerevisiae* (4–6). In addition, we have shown that *S. cerevisiae* has acyl DHAP reductase activity as well, which suggests that PtdOH can be synthesized by either the G3P- or the acyl DHAP-pathway in this organism (4).

Several aspects of PtdOH biosynthesis in *S. cerevisiae* are not clear. First, the relative contribution of the G3P and the DHAP pathways to the synthesis of PtdOH and PtdOH-derived phospholipids remains to be elucidated. Thus, while the acyl DHAP pathway is required for ether

Abbreviations: PtdOH, phosphatidic acid; GPAT, glycerophosphate acyltransferase; DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetone phosphate acyltransferase; Gro, glycerol; G3P, glycerol 3-phosphate; Ins-1-P, inositol-1-phosphate; CDP-DG, CDP-diacylglycerol; PtdSer, phosphatidylserine; PtdGro-P, phosphatidylglycerolphosphate; TLC, thin-layer chromatography.

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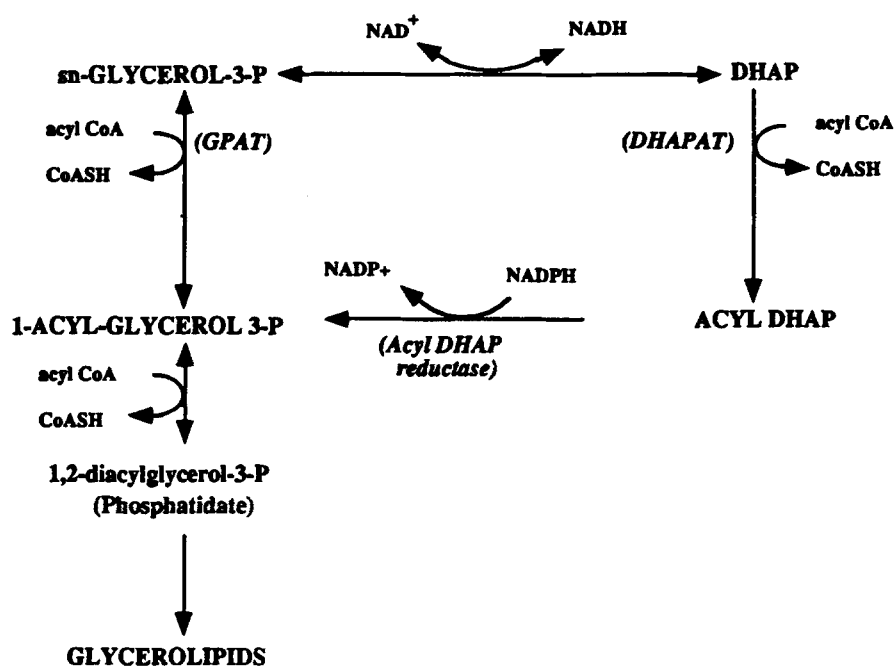


Fig. 1. Pathways of phosphatidate biosynthesis.

lipid synthesis in higher eukaryotes, the role of this pathway in yeast, which lack ether lipids, is unclear. Second, the enzymes involved in PtdOH biosynthesis have not been characterized well. It is not clear whether GPAT and DHAPAT activities are due to the same or to two separate enzymes. Schlossman and Bell (5) reported that these enzymes have identical properties and suggested that the same enzyme may have dual catalytic functions. This is supported by the findings of Tillman and Bell (7), who identified a mutation (*tpa1*) that causes a decrease in both GPAT and DHAPAT activities (7). Racenis et al. (4), however, showed that these two activities have different pH optima and different degrees of sensitivity to inhibition by N-ethylmaleimide. These findings could be consistent with a single enzyme containing two active sites or two separate enzymes.

Inositol, growth phase, and carbon source are important regulatory factors in the expression of yeast biosynthetic enzymes involved in the synthesis of phospholipids derived from PtdOH. PtdOH phosphatase is derepressed 2-fold in *S. cerevisiae* cells grown in the presence of inositol. In contrast, inositol represses inositol-1-phosphate (Ins-1-P) synthase about 50-fold, and causes 2- to 5-fold repression of CDP-diacylglycerol (CDP-DG) synthase, phosphatidylserine (PtdSer) synthase, PtdSer decarboxylase, the phospholipid N-methyltransferases, phosphatidylglycerolphosphate (PtdGro-P) synthase, and choline kinase (8, 9). These enzymes are also regulated during the course of the growth phase. Activities of CDP-DG syn-

thase, PtdSer synthase, and the N-methyltransferases are maximal in the exponential phase (10), while PtdGro-P synthase (11), PtdSer decarboxylase (12), and PtdOH phosphatase (13) activities are maximal as yeast cells enter the stationary phase of growth. The mitochondrial enzyme PtdGro-P synthase is also derepressed 2- to 4-fold in cells grown in a nonfermentable carbon source compared to glucose-grown cells (11).

Much less is known about the regulation of enzymes involved in the synthesis of PtdOH. GPAT, DHAPAT, and acyl DHAP reductase activities are reduced by 50% in *S. cerevisiae* grown under anaerobic conditions compared to cells grown in the presence of oxygen (4). This observation suggests that these three activities may be co-regulated, and prompted us to further examine the regulation of expression of these activities in yeast. The present study addresses the effects of carbon source, growth phase, inositol, and the mitochondrial genome on the regulation of GPAT, DHAPAT, and acyl DHAP reductase activities. Our results indicate that GPAT, DHAPAT, and acyl DHAP reductase activities are regulated by factors affecting the respiratory state of the cell, but not by inositol. Interestingly, while both GPAT and DHAPAT activities were increased during respiratory growth, the extent of increase was different for the two enzymes. Furthermore, the relative decrease in these two activities differed in the *tpa1* mutant. These results suggest that these two activities may be due to separate acyltransferases.

## MATERIALS AND METHODS

### Strains

The *S. cerevisiae* strains used in this study are listed in **Table 1**. The *tpa1* mutant was isolated and kindly provided by Tillman and Bell (7). Strain WTA1-5 is a *tpa1* mutant strain derived by twice backcrossing the original mutant to a wild type strain (W303) (Tillman and Bell, personal communication).

### Growth media

*S. cerevisiae* strains were maintained in 15% glycerol at  $-80^{\circ}\text{C}$  for long-term storage and on YEPD (1% yeast extract, 2% peptone, 2% dextrose) plates (2% agar) at  $4^{\circ}\text{C}$  for short-term storage. For experiments, cells were grown on YEP or complete synthetic medium. The YEP medium used for experiments contained one of the following carbon sources: 2% glucose, 2% fructose, 2% galactose, 3% glycerol plus 1% ethanol, 3% glycerol, 1% ethanol, or 2% dihydroxyacetone. Complete synthetic medium contained vitamin-free yeast base (2.7 g/liter), vitamins (described in reference 14), glucose (2%), and amino acids. Vitamin-free yeast base was prepared in the laboratory (similar to Difco vitamin-free yeast base) and consisted of ammonium sulfate (2 g/liter), boric acid (200  $\mu\text{g}$ /liter), calcium chloride (0.04 g/liter), copper sulfate (16  $\mu\text{g}$ /liter), ferric chloride (80  $\mu\text{g}$ /liter), magnesium sulfate (0.2 g/liter), manganese sulfate (160  $\mu\text{g}$ /liter), potassium iodide (40  $\mu\text{g}$ /liter), potassium phosphate, monobasic (0.4 g/liter), sodium chloride (0.04 g/liter), sodium molybdate (80  $\mu\text{g}$ /liter), and zinc sulfate (160  $\mu\text{g}$ /liter). Complete synthetic medium (complete medium) contained the following amino acids and nucleotides: arginine (0.02 g/liter), histidine (0.01 g/liter), lysine (0.02 g/liter), leucine (0.06 g/liter), methionine (0.02 g/liter), threonine (0.3 g/liter), tryptophan (0.02 g/liter), adenine (0.02 g/liter), and uracil (0.04 g/liter).

### Materials

Components of synthetic medium, palmitoyl-CoA, EDTA, ATP, Tris base, triethanolamine-HCl, dihydroxyacetone, and DHAP were purchased from Sigma Chemical Company (St. Louis, MO). Yeast extract, peptone, and agar were purchased from Difco (Livonia, MI). Glycerol kinase was purchased from Boehringer Mannheim

(Indianapolis, IN).  $[2\text{-}^3\text{H}]\text{glycerol}$  and  $[2\text{-}^3\text{H}]\text{glycerol-3-phosphate}$  were obtained from DuPont, NEN Research Products (Boston, MA).  $[2\text{-}^3\text{H}]\text{glycerol-3-phosphate}$  ( $2 \times 10^6/\text{dpm}/\mu\text{l}$ ) was also synthesized by the method of Hajra and Burke (15, 16) as follows.  $[2\text{-}^3\text{H}]\text{glycerol}$  (DuPont, NEN; 30 Ci/mmol) was incubated with 16.7 mM ATP, 50 mM triethanolamine-HCl (TEA) (pH 8.0), 5.2 mM  $\text{MgCl}_2$ , and 3.3 mM glycerol in the presence of glycerol kinase (8.6 mg/liter) in a total volume of 3 ml at room temperature for at least 2 h. The reaction appeared to be 100% complete as determined by chromatography on Whatman no. 1 paper in butanol-acetic acid-water 25:4:10, followed by autoradiography.  $[^{32}\text{P}]\text{DHAP}$  and  $[^{32}\text{P}]\text{G3P}$  were prepared by the enzymatic phosphorylation of dihydroxyacetone and glycerol, respectively, with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as described previously (16). B- $[4\text{-}^3\text{H}]\text{NADPH}$  was prepared and purified according to procedures described by Das and Hajra (17). Palmitoyl DHAP and hexadecyl DHAP were chemically synthesized as previously described by Hajra, Saraswathi, and Das (18).

### Growth conditions

Liquid cultures were inoculated from YEPD plates and grown overnight at  $30^{\circ}\text{C}$  in a rotary shaker at 200 rpm. Experimental cultures were inoculated from these overnight cultures and grown at least 18 h to the  $A_{550}$  indicated. Experimental cultures contained the same media as the overnight cultures. Cells were harvested by centrifugation ( $4^{\circ}\text{C}$ , 3,000 g, 5 min) and washed once with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 mM sucrose, and 10 mM  $\beta$ -mercaptoethanol (buffer 1). Cell pellets were frozen immediately at  $-80^{\circ}\text{C}$ .

### Viable cells

For determination of the number of viable cells, serial dilutions of cultures in sterile water were spread onto YEPD plates and incubated for several days at  $30^{\circ}\text{C}$ .

### Preparation of cell extracts

Cell extracts were prepared as described previously (11). Cell pellets were suspended in buffer 1 (1 g wet weight of cells plus 1 ml buffer), and cells were broken open by vortexing with pre-chilled glass beads for five 1-min intervals, with cooling of the cells on ice between intervals. Extracts remained on ice throughout the procedure, and all centrifugations were at  $4^{\circ}\text{C}$ . Extracts were then centrifuged at 3,000 g for 5 min, and supernatants were transferred to fresh test tubes. The pellet was washed twice in the buffer as described above, transferring the supernatants each time. The washed pellet was discarded, and the supernatants were combined and stored at  $-80^{\circ}\text{C}$ .

### Preparation of mitochondrial extracts

Mitochondrial extracts were prepared as previously described (19). Cell extracts prepared as described above

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
Ade5	MAT $\alpha$ <i>ade5</i>	7
W303-1B	MAT $\alpha$ <i>leu2 his3 ura3 trp1 ade2 can1 TPA1</i>	26
WTA1-5	MAT $\alpha$ <i>leu2 his3 ura3 trp1 ade2 can1 tpa1</i>	26
D273-10B, <i>rho</i> <sup>+</sup>	MAT $\alpha$ <i>met6</i>	
D273-10B, <i>rho</i> <sup>o</sup>	MAT $\alpha$ <i>met6</i>	

were centrifuged at 27,000 *g* for 10 min. The mitochondrial pellets were washed twice in buffer 1, suspended in 50 mM Tris-HCl (pH 7.5), 20% glycerol, and 10 mM  $\beta$ -mercaptoethanol (buffer 2) to a concentration of 2.5 mg/ml (wet weight), and stored at  $-80^{\circ}\text{C}$ .

#### Assay for protein concentration

Protein concentration was determined by the method of Bradford (20) with a protein assay kit (Bio-Rad Laboratories, Mellville, NY) and bovine serum albumin as the standard.

#### Enzyme assays

Glycerophosphate acyltransferase (GPAT) and DHAP acyltransferase (DHAPAT) were assayed by measuring the radiolabeled lipids formed from [ $^{32}\text{P}$ ]G3P or [ $^3\text{H}$ ]G3P, and [ $^{32}\text{P}$ ]DHAP, respectively, and palmitoyl-CoA as described before (4, 21). The products of a GPAT assay were shown to be lyso-phosphatidic acid (5–10%) and phosphatidic acid by thin-layer chromatography (TLC) analysis with chloroform-methanol-acetic acid-water 25:15:2:4 as the solvent system, comparing the radiolabeled products to standards. For the DHAPAT assay only acyl [ $^{32}\text{P}$ ]DHAP was produced as characterized by TLC analysis. Specific activities (SA) were defined as units per mg protein, where 1 unit (U) is the amount of enzyme that catalyzes the synthesis of 1 nmol of product per minute.

Acyl DHAP reductase activity was assayed by measuring the formation of  $^3\text{H}$ -labeled lipid from *O*-hexadecyl DHAP and B-[4- $^3\text{H}$ ]NADPH as described (4, 22). The product was characterized as 1-*O*-hexadecyl-*sn*-[2- $^3\text{H}$ ]glycerol-3-phosphate by TLC analysis (4).

## RESULTS

#### Regulation of GPAT and DHAPAT activities by carbon source

In order to examine the carbon source effect on GPAT and DHAPAT, wild type cells (Ade5) were grown in glucose or glycerol/ethanol, harvested in the exponential phase of growth, and assayed for expression of these two activities (Table 2). Both GPAT and DHAPAT activities were derepressed in cell extracts prepared from cells grown on the nonfermentable carbon source glycerol/ethanol versus the fermentable carbon source glucose. Interestingly, the extent of derepression of these two activities in cell extracts differed. GPAT activity was increased approximately 3-fold, while DHAPAT activity was increased approximately 9-fold. The ratio of GPAT to DHAPAT activity in glucose-grown cells was about 12, compared to a ratio of 4 in the cells grown in glycerol/ethanol (Table 2).

TABLE 2. Effect of carbon source on GPAT and DHAPAT activities in cell extracts

	GPAT (S.A.)	DHAPAT (S.A.)	GPAT/DHAPAT
Glucose	3.24 $\pm$ 1.21	0.27 $\pm$ .11	12.0
Glycerol/ethanol	10.98 $\pm$ 2.95	2.54 $\pm$ 0.74	4.3

Ade5 cells were grown in YEP medium with glucose or glycerol/ethanol to the mid-exponential phase of growth (avg  $A_{550}$  = 0.5;  $2.8 \times 10^7$  cells/ml) and assayed for GPAT and DHAPAT activities in whole cell extracts as described in Materials and Methods. The results represent an average of five separate experiments and specific activities (S.A.) are expressed as mean  $\pm$  SEM.

#### Regulation of GPAT and DHAPAT activities during growth phase

In order to analyze the effect of growth phase on GPAT and DHAPAT activities, wild type cells (Ade5) were assayed for these two activities during logarithmic and stationary phases of growth (Fig. 2). In glucose-grown cells, GPAT activity was derepressed over 2-fold, while DHAPAT activity was derepressed 3.6-fold as cells entered the stationary phase. In glycerol/ethanol, GPAT and DHAPAT activities were relatively constitutive throughout the exponential and stationary phases of growth. The extent of derepression in glycerol/ethanol was greater for DHAPAT than for GPAT throughout the growth phase.

#### GPAT and DHAPAT activity in cells grown in different carbon sources

To further analyze the effect of carbon source on acyltransferase activities, Ade5 cells were grown in a variety of carbon sources to the mid-exponential phase of growth. Acyltransferase activities were assayed in cell extracts (Fig. 3). The results show that both GPAT and DHAPAT activities are lowest in carbon sources glucose and fructose. These acyltransferases were derepressed 3- to 9-fold in cells grown in nonfermentable carbon sources such as glycerol, ethanol, or dihydroxyacetone (Fig. 2). A difference in the extent of derepression of these two enzyme activities was likewise observed (Fig. 3). GPAT and DHAPAT activities were also increased 3-fold in cells grown in galactose compared to those grown in glucose.

#### Effect of inositol on GPAT and DHAPAT activities

As inositol is a major regulator of phospholipid metabolism in yeast (8, 9), the effect of inositol on GPAT and DHAPAT activities was investigated. Ade5 cells were grown to the mid-exponential phase of growth (avg  $A_{550}$  = 0.5;  $2.5 \times 10^7$  cells/ml) in complete synthetic medium containing glucose in the presence or absence of inositol, and enzyme activities were assayed in whole cell

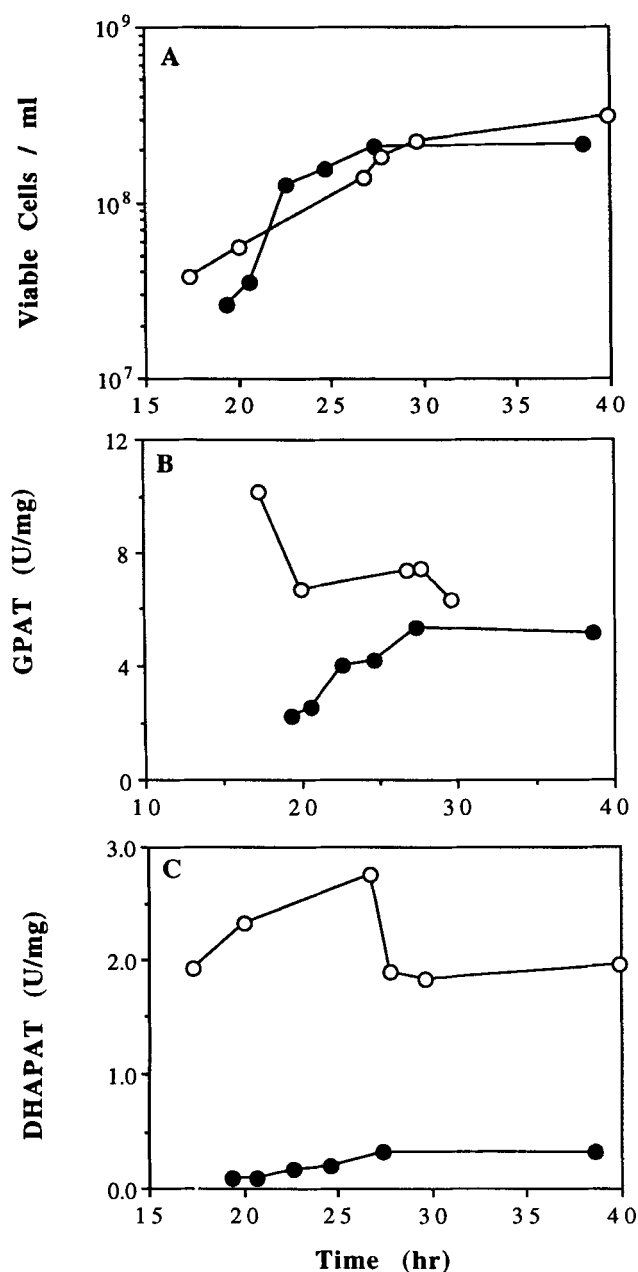


Fig. 2. Effect of growth phase on GPAT and DHAPAT activities in cell extracts. Wild type cells (Ade5) were grown in YEP medium containing glucose or glycerol/ethanol and harvested throughout the growth phase. GPAT and DHAPAT activities were assayed in whole cell extracts as described in Materials and Methods. (A) Viable cell count at the time of harvest; (B) GPAT specific activity; (C) DHAPAT specific activity; glucose (closed circles); glycerol ethanol (open circles). These data are representative of four separate experiments.

extracts. Neither GPAT nor DHAPAT had significantly different specific activities in cells grown in the presence (GPAT,  $7.75 \pm 0.88$  U/mg; DHAPAT,  $0.19 \pm 0.02$  U/mg; mean  $\pm$  SE,  $n = 3$ ) or absence (GPAT,  $9.09 \pm 0.47$  U/mg; DHAPAT,  $0.26 \pm 0.02$  U/mg; mean  $\pm$  SE,  $n = 3$ ) of  $75 \mu\text{M}$  inositol.

## Effect of the mitochondrial genome on GPAT and DHAPAT activities

To determine the dependence of GPAT and DHAPAT on the mitochondrial genome, we assayed activities in wild type cells containing mitochondrial DNA (D273-10B, *rho*<sup>+</sup>) and isogenic cells lacking mitochondrial DNA (D273-10B, *rho*<sup>-</sup>). These two enzyme activities were assayed in extracts from cells grown in YEP glucose and harvested throughout the growth phase. In both whole cell and mitochondrial extracts, GPAT and DHAPAT activities in *rho*<sup>-</sup> cells were similar to those in *rho*<sup>+</sup> cells harvested in the exponential and stationary phases of growth (data not shown). Therefore, these two enzyme activities were not significantly affected by the mitochondrial genome.

## GPAT and DHAPAT activities in the *tpa1* mutant

The mutant *tpa1*, which is deficient in GPAT and DHAPAT activities, was isolated by Tillman and Bell (7) using a replica printing colony autoradiography screen for mutants defective in GPAT activity. We assayed GPAT and DHAPAT in *tpa1* cells grown in glucose or ethanol in order to determine the relative deficiencies of these enzymes. As shown in Table 3, the extents of reduction of GPAT and DHAPAT activities were strikingly different in the mutant. GPAT activity in the mutant was decreased

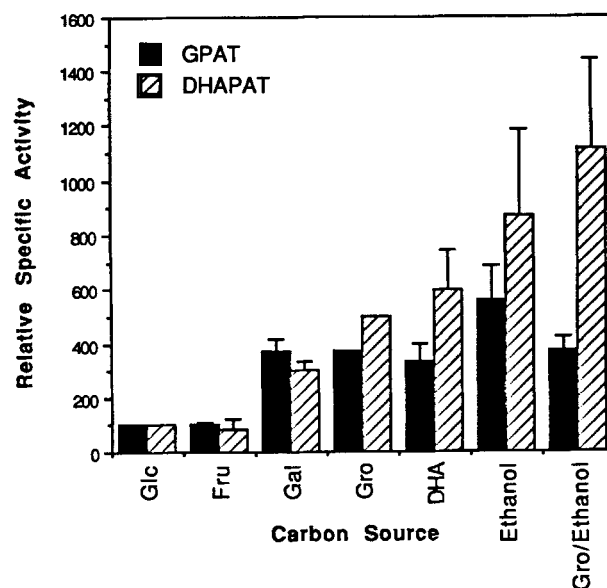


Fig. 3. Effects of carbon source on GPAT and DHAPAT activities in cell extracts. Wild type (Ade5) cells were grown in YEP medium with the indicated carbon source to the mid-exponential phase of growth ( $A_{550} \sim 0.5$ ;  $3.0 \times 10^7$  cells/ml). GPAT and DHAPAT activities were assayed in whole cell extracts as described in Materials and Methods. The results are shown as averages ( $n = 2$  or  $3 \pm$  range) of percentages of specific activities compared to that of glucose-grown cells. The specific activities (mean  $\pm$  SEM,  $n = 3$ ) of GPAT and DHAPAT from glucose grown cells were  $1.72 \pm 0.79$  nmol/min/mg protein and  $0.18 \pm 0.05$  nmol/min/mg protein respectively.

TABLE 3. Effect of carbon source on GPAT and DHAPAT activities in cell extracts of the mutant strain *tpa1*

Strain	Carbon Source	GPAT (% S.A.)	DHAPAT (% S.A.)
Wild type	Glucose	100	100
Wild type	Ethanol	186 ± 40	465 ± 168
<i>tpa1</i>	Glucose	42 ± 9.0	3.7 ± 1.8
<i>tpa1</i>	Ethanol	46 ± 2.0	7.4 ± 1.9

Wild type (W303-1B) and mutant (WTA1-5) cells were grown in YEP medium with the indicated carbon source to the mid-exponential phase of growth (avg  $A_{550} = 0.64$ ;  $2.9 \times 10^7$  cells/ml). The results are shown as averages of percentages of specific activities (S.A.) compared to that of glucose-grown wild type cells (mean ± range,  $n = 2$ ). The specific activities of GPAT and DHAPAT (mean ± range,  $n = 2$ ) from wild type cells grown on glucose were  $46.7 \pm 26.1$  U/mg and  $0.69 \pm 0.22$  U/mg, respectively.

to 42% in glucose-grown cells and to 46% in ethanol-grown cells, while DHAPAT activity was reduced to 3.7% and 7.4% in mutant cells grown on glucose and ethanol, respectively.

#### Regulation of acyl DHAP reductase activity

Previous experiments showed that acyl DHAP reductase activity is high in *S. cerevisiae* membrane fractions, and that activity is reduced 50% in cells grown under anaerobic conditions (4). In order to determine whether this enzyme may be co-regulated with DHAPAT and GPAT, we investigated the effects of carbon source, growth phase, inositol, and the mitochondrial genome on reduc-

tase activity. Results indicated that reductase activity was 1.5- to 2-fold greater in extracts prepared from cells grown on the nonfermentable carbon sources glycerol, ethanol, glycerol/ethanol, and dihydroxyacetone compared to activity in glucose-grown cells (Fig. 4). Reductase activity was also measured as a function of growth phase in glucose or glycerol/ethanol-growth cells (data not shown). Activity was derepressed 3.4-fold as glucose-grown wild type cells entered stationary phase, but only 1.6-fold in glycerol/ethanol-grown cells entering stationary phase. This is similar to regulation of the two acyltransferases described above.

Extracts of wild type cells grown on glucose to the mid-exponential phase of growth in the presence or absence of inositol (75  $\mu$ M) exhibited only a small, although significant ( $P < 0.05$ ) difference in reductase activity ( $1.54 \pm 0.74$  vs.  $2.28 \pm 0.047$ , U/mg, respectively). Reductase activity was not affected by deletion of the mitochondrial genome (data not shown). Reductase activity was also assayed in cell extracts of the *tpa1* mutant. Interestingly, activity was significantly reduced in *tpa1* cells. This decrease (40–50%) was observed in both glucose- and ethanol-grown cells in the late exponential and stationary phase of growth (Fig. 5).

#### DISCUSSION

All of the glycerolipids in both prokaryotic and eukaryotic cells, including yeasts, are derived from PtdOH.

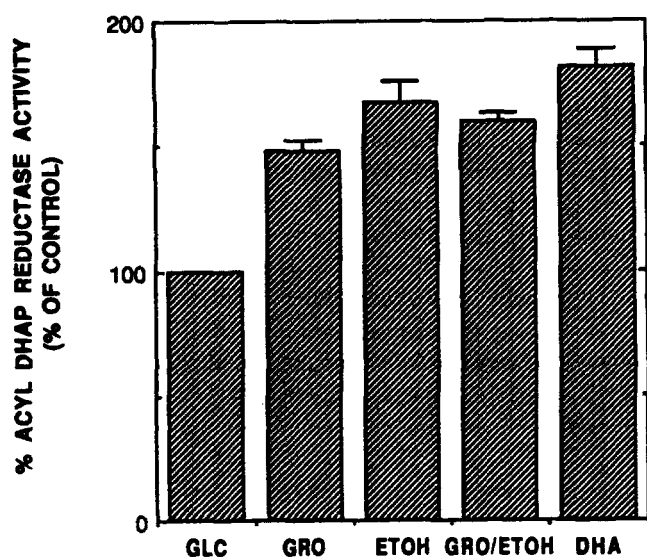


Fig. 4. Effect of carbon source on acyl DHAP reductase activity in cell extracts. Wild type cells (Ade 5) were grown in YEP medium containing the indicated carbon source and harvested in mid-logarithmic phase of growth ( $A_{550} \sim 0.5$ ;  $3.0 \times 10^7$  cells/ml). Acyl DHAP reductase activity was assayed in whole cell extracts as described in Materials and Methods. The results are expressed as the percentage of the specific activity of glucose-grown cells ( $3.93$  nmol/min per mg protein  $\pm 0.383$ ; mean  $\pm$  SEM).

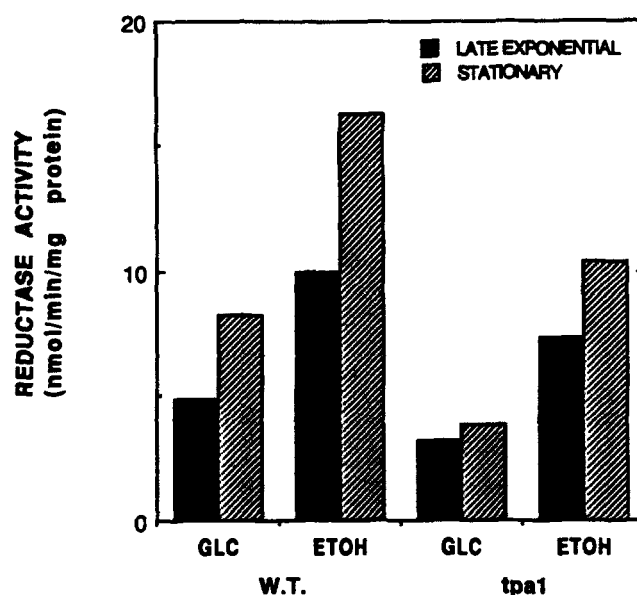


Fig. 5. Acyl DHAP reductase activity in the *tpa1* mutant. Wild type (W303-1B) and *tpa1* (WTA1-5) cells were grown in YEP medium with glucose or ethanol and harvested at logarithmic and stationary phases of growth. Reductase activity was measured in cell extracts as described above. The results ( $n = 2$  or 3) ranged between 0.5 and 15.4 per cent of the average.

A great deal has been learned about the function of these lipids in *S. cerevisiae*, primarily from characterizing the enzymes involved in their synthesis and the regulatory factors controlling expression of these enzymes (8, 9). Because PtdOH is the precursor to cellular phospholipids, knowledge of the route(s) of PtdOH synthesis and the mechanism of regulation of its synthesis is clearly important to understanding how the biosynthesis of glycerolipids is controlled. However, regulation of PtdOH biosynthesis has not been extensively studied. We addressed this topic and conclude the following from the experiments described above. 1) The activities of the PtdOH biosynthetic enzymes GPAT and DHAPAT, and the acyl DHAP reductase, are increased during respirative growth, but are not significantly affected by the presence of exogenous inositol or by deletion of the mitochondrial genome. 2) The extent of derepression differs for GPAT and DHAPAT in the wild type cell. 3) The extent of reduction of the two enzyme activities is strikingly different in the *tpa1* mutant. These data suggest that PtdOH can be synthesized by two different routes, and that GPAT and DHAPAT are probably different enzymes.

Maximal GPAT and DHAPAT activities were observed in cells under metabolic conditions that require mitochondrial function. Thus, activities were highest in extracts of cells grown in nonfermentable carbon sources compared to glucose-grown cells (Table 2 and Figs. 2 and 3). Activities in galactose and dihydroxyacetone were similar to those of the nonfermentable carbon sources. This is true for galactose because metabolism of galactose requires mitochondrial function (23–26). However, it is surprising to see that dihydroxyacetone (DHA) also acts as a nonfermentable carbon source in regulating the GPAT and DHAPAT activities. DHA is metabolized by phosphorylation (ATP) to DHAP, catalyzed by the cytosolic glycerokinase. Because DHAP is a glycolytic intermediate, it seems that DHA metabolism should not require any mitochondrial involvement. However, Sprague and Cronan (6) have shown that glucose represses glycerokinase, and that the regulation of this enzyme is linked to glycerophosphate dehydrogenase, a mitochondrial enzyme. Therefore, it is possible that mitochondrial function is necessary for the metabolism of DHA. Consistent with this possibility is our observation that growth of cells on DHA is very slow, as is growth on the nonfermentable carbon sources. GPAT and DHAPAT activities measured in cell extracts were also derepressed approximately 3-fold as cells grown on glucose entered the stationary phase, when cells presumably switch from fermentation to respiration. These activities were constitutively high throughout growth in glycerol/ethanol, when cells are continually respiring (Fig. 3). Previously we have shown that GPAT and DHAPAT activities are greater in cells grown aerobically than anaerobically (4). One explanation for derepression during respirative growth is that

these enzymes might be located in the mitochondria. Earlier studies on the localization of GPAT have not been definitive, most likely due to different procedures used in subcellular fractionation. Kuchler, Daum, and Paltauf (27) detected GPAT activity in both the mitochondrial and microsomal fractions of *S. cerevisiae*. However, a later study by Zinser et al. (28) detected significant levels of GPAT expression only in the microsomal fraction. In preliminary experiments, we observed enrichment of GPAT and DHAPAT activities in mitochondrial extracts (data not shown). It is possible that these enzymes are located in more than one organelle. Alternatively, the increase could simply be due to the cells' response to increased mitochondrial volume and concomitant need for more membrane phospholipid. In this respect, it is noteworthy that we found that *rho*<sup>o</sup> mutants have the same GPAT and DHAPAT activities as do *rho*<sup>+</sup> cells.

The phospholipid precursor inositol, which is a key regulator of expression of enzymes involved in the synthesis of PtdOH-derived lipids, does not appear to regulate the enzymes of PtdOH synthesis. Acyl DHAP reductase activity in *S. cerevisiae* is only slightly affected by exogenous inositol. In contrast, respiration plays a role in the synthesis of PtdOH as well as PtdOH-derived lipids in this yeast. Activities of CDP-DG synthase, PtdSer synthase, and the phospholipid N-methyltransferases are maximal in exponential phase cells and are repressed as cells enter the stationary phase of growth in media lacking inositol (10). In the presence of inositol, these enzyme activities remain relatively constant at repressed levels in the log and stationary phases of growth (10). In contrast, PtdOH phosphatase, PtdSer decarboxylase, and PtdGroP synthase were shown to be derepressed as glucose-grown cells entered the stationary phase of growth, independent of the presence of inositol (11–13). These latter three enzymes are found in the mitochondria (13, 27, 28), which could explain increased expression as cells switch from glycolysis to respiration. In summary, the entire phospholipid pathway appears to respond to fermentative versus respirative growth changes, while inositol regulation is specific to phospholipid synthesis from PtdOH.

The most striking observation of the present study is that GPAT and DHAPAT are regulated differently by the carbon source supporting growth (Figs. 2 and 3 and Table 2). DHAPAT activity was derepressed up to 3-fold more than GPAT activity in glycerol/ethanol-grown cells. Furthermore, the ratio of GPAT to DHAPAT activity in whole cell extracts from cells grown on glucose was 3-fold higher than the ratio in glycerol/ethanol-grown cells. These results can most easily be explained if the two activities are due to different enzymes. This conclusion is further supported by the differential decreases of these enzyme activities in the *tpa1* mutant. While GPAT activity was decreased only 2-fold, DHAPAT was reduced up to 27-fold in the mutant, compared to wild type cells (Table

3). These results are not in agreement with Tillman and Bell's data (7) showing that, in the mutant, both GPAT and DHAPAT activities and kinetic parameters were altered to the same extent. We note that while we assayed enzyme activities in whole cell extracts, which consist of both membrane-associated and soluble proteins, Tillman and Bell measured GPAT and DHAPAT activities in membrane preparations. The difference in subcellular fractions assayed may thus account for the discrepancy. The subcellular localization of GPAT and DHAPAT activities and the cloning of the structural genes encoding these enzymes may help to resolve this conflict. Experiments addressing these questions are in progress.

If the acyl-DHAP pathway is an important route to the synthesis of PtdOH in *S. cerevisiae*, then one might expect that acyl DHAP reductase would also be regulated by factors affecting GPAT and DHAPAT expression. In fact, acyl DHAP reductase activity in yeast cell extracts was derepressed 2-fold in the presence of oxygen (4), in cells grown on a nonfermentable carbon source (Fig. 4), and as glucose-grown cells entered the stationary phase of growth (Fig. 5). Acyl DHAP reductase activity was also enriched 3.5-fold in mitochondrial extracts. In addition, acyl DHAP reductase was likewise not affected by deletion of mitochondrial DNA. These results suggest that GPAT, DHAPAT, and acyl DHAP reductase may be co-regulated in *S. cerevisiae*.

The implication of separate enzymes for GPAT and DHAPAT activities in *S. cerevisiae* is that the acyl DHAP pathway may play a significant role in PtdOH biosynthesis in this yeast. As *S. cerevisiae* cells do not appear to contain ether lipids, this organism would be an excellent model system in which to study the contribution of this pathway to the biogenesis of non-ether lipids in eukaryotic cells. ■

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