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# Regulation of Phospolipid Synthesis in Phosphatidylserine Synthase-Deficient (*chol*) Mutants of *Saccharomyces cerevisiae*

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***chol* mutants of *Saccharomyces cerevisiae* are deficient in the synthesis of the phospholipid phosphatidylserine owing to lowered activity of the membrane-associated enzyme phosphatidylserine synthase. *chol* mutants are auxotrophic for ethanolamine or choline and, in the absence of these supplements, cannot synthesize phosphatidylethanolamine or phosphatidylcholine (PC). We exploited these characteristics of the *chol* mutants to examine the regulation of phospholipid metabolism in *S. cerevisiae*. Macromolecular synthesis and phospholipid metabolism were examined in *chol* cells starved for ethanolamine. As expected, when *chol* mutants were starved for ethanolamine, the rates of synthesis of the phospholipids phosphatidylethanolamine and PC declined rapidly. Surprisingly, however, coupled to the decline in PC biosynthesis was a simultaneous decrease in the overall rate of phospholipid synthesis. In particular, the rate of synthesis of phosphatidylinositol decreased in parallel with the decline in PC biosynthesis. The results obtained suggest that the slowing of PC biosynthesis in ethanolamine-starved *chol* cells leads to a coordinated decrease in the synthesis of all phospholipids. However, under conditions of ethanolamine deprivation in *chol* cells, the cytoplasmic enzyme inositol-1-phosphate synthase could not be repressed by exogenous inositol, and the endogenous synthesis of the phospholipid precursor inositol appeared to be elevated. The implications of these findings with respect to the coordinated regulation of phospholipid synthesis are discussed.**

In the yeast *Saccharomyces cerevisiae*, many enzymes of phospholipid biosynthesis are regulated in a coordinated fashion (14). These enzymes (see Fig. 1 for the pathways) include the cytoplasmic enzyme inositol-1-phosphate synthase (I-1-PS) (7, 9) and the membrane-associated phospholipid *N*-methyltransferases, which are responsible for the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) (22, 24, 25). Other coordinately regulated enzymes include phosphatidylserine (PS) synthase (5, 17), the gene product of the *CHO1* gene (20), and PS decarboxylase (6). The phospholipid methyltransferases, PS synthase, and PS decarboxylase are all repressed by the addition of choline to medium which already contains inositol (5, 6, 17, 24). Recently it was demonstrated that the presence of inositol, in addition to choline, is required for the repression of the phospholipid methyltransferases and PS synthase (14, 17, 25). I-1-PS is repressed by the addition of inositol alone (7, 9), but it is believed to be regulated in coordination with the other enzymes mentioned above, because it is controlled by the same set of positive and negative regulatory genes (14, 17, 22).

The biological function of this regulation is not known. However, the regulation appears to control the synthesis of inositol-containing lipids, including phosphatidylinositol (PI), by regulating the synthesis of the precursor inositol (Fig. 1). Simultaneously, the regulation controls the sequence of reactions culminating in the synthesis of PC (i.e., cytidine disphosphate-diacyl glycerol [CDP-DG]→PS→PE→→→PC; Fig. 1). The net result of this regulation is coordination of the relative rates of synthesis of PI, a major anionic phospholipid of the yeast membrane, and PC, a major neutral (dipolar ionic) phospholipid (12). It is, therefore, possible that the biological role of the regulation is control of phospholipid net charge. In mutants of *S. cerevisiae* with defects in phospholipid synthesis, phospholipid compositions vary widely, but the overall net charge of the phospholipids is largely conserved. For example, in the *opi3* mutant of yeast, which synthesizes very little PC, the net charge is maintained by the accumulation of the methylated intermediates phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) (Fig. 1) (10). Likewise, in the *chol* mutants which synthesize little or no PS (a negatively charged lipid), there is a compensatory elevation in the proportion of PI (1, 2). In addition, during inositol starvation in *S. cerevisiae*, phospholipid composition changes dramatically, but charge compensation is observed (3). Charge compensation has also been observed in the phospholipid compositions of inositol- and choline-requiring mutants of *Neurospora crassa* (15).

In the present study, we analyzed the regulation of phospholipid biosynthesis by analyzing phospholipid metabolism in yeast *chol* mutants. *chol* mutants cannot synthesize the phospholipid PS owing to a lesion in PS synthase (1, 2, 18; V. A. Letts, Ph.D. thesis, University of Edinburgh, Edinburgh, Scotland, 1980). The mutants have an auxotrophic requirement which is satisfied by either of the precursors ethanolamine or choline, which are incorporated into lipid via the pathway first described by Kennedy and Weiss (16) (Fig. 1), bypassing PS as an intermediate. In the absence of choline or ethanolamine, *chol* mutants cannot synthesize PE or PC. In the present study, we made use of a *chol* mutant to examine the regulation of phospholipid metabolism under conditions in which synthesis of the lipids PS, PE, and PC was blocked.

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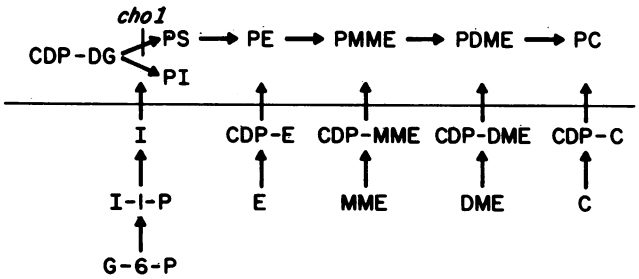


FIG. 1. Pathways for phospholipid synthesis in *S. cerevisiae*. Reactions which take place in the membrane are shown between the double lines. Abbreviations: I, inositol; E, ethanolamine; C, choline; CDP-E, CDP-MME, CDP-DME, and CDP-C, cytidine diphosphate derivatives of E, MME, DME, and C, respectively. The pathway for the incorporation of E, MME, DME, and C into lipids was first described by Kennedy and Weiss (16). The pathways for de novo synthesis of the lipids PS, PI, PE, PMME, PDME, and PC in *S. cerevisiae* were demonstrated in vitro by Steiner and Lester (23). The endogenous synthesis of inositol from glucose-6-phosphate in *S. cerevisiae* was described by Culbertson et al. (7). The position of the genetic lesion in the *cho1* mutant is illustrated.

## MATERIALS AND METHODS

**Strains and media.** The wild-type yeast strain (SHID5C) has the genotype *ade6 ura1 MAT*α. The mutant *cho1* strains were isolated from this parent strain after mutagenesis induced with ethyl methanesulfonate by using the density enrichment procedure described by Letts and Dawes (19).

Strains were maintained on YEPD medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 2% glucose), YEPD plates (YEPD medium, 2% agar), and synthetic complete medium which contained 2% glucose, 0.67% Difco yeast vitamin-free nitrogen base, and supplements including vitamins, amino acids, adenine, and uracil as described by Culbertson and Henry (8). Inositol was added at concentrations ranging from 10 to 75 μM, and ethanolamine or choline (1 mM) was added as indicated.

**Immunoprecipitation of I-1-PS.** Strains were tested by immunoprecipitation for the presence of the 62,000-dalton subunit of I-1-PS. The technique, which has been described previously in more detail (9), involved preparing a crude extract (by Braun homogenization of pelleted cells) of mutant or wild-type yeast cells suspended in a buffer consisting of 20 mM Tris hydrochloride (pH 7.2) containing 2 mM phenylmethylsulfonyl fluoride (to inhibit proteolysis) in 1% dimethyl sulfoxide–10 mM $NH_4Cl$–10 mM 2-mercaptoethanol. The lysate was centrifuged at 4°C for 5 min at 7,000 × *g* (to remove intact cells) and at 27,000 × *g* for 20 min. Immunoprecipitations of the extracts were carried out with rabbit anti-I-1-PS serum prepared as described by Donahue and Henry (9). Volumes of crude extracts corresponding to 1.0 to 2.0 mg of protein were incubated with 10 μl of rabbit antisynthase serum for 3 h at 4°C in 1.5-ml Eppendorf centrifuge tubes and then indirectly precipitated with *Staphylococcus aureus* cells (10% of the volume of the incubation mixture) for 1 h at 4°C. Samples were centrifuged, and the precipitates were washed twice with phosphate-buffered saline containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1% deoxycholate, and 0.5 M NaCl. The washed precipitates were then separated by electrophoresis through 10% sodium dodecyl sulfate-polyacrylamide gels under fully dissociating conditions as described in detail by Donahue and Henry (9). On such gels, the 62,000-dalton subunit of I-1-PS, when present in crude extracts at wild-type derepressed levels, is readily visualized by Coomassie blue staining of the gels. The band is absent in extracts prepared from wild-type cells grown under repressing conditions (9).

**Analysis of phospholipid composition and synthesis.** Phospholipid compositions were determined as previously described (1, 2) by labeling cells with $^{32}P$ for five to six generations until steady-state labeling was achieved. Cultures in synthetic complete medium at 30°C were labeled with [$^{32}P$]orthophosphate (final specific activity in the medium, 0.544 mCi/mmol). To determine the net synthesis of phospholipids and their composition under changing growth conditions (i.e., during ethanolamine starvation), we first labeled phospholipids to steady state as described above. The specific activity of $^{32}P$ in the growth medium was then maintained in all subsequent experimental manipulations as previously described by Atkinson et al. (1). To analyze the relative rates of synthesis of the phospholipids, we pulse-labeled cultures for 20 min in synthetic medium at 30°C with [$^{32}P$]orthophosphate (50 μCi/ml).

The labeled cells were harvested by centrifugation and washed twice in distilled water. Lipids were extracted as described by Atkinson et al. (1). Samples were evaporated to dryness in a stream of nitrogen gas, suspended in 50 μl of chloroform-methanol (2:1 [vol/vol]), and applied to Whatman SG-81 chromatography paper (Whatman, Inc., Clifton, N.J.) treated as described by Steiner and Lester (23). Two-dimensional chromatographic separation of the phospholipids were achieved by using the following solvent systems originally described by Steiner and Lester (23): direction 1, chloroform-methanol-ammonium hydroxide-water (165:67.5:7.5:2 [vol/vol]); direction 2, chloroform-methanol-acetic acid-water (160:20:25:5 [vol/vol]). The labeled lipids were identified by autoradiography, and the labeled areas were cut from the paper and counted by liquid scintillation. Phospholipids were identified by comparison with authentic phospholipid standards as previously described (1, 2, 10).

**Preparation of crude membranes and in vitro assay of phospholipid methylation.** Cells were grown in synthetic complete medium at 30°C and harvested by centrifugation at 2,500 × *g* for 10 min. The cells were then washed in 0.2 M sucrose–0.05 M $KH_2PO_4$ (pH 7.2) and suspended in this buffer at a concentration of 1 g (wet cell weight) per ml before cell rupture with a Braun homogenizer. Whole cells and glass beads were removed by centrifuging the lysate twice at 5,000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 60 min, and the pellet was retained and suspended in buffer. The sample was centrifuged again at 100,000 × *g* for 60 min. The resulting pellet, containing most of the cellular methyltransferase activity, was suspended in sucrose buffer on ice and used immediately for enzyme assays. Protein was determined by the Coomassie blue dye-binding method of Bradford (4) with serum albumin as the standard.

Phospholipid methyltransferase activity was measured in these membrane preparations by a modification of the procedure of Waechter and Lester (24). The assay mixture contained 15 mM potassium phosphate (pH 7.2), 60 mM sucrose, 0.25 mM *S*-adenosylmethionine, and 1 μCi of [*methyl*-$^3H$]*S*-adenosylmethionine per ml. The assay was started by the addition of approximately 0.5 mg of crude membrane preparation per ml. Each sample contained 0.5 ml total volume, and the reaction temperature was 30°C. The reaction was terminated by the addition of 3 ml of chloroform-methanol (2:1 [vol/vol]). This mixture was vortexed

TABLE 1. Phospholipid composition of *chol* mutants compared with that of the wild-type strain

| Strain (genotype)[a] | Phospholipid composition (%)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | PA | PI | PS | PE | PMME and PDME | PC | Other |
| Wild type (*ade6 ura1 MAT*α) | 1.7 | 23.3 | 8.5 | 12.6 | 4.9 | 33.2 | 4.8 |
| KA101 (*chol-1 inol-13 lys2 MAT*α) | 2.6 | 33.6 | ND | 15.0 | 2.0 | 41.7 | 5.1 |
| 214 (*chol-3 ade6 ura1 MAT*α) | 1.9 | 39.1 | 1.0 | 16.4 | 0.8 | 34.3 | 6.5 |
| 2212 (*chol-4 ade6 ura1 MAT*α) | 2.0 | 36.1 | 1.4 | 18.6 | 1.7 | 36.4 | 3.8 |
| 12 (*chol-5 ade6 ura1 MAT*α) | 2.5 | 33.9 | 1.1 | 19.2 | 1.6 | 38.0 | 3.3 |
| 303 (*chol-6 ade6 ura1 MAT*α) | 2.6 | 34.1 | 0.7 | 14.9 | 2.8 | 42.0 | 2.9 |
| 631 (*chol-7 ade6 ura1 MAT*α) | 2.5 | 34.1 | 2.0 | 19.4 | 2.8 | 32.8 | 6.4 |
| 1122 (*chol-8 ade6 ura1 MAT*α) | 2.5 | 38.7 | 0.8 | 16.4 | 2.1 | 34.1 | 5.4 |

[a] The strain numbers correspond to those referred to in reference 19 and by Letts (Ph.D. thesis). Allele designations of the new *chol* mutants start with *chol-3*. The allele designation *chol-1* is used for the strain described by Atkinson et al. (1,2), and *chol-2* refers to the strain originally isolated by Lindegren et al. (21) and analyzed by Kováč et al. (18).

[b] Phospholipid compositions were determined as described in the text. The cells of all strains were grown in synthetic complete medium with 75 μM inositol and 1 mM ethanolamine. Numbers given in the body of the table are percentages of total lipid phosphorus determined for each lipid by chromatographic analysis. Total lipid phosphorous was 9.5 to 13.9 nmol/$10^7$ cells and was not significantly different in any of the strains tested. Abbreviations: PA, phosphatidic acid; ND, no lipid detected. The category "other" includes the pooled percentages of cytidine diphosphate-diglyceride, cardiolipin, phosphatidylglycerol, and other minor lipid species.

vigorously and left at room temperature for 1 h. The lower (chloroform) phase containing the lipid was retained, and the lipids were separated by one-dimensional chromatography as described by Waechter and Lester (24). The radioactively labeled areas were located by comparison with $^{14}$C-labeled phospholipid standards spotted alongside the $^{3}$H-labeled samples. The lipids were cut from the paper and counted by liquid scintillation spectrometry.

**Measurement of the rates of nucleic acid and protein synthesis.** The synthesis of nucleic acid was measured by monitoring the incorporation of 2-[$^{3}$H]adenine (specific activity, 20 Ci/mmol; added to the medium at 1 μCi/ml of medium) into trichloroacetic acid-precipitable material. Protein synthesis was measured by the incorporation of [U-$^{14}$C]lysine (specific activity, 342 mCi/mmol; added to the medium at 0.1 μCi/ml) into trichloroacetic acid-precipitable material. Cells were grown to mid-exponential phase, and 0.5-ml samples were labeled for 10-min periods. Incorporation was stopped by the addition of 0.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid. After 30 min of incubation on ice, the samples were filtered through glass fiber disks (Whatman GFA; 25-mm diameter) and washed twice with cold 5% trichloroacetic acid. The radioactivity retained in the dried filters was determined by liquid scintillation spectrometry.

## RESULTS

The original biochemical analysis of *chol* mutants was limited to one mutant from the series of mutants described by Atkinson et al. (1, 2) and one mutant (18) from the mutant originally isolated by Lindegren et al. (21). We analyzed the phospholipid compositions of five additional independently isolated *chol* mutants selected by the density centrifugation enrichment procedure of Letts and Dawes (19; Letts, Ph.D. thesis). The mutants were shown by complementation analysis to be allelic to the *chol-1* allele present in the KA101 strain characterized by Atkinson et al. (1, 2). All the new *chol* mutants had an absolute auxotrophic requirement satisfied by ethanolamine, choline, or either of the methylated intermediates monomethylethanolamine (MME) or dimethylethanolamine (DME). In this regard, the new *chol* mutants were identical to the previously described *chol*

TABLE 2. Pulse-labeling of phospholipids with $^{32}$P in mutant *chol-3* and the wild-type strain[a]

| Strain (genotype) | Growth supplement | Total cpm per $10^4$ cells | % $^{32}$P in phospholipid: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | PA | PI | PS | PE | PMME and PDME | PC | Other |
| Wild type (*ade6 ura1 MAT*α) | 10 μM inositol | 16.2 | 5.6 | 26.6 | 32.3 | 13.3 | 1.5 | 15.4 | 5.3 |
| | 10 μM inositol–1 mM ethanolamine | 15.6 | 3.6 | 28.9 | 33.1 | 16.7 | 0.4 | 10.2 | 7.1 |
| | 75 μM inositol | 23.3 | 3.3 | 57.4 | 15.3 | 9.6 | 0.8 | 8.3 | 5.3 |
| | 75 μM inositol–1 mM ethanolamine | 20.8 | 2.7 | 51.3 | 13.9 | 16.7 | 0.4 | 10.3 | 4.7 |
| 214 (*chol-3 ade6 ura1 MAT*α) | 10 μM inositol | 4.1 | 3.7 | 53.2 | 1.8 | 4.5 | 0.4 | 34.0 | 2.8 |
| | 10 μM inositol–1 mM ethanolamine | 10.7 | 4.2 | 53.4 | 1.7 | 13.6 | 0.5 | 25.3 | 1.3 |
| | 75 μM inositol | 5.2 | 3.2 | 54.0 | 1.3 | 4.2 | 0.2 | 35.1 | 2.0 |
| | 75 μM inositol–1 mM ethanolamine | 11.7 | 5.8 | 54.1 | 2.0 | 15.1 | 0.5 | 19.3 | 3.2 |

[a] Cells were pulse-labeled with $^{32}$P for 20 min as described in the text. Data in the body of the table represent the percentage of lipid-soluble $^{32}$P recovered in each phospholipid. Cells were pregrown in synthetic complete medium at the concentration of inositol indicated with 1 mM ethanolamine. Before labeling, the cultures were split, and one-half of each was washed with ethanolamine-free medium and placed in ethanolamine-free medium in which the indicated concentration of inositol was maintained. The cultures were incubated for 2 h at 30°C; label was then added, and the cultures were incubated for 20 min. Lipids were extracted and separated as described in the text. PA, Phosphatidic acid.

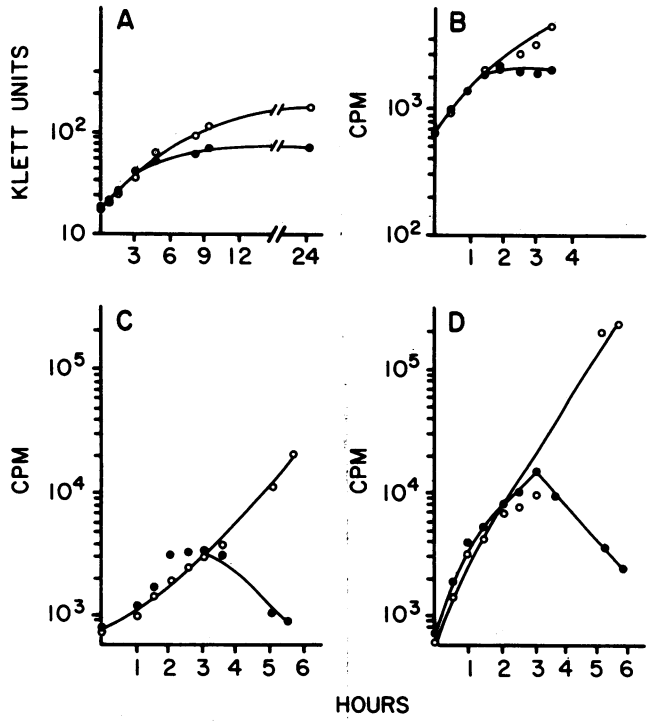


FIG. 2. Growth and metablism of *chol* cells with (○) and without (●) exogenous ethanolamine. At time zero, growing cultures of *chol* cells were placed in synthetic complete medium with and without ethanolamine as described in the text. Time is shown in hours. A, Optical density of the two cultures measured in Klett units; B, net synthesis of phospholipids measured by steady-state incorporation of $^{32}P$ into lipid-soluble material, as described in the text; C, synthesis of protein measured by pulse-labeling with [$^{14}C$]lysine as described in the text; D, synthesis of nucleic acids measured by pulse-labeling with [$^{3}H$]adenine as described in the text.

mutants (1, 18). The phospholipid compositions of the mutants are displayed in Table 1. The wild-type parent and *chol* mutant strains had similar amounts of total phospholipid on a per-cell basis (Table 1), but *chol* strains all had a reduced proportion of PS in their phospholipids. Whereas KA101 (*chol-1 inol-13 lys2 Matα*), originally analyzed by Atkinson et al. (1, 2), had no detectable PS in its phospholipid composition, the five new *chol* mutants had reduced but detectable PS levels ranging from 0.7 to 2.0% of their total phospholipid composition. The reduced proportion of PS in *chol* strains could be compared with the proportion of this lipid in the parent strain, where PS accounted for 8.5% of the total phospholipids (Table 1). The *chol* mutants also exhibited a slightly elevated proportion of PI in their phospholipids—33 to 39% compared with 23% in the wild-type strain (Table 1).

The rate of incorporation of $^{32}P$ into the individual phospholipids during a 20-min pulse was examined in *chol-3* and wild-type strains (Table 2) grown in the presence of 10 or 75 μM inositol in the presence or absence of 1 mM ethanolamine. In wild-type yeast, 75 μM inositol represses the endogenous synthesis of inositol in *S. cerevisiae*, whereas 10 μM inositol does not (7, 9). In the wild-type strain, 32% of the lipid-soluble label from $^{32}P$ was recovered in PS, and 27% of the label was recovered in PI after a 20-min pulse-labeling period when the cells were grown in the lower concentration of inositol. At the higher concentration of inositol in wild-type cells, more than 50% of the label was recovered in PI, and only 14 to 15% was recovered in PS in the same pulse-labeling period (Table 2). In wild-type cells grown in the presence of ethanolamine, a slightly elevated proportion of label was incorporated into PE (approximately 17% compared with 10 to 13% in the absence of ethanolamine; Table 2). In contrast, in the *chol-3* mutant the proportion of label recovered in PS never exceeded 2.0% during a 20-min pulse, and the proportion of label recovered in PI always exceeded 50%, whether inositol was present at 10 or 75 μM (Table 2). In the *chol* cells, compared with the parent strain, a substantially greater proportion of the $^{32}P$ label was recovered in PC in a 20-min pulse. When ethanolamine was supplied to *chol* cells, total incorporation of $^{32}P$ into phospholipid was elevated, and the proportion of label recovered in PE was at least threefold greater than in cells starved for ethanolamine. The effect of ethanolamine deprivation on overall phospholipid metabolism is examined in greater detail below.

The rapid labeling of PC with $^{32}P$ in *chol* cells suggested that the overall rate of conversion of PE to PC might be elevated in the *chol* mutants. Therefore, we examined the activity levels of the phospholipid *N*-methyltransferases in the *chol* mutants. The data (Table 3) show that the specific activities of the phospholipid *N*-methyltransferases were not elevated in the *chol-3* mutant compared with those of wild-type cells.

**Growth and metabolism of ethanolamine- and choline-deprived *chol* cells.** The rates of protein and nucleic acid synthesis were examined in mutant strain 214 (*chol-3*) during choline and ehtanolamine deprivation. The data (Fig. 2) show that the ethanolamine-starved *chol* cells incorporated label into nucleic acid and protein at rates comparable to those of supplemented cells for a period of about 3 h. Thereafter, the rates of macromolecular synthesis declined rapidly. The cells stopped increasing in optical density shortly after macromolecular synthesis stopped (Fig. 2), but they remained viable for at least 24 h as previously reported (1). However, net accumulation of $^{32}P$ in phospholipid ceased between 1 and 2 h after ethanolamine and choline deprivation (Fig. 2).

The data (Table 2) suggest that after 2 h of ethanolamine deprivation, the rate of $^{32}P$ incorporation into total lipid was reduced by at least 50% in comparison with total incorporation in the supplemented *chol* cells. However, there was relatively little change in the relative proportions of the various lipids synthesized. The proportion of label recovered

TABLE 3. Methylation of phospholipids assayed in vitro[a]

| Phospholipid | Incorporation (nmol/mg of protein) by strain after no. of min: | | | | | |
|---|---|---|---|---|---|---|
| | Wild type (*ade6 ural MATα*) | | | 214 (*chol-3 ade6 ural MATα*) | | |
| | 5 | 10 | 20 | 5 | 10 | 20 |
| PMME | 0.1 | 0.2 | 0.3 | 2.1 | 2.2 | 2.0 |
| PDME | 5.6 | 9.9 | 12.7 | 2.8 | 4.6 | 6.5 |
| PC | 7.2 | 20.3 | 33.9 | 4.7 | 12.2 | 28.6 |

[a] Membranes were prepared from *chol* and wild-type strains as described in the text. The cells were grown in medium containing 75 μM inositol and 1 mM ethanolamine. The in vitro assay for phospholipid methylation was carried out as described in the text by measuring incorporation of $^{3}H$ label from methyl-labeled *S*-adenosylmethionine into each phospholipid over the time course indicated. Label recovered from each lipid reflects the cumulative incorporation of methyl groups in the three sequential methylation reactions. Therefore, incorporation into each lipid reflects overall activity in the pathway rather than the individual specific activities of each reaction.

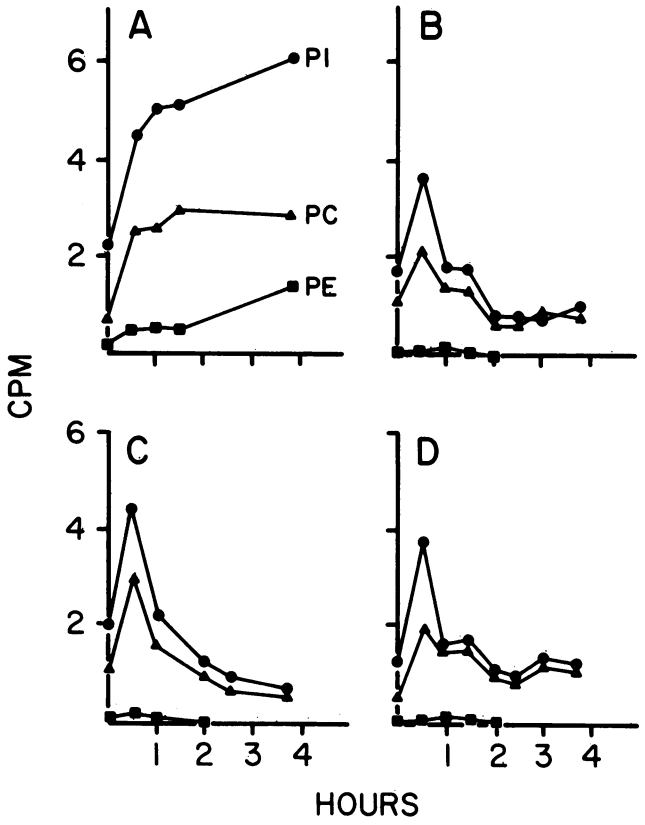


FIG. 3. Synthesis of individual phospholipids in the *chol* mutant during ethanolamine and choline deprivation measured, as described in the text, by pulse-labeling with $^{32}P$ at the times indicated. Lipids were extracted and separated as described in the text. Symbols: PI, ●; PC, ▲; PE, ■. A, *chol* cells supplemented with ethanolamine and 10 μM inositol; B, *chol* cells deprived of ethanolamine but supplemented with 10 μM inositol; C, *chol* cells supplemented with 25 μM inositol but no ethanolamine; D, *chol* cells supplemented with 75 μM inositol but no ethanolamine.

in PI, for example, remained constant at about 50% of the phospholipid synthesized in ethanolamine-deprived cells. A decline in the proportion of label recovered in PE was offset by an increase in the proportion of label recovered in PC. To examine phospholipid synthesis in ethanolamine-starved *chol* cells further, we measured the rates of synthesis of the phospholipids individually. In the ethanolamine-supplemented culture, after a 20-min pulse with $^{32}P$, label was detected in PI, PE, and PC (Fig. 3; Table 2). In this short labeling period, the most heavily labeled phospholipid was PI. (In wild-type cells, substantial label was also observed in PS, but in the *chol* mutant less than 2% of the label was recovered in PS as previously discussed [Table 2].) However, in *chol* cells deprived of ethanolamine, accumulation of label in PE was reduced from the start of ethanolamine starvation. The rate of synthesis of PC increased for the first 30 min (as it did in the control ethanolamine-supplemented culture), but PC synthesis decreased after 30 min. Interestingly, the rate of PI biosynthesis also decreased after about 30 min of ethanolamine starvation. The rates of synthesis of PI and PC appeared to decline in parallel (Fig. 3), and the decline in PI biosynthesis occurred whether exogenous inositol was supplied or not (Fig. 3). The decrease in the rates of PC and PI biosynthesis in the starved *chol* cells was a reflection of an overall decrease in phospholipid synthesis, since PI and PC together represented more than 80% of the phospholipid synthesized under these conditions (Table 2). The parallel decrease in the rates of synthesis of these two lipids explains the relatively constant proportion of PI seen in the data (Table 2). Furthermore, the overall decrease in the synthesis of PI under these conditions did not appear to be accompanied by a buildup of the lipid precursors CDP-DG or phosphatidic acid (Table 2), as is observed during inositol starvation (3).

**Regulation of inositol biosynthesis in the *chol* mutant.** The mutant strain 214 (*chol-3*) used in the ethanolamine deprivation study described above is not an inositol auxotroph. In wild-type cells grown in 10 μM inositol, the endogenous synthesis of inositol via the conversion of glucose-6-phosphate to inositol-1-phosphate is not repressed (7). However, *chol* cells deprived of ethanolamine synthesized PI at a reduced rate regardless of the concentration of exogenous inositol (Fig. 3). Since it was not clear to what extent the ethanolamine-deprived cells were capable of endogenous synthesis of inositol, the synthesis of inositol in these cells was examined.

Cells of strain 214 (*chol-3*) were examined for inositol excretion by the plate assay of Greenberg et al. (11) as described above. The *chol-3* mutant (and all other *chol* mutants) produced a very weak ring of inositol excretion (about 1 mm in radius surrounding the *chol* colony) when the medium was supplied with ethanolamine, choline, or the intermediate MME or DME. The wild-type strain produces no excretion ring under these circumstances (11). When the *chol* strain was replicated onto inositol-deficient medium in the absence of ethanolamine (or other supplements), how-

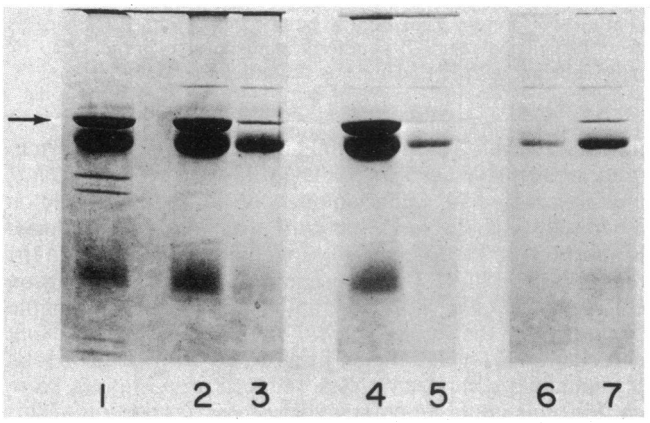


FIG. 4. Immunoprecipitation of I-1-PS from *chol* cells grown under various conditions. Immunoprecipitation was carried out as described by Donahue and Henry (9), as stated in the text. The arrow indicates the position of the I-1-PS subunit (62,000 daltons). The band immediately below the enzyme subunit is the heavy chain of immunoglobulin. Lanes: 1, immunoprecipitation of partially purified I-1-PS (standard); 2, immunoprecipitation of a crude extract of *chol* cells grown in the absence of ethanolamine for 6 h as described in the text, with no inositol present; 3, crude extract of *chol* cells grown in the presence of ethanolamine, but with no inositol present; 4, crude extract of *chol* cells grown in the absence of ethanolamine for 6 h, with inositol (75 μM) present; 5, crude extract of *chol* cells grown in the presence of ethanolamine and 75 μM inositol; 6, crude extract of wild-type (*CHO1*) cells grown in the presence of ethanolamine and 75 μM inositol; 7, crude extract of wild-type cells grown in the presence of ethanolamine, with no inositol present. Results identical to those shown in lanes 6 and 7 are obtained with wild-type cells (with and without inositol) when ethanolamine is absent (9).

ever, it produced a very intense excretion ring (10 to 20 mm in radius). Since the excretion ring required several days of incubation to produce, it appeared likely that the ethanolamine-deprived cells continued to synthesize inositol endogenously for some time after being exposed to ethanolamine deprivation.

The excretion of inositol (as described above) has been observed in strains in which the biosynthetic enzyme I-1-PS is constitutively expressed (11). Therefore, we examined the regulation of I-1-PS in the *chol-3* mutant grown in the presence and absence of ethanolamine and inositol by using the immunoprecipitation technique of Donahue and Henry (9). The presence (or absence) of ethanolamine had no effect on the regulation of I-1-PS in wild-type cells. The expression of the enzyme subunit in wild-type cells grown in the presence of ethanolamine (Fig. 4) is indistinguishable from the pattern of expression observed in the same cells grown in the absence of ethanolamine, as previously described (9). In wild-type cells, the I-1-PS subunit can be detected when the cells are grown under derepressing conditions (10 [or less] μM inositol). The enzyme subunit is not detectable when the cells are grown at higher concentrations of inositol (25 μM or greater) (9; Fig. 4) whether ethanolamine is present or not.

To examine the expression of I-1-PS during ethanolamine deprivation, *chol* cells (and wild-type controls) were grown in preculture to a density of $2 \times 10^6$ to $5 \times 10^6$ cells per ml in the presence of ethanolamine and 10 μM inositol (derepressed condition). The cultures were divided and washed twice in the medium into which they were to be transferred. The cells were then suspended at a density of $1 \times 10^6$ to $2 \times 10^6$ cells per ml in medium with or without ethanolamine. The cells were cultured for an additional 6 h in the presence or absence of inositol. In wild-type cells, the addition of 75 μM inositol resulted in complete repression of the enzyme subunit during the final incubation period whether or not ethanolamine was present (Fig. 4). In the *chol* mutant supplemented with ethanolamine, expression of the enzyme subunit was also repressed if inositol was present (Fig. 4, lane 5). However, when the *chol* mutant was incubated in the absence of ethanolamine, the I-1-PS subunit could be detected at both concentrations of inositol. The amount of I-1-PS subunit immunoprecipitable from crude extracts appeared to be substantially increased in ethanolamine-deprived *chol* cultures (Fig. 4). Thus, in ethanolamine-deprived *chol* cells, I-1-PS appeared to be overproduced and could not be repressed by the addition of repressing amounts of exogenous inositol.

## DISCUSSION

The *chol* mutants of yeast were first isolated by Lindegren et al. (21) as choline auxotrophs. A later series of *chol* mutants had an auxotrophic requirement satisfied by ethanolamine, choline, or one of the methylated intermediates, MME or DME (2). Another series of mutants isolated as ethanolamine auxotrophs after enrichment by a density centrifugation procedure (19; Letts, Ph.D. thesis) also had lesions in the *CHO1* gene. Previously, biochemical analysis of *chol* mutants revealed that they were deficient in the synthesis of the phospholipid PS (Fig. 1) owing to reduced activity of the membrane-associated enzyme PS synthase (1, 18; Letts, Ph.D. thesis). The defect in PS synthesis in these mutants is not conditional. The *chol* mutants grow in the presence of ethanolamine, choline, or one of the methylated intermediates, MME or DME, by incorporating these precursors directly into phospholipid, bypassing PS as an intermediate (see Fig. 1 for pathway and abbreviations). The *CHO1* gene is believed to be the structural gene for the membrane-associated enzyme PS synthase (1, 18, 20).

The new series of mutants examined here reveals that some *chol* mutants retain limited synthesis of PS (Tables 1 and 2). However, all *chol* mutants have an absolute requirement for ethanolamine, choline, MME, or DME. In the absence of these supplements, *chol* strains will not grow, and the synthesis of lipids in the reaction sequence CDP-DG→PS→PE→→→PC is largely blocked (Table 2; Fig. 3). This fact permitted us to examine overall phospholipid synthesis and the synthesis of inositol-containing phospholipids in particular, under conditions in which the rate of synthesis of PE and PC could be experimentally manipulated. Analogous experiments had already been performed in inositol-requiring mutants in which the synthesis of inositol-containing lipids were blocked by inositol starvation (3, 13). The inositol starvation experiments had clearly demonstrated that under conditions in which PI synthesis was severely reduced or completely blocked, the rate of total phospholipid biosynthesis was unaffected (3, 13). In particular, in the inositol-starved cells the synthesis of PE and PC was unaffected for as long as macromolecular synthesis continued (13). Therefore, the finding in *chol* cells that a block in the synthesis of PE and PC results in an overall decline in phospholipid synthesis (Fig. 3; Table 2) was unexpected. It was particularly surprising that the decrease in PC synthesis resulted in a parallel decrease in PI synthesis, since the block in PI biosynthesis produced by inositol starvation has no effect on PC synthesis (3, 13). Clearly, the decline in phospholipid biosynthesis was not due to early metabolic failure in the ethanolamine-starved cells. A decline in the rates of synthesis of individual phospholipids was detected after 1 h (Fig. 3) in ethanolamine-starved cells, but the rate of synthesis of macromolecules was unaffected for at least 3 h (Fig. 2). In inositol-starved cells, in contrast, overall phospholipid synthesis continues unaffected until general metabolic failure occurs and overall macromolecular synthesis ceases (13). Furthermore, in inositol-starved cells a loss of viability occurs starting at about the time that macromolecular synthesis ceases (13), whereas ethanolamine-starved *chol* cells remain viable for up to 24 h (2). In the present study, no loss of viability was detected during any of the starvation experiments reported.

Inositolless death has been attributed to unbalanced growth, that is, the continuation of some metabolic processes when others have been interrupted. In particular, synthesis of PC and other phospholipids occurs even after PI synthesis is completely blocked (3, 13). Under these circumstances, the phospholipid composition becomes highly abnormal, with depletion of PI relative to PC and other phospholipids. In contrast, during ethanolamine and choline starvation of *chol* cells, overall phospholipid synthesis ceases in a relatively balanced fashion. In particular, PC and PI biosyntheses decline simultaneously during ethanolamine and choline starvation in *chol* cells. (Table 2; Fig. 3). These observations suggest that the cell has a mechanism(s) for controlling overall phospholipid synthesis relative to the rate of ongoing synthesis of PC. It is not clear at what level this control is exerted. The metabolic effects are immediate, since PI biosynthesis drops off in parallel to the decline in PC biosynthesis. However, preliminary results of enzyme assays of the membrane-associated PI synthase suggest that its activity is normal in starved *chol* cells (G. Carman, personal communication). The control, in fact, may be exerted at an early step in phospholipid synthesis, such as the production of some common precursor such as CDP-DG (Fig. 1). Future

studies will be directed at determining the step at which the regulation takes place.

The decline in PI biosynthesis in ethanolamine- and choline-starved *cho1* cells certainly cannot be attributed to unavailability of the inositol precursor. Not only is the decrease in synthesis of PI independent of the concentration of exogenous inositol, but it is apparent that endogenous synthesis of inositol is occurring in the ethanolamine- and choline-starved *cho1* cells. The starved cells, in fact, excrete inositol into the growth medium. Furthermore, the starved cells produce the biosynthetic enzyme I-1-PS constitutively (Fig. 4). The fact that I-1-PS is not subject to normal regulation during ethanolamine and choline starvation of *cho1* cells is somewhat surprising. Not only did the enzyme fail to be repressed by exogenous inositol in starved *cho1* cells, it actually appeared to be overproduced relative to its production in wild-type cells and in ethanolamine-supplemented *cho1* cells (Fig. 4). Whereas immunoprecipitation experiments are not quantitative, the apparent overproduction of I-1-PS which is detected by this method correlates with the excretion of inositol in starved *cho1* cells. A similar correlation of excretion of inositol and overproduction of this enzyme has been reported previously for mutants constitutive for I-1-PS (11). These results suggest that normal regulation of I-1-PS is contingent on the biosynthesis of PC or the normal functioning of some aspect of the reaction series PE→→→PC. This reaction series has already been shown to be regulated coordinately with I-1-PS (14, 17, 22). The results reported here suggest that ongoing synthesis of PC is necessary for the normal regulation of I-1-PS.

Coordinated regulation of enzymes involved in the synthesis of inositol- and choline-containing lipids has been shown to occur via the simultaneous repression of the enzymes in response to exogenous inositol and choline (5, 6, 7, 17, 24, 25). In addition, these same enzymes, including I-1-PS, PS synthase, and the phospholipid methyltransferases, are all controlled by the same set of positive and negative regulatory genes (17, 22). I-1-PS is the product of the *INO1* gene, which has recently been cloned (L. S. Klig, Ph.D. thesis, Albert Einstein College of Medicine, Bronx, N.Y., 1983). It has been shown that the genes which are involved in the regulation of phospholipid biosynthesis have major effects on the steady-state levels of the transcript encoded by the structural gene (Klig, Ph.D. thesis). Thus, it is possible that the coordinated regulation of phospholipid synthesis, controlled by these regulatory genes occurs via a transcriptional mechanism. Since the expression of I-1-PS is under the control of this coordinated regulation, it seems possible that some process essential to the functioning of this regulation has been blocked in starved *cho1* cells. Since several other enzymes are subject to this same coordinated control, it should be possible to test this hypothesis by examining the expression of these enzymes in *cho1* cells.

However, the coordinated repression of I-1-PS and other phospholipid-biosynthetic enzymes appears to represent a different level of control than the balanced decline of overall phospholipid biosynthesis observed in ethanolamine- and choline-starved *cho1* cells. The direct coupling of the overall rate of phospholipid synthesis to the rate of PC biosynthesis is too rapid to be ascribed to a repression mechanism. The decline in PI biosynthesis which parallels the decline in PC synthesis occurs with no apparent lag (Fig. 3). Furthermore, the decrease in overall phospholipid synthesis occurs under conditions (i.e., the absence of inositol and choline) which produce maximum derepression of the coordinately regulated enzymes (14). All of these results argue that the overall balanced decline in phospholipid synthesis, which parallels the decline in PC biosynthesis in *cho1* cells, represents another level of regulation by an as yet unknown mechanism. This regulation, like the previously described coordinated repression (14), presumably maintains balanced synthesis of the different classes of phospholipids under changing metabolic conditions.

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