REGULATORY MUTATIONS OF INOSITOL BIOSYNTHESIS IN YEAST: ISOLATION OF INOSITOL-EXCRETING MUTANTS

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Manuscript received August 14, 1981
Revised copy accepted October 5, 1981

ABSTRACT

The enzyme inositol-1-phosphate synthase (I-1-P synthase), product of the *INO1* locus, catalyzes the synthesis of inositol-1-phosphate from the substrate glucose-6-phosphate. The activity of this enzyme is dramatically repressed in the presence of inositol. By selecting for mutants which overproduce and excrete inositol, we have identified mutants constitutive for inositol-1-phosphate synthase as well as a mutation in phospholipid biosynthesis. Genetic analysis of the mutants indicates that at least three loci (designated *OPI1*, *OPI2* and *OPI4*) direct inositol-mediated repression of I-1-P synthase. Mutants of these loci synthesize I-1-P synthase constitutively. Three loci are unlinked to each other and to *INO1*, the structural gene for the enzyme. A mutant of a fourth locus, *OPI3*, does not synthesize I-1-P synthase constitutively, despite its inositol excretion phenotype. This mutant is preliminarily identified as having a defect in phospholipid synthesis.

INOSITOL is a precursor of several membrane phospholipids in yeast (STEINER and LESTER 1972a). These phospholipids appear to play some essential role, since failure to synthesize them leads rapidly to cell death (HENRY *et al.* 1977; BECKER and LESTER 1977). The characterization of inositol auxotrophy in a mammalian cell line has revealed striking similarities between the effects of inositol deprivation in cells of higher eukaryotes and in yeast (JACKSON and SHIN 1980). The synthesis of the precursor, inositol, is highly regulated in *Saccharomyces cerevisiae*. The key biosynthetic enzyme, inositol-1-phosphate synthase, is repressed over 50 fold when cells are grown in the presence of 50μ M inositol (CULBERTSON, DONAHUE and HENRY 1976a; DONAHUE and HENRY 1981a). Because inositol biosynthesis is highly regulated and inositol is a vital membrane component, the study of this pathway affords a unique opportunity to approach two fundamental questions pertaining to eukaryotic genetic regulation: (1) How do structural and regulatory genes interact in a major biosynthetic pathway to bring about enzymatic changes in response to nutritional changes in the environment? (2) How does regulation of the biosynthesis of membrane precursors affect the utilization of these precursors in membrane biogenesis?

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In this study, we report the isolation of a new class of mutants that are defective in the regulation of inositol biosynthesis. Previously, mutants have been described which lack inositol-1-phosphate synthase activity (CULBERTSON and HENRY 1975; CULBERTSON, DONAHUE and HENRY 1976b). The inositol-1-phosphate synthase mutants were isolated on the basis of inositol auxotrophy and fall into at least 15 genetic complementation groups (CULBERTSON and HENRY 1975; DONAHUE *et al.* 1978; DONAHUE and HENRY 1981b). However, the majority of the mutants are alleles of the *INO1* locus. This locus has been shown to be the structural gene for the 62,000 dalton subunit of inositol-1-phosphate synthase, a tetrameric enzyme consisting of identical subunits (DONAHUE and HENRY 1981a). The other INO loci may be involved in regulation of the enzyme and mutants of two loci; *INO2* and *INO4* have been shown to exhibit pleiotropic defects in phospholipid biosynthesis (HENRY *et al.* 1981).

The identification of regulatory mutants in yeast is hampered by a number of technical problems. Such mutants are frequently indistinguishable from the wild type in nutritional requirements, precluding auxotrophy as a means of selection. Furthermore, functionally related genes are not associated in operons, so that polar mutations do not affect all the enzymes in a related pathway. However, a variety of techniques have been exploited successfully in the isolation of regulatory mutations in yeast, and these have been reviewed by GREER and FINK (1975). In this report we describe the identification of regulatory mutations of inositol metabolism by selecting for inositol-excreting mutants. The phenotype of metabolite excretion has previously been used in the isolation and/or characterization of regulatory mutants, particularly in the regulation of amino acid metabolism (HOLDEN 1962; DEMAIN 1966; DEMAIN and BIRNBAUM 1968; SATYANARAYANA, UMBARGER and LINDEGREN 1968; CHAMPNEY and JENSEN 1970; KASHMIRI and GROSS 1970; RASSE-MESSENGUY and FINK 1974; and LAX, FOGEL and CRAMER 1979). Genetic characterization of the inositol excreting mutants reported here demonstrates that at least three unlinked loci regulate the biosynthesis of inositol. Use of the inositol-excretion phenotype in the identification of mutants defective in phospholipid synthesis is also discussed.

MATERIALS AND METHODS

Yeast strains: The parental wild type, (nonexcreting, INO+) strain (*ade5 MAT***a**) has been previously described (CULBERTSON and HENRY 1975). Other strains used in the genetic characterization of the inositol-excreting mutants are listed in Table 1.

Inositol analogs: The inositol analogs described in this study were the generous gift of Dr. Henry Sable, Biochemistry Department, Case Western Reserve University.

Media: The media used in this study have been previously described in detail (CULBERTSON and HENRY 1975; HENRY *et al.*, 1977). For routine culture, YEPD medium (containing 2% glucose, 2% bactopeptone, 1% yeast extract) was used. (Solid plates contain 2% agar in addition to listed ingredients.) The components of complete defined media are Difco Yeast Nitrogen Base (YNB) without amino acids (6.7 g/l), glucose (2%), lysine (20 mg/l) arginine (10 mg/l), leucine (10 mg/l), histidine (10 mg/l), tryptophan (10 mg/l), methionine (10 mg/l), threonine (60 mg/l), aspartate (100 mg/l), adenine (10 mg/l), uracil (10 mg/l), and agar (2%). Difco Yeast Nitrogen Base without amino acids contains a full vitamin supplement including inositol (1.8 mg/l). Medium used to test for auxotrophy was complete defined medium from which one component was omitted ("drop out media"). Inositol auxotrophy was tested on medium, similar

TABLE 1

List of strains of Saccharomyces cerevisiae *used in this study*

Strain	Genotype	Source
MC13	*MAT***a**, *ino1–13*, *ade1* *MATα*, *ino1–13*, *lys2*	Laboratory strains
AID-1	*MAT***a**/*MATα*, *ade1/ade1*, *ino1–13/ino1–13*, *lys2/+*	This study
MC21	*MAT***a**, *ino2–21*, *ade1* *MATα*, *ino2–21*, *lys2*	Laboratory strains
MC26	*MAT***a**, *ino4–26*, *ade5*	Laboratory strains
ade5	*MAT***a**, *ade5* *MATα*, *ade5*	Laboratory strains
P49	*MATα*, *lys2*	Berkeley Stock Center
S5	*MAT***a**, *lys2* *MATα*, *ade1*	Berkeley Stock Center
OP1	*MATα*, *opi1–1*, *lys2* *MAT***a**, *opi1–1*, *ade5*	This study
OP12	*MATα*, *opi1–12*, *lys* *MAT***a**, *opi1–12*, *ade5*	This study
OP3	*MATα*, *opi3–3*, *lys1* *MAT***a**, *opi3–3*, *ade5*	This study
OP4	*MATα*, *opi4–4*, *lys2* *MAT***a**, *opi4–4*, *ade5*	This study
OP37	*MATα*, *opi2–37*, *lys1* *MAT***a**, *opi2–37*, *ade5*	This study

to complete medium, except that Difco Vitamin Free Yeast Base was substituted for YNB without amino acids, and the following vitamins were added separately: biotin (2 μg/l), calcium pantothenate (400 μg/l), folic acid 2 μg/l), niacin (400 μg/l), p-aminobenzoic acid (200 μg/l), and pyridoxine hydrochloride (400 μg/l).

Inositol-free minimal medium contained Difco Vitamin Free Yeast Base (6.7 g/l), glucose (2%), agar (2%) and the vitamins listed above. Minimal medium with inositol consisted of Difco YNB without amino acids (6.7 g/l), glucose (2%) and agar (2%).

Diploids were induced to undergo meiosis by starvation for nitrogen on medium containing potassium acetate (9.8 g/l), yeast extract (1.25 g/l), inositol (4 mg/l), glucose (1.0 g/l), and agar (2%).

Mutagenesis, selection of mutants and genetic analysis: The parental strain (*ade5 MAT***a**) was mutagenized with ethyl methane sulfanate (EMS) according to the method of LINDEGREN *et al.* (1965). Mutagenized cells were plated on YEPD (approximately 100 colonies per plate) and incubated at 30° for 3–4 days.

Selection plates were prepared by spreading lawns of the indicator strain (*ino1–13, ade1 MAT***a**, Table 1) onto plates containing complete medium minus inositol and refrigerated until needed. The *ade1* marker in the indicator strain confers a red phenotype and the *ino1* marker confers inositol auxotrophy. Mutagenized colonies were replicated from YEPD plates to the selection plates and incubated 5–10 days at 30°. Inositol-excreting mutants were identified as those phenotypically white colonies of the mutagenized strain that were surrounded by a red halo. The red halo represents growth of the red indicator strain in the circular area around the inositol-excreting mutant.

Potential excretor mutants were crossed initially to strain P49 *lys2 MAT* (Table 1) and diploids were selected on minimal medium. Recovered diploids were sporulated and subjected to tetrad analysis according to standard methods (MORTIMER and HAWTHORNE 1969).

Biochemical analysis of mutants: Inositol excreting mutants were assayed for the presence of inositol-1-phosphate synthase (I-1-P synthase) under repressing (75 μM inositol) and derepressing conditions (10 μM inositol or less, CULBERTSON, DONAHUE and HENRY 1976) using the specific antibody and immunoprecipitation techniques described by DONAHUE and HENRY (1981a). Basically, the technique involves preparing a crude extract by Braun homogenization of pelleted cells of mutant or wild type yeast suspended in a buffer consisting of 20 mM Tris HCl; pH 7.2 containing 2 mM α-tolulene-sulfonyl fluoride (PMSF; to inhibit proteolysis) in 1% dimethyl sulfoxide (DMSO); 10 mM NH_4Cl and 10 mM 2-mercaptoethanol. The lysate is then centrifuged at 4° for 5 min at 7,000 × g (to remove intact cells) and then at 27,000 × g for 20 min. The supernatant is then assayed for protein by the commercial Bio Rad method.

Immunoprecipitation of this extract is carried out using rabbit anti-inositol-1-phosphate synthase antiserum (anti I-1-P synthase) prepared as described by DONAHUE and HENRY (1981a). Volumes of crude extracts corresponding to 0.5–1.0 mg protein are incubated with 10 μl of rabbit anti-synthase antiserum for 3 hr at 4° in 1.5 ml Eppendorf centrifuge tubes, and then indirectly precipitated by *Staphlococcus aureus* cells (10% of volume of incubation mixture) for 1 hr at 4°. Samples were then centrifuged and precipitates were washed twice with PBS containing 1% Triton X-100, 0.1% SDS, 0.1 deoxycholate, and 0.5 M NaCl. The washed precipitates were then subjected to electrophoresis through 10% polyacrylamide gels under fully dissociating conditions (LAEMMLI 1970) as described in detail by DONAHUE and HENRY (1981a). On such gels, the 62,000 dalton subunit of I-1-P synthase, when present in a crude extract at wild type derepressed levels, is readily visualized by Coomassie blue staining of the gel, whereas the band is absent in extracts prepared from wild type cells grown under repressing conditions (DONAHUE and HENRY 1981a).

Analysis of phospholipids: Cells were labelled with ^{32}P-orthophosphate in order to determine the rate of phosphatidylinositol (PI) synthesis relative to total membrane phospholipid synthesis. Cells were grown in complete synthetic medium containing 75 μM inositol (fully repressing conditions) for 5–6 generations. They were then washed 3 times in inositol-minus media, resuspended at a cell density of 4–6 × 10^6/ml in 10 ml of complete medium containing 0, 10, 50, or 75 μM inositol and then incubated for 1 hr at 30°. 10 ml cultures were then pulse-labelled with 0.5 mCi ^{32}P-orthophosphate (final specific activity of ^{32}P in culture: 5.33 mCi/mM for 15 min. The cells were pelleted and washed once with distilled water, and spheroblasts were prepared as described previously (ATKINSON, FOGEL and HENRY 1980). Lipids were extracted from the spheroblasts by a modification of the method described by FOLCH, LEES and STANLEY (1957). Pelleted spheroblasts were resuspended by vortexing in 5 ml chloroform methanol (2:1), and extracted for 45 min–1 hr at room temperature. Distilled water (1 ml) was then added and the tubes were vortexed again and centrifuged briefly to separate the aqueous and organic phases. The organic (bottom) phase was then removed and dried under N_2.

The two dimensional silica-impregnated paper chromatography method of STEINER and LESTER (1972) was used to separate the labelled phospholipids. Lipid residues were resuspended in 100 μl of cloroform:methanol (2:1) and spotted on Whatman SG81 silica-impregnated paper which had been dipped in 2% EDTA, pH 7, and air dried. The lipids were then separated by ascending chromatography first in the solvent chloroform:methanol:ammonium hydroxide (30%):H_2O (66:27:3:0.8) and in the second dimension, in the solvent chloroform:methanol: acetic acid:H_2O (32:4:5:1). Chromatograms were autoradiographed by exposing to Kodak no screen x-ray film for 24 hrs. Radioactive spots located by autoradiography were cut out and counted by liquid scintillation in 10 ml of scintillation cocktail (toluene-PPO-POPOP).

RESULTS

Screening of inositol analogs: In an attempt to find a compound that would be useful in selection of regulatory mutants of inositol biosynthesis, we screened 18 potential analogs in hopes of finding a compound that would repress inositol-1-phosphate synthase, thus preventing endogenous synthesis of inositol, but that would not substitute for inositol in the membrane phospholip-

ids. Since inositol is a vital membrane component such a compound should inhibit growth of wild-type yeast. Among the compounds screened for inhibition of growth of wild-type yeast were cyclohexanol, hexaketocyclohexane octahydrate, hexamethyl 1,3,5 cyclohexane trione, inosose, cyclohexanediol, isomytilitol, cyclohex-4-ene 1,2-*cis* diol, cyclooctenediol, cyclooctane-1,2-*cis* diol, cyclooctane tetrol, norbornane-2,3 diol, norbornane-2,3,5,6-tetrol, mannotetrol, and the following epimers of cyclohexamine: 1,2,3/4 tetrol, 1,2,4/3 tetrol 1,2,4/5 tetrol, 1,2/4,5 tetrol and 1,2-*cis* diol. None of these cyclitols inhibited the growth of wild-type yeast when added to inositol-free media in concentrations up to 1 mM (data not shown).

Isolation of inositol excretor mutants: Since no analogs of inositol were identified which would be useful in selecting regulatory mutants, we decided to search for inositol overproducer mutants. The selection protocol employed in this study is described in detail in the MATERIALS AND METHODS and is designed to detect mutant yeast colonies that excrete inositol into the medium. The rationale underlying this selection is that mutations resulting in higher than normal amounts of inositol might include, among others, defects in the I-1-P synthase repression mechanism(s). Mutants that overproduce inositol might then excrete inositol into the growth medium. In order to detect such mutants, colonies of the mutagenized parental strain were replicated onto selection plates (described in the MATERIALS AND METHODS) and incubated 5–10 days at 30°. An inositol-excreting mutant of the parental strain was identified as a phenotypically white colony surrounded by a red halo. (The halo signifies growth of the red indicator strain MC13 (*ino1–13*, *ade1*, *Mat***a**, Table 1), in the circular area into which the mutant is secreting inositol.)

Of 61 mutants that were isolated by this procedure, nine retained the mutant (excretor) phenotype after retesting. These nine were then crossed to strain P49 *lys2 MAT*α (Table 1) and tetrad analysis was carried out in order to ascertain the number of genes associated with the excretor phenotype in each mutant. Segregation of the mutant phenotype was determined by replica plating the four colonies derived from a given tetrad to an excretor-detection plate. (No more than the four colonies of a single tetrad can be tested on a given excretor-detection plate due to the technical problem of the diffusion and overlapping of the inositol excretion rings.) Basically these plates were similar to those used in the original selection (described in MATERIALS AND METHODS) and consisted of inositol deficient complete defined agar medium spread with a lawn of the diploid strain, AID, (Table 1 genotype: *ino1–13 ade1 lys2 MAT***a**/*ino1–13*, *ade1 MAT*α). (The use of the diploid detection strain avoids the potential problem of mating between spore colonies and the detection strain). Segregation of the excretor phenotype is readily observed in this fashion. Five of the mutants subjected to tetrad analysis segregated the mutant phenotype in the 2^+ (nonexcretor):2^-(excretor) pattern expected for single gene mutations. These mutants carried the laboratory strain designations OP1, OP3, OP4, OP12 and OP37 (Table 1).

Dominance/recessiveness of excretor phenotypes: The dominance *vs.* recessiveness of the mutations in the excretor mutants was determined by crossing the mutants (in the parental *ade5 MAT***a** background) with the P49 *lys2 MAT*α

(nonexcretor) strain, as decribed above. Individual diploid clones were tested for the excretor phenotype. The results were the following:

1. The mutations in strains OP3, 4 and 37 were found to be recessive. The red rings that surrounded haploid colonies of the mutants four to seven days following plating on detection plates were not seen around colonies of the heterozygotes nine days after plating.

2. The excretion phenotype in the OP1 and 12 strains was codominant. The excretor phenotype in the haploids was apparent by the fourth day after plating on the detector plates. In the heterozygotes, a faint red ring surrounded the OP1/+ diploid colony after seven days, and the OP12/+ colony after nine days.

Complementation analysis: In order to determine the number of independent loci represented by the mutations in the five excretor mutants, complementation analysis was performed. Complementation analysis was complicated by two factors: (1) The mutant and wild-type phenotypes were codominant in two of the mutants as described above; and (2) complementation could not be measured in the presence of haploid cells, since a positive complementation response is expressed by the absence of the excretor phenotype. With these factors in mind, complementation was measured in the following manner:

Excretor strains bearing the mutations and *MAT***a** and *ade5*: *MAT*α and *lys1* or *lys2* were constructed. All possible diploid combinations were constructed and diploids were selected on the basis of growth on minimal medium using the complementing *lys* and *ade* markers. Individual clones were then picked, retested on minimal medium to confirm that they were diploids and then tested for the excretor phenotype. Complementation responses given by heterozygotes (OP/+) were used as negative controls. Positive controls were the responses seen for mutant homozygotes (*i.e.*, OP1/OP1 etc.). The results of the complementation tests indicated that the five mutations represented four complementation groups, with OP1 and OP12 representing the same group.

Recombination analysis: Strains bearing excretor mutations were crossed pairwise to each other and tetrad analysis was carried out as described previously. The results are described in Table 2. No recombination occurred between the

TABLE 2

Genetic linkage among overproducer mutants

Parental alleles	Number of asci			
	2+/2-	3-/1+	4-/0+	2+/2- Controls
OP1 × OP12	0	0	11	*ade5 lys1*
× OP3	6	7	0	*ade5 lys2*
× OP4	4	6	0	*ade5 lys1*
× OP37	1	8	2	*ade5 lys1*
OP12 × OP3	2	7	2	*ade5 lys2*
× OP4	2	1	1	*ade5 lys2*
× OP37	1	5	0	*ade5 lys2*
OP3 × OP4	3	7	0	*ade5 lys2*
× OP37	2	4	2	*ade5 lys2*
OP4 × OP37	3	3	0	*ade5 lys2*

mutations present in strains OP1 and OP12 in 11 tetrads analyzed. Thus, since these mutants also represent a single complementation group they are presumed to be allelic. The locus represented by mutants OP1 and OP12 will be designated *OPI1* (OPI for overproducer of inositol); the mutations present in strains OP1 and OP12 will be designated *opi1–1* and *opi1–12*, respectively. Mutants OP37, OP3 and OP4 each clearly represent different loci unlinked to each other and to *OPI1*. These loci will be designated *OP12*, *OP13* and *OP14* respectively. The mutants representing them will be designated *opi2–37* (OP37); *opi3–3* (OP3) and *opi4–4* (OP4). Since *opi1–1* and *opi1–12* are alleles of the same locus, the tetrad data from crosses involving these two mutations can be pooled.

The mutants were then crossed to mutants representative of loci *INO1*, *INO2* and *INO4*. The tetrad data are displayed in Table 3. A comment is necessary concerning analysis of these crosses. Three phenotypes were distinguishable among the progeny spores of $opi^- \times ino^-$ crosses. They are: inositol excretion (genotype: INO^+, opi^-), inositol requiring (ino^-, OPI^+ or ino^-, opi^-), and nonrequiring, nonexcreting (genotype: INO^+ OPI^+). Thus the inositol-requiring spores are expected to be of two genotypes that cannot be distinguished without further analysis. The existence of the recombinant ino^-, opi^- spores can be inferred by the observation of the reciprocal class, INO^+ OPI^+, in a given tetrad. Thus a tetratype (T) ascus has the following spore phenotypes; 2 ino^-: 1 Ino^+ excretor: 1 Ino^+ nonexcretor, while a nonparental ditype (NPD) has 2 ino^-: 2 Ino^+ nonexcretors. In an NPD ascus *both* ino^- spores are predicted to have the genotype ino^-, opi^-. To demonstrate that this is indeed the case in every instance would require complementation analysis with opi^- INO^+ haploid tester strains (*i.e.*, excretion of inositol by the diploid ino^-, $opi^- \times INO^+$ opi^- would prove the existence of the opi^- mutation in the haploid spore). As explained previously, complementation analysis requires selection and cloning of diploids.

TABLE 3

Genetic linkage of overproducer mutants with INO1, INO2 *and* INO4 *loci*

Parental alleles	Number of asci			2+/2– Controls
	PD	NPD	T	
opi1–1 × *ino1–13*	2	1	7	*ade1*, *lys1*
opi1–1 × *ino2–21*	1	2	8	*ade1*, *lys1*
opi1–1 × *ino4–26*	1	2	14	*ade5*, *lys1*
opi1–12 × *ino1–13*	5	1	5	*ade1*, *lys2*
opi1–12 × *ino2–21*	3	1	6	*ade1*, *lys2*
opi1–12 × *ino4–26*	3	2	3	*ade5*, *lys2*
opi2–37 × *ino1–13*	2	0	8	*ade1*, *lys2*
opi2–37 × *ino2–21*	3	5	4	*ade1*, *lys2*
opi2–37 × *ino4–26*	3	1	5	*ade5*, *lys2*
opi4–4 × *ino1–13*	5	1	9	*ade1*, *lys2*
opi4–4 × *ino2–21*	2	2	5	*ade1*, *lys2*
opi4–4 × *ino4–26*	4	0	5	*ade5*, *lys2*

PD = parental ditype; NPD = nonparental ditype; T = tetratype.
Only tetrads in which all four spores survived were employed in this analysis. Total spore viability in these crosses was greater than 85%.

However, we selected three NPD tetrads on the basis of the phenotypic criteria discussed above: one tetrad each from the *opi1–1* crosses involving *ino1*, *ino2* and *ino4* alleles. Complementation analysis demonstrated the existence of the *opi1–1* allele in each of the six *ino*⁻ spores from the NPD asci. Further biochemical and genetic analysis is being performed on these recombinants and will be presented in a subsequent report. The data reported here are sufficient to rule out tight linkage between the three *opi* loci and the three *ino* loci. Of particular importance, tight linkage to *INO1*, the structural gene for I-1-P synthase, has been ruled out for these overproducer loci.

The linkage data for mutation *opi3–3* are not shown here because they will be the subject of another report. Preliminary data suggest that *opi3* is linked to locus *INO4* (HENRY *et al.* 1981).

Immunoprecipitation of inositol-1-phosphate synthase (I-1-P synthase) from crude extracts: Cell-free crude extracts prepared from cultures of OP4 (*opi4–4*), OP12 (*opi1–12*) and OP37 (*opi2–37*), grown under repressing and derepressing conditions (75 μM and 0 μM inositol, respectively), were immunoprecipitated with rabbit anti- I-1-P synthase antisera. The immunoprecipitates were subjected to electrophoresis on an SDS-polyacrylamide gel, as seen in Figure 1. All three mutants synthesized the 62,000 dalton I-1-P synthase subunit under conditions which repress synthesis of the enzyme in the wild type (75μM inositol). From these data we conclude that the mutations in the three loci (*opi1*, *opi2* and *opi4*)

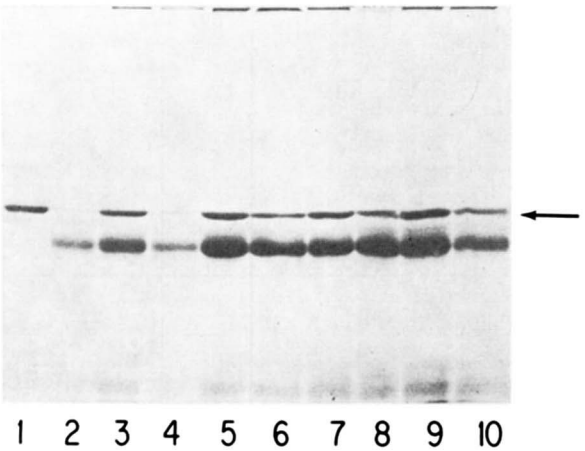

FIGURE 1.—*Immunoprecipitation of crude extracts of strains OP4* (opi4–4), *OP12* (opi1–12) and *OP37* (opi2–37). Crude extracts were prepared from cells grown under repressing (75 μM inositol) or derepressing (10 μM inositol) conditions and 1 mg protein was immunoprecipitated with anti-synthase antibody. Samples were electrophoresed under fully dissociating conditions in an SDS polyacrylamide gel as detailed in the Methods. Gel is shown stained with Coomassie blue. Arrow indicates the position of the 62,000 MW subunit of inositol-1-phosphate synthase. Band immediately below is the heavy chain of the immunoglobulin. Partially purified wild type synthase (lane 1) and anti-synthase antibody (lane 2) are included as references. Lanes 3–10 contain immunoprecipitates of crude extracts from: wild type, derepressed (lane 3) and repressed (lane 4); OP4 derepressed (lane 5) and repressed (lane 6); OP12, derepressed (lane 7) and repressed (lane 8); and OP37, derepressed (lane 9) and repressed (lane 10).

represented by strains OP37, OP12, OP4, lead to constitutive synthesis of the enzyme I-1-P synthase.

Mutant OP3, however, did not exhibit constitutive synthesis of I-1-P synthase. As seen in Figure 2, immunoprecipitable I-1-P synthase is not detected in crude extracts of this mutant when grown in the presence of 75 μM inositol (repressing condition).

Dominance/recessiveness of the constitutive phenotype: As described above diploids heterozygous for the mutations *opi4–4* and *opi2–37* did not excrete inositol, as measured by red ring assay, while diploids that were *opi1–1/OPI1* or *opi1–12/OPI1* exhibited a phenotype intermediate between homozygous mutant and homozygous wild-type controls when tested by the ring assay.

The question of dominance *vs.* recessiveness was explored further by analysis of immunoprecipitable I-1-P synthase from crude extracts of diploids heterozygous for the mutations *opi1–1*, *opi4–4*, *opi1–12* or *opi2–37*. The absence of immunoprecipitable enzyme in crude extracts from the heterozygous diploids grown in the presence of 75μM inositol indicated that synthase was repressed. Thus, the constitutive phenotype is clearly recessive for all of the mutants while the excretion phenotype is codominant for mutants OP1 and OP12. The excretion phenotype is measured on inositol deficient media and thus measures inositol overproduction under conditions that are derepressing for wild type. The constitutive phenotype, on the other hand, is determined under conditions that are repressing for wild-type yeast. It is, thus, not surprising that the two phenotypes do not coin-

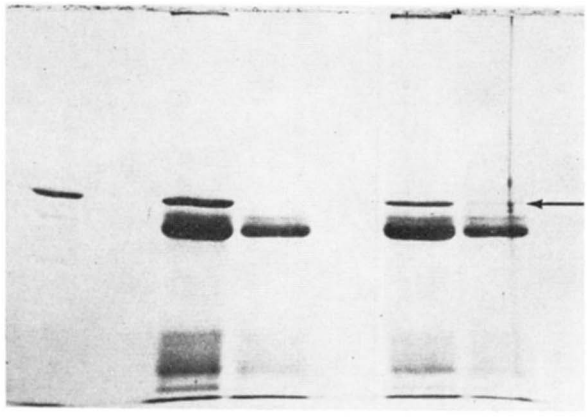

FIGURE 2.—*Immunoprecipitation of crude extracts of OP3* (opi3–3) *and wild type strains.* Samples were electrophoresed under fully dissociating conditions in SDS polyacrylamide as detailed in MATERIALS AND METHODS Gel is shown stained with Coomassie Blue. Arrow marks the position of the 62,000 MW subunit of inositol-1-phosphate synthase. Band immediately below is the heavy chain of the immunoglobulin. Crude extracts were prepared from cells grown under repressing (75 μM inositol) or derepressing (10 μM inositol) conditions and immunoprecipitated with anti-synthase antibody. Partially purified synthase shown in lane 1. Lanes 2–7 depict immunoprecipitates from: wild type, derepressed (lane 2) and repressed (lane 3); OP3, derepressed (lane 4) and repressed (lane 5).

cide absolutely in all cases, but probably reflect slightly different aspects of the underlying regulation.

Analysis of phospholipids synthesized by strains OP4, OP12 and OP37: Inositol is the immediate precursor in the synthesis of the membrane phospholipid, phosphatidylinositol (PI). The synthesis of phospholipids was examined in the *opi*⁻ mutants as described in MATERIALS AND METHODS in order to determine whether the alterations in inositol regulation found in these strains is reflected in subsequent phospholipid synthesis.

The membrane phospholipids of strains OP1 (*opi1–1*), OP3 (*opi3–3*) OP4 (*opi4–4*), and OP37 (*opi2–37*) were pulse labelled with ^{32}P by the procedure described in MATERIALS AND METHODS. The phospholipids were extracted, separated chromatographically, and the chromatograms autoradiographed. The phospholipid quantitations are summarized in Table 4 and representive autoradiograms are illustrated in Figure 3.

Since the lipids were labelled in a 15 minute pulse, the percentages of ^{32}P incorporated into each lipid do not reflect the total membrane phospholipid composition. Rather, they represent the relative rates of incorporation of ^{32}P into each lipid during the pulse. Lipids such as PI and phosphatidylserine (PS) which are

TABLE 4

Phospholipid synthesis in inositol excreting mutants

Strain	Inositol supplement[a] (μM)	% Phospholipids[b]					
		PI	PS	PC	PE	PMME	Others[c]
wild type	0	11	28	4	32	1	24
(*ade5*)	10	34	25	7	18	<1	16
	75	38	19	7	26	1	9
OP1	0	31	20	10	31	1	7
(*opi1–1*)	10	37	18	9	29	1	6
	75	38	18	11	23	1	9
OP37	0	25	20	8	29	1	17
(*opi2–37*)	10	37	15	6	31	4	7
	75	46	22	8	15	1	8
OP4	0	19	31	7	24	1	18
(*opi4–4*)	10	34	21	10	23	2	10
	75	38	28	9	13	1	11
OP3	0	28	20	3	23	9	17
(*opi3–1*)	10	46	17	2	16	7	12
	75	31	22	3	18	11	15

The phospholipids were pulse labeled with ^{32}P for 15 minutes as described in the METHODS. The data in each case represents the average of 2–3 experiments.

[a] Inositol supplement refers to supplementation during the pulse labelling period and for the 1 hour immediately preceding the labelling period.

[b] The figures in the body of the table are expressed as a % of total label extracted from 10^7 cells which ranged from $6.5 - 4.5 \times 10^3$ CPM, with no consistent variation in total labelling associated either with inositol supplementation or with strain.

[c] Others include phosphatidic acid, cardiolipin, sphingolipids and cytidine diphosphate diglyceride.

[d] Abbreviations used in the Table: phosphatidylinositol (PI); phosphatidylserine (PS); phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylmonomethylethanolamine (PMME).

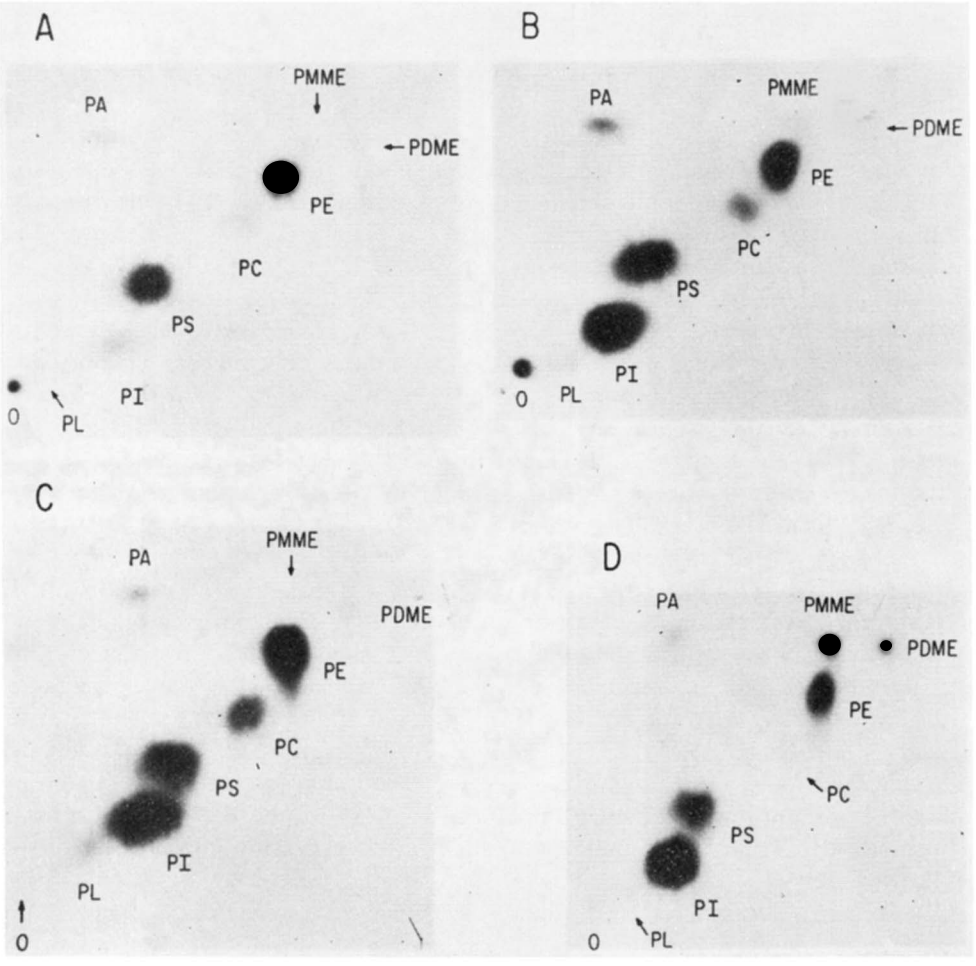

FIGURE 3.—*Two dimensional chromatographic separation of ^{32}P pulse labelled phospholipids.* Panel A: wild type (*ade5*a) labelled in inositol free media. Panel B: wild type labelled in presence of 75 μM inositol. Panel C: strain OPI (*opi1–1*) labelled in inositol free media. Panel D: strain OP3 (*opi3–3*) labelled in inositol free medium.

Phospholipid abbreviation as follows: PL, polar lipids; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PA, phosphatidic acid.

synthesized directly from the precursor CDP-diglyceride (STEINER and LESTER 1972b) are rapidly labelled. On the other hand, phosphatidylcholine (PC) is synthesized primarily via a pathway whch requires decarboxylation of PS to form phosphatidylethanolamine (PE) followed by three sequential methylations of PE. Thus PC is not labelled as rapidly with ^{32}P and this is reflected in the relative proportions of label shown in Table 4. In the wild type, the relative proportion of phosphatidylinositol (PI) synthesized in a 15 minute pulse labelling period is low (11%) when the cells are grown in the absence of inositol. In the

wild type strain the proportion of PI synthesized in a 15 minute pulse labelling period rises more than 3 fold to 38% in response to increasing exogenous inositol concentration. The increase in the proportion of PI synthesized in wild type in the presence of inositol is evident on the autoradiogram of the separated lipids illlustrated in Figure 3. In contrast, the *opi1*, *opi2*, *opi4* and *opi3* mutants are characterized by a relatively high proportion of PI synthesis even in the absence of exogenous inositol. This is most evident in mutant OPI (*opi1–1*) in which PI constitutes 31% of the phospholipid synthesized in the absence of exogenous inositol. The addition of exogenous inositol causes the proportion of PI in mutant OPI to rise only a few percentage points to 38% of total phospholipid synthesized in a 15-minute pulse. In mutants *opi2–37*, *opi3–3* and *opi4–4* the proportion of PI synthesized in the absence of inositol supplementation is also elevated compared to wild type, though not as dramatically as in mutant *opi1–1*. No other consistent differences were observed in the pulse labelled phospholipids of mutants *opi1–1*, *opi1–12*, *opi4–4* and *opi2–37* compared to wild type. The elevated proportion of PI synthesized in the absence of exogeneous inositol supplementation in these mutants is consistent with the presence of a high endogenous pool of inositol produced by consitutive synthesis of inositol.

The phospholipids of mutant *opi3–3*, one the other hand, differ significantly from wild type in an additional respect. Consistently, an increased amount of the phospholipid phosphatidylmonomethylethanolamine (PMME) was detected in the chromatographic separations of lipids from this strain. This is strikingly evident in the autoradiogram illustrated in Figure 3 and is reflected in the data in Table 4. This lipid constitutes approximately 9% of the ^{32}P incorporated into phospholipid in a 15 minute pulse as compared to 1% or less in wild type and in the other *opi* mutants. PMME is an intermediate in the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE). The accumulation of this intermediate suggests a defect in PC biosynthesis. Preliminary studies suggest that mutant *opi3* is indeed quite deficient in PC biosynthesis (HENRY *et al.* 1981) and further analysis of its defect(s) is in progress.

DISCUSSION

The purpose of the work described in this report was to identify mutants altered in their regulation of inositol-1-phosphate synthase. Prior to using the inositol excretion selection protocol described in the present report, we attempted to find an analog(s) of inositol that would serve as a false repressor of the enzyme. However, as reported, we screened 18 potential analogs and found none that affected growth of wild-type yeast in the manner expected for a compound having the appropriate characteristics. Previously, CULBERTSON (1975) reported that hexaketocyclohexane, mesoerythritol, quinic acid, quercitin, hexamethylcyclohexane, quebrachitol and shikimic acid were also ineffective in inhibiting yeast cell growth in concentrations up to 0.5 mM. The failure of this large spectrum of analogs to inhibit growth of wild type cells may be explained in at least two ways. The compounds may be capable of substituting for inositol in cell membrane

without deleterious effects; or more likely, they may not be recognized by the cell as inositol and would thus be unable to effect repression of I-1-P synthase. This latter explanation would require that a very precise stereospecific recognition mechanism exists for the regulation of synthesis of I-1-P synthase.

Since inositol analogs useful in the selection of regulatory mutants could not be identified, we used the inositol excretion phenotype described in this report to select regulatory mutants. This approach proved highly successful and resulted in the identification of mutants constitutive for I-1-P synthase whose mutations map to three distinct loci, *OPI1*, *OPI2* and *OPI4*. These mutants appear to be true regulatory mutants and not mutants defective in the uptake of inositol. In the present study we did not attempt to do direct inositol uptake experiments, since the high endogenous levels of inositol present in these mutants will make such experiments quite difficult to interpret. However, genetic evidence presented in this report tends to rule out the possibility that these are uptake mutants. Since inositol is absolutely required by the yeast cell (HENRY *et al.* 1977), the combination of inositol auxotrophy and defective inositol transport should be lethal. The spore colonies of crosses of *opi1–1* × *ino1–13*; *opi1–1* × *ino4–26* and *opi1–1* × *ino2–21* selected from putative nonparental ditype asci were proven by complementation to be *ino1*, *ino2* or *ino4*, *opi1–1* recombinants. Thus, it is certain that the *opi1* genotype is viable in an *ino*$^-$ background. Similarly, lethality was not observed in selective loss of any tetrad class in the crosses performed with other *opi* mutations to *ino1*, *ino2* and *ino4* strains (Table 3). Thus, it is unlikely that any of the *opi* mutations represent inositol transport defects. However, this possibility will be further investigated using the *ino*$^-$, *opi*$^-$ double mutants.

The *opi1*, *opi2* and *opi4* mutations are recessive and are unlinked to the structural gene for I-1-P synthase (*INO1*). The mechanism by which these three genes exert control over inositol biosynthesis is not known at present. It is possible that all three gene products are components of a single complex repressor molecule. Alternatively, it is conceivable that only one of the gene products (or indeed the product of some as yet unidentified locus) serves as a repressor and that the products of the other loci are required for synthesis or activity of the repressor. In fact even more complex models of interaction of positive and negative regulatory elements are possible since many of the *INO* loci may also serve a regulatory function. The *ino*$^-$ mutants all lack I-1-P synthase activity (CULBERTSON, DONAHUE and HENRY 1976b), yet only the *ino1* mutants have defects in the structure of the inositol-1-phosphate synthase enzyme itself. Many of the other *INO* loci may be involved in positive regulation of enzyme synthesis. Genetic and biochemical analysis of the constitutive *opi*$^-$ mutants described here, together with further studies of the *ino*$^-$ mutants, may eventually lead to an understanding of the mechanism(s) by which this complex regulation is carried out.

A final and unexpected outcome of this work was the isolation of a nonconstitutive excretor mutant, *opi3–3*. This mutation appears to affect synthesis of phosphatidylcholine (HENRY *et al.* 1981) and a much more extensive analysis of the defect in phospholipid biosynthesis in this mutant is being conducted. However, the observation that a mutant with a primary defect in phosphatidyl-

choline biosynthesis exhibits the phenotype of inositol excretion is unexplained. This result suggests that inositol biosynthesis, as one of the most highly regulated aspects of phospholipid biosynthesis, is easily perturbed by mutations affecting other aspects of the utilization of precursors in phospholipid synthesis. Thus, the identification of this mutant suggests that the inositol excretion phenotype provides a powerful tool for the isolation of mutants defective in the synthesis or regulation of membrane phospholipids, as well as mutants with defects in regulation of inositol biosynthesis.

We are greatly indebted to P. GOLDWASSER for his expert technical assistance in these experiments. These studies were supported by grant GM 19629. M. GREENBERG was supported by training grant GM 07491 and S. HENRY by Research Career Development Award GM 00024, all from the National Institutes of Health. S. HENRY is also the recipient of a faculty award from the Irma T. Hirschl Trust. This report is taken in part from a thesis submitted by M. GREENBERG in partial fulfillment of the requirements of the Ph.D. degree from the Albert Einstein College of Medicine, 1980.

LITERATURE CITED

ATKINSON, K., S. FOGEL and S. HENRY, 1980 Yeast mutants defective in phosphatidylserine synthesis. J. Biol. Chem. **255:** 6653–6661.

CHAMPNEY, W. S. and R. A. JENSEN, 1970 Metabolic influences in tyrosine excretion in *Bacillus subtilis*. J. Bacteriol. **104:** 351–359.

CULBERTSON, M. and S. HENRY, 1975 Inositol requiring mutants of *Saccharomyces cerevisiae*. Genetics **80:** 23–40.

CULBERTSON, M., 1975 Regulation of inositol biosynthesis in *Saccharomyces cerevisiae*. Ph.D. thesis, Albert Einstein College of Medicine, Bronx, New York.

CULBERTSON, M., T. DONAHUE and S. HENRY, 1976a Control of inositol biosynthesis in *Saccharomyces cerevisiae*. I. Properties of a repressible enzyme system in extracts of wild-type (Ino+) cells. J. Bacteriol. **126:** 232–242. ——, 1976b Control of inositol biosynthesis in *Saccharomyces cerevisiae*. II-Inositol-1-phosphate synthetase mutants. J. Bacteriol. **126:** 243–250.

DEMAIN, A. L. and J. BIRNBAUM, 1968 Alteration of permeability for the release of metabolites from the microbial cell. Curr. Top. Microbiol. Immunol. **46:** 1–25.

DEMAIN, A. L., 1966 Industrial fermentations and their relation to regulatory mechanisms. Adv. Appl. Microbiol. **8:** 9–27.

DONAHUE, T., K. ATKINSON, A. KOLAT and S. HENRY, 1978 Inositol-1-phosphate synthase mutants of yeast *Saccharomyces cerevisiae*. p. 311. In: *Cyclitols and Phosphoinositides*, Edited by F. EISENBERG and W. WELLS. Academic Press, New York.

DONAHUE, T. and S. A. HENRY, 1981a L-myo-inositol-1-phosphate synthase: characteristics of the enzyme and identification of its structural gene in yeast. J. Biol. Chemistry **256:** 7077–7085. ——, 1981b Inositol mutants of yeast: Mapping of the *ino*1 locus and characterizing alleles of the *ino*1, *ino*2 and *ino*4 loci. Genetics **98:** 491–503.

FOLCH, J., M. LEES and C. H. S. STANLEY, 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:** 497–509.

GREER, H. and G. R. FINK, 1975 Isolation of regulatory mutants in *Saccharomyces cerevisiae*. pp. 247–272. In: *Methods in Cell Biology*, Vol. 11, Edited by D. M. PRESCOTT. Academic Press, New York.

HENRY, S., K. ATKINSON, A. KOLAT and M. CULBERTSON, 1977 Growth and metabolism of inositol starved cells of *Saccharomyces cerevisiae*. J. Bacteriol **140:** 472–484.

HENRY, S., M. L. GREENBERG, V. A. LETTS, B. SHICKER, L. KLIG and K. D. ATKINSON, 1981 Genetic regulation of phospholipid synthesis in yeast. Proc. of the VI Int. Yeast Symposium pp. 311–316. In: *Current Developments in Yeast Research.* Edited by G. STEWART and I. RUSSELL. Pergamon Press, New York.

HOLDEN, J. L., 1962 The composition of microbial amino acid pools. pp. 73–108. In: *Amino Acid Pools.* Edited by J. T. HOLDEN. Elsevier, New York.

JACKSON, M. and S. SHIN, 1980 Analysis of inositol starvation in an inositol requiring mutant of CHO-K1. J. Cell Biol., **87:** 293a.

KASHMIRI, S. V. S. and S. R. GROSS, 1970 Mutations affecting the regulation of production of the enzymes of leucine synthesis in *Neurospora.* Genetics **64:** 423–440.

LAEMMLI, U. K., 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

LAX, C., S. FOGEL and C. CRAMER, 1979 Regulatory mutants at the *his1* locus of yeast. Genetics **92:** 363–382.

LINDEGREN, G., W. L. HWANG, W. OSHIMA and C. C. LINDEGREN, 1965 Genetical mutants induced by ethylmethane sulfonate in *Saccharomyces.* Can. J. Genet. Cytol. **7:** 491–499.

MORTIMER, R. K. and D. C. HAWTHORNE, 1969 Yeast genetics. pp. 386–460. In: *The Yeasts.* Edited by A. H. ROSE and J. S. HARRISON. Academic Press, New York.

RASSE-MESSENGUY, F. and G. R. FINK, 1974 Feedback-resistant mutants of histidine biosynthesis in yeast. pp. 85–95. In: *Genes, Enzymes and Populations.* Edited by A. M. SRB. Plenum Press, New York.

SATYANARAYANA, J. H. E., G. UMBARGER and G. LINDEGREN, 1968 Biosynthesis of branched-chain amino acids in yeast: regulation of leucine biosynthesis in prototrophic and leucine auxotrophic strains. J. Bacteriol. **96:** 2018–2024.

STEINER, M. and R. LESTER, 1972a Studies on the diversity of inositol-containing yeast phospholipids: Incorporation of 2-deoxyglucose into lipid. J. Bacteriol. **109:** 81–88. ——, 1972b *In vitro* studies of phospholipid biosynthesis in *Saccharomyces cerevisiae.* Biochem. Biophys. Acta. **260:** 222–243.

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