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Control of Inositol Biosynthesis in *Saccharomyces cerevisiae*: Properties of a Repressible Enzyme System in Extracts of Wild-Type (*Ino*$^+$) Cells

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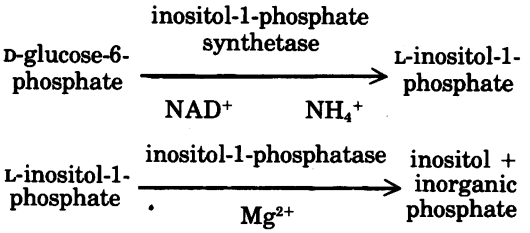
Inositol biosynthesis was studied in soluble, cell extracts of a wild-type (*Ino*$^+$) strain of *Saccharomyces cerevisiae*. Two reactions were detected: (i) conversion of D-glucose-6-phosphate to a phosphorylated form of inositol, presumably inositol-1-phosphate (IP synthetase, EC 5.5.1.4), and (ii) conversion of phosphorylated inositol to inositol (IP phosphatase, EC 3.1.3.25). The in vitro rate of conversion of glucose-6-phosphate to inositol was proportional to incubation time and enzyme concentration. The pH optimum was 7.0. The synthesis of inositol required oxidized nicotinamide adenine dinucleotide (NAD^+) and was stimulated by NH_4Cl and $MgCl_2$. $NADP^+$ substituted poorly for NAD^+, and NADH inhibited the reaction. Phosphorylated inositol accumulated in the absence of $MgCl_2$, suggesting that inositol-phosphate is an intermediate in the pathway and that Mg^{2+} ions stimulate the dephosphorylation of inositol-phosphate. IP synthetase was inhibited approximately 20% in the presence of inositol in the reaction mixture at concentrations exceeding 1 mM. The enzyme was repressed approximately 50-fold when inositol was present in the growth medium at concentrations exceeding 50 μM. IP synthetase reached the fully repressed level approximately 10 h after the addition of inositol to logarithmic cultures grown in the absence of inositol. The specific activity of the enzyme increased with time in logarithmically growing cultures lacking inositol and approached the fully derepressed level as the cells entered stationary phase.

Saccharomyces cerevisiae grows on defined medium in the absence of inositol, and mutants that require inositol for growth have been isolated, suggesting the existence of a pathway for de novo inositol biosynthesis (10). Furthermore, inositol is known to be required for the synthesis of seven inositol-containing lipids (22), including phospholipids (phosphatidylinositol; di- and triphosphoinositide) and sphingolipids [ceremide-(P-inositol)$_2$-mannose and several others whose composition has not been determined with certainty (22, 23). The metabolic relationships among these lipids are such that free inositol, which is shown in this communication to be synthesized by a soluble enzyme system, is transferred to cytidine 5'-diphosphate-diglyceride to form phosphatidylinositol, which serves as precursor in subsequent steps of a branched biosynthetic pathway leading to the formation of the polyphosphoinositides and the spingolipids (1, 21).

Several lines of evidence point to an important role for inositol in the phospholipids of membranes. For example, rapid adenosine 5'-triphosphate-dependent formation and turnover of phosphomonoesters in polyphosphoinositides of *S. cerevisiae* suggest a plausible mechanism for controlling the availability of an adenosine 5'-triphosphate energy source at sites of active membrane transport (21). In addition, physiological studies of *S. cerevisiae* mutants that require inositol for growth revealed some unusual properties that may be related to the function of inositol in membranes (10). Starvation of these mutants for inositol results in logarithmic cell death, which can be prevented by supplementation with inositol or by inhibition of protein synthesis (10; S. A. Henry, T. F. Donahue, and M. R. Culbertson, Genetics 80:s41, 1975). Mutants auxotrophic for saturated fatty acids behave similarly when starved for fatty acids (13, 14). Evidence from studies on inositol-requiring whole cells and protoplasts suggest that starvation for inositol may lead to uncoupling of cell division and macromolecular synthesis, possibly through the loss of normal assembly, function, or integrity of the cellular membranes (A. Brotsky, K. D. Atkinson, M. R. Culbertson, and S. A. Henry, Genetics 80:s17, 1975).

The availability of inositol auxotrophs affords an opportunity to study gene-enzyme relationships in the biosynthesis of inositol; however, the characterization of wild-type inositol biosynthetic enzymes, which has not previously been attempted in *S. cerevisiae*, is an essential prerequisite to this approach. Chen and Charalompous (4-8) have shown that inositol is synthesized in the yeast *Candida utilis* by cyclic aldol condensation of glucose-6-phosphate and that inositol-1-phosphate is an intermediate in the pathway, as follows:



Studies on the purification and separation of inositol-1-phosphate synthetase (IP synthetase, EC 5.5.1.4) and inositol-1-phosphatase (IP phosphatase, EC 3.1.3.25) in *C. utilis* indicate that the two reactions described above are catalyzed by physically separate enzymes (7).

However, the reaction catalyzed by IP synthetase is of sufficient complexity to suggest that several enzymes or an enzyme aggregate may be involved. In support of this view are the facts that oxidized nicotinamide adenine dinucleotide (NAD^+) is required in the reaction (5) but NADH does not accumulate (4), suggesting at least two steps which involve coupled NAD^+-dependent oxidation and NADH-dependent reduction in the formation of inositol-1-phosphate. In addition, evidence from studies on IP synthetase in bovine and rat testes indicate the existence of at least two additional intermediates that are tightly enzyme bound, 5-keto-glucose-6-phosphate and inosose-2-1-phosphate (2, 11, 20). Since 10 unlinked loci were shown to be represented among inositol-requiring mutants in *S. cerevisiae*, the genetic evidence suggests that a large number of polypeptides may be involved in these reactions (10).

The present work concerns the characterization of inositol biosynthetic enzymes in soluble, cell extracts of a wild-type (*Ino*$^+$) strain of *S. cerevisiae*. Two reactions in inositol biosynthesis are described which presumably correspond to the reactions catalyzed in other organisms by IP synthetase and IP phosphatase. In addition, evidence is presented that the inositol biosynthetic pathway is regulated by inositol-mediated repression of enzyme synthesis. The accompanying paper (9) presents evidence on the biochemical characterization of inositol-requiring mutants.

(This work was taken from a dissertation to be submitted by M.R.C. in partial fulfillment of the requirements for the Ph.D. degree in the Sue Golding Division of the Albert Einstein College of Medicine, Bronx, N.Y., and was reported in preliminary form [M. R. Culbertson, T. F. Donahue, and S. A. Henry, Genetics 80: s24, 1975].)

MATERIALS AND METHODS

Materials. D-[U-^{14}C]glucose-6-phosphate (specific activity, 220 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc. *myo*-[U-^{14}C]inositol (specific activity, 215 mCi/mmol) was obtained from New England Nuclear Corp. Cation-exchange resin AG 50W-X8 H^+ (100 to 200 mesh) and anion-exchange resin AG 1-X8 acetate (100 to 200 mesh) were obtained from Bio-Rad. Purified bacterial alkaline phosphatase (*Escherichia coli*) (specific activity, 15.6 μmol of phosphate hydrolyzed/min per mg of protein) was obtained from Worthington Biochemicals Corp.

Source of wild-type enzyme. A haploid strain of *S. cerevisiae* (*ade5*, *Ino*$^+$,*α*) from which a series of inositol auxotrophs was derived previously (10) served as the source of wild-type enzyme.

Preparation of enzyme extracts. The composition of defined growth medium was described previously (10). Cell cultures harvested for the purpose of enzyme extraction were grown in medium containing 10 μM inositol, which maximally derepresses the enzymes responsible for inositol biosynthesis. Cultures (1,500 ml) were inoculated from 25-ml precultures to give a density of 5×10^5 cells/ml and placed in a gyratory shaker for 24 h at 30 C. Cells were harvested by centrifugation at a density of 5×10^7 cells/ml, washed three times in cold deionized water, and suspended in 30 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 5×10^{-4} M reduced glutathione. The buffered cell suspension was disrupted in a Braun homogenizer containing 40 g of glass beads (0.45- to 0.50-mm diameter), which had been prewashed in 1 N NaOH and 1 N HCl. The homogenate was centrifuged at 48,000 × *g* in a Sorvall RC-2 refrigerated centrifuge (4 C), followed by centrifugation of 9 ml of the supernatant at 100,000 × *g* (4 C) in a Beckman model L2-B preparative ultracentrifuge. The clear supernatant resulting from this procedure was dialyzed, unless otherwise indicated, in 1 liter of 5 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2) for 48 h at 4 C. The medium was changed at 12-h intervals. After dialysis, the amount of protein was determined by the method of Lowry et al. (16), with bovine serum albumin as the standard. The final yield was 7 to 10 mg of protein per ml. The lysates were assayed immediately or stored at −20 C for not more than 1 week. No significant loss in enzyme activity was observed in preparations that had been stored frozen for this length of time.

Reaction conditions. The complete rection mix-

ture contained protein from the lysates (specific amounts given in each experiment), D-[^{14}C]glucose-6-phosphate (concentration and specific activity given in each experiment), 0.8 mM NAD^+, 14 mM NH_4Cl, and 2.7 mM $MgCl_2$ in 100 mM Tris-acetate buffer (pH 7.0). Final volume was 1.0 ml. The reaction was allowed to proceed for 3 h at 30 C and was terminated by immersion in a 100 C water bath for 3 min.

Enzyme assays. Inositol biosynthesis was assayed by a variation of the end product method of Charalompous and Chen (3). IP synthetase was assayed as follows. After termination of the reaction, 10 μg of unlabeled carrier inositol was added to each tube, and the precipitated proteins were removed by centrifugation. The pellet was washed twice with 0.5 ml of 200 mM Tris-acetate buffer (pH 8.0), and the reaction mixture and washes were combined in a total volume of 2.0 ml. Commercially prepared bacterial alkaline phosphatase (40 μg in 20 μl) was added to each tube, and the samples were incubated for 1 h at 30 C. The reaction was terminated by the addition of 0.2 ml of 50 mM ethylenediaminetetraacetate (EDTA). This procedure was sufficient to allow complete dephosphorylation of all radioactive, phosphorylated compounds. The purpose of this procedure was to convert the intermediate [^{14}C]inositol-phosphate to [^{14}C]inositol, thereby combining all products of IP synthetase in the form of dephosphorylated inositol.

The dephosphorylated samples were diluted to 10 ml with deionized water and passed through an ion-exchange column. The columns were constructed in Pasteur pipettes with a cation-exchange resin (AG 50W-X8 H^+) on the bottom and an anion-exchange resin (AG 1-X8 acetate) on the top. The resins were preequilibrated by being washed in 0.1 N HCl and 0.5 M sodium acetate, respectively, and each resin was layered to a height of approximately 3 cm. After deionization, the eluates were concentrated by rotary evaporation at 60 C until dry. The residue was redissolved in 3 ml of water and transferred to a test tube containing 1 ml of 0.3 N $Ba(OH)_2$ to hydrolyze radioactive glucose. After boiling for 15 min, the samples were diluted to 10 ml and passed through columns identical to those described above. The samples were finally concentrated to a volume of 50 μl.

The entire 50-μl volume from each sample was spotted on Whatman no. 1 paper (10 by 10 inches [25.4 by 25.4 cm]) and chromatographed for 12 h in an ascending, one-dimensional system with either acetone-water (85:15, vol/vol) or propanol-pyridine-water (3:1:1, vol/vol) as the solvent. Authentic standards for glucose-6-phosphate and inositol were chromatographed along with the samples as controls. After chromatography, the paper was air-dried.

The inositol area on the chromatogram was identified by two methods. In preliminary experiments and controls, nonradioactive inositol was identified by staining the chromatogram with a saturated solution of silver nitrate dissolved in acetone-water (100:3, vol/vol), followed by development in a solution containing 10 ml of 10 N NaOH and 190 ml of 95% ethanol and subsequent fixation in 6 N NH_4OH (24). When treated as described, the inositol area turns dark brown. Radioactive inositol isolated from the enzyme reaction mixtures was identified by autoradiography. Kodak No-Screen X-ray film was overlaid on the chromatogram and placed in the dark for 24 h. The radioactive inositol area of the chromatogram was cut out and counted by liquid scintillation in vials containing 10 ml of toluene with 0.4% PPO (2,5-diphenyloxazole) and 0.041% POPOP {*p*-bis-[2-(5-phenyloxazolyl)]benzene}.

The overall pathway from glucose-6-phosphate to inositol (IP synthetase plus IP phosphatase) was assayed in a manner similar to the procedure outlined above, except that treatment with bacterial alkaline phosphatase was omitted. After removal of precipitated proteins, the samples were treated directly with $Ba(OH)_2$. Using this method, any phosphorylated inositol would be retained by the ion-exchange column and would not be recovered. Thus, the inositol recovered by this procedure is dependent for its formation on IP synthetase and IP phosphatase present in the reaction mixture.

In addition, a method was devised for separately measuring the amount of [^{14}C]inositol and [^{14}C]inositol-phosphate. Separation of phosphorylated inositol and inositol was accomplished by passing samples through ion-exchange columns identical to those described previously. The inositol passing through the column was isolated by the procedures outlined above. Phosphorylated inositol bound to the column was released with 20 ml of 0.1 N HCl, concentrated to 2.0 ml, adjusted to pH 8.0 with Tris-base, converted to inositol with bacterial alkaline phosphatase, and isolated by paper chromatography. Since phosphorylated inositol is isolated in this procedure in its dephosphorylated form, the method cannot provide information on the position of the phosphate. Although Chen and Charalompous (6, 8) showed that the true phosphorylated intermediate in inositol biosynthesis in *C. utilis* is inositol-1-phosphate, the phosphorylated intermediate in *S. cerevisiae* will be referred to throughout the remainder of this communication simply as inositol-phosphate.

The assay method of Charalompous and Chen (6, 8) for measurement of inositol-1-phosphatase activity (release of inorganic phosphate from inositol-1-phosphate) was not used because the substrate inositol-1-phosphate is no longer commercially available. However, the above procedure is expected to indirectly provide evidence for the presence or absence of IP phosphatase activity since, under conditions in which IP phosphatase is inactive but IP synthetase is active, inositol-phosphate is expected to accumulate.

The recovery of [^{14}C]inositol at all steps in the chromatographic procedure was 98%, and [^{14}C]inositol was found to be completely stable in the reaction mixture. [^{14}C]glucose-6-phosphate was completely eliminated in the purification of inositol from the extract.

Enzyme activity is expressed as the amount of enzyme that catalyzes the formation of 1 nmol of inositol per h at 30 C. Enzyme activities, expressed as counts per minute in radioactive inositol, were

converted to mole amounts of inositol on the assumption that the substrate D-[U-^{14}C]glucose-6-phosphate is converted stoichiometrically to [^{14}C]inositol as an intact 6-unit, as demonstrated by Chen and Charalompous (5).

Enzyme repression and inhibition. Repression of the synthesis of IP synthetase was examined in cultures containing several different concentrations of inositol. These cultures were inoculated at a density of 5×10^5 cells/ml from precultures lacking inositol, grown continuously for 24 h, and harvested at a density of 5×10^7 cells/ml. IP synthetase activity in cell extracts prepared from these cultures was determined in reaction mixtures lacking inositol. Under these conditions, differences in the level of enzyme activity measured in vitro were presumed to reflect differences in the rate of enzyme synthesis in vivo. The kinetics of repression by inositol was studied by inoculating cultures lacking inositol at a density of 5×10^5 cells/ml from precultures lacking inositol. The cultures were allowed to grow to a density of 6×10^6 cells/ml, at which time inositol was added to give a concentration of 50 μM. Comparable cultures to which no inositol was added were included as controls. Cultures with and without inositol were harvested at various times after the addition of inositol, and IP synthetase activity was assayed in extracts of each culture as described previously.

Inhibition of IP synthetase was studied using 24-h cultures grown as described above in medium lacking inositol. Cell extracts prepared from these cultures were added to reaction mixtures containing several different concentrations of inositol. This procedure was limited by the fact that large amounts of inositol in the reaction mixtures caused severe overloading of the paper chromatograms, resulting in poor separation of radioactive inositol and other labeled compounds. However, at concentrations of 1 mM or less, reasonable separation was achieved.

RESULTS

When substrate (D-[^{14}C]glucose-6-phosphate) and end product ([^{14}C]inositol) were added to the reaction mixture in the absence of enzyme, glucose-6-phosphate could be eliminated from the mixture by passage through an ion-exchange column, and the free inositol could be recovered with an efficiency of 98%. The efficiency of the method described for detecting the intermediate inositol-phosphate was estimated to range between 80 and 85% on the basis of determinations made with glucose-6-phosphate. The efficiency of recovery probably represents the efficiency of elution of phosphorylated compounds from the column. It is assumed for these calculations that phosphorylated glucose and phosphorylated inositol elute with the same efficiency.

Incubation time, enzyme concentration, and pH. The overall conversion of glucose-6-phosphate to inositol (which involves the sequential action of at least two enzymes, IP synthetase and IP phosphatase) proceeds at a constant rate for at least 3 h and is proportional to the concentration of enzyme added in amounts ranging from 1 to 3 mg of protein from the cell extract (Fig. 1A and B).

Dependence of the initial reaction velocity on the pH of the reaction mixture is shown in Fig. 2. The maximum reaction velocity was obtained at pH 7.0 to 7.1 in 100 mM Tris-acetate buffer.

Cofactor requirements. Figure 3 (A and B) shows the effect of NH_4Cl and NAD^+ on the conversion of glucose-6-phosphate to inositol. Stimulation by ammonium ions reached a maximal fivefold level when the NH_4Cl concentration was 14 mM. At concentrations exceeding 14 mM, the initial reaction velocity was inhibited. The requirement for NAD^+ was absolute, and the maximum rate of reaction was achieved at concentrations exceeding 0.3 mM. Substitution of $NADP^+$ for NAD^+ gave 23% of the NAD^+-stimulated activity. When 0.8 mM NAD^+ and 0.8 mM NADH were added together in the reaction mixture, the resulting activity was 57% of that obtained when 0.8 mM NAD^+ was added alone, suggesting that NADH inhibits the reaction significantly.

In contrast to the work of Chen and Charalompous (5) on *C. utilis*, the conversion of glucose-6-phosphate to inositol in *S. cerevisiae* did not show an absolute requirement for magnesium. Magnesium ions gave approximately a twofold stimulation at a concentration of 2.7 mM in reaction mixtures containing cell extracts dialyzed against Tris-hydrochloride buffer. Thus, roughly half of the enzyme activity remained when magnesium was omitted from the reaction mixture. The addition of 5 mM EDTA to the reaction mixture further reduced activity to 34% of the complete system ($+MgCl_2, -EDTA$).

Since the routine method of enzyme preparation may not have been successful in removing all divalent metal ions, the effect of magnesium was investigated by using enzyme preparations dialyzed under a variety of conditions (Table 1). In general, the enzyme activities, as measured in the complete reaction mixture, were significantly reduced when the cell extract was dialyzed in the presence of 1 mM EDTA and/or 100 mM KCl in 5 mM Tris-hydrochloride or 20 mM potassium phosphate buffer. Regardless of the dialysis system, the maximum stimulation occurred at a concentration of 2.7 mM $MgCl_2$. At concentrations exceeding 3.0 mM $MgCl_2$, the initial reaction velocity was slightly inhibited. Enzyme activity was reduced to the greatest degree (28% of the complete reaction mixture) in reaction mixtures lacking $MgCl_2$ when the

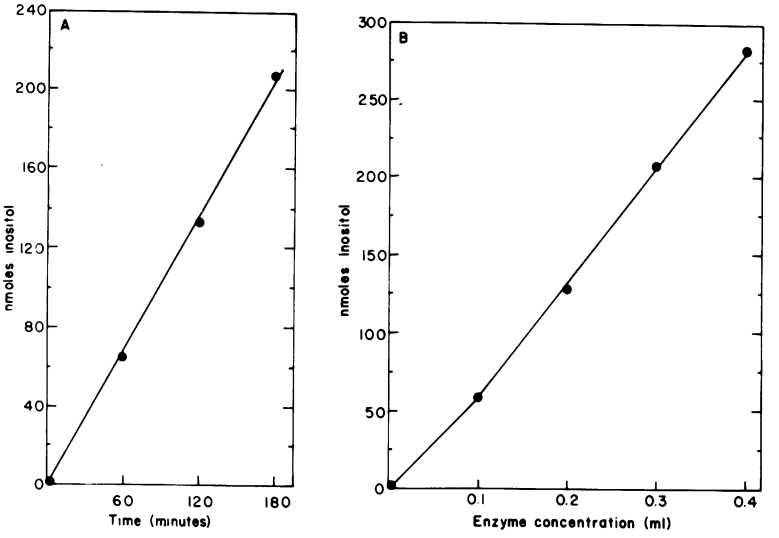


FIG. 1. *Effect of incubation time and enzyme concentration on the rate of conversion of glucose-6-phosphate to inositol. (A) The complete reaction system is described in the text. Reaction mixtures contained 5.00 mM* D-*[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2.3 mg of protein. (B) Reaction mixtures were identical to those given for (A), except that aliquots of increasing volume were added from an enzyme preparation containing 7.6 mg of protein per ml. Incubation time was 3 h.*

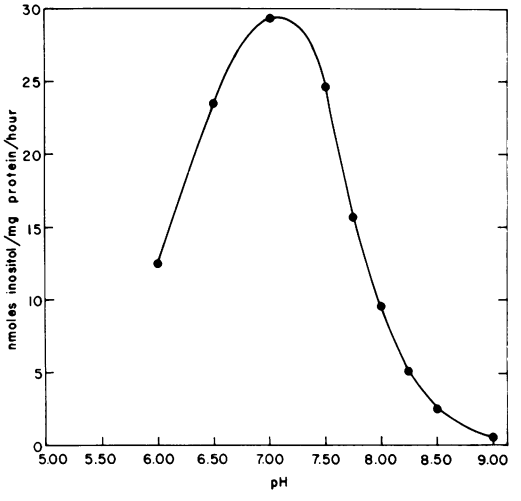


FIG. 2. *Effect of pH on the rate of conversion of glucose-6-phosphate to inositol. The complete reaction system is described in the text. Reaction mixtures contained 4.25 mM* D-*[^{14}C]glucose-6-phosphate (specific activity, 177,000 counts/min per μmol) and 2.7 mg of protein.*

cell extract was dialyzed for 24 h in 5 mM Tris-hydrochloride buffer (pH 7.2) plus 1 mM EDTA and 100 mM KCl, followed by further dialysis for 24 h in the absence of KCl (Fig. 4).

A summary of the cofactor requirements for the conversion of glucose-6-phosphate to inositol is given in Table 2.

Substrate concentration. The biosynthesis of inositol from glucose-6-phosphate is roughly consistent with Michaelis-Menten kinetics over a range of glucose-6-phosphate concentrations between 1.25 and 5.00 mM (Fig. 5). At higher concentrations, the reaction shows an apparent substrate inhibition.

Phosphorylated inositol as an intermediate in inositol biosynthesis. Preliminary experiments indicated that inositol could be recovered from the reaction mixture in amounts greatly exceeding the amount routinely observed in the assay for the overall conversion of glucose-6-phosphate to inositol if the sample was treated prior to ion-exchange chromatography with bacterial alkaline phosphatase. This result suggested that inositol was being released from a phosphorylated precursor by the action of the bacterial phosphatase.

Since it was anticipated that magnesium might be involved in the dephosphorylation step (6, 8) the method for separation of the substrate (inositol-phosphate) and product (inositol) of IP phosphatase was applied to reaction mixtures which either contained or lacked $MgCl_2$. On the assumption that magnesium ions stimulate IP phosphatase activity, it was expected that, in the absence of magnesium, phosphorylated inositol should accumulate. In accord with previous results for cell extracts dialyzed in Tris-hydrochloride buffer, the rate of synthesis of inositol was reduced approximately 50% in the absence of $MgCl_2$ (Table 3)

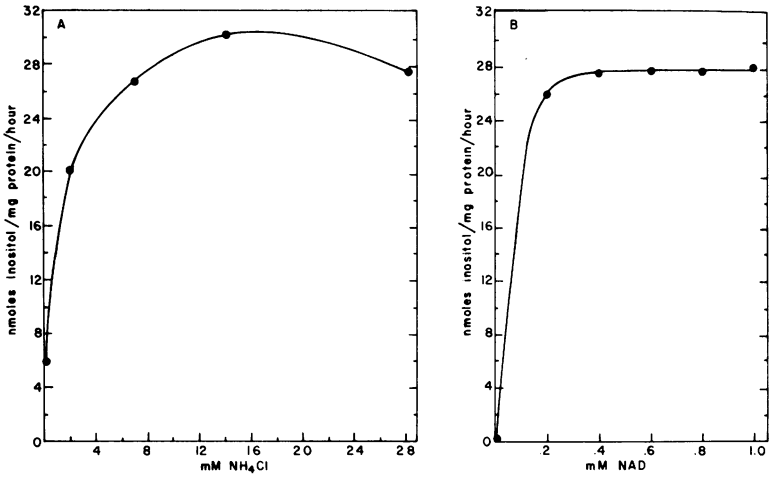


FIG. 3. *Effect of NH_4Cl and NAD^+ on the rate of conversion of glucose-6-phosphate to inositol. (A) The complete reaction system is described in the text. Reaction mixtures contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2.3 mg of protein. (B) Reaction mixtures were identical to those given for (A), except that the NH_4Cl concentration was 14 mM.*

TABLE 1. *Effect of dialysis on stimulation of enzyme activity by magnesium*[a]

Dialysis buffer	Reaction components[b]				
	Complete	$-Mg^{2+}$		$-Mg^{2+}$ + 5 mM EDTA	
	Sp act[c]	Sp act	% of control	Sp act	% of control
Tris-hydrochloride	29.7	14.0	47	10.0	34
Tris-hydrochloride + EDTA	15.9	11.4	72	9.9	62
Tris-hydrochloride + EDTA + KCl	14.1	10.6	75	7.8	55
Potassium phosphate + EDTA	12.4	3.8	31	3.5	28
Potassium phosphate + EDTA + KCl	9.2	3.8	41	3.3	36

[a] The overall conversion of glucose-6-phosphate to inositol (synthetase plus phosphatase) was assayed as described in the text. In the preparation of cell extracts, all dialysis media were adjusted to pH 7.2. Tris-hydrochloride, when present, was at 5 mM; potassium phosphate was at 20 mM; EDTA was at 1 mM; and KCl was at 100 mM. Extracts dialyzed in KCl were re-dialyzed for 24 h in the absence of KCl.

[b] The complete reaction mixture is described in the text. Reaction mixtures contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2 to 3 mg of protein.

[c] Specific activity is expressed as nanomoles of inositol per milligram of protein per hour.

This experiment shows that, in the absence of magnesium, the reduced rate of inositol biosynthesis coincides with the accumulation of phosphorylated inositol, suggesting that magnesium ions may play a role in stimulating IP phosphatase activity.

In addition, the results indicate that *S. cerevisiae* is probably similar to *C. utilis* in that phosphorylated inositol serves as an intermediate in the biosynthesis of inositol. Since phosphorylated inositol was not isolated directly, but rather dephosphorylated and isolated as inositol, it was not possible to determine the position of the phosphate on the inositol carbon skeleton.

Regulation of IP synthetase by inositol. Two aspects of regulation were studied: in vivo repression of enzyme synthesis and in vitro inhibition of enzyme activity.

It can be seen both from the density of [^{14}C]inositol spots in the autoradiogram of Fig. 6 and the graph of IP synthetase activity versus inositol concentration in the growth medium in Fig. 7A that the enzyme system is severely repressed by inositol. Under fully repressed conditions, approximately 1 nmol of inositol is synthesized in vitro per mg of protein per h of incubation compared with fully derepressed conditions in which approximately 50 nmol of inositol is synthesized under identical assay conditions.

As a control for the repression experiment, cells were grown under fully repressed and fully derepressed conditions. Cell extracts prepared from each batch of cells were assayed

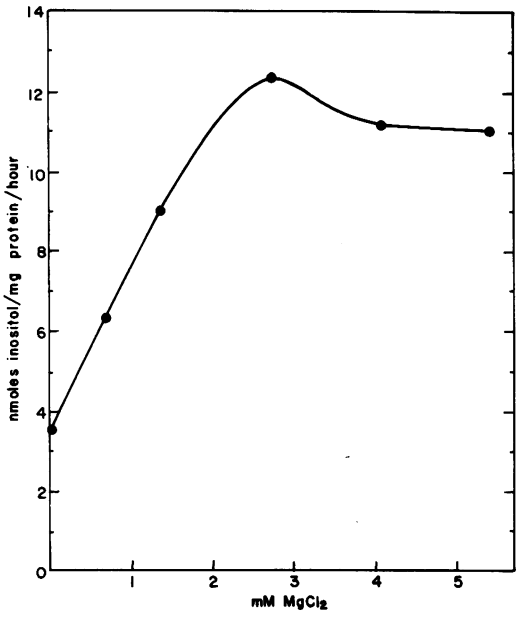


FIG. 4. *Effect of $MgCl_2$ on the rate of conversion of glucose-6-phosphate to inositol. The complete reaction system is described in the text. Reaction mixtures contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2.6 mg of protein. The reaction mixture lacking magnesium contained 5.00 mM EDTA. The extract was dialyzed for 24 h in 5 mM Tris-hydrochloride buffer (pH 7.2) plus 1 mM EDTA plus 100 mM KCl. The extract was redialyzed for 24 h in the absence of KCl.*

TABLE 2. *Requirements for the enzymatic conversion of glucose-6-phosphate to inositol*

Reaction system[a]	Total inositol formed	
	nmol	% of control
Complete	258.8	100
$-NH_4Cl$	52.7	20
$-NAD^+$	1.5	0.006
$-MgCl_2$	121.9	47
$-MgCl_2$ + 5 mM EDTA	87.1	34
Complete, boiled enzyme	0.8	0.003

[a] The conversion of glucose-6-phosphate to inositol (synthetase plus phosphatase) was assayed as described in the text. The complete reaction system contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2.9 mg of protein.

separately for IP synthetase activity. In addition, the synthetase activity was assayed after the two enzyme preparations were mixed in the same reaction vessel (2 mg of protein from each lysate was added). On the assumption that reduced activity in the repressed lysate reflects a

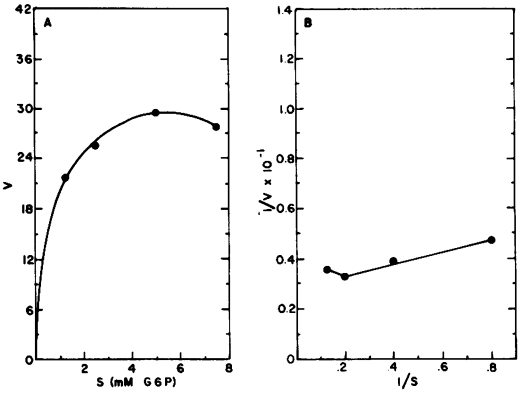


FIG. 5. *Effect of substrate concentration on the rate of conversion of glucose-6-phosphate to inositol. The complete reaction system is described in the text. Reaction mixtures contained substrate (D-[^{14}C]glucose-6-phosphate) at different concentrations, as follows: 1.25 mM (608,000 counts/min per μmol); 2.50 mM (304,000 counts/min per μmol); 5.00 mM (152,000 counts/min per μmol); and 7.50 mM (101,000 counts/min per μmol). Each reaction mixture contained 2.9 mg of protein. (A) Velocity versus substrate concentration. (B) Double-reciprocal plot of velocity versus substrate concentration. Velocity (V) is expressed as nanomoles of inositol produced per milligram of protein per hour.*

TABLE 3. *Accumulation of phosphorylated inositol*[a]

Reaction system[b]	Product formed[c]		
	Inositol-phosphate	Inositol	Total product
$+MgCl_2$	24.60	28.05	52.65
$-MgCl_2$	38.70	14.02	52.72

[a] Radioactive inositol and phosphorylated inositol were separated by ion-exchange chromatography as described in the text. After separation, phosphorylated inositol was treated with bacterial alkaline phosphatase, and the dephosphorylated product (inositol) was isolated.

[b] The complete reaction system is described in the text. Reaction mixtures contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2.9 mg of protein. $MgCl_2$, when present, was at 2.7 mM. The cell extract was dialyzed in 5 mM Tris-hydrochloride buffer (pH 7.2).

[c] Expressed as nanomoles per milligram of protein per hour.

change in the rate of enzyme synthesis, it follows that the sum of activities observed in repressed and derepressed lysates should equal the activity observed in the mixed reaction vessel. The results of this experiment indicate that reduced activity in repressed lysates very likely represents an effect on the rate of enzyme synthesis, since it was observed that enzyme activ-

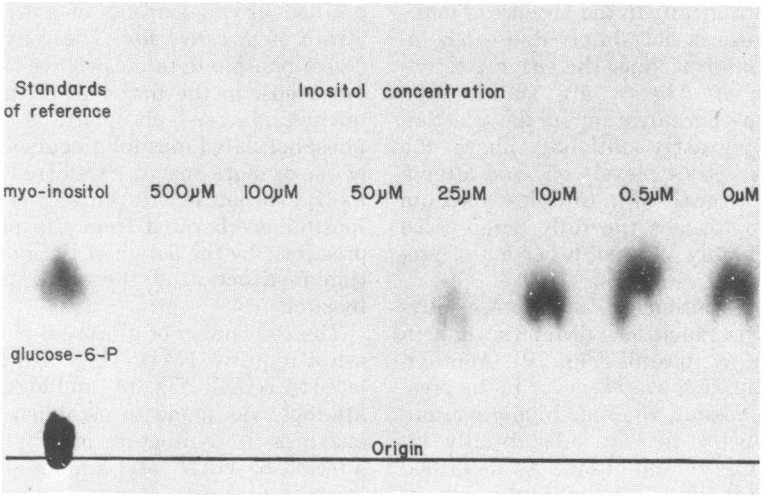


FIG. 6. *Inositol-mediated repression of IP synthetase. Cell cultures were grown continuously for 24 h in medium containing different amounts of inositol. Cell extracts prepared from these cultures were added (2.5 mg of protein) to reaction mixtures containing 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and other components as described in the text. After incubation of the mixtures, radioactive inositol was isolated by the IP synthetase assay procedure. Samples were chromatographed on Whatman no. 1 paper for 12 h in propanol-pyridine-water (3:1:1, vol/vol/vol). The chromatogram was exposed to Kodak No-Screen X-ray film for 24 h. The resulting autoradiogram indicates that the density of radioactive inositol spots is dependent on the amount of inositol in the growth medium. Repression of enzyme synthesis takes place at concentrations of inositol exceeding 50 μM.*

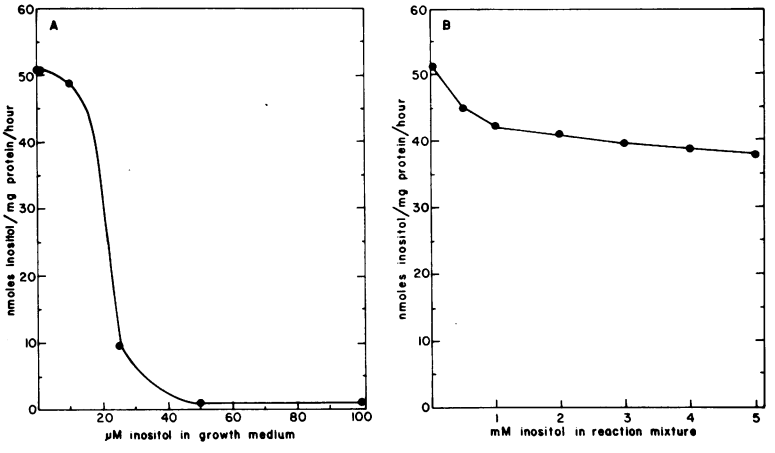


FIG. 7. *Inositol-mediated repression and inhibition of IP synthetase. The complete reaction system, method of assay for IP synthetase, and criteria for distinguishing between repression of enzyme synthesis and inhibition of enzyme activity are given in the text. Reaction mixtures contained 5.00 mM D-[^{14}C]glucose-6-phosphate (specific activity, 152,000 counts/min per μmol) and 2 to 3 mg of protein. (A) Repression of IP synthetase resulting from the presence of inositol in the growth medium. (B) Inhibition of IP synthetase resulting from the presence of inositol in the reaction mixture.*

ity in the mixed reaction was 98% of the sum of repressed plus derepressed activities, as assayed separately.

IP synthetase activity in cultures to which inositol was added (50 μM concentration) is comparable to the activity observed in inositol-free cultures for the first hour after addition of inositol, followed by a dramatic decrease in activity (Fig. 8). After 10 h in the presence of inositol, IP synthetase activity approaches the fully repressed level. However, IP synthetase activity increases dramatically in cultures

growing logarithmically in the absence of inositol. This increase is not simply due to an increase in cell number, since the enzyme activities presented in Fig. 8 are standardized against protein concentration in the reaction mixtures. During early stationary phase, the rise in enzyme activity levels off, and after a total of 22 h of growth in inositol-less medium the activity approaches the fully derepressed level (approximately 50 nmol/h per mg of protein).

The in vitro inhibition of IP synthetase activity by inositol is much less dramatic than in vivo repression by inositol (Fig. 7B). Approximately 20% inhibition was observed in the presence of 1 mM inositol, whereas higher concentrations of inositol did not substantially increase the degree of inhibition. As described above, values for the higher concentrations of inositol may be inaccurate due to technical difficulties resulting in incomplete separation of radioactive inositol from other labeled compounds during chromatography.

DISCUSSION

The biosynthesis of inositol from glucose-6-phosphate by a soluble enzyme system was examined in cell extracts of a wild-type (*Ino*$^+$) strain of *S. cerevisiae*. The conversion of glucose-6-phosphate to inositol was separated into two steps. In the first half of the reaction sequence, glucose-6-phosphate is converted to a phosphorylated inositol precursor by the action of one or more enzymes (referred to collectively as IP synthetase). In the second reaction, free inositol is liberated from the phosphorylated precursor by the action of IP phosphatase. The combined action of these enzymes generates inositol.

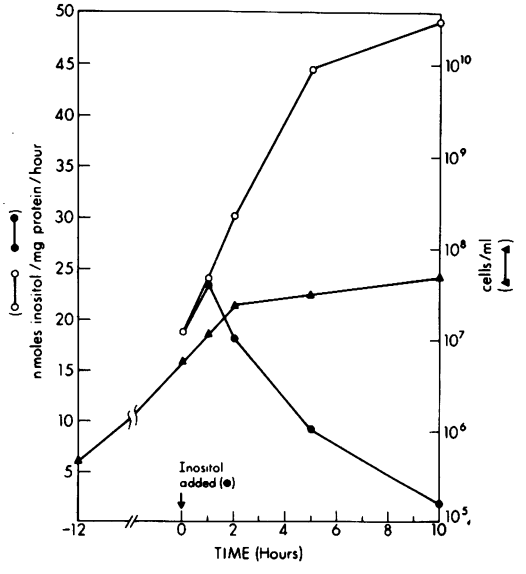


FIG. 8. *Kinetics of inositol-mediated repression. Cultures were grown as described in the text. During logarithmic growth, inositol was added to give a concentration of 50 μM. At various times after the addition of inositol, cells were harvested, cell extracts were prepared, and IP synthetase was assayed. Control cultures to which no inositol was added were included as controls. Symbols: ○, inositol-less cultures; ●, cultures containing 50 μM inositol; ▲, cells per milliliter.*

The conversion of glucose-6-phosphate to inositol requires NAD^+ (Fig. 3B) and is stimulated by NH_4Cl (Fig. 3A) and $MgCl_2$ (Fig. 4). No attempt was made to establish which of the enzymes (IP synthetase or IP phosphatase) is affected by NAD^+ and ammonium ions. However, in *C. utilis*, it was shown that both of these cofactors are involved specifically in the synthetase reaction (8). In *S. cerevisiae*, magnesium ions are involved in the reaction catalyzed by IP phosphatase (Table 3).

In *C. utilis*, the requirement for magnesium in the phosphatase reaction is absolute, and this fact was used to demonstrate the accumulation of inositol-1-phosphate in mixtures lacking magnesium and to establish the role of this compound as an intermediate in the pathway (6, 8). In *S. cerevisiae*, the amount of inositol formed when magnesium is omitted from the reaction mixture varies between 28 and 50% of the complete system, depending on the manner in which the cell extract is dialyzed (Table 1). These results might be explained by two alternative hypotheses. It is possible that the crude cell extracts used in this study contain substrate-nonspecific, magnesium-independent phosphatases. Although the phosphatase which is physiologically relevant for inositol biosynthesis in vivo may, in fact, have a stringent requirement for magnesium, this would not necessarily be apparent in crude extracts containing other phosphatases with less stringent or different metal requirements. On the other hand, it is possible that the results obtained in this study represent a real difference between the enzyme from *C. utilis* and *S. cerevisiae*. Purification of the enzyme system from *S. cerevisiae* should aid in answering questions of this nature.

IP synthetase is repressed by a magnitude of 50-fold in the presence of inositol in the growth medium at concentrations exceeding 50 μM (Fig. 7A). The results of reconstituting repressed and derepressed lysates in single reaction mixtures indicate that the inositol-mediated decrease in IP synthetase activity is very likely the result of lowered rates of enzyme

synthesis. The large net difference between repressed and derepressed levels of enzyme synthesis stands in contrast with the observations of numerous investigators that the difference between minimal and maximal levels of enzyme synthesis is generally unimpressive in the fungi, especially when compared with enzyme systems in the enteric bacteria (12). This fact is illustrated, for example, by the threefold net difference between repressed and derepressed levels of aspartic transcarbamylase in *S. cerevisiae* (15). However, examples in the fungi, in which the change in amplitude of enzyme production is large, do exist, as in nitrate reductase (40-fold) or nitrite reductase (140-fold) in *Aspergillus nidulans* or aryl sulfatase (500-fold) in *Neurospora crassa* (17, 18). Thus, the 50-fold net difference between repressed and derepressed levels of IP synthetase does not represent a unique finding among fungal enzyme systems.

The addition of inositol to logarithmically growing cultures results in a time-dependent decrease in IP synthetase activity (Fig. 8). Ten hours after the addition of inositol to the cultures, the activity of the enzyme approaches the fully repressed level. This result can be interpreted to mean that the addition of inositol to the growth medium prevents further synthesis of the enzyme, whereas the actual rate of decrease in enzyme activity represents a composite function of enzyme turnover and enzyme dilution.

In addition, the specific activity of IP synthetase increases dramatically for time periods ranging from the mid-logarithmic to the early stationary phases of growth in cultures lacking inositol (Fig. 8). The rate of increase in specific activity in these cultures levels off as the cells enter stationary phase, and after a total of 22 h of growth the enzyme approaches the fully derepressed level.

Similar kinetics of derepression have been observed for mitochondrial enzymes, which are sensitive to glucose repression (19). Since all of the experiments reported in this study were conducted in glucose-containing medium (2%, wt/vol), it is possible that the time-dependent increase in IP synthetase activity may represent a response of the cells to glucose depletion as the cell density increases.

The low degree of inositol-mediated inhibition of IP synthetase activity and the relatively high concentration of inositol required to mediate the effect in cell-free systems (Fig. 7B) leave doubt as to whether feedback inhibition plays a physiologically significant role in the regulation of the pathway. In general, the combined evidence supports the conclusion that the pathway is regulated primarily by the repression of enzyme synthesis.

The availability of mutants specifically lacking the capacity to synthesize inositol may prove instrumental in providing an approach toward understanding the mechanism through which inositol biosynthesis is controlled and coordinated with the demand for inositol in the synthesis of phospholipids. In the following communication (9), an attempt is made to correlate some parameters of inositol biosynthesis with the metabolism of inositol in membrane phospholipids through the biochemical characterization of inositol-requiring mutants.

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