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Secretion Can Proceed Uncoupled from Net Plasma Membrane Expansion in Inositol-Starved *Saccharomyces cerevisiae*

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Secretion of acid phosphatase and invertase was examined in an inositol-requiring *ino1* mutant of the yeast *Saccharomyces cerevisiae*. Inositol starvation is known to block plasma membrane expansion, presumably due to restricted membrane phospholipid synthesis. If membrane expansion and extracellular protein secretion are accomplished by the same intracellular transport process, one would expect secretion to fail coordinately with cessation of plasma membrane growth in inositol-starved cells. In glucose-grown, inositol-starved cells, plasma membrane expansion and acid phosphatase secretion stopped coordinately, and intracellular acid phosphatase accumulated. In sucrose-grown, inositol-starved cells, plasma membrane growth halted, but secretion of both acid phosphatase and invertase continued until the onset of inositol-less death. Although glucose-grown and sucrose-grown cells differ in their ability to secrete when deprived of inositol, they exhibited the same disturbances in phospholipid synthesis. Phosphatidylinositol synthesis failed, and its precursors phosphatidic acid and CDP-diglyceride accumulated equally in both cultures. Sucrose-grown yeast cells appear to accomplish normal levels of extracellular protein secretion by an inositol-independent mechanism. In glucose-grown yeasts, both plasma membrane expansion and secretion are inositol dependent.

The *Saccharomyces cerevisiae* temperature-sensitive secretion mutants, reviewed by Schekman and Novick (28), provide evidence that a single subcellular transport system controls the synthesis, processing, and transport of proteins to and through the plasma membrane, as envisioned by Palade (25). At least 25 different cell functions have been genetically defined (*sec* mutants), all simultaneously required for successful secretion (21–23), integration of plasma membrane proteins (24), and overall expansion of the plasma membrane (27). If a single transport pathway supports both membrane growth and secretion in yeast, we would expect that other mutants that cause specific blocks in plasma membrane growth, without restricting intracellular metabolism, would also block secretion.

The yeast inositol auxotrophs (*ino1*) are blocked in plasma membrane growth, due to restricted synthesis of the inositol-containing phospholipids (4, 5, 15). Internal metabolism and macromolecule accumulation continue within the physically limited cell volume, causing lethal congestion (15, 17). A short period of time exists during the course of inositol starvation when cell surface expansion is blocked, but protein synthesis continues at a nearly normal rate. During this 2-h period, it is possible to determine whether extracellular proteins are secreted when overall surface growth is blocked. In a preliminary report, Novick and Schekman (23) found that invertase was secreted in inositol-starved yeast after surface growth stopped. In their study, the conditions for inducing invertase could have prevented key consequences of inositol starvation (15, 16).

We have examined acid phosphatase and invertase secretion in an inositol-starved *ino1* mutant that was provided with growth-supporting phosphatase and invertase substrates. In these cells, with plasma membrane expansion demonstrably restricted, secretion proved to be dependent on the carbon source offered. In glucose-containing medium, acid phosphatase secretion stopped at the same time that plasma membrane expansion was restricted. Acid phosphatase accumulated internally. In glucose-free medium, with sucrose as the carbon source, both invertase and acid phosphatase were secreted after inositol deprivation had restricted plasma membrane growth.

MATERIALS AND METHODS

***S. cerevisiae* strain and growth media.** Strain FAI-4C (*MATαino1-13 fas1-100 ole1-1 SUC2 PHO*$^+$) was derived from a cross of BW1-1C (*MATa fas1-100 ole1-1 SUC2* [14]) and MC13 (*MATαino1-13 lys2 SUC2* [7]). FAI-4C requires inositol and both saturated and unsaturated fatty acids. The strain was maintained on YTTF agar plates (1% yeast extract, 2% peptone, 2% glucose, 1.5% Tween-80, 1.5% Tween-40, 2 mM myristate, 2 mM oleate, 2% agar). Liquid cultures were grown at 30°C in YTF (1% yeast extract, 2% peptone, 2% glucose, 3% Tergitol NP-40, 2 mM myristate, 2 mM oleate). Synthetic media contained a nitrogen base containing the following (per liter): 5 g of ammonium sulfate, 2 μg of biotin, 400 μg of calcium pantothenate, 2 μg of folate, 400 μg of niacin, 200 μg of *p*-aminobenzoate, 400 μg of thiamine hydrochloride, 200 μg of riboflavin, 400 μg of pyridoxine hydrochloride, 500 μg of borate, 40 μg of copper sulfate, 100 μg of potassium iodide, 200 μg of ferric chloride, 400 μg of manganese sulfate, 200 μg of sodium molybdate, 400 μg of zinc sulfate, 0.5 μg of magnenium sulfate, 0.1 g of calcium chloride. Regular nitrogen base contained 1 g of monobasic potassium phosphate per liter, whereas phosphate-free base contained 1 g of potassium chloride per liter instead. All media were supplemented with 300 mg of threonine per liter, 40 mg each of leucine and lysine per liter, 30 mg each of adenine, arginine, and tryptophan per liter, 20 mg each of histidine, methionine, tyrosine, and uracil per liter, 3% (wt/vol) Tergitol NP-40 per liter, and 2 mM each myristate and oleate. Inositol-containing media had 5 mg of *myo*-inositol per liter. Glucose-containing media had 20 g of glucose per liter. Phosphate-free media had 1.5 g of β-glycerol-phosphate per liter added (12). Invertase-induction media had only 25 mg of glucose and 20 g of sucrose per liter, whereas media for continued invertase secretion contained no glucose (8, 10, 20). Both sucrose and β-glycerol-phosphate were added after autoclaving, and the media were

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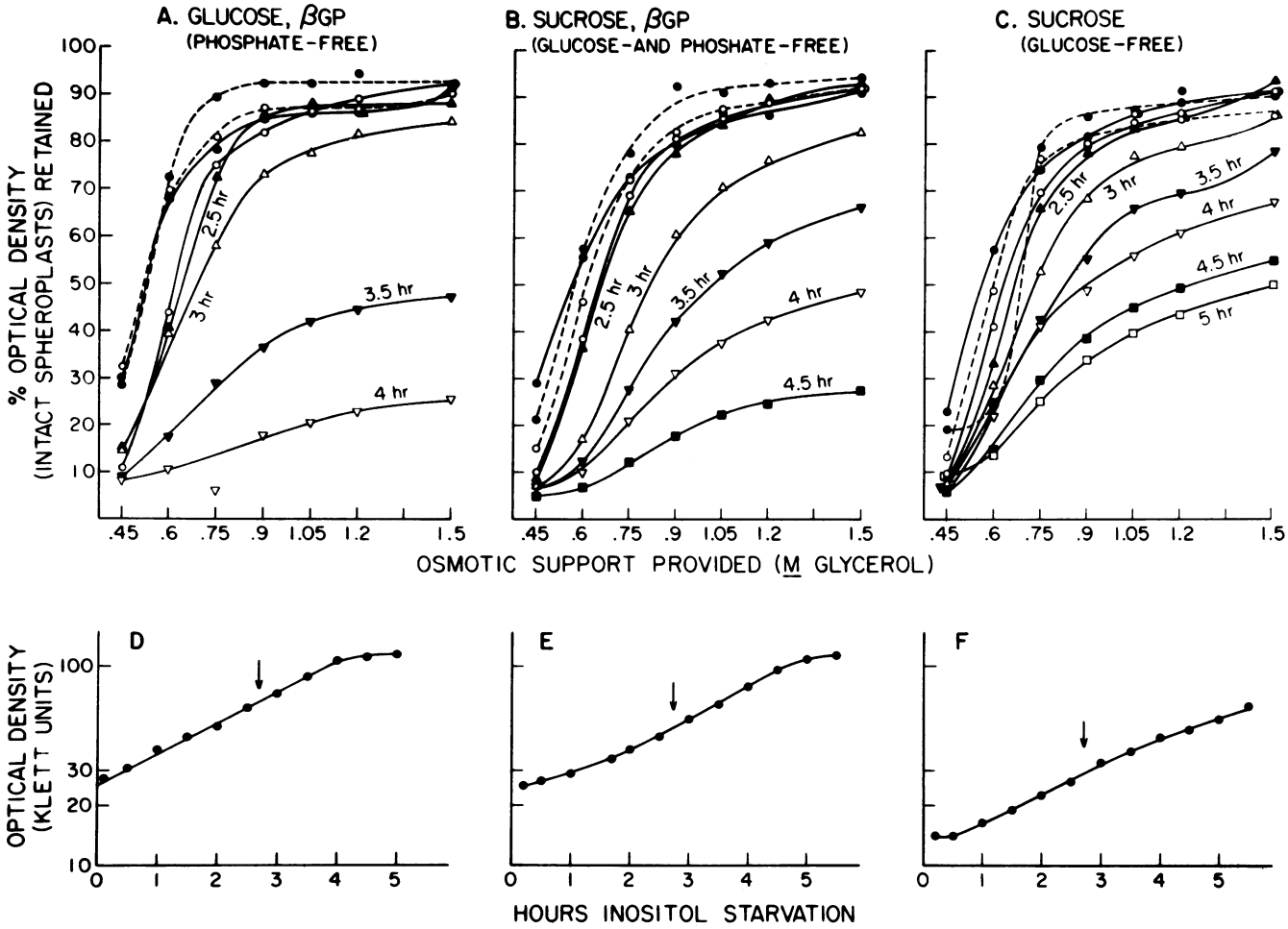

FIG. 1. Osmotic sensitivity shifts show restricted plasma membrane expansion. Cultures were transferred to inositol-less medium with glucose and β-glycerol-phosphate (A), with sucrose and β-glycerol-phosphate (B), or with sucrose and PO_4 (C). Cell samples were taken, their walls were enzymatically removed, and their osmotic sensitivity patterns were determined. Before removing inositol (●– – –● and ○– – –○) and after 1 h (●———●), 2 h (○———○), or 2.5 h (▲———▲) of inositol deprivation, osmotic sensitivity remained constant. After 3 h (△———△), 3.5 h (▼———▼), 4 h (▽———▽), 4.5 h (■———■), or 5 h (□———□) of inositol deprivation, spheroplasts became more osmotically sensitive. Parallel determinations of culture optical density (D, E, and F) showed optical density increases after the point, marked with an arrow, when cell multiplication ceased.

resterilized by filtration to prevent breakdown of these compounds.

Growth and induction conditions. FAI-4C overnight cultures were grown at 30°C in YTF. Cells were harvested by centrifugation, but not washed, and placed in synthetic medium with glucose, phosphate, and inositol at a density of 5×10^6 to 6×10^6 cells per ml. After 4 h of adaptation and growth at 30°C, cells were harvested by centrifugation, washed with secretion-induction medium, and placed in secretion-induction medium with inositol at 5×10^6 cells per ml at 30°C. Induction medium for invertase contained 20 g of sucrose and 25 mg of glucose per liter. Induction medium for acid phosphatase contained β-glycerol-phosphate replacing all PO_4 and either 20 g of glucose per liter or 20 g of sucrose and 25 mg of glucose per liter. After 2 h, the culture volume was doubled with fresh medium, except that the fresh medium did not contain the 25 mg of trace of glucose per liter for cultures with sucrose. After a further 2 h the cells were harvested by centrifugation, washed twice with inositol-containing or inositol-free medium and placed in the same medium at 5×10^6 cells per ml at 30°C. After 2 h an equal volume of fresh medium was added. In some experiments 10 mM disodium cyclic AMP (Sigma Chemical Co.) was added to glucose-containing phosphate-free medium (18). Cultures in which any of the adaptive growth steps were shortened or omitted (including the trace amounts of glucose in the first sucrose-containing growth phase) failed to grow or respond to inositol starvation. Cell growth was monitored by counting in a hemacytometer and by determining optical density in a Klett-Summerson spectrophotometer with a 600-nm red filter.

Spheroplast osmotic sensitivity. Samples were removed from cultures that had been established as described above, washed twice with water, and digested for 15 min at 25°C with 0.5 mg of Zymolyase 5000 (Miles Laboratories) per ml in 1.5 M glycerol–100 mM sodium thioglycolate–50 mM Tris (pH 7.5). Samples were diluted with 5 volumes of thioglycolate-Tris with 0.2 to 1.5 M glycerol. After 20 min at 25°C, spheroplasts remaining intact were estimated by determining optical density at 600 nm (1, 27). Corrections for spheroplast lysis during the zymolyase digestion period were made by direct counting of whole cells and surviving spheroplasts in a

hemacytometer, at the beginning and end of the digestion period.

Secretory enzyme assays. Samples were removed from cultures and washed twice with water. For acid phosphatase determinations, cells were suspended by gentle agitation, since surface phosphatase activity was dislodged from FAI-4C cells after ordinary vortex mixing. External invertase was measured by the method of Goldstein and Lampen (10), and external acid phosphatase was measured by the method of VanRijn et al. (30). Internal activities were measured by the same reactions with a spheroplast lysate sample (23). Spheroplasts digested with zymolyase (as described above) for 20 min were washed with 2.5 M glycerol and resuspended with gentle pipetting. Whole cells were counted before digestion, and spheroplasts surviving the washes were determined by counting in a hemacytometer. The recovered spheroplasts were suspended in the buffer appropriate for either enzyme assay and broken by vigorous vortex mixing with glass beads.

Phospholipid labeling. Parallel inositol-starved cultures adapted to growth with glucose or with sucrose as the carbon source were established as described above. At intervals, 5-ml samples were removed from the culture and incubated for 15 min at 30°C with 1 mCi of carrier-free $^{32}P_i$, (ICN Pharmaceuticals). Labeled cells were harvested by centrifugation, suspended in 5% trichloroacetic acid, and kept on ice for 20 min. Cells were washed with water and extracted with 1.0 ml of solvent at 60°C for 20 min. The solvent used was ethanol–water–diethyl ether–pyridine–28% NH_4OH–5% butylated hydroxytoluene in chloroform (15:15:5:1:0.001:0.001, vol/vol) (13). Samples were cooled, and 0.5 ml of water and 5.0 ml of chloroform-methanol (2:1, vol/vol) were added for a modified Folch et al. wash (9). After vigorous mixing, phase separation was enhanced by brief centrifugation, and the lower organic phase was withdrawn. Organic extracts were dried and spotted onto silica chromatography paper (EDTA-treated Whatman SG81 paper [29]). Phospholipids were resolved by ascending chromatography in two dimensions (45 min for each dimension with drying between dimensions). The solvent for the first dimension was chloroform–methanol–28% NH_4OH–water (66:27:3:0.9, vol/vol), and the solvent for the second dimension was chloroform-methanol-glacial acetic acid-water (32:4:5:1, vol/vol) (29). Radioactive spots were located by autoradiography with Kodak SB-5 X-ray film, and spots were cut out and counted in a scintillation counter.

RESULTS

Growth adaptation is required to induce valid inositol deficiency. External invertase is induced in the absence of glucose (6, 8, 26), and external acid phosphatase is induced in the absence of free phosphate (30). Lacking a carbon source or a phosphate source, yeast cells undergo metabolic arrest that prevents the usual consequences of inositol starvation (15, 16). By providing the inositol-requiring strain with sucrose as a growth-supporting invertase substrate and β-glycerol-phosphate as an acid phosphatase substrate (12), cultures were established that were metabolically active and secreting at a constant rate, parallel to growth, before removing inositol.

Figure 1 shows the osmotic sensitivity profiles of spheroplasts derived from inositol-starved cells grown in phosphate-free, glucose-free, and doubly free media with the appropriate β-glycerol-phosphate or sucrose substitutes. In each medium, cell multiplication proceeded normally for 2 to 2.5 h. Thereafter, cell multiplication halted, but the culture

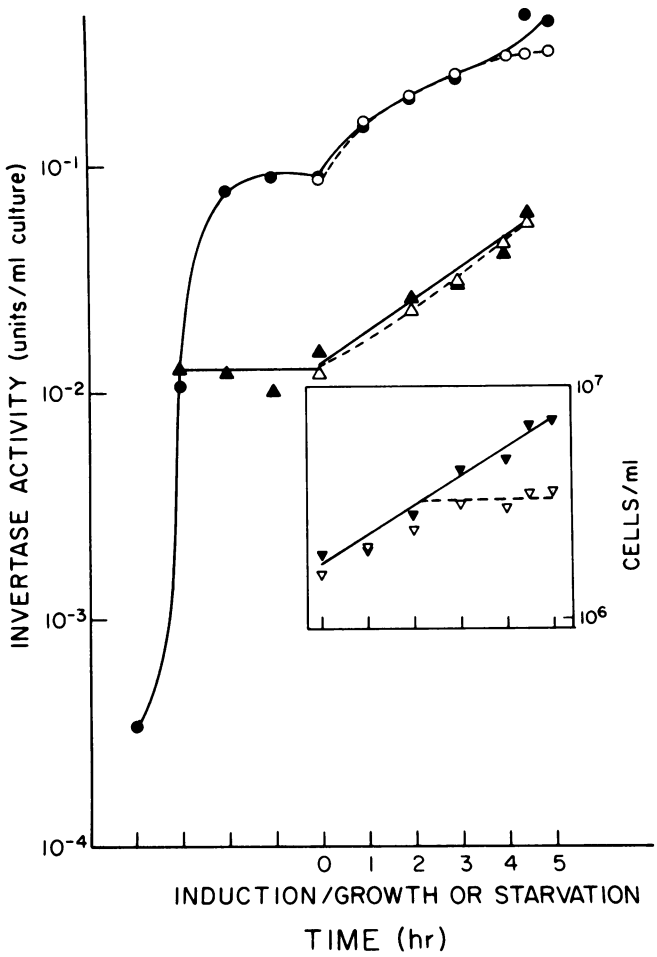

FIG. 2. Invertase secretion continues in inositol-starved cells. Induction and continued accumulation of external and internal invertase activities was determined. Filled-in symbols indicate a culture continuously supplemented with inositol, whereas open symbols indicate a parallel inositol-less subculture. External invertase (● and ○) was determined with whole cells, and internal invertase (▲ and △) was determined with spheroplast lysates. Cell multiplication (▼ and ▽) was determined by hemacytometer counts.

optical density continued to rise until the onset of inositol-less death at 5 h. The data placed together in Fig. 1, 2, and 3 were collected simultaneously on the same culture. Figure 1 represents the osmotic fragility of spheroplasts derived from inositol-starved cultures. The intact cells were not fragile; in fact, the walls of starved cells became progressively resistant to enzymatic degradation unless high concentrations of reducing agent were used. Repeated samplings before the point where cell multiplication stopped revealed that spheroplasted cells had a normal osmotic fragility profile. Samplings taken after multiplication stopped showed spheroplasts with increasingly greater osmotic sensitivity. In accordance with earlier studies (4) osmotic fragility, developing in protoplasts from cells that had ceased multiplying, but whose optical density continued to rise, was taken as evidence that the characteristic feature of inositol starvation had occurred: restriction of plasma membrane expansion.

Cultures shifted directly from rich medium to the inositol-free media with sucrose or β-glycerol-phosphate to initiate both starvation and secretory induction simultaneously failed to exhibit various features that indicate effective

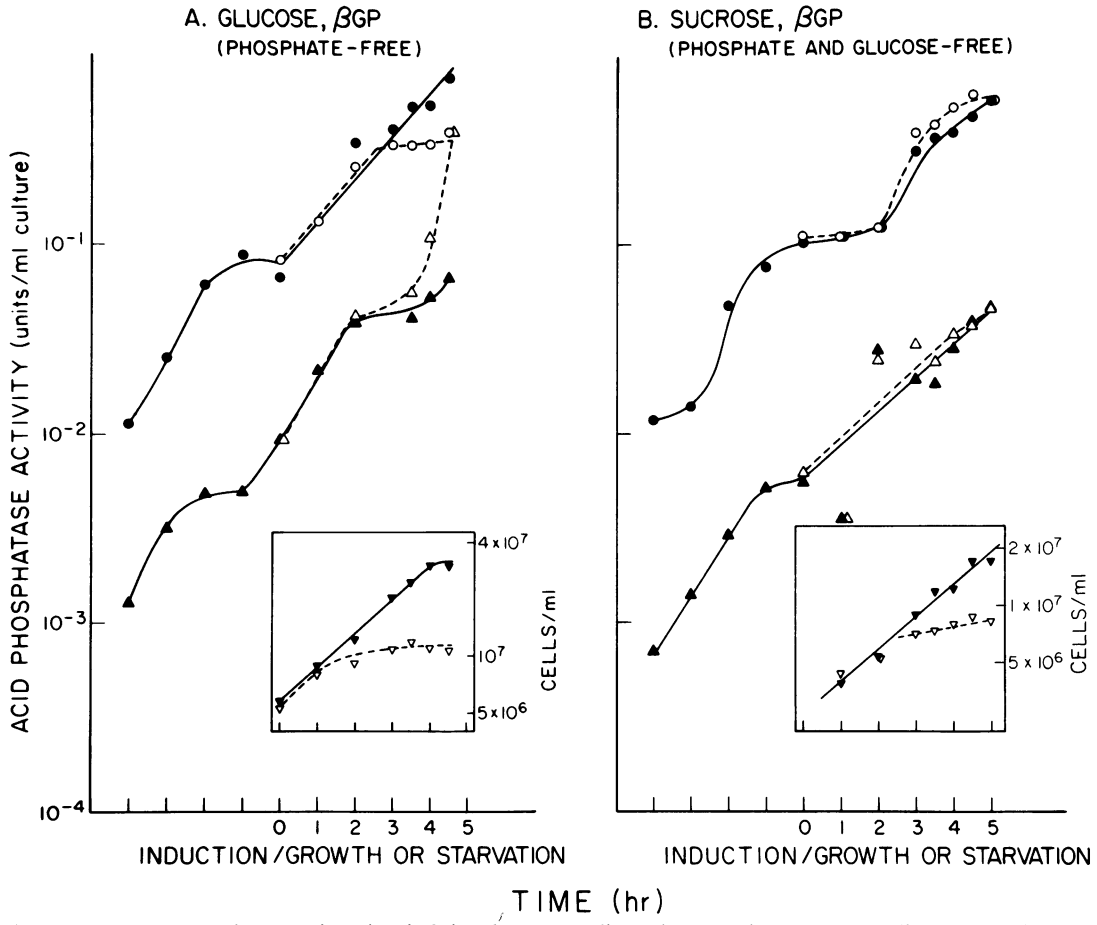

FIG. 3. Acid phosphatase secretion requires inositol in glucose medium, but not in sucrose medium. Induction and continued accumulation of external and internal acid phosphatase activities was determined in PO_4-free cultures with either glucose (A) or sucrose (B) as the carbon source. Filled-in symbols indicate cultures continuously supplemented with inositol, whereas open symbols indicate a parallel inositol-less subculture. External acid phosphatase (● and ○) was determined with whole cells, and internal activity (▲ and △) was determined with spheroplast lysates. Cell multiplication (▼ and ▽) was determined by hemacytometer counts.

inositol starvation. They showed no increase in optical density, no cell multiplication, a high proportion of unbudded cells arrested at the G_1 cell cycle stage, no changes in spheroplast osmotic sensitivity, and very little inositol-less death (data not shown).

Invertase secretion proceeds after plasma membrane growth is restricted. External invertase, measured in intact cells (10), is shown in Fig. 2. The data obtained from culture samples after the culture was diluted were multiplied by the dilution factor. Dilution was necessary to maintain logarithmic-phase cultures throughout the 9-h culture period. These data are expressed as enzyme activity per milliliter of culture, as actually measured. The traditional form of expression as activity per milligram of protein raised a problem because inositol-starved cells gradually increase in cellular protein content (4, 15). We show cell counts as a means to compare the starved and supplemented cultures. Cells were grown in synthetic medium with sucrose substituting for glucose until the activity of external invertase increased in parallel with the optical density and cell number. Transfer to inositol-less medium did not alter the pattern of continual increase of extracellular invertase, even after cell multiplication and plasma membrane growth ceased. Aside from the low constitutive levels found in inositol-supplemented cells, no internal invertase was detected (Fig. 2). Normal levels of invertase were produced and fully secreted in the inositol-starved culture.

Acid phosphatase secretion: two different results. Acid phosphatase secretion was induced in medium with β-glycerol-phosphate replacing all free phosphate; both external (secreted) and internal enzyme levels were measured. Different results were obtained when inositol was removed from cultures that contained either glucose or sucrose as the carbon source (Fig. 3). Acid phosphatase induced in the presence of glucose was accumulated and not secreted after inositol starvation had blocked cell multiplication. In glucose-free medium, with sucrose as the carbon source, inositol starvation did not impair acid phosphatase secretion, even though cell multiplication stopped after the usual interval. Treatment with 10 mM cyclic AMP was not sufficient to induce inositol-independent acid phosphatase secretion in glucose cultures (data not shown).

Phospholipid synthesis. ^{32}P pulse-labeling of the different phospholipid species in parallel inositol-starved cultures grown with glucose or with sucrose as the carbon source is shown in Fig. 4. The labeling pattern in glucose medium was similar to that reported for other starved *inol* yeast stains (5). The culture grown in sucrose medium displayed a transient difference in the synthesis of all phospholipids. Immediately after transfer to the inositol-free, glucose-free

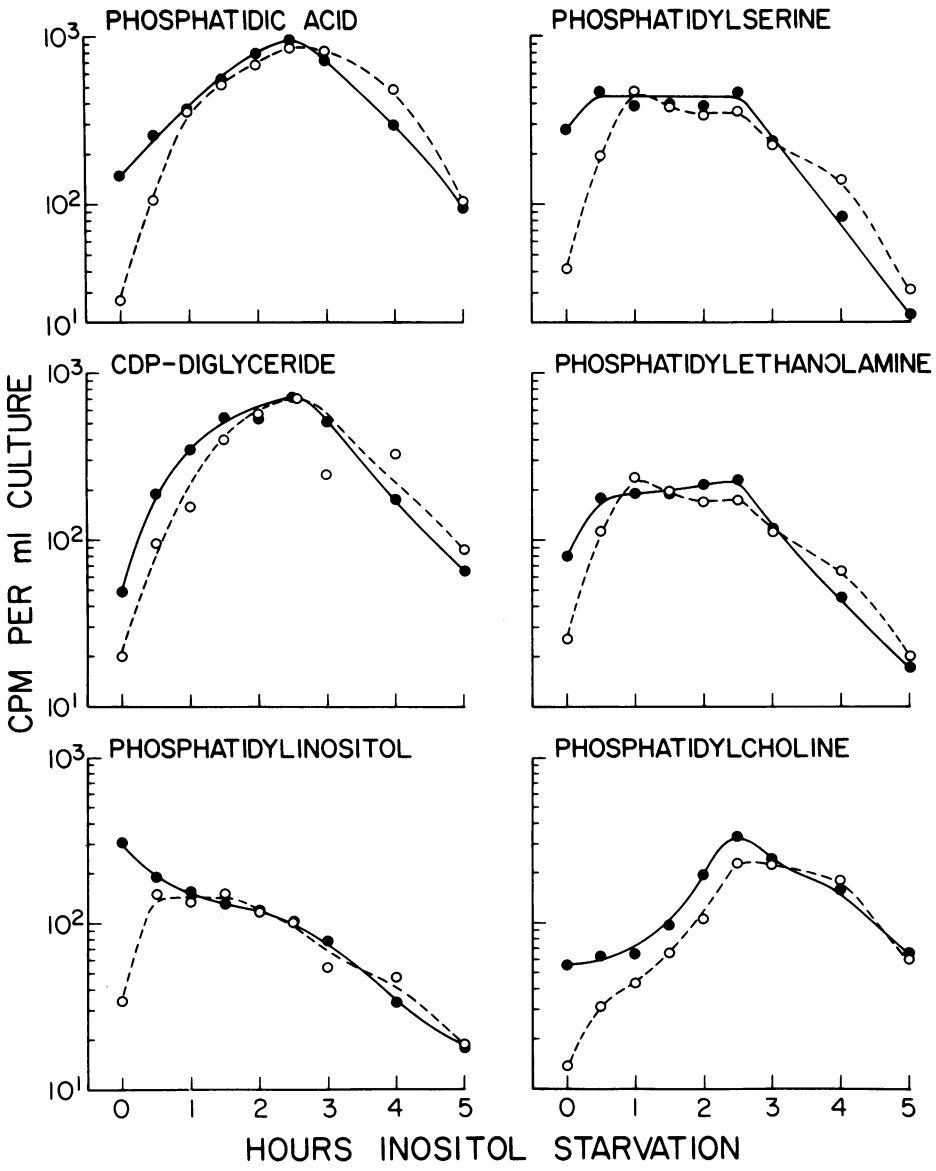

FIG. 4. Rates of phospholipid synthesis. Parallel cultures grown in glucose or sucrose medium were transferred to inositol-less medium. At intervals, culture samples were labeled for 15 min with $^{32}PO_4$, and lipids were extracted. Labeling of each major phospholipid species is indicated in a separate panel. Symbols: inositol-less culture with glucose as the carbon source (●———●), inositol-less culture with sucrose as the carbon source (○- - -○).

medium, these cells exhibited reduced lipid synthetic capacity that rose to match the parallel glucose-containing culture within 1 h. An overall growth lag of the same magnitude is evident from culture optical density (Fig. 1F). Both the growth lag and the transient reduction in lipid synthesis in sucrose-grown cultures were eliminated by shifting sucrose-grown cells to inositol-less medium with 25 mg of glucose per liter and 2% sucrose (data not shown). Well before the cessation of cell multiplication and plasma membrane expansion, the growth lag of the sucrose-grown culture was completed, and rates of phospholipid synthesis compared very closely with the parallel glucose-grown, inositol-free culture. Between 2.5 and 4 h, when the cultures differ in secretory activity, no difference in rates of phospholipid synthesis was detected.

DISCUSSION

Membrane growth is restricted. The imbalance between cell surface expansion and accumulation of internal metabolities and macromolecules is the key feature of inositol-starved yeast, eventually causing cell death (4, 15, 17). Inositol-starved cells do not become leaky or unstable if the cell wall is left intact, but protoplasts cannot withstand progressively increasing internal pressure without high osmotic support. In previous studies, protoplasts were found to have a strictly limited cell volume, indicating complete cessation of plasma membrane expansion (4). In these studies, under all three growth conditions examined, protoplasts exhibited normal osmotic stability before cell multiplication stopped and showed progressively increasing osmotic

fragility after cell division stopped. It is difficult to say whether plasma membrane expansion stops completely or merely slows down. The quantitative shifts in osmotic fragility differ in each of the three growth conditions examined (Fig. 1). Since osmotic fragility reflects an imbalance in plasma membrane expansion and internal metabolite accumulation, differences in either the rate of membrane expansion or the rate of metabolism could alter the rate at which protoplasts exhibit fragility. The rates of optical density increase in the three cultures (Fig. 1), a crude measure of metabolic accumulation, did not differ detectably. It is therefore likely that very rapid development of osmotic sensitivity in cultures with glucose as the carbon source (Fig. 1A) reflects nearly complete cessation of plasma membrane expansion, whereas less rapid development in sucrose cultures (Fig. 1B and C) means that plasma membrane growth is restricted, but not fully halted.

Previous studies suggested that specific failure of phosphatidylinositol synthesis led to failure of cell surface growth (15). No differences between glucose and sucrose cultures were detected in rates of phosphatidylinositol synthesis (Fig. 4). The possibility that membrane expansion stops in inositol-starved, glucose-grown cells, but merely slows down in sucrose-grown cells, suggests that phosphatidylinositol synthesis is not the primary factor restricting membrane expansion. Phosphatidylinositol turnover, associated with secretory activity in higher eucaryotes (19), is known to differ in normal yeast grown with or without glucose (2, 3).

Differential inhibition of secretion and membrane growth. In inositol-starved cells grown with glucose as the carbon source, acid phosphatase secretion fails at the same time that plasma membrane expansion is restricted. Normal levels of active acid phosphatase are made and accumulated inside. Preliminary cytochemical examination reveals acid phosphatase in vesicles, golgi-like bodies, endoplasmic reticulum, and vacuoles with no distinctive exaggeration of one particular secretory organelle.

By contrast, both acid phosphatase and invertase are secreted in inositol-starved cells grown with sucrose as the carbon source. After cell multiplication is blocked, these cells exhibit restricted plasma membrane growth, but are actually secreting at a higher rate per cell than normal growing inositol-supplemented cells. Plasma membrane growth has slowed down, but secretion has not. Preliminary cytochemistry shows that acid phosphatase achieves a normal cell wall location in these cells. Inositol starvation differentially affects net plasma membrane expansion and secretory activity in sucrose-grown cells.

Two different models for the effects of inositol starvation on secretion and membrane expansion can be envisioned. Model A has one pathway. Normally, membrane expansion and secretion are accomplished by a single intracellular pathway of material transport, the secretory pathway defined by Novick and Schekman's *sec* mutants. In glucose-grown cells, inositol starvation cuts off the pathway completely. In sucrose-grown cells the pathway slows down substantially; plasma membrane growth slows, but limited intracellular traffic sustains successful exit of all secretory proteins. In other words, secretory proteins can commandeer a limited cellular transport resource. Model B has two pathways. In higher eucaryotes, distinct post-golgi transport pathways exist. Mouse pituitary cells have a constitutive pathway that is responsible for plasma membrane growth and a distinct catabolite-repressed secretory pathway that does not add much material to the surface membrane (11). Perhaps yeast cells, relieved of catabolic repression in sucrose growth medium, develop a second transport pathway that is not sensitive to inositol depletion and that can transport all secretory material, but that does not sustain normal levels of plasma membrane expansion.

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