INHIBITION OF PHOSPHOMANNAN SYNTHESIS IN HANSENULA HOLSTII BY CYCLOHEXIMIDE

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ABSTRACT

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The ascomycetous yeast *Hansenula holstii* Y-2448 elaborates a phosphorylated mannan which is found both in the medium and as a covalently attached capsule. Cycloheximide did not inhibit reproduction of whole cells, a phenomenon which could not be related to the presence of phosphomannan. However, the uptake of [1-^{14}C] leucine and [2,3-^{3}H] valine into cellular protein of protoplasts was inhibited 36.3% and 50.4% respectively by cycloheximide. The incorporation of glucose into phosphomannan in the presence of cycloheximide was also inhibited in both protoplasts (70.5%) and whole cells (49.1%). This effect was not due to a lack of necessary enzyme, since polysaccharide synthesis is not affected by cycloheximide within the time interval used. It is concluded that a step in the synthesis of phosphorylated mannan is dependent on protein synthesis. This is consistent with studies on the synthesis of cell wall mannan in other yeasts.

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Gary J. Van Haitsma

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DEDICATION

This thesis is dedicated to my mother and father for their patience, understanding, and support.

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INTRODUCTION

Phosphomannans are phosphorylated mannans characteristically produced by yeasts of the genus *Hansenula*; the quality of the extracellular phosphomannan has, in fact, been used in a phylogenetic classification of this genus (57). The phosphomannans have useful physical properties: in aqueous solution, they are highly viscous, thixotropic substances which may be of considerable value as thickeners and suspending agents (57). The phosphomannans also have unique biological properties: they have the ability to bind complement in vitro (26) and they induce the production of interferon in rabbits (50). Pilot plant studies have shown that phosphomannans may be economically produced (39). Therefore, information regarding the synthesis of these potentially important polysaccharides would be useful.

In addition to occupying an extracellular location as in *Hansenula*, phosphorylated mannans are found in all yeast cell walls. These mannans, displaying a wide range in the mannose to phosphate ratio (37), are present as a protein-mannan complex (35) with the protein and mannan synthesis being interdependent (13). Moreover, by virtue of their thixotropic character, phosphomannans might, after being synthesized by the cells, form a capsular layer prior to their release.

The hypothesis has been suggested for *Candida* that extracellular and cell wall polysaccharides are synthesized by the same pathway (6). It is reasonable, therefore, to propose for *Hansenula* a comparable biosynthesis of phosphorylated mannans which are present both extracellularly and in the cell walls. It is the purpose of this study to show a requirement for protein synthesis in the production of these phosphorylated mannans produced by *Hansenula*. This should provide information on pathway coincidence.

LITERATURE REVIEW

Capsular Material

Although much is known about bacterial capsules, little information is available on the subject of yeast capsules. With the exception of *Cryptococcus neoformans*, no direct evidence for capsule is given in the literature. Extracellular polysaccharides produced by yeasts are referred to as capsular material irrespective of their relationship to the cell surface. According to Wickerham (55), *Hansenula capsulata* was so named due to "the capsular material surrounding the cells" but no further information is presented except that the colonies are extremely mucoid. Subsequently Wickerham (56) referred to capsules and capsular material in *Hansenula holstii*, but he presented no direct evidence for a capsule. A layer resembling a capsule is shown surrounding *Hansenula wingei* cells in electronmicrographs (6). A surrounding capsular layer may also be seen in electronmicrographs of *H. holstii* (Dr. S. H. Black, personal communication).

Phosphomannan Production

Many species of *Hansenula* produce extracellular phosphomannans (15,46). Anderson et al. (2) demonstrated that phosphomannans produced by *Hansenula holstii* are generated

extracellularly in high yields from glucose: if the initial glucose concentration in the medium is between 5 and 6 percent, greater than 50 percent of this glucose is incorporated into the phosphomannan. Other medium constituents for optimal laboratory production of the phosphomannan have been reported (2).

Phosphomannan Structure

The structure of this polysaccharide is characterized by its phosphodiester linkages between the carbon-1-hemiacetal position of one mannose unit and the carbon-6 position of another (16,48). The mannose to phosphate ratio in the phosphomannan of *H. holstii* is approximately 5:1 (2,16,47); however, this ratio varies for other species within the genus (47). Using periodate oxidation, Jeanes and Watson (17) investigated the glycosidic linkages which connect the α-mannosyl residues in *H. holstii* and reported these to be 1→2 and 1→3. The fact that these linkages are found in a 3:5 ratio led them to propose a repeating unit containing 10 α-mannosyl residues (17). A subsequent analysis of phosphomannan products produced by mild acid hydrolysis showed 3 different types were present. These products were a phosphorylated pentasaccharide (65%), a high molecular weight fragment which was resistant to further mild acid hydrolysis (9%), and other small fragments which were not characterized. It was demonstrated that the pentasaccharide had the structure P-6-Manp-α-(1→3)-Manp-α-(1→3)-Manp-α-(1→3)-Manp-α-(1→2)-Man. The high molecular weight portion is thought to be the "core" to which the other oligosaccharides

are linked (10). Further analysis is necessary to clarify the structure of the phosphomannan from *H. holstii*.

Cell Wall Structure

The polysaccharide content of isolated cell walls from Baker's Yeast was investigated by Northcote and Horne (35). The cell wall composition was found to be 60% polysaccharide distributed as 29% glucan and 31% mannan. Northcote and Horne also reported 13% protein and 8.5% lipid in the cell wall; however, the high percentage of lipid may be due to cell membrane contaminants. Two layers in the cell wall were observed, one of which was thought to be pure glucan and the other a protein-mannan mixture (35). Mundkur (29) also reported the occurrence of two distinct layers. In addition, he demonstrated the mannan nature of the outer layer and concluded that the inner layer must consist of glucan. Using the freeze-etch technique, Moor and Muhlethaler (27) rarely observed a distinct boundary between layers in the cell wall. The "cross fractured" cell walls showed a change in the granular structure which grows coarser toward the inner surface. This may be due to the mannan impregnating the coarser glucan structure near the cell surface. Cell wall regeneration studies of *S. cerevisiae* protoplasts in gelatin have shown that a glucan fibrillar network is formed on the surface of the protoplasts. This network is then masked by a "cement substance" of mannan which is produced between and over the fibrils (32). This information may explain why Moor and Muhlethaler (27) did

not observe a sharp change in the granularity of the wall but rather a gradual blending.

The regeneration of cell walls by protoplasts of yeast cells provides a useful tool in the study of cell wall structure and biosynthesis. When protoplasts of *S. cerevisiae* are cultivated in liquid media, they form only a basic fibril mesh of glucan over the cell surface; but they do not revert to normal cells (32). Necas (30,31) demonstrated that, when impregnated into gelatin, most *S. cerevisiae* protoplasts could regenerate their cell walls within 24 hr. The regeneration of the cell wall in protoplasts of all budding yeasts examined proceeds primarily in the same way as that for *S. cerevisiae* (34). This information indicates two distinct layers in the yeast cell wall synthesized by two different mechanisms.

Phosphomannan and Mannan Biosynthesis

Basic information concerning synthesis of extracellular phosphomannan and the cell wall mannans has been determined. It seems useful to present the biosynthetic pathways which produce these similar polysaccharides. A definite parallel in the early stages may be observed.

Biosynthetic studies of phosphomannan produced by *H. holstii* showed that glucose is incorporated with the carbon chain intact (7). The glucose is epimerized to mannose as a hexose phosphate and then converted to guanosine diphosphate mannose (7). The transfer of mannose from GDP-mannose to particle bound acceptors is catalyzed by a particulate enzyme fraction in *H. holstii*. This same enzyme fraction also

transfers some of the β-phosphoryl groups from the GDP-mannose (8,20). These phosphoryl groups may be the source of the phosphates involved in the phosphodiester linkages in mannan and phosphomannan.

Mayer (24) showed that GDP-mannose serves as a substrate for phosphomannan synthesis in *Hansenula capsulata* and that both the mannosyl and phosphate residues of the polysaccharide are derived from the nucleotide mannan. GDP-mannose has also been reported to be a precursor of cell wall mannan in *Saccharomyces carlsbergensis* (1), and, as is the case with *Hansenula*, the mannan is bound to particles by a particulate preparation (4). In further studies, Bretthauer and Irwin (9) observed that mannose and β-phosphoryl group from GDP-mannose are transferred to particle bound acceptors by a particulate enzyme fraction in *H. holstii* so that the ratio of bound mannose to phosphate is about 7:1. Bretthauer and Irwin (9) also suggested that the mannose and phosphate are present as glycoproteins. Oligosaccharides through mannotetrose containing a terminal mannose-6-phosphate have been isolated. It has been proposed that mannose and mannose-1-phosphate from GDP-mannose are transferred to certain acceptors where both mannosyl-mannose and mannose-1-P-6-mannose linkages are synthesized (9).

Using *S. cerevisiae*, Tanner (51) presented evidence for a lipophilic mannosyl intermediate in mannan biosynthesis. Sentandreu and Lampen (43) also reported the presence of a lipid intermediate in the synthesis of *S. cerevisiae* mannan. More recently it has been reported that dolichol

monophosphates serve as acceptor lipids in the transfer of mannose from GDP-mannose in *S. cerevisiae*. This process is catalyzed by the same particulate preparation that has been shown to synthesize mannan and phosphomannan (52).

Effect of Cycloheximide on Cell Wall Biosynthesis

The antibiotic cycloheximide has proved useful in studies of cell wall synthesis. Cycloheximide halts protein synthesis to a variable extent in eukaryotic cells (18,44) by preventing translocation of ribosomes on m-RNA (25). In the more resistant organisms, resistance has been shown to be a property of the 60s ribosomal subunit (38).

Although the protein content of the yeast cell wall is relatively low (36), cycloheximide has been shown to inhibit the regeneration of the cell wall by sensitive protoplasts (49). Necas et al. (33) reported that cycloheximide did not block the synthesis of the entire cell wall. Electronmicrographs showed that only the mannan matrix was not produced; however, the fibrillar network of glucan was readily regenerated in the presence of cycloheximide.

Studies of cell wall incorporation in *S. cerevisiae* showed that cycloheximide inhibits the incorporation of labeled amino acid but only partially inhibits the incorporation of labeled glucose (41). Subsequently it was demonstrated that the percentage of label from ^{14}C-glucose incorporated into the glucan portion of the cell wall was greatly increased in the presence of cycloheximide, while the percentage in the mannan was decreased radically (12).

Cycloheximide greatly reduces the amount of threonine incorporated into the cell wall, as well as terminating glucose incorporation into the mannan layer after 5 minutes (12). Morris (28) reported that in *Chlorella*, although protein synthesis is inhibited almost immediately, cycloheximide does not affect polysaccharide synthesis for several hours. The influence of cycloheximide on the enzyme mannan synthetase has also been studied and no effect was detected (13).

Effect of 2-deoxy-D-glucose on Cell Wall Biosynthesis

A glucose analog, 2-deoxy-D-glucose, has also been used to study cell wall synthesis. Although 2-deoxy-D-glucose has little effect on protein synthesis it strongly inhibits the appearance of both mannan and protein in the cell wall (13).

Relationship of Peptide and Polysaccharide Synthesis

Analysis of the matrix material in the cell wall has shown it to consist mainly of mannan covalently linked to polypeptides to yield a glycopeptide (40).

Considering the effects of cycloheximide and 2-deoxy-D-glucose, it appears that there is a parallel inhibition in the synthesis of protein and polysaccharide in the cell wall. Farkas et al. (13) suggested that both components of the mannan-protein complex are necessary for the secretion of either part of that complex.

Sentandreu and Lampen (42) investigated the nature of this block and found that cycloheximide caused the

accumulation of GDP-mannose. Cycloheximide neither blocks the epimerization of glucose to mannose nor the conversion of mannose to its nucleotide. Rather it blocks the glycosylation or synthesis of the peptide as would be expected. Sentandreu and Lampen suggested that in cell wall synthesis the glucan fibrillar network is produced independently of protein synthesis. Since the amorphous matrix of the wall is primarily a mannan-polypeptide, it is suggested that the protein moiety is synthesized first and is subsequently glycosylated during transport to its site of incorporation (42). This hypothesis would explain why both cycloheximide and 2-deoxy-D-glucose inhibit the matrix formation.

MATERIALS AND METHODS

Organisms

A diploid strain (NRRL Y-2448) of *Hansenula holstii*, which produces copious extracellular phosphomannan, and a haploid strain (VIA) of *Hansenula wingei* were obtained from Dr. M. E. Slodki, Northern Regional Research Laboratory, Peoria, Illinois. The strain of Baker's Yeast used was from a dried commercial source.

Cultural Methods and Media

Stock cultures of Baker's Yeast and VIA were maintained on yeast maintenance medium (YM) as described by Haynes et al. (14). In order to maintain the phosphomannan producing characteristic of Y-2448, phosphomannan maintenance medium (PMM) was employed (Dr. M. E. Slodki, personal communication). For the same reason, the transfer interval (2 months) of Y-2448 was twice that used for the other organisms. PMM contained 0.1% glucose, 0.1% yeast extract, 0.1% malt extract, and 0.15% peptone. For optimum phosphomannan production, OP medium was used. This medium contained 6% glucose, 0.1% corn steep liquor, 0.1% tryptone, 0.5% monobasic potassium phosphate, and 0.5% (v/v) Speakman's Salt Solution B (2). Winge's medium (2% glucose and 0.3% yeast extract) was used for the antibiotic testing. For

all media, agar (2%) was added when solid medium was required.

Phosphomannan Preparation

Phosphomannan was isolated and purified according to the method of Jeanes et al. (16) with modifications. The cells were grown in OP medium for 120 hr at 25° C with gentle shaking on a New Brunswick rotary incubator. After this time it was assumed that the glucose had been depleted and the maximum amount of phosphomannan had been produced (2). The culture appeared very smooth and viscous at this time. The culture was diluted with 1/2 vol water and the pH was adjusted from 3.6 to about 6 with potassium hydroxide. Potassium chloride and ethanol were added with stirring to give final concentrations, in percent, of 0.5 (w/v), and 25 (w/v) respectively. The salt and alcohol thinned the culture to facilitate the removal of the cells. After standing for 75 min, the solution was centrifuged until all the cells were removed. The supernatant fluid was a brilliantly clear yellow. After increasing the concentration of potassium chloride to 1% (w/v), the total concentration of ethanol was brought to 50% (v/v) which caused the complete precipitation of the phosphomannan. The precipitate was a soft, cohesive gumlike mass which settled rapidly. The supernatant fluid was decanted.

The soluble materials that were enmeshed in the gum mass were partially removed by kneading and decantation. The phosphomannan was further purified by reprecipitation twice from an aqueous solution containing 2.5% of the gum

and 1% of potassium chloride with ethanol at a concentration of 50%.

For later use, Phosphomannan Y-2448 was then dehydrated by adding, with vigorous stirring, a 10% aqueous solution of the gum to 15 vol absolute methanol containing 0.05% potassium chloride. The precipitate was filtered, washed by resuspension in methanol, and dried in vacuo under anhydrous conditions at room temperature.

Cycloheximide

A stock solution of cycloheximide (Calbiochem) was made up in absolute ethanol to a concentration of 10 mg/ml.

Preparation of Protoplasts

Protoplasts were prepared according to the method of Kozak and Bretthauer (19) with minor modifications. The cells were grown in OP medium with 2% glucose in a New Brunswick rotary incubator for 24 hrs and harvested by centrifugation (10,000xg, 10 min). They were washed in 3 vol of 1% potassium chloride to remove the adhering phosphomannan and then brought to a concentration of $1x10^9$ cells/ml in 0.02M Tris pH 7.6 and 0.06M 2-mercaptoethanol. After incubating at room temperature for 1.5 hr, the cells were centrifuged and washed with 4 vol of potassium phosphate buffer pH 6.5 containing 1.25M potassium chloride. Subsequently the cells were resuspended in the same buffer at a concentration of $1x10^9$ cells/ml and 0.04 vol snail gut juice (Industrie Biologoque Francaise) was added. This suspension was then incubated with gentle shaking at 30° C. After 7 hr,

practically all the cells were protoplasts as determined by plate counts and by examining the cells in hypotonic solution under a phase microscope.

Incorporation of Labeled Amino Acids

Protoplasts were prepared as described above, removed by centrifugation (1000xg, 10 min), and washed in 1 vol of 1.25M potassium chloride in 0.04M potassium phosphate buffer pH 6.5. The cells were then suspended in a medium containing 1.25M potassium chloride, 0.04M monobasic potassium phosphate, and 1% glucose at a concentration of $5x10^8$ cells/ml (19) and 2 ml of this was dispensed into each of six 25 ml Erlenmeyer flasks. The flasks were chilled on ice and 10 µl of the cycloheximide stock solution were added to three of the flasks to give a final concentration of 50 µg per ml. These flasks, along with three controls which had 10 µl of ethanol added, were then incubated at 25° C for 15 min with shaking. After being rechilled, 1 µCi of [1-^{14}C] leucine and 10 µCi of [2,3-^{3}H] valine were added to each flask. After mixing well, 100 µl portions were removed and the flasks were incubated at 25° C with shaking. Portions of 100 µl were removed every half hour for the first 3 hr and a final one was taken after 4 hr.

The portions were handled according to the method of Mans and Novelli (23) as adapted. Each portion was pipetted onto a 2.4 cm GF/c glass fiber disc (Whatman) and exposed to a stream of warm air for 15 sec. The discs were then placed in an ice-cold solution which contained 10% TCA (w/v), 0.1M leucine, and 0.1M valine. Approximately 3 ml

of the above solution was used per disc for the first wash. After standing for 60 min with occasional swirling, the TCA solution was decanted and the discs were washed in the same volume of the solution for 15 min. The liquid was then decanted and the discs were plunged into 5% TCA (w/v) at 90° C and held at this temperature for 30 min. The TCA was removed. The discs were suspended in ether-ethanol (1:1) and incubated at 37° C for 30 min. Finally the discs were suspended in ether for 15 min at room temperature. They were then air dried, placed in scintillation vials, and counted.

Incorporation of Labeled Glucose into Phosphomannan by Protoplasts

The procedure for incorporation of labeled glucose was the same as that for the incorporation of labeled amino acids up to the point where the amino acids were added. Here, rather than labeled amino acids, 5 μCi of [U-^{14}C] glucose were added to each flask and the flasks were incubated in a water bath at 25° C with shaking. After 3 hr, the cells were removed and chilled on ice. The protoplasts were removed by centrifugation (1000xg, 10 min) and the supernatant fluid (M) was removed and retained. Portion M was cleared of all debris by centrifugation (10,000xg, 10 min). The protoplasts were washed twice with buffer and then lysed by repeated freezing and thawing. The lysate was centrifuged (10,000xg, 10 min) and the supernatant fluid (L) removed. Fractions L and M were then centrifuged (25,000xg, 1 hr) and the supernatant fluids removed. Carrier

phosphomannan, 50 µg/ml, was then added to each sample. Portions of 100 µl were pipetted onto GF/c discs (2.4 cm), dried in a stream of warm air for 10 sec, and immersed in 75% ethanol containing 0.1M glucose and 0.5% potassium chloride. After standing for one hr, the ethanol was decanted and the discs were washed for another hour in the same solution. Finally the discs were washed for 30 min in ether-ethanol (1:1) and 15 min in absolute ether. After drying, the discs were placed in scintillation vials and counted.

Incorporation of Labeled Glucose into Phosphomannan by Whole Cells

The cells were prepared in the same manner used to make protoplasts. After being washed with 3 vol of 1% potassium chloride, the cells were centrifuged (10,000xg, 10 min) and suspended in 1.25M potassium chloride in 0.04M potassium phosphate buffer pH 6.5 to a concentration of $5x10^8$ cells/ml. The cells were centrifuged again (5000xg, 10 min) and resuspended to the same concentration in a medium containing 1.25M potassium chloride, 0.04M monobasic potassium phosphate, and 1% glucose. Two ml of this suspension were dispensed into each 25 ml Erlenmeyer flask used, and these were then chilled on ice. To each flask, cycloheximide was added to give a final concentration of 50 µg/ml, and the flasks were incubated in a water bath at 25° C with shaking for 15 min. They were then rechilled and 5 µCi of [U-^{14}C] glucose were added to each flask. Controls were prepared in the same manner except ethanol was added rather than cycloheximide stock

solution. The flasks were then reincubated at 25° C with shaking. After 3 hr the flasks were removed from the water bath and chilled. The cells were removed by centrifugation (10,000xg, 10 min), and the supernatant fluid was treated as stated in the preceding section and counted.

Radioactivity

Radioactivity on glass fiber discs was measured with a Packard Tri-carb liquid scintillation spectrometer, model 3320. The discs were immersed in 15 ml scintillation fluid [4.0 gms 2,5-bis-2-(5-tert-Butylbenzoxazolyl)-Thiophene (BBOT) made up to 1 liter with scintillation grade toluene] and counted repeatedly to obtain mean values. The BBOT was purchased from Packard Instrument Company, Inc., and the toluene was a product of J. T. Baker Chemical Company.

RESULTS

Characterization of the Capsule

Hansenula holstii Y-2448 cells grown for 30 hr in OP medium were harvested by centrifugation, suspended in Pelikan India ink and observed with a phase contrast microscope. A large clear area that represented the capsule surrounded the cell. This area had a diameter of 1.5 to 2 times that of the cell. Extensive clumping of the ink was observed in the intercellular space; however, this effect was not seen if the cells were washed prior to their suspension in the ink. This clumping effect was probably due to some constituent of the medium.

Since washing the cells with water did not remove the capsule, further studies were done to determine the nature of the junction between the capsule and the cell wall (Table 1). The results are based on the majority of the cells that initially exhibited large capsules. In the cases of treatment with water, salt, or heat, the reduction of the capsular size was proportional to the initial size of the capsule; however, when the capsules were treated with acid the rate of removal with respect to size was constant. These results indicate that the capsule is covalently bound to the cell surface.

TABLE 1.--Effect of various treatments on the cell capsule*

Treatment	Conditions	Reduction of Capsular Size
Water	1x (1vol)	None Observable
	2x (1vol)	None Observable**
	5x (1vol)	None Observable
Salt Solutions	1M $CaCl_2$ (1 vol)	None Observable
	1% KCl; 24hr (10vol)	None Observable
Heat	121° C; 15psi; 25min	+
Acid	2.0N HCl; 50° C, 2hr	++++
	2.0N HCl; 25° C, 7hr	+++
	1.0N HCl; 50° C, 2hr	++
	1.0N HCl; 50° C, 3hr	+++
	1.0N HCl; 50° C, 4hr	++++
	0.7N HCl; 50° C, 2hr	++
	0.7N HCl; 25° C, 7hr	+
	0.1N HCl; 50° C, 1hr	None Observable

*Total capsular removal is indicated by ++++.

**Agitation each time in a vortex mixer causes some capsular removal and also causes the capsule's edge to appear more sharply defined.

Toxicity of Cycloheximide for Whole Cells

Auxanographic technique. In order to determine the lethal effect of cycloheximide on cells of *H. holstii* Y-2448, a Sabouraud Dextrose Agar plate was inoculated with an even layer of cells from a 24 hr culture of Winge's medium. Similar plates were inoculated with *H. wingei* VIA and Baker's Yeast. Discs of Whatman #1 filter paper (1 cm) were saturated with cycloheximide stock solution, allowed to dry, and placed on each plate immediately after the plates were inoculated. The plates were read after 36 hr. Results are shown in Table 2. It is clear that Y-2448 is substantially more resistant to cycloheximide than VIA or Baker's Yeast.

Tube dilution tests. Different concentrations of cycloheximide were tested in Winge's medium for their inhibitory effect on the same organisms used in the preceding test. The results are shown in Table 3. These results confirm Y-2448's resistance to cycloheximide.

Effect of cycloheximide in the presence of phosphomannan. Since *H. holstii* Y-2448 showed a high tolerance for cycloheximide compared to the other two organisms, it was thought that perhaps the copious phosphomannan produced by Y-2448 protected it in some way. In order to study this, *H. wingei* VIA was picked because of the previous information and because it belonged to the same genus as Y-2448. Before proceeding, it was necessary to determine the minimal inhibitory concentration (MIC) of cycloheximide on VIA in Winge's medium (Table 4).

TABLE 2.--Effect of cycloheximide on growth as determined by auxanographic technique

Organisms	Zone of Inhibition diameter in mm.
Y-2448	∿20
VIA	∿60
Baker's Yeast	∿50

TABLE 3.--Effect of various concentrations of cycloheximide on growth as determined by tube dilution tests

Cycloheximide	Organisms		
µg/ml	Y-2448	VIA	Baker's Yeast
10	+	-	-
20	+	-	-
40	+	-	-
60	+	-	-
80	+	-	-
100	+	-	-
Ethanol only	+	+	+
0	+	+	+

TABLE 4.--Effect of various concentrations of cycloheximide on the growth of *H. wingei* VIA

Cycloheximide (µg/ml)	Growth in 36 Hours
10.00	-
5.00	-
2.50	-
1.25	-
0.63	±
0.31	+
0.16	+
0.00	++++

Taking the MIC to be about 1 µg/ml for VIA, cycloheximide at this concentration was used to test the effect of various levels of phosphomannan on the growth of VIA in Winge's medium. Due to the problems of sterilizing the phosphomannan, chloramphenicol was added. Results are shown in Table 5. It is clear that phosphomannan conferred no protective effect against cycloheximide.

Preincubation with cycloheximide. Previous studies have shown that when cells are incubated with cycloheximide for two or more hours the effect becomes irreversible (33). Considering this, *H. holstii* Y-2448 cells from a 24 hr culture were suspended in phosphate buffer pH 6.0 containing 100 µg/ml of cycloheximide. The cells were then incubated, with shaking, at 25° C. A one ml portion was removed at intervals of 0, 1, 6, and 24 hr. These portions were washed in phosphate buffer and resuspended in one ml of Winge's medium. One drop of this was used to inoculate 10 ml of Winge's medium in each case. After 24 hr, approximately equal growth was observed in all tubes.

The data on cycloheximide activity indicate that cells are not killed by cycloheximide even at concentrations of 100 µg/ml. Since all the above tests are qualitative, the possibility of a quantitative test remains.

Effect of Cycloheximide on Protein Synthesis in Protoplasts

The inhibition of protein synthesis by cycloheximide in *H. holstii* Y-2448 was determined by studying the

TABLE 5.--Effect of phosphomannan on cycloheximide activity

Phosphomannan (%)	Chloramphenicol (µg/ml)	Cycloheximide (µg/ml)	Growth (36 hr)
0.0	0	0.0	++++
0.0	0	1.0	-
0.0	50	1.0	-
0.8	50	0.0	++++
0.1	50	1.0	-
0.2	50	1.0	-
0.4	50	1.0	-
0.8	50	1.0	-

incorporation of the labeled amino acids [1-^{14}C] leucine and [2,3-^{3}H] valine into the cellular protein. It is apparent, from Figure 1, that the incorporation of both leucine and valine was inhibited in the presence of cycloheximide. The radioactivity found at zero time was subtracted from the measured values.

Effect of Cycloheximide on Phosphomannan Synthesis

In order to determine the effect of cycloheximide on the biosynthesis of phosphomannan by cells of *H. holstii* Y-2448, the incorporation of [U-^{14}C] glucose into the phosphomannan was studied. Both protoplasts and whole cells were used.

In the case of protoplasts, the production of free extracellular phosphomannan was greatly inhibited by the presence of cycloheximide (50 μg/ml) as shown in Table 6. Intracellular phosphomannan was also determined in order to discover if there was an accumulation of the phosphomannan within the cells. The data in Table 6 would contraindicate this.

Although data presented previously in this paper indicate that cycloheximide has little effect on whole cells, a study was done to determine its effect on the free extracellular phosphomannan produced by whole cells. As may be seen in Table 6, the phosphomannan production is decreased in this case as well, although not to as great an extent as in protoplasts.

Figure 1.--Incorporation of [1-^{14}C] leucine and [2,3-^{3}H] valine into protoplast protein in the presence of cycloheximide (50 µg/ml). Leucine with cycloheximide, ● ; without cycloheximide, ○ ; valine with cycloheximide, ▲ ; without cycloheximide, △ .

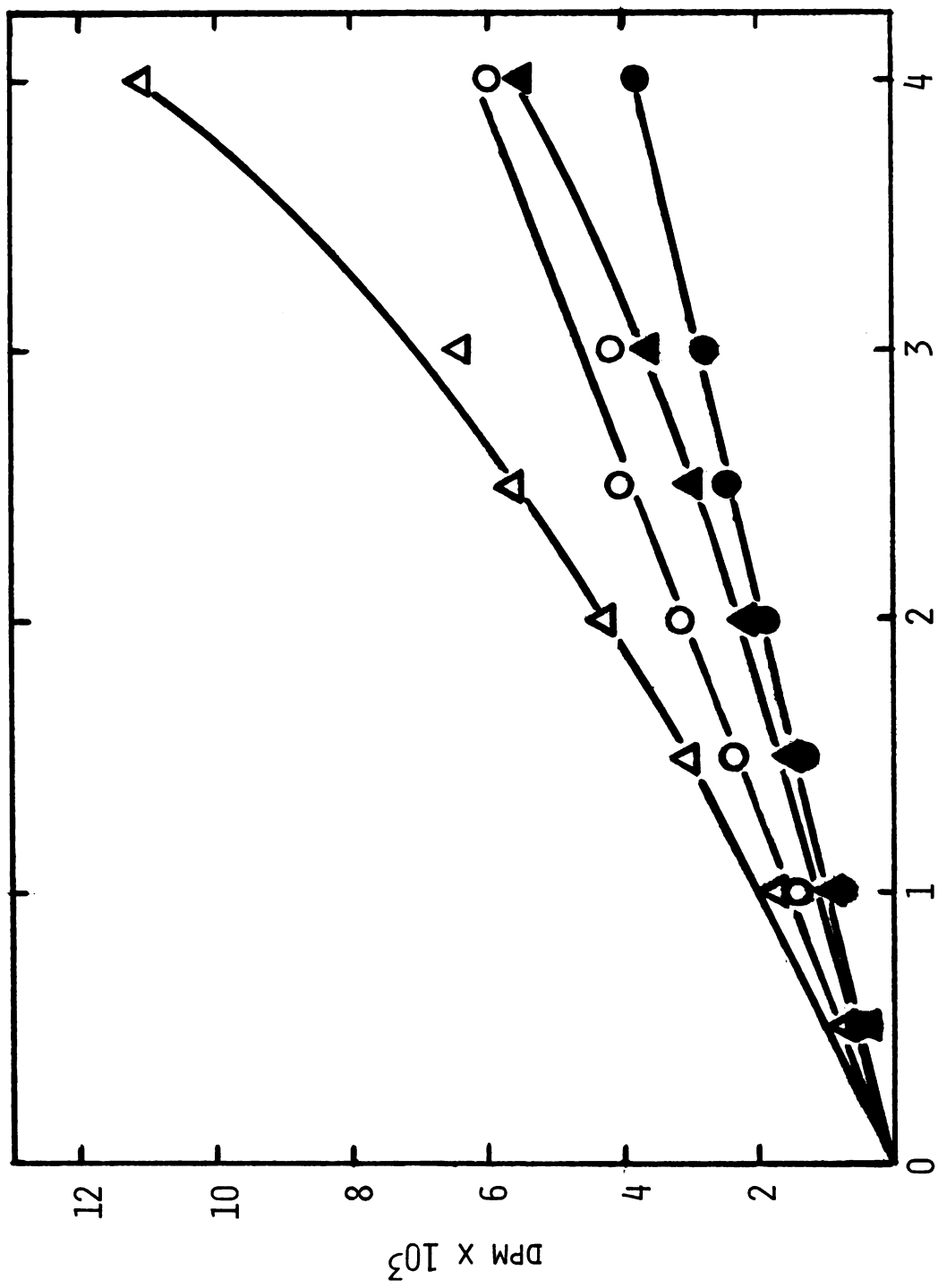

Figure 1

TABLE 6.--Effect of cycloheximide on phosphomannan synthesis

	No. of Determinations	Counts per Minute ± σ		Percent Inhibition
		Cycloheximide Present	Control	
Free phosphomannan excreted by protoplasts	6	1472±104	4987±183	70.5
Phosphomannan extracted from protoplasts	9	473±54	711±71	33.5
Free phosphomannan excreted by whole cells	9	901±167	1771±201	49.1

The analysis of inhibition of phosphomannan synthesis in protoplasts by cycloheximide was repeated three times. In each case similar results were obtained.

From these results it is clear that phosphomannan (a polysaccharide) synthesis is inhibited by cycloheximide (a known inhibitor of protein synthesis).

DISCUSSION

The effect of various treatments on the cell capsule of *Hansenula holstii* Y-2448 demonstrated quite clearly that the capsule was covalently bonded to the cell. Since phosphomannan is extremely water soluble, a thorough washing of the cells with water should remove any adherent nonbonded phosphomannan; however, only a slight result was noticeable and that only with vigorous agitation. A 1% aqueous solution of phosphomannan is extremely viscous due to intermolecular hydrogen bonding. If salts, such as potassium chloride, are added to this solution there is a large decrease in the viscosity because the ions effect a weakening of the hydrogen bonds. When the cells were washed with salt solutions, no effect could be discerned. This excludes the possibility that the capsules are hydrogen-bonded to the cell surface. Since repeated vigorous agitation with water did remove a barely noticeable fraction of the capsule and cause the edges to appear more sharply defined, there was some phosphomannan loosely attached to the capsule, probably by hydrogen bonding. This removal was not seen with the salt washings; but, in these cases, vigorous agitation was not employed. Autoclaving the cells caused some reduction in the size of the capsule. This was most likely due to the dissolution of the hydrogen bonded phosphomannan. The

possibility of partial autohydrolysis cannot be excluded. Of the methods tried the only effective one was the treatment of the capsule with dilute acid. Since the phosphate linkage to the hemiacetal position of the mannose is extremely acid labile, it is reasonable to assume that the capsular disappearance was due to its hydrolysis. Considering the fact that it is necessary to break a bond in order to remove the capsule, the conclusion that the capsule is covalently bonded to the cell is justified.

Extreme differences in sensitivity to cycloheximide have been observed in yeasts. Studies by Whiffen (54) showed that growth of *Saccharomyces fragilis* was not prevented by 1000 µg/ml of the antibiotic. In contrast, growth of *Saccharomyces pastorianus* was completely inhibited by 0.17 g/ml (54). Using a cell free system, Sigel and Sisler (45) found no inhibition of protein synthesis in *S. fragilis* at 500 µg/ml cycloheximide; however, using a similar system from *S. pastorianus* they found that 0.2 µg/ml of the antibiotic produced 50% inhibition.

The results in this paper also show a variable range of effect for cycloheximide. *H. holstii* Y-2448 shows substantial resistance to the antibiotic when compared to *Hansenula wingei* VIA and Baker's Yeast as is shown by auxanographic and tube tests.

Since *H. holstii* Y-2448 produces abundant free extracellular phosphomannan, the possibility that this was a factor in the resistance of the organism to cycloheximide was considered. This does not appear to be the case. When

H. wingei VIA was incubated in a medium containing 0.8% phosphomannan, 1 µg/ml cycloheximide (this was determined to be the MIC), and 50 µg/ml chloramphenicol (because of the difficulty sterilizing phosphomannan) no protective effect was discerned. In addition, it is not likely that capsular phosphomannan was responsible for Y-2448's tolerance inasmuch as VIA also produced a capsule, although without significant free extracellular phosphomannan. Thus if cycloheximide is not blocked from entering the cell then resistance must be a function of an intracellular component. This is in agreement with the results of Westcott and Sisler (53). They have demonstrated that cells of *S. fragilis* do not concentrate cycloheximide, but do allow it to penetrate the cell. In contrast, *S. pastorianus* cells do concentrate the antibiotic 13-14 fold when the external concentration is in the 0.1-1.0 µg/ml range (53). This concentration effect is probably due to a large number of binding sites within the cell.

Because of the need for quantitative knowledge about the inhibition of protein synthesis by cycloheximide in *H. holstii* Y-2448, label incorporation studies were undertaken. Protoplasts were used because of the intention to use them in further experiments and because of their susceptibility to lysing. After a 4 hr incubation with cycloheximide, there was a 50% inhibition in the uptake of valine and a 36% inhibition in the uptake of leucine. It is clear that cycloheximide (50 µg/ml) does cause a clear although limited effect on protein synthesis.

Since the cells were incubated with the labeled amino acids for an extended period of time, the problem of metabolic products of these labeled compounds must be considered. The amino acids, [1-^{14}C] leucine and [2,3-^{3}H] valine, were chosen in order to minimize the possibility of interference by labeled metabolic products. In the cell the catabolism of leucine includes first a transamination followed by decarboxylation to yield CO_2 and isovaleryl CoA (21). By labeling leucine at the C-1 position this amino acid is metabolized in such a way that the only labeled product is CO_2. Labeled [2,3-^{3}H] valine was used simultaneously with the leucine to study incorporation. Although there is an exchange of the tritium into water, the tritiated valine yields fewer labeled metabolic products than [4,5-^{3}H] leucine or [3,5-^{3}H] tyrosine (3), all of which follow a catabolic pathway similar to leucine. Double label counting was used so that both amino acids would be exposed to the same cell population.

Protoplasts of *H. holstii* are able to produce extracellular phosphomannan (19). Since the cell wall is absent, it seems reasonable to assume that enzymes associated with the cell wall are not responsible for phosphomannan biosynthesis. This leaves two possible methods by which synthesis may occur. The polysaccharide may be synthesized at the plasma membrane and then released into the cellular environment or it may be synthesized within the cell and transported to and across the plasma membrane, as is the case for invertase (5).

The advantage in using protoplasts is that there is no cell to which the phosphomannan may become attached and complicate the experiment.

The results presented in this paper demonstrate that cycloheximide inhibits the production of free extracellular phosphomannan. Cycloheximide also blocks the synthesis of cell wall mannan (33,12). Considering the structural and spatial relationships of mannan and phosphomannan along with the battery of synthetic evidence (see literature review) it is probable that both polysaccharides are synthesized primarily by the same or parallel pathways. Sentandreu and Lampen (42) have shown that the blockage in the synthesis of cell wall mannan by cycloheximide is due to the fact that no protein is synthesized. Since they found an accumulation of GDP-mannose (a precursor of both mannan and phosphomannan), they concluded that the protein must be synthesized first and the mannose units are subsequently attached. Because cycloheximide inhibits phosphomannan synthesis, it is reasonable to assume that phosphomannan production is also dependent on protein synthesis. This is further evidence for the similarity in the biosynthetic pathways of mannan and phosphomannan.

The inhibition of phosphomannan synthesis by cycloheximide was greater with protoplasts than with whole cells. The reason for this phenomenon is not understood. Perhaps it is because cycloheximide more rapidly penetrates the protoplasts, or it may be due to the contamination of the phosphomannan by soluble protein from protoplasts which ruptured during the incubation period. Contamination seems

unlikely, however, since analysis of phosphomannan produced by protoplasts of *H. holstii* Y-2448 showed no appreciable DNA, RNA or protein when studied by Kozak and Bretthauer (19). Another possibility is that a certain amount of phosphomannan remained attached to the capsule of the whole cell. This probably accounts for at least part of the difference since it is shown in this paper that some phosphomannan does remain attached to the capsule even after washing.

The analysis of lysed protoplasts showed no accumulation of intracellular phosphomannan when cycloheximide was present; in fact, there was a decrease in the number of counts compared to the control. It must be noted that in the control 12.5% of the total phosphomannan was intracellular. This conflicts with the data presented by Kozak and Bretthauer (19) who found the intracellular percentage to be only 1.4. Again the possibility of contamination must be considered although Kozak and Bretthauer found it to be no problem. It is of interest to note that the intracellular inhibition of phosphomannan is 33.5% which is close to that shown for leucine incorporation (36%). If, in fact, the high percentage value of intracellular phosphomannan was due to protein contamination then a minor accumulation of phosphomannan may have been masked.

Kozak and Bretthauer (19) suggest that protoplasts may produce and release glucan and mannan as well as phosphomannan. The basis of this suggestion was the fact that the heaviest portion produced by mild acid hydrolysis of a phosphomannan synthesized by protoplasts had a mannose to

phosphate ratio substantially greater than was found for normally produced phosphomannan. However a similar product was found when normal phosphomannan was hydrolyzed in the same way. Subsequent analysis of this material by Bretthauer et al. (10) showed it to be a high molecular weight "core" fragment of phosphomannan containing fewer phosphate linkages than the other fragments. The possibility that mannan and glucan are produced and released cannot be excluded; however, it appears that if they are produced, it is not in sufficient quantity to be detected, as shown by the results of Kozak and Bretthauer (19).

The data presented here indicate that the synthesis of phosphomannan is dependent on protein synthesis. This is in agreement with studies on the synthesis of yeast invertase where both protein and polysaccharide synthesis are necessary for secretion of the enzyme (22). The theory of Sentandreu and Lampen (42) stating that, in mannan synthesis, protein is synthesized first and subsequently glycosylated, is applicable to phosphomannan synthesis. Although mannan and phosphomannan do not have the same biosynthetic pathways in toto, it is probable that initially they are the same. The exact point of divergence remains to be identified. The fact that the synthesis of both mannan and phosphomannan is inhibited by cycloheximide indicates that the divergence occurs after protein glycosylation. However, further evidence is necessary to pinpoint this biosynthetic fork.

LITERATURE CITED

LITERATURE CITED

1. Algranati, I. D., H. Carminatti, and E. Cabib. 1963. The enzymic synthesis of yeast mannan. Biochem. Biophys. Res. Commun. 12:504-509.

2. Anderson, R. F., M. C. Cadmus, R. G. Benedict, and M. E. Slodki. 1960. Laboratory production of a phosphorylated mannan by *Hansenula holstii*. Arch. Biochem. Biophys. 89:289-292.

3. Banker, G., and C. W. Cotman. 1971. Characteristics of different amino acids as protein precursors in mouse brain: Advantages of certain carboxyl-labeled amino acids. Arch. Biochem. Biophys. 142:565-573.

4. Behrens, N. H., and E. Cabib. 1968. The biosynthesis of mannan in *Saccharomyces carlsbergensis*. J. Biol. Chem. 243:502-509.

5. Beteta, P., and S. Gascon. 1971. Localization of invertase in yeast vacuoles. FEBS Lett. 13:297-300.

6. Black, S. H., and C. Gorman. 1971. The cytology of *Hansenula*. III. Nuclear segregation and envelopment during ascosporogenesis in *Hansenula wingei*. Arch. Mikrobiol. 79:231-248.

7. Bretthauer, R. K., D. R. Wilkin, and R. G. Hansen. 1963. The biosynthesis of guanosine diphosphate mannose and phosphomannan by *Hansenula holstii*. Biochim. Biophys. Acta 78:420-429.

8. Bretthauer, R. K., L. P. Kozak, and W. E. Irwin. 1969. Phosphate and mannose transfer from guanosine diphosphate mannose to yeast mannan acceptors. Biochem. Biophys. Res. Commun. 37:820-827.

9. Bretthauer, R. K., and W. E. Irwin. 1970. Biosynthesis of phosphorylated mannans in *Hansensula holstii*. Fed. Proc. 29:338.

10. Bretthauer, R. K., G. J. Kaczorowski, and M. J. Weise. 1973. Characterization of a phosphorylated pentasaccharide isolated from *Hansenula holstii* NRRL Y-2448 phosphomannan. Biochemistry 12:1251-1256.

11. do Carmo-Sousa, L., and C. Barroso-Lopes. 1970. A comparative study of the extracellular and cell-wall polysaccharides from some *Candida* species. Antonie van Leeuwenhoek J. Microbiol. Serol. 36:209-216.

12. Elorza, M. V., and R. Sentandreu. 1969. Effect of cycloheximide on yeast cell wall synthesis. Biochem. Biophys. Res. Commun. 36:741-747.

13. Farkas, V., A. Svoboda, and S. Bauer. 1970. Secretion of cell-wall glycoproteins by yeast protoplasts. Biochem. J. 118:755-758.

14. Haynes, W. C., L. J. Wickerham, and C. W. Hesseltine. 1955. Maintenance of cultures of industrially important microorganisms. Appl. Microbiol. 3:361-368.

15. Jeanes, A., R. G. Benedict, M. C. Cadmus, P. R. Watson, S. P. Rogovin, J. E. Pittsley, R. J. Dilmer, R. W. Jackson, and F. W. Senti. 1958. Phosphorylated mannan, a new extracellular polysaccharide from a yeast. Abstracts of Papers, 134 Meeting Am. Chem. Soc. Sept.:23D.

16. Jeanes, A., J. E. Pittsley, P. R. Watson, and R. J. Dilmer. 1961. Characterization and properties of the phosphomannan from *Hansenula holstii* NRRL Y-2448. Arch. Biochem. Biophys. 92:343-350.

17. Jeanes, A., and P. R. Watson. 1962. Periodate-oxidized phosphomannan Y-2448: Structural significance of its reaction with alkali. Can. J. Chem. 40:1318-1325.

18. Kerridge, D. 1958. The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*. J. Gen. Microbiol. 19:497-506.

19. Kozak, L. P., and R. K. Bretthauer. 1968. Synthesis of exocellular phosphomannan by protoplasts of *Hansenula holstii*. Arch. Biochem. Biophys. 126: 764-770.

20. Kozak, L. P., and R. K. Bretthauer. 1970. Studies on the biosynthesis of *Hansenula holstii* mannans from guanosine diphosphate mannose. Biochemistry 9:1115-1122.

21. Lehninger, A. L. 1970. Biochemistry. Worth Publishers, Inc. New York.

22. Liras, P., and S. Gascon. 1971. Biosynthesis and secretion of invertase. Eur. J. Biochem. 23:160-165.

23. Mans, R. J., and G. D. Novelli. 1960. A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem. Biophys. Res. Commun. 3:540-543.

24. Mayer, R. M. 1971. The enzymatic synthesis of the phosphomannan of *Hansenula capsulata*. Biochim. Biophys. Acta 252:39-47.

25. McKeehan, W., and B. Hardesty. 1969. The mechanism of cycloheximide inhibition of protein synthesis in rabbit reticulocytes. Biochem. Biophys. Res. Commun. 36:625-630.

26. Miler, I., J. Milerova, and M. A. Leon. 1970. The inhibition of bactericidal activity of sera of newborn germ-free piglets to rough *Escherichia coli* by yeast mannan. Folia Microbiol. (Praha) 15:1-8.

27. Moor, H., and K. Muhlethaler. 1963. Fine structure in frozen-etched yeast cells. J. Cell Biol. 17: 609-628.

28. Morris, I. 1967. The effect of cycloheximide (actidione) on protein and nucleic acid synthesis in *Chlorella*. J. Exp. Bot. 18:54-64.

29. Mundkur, B. 1960. Electron microscopical studies of frozen-dried yeasts. I. Localization of polysaccharides. Exp. Cell Res. 20:28-42.

30. Necas, O. 1961. Physical conditions as important factors for the regeneration of naked yeast protoplasts. Nature (London) 192:580-581.

31. Necas, O. 1962. The mechanism for regeneration of yeast protoplasts. I. Physical conditions. Folia Biol. (Praha) 8:256-262.

32. Necas, O. 1965. The mechanism for regeneration of yeast protoplasts. II. Formation of the cell wall de novo. Folia Biol. (Praha) 11:97-102.

33. Necas, O., A. Svoboda, and M. Kopecka. 1968. The effect of cycloheximide (actidione) on cell wall synthesis in yeast protoplasts. Exp. Cell Res. 53: 291-293.

34. Necas, O. 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35:149-170.

35. Northcote, D. H., and R. W. Horne. 1952. The chemical composition and structure of the yeast cell wall. Biochem. J. 51:232-236.

36. Phaff, H. J. 1963. Cell wall of yeasts. Ann. Rev. Microbiol. 17:15-30.

37. Phaff, H. J. 1971. Structure and biosynthesis of the yeast cell envelope. In A. H. Rose and J. S. Harrison (ed.), The Yeasts, vol. 2. Academic Press, Inc., New York.

38. Rao, S. S., and A. P. Grollman. 1967. Cycloheximide resistance in yeast: A property of the 60s ribosomal subunit. Biochem. Biophys. Res. Commun. 29: 696-704.

39. Rogovin, S. P., V. E. Sohns, and E. L. Griffin, Jr. 1961. A fermentation pilot plant study for making phosphomannan. Ind. Eng. Chem. 53:37-40.

40. Sentandreu, R., and D. H. Northcote. 1968. The structure of a glycopeptide isolated from the yeast cell wall. Biochem. J. 109:419-432.

41. Sentandreu, R., and D. H. Northcote. 1969. Yeast cell wall synthesis. Biochem. J. 115:231-240.

42. Sentandreu, R., and J. O. Lampen. 1970. Biosynthesis of yeast mannan: Inhibition of synthesis of mannose acceptor by cycloheximide. FEBS Lett. 11:95-99.

43. Sentandreu, R., and J. O. Lampen. 1971. Participation of a lipid intermediate in the biosynthesis of *Saccharomyces cerevisiae* LK2G12 mannan. FEBS Lett. 14: 109-113.

44. Siegel, M. R., and H. D. Sisler. 1964. Site of action of cycloheximide in cells of *Saccharomyces pastorianus*. I. The effect of the antibiotic on cellular metabolism. Biochim. Biophys. Acta 87:70-82.

45. Siegel, M. R., and H. D. Sisler. 1965. Site of action of cycloheximide in cells of *Saccharomyces pastorianus*. III. Further studies on the mechanism of action and the mechanism of resistance in *Saccharomyces* species. Biochim. Biophys. Acta 103:558-567.

46. Slodki, M. E., M. C. Cadmus, R. F. Anderson, and R. W. Jackson. 1959. Phosphomannans produced by yeasts of the genus *Hansenula*. Abstracts of Papers, 134 Meeting Am. Chem. Soc. Sept.:6D.

47. Slodki, M. E., L. J. Wickerham, and M. C. Cadmus. 1961. Phylogeny of phosphomannan-producing yeasts. II. Phosphomannan properties and taxonomic relationships. J. Bacteriol. 82:269-274.

48. Slodki, M. E. 1962. Phosphate linkages in phosphomannans from yeast. Biochim. Biophys. Acta 57:525-533.

49. Soskova, L., A. Svoboda, and J. Soska. 1968. The effect of actidione on the regeneration of yeast protoplasts. Folia Microbiol. (Praha) 13:240-244.

50. Suzuki, S., M. Suzuki, and M. Imaya. 1971. Interferon-inducing activity of acid polysaccharides. I. Induction of rabbit serum interferon by *Hansenula* phosphomannans. Jap. J. Microbiol. 15:485-492.

51. Tanner, W. 1969. A lipid intermediate in mannan biosynthesis in yeast. Biochem. Biophys. Res. Commun. 35:144-450.

52. Tanner, W., P. Jung, and N. H. Behrens. 1971. Dolichol-monophosphates: Mannosyl acceptors in a particulate in vitro system of *S. cerevisiae*. FEBS Lett. 16:245-248.

53. Westcott, E. W., and H. D. Sisler. 1964. Uptake of cycloheximide by sensitive and resistant yeast. Phytopathology 54:1261-1264.

54. Whiffin, A. J. 1948. The production, assay, and antibiotic activity of actidione, an antibiotic from *Streptomyces griseus*. J. Bacteriol. 56:283-291.

55. Wickerham, L. J. 1956. Taxonomy of yeasts. 1. Techniques of classification. 2. A classification of the genus *Hansenula*. U. S. Dept. Agr. Tech. Bull. No. 1029. 56pp.

56. Wickerham, L. J. 1960. *Hansenula holstii*, a small yeast important in the early evolution of heterothallic species of its genus. Mycologia 52:171-183.

57. Wickerham, L. J., and K. A. Burton. 1961. Phylogeny of phosphomannan-producing yeasts. I. The genera. J. Bacteriol. 82:265-268.