Biosynthesis of Glycogen and Starch in Cryptococcus laurentii

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Cells of Cryptococcus laurentii, when grown in liquid culture on 2% glucose close to neutral pH, showed glycogen granules throughout the cytoplasm. Glycogen levels of C. laurentii cells reached maximal levels just before onset of stationary phase. Concomitantly, a sharp rise in total and specific activity of glycogen synthetase was observed. Conversely, glycogen phosphorylase reached its highest specific activity approximately 3 hr after the glycogen peaked and remained high until most of the endogenous glycogen was utilized. Uridine diphosphoglucose pyrophosphorylase activity was always an order of magnitude higher than glycogen synthetase during log phase, but fell off rapidly after the cells reached stationary growth. Kinetic properties of the glycogen synthetase showed that the enzyme is always activated by glucose-6-phosphate, although the degree of activation by glucose-6-phosphate was found to be somewhat variable. The accelerated uptake of glucose commencing with the onset of stationary phase is explained by the rapid formation of extracellular acidic polysaccharide, which continues as long as there is glucose in the medium. In cells grown at pH 3.4, where no detectable extracellular acidic polysaccharide was formed, glucose uptake drastically declined when the cells reached stationary phase. These cells also contained glycogen-like granules in the cytoplasm. The evidence presented indicates that these granules are in fact glycogen, and that its structure does not resemble that of the starch excreted by cells grown at acidic pH.

Fungi of the genus Cryptococcus are encapsulated, nonfermentative, yeastlike organisms that multiply by budding and are classified among the fungi imperfecti (12). These organisms excrete starch when grown at low pH; when grown close to neutral pH, where starch production is not observed, they excrete an acidic polysaccharide, (1, 6). The acidic polysaccharide of C. laurentii is composed of mannose, xylose, and glucuronic acid in a ratio of 5:2:1 (1, 9) and resembles the capsular material of the organism. Our earlier studies with C. laurentii (20), in agreement with findings by Ruinen et al. (19), showed the presence of glycogen granules throughout the cytoplasm of cells grown close to neutral pH. Furthermore, they revealed that most of the cell's glycogen phosphorylase and some of its glycogen synthetase are bound to these glycogen granules (20). This communication provides information on the accumulation and utilization of glycogen in C. laurentii and on activity changes of uridine diphosphoglucose (UDP-glucose) pyrophosphorylase, glycogen synthetase, and glycogen phosphorylase during growth close to neutral pH. We also present data that compare cells grown at low pH with those grown at neutral pH. These data indicate that acidic polysaccharide accumulates in the medium only when the pH is close to neutrality. They also demonstrate that the extracellular starch of cells grown at low pH is structurally different from the glucose polymer comprising the granular material within these cells which resembles glycogen.

MATERIALS AND METHODS

Organism. C. laurentii var. flavescens (NRRL-Y-1401) was grown and harvested as described by Ankel and Feingold (4).

Media. The following liquid media were employed. Medium I contained 2% (w/v) glucose,
0.1% urea, 0.1% KH₄PO₄, 0.05% MgSO₄, H₂O, and 0.2 mg of thiamine hydrochloride per liter (14). The pH of this medium was 6.8. Unless mentioned otherwise, medium I was employed. Medium II contained 1.2% (w/v) glucose, 0.12% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·H₂O, and 0.2 mg of thiamine hydrochloride per liter. The pH of the medium was brought to 3.4 with HCl (5).

Fractionation of cell-free extracts. All operations were carried out at 0 to 4 °C. The cells were harvested by centrifugation at 12,000 × g for 15 min, and were washed with 10 volumes of 1% NaCl and with 10 volumes of distilled water; the latter washing was repeated a second time, and the cells were finally washed with 10 volumes of 0.1 M tri(hydroxy-methyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) containing 0.5 g of ethylene diaminetetra-acetate (EDTA)/liter (buffer A). The packed, washed cells (5 g, dry weight) were suspended in an equal volume of buffer A and disrupted by sonic oscillation (4). The broken cell suspension was centrifuged at 28,000 × g for 15 min. The resulting supernatant fluid (S-1) was centrifuged at 100,000 × g for 30 min. After removal of the supernatant fluid (S-2), the particulate fraction was suspended in 10 ml of 0.10 M Tris-hydrochloride buffer (pH 6.5) containing 0.05 g of EDTA/liter (buffer B) and centrifuged again at 100,000 × g for 45 min. The sediment separated into three distinct layers (8). Top, middle, and bottom layers were carefully isolated and resuspended in approximately 2 volumes of buffer B.

Chemicals, enzymes, and substrates. 2-(N-morpholino)-ethanesulfonic acid (MES) was purchased from Calbiochem (Los Angeles, Calif.). Rabbit liver glycogen type III and glucose-6-phosphate were obtained from Sigma Chemical Co., (St. Louis, Mo.). C. laurentii extracellular acidic polysaccharide was a gift from D. S. Feingold, University of Pittsburgh. NAD (297 μCi/m mole), adenosine diphosphate (ADP)-14-C-glucose (228 μCi/m mole), guanosine diphosphate (GDP)-14-C-glucose (28 μCi/m mole), cytidine diphosphate (CDP)-14-C-glucose (8 μCi/m mole), and thymidine diphosphate (TDP)-14-C-glucose (40 μCi/m mole) were purchased from New England Nuclear Corp. and International Chemical and Nuclear Corp. All other chemicals not specifically listed were obtained from commercial sources and were of the purest grade available. Crystalline sweet potato β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2), crystalline swine pancreas α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1), Aspergillus niger glucohydrolase, EC 3.2.1.3, and α-1,6-glucano-hydrolase, EC 3.2.1.11), maltase (α-1-glucoside glucohydrolase, EC 3.2.1.20), UDP-glucose dehydrogenase (UDP-glucose:nicotinamide adenine dinucleotide [NAD] oxidoreductase, EC 1.1.1.22) were purchased from Sigma Chemical Co.

Chromatography. Paper chromatography was carried out on Whatman no. 1 or 3 MM paper with the following solvents: solvent A, 1-propanol-ethanol-acetate-water (7:1:2, v/v); solvent B, 95% ethanol-1 mM ammonium acetate, pH 7.5 (7:3, v/v) containing 1 mM EDTA; and solvent C, n-butanol-95% ethanol-water (5:1:4, v/v). Carbohydrates on paper chromatograms were detected with p-anisidine phthalate or silver nitrate-acetone followed by alcoholic sodium hydroxide spray (25).

Paper chromatograms were analyzed for radioactivity with a Nuclear-Chicago Actigraph III or by radioautography.

Analytical methods. Glucose was determined with glucose oxidase (Glucostat reagents of Worthington Biochemical Corp.). Protein was measured according to Lowry et al. (13) with bovine serum albumin (Sigma Chemical Co.) as a standard.

The dry weight of the cells was determined after repeated washing of the cells with cold water, followed by drying in vacuo to constant weight.

Polysaccharide analysis. Glycogen was extracted from the cells according to the method of Rothman and Cabib (17). The final pellet was suspended in water, and the glycogen content was determined according to the iodine method of Krisman (11). Absorption maximum and specific absorption of 14C-glycogen complexes were determined according to the method of Chester and Byrne (7). In the growth experiments, the amount of starch was determined by weighing the amount of starch isolated according to the procedure below and by measuring the absorption of the iodine complex at 690 nm (11).

Determination of the acidic polysaccharide. The concentration of acidic polysaccharide in culture supernatant fluids was determined by measuring its relative viscosity. A standard curve was prepared by use of known concentrations of authentic acidic polysaccharide. The relative viscosity of a solution of the acidic polysaccharide is directly proportional to its concentration up to 0.02% (w/v), as described by A. Cohen (Ph.D. thesis, Univ. of Pittsburgh, Pittsburgh, Pa., 1966). Samples of the culture medium were taken at timed intervals, the cells were removed by centrifugation, and the viscosity of 3 ml of supernatant fluid was measured with an Ostwald-type viscometer at 37°C. All dilutions were made with uninoculated growth medium. To eliminate viscosity changes due to pH difference, samples from low pH cultures were brought to pH 6.4 prior to the viscosity measurements. The viscosity of the acidic polysaccharide is essentially unchanged between pH 5 and 8, but decreases below pH 4.5 (9).

Isolation and purification of starch. The extracellular starch was isolated from 3 liters of the culture medium of C. laurentii grown in medium II. The method used to isolate the crude polysaccharides was that of Koosman (10). This material was dried in vacuo. Its yield was 1.46 g.

To a 25% solution of crude polysaccharides in water (600 ml), 40 ml of a 2.5% (w/v) Cetavlon (cetyltrimethyl ammonium bromide) solution was added with stirring to precipitate all of the acidic polysaccharide as the Cetavlon salt. The resulting precipitate was removed by centrifugation and discarded. Four volumes of 95% ethanol were added to the supernatant fluid, and the suspension was allowed to stand at 4°C overnight. The precipitated starch was collected by centrifugation, washed by...
resuspension in 95% ethanol, and recentrifuged. The final precipitate was dried in vacuo. Its yield was 0.106 g.

**Enzyme assays.** Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1.) activity was measured in the synthetic direction by following the rate of incorporation of "^1^C-glucose from "^1^C-glucose-1-phosphate into glycogen as described previously (20).

Glycogen synthetase (UDP-glucose:glycogen α-1,4-glucosyl transferase, EC 2.4.1.11.) activity was determined by following the rate of incorporation of "^1^C-glucose from UDP-"^1^C-glucose into glycogen. The reaction mixtures contained 13 mg of rabbit liver UDP-glucose-i-phosphate (237 mCi/m mole), 7.2 mM glucose-6-phosphate (when present), and enzyme in 0.21 M glycylglycine buffer, pH 7.5, in a final volume of 70 μl. The reaction mixtures were incubated at 25 C for 10 min. Reaction mixtures containing heat-denatured enzyme served as controls. The reaction mixtures were subjected to descending chromatography in solvent B. Appropriate areas at the chromatographic origin were excised and counted in a gas-flow counter.

UDP-glucose pyrophosphorylase (uridine triphosphate [UTP]: α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9.) activity was measured by spectrophotometric determination of the amount of UDP-glucose formed from UTP and glucose-1-phosphate in a two-step assay employing NAD^+ and UDP-glucose dehydrogenase (UDP-glucose:NAD^+ oxidoreductase, EC 1.1.1.22.). The reaction mixture consisted of 0.25 M Tris-hydrochloride buffer, pH 7.5, 2.5 mM MgCl2, 2.5 mM UTP, 1.25 mM glucose-1-phosphate and enzyme, contained in a final volume of 0.2 ml. After incubation for 5 min at 30 C, the reaction was stopped by immersing the tubes into boiling water for 90 sec. Suitable samples were incubated in a microcuvette at 30 C in the presence of 0.5 M glycine-KOH buffer, pH 8.7, 1.2 mM NAD^+, 5 mM EDTA, and 400 units of UDP-glucose dehydrogenase, contained in a final volume of 0.2 ml. The reaction was initiated by the addition of NAD^+ and the amount of UDP-glucose originally present was determined from the change in absorbancy at 340 nm after completion of the reaction.

**Measurement of the pH of broken C. laurentii cells.** The cells were collected by centrifugation and washed four times with 10 volumes of cold CO2-free distilled water. After breaking a 50% suspension of the cells (v/v) in cold CO2-free water by sonic treatment, the pH of the broken cell suspension was measured with a glass electrode (15).

**RESULTS**

Accumulation and utilization of glycogen by C. laurentii during growth. C. laurentii cells were grown in medium I as described in Materials and Methods over a period of 120 hr. The levels of glycogen, glycogen synthetase, and UDP-glucose pyrophosphorylase of the cells and the glucose content of the medium are shown in Fig. 1A and B. Glycogen accumulation was maximal (16.5% of the cells' dry weight) just before the onset of the stationary phase of growth. After that time, the cells began to utilize the stored glycogen. These results are similar to those described for Saccharomyces cerevisiae by Rothman-Denes and Cabib (18), but the C. laurentii cells began to accumulate glycogen considerably earlier. The appearance of appreciable amounts of glycogen in log-phase cells was initially observed by electron microscopy (20). It is striking that endogenous glycogen is utilized even before exogenous glucose is significantly depleted. This will be discussed below.

**Glycogen synthetase activity during growth.** Figure 1B indicates that glycogen synthetase activity, when assayed immediately after disruption of the cells, was almost entirely glucose-6-phosphate-dependent, although a measurable increase in glucose-6-phosphate-independent activity, as well as in the total amount of enzyme (not shown), was observed coincident with the simultaneous rise in glycogen levels. The decrease in glycogen content after 30 hr was accompanied by a simultaneous decrease in total and specific glucose-6-phosphate-dependent and -independent synthetase activity.

**Glycogen phosphorylase activity during growth.** Previous studies (20) have shown that in late log-phase cells 73% of the phosphorylase activity is associated with the bottom layer preparation and that the specific activity of this fraction is 65 times that of the crude extract. The activity of glycogen phosphorylase was also measured in the crude cell extracts and in the bottom layer fraction during growth of the organism. Activities were measured in the direction of glycogen synthesis with saturating concentrations of both glycogen and glucose-1-phosphate present in the assay. Figure 1C illustrates the results obtained in these studies. The specific activity of glycogen phosphorylase in the bottom layer was low in log-phase cells, but increased 10-fold concomitantly with glycogen breakdown and rapid utilization of the exogenous carbon source. Similar results were obtained for the crude extracts. The specific activity in the crude extracts likewise increased when the cells entered the stationary phase, although less dramatically than observed with the bottom layer. Total phosphorylase activity (not shown) was likewise lower in log and early stationary phase. At 68 hr of growth, there is a 38% decrease in total phosphorylase activity as...
Fig. 1. Accumulation and utilization of glycogen by Cryptococcus laurentii during growth. In A, samples were taken at the times indicated, and glucose and glycogen were determined as described in Materials and Methods. In B, the specific activities of glycogen synthetase in the crude extracts were measured in the presence (7.2 mM; O) and absence (□) of glucose-6-phosphate. The specific activity of UDP-glucose pyrophosphorylase in the crude extracts (△) was measured as described in Materials and Methods. In C, the specific activity of glycogen phosphorylase in the bottom layer fraction (O) and in the crude extracts (□) is shown. Phosphorylase activity was measured as described (20), except that 7.3 mM glucose-1-phosphate was employed.

compared with that measured at 33 hr, when the total activity was highest. The specific activity, however, during this period remained essentially constant.

**UDP-glucose pyrophosphorylase activity during growth.** UDP-glucose pyrophosphorylase, as can be seen in Fig. 1B, was highest in the early stages of growth, when glycogen accumulation was greatest, and decreased quite rapidly after late log phase. When late stationary-phase fraction S-2, which did not show demonstrable UDP-glucose pyrophosphorylase activity, was added to active fractions from log-phase cells, no effect on the activity was observed. This indicates that the lack of activity in late stationary-phase preparations is not due to inhibitors of UDP-glucose pyrophosphorylase activity.
Exogenous glucose utilization and production of extracellular polysaccharides. It was surprising to observe that C. laurentii cells reaching stationary phase started breaking down endogenous glycogen, despite the fact that ample glucose was still present in the medium (82% after 30 hr, Fig. 1A), and that this glucose was taken up in an accelerated fashion during stationary growth. (In the next 10 hr, the glucose concentration in the medium decreased to 15% of that originally present.) As can be seen in Fig. 2, this glucose uptake can be quantitatively related to the production of extracellular acidic polysaccharide, which starts to accumulate in the medium once stationary phase is reached.

It has been observed that cells grown at lower pH do not excrete extracellular acidic polysaccharide, but excrete starch instead. In Fig. 2, the production of extracellular polysaccharide is measured by determining the viscosity of the medium of cells originally at pH 3.4 (medium II). These data show only a very slight change in viscosity up to 270 hr of growth of C. laurentii in medium II, which is in sharp contrast to the data obtained with cells grown in medium I. Figure 3 shows the growth curve of cells grown in medium II, the amount of starch found in the medium, and the levels of glucose in the medium. These data reveal that, once the cells reach stationary phase, the glucose content in the medium does not change appreciably and that the starch concentration in the medium does not increase. It should be pointed out that the glucose concentration of medium II originally was 1.2%, whereas medium I contained 2% glucose. However, C. laurentii cells when grown in medium I with 1% glucose behaved essentially the same as those described here, except that the amount of extracellular acidic polysaccharide which accumulated in the medium was correspondingly less (A. Cohen, Ph.D. Thesis, Univ. of Pittsburgh, 1966).

Electron micrographs of C. laurentii cells grown in medium II revealed glycogen-like granules in the cytoplasm of log-phase and of stationary-phase cells, but must less in the latter case. These cells also had no distinct capsule, in contrast to cells grown in medium I. (Electron microscopic observations were made by J. C. Garancis, Department of Pathology, The Medical College of Wisconsin.) The granules from cells grown in medium II were isolated as described in Materials and Methods. The isolated material gave a brown color with iodine with an absorption maximum at 493 nm and a specific absorption for a 0.01% solution (w/v) of 0.26. Both values are similar to those obtained for glycogen of C. laurentii cells grown in medium I (457 nm, 0.24) and for rabbit liver glycogen (492 nm, 0.24), which were simultaneously measured. They are clearly different from the values obtained for cryptococcal starch found in the medium (610 to 630 nm and 0.31, calculated from the Blue Value; see 10). They thus indicate that the granules found inside the cells grown in medium II are not starch granules, but represent glycogen. Our data show that C. laurentii cells, when grown in medium I (neutral pH), continue to take up exogenous glucose in stationary phase, but use this glucose primarily to produce extracellular acidic polysaccharide. In contrast, the same cells grown in medium II (acidic pH) cease to take up exogenous glucose once they reach stationary phase. But, under both growth
conditions, endogenous glycogen is depleted during stationary phase. These differences are undoubtedly due to the difference in pH, both in the medium as well as inside the cells. Cultures grown for 96 hr in medium I showed a pH of 7.5 in the medium, and a pH of 6.2 in the broken cell suspension. Cultures grown for 48 or 120 hr in medium II showed a pH of 2.8 in the medium and of 3.9 to 3.95 in the broken cell suspension. It appears that the low pH caused starch formation during log phase, but prevented its formation and that of extracellular acidic polysaccharide during stationary phase. In contrast, neutral pH impaired starch formation during log and stationary phase, but caused the formation of extracellular acidic polysaccharide during stationary phase. In both media, however, the cells remained viable for a long time and utilized endogenous glycogen. We have no explanation for this peculiar behavior at the present time.

**Distribution of glycogen synthetase.** Various fractions from extracts of cells grown in medium I were assayed for glycogen synthetase. As can be seen in Table 1, the majority of the activity was associated with the 100,000 x g supernatant fraction (S-2). Glycogen synthetase activity in crude extracts was found to be very unstable, and essentially all activity was lost within a few hours of storage at 0 C. In contrast, the glycogen-bound fraction was found to be considerably more stable. Increased stability could be achieved if the glycogen pellet was stored at -20 C as such and suspended in buffer prior to the experiments. Under these conditions, losses of 50 to 75% activity were observed over a period of 24 hr at -20 C. The losses in the glucose-6-phosphate-dependent and -independent activity were nearly identical. It was subsequently found that the glycogen synthetase activity in the 100,000 x g supernatant fraction (S-2) could be almost quantitatively precipitated at 100,000 x g for 30 min in the presence of 40 mg of cryptococcal glycogen/ml. The total activity was actually found to increase two-fold when precipitated with exogenous glycogen, which may indicate that the soluble fractions contain inhibitors of the enzyme.

**Characteristics of glycogen synthetase.** Glycogen synthetase of C. laurentii has thus far not been described. In the following, we present some of the characteristics of the enzyme, employing the glycogen-bound synthetase unless otherwise mentioned. It should be pointed out that what we are describing is essentially the properties of the glucose-6-phosphate-dependent enzyme. We have not been able to demonstrate conclusively the existence of a separate glucose-6-phosphate-independent form, nor have we been able to separate two such forms, as in the case of S. cerevisiae (18) or Neurospora crassa (23).

Of the nucleotide sugars tested, UDP-glucose was the best glucosyl donor. TDP-glucose was approximately 50% as active as UDP-glucose. Glucose-6-phosphate stimulated the incorporation of glucose from UDP-glucose as well as from TDP-glucose to a similar extent. We have consistently observed stimulations of between 4- and 16-fold by glucose-6-phosphate of glucosyl transfer from UDP-glucose to glycogen primer in different enzyme preparations. Although we think that these differences might be due to the interconversion of two forms of the enzyme, we have so far not been able to demonstrate such a process. There was no incorporation of glucose from CDP-glucose and GDP-glucose in the presence or in the absence of glucose-6-phosphate and only slight incorporation from ADP-glucose, which was not stimulated by glucose-6-phosphate.

As shown in Fig. 4, the incorporation of 14C-glucose from UDP-14C-glucose into a glycogen primer varies linearly with the enzyme concentration.

Figure 5 shows the effect of pH on enzyme activity. The highest activity was obtained with glycyglycine buffer at pH 7.5. The activities obtained with glycyglycine buffer were much higher than those obtained with

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**Table 1. Distribution of glycogen synthetase in various fractions of Cryptococcus laurentii cells**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonically disrupted cells</td>
<td>25,330</td>
<td>127</td>
<td>100</td>
</tr>
<tr>
<td>S-1</td>
<td>29,120</td>
<td>173</td>
<td>115</td>
</tr>
<tr>
<td>S-2</td>
<td>21,850</td>
<td>190</td>
<td>88</td>
</tr>
<tr>
<td>Top layer</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Middle layer</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>300</td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td>Glycogen pellet</td>
<td>43,700</td>
<td>1206</td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as nanomoles of [14C]glucose incorporated per 10 min.

*Expressed as nanomoles of [14C]glucose incorporated per 10 min per mg of protein.

*Obtained by addition of cryptococcal glycogen (final concentration, 4%) to fraction S-2 followed by centrifugation at 100,000 x g for 30 min.
The reaction mixtures were as described in Materials and Methods except that the glucose-6-phosphate concentration was 15 mM and the UDP-glucose concentration was 6 μM.

Tris-hydrochloride buffer under the same experimental conditions.

Figure 6 illustrates the linear relationship between time of incubation and glucose incorporation in the absence and presence of glucose-6-phosphate. The reaction is linear for at least 30 min in the presence and absence of glucose-6-phosphate. In this experiment, glucose-6-phosphate stimulated the activity 15-fold.

Figure 7 shows that the concentration of glucose-6-phosphate required for half maximal activity is 2.5 mM.

Addition of glycogen in excess of 13 mg/ml (see Materials and Methods) had no effect on the glucose-6-phosphate-dependent or -independent activity, indicating that the glycogen concentration is saturating. Transfer of 14C-glucose from TDP-14C-glucose and from ADP-14C-glucose likewise was not stimulated by increasing the glycogen concentration.

Figure 8 shows the dependence of the rate of glycogen synthesis on UDP-glucose concentration in the presence of glucose-6-phosphate (16.6 mM). From the reciprocal plot, an apparent Km value of 1.0 mM was calculated.
substrate were obtained from maltose, again as radioactive products obtained (20). These data indicate that the cryptococcal glycosynthetase employed in these studies catalyzes transfer of glucosyl units from UDP-glucose to non-reducing terminals of a glycogen primer, forming new α-1,4-linkages. It should be pointed out that, when the radioactive products obtained with TDP-14C-glucose and ADP-14C-glucose as the substrate were treated with α- or β-amylase, again all of the radioactivity was released as maltose or as glucose and maltose. It therefore appears that similar products are formed from all three sugar nucleotides.

**DISCUSSION**

The data presented here demonstrate that glycogen metabolism in *C. laurentii* proceeds according to the pathway shown in Fig. 9, which is analogous to that reported for mammalian cells (26), *S. cerevisiae* (2, 3, 17), and *N. crassa* (21–24). Since such a sequence of reactions represents a “short circuit,” effective control mechanisms must regulate glycogen synthesis and breakdown in this organism: when glycogen synthesis prevails, phosphor-yllase activity should be turned off, whereas under circumstances where glycogen breakdown is predominant synthetase activity should be curtailed.

Measurements carried out with cell homogenates obtained during various phases of growth of the organism close to neutral pH reveal the following: glycogen starts to accumulate in late log-phase cells and begins to be rapidly utilized at the beginning of the stationary phase. This occurs in spite of the fact that exogenous glucose is still present and is still taken up. Simultaneously, the specific activity of glycogen synthetase in crude cell extracts is highest when the endogenous glycogen concentration peaks and falls off concomitantly with glycogen utilization. Conversely, glycogen phosphorylase reaches its highest specific activity approximately 3 hr after the glycogen peak and remains high until most of the endogenous glycogen is utilized. It is striking that the specific activity of UDP-glucose pyrophosphorylase rapidly falls off at the end of log phase and shows only 60% of its peak activity when the specific activity of glycogen synthetase is maximal. This might be related to the fact that UDP-glucose is also involved in cell wall biosynthesis, a process that slows down when the cells cease to multiply exponentially. Glycogen synthetase

**FIG. 8. Reciprocal plot of velocity versus UDP-glucose concentration in the presence of glucose-6-phosphate.** The assay was as described under Materials and Methods except that the amounts of UDP-glucose were varied as indicated. The reaction mixtures contained 5 μlites of enzyme (5 μg of protein).

**FIG. 9. Pathway of glycogen metabolism in Cryptococcus laurentii.** UDP, uridine diphosphate; UTP, uridine triphosphate; PPI, inorganic pyrophosphate; P, inorganic orthophosphate; glucose-1-P, glucose-1-phosphate.
and UDP-glucose pyrophosphorylase activities approach zero when the exogenous glucose becomes exhausted, whereas glycogen phosphorylase remains relatively constant as long as there is glycogen in the cells.

As shown in Fig. 1B, UDP-glucose pyrophosphorylase activity in crude cell homogenates is always more than an order of magnitude higher than glycogen synthetase. As UDP-glucose is also converted to precursors of other polysaccharides of *C. laurantii* (UDP-glucuronic acid, UDP-xylene [4, 8]), its synthesis is not a step unique in glycogen synthesis, and thus control of UDP-glucose pyrophosphorylase activity at the substrate level would be an unlikely regulating mechanism of glycogen synthesis in *C. laurantii*.

Glycogen synthetase at all levels of growth, when measured in crude homogenates after limited manipulation of the cells at temperatures close to zero, shows profound activation by glucose-6-phosphate, indicating that most of the enzyme is always glucose-6-phosphate dependent. We have, however, found that the degree of activation by glucose-6-phosphate in a given preparation is generally less (4 to 10 times) in the bottom layer than in crude homogenates (10 to 16 times). It should be pointed out that the glycogen synthetase in crude homogenates was assayed approximately 1 hr after harvesting of the cells and approximately 15 min after breaking of the cells, whereas it took more than 24 hr from the time the cells were broken until the bottom layer was assayed for glycogen synthetase activity. The differences in this effect of glucose-6-phosphate could be due to a slow transformation of the enzyme to a different form similar to the D → I transformation in muscle, which has also been shown for other fungi [23] and for yeast [18]. However, in an experiment in which we have assayed crude cell homogenates over a period of time in the presence and in the absence of glucose-6-phosphate, we were unable to show a change in the ratio of both activities for more than 24 hr. The fact that the total activity measured after precipitation in the presence of excess cryptococcal glycogen is twice the activity measured in the crude extract points to the presence of an endogenous inhibitor(s) in crude homogenates. The presence of inhibitory material in crude homogenates may provide a possible explanation for the observed differences in stimulation of glycogen synthetase activity. Rothman and Cabib [16] have shown that the relative stimulation of *S. cerevisiae* glycogen synthetase by glucose-6-phosphate is much larger in the presence of inhibitors. Separation of the enzyme from inhibitors by centrifugation should therefore result in decreased activation by glucose-6-phosphate, which in fact has been observed. From the data in Fig. 1A, it appears that glucose uptake towards the end of log phase accelerates. This fact might lead to an accumulation of glucose-6-phosphate in the cells and thereby increase glycogen synthesis, while at the same time glycogen phosphorylase becomes inhibited by glucose-6-phosphate. Such inhibition has in fact been demonstrated [20].

Electron microscope and chemical evidence reveals that glycogen granules occur also in *C. laurantii* cells grown in acidic medium. The acidic conditions, however, appear to impair capsule and extracellular acidic polysaccharide formation. The relationship of these observations to starch production, however, remains obscure. It is clear that the findings reported here do not provide an explanation for the biosynthetic mechanism by which starch is formed at low pH. Failure of cryptococcal cells to produce starch when grown close to neutral pH might be due to the fact that acidic conditions result in the induction of an entirely new enzyme system. Another possibility might be that during growth at neutral pH the starch-synthesizing system is inhibited by some metabolite(s) that is not present at effective concentrations during growth at low pH. It appears certain, however, that *C. laurantii* cells grown in acidic medium are able to synthesize glycogen. Extracellular starch production, therefore, cannot be the consequence of an impairment of glycogen synthesis during growth at acidic pH.

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**LITERATURE CITED**