

# Carbohydrate Accumulation During the Sporulation of Yeast

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The sporulation of *Saccharomyces cerevisiae* is characterized by an increase in dry weight without cell division. At least 67% of the dry weight increase is due to the synthesis of cellular carbohydrates consisting of trehalose and insoluble components. The insoluble carbohydrates accumulate only during the period preceding the actual formation of visible ascospores. The trehalose accumulates throughout the sporulation cycle and is specifically localized in the ascospore.

Sporulation in yeast offers a model system for examining the biochemical events accompanying cellular development in a eukaryotic organism. In yeast, sporulation is initiated by placing a washed suspension of vegetative cells into a simple potassium acetate medium (14). Under these conditions, cell division stops abruptly and is followed by an initial "latent" period of little outward morphological change except for a gradual increase in size (4, 5). Internally, however, two meiotic divisions are taking place. The latent period ends with the actual process of free spore formation, during which the population of cells are converted into asci containing up to four ascospores (8).

A number of the biochemical and physiological changes accompanying the sporulation cycle have recently been resolved (6, 8). One of the most dramatic quantitative changes was the demonstration by Croes (4) of a large increase in dry weight during the early stages of sporulation. However, the identity of the material(s) responsible for this increase in weight was not examined.

The purpose of this work was to determine the compounds responsible for the increase in dry weight during the sporulation of yeast.

## MATERIALS AND METHODS

*Saccharomyces cerevisiae* strain Z113 was maintained and grown on media previously described (14). For this study, cells were cultivated in 500 ml of pre-sporulation medium in 2-liter Erlenmeyer flasks. Sporulation was carried out in 500-ml flasks containing washed cells suspended in 100 ml of 1% potassium acetate (14). At intervals, a flask was removed from the shaker, and samples were taken for the determination of dry weight, cell count, optical density at 600 nm, and the percentage of asci (14). The remainder of the culture was collected by centrifugation, washed

with distilled water, and frozen for subsequent carbohydrate analysis.

**Dry weight determinations.** Duplicate samples of  $2 \times 10^8$  cells were collected on individual preweighed, 47-mm membrane filters (Millipore Corp., 0.45  $\mu$ m pore size, N°HAWP) and washed with 100 ml of cold water. Control filters containing no cells, but washed with water, were run in each experiment. The filters were dried at 80 C to constant weight.

**Analysis for total carbohydrates.** The procedure for the analysis of total carbohydrates was adapted from that developed by Trevelyan and Harrison (12, 20-22) for vegetative yeast. The thawed cell pellets were brought to 12 ml with 0.5 M trichloroacetic acid and were incubated with mild agitation at room temperature for 40 min. These suspensions were then analyzed for total carbohydrates by the standard heat-of-mixing anthrone procedure (9, 22). Absorbancy was determined with a Klett colorimeter by using a 540-nm filter. Figure 1 shows the results of a typical analysis for total carbohydrate with cells harvested at 0, 4, and 24 hr after being placed in sporulation media. With each extract (Fig. 1), the absorbancy was proportional to the volume of suspension assayed. When mixtures were prepared between extracts showing high anthrone reactivity (24 hr) and those showing low anthrone reactivity (0 hr), the absorbancies obtained were exactly the sum expected from the individual extracts. This indicated that the values obtained with the individual extracts were not affected by detectable inhibitors or activators of the anthrone reaction.

**Carbohydrate fractionation.** The cells were fractionated into trichloroacetic acid-soluble (trehalose) and -insoluble fractions by the procedure of Trevelyan and Harrison (12, 21, 22). The samples, already in 0.5 M trichloroacetic acid, were centrifuged and the supernatant fluid was removed. The cells were extracted two additional times with trichloroacetic acid, as above, and the supernatant fluids of the three extractions were pooled. The trichloroacetic acid-soluble carbohydrate (trehalose) was determined in this fraction. A fourth extraction with trichloroacetic acid con-

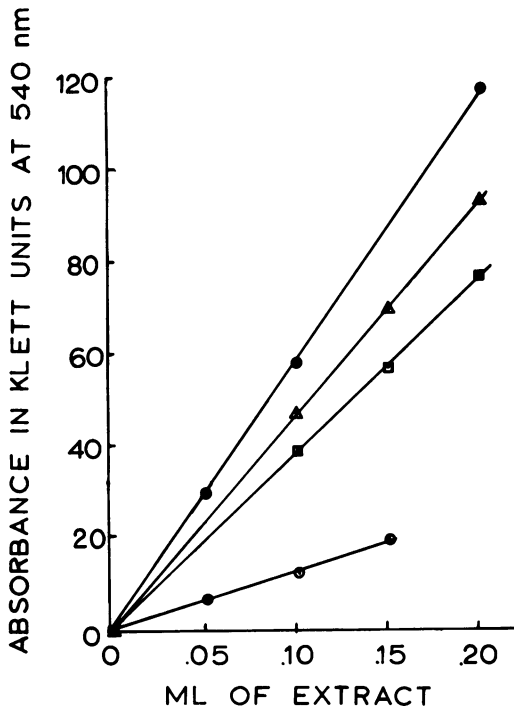


FIG. 1. Anthrone reactivity of extracts prepared from yeast harvested during the sporulation cycle. Trichloroacetic acid extracts were prepared from cells harvested immediately after suspension ( $\odot$ ) and then 4 ( $\square$ ) and 24 ( $\triangle$ ) hr after suspension in 1% potassium acetate sporulation media. The carbohydrate content of each was then determined by analyzing increasing amounts of the extract by the anthrone procedure. A glucose solution (500  $\mu$ g of glucose/ml in 0.5 M trichloroacetic acid) was analyzed for comparison ( $\bullet$ ). Each point is the average of duplicate determinations.

tained negligible anthrone-positive material. The extracted cell pellets were resuspended in 0.5 M trichloroacetic acid for analysis of the insoluble fractions.

Traveyan and Harrison (20-22) and others (1, 12) have shown that trehalose is the sole carbohydrate in the trichloroacetic acid-soluble fraction of vegetative yeast. The presence of trehalose as the only detectable carbohydrate in the trichloroacetic acid-soluble fraction of sporulating cells and isolated ascospores was confirmed by chromatography and hydrolysis as described (1, 15, 21).

The carbohydrate content of both the soluble and insoluble fractions was determined with anthrone as above.

**Preparation and analysis of free ascospores.** Washed, unfrozen asci ( $10^8$  to  $2 \times 10^9$ ; 31 hr in sporulation media) were digested in 1 ml of 10% "Glusulase" (Endo Laboratories, Garden City, N.Y.) for 30 to 40 minutes at 22 C with mild agitation. The suspension was then diluted to 10 ml with ice-cold water and passed through a cold French pressure cell at 10,000 psi. This treatment yielded a suspension of single ascospores free from vegetative cells and intact asci. The

concentration of spores was determined with a hemocytometer, and the spores were then harvested by centrifugation and washed with distilled water. The resulting washed spores were analyzed for trehalose as above.

## RESULTS

The kinetics of sporulation of *S. cerevisiae* strain Z113 in acetate sporulation media are shown in Fig. 2. Cells were harvested from cultures in exponential growth, washed once, and suspended in 1% potassium acetate solution. Cell counts and the percentage of asci were determined by phase-contrast microscopy during the first 40 hr in sporulation media (14). The cell number remained constant throughout the 40-hr period of observation. No asci were detectable until 11 hrs, after which time asci appeared rapidly for the next 12 hr and then more slowly thereafter. By 23 hr, there were about 60% asci, and, by 40 hr, there were about 75% asci. Although the cell number remained constant during the sporulation cycle, the optical density of the cultures increased 57% over the first 10 hr (Fig. 2) and then remained constant. This indicated an increase in mass by the cells.

Croes (4, 5), employing a different strain of yeast, observed an increase in both dry weight and size of the cells during sporulation. As the turbidity had indicated, the dry weight of strain

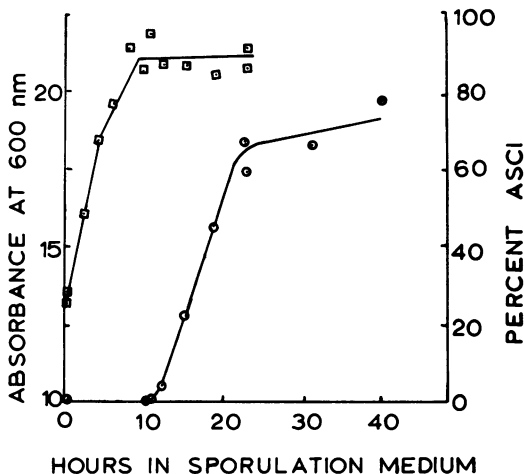


FIG. 2. Absorbance changes during sporulation of *Saccharomyces cerevisiae* strain Z113. Washed cells were placed in 1% potassium acetate solution (sporulation media) to initiate the sporulation cycle. At intervals, samples were withdrawn for the microscopic determination of the percentage of asci ( $\odot$ ) and the absorbance at 600 nm ( $\square$ ). For each point, the absorbance was determined in duplicate, averaged, and corrected so as to correspond to the equivalent of  $10^8$  cells/ml. At least 500 cells, buds, or asci were examined for each point of the sporulation curve.

Z113 also increased during the first 10 hr in sporulation media (Fig. 3).

A number of observations with vegetative yeast (1, 11, 21) have shown that placing a suspension of glucose-grown cells in nitrogen-free, glucose-containing media resulted in a rapid increase in cellular carbohydrates, among which was the disaccharide trehalose. Whether an analogous increase in carbohydrates occurred when acetate-grown cells were resuspended in the nitrogen-free acetate sporulation media was examined; the results are shown in the lower curve of Fig. 3. Total carbohydrates increased rapidly during the first 10 hr of incubation of the cells in sporulation media. Using Fig. 3 and comparing the increase in dry weight (2.55 mg per  $10^8$  cells) with the increase in total carbohydrate (1.7 mg per  $10^8$  cells) showed that 67% of the increase in dry weight could be accounted for as anthrone-reactive carbohydrate. Total carbohydrates, which accounted for 22% of the dry weight of vegetative cells, accounted for 43% of the dry weight in the sporulated cells.

Trehalose, a constituent of the yeast carbohydrates (17), has been observed in both vegetative cells and asci (12). It is known to accumulate in the spores of a number of other fungal species (2, 18), including another ascomycete, *Neurospora* (19). Trevelyan and Harrison (21, 22) have developed a procedure to extract selectively and to measure trehalose from yeast. Whole cells are fractionated with trichloroacetic acid into soluble and insoluble fractions; the soluble fraction contains trehalose, whereas the insoluble fraction contains components of the cell wall and glycogen. The carbohydrate content of each fraction is then determined with the anthrone reagent. The results of such a procedure applied to samples of cells harvested throughout the sporulation cycle are shown in Fig. 4. There was an increase in both the trehalose and insoluble carbohydrate fractions starting as soon as the cells were placed in sporulation media. Since the number of cells remained constant, the average trehalose content of the cells increased, in 31 hr, from 0.034 mg per  $10^8$  cells to 0.610 mg per  $10^8$  cells, an 18-fold increase in trehalose. The insoluble carbohydrates increased from 0.455 mg per  $10^8$  cells to 1.62 mg per  $10^8$  cells, or a 3.6-fold increase. Trehalose, which accounted for about 7% of the total carbohydrates of the vegetative cells, represented 27% of the total carbohydrates in the sporulated culture.

Between 10 and 11 hr after cells were placed in sporulation medium, the increase in both dry weight and total carbohydrate ceased rather abruptly (Fig. 3). As indicated in Fig. 4, the cessation in total carbohydrate accumulation re-

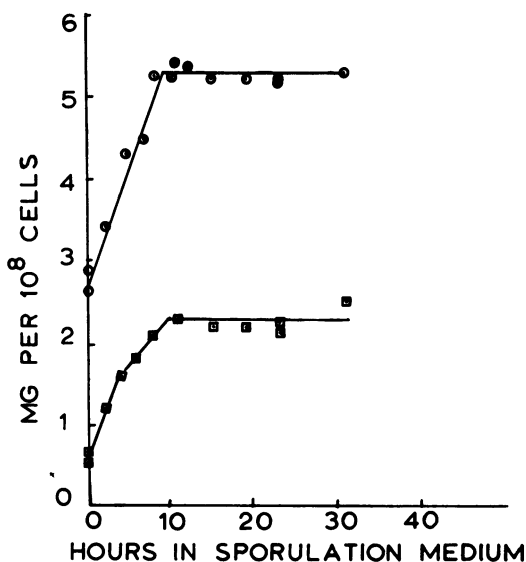


FIG. 3. Increase in dry weight and total carbohydrate during the sporulation cycle. At intervals during the sporulation cycle, samples of cells were collected for the subsequent determination of both dry weight ( $\odot$ ) and total carbohydrate ( $\square$ ). Each point is the average of duplicate determinations. The values were corrected so as to correspond to  $10^8$  cells for both sets of data. Carbohydrates are expressed as milligrams of glucose determined with the anthrone procedure.

sulted mainly from a stop in the synthesis of the insoluble fraction. Therefore, these results show that there was no increase in either the dry weight or the insoluble fraction during the period when visible ascospore cell walls were appearing (this began at 11 hr). In contrast, trehalose synthesis continued at a slow but measurable rate during spore formation (Fig. 4).

Sporulation did not reach 100% (Fig. 2). As Croes observed (4), the majority of these non-sporulating cells were small buds attached to sporulated mother cells. The question arose as to whether the trehalose accumulated during sporulation was localized within the asci or in the residual unsporulated buds. In addition, it was conceivable that the trehalose may have been localized in the ascus but situated external to the ascospores themselves (i.e., between the ascospores and the mother cell wall). These questions were resolved by isolating free ascospores from the asci, washing them, and analyzing them for trehalose. Free washed ascospores were found to contain 0.151 mg of trehalose per  $10^8$  spores. This is about one quarter of the content of trehalose in  $10^8$  cells, but each ascus contains, on the average, four such spores.

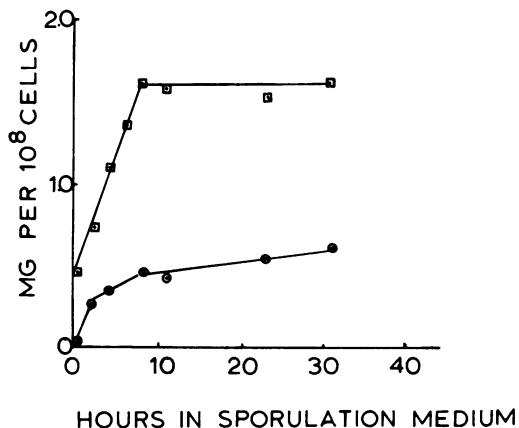


FIG. 4. Accumulation of trehalose and insoluble carbohydrate during the sporulation cycle. At intervals during the sporulation cycle, samples of cells were harvested and later fractionated into trehalose (○) and insoluble carbohydrate (□) fractions. The carbohydrate content of each fraction was then determined with the anthrone procedure. Each point is the average of duplicate determinations. The values are expressed as milligrams of glucose per  $10^8$  cells.

## DISCUSSION

The sporulation of *S. cerevisiae* is characterized by an increase in dry weight in the absence of cell division. Much of this increase is due to the formation of cellular carbohydrates consisting of both trehalose and insoluble components. The accumulated trehalose is localized in the ascospores.

Approximately 67% of this increase in dry weight during sporulation was accounted for as anthrone-reactive carbohydrate. This probably represents a minimum figure, since certain yeast carbohydrates yield substantially smaller amounts of anthrone color per unit weight than glucose. [For example, mannose, the primary constituent of mannan, gives only 55% of the color of an equivalent quantity of glucose (22), whereas glucosamine, a component of chitin, shows no reaction with the typical anthrone reagents (3).] The synthesis of carbohydrates probably accounts for a large fraction of the acetate which is assimilated during sporulation (7).

The presence of trehalose in spores has been observed in *Neurospora* and a number of other fungi (2, 18). Where it has been examined (18), trehalose served as a source of carbon and energy for spore germination. Possibly it serves the same role in yeast spores.

A number of authors (1, 11, 21) have examined trehalose metabolism in vegetative yeast and have proposed a mechanism to explain rapid trehalose accumulation. Trevelyan and Harrison (21)

showed that trehalose was synthesized only in the absence of an assimilable nitrogen source. Panek (11) later observed that trehalose synthesis could take place in the presence of usable nitrogen provided that no cell division or growth occurred. In both cases, an utilizable carbon source was present. On this basis, it was proposed (11) that there is competition between the synthesis of amino acids and trehalose for a common intermediate metabolite, perhaps glucose-6-phosphate. In the absence of appreciable amino acid synthesis, trehalose is rapidly formed from the intermediate. The accumulation of trehalose reported here, under experimental conditions leading to sporulation (i.e., absence of both growth and usable nitrogen), is therefore in agreement with the previous observations concerning trehalose formation under nonsporulating conditions.

The components of the trichloroacetic acid-insoluble fraction of vegetative yeast have been identified as glycogen and the cell wall polysaccharides mannan and glucan (10, 12, 22). Chitin is also present in this fraction but does not react with the anthrone reagent (3) and consequently was not measured in this study.

Little is known about the composition of the ascospore cell wall, except that spores have different surface properties than vegetative cells (they are extremely hydrophobic; 16). In this study, the formation of microscopically detectable ascospore cell walls began 11 hr after the cells were placed in sporulation medium. It was surprising, therefore, that there was no observed increase in either the dry weight or the insoluble carbohydrate fraction during the period of visible ascus wall formation. This suggests that there was conversion of some previously formed, insoluble carbohydrate(s) into the visible walls.

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