Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4351–4355, October 1977
Biochemistry

Developmental defects associated with glucosamine auxotrophy in *Saccharomyces cerevisiae*

(mitosis/meiosis/glucan/mannoprotein/chitin)

CLINTON E. BALLOU, SHYAMAL K. MAITRA, JEFFERY W. WALKER, AND WILLIAM L. WHELAN

Department of Biochemistry, University of California, Berkeley, California 94720

Contributed by Clinton E. Ballou, August 4, 1977

ABSTRACT *Saccharomyces cerevisiae* mutants, unable to make D-glucosamine owing to a defect in the enzyme 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring) (EC 5.3.1.19), show aberrations both in sporulation and in vegetative growth. They grow normally on a medium of yeast extract, peptone, and dextrose (YEPD) containing D-glucosamine (1 mg/ml), and such cells accumulate 4 to 5 times the amount of D-glucosamine present in wild-type cells cultured on YEPD alone. When such mutant cells are shifted to YEPD alone, they continue to increase in cell mass for about 10 hr (three to four cell cycles) and produce strings of beads in which the cells fail to separate. Although each of the "cells" contains a nucleus, electron micrographs of thin sections reveal that septation is defective apparently owing to the inability to synthesize chitin, which forms the primary septum in *S. cerevisiae*. The viability of such cultures drops rapidly after 3–5 hr, a fact attributable to lysis of the cells through wall defects in the septum region where gross disorganization is apparent. When the mutant cells grown on YEPD plus D-glucosamine are transferred to sporulation medium (1% potassium acetate), they proceed through meiosis to produce viable spores that appear to be altered only in the nature of the spore wall. The spores lack a dark-staining surface layer that is visible in thin sections prepared from wild-type cells, they are notably less hydrophobic than wild-type spores, and they are digested and lysed by glucanases that do not affect normal spores. All of these properties suggest that D-glucosamine is required for spore maturation and is used to synthesize a glucanase-resistant hydrophobic surface layer on the primary glucan spore wall. In agreement with this postulate, D-glucosamine synthesis and the activity of the isomerase do not appear until late in meiosis when tetranucleate cells are abundantly present in the sporulation culture.

Many mutants of the yeast *Saccharomyces cerevisiae* are known that show defects in cell division (1) or sporulation (2), but rarely has the biochemical lesion been identified. A recent report from this laboratory (3) described the isolation of *S. cerevisiae* mutants that were unable to synthesize D-glucosamine owing to a defective enzyme 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring) (EC 5.3.1.19). Such D-glucosamine auxotrophs proceed successfully through meiosis but they produce defective ascospore walls. In that report (3) it was stated "we expect that cells unable to synthesize D-glucosamine would grow in its absence with the formation of a colony of 'clumpy cells.' This was not observed." In fact, what we have observed, and document in this report, is that the D-glucosamine auxotroph does grow for several cycles to produce undivided strings of two to eight cells in which defective septation prevents cell separation. Nuclear division also occurs, but the cells lose viability and can no longer be rescued by the addition of D-glucosamine after about 20 hr on the deficient medium.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

We have developed procedures for isolation of the mutant spores and demonstrate directly that they are susceptible to digestion by β-glucanases. Further investigation of sporulation in the wild-type *S. cerevisiae* strain reveals that the activity of the isomerase and the synthesis of D-glucosamine do not appear until late in meiosis when tetranucleate cells make up a significant fraction of the population. Thus, this enzyme activity serves as a landmark for the onset of spore maturation.

MATERIAL AND METHODS

Materials. *S. cerevisiae* diploid strains of the following genotypes were derived from strain X2180: XW290 (*a/α,lys2-1/lys2-2*) and XW285 (*a/α,lys2-1/lys2-2,ade2/+,gcn1-1/gcn1-1*). The latter gave only about 25% asci on sporulation. To obtain a strain with a higher frequency of sporulation, we crossed the *gcn1-1* mutation into the homothallic *S. cerevisiae* Y55 strain, and a homothallic recombinant was recovered. This strain (XW471-2D) had the genotype *a/α,gcn1-1/gcn1-1*, and it gave up to 60% asci.

Culture media were prepared from Bacto-Yeast Extract, Bacto-Peptone, and Bacto-Agar, all obtained from Difco, whereas D-glucosamine·HCl came from Sigma and dextrose (D-glucose) was reagent grade. Media were prepared as described previously (3). YEPD contains 1% yeast extract, 2% peptone, and 2% D-glucose.

General Methods. Carbohydrate was determined with a phenol/sulfuric reagent (4) and protein by the Lowry method (5). D-Glucosamine and the activity of the isomerase, which produces D-glucosamine 6-phosphate, were assayed as described (3) by a modified Morgan–Elson procedure according to Ghosh *et al.* (6). Total glucosamine was estimated by removing samples, collecting the cells by centrifugation, washing them in distilled water, and either counting them or drying them to constant weight and hydrolyzing them in 6 M HCl in a sealed tube at 110° for 6 hr. The acid was removed in a desiccator over NaOH, and the glucosamine content of the dry residue was determined. For isomerase assays, the cells were collected by centrifugation and resuspended in an equal volume of buffer (15 mM phosphate, pH 7, containing 2.5 mM EDTA); 0.8 ml of the suspension was broken in a Braun homogenizer with 1.25 g of Microbeads (Class V-A) (Cataphote Division, Ferro Corp.). Three cycles of 2 min each were required, using a microcell of 2.2 ml volume (10-mm Pyrex combustion tube) in a special aluminum adapter that was cooled by CO_2 gas. The supernatant liquid was removed and centrifuged, and the clear extract was assayed for isomerase activity in a 30-min incubation at 30° (3, 6).

Light and fluorescence microscopy were done on a Zeiss

Abbreviations: YEPD, 1% yeast extract/2% peptone/2% D-glucose; isomerase, 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring) (EC 5.3.1.19).

FIG. 1. Formation of "strings of beads" by an *S. cerevisiae* glucosamine auxotroph grown on YEPD without glucosamine. (*Upper*) Photograph was taken by differential interference contrast at a magnification of about 700-fold; (*Lower*) fluorescence photograph of the same cells stained with mithramycin to reveal the nuclei.

Photomicroscope III with a Planapo 63 objective. Specimens were mounted in glycerol for viewing and for photography, which was done with Kodak S0-410 black-and-white film (ASA 160). Nuclei were stained with Gurr Giemsa stain or, preferably, with mithramycin (7), because the strong alkali treatment of the Giemsa procedure extensively disrupted cells of the glucosamine auxotroph grown in the absence of the hexosamine. Although multinucleate cells and mature asci both show up to four stainable nuclei, the latter are clearly distinguishable owing to the refractile nature of the wild-type ascospores. Electron microscopy was performed as described (3).

For sporulation, cells from logarithmic phase cultures on YEPD or YEPD plus D-glucosamine were washed in distilled water and resuspended at a density of 2 to 5 × 10^{10} cells per ml in 1% potassium acetate solution; the suspensions were shaken gently at room temperature (25°). Addition of D-glucosamine to the sporulation medium in amounts from 0.1 to 1 mg/ml at various times failed to promote normal sporulation and led instead to a loss in viability of both mutant and wild-type cultures.

To isolate mutant spores, we partially digested the isolated and washed asci with Zymolyase 5000 (Kirin Brewing Co., Takasaki, Japan), 5 mg/ml in 66 mM potassium phosphate buffer (pH 7), for 15 min at 30°, and the asci and spores were collected by centrifugation. The material was resuspended in aqueous 1% Triton X-100 and sonicated gently to disrupt intact asci; the released spores were washed by repeated centrifugation. Finally, the spore suspension, 10^6 per ml, was layered on a step gradient of 20, 40, and 60% sucrose and centrifuged in a clinical centrifuge at full speed for 4 min. The spores collected on the 60% shelf; after recovery they were washed with water. The same procedure may be used to isolate wild-type spores, but the conditions of Zymolyase digestion need not be controlled because the spores are resistant to attack by the enzyme.

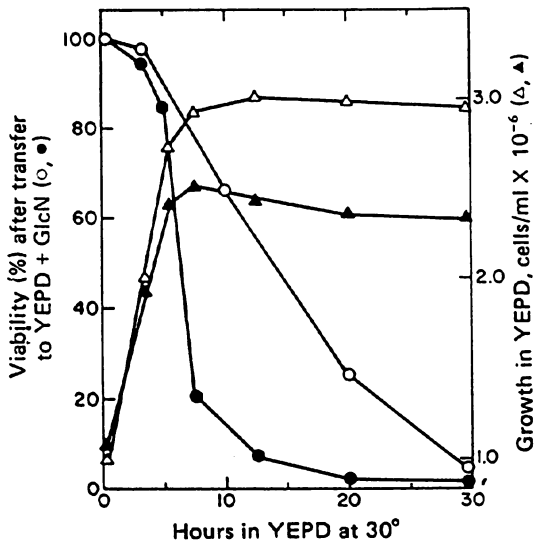

FIG. 2. Growth and viability of the *S. cerevisiae* glucosamine auxotroph. The cells of strain XW285 were cultured in YEPD with 0.6 M KCl (open symbols) and without KCl (closed symbols), and the increase in cell number was followed (△, ▲). At each point, samples were removed and spread on YEPD-agar plates containing 1 mg of D-glucosamine per ml and 0.6 M KCl to evaluate the percent of viable cells remaining in the culture (○, ●).

RESULTS

S. cerevisiae glucosamine auxotrophs form strings of beads and lose viability

The homozygous *S. cerevisiae* glucosamine auxotroph, strain XW285, grows normally on YEPD medium supplemented with D-glucosamine with a doubling time of about 2 hr, but when the cells are harvested and resuspended in YEPD without the hexosamine, they develop the aberrant morphology shown in Fig. 1 *upper*. That nuclear division has accompanied cell division is demonstrated by mithramycin staining (Fig. 1 *lower*).

Viable cells can be recovered from such cultures for an extended period of time if subcultured onto a glucosamine-containing medium (Fig. 2). That the length of this period is increased if an osmotic stabilizer is added to the medium suggests that cell death is due in part to cell lysis. These results demonstrate that glucosamine deficiency does not stop growth, but rather it leads to a condition of "glucosamineless death."

S. cerevisiae glucosamine auxotrophs are defective in septation

Electron micrographs of thin sections prepared from strain XW285 grown on YEPD without glucosamine show several unusual features (Fig. 3). Most notable is the progressive disorganization of the region between the cells where septation would normally occur. Because the mutant cells, when grown on YEPD containing glucosamine, accumulate much more of the hexosamine than is present in wild-type cells grown on YEPD alone (Table 1), we assume that the first one or two cell cycles may exploit this pool and lay down a more or less normal septum. However, most strings of beads show at least one highly disorganized septum region (Fig. 3), and we expect that cell lysis may occur through this part of the wall.

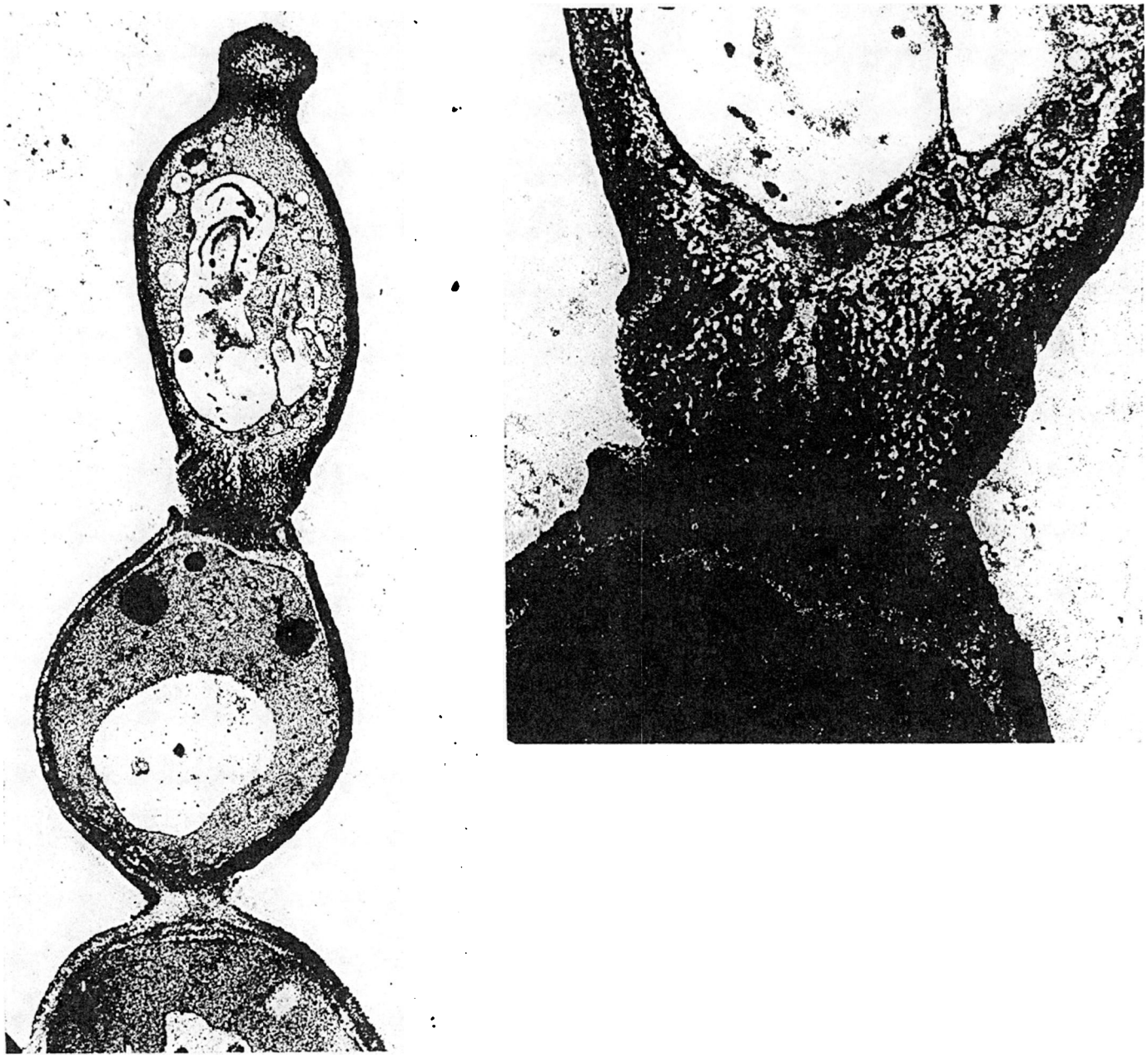

FIG. 3. Electron micrographs of thin sections of beads, such as shown in Fig. 1, obtained by culturing *S. cerevisiae* XW285 on YEPD without glucosamine. (*Left*) Portions of a string of three cells enlarged 16,200-foid; (*Right*) a 54,000-fold enlargement of the top septum in the left photograph.

This figure illustrates one other unusual feature, namely, that most of the internal membranous organelles of the group of cells are accumulated in the cell that appears to represent the last bud to form. This suggests that as each new bud forms there is a directional and unequal migration of these organelles. This could mean that the glucosamine deficiency prevents duplication of some of the organelles and that the available organelles migrate into the new bud in preference to re with the mother cell.

Glucosamine auxotrophy does not prevent meiosis

We have shown previously that *S. cerevisiae* strain XW285, when placed in a medium of 1% potassium acetate, will sporulate to the extent of about 25% as assessed by Giemsa staining (3). That meiosis is essentially normal is confirmed by the formation of the expected recombinant haploid nuclei, but an obvious abnormality is apparent in that the asci lack their usual refractility and can be easily visualized only after staining. In contrast, the homothallic glucosamine-requiring strain, XW471-2D, produces asci with a frequency (0.6) approaching that of the wild-type Y55 strain (about 0.8). The asci are clearly distinguishable by light microscopy, although electron micrographs of thin sections still show that their spores lack the dark-staining surface layer of the wild-type asci (see figure 5 of ref. 3).

A clear distinction exists between the mutant and wild-type spores in their resistance to digestion by β-glucanases (Zymolyase). As illustrated in Fig. 4, treatment of both wild-type and mutant asci releases the spores; but, after an initial increase in colony count, the viability of the mutant spore culture decreases. If digestion of the mutant asci is terminated at the appropriate time, the spores can be recovered in a viable state. Such spores are notably less "sticky" than the wild-type spore and can be purified by differential centrifugation. If again subjected to Zymolyase digestion under the conditions in *Material and*

Table 1. D-Glucosamine (GlcN) content and sporulation frequency of cells grown under different conditions

Yeast strain and conditions	D-Glucosamine content μmol/10^{10} cells Total*	Free†	Bound*	Sporulation frequency
Y55				
Grown on YEPD	5.1	0.3	4.8	0.80
Grown on YEPD + 1 mg/ml GlcN	24.0	9.4	14.4	0.75
XW471-2D				
Grown on YEPD + 1 mg/ml GlcN	23.7	9.2	15.6	0.60
Grown on YEPD + 0.5 mg/ml GlcN	12.9	5.4	8.2	0.35
Grown on YEPD + 0.25 mg/ml ClcN	12.2	3.4	8.2	0.30
XW285				
Grown on YEPD + 1 mg/ml GlcN	28.0	—	—	0.25

* Assayable as glucosamine after hydrolysis in 6 M HCl at 110° for 6 hr. The values are corrected for a 20% destruction of glucosamine during hydrolysis.

† Assayable as glucosamine in a water extract of the cells obtained by heating at 110° for 2 hr. This value is quite variable owing to the leaching of glucosamine during washing of the cells.

Methods, they lose viability with a half-time of about 30 min.

Glucosamine synthesis is a late landmark in meiosis

That the *S. cerevisiae* glucosamine auxotroph can proceed through meiosis, with the only apparent defect being in spore wall synthesis, suggests that synthesis of this hexosamine may not occur until late in the process. This was found to be true, as illustrated in Fig. 5. The cells of *S. cerevisiae* strain Y55 have a relatively low total D-glucosamine content when grown on YEPD, which probably represents mainly the chitin content of the bud scars (8). Multinucleate cells first appear at about 8–10 hr and increase to a frequency of about 0.25 of the sporulation culture. Mature asci begin to appear at about 10–12 hr and reach a frequency of 0.6–0.8. D-Glucosamine synthesis occurs at a very low rate, if at all, until tetranucleate cells are abundant in the culture, and it then parallels the formation of

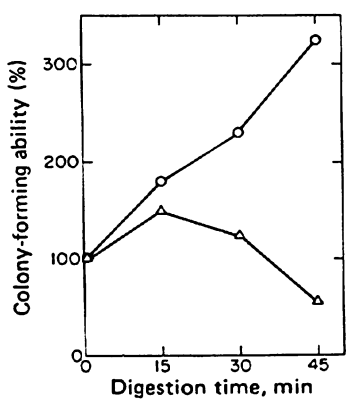

FIG. 4. Resistance of spores from *S. cerevisiae* strain Y55 and the mutant XW471 to digestion by Zymolyase. At various times, samples were removed, sonicated lightly to break the digested asci apart and release the spores, and then spread on YEPD-agar plates containing glucosamine to evaluate colony-forming ability. Since each cell has the potential to produce four spores, the maximum increase in colony-forming ability is 400%.

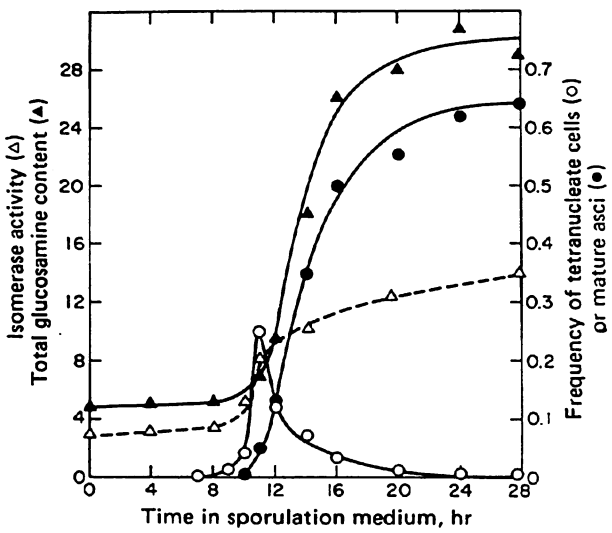

FIG. 5. Time course of glucosamine synthesis during sporulation. The curves show the frequency of immature tetranucleate cells (○) and of mature asci (●), the isomerase activity in μmol/hr per 10^{10} cells (△), and the total glucosamine content in μmol/10^{10} cells (▲).

mature asci until meiosis is completed, at which time the total glucosamine content of the culture has increased about 7-fold. The increasing activity of the isomerase almost directly parallels the formation of glucosamine, although it may precede the latter curve by a short interval.

A similar analysis of the glucosamine auxotroph XW471-2D demonstrated that glucosamine synthesis does not occur throughout meiosis, but the fact emerges that such cells, which must first be grown on YEPD containing D-glucosamine before they are placed in sporulation medium, accumulate much higher levels of the hexosamine than do the wild-type cells grown on YEPD alone (Table 1). Although reducing the level of D-glucosamine in the presporulation growth medium reduces this accumulation, it also significantly reduces the fraction of cells that is able to produce asci. We have attempted to deplete the glucosamine pool in such cells by starving them on YEPD alone before placing them in sporulation medium, but the cells continue to grow mitotically and produce the "beads" described earlier in this report, and such beads sporulate very poorly.

DISCUSSION

From what is known of the composition of *S. cerevisiae*, D-glucosamine is required at least for synthesis of chitin (9) and the core structure of glycoproteins, including the cell wall mannoproteins (10). The bulk of chitin synthesis during mitotic growth occurs in a stepwise manner closely correlated with septation and cell separation (11, 12), but a low level of synthesis must occur throughout the cell cycle to accommodate the needs for glycoprotein synthesis, assuming that these macromolecules are made continuously. Our study of the D-glucosamine auxotroph suggests that the cells give first priority to glycoprotein synthesis, because they continue to lay down cell wall and form unseparated beads that are clearly defective in septation. We conclude that chitin synthesis is the first process to suffer, and that any available D-glucosamine is used instead to make cell wall mannoproteins and other metabolically important macromolecules. The morphological defects we observe are similar to those obtained when *S. cerevisiae* is grown in the presence of Polyoxin D (12), an inhibitor of chitin synthetase.

A similar interpretation may be given to the results of our investigations of the effects of glucosamine auxotrophy on

meiosis. Again glucosamine deficiency does not prevent meiosis, and it is probable that the cells are able to obtain enough of the hexosamine from internal pools or from the degradation of cellular materials to allow for synthesis of vital components in the early steps of meiosis. However, they do fail to carry out a late stage of meiosis that uses by far the majority of the D-glucosamine made during sporulation, a process that we conclude involves the deposition of a glucosamine-containing surface layer on the spore and is the final step in spore maturation. This surface layer gives the spore its characteristic hydrophobic property and protects it from the action of external β-glucanases. Thus, although it is probably important for the long-term maintenance of spore viability, the surface layer is not essential and appears to be given low priority under conditions of glucosamine deprivation. A glucosamine auxotroph of *Bacillus subtilis* shows a similar sporulation defect in that endospores are formed that lack the normal cortex layer (13). The spores show a reduced resistance to environmental stress, and normal sporulation cannot be obtained by adding glucosamine to the sporulation medium.

We cannot say at present whether some D-glucosamine is essential for early steps in meiosis because the mutant cells accumulate abnormally large amounts of unbound hexosamine when grown in its presence, and this pool would be more than adequate for mannoprotein synthesis. If such cells are starved for glucosamine before they are placed in sporulation medium, they form aberrant beads that sporulate poorly, and we do not know whether the result is a consequence of glucosamine deficiency or unbalanced growth. Presumably this question can be settled by a study of conditional mutants, but it would be surprising if some glucosamine-containing molecules were not essential for early meiotic processes.

The composition of the *S. cerevisiae* spore coat is only poorly defined, although Kane and Roth (14) have provided tentative evidence that the spore contains mannan and glucan. As reviewed by Haber and Halvorson (15), there is other evidence that the surface layer of the spore may contain lipid and/or protein. Our study suggests that the surface layer requires D-glucosamine for its synthesis, which could implicate chitin, a glucosamine-containing lipid, or a glycoprotein. That the mutant spores are readily digested by β-glucanases provides strong confirmation that the bulk of the wall is composed of β-glucan. Whether this glucan is similar to that of the vegetative cell wall glucan (16) is still to be determined. The observation that the mutant spores are susceptible to lysis by β-glucanases suggests that they might be a ready source of undegraded spore DNA because the spore wall can be removed and the cells lysed under very mild conditions.

We thank Ms. Alice Taylor for assistance in preparation of the electron micrographs. This work was supported by National Science Foundation Grant BMS74-18893 and U.S. Public Health Service Grant AI-12522.

1. Hartwell, L. (1974) *Bacteriol. Rev.* **38**, 164–198.
2. Moens, P. B., Esposito, R. E. & Esposito, M. S. (1974) *Exp. Cell Res.* **83**, 166–174.
3. Whelan, W. L. & Ballou, C. E. (1975) *J. Bacteriol.* **124**, 1545–1557.
4. Dubois, M., Gilles, K. A., Hamilton, J. K. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356.
5. Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
6. Ghosh, S., Blumenthal, H. J., Davidson, E. & Roseman, S. (1960) *J. Biol. Chem.* **235**, 1265–1273.
7. Slater, M. L. (1976) *J. Bacteriol.* **126**, 1339–1341.
8. Cabib, E. & Bowers, B. (1971) *J. Biol. Chem.* **246**, 152–159.
9. Keller, F. A. & Cabib, E. (1971) *J. Biol. Chem.* **246**, 160–166.
10. Nakajima, T. & Ballou, C. E. (1974) *J. Biol. Chem.* **249**, 7685–7694.
11. Cabib, E. & Farkas, V. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2052–2056.
12. Cabib, E., Ulane, R. & Bowers, B. (1974) *Curr. Top. Cell. Regul.* **8**, 1–32.
13. Freese, E. B., Cole, R. M., Klofat, W. & Freese, E. (1970) *J. Bacteriol.* **101**, 1046–1062.
14. Kane, S. M. & Roth, R. (1974) *J. Bacteriol.* **118**, 8–14.
15. Haber, J. E. & Halvorson, H. O. (1975) *Methods Cell Biol.* **11**, 45–69.
16. Manners, D. J. & Masson, A. J. (1969) *FEBS Lett.* **4**, 122–124.