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Hyphal Wall Synthesis in *Aspergillus nidulans*: Effect of Protein Synthesis Inhibition and Osmotic Shock on Chitin Insertion and Morphogenesis

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Pulse-labeling with *N*-[*acetyl*-^3H] glucosamine and radioautography were used to follow the sites of chitin incorporation in hyphae of an *Aspergillus nidulans* mutant blocked in amino sugar synthesis. Growing hyphae incorporated *N*-acetylglucosamine almost exclusively at the tip. Cycloheximide addition greatly increased the label in subapical regions of the hyphae and reduced that at the tip. This effect of cycloheximide was immediate, could be reversed by removing the inhibitor, and did not appear to be due to chitin turnover. A similar change from apical to subapical *N*-acetylglucosamine incorporation occurred after hyphae were subjected to an osmotic shock which did not inhibit protein synthesis. The two treatments induced morphogenetic changes in the hyphae which produced abnormally large numbers of branches and septa.

A number of publications have described the growth and extension of vegetative fungal hyphae. As shown by microscope observation (18), radioautography (3, 9), and studies with fluorescent antibodies specific for wall components (14), hyphae extend by synthesizing new wall at their apex. As the hyphae increase in length, additional and localized sites of wall synthesis are formed in subapical positions. These then give rise to lateral branches, with wall synthesis again confined to the tip. Alternatively, the subapical sites may function for a short period only and form transverse septa (4). Although wall extension is limited to the apex of each growing hypha, wall thickening may occur for a short distance behind the apex (14), and a very low level of wall synthesis may be maintained in regions distant from the tip (3).

Although the general features of hyphal extension have thus been established, little is known about the biochemistry of wall synthesis or about the nature of the controls that limit wall growth to a small number of sites (1). These controls could be of considerable complexity, as electron microscope observations suggest that wall polymers are formed in cytoplasmic vesicles which move to the hyphal tip and then insert their contents into the wall (5, 12). Vesicle formation, polymer synthesis, transport, and insertion may thus be distinct sequences subject to different controls.

One approach to studying the factors involved in hyphal extension and branching would be to look for conditions that change the normal pattern of wall formation. We have attempted such a study by using a glucosamine-requiring mutant of the ascomycete *Aspergillus nidulans* (10) and radioautography to follow *N*-acetylglucosamine incorporation into hyphal walls. We find that inhibition of cell protein synthesis or an osmotic shock rapidly and drastically alters the normal pattern of apical incorporation to one of insertion along the length of the hypha. These treatments also produce hyphae with abnormally large numbers of branches and septa.

MATERIALS AND METHODS

Organism. The strains of *A. nidulans* used had the genotypes (for gene symbols, *see* reference 2) paba 1 y; and ad 15 bi 1; w; cnx ts6 glc N Ac⁻. The ts6 mutation has been described by us (10) and leads to inability to synthesize glucosamine and hence chitin at 41 C, whereas growth is normal at 30 C. Mutation glc N Ac⁻ results in a marked reduction in the wild-type ability to use *N*-acetylglucosamine as sole carbon source. We obtained this mutant by treating conidia of an ad 15 bi 1; w; cnx ts6 strain with 0.5 mg of nitrosoguanidine per ml at *p*H 6.0 and replicating colonies from glucose-agar plates to agar containing *N*-acetylglucosamine as sole carbon source. Colonies showing poor growth on the amino sugar were selected and retested for the other characters described above.

Media and growth conditions. The basal medium was as previously described (10). For growth of the ts6 strain, glucose, *N*-acetylglucosamine, and sodium ace-

tate were added to give final concentrations of 0.5% (w/v), 0.5% (w/v), and 0.1% (w/v), respectively. Acetate was included in the medium to minimize metabolism of any radioactive acetate split from *N*-[*acetyl*-^{3}*H*]D-glucosamine by residual activity of the mutated system catabolizing *N*-acetylglucosamine. Growth factor requirements were met by adding 30 mg of adenine and 1 mg of biotin per liter. For the paba y strain, 0.1% sodium acetate and 1 mg of *p*-aminobenzoic acid per liter were added to the basal medium.

Labeling with *N*-[*acetyl*-^{3}*H*]D-glucosamine (specific activity, 243 mCi/mM; Radiochemical Centre, Amersham, England) was carried out under two different sets of conditions. To determine the distribution of label between the wall and cytoplasm and between the various wall sugars, cultures were grown in the above medium with the addition of 0.5 μCi of [^{3}H]*N*-acetylglucosamine per ml for 12 hr, the mycelium was harvested by filtration, and the distribution of the label was examined. For radioautographic experiments excepting those of the osmotic shock, cultures were grown for 12 hr in the above medium with no labeled compound added, filtered, and transferred to medium containing 60 μg of nonradioactive *N*-acetylglucosamine per ml. From this culture, samples were taken at intervals, 20 μCi of *N*-[*acetyl*-^{3}*H*] glucosamine per ml was added, and the sample was incubated for 5 or 10 min as indicated. After labeling, the mycelium was processed for radioautography. Cultures were labeled with [*1*-^{14}*C*]L-leucine (specific activity, 62 mCi/mM; Radiochemical Centre, Amersham, England) by adding 0.1 μCi of medium per ml containing 10 μg of leucine/ml.

To determine the number of branches and septa in hyphae, conidia were inoculated onto cellophane sheets resting on agar media. Sheets were transferred to other agar plates to change growth conditions and to solutions in petri dishes for washing or osmotic shock. Finally, they were stained with fuchsin, and hyphal length was measured with an eyepiece micrometer standardized against a stage micrometer.

The preparation of conidial suspensions, inocula, and conditions of cultivation were as previously described (9, 10). Unless indicated otherwise, cultures were incubated at 41 C.

Osmotic shock. Cultures of the ts6 strain were grown in basal medium plus glucose, sodium acetate, *N*-acetylglucosamine, and 6% (w/v) NaCl for 32 hr, filtered, and suspended in distilled water containing 5×10^{-3} M ethylenediaminetetraacetic acid (EDTA), *p*H 6.0. After 10 min at 4 C, the mycelium was collected by filtration and suspended in growth medium. The paba 1 y strain was grown on cellophane on acetate-6% NaCl agar, and the cellophane was placed in 5×10^{-3} M EDTA in the cold for 10 min.

Chemical analyses. The purification of hyphal walls, their hydrolysis, and estimation of the sugar monomers in acid hydrolysates were carried out as described previously (9, 10). *N*-acetylglucosamine was isolated from hyphal walls by fractionating the partially purified walls (13) and incubating fraction IV with 10 mg of Helicase (Industrie Biologique Française, Gennevilliers, France) per ml in 0.1 M citrate buffer, *p*H 5.0, for 48 hr at 37 C. The digest was chromatographed on paper with butanol-pyridine-water (6:4:3) and the spots were localized with $AgNO_3$.

Radioactivity in wall and cytoplasmic fractions was estimated as described previously (9, 10). The distribution of radioactivity among sugars in wall hydrolysates was determined by preparing paper chromatograms (9, 10) and scanning these with a Gas-Flow chromatogram scanner (Packard Instrument Co., Inc.). To measure ^{14}C-leucine incorporated into proteins, the mycelium was washed with cold 5% trichloroacetic acid and water and extracted with 0.5 N NaOH for 15 min at 100 C. Next, 0.1 ml of the extract was dried on Whatman no. 3 filter strips and counted in a Tri-Carb scintillation counter by using a toluene-based scintillation fluid. Protein was estimated by the method of Lowry et al. (11).

Radioautography. Labeling with *N*-[*acetyl*-^{3}*H*] glucosamine was terminated by adding NaOH (0.5 N final concentration). The sample was heated at 100 C for 10 min, and the mycelium was washed with water and extracted with NaOH. The hyphae were washed successively in 1 M NaCl, 0.2% sodium dodecyl sulfate, and water and finally suspended in water and spread on clean slides. After drying, the slides were placed in chloroform-methanol (2:1) for 30 min at 18 to 22 C, washed with water, and dried. The hyphae retained their shape after these treatments and were clearly visible under the phase microscope.

The slides were covered with Ilford K5 emulsion and the radioautograms were developed as described previously (9). To count the grains in measured segments, we used an eyepiece micrometer previously calibrated with a stage micrometer and a magnification of $\times 1,600$ on a Zeiss GFL microscope. The counts were not corrected for background which was always very low.

RESULTS

Labeling of wall chitin with N-[acetyl-^{3}H] glucosamine. To explore the specificity of wall labeling, the ts6 strain, unable to synthesize *N*-acetylglucosamine and impaired in amino sugar catabolism, was grown with *N*-[*acetyl*-^{3}*H*] glucosamine and the mycelium was broken and separated into particulate and soluble fractions (9, 10). A considerable part of the total radioactivity was present in the soluble fraction, partly as a polymer which could be degraded to *N*-acetylglucosamine by Helicase (*results not shown*). To reduce the cytoplasmic radioactivity, we extracted labeled hyphae with NaOH, detergent, and chloroform-methanol as described above (but treating with chloroform-methanol in tubes and not on slides). The extraction did not change hyphal shape and yielded mycelium with 93% of the total radioactivity in the particulate fraction.

To determine which of the wall monomers was radioactive, the particulate fraction from labeled unextracted hyphae was hydrolyzed with 3 N HCl and enzymatically. Scanning chromatograms of acid hydrolysates showed that the neutral sugars glucose, galactose, mannose, and arabinose contained little radioactivity, although they constitute the major part of the wall (10). A

small peak of radioactivity was found near the chromatogram origin where partially hydrolyzed chitin would be located. Chromatography and scanning of enzymatic digests showed a strong radioactive peak corresponding to *N*-acetylglucosamine, whereas the neutral sugar spots, clearly visible when sprayed with $AgNO_3$, contained negligible radioactivity.

Site of chitin incorporation in growing hyphae. Inspection of radioautograms prepared from normally growing hyphae showed that in all cases the grains were located almost exclusively at the hyphal tip (Fig. 1a). Quantitative grain counts from 30 hyphae selected at random fully supported this view (Fig. 2). The pattern of incorporation is what would be predicted from apical growth of hyphae and constitutes a further check on the validity of our method of labeling. We compared hyphae growing with 6% NaCl with those in media of normal salt concentration because such conditions were used in later experiments involving osmotic shocks. NaCl slowed the growth rate and reduced the number of grains by approximately half.

Inhibition of protein synthesis and its effect on N-acetylglucosamine incorporation. Addition of cycloheximide to cultures almost immediately stopped protein synthesis as shown by the incorporation of ^{14}C-leucine and did not affect the rate of *N*-acetylglucosamine incorporation (Fig. 3 and 4). The inhibitor rapidly changed the sites of *N*-acetylglucosamine incorporation so that increased numbers of grains appeared subapically, whereas those at the tip were reduced (Fig. 5).

After washing the mycelium free of cycloheximide, incorporation again became apical. Thirty minutes after removing cycloheximide from cultures, protein synthesis was still much slower than in exponentially growing cultures (Fig. 3) and *N*-acetylglucosamine incorporation, though reduced in rate, was already apical (Fig. 5).

To determine whether subapical incorporation was due to chitin turnover, we labeled cultures with ^{3}H-*N*-acetylglucosamine, washed the mycelium, suspended it in medium containing 0.1% cold *N*-acetylgucosamine, and after 15 min of incubation added cycloheximide. The radioactivity in the wall fraction, calculated per micro-

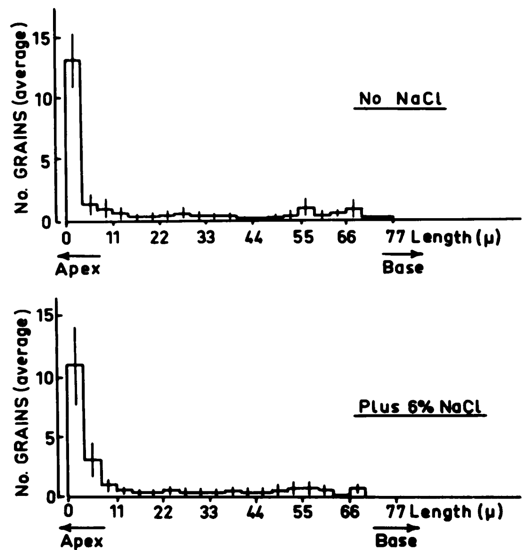

FIG. 2. *Sites of chitin incorporation in growing hyphae. After labeling with N-acetylglucosamine, radioautograms were exposed for 2 days, and the grains in segments (3.67 µm long) of the hypha were counted starting at the tip. Each column represents the average grain count from corresponding segments of 30 different hyphae, and the line shows the standard error of the mean. No NaCl, medium without added NaCl and hyphae labeled for 5 min; 6% NaCl: 6% NaCl added to medium and hyphae labeled for 10 min.*

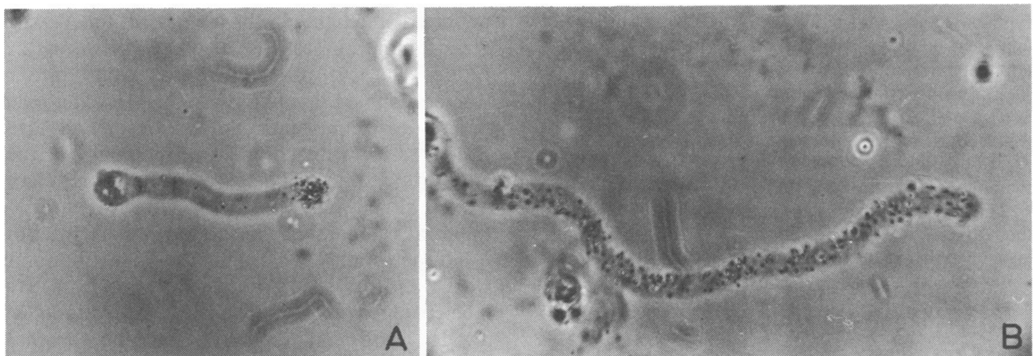

FIG. 1. *Radioautograms of hyphae labeled with N-(acetyl-^{3}H) glucosamine. (A) Hypha growing in medium without NaCl, 10-min label; (B) hypha 30 min after osmotic shock, 10-min label. Both radioautograms were exposed for 2 days.*

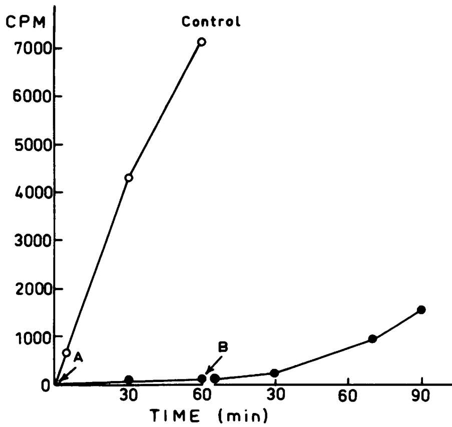

FIG. 3. *Effect of cycloheximide on protein synthesis. A culture was divided into two parts, and* ^{14}*C-leucine was added to both. Samples (2.5 ml) were taken at intervals, the hyphae was extracted with a total of 2 ml of 0.5* N *NaOH, and the counts per min in 0.1 ml of the extract were determined.* (○) *Control;* (●) *plus 10 μg of cycloheximide per ml. A, addition of cycloheximide; B, hyphae washed free of cycloheximide and suspended with* ^{14}*C-leucine.*

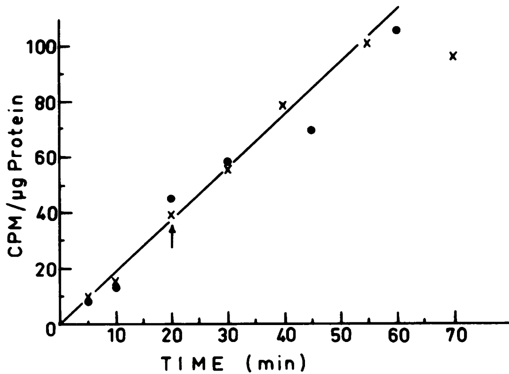

FIG. 4. *N-acetylglucosamine incorporation in the presence of cycloheximide. A culture was divided into two parts and N-(acetyl-*^{3}*H) glucosamine was added to both. Samples were taken at intervals, the hyphae were extracted with 0.5* N *NaOH, washed, collected on a membrane filter, and counted in a scintillation counter. Protein was estimated on the NaOH extracts.* (●) *Control;* (×) *10 μg of cycloheximide per ml was added as indicated by arrow.*

gram of hyphal protein to correct for sampling errors due to culture inhomogeneity, remained constant during 1 hr of incubation with cycloheximide. Of the radioactivity in the wall fraction, 4.5% was liberated into the medium during 1 hr of incubation with cycloheximide and 4% in controls with no added cycloheximide. Additional controls showed that *N*-acetylglucosamine uptake during 1 hr with cycloheximide was equivalent to 10% of that in the mycelium at zero time.

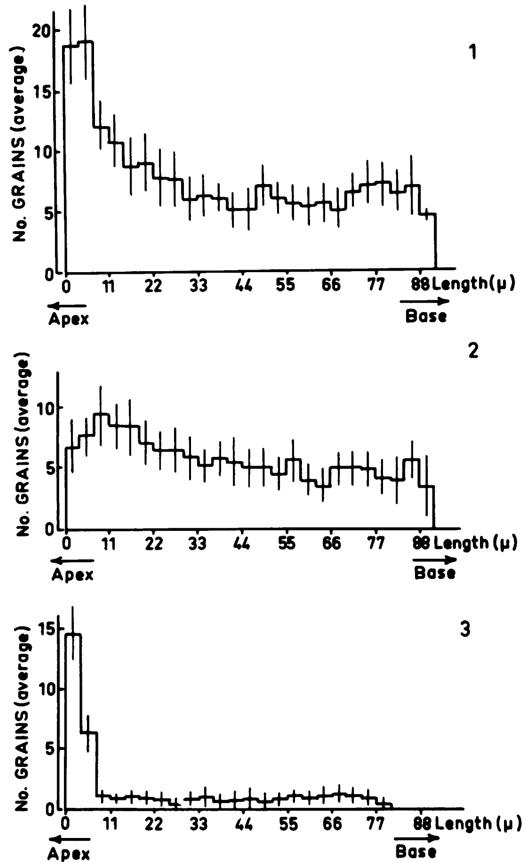

FIG. 5. *Sites of chitin incorporation in the presence of 10 μg of cycloheximide per ml. Hyphae were labeled with N-acetylglucosamine and the distribution of label along the hypha was determined from a random sample of 30 hyphae. 1, Labeled during 0- to 10-min period with cycloheximide; 2, labeled during 10 to 20 min with cycloheximide; 3, hyphae washed free from cycloheximide and labeled during 30- to 40-min period after removal of inhibitor. Radioautograms were exposed for 2 days.*

Any chitin turnover that did occur was thus too small to be detected by our methods.

N-acetylglucosamine incorporation after osmotic shock. The following variations of the growth and suspension medium were tested in conjunction with the osmotic shock treatment: (i) growth in 6% NaCl, osmotic shock, suspension in medium plus 6% NaCl; (ii) growth in 6% NaCl, osmotic shock, suspension in medium without NaCl; (iii) as above but with 40% sucrose replacing 6% NaCl. Similar results were obtained with all the combinations, and data are given for growth in 6% NaCl, followed by osmotic shock and suspension in medium containing no added salt.

The osmotic shock treatment did not alter the rate of hyphal protein synthesis as measured by ^{14}C-leucine incorporation (*results not shown*). Label from *N*-acetylglucosamine also continued to be incorporated by hyphae after an osmotic shock, but grains were present along the whole length of the hyphae instead of being confined to the tip (Fig. 1b and 6). Two hours after the shock, incorporation had again reverted to apical.

To check that we were indeed measuring *N*-acetylglucosamine incorporation in the radioautograms, mycelium was subjected to an osmotic shock and after 30 min labeled with *N*-[*acetyl*-^{3}*H*] glucosamine for 10 min. The wall fraction was purified and digested with Helicase, and the sugars in the digest were chromatographed. Scanning the chromatograms for radioactivity showed that this was confined to the spot corresponding to *N*-acetylglucosamine (*results not shown*).

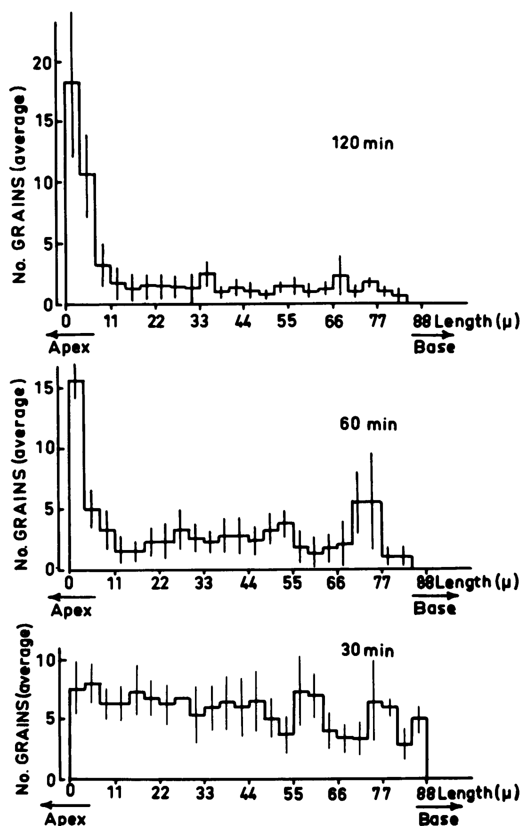

FIG. 6. *Sites of chitin incorporation in hyphae after an osmotic shock. Hyphae were labeled for 10 min at the various times after the shock indicated on the figure. Distribution of label was determined as in Fig. 3. Radioautograms were exposed for 2 days.*

Effect of cycloheximide and osmotic shock on branching and septum formation. Conidia were germinated on cellophane sheets placed on agar to obtain hyphae that were well separated and easy to examine and measure under the microscope. After germination, the sheets could be transferred to liquid for washing or osmotic shock without the hyphae being washed off. Hyphae of the ts6 mutant became highly branched when returned to growth conditions after the treatments. To determine whether this was a feature of the ts6 strain, we examined a second strain with genotype paba 1 y. Four hours after exposure to cycloheximide, the hyphae were much more highly branched than control hyphae of similar length (Fig. 7), and increased

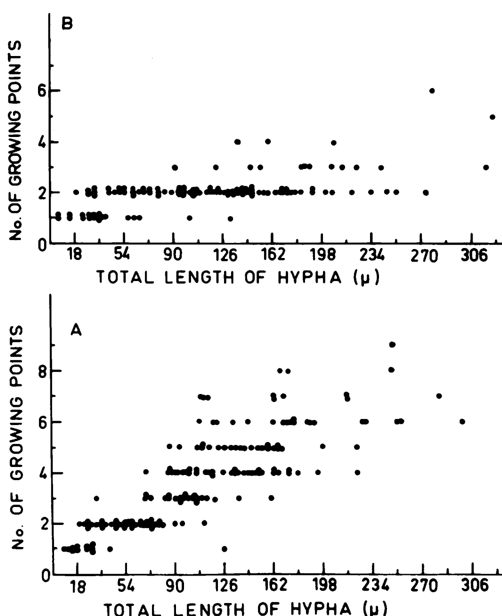

FIG. 7. *Degree of branching in hyphae after cycloheximide treatment. Conidia of strain paba y were grown at 30 C, transferred for 1 hr to medium containing 10 μg of cycloheximide per ml, and retransferred to growth medium. After 5 hr of growth at 30 C, the hyphae were stained, their whole length from conidium to tip was measured with an eyepiece micrometer, and the number of branches was counted. Each point represents one hypha. A, Cycloheximide treated; B, untreated hyphae of similar length.*

branching was found in hyphae 3 hr after an osmotic shock. After these treatments, hyphae also contained increased numbers of septa (Fig. 8).

DISCUSSION

Cycloheximide induced *N*-acetylglucosamine

incorporation along the whole length of hyphal walls. Since it is an inhibitor of protein synthesis (6, 21), this could not have been due to the action of newly formed enzymes. The activation of preexisting enzymes, located along the length of the wall and constituting one of the factors in wall synthesis, could, however, explain the changed labeling pattern. Subapical incorporation was rapidly induced by cycloheximide and readily reversed by its removal. This supports the view of an enzyme activation mechanism. The nature of the postulated activation remains quite undefined, and its basis could be as different as exposure of a primer, an increased supply of substrates, the accumulation of activators, or removal of inhibitors. The effects of an osmotic shock were very similar to those of inhibiting protein synthesis, and it appears reasonable to conclude that the same enzymes were activated by this treatment as were by cycloheximide.

N-acetylglucosamine is found in hyphal walls as its β 1-4 polymer, chitin (1). The chitin is embedded in a matrix of other wall polymers, as shown by its inaccessibility to purified chitinase unless other wall components are removed (8, 17) and by electron microscope studies (8). The simplest ways in which ^{3}H-*N*-acetylglucosamine could be inserted into subapical walls would appear to be (i) chitin present in the wall undergoes turnover, (ii) existing chitin chains are lengthened, (iii) new chitin chains are synthesized in their usual or in abnormal wall locations, and (iv) new pieces of wall containing polymers besides chitin are inserted.

We have failed to detect any chitin turnover after cycloheximide addition. To conclude that turnover does not occur, however, it must be assumed that excess cold amino sugar is an efficient trap for *N*-acetylglucosamine split from the labeled polymer. Regarding the other possibilities of incorporation, the present data are insufficient to distinguish between them. Specific labeling of wall polymers other than chitin should indicate whether complete segments of wall are formed, and we are attempting such experiments with a system that labels galactose- and glucose-containing polysaccharides (9). The availability of subapically formed chitin to hydrolysis by chitinase may help to clarify its location in the wall, and this also is being examined. It may be relevant that inhibition of protein synthesis in gram-positive bacteria stops peptidoglycan synthesis at the point where wall extension normally takes place and triggers greatly increased peptidoglycan formation around the whole cell (16, 20). The polymer does not undergo turnover and the whole wall is thickened.

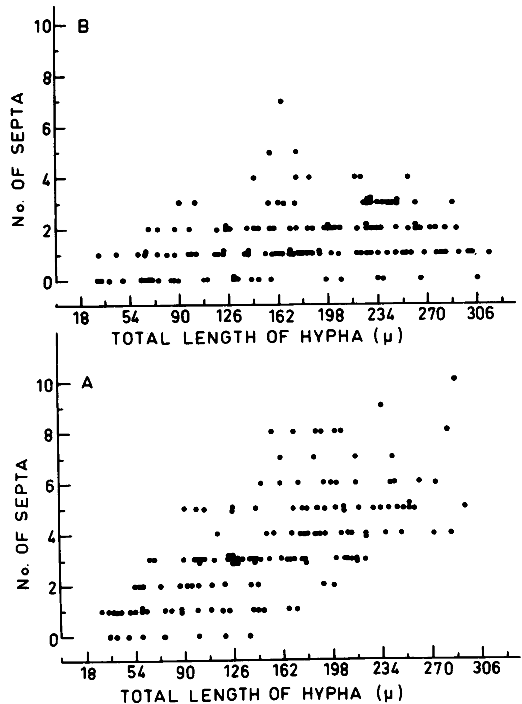

FIG. 8. *Septation in hyphae after an osmotic shock. Each point represents one hypha.*

During fungal growth, wall synthesis in positions other than the apex results in a morphogenetic change of the hypha (18). Such wall formation is, however, confined to the specific loci where branches or septa are formed. Inhibition of protein synthesis or osmotic shock led to a uniform incorporation of *N*-acetylglucosamine along the length of the hyphae, and the question arises if this has any relation to the mechanisms initiating synthesis in specific, subapical loci. The increased numbers of branches and septa in hyphae after cycloheximide addition or osmotic shock indicate that such a relation does indeed exist. The kinetics of branch and septum formation were not studied exhaustively, but 2 to 4 hr of normal growth after the treatments were required to produce visible changes. Subapical incorporation could thus be part of an early trigger for a complex morphogenetic sequence.

The osmotic shock did not inhibit protein synthesis, and its immediate effects on hyphal metabolism were therefore different from that of cycloheximide. In bacteria and fungi, osmotic shocks produce losses of proteins associated with the cell envelope (7, 15, 22), and in fungi they

also result in temporary inhibitions of hyphal extension at the tip (19). A disarrangement of normal extension at the tip could thus be the factor common to both treatments. As the steps in the synthesis of hyphal walls are barely understood at present and the controls governing them practically unknown, it does not appear fruitful to speculate in greater detail.

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