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Cytodifferentiation and Morphogenesis in *Schizophyllum commune*

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INTRODUCTION

> "The delicate adaptations of structure to function, as revealed by a study of the fruit-body of a mushroom . . . have provided me with no small cause for wonderment and delight."
>
> A. H. Reginald Buller

Studies of regulatory devices in diverse microbial systems have profoundly affected contemporary biology. Yet, in the area of microbial morphogenesis, the challenge remains to define the molecular bases of control mechanisms governing patterns of form. In eukaryotic microorganisms, experimental systems include fruiting in cellular slime molds (94, 123), resistant sporangium formation in *Blastocladiella emersonii* (15), yeast-mold dimorphism in *Mucor rouxii* (4, 5, 98), phototropisms in *Phycomyces* (6, 16), hyphal differentiation in *Neurospora crassa* (12, 13, 20), sexual hormones in *Achlya* (3) and *Allomyces* (50), and ascospore activation in *Neurospora tetrasperma* (92), to mention only a few. Absent here are the Basidiomycetes such as the mushrooms, which grow slowly compared with *Neurospora* and often fail to complete their development on defined media. Thus, the fruiting Basidiomycetes have not become popular tools for students of microbial development. An exception to this is the wood-rotting mushroom *Schizophyllum commune*, which fruits regularly on synthetic medium. Genetic studies have also progressed to the point where considerable information exists concerning the control of sexual morphogenesis of *S. commune*. Consequently, this microbial system is well suited for biochemical investigations of regulatory devices governing patterns of form. The purpose of this review is to correlate ultrastructural features of *S. commune* with biochemical aspects of development.

GENETIC REGULATION OF MORPHOGENESIS

The genome of the cell is the most critical factor determining patterns of differentiation and morphogenesis. Genetic studies of *S. commune*

are mainly concerned with the structure and function of the bifactorial incompatibility system governing sexual interaction between self-sterile mycelia (75, 79, 80). We shall discuss these matters only as they bear upon the biochemical aspects of morphogenesis.

The haploid phase of *S. commune* is a homokaryotic mycelium (i.e., genotypically identical nuclei) made up of uninucleate cells. The homokaryon is self-sterile and capable of indefinite vegetative growth. The colonial morphology on agar medium is shown in Fig. 1.

When two mycelia grow into contact, fusions occur between the hyphae. Subsequent development depends on the mating types of the paired homokaryons. Mating between homokaryons

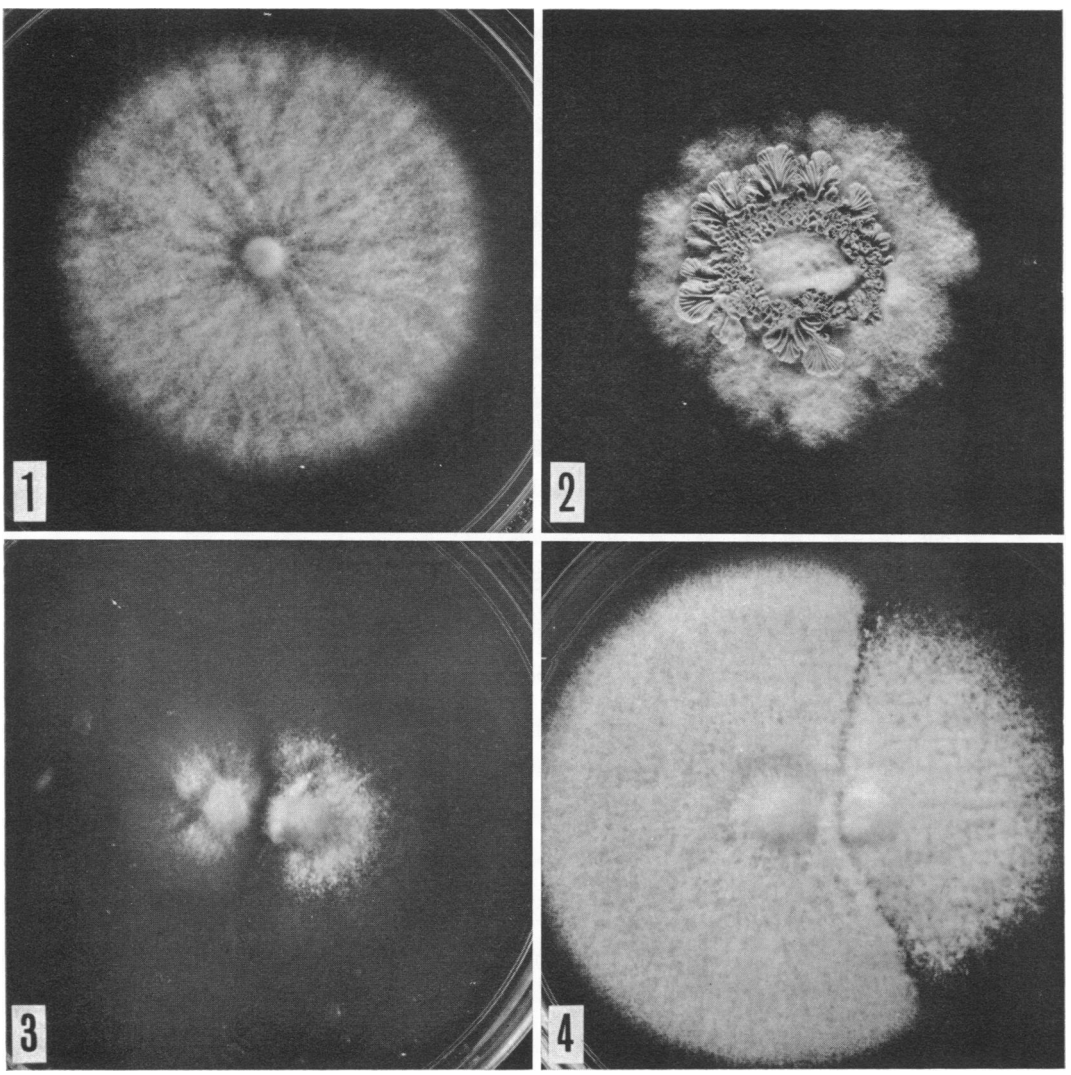


FIG. 1. *Colony morphology of vegetative, homokaryotic mycelium of S. commune (strain 699 A41B41) grown for 7 days on glucose-asparagine minimal agar medium.*

FIG. 2. *Sporulating fruit-bodies of S. commune (strain 699 A41B41 × strain 845 A51B51) after 10 days on minimal agar medium incubated inverted.*

FIG. 3. *Colony morphology of common-A heterokaryon of S. commune (strain 845 A51B51 × strain 1 A51B41) grown for 10 days on minimal agar medium. Note "flat" appearance due to absence of abundant aerial hyphae in periphery.*

FIG. 4. *Colony morphology of common-B heterokaryon of S. commune (strain 699 A41B41 × strain 1 A51B41) grown for 10 days on minimal agar medium. Note typical "barrage" reaction at line of confrontation.*

leads to the normal morphogenetic sequence (formation of a dikaryon and subsequent fruiting) only if the two interacting homokaryons are heteroallelic for two incompatibility factors ($A \neq B \neq$). In such a dikaryon, the hyphae are composed of binucleate cells with clamp connections. The formation of a clamp connection is shown in Fig. 5. Dikaryotic mycelia produce sporulating fruit-bodies (Fig. 2).

In a mating between strains possessing different *B* factors but similar *A* factors ($A = B \neq$), a so-called common-*A* heterokaryon is established by exchange of nuclei from each mycelium by nuclear migration (68). The common-*A* heterokaryon has little aerial mycelium (Fig. 3). Importantly, its septa show various degrees of disruption (33). Some mutations in the *B* factor of *S. commune* (e.g., *AxBmut*) mimic the common-*A* heterokaryon (70). In another Basidiomycete, *Coprinus lagopus*, electron microscopic evidence suggests that dissolution of complex septa facilitates nuclear migration in a compatible mating before the dikaryon is formed (26).

The cytological events which take place in the common-*A* heterokaryon are called the *B-morphogenetic sequence*. The observations reported above indicate that the *B*-morphogenetic sequence of *S. commune* primarily involves extensive nuclear migration, because this event invariably occurs under specific genetic conditions of *B*-locus functionality (e.g., $A = B \neq$; *AxBmut*).

In matings involving common *B* but different *A* factors ($A \neq B =$), nuclei of each homokaryon do not migrate extensively into the opposing mycelium. Where the two mycelia join, however, a common-*B* heterokaryon is established, and the culture exhibits a "barrage" appearance in this zone (Fig. 4). In common-*B* heterokaryotic tip cells, the resident and migrant nuclei pair, conjugate nuclear division of these nuclei occurs, and at least the *initiation* of a clamp connection follows. After delimitation, one daughter nucleus is trapped in the clamp initial. These events, which comprise part of the formation of a dikaryon (Fig. 5), have been called the *A-morphogenetic sequence*. However, because the hook cell containing the trapped nucleus fails to fuse with the newly delimited penultimate cell in the common-*B* heterokaryon, it is assumed that cell wall dissolution to complete the clamp connection is part of the *B*-morphogenetic pathway. Mutations in the *A* factor (e.g., *AmutBx*) are known which elicit the *A*-morphogenetic sequence in homokaryons of *S. commune* (76). These mutant homokaryons have all the features of a common-*B* heterokaryon.

The functional equivalence of mutant incompatibility factors to heteroallelic factors is demonstrated by an *AmutBmut* homokaryon which has a phenotype indistinguishable from that of the dikaryon and which produces fruit-bodies regularly (76).

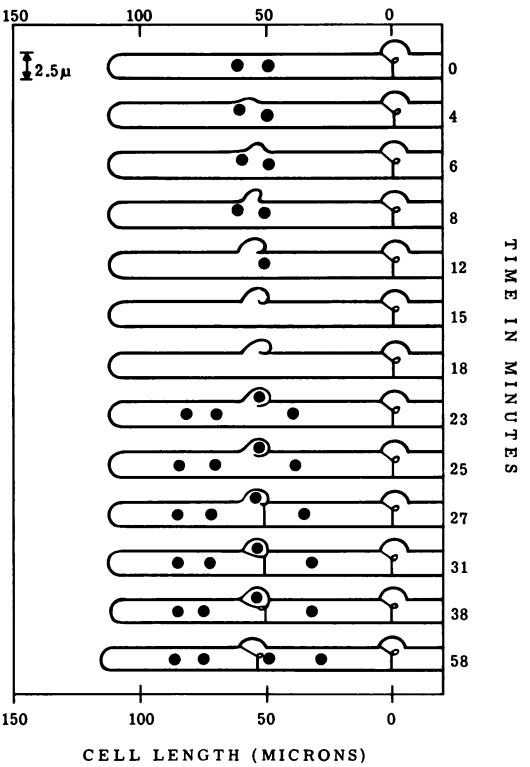


FIG. 5. *Clamp-connection formation in the dikaryon of S. commune. Lateral outgrowth of presumptive clamp precedes mitosis. Following conjugate nuclear division, establishment of septa, and production of a basal portal of entry, the daughter nucleus entrapped in the hook subsequently migrates into the penultimate cell, thus restoring the dikaryotic condition.*

In summary, when both *A* and *B* incompatibility factors are common in a mating ($A = B =$), none of the cytological events described for the *A* or *B* morphogenetic sequences ordinarily occurs. Partial sequences transpire in the common-*A* heterokaryon ($A = B \neq$) or the common-*B* heterokaryon ($A \neq B =$). The *B*-morphogenetic sequence is operative in the former situation and only involves nuclear migration. The *A*-morphogenetic sequence occurs in the latter and involves nuclear pairing, conjugate nuclear division, and the *initiation* of a clamp connection endowed with septa. The completion of the clamp connection is only realized in a dikaryon ($A \neq B \neq$) wherein the trapped daughter nucleus in the hook cell eventually migrates into the subapical cell (*see* Fig. 5). The complete series of events which occurs

in the ($A \neq B \neq$) heterokaryon is called the normal, or *AB-morphogenetic sequence*.

Recently, Koltin and Raper (39) showed that the establishment of the dikaryon also depends on a dominant gene *dik*$^+$. Interaction between compatible homokaryons, both of which carry the recessive allele *dik*, leads to the formation of a diploid mycelium resembling the morphology of the homokaryon.

Many hypotheses have been offered to explain how the incompatibility factors influence hyphal morphogenesis [some of these have been summarized by Parag (71)]. The model that fits most of the genetic data was proposed independently by Prévost (72) and Raper (75, 80; *see also* 45). In a homoallelic condition, the products of the incompatibility genes repress the expression of morphogenesis leading to a dikaryon. Such a situation prevails in the homokaryon. Heteroallelic conditions, for either the *A* factor or the *B* factor, or for both, derepress the corresponding morphogenetic pathways via specific interaction and inactivation of the repressing gene products. Mutations in the *A* and *B* factor therefore cause a loss in repressor function. Consequently, the corresponding morphogenetic sequences are expressed in homokaryons bearing these mutations.

Two other types of mutations can be classified either as secondary mutations in the *B* factor (38, 76) or as modifier mutations outside the incompatibility loci (74). The first type restores homokaryotic morphology in a homokaryon with a primary mutation in the *B* factor, although there are some deviations from a wild-type homokaryon when mated with a compatible homokaryon. The modifier mutations fall in five classes (*I–V*), each influencing different parts of the *A*, *B*, or *AB* morphogenetic sequence. For instance, a mutation of class *IV* blocks nuclear migration induced by heteroallelic *B* factors or a mutated *B* factor. Thus, a common-*A* heterokaryon with a double dose of mutation *IV* mimics a common-*AB* heterokaryon; the formation of this heterokaryon depends on nutritional forcing. Similarly, the phenotype of a homokaryon carrying both a primary mutation in the *B* factor and a mutation *IV* mimics a normal wild-type homokaryon.

The general pattern of development of the fruit-body of *S. commune* has been outlined by Essig (24) and Putterill (73). A tiny knot of hyphae develops into a cup-shaped structure lined with hymenium. Foldings of the hymenium produce the gills which are continuously formed and extend radially as indeterminate growth enlarges the sporulating surface. Enlargement occurs unilaterally so that in the fully developed fruit-body the gills radiate out from the point of attachment of the fruit-body to the substrate.

Fruiting in *S. commune* is sometimes observed in "wild-type" homokaryons but is rare in freshly collected material (78). Fruiting has rarely been observed in common-*AB* heterokaryons (53), common-*A* or common-*B* heterokaryons, or their homokaryotic mimics carrying mutations at the incompatibility loci. Dikaryotic mycelia or homokaryons having mutations at both incompatibility loci fruit regularly, indicating that heteroallelic *A* and *B* factors provide the most favorable genetic background for fruiting.

Additional genetic determinants are required for normal dikaryotic fruiting, as born out in several earlier studies with *S. commune* (36, 126), and studied most extensively by Raper and Krongelb (78). A sample of more than 3,000 dikaryons showed a range of fruiting expression from nonfruiting to abundant fruiting. Fruiting competence was heritable but polygenically determined.

In the same study (78), several aberrant fruit-body forms were described. Some were due to a single dominant gene (e.g., *bug's ear*, *coralloid*) and others were polygenic (e.g., *cauliflower* and *medusoid*). Zattler (126) earlier described a fruit-body abnormality called Knauél-Fruchtkörper (i.e., gnarled, similar in appearance to *cauliflower*) which behaved as a single recessive.

Aged stock cultures of dikaryotic mycelia of *S. commune* often carry morphological mutations (54, 78). Wessels (113) isolated a mutant with abnormal pileus development from the old "Kniep stock culture" of dikaryotic mycelium, which was useful for studying the mechanism of normal pileus development.

ENVIRONMENTAL CONTROL OF FRUITING

In certain fungi, morphogenesis can be controlled by adjusting chemical or physical environmental factors. Once recognized, such a factor can be used as a tool for tracing biochemical patterns related to the morphogenetic effect. For fruit-body formation, however, analyses of environmental stimuli have seldom proceeded beyond a demonstration of the effect (cf. 96). Investigations with *S. commune* provide some additional insight.

Carbon and Nitrogen Sources

S. commune grows and fruits on a simple, defined medium containing D-glucose, L-asparagine, and thiamine (81, 86). A wide variety of carbon sources, including certain disaccharides, monosaccharides, xylose, sugar alcohols, and ethyl alcohol, are also satisfactory, whereas lactose, L-sorbose, and inositol are poor carbon sources (62). Tricarboxylic acid cycle intermedi-

ates were reported as poor carbon sources (62), but this may be due to cellular permeability barriers at the neutral *p*H examined. In this connection, succinic dehydrogenase and malic dehydrogenase occur in cell extracts of *S. commune* (112; T. Ratts et al., Bacteriol. Proc., p. 111, 1964), although malate and succinate are good carbon sources for mycelial growth at *p*H 5 (Wessels, *unpublished*).

Peptone, tryptone, certain L-amino acids, urea, and various ammonium salts are good nitrogen sources for growth and fruiting of *S. commune* (62). Neither nitrate nor nitrite can be utilized as nitrogen sources. No differential effect on mycelial growth and fruiting was observed in these studies. These data should be compared with nutritional studies of fruiting initiated with a preformed dikaryon of *S. commune*.

Figure 6 shows total nitrogen and carbohydrate in mycelium and fructifications of a dikaryon of *S. commune* growing on synthetic medium (113). Approximately 100 hr after inoculation, the surface of the culture is covered with fruit-body primordia. The number and size of the primordia then increase rapidly, coinciding with rapid nitrogen assimilation. At the time of maximal nitrogen content, most of the nitrogen occurs in the mycelium. In the following period, which ends when the glucose supply is exhausted, much of this mycelial nitrogen appears to be transferred to the primordia, which become larger and attain a cup-shaped appearance. This period is charac-

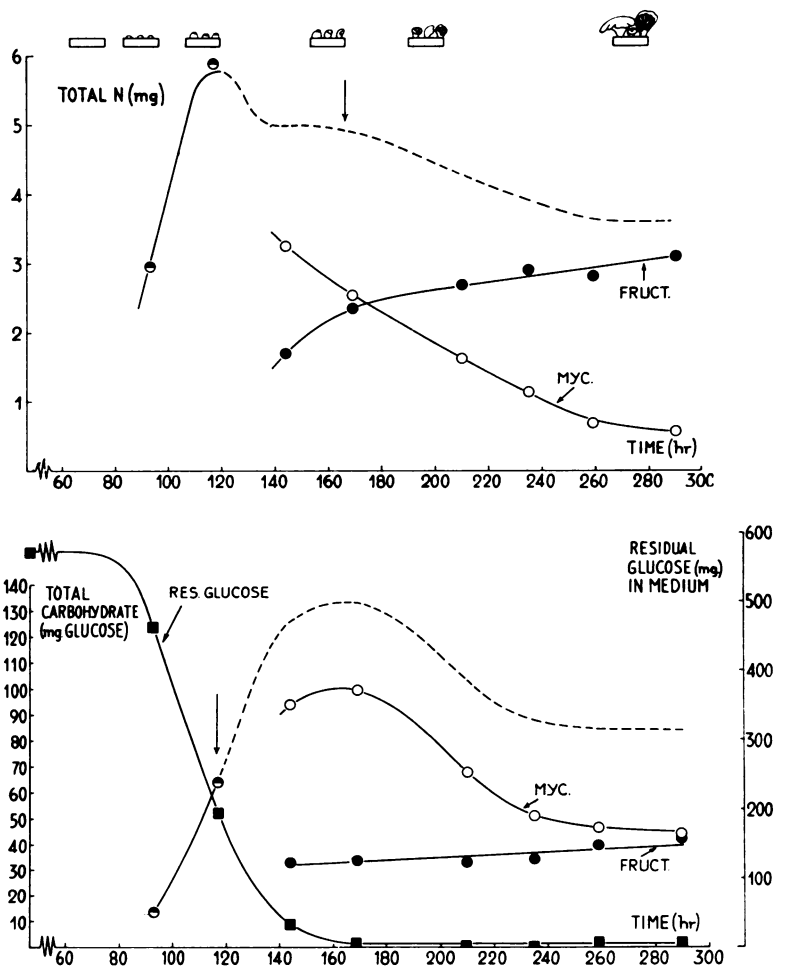


FIG. 6. *Changes in total carbohydrate and nitrogen of mycelium and fructifications of S. commune K.8 accompanying development. The schematic drawings at the top represent the various stages of development. After Wessels (113).*

terized by an intensive accumulation of cellular carbohydrate. Thereafter, under conditions of both carbon and nitrogen starvation, carbohydrate and nitrogen are lost from the mycelium but remain constant or even increase somewhat in the fruiting structures. Simultaneously, a few of the cup-shaped fruit-bodies form large pilei (113).

This developmental pattern suggests that only certain stages of fruit-body formation depend on exogenous nutrients. Use of a replacement culture technique shows that: (i) initiation of fruit-body primordia requires an external carbon and nitrogen source; (ii) growth of primordia requires a carbon source in the medium but no exogenous nitrogen; and (iii) the asynchronous formation of pilei proceeds in the absence of an external carbon and nitrogen source (113).

The significance of degradation of cellular carbohydrate for pileus expansion is indicated by the following observations (113). A mutant dikaryon, inhibited in normal pileus expansion (cup mutant), shows a marked decrease in the ability to catabolize its cellular carbohydrate after exhaustion of the glucose supply. However, the cup-shaped fruit-bodies of this mutant can form small pilei if, after exhaustion of the major glucose supply, a continuous low concentration of glucose is applied via a gradient. On the other hand, continuous application of glucose at high concentrations (2%) completely inhibits pileus expansion, even in the wild-type dikaryon. These results suggest that the process of pileus expansion, though dependent on a continuous supply of carbohydrate as provided by slow degradation of cellular carbohydrate, is repressed by high concentrations of glucose.

These findings substantiate the old observation that exhaustion or sudden reduction of exogenous nutrients favors fruiting in *S. commune* (105). That unfavorable culture conditions are conducive to fruit-body formation was supported further by the fact that either low *p*H or dilute culture medium promotes homokaryotic fruiting of *S. commune*; only 2 homokaryons of 16 examined fruited under the latter condition and basidiospore production was scanty (62). Recently, a haploid fruiting-inducing substance (FIS) active with *S. commune* has been isolated from *Hormodendrum* (43). Further genetic work showed that a single gene functions in FIS-induced haploid fruiting of *S. commune* (44).

Thiamine

The only growth factor required by *S. commune* is thiamine or its pyrimidine moiety (83, 84). Schopfer and Blumer (86) claimed that an additional natural compound is required for normal fruiting, an observation which has not been confirmed. Raper and Krongelb (78) stated that at low concentrations of thiamine "there was good vegetative growth but no fruiting." This observation was explored to test the possibility of using thiamine as an external factor to regulate fruit-body production (113). The effect of thiamine concentration on a culture of a particular dikaryon is shown in Fig. 7.

At a thiamine concentration of 6 ng/ml, fruit-body primordia are initiated, although the cells contain only about 20% of their maximal nitrogen content. Above 20 ng/ml, nitrogen content, total glucose consumption, and final *p*H are not influenced. The distribution of nitrogen between mycelium and fructifications, however, suggests that transfer of nitrogen from the mycelium into the developing fruit-bodies is influenced by concentrations of thiamine exceeding 20 ng/ml. The low final *p*H at the lower thiamine concentrations seems to be due primarily to the excretion of large quantities of pyruvic acid into the medium (113).

Although growth of *S. commune* is influenced by thiamine, it can occur in a thiamineless medium (113). Best results were obtained with a glucose-asparagine medium to which $CaCO_3$ and citrate were added. Repeated transfer on this thiamineless medium (Norit-treated) does not diminish the yield, which is about 30% of that in a thiamine-containing medium, and no thiamine is detected in the mycelium.

Thiamineless growth of the dikaryon of *S. commune* is characterized by the absence of aerial hyphae and primordia. Replenishment by a medium containing thiamine but lacking a nitrogen source fails to induce primordia; a minimal amount of exogenous nitrogen is necessary. Once the differentiated cells emerge, however, the primordia grow without an exogenous nitrogen source (113). The need for nitrogenous compounds is evidently met by transport of cell material from the undifferentiated cells of the mycelium.

Carbon Dioxide

Incubation of *S. commune* under conditions of restricted aeration leads to decreased fruiting (78). By use of various $A \neq B \neq$ crosses, reversal of this effect was demonstrated with alkali but not with other trapping agents (56). This indicates that oxygen deficiency per se cannot explain this effect, but rather that a respiratory gas such as CO_2 accumulates under these conditions. In fact, 5% CO_2 completely inhibits fruiting of *S. commune* (56). Good mycelial growth and dikaryotization under these conditions indicate that these processes are not inhibited by CO_2. The general failure of various common-*A* and com-

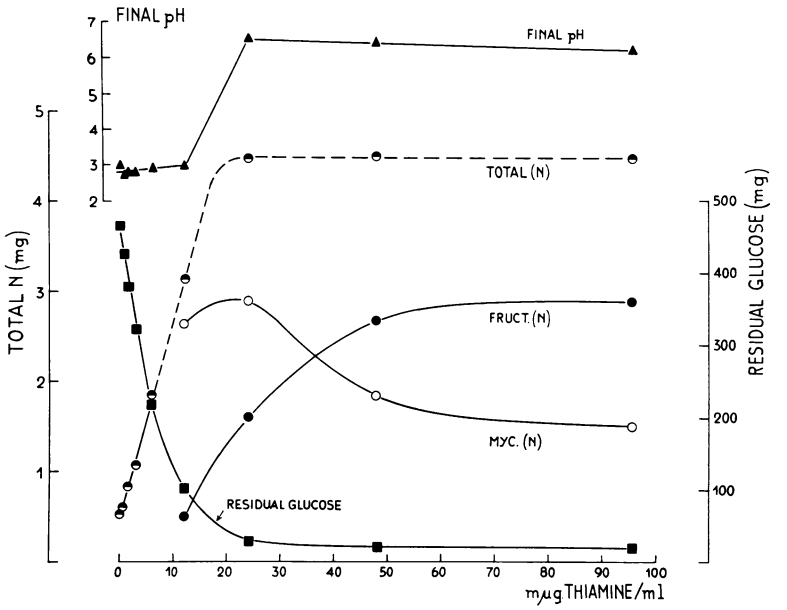


FIG. 7. *Effect of thiamine concentrations on total nitrogen content of mycelium and fructifications of S. commune K.35, on final pH, and on residual glucose in the medium. Glucose initially present, 619 mg. Incubation time, 11 days. After Wessels (113).*

mon-*B* matings of *S. commune* to fruit is probably not due to an unusually high sensitivity to CO_2, because these crosses do not fruit in sealed chambers containing alkali reservoirs either (56). On the other hand, nutritionally induced homokaryotic fruiting of *S. commune* is sensitive to respiratory CO_2 (62), as perhaps is FIS-activated development, because an alkali reservoir is routinely included in the bioassay of this system (43). Exactly what stage of morphogenesis is affected by CO_2 remains to be established, as does the biochemical basis of this control device in *S. commune*.

Tachibana et al. (97) have studied CO_2 assimilation into malate with essentially thiamineless submerged cultures of *S. commune*. Since fruit-bodies are never produced in such cultures, this probably does not bear directly upon the effect of CO_2 on fruiting. Rast and Bachofen (82), studying the effect of CO_2 on fruiting in *Agaricus bisporus*, have discussed the possibility that acetone is the CO_2 acceptor.

Light

Light has long been implicated in fruiting of *S. commune* (36, 86, 105). Raper and Krongelb (78) reported that fruit-body primordia are formed in total darkness but their normal development requires light, although intensity and duration are not critical. Jürgens (35) found a dominant gene in the "Kniep stock" of *S. commune* determining independence from light for normal fruit-body development. Trione et al. (99) isolated sporogenic substances which induce fruiting in the dark from a number of fungi but were unable to isolate such a substance from *S. commune*.

Temperature

Fruiting normally occurs at 20 to 25 C (62, 69, 113) but is inhibited by elevated temperature (30 to 37 C). A possible clue to the effect of elevated temperature on fruiting is the observation that cultivation of the dikaryon K.8 of *S. commune* at 30 C results in a decreased ability to degrade cell wall glucan (113). This is not due to a decrease in the activity of degrading enzyme but rather to a decreased susceptibility of the glucan in the cell wall to enzymatic attack (114).

None of the environmental factors mentioned above has been explored at a biochemical level except for the role of carbon starvation in pileus development and in the initiation of primordia on a thiamineless mycelium. A review of these studies follows.

BIOCHEMICAL CHANGES IN FRUITING

Initiation of Fruit-Body Primordia

Young primordia are largely obscured by aerial mycelium. This difficulty may be overcome by seeding the surface of a nutrient medium with a

mycelial suspension of the dikaryon (113). The hyphae at first grow submerged, and within a few days primordia are seen as little knots of intermingled hyphae. Primordia do not develop without thiamine or at oxygen tensions below 1%. An outer cell wall layer in the primordial hyphae, which stains metachromatically red with thionine or Toluidine Blue (113), may be implicated in hyphal aggregation.

Changes in protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) were measured during the transition from purely vegetative mycelium to primordia-bearing mycelium with thiamine as the inducing factor (113). Primordia are produced within 24 hr, and simultaneously a drop in the ratio of RNA to protein is observed, which is correlated with accumulation of trichloroacetic acid-soluble substances specifically absorbing at 260 nm. In the next 24 hr, RNA is synthesized faster than protein, causing a rise in the RNA to protein ratio and a decrease of soluble substances absorbing at 260 nm. The significance of these changes for differentiation of primordia is not clear. With the use of a minimum of nitrogen in the induction medium, primordia are induced without net synthesis of RNA and with only a twofold increase in protein.

Respiration and Fruit-Body Formation

A number of studies have demonstrated the reactions of the tricarboxylic acid cycle and a conventional electron transport chain in *S. commune* (22, 60, 112). This work was mostly done with submerged cultures of vegetative mycelium and does not bear directly upon the problem of fruit-body formation.

After transfer of dikaryotic mycelium bearing fruit-body primordia to a medium lacking nitrogen, glucose supports primordium growth and a high constant rate of respiration (113). The respiratory quotient (RQ) of approximately 2 and the rate of anaerobic carbon dioxide production, however, drop steadily. In contrast, if the experiment is performed at 30 C instead of 25 C, where no growth of the primordia occurs, the respiration rate also drops with time even in the presence of glucose. This suggests that high respiratory activity, as sustained by external glucose after exhaustion of the nitrogen supply in the medium, is related to the growth of the fruit-body primordia. After the glucose supply is exhausted, the respiratory rate drops, with an RQ around unity, and pileus formation follows.

Cell Wall Polysaccharide and Pileus Formation

After the glucose in the medium is depleted, pileus formation can occur in some fruit-body primordia. This is accompanied by a decrease in cellular carbohydrate (Fig. 6). Breakdown of cell wall polysaccharide contributes considerably to this decrease, and therefore prompted a study of cell wall composition in *S. commune* (113).

The composition of the wall changes with development. Analysis of enzymatically cleaned walls showed the following composition: glucan (86.8%), chitin (3.1%), "protein" (2.6%), and ash (0.4%), accounting for 92.9% of the dry weight of the wall. Xylose and mannose were also present. Chitin was identified by its X-ray diffraction pattern (113).

The glucan fractions of the wall have not been completely characterized, but two distinct fractions were isolated on the basis of solubility in 1 N KOH at room temperature (113). The alkali-insoluble glucan fraction, called R glucan, contains laminaribiose and gentiobiose as partial hydrolysis products. After removal of chitin (40), powder X-ray diagrams of the hydroglucan are identical to those of yeast glucan and paramylon. Although we have no further information about the detailed structure or the relative distribution of β-(1 $\rightarrow$ 3) and β-(1 $\rightarrow$ 6) bonds in R glucan, the data point to similarity of the R glucan of *S. commune* to the cell wall glucan of yeast. The action of glucanases on R glucan and yeast glucan, however, reveals that these glucans are not identical (115). Approximately 80 to 96% of the *S. commune* glucan solubilized by alkali is precipitated by neutralization. This glucan was called S glucan, and its X-ray diagram is identical to that of a glucan found in the yeast *Schizosaccharomyces* sp. and in a number of filamentous fungi (40). Preliminary studies (113) suggested β-(1 $\rightarrow$ 6) linkages. Johnston (34), however, has isolated an α-(1 $\rightarrow$ 3)-linked dextrin series from a glucan in *Aspergillus niger* with solubility characteristics similar to those of S glucan. Recently, Bacon et al. (1) used both X-ray diffraction analysis and partial hydrolysis and, comparing the X-ray diagrams, suggested that S glucan is predominantly α-(1 $\rightarrow$ 3) linked. This has been confirmed (deVries and Wessels, *unpublished*). The S glucan of *S. commune* is a highly dextrorotatory polysaccharide (α_D^{25} + 202 in 1 N NaOH). Apart from a small amount of xylose, only glucose, nigerose, and other members of the α-(1 $\rightarrow$ 3)-linked series are detected after partial hydrolysis with formic acid.

Wang and Miles (107) surmised β-(1 $\rightarrow$ 4) linkages in the cell wall glucans of *S. commune*. Their evidence rests entirely on the fact that a commercial cellulase preparation liberates glucose from the wall. It is entirely possible that this crude cellulase preparation also contained other glucanases.

In addition to these cell wall polysaccharides,

most strains of *S. commune* manufacture a slimy material which surrounds the hyphae and diffuses into the medium (86, 106). No data on its composition and structure are available. Morphological mutants have been reported not to produce extracellular slime (87). The above data on cell wall composition and the following data were obtained with dikaryons derived from the "Kniep stock" which exhibit normal hyphal morphology but do not produce slime.

Figure 8 shows quantitative changes in cellular polysaccharides in a wild-type stock (K.8) and in a mutant stock (K.35, cup mutant) with abnormal pileus development (113). The R glucan fraction (i.e., the material insoluble in 1 N KOH at room temperature) is further differentiated into two fractions: one is soluble in boiling 2 N KOH (R_s glucan), and the other resists such treatment (R_i glucan). Both cultures exhaust their exogenous glucose at the 7th to 8th day of culture. The decrease in cellular carbohydrate, concomitant with pileus formation, is due largely to disappearance of R glucan, notably, R_i glucan. In the cup mutant, there is less breakdown of R glucan, and it is tempting to relate this deficiency to the inability of this mutant to form pilei.

This difference in development and R glucan degradation depends, however, on cultural conditions (Wessels, *unpublished*). When the two dikaryons are not grown on the surface of a layer of sand flooded with liquid medium but in static liquid culture, their morphological development is similar. Instead of the wild-type stock producing a few large pilei and the cup mutant none, all fruit-body primordia in both dikaryons produce small pilei. Both cultures can degrade R glucan under these conditions. Other observations (113) support the idea that R glucan degradation may be coupled to normal pileus formation: little R glucan degradation occurs in the wild-type stock growing at 30 C, a temperature at which pileus formation is blocked, nor in a dikaryon with no fruiting capacity (v. Luyk stock).

During carbon starvation, R glucan is broken down in nondeveloping fruit-body primordia and in the dense layer of surface mycelium (i.e., stroma; 113). Presumably, a portion of the degradation product is used as precursors for constructing fully expanded fruit-bodies; degradation of R glucan may fulfill the need for a slow steady supply of carbohydrate to allow for pileus expansion.

In mycelial extracts and in the culture medium of *S. commune*, an enzyme is present which hy-

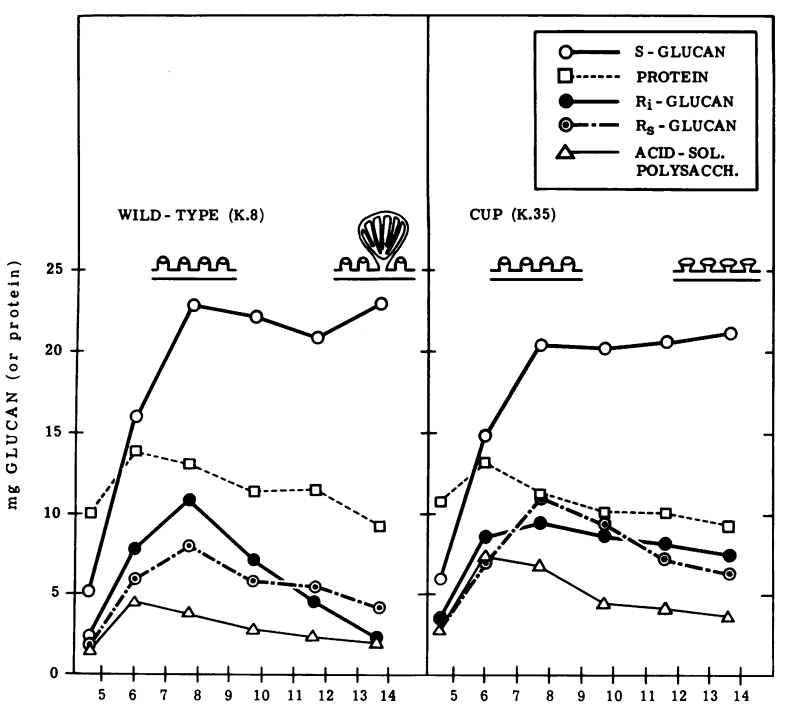


FIG. 8. *Quantitative changes in various polysaccharide fractions and protein during development of S. commune K.8 and K.35 dikaryons on sand-liquid medium (Wessels, unpublished).*

drolyzes isolated R glucan (i.e., R glucanase; 114). Little, if any, activity against S glucan is found. Fractionation of enzyme preparations obtained from the culture medium on Sephadex G-100 and heat-inactivation kinetics (115) have shown that R glucanase is not a laminarinase, and it also seems distinct from a pustulanase normally present. However, since R glucanase also acts on pustulan, it appears that the enzyme is specific for certain 6-substituted glycosyl units in R glucan. The action of R glucanase primarily results in the production of large soluble glucan fragments in which β-(1 $\rightarrow$ 3) linkages are preserved.

R glucanase activity in mycelial extracts and in the culture fluid increases when glucose is exhausted in the medium (114). At several stages of development, the appearance of R glucanase can be enhanced or suppressed by removing or adding glucose. No external nitrogen is necessary for the appearance of R glucanase, nor does the addition of a nitrogen source preferentially enhance the activity of R glucanase (Dons and Wessels, *unpublished observation*). Changes in R glucanase activity are not parallelled by changes in laminarinase, pustulanase, and β-glucosidase activity (117).

Although the appearance of R glucanase explains the degradation of R glucan during pileus expansion, R glucanase is also formed in the wild-type stock K.8 growing at 30 C and in the cup mutant K.35 growing at 25 C (114). In these cases, little R glucan degradation occurs and no pilei are formed. It appears that degradation of R glucan in vivo is also determined by the susceptibility of R glucan in the cell wall to enzymatic attack. Table 1 shows that enzyme preparations from K.8 and K.35 are equally effective in liberating carbohydrate from cell walls of K.8, but both enzyme preparations display much less activity towards cell walls of K.35. Similarly, cell walls prepared from mycelium of stock K.8 grown at 30 C and from young, expanded fruit-bodies are less susceptible to R glucanase than are cell walls from the mycelium of K.8 grown at 25 C (Table 2). Table 2 gives data pertaining to the question of what determined susceptibility of the wall to enzymatic degradation. This may occur in at least three ways: (i) protection of R glucan by a hot water-soluble component, (ii) protection of R glucan by S glucan, and (iii) differences in susceptibility to enzymatic attack of the R glucan proper.

TABLE 1. *Liberation of trichloroacetic acid-soluble carbohydrate from cell walls of S. commune K.8 and K.35 by enzyme preparations from 13-day-old cultures of K.8 and K.35*[a]

Enzyme prepn	Liberation of soluble carbohydrate[b]	
	K.8 cell walls[c]	K.35 cell walls
Intracellular, K.8	19.3	1.9
Intracellular, K.35	20.8	1.9
Extracellular, K.8	21.9	2.7
Extracellular, K.35	21.9	3.3

[a] Data calculated from Wessels (114).

[b] Reactions took place in 0.0365 M phosphate buffer, *p*H 7.0. Liberated carbohydrate is expressed as percentage of the amount produced by action of the same enzyme preparation on isolated R glucan from K.35.

[c] Both cell wall preparations contain the same amount of R glucan.

TABLE 2. *Liberation of trichloroacetic acid-soluble carbohydrate from various cell-wall preparations and extracted products by an extracellular enzyme preparation from S. commune K.8 (Wessels, unpublished)*

Cell walls from[a]	Culture age (days)	Ratio of S glucan to R glucan	Liberation of soluble carbohydrate[b]		
			No treatment	Hot-water extraction[c]	Alkaline extraction[c]
K.8	6	1.18	21.7	51.6	116.0
K.8	10	1.51	24.4	44.8	106.9
K.8, pilei removed	14	2.70	8.0	31.4	94.8
K.8, pilei	14	0.59	15.2	60.8	83.7
K.8, 30 C	10	0.99	16.2	42.7	68.2
K.35	10	0.73	7.4	43.1	100.0

[a] All cell wall preparations were standardized on the same amount of R glucan.

[b] Reactions were performed in 0.05 M McIlvain buffer, *p*H 5.0. Liberated carbohydrate is expressed as percentage of the amount produced by action of the enzyme preparation on isolated R glucan from K.35.

[c] Cell walls were successively extracted with water at 100 C for 10 min and with 1 N KOH at 60 C for 10 min.

In summary, it can be said that exhaustion of the carbon supply in the medium enhances the activity of R glucanase in *S. commune* dikaryons. This enzyme is responsible for R glucan degradation, which appears to be requisite for pileus expansion. Decreased susceptibility of pileus cell walls to enzyme hydrolysis may allow for the construction of pilei while R glucan is being broken down in the cell walls of nondeveloping primordia and of the mycelium.

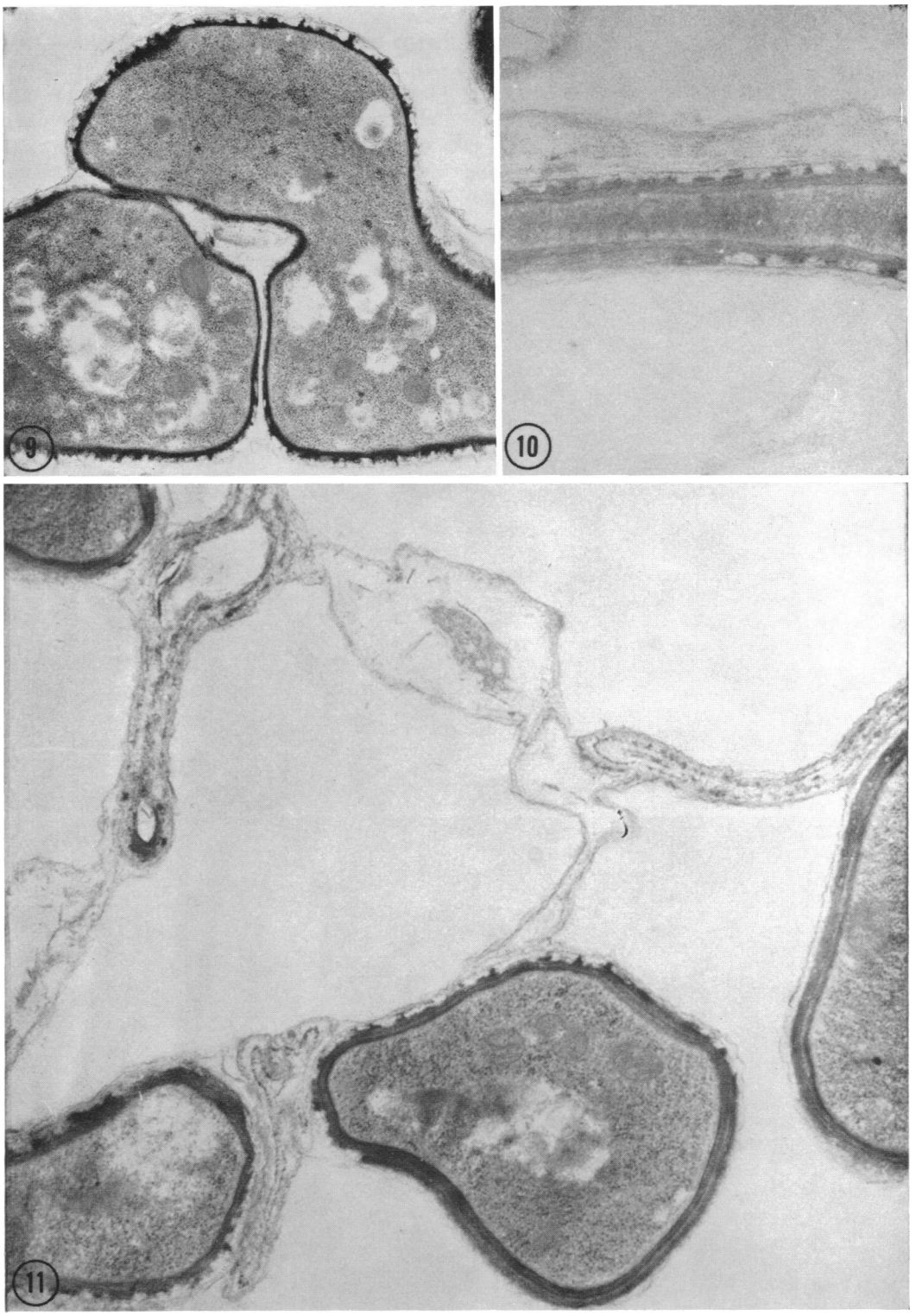


FIG. 9. *Clamp connection of a dikaryotic cell of a fruiting culture of S. commune showing the altered cell wall and normal cytoplasmic contents. Fixation, glutaraldehyde-OsO_4 for Fig. 9–16 (103).* ×*13,000.*

FIG. 10. *Detail of cell wall.* ×*21,000 (103).*

FIG. 11. *Normal cells showing partial cell-wall alteration and collapsed filaments in fruiting culture of S. commune.* ×*21,000 (103).*

ULTRASTRUCTURAL FEATURES OF FRUIT-BODY FORMATION AND SPOROGENESIS

Electron microscopic studies on the transition from vegetative dikaryotic mycelium to sporulating fruit-bodies of *S. commune* have provided additional information concerning the nature of specific cell wall polysaccharide breakdown during morphogenesis (103). Alteration of a cell wall layer occurs in dikaryotic cells of fruit-body

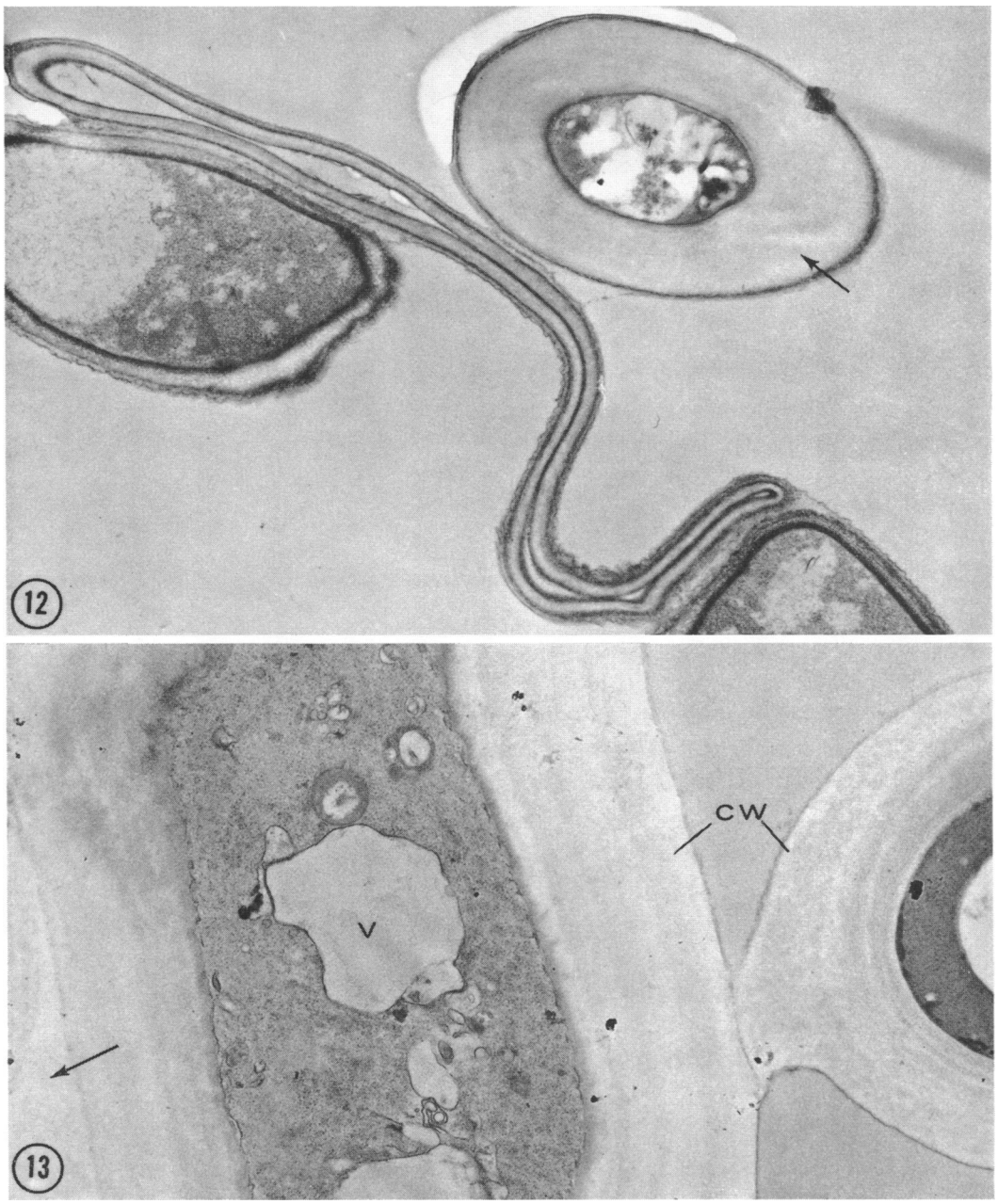


FIG. 12. *Thick-walled stipe cell (arrow) adjacent to a collapsed filament.* ×*10,000 (103).*

FIG. 13. *Stipe cells of dikaryotic fruit-bodies of S. commune. Note thick concentric layering of cell walls (CW) and amorphous extracellular matrix (arrow). Large vacuoles (V) are present in the cytoplasm.* ×*12,000 (103).*

primordia (Fig. 9 and 10). This material rests on a basal layer of cell wall material and is overlaid by a loose fibrous substance. At this stage, the protoplast is still intact whereas neighboring cells change in a manner that leads to collapse of vestigial filaments (Fig. 11). This loss of internal structure may be correlated with previous estimates of cellular nitrogen depletion during fruiting, and it provides insight into the specific cellular events involved. Collapsed threadlike cells occur in the stipe cells of the mushroom *Agaricus campestris* (51). Similar indications of this rela-

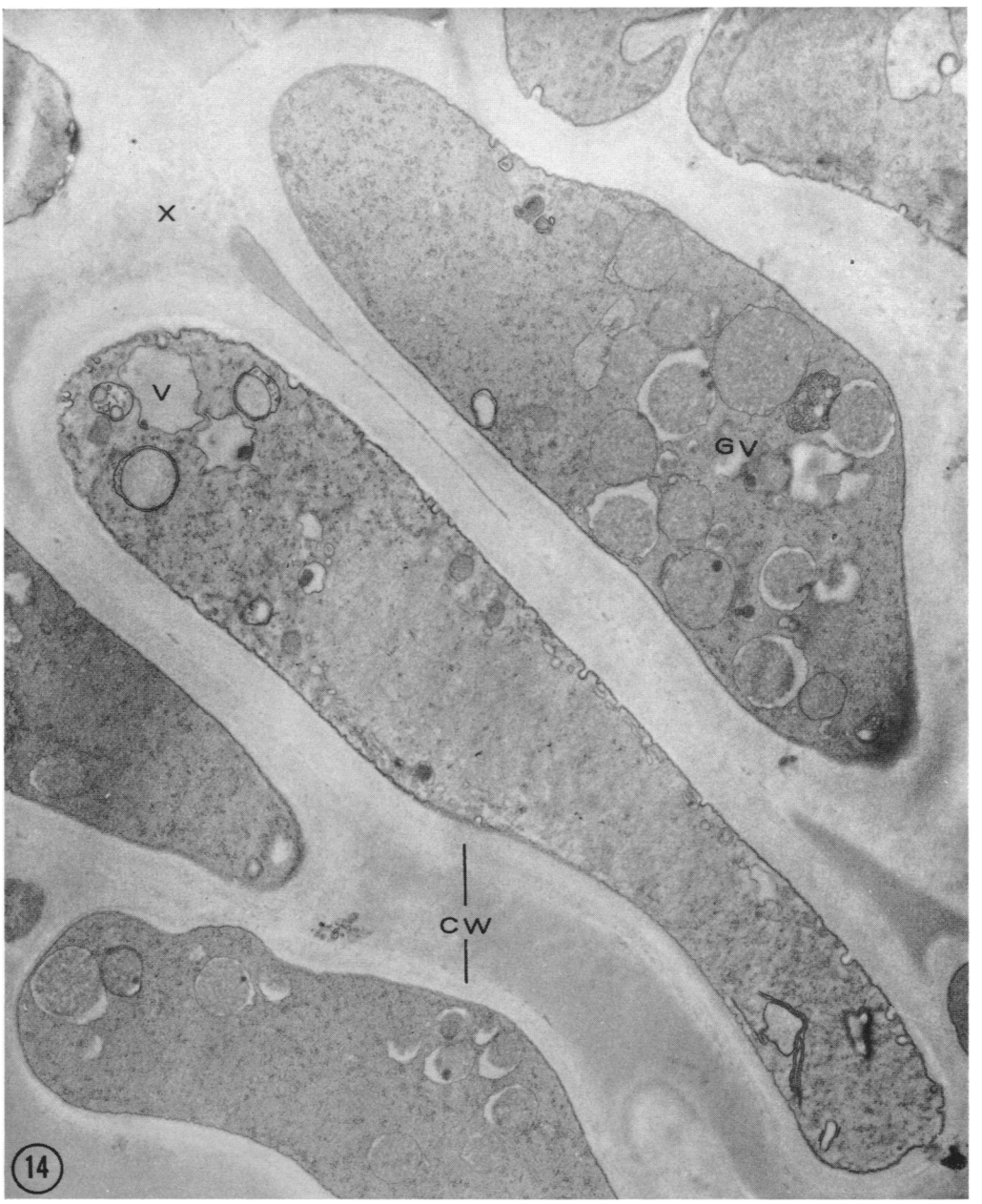


FIG. 14. *Section of cells comprising the subhymenium of dikaryotic fruit-body. Note conventional vacuoles (V) and special vacuoles with granular contents (GV). Thick cell walls (CW) and amorphous extracellular matrix (X) are present.* ×*10,000 (103).*

tionship were seen in *S. commune* (103). A presumptive stipe cell with a thick cell wall is in the vicinity of a collapsed filament (Fig. 12). Ultrastructural studies of the stipe of *S. commune*, as it appears in upright cultures, showed that excessive cell wall biosynthesis is an important feature at this stage of fruiting (Fig. 13). Concentric layers of cell wall are conspicuous, as is amorphous material comprising the extracellular matrix. However, aged vegetative hyphae of *S. commune* do not exhibit serration, excessive cell wall layering as in fruits, or the extracellular material.

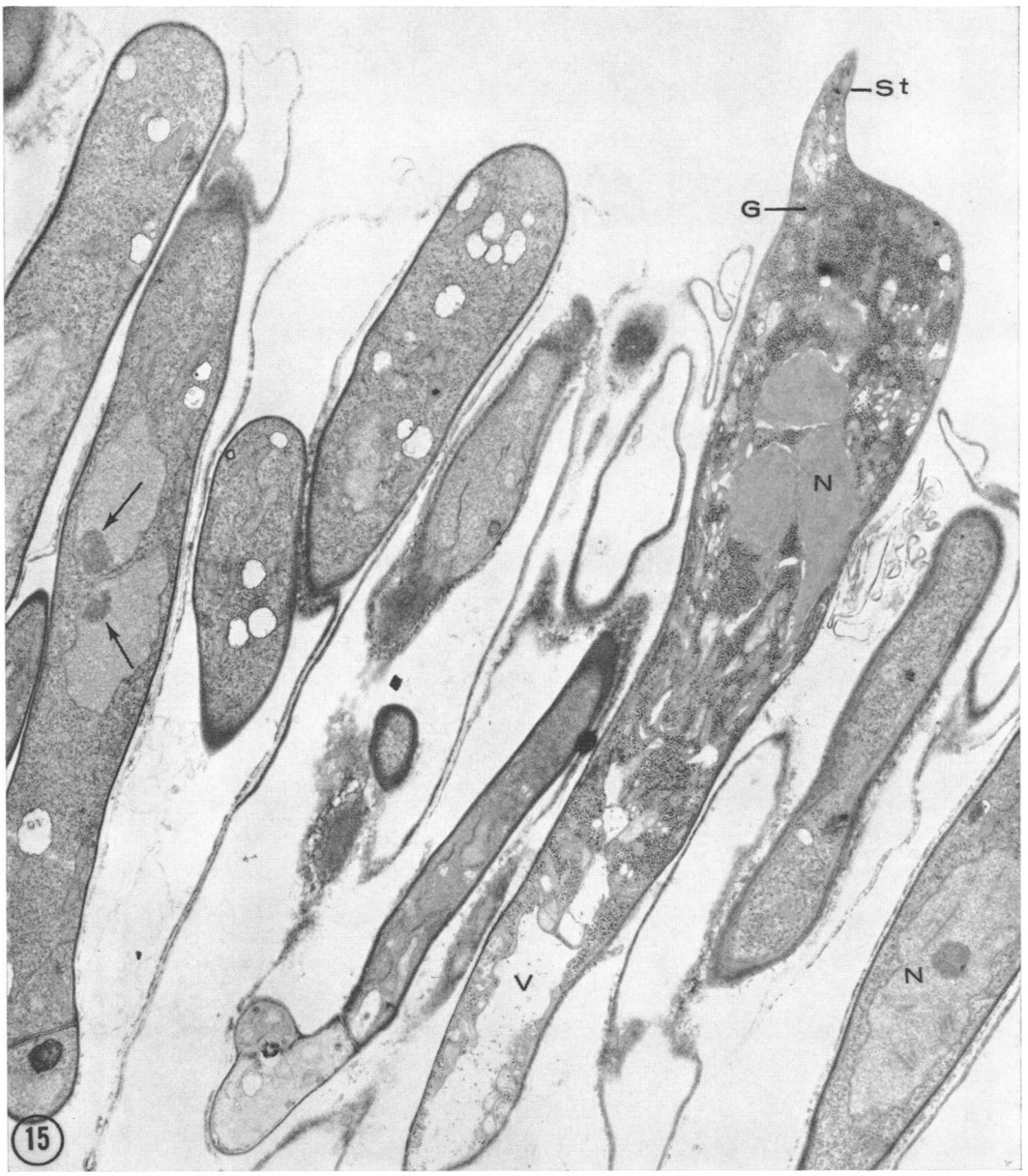


FIG. 15. *Section of hymenium of dikaryotic fruit-body. Nuclei contain a single nucleolus (arrows). The nucleus at the lower right (N) shows tripartite synaptinemal complexes and a persistent nucleolus. The basidium with a sterigma (St) contains abundant glycogen (G), three apical nuclei (N) visible in this section, and a prominent basal vacuole (V).* ×*8,000 (103).*

Increased vacuolation is observed in either stipe cells of the dikaryon or aged hyphae of the homokaryon.

Cells comprising the subhymenium of dikaryotic fruit-bodies of *S. commune* are shown in Fig. 14. Amorphous extracellular matrix is present, and cells of the subhymenium have special vacuoles containing moderately dense granular material. Thinner cell walls also occur here. There is no indication of collapsed hyphae or cell wall alterations.

The nuclei of dikaryotic basidia each contain a

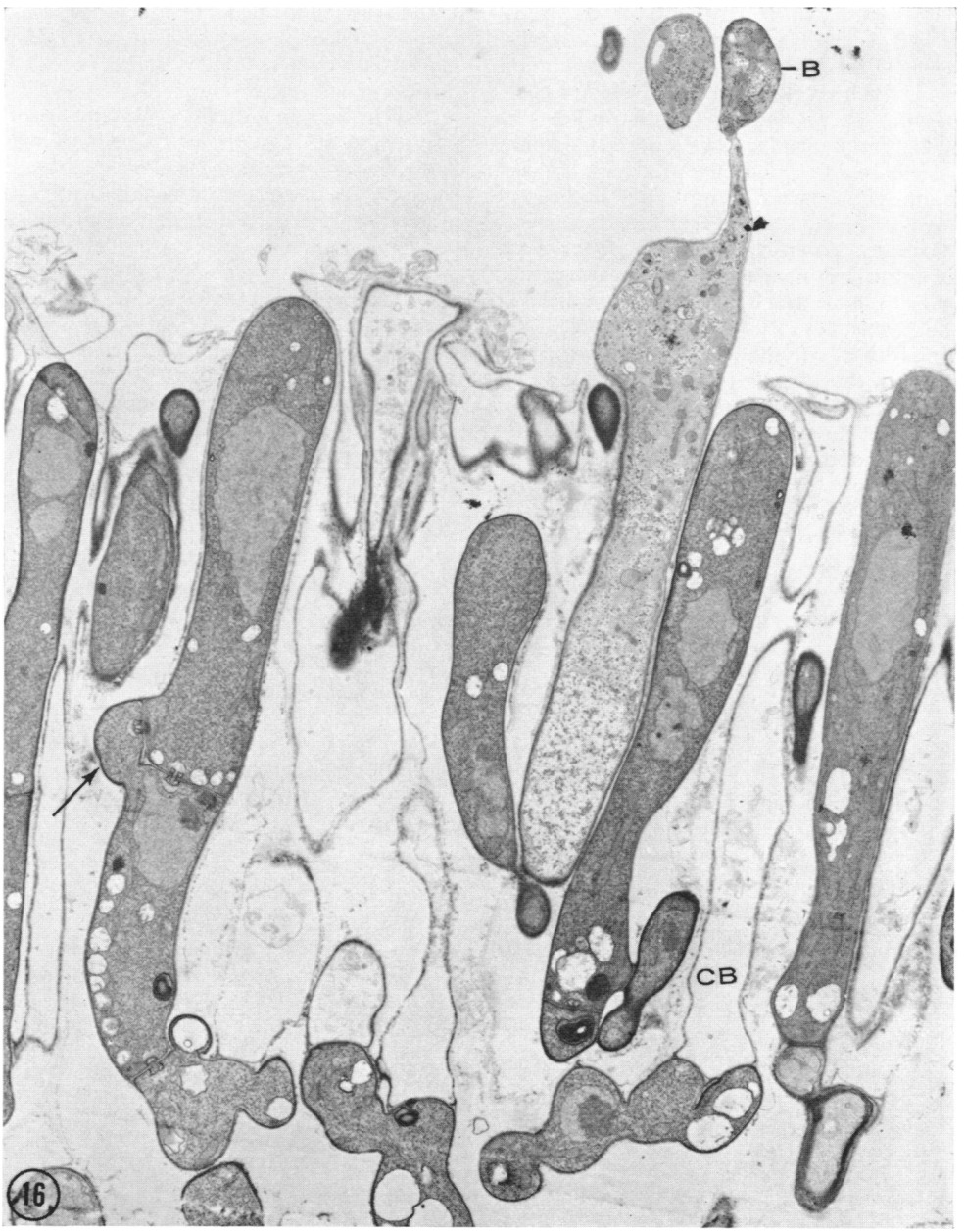


FIG. 16. *Late sporogenesis in hymenium of dikaryotic fruit-body. Note basidiospore (B) attached to sterigma. The clamp connection (arrow) contains complex septa. Cell wall layering is reduced to normal. Collapsed basidia (CB) are numerous.* ×*6,000 (103).*

single nucleolus. Synaptinemal complexes (102) and a persistent nucleolus are present in the nucleus of a basidium undergoing meiosis (Fig. 15). The originally binucleate basidium undergoes nuclear fusion and meiosis to produce four haploid nuclei. Upon formation of sterigmata (*see* Fig. 15), the haploid nuclei migrate to the apex of the basidium and pass through the four sterigmata to enter the basidiospore initials. Glycogen accumulates at this stage of cytodifferentiation, as was noted by Wells (111). Once migrant nuclei are within the presumptive basidiospore, each haploid nucleus divides once, and four haploid binucleate basidiospores are produced by each basidium. The attached presumptive basidiospore is noted in Fig. 16. Collapsed basidia indicate basidiospore discharge. Typical complex septa are in the clamp connections. The extracellular ground substance which is prominent in the stipe and subhymenium is not obvious, and cell wall layering is similar to that in vegetative mycelium. Contrary to the supposed occurrence of a Golgi apparatus in the sporulating fruits of *Coprinus lagopus* (49), no evidence has been obtained for the presence of dictyosomes in *S. commune*.

CELLULAR FEATURES OF BASIDIOSPORE GERMINATION

Characteristics of Germination In Vivo

The basidiospores of *S. commune* are 2.5 μm in diameter and approximately 5 to 6 μm long. They appear white *en masse* (24). Variously described as cylindrical and obliquely apiculate (47), narrowly elipsoid or long ovate, and cylindrical or suballantoid (17), the spores occasionally possess small vacuoles (24). They germinate readily in nutrient solutions (24) but poorly in distilled water (73). The width of the germ tube approximates that of the basidiospore and may emerge at both ends, so that the identity of the original spore is soon lost (2, 24). In this discussion, *germlings* will refer to germinated spores which are undergoing elongation and which have not yet formed a septum to produce the vegetative hyphae.

Basidiospore germination in *S. commune* begins with a characteristic lag phase in outgrowth (59), followed by germ tube extension, nuclear division(s) of the aseptate, binucleate germling, and formation of septa (101). Some of the variation

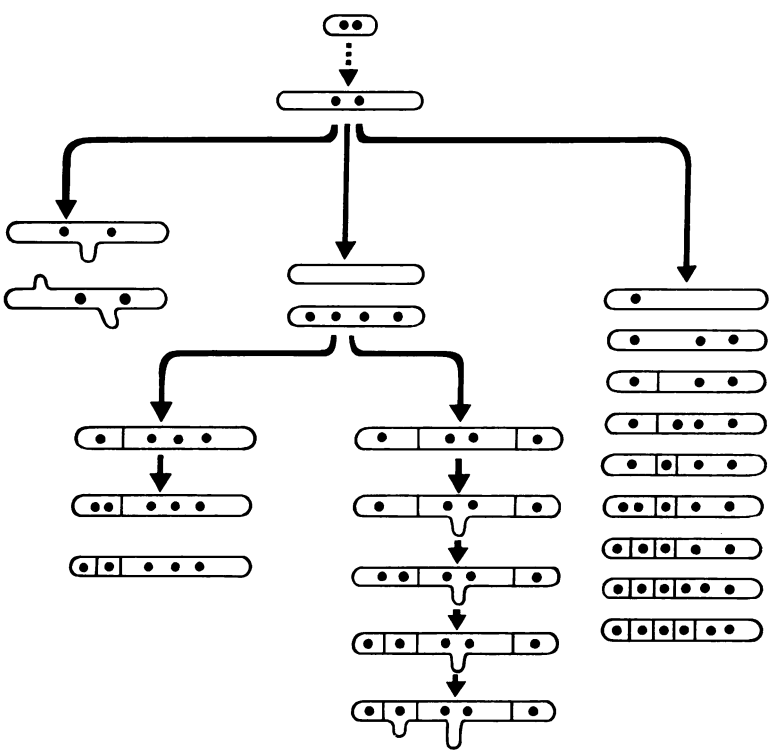


FIG. 17. *Alternate routes of cytodifferentiation in basidiospore germination of S. commune (104).*

which occur among basidiospore progeny of dikaryotic fruit-bodies are shown in Fig. 17. Basidiospores produced from homokaryotic fruit-bodies also show varied routes of cytodifferentiation (Niederpruem, *unpublished*). Eventually, however, the normal homokaryon is established in both cases.

In elongating germlings and vegetative homokaryotic or dikaryotic hyphal apices of *S. commune* seen by phase-contrast microscopy, the nuclei gradually become invisible prior to division and cross-wall formation (33). Several minutes later, nuclear division products appear, often distant from the parental nuclei. Thus, the previously aseptate, binucleate germling now contains either three or four nuclei, depending on whether one nucleus or both nuclei of the original cell divided (*see* Fig. 17). At this point, aggregations of oscillating granules and lashing filaments occur at the site soon to contain a forming cross-wall. Annular ingrowth of wall material ensues, and within a few minutes no further opening is apparent in the septum. Once cross-walls appear, it is possible to measure elongation kinetics and record nuclear behavior of every cell in the young hypha, with the hyphal apices and septa as reference points (104). The functional relationships between all the cells of a septate hypha are frequently neglected because hyphal apices of a colony periphery are most easily studied. In young hyphae of *S. commune*, elongation occurs solely by apical extension of either the main axis or associated branches; no elongation of subterminal cells of *S. commune* has ever been recorded. Usually one main axis-apex predominates, and often a primary branch becomes a leading center of vegetative growth. Moreover, synchronous nuclear division in every cell of a young septate filament has never been observed, even though cytoplasmic continuity could occur by virtue of the septal pore (55, 101).

Although none of the cells behind the hyphal apex of *S. commune* elongates along its main axis, they can initiate primary branches and undergo further nuclear divisions to accommodate these branches. Multicellular hyphae of *S. commune* also contain small subterminal cells, often measuring only 2.5 to 5 μm long; these arise by "intercalary cell division" as shown in Fig. 18 (104). Annular ingrowth of forming septa follows nuclear division(s). Of 110 observations dealing with septum formation in *S. commune*, 7 were delimitations of subterminal cells in young hyphae (e.g., 24-hr cultures) derived from basidiospores. Intercalary cell division of this sort therefore provides a mechanism which explains the origin of very small cells within a highly multicellular filament of *S. commune*. This interesting phenomenon was also described earlier as "secondary septum formation" by Harder (28). The reason

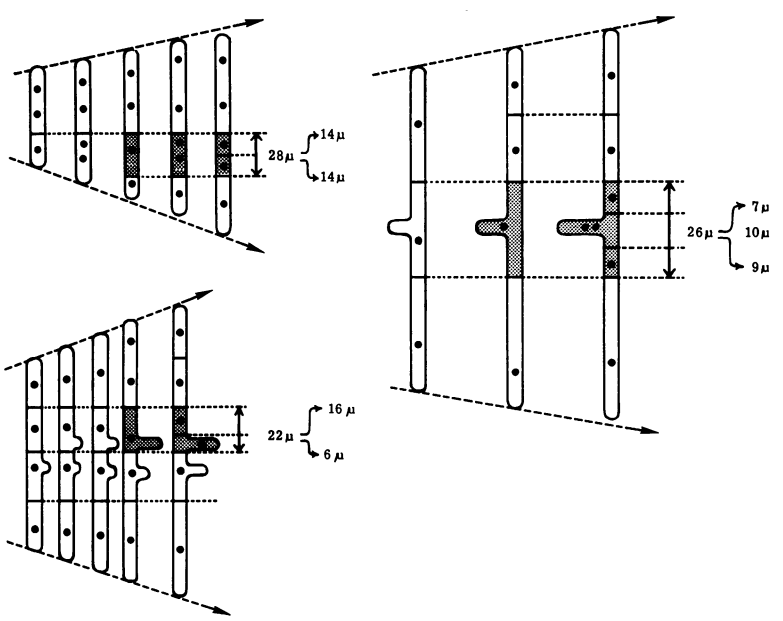


FIG. 18. *Intercalary cell division patterns in young septate hyphae derived from basidiospore germlings of S. commune (104).*

that a mature vegetative colony cleaves successively into smaller and smaller cells within an established hypha is not apparent.

Ultrastructural Aspects of Basidiospore Germination

There is little information on the ultrastructure of germinating basidiospores of *S. commune*. Longitudinal sections of ungerminated basidiospores are shown in Fig. 19 and 20. The cell surface consists of at least two well-defined layers, including an outer, more electron-lucent substance and an inner electron-dense material which, at times, also appears layered. The outer surface is generally amorphous. Occasional glycogen aggregates, some small lipid vacuoles, sparse profiles of endoplasmic reticulum, and conventional mitochondria occur in the cytoplasm. Two nuclei are present in each basidiospore of *S. commune*, and each nucleus appears to have a single nucleolus, as shown in Fig. 20. The nuclei are enclosed by

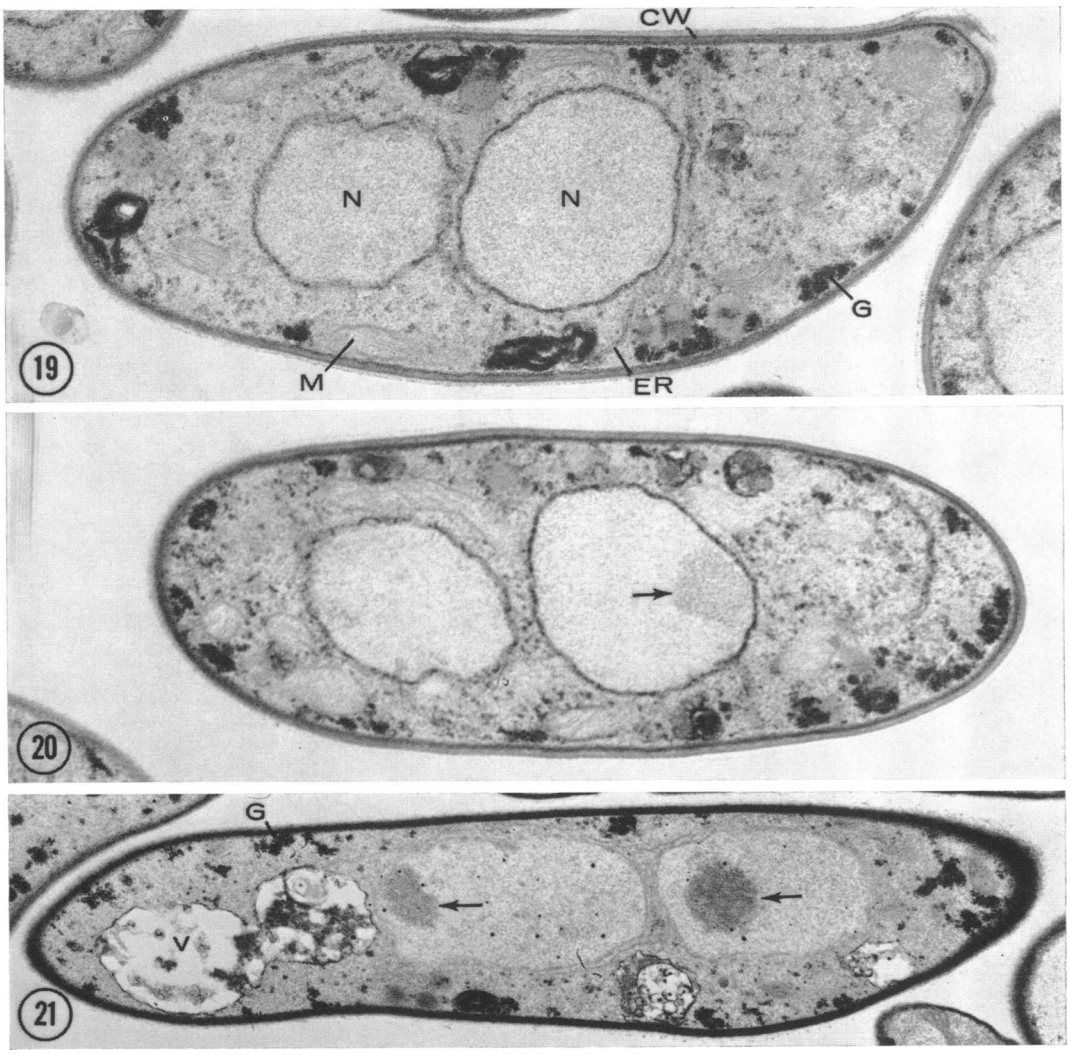


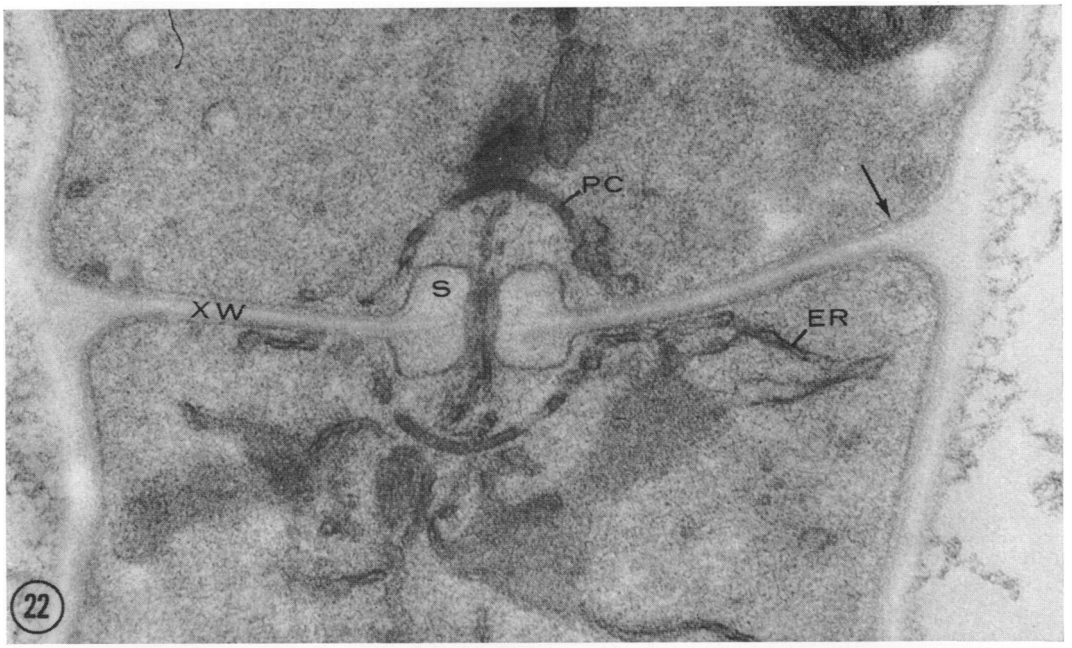
FIG. 19. *Section of ungerminated basidiospore of S. commune. Note two layers in cell wall (CW). The cell contains two nuclei (N), mitochondria (M), endoplasmic reticulum (ER), and glycogen (G). Fixation, glutaraldehyde-OsO_4 for Fig. 19–21. ×23,000 (Fig. 19–25, Jersild and Niederpruem, unpublished).*

FIG. 20. *Section of binucleate basidiospore. Note nucleolus (arrow) in one nucleus (N). ×23,000.*

FIG. 21. *Basidiospore germling of S. commune. Note single nucleolus (arrow) in each nucleus, glycogen (G), vacuoles (V), and absence of complex septa. ×12,000.*

double membranes, and only occasionally are nuclear pores evident.

During the basidiospore germination of *S. commune*, the elongated germling has two nuclei, each with a single nucleolus (Fig. 21). Vacuoles are prominent, as are endoplasmic reticulum and glycogen aggregates. The wall layers are the same as those of the basidiospore. Ribosomes are



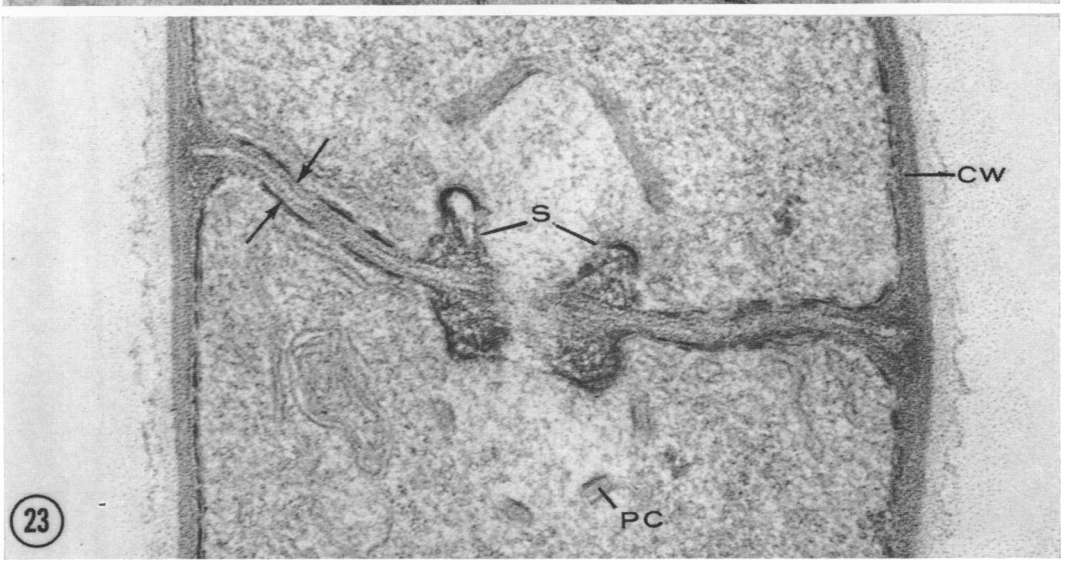


FIG. 22. *Section of a complex septum in basidiospore germling fixed with $KMnO_4$. The septal pore apparatus is a partial elaboration of the cross wall (XW) and consists of a septal swelling (S) and pore cap (PC) which appears continuous with the endoplasmic reticulum (ER). The plasma membrane (arrow) covers the cross-wall and septal swelling.* ×47,000.

FIG. 23. *Section of complex septum in basidiospore germling fixed with glutaraldehyde-OsO_4. Lamellated plates of the cross-wall (arrows) extend into the septal swelling (S). An amorphous substance coats the main hyphal cell wall (CW).* ×68,000.

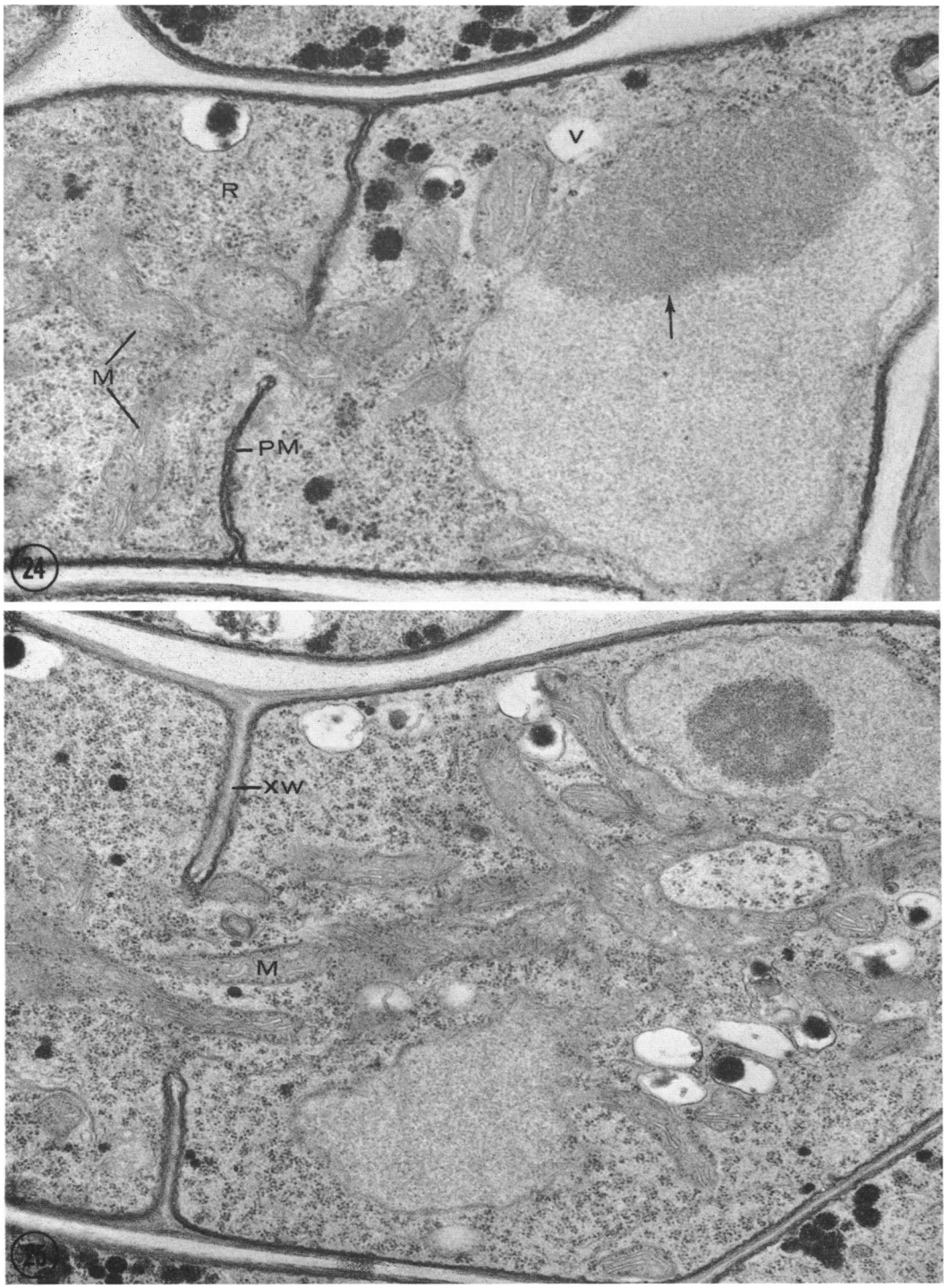


FIG. 24. *Early stage in septum formation. Fixation, glutaraldehyde-OsO_4. Note apparent ingrowth of plasma membrane (PM). The cytoplasm contains numerous mitochondria (M), free ribosomes (R), small vacuoles (V) some with dense granular content, and nucleus with contained nucleolus (arrow).* ×30,000.

FIG. 25. *Later stage in septum formation. Fixation, glutaraldehyde-OsO_4. Note primary cross-wall elaboration (XW) and abundant mitochondria (M) in septal opening.* ×22,000.

abundant in basidiospores and germlings, but no clear evidence for polysomes has been obtained.

The formation of cross-walls marks the transition from an elongate, binucleate germling to a septate hypha of *S. commune* (Fig. 22 and 23). In $KMnO_4$-fixed material (Fig. 22), the lack of the cell wall character and septum detail stands in contrast to the delineation of the cell membrane and dome-shaped membranes (i.e., septal pore caps) which form regularly perforated enclosures over the pore. These membranes are continuous with the endoplasmic reticulum, an attribute shared by many Basidiomycetes (cf. 9). Material is trapped within the septal pore and subcap matrix. Further ultrastructural aspects of the complex septum of *S. commune* are revealed by aldehyde-osmium (Fig. 23). Electron-dense lamellated plates, which arose presumably from the similarly dense inner wall of the main hyphal axis, extend into the septal swellings.

Details of complex septum formation in germlings of *S. commune* are not known, although sections of vegetative hyphae reveal the possibility that annular ingrowth of the cell membrane (Fig. 24) precedes cross-wall formation (Fig. 25). Mitochondria in the region of septum formation correlate with in vivo observations of oscillating granules and filaments in this area. No evidence is available concerning the mode of origin of either the septal swellings or septal pore caps in *S. commune*. In *Rhizoctonia solani*, the septal pore complex does not form until annular ingrowth of the cross-wall ceases, and then subsequent swelling completes the septal pore apparatus (10). The nature of the biochemical regulatory devices governing the appearance of complex septa only after considerable cell elongation and subsequent nuclear division(s) is unknown.

BIOCHEMICAL CHANGES IN GERMINATION

Respiratory Physiology of Basidiospores

Whereas Q_{O_2} values for resting spores of *Phycomyces blakesleanus*, *Neurospora tetrasperma*, and *Puccinia graminis tritici* are 1.9, 0.4, and 1.6, respectively (cf. 93), the basidiospores of *S. commune* show Q_{O_2} values of 10 to 20 (57). Consequently, metabolic dormancy, relative to basal respiration, is not a feature of *S. commune*. The fact that the uncoupling agent 2,4-dinitrophenol (DNP) accelerates oxygen consumption suggests that respiration and oxidative phosphorylation are already tightly coupled in the ungerminated basidiospore. Respiratory capacity is further indicated by oxidative responses to exogenous substrates. Respiratory rates are highest with glucose, fructose, mannose, L-sorbose, xylose, and acetate, although sucrose and maltose are also stimulatory (57). Other sugars are inactive, as are various amino acids and sugar alcohols (polyols). However, ungerminated basidiospores of *S. commune* readily incorporate labeled amino acids into trichloroacetic acid-precipitable material, and this process is arrested by cycloheximide. Thus, the potential for protein synthesis also occurs in these propagules. The inhibitory effects of metabolic poisons on glucose-enhanced respiration of basidiospores are similar to those shown previously for vegetative mycelium of *S. commune* (60); oxygen consumption is sensitive to cyanide, antimycin A, Atabrine, and phenylmercuric acetate. These findings implicate the operation of cytochrome oxidase, *b*- and *c*-type cytochromes, flavoprotein, and enzyme(s) dependent on sulfhydryl groups in the ungerminated basidiospore. Thus, initial attempts to discern respiratory peculiarities in germination confirmed that the components of a conventional respiratory chain also occur in ungerminated basidiospores of *S. commune* (57). The functional role of these components in cytodifferentiation is indicated by the finding that germination is curtailed by azide and DNP (59).

Respiratory Changes in Germination

The respiratory characteristics of ungerminated basidiospores provide a basis for examining similar features in germlings of *S. commune*. Although stimulatory sugars for spore respiration are also active in germling oxidation, some sugar alcohols only enhance the oxygen consumption of cells which have passed through the lag phase of germination. Active substrates include mannitol, arabitol, and xylitol, but galactitol and inositol are not stimulatory. Moreover, enzymes active in carbohydrate metabolism (65) and polyol oxidation (61) already exist in the ungerminated basidiospore of *S. commune*. Consequently, there is no support for the de novo origin of polyol oxidation systems during germination on glucose. On the other hand, germination on *meso*-erythritol as sole carbon source leads to a nicotinamide adenine dinucleotide (NAD)-dependent erythritol dehydrogenase (32). We view this as a conventional example of enzyme induction, and it probably bears no direct relation to changes in polyol oxidation during glucose germination.

Although there is no evidence that polyol oxidation systems arise during germination on glucose, other explanations are possible. For example, activation in vivo of preformed polyol oxidation systems, changes in cellular permeability to sugar alcohols, the de novo origin of ancillary enzymes of polyol and carbohydrate metabolism, and changes in the intracellular ratio of reduced/oxidized coenzymes have not been excluded. Further

insight into the role of sugar alcohols in *S. commune* has been gained from nutritional studies of basidiospore germination.

Nutritional Correlates in Germination

Although inferior to glucose, polyols are also used as sole carbon sources for germination. Active polyols include mannitol, sorbitol, ribitol, xylitol, arabitol, and *meso*-erythritol (59). With the exception of erythritol dehydrogenase, oxidative enzymes for all of these polyols already occur in the basidiospore. Consequently, specific uptake systems for polyols may arise under these circumstances. In this connection, a lag phase related to uptake of mannitol but not glucose is observed in vegetative mycelium of *S. commune*, whereas subsequent incorporation of mannitol is arrested by prior incubation with cycloheximide (Braun and Niederpruem, *unpublished*). Thus, enzymes of polyol oxidation are present throughout development, although specific uptake systems may arise in *S. commune* when exogenous polyols are present. In *Coccidioides immitis*, mannitol supports mycelial growth although it is inactive for spherules of this dimorphic fungus; mannitol-1-phosphate dehydrogenase occurs in extracts of both forms (48).

Carbohydrates and Polyols

Alternatively, the failure of exogenous polyols to stimulate basidiospore respiration in *S. commune* may reflect an intracellular reserve of sugar alcohol(s), thereby saturating extant polyol oxidation systems. Glucose germlings would then be expected to deplete their sugar alcohol reserves during the lag phase of outgrowth.

Glucose, fructose, glycerol, mannitol, and arabitol are present in the free carbohydrate pool of vegetative mycelium of *S. commune*. During basidiospore formation, reducing sugars and glycerol decline and mannitol and arabitol predominate in association with trehalose (63; W. B. Aitken and D. J. Niederpruem, Bacteriol. Proc., p. 31, 1968). The important role of these substances in dormancy is indicated by their wide distribution in fungi (46). However, metabolic roles of trehalose and polyols in germination and the nature of cellular controls governing their synthesis and catabolism are unknown.

In germinating basidiospores of *S. commune*, changes in intracellular polyols and trehalose stores can be detected, because little or no germination ensues if either the sole nitrogen source or carbon source is withheld from liquid medium (59). Mannitol and arabitol account for approximately 10% of the spore dry weight, and trehalose amounts to an additional 12%. The amount of glucose or fructose is insignificant. Decreases in total polyols and trehalose occur in the presence of exogenous carbon and nitrogen sources in liquid medium (Aitken and Niederpruem, Bacteriol. Proc., p. 31, 1968) so that starvation cannot explain the mobilization of these reserves.

The major decrease in polyols and trehalose occurs during the lag phase in germination. Germlings on glucose medium show respiratory stimulation by exogenous polyols. After the lag phase in germination on glucose, "glycogen" increases, and the ratio of the two cell wall glucans of *S. commune* changes (Aitken and Niederpruem, Bacteriol. Proc., p. 31, 1968). Thus, mobilization of intracellular reserves precedes cell wall glucan biosynthesis.

Dormant conidia of *Aspergillus oryzae* contain large amounts of mannitol relative to trehalose (30). Since trehalase occurs in conidial extracts and is competitively inhibited by mannitol in vitro, trehalase may be active only after mannitol depletion during germination (31). In mycelial extracts of *S. commune*, trehalase activity in crude or dialyzed extracts from glucose or trehalose cultures is not affected by mannitol and other sugar alcohols (120). Since even cellular growth on trehalose is unaffected by mannitol in *S. commune* (120), it is unlikely that sugar alcohols regulate glycosidase in vivo. Therefore, other regulatory mechanisms must be sought to explain trehalose mobilization in basidiospore germination.

Regulation of Glutamate Dehydrogenases

Ammonia (NH_3) can serve as the sole nitrogen source for pigment formation (95), mycelial growth and dikaryotization (62), and basidiospore germination (59) in *S. commune*. However, information was lacking to explain the route of NH_3 assimilation and the nature of associated regulatory devices throughout cytodifferentiation. Nicotinamide adenine dinucleotide phosphate (NADP)-dependent glutamate dehydrogenase (GDH) was found in mycelium of *S. commune* cultured on glucose-yeast extract (22), but the question remained regarding the role of exogenous ammonia in governing GDH. Unlike mammalian GDH which functions with either NAD or NADP (cf. 25), separate GDH proteins occur which are specific for either NAD or NADP in fungi. Moreover, the nature of ammonia regulation of GDH is related to specific stages of fungus development. Conidia of *Neurospora crassa* lack the NAD-GDH and only contain low NADP-GDH; upon germination, the NAD-GDH emerges but the NADP-GDH remains higher in specific activity (85).

Since two separate GDH activities of *S. com-*

mune show parallel increases in specific activity during mycelial growth on glutamate medium, as compared with increased NAD-GDH and depressed NADP-specific GDH on ammonia (18), the situation in *N. crassa* is not generally valid for other fungi. Moreover, ammonia represses the NAD-specific GDH in yeast (7, 8, 29, 67, 118), which is also contrary to that of mycelium of *S. commune*. Since these comparative biochemical studies revealed basic differences in the regulation of GDH in *N. crassa*, yeast, and *S. commune*, control of GDH activities was studied during basidiospore germination, mycelial growth, and dikaryotization of *S. commune* (19).

Role of Glutamate Dehydrogenases

The changes in specific activity of the two separable glutamate dehydrogenases during basidiospore germination of *S. commune* on either ammonia or glutamate as sole nitrogen sources are shown in Fig. 26. During the lag phase in germination, an increase in the specific activity of the NADP-GDH is striking, and the NAD-specific enzyme shows a marked depression. Similar changes occur during glucose germination sustained by other nitrogen sources as well. It appears that ammonia regulation of the NADP-GDH does not operate early in basidiospore germination, and the depression of the NAD-GDH by ammonia resembles that in yeast (29). As germling elongation continues, the NADP-GDH decreases (*see* Fig. 26). This continues in vegetative mycelium of several strains of *S. commune* in that each of these glucose-ammonia cultures has a depressed NADP-GDH and higher specific activity of the NAD-coupled enzyme. These data suggest that ammonia regulation of the NADP-GDH occurs in vegetative mycelium of *S. commune* but is absent during early differentiation of the basidiospore.

Homokaryotic mycelium cultured on glutamate medium shows parallel increases in both GDH activities of *S. commune*, in contrast to a depressed NAD-specific GDH seen during glutamate germination. Dikaryotic mycelium of *S. commune* cultured on glucose-ammonia shows ratios of near unity for NAD- and NADP-coupled GDH (19). The observation of parallel GDH activities also rules out the possibility that reciprocal regulation occurs throughout development. Recent studies of the two GDH activities in *N. crassa* development also fail to support the idea of invariable reciprocal regulation (91). As a whole, these investigations show that the regulation of two separate GDH activities in both *Neurospora* and *Schizophyllum* is qualitatively different during cytodifferentiation.

Ammonia regulation of the NADP-specific GDH in mycelium of *S. commune* appears to operate at the level of protein synthesis, because the shift from a low NADP-GDH in ammonia

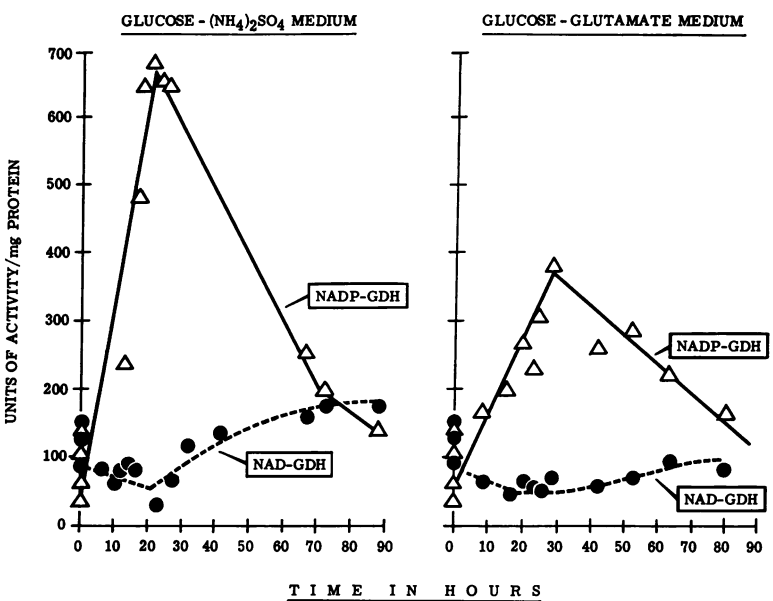


FIG. 26. *Changes in specific activities of two glutamate dehydrogenases of S. commune during basidiospore germination on culture medium containing either* $(NH_4)_2SO_4$ *or* L*-glutamate as sole nitrogen source. After Dennen and Niederpruem (19).*

medium to a high enzyme activity in glutamate medium is depressed by cycloheximide, 5 μg/ml (19). This drug also causes total cessation of the development of NADP-GDH in conidia of *N. crassa* (100). However, the rate of glutamate oxidation in vivo during differentiation of the slime mold *Dictyostelium discoideum* is thought to be governed by endogenous substrates (14, 124). The physiological basis for the depressed NAD-GDH in basidiospore germination of *S. commune* is not known, although the lack of ammonia regulation of the NADP-GDH could be explained by low amounts of intracellular ammonia at this stage (19).

Further control of glutamate metabolism may involve availability of coenzymes; the NADP nucleotidase of *Neurospora* conidia is elevated in activity compared with mycelia (110, 125), whereas the NAD nucleotidase decreases as the two GDH enzymes increase in *Neurospora* development (91). In addition to these regulatory devices and subsequent changes during development, it has been suggested that glutamic acid, not ammonia, is the repressor for NADP-GDH synthesis in the yeast *Hansenula subpelliculosa* (R. Ambrosine and L. R. Hedrick, Bacteriol. Proc., p. 127, 1967).

As in *Neurospora crassa* (90), guanosine triphosphate (GTP) specifically inhibits the NAD-GDH of *S. commune*. Moreover, L-glutamine inhibits the NADP-dependent oxidation of L-glutamate (19). Whereas GTP inhibition of the NAD-GDH occurred throughout development, germling extracts of *S. commune* showed an NADP-GDH that was resistant to L-glutamine (19). Thus, in addition to mechanisms involving protein synthesis, endogenous metabolites may also play differential roles in governing preformed GDH during development of *S. commune*.

ULTRASTRUCTURE AND METABOLISM IN HETEROKARYOSIS

The establishment of a dikaryon, composed of binucleate cells and clamp connections, occurs only after anastomosis of hyphae between two strains bearing nuclei containing dissimilar *A* factors and dissimilar *B* factors. The ultrastructural features of the complex septa in a clamp connection are shown in Fig. 27. The septal swellings and associated pore caps are similar to the complex septa seen in the homokaryon (33). Reciprocal nuclear exchange and migration occur during such a mating (88, 89), and important questions arise regarding the motive forces and guiding influences for nuclear movement as well as the manner in which nuclei traverse complex septa. Little is known of the motive forces responsible for nuclear migration in *S. commune*, and

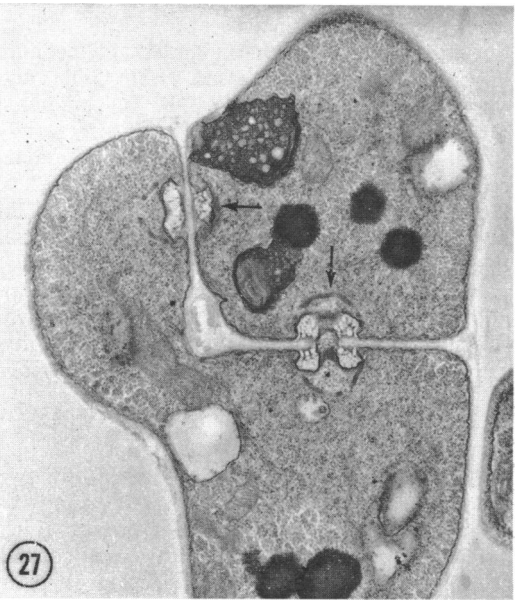


FIG. 27. *Section of a clamp connection of a vegetative dikaryon of S. commune. Fixed with glutaraldehyde-OsO_4. A complex septum occurs in both portions of the clamp (arrows).* ×*15,000 (Jersild and Niederpruem, unpublished).*

there is no evidence for vigorous protoplasmic streaming. Lange (41) reported simultaneous movement of nuclei and cytoplasmic granules after dissolution of septa in *Polystictus versicolor*. In *Rhizoctonia solani*, in comparison, cytoplasmic streaming may expedite the passage of organelles via intact basidiomycetous septa. The diameter of the septal pore apparently increases during streaming, and organelles can be seen passing through the pore and pore caps in living hyphae (10, 11). The possibility that the "centriole" is responsible for motility of fungal nuclei has also been proposed (121), but there is no ultrastructural evidence for true centrioles in basidiomycetous fungi. Surveys of other fungi exhibiting nuclear movements are presented elsewhere (27, 119).

In contrast to the idea that nuclei are squeezed through elastic septal pores is the view that the septal pore apparatus degenerates to accommodate the passage of migrant nuclei. The occurrence of "half" or "three-quarter" septa is documented during nuclear migration in *Typhula erythropus* (42), *T. trifolii* (66), *S. commune* (74), *Puccinia recondita* (23), and *P. versicolor* (41). Recent ultrastructural studies of heterokaryotic mycelia of *Coprinus lagopus* show that, in addition to complex septa, intermediate stages in septum disorganization occur in every mating

where the two parental mycelia bear different *B* factors (26). These findings led to the suggestion that a primary function of the *B* mating-type factor may be complex septum disorganization, which would thus facilitate subsequent nuclear migration.

In *S. commune*, at least two mating situations are possible in addition to a cross which yields a dikaryon; these include the common-*A* mating ($A = B\neq$) which shows extensive bilateral nuclear migration and the common-*B* mating ($A \neq B=$) which exhibits little or no migration. The additional finding that an *AxBmut* homokaryon of *S. commune* mimics the common-*A* heterokaryon by virtue of an erratic nuclear distribution and extensive disruption of septa (74)

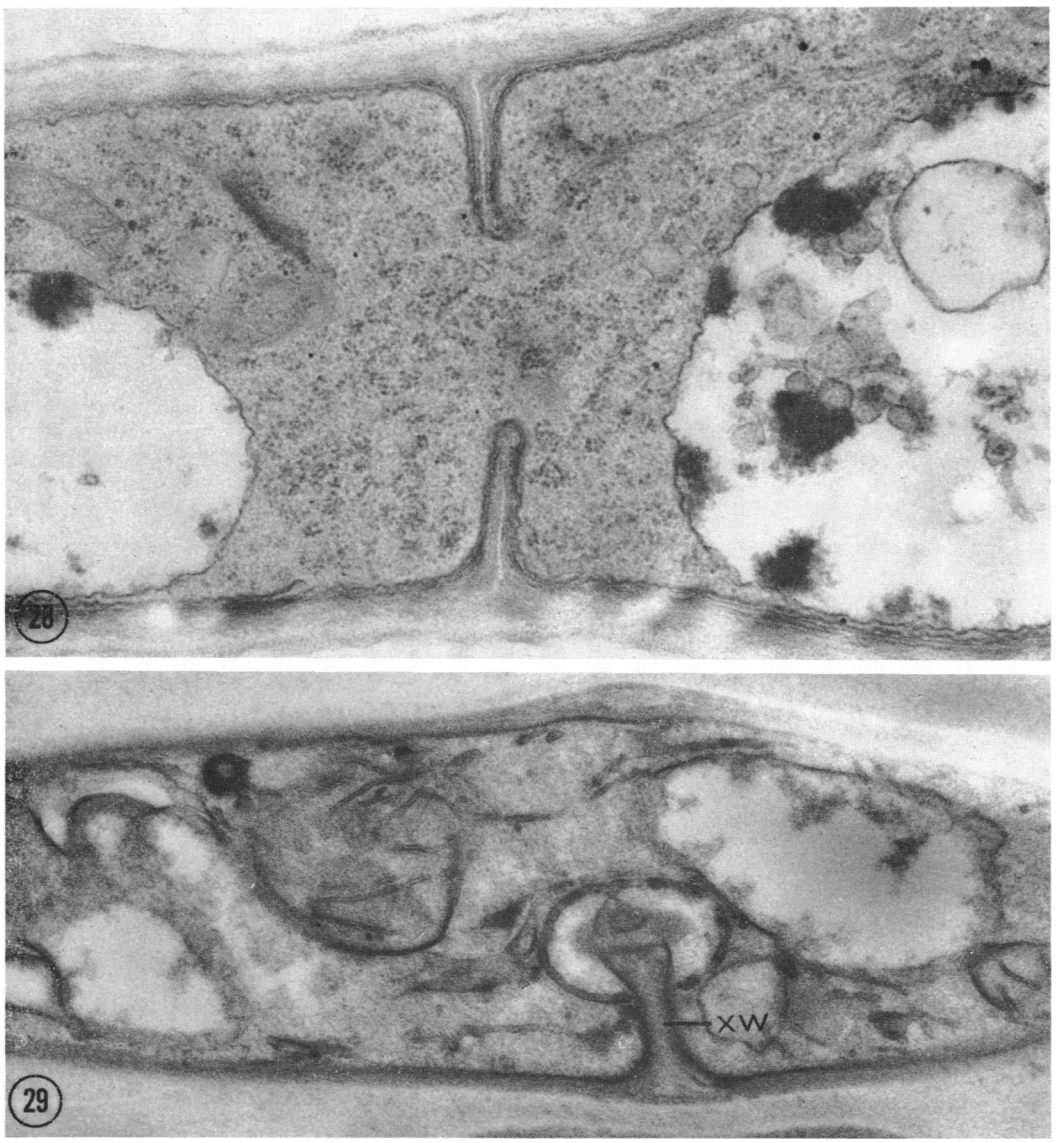


FIG. 28. *Section of a simple septum of a common-A heterokaryon of S. commune fixed with $KMnO_4$. An absence of the septal swelling and septal pore cap is evident. ×30,000 (Jersild and Niederpruem, unpublished).*

FIG. 29. *Partial septum of a common-A heterokaryon of S. commune fixed with $KMnO_4$. The cross-wall (XW) is present only along the lower margin of the cell. A portion of the septal pore complex remains, whereas upper components are absent. ×30,000. After Jersild, Mishkin, and Niederpruem (33).*

lends further credence to the idea that the *B* incompatibility factor governs septum disorganization and hence expedites nuclear migration.

Information on septum disruption came initially from comparative ultrastructural studies of septa in the homokaryon and heterokaryons of *S. commune* (33). Whereas complex septa occur in the homokaryon and established dikaryon, simple septa are abundant in the common-*A* heterokaryon (Fig. 28). Since cross-wall formation occurs by annular ingrowth in living cells of *S. commune*, this may merely represent a formative stage in septum synthesis. The common-*A* heterokaryon is also endowed with partial or "three-quarter" septa (Fig. 29). Because cross-walls do not arise in this unequal fashion (33), the conclusion seems inescapable that partial dissolution of complex septa results in these structures in *S. commune*. Studies of nuclear behavior in aberrant multinucleate basidiospore-germlings of *S. commune* demonstrate that nuclei can pass between adjoining cells at the juncture of the septum with the main hyphal wall in vivo (64). "Half-septa" also accommodate passage of organelles and nuclei in hyphae of the Basidiomycete *Puccinia recondita* (23) and in the *B*-mutant strains of *S. commune* (37). Serious alternatives are therefore offered to the ideas that flexible nuclei squeeze through membrane discontinuities and penetrate intact, elastic septal pores in *S. commune*.

Recent kinetic studies involving cytological observations of nuclear behavior in a living *AxBmut* homokaryon of *S. commune* reveal that extensive disruption of septa occurs in subterminal cells before nuclear movements. This is followed by rapid basipetal migration of several nuclei at nearly 1 mm/hr (58). During these events, no cytoplasmic streaming was seen nor were any correlations noted between nuclear migration and the activities of cytoplasmic granules and filaments. The nature of motive forces for nuclear migration in *S. commune* remains open. On the other hand, genetic control of a specific cell wall lytic enzyme, possibly active in disrupting complex septa, has been demonstrated in *S. commune* (117).

Although genetic and morphological information has clearly shown the role of incompatibility factors in sexual morphogenesis of *S. commune*, only a beginning has been made in studying the biochemistry of the system. There are differences in the proteins of a homokaryon and a dikaryon, as demonstrated by comparing protein patterns by use of immunological or electrophoretic techniques (21, 77, 108). However, an understanding of the biochemical events underlying morphogenesis and its genetic control requires the identification of specific proteins which are involved. Such proteins should meet two criteria: they must be formed under the influence of the incompatibility factors, and their activity must be causally related to a morphological effect.

R glucan degradation in the mycelium is a prerequisite for pileus expansion (113), and R glucanase is subject to catabolite repression (114). Normal fruiting is usually a prerogative of the dikaryon. Therefore, the possibility that the pattern of R glucanase regulation in the dikaryon is confined to this type of mycelium was investigated (117).

In enzyme extracts from several homokaryons, R glucanase activity is low and, unlike the dikaryons, there is little or no increase in activity after glucose is exhausted from the culture medium. In a common-*A* mating, but not in a mating leading to a common-*B* heterokaryon, the specific activity of R glucanase increases 30-fold while glucose is present in the medium (117). Similarly, various common-*A* heterokaryons exhibit a high R glucanase activity in the presence of extracellular glucose (117). These data, if interpreted in terms of enzyme synthesis, suggest permanent repression of R glucanase in the homokaryons and probably also in the common-*B* heterokaryon, derepression in the common-*A* heterokaryon, and catabolite repression in the dikaryon.

Similar differences in enzyme regulation are not found for enzymes hydrolyzing laminarin [β-D-(1 $\rightarrow$ 3)-glucan], pustulan [β-D-(1 $\rightarrow$ 6)-glucan], cellobiose [β-D-(1 $\rightarrow$ 4)-linked], and *p*-nitrophenyl-β-D-glucoside (117). Apart from showing that the pattern of regulation for R glucanase can be assigned at the most to only a few enzymes, this emphasizes the specificity of the enzyme R glucanase that cannot be assayed with laminarin or pustulan as substrates, although apparently it hydrolyzes either a β-(1 $\rightarrow$ 3) or a β-(1 $\rightarrow$ 6) linkage in R glucan (115).

In a mutant dikaryon K.35, a high R glucanase activity does not result in extensive R glucan degradation in the wall; differential protection of R glucan in the wall also plays a role (114; *see also* Tables 1 and 2). Therefore, it is important to note that after interaction of the homokaryons in mating combinations, the ratio of S glucan to R glucan in the cell wall of the dikaryon is similar to that of the homokaryons but is elevated almost threefold in the common-*A* heterokaryon. A smaller increase in the ratio of S glucan to R glucan in a common-*B* mating cannot be attributed to increased activity of R glucanase (117).

Homokaryons carrying mutations in the *B* factor, in the *A* factor, or in both factors are

morphological mimics of common-*A* heterokaryons, common-*B* heterokaryons, or dikaryons, respectively (70, 76). There is a direct relationship among the *B* factor, R glucanase activity, the ratio of S glucan to R glucan, and hyphal morphology (116). Extracts of the *AxBmut* homokaryon display exceptionally high R glucanase activities throughout development. At the same time, the ratio of S glucan to R glucan is about twice that of a normal homokaryon. Wild-type homokaryotic morphology can be restored in these mutants by a secondary mutation in the *B* factor (*AxBmut-mut*; 76) or by the simultaneous presence of an unlinked modifier mutation (*AxBmut-MIV-11*; 74). Both mutations have been found to lower the R glucanase activity in extracts, and after these mutations the cell wall contains normal, wild-type proportions of S glucan and R glucan. The fact that the *MIV* mutation does not prevent a rise in R glucanase activity after exhaustion of the glucose supply in the medium suggests that it is not a mutation in a structural gene for R glucanase. A mutated *A* factor in a homokaryon with a mutated *B* factor (*AmutBmut*) also restores low R glucanase activities in extracts and lowers the S glucan to R glucan ratio. Homokaryons with a mutation only in the *A* factor resemble the common-*B* heterokaryon with regard to an elevated ratio of S glucan to R glucan (although not as high as in a common-*A* heterokaryon or an *AxBmut* homokaryon) that is not correlated with enhanced R glucanase activities. None of the examined mutations significantly influences the net synthesis of the cell wall polymers S glucan and chitin. All homokaryons used in this investigation (116), with the exception of the secondary mutant of the *B*-factor, were isogenic otherwise. Therefore, the biochemical differences observed are attributed to the specified mutations.

Cell wall chemistry has often been implicated in fungal morphology (4, 5, 20). Restricted growth in *S. commune*, as caused by cellobiose, is correlated with a high S glucan to R glucan ratio and elevated R glucanase activities (122). Cell wall softening was surmised as a reason for frequent branching in cellobiose-grown mycelium. Many morphological mutants of *S. commune* occur (79). Recently, the aberrant morphology of some of these strains ("puff" and "thin") was related to quantitative differences in cell wall composition (109).

Removal of S glucan from the cell wall of *S. commune* leaves normally shaped hyphae, indicating that R glucan (probably together with chitin) is a form-determining substance in the wall (113). The correlations observed in the common-*A* heterokaryon and in the various *B*-factor mutants suggest that high R glucanase activities prevent the proper amount of R glucan from being deposited in the wall. Such interference with cell wall biosynthesis in growing hyphae, presumably at the tip, may prevent the construction of normally shaped hyphae. This would account for the inflated or "knobby" hyphae observed in common-*A* heterokaryons and *AxBmut* homokaryons. Disrupted septa are found only in hyphae in which R glucanase activities are enhanced. Therefore, one can also conjecture on a causal relationship between high R glucanase activities and dissolution of complex septa, although other enzymes, such as those required for membrane disorganization, may also be required for this process. Finally, localized action of R glucanase may take part in the fusion of the hook cell with the subterminal cell which is the last stage in clamp formation (*see* Fig. 5).

Some evidence for the direct involvement of glucanases in septum dissolution is available (Janszen and Wessels, *unpublished*). Cell wall preparations from a homokaryon (strain 699a) obtained by breaking the cells and removing the cytoplasm by repeated washing still contain intact septa but are devoid of the parenthesome membranes. Electron microscopic examination of sections of these walls treated with KOH (1 N), Pronase, lipase, or chitinase does not reveal dissolution of septa, except for the removal of the septal swelling by alkali. However, after treatment with chitinase, incubation of the cell walls with an R glucanase preparation results in complete dissolution of septa. At the same time, little change can be observed in the structure of the cell wall. Unfortunately, the crude R glucanase preparation used here also contained other glucanases, notably, laminarinases. Consequently, the results are still inconclusive.

The morphogenetic sequences which can be studied in isolation in common-factor heterokaryons and factor mutants are precisely integrated in the formation of the dikaryon. For instance, before an invading nucleus reaches a terminal cell in the recipient mycelium, septal dissolution occurs along the path of transport (26, 42, 66). At this time, the *B*-morphogenetic pathway prevails. Once the binucleate tip cell is established, further septal dissolution is arrested and complex septa are formed. The results with R glucanase suggest an interesting parallel at the biochemical level. It appears that during nuclear transport the R glucanase activity is increased, possibly due to derepression of the enzyme. As surmised above, this could be instrumental in the dissolution of septa. The transition to catabolite repression of R glucanase may come into

effect after the migrant nucleus reaches an apical cell and becomes associated with the resident nucleus. This derepression-repression sequence during dikaryon formation might work if the release of *B*-factor products by the invading nucleus in the recipient cytoplasm were more rapid than the formation of *A*-factor products. This would temporarily relieve R glucanase repression. Eventually, repression would be restored again in the dikaryotic cell when the products of the *A* factor accumulate.

The observed variations in R glucanase activities fit Raper's model of the mode of action of the incompatibility factors in sexual morphogenesis (75). The results also indicate that both the *A* and *B* factors are involved in regulating R glucanase. However, there is no suggestion as to the role of the *A* factor in regulating such events as nuclear pairing, conjugate nuclear division, hook-cell formation, and hook-cell septation.

No definite statements can be made concerning derepression or activation of enzyme synthesis as the basis for expression of the morphogenetic pathways. The mutated *B* allele is universally compatible and dominant to all wild-type alleles (75). Presumably, it produces an activator. However, the cytoplasmic products of the mutated *B* allele may retain their competence to interact with the product of a wild-type allele (75), thus neutralizing the repressing activity of this product. The increasing examples of positive control of enzyme synthesis in eukaryotes (cf. 52) caution against too rapid assertions on this point. It is useful to recall the early experiments of Harder (28), who removed one of the nuclei from a dikaryotic cell of *S. commune* and observed pseudoclamped hyphae in the homokaryotic offspring from the manipulated cell. Similar observations by Lange (41) indicate that pseudoclamp formation in *Polystictus versicolor* depends on the nuclear type in the derived homokaryotic mycelium. The operation of the *A*-morphogenetic sequence in these cases is explained by assuming prolonged activity of an activator in the cytoplasm of the originally dikaryotic cell. Another possibility is that the *A*-morphogenetic pathway, once set in operation, exemplifies a case in which differentiation is not readily reversible.

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