BACTERIOLOGICAL REVIEWS, Mar. 1976, p. 1-41
Vol. 40, No. 1

Printed in U.S.A.

Biochemical Genetics of *Neurospora crassa* Conidial Germination

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INTRODUCTION

Neurospora crassa has many properties that are advantageous for studying development (16). First, it is a eukaryotic organism that can be grown in a defined medium under controlled conditions. Second, the developmental sequences are relatively simple, and morphologically distinguishable cells can be obtained in large quantities. Third, *N. crassa* is well characterized from a genetic standpoint. A collection of nearly 2,000 auxotrophic, morphological, and developmental mutant strains are available from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. Finally, many biochemical regulatory mechanisms that could be involved in development have been studied in this organism. These include the regulation of gene expression (127), compartmentalization (33), molecular channeling (61), and feedback inhibition (68).

N. crassa has both sexual and asexual life cycles. Developmental studies on ascospore germination (200, 202) and studies on the conidiation phase of the asexual cycle (186, 213) have been reviewed. This review will be concentrated predominantly on the conidial germination phase of the asexual life cycle. The differences between conidia and vegetative mycelia are described as well as the morphological, physiological, and biochemical changes that occur during conidial germination.

A model for the activation of dormant spores is discussed in reference to conidial germination as well as to possible analogous activation of other dormant cells. An evaluation is given of the various genetic and biochemical approaches that can be used to study a developmental process, such as germination. Guidelines are discussed that can be used to determine the developmental significance of a particular genetic or biochemical observation.

THE THREE PHASES OF THE ASEXUAL LIFE CYCLE OF *N. CRASSA*

Neurospora can produce two types of asexual spores, macroconidia and microconidia. The usual laboratory wild-type strains produce abundant amounts of macroconidia. These are orange, multinuclear spores, produced by budding from the tips of specialized aerial hyphae (212). In contrast, microconidia contain only one nucleus and are produced as single spores from vegetative hyphae (64, 65). Microconidia appear in late stationary-phase agar cultures. Some mutant strains produce only microconidia (65). Because of their very poor viability (60), microconidia have not been very useful for studying development. All of the studies in this review were done with macroconidia. Therefore, the terms conidia and macroconidia will be used interchangeably.

Conidial Germination

There are three distinct phases of the asexual life cycle: conidial germination, vegetative growth, and conidiation (Fig. 1). Conidial germination is defined in this review as all of those events culminating in, and including, the formation of a rapidly growing germ tube. The process of conidial germination includes the following (118).

(i) Hydration. Mature conidia, before being suspended in water, appear wrinkled and shrunken (178). Once suspended in water, the conidia become relatively smooth and have been described as looking like truncated footballs (184). Presumably, hydration does not require metabolic energy and includes the loss of a hydrophobic surface layer and the solubilization of exocellular enzymes as well as the uptake of water.

(ii) Internal changes. Internal changes are changes that occur within the confines of the cell wall (118) and require metabolic energy. Both endogenous and exogenous energy sources could be used.

(iii) Germ tube emergence. The formation of a cylindrical germ tube from a relatively spherical conidium is the major morphological change associated with conidial germination. This process requires an exogenous carbon source as well as some inorganic salts (132). Germ tubes can emerge from either the side or end of the conidium (178).

(iv) Germ tube elongation. Germ tube elongation includes any of those events that might be required for the initial stages of germ tube elongation but which are dispensable for subsequent mycelial growth.

Because germ tube emergence is not synchronous (Fig. 2), these processes will overlap. Some individual conidia will be forming germ tubes while others will still be undergoing internal changes. Hydration and internal changes can be considered analogous to activation and germination, respectively, of bacterial spores (191). Germ tube emergence and elongation may be analogous to outgrowth in bacterial systems.

Vegetative Growth

The vegetative mycelial growth phase of the asexual cycle can be maintained almost indefinitely by periodically transferring a small

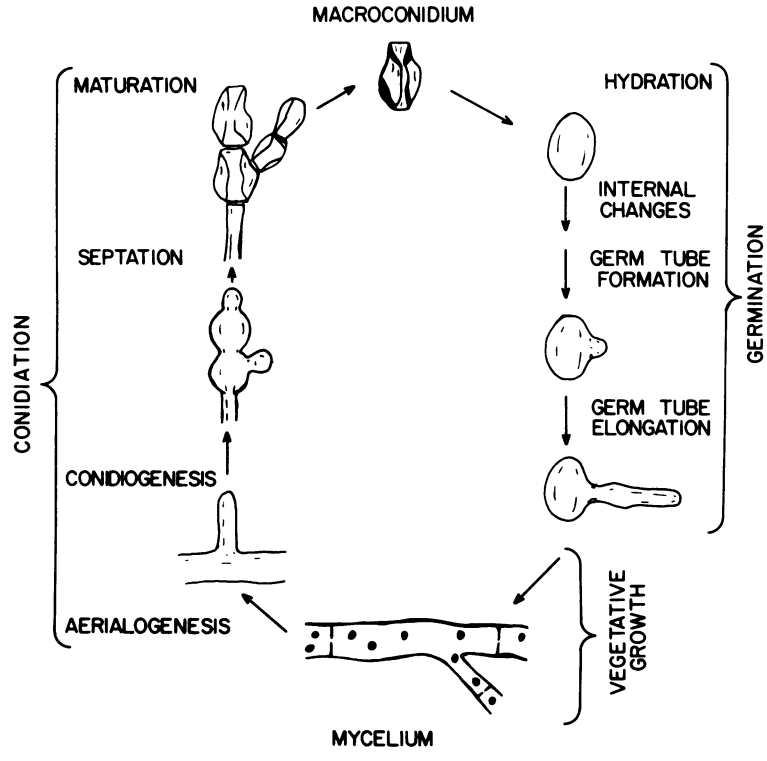

FIG. 1. *Asexual life cycle of N. crassa.*

amount of mycelia to fresh medium. Some of the enzymatic and chemical properties of mycelia are dependent on the composition of the medium (31) and on whether the mycelia were obtained from exponential- or stationary-phase cultures (69). Unless otherwise indicated, the properties of conidia will be compared to those of mycelia from exponentially growing, liquid shake cultures.

Regional differentiation occurs within the mycelial mat itself. The cross walls for the hyphae are incomplete and have a central pore. Nuclei and other granular material can be seen with a light microscope streaming from the older regions of the hyphae toward the growing tips (240). The principle mode of vegetative growth is by apical extension (240). Eventually, the older regions of the hyphae become vacuolated and are laden with fat droplets (240). In addition, the growing tips have been shown to have different enzymatic and chemical properties than older regions of the hyphae (239).

The morphology of the mycelia (variations in the shape of the hyphae and the branching frequency [16]), can be altered by environmental conditions and by mutation. Normally *N. crassa* mycelia grow rapidly with hyphal tips extending at a rate of about 1 mm/h at 22 C in minimal glucose medium. If sorbose, a non-metabolizable sugar, is included in the medium, the hyphal tips grow slowly and mycelia become highly branched (203). More than 120 different genetic loci have been identified that alter the mycelial morphology. This class of mutant strains does not include most of the auxotrophs which have normal "wild-type" morphology and simply grow slowly when starved for the required metabolite. The subject of mycelial morphogenesis and the types of enzymatic defects that lead to altered morphology has recently been reviewed (16).

Conidiation

The process of conidiation (Fig. 1) includes all of the events involved in the production of mature conidia from vegetative mycelia and can be divided into the following four stages.

(i) Aerialogenesis. Conidiation begins with the formation of specialized aerial hyphae. Aerial hyphae have been isolated and some of their properties are distinct from those of vegetative hyphae (184).

(ii) Conidiogenesis. Conidia are formed by budding from the tips of these aerial hyphae (212). After the first conidium is formed it produces one or more buds. Eventually, long, branched chains of conidia are formed.

(iii) Septation. Complete cross walls form

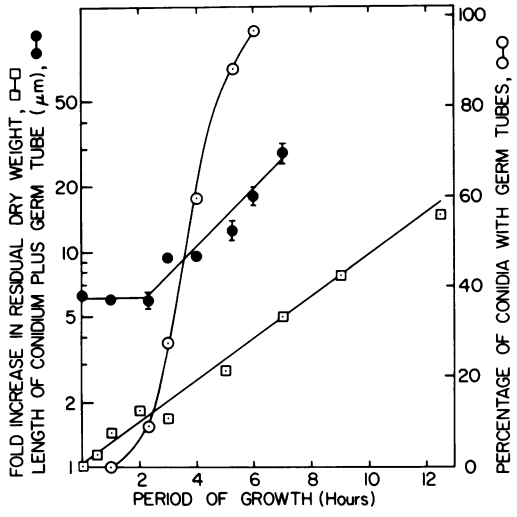

FIG. 2. *Comparison of the time course for the formation of germ tubes, the increase in germ tube length, and the residual dry weight. The percentage of conidia with germ tubes and the average length of conidium plus germ tube were obtained from the same culture. The residual dry weight (alcohol-insoluble material) was obtained in a separate experiment. In both experiments the conidia from the wild-type strain, RL3-8A, were incubated in shake cultures containing glucose minimal medium at 21 C. The procedures used have been described in detail (169). Symbols: O, Percentage of conidia with germ tubes; □, fold increase in residual dry weight; ●, average length of conidium plus germ tube. The bars indicate standard error.*

between each individual conidium and they become separate cells (186).

(iv) Maturation. The conidia undergo a maturation period that takes about 3 days (195). This was measured by the ability of the conidia to form germ tubes at their maximum rate when put in germination medium.

Mutant strains have been isolated that specifically affect each of the first three stages of conidiation. These mutations do not appear to affect the other phases of the life cycle (124, 179, 186).

Four different systems have been devised for studying the process of conidiation in *N. crassa*. Each of these systems has advantages and disadvantages, and the choice of which to use is dependent on the kinds of questions that are to be answered. Briefly, these systems are as follows: (i) liquid media have been devised which favor either conidiation (C medium) or mycelial growth (M medium) (209). However, because these media are different, biochemical changes that occur may be due to adaptation to the media as well as to the induction of conidiation. (ii) Procedures for the synchronous production of conidia have been reported which induce conidiation by starvation (185, 196). In this case it must be determined if the biochemical changes that occur are specific just to conidiation or are the result of starvation. This is a general problem of studies of all "catabolic development" where development occurs during starvation. (iii) A mutant strain, *bd*, has been employed which conidiates at the edge of the mycelial mat when growing on the surface of agar medium (158). In this strain, conidiation responds to an endogenous signal and does not have to be induced by starvation or by changing the composition of the medium (157). (iv) A system for microcycle conidiation has been described (32). Conidia incubated in liquid cultures at 46 C for 15 h and then shifted to 25 C germinate directly into presumed conidiophores and produce conidia. Whether these conidia have the same properties as conidia produced on aerial hyphae remains to be determined.

CONIDIA ARE DORMANT SPORES

Conidia have many of the properties of dormant cells (200, 202). These include longevity, low metabolic rate, and resistance to environmental stress.

Conidia are resistant to dessication, and, once dry, can be stored for long periods of time without loss of viability. Lyophilized conidia and conidia dehydrated on activated silica gel (W. N. Ogata, *Neurospora* Newsletter 1:13, 1962) remain viable for at least 3 years at 4 C and probably for much longer. Conidia have been stored in a dessicator at 22 C for 6 months without any loss of viability (R. Fahey, personal communication).

One of the factors that does affect conidial viability is humidity. Conidia stored at 100% humidity begin to die after only 9 days at 22 C (Fahey, personal communication). Humidity may also be a factor which causes conidia in agar slant cultures to begin to lose their viability after 18 days at 22 C (K. Haard, *Neurospora* Newsletter 11:12-13, 1967).

The resistance of conidia to dry heat appears to be dependent in some way on their water content. Dehydrated conidia are extremely resistant to dry heat. More than 90% of dessicated conidia survived being heated for 3 min at 124 C (Fahey, personal communication). If the dehydrated conidia were stored at 50% humidity for 1 day, only 38% of the conidia survived this same treatment. Less than 10% of the conidia survived being heated to 100 C for 5 min after being stored at 100% humidity for 1 day.

Mature conidia from slant cultures are resist-

ant to being heated in water at 55 C for 10 min (J. C. Schmit, unpublished observations). During germination, heat resistance is lost. By 2 h after initiating germination (germ tubes had not yet appeared), only 17% of the conidia survived being heated at 54 C for 2 min (109). Exogenous energy sources do not appear to be necessary for the loss of heat resistance since heat resistance is lost if conidia are suspended in deionized water at 22 C (Schmit, unpublished observations). Other metabolic events such as changes in free amino acid pools (168) and assembly of polysomes (132) also occur in deionized water.

Conidia can also be stored in cold water for many days without apparent loss of viability and will survive freeze-thaw cycles. Seventy percent of the conidia formed germ tubes after being stored in water at 4 C for 81 days (G. J. Stine, *Neurospora* Newsletter 11:7-8, 1967). However, this result must be interpreted with caution as the ability to form a germ tube is not always correlated with the ability to form a viable colony (109). Conidia that had been suspended in water and quick-frozen in a dry ice-acetone bath retained their viability for at least 6 days (197). If the conidia were incubated for 2 h in minimal medium before being frozen, only 20% survived. It was concluded that "cold death" was related to metabolism and not to the uptake of water (197). The mechanisms for "cold death" and loss of heat resistance are apparently different since exogenous energy sources are not necessary for loss of heat resistance. In other studies conidia were not damaged by fast or slow freezing as long as the rate of thaw was rapid (400 C/min) (5).

Conidia are also resistant to acid. Twenty percent of the conidia survived treatment with concentrated HCl for 10 min (J. D. Graham, *Neurospora* Newsletter 11:14, 1967). Conidia can also survive treatment with 0.05 N HCl for 1 h at 0 C (W. D. Scott and R. L. Metzenberg, *Neurospora* Newsletter 11:8-9, 1967), 0.1 N HCl for 5 min (47), and 0.2 N HCl for 8 min at 37 C (M. L. Sargent and H. D. Braymer, *Neurospora* Newsletter 14:11-12, 1969).

The endogenous respiratory rate of conidia is considerably less than that of mycelia. The oxygen quotient of conidia in water is 0.74 (145). Mycelia growing on sucrose can have an oxygen quotient of 9.0 (99). Because the metabolism of some of the endogenous stored compounds in conidia begins as soon as the conidia are suspended in water (168), the respiratory rate of dormant conidia (before being suspended in water) may in fact be much lower than has been reported.

COMPARISON OF THE STRUCTURE AND COMPOSITION OF CONIDIA AND MYCELIA

Ultrastructure

Conidia are roughly spherical and average 6.5 μm (4.7 to 10 μm) in length and 5.7 μm (3 to 8 μm) in width (169; G. J. Stine, *Neurospora* Newsletter 11:7-8, 1967). Mycelia are cylindrical and vary in diameter from 4 to 10 μm. Neither conidia nor mycelia have any distinguishing surface structures. Both types of cells appear relatively smooth in scanning electron micrographs (178). Flattened regions can be distinguished on the conidia where they had been attached together during conidiation.

Most of the ultrastructural features of conidia and mycelia are very similar. In electron micrographs, the cell walls of conidia and mycelia appear to be identical (62, 119, 226) with the exception that conidia have an additional electron-dense surface layer (62). This layer is lost when conidia are suspended in water. The cell wall of the germ tube is formed by extension of the existing conidial cell wall.

The nuclei and mitochondria of conidia and mycelia are morphologically indistinguishable (136, 226). Conidia contain on the average about 2.5 nuclei with some individual conidia containing up to 10 (62, 119, 224; M. Kihara, *Neurospora* Newsletter 2:8-9, 1962; and T. H. Pittenger, *Neurospora* Newsletter 11:10-12, 1967). Four- to five-day-old conidia contain very few vacuoles (136), but large vacuoles are found in conidia from cultures that are 2 or more weeks old (136). Large vacuoles have also been observed in hyphae from older regions of the mycelial mat but are usually absent from the growing tip (136, 240). Small dense bodies that are usually associated with the endoplasmic reticulum have been observed in conidia and are probably lipid (119, 226) though their composition has not been chemically determined.

The endoplasmic reticulum in conidia was sparse and discontinuous, whereas in mycelia it was well defined and plentiful (119, 136, 226, 227). The ribosomes in conidia were freely dispersed in the cytoplasm (226). In mycelia, most of the ribosomes are associated with polysomes (78, 132). Dispersed ribosomes have been observed in spores from many different organisms (217).

Cell Wall

Both conidial (113, 115) and mycelial (116, 169) cell walls have been isolated and fractionated. The cell walls from both types of cells contained β-1,3-glucan, chitin, and protein (for

review see reference 169). However, the percentage of the cell wall obtained in each of the cell wall fractions was different for conidia and mycelia (113, 116). In addition, conidial cell walls contained only trace amounts of galactosamine (62, 169), whereas up to 10% of the mycelial cell wall can be composed of this amino sugar (169). The galactosamine in the mycelial cell wall is predominantly a high-molecular-weight homopolymer of partially acetylated galactosamine (151). Galactosamine polymers (in the form of a mucopeptide), that appear to be identical to those in the cell wall, have been isolated from the growth medium of stationary-phase cultures (151).

Conidia contain a unique hydrophobic surface layer that is not found in mycelia (119, 213). The composition of the hydrophobic material is not known. Small amounts of many different fatty acids and long-chain alkanes have been extracted from the surface of conidia with petroleum ether (54). However, these conidia had been suspended in water before being extracted and many of the surface components may have been lost. Many different enzymes have been recovered in water or saline washes of conidia. From 0.2 to 0.3% of the dry weight of the conidia was soluble in 4.5 M potassium chloride (198). To what extent these extracellular proteins contribute to the hydrophobic nature of the conidia remains to be determined. A mutant strain, *sponge,* has been isolated that produces conidia that have lost their hydrophobic properties (C. P. Selitrennikoff, personal communication). These conidia can be easily suspended in water. This strain might be useful for determining the biosynthetic pathways involved in the formation of the hydrophobic surface layer of conidia. Because the conidia produced by the *sponge* strain are morphologically "normal," the hydrophobic surface layer does not appear to play a causal role in the formation of spherical conidia. Also, there appears to be a reciprocal relationship between galactosamine polymers and the hydrophobic surface layer, both of which are located on the outer surface of the cell wall. Perhaps both of these components bind to the same cell wall structures and, therefore, are mutually exclusive.

Lipids

Conidia contain phospholipids, carotenoids, and the steroid, ergosterol. More than 90% of all of the lipid in conidia is phospholipid which comprises about 17% of the conidial dry weight (12, 146). In contrast, only 3 to 4% of the mycelial dry weight is phospholipid (84, 165). All five of the major classes of phospholipids that are found in mycelia (84), phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin, are found in conidia and are at about the same relative proportions (Schmit, unpublished observations). The amount of phospholipid in conidia is dependent to some extent on the growth conditions. Higher levels of phospholipid were accumulated when the cultures were grown on rich medium (12). As the conidia aged, the total phospholipid content decreased slowly (12). This suggests that phospholipids may be consumed during dormancy as a source of maintenance energy. The high levels of phospholipid in conidia may be associated with the electron-dense bodies that have been observed in electron micrographs (119, 226).

Conidia do not contain many triglycerides (12, 146), whereas these lipids can account for more than 8% of the mycelial dry weight (17, 143). The triglycerides are found predominantly in the older regions of the mycelia (240). Carotenoids, which account for the bright orange color of conidia, accumulate in conidia when they are grown in the light (238). Even though carotenoids are usually associated with conidia, they are not required for conidiation or for conidial germination. Albino strains which lack carotenoids (238) and cultures grown in the dark will produce conidia that are white and that germinate normally.

The steroid, ergosterol, is found at about the same level in both conidia and mycelia (12). In mycelia, a large proportion of the ergosterol is associated with the plasma membrane (165).

Protein

The types of proteins found in conidia and mycelia may be different. The predominant amino terminal amino acid in conidial proteins was phenylalanine and in mycelial proteins it was glycine (152). It still remains to be determined whether this difference in amino terminal amino acids represents completely different classes of proteins or changes in specificity of the post-translational proteolytic trimming of the amino end of the proteins.

Nucleic Acids

Because the subject of nucleic acids in fungal spores has recently been reviewed (218), the properties of *N. crassa* nucleic acids will be described only briefly.

DNA and chromatin. The haploid genome of *N. crassa* has from 1.4×10^{10} to 2.3×10^{10} daltons of deoxyribonucleic acid (DNA) (18, 26, 42, 44). There are seven linkage groups in *N. crassa,* and if each contained an equal amount

of DNA, the average chromosome would be approximately the same size as the *Escherichia coli* genome (218). From 10 to 20% of the total cellular DNA is not single-copy DNA (18, 42). About 1% of the reiterated DNA is mitochondrial DNA (110). Another 1% is in ribosomal ribonucleic acid (rRNA) cistrons (18, 26), and 0.3% is in transfer RNA (tRNA) cistrons (18, 44). Each haploid genome contains 100 to 200 rRNA cistrons and from 2,600 to 3,000 tRNA cistrons. There is no evidence for any differences between conidial and mycelial DNA. The base composition of DNA from conidia, germinating conidia, and mycelia is identical (131).

The composition of *Neurospora* chromatin is still somewhat controversial (218). Chromatin has been isolated from both mycelia (45) and conidia (43) of the wild-type strain of *N. crassa* and from a cell wall-less mutant strain, *slime* (83). Histones were not found in the chromatin from either mycelia or conidia (43, 45, 106). However, two slightly lysine-rich histones were isolated from chromatin preparations from the *slime* mutant (83). The ratio of histone to DNA in the *slime* mutant was 0.24, which is about one quarter of the amount found in other eukaryotes (83). It still remains to be determined if there are any significant changes in chromatin proteins during asexual development.

RNA. The rRNA and tRNA from conidia and mycelia have identical sedimentation properties and base composition (76, 77). The tRNA's from conidia and mycelia, however, differ in their ability to accept methyl groups (236). The tRNA from conidia have a higher capacity to become methylated than tRNA from mycelia. A unique species of $tRNA_{phe}$ has been isolated from conidia (218). Conidia also contain a species of "soluble" RNA that is not found in mycelia (222).

Conidia probably contain some messenger RNA (mRNA) since polysomes are assembled very rapidly when conidia are suspended in water (132). An RNA fraction, which has the properties of mRNA, has been isolated from *N. crassa* conidia (10). This isolated mRNA hybridized to 2% of the cellular DNA and stimulated amino acid incorporation into protein in a cell-free protein synthesizing system from *N. crassa* mycelia (11).

Low-Molecular-Weight Compounds

The levels of many water-soluble small molecules have been measured in conidia. These include free amino acids and other ninhydrin-positive compounds (168), the two nucleotide sugars uridine diphospho-2-acetamido-2-deoxy-D-glucose (UDP-GlcNAc) and uridine diphospho-2-acetamido-2-deoxy-D-galactose (UDP-GalNAc) (169), reduced and oxidized pyridine nucleotides (170), adenine nucleotides (188), reduced and oxidized glutathione (53), and trehalose (69).

Conidia contain high levels of readily metabolizable compounds such as glutamic acid (170) and trehalose (69). About 2.5% of the conidial dry weight is glutamic acid and up to 10% is trehalose. Conidia can also contain relatively high levels of glutamine and alanine (168). These compounds may be stored in conidia during conidiation for consumption during dormancy or germination.

Conidia contain low levels of three amino acids of the urea cycle, arginine, ornithine, and citrulline (168). In mycelia, arginine and ornithine are compartmentalized in discrete, membrane-enclosed vesicles (199, 228). Either these vesicles are not present in conidia or they are nearly empty of basic amino acids. Dry-harvested conidia did not contain detectable levels of γ-aminobutyric acid. As soon as the conidia were suspended in water, this amino acid appeared (168).

The levels of UDP-GlcNAc, the precursor of chitin, were nearly identical in conidia and mycelia (167, 169). Conidia contained lower levels of UDP-GalNAc than mycelia which is consistent with the observation that conidial cell walls contain very low levels of galactosamine-containing polymers (62, 169).

Conidia contain about the same level of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide, reduced form (NADH) and twice the level of nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) as mycelia (170). The ratios of NADPH/NADP and NADH/NAD were nearly identical for both cell types. This is in contrast to *Bacillus megaterium* spores which have very low levels of reduced pyridine nucleotides (182).

The total adenine nucleotide pool adenosine 5'-triphosphate plus adenosine 5'-diphosphate plus adenosine 5'-monophosphate (ATP + ADP + AMP) of conidia was less than 40% of that from log-phase mycelia (188). The energy charge (ATP + 1/2ADP)/(ATP + ADP + AMP) (4) was 0.7 for both conidia and mycelia (37, 188). Again, these results are in contrast to those for *B. megaterium* spores which have low levels of ATP and an energy charge of 0.1 (181).

Conidia contain higher levels of oxidized glutathione (GSSG) than mycelia (53, 170). The role that disulfide compounds might play in conidial germination will be discussed as part of a general model for dormancy.

The levels of tricarboxylic acid cycle intermediates in *N. sitophila* conidia have been measured (146).

Exocellular Enzymes

Conidia contain relatively high levels of many hydrolytic enzymes which can be removed, at least partially, by washing the conidia with water. Some of these exocellular enzymes have been studied with regard to their subcellular localization and whether or not they are unique to conidia. A review of these studies is included.

NADase [NAD(P) glycohydrolase; EC 3.2.2.5] is uniquely associated with aerial hyphae and conidia when grown on normal media (31, 193, 195, 215, 245). However, this enzyme can be induced in mycelia by zinc starvation (52) and thus is not absolutely unique to conidia. NADase, which was first assayed by Kaplan et al. (90), splits the oxidized pyridine nucleotides, NAD and NADP, into nicotinamide and adenosine diphosphoribose and adenosine-3′-phosphate-5′-diphosphoribose, respectively. The enzyme as isolated from zinc-starved mycelia is a glycoprotein and has a ratio of carbohydrate to protein of about 6:1 (52). Conidia contain high levels of this enzyme activity which can be almost completely removed by washing with water (194, 241). NADase cannot be detected in log-phase vegetative mycelia (186, 215) or in *N. crassa* ascospores (186). Four different aconidial mutants also do not have NADase activity (80, 215). A mutant strain, that was temperature sensitive for conidiation, was also temperature sensitive for NADase formation (80). Another mutant strain, fluffy *(fl)*, that forms aerial hyphae but no conidia, accumulated NADase in aerial hyphae (193, 195). Thus, NADase is usually associated with the conidiation phase of the asexual cycle.

Five mutant strains have been isolated that have very low levels of NADase activity (139). All five mutants are allelic and are located on linkage group IV. These mutations *(nada)* are probably in the structural gene for NADase since one of these strains had low levels of temperature-sensitive NADase activity. The absence of this enzyme did not affect aerialogenesis, conidiogenesis, or conidial germination (139). The growth and fertility of the *nada* strains were normal. NADase has been described as a "luxury molecule" (186) whose synthesis is in some way correlated with conidiation but which is not required for asexual development. The physiological role of this enzyme is still unknown.

Trehalase (α,α'-glucoside 1-glucohydrolase; EC 3.2.1.28) is induced during conidiation and is preferentially accumulated in conidia (69, 79). Both the substrate, trehalose, and the degradative enzyme, trehalase, are synthesized simultaneously. Conidiation is not essential for trehalase synthesis since this enzyme can be induced by carbon starvation in shake cultures where conidiation does not occur (69). The synthesis of trehalase is repressed in shake cultures by glucose and other easily metabolized carbon sources (70).

Mutant strains *(tre)* which lack trehalase activity have been isolated (201). These may be regulatory mutants rather than structural gene mutants (127). Conidia from these mutant strains still germinate in minimal glucose medium (201; M. Sargent, personal communication). However, there is the possibility that the degradation of the large trehalose pool (10% of the conidial dry weight) by trehalase may serve as an energy source for some of the early biochemical events that occur in the absence of exogenous carbon sources (132, 170). This possibility remains to be tested. The mutant strains, *exo-1* and *exo-2*, are derepressed for trehalase as well as for some other carbohydrases (62, 63). The effect of these mutations on conidiation and conidial germination has not been reported.

Some invertase activity (β-D-fructofuranosidase fructohydrolase; EC 3.2.1.32) can be removed from conidia by washing with water (46). However, most of the conidial invertase is a cell-bound exoenzyme which is freely accessible to substrate, protons, and inhibitors (125). Invertase is uniformly distributed along the cell periphery of the conidia with 8% of the total cellular activity in the conidial cell wall itself (23, 29). Most of the remaining activity is in the periplasmic space or perhaps associated with the outside of the plasma membrane (23). In mycelia, 24% of the enzyme is bound to the cell wall with the highest concentration of enzymatic activity in the growing hyphal tip (29). Two different invertase activities have been isolated from *Neurospora* (49, 126). Mutations have been isolated that have low levels of invertase activity (159). These mutant strains will neither grow nor form germ tubes when sucrose is used as a carbon source.

Conidia contain two different β-glucosidases, aryl-β-glucosidase and cellobiase (47) both of which are designated as β-glucoside glucohydrolases (EC 3.2.1.21). About 50% of the aryl-β-glucosidase activity in conidia was exocellular and could be extracted from dry-harvested conidia with water (9, 46, 47). The remaining aryl-β-glucosidase activity was bound to the surface of the cells and was freely accessible to

substrates. It could be inactivated by mild acid treatment (47). In contrast, all of the cellobiase activity was intracellular (47).

Both β-glucosidase activities are induced in mycelia with cellobiose (48). The mutant strain, *cell-1*, is constitutive for cellobiase activity (135). During growth of the wild-type strain, aryl-β-glucosidase, but not cellobiase, became constitutive as the cultures entered stationary phase, before conidiation (48). Mutant strains (*gluc-1* and *gluc-2*) that lack aryl-β-glucosidase activity (112) apparently produce conidia that germinate normally.

About 30% of the aryl sulfatase activity (W. A. Scott and R. L. Metzenberg, *Neurospora* Newsletter 11:8, 1967) in conidia can be extracted by suspending the conidia in buffer. About 40% of the remaining activity can be inactivated with acid (0.05 N HCl, 1 h, 0 C), and is therefore located external to the plasma membrane. The remaining activity is intracellular, and can be assayed only after the cells are broken.

Structural gene mutations of aryl sulfatase (129, 130) and the regulatory gene (*cys-3*), both of which lack this enzyme (123, 128), do not appear to affect conidiation or conidial germination specifically. A mutant strain, *scon*^{c} (sulfur-controlling, constitutive), produces constitutive levels of all of the enzymes, including aryl sulfatase, that are missing in *cys-3* (19). The *scon*^{c} strain produces morphologically normal conidia, even though, only 2 to 8% of the conidia germinate (Schmit et al., *Neurospora* Newsletter 21:17-18, 1974). The conidia from this strain appear to be osmotically fragile. A large amount of ultraviolet-absorbing material and almost all of the intracellular free amino acids were lost when the conidia were suspended in water. Sucrose (20%) prevented the loss of some of the ultraviolet-absorbing material and improved the viability of the conidia by nearly fourfold (Schmit et al., *Neurospora* Newsletter 21:17-18, 1974). Perhaps the constitutive synthesis of the enzymes of sulfur metabolism during conidiation results in the formation of either a defective plasma membrane or an altered cell wall.

Large amounts of both laminarinase [β-1, 3(4)-glucan glucanohydrolase; EC 3.2.1.6] and α-amylase (1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) (62) are exocellular and can be washed from conidia with water. Most, if not all, of the laminarinase activity that remains after the conidia have been washed is associated with the conidial cell wall (46). Neither of these enzymes is apparently unique to conidia and both can be found in mycelia (62, 114).

Other extracellular enzymes such as nucleases (74, 86), phosphatases (208), and proteases (40, 41) have been studied in vegetative cultures. Whether these enzymes are also associated with conidia has not been reported.

It is interesting that the levels of many of these enzymes, i.e., trehalase, invertase, and aryl-β-glucosidase, are repressible by glucose. One explanation for the high levels of these enzymes in conidia is that they may accumulate because conidiation occurs under conditions where glucose repression is released. Perhaps both the initiation of conidiation and the increased synthesis of exocellular enzymes share a common component of the glucose repression mechanism.

In summary, none of the exocellular enzymes that have been studied have been shown to be essential for either conidiation or for conidial germination under laboratory conditions. The accumulation of high levels of carbohydrase activities on the conidial surface may simply be coincidental, or these enzymes may be stored for hydrolysis of potential carbon sources during germination. These enzymes would be released as soon as the conidia contact an environment suitable for germination and could supply energy for the initial stages of growth. A summary is given in Table 1 of some of the differences between conidia and mycelia.

CONIDIAL GERMINATION

The initiation of the germination sequence begins as soon as the conidia contact water or media. The precise end of the germination period is more difficult to define. The elongation of germ tubes and the growth of vegetative hyphae probably share many of the same biochemical processes. It is convenient to consider an individual conidium germinated when its germ tube is as long as it is broad (118). It should not be inferred from this definition that some germination-specific events could not occur after the formation of a germ tube.

In minimal glucose medium, conidia germinate asynchronously with the larger conidia forming germ tubes first (Fig. 2 and 4). By 2 h, a few conidia had germ tubes and by 5 h, more than 90% had germinated. Perhaps the larger conidia form germ tubes first because they have a greater potential capacity to synthesize protein, RNA, etc. Even though germ tube formation is asynchronous, hydration and many of the internal changes that are associated with the early stages of conidial germination may be relatively synchronous.

Almost complete synchrony of the event of germ tube formation can be obtained by first

TABLE 1. *Distinctive characteristics of conidia and mycelia*

Property	Conidia	Mycelia (log phase)	Reference
Resistance			
Heat	High	Low	109
Acid	High	Low	47
Ultrastructure			
Endoplasmic reticulum	Sparse	Plentiful	119, 136, 226, 227
Ribosomes	Dispersed	Polysomes	78, 132
Cell wall			
Hydrophobic layer	+	−	119, 213
Galactosamine-polymers	−	+	62, 169
Low-molecular-weight compounds			
Trehalose	High	Low	69
Glutamic acid	High	Low	170
Arginine, ornithine	Low	High	168
GSSG	High	Low	53
Adenine nucleotides	Low	High	188
Proteins			
Amino terminal amino acid	Phe	Gly	152
Lipids			
Percentage of dry weight as phospholipid	17	4	12, 84, 146, 165
Carotenoids	High	Low	238
Respiration			
O_2 consumption	Low	High	99, 145
Cyanide sensitive	Low	High	30
Hydroxamate sensitive	High	Low	30
P/O ratio	0	2	66
Enzymes			
Exocellular	High	Low	Table 3
Intracellular	Low	High	Table 2
Molecular transport			
Amino acids, nucleosides, glucose	Low	High	164
Sulfate permease	Type I	Type II	122

incubating the conidia in minimal medium with 3.22 M ethylene glycol for 49 h and then diluting the ethylene glycol 10-fold within a period of 35 min (6). About 70% of the conidia formed germ tubes between 25 and 35 min. Since the conidia enlarge and the number of nuclei increase during incubation in high concentrations of ethylene glycol, most of the early internal events associated with germination can occur. Therefore, this may be a good system for studying germ tube formation itself. Similar inhibition of germ tube formation was also obtained with glycerol (6).

Conidia swell but do not form germ tubes when incubated in minimal glucose medium with 1 mM cysteine (Schmit, unpublished observations). Perhaps the removal of cysteine would result in the synchronous formation of germ tubes but this has not been tested. Attempts to synchronize the appearance of germ tubes with heat shocks (13) or by flushing the culture with nitrogen (S. W. Bradford and B. I. Gibgot, *Neurospora* Newsletter 4:17-19, 1963) have not been very successful.

For conidia from the wild-type strain, the requirements for the formation of germ tubes are simply a salt solution, a carbon source (132), and a supply of oxygen (82; Bradford and Gibgot, *Neurospora* Newsletter 4:17-19, 1963). An exogenous nitrogen source is not required for germination (Schmit, unpublished observations). Some unidentified component of Vogel's salts (221) may be required for germination in liquid medium (132) but not for germination on agar (Schmit, unpublished observations). A factor essential for germination can be removed from the conidia with high concentrations of NaCl, glucose, glycerol, or sucrose (24). This factor can also be removed from mycelia (25) and may be required for both germination and vegetative growth.

Many things affect the rate of germ tube formation. These include the "age" of the conidia, the harvesting procedure, the concentration of conidia, as well as the temperature and the composition of the germination medium.

Conidia require a maturation period of about 3 days after being formed before they are able to germinate quickly (195, 196). The age of the conidia also affects their colony-forming ability.

About 50% of the conidia from 1-day-old cultures formed colonies, whereas 80% of the conidia from 2- to 18-day-old cultures formed colonies (Haard, *Neurospora* Newsletter 11:12-13, 1967). After 18 days, the colony-forming ability began to decrease. The optimum age for maximum germination and viability is approximately 7 days if the conidia are maintained in slant cultures at room temperature (156).

Conidia do not appear to contain self-inhibitors of germination. However, optimum germination rates in shake cultures are obtained at concentrations of less than 1×10^7 conidia/ml of medium (Schmit and S. Brody, unpublished observations). Conidial concentrations of about 5×10^6/ml can be used conveniently to study biochemical changes that occur during germination (53, 170).

Conidia do not form germ tubes in deionized water, but preincubating the conidia in water does affect their rate of germination in glucose minimal medium. After 2 h at 21 C in deionized water, the time required for 50% of the conidia to germinate decreased from 3.6 to 2.6 h (Fig. 3). After 5 h in water, 50% of the conidia germinated in 3.1 h. These results strongly suggest that some events required for conidial germination can occur without exogenous metabolites. Thus, it is important that dry-harvested conidia be used as controls when measuring biochemical changes during germination.

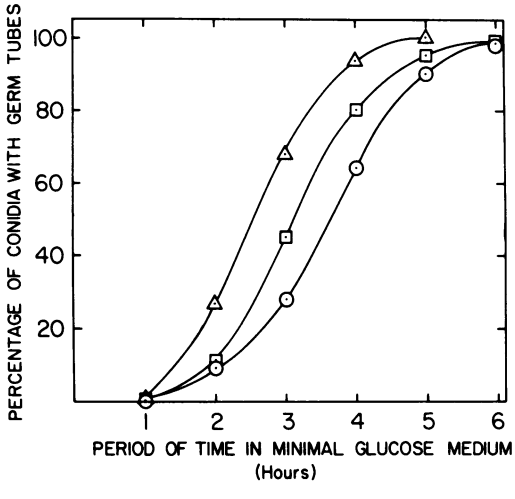

FIG. 3. *The effect of preincubating conidia in water on the rate of germ tube formation. The conidia (RL3-8A) were dry harvested and preincubated in distilled water at 21 C. The conidia were then put into shake cultures containing minimal glucose medium and incubated at 21 C. The percentage of the conidia with germ tubes was measured as described previously (169). Conidia were preincubated in water for 0 min (○); 1 h or 5 h (□); and 2 h (△).*

Both the temperature and composition of the germination medium affect the rate of germ tube formation (Fig. 4). As would be expected, increasing the temperature increased the rate of germination. An unexpected observation was that germination was inhibited in a complete medium containing 0.4% glycerol, 0.4% sucrose, 0.1% Casamino Acids, and 0.25% yeast extract (169) as compared to germination in a minimal medium with 2% glucose (Fig. 4). This complete medium does not inhibit mycelial growth (Brody and Schmit, unpublished observations). In the complete medium, the conidia swell initially. When the germ tubes finally do form, they are larger in diameter than those in minimal medium. The reason for the inhibition of germ tube formation in complete medium is not known. Glycerol has been shown to inhibit germ tube formation of *Neurospora* conidia while allowing them to swell (6), but the concentrations that were used were 50 times the glycerol concentration that is in the complete medium.

Besides the rate of germ tube formation, four other parameters have been used to measure germination. These are the total dry weight; the residual dry weight, which is the material that can be precipitated with either boiling 80% ethanol (169) or 10% trichloroacetic acid (170); the optical density at 660 nm (Schmit et al., *Neurospora* Newsletter 21:17-18, 1974); and the average germ tube length (169). Two of these parameters, residual dry weight and average germ tube length, are shown in Fig. 2. The residual dry weight begins to increase before germ tubes appear. Thus, high-molecular-weight components (protein, RNA, cell wall, etc., which are insoluble in hot ethanol) are synthesized before there are any visible signs of germination. Once germ tubes appear, their average length increases with about the same doubling time as the residual dry weight.

BIOCHEMICAL CHANGES THAT OCCUR DURING CONIDIAL GERMINATION

One of the primary purposes for making biochemical measurements is to identify some of the unique events that occur during germination. The biochemical studies that have been made on germinating conidia include measurements of (i) the levels of low-molecular-weight metabolic intermediates (free amino acids, ATP, etc.); (ii) the rate of synthesis of macromolecules (protein, nucleic acids, and cell wall polymers); (iii) the specific activity of enzymes;

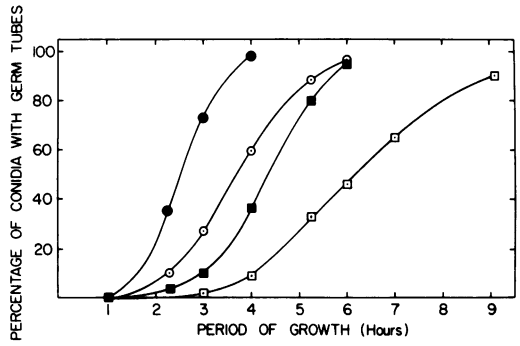

FIG. 4. *The effect of temperature and composition of the medium on germ tube formation. The germination of dry-harvested conidia from the wild-type strain of N. crassa, RL3-8A, was measured in liquid shake cultures as described previously (169). Either minimal glucose medium or "complete" medium (yeast extract, Casamino Acids, sucrose, glycerol) (169) were used. Symbols: ○, Minimal medium at 21 C; ●, minimal medium at 34 C; □, complete medium at 21 C; ■, complete medium at 34 C.*

(iv) the rate of molecular transport; and (v) the increase in oxygen consumption.

Metabolic Intermediates

Measuring the levels of low-molecular-weight intermediates during a developmental sequence, such as conidial germination, has several distinct advantages. First, a large number of molecules can be rapidly and accurately measured. Second, unknown compounds that may be detected can be identified by standard chemical techniques. Finally, a change in the steady state level of a particular small molecule is an in vivo indication of changes in the activity of related enzymes. Thus, observations of unique changes in the levels of small molecules could lead to the identification of developmentally significant biochemical pathways. One obvious problem with this approach is that it may be difficult to determine what change in enzymatic activity actually caused the change in the level of a particular small molecule. This is especially true for compounds such as coenzymes which are produced and consumed in many different enzymatic reactions.

Changes in the levels of free amino acid pools, pyridine nucleotides, and glutathione that occur during germination are reviewed in this section. The levels of the adenine nucleotides (188), and the nucleotide sugars, UDP-GlcNAc and UDP-GalNAc (169), have also been measured during germination. No unique changes were observed in the adenine nucleotide pools (188). Changes in the levels of the nucleotide sugars will be discussed in the section on cell walls.

Endogenous amino acid pools. In conidial extracts all of the common amino acids were detected except proline, cystine, and methionine (168, 170). The levels of these three amino acid pools were also very low in mycelia. During the first hour of germination in minimal medium, the levels of most of the free amino acid pools decreased. Following this initial decrease, the levels of many of these amino acid pools began to increase by about 3 h. This was approximately the same time germ tubes began to appear. For example, the arginine, ornithine, and citrulline pools, which were low in conidia, increased from 4- to 20-fold during germ tube formation (168). This was a particularly interesting observation since it has been reported that more than 90% of the free arginine and ornithine in mycelia is located in discrete, membrane-enclosed vesicles (199, 228). Either these vesicles were being formed during germ tube formation or the vesicles were already in the conidia and were being refilled.

The glutamic acid pool, the predominant amino acid pool in conidia (2.5% of the conidial dry weight) was rapidly consumed within 1 h of germination (168, 170). Less than 5% of the glutamic acid pool was excreted into the medium (168). During germination, there was a rapid increase in the aspartic acid pool (168, 170). The level of the aspartic acid pool remained high for only a few minutes (Fig. 5A). Within the initial 20 min of germination, the increase in the aspartic acid pool was nearly equivalent to the decrease in the glutamic acid pool (Fig. 5A). The formation of aspartic acid also occurred in deionized water, and was not inhibited by cycloheximide (168). These results suggest that during the initial stages of germination, the large endogenous pool of glutamic acid in conidia is degraded to aspartic acid.

Two pathways have been proposed that could account for the simultaneous degradation of glutamic acid and the formation of aspartic acid during germination (168, 170). The first pathway involves a glutamic-oxaloacetic transaminase and some of the enzymes of the tricarboxylic acid cycle (170). The second pathway (168) involves the decarboxylation of glutamic acid to γ-aminobutyric acid (GABA), and the degradation of GABA to aspartic acid by the enzymes of the GABA pathway (39) and the tricarboxylic acid cycle. GABA could not be detected in dry-harvested conidia, but a small amount appeared after the conidia had been put in water (168). The metabolic products of the degradation of glutamic acid by either pathway would be aspartic acid, CO_2, and the reduced coenzymes NADH, NADPH, and flavine adenine dinucleotide, reduced form. Thus, glutamic acid

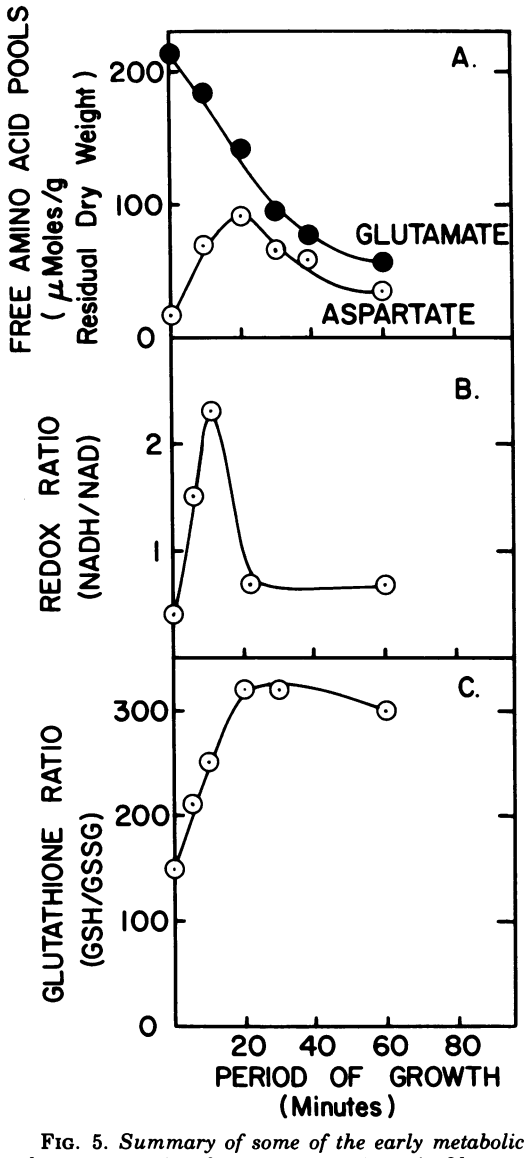

FIG. 5. *Summary of some of the early metabolic changes occurring during germination. (A) Changes in glutamic acid and aspartic acid pools; (B) changes in the ratio of NADH to NAD; (C) changes in the molar ratio of GSH to GSSG. The experimental details have been described (170).*

may be stored in conidia during conidiation as a reservoir for the production of reduced coenzymes during the early stages of germination.

Pyridine nucleotides. Dry-harvested conidia and vegetative mycelia contained nearly identical levels of NAD and NADH (170). The levels of NADP and NADPH in conidia were nearly twice those in mycelia. During the first 11 min of germination, the levels of the reduced forms of both pyridine nucleotides increased rapidly. The ratio of NADH to NAD increased from 0.4 in conidia to more than 2.0 at 11 min (Fig. 5B). The levels of NADPH increased from two- to seven-fold by 11 min. By 22 min after the initiation of germination, the levels of both NADH and NADPH had decreased to the original conidial levels.

Because many enzymatic reactions would be affected by changes in the ratios of NADH/NAD and NADPH/NADP, the shift toward the reduced forms of these coenzymes during the early stages of germination could be an important metabolic event associated with the breaking of dormancy. Those enzymatic reactions that require reduced pyridine nucleotides would be favored whereas those that require the oxidized forms of the coenzymes would be inhibited. The degradation of the endogenous glutamic acid pool could produce some of these reduced coenzymes.

Glutathione thiol-disulfide status. Conidia contained higher levels of oxidized glutathione (GSSG) and protein-bound glutathione (PSSG) than log-phase mycelia (53, 170). The levels of both of these disulfide compounds decreased rapidly during the first few minutes of germination. The molar ratio of reduced oxidized glutathione (GSH:GSSG) increased from 150 to 300 within the first 10 min of germination (170, Fig. 5C). The absolute levels of both GSSG and PSSG in conidia varied depending on the age of the conidia (53) and on the humidity at which the conidia were stored (Fahey, personal communication). However, the time required during germination for the levels of both of these disulfide compounds to decrease was relatively independent of the absolute levels.

The high levels of disulfide compounds in conidia are thought to play an important role in conidial dormancy. For germination to occur, these disulfide linkages may need to be reduced. The high levels of reduced pyridine nucleotides, especially NADPH, may occur to insure that all of these disulfide linkages are reduced during the early stages of germination. A more detailed discussion of this model for the breaking of dormancy is presented in a later section of this review.

Macromolecules

Studies of the synthesis of macromolecules (protein, nucleic acids, and cell wall components) during conidial germination have been mainly concerned with two questions. First, when are the syntheses of the various macromolecules initiated during germination? Second, is the synthesis of each of these classes of macromolecules essential for germination?

Protein synthesis and polysome assembly. During germination, the synthesis of protein,

as detected by the incorporation of radioactive precursors into trichloroacetic acid-insoluble material, begins within the first few minutes after the conidia have been suspended in media (10, 66, 132). Conidia, that had been harvested in water, incorporated a significant amount of radioactive leucine into protein within the first 5 min (10). In another study, mitochondrial as well as cytoplasmic protein synthesis was detected within the first 15 min after initiating germination (66).

Four lines of evidence indicate that protein synthesis is obligatory for germ tube formation. First, low levels of cycloheximide (5 to 10 μg/ml) effectively block both the incorporation of radioactive precursors into protein and the formation of germ tubes (81, 85; S. E. Hitchcock and V. W. Cochrane, *Neurospora* Newsletter 15:18–19, 1969). Because germ tubes appear after a few hours' delay when cycloheximide is removed, the effect of the inhibitor on germination is reversible (85). Second, ethionine, a methionine analogue, also inhibits both protein synthesis and germ tube formation (197). Third, the conidia from a mutant strain, *psi-1* (temperature sensitive for protein synthesis [108]), do not form germ tubes (109) at the restrictive temperature. Finally, some amino acid auxotrophs will not form germ tubes on unsupplemented media (156; 197; Schmit, unpublished observations).

In dry-harvested conidia, most of the ribosomes are freely dispersed. Less than 3% sedimented as polysomes (78, 132). Within 15 min after the conidia had been suspended in deionized water, from 20 to 40% of the ribosomes were assembled into polysomes. After 30 min in glucose medium, 60% of the ribosomes were in the polysome fraction. Because radioactive leucine was incorporated into polysomes within the first 15 min, the polysomes were actively engaged in protein synthesis. Thus, both polysome assembly and protein synthesis are early events in the process of conidial germination.

An interesting question is, what controls the initiation of protein synthesis during conidial germination? Ribosomes from conidia and mycelia have nearly identical sedimentation coefficients and RNA compositions (77, 154), and RNA with the properties of mRNA has been isolated from conidia (10). Perhaps protein synthesis is controlled by regulating the assembly of ribosomes into polysomes (53, 170). It has been reported that oxidized glutathione (at levels that are found in dormant conidia [53]) inhibits in vitro protein synthesis (101, 142). Oxidized glutathione can cause the dissociation of the ribosomes from the mRNA (101). The rapid reduction of oxidized glutathione during conidial germination may create the conditions favorable for polysome assembly, and thus, for the initiation of protein synthesis.

RNA synthesis. The initiation of the synthesis of all three major classes of RNA, rRNA, tRNA, and mRNA, occurs within the first 15 min of conidial germination. The synthesis of RNA has been measured by germinating the conidia in media containing radioactive uracil or adenine. During germination, most of the radioactive uracil that is associated with RNA is incorporated into rRNA (10, 132). Radioactive rRNA was found associated with polysomes within 5 min after the initiation of germination (132). Thus, both the synthesis and the processing of rRNA occurs very rapidly during germination. Newly synthesized tRNA can be detected during the early stages of germination (10, 132). Mirkes (132) isolated radioactive tRNA from polysomes after the first 15 min. With radioactive uracil, mRNA synthesis could be detected after the first 20 min (10). However, tritiated adenine was incorporated into the mRNA fraction within the first 5 to 15 min of germination (132). Because this RNA fraction was isolated from polysomes, the synthesis and association of mRNA with ribosomes apparently also occurs very rapidly during germination (218).

The rate of the incorporation of radioactive precursors into RNA appears to accelerate during germination (10, 81). However, these results are difficult to interpret because changes in either the levels of the intracellular pools or the rate of transport of the radioactive precursors could affect the apparent rate of RNA synthesis. It is interesting that some incorporation of uracil into RNA can occur in deionized water (132; S. E. Hitchcock, *Neurospora* Newsletter 11:15, 1967). Presumably, endogenous energy sources were being used.

Attempts to determine if RNA synthesis is obligatory for conidial germination have been hampered by technical difficulties. Neither RNA synthesis nor germ tube formation is inhibited by either actinomycin D or α-amanitin (207). Conidia appear to be quite impermeable to both of these inhibitors (207). Both conidial germination and RNA synthesis are inhibited by proflavin (81, 85, 207), but the specificity of this inhibitor is questionable. The concentrations of proflavin required to inhibit germination are higher than those required to inhibit RNA synthesis (85). Proflavin may inhibit germination by altering conidial permeability, rather than by specifically inhibiting RNA synthesis (207). Many different adenine auxotrophs

can form germ tubes on unsupplemented media (R. A. Meaden and A. M. Wellman, *Neurospora* Newsletter 11:19-20, 1967; Schmit, unpublished observations). Only conidia from the *ad-8* strain did not form germ tubes. Of two pyrimidine-requiring strains, *pyr-1* (Schmit, unpublished observations) and *pyr-3d* (109), only conidia from the *pyr-3d* strain did not form germ tubes on unsupplemented media (109). Because both adenine and pyrimidine nucleotides have many functions in the cell, such as the formation of ATP and sugar nucleotides, the inability of the conidia from the *ad-8* and *pyr-3d* strains to form germ tubes cannot be interpreted to mean that RNA synthesis is required for germination.

Four different RNA-polymerase activities have been isolated from the cell wall-less mutant strain (*slime*) of *N. crassa* (204). RNA polymerase activities have been isolated from the mitochondria (102, 231). It has not been reported if there are any changes in subunit structure or template specificity of any of these RNA polymerase activities during conidial germination.

In summary, it is concluded that RNA synthesis probably begins within 5 min after the initiation of conidial germination. All three major classes of RNA are synthesized. It still remains to be determined if RNA synthesis is essential for germination. Because mRNA is apparently in conidia (10, 132), it is possible that conidia are packaged with all of the components necessary to complete germination. This question will not be settled until reliable RNA synthesis inhibitors are found, or until mutant strains are isolated with defective RNA polymerase activities.

DNA synthesis and nuclear division. Measurement of DNA synthesis during conidial germination has been hampered by the inability of *N. crassa* to incorporate thymidine directly into DNA. *Neurospora* lacks thymidine kinase activity (67). Based on the increase in the total amount of DNA that could be extracted during conidial germination, Weijer (224) concluded that the DNA content begins to increase slightly before germ tube formation. Loo (109) came to the same conclusion by measuring the incorporation of [^{14}C]uracil into sodium hydroxide-resistant, trichloroacetic acid-precipitable material.

DNA synthesis does not appear to be essential for germ tube formation. About 40% of the conidia formed germ tubes with concentrations of hydroxyurea that inhibited DNA synthesis (109). The observation that DNA synthesis is not necessary for germ tube formation appears to be a general phenomenon for most fungal spores (218).

A small but steady increase in the number of nuclei occurred during conidial germination (109, 197). The number of nuclei doubled by the time germ tubes were three times the length of the conidia (13). About 30% of the nuclei in the conidia could still divide when DNA synthesis was inhibited with either hydroxyurea or by incubating the mutant strain, *psi-1*, which is temperature sensitive for protein synthesis at the restrictive temperature (109). This indicates that some of the nuclei in the conidia were probably arrested at the G2 phase of the cell cycle. Similar conclusions have been made with germinating spores from *Phycomyces blakesleeanus* (216).

In summary, neither DNA synthesis nor nuclear division appear to be essential for conidial germination. DNA synthesis is a late event in conidial germination and begins just before germ tube formation (2 to 3 h after the initiation of germination). Some of the nuclei in the dormant conidia appear to be in the G1 and some in the G2 phase of the cell cycle. There is no evidence for gene amplification or loss of genetic material during any phase of the asexual developmental cycle in *Neurospora* (218).

Cell wall polymers. The most striking morphological change that occurs during conidial germination is the formation of a tubular germ tube from a relatively spherical conidium. Because the major shape-determining element in fungi are its cell wall (16), there should be changes in the composition and/or the orientation of the structural components of the cell wall during germination.

The levels of two cell wall components, galactosamine and glucosamine, have been quantitatively measured during conidial germination (169). Cell wall galactosamine is predominantly a high-molecular-weight homopolymer of partially acetylated galactosamine (72, 151). Cell wall glucosamine is predominantly chitin, a β-1,4 *N*-acetyl glucosamine polymer (116, 117). Conidia contain very low levels of galactosamine (62, 169), whereas up to 10% of the mycelial cell wall can be this amino sugar (169). Galactosamine was detected in the cell wall-containing fractions when the shake cultures had reached a cell density of about 0.6 mg (dry weight) per ml of medium, regardless of the time required to reach this cell density. In some experiments, long germ tubes had been formed before galactosamine was detected (169). Because germ tubes can be formed without galactosamine-containing polymers, the accumulation of these polymers in the cell wall cannot be

responsible for the tubular structure of germ tubes.

About 10% of both conidial and mycelial cell walls is chitin (113, 116). During germination, the glucosamine content (chitin) increased more slowly than either the residual dry weight or the total surface area of the germ tubes (169). Thus, germ tube cell walls contain considerably less chitin that either conidial or mycelial cell walls. One possibility is that germ tubes may be initially formed without chitin (169). Chitin may be deposited as a secondary wall (120) behind the growing tip. Thus, chitin accumulation would lag behind the increases in both the total surface area and the dry weight. The hypothesis that chitin synthesis is not required for initial germ tube formation is supported by the observation that polyoxin D, a competitive inhibitor of chitin synthase (51), does not inhibit germ tube formation but does inhibit hyphal elongation (50). Since the germ tubes that were formed with polyoxin D were distorted, chitin probably does play some role in hyphal morphology.

Another compound, benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate], also inhibits hyphal elongation at concentrations that do not inhibit germ tube formation (K. S. Borck and H. D. Braymer, *Neurospora* Newsletter **20**: 16–17, 1973). Perhaps benomyl also inhibits the synthesis of chitin or some other cell wall component.

The nucleotide sugar precursors of chitin (UDP-GlcNAc) and galactosamine polymers (UDP-GalNAc) have been measured during germination (169). UDP-GlcNAc can be detected at all times. Thus, the relatively slow initial rate of chitin synthesis during germination is not due to the absence of this nucleotide sugar. The levels of UDP-GalNAc were lower in conidia than in mycelia from late log-phase cultures. However, only low levels of UDP-GalNAc were necessary for the synthesis of galactosamine-containing polymers in mycelia that were growing on agar medium (167). Additional experiments will be required to determine if the synthesis of galactosamine polymers can be regulated by controlling the production of UDP-GalNAc.

Both the appearance of galactosamine-containing polymers and the increase in the glucosamine concentration occurred simultaneously during germination (169). Thus, these two events may be in some way co-regulated. In yeast (21), chitin synthase is formed as a zymogen that is activated by proteolytic cleavage. Perhaps the formation of chitin and galactosamine polymers in *Neurospora* also involves zymogen activation. In shake cultures, a specific cell density may be required for the appearance of the proteolytic activities that are necessary for zymogen activation. Other explanations, such as the appearance of an inducer or perhaps cell contact, are also possible.

In summary, since the accumulation of galactosamine-containing polymers can occur well after germ tubes have appeared, the synthesis of this cell wall component does not appear to be essential for germ tube formation. Germ tubes can also form when chitin synthesis is inhibited, but the morphology of the germ tubes is altered. Thus, chitin synthesis also does not appear to be required for germ tube formation, but may be necessary for germ tube elongation. The precise changes in the composition and/or orientation of the cell wall structural components that are responsible for the different morphologies of conidia and mycelia still remain to be determined.

Specific Activity of Enzymes

The specific activities of 21 different enzymes have been measured in both conidia and mycelia. Those enzyme activities that are lower in conidia than in mycelia are given in Table 2, and those that are higher in conidia than in mycelia are given in Table 3. Many different kinds of enzymes, including biosynthetic, degradative, tricarboxylic acid cycle, and glycolytic enzymes, are represented in these Tables. Changes in the activity of some of these enzymes will be discussed in this section, as well as some of the considerations that must be made when interpreting changes in the specific activities of enzymes during germination or during any other developmental sequence.

The specific activities of three enzymes of the isoleucine-valine pathway, acetohydroxy acid synthetase, dihydroxy acid dehydratase, and amino-transferase,were lower in conidia than in mycelia (88). Also, the overall synthesis of valine from pyruvate was from 10- to 100-fold lower in conidia than in mycelia. An interesting observation was that the subcellular localization of the enzymes of the isoleucine-valine pathway changed during germination (88). In conidia these enzyme activities were located predominantly in the cytosol. In mycelia they were associated predominantly with the mitochondria (88). During germination, both the acetohydroxy acid synthetase and the dihydroxy acid dehydratase activities stayed relatively constant in the cytosol fraction, but increased in the mitochondrial pellet. In the mitochondria, the specific activity of the acetohydroxy acid synthetase and the dihydroxy acid dehydratase began to increase by 60 and 150 min, respectively. Cycloheximide prevented

TABLE 2. *Enzyme activities that increase during conidial germination*

Enzyme	Relative specific activity				Reference
	Conidia[a]	Germinating conidia[b]	Log-phase mycelia	Stationary-phase mycelia	
Acetohydroxy acid synthetase	1	40	~50		88
Aldolase	1		1.9	3.5	239
Aminotransferase	1	5			88
Aspartate transcarbamylase	1	2	2		88
	1	2			109
Cytochrome oxidase	1		1.4	1.2	202
Dihydroxy acid dehydratase	1	6	~30		88
Glucose 6-phosphate dehydrogenase	1		1.4	3.8	31
Glutamate dehydrogenase (NAD)	1	1.2	20	21	195
	1	1.1			214
Glutamate dehydrogenase (NADP)	1	5.0	100	50	195
	1	5.2			109
	1	20 (37 C)			109
	1	460	1000		214
Malate dehydrogenase	1		10	32	31
	1	1.1			214
Malic enzyme	0	60[c]	250	0	242
Ornithine transcarbamylase	1	2	4		88
Succinate dehydrogenase	1	0	55	50	195
	1		1	2	31
	1		10	9	239
	1		0.7	3.7	202
tRNA methylase[d]	1	2.5	2.3	1	236
Tryptophan synthetase	1		1.3	1.9	239
Tyrosinase	1		1.9	3.5	239

[a] The specific activity of the enzyme in the extract from the conidial inoculum was taken as 1.
[b] The enzyme activity from extracts prepared between the 3 and 7 h of germination.
[c] These are specific activities since no activity was detected in the conidia (242).
[d] This is the combined activity of three different tRNA methylases with *E. coli* tRNA as substrate. The ratios of the different methylase activities to one another changed during germination and growth (236).

TABLE 3. *Enzyme activities that decrease during germination*

Enzyme	Relative specific activity				Reference
	Conidia	Germinating conidia	Log-phase mycelia	Stationary-phase mycelia	
NADase	100	37	1	15	241
	100	43	0	0	194
	100		0	10	31
Trehalase	100	90	20	48	79
Invertase	100	120	1.7	7	79
β-Galactosidase	100		6	25	239
Malate synthetase	100		13	100	31

the increases in both enzyme activities. The overall ability to synthesize valine from pyruvate first increased in the cytosol and then, by about 3 h, increased in the mitochondria. One possible explanation for the change in the subcellular localization of these enzymes is that there may be a general membrane reorganization occurring during germination. Other observations which lend support to this hypothesis will be discussed in more detail in a later section of this review.

N. crassa has two different glutamate dehydrogenases, one specific for NAD (NAD-GDH) (EC 1.4.1.2) and one specific for NADP (NADP-GDH) (EC 1.4.1.4 [161]). Both forms of GDH were easily detected in extracts of conidia that

had been prepared from cultures that were 7 days old (214). However, after 15 days, the activity of the NADP-GDH had decreased to barely detectable levels, whereas the activity of the NAD-GDH had increased slightly. When conidia from 23-day-old cultures were put in minimal medium, the specific activity of the NADP-GDH increased more than 100-fold within the first 3 h. During this same period of time, the specific activity of the NAD-GDH increased only slightly. The increase in the specific activity of the NADP-GDH required a carbon but not a nitrogen source, and was inhibited by cycloheximide. A mutant strain, *am-1*, which lacks NADP-GDH activity and therefore requires a source of α-amino groups for maximum mycelial growth (161), germinates rapidly and produces long germ tubes in unsupplemented medium (109; Schmit, unpublished observations). Perhaps the large glutamic acid pool (168, 170) in the conidia supplies all of the amino groups that are necessary for the germination of the *am-1* strain. These results indicate that the synthesis of an active NADP-GDH is not essential for germination.

Different isozyme patterns of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) have been detected in conidia and in vegetative mycelia (31). Conidia had three electrophoretically distinguishable glucose-6-phosphate dehydrogenase activities, whereas only two were detected in mycelia harvested after 3 days of growth in sucrose medium (31). However, three distinguishable activities have been found in mycelia grown in glucose minimal medium (174, 175) and in mycelia from cultures grown for 3 days in acetate medium (31).

Conidia contained only one electrophoretically distinguishable malate dehydrogenase activity (EC 1.1.1.37) whereas four were detected in mycelia from cultures grown for 1 to 3 days in sucrose medium (31). Both the number of different malate dehydrogenase activities and their electrophoretic mobility varied with the age of the mycelia. On acetate medium, only two different activities were detected. It has been reported that malate dehydrogenase was a bifunctional enzyme containing both malate dehydrogenase and aspartate aminotransferase (EC 2.6.1.1) activities (133). Two unlinked genetic loci, *ma-1* and *ma-2*, affect these enzyme activities (133). However, these results are questionable since Kitto et al. (96) have been able to physically separate malate dehydrogenase from aspartate transaminase. They reported that there are both mitochondrial and cytoplasmic forms of both of these enzymes. The relationships of the various electrophoretic forms of malate dehydrogenase to the mitochondria and cytoplasmic forms of the enzyme have not been reported.

Malic enzyme (malate dehydrogenase [decarboxylating]; EC 1.1.1.40), which catalyzes the oxidative decarboxylation of L-malate to yield pyruvate and CO_2, has only been found in mycelia (242). Three different electrophoretically distinguishable isozymes have been detected (244). Two of these isozymes appear during the early stages of mycelial growth and disappear after 24 h. The third isozyme appears after 12 h, and its activity increases with the age of the culture. The first two isozymes were isolated from the third isozyme, and at least one of them could be inhibited by fructose-1,6-diphosphate (243). The third isozyme could be inhibited by aspartate. All of the enzymes are repressed by acetate and pyruvate, and their activities are inhibited by glyoxylate and oxaloacetate. Malic enzyme is interesting since it is the only enzyme activity assayed so far that is present in vegetative mycelia but that has not been detected in conidia.

As might be expected, the specific activity of those enzymes in conidia that are at least partially exocellular such as NADase, trehalase, and invertase, decreased during germination (Table 3). Some of the initial enzyme activity may have been lost into the water wash when the conidia were harvested, and additional enzyme may have been lost because it was bound to the cell wall (see Exocellular Enzymes). Measurements of the specific activity of these enzymes in extracts may not be a true indication of the amount of activity present in the conidia.

Changes in the specific activity of enzymes during conidial germination (or any other developmental sequence) must be interpreted carefully. Specific activity is expressed as units of activity per unit of protein. Decreases in the amount of extractable protein have been reported to occur during germination by some workers (79, 214), but not by others (88). Thus, the increase in the specific activity of some of the enzymes in Table 2 during germination could be due, at least in part, to decreases in the amount of extractable protein. The presence of inhibitors, activators, and proteolytic activities in the extracts must also be carefully considered. Many factors other than the rate of protein synthesis and the rate of degradation can affect the specific activity of an enzyme. These include post-translational modifications of the enzyme (phosphorylation, adenylation, specific proteolytic cleavage, glycosylation, etc.) and excretion of the enzyme into the media. A word

of caution also about the use of inhibitors of protein synthesis (i.e., cycloheximide) to establish that an enzyme is synthesized de novo during germination. Rather than inhibiting the synthesis of the enzyme itself, it is possible that the inhibitor could affect the synthesis of a second enzyme that may be required for activating the enzyme being studied. Another complexity is that, if the enzyme and its substrate are located in different organelles, changes in the specific activity of the enzyme in an extract might not reflect the in vivo activity of the enzyme. Finally, the observation of even a dramatic change in enzyme activity during a particular phase of development does not necessarily mean that this enzyme activity is needed for this phase of development. The enzyme may not be needed at all or may be needed for the subsequent phase of development. For example, NADP-GDH activity, which increases rapidly during germination (195, 214), is not required for germination since mutant strains, *am-1*, which lack this enzymatic activity germinate and produce long germ tubes on unsupplemented media. Another example is trehalase which may accumulate during conidiation, not because it is required for conidiation, but because it may be necessary for some aspect of the next phase of development, conidial germination.

In summary, conidia contain most of the enzymes found in vegetative mycelia. Both biosynthetic as well as degradative enzymes can be detected. The specific activity of most of the enzymes that have been measured (exocellular enzymes being the major exception) increase during germination. The increase in the specific activity of these enzymes probably reflects the general activation of metabolism that is associated with the breaking of dormancy.

Molecular Transport

The transport of amino acids, nucleosides, sulfate ions, and glucose has been studied in both conidia and mycelia. Recent reviews on molecular transport in *Neurospora* have appeared (164, 189). The reader is referred to these reviews for a more extensive discussion of this subject area.

Four different amino acid transport systems have been described in *Neurospora*. A general amino acid transport system transports D-, L-, basic, neutral, and acidic amino acids (147). The other three transport systems are specific for classes of amino acids based on net charge. A basic amino acid transport system is specific for L-arginine, L-lysine, and the arginine analogue, L-canavanine (7). A neutral amino acid transport system transports neutral and aromatic amino acids (36), including L-histidine (111). An acidic amino acid transport system that will transport glutamic and aspartic acids can be induced in "starved" mycelia (149). However, during conidial germination and log phase, the acidic amino acids are predominantly transported by the general amino acid transport system (91, 234, 235).

The general amino acid transport system in mycelia is repressed by ammonia and can be inactivated by high temperatures or by high concentrations of glycerol (160). This transport system is in wet-harvested conidia (111, 91), but is less active than in early log-phase mycelia (206).

Conidia which have been harvested and stored in water have constitutive neutral and basic amino acid transport systems (198). Conidia can transport both arginine and phenylalanine against a concentration gradient without an exogenous carbon source (232). In fact, glucose decreased the final level of phenylalanine accumulated in the conidia (36). Phenylalanine can be concentrated 1,500-fold within 1 h after the addition of this amino acid (232). The final intracellular concentration of free phenylalanine can reach 150 mM, which is about 100 times the level found at any time during conidial germination or vegetative growth in minimal glucose medium (168).

Many mutant strains have been isolated that have defective or altered transport of either neutral (87, 91, 107, 111, 148, 160, 190, 232) or basic (28, 111, 148, 153, 160, 205, 232, 237) amino acids. No mutant strains have been reported with defective general or acidic amino acid transport systems.

Two arginine-binding glycoproteins have been isolated from potassium chloride washes of dry-harvested conidia (198). Both binding proteins were missing in conidia from a mutant strain, $Pm^{-}B^{37}$, which is defective in the transport of basic amino acids. One of the binding proteins was missing in strain $Pm^{-}N^{22}$ which is defective in the transport of neutral amino acids. A tryptophan-binding protein has been isolated from germinated conidia by cold osmotic shock (230). This protein may be similar to the second arginine-binding protein isolated from dry-harvested conidia (198).

There are two nucleoside transport systems in conidia (166). One system is specific for purine nucleosides and the other is a common system for both purine and pyrimidine nucleosides. The activities of both transport systems are sensitive to sodium cyanide and 2,4-dinitro-

phenol. During the first 2.5 h of conidial germination (166), the activity of both nucleoside transport systems increased. Cycloheximide inhibited the increase in the transport activities of both of these systems.

The uptake of inorganic sulfate in *Neurospora* occurs by two different energy- and temperature-dependent transport systems (122). Permease I is found predominantly in conidia and is characterized by a relatively high K_m for sulfate. Permease II is found in mycelia and has a K_m for sulfate one order of magnitude lower than the permease in conidia. The unlinked genetic loci, *cys-13* and *cys-14*, affect permease I and II, respectively (122).

Two glucose transport systems, designated I and II (171), have been found in *Neurospora* mycelia (17, 162, 163, 197). System I is constitutive and has a low affinity for glucose (162, 171). System II is induced by starvation (163, 172, 173) and has a high affinity for glucose (163, 171). Ungerminated conidia appear to have both the low- and the high-affinity glucose transport systems (140).

In summary, molecular transport systems for amino acids, nucleosides, glucose, and sulfate ions have been detected in conidia. These transport systems appear to be identical with those in mycelia, with the exception of the sulfate permease system. Conidia have a different sulfate permease system than mycelia (122). The rate of transport of amino acids, glucose, and nucleosides increases during germination (164). However, the time when the increase in the rate of transport occurs during germination is not well understood. Careful studies using dry-harvested conidia are needed. It is interesting that conidia, even after being stored in cold water for many days, can accumulate large free pools of amino acids such as phenylalanine and arginine (232). This indicates that conidia have endogenous storage compounds that can supply energy for molecular transport.

Electron Transport and Oxidative Phosphorylation

N. crassa has two different respiratory pathways which can be distinguished by their sensitivity to cyanide (30, 103, 105). The cyanide-sensitive respiratory pathway (terminal respiration) transfers electrons to oxygen via the mitochondrial cytochrome chain. This pathway is also sensitive to antimycin and azide. The second respiratory pathway is resistant to inhibitors of terminal respiration, but is sensitive to salicyl hydroxamate (103). This "alternate" pathway proceeds by the initial portion of the electron transfer chain and does not appear to involve cytochromes (104). Mycelia from wild-type *Neurospora* normally respire by the mitochondrial cytochrome chain. However, the alternate pathway, which branches from the electron transfer chain before cytochrome *b* (104), can be induced by incubating the mycelia with antimycin A, cyanide, or chloramphenicol (103, 183). In addition, mutant strains *(poky)*, which have a defective terminal respiratory pathway, respire predominantly by the alternate pathway (105).

Conidia (in water) have relatively low rates of oxygen consumption (oxygen quotient from 20 to 30 μl of O_2/mg [dry weight] per h) (30, 66, 188) and respire predominantly via the alternate, cyanide-resistant pathway (30). From 2 to 4 h after initiating germination, the oxygen consumption of whole cells (188) and isolated mitochondria (66) increased 10- and 6-fold, respectively. The increased rate of oxygen consumption was correlated with the appearance of cyanide-sensitive respiration (30).

Oxidative phosphorylation also increased between 2 and 4 h of germination (66). Mitochondria isolated from conidia had a P/O ratio of 0 (66), which is consistent with the report that the alternate respiratory pathway is nonphosphorylating (105). By 4 h, the P/O ratio had increased to 0.6 (66). This is still somewhat lower than the P/O ratio of 1.5 to 2.0 obtained with mitochondria from mycelia (105).

In summary, *N. crassa* conidia respire predominantly by an alternate, noncytochrome, electron transport pathway. This is similar to resting cells from other organisms. Both seeds (8) and bacterial spores (at least immediately after initiating germination) (38, 94) respire by noncytochrome pathways. Because the alternate pathway in *Neurospora* is a nonphosphorylating pathway, conidia must obtain most of their ATP from substrate level phosphorylation for the first 2 h of germination. The increase in the rate of respiration during germination is accompanied by changes in the composition of the mitochondria (66). The ratios of phospholipid to both total lipid and protein increased five- to six-fold between 3 and 6 h of germination. Perhaps there is a causal relationship between the change in the lipid composition and the appearance of the cytochrome electron transport chain.

GENETIC ANALYSIS OF CONIDIAL GERMINATION

In the past it has proven useful to employ the mutational approach to unravel metabolic pathways and regulatory systems. Genetic ap-

proaches should also be very useful in understanding the mechanisms that control conidial germination.

There are three genetic approaches that can be applied to conidial germination. The first approach is to isolate mutant strains that specifically affect conidial germination and no other phase of the asexual cycle. The initial purpose of this purely genetic approach is to determine the number of loci that are both unique and essential for germination. Perhaps eventually, through diligent biochemical analysis, the biochemical defect in some of these strains might be discovered, but this may prove to be a very difficult task. The techniques for both selecting and characterizing conidial germination mutants should be developed before this class of mutant strains can be isolated. Some of these techniques will be described in this section of this review.

The second genetic approach is to characterize the conidial germination of the available mutant strains. Those strains that conidiate include auxotrophs, temperature-sensitive strains, and some of the morphological and regulatory mutants. Characterizing the germination of conidia from these strains will help to define more precisely those biochemical functions that are required for germination and may lead to the discovery of mutant strains that produce totally defective conidia.

The third approach is to isolate mutant strains in a specific biochemical function that appears to be unique to germination. These strains can then be used to answer the question of whether or not this particular function is essential for germination. This would be identical to the type of analysis used by Nelson et al. (139) to determine that NADase was not essential for conidiation or conidial germination.

A brief discussion is given of some of the results that have been obtained by using the first two genetic approaches described above. Consideration of potential biochemical functions where the third approach might be applied are presented in the next section of this review.

Methods for Characterization of Mutants

The success of genetic approaches to conidial germination relies heavily on a thorough characterization of mutant strains. This must include a thorough analysis of the other phases of the asexual cycle, as well as the germination process itself. A series of four different methods for characterizing the process of conidial germination are described below. These tests allow an extensive comparison of mutant and wild-type strains.

Spot test, microscopy observation of germ tube formation. A spot test involves the visual observation of a drop of conidial suspension placed in a spot on the surface of an agar medium. Estimates are made of the percentage of conidia with germ tubes at 6 and at 24 h. In addition, measurements are made with a calibrated ocular micrometer on the length of any germ tubes that are formed. The density of the conidia in the spot varied from 10^3 to 10^4 conidia/mm^2. This test is usually employed as the initial screening technique for new mutants because it is rapid and simple.

Colony formation, plating efficiency. In colony formation the number of conidia in conidial suspensions are counted in a hemacytometer, and then appropriate dilutions are made to insure the plating of approximately 100 conidia/plate. The conidia are plated on the surfaces of agar media, and the plates are incubated at 34 C. The agar contains sorbose (203) to restrict the growth of the hyphae, and therefore allows individual colonies to develop. Because this method measures colony formation, and not just the germ tube formation, it is a better criterion for the completeness of germination. For instance, many auxotrophic mutants (see below) will form germ tubes, but not colonies, on minimal media. This method would then indicate that they were indeed blocked in germination. However, this method does suffer from the fact that the conditions for colony formation may be slightly restrictive, i.e., high temperature, and high concentrations of sorbose.

Growth of individual conidia in liquid media. The testing of individual conidia grown in liquid media was designed to measure the germination of individual conidia without resorting to the use of sorbose or incubation at 34 C. The number of conidia in conidial suspensions are counted and then diluted into minimal medium. Dilutions are made so as to obtain a Poisson distribution around 1.0 conidium/tube. Because some of the tubes will have two or more conidia, and some will have none, about 50% of the tubes will have visible growth when incubated with conidia of the wild-type strain. By comparing the number of tubes containing growth from a mutant strain with those of the wild-type strain, a defect in germination can be detected. This method also measures conidial germination under submerged conditions, where the O_2 tension may be lower than on the surface of agar media.

Germination in shake cultures. The germi-

nation in shake cultures technique involves the inoculation of a sizeable number of conidia (10^4 to 10^7/ml) into liquid media, and the subsequent measurement of the dry weight of the culture as a function of time. This method allows variations in the density of the inoculum, as well as a direct comparison to an equivalent amount of mycelial inoculum. Germination is also under submerged condition. Samples can be removed at different times, fixed with Formalin, and the percentage and length of germ tubes measured.

Selection of Mutants

In *Neurospora,* the direct selection of conidial germination mutants has been attempted by several laboratories (85; Loo and Stadler, personal communication; Schmit and Brody, unpublished observations). In other microorganisms, specific spore germination mutants have been isolated and characterized. A partial listing includes *B. megaterium* (219), *B. subtilis* (56), and *Dictyostelium discoideum* (92). A general approach has been to look for temperature-sensitive germination mutants because this technique allows a strain with an otherwise lethal mutation to be propagated easily. In addition, the temperature-sensitive property allows an interesting set of environmental manipulations to be performed.

Although it is not clear why there has been only limited success in the isolation of mutants specifically blocked in conidial germination, a few reasons suggests themselves. One, there may be only a few genes and gene products involved just in the germination process. Thus the majority of mutations that block germination would also be expected to affect vegetative growth. A second conceivable stumbling block is the possibility that those gene products truly unique to the germination process are already present in the spore before germination, and therefore, do not require de novo synthesis upon germination. If this were the case, then a straightforward mutagenesis and plating of spores would not be very successful. Even those spores bearing a new mutation in a critical gene would still contain enough of that existing gene product to germinate. A possible ploy to get around this problem is, of course, to employ an "expression cycle" between the time of mutagenesis and the time of mutant selection. For instance, in *Neurospora*, mutagenized conidia can be allowed to form individual colonies and then to conidiate. The conidia, which are collected from these colonies, are then tested for germination. If temperature-sensitive mutations are being hunted, then the intervening vegetative growth cycle can be performed at the high temperature. This latter technique has the added advantage that the large number of general temperature-sensitive mutants often found (86) are excluded because they will not form colonies or conidiate at the high temperatures. Theoretically, the only mutants eventually isolated should be blocked specifically in the germination process. The defect in mutants of this type would be analogous to a temperature-sensitive starter on a car, a well-known phenomenon.

Heterocaryon Analysis

The formation of heterocaryons in *Neurospora* and other fungi has been employed primarily for the studies on the complementation of auxotrophic mutants. It can also be of some value when applied to the studies on germination mutants. As a quick review, heterocaryons are formed by the fusion of hyphae of two different genotypes. Both types of nuclei are found in this common cytoplasm. The nuclei do not fuse, so a diploid state is not formed. The heterocaryotic strain can conidiate, and the conidia so formed are of three types. Usually, the predominant type contains nuclei of both genotypes, whereas the two minor varieties of conidia contain only one or the other of the genotypes. The proportion of these three classes depends on the nuclear ratio of the two genotypes in the cytoplasm and on the average number of nuclei per conidium. The use of this technique in studying germination lies in the fact that heterocaryons can be constructed between normal strains and germination mutants, and a type of conidia can be obtained from this heterocaryon which contains all of the normal gene products, but which is genotypically mutant. In other words, some of the conidia contain mutant nuclei, but still have the cytoplasmic gene product produced by the nuclei of the normal strain. In practice, this technique works as follows: mycelia from an auxotrophic strain, say *arg*$^-$, and from a tryptophan-requiring, nongerminating strain, designated *cog*$^-$ *trp*$^-$, are placed on minimal media. After fusion of the mycelia, the resulting balanced heterocaryon is propagated on minimal media and allowed to conidiate. The conidia are plated on media containing arginine and tryptophan, under conditions where the *cog*$^-$ strain would not be able to germinate. The colonies that arise from individual conidia are then tested for their tryptophan requirement. Any *trp*$^-$ colonies that arose from conidia obtained from the heterocaryotic strain are due to *cog*$^-$ *trp*$^-$ conidia that somehow germinated. Persumably, these conidia were able to germinate

because they had some cytoplasmic factor, originally produced by the nuclei of the *arg*$^-$ component, which allowed them to germinate. This factor could be a critical enzyme, the mRNA for that enzyme, or even the product of that enzyme. If on the other hand the *cog*$^-$ *trp*$^-$ conidia were unable to germinate from a heterocaryotic strain, then the *cog*$^-$ mutation was either dominant in heterocaryons, or, more likely, the critical gene product had to be made de novo upon germination, and therefore was not present in the common cytoplasm of the heterocaryon. In principle then, this heterocaryon test can distinguish between mutations which affect those gene products synthesized de novo and those prepackaged into spores at the time of conidiation.

Mutations That Affect Germination

In *Neurospora*, a large number of mutations of different types have been tested for their effects on conidial germination. These mutations can be roughly classified as auxotrophic, morphological, temperature sensitive, and miscellaneous. The auxotrophic mutants that have been tested so far consist of 54 different mutant strains. These mutations lead to blocks in 26 different pathways, such as the synthesis of amino acids, purines, pyrimidines, vitamins, lipids, etc. These results will be reported in some detail elsewhere, and are just summarized here. Conidia from these different mutant strains were all tested for germ tube formation on minimal media, and in many cases, for colony formation on minimal media. None of the auxotrophic strains formed colonies, but most of the conidia from each of 37 of these strains did form germ tubes on unsupplemented media. This suggests that for most of these strains, the existing pools of conidial compounds were at least sufficient for the early stages of germination, including the formation of a visible germ tube. In addition, some of the auxotrophic strains had very long germ tubes, which can be interpreted to mean that either a large amount of nutrients were present in the conidia, or that the mutational block was not complete, i.e., "leaky." The strains whose conidia showed few, if any, germ tubes are as follows: *ace*-1, *ad*-8, *arg*-6, *aro*-1, *cys*-4, *his*-3, *his*-6, *lys*-1, *lys*-2, *met*-2, *phe*-2, *pro*-1, *thr*-1, *trp*-2, *tyr*-1. It is interesting that defects in the synthesis of the aromatic amino acids or basic amino acids (arginine, lysine, and histidine) lead to inability to form germ tubes.

A large number of morphological mutants of *Neurospora* have been described (16). Mutations at 120 different loci have already been detected. Most of these morphological mutants grow more slowly than the wild-type strain, have abnormally shaped hyphae, and do not conidiate. Of the 19 morphological mutants that do conidiate, all produce conidia that will form germ tubes (Schmit and Brody, unpublished observations). Colony formation was also not impaired in these strains. The rate of germ tube elongation was slower in these strains, suggesting that the defect in the metabolism of the vegetative phase was also expressed during the germination process.

Many temperature-sensitive mutants of *Neurospora* have been analyzed with respect to conidial germination (85, 108). Out of a group of 47 non-supplementable temperature-sensitive strains, only 7 did not form germ tubes at 35 C, the nonpermissive temperature (85, 108). All of these seven strains were also defective in vegetative growth at 35 C. Thus, temperature-sensitive mutant strains that blocked just a step in the germination sequence were not obtained. It is interesting that most of the temperature-sensitive mutants would form germ tubes at high temperatures. This suggests that those particular genes and gene products affected by these mutations were either not required for germ tube formation or that the existing pools of compounds were sufficient for germ tube formation. It is also interesting that conidia of the wild-type strain will form germ tubes at 4 C and 44 C, temperatures which do not allow hyphal growth (156).

Two mutations which appear to affect conidial germination more severely than vegetative growth have been studied. One of these, a mutation affecting the regulation of sulfur metabolism, designated *scon*^{c} (19), produces defective conidia (Schmit et al. *Neurospora* Newsletter 21:17–18, 1974). The conidia appear to be osmotically fragile. No obvious morphological change in the vegetative phase of the life cycle was detected, even though the enzymes of sulfur metabolism are derepressed (19). The connection between the constitutive nature of the sulfur metabolism and the germination defect requires further study.

A second temperature-sensitive mutant strain, JS-K3, shows some interesting properties (Schmit and Brody, manuscript in preparation). Under normal conditions, this mutant strain is nearly identical at all temperatures to the wild-type strain, with respect to growth rate and morphology of the mycelia. Conidiation proceeds readily at both temperatures, but the conidia of the mutant strain formed at any temperature produce only short germ tubes at

34 C. Germination proceeds rapidly at room temperature, however. Although in initial studies this strain appears to be defective only in the later stages of germination, extensive testing indicated otherwise. Under conditions of high cell density, conidia from this strain will germinate at 34 C in liquid cultures. Under certain abnormal conditions, the morphology of the vegetative hyphae is also affected by the mutation in this strain. Additional details of this strain will be published later. Perhaps the important point to be made is that extensive and multiple criteria must be employed to establish that a particular mutation affects only the germination phase of asexual development.

Phase-Specific Mutations

In the previous paragraphs, there has been some mention of mutations which might affect just the germination process. The term "phase-specific mutations" could be applied to this type of mutation if it could be rigorously shown that only one phase of the life cycle was affected. This term was adapted from the description of stage-specific mutations used by the developmental geneticists. The term "phase specific" should be defined on the basis of gene products and not just on observable phenotypes. That is, it would have to be shown that the gene product which was altered in a given mutant and lead to a defect in one phase, was not present in the other phases of the life cycle. Gross characterization of the external phenotype would not be enough, since one could imagine some untested combination of environmental conditions that could still distinguish the mutant from the wild-type strain. Perhaps a slightly more flexible term, such as "phase-critical mutations", would be more useful. This term is defined as those mutations affecting a gene product which is present throughout most of the phases of the life cycle, but whose function is more critical to one given phase. Phase-critical mutations are those which severely affect germination, but have only trivial effects in other phases. In these cases, perhaps the gene product may be present at low levels during other phases of the life cycle, or the metabolism during other phases is not as critically dependent upon that particular gene product. In any event, the term "phase-critical mutations" could initially apply to all of germination mutations until some of them were rigorously proved to be "phase specific".

Some examples of "phase-critical mutations" in *Neurospora* are the aconidial mutants (179), and a mutation affecting a protein found only in the sexual cycle (137). Other examples are the mutations which lead to male sterility in conidia, but do not affect their germination ability (225).

Conclusions

The results of the screening of many (>100) different mutant strains for conidial germination are somewhat disappointing. The conidia from most of the auxotrophic mutants would form germ tubes on minimal media, as did the conidia from most of the morphological mutants. Similar studies performed on temperature-sensitive mutants also yielded no obvious clues as to the nature of the germination process. However two strains that were studied, *scon*^{c} and JS-K3, showed some promise in terms of further characterizing their defective germination process. No phase-specific germination mutants have been isolated so far. It would probably be helpful to select mutants blocked in those steps postulated to be unique to germination, such as: (i) the generation of large amounts of reduced pyridine nucleotides; (ii) the disappearance of soluble and protein disulfide bonds; (iii) the reorganization of the mitochondrial oxidative phosphorylation system; (iv) the creation of a site for germ tube appearance. In addition, any germination mutants that are found could be screened for defects in these steps.

CLASSIFICATION OF EVENTS IN GERMINATION

The approximate time when various morphological and biochemical events occur during conidial germination is given in Fig. 6. The major morphological events are hydration and then the formation and growth of a germ tube. Hydration occurs within 2 min after the conidia are suspended in either water or media. Some of the conidia begin to form germ tubes approximately 2.5 h after the initiation of germination. Many factors affect the formation of germ tubes including the age of the conidia, the composition of the media, the temperature, the harvesting conditions (dry harvested versus wet harvested), the concentration of conidia in the media, and the genetic background of the strain being tested. After 5 h in shake cultures containing glucose minimal medium at 22 C, most of the conidia will have formed germ tubes. Macromolecular synthesis begins before germ tubes appear, since the dry weight (ethanol-insoluble material) had increased more than 1.6-fold by 2.5 h (Fig. 2). The average germ tube length increased exponentially with about the same doubling time as the residual dry weight.

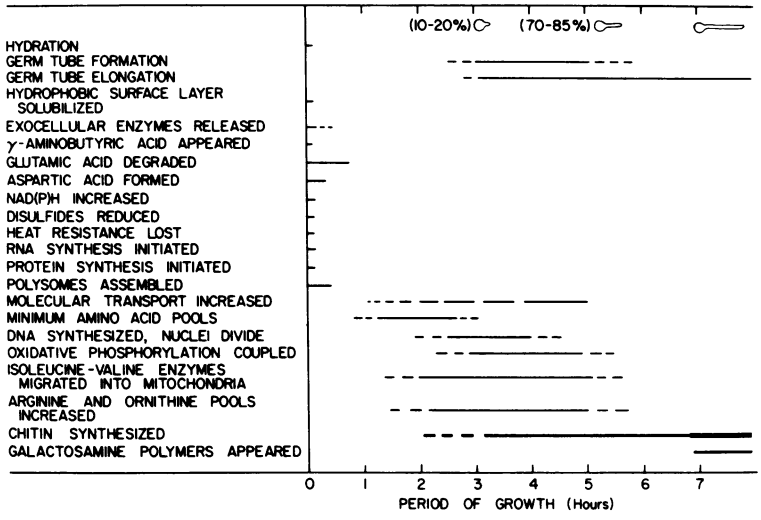

FIG. 6. *Summary of some of the morphological and biochemical events that occur during conidial germination. The time each event occurs is approximate (dashed lines) since different conditions were used by different investigators. By 3 h, about 10 to 20% of the conidia had germ tubes, and by 5 h, 70 to 85% had germinated.*

Many different biochemical events have been observed during conidial germination. These events fall into three groups, depending on when they occur during germination (Fig. 6). The largest group of biochemical events that have been described are those that occur within the first 20 min of germination. These are considered early events and include the loss of heat resistance, the degradation of free glutamic acid, the formation of aspartic acid and γ-aminobutyric acid, the conversion of most of the pyridine nucleotides to their reduced forms, the reduction of oxidized glutathione, and the initiation of protein and RNA synthesis. The changes that occur in the levels of the low-molecular-weight compounds, glutamic acid, pyridine nucleotides, and glutathione, deserve special consideration because these changes are both initiated and completed within the early stages of germination. This is in contrast to protein and RNA synthesis which are initiated during the early stages of germination but then continue throughout vegetative growth. A model for the breaking of dormancy that is based in part on these early biochemical events is presented in the next section of this review.

A second group of biochemical events that have been described occurs approximately coincident with germ tube formation (Fig. 6). These include the initiation of DNA synthesis and nuclear division, the appearance of coupled oxidative phosphorylation in the mitochondria, the migration of the enzymes of the isoleucine-valine pathway into the mitochondria, and the accumulation of high levels of free arginine and ornithine. In the last case, the arginine and ornithine are probably being accumulated in discrete membrane-enclosed vesicles (228). These events, including the formation of a germ tube itself, are presumably associated with some change in membrane structure. This is consistent with the observation that the phospholipid content of conidia decreases sometime during germination (12). Thus, it is proposed that there is some type of general reorganization of the membrane structure that occurs coincident with germ tube formation.

The third group of biochemical events are those that occur relatively late in germination. These include the synthesis and accumulation of chitin and galactosamine-containing polymers in the cell wall. The synthesis of chitin is not necessary for germ tube formation but may be required for germ tube elongation. Galactosamine-containing polymers, which are essentially absent from conidia, begin to accumulate well after germ tubes have formed under some growth conditions (169). Thus, these polymers are not required (at least not at high levels) for germ tube formation. It is interesting that galactosamine polymer formation and accelerated formation of chitin appear to occur simultaneously during germination (169). This suggests that both of these amino sugar polymers may respond to changes in the same set of environmental conditions.

At least some of the early germination events can occur upon the addition of water and, thus,

do not require exogenous carbon sources. These include the formation of γ-aminobutyric acid, the simultaneous degradation of glutamic acid and formation of aspartic acid, the assembly of ribosomes into polysomes, and a limited amount of protein and RNA synthesis. The reduction of GSSG to GSH can be accomplished simply by incubating conidia at 100% humidity. Preliminary results also indicate that the pyridine nucleotides can be converted to their reduced forms during incubation in deionized water (Brody, unpublished observations). The appearance of germ tubes can be accelerated by preincubating the conidia in water (Fig. 3). Also, conidia that have been stored in cold water for several days are capable of transporting and concentrating amino acids without an exogenous metabolizable carbon source. All of these observations indicate that conidia contain reservoirs of readily available energy sources that can be metabolized as soon as the conidia become hydrated. These storage compounds include glutamic acid (2.5% of the dry weight), trehalose (10% of the dry weight), and perhaps phospholipids (17% of the dry weight). Thus, most of the early biochemical events that have been measured thus far can also occur in deionized water. Of the other events that occur during germination only germ tube formation itself has been shown to require an exogenous carbon source.

The biochemical events that occur during germination can also be classified depending on whether or not they are unique to the germination phase of the asexual developmental cycle. Some of the events that may be unique to germination are the simultaneous degradation of glutamic acid and formation of aspartic acid, the conversion of most of the pyridine nucleotides to their reduced forms, the reduction of GSSG to GSH and the loss of heat resistance. Both the formation of large endogenous aspartic acid pool and extremely high levels of reduced pyridine nucleotides have not been observed at any other time during the asexual cycle. The rapid decrease in the level of GSSG has only been observed during conidial germination. It is not yet known whether other events such as the initiation of both protein and RNA synthesis are unique to germination. The molecular mechanisms that result in the initiation of the synthesis of these macromolecules during germination may be identical to those used during vegetative growth. Perhaps simply the production of energy or the reduction of disulfide linkages (GSSG) are all that is necessary to initiate these events. The mechanisms associated with the appearance of coupled oxidative phosphorylation may be unique to germination, but additional experiments are necessary. Events that are probably not unique to germination are the syntheses of DNA, galactosamine-containing polymers, and chitin.

The data collected so far can be interpreted to indicate that more than one developmental program is operating during germination. For example, germ tubes can be formed without DNA synthesis, and conversely, DNA synthesis can occur when germ tube formation is inhibited. Both of these events normally occur at about the same time during germination. This may be analogous to DNA replication and budding in the yeast cell cycle, which appear to operate on different programs (73).

BREAKING OF DORMANCY

Proposed Model for the Germination of *Neurospora* Conidia

On the basis of the data published here and elsewhere, a four-point model is proposed which deals with *Neurospora* conidial dormancy and the initial events in germination. This model, although formally discussed here, should still be considered as a "working hypothesis" and not a theory. It obviously requires considerable experimental verification and it is surely expected to undergo substantial modifications. It is presented here because it makes certain experimental predictions for the process of conidial germination in *Neurospora* and because of its potential general applicability towards understanding the germination process in other organisms.

Dormancy and oxidized sulfhydryls. When compared to mycelia, dormant conidia contain a higher level of oxidized sulfhydryl groups (53). These include both oxidized glutathione (GSSG) and glutathione associated with protein. Because of these findings, the first proposal in the model is that the high levels of these oxidized compounds play a role in the maintenance of the dormant state. It is expected that the high levels of GSSG in conidia (53) affect the sulfhydryl groups of the conidial proteins. This expectation is based on the studies of the interaction of oxidized and reduced glutathione (GSH) with purified lysozyme (229). The addition of various mixtures of oxidized and reduced glutathione or cystine and cysteine caused a significant proportion of the sulfhydryl groups of lysozyme to be converted to their disulfide form. The reaction with oxidized glutathione did not appear to be enzymatically catalyzed, but it was affected by the GSH:GSSG ratio. By analogy to this work, an increase in the intracellular level of oxidized glutathione

might lead to an increase in protein disulfide bonds. Enzymes either internally or externally cross-linked in this way might be relatively inactive and in a much more rigid, heat-stable form. Thus, the formation of protein disulfide linkages could account in part for the low metabolic activity of spores and for the apparently high heat resistance of the protein. Direct measurements on the protein disulfide content of conidia versus mycelia will be important data to obtain.

Clearly, alternative explanations for the appearance of high GSSG in conidia are possible. For instance, high levels of GSSG may merely be coincidentally associated with conidia and/or the mechanisms which enzymatically reduce these compounds may be relatively inactive in conidia. In addition, other roles for the high levels of GSSG could be the prevention of lipid oxidation or the regulation of polysome assembly (53, 170).

Germination and the reduction of disulfide bonds. In the first few minutes in the conidial germination process, most of the oxidized glutathione and protein-bound glutathione is reduced (53). The second postulate of this model is that this disulfide reduction is one of the initial requirements for germination. It is not postulated to be the only initial requirement for germination and there is no evidence at this time which indicates that this step must obligatorily precede any other early event. Nevertheless, the reduction of oxidized glutathione occurs almost immediately during conidial germination and it also occurs during the activation of other dormant organisms (see below). If proteins were in an inactive, disulfide form in conidia, then one might expect metabolic activation of these proteins to occur quite early in the germination process. The mechanism of this reduction could again involve some type of interchange between soluble thiol and protein disulfides. It is interesting to note that the heat resistance properties of *Neurospora* conidia are also lost in the first few minutes of germination. An alternative explanation for the early reduction of the disulfides would be that it was not at all necessary for germination, but was merely coincidental with germination.

Initial requirement for NADH and NADPH. One of the other events that occur in the first few minutes of conidial germination is a rapid shift towards the reduced state of the pyridine nucleotides (170). Five to ten minutes after the onset of germination, there is a threefold increase in NADH and NADPH content, and a decrease in the NAD content. This sharp increase in the levels of NADH and NADPH is only temporary. By 20 min after germination, the levels and ratios of these compounds had returned to their ultimate mycelial levels. The third postulate of the model is that a surge of reducing power, in the form of NADH and NADPH, is a key initial requirement for conidial germination. It is not postulated to be the only requirement for germination and it is not known where it comes in a sequence of germination events. This surge in reducing power could be used in part to reduce GSSG (H^+ + NADPH + GSSG $\rightarrow$ 2 GSH + $NADP^+$). This could provide a feasible explanation for the decrease in GSSG observed during germination. The high levels of reduced coenzymes could also be used for biosynthetic reactions, such as fatty acid synthesis and desaturation, etc. Those enzymatic reactions that require reduced coenzymes would be favored whereas those that require oxidized coenzymes would be inhibited. In addition, the reduced coenzymes might also be consumed for the generation of some ATP during the initial stages of germination via some type of noncytochrome-mediated mitochondrial electron transport system.

It is also significant to note that changes in the pyridine nucleotide redox ratio of this magnitude have not been observed before in *Neurospora,* even under a variety of conditions. For instance, mutations in the pyridine nucleotide synthesis pathway and supplementation with niacin have no effect on these ratios (15). Even the influence of the biological clock on these ratios (16a; Brody, unpublished observations) is relatively small compared to the changes observed during germination.

Endogenous source for the generation of NADH and NADPH. The fourth postulate of this model is that spores contain endogenous reservoirs of storage compounds that are rapidly metabolized during germination. The important metabolic consequence of the consumption of these storage compounds would be the generation of the reduced form of the coenzymes. Conidia contain a large pool of free glutamic acid that is rapidly consumed during the first hour of germination (168, 170). Glutamic acid could be degraded via one of two pathways, either of which would generate NADH and NADPH. Other stored compounds such as trehalose and alanine could also produce reduced coenzymes during conidial germination.

Regardless of the actual source of these reduced coenzymes, some general prediction about the nature of the generation process might be made. It is postulated above that disulfide reduction is the activation reaction for the breaking of dormancy. The generation of

some of the reduced coenzyme (especially NADPH since glutathione reductase requires this coenzyme) would precede this reduction. Therefore, one might expect that the few enzymes involved in NADH and NADPH generation would have to be active in the conidia. Either they don't contain reactive sulfhydryl groups or they remain active upon oxidation and reduction of their disulfides. These enzymes could also be stabilized in some other way, such as by binding to metal ions or other compounds. It is interesting to note that glutathione reductase is one of the few intracellular enzymes known to contain disulfide bonds (89). Whether this will also be true for the enzymes involved in the degradation of glutamic acid will need to be experimentally tested. Another prediction about these enzymes is that they should already be present in the conidia before germination, and would not have to be synthesized de novo. Therefore, their specific activity may increase during conidiation (i.e., trehalase). Perhaps also, the enzyme(s) involved in generating NADH and NADPH will be different from most dehydrogenases in terms of their sensitivity to inhibition by reduced coenzymes or in terms of their response to the ratio of NAD/NADH or NADP/NADPH. This point is raised because a drastic shift in the redox ratio toward the reduced side as seen in the first few minutes of germination might be interpreted to mean that a special set of enzymes was involved in NADH and NADPH generation at that time.

The overall model requires some further comment. The proposed inactivation and stabilization of proteins via disulfide formation is readily reversible and possibly a non-enzymatic process. No changes in the primary structure of the cellular proteins are postulated to be involved, only possible changes in the conformation of the proteins. The metabolic event which is postulated to activate the enzymes (i.e., sulfhydryl interchange with reduced glutathione) is quite different from those events which are proposed to initiate the process (i.e., degradation of a storage compound such as glutamate). The sum total of all these proposed events would constitute a multistep "trigger reaction" (Fig. 7). The model does not exclude other reactions from being requirements for germination or from grouping these other events into multistep trigger reactions. The model makes no predictions about the events that might occur subsequent to the proposed activation of the cellular proteins. This point is raised in reference to the release or activation of cell wall degradative enzymes, a process

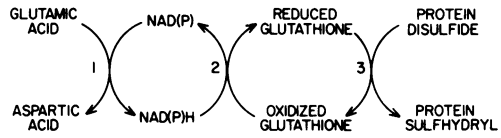

FIG. 7. *Proposed model for the initial events in Neurospora conidial germination. (1) Glutamate could be converted to aspartate by either of two pathways (168, 170), both of which would produce NADH and NADPH. (2) The reduction of GSSG to GSH is catalyzed by glutathione reductase and would require NADPH. (3) The reduction of protein disulfide linkages could occur by thiol-disulfide interchanges. It is proposed that proteins in the dormant spore may at least be partially inactivated and stabilized by internal and external disulfide linkages.*

sometimes called the "can-opener" step. Likewise, the model does not deal with the other key elements in the germination process, such as gene activation, translational controls, etc. This model is concerned only with some of the very early events in germination. Whether these events are actually the initial events is still to be determined.

The breaking of dormancy as currently envisioned for conidial germination is summarized in Fig. 7. Briefly, this model for the initial events in *Neurospora* conidial germination has four major postulates, each of which is based on new experimental observations. As pointed out above, each of these observations can be interpreted in alternate ways. Therefore, it is possible that the entire sequence of events outlined above is just coincidental with dormancy and germination, and is not at all critical to germination. Even when viewed in this light, however, this model could still be employed as a framework for understanding some of the events in germination and for stimulating the undertaking of better experiments. It is clear that quite a few decisive experiments are needed at this time, since most of the existing observations are really just phenomenology, whose significance is unknown. It is hoped that the proposal of this model does not distract from the new experimental observations that have, in fact, been made about the early stages of germination.

Possible Relationships to the Germination of Other Organisms

Some of the points in the model as presented above might be analogous to events that occur in the breaking of dormancy of other organisms. Three aspects of the model are worth exploring, namely, the role of disulfides, the production of NADH and NADPH, and the

presence of endogenous storage compounds. Naturally, there will be limits to these analogies since the germination of spores of certain organisms require conditions not necessary for conidial germination, such as heat shocks, specific compounds, exposure to light, etc. (202). Nevertheless, some interesting parallels can be pointed out.

Increased levels of disulfides are also found in the dormant stage of other organisms. Fahey (personal communication) has evidence indicating that wheat seeds contain 10-fold more GSSG than germinated wheat seeds. In the spores of certain bacilli, the spore coat contains a high content of disulfide bonds (2, 100). In addition, the overall sulfhydryl content has been examined in *B. megaterium* spores (180, 220). One report of an increase in SH content upon germination (220) was not confirmed when ruptured dormant spores were assayed (180). It is difficult to make this type of measurement of the true dormant state because the addition of liquid to spores or ruptured spores may in itself trigger some activation reactions. Some attempts were made to rule out any enzyme-mediated changes in SH content during the preparation of a ruptured spore extract (180). However, some type of non-enzymatic thiol-disulfide interchange, as proposed in the model, may have occurred. It is unlikely that any reaction of this type during bacterial germination involved glutathione, since hardly any glutathione can be detected in *B. cereus* spores (27). Whether some other soluble sulfur-containing compound might be involved is simply a matter of conjecture. It seems quite plausible that the stabilization and inactivation of proteins in the bacterial spore could occur by completely different mechanisms other than that proposed for conidia. The theories for the bacterial protein changes have been concisely reviewed by Keynan (93). The presence of large amounts of calcium-dipicolinic acid in many bacterial spores is an aspect of their dormancy not found in the *Neurospora* conidia, and should not be overlooked.

The proposal that proteins in their disulfide form may be relatively stable to heat or other environmental insults has been pointed out previously. In *B. stearothermophilus,* a heat-stable α-amylase and a heat-stable glyceraldehyde-3-P-dehydrogenase, were both found to have a high disulfide content (187). It is well known that extracellular enzymes from a variety of sources have a high disulfide content (89). These lines of evidence do not indicate that the only way to stabilize proteins is by the formation of internal disulfide bonds, but it does indicate that this is one possible mechanism. Another possibility could be the formation of disulfide linkages between proteins or between protein and some other cellular component. It would be informative to do further studies on the relative heat stability of the reduced forms of these disulfide-rich proteins. Many studies have been conducted on the effect on the activity of proteins due to disulfide reduction. Two cases in particular may be pertinent here. Studies on β-amylase in germinating barley seeds have shown that the enzyme is present in an inactive form in the seeds, and can be activated by reducing agents, such as mercaptoethanol (14). Upon germination, the enzyme activity is found to increase. A second interesting case is the studies on streptococcal proteinase (20). This protein can be isolated as a zymogen in the disulfide form. Reduction activates the enzyme, and then proteolysis takes place. If proteolysis occurs first, then disulfide reduction occurs more rapidly. It might be interesting to determine if the proteases activated during germination have similar properties to this proteinase.

Another similarity between the germination events in *Neurospora* and in other organisms is the apparent need for reduced pyridine nucleotides during germination. Freese and his coworkers (150) have inferred this for *B. subtilis* germination, based on work with mutant strains and initiator compounds. However, no actual measurements of NADH or NADPH during germination have been reported. Setlow and Kornberg (182) reported that *B. megaterium* spores contained NAD and NADP but very little NADH or NADPH. Vegetative cells had the reduced forms of these coenzymes. It is assumed from their work that some time in the transition from spore to vegetative cell there must have been a shift towards the reduced forms of these coenzymes. It is not known whether this shift in the ratio occurs early in germination. It is also possible that the formation of NADH and NADPH is employed for some purpose other than the reduction of disulfides. In seeds, recent evidence on the effects of germination initiator compounds has been interpreted to indicate the importance of the pentose phosphate shunt in germination (75). This pathway is a well known source for the generation of NADPH.

Conidia contain storage compounds such as glutamic acid and trehalose which could be used to generate reduced pyridine nucleotides. Possibly analogous situations have been reported in a variety of different organisms. In *Schizophyllum commune,* the basidiospores contain high levels of polyols, such as mannitol

and arabitol (1, 141). Arabitol was rapidly metabolized during germination, presumably by a dehydrogenase shown to be present in spore extracts (1). Spores from a considerable number of other fungi contain polyols (see 141). In the ascospores of *S. cerevisiae*, high quantities of proline are present which disappear upon germination (155). Proline can be metabolized via proline dehydrogenase. In certain bacterial spores, high quantities of glutamic acid are present (138). In the spores of *D. discoideum*, large stores of trehalose have been reported (22). In various plants in a quiescent state, high quantities of proline have been found (192). All of these seemingly unrelated compounds could serve the same biological function, i.e., the initial generation of NADH and NADPH during recovery from their quiescent or dormant states.

Recent studies on the degradation of the endogenous pool of free glutamic acid in *B. megaterium* are especially interesting. The large glutamic acid pool in *B. megaterium* (138) is rapidly metabolized during the initial stages of spore germination (57). A mutant strain of *B. megaterium* has been isolated that requires γ-aminobutyric acid for germination (56) and cannot degrade its endogenous glutamic acid pool (57). Mutant spores require exogenous γ-aminobutyric acid for the losses of refractility, dipicolinic acid, and heat resistance (H. F. Foerster, personal communication). During the early stages of germination, glutamic acid decarboxylase activity is lower in the mutant than in the parent strain (55). The pathway for glutamic acid degradation may be the same as proposed for the degradation of glutamic acid during *N. crassa* conidial germination (168). It is interesting that glucose and alanine alone could not initiate germination in this mutant strain (56). The degradation of either of these compounds could readily yield NADH. Perhaps the critical metabolite is NADPH, which could be produced by the degradation of γ-aminobutyric acid (39, 168).

It is also possible that NADH and NADPH could be generated via exogenous compounds in certain organisms. This has been proposed for the initiator compound alanine (150). In addition, various other organic initiator molecules may be metabolized to yield NADH and NADPH via dehydrogenase reactions. However, some initiator compounds do not appear to be substrates for dehydrogenases. With these facts in mind, it might be worthwhile to attempt to group initiator compounds into five classes. One class would be those compounds which directly generate NADH and NADPH via dehydrogenases. A second class of compounds might be those that activate an endogenous pathway for generating reduced coenzymes. This could occur in a variety of ways, such as activation of an enzyme, complexing with a bound inhibitor, or release of a substrate from a compartment, etc. A third group of compounds, such as mercaptoethanol or cysteine, might be those that directly reduce disulfide bonds. A fourth class might be those that activate an endogenous sytem for disulfide reduction. A fifth class might be those that activate cell wall degradative steps. It should be kept in mind that certain organisms will have other types of requirements besides those postulated above, such as requirements for an amino group, or carbon source, etc. It is also known that many organisms require two or more compounds to germinate. This grouping into five classes is presented even though the idea of one single trigger sequence in any given organism is probably an over simplification.

There are other parallels between the germination of conidia and of other organisms. The respiration of bacterial spores is also relatively cyanide insensitive and mediated by a flavin complex (38). In conidia, respiration proceeds mainly via an alternate oxidase pathway. It is interesting that the presence of this alternate pathway is often found in organisms that have a dormant stage. Other parallels are found in the rapid formation of polysomes upon the germination of bacteria (98) and seeds (121), and in the rapid loss of heat resistance in bacterial spores upon germination. In bacteria, the germination process is sometimes divided into three stages: activation, germination, and outgrowth. In conidial germination, a stage similar to the bacterial activation stage seems to occur during the first 20 min and only requires hydration. In conidia, the events that occur after this stage, and that require a carbon source, may be similar to the germination stage of bacterial spores. Finally, the germ tube elongation stage in conidia, a period requiring active macromolecular synthesis, may be analogous to the outgrowth stage of bacterial spores.

MOLECULAR CONTROL OF DEVELOPMENT

Because *N. crassa* can be easily manipulated, both biochemically and genetically, it has potential as an experimental organism for studying eukaryotic development. Development can be defined as a series of genetically programmed events that lead to the production of morphologically, physiologically, and biochemically distinct cell types from genetically

identical cells. Thus, development is the ultimate result of a sequence of differential gene activations and is therefore, essentially, a regulatory phenomenon. The eventual elucidation of the molecular mechanisms that control the activation of developmentally significant genes is one of the major problems facing developmental biologists. This problem can be separated into two parts. The first is to identify some of the developmentally significant gene products that are required for a particular developmental sequence, such as conidial germination, and the second is to determine the mechanisms that control the expression of these genes. This section of this review is included for three reasons: first, to present the criteria that might be used to determine if a particular biochemical event is developmentally significant; second, to apply these criteria to some of the biochemical events that occur during germination; and third, to propose experiments that might yield some insight into the molecular mechanisms that control the asexual developmental cycle in *N. crassa*.

Interpretation of Experimental Observations

Most biochemical approaches to development are based on the assumption that the properties of the cell are determined by the nature of the cells enzymatic activities. Thus, development can be considered the result of a series of genetically programmed changes in enzymatic activity. Changes in enzymatic activity can be detected in many ways including the measurement of the in vivo levels of low-molecular-weight intermediates, the specific activity of enzymes, and the synthesis of macromolecules.

A developmentally significant biochemical event has at least two distinctive characteristics. First, the event is unique to one, or at most, a few phases of the developmental sequence being studied. Second, the particular event must be essential for the continuation of development. Both criteria, unique and essential, need to be established before it can be assumed that a particular biochemical change is developmentally significant. Similar criteria have been described for bacterial sporulation (71) and for development of *D. discoideum* (95).

Any biochemical change that is measured during development could be put into one of the following four categories. It could be (i) unique and essential; (ii) unique but not essential; (iii) essential but not unique; and (iv) neither unique nor essential for development. The first category contains, by definition, the developmentally significant events. Thus far, none of the unique biochemical changes that occur during conidial germination (see CLASSIFICATION OF EVENTS IN GERMINATION) have been shown to be essential for germination. Further experiments including the isolation of appropriate mutant strains and the careful use of inhibitors are required to establish that any of these events are essential for this developmental sequence. The second category, unique but not essential, consists of those events that are associated with the developmental sequence but are not necessary for development to continue. Examples are the synthesis of both carotenoids and the enzyme NADase. Both of these events occur during conidiation. However, albino mutant strains that cannot produce carotenoids, and strains that lack NADase activity, still produce conidia and the conidia germinate normally. The synthesis of carotenoids and NADase may respond to the same signal(s) that induces conidiation, but they apparently are on side pathways, and the other developmental events are not dependent upon their synthesis. There are many examples that fall into the third category, essential but not unique. This category contains those events that are essential for all phases of development and includes protein synthesis, energy production, cell wall synthesis, etc. The fourth category, neither unique nor essential, includes those events that are simply coincidental with development. These are biochemical changes that may be induced by the same set of environmental conditions that induced the developmental sequence but are not themselves required for development. For example, aryl-β-glucosidase accumulates during stationary-phase growth and during conidiation but it is not required for conidiation (see Exocellular Enzymes).

In addition to these four categories, a fifth possibility exists. The accumulation of high levels of an enzyme or a low-molecular-weight compound may not be essential for the particular phase of development where the accumulation occurred (i.e., conidiation), but rather for a subsequent stage of development (i.e., germination). A potential example may be trehalose and trehalase, both of which appear during *Neurospora* conidiation. The storage compound, trehalose, and the degradative enzyme, trehalase, may be stored in conidia during conidiation to supply energy for the initial stages of germination.

It should be emphasized that before it can be concluded that a particular event is developmentally significant it must be shown that the event is both unique and essential to the developmental sequence. This is not to imply that

other events that do not fall into this stringent category have nothing at all to do with development. However, unless these two criteria are established it could always be argued that the event being measured (change in enzyme activity, etc.) was simply coincidental with development. Because one of the eventual objectives is to understand the molecular mechanisms that control the expression of developmentally significant genes, it must be established beyond doubt that the enzymatic activities being studied are actually part of the developmental sequence.

Once some of developmentally significant changes in enzyme activity have been identified, it then becomes feasible to begin to decipher the regulatory mechanisms that are involved. This is a complex problem because enzyme activity can be controlled at three or more different levels. The in vivo enzyme activity can be affected by changes in the microscopic environment of the enzyme. This includes the concentration of substrates, cofactors, products, activators, and inhibitors. The activity of an enzyme can be regulated at the level of the protein itself. This includes changing the rate of translation, the rate of degradation, the aggregational state of the enzyme, and the cellular localization (compartmentalization). Post-translational modification of an enzyme (phosphorylation, adenylation, specific proteolysis, glycosylation, formation of disulfide bonds, etc.) would function at this level. Also, enzyme activity can be regulated at the level of mRNA. This may involve changes in the specificity of RNA polymerase(s), as well as changes in the rate of synthesis, the rate of degradation, and the post-transcriptional processing of the mRNA. In addition, many of these potential regulatory points could be affected by the general metabolic state of the cell. For example, the rates of both protein and RNA synthesis would be affected by the availability of energy. Thus, a great deal of detective work will be required before we will begin to understand regulation of development at a molecular level.

Proposed Experiments

For eventually elucidating some of the regulatory systems that control the asexual cycle of *N. crassa*, four areas of research appear promising. These are the study of the early biochemical events in germination, the possible role of membrane reorganization in germination, the involvement of arginine and ornithine in conidiation, and the relationship of conidiation to catabolite repression. This discussion has purposely not been restricted to germination since many of the initial germination events may be programmed into the conidia during conidiation. These four areas were chosen because of the experiences and prejudices of the authors and should be considered as only suggestions.

The mechanism for the activation of dormant spores, as described in the model for breaking dormancy, probably involves enzymes that are unique to germination. These may include enzymes for the degradation of glutamic acid, for the formation of reduced coenzymes, and perhaps for the reduction of disulfide bonds. Mutant strains are needed that specifically affect one or more of these processes to establish that these changes are essential for germination. The isolation of mutant strains blocked in the degradation of glutamic acid may be potentially feasible. It has been proposed that glutamic acid is degraded by first being decarboxylated to γ-aminobutyric acid (168). Mutant strains have been isolated in *B. megaterium* which have low glutamic acid decarboxylase activity and require γ-aminobutyric acid for germination (55–57). This is one of the few examples of an enzyme that has been established to be developmentally significant in bacteria. Hopefully, procedures can be developed for isolating analogous mutant strains in *Neurospora* that lack glutamic acid decarboxylase.

There appears to be a membrane reorganization that occurs during germination (see CLASSIFICATION OF EVENTS IN GERMINATION). At this time, the evidence for general changes in membrane structure and function are only circumstantial. Comprehensive studies are needed on the changes in the composition of both the total lipid fraction (fatty acids, phospholipids, etc.) and of the various membranes themselves (mitochondrial, plasma, etc.). Because membrane reorganization presumably occurs sometime during conidiation, the complete asexual cycle should be studied. If there is a general membrane change, this could account for many of the properties of dormant conidia. Once this information is available, some more specific proposals can be made.

A third area that has not been exploited is the apparent involvement of arginine and ornithine in conidiation. The arginine and ornithine pools are relatively low in conidia (168). The high levels of these pools in mycelia are depleted during conidiation (Schmit and Brody, unpublished observations). Perhaps these amino acids are stored in mycelia because they are required for "competence" to conidiate. Two observations support the hypothesis that arginine and ornithine are in some way involved in conidiation. First, a mutant strain has been isolated that affects the utilization of ornithine and is aconidial (34). No other biochemical mu-

tations have been described in *N. crassa* that are aconidial. Second, arginine added to solid media greatly enhances conidiation (157). Thus, some aspect of arginine and ornithine metabolism appears to be related to conidiation. The nature of these biochemical events remains to be determined.

The last area is the relationship between the release of catabolite repression and conidiation. Certain enzymes found at high levels in conidia can be repressed in mycelia by glucose or other readily metabolizable carbon sources (see Exocellular Enzymes). The process of conidiation itself can also be repressed by glucose (144, 157, 209–211). Thus, conidia apparently are formed under conditions where catabolite repression is relaxed. Presumably cyclic AMP is in some way involved in these processes. The observation that during conidiation oxidative phosphorylation becomes partially uncoupled in the mycelia (AMP levels are elevated) is consistent with this proposal (37).

SUMMARY

A compilation of data has been presented comparing the conidia of *Neurospora* with the vegetative mycelia. These two phases of the asexual life cycle were compared with respect to their content of DNA, RNA, protein, lipid, 20 different low-molecular-weight compounds, and cell wall composition (Table 1). The specific activities of 21 enzymes from both phases are compared (Tables 2 and 3). In general, conidia have more similarities than differences to vegetative mycelia. Some of the important differences found between them so far are as follows: conidia have few polysomes, little oxidative phosphorylation, no cell wall galactosamine, and low cellular pool levels of arginine and ornithine. They contain higher amounts of glutamic acid, oxidized glutathione, phospholipid, and a hydrophobic surface layer. The biochemical changes that occur during conidial germination are summarized in Fig. 6. This Figure indicates that the known biochemical events can be grouped into three classes. The first group of events occurs in the first 10 to 20 min, and only requires hydration of the conidia. Some of these early events can be grouped into a temporal sequence (Fig. 7), similar to a multistep trigger reaction, and a model for the breaking of dormancy is proposed based on these events. The second group of events occurs after a few hours (Fig. 6), and requires a carbon source. This group of events, such as mitochondrial changes and transport changes, appear to be related in some way to changes in the structure of the membranes. A third group of events occurs considerably later in germination and involves the activation of the synthesis of two cell wall polymers. The prominent events that occur very early in germination are the degradation of a large endogenous glutamic acid pools, a surge in the level of NADH and NADPH, the enzymatic reduction of the high content of oxidized glutathione, and the formation of polysomes. The first three of these events are, in some sense, unusual biochemical events. First, they appear to be unique to germination and were not detected at any other time. Second, they all start and finish during the initial phases of germination, as opposed to many events, such as RNA synthesis which may start in the first few minutes, but then continue throughout vegetative growth. A four-point model for dormancy and conidial germination is proposed which is based primarily on these three early events. One prediction of this model is that cellular proteins in the conidia have a high content of disulfide bonds. An elaboration of this idea is put forth with respect to how internal cross-linking of this type could stabilize and inactivate enzymes in a readily reversible manner. Other aspects of the model are discussed with reference to parallels found in the germination of bacterial spores and plant seeds. There is also a discussion of the previous and potential uses of genetic approaches to studies on a particular phase of development, such as germination.

ACKNOWLEDGMENTS

This study was supported by grant GB 21227 from the National Science Foundation and by Public Health Service research grant GM 19308 from the National Institute of General Medical Sciences.

We extend our appreciation to Doris Williams for her help in preparing this manuscript. We are especially grateful to Robert Fahey for contributing his ideas on the possible roles of sulfhydryl compounds in spore germination.

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