

CHAPTER 23. Biochemistry: Biosynthesis and Regulation

The energy incorporated and subsequently released by metabolism becomes a reality in the most complex activity of the cell, that of biosynthesis. Moreover, it can be argued defensibly that *all* of the cell (or hypha) is the product of self-regulated assembly of its components from elemental constituents. It happens that research on biological fabrication in members of the Saprolegniaceae falls into five broad areas: amino acid, protein, lipid, and sterol synthesis, and the immunochemical responses of some species associated with freshwater fish. These aspects are treated in this chapter.

Biosynthesis alone does not establish the continuity and interdependence of processes that is the hallmark of the functional cell. Therefore, in common with what occurs in all other eukaryotic organisms, assembly of the constituent parts of water molds is regulated in very precise ways. The specifications for the protein structure of these fungi (no less than in any other organism) reside in the nucleotide base sequences of DNA. The information conveyed by these sequences is transcribed into messenger RNA, which is “decoded” by translation at the ribosomal level into the structure of proteins. This chapter, then, also will concern itself with the structure and operation of the genetic regulatory system in the Saprolegniaceae.

AMINO ACID BIOSYNTHESIS

Lysine, a basic (positively charged) essential amino acid, is synthesized through one of two pathways (Vogel, 1959), either that involving α -aminoadipic acid (AAA), or that including α - ϵ -diaminopimelic acid (DAP) as an intermediate. By tracer labeling, Vogel (1960, 1964) demonstrated that *Achlya bisexualis*, *A. americana*, *Saprolegnia ferax*, *S. parasitica* (= *diclina*), and *Thraustotheca clavata* synthesized lysine through the DAP route. This being so, at least these members of the Saprolegniaceae are equipped with the identical pathway that provides lysine for bacteria and the green algae, and sets the water molds off sharply from the Chytridiomycotina, Zygomycotina and high fungi in which this amino acid is synthesized via the AAA pathway (Gleason, 1976; Vogel, 1964).

PROTEIN SYNTHESIS

From an analysis of rates of amino acid uptake and incorporation, protein turnover, and degradation, Timberlake *et al.* (1973) determined that during sporangium differentiation *Achlya bisexualis* actively synthesizes protein in the mycelium at a rate demonstrably greater than that extant at the onset of sporulation. Of particular significance is the fact that in this water mold the amino acids necessary for synthesis of proteins are derived from the degradation and reassembly of preexisting proteins (Timberlake *et al.*, 1973; Gleason, 1976).

It has been shown (L. E. Cameron and LéJohn, 1972a, b) that calcium (Ca^{2+}) regulates amino acid transport in a nonsexual *Achlya*. Since in this fungus the uptake of

Ca²⁺ proceeds concomitantly with protein synthesis, L. E. Cameron and LéJohn (1972a) have suggested that the cations may couple amino acid transport and protein synthesis at the hyphal membrane. They also have demonstrated (1972b) that if protein synthesis is blocked, as by cycloheximide, but calcium-mediated transport is left intact, amino acid uptake continues.

Cytochalasin A, a third reagent capable of interrupting cytoplasmic streaming, has been implicated by Thomas *et al.* (1974) as an inhibitor in cellulase (secretory protein) production and secretion by the male strain of *Achlya ambisexualis*. In a continuation of the work by Thomas and his associates, with the same fungus, Manavathu and Thomas (1976) investigated the action of cytochalasin A on cellulase secretion. They tested the effect of two other thiol reagents, one penetrating, and one superficial (largely nonpenetrating) on labeled leucine incorporation into mycelial protein. The nonpenetrating thiol reagent *p*-chloromercuribenzenesulfonate (*p*CMBS) in effect was a mimic of cytochalasin A in that it selectively inhibited synthesis of cellulase without affecting general protein manufacture in the mycelium. The penetrating thiol reagent, N-ethylmaleimide, also inhibited cellulase synthesis and secretion. Glutathione effectively antagonized the inhibitory influence of *p*CMBS.

LIPID SYNTHESIS

In 1972(a) Darnaud reported on some aspects of lipid synthesis and the identification of compounds in *Achlya bisexualis* grown on Barksdale's (1962a) medium, and uncovered a parallelism in this species between growth and the assembly of endogenous lipids. Two phases were evident. In the first, coming at the early stages of mycelial development, lipids contributed little to growth (lipids and proteins constituted 16% of the mycelial dry weight). Subsequently, in a second phase of growth, lipids accumulated in *A. bisexualis* such that by the time mycelial autolysis had begun, this fraction accounted for 94% of the weight loss. It would appear from Darnaud's analysis that lipids are highly functional during the latter stages of development in this water mold, at least.

Of the lipids identified by Darnaud (1972a) in *Achlya bisexualis*, 80-90% were triglycerides. Phosphatidylcholine and lyso-phosphatidylcholine were the principal phospholipids, and palmitic and oleic acid constituted 60% of the total fatty acids. Myristic acid also was present in the mycelium of *A. bisexualis*, but contrary to what is found in other filamentous fungi, there was very little linolenic acid.

In an attempt to differentiate between *Aphanomyces laevis* and *A. cochlioides*, Beiss and Schäfele (1973) analyzed chromatographically the lipid and phospholipid content of the vegetative thalli. *Aphanomyces cochlioides* had a higher total lipid content (percent dry weight basis) than *A. laevis*, but the reverse was true for the amount of phospholipid. The investigators could not detect glycolipids, sterol glycosides, phosphatidic acids, or phosphatidyl glycerin in either species. Nine of 16 lipids chromatographed from ruptured mycelium of the isolates of *Aphanomyces* were phospholipids, of which only four -- three in the phosphatidyl series -- were identified.

The four were choline, serine, and ethanolamine phosphatidyl esters, and diphosphatidyl glycerin.

STEROL SYNTHESIS

The sterols (one class of steroids) are nonsaponifiable lipids that occur either as long-chain fatty acid esters or free alcohols. Various sterols have been identified in water molds, and as Popplestone and Unrau (1973a, b) show, these particular compounds are more closely akin to those in algae (Bu'Lock, 1976) than in other fungi. Some of the principal sterols that have been identified in water molds are listed in Table 40.

McCorkindale and his associates (1969) compared chromatographically the sterol composition of fungi with cellulosic hyphal walls -- including six species of Saprolegniaceae -- with those having chitinous walls. The amounts of sterols detectable in the six species of water molds fell into two groups. In *Saprolegnia ferax*, *S. megasperma*, *Leptolegnia caudata*, and *Aplanopsis terrestris*, 0.1-0.25% of the dry weight of mycelium was sterol; that of *Achlya caroliniana* and *Pythiopsis cymosa* was only 0.01-0.025%. None of the species of Saprolegniaceae tested for sterols (Goodwin, 1973) has been shown to synthesize either ergosterol or 22-dihydroergosterol. The sterol composition similarities among the fungi analyzed by McCorkindale *et al.* (1969) suggest that the Saprolegniales are more closely akin in constituent sterols to the leptomitaceous forms than to the Mucorales.

There are two pathways in the biosynthesis of sterols (Bu'Lock, 1973), namely, the cyclization of squalene oxide to lanosterol (fungi and animals) or to cycloartenol (plants). The Oomycetes -- and *Saprolegnia ferax* is a specific example -- can convert both of these precursors into sterols (Bu'Lock and Osagie, 1976; McCorkindale, 1976). *Saprolegnia ferax* also synthesizes cyclosteroid isomerase (Bu'Lock and Osagie, 1976), an enzyme functional only in organisms that obtain sterols via the cycloartenol pathway using lanosterol as a precursor. Bu'Lock (1973) has proposed that the identifiable sterols in the saprolegniaceous fungi (Table 40) are so unlike the usual ones in plants that they can be designated as fungal phytosterols.

IMMUNOLOGICAL PROPERTIES

Animal serum can synthesize antibodies in response to challenge from some foreign protein: an antigen (or immunogen). The antigen influences the amount of specific antibody that is formed, but does not modify its structure or amino acid sequence, for it is in the sequence that the specific binding site for the antigen resides. One of the ways by which antibodies react is to precipitate soluble antigens.

Antigen-antibody responses are known to occur between certain water molds identified as fish pathogens and the host serum. The work of H. J. Fletcher and associates (1979) suggests that agglutinins (lectins) from snails (of the genera *Helix* and *Caucasotachea*) and the lectin concanavalin A (from jack-bean, *Canavalia ensiformis* DC)

might be useful in selectively isolating antigenic compounds from members of *Saprolegnia* associated with fish. The lectins reacted strongly with extracts from *S. parasitica* and *S. ferax*. The bulk of immunochemical work on saprolegniaceous fungi, however, resides in studies by Peduzzi (1973, 1975) and colleagues, but Hodkinson and Hunter (1970a) have also been contributory. The immune responses involving freshwater fish and water molds are reviewed by J. G. M. Wilson (1976).

In experimental work on ulcerative dermal necrosis (U.D.N.) of *Salmo salar* (salmon), Hodkinson and Hunter (1970a) explored antigen-antibody reactions with a nonsexual *Saprolegnia* associated with diseased fish. By homogenizing and centrifuging host tissue or fungal mycelium, Hodkinson and Hunter prepared crude antigen extracts, which then were tested by the double gel diffusion method for antibody inducement in blood serum. The resulting precipitin bands demonstrated that the salmon serum responded to more than one antigenic fraction in the extract from the fungus mycelium. Antibodies were produced in adults, kelt, and parr (individuals prior to migration to the sea) of salmon, but were not necessarily associated either with U.D.N. or fungus colonization. Further exploration with cultures of *Saprolegnia* sp. led Hodkinson and Hunter (1971, 1974) to conclude that the antigenic properties of the mycelium reflected the nature of the medium in which the fungus was grown. In their initial study (1971), they found precipitation zones in Ouchterlony double diffusion plates only when the antigens came from fungal mycelium grown on agar containing salmon extract. They subsequently (1974) demonstrated that β -sitosterol was essential for the production of soluble antigens by the *Saprolegnia* (precipitation bands between the extracted antigens and salmon serum). A low carbon to nitrogen ratio in the growth medium favored antigen production.

Peduzzi (1973, 1975) experimented at length with antigen-antibody reactions involving *Saprolegnia parasitica*, *S. ferax*, *S. diclina*, and *S. delica* (= *diclina*). Antigen extracts from mycelium of these species were injected into rabbits to produce four immune sera (anti-*S. parasitica*, and others). The resulting immune sera were then tested (Ouchterlony plates) against antigenic extracts of each of the four water mold isolates and some other fungi. Four of the reactions between the antisera and antigens (in various combinations) were homologous, and twelve were heterologous. Precipitation distribution on immunophoregrams thus confirmed (Peduzzi and Bizzozero, 1977) that the antigenic properties of the four isolates were remarkably homogeneous.

The protein homogeneity of the foregoing four species of *Saprolegnia* also was demonstrated by Grimaldi *et al.*, in 1973. They prepared antiserum of *S. diclina* and tested for precipitation of soluble proteins. Precipitation rings were formed between the serum and the antigen-containing extracts of the four water molds. The implications (to the pathology of saprolegniosis) of these results and those assembled by Peduzzi (1973, 1975) are treated in Chapter 29.

REGULATORY MECHANISMS

The metabolic and biosynthetic activities of the water molds do not operate without regulatory constraints being put upon them. In the absence of regulation or orderliness, the mycelium of these fungi, no less than the cells of other organisms, is stricken with degeneration. Two regulatory mechanisms have been explored in the Saprolegniaceae, namely cytokinin influence and genetic control.

CYTOKININS AND CONTROL

That calcium (Ca^{2+}) plays a significant part in amino acid transport in species of Saprolegniaceae is firmly documented. This ion is essential for growth of a nonsexual *Achlya*, LéJohn *et al.* (1974) found, but its uptake by the mycelium is evidently not necessarily an active process because metabolic inhibitors have no effect on transport. In any event, it appears that Ca^{2+} transport involves a calcium-binding phosphorylated proteoglycan in the hyphal wall matrix, and a component of similar function associated with the plasmalemma.

Calcium sequestering in the presence and absence of cytokinin (a plant growth regulator) was studied by LéJohn and Cameron (1973). They prepared a purified phosphorylated proteoglycan from germlings of *Achlya* sp. and by means of equilibrium dialysis tested for the capacity of this compound to bind the cation in the presence of cytokinin. When cytokinins (N^6 -substituted adenine derivatives) were added to preparations, the sequestering action of the proteoglycan was markedly reduced.

“Normal” germlings of *Achlya* sp. -- ones not osmotically shocked to free the wall matrix and plasma membrane of proteoglycan -- bind large amounts of calcium when mercury, iodine, silver, or organic mercurials are added to the transport medium (LéJohn, *et al.*, 1974; LéJohn and Cameron, 1973.). LéJohn and Stevenson (1973) reported that cytokinins were effective in releasing calcium from proteoglycan bonding in the presence of Hg^{2+} . The most effective of the eight cytokinins tested was N^6 -(Δ^2 isopentyl) adenine, but xanthine (which is not a cytokinin) was nearly as effective. Caffeine, proflavins, cyclic adenosine monophosphate, guanosine diphosphate, and purine or pyrimidine nucleosides were unable to uncouple Ca^{2+} from its sequestering bonds. In osmotically shocked germlings, LéJohn and Stevenson (1973) found, Ca^{2+} uptake was increased by cytokinins in the transport medium, and Hg^{2+} , Ag^+ , and I_2 facilitated cytokinin activation. Cytokinin-assisted transport was antagonized, however, by the presence of magnesium. LéJohn and his associates concluded (1974) that the role of cytokinin was to prevent Mg^{2+} from competing with the Ca^{2+} for a binding site. In normal hyphae, cytokinins also obstruct the ability of the thallus to concentrate metabolites (LéJohn and Stevenson, 1973).

LéJohn (1975a) succeeded in isolating the phosphorylated proteoglycan to which calcium binding occurs at the plasma membrane level. This proteoglycan also bound (stimulated uptake of) cytokinins, auxins, and tryptophan. It has been demonstrated, then, that the same cytokinins (and their analogs) inhibiting amino acid transport stimulate tryptophan uptake, and are antagonistic to Ca^{2+} binding. As a tentative

hypothesis, LéJohn (1975a) proposed that calcium cations, by themselves or coordinately with cytokinin bound to the membrane-level proteoglycans are uncoupled by auxin activity. If this hypothesis is correct, it follows that release of the calcium could change the hyphal wall structure and thus facilitate wall extension (LéJohn, 1975a). We would suggest that lateral branching (which must begin by some mechanism that softens the rigid wall) could also be promoted. The contribution of cellulases then becomes an intriguing question in light of calcium binding. In any case, the cytokinins appear to initiate the release of proteoglycan-sequestered calcium, and to stimulate its uptake (LéJohn and Cameron, 1973; LéJohn and Stevenson, 1973).

Cytokinins have been used to explore certain aspects of energy-related transport of glucose by germ hyphae from spores of *Achlya* sp. (Goh and LéJohn, 1978). These modified adenines, without penetrating the hyphal membrane, block active transport of glucose by interacting with membrane phosphorylated proteoglycan (PPG) and displacing the phosphorylated dinucleoside HS compounds (*see* section on RNA polymerase). It is suspected (Goh and LéJohn, 1978) that there is a link between calcium, PPG-associated HS molecules, and energized solute uptake. Perhaps these various elements act together to help maintain membrane integrity so that glucose transport can occur.

In addition to their use in analysis of glucose movement, cytokinins have been employed (R. M. Stevenson and LéJohn, 1978) to investigate kinetically the transport of nucleosides (adenosine, guanosine, uridine, cytidine, and thymidine). The cytokinins inhibit nucleoside transport without concomitantly preventing the movement of nucleic acid bases. Osmotically-shocked hyphae of *Achlya* sp. do not transport nucleosides (a system that can be dependent upon energy), and this is a failure that can be correlated with the release of membrane-sequestered PPG associated with calcium and the HS compounds (LéJohn *et al.*, 1975, 1977) also in the plasmalemma.

GENETIC REGULATION

The realization that genetic information in a living organism flows from DNA to RNA to proteins is so deeply entrenched in biological thought that it hardly seems necessary to review the part played by DNA in carrying the specifications for growth and reproduction. In this section we shall look at those aspects of the molecular nature of genetic material of water molds that have been explored.

Structure and Composition: -- During the infant days of the application of information on DNA content to the taxonomy of fungi, it became clear that the base composition of the DNA molecule varies from one species to another. The tool most valuable in analyses that provided this knowledge was equilibrium centrifugation of DNA in cesium chloride. This method determines buoyant density, a condition that increases as the guanine-cytosine (G+C) content rises.

The earliest reports of DNA base composition in the Saprolegniaceae were those of Storck and Alexopoulos (1970), and B. R. Green and Dick (1972). Their measurements of buoyant density, converted to percent G+C content, are given in Table 41. On the basis of such data certain of the species were seen to cluster into groups that presumably had some taxonomic significance. For example, the species of *Saprolegnia* for which buoyant density determinations have been made (Table 41) fall into three categories: G+C ranges of 40.5-49.5, 54.5-55.5, and 60.5-62 (Storck and Alexopoulos, 1970). The racemose species of *Achlya*, on the other hand, are nearly identical with respect to their DNA buoyant densities (B. R. Green and Dick, 1972), but differ noticeably from those taxa having eccentric oospores. Subsequently, Horgen *et al.* (1973) showed by amino acid analysis on carboxymethylcellulose that *A. bisexualis* possessed nuclear- and chromatin-associated basic histone-like nucleoproteins. On acid-urea and polyacrylamide gels the mobility of proteins of *A. ambisexualis* is identical to that of rabbit kidney histones H3, H4, and H2A (Horgen and Silver, 1978).

The analysis by B. R. Green and Dick (1972) is evidence that species of *Saprolegnia* contain satellite DNA in an amount equal to about 10-20% of the total DNA. They suggested that the satellite fraction might be mitochondrial in origin or could perhaps represent "contaminant" DNA from endobiotic parasitic fungi that were not detected by light microscopy. In fractions from pellet preparations (rich in mitochondria) from mycelium of an unidentified, nonsexual *Saprolegnia*, Clark-Walker and Gleason (1973) detected a main DNA component and a closed, circular satellite fraction. There was no evidence that the DNA in this fungus occurred anywhere but in the nucleus and mitochondria. The main DNA of *S. diclina* was determined by Neish and Green (1976, 1977) to have a buoyant density of 1.717 g cm⁻³, and a nuclear base composition of 58% G+C (compare with Table 41). There also was in this species a satellite DNA with a buoyant density of 1.707 g cm⁻³, which on purification yielded a base composition of 48% G+C. Alkaline AgNO₃ / Cs₂SO₄ gradient determinations gave two additional minor satellite fractions with buoyant densities of 1.701 ± 0.001 and 1.682 ± 0.001 g cm⁻³. The major satellite fraction perhaps represented ribosomal DNA cistrons. From a more extensive analysis of DNA base composition in species of *Saprolegnia*, Neish and Green (1976) recorded 3 satellite DNA buoyant densities (in 11 isolates) of 1.706-1.709 g cm⁻³, or G+C bases of 47-50%. It may be mentioned in passing that the ligands distamycin A and netropsin enhance (Tatti *et al.*, 1978) the base composition-dependent buoyant separations of duplex DNA (*Achlya* isolate and *Serratia marcescens* Bizio).

The molecular weights of two fractions of RNA in *Achlya ambisexualis* 734 (♀) were determined by Lovett and Haselby (1971) from electrophoretic migration patterns. The larger RNA species (25S) weighed 1.40 x 10⁶ daltons, while that of the smaller species (18S) was 0.72 ± 0.001 X 10⁶ daltons.

In what is the first investigation of oomycetous DNA by reassociation kinetic analysis, Dodd *et al.* (1975) characterized DNA of the male element (E87) of *Achlya ambisexualis*. The G+C content of this strain was 40%, a substantially lower figure than Storck and Alexopoulos (1970) recorded in their analysis of this same species (mating strain was not identified). The repeated portion of the genome in *A. ambisexualis* E87

constituted 17.6% of the nuclear DNA, while 80.1% were single copy transcripts (Dodd, *et al.*, 1975). Some characteristics of the RNA fraction of this male mating strain of *A. ambisexualis* have also been determined. Timberlake *et al.* (1977) explored possible differences between poly(A) heterogeneous RNA of the nucleus (hnRNA) and poly(A) mRNA, but found that these molecules were not at variance as to size. Hybridization experiments (RNA excess in DNA/RNA) indicated that about 1900 diverse RNA species were present in the isolate. That the composition of the hnRNA and mRNA in the one male strain of *A. ambisexualis* is virtually identical seems clear: both sources of RNA contain about 10% repetitive transcripts (Rozek *et al.*, 1978) and 44% single-copy ones.

Two studies (D. J. Law *et al.*, 1978; Rozek *et al.*, 1978) on the kinetics of hybridization of poly (A⁺) RNA with complementary DNA demonstrate that there are three frequency classes -- approximately 50, 30, and 15 nucleosides -- of this compound in *Achlya ambisexualis* (♂). The polyadenylated sequences in the nuclei of this strain generally are in the 50-nucleotide class, while those in mRNA that is active in translation are predominantly in the two smaller (30 and 15 nucleotide) size classes (D. J. Law *et al.*, 1978).

In *Achlya bisexualis* two components appeared when the total DNA was fractionated (Jaworski and Horgen, 1973). The major component had a buoyant density of 1.697 g cm⁻³, while that of the minor or mitochondrial portion was 1.685 g cm⁻³. Two calculations done by Jaworski and Horgen (1973) are of interest. Their analyses indicate that each haploid chromosome of *A. bisexualis* contains approximately 435 copies of ribosomal cistrons. The total weight of DNA in each diploid spore was calculated at 2.8 x 10¹¹ daltons.

It is known that two types of nucleotide sequence arrangements exist in DNA molecules. In one type, the repetitive sequences average about 200-400 nucleotide pairs, and represent the short-period interspersion pattern. The second type has sequences with more than 4000 nucleotide pairs in each, and is accordingly a long-period interspersion. The pattern of DNA sequence organization has been investigated in but one species, a female mating strain of *Achlya bisexualis* (Hudspeth *et al.*, 1977). Reassociation kinetic analyses on sheared DNA (single strand fragment lengths were determined by alkaline band sedimentation ultracentrifugation, or alkaline sucrose density centrifugation) provided Hudspeth and his associates with data showing that this strain of this species has a long-period interspersion pattern with repetitive sequences extending for at least 6000 nucleotide pairs. *Achlya bisexualis* thus is the first fungus known to be equipped with the long-period sequences, and it certainly has longer spacing intervals than recorded for most plant and animal genomes. In a study of chromatin organization in nuclease-digested nuclei of *A. ambisexualis* Silver (1979a) discovered a short DNA repeat size (length of DNA in a core plus linker region) averaging 159 ± 1.2 base pairs. The average repeat size for the higher eukaryotes is about 200 base pairs. The measurement on *A. ambisexualis* lacks an explanation.

Timberlake and his associates (1977) have summarized the general nature of the information-coding nucleic acids in *Achlya* species. (1) About 7% of the total genome in

vegetative hyphae is represented by some 2000 single copy sequences, and most mRNA molecules are transcribed from these sequences. (2) Repeated DNA is capable of coding for both messenger and ribosomal RNA. (3) The sequences in nuclear RNA are virtually identical to those sequences translated on polysomes. (4) Polycistronic mRNA is absent in these fungi. (5) The transcription and maturation of mRNA is much less complex in the *Achlyas* than in animal cells.

Macromolecular Synthesis: -- Correct transcription is essential to the proper translation of information coded by DNA into the realities of structure and operation. For transcription to occur with high fidelity -- barring some mutation, of course -- a DNA-dependent enzyme system capable of participating in the process and binding to the DNA template must be present. RNA polymerases are the enzymes that insure creation of RNA polymers. The bulk of the work on regulatory aspects of the genome in water molds has quite properly focused on RNA and the polymerases that operate in transcription, and much emphasis has been given to inhibition of synthesis and function.

Inhibition of DNA and RNA: -- As early as 1969, Griffin and Breuker demonstrated that ribosomal RNA synthesis in a nonsexual *Achlya* continued throughout the growth of hyphae and differentiation of sporangia. When the antibiotic actinomycin D was added exogenously to mycelium of the water mold in which differentiation was occurring, this process halted at once, indicating that DNA-dependent RNA synthesis was required for the activity to proceed. It was later demonstrated by L. E. Cameron and LéJohn (1972b) that amino acid transport could be blocked if calcium metabolism was interrupted, but DNA and RNA synthesis were not hindered. These latter two essential processes were thus independent of Ca^{2+} .

That synthesis of whole cell and ribosomal RNA in a water mold can be interrupted effectively by prohibiting protein synthesis has been shown by Timberlake and Griffin (1974a, b), and Sullia and Griffin (1977). Timberlake and Griffin (1974a) analyzed the effect on an isolate of *Achlya* of eight glutarimide analogs of cycloheximide (known to block the assembly of protein) modified by chemically-induced changes in the ketoncarbonyl, hydroxyl, or cyclohexone portions of the molecule. Cycloheximide and cycloheximide oxime prevented protein and RNA synthesis, while the analogs streptovitacin A and streptimidone only hindered the former process. Cycloheximide acetate and dehydro- and anhydrocycloheximide were favorable to the incorporation of uridine into RNA (one measure of RNA synthesis). It also has been found (Timberlake and Griffin, 1974b) that RNA synthesis is inhibited (uptake of [5- 3H] uridine into rRNA) by the application of *dl-p*-fluorophenylalanine, puromycin, and blasticidin-S, all three of which prevent the assembly of protein.

The results of further study on the effect of analogs of cycloheximide on DNA synthesis were reported in 1977 by Sullia and Griffin. They experimented with the uptake of labeled thymidine by *Achlya bisexualis*, using the thymidine incorporation into DNA as an index of DNA synthesis. The results essentially corroborated those reported

earlier by Timberlake and Griffin that cycloheximide and some analogs (isocycloheximine and streptimidone, for example) prevented the incorporation of thymidine into DNA while others such as puromycin and cycloheximide acetate did not.

RNA Polymerases: -- Crude nuclear pellets prepared from centrifuged, homogenized mycelium of *Achlya bisexualis* were subjected by Timberlake *et al.* (1972) to an analysis of RNA polymerase activity by means of ATP incorporation into polynucleosides insoluble in trichloroacetic acid. Three enzymes were eluted from the pellets: RNA polymerase I (nucleolar in origin; probably transcriptional of rRNA cistrons), II (in the nucleoplasm, and possibly responsible for mRNA synthesis), and III (located in the nuclear-nucleolar interface, and possibly involved in synthesis of tRNA). Cycloheximide inhibited RNA polymerase I but not II or III, and Griffin *et al.* (1975) showed that some glutarimide analogs of cycloheximide also had a direct regulating effect on RNA polymerase I.

The regulation of RNA polymerases has been investigated most extensively by LéJohn and his associates, experimenting with a nonsexual *Achlya*. McNaughton *et al.* (1975) isolated RNA polymerases from nuclei or intact germling hyphae of *Achlya* sp., and by limited characterization showed that the three classical types of eukaryotic polymerases had been resolved. The activities of these polymerases were affected by the presence of three compounds which had been discovered in formic acid extracts of *Achlya* sp. mycelium and designated HS ("hot spot") 1, 2, 3 (LéJohn *et al.*, 1975). The identity of the HS compounds, and a complete characterization of them remain to be settled, (*see also*, LéJohn *et al.*, 1977).

LéJohn and his colleagues (1977) had proposed that the so-called "hot spots" in preparations from the mycelium of *Achlya* sp. were unusual forms of guanosine polyphosphates. This suggestion was explored by Warner *et al.* (1977), using *A. ambisexualis* E87 as the test organism. They analyzed (radioactive labeling followed by extraction, purification, and elution from Dowex-I-formate columns) preparations of mycelium of the *Achlya* for tri- and tetraphosphate diguanosine nucleotides. (Diguanosine nucleotides are a source of cellular purines, and also function in phosphate bond energy; they also may play a part in DNA synthesis.) Judging from the regions at which ³²P compounds appeared on elution, and from enzymatic hydrolyses of them, Warner and his collaborators found no diguanosine tetraphosphate, and very little, if any, of the triphosphate form. They concluded that their experiments cast doubt on the importance of nucleosides as metabolic regulators in aquatic fungi.

The study by Warner *et al.* (1977) seems to have confirmed the contention of Goh and LéJohn (1977) that the HS compounds were not diguanosine nucleotides (LéJohn *et al.*, 1975) but were polyphosphorylated dinucleotides. In 1977, W. H. Lewis and his associates reported that HS-3 from ovary cells of the Chinese hamster was similar in its physical and chemical properties to that found in *Achlya* sp. A year later, McNaughton and colleagues (1978) published an account that further characterized the three highly phosphorylated dinucleotides. All three HS compounds are rich in uracil. Partial

characterization of HS-3 suggests that it may be ATP ribitol (glutamate) covalently linked to UDP-mannitol tetrphosphate. Results of a study by LéJohn, Klassen, and Goh (1979) support the tentative identification of HS-3 proposed by McNaughton *et al.* (1978).

What are the chief properties of the HS compounds, and what relationships do they exhibit to other elements in the mycelium of *Achlya* sp.? Of the three compounds in *Achlya* sp., HS-3 appears to be the most active (and potent, but see, to follow, LéJohn *et al.*, 1978) since it inhibits all three RNA polymerases (McNaughton *et al.*, 1975). HS-2 is moderately effective, but although HS-1 activates RNA polymerase III, it is partially inhibitory to I and II. Some correlation exists between growth stages of *Achlya* sp. (20 hour asexual "cycle") and the activity of the polymerases. When all three dinucleoside derivatives (HS) are maximum (at 15 hours growth) no RNA polymerase I can be isolated from the nuclei of *Achlya* sp., but when the HS compounds are minimal in preparations, all three polymerases can be recovered. Some data given by McNaughton and his associates (1975) have been superseded by results of studies by LéJohn *et al.* (1978: table 4). All three compounds are uniformly inhibitory to all RNA polymerases of *Achlya* sp., with K_i values of 1-4 μ M. HS-1 and 2 are slightly more potent than HS-3.

Further analysis of the HS polyphosphates has been provided by LéJohn *et al.* (1975, 1978). These compounds accumulate in the young germ hyphae from spores of *Achlya* sp. in an inverse proportion to RNA synthesis. When the rate of RNA assembly in the mycelium is at a high level, the synthesis of the "hot spots" is low. At accelerated rates of protein synthesis (synchronous growth experiments using interval pulsing with labeled compounds), however, the HS compounds are synthesized. Two of the three phosphorylated dinucleosides, HS-2 and HS-3, obstruct pyrimidine reduction, and the latter also inhibits the reduction of ribonucleotides (W. H. Lewis *et al.*, 1976). Both HS-1 and HS-3 prevent the reduction of ADP.

From the various studies on the HS compounds certain essential facts emerge (LéJohn *et al.*, 1977, 1978; LéJohn, Klassen, and Goh, 1979). First, each of the compounds evidently has a regulatory role that is expressed at different times during the development of *Achlya* sp. During starvation, the mycelium synthesizes HS-1, HS-2, and HS-3, and the metabolism of the latter is directly correlated with vegetative (low HS-3) and sporulating (high HS-3) activity in the fungus. During the growth and asexual cycle of *Achlya* sp. there are three periods when RNA, DNA, and polyphosphates (in that order) display high rates of synthesis. This correlates with the fact that in the first eight hours of development the mycelium has a low transcriptional rate. In the subsequent period (up to 16 hours of age) the fungus still exhibits a low transcriptional rate, but the level of translation is high. Intense transcriptional activity precedes and partially overlaps DNA synthesis. Second, since all DNA-dependent RNA polymerases and ribonucleoside diphosphate reductases are inhibited by the HS compounds, the latter change from a high to a low level as the various RNA polymerases appear. RNA polymerase I_A and I_B predominate in the young mycelium, while types II and III are detected in older hyphae. Third, if zinc cations (Zn^{2+}) are added to the reaction medium, the level of the HS compounds increases; when iron

(Fe³⁺) is incorporated they are depressed. The sensitivity of the highly phosphorylated compounds to the cation of iron suggests for them a function in nucleic acid synthesis. Fourth, in the absence of phosphate, glutamine inhibits the synthesis of HS-3, promotes its decay, and blocks sporulation by the *Achlya*. The glutamine effect (mimicked by ammonia and glucosamine) is antagonized by phosphate.

Obviously the literature dealing with nucleic acid and protein synthesis in the water molds is not extensive, and many additional and varied analyses need to be carried out. The review by D. R. Berry and Berry (1976) is a particularly valuable reference on this aspect of fungal metabolism.

Amatoxins: -- Certain extracts from *Amanitas* have been tested for their effects on *Achlya ambisexualis* (Horgen *et al.*, 1976, 1978) through techniques of assaying the ability of isolated nuclei and chromatin to incorporate the nucleotide ³H-uridine monophosphate into RNA. Both α - and β -amanitin block *in vitro* transcriptional ability of the RNA of this species. It also has been established experimentally (Horgen *et al.*, 1978) that α -amanitin prevents RNA synthesis by inhibiting RNA polymerase II, the enzyme responsible for transcription of mRNA.