

CHAPTER 22. Biochemistry: Molecules and Metabolism

A well-constituted plant or animal cell is most certainly equipped with a functional, regulated organization of its impressive array of molecules, and there is no reason to suppose that the coenocytic hypha of a watermold is constructed differently from the cells of other eukaryotes in this regard. Unfortunately, there is a vexatious shortcoming to the collection of facts surrounding the biological occurrence of organic compounds in the Saprolegniaceae. The experimental work at hand is of bits and pieces, and we are thus forced to consider these fragments without a unifying thread of continuity. We have found it expeditious, moreover, to fracture the fragile limits between physiology and biochemistry in the account to follow, although this should not be harmful even if discordant. It will be quite apparent that seemingly limitless opportunities exist for biochemical inquiries into the growth and reproduction of the watermolds, and it is to be hoped that the impetus for exploring the gaps in knowledge will not fall between the physiologist and biochemist and thus be lost.

In this chapter and the succeeding one we consider the molecular organization of those few watermolds that have been subjected to the cell biologist's probing and analysis. We have grouped what is known into a recital of some biological molecules identified in these fungi, their involvement in metabolism, their biosynthesis, and finally their replication. The preoccupation of comparative biochemistry with evolution (the truly unifying theme of consequence in biology) is treated in Chapter 25. Some of the data to which we refer may have little current significance except as historical background, but this impact is sufficient, in our view, to warrant their inclusion.

BIOLOGICAL MOLECULES -- ENZYMES

Not unexpectedly, the earliest papers dealing with the biochemistry of the watermolds are relics of the days when identifying enzymes in organisms was considered frontier research. Among the first of such accounts was that of Emoto (1923) reporting some analyses of the mycelium of *Saprolegnia tokugawana* (= *ferax*) and a nonsexual *Achlya*. He identified numerous glycolytic and proteolytic endo- and exoenzymes (some no longer recognized) associated with both species, but in each case, found no "lipases". Wolf (1937b), on the contrary, reported that both *S. ferax* and *A. bisexualis* could hydrolyze fats, and therefore must be equipped with lipolytic enzymes. *Achlya dubia* and *Thraustotheca clavata* also produced lipase, according to R. K. Saksena and Bose (1944), although the enzyme system was alleged to be located only within the hyphae, and was only capable of feeble activity. In general, their data agreed with those reported by Bhargava, in 1943, who detected a number of endo- and exoenzymes in four species of Saprolegniaceae (excluding *Brevilegnia gracilis*, which is a *Pythium*). In common with methodologies employed by his predecessors, Bhargava sought to identify enzymes by such techniques as reducing sugar analyses, colorimetric reactions, or polarimetric rotations of substrates.

With the advent of precise (although in some cases indirect) methods of detecting enzymes and their action, the gross identification of these proteins has given way to accurate analyses. Sucrose density gradient determinations (Ahmed, 1973), spectrophotometry (Gleason, 1972; Fox and Wolf, 1977b), protamine sulfate fractionation (Hütter and DeMoss, 1967), polyacrylamide gel electrophoresis (O'Day and Horgen, 1974; Wang and LéJohn, 1974a), and lysolecithin- ³²P recovery (Querci *et al.*, 1973, 1974), are among the methods that have revealed pertinent details about enzymes in water molds. Some of the enzymes detected in saprophytic species of the family by these methods are listed in Table 39. Other enzymes occur in these fungi, of course, and have been investigated in far greater depth than mere identification.

Several papers have been published on the ultrastructural location of enzyme activity in a few water molds: Choinski and Mullins (1977), Dargent (1975), Dargent and Denisse (1976), Murrin and Nolan (1977), Palczewska (1965), and Palczewska and Jagodzka (1972). These accounts are treated in the prior chapters on ultrastructure.

ENZYMES IN THE SAPROTROPHIC SPECIES

Glutamate Dehydrogenase¹: -- The mitochondrial matrix is the site of the allosteric enzyme glutamate dehydrogenase (GDH) functioning in oxidative deamination. The reaction catalyzed by the enzyme uses the *l*-isomer of glutamic acid with NADP⁺ serving as the electron acceptor, and the product, NADPH, acting as a reducing agent in synthesis reactions. Thus, GDH holds a key function in carbohydrate metabolism. Work primarily from the laboratory of H. B. LéJohn and his associates deals with some aspects of this enzyme in a nonsexual *Achlya* isolate.

From sucrose density gradient determinations and kinetic measurements of optical density changes during oxidation and reduction of NAD⁺ and NADH, LéJohn and Stevenson (1970) found glutamate dehydrogenase in *Achlya* sp. to be NAD-dependent. Glucose metabolism represses the synthesis of GDH as does sucrose (LéJohn, 1971b), and both citrate and isocitrate in the hyphal system are toxic to the enzyme's activity. However, LéJohn and Stevenson (1970) discovered that phosphoenolpyruvate counteracted the poisoning effect of citrate. Thus, the multivalent modulators NADP⁺, NADPH, and phosphoenolpyruvate release GDH from citrate inhibition, and are antagonistic to it.

Further evidence for multivalent control of glutamate dehydrogenase has been assembled by LéJohn *et al.* (1970). In both *Saprolegnia parasitica* (= *diclina*) and *Achlya* sp. guanosine nucleotides, short-chain acyl-CoA derivatives, ATP, and ADP are allosteric activators of GDH. Conversely, ammonia and α -ketoglutarate are allosteric inhibitors of the enzyme. The short-chain fatty acid coenzyme A esters that activate GDH in these two species are CoA, acetyl-CoA, aceto-acetyl-CoA, succinyl-CoA, *n*-butyryl-CoA,

¹ In conformity with current practice, we are following the spelling of enzyme names as recommended by Dixon and Webb (1979).

n-propionyl-CoA, malonyl-CoA, *n*-hexyl-CoA, and *n*-valeryl-CoA. In several other species of Saprolegniaceae [*Achlya* sp., *A. flagellata*, *Saprolegnia* sp., *S. ferax*, *Isoachlya itoana* (= *S. itoana*), *Thraustotheca* sp., *T. clavata*, and *Aphanomyces euteiches*] glutamate dehydrogenase is activated (LéJohn, 1971a, b) by uridine nucleotides (UDN), uridine nucleotide sugars and amino sugars, triphosphopyridine nucleotide (=NADP) and its analog deamino-TPN⁺ (=NADP⁺).

LéJohn (1971a) puts forth an intriguing hypothesis regarding glutamate dehydrogenase. Those organisms capable of synthesizing chitin -- uridine nucleotides activate the enzyme to provide the glutamine necessary for the biosynthesis of amino sugars -- should be insensitive to uridine nucleotides. On the other hand, organisms that do not produce chitin, and this includes the water molds, should indeed possess glutamate dehydrogenases that are UDN-insensitive. In a later study of NAD-specific GDH isozymes, however, Wang and LéJohn (1974a) determined that some species of the family were sensitive to uridine-5'-triphosphate, and thus, according to LéJohn's (1971a) proposition, would be expected to produce some chitin. Dietrich (1973), it will be recalled (Chapter 6), thought it possible that the glucosamine in hyphal walls of some saprolegniaceous fungi could originate from a chitin fraction.

Experimental data assembled by Wang and LéJohn (1974a) indicate that with but two exceptions, species of water molds possess only a single isozyme of NAD-dependent glutamate dehydrogenase. This determination was based on analysis of the mating strains of the dioecious *Achlyas*, four species of *Aphanomyces*, ten of *Saprolegnia*, four of *Isoachlya* (= *Saprolegnia*), *Aplanopsis terrestris*, *Leptolegnia caudata*, *Protoachlya paradoxa*, and *Thraustotheca clavata*. Only *S. litoralis* and *S. monoica* (= *ferax*) exhibited two isozymes of GDH. There were differences, Wang and LéJohn also discovered, in reaction of the species to certain allosteric modulators (activators), namely, NADP⁺, phosphoenolpyruvate, guanosine-5'-triphosphate, ATP, UTP, and short-chain acyl Co-A derivatives. All species tested by these investigators were sensitive (GDH activation) to NADP⁺ and some of the other six modulators, but of the *Achlyas*, only *A. racemosa* and *A. colorata* responded to all six, and three species -- *A. flagellata* (= *debaryana*), *A. intricata*, *A. radiosa* -- were not activated by phosphoenolpyruvate. In the genus *Saprolegnia*, *S. asterophora* alone responded to all six modulators; *S. monoica* and *S. megasperma* were stimulated by phosphoenolpyruvate to produce GDH. It seems quite clear from these data that there is no great uniformity among the water molds with respect to the allosteric controls that act on GDH.

The taxonomist marvels at the inconsistencies between the traditional view of taxa in the Saprolegniaceae and the varied sensitivities of some of its species to modulators of glutamate dehydrogenases as determined by Wang and LéJohn (1974a). The GDH of *Saprolegnia ferax* is allosterically controlled by five modulators, but that of *S. mixta* (= *ferax*) responds to only one; morphologically, these taxa are indistinguishable. The male strain of *Achlya ambisexualis* has GDH responding to two activators; its female companion replies to five modulators.

Two characteristics of glutamate dehydrogenase from *Achlya* sp. have been determined by R. M. Stevenson and LéJohn (1971). The GDH is heat labile (it denatures at 37 °C), and its activators and inhibitors do not stabilize its protein structure against heat denaturation. Moreover, the enzyme appears to be maximally activated or inhibited at the pH that is minimum for its activity. Conversely, at the pH that is optimum for GDH activity, inhibition or activation is at low points.

Lactate Dehydrogenase: -- The last step in glycolysis is the conversion of pyruvate to lactate, in which isozymes of lactate dehydrogenase (LDH) are effective in reoxidizing NADH with pyruvate. This enzyme system is also a key one in anaerobic energy metabolism. LéJohn (1971c) and Wang and LéJohn (1974b) have assembled most of what is known of this enzyme in members of the Saprolegniaceae. Using starch gel electrophoresis to determine the location of the activity of D(-)lactate dehydrogenase, Gleason and Price (1969) could not detect this enzyme either in *Aphanomyces euteiches* or *Saprolegnia* sp.

LéJohn (1971c) found that six species in the family (*Achlya* sp., *A. americana*, *Aphanomyces euteiches*, *Saprolegnia ferax*, *S. parasitica*, and *Thraustotheca clavata*) produced the NAD-dependent lactate dehydrogenase under the allosteric control of guanosine triphosphate acting as an inhibitor. In addition, LDH was also modulated by lactate and NADH. A study by Wang and LéJohn (1974b) of the electrophoretic mobility (R_e values) of the D(-)lactate dehydrogenase isozymes added to LéJohn's determinations. They studied this enzyme complex in ten species of *Saprolegnia*, eleven of *Achlya* (they record 14 species, but regard the male and female strains of dioecious *Achlyas* as two species), four of *Aphanomyces*, and several others. The data show patterns both of dissimilarity and uniformity among the species tested, just as had been found for glutamate dehydrogenase (Wang and LéJohn, 1974a). All of the *Achlya* species, for example, had identical R_e values, and differences among the species of *Saprolegnia* were only slight. The four taxa of *Aphanomyces* differed from one another in electrophoretic mobility of their LDH isozymes. Only *Leptolegnia caudata* had two isozymes of the parent enzyme; all others exhibited but one. Patterns of similarity and dissimilarity among taxa also appeared when the allosteric inhibitory effects of guanosine triphosphate (GTP) were determined. *Achlya flagellata* cultures were 100% sensitive to GTP, but all other *Achlyas* were only partially inhibited. Some additional GTP-inhibition percentages determined by Wang and LéJohn were: *S. mixta*, 100%; *S. ferax*, 57%, *A. ambisexualis* ♂ 60%, ♀ 33%; *A. bisexualis* ♂ 25%, ♀ 67%.

Malate and Isocitrate Dehydrogenases: -- LéJohn (1971b) reported that NAD-linked isocitrate dehydrogenases (unique in that these enzymes are themselves catalyzed by an allosteric enzyme) were absent in Oomycetes. In the TCA cycle, catalysis of isocitrate to α -ketoglutarate is accomplished by NAD⁺- or NADP⁺-dependent isozymes. Later experiments by Wang and LéJohn (1974c) showed that isocitrate dehydrogenase was indeed present in the same water molds that had been tested for glutamate and lactate dehydrogenases (Wang and LéJohn, 1974a, b).

The R_e values for isozymes of isocitrate dehydrogenase in eleven species of *Achlya* were very similar, and members of the genus *Saprolegnia* (*S. ferax*, *S. litoralis*, and *S. parasitica*, among others) were even more uniform in this respect. Extracts from *Aphanomyces cladogamus* and *Aplanopsis terrestris* exhibited two bands in slab gel electrophoresis, but the main bands from isocitrate dehydrogenase isozymes produced by the other *Aphanomyces* species, and by *Thraustotheca clavata* and *Leptolegnia caudata*, were nearly identical. The IDH isozymes of *Isoachlya* (= *Saprolegnia*) *eccentrica* and *I.* (= *Saprolegnia*) *unispora* were not the same (R_e values) as those synthesized by *I.* (= *Saprolegnia*) *intermedia* and *I. toruloides* (= *S. torulosa*). Wang and LéJohn suggested that further work on this dissimilarity between these two groups of species might show that they should be assigned to different genera. In view of the inconsistencies in dehydrogenase production displayed by seemingly closely related water molds (for example, the mating strains of *A. bisexualis*; Wang and LéJohn, 1974b), such a suggestion is at best speculative.

With respect to the possession of isozymes of malate dehydrogenase (MDH, catalyzes the final TCA cycle reaction, the oxidation of malate to oxaloacetate) there is much diversity among the species of Saprolegniaceae tested by Wang and LéJohn (1974c). Preparations from mycelium of *Saprolegnia ferax*, *S. mixta*, *S. terrestris*, and *S. furcata* produced R_e values indicative of a single MDH isozyme; *S. diclina*, on the contrary, exhibited three electrophoretic bands. The number of isozymes synthesized by species of *Aphanomyces* reflects even greater variability among water molds. *A. laevis* has but one isozyme of MDH, *A. euteiches* has two, *A. stellatus* three, and five are present in *A. cladogamus*. The pattern of IDH isozyme distribution in the four species of *Isoachlya* parallels precisely that of MDH.

Phosphatase and Other Enzymes: --The phosphatases are broadly specific enzymes capable of hydrolyzing two phosphate or phosphoric esters. By the use of *dl*-parafluorophenylalanine (suppressant of lateral branching by hyphae), Fèvre (1974) was able to inhibit phosphatase activity in *Saprolegnia monoica*. O'Day and Horgen (1974) studied the pattern of acid phosphatase activity in calcium-induced sporangium formation in *Achlya bisexualis*. Their data suggest that acid phosphatase is synthesized *de novo* by the hyphae of this species during differentiation. This enzyme also accumulates extracellularly during differentiation. The transcriptional inhibitor actinomycin D prevents acid phosphatase activity. It is thus possible (O'Day and Horgen, 1974) that RNA synthesis is essential for increased action by this enzyme.

Not surprisingly, the activities of certain enzymes are localized during morphogenesis, as Palczewska (1973) has found by colorimetric analyses. The sex cells of *Achlya flagellata* yielded indoxyl esterase, acid and alkaline phosphatases, and β -galactosidase. Cytoplasmic granules 0.2-1.0 μm in diameter were associated with the measurable enzyme activity in the antheridial cells of this species. Palczewska uncovered an interesting aspect of acid phosphatase activity. The action of this enzyme was elevated in those antheridial cells attached to oogonia in which the oospheres had

cleaved. The oospheres themselves also exhibited enhanced activity of acid phosphatase as well.

The role of cellulase in hyphal branching in the water molds has been explored in some depth (Chapter 6), but exogenous cellulolytic ability is not a property common to all species in the family. Miele and Linkins (1978) demonstrated that *Achlya bisexualis* produced cellulase (even if no substrate was present) as a soluble, extracellular enzyme. As has been seen (Chapter 6), *A. ambisexualis*, on the contrary, is reported to be unable to degrade either cellulose or the soluble carboxymethylcellulose (CMC). *Thraustotheca clavata* hydrolysed both filter paper and CMC, Berner and Chapman (1977) reported, but *A. americana* did not degrade CMC at all, and *S. parasitica* hydrolyzed this substrate only very poorly. Cell-free filtrates from all three species failed to hydrolyze CMC.

ENZYMES IN THE PARASITIC SPECIES

Plant Pathogens: -- It is known from the work of Wang and LéJohn (1974a-c) that *Aphanomyces euteiches* (causal agent of pea root rot) and *A. cladogamus* (injurious to roots of *Lycopersicum esculentum* Mill.) produce isozymes of glutamate, lactate, malate, and isocitrate dehydrogenases. Most of the biochemical experimentation on parasitic species in the genus has centered, however, on the synthesis, assay, and activity of enzymes capable of hydrolyzing pectic substances (linear polymers of *d*-galacturonic acid in α -1, 4 linkages). Penetration by the pathogens into and through plant tissue must surely involve pectinolytic action, hence it is logical that such enzymes should be sought in the invading fungi.

Filtrates from the mycelium of *Aphanomyces euteiches* were assayed viscometrically by Ayers and Papavizas (1965) for their ability to degrade pectin. The filtrates showed no evidence of pectin methylesterase, but extracts from infected or noninfected pea roots (*Pisum sativum* L.) contained this enzyme. These investigators concluded that either the fungus produced this enzyme *in vivo* only, or induced the invaded tissue to synthesize it. On the other hand, culture filtrates of *A. euteiches* reduced the viscosity of sodium polypectate and pectin, yet left no galacturonic acid residues. Ayers and Papavizas suggested that this was evidence for the absence of polygalacturonase in *A. euteiches* and the presence of a pectic depolymerase. Independently, by standard viscometric analyses, Temp (1966) coincidentally confirmed the results obtained by Ayers and Papavizas (1965), but considered the pectinolytic enzyme system in this species to be an endopolygalacturonase.

While it had been demonstrated earlier that *Aphanomyces euteiches* produced pectin-hydrolyzing enzymes, it remained for Ayers and associates (1969a, b) to assay this activity in depth. Of nine isolates of the species, all produced polygalacturonase constitutively. Pectin alone (or pectin plus calcium) in the growth medium enhanced enzyme accumulation in the early stages of mycelial growth of *A. euteiches* but this effect was not additive with prolonged incubation. With sodium polypectate as the substrate, the optimum pH for polygalacturonase activity was 6.7. If pectin was used as the substrate, the optimum pH range for action by this enzyme was 5.0-6.0.

Polygalacturonase was highly sensitive to temperatures above 30 °C. Ayers *et al.* (1969b) purified the endopolygalacturonase by gel filtration chromatography on Sephadex columns. Further experimentation gave presumptive evidence that pectin-degrading enzymes were indeed operable in the spread of the pathogen in invaded pea roots. The purified enzyme released galacturonic acid only upon prolonged incubation, although it did release early several oligomers of this acid.

Temp (1966) tested filtrate from the growth of *Aphanomyces euteiches* for its cellulolytic ability (carboxymethylcellulose method). The soluble substrate CMC was hydrolyzed, but the preparations from colonies of this species were unable to hydrolyze native cellulose. Cellulolytic activity was optimum at pH 5.0 or 6.0, depending upon the medium from which the filtrate was prepared. Papavizas and Ayers (1964) earlier had reported that *A. euteiches* had little or no cellulolytic activity, but Temp (1966) found the ability of this *Aphanomyces* to hydrolyze CMC to be variable among isolates.

There appears to be but one major account -- that by Herr (1977) -- of pectin-hydrolyzing enzymes in the beet root rot organism, *Aphanomyces cochlioides*. Filtrates from liquid culture of the species were used, and activity was assayed by viscometric means and chromatographic analysis of end products. There was no correlation between substrate viscosity reduction and the amount of mycelium produced by *A. cochlioides* in shake culture. However, the capacity of the species to lower the viscosity was increased 10-fold if cultures were propagated in stationary vessels. Constituents of the medium also influenced the final pectin-lysing capacity of the filtrate. The filtrate from a seeded medium containing the *l*-isomer of asparagine was less effective in changing the viscosity of the pectin substrate than was the filtrate from a medium with the *dl*-isomer, (Herr, 1977). As a substrate, sodium polypectate was more susceptible to hydrolysis by the filtrate from cultures of *A. cochlioides* than was pectin. Herr detected pectin methylesterase in the culture medium only if cysteine·HCl was included in the extraction fluid. *Aphanomyces cochlioides* did not produce *trans*-eliminase, but did elaborate endopolygalacturonase, judging from the fact that the latter was found in the culture filtrate. Herr (1977) concluded that the hyphae of *A. cochlioides* probably play a part in dissolution of the middle lamella as they traverse the roots of sugar beets, but pectin methylesterase is unimportant in this role.

Another study of the foregoing three phytopathogens was conducted by Unestam (1966a) as part of an analysis of enzyme production by the crayfish pathogen *Aphanomyces astaci*. He concluded that (1) *A. euteiches* had little chitinolytic, cellulolytic, or pectinolytic activity; (2) *A. cladogamus* was clearly capable of lysing chitin, but its ability to degrade pectin and cellulose was much less prominent, and (3) *A. cochlioides* was effective to a modest degree in hydrolyzing all three substrates tested (native cellulose, beetle chitin, and pectin).

Animal Pathogens: -- The water molds associated with diseases of fish, and the notorious *Aphanomyces astaci* (the cause of crayfish plaque) constitute the chief invaders of animal tissue about which some biochemical information has been assembled. The bulk of such knowledge rests in work that has been done on *A. astaci*.

The early biochemical work on *Aphanomyces astaci* by Unestam (1966a; 1969a) demonstrated that this fungus produced chitinase independent of an exogenous supply of chitin, that is, the enzyme is constitutive. By contrast, *A. laevis*, a species readily collected on chitinous substrates, has only inductive chitinase. Unestam concluded that certain nutritional lines of evidence singled out *A. astaci* as a fungus adapted to a parasitic existence (in crayfish) rather than to a more carefree life. First, it uses only glucose, the sugar in animal blood, and also can utilize amino acid carbon. Second, it has little or no pectinase activity. Third it is constitutive with respect to chitinase. Unestam (1968a) demonstrated by turbidimetric assay of a culture filtrate that chitin, the substrate (a homopolymer of N-acetyl-D-glucosamine) for *A. astaci*, "protected" the chitinolytic enzyme against heating, agitation, and extremes in pH.

Of course, chitin-degrading enzymes are not the only ones synthesized by *Aphanomyces astaci*. Various methodologies employed by Söderhäll and Unestam (1975) -- casein digestion for the measurement of proteolysis, viscosity reduction to determine the presence of hyaluronidase, for instance -- demonstrate that this species synthesized constitutive proteases, hyaluronidase, and in the germinating spores, esterases. The tests for enzymes did not detect any lipase in *A. astaci*.

Melanization in the hyphae of *Aphanomyces astaci* is effected by phenol oxidase in the crayfish, a condition that is correlated with disease resistance. In the relationship between fungus and host, activation of prophenol oxidase (inactive form of the enzyme) is a critical factor. Söderhäll and associates (communication; Söderhäll and Unestam, 1979) also have demonstrated that β -1, 3-glucans activate prophenol oxidase, but precisely how this is accomplished remains to be discovered. Extracellular glycoproteins purified from culture liquid in which *A. astaci* had grown also are known to stimulate prophenol oxidase to its functional state. Söderhäll and Unestam (1979) tested a number of mono-, di-, and polysaccharides (for instance, glycogen, starch, glucose, trehalose, laminarin, dextrin, carboxymethylcellulose) for their ability to activate blood serum prophenol oxidase. Trehalose was effective in this regard, and although laminarin did not serve as an enzyme stimulator, a hydrolytic product of yeast glucan, laminaropentaose, did so. Treatment of the glycoproteins with exo- β -1, 3-glucanase decreases their activating capacity (Söderhäll and Ajaxon, communication; Söderhäll and Unestam, 1979). The pathology associated with the defense mechanism in *Astacus astacus* is well documented (Chapter 30), but the significance of the glucan and glycoprotein activation of the serum prophenol oxidase is obscure.

Through double gel diffusion and immunoelectrophoretic methods, Peduzzi *et al.* (1976) demonstrated that species of *Saprolegnia* (*S. parasitica*, *S. diclina*, *S. ferax*) associated with diseased fish possessed a chymotrypsin-like proteolytic enzyme system present both in culture filtrates and in extracts from the mycelium. The investigators hypothesized that the production and extracellular secretion of proteolytic enzymes by these fish-associated fungi would perhaps assist hyphae in penetrating deeply into the invaded animal tissue. They also suggested that proteolytic enzymes might be of adaptive significance by conferring a parasitic potential on these Saprolegnias.

METABOLISM

It can be supposed that a recital of the reaction sequences in the central metabolic pathways of organisms in general would apply also to members of the Saprolegniaceae. Certainly these fungi are known to have some of the intrinsic and allosteric enzyme systems that operate in intermediary metabolism. The information on specific metabolic functions in the water molds is, however, rather thoroughly fragmented -- a fact not congenial to an adequate treatment of the subject.

RESPIRATION

Applying respirometry, spectrophotometry, and specific enzyme inhibitors, C. O. Warren (1966) and C. O. Warren and Mullins (1969) experimented with respiratory metabolism in *Achlya ambisexualis* (E87, the Γ strain, and 734, the E one). The very high rates of endogenous respiration measured in the mycelium of this species were increased as the pH of the suspending fluid was raised sequentially from 5.0 to 7.9. Oxygen uptake was more than doubled in the male strain of *A. ambisexualis* in the presence of antheridiol. The RQ data, however, suggested that the substrate for endogenous respiration was not a carbohydrate but possibly could have been lipid. Enzymatic analyses performed on the mycelial extracts demonstrated that the Embden-Meyerhof-Parnas scheme of glycolysis operated in *A. ambisexualis* as (very likely) did the hexose monophosphate shunt. In 1966, C. O. Warren reported having found two enzymes of this latter pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase. As evidence for the former pathway, he identified such enzymes in extracts of mycelium from *A. ambisexualis* as 6-phosphofructokinase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase.

The age of the mycelium evidently is instrumental in some fashion in determining the magnitude of activity of certain respiratory enzymes (C. O. Warren and Mullins, 1969) of *Achlya ambisexualis*. Glucose-6-phosphate dehydrogenase and glycerolphosphate dehydrogenase evidently are more active in three-day than in one-day-old mycelium. Warren and Mullins noted that the activity of five other enzymes, however, decreased as the fungus aged.

Gleason (1974) studied the factor of aging and its influence on respiration by subjecting mycelium of *Saprolegnia* sp. to the inhibitory properties of potassium cyanide, antimycin A, and salicylhydroxamic acid. Up to about 30 hours, the hyphae from germinating spores of the water mold were sensitive to cyanide and antimycin A but this response then decreased sharply with further aging. Sensitivity of the fungus declined concomitantly with the utilization of glucose from the medium. Thus, Gleason concluded, the rate of endogenous respiration is a function of age. He found salicylhydroxamic acid to cause nearly a 50% lowering of the cyanide-insensitive component of respiration. Gleason (1976) also noted that in isolated mitochondria of Saprolegnians intermediates of the tricarboxylic acid cycle acted as substrates for respiration.

Manometric and spectrophotometric data assembled by C. O. Warren (1966) from experiments with *Achlya ambisexualis* give evidence that there is a functional TCA cycle in the respiratory metabolism of this species. Furthermore, the fungus has a terminal oxidative system mediated in part by cytochrome oxidase (C. O. Warren, 1966; C. O. Warren and Mullins, 1969).

Achlya flagellata and *Brevilegnia unisperma* var. *delica* (= *minutandra*) have been examined for certain aspects of energy-yielding metabolism. Barbier (1969) suggested that if proper hydrogen acceptors were present, *A. flagellata* might exist in a situation very closely approaching an anaerobic one. The work of Thakur Ji and Dayal (1971) on the *Brevilegnia* centered on respiratory inhibitors. Iodoacetate, sodium flouride, and sodium malonate influenced respiratory metabolism in the watermold in different ways. The former two limited glucose metabolism severely, but endogenous respiration was relatively insensitive to the latter. Thakur Ji and Dayal found, as would be expected, that as the age of the fungus increased, endogenous respiratory rates declined sharply and progressively.

A comparative study of endogenous respiration in six species of watermolds -- *Achlya* sp., *Aphanomyces astaci*, *A. euteiches*, *Dictyuchus* sp., *Saprolegnia* sp., and *Thraustotheca* sp. -- was reported by Unestam and Gleason in 1968. Glucose stimulated the uptake of oxygen by all the isolates, and for *A. euteiches* fructose and acetate also augmented respiration. Both species of *Aphanomyces* utilized endogenous protein of amino acids as carbon sources (measured manometrically), a not unexpected ability in view of the parasitic nature of these fungi under natural conditions. Of all the species tested, Unestam and Gleason found *Saprolegnia* sp. to be unusually resistant to respiratory inhibitors such as antimycin A and HCN. They concluded that the crayfish plague fungus was not unique in any way as far as its endogenous respiration was concerned. Indeed, *A. astaci* clearly was oomycetous in this respect.

Manavathu and Thomas (1980) studied cytochalasin A (CA) as a respiratory inhibitor in five species of Saprolegniaceae: *Achlya ambisexualis*, *A. flagellata*, *A. mucronata* (= *Protoachlya mucronata*), *Saprolegnia* sp., and *S. ferax*. The alkaloid was added to cultures of the fungi and the rates of oxygen consumption at 30 °C determined after a period of incubation. Cytochalasin A was a potent inhibitor of respiration in these watermolds, and its effect was irreversible (as determined by washing CA-exposed mycelium on 0.01 M tris-phosphate buffer). The inhibitory action of cytochalasin B, on the other hand, was reversible.

PRODUCTS OF METABOLISM

There are a few reports of metabolic products released into growth media during the development of watermolds, or of compounds identified in their mycelium on extraction during active growth. *Saprolegnia megasperma* produces ammonia and urea during active growth in media with or without glucose (Nolan, 1975b). When propagated in a casamino acid medium *S. ferax* also liberates urea and ammonia (Nolan, 1976) but in addition releases *l*-1-methylhistidine, *o*-phosphoethanolamine, glutamine,

asparagine, spermidine (with prolonged incubation), and γ -aminobutyric acid. Cultures of *Pythiopsis cymosa* grown by Nolan and Lewis (1974) on a casamino acid-mineral salts medium with EDTA and micronutrients were analyzed for products of metabolism. When the mycelium had been growing 48 hours, glutamine, asparagine, and α -aminobutyric acid had been released, and at 72 hours, some additional compounds were present: α -aminoadipic acid, β -alanine, γ -aminobutyric acid, carnosine, and citrulline. One water mold, *S. litoralis*, of 31 species of fungi tested by means of gas-liquid chromatography of extracted and saponified lipids (Shaw, 1965) contained γ -linolenic acid but not the α -form. This fatty acid apparently has no metabolic function in this species, and, Shaw concluded, is likely present as a storage product. Rioux and Achard (1956) grew *S. diclina* -- isolated from the larvae of *Aedes berlandi* Seguy -- on Sabouraud medium, noting that it produced there a weak citron-yellow fluorescence. Should this discovery be confirmed, it will stand as the first report of fluorescence in the Saprolegniaceae. Agar gel electrophoresis utilized by Dietrich (1976) failed to uncover any evidence of polyphosphates in *Achlya pseudoradiosa* (= *radiosa*) and in this same species, Pfyffer and Rast (1980) could not detect any free ribitol (polyol fractions).

CYTOCHROMES

Certain absorption spectra derived from hypha-free extracts of *Achlya radiosa* and *Saprolegnia* sp. led Boulter and Derbyshire (1957) to conclude that there were no additional cytochromes in these fungi beyond the ones detectable in yeasts. In *A. flagellata*, the cytochrome-oxidase system is disrupted by a redox potential below +310 mV, and Barbier (1969) has suggested that an azide-insensitive cytochrome b_7 may operate in this species.

Spectrophotometric analyses (Unestam and Gleason, 1968) coupled with antimycin A application (Gleason and Unestam, 1968) give evidence that *Aphanomyces astaci* synthesizes two cytochrome *b* types, cytochrome *c*, and cytochrome *a-a₃*. Data provided by Gleason and Unestam show that *Saprolegnia* species have cytochrome *a-a₃*, b_{564} and b_{557} as well as *c*.

AMINO ACID TRANSPORT

Gleason (1973b) demonstrated that certain amino acids were taken up from the medium during the growth of a nonsexual *Saprolegnia*: proline and phenylalanine (absorbed most rapidly), arginine, aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, methionine, isoleucine, leucine, and lysine (absorbed most slowly). Diauxy did not operate during absorption. Data more analytical than the foregoing, however, appear in two accounts published by L. E. Cameron and LéJohn (1972a, b) on calcium and its role in amino acid transport.

To determine if calcium (Ca^{2+}) alone or in combination with other cations was necessary for amino acid transport (and coupling into proteins at the membrane level) in a nonsexual *Achlya*, L. E. Cameron and LéJohn (1972a) used radioisotope techniques

coupled with the stain for mucopolysaccharide ruthenium red (obstructs calcium transport in mitochondria) and citrate (excludes Ca^{2+} from the plasmalemma). Mycelium of *Achlya* sp. in the presence of these two inhibitors was unable to take up any of 15 amino acids from the medium, but Ca^{2+} released the hyphae from the effect of both. Cameron and LéJohn also found that Mg^{2+} and Mn^{2+} were antagonistic to citrate inhibition when applied to the hyphae of this fungus, and that uracil and thymidine uptake was independent of calcium metabolism.

Calcium activates membrane-associated ATPase, and participates in ion transport and the functional and structural integrity of the hyphal (or cell) membranes. Using labeled compounds, L. E. Cameron and LéJohn (1972b) assayed *Achlya* sp. for a possible calcium-mediated transport of amino acids. When Ca^{2+} entry into the mycelium of the isolate was arrested by citrate or chelating agents amino acid transport also stopped. If Ca^{2+} was added to a calcium-deficient medium, the uptake of amino acids was enhanced nearly 100 per cent. Evidently the requirement for cations in the system was absolute, although the bivalent ions of manganese, magnesium, cobalt, or iron could be substituted for Ca^{2+} . Cameron and LéJohn have thus demonstrated conclusively that amino acid uptake in one watermold, at least, is cation-dependent (*see* also, in Chapter 23, protein synthesis).

Amino acid transport (and the movement of nucleosides and sugars) in the mycelium of *Achlya* sp. is arrested by removal of a low molecular weight phosphorylated proteoglycan (PPG). This PPG was isolated by L. E. Cameron and LéJohn (1978) by cold osmotic shock of newly germinated spores. The compound (possessing a uracil moiety) was associated with three phosphorylated dinucleosides (*see* Chapter 23). Cameron and LéJohn propose that the PPG in *Achlya* sp. is a polymer of glucose phosphate with the sugar moieties linked via phosphate (such linkage is not characteristic of cellulose). As the phosphorylated proteoglycan has strong cation-binding properties, it may have a role somewhat like that suggested for teichoic acid, that is, to function as a repository for ions needed for the maintenance of membrane integrity and membrane-sequestered enzymes (L. E. Cameron and LéJohn, 1978).

LIPID METABOLISM

One of the readily observable characteristics of the watermolds is the abundance of nearly anhydrous lipid -- usually in the form of globules or droplets -- in their hyphae and reproductive cells (most notably in the oospores). It would seem likely, then, that these fungi metabolize endogenous lipids, and particularly so in germination.

By means of hyphal-free extracts prepared from cultures of the same non-fruiting *Achlya* utilized earlier by LéJohn and his associates, S. W. T. Law and Burton (1976a) assayed for fatty acid synthetase activity, *in vivo* incorporation of labeled sodium acetate into lipids, fatty acid oxidation, and lipid turnover in encysted spores and germlings. As has been mentioned in an earlier chapter, lipid synthesis is balanced by growth of the mycelium of *Achlya* sp., but when the hyphae differentiate to form sporangia, lipid synthesis increases, suggesting that lipid catabolism is in progress (S. W. T. Law and

Burton, 1976a). Quite the reverse obtains during growth of the germ hyphae from encysted spores, for at this time the ^{14}C -labeled lipids increase. Data from the Law and Burton experiments on incorporation of the sodium acetate isotope into lipids during the growth and development of the hyphae corroborated these findings.

Fatty acid synthetase activity in *Achlya* sp. was determined by S. W. T. Law and Burton (1976a) to be relatively low during germination and germ hypha elongation, but to increase some 15-fold during sporulation. Labeled hexanoic and palmitic acids released $^{14}\text{CO}_2$ when oxidized by the fungus. This process also was highest during sporulation. Law and his associates determined (by thin-layer chromatography) that the bulk of the total lipid in the mycelium was in the form of glycerides.

The lipid constituents of *Achlya* sp. were further explored and the results reported by S. W. T. Law and Burton in a second paper published in 1976(b). By means of fractionation and thin-layer chromatography they found the chief components of neutral lipids in *Achlya* sp. to be triglycerides (no mono- or diglycerides were detected), free fatty acids (about 50%), and cholesterol. Their data suggest that free fatty acids (nonesterified) are synthesized by the mycelium during sporulation, and incorporated into glycerides which then accumulate. More than one-third of the total fatty acids synthesized were the saturated hexa- (palmitic) and octadecanoic (stearic) acids; these were present throughout all the stages of development of the hyphae. The fatty acid composition in *A. flagellata* evidently is almost identical (Clausz, 1979) to that in *Achlya* sp. except that the mycelium of the latter possesses 18:3, 20:5, and 22:0 components. Three fatty acids seem to be lacking in the vegetative (reproductively undifferentiated) hyphae of *Achlya* sp.: eicosatetraenoic, hydroxyhexadecanoic, and an unidentified long-chain acid. In the active stage of hyphal elongation, there is maximum incorporation of polar lipids into the mycelium (S. W. T. Law and Burton, 1976a), a feature consistent with the idea that such lipids are required for the synthesis of new plasmalemma during vegetative growth.

It should be noted that *Achlya flagellata* evidently has a lower lipid content (Clausz, 1979) than *A. bisexualis* (Darnaud, 1972b). Contrary to Weete's report (1974), Clausz found that there was more unsaturation of fatty acids in *A. flagellata* at higher than at lower temperatures of incubation. The carbon:nitrogen ratio (which Weete cited as being quite important in determining lipid levels) had no influence on the lipid content of the mycelium of this species.

Stearic acid conversion to 5, 8, 11, 14, 17-eicosapentaenoic acid in *Saprolegnia parasitica* was investigated by Gellerman and Schlenk (1979). Oleic, linoleic, and (6, 9, 12)-linolenic acids are intermediates, with methyl-directed desaturation of arachidonic acid to eicosapentaenoic acid being the last step in the conversion.