CHAPTER 21. The Physiology of Reproduction: Hormonal Control

As early as 1881, de Bary proposed that chemical substances emanating from the oogonia of watermolds triggered the development of antheridial branches and directed their growth toward these cells. This was, on the face of it, an unusually perceptive theory because de Bary did not fully accept the idea that fertilization by antheridia took place in any of the species then known in the family. Klebs (1899) also surmised that the presence of oogonia stimulated antheridial branches to form. Nutrients, Kauffman (1908) conjectured, lead to the synthesis of substances -- he referred to them variously as enzymes and hormones -- that were set free in the medium and there, at many points of "equilibrium" induced antheridial hyphae to form and to grow toward the oogonia.

In his study on "heterothallism" (Whitehouse, 1951, refers to this condition as haplodioecism) in *Dictyuchus*, J. N. Couch (1926b) sought possible chemical stimulants for the matings obtained in two-member cultures. Since antheridial branches in some of his isolates grew rather long distances before contacting oogonia, he suspected that a diffusible substance was operating in some fashion. However, the mating strains of *Dictyuchus* had to make actual contact, he found, or the sexual process would not manifest itself. If as many as six hyphae from an antheridium-producing thallus were simply positioned across the hyphae of a "female" thallus sexual stimulation resulted. On the other hand, one female colony immersed in the same culture water with eight "male" thalli would not respond unless there was direct contact, and plants potentially able to form oogonia simply would not do so in "juices" expressed from male mating strains (J. N. Couch, 1926b). In sum, Couch uncovered three lines of evidence to suggest that there were diffusing chemical substances in various individuals of *Dictyuchus*. First, antheridial hyphae definitely grew toward the oogonia. Second, oogonial initials and antheridial branches sometimes were formed on portions of reacting hyphae far from regions of actual contact, and third, sex cell production was noticeably stimulated in matings among dioecious members of *Dictyuchus* and *Thraustotheca primoachlya* (=*Achlya primoachlya*).

About four decades after Couch's paper appeared, W. A. Sherwood (1966a) assembled experimental evidence that *Dictyuchus monosporus* in fact did produce a diffusible hormone system. His observations suggested that the mechanism was induced under the combined influence of the male and female strains. Sherwood utilized a perfusion system in "microaquaria" in which the male and female thalli could be separated but kept in alternating order. Female thalli receiving water that had perfused over male and female colonies together in one microaquarium produced oogonia. Antheridia were not induced in the cultures, however, so there were no oospores.

Couch's early work was a pioneering effort, but the reality of hormonal control of sexuality in some watermolds was proven by J. R. Raper (1939a, b; 1940a; 1942a, b). In a series of carefully conducted experiments coupled with a fortunate choice of organisms, Raper demonstrated conclusively that diffusible substances are produced in

the mating process in certain species of *Achlya*. Others have built on this basic work so that it is now respectable to claim that sex hormones are produced by these structurally simple eukaryotes. Arguing that because the term hormone originally was applied specifically to secretions from endocrine glands, Karlson and Lüscher (1959) proposed that a new name, pheromone, should be adopted for biologically active compounds having a function similar to that of hormones, but different in their source. By their definition, the active agents in sex cell excitation in the watermolds should be referred to by this substitute name. Kochert (1978) used the term pheromone exclusively in reference to the sex "hormones" of algae and fungi. Machlis (1972) proposed that the generic term "erogen" be used for those compounds that control induction and differentiation of the sexual apparatus. Terminology notwithstanding, however, it is true, as Raper (1960) has pointed out, that there are two factors controlling sex in the fungi: genetic predesignation of sex capacity, and provision for a genetic and metabolic apparatus to insure normal development. It is this latter packet to which he contributed so much basic information on the sexuality of watermolds.

THE ORIGIN OF THE HORMONAL (PHEROMONE) CONCEPT

Several papers, among them the pioneering ones of Raper himself, review the physical and chemical nature of hormonal induction in the watermolds, and incidentally recount the historical milestones in the advance of knowledge about the process (Bu'Lock, 1976; C. G. Elliott, 1977; Gooday, 1974; Horgen, 1977a; McMorris, 1978a, b; Mullins, 1968; Raper, 1951a, 1952, 1957, 1970). The clues to hormone-induced sexuality as uncovered by Raper's early studies are reviewed in this section.

Some aspects of matings between the males and females, and between hermaphroditic isolates and the males of isolates of *Achlya* were reported by Raper in 1936, but it was not until 1939 (a, b) that the concept of hormonal control was developed. In matings between male and female colonies of *Achlya ambisexualis* (Raper, 1939b), five early stages in sex cell development are evident: (1) appearance of antheridial hyphae, (2) materializing of oogonial initials, (3) growth of the former toward the latter, (4) contact between the antheridial filament and the oogonium wall and delimitation of an antheridial cell, and (5) separation of the oogonial cell from its stalk by a septum. These events Raper argued, occurred in a definite sequence at specific time intervals, hence were probably under hormonal control. When matings between *A. ambisexualis* and *A. bisexualis* were attempted, some but not all of these five morphogenetic phases took place, hence the sexual pattern was never completed. This, too, Raper cited as evidence for a hormonal mechanism. By varying to some extent the nature of the medium in which dioecious *Achlya* isolates were "crossed" he could interrupt the mating sequence (the five stages) at any point. For example, when phosphate was decreased in the growth medium antheridial hyphae and oogonial initials appeared, but the physical aspects of mating never went beyond this point. Taken by itself, such a reaction suggested that stage 3 -- attraction of the antheridial hypha to the oogonium -- was under the control of a substance produced by the

oogonial initials, and not by the vegetative mycelium (this, of course, was basically de Bary's postulate nearly 60 years earlier. In any event, Raper (1939b) postulated that four primary hormones (Table 38) were present in the sexual system. There is proof for the existence of at least two of these, A (antheridiol), and B (oogoniol).

A series of experiments directed at uncovering any telemorphotic responses by the dioecious Achlyas were described by Raper in 1940(a). The results of matings of isolates on agar, in water, and in membrane-separated cultures, the data from perfusion experiments, agar block diffusion techniques, and from reactions when one strain was washed in the water in which the other "sex" had bathed, as it were, proved that diffusible substances were involved in the sequential induction of sex cells. When "female" mycelium was put in water in which the compatible male culture had developed, oogonial initials were formed by the female provided the male had formed antheridial branches. Thus, it was evident that initiation of sex cell induction began with the female thallus. The intensity of the first reaction -- hormone A -- could be measured (Raper, 1942a) to some degree by the relative abundance of antheridial hyphae induced at particular concentration levels of the hormone.

What was the nature of this hormone A? The unpurified form, J. R. Raper and Haagen-Smit (1942) found, had several characteristics. The diffusing substance was soluble in methyl and ethyl alcohols, acetone and acetic acid; insoluble in petroleum ether, benzene, and cold toluene; not precipitable by heavy metals; could be inactivated by temperatures in excess of 130 ^{o}C, and chemically was of the nature of a ketone. These investigators concentrated the crude agent to a level some 70,000 times greater than that in the starting material. This residue of the fractionation process induced antheridial hyphae even in a concentration as low as 10^{-12} g mL^{-1}. Later studies on more highly purified material were to confirm that very small amounts of the hormone were extremely active biologically.

It had been shown by Raper, in 1940(a), that the male thalli of *Achlya ambisexualis* reacted more strongly -- that is, produced antheridial filaments -- when placed in water where the female of *A. bisexualis* had been growing than in water from cultures of its own female counterpart. Further exploration into the male response was to indicate to Raper (1942b) that this hormone was part of a complex. Thus he postulated that there was a fifth hormone, which he designated as A prime. An analysis was to show that A^1 (Raper, 1950b) probably was produced by the male thallus. Further differential extractions and matings led Raper (1950a) to suppose that two additional hormones made up the hormone A complex. He interpreted increased male response by the *Achlya* to be a function of the male-secreted hormone A^1. Simultaneous with the liberation of hormone A, the female thallus provided hormone A^2, Raper said. However, he believed that A^1 augmented A^2. Concurrent with the production of A^1 by the male mycelium, another compound, A^3 differing from A^1 by its solubility characteristics was secreted by the male thallus. This fourth segment of the A complex, unlike the other three, was presumed to be inhibitory and in this sense functioned to limit the number of antheridial hyphae produced by the male mycelium. Thus, Raper (1951a) saw the hormone A complex to be constructed as shown in Table 38. Gooday

(1974) has pointed out, however, that it is the concentration of the hormone that actually determines the sexual response in the Achlyas: successively higher concentrations than those needed to elicit branching are necessary for chemotropism and antheridial cell delimitation (see also Barksdale, 1963b)

Other responses by the *Achlya* isolates came to light in Raper's membrane barrier experiments (1940a). In cultures where the male and female mycelium of *Achlya ambisexualis* were separated physically by a cellophane membrane, the antheridial branches contacting the membrane would delimit antheridial cells. The oogonial initials, on the other hand, proliferated unless contacted by an antheridial hypha (Raper, 1951a). For the antheridia, at least, antheridial cell formation appeared to require both a hormonal inciter and physical contact with a surface (J. R. Raper, 1952).

A summation of Raper's work (1957, 1970) necessarily would include the following concepts which he generated during his studies. Sexual apparatus regulation in the dioecious (heterothallic) Achlyas requires four male-secreted hormones, and three female-produced ones (Table 38). These provide a basic intercellular regulation consisting of six segments: (1) initiation (by the female thallus in the Achlyas); (2) differentiation (contributed by both sexes in the Achlyas); (3) sequential regulation; (4) spatial orientation (chemotropic response-hormone "C"); (5) quantitative control (provided by elements of the A complex), and (6) qualitative control.

Possible hormonal mechanisms in monoecious (homothallic) species of *Achlya* (*A. apiculata, A. americana, A. recurva, Achlya* spp.), and in *Thraustotheca clavata* and *T. primoachlya* also were sought by Raper (1950b). Of the 12 homothallic isolates he tested, three reacted as strong male thalli and nine as weak males when mated with the male or female of the two dioecious species, *A. bisexualis* and *A. ambisexualis*. Results from the various combinations of matings led Raper to conclude that monoecious species possessed hormonal coordinating mechanisms conforming at least in principle to the system possessed by the dioecious ones. Not unexpectedly, compatibility was expressed among the various matings in more than one degree of "intensity". Matings between *A. ambisexualis* and *A. americana* or *T. clavata* resulted in the production of hybrid oospores. Crosses such as those between *T. clavata* and the male counterparts of the two dioecious species exhibited partial compatibility, that is to say, at some point prior to oospore formation, the sexual process was interrupted. In still other matings -- as, for example, between *T. primoachlya* and the male or female thallus of *A. bisexualis* -- incompatibility was expressed.

ANTHERIDIOL AND ITS ACTION

Several years were to pass following Raper's active experimentation on the hormonal mechanisms in dioecious Achlyas before his work was again taken up, this time (1963a, b) by Barksdale. It had been discovered earlier that the concentration of hormone A in a medium in which both the male and female thalli had grown equaled that in the medium containing the "male" mycelium alone. It was evident that the male thalli of dioecious species actively removed the hormone from the medium. Barksdale

(1963a) prepared a stock solution of crude hormone A, and assayed its ability to induce antheridial filaments in terms of "units of A." Each unit was the smallest amount of hormone A in 1 mL of suspending liquid that would induce antheridial branch initials on male plants in two hours at 30 °C. The various isolates responded to different numbers of units of A. Of the six isolates Barksdale tested, five took up hormone A from the medium, and these were precisely the individuals that produced antheridial branches when mated with the compatible thalli. By the ingenious use of inert plastic material (simulated "oogonia") and resin particles coated with a given number of units of hormone A, Barksdale (1963b; 1967) demonstrated that this chemical excitant not only functioned to induce antheridial branches, but also to entice those branches to the oogonia and to insure the delimitation of antheridial cells. Raper's hormones A and C (Table 38), then, well could be one compound.

In a review article published in 1967, Barksdale reported that there were relative rather than absolute differences between *Achlya ambisexualis* and *A. bisexualis* with respect to their production of and sensitivity to hormone A which, she postulated, was a steroid. For example, the female of *A. bisexualis* secreted 3-10 times more hormone A than did the counterpart of *A. ambisexualis*. The male thallus of the former, on the other hand, was relatively insensitive to the hormone, while the male of *A. ambisexualis* was very sensitive. Moreover, the male plant of *A. bisexualis* behaved as a male only when mated with its own female; when mated with the male of *A. ambisexualis*, it functioned as a female. In *A. ambisexualis* a "range" of sexuality obviously existed, with the male at one extreme acting only as a male, and the female at the other acting only as a female. Thus, the dioecious species of *Achlya* are not strictly dioecious, although individual isolates may be so.

Hormone A was isolated by McMorris and Barksdale (1967) as a crystalline substance, and designated by the new name antheridiol. Filtrate from a growth medium was concentrated *in vacuo*, and active material extracted and further purified by chromatograph on silica gel, followed by counter-current distribution using four solvents. At very high dilutions this crystalline material induced antheridial branch formation in male strains of the Achlyas. It had already been shown by Barksdale *et al.* (1965) that the bicyclic sesquiterpenediol sirenin (Nutting *et al*., 1968) -- a male gamete attractant produced by the female gametes of species of *Allomyces* -- did not incite antheridial branches in species of *Achlya*, and furthermore that hormone A was unattractive, as it were, to the male gametes of an *Allomyces* hybrid. In any case, with a renewed interest there followed a flurry of experimentation with this sex hormone, and much information subsequently was obtained using two strains of *Achlya ambisexualis* in particular, E87 (♂) and 734 (♀), collected by Raper (1950b).

BRANCHING AND CELLULASE PRODUCTION

Since antheridiol was known to elicit antheridial hypha formation, the physiology of branching was among the first of the induced behavioral patterns to be investigated (Thomas, 1966; Mullins, 1968). By means of chemical isolation methods

and hyphal wall hydrolysis, Thomas (1966) explored the production of cellulase and its role in wall softening both in antheridiol-induced and noninduced mycelium of *Achlya ambisexualis*. The cellulase in the Achlyas, unlike that typical of fungi, is not induced by cellulosic substances, nor is it effective in degrading them. Thomas also found that vegetative and induced cellulase differed in certain extraction and thermostability properties. Exogenously applied antheridiol caused considerable cellulase production in the various male strains of *A. ambisexualis* and in *Achlya conspicua* (=*debaryana*), but only very slightly in *A. ambisexualis* 734 (♀). Either casein hydrolysate or a mixture of amino acids also induced branching in the male strain (E87) of the latter species. Mullins (1973) likewise reported this function for casein. It has been shown that cellulase also can be induced by shake culture (Thomas *et al*., 1974). In sum, antheridiol-induced branching unmistakably is dependent on cellulase activity, and there apparently is no branching in the male strains of dioecious Achlyas without a concomitant rise in the activity of this enzyme (Thomas, 1966). Indeed, Thomas and Mullins (1967), and Mullins (1968) demonstrated that the peak of cellulase activity in antheridiol-sensitive males coincided precisely with the time at which antheridial branch primordia appeared.

As would be expected, certain exogenous and endogenous factors effect the action of antheridiol. Thomas (1966), and Thomas and Mullins (1969) demonstrated that puromycin and *dl*-p-fluorophenylalanine inhibited cellulase production and branching in male test strains of dioecious Achlyas. If *dl*-phenylalanine was added simultaneously with the *dl*-p-fluorophenylalanine, the inhibitory effect of the latter compound was counteracted (Thomas and Mullins, 1969). By use of the carboxymethylcellulase (CMC) assay method, C. O. Warren and Sells (1971) tested the sensitivity of *Achlya ambisexualis* E87 to antheridiol in three stages of mycelial growth: early log, late log, and plateau (94-96 hours). Cellulase levels in the hyphae generally were stable throughout the log phase, but increased substantially during the plateau. Thus, age of the mycelium is a critical factor in the initial stages of antheridiol induction. Later, Mullins (1973) was to discover that when labeled amino acids were "fed"to antheridiol-sensitive hyphae, and cycloheximide then was added, cellulase production ceased. Inasmuch as one of the functions of cycloheximide is inhibiting amino acid incorporation into proteins, the cellulase that is produced in response to antheridiol appears to be the result of new protein synthesis. It has been demonstrated by Sullia (1977) that spore germination by *A. bisexualis* can be used as a bioassay for cycloheximide.

Achlya ambisexualis E87 (♂) was used by Thomas and his associates (1974) in a study of cytoplasmic streaming and cellulase production, the latter being determined by the CMC method. Colchicine and vinblastine (known to disrupt microtubule function) and cytochalasin A and B, were tested for their effect on enzyme production. Cytochalasin A inhibited cyclosis and cellulase activity, but streaming would commence again within thirty minutes if the mycelium was transferred to a medium free of this agent. Slight retardation or stimulation of cellulase production accompanied exposure of the colonies of the watermold to certain concentrations of cytochalasin B,

but this chemical, like colchicine and vinblastine, had no effect on cyclosis. The results of this study (Thomas *et al*., 1974) indicate that there is a coupling between cellulase secretion and cytoplasmic streaming. Additionally, it is possible that secretory protein synthesis also might be modified by cytochalasin treatment.

PROTEIN AND RNA SYNTHESIS

At what level does antheridiol exert its control over the sequential events of sexual reproduction? The immediately obvious levels, of course, are (among others) those of enzyme activation, nucleic acid metabolism, transcription, and the assembly of polysomes for protein synthesis and translation. The mechanisms involved in control were first explored by B. E. Kane (1971) using selective inhibitors on antheridiol-induced thalli of *Achlya ambisexualis*. He found that actinomycin D inhibited DNA-dependent RNA synthesis; 5-fluorouracil blocked DNA replication and prevented the formation of new ribosomes. The results of Kane's experiments suggest that there is but one site for branch induction. Actinomycin D simultaneously inhibited the transcription and antheridiol-stimulated production of cellulase. Since branching subsequently occurred if hyphae were washed free of the agent, it follows that the chemical either was not specific or did not affect the system operating for branch induction. Accordingly, Kane proposed that the influence of actinomycin D was on RNA transcription, and suggested that hormonal control of cellulase activity (necessary for branching) was exercised at this level. Fluorouracil evidently has no influence on antheridial branch initiation in *A. ambisexualis*, and there is no evidence (deoxycholate treatment) of polysomes (Kane, 1971) in this species. From the study of *A. ambisexualis* by Horowitz and Russell (1974), it is now known that continued RNA synthesis is necessary for antheridial branch elongation.

Acting on the premise that sex hormone control in the Achlyas could take effect either at transcription or translation, Reiskind (1970) studied protein synthesis in antheridiol induction of branching in *Achlya ambisexualis*. She grew isolates on a chemically defined, complete medium, and analyzed protein synthesis by the use of inhibitors -- cycloheximide, and the analog of phenylalanine, *p*-fluorophenylalanine -- and by incorporation of ^{14}C phenylalanine into branch-induced, branch-inhibited, and vegetative mycelium. Cycloheximide inhibited both induced branching and protein synthesis. The effect of cycloheximide (it influences more than protein synthesis, of course) was not permanent since mycelium of the male strain of *A. ambisexualis* could recover, in time, to complete the sex process even if the colonies were not removed from the presence of the agent. Reiskind's (1970) work was essentially confirmed by B. E. Kane *et al.* (1973). Actinomycin D blocked incorporation of uridine into RNA and concomitantly prevented antheridial hyphae from forming in the male strain (E87). Cycloheximide prevented amino acid assembly into protein and obstructed the effect of antheridiol. Kane and his associates concluded that both transcription and translation are required for hormonal induction by this *Achlya*.

Groner *et al*. (1976) used a double labeling method (^{3}H-leucine and ^{14}C-leucine) to demonstrate that a major protein -- of approximately 69,000 daltons -- was made preferentially by antheridiol-induced males of *A. ambisexualis*. This protein was synthesized within the first 30-60 minutes after the addition of the hormone to the culture, and preceded the appearance of the antheridial branch initials. The fraction in which this protein occurred decreased about three hours after the mycelium had been exposed to antheridiol.

Rozek and Timberlake (1980) investigated experimentally the mRNA sequences during antheridial branch differentiation in antheridiol-induced mycelium of the male strain of *Achlya ambisexualis*. They found that the administration of antheridiol did not cause any major alterations in the spectrum of mRNA sequences transcribed and accumulated during differentiation. Changes in gene activity in induced branching thus appear to be subtle rather than major ones. It is obvious that the full biochemical role of antheridiol is not yet fully understood.

The effect of antheridiol on transcription in *Achlya ambisexualis* has been explored extensively by Sutherland and Horgen (1977). They used a rifampicin (antibiotic) challenge assay method, and measured the number of RNA polymerase binding and initiation sites on isolated chromatin. Antheridiol increased chromatin template activity, chromatin-bound RNA polymerase, and rifampicin-resistant initiation sites in this watermold. At the transcriptional level, then, the regulatory capacity of antheridiol is effected by increasing the number of RNA polymerase initiation sites on the nuclear chromatin. It is known that in animal tissues steroid-receptor interactions occur, and some experiments by Horgen (1977b) suggest that in the Achlyas there is a similar mechanism operating. When cytosol (centrifuged from hyphal homogenates) from the male thallus of *A. ambisexualis* was coupled with antheridiol and the chromatin preincubated in this mix, heterologous polymerase transcription by isolated chromatin was increased dramatically over that measured in chromatin preparations with antheridiol or cytosol alone. Cytosol from the female mycelium of the same species added to antheridiol and mixed with an isolated chromatin preparation did not stimulate transcription.

Silver and Horgen (1974) isolated a poly (A)-rich fraction of cellular RNA (polyadenylic acid at the 3' ends of the RNA molecule) in *Achlya ambisexualis* and found that antheridiol had a noticeable effect on the accumulation of this compound. After an initial lag period, antheridiol applied to the mycelium of the fungus stimulated a three or four-fold increase in the endogenous poly (A)-rich fraction, and this rise coincided with the appearance of branch initials on the hyphae. Silver and Horgen suggested that antheridiol initiated the synthesis of this poly (A)-rich compound which was then translated into the proteins required for the differentiation of a male filament. They pointed out also that the poly (A) segment of the mRNA molecule in the Achlyas is smaller than that in animals, but comparable to that of yeasts.

From precipitated RNA, stimulated by exposing hyphae of *Achlya ambisexualis* E87 (♂) to antheridiol, Horgen, Smith, and Silver, and Craig (1975) and Horgen, Smith, and Silver (1975) studied the pathway of ribosomal RNA synthesis with actinomycin D

chase experiments. An initial high molecular weight rRNA precursor cleaves and matures into 26S and 186 rRNA. In the presence of antheridiol the synthesis of these rRNA precursors was enhanced 3-5 times over that in noninduced mycelium, and the total rRNA likewise was increased. Hence, one of the primary macromolecular changes brought about by steroid (antheridiol)-induced differentiation appears to be a rise in the number of ribosomes (as RNA synthesis totally is augmented). Ribosomal protein is known to increase in *A. ambisexualis* E87 in response to hormone stimulation, as are levels of cellular proteins. There is at least a suggestion in the study of this species by Michalski (1978) that antheridiol induces some -- perhaps only minor -- alterations in gene expression.

There is indirect evidence with the sensitive Achlyas that antheridiol functions in RNA and protein synthesis (Michalski, 1978, and others), but is its involvement of long- or short-term duration? Gwynne and Brandhorst (1980) concluded that the responses to antheridiol were very subtle. They found that antheridiol-induced differentiation was accompanied only by minor transcriptional and translational changes, if any, and that post-translational regulatory events may be required for differentiation. Induced antheridial branch development in *A. ambisexualis*, Timberlake (1976) has shown, follows a temporal sequence: the first branches appear on the male strains of the species 60-90 minutes after the mycelium is exposed to antheridiol; by 300 minutes, 90-100% of the hyphae show morphological evidence of antheridial branching. Most of the necessary RNA synthesis is completed by the time the morphological changes (branch development) appear, but there is continued transcription and translation (Timberlake, 1976) after this event.

As the hormone-sensitive isolates of *Achlya* respond to antheridiol, a precedential modification in chromosomal protein structure takes place involving acetylation of particular basic nuclear proteins. It is possible, or course, that histone acetylation is one modifying mechanism, and is associated with the activation of new genes during differentiation in response to hormonal stimulus. This being so, the dioecious Achlyas should possess histones. Horgen *et al*. (1973) demonstrated that both *A. bisexualis* and *A. ambisexualis* have intranuclear histone-like nuclear proteins. A reasonable assumption is that these Achlyas have basic chromosomal proteins with some characteristics in common with histones of higher plants and animals, and Silver (1979b) has demonstrated by electrophoretic mobilities that this is indeed the case. In *A. ambisexualis*, there are acid-extractable nuclear proteins having molecular weights and charge properties similar to histones H3 and H4 from plant or mammalian tissue. This same fungus also has two other basic nuclear proteins. One of these, α, migrates on dodecyl sulfate gels between H2A and H4. This protein is not found in acid/urea gels, but a second one, β, migrates in a region similar to plant histones H2A and H2B. Both *A. ambisexualis* and *Saprolegnia ferax* show nuclear protein bands on acid/urea gels that are more like the H1 histones of rye plants than the H1 histones of rabbit.

Several other events are associated with hormone-induced differentiation in the dioecious watermolds. In addition to cellulase induction and activity in hormone-sensitive specimens oxygen uptake is accelerated, and there also are responses in RNA

and protein synthesis (Barksdale, 1969; Gooday, 1974; Mullins, 1968). Glucan metabolism also is influenced by hormonal control in *Achlya ambisexualis* and *A. heterosexualis* (Faro, 1972b). Very shortly after the appearance of antheridiol, glucan consumption exceeds synthesis, but when branching subsequently is initiated, the rate of manufacture increases, and this compound accumulates. The main events under hormonal control occur in a time-ordered sequence (Fig. 49) such that in the final analysis it is gene activation and gene product accumulation (Horgen, 1977a) that are affected by "hormone A."

THE PRODUCTION OF ANTHERIDIOL

The culture environment -- exogenous factors such as pH, temperature, and nutrition -- influence the hormonally induced sex act in the Achlyas. In a paper published in 1942(a) J. R. Raper reported that a pH of 6.0 was optimum for a mating reaction to occur in dioecious strains, and the most intense reaction appeared when mating cultures were incubated at 23-28 ^{o}C. He found there was no reaction at 37 ^{o}C (although the isolates would grow at this temperature), but if mated cultures were brought to a lower temperature, the reaction commenced. Medium constituency undeniably influences strongly the intensity of the mating reaction, as Barksdale (1962a) demonstrated.

The most extensive work on the relationship between the growth medium and sex hormone activity was that reported by Barksdale in 1970, who experimented with the effect of crude and refined antheridiol on *Achlya ambisexualis*. The tests were carried out on a basic mineral salts medium (with and without antheridiol) to which substrates could be added. The results were expressed in terms of numbers of branches (20-50 μm long) produced by the male strain of *A. ambisexualis* in the various nutrient solutions. Barksdale found that the maximum number of antheridial initials developed on mycelium grown in the basal medium with glucose and a hydrolysate of lactalbumin plus 2.5 ng of antheridiol mL^{-1}. Few branches were produced by *A. ambisexualis* when glucose was omitted from the medium, and fewer still when the hydrolysate was absent. In addition, the hormone concentration coupled with that of constituents in the medium actually determined the number of branches that would form. Cysteine·HCl was the most effective carbon and nitrogen source of five compounds tested, and it was the nitrogen supply which appeared to be the key to the degree of hormonal activity. Antheridiol-induced branches on mycelium grown in the highest levels of nitrogen used (Barksdale, 1970) were analogous to vegetative filaments, but when the nitrogen content of the medium was lowered, the induced branches functioned as antheridial hyphae.

The study by Mullins and Warren (1975) yielded nutritional influence determinations somewhat at variance with those reported by Barksdale (1970), even though the isolates used (subcultures of *Achlya ambisexualis*) were the same in both investigations. In the absence of exogenous nutrients the male isolates began forming antheridial branch initials about two hours after mating. If the medium contained low

levels of nutrients, branch initiation was retarded, and increasing the nutrient simply further delayed induction.

One of the unique aspects of antheridiol that has been reported is its ability to induce the fungus to metabolize this steroid. Musgrave and Nieuwenhuis (1975) fed radioactive antheridiol (in what appears to have been extraordinarily high concentrations) to dioecious and monoecious species of *Achlya* in the presence of cycloheximide and actinomycin D (inhibitors of protein and RNA synthesis, among other effects). They found that some isolates could convert antheridiol to two metabolites, designated simply as A and B, recognizable by their appearance on chromatograms. Any *Achlya* strain capable of functioning readily as a male was induced by antheridiol to metabolize that steroid; strong females were inert. The monoecious *A. conspicua*, for example, was sensitive to the branch-induced capacity of antheridiol and also metabolized it. The ability of the Achlyas to metabolize antheridiol, these investigators concluded, contributes to the establishment of a gradient that, in turn, may amplify the chemotropic signal (antheridial branch attraction). Musgrave, Ero, and Scheffer (1978) set about to determine if metabolism of the sex hormone is actually antheridiol-specific. They experimented with eight species of Saprolegniaceae, and three labeled steroids, ^{3}H-antheridiol, 7-deoxy-antheridiol-3-acetate (an isomer), and progesterone. Neither of the latter two compounds induced its own metabolism in any of the watermolds tested, because these steroids were degraded by the test fungi without any lag time. *Thraustotheca clavata*, *Dictyuchus sterile* (excluded name; *see* systematic account), *Aphanomyces cladogamous* and *Saprolegnia ferax* metabolized the steroids without delay, and furthermore, this act was insensitive to cycloheximide. Neither *S. parasitica* (=*diclina*) nor *Isoachlya* (=S*aprolegnia*) *unispora* were able to metabolize antheridiol to any major degree. Musgrave and his associates concluded that self-induced metabolic inactivation of antheridiol is a natural hormonal effect inherent in those species capable of responding sexually to antheridiol. The role, if any, of oogoniol in this self-destruct phenomenon is yet to be determined.

Do other compounds mimic the inductive capacity of antheridiol? It was early shown by Raper (1942a) that certain chemicals do in fact elicit a "hormone A" reaction in male thalli, but the magnitude of the response is slight. Among the many compounds tested, Raper found that representative saturated dicarboxylic acids (malonic, glutaric, pimelic) and barbituric acids and related compounds were effective to some degree.

The mycelium of *Achlya bisexualis* contains a mixture of sterols with 27-, 28-, and 29-carbon skeletons-24-methylene cholesterol (C_{28}), and 7-dehydrofucosterol (C_{29}), for example (Popplestone and Unrau, 1973a) with fucosterol as a major component (Popplestone and Unrau, 1973b). Whether *A. ambisexualis* is similarly equipped, has not been discovered but the reaction of this species to exogenously supplied steroids certainly has been explored thoroughly. Barksdale, *et al*. (1974) exposed mycelial mats of the male thallus to preparations of 53 steroids. Thirty-four compounds, including such familiar ones as esterone, cortisone, stigmasterol, progesterone, and cholesterol, were inactive. The C_{22} and C_{23} stereoisomers of antheridiol showed an activity in inducing

branching in the male strains at a level about 0.1-0.0001% of that of antheridiol. The *erythro* (22R, 23S) and *threo* (22S, 23R) isomers of antheridiol were also active, but much less so than antheridiol itself.

THE STEREOCHEMISTRY OF ANTHERIDIOL

There seemed little doubt from the earliest studies on reactions in the dioecious Achlyas that control of the sexual response was not unlike that effected by steroid sex hormones in other biological systems. Credit for the discovery of such hormones in aquatic fungi must go to Bishop, who, in his thesis of 1937 reported these in *Sapromyces reinschii* [=*S. elongatus* (Cornu) Coker]. The substance of Bishop's work did not appear in print until 1940, and in the meantime J. R. Raper (1939a, b) had reported sex hormones in the Achlyas.

It was J. R. Raper and Haagen-Smit (1942) who characterized a crude filtrate containing hormone A, and in 1967, McMorris and Barksdale reported isolation from *Achlya bisexualis* of a compound that achieved the stimulatory properties of this hormone. Deducing largely from spectroscopic analyses, Arsenault, *et al*. (1968) proposed that crystalline antheridol had an elemental composition of $C_{29}H_{42}0_5$; its structure is shown in Figure 50. A year later, Edwards and associates (1969) synthesized a steroid which in its physical and biological properties was virtually identical to natural antheridiol. They concluded that the hormone must possess either a 22*R*, 23*S*, or a 22*S*, 23*R* configuration. This was not the first structurally-characterized fungal sex hormone; that distinction belongs to sirenin, reported by Machlis, *et al.* in 1968.

In 1971, D. M. Green and associates reported the isolation of 23-deoxyantheridiol from culture filtrates, and were able to synthesize the 22-isomer. On the basis of this analysis, they proposed that the stereochemistry of antheridiol was 22*S*, 23*R* (22βF, 23βF) and this was confirmed in 1972 by Edwards and his associates. They prepared the four 22, 23-isomers of an intermediate, and obtained synthetic antheridiol by photo-oxygenation of the 22*S*, 23*R*-butenolide intermediate followed by treatment of the resulting crude Δ^6-5α-hydroperoxide with $Cu(OAc)_2$. Stereo-selective synthesis of (22*R*)-3β, 22-dihydroxy-7-oxostigmasta-5, 24(28)-dien-29-oic acid δ lactone was reported in 1978 by Weihe and McMorris. This synthetic 23-deoxyantheridiol had the same structural properties as the natural product, but was less active biologically (possibly was contaminated with antheridiol). It is possible (Weihe and McMorris, 1978) that 23-deoxyantheridiol is a biosynthetic precursor of antheridiol.

Various isomers of antheridiol have been characterized. In 1970, McMorris synthesized 3β–acetoxy-22, 25-dihyroxy-5, 24 (28)- stigmastadien-29-oic acid γ-lactone, and also a biologically inactive 7-keto analog isomeric with antheridiol. From a C_{22} aldehyde, McMorris *et al*. (1972, 1974) synthesized stereochemically pure antheridiol in a yield of approximately 20%. The three 22, 23-isomers of antheridiol also were obtained. An epimeric mixture of antheridiol was developed by McMorris and Seshadri (1971), and McMorris (1970) synthesized an isomer of antheridiol with the γ-

lactone ring at C-24 instead of at the C-23 position. Popplestone and Unrau (1973b) prepared a structural isomer, (22*R*, 23*S*)-3β22, 23-trihydroxy-7-oxostigmasta-5,24(28)-dien-29-oic acid δ-lactone. By reduction of 22-oxo-23-hydroxy γ-lactone followed by hydrolysis, photooxygenation, and cupric chloride-catalyzed rearrangement, McMorris and Arunachalam (1975) succeeded in assembling antheridiol-(22, 23-^{3}H), a radioactive compound of high specific biological activity.

Popplestone and Unrau (1973b) identified fucosterol as the major naturally occurring sterol in *Achlya bisexualis*. Tracer study demonstrated to them (Popplestone and Unrau, 1974) that this steroid could, in fact, serve as the necessary carbon skeleton for the biosynthesis of antheridiol. Enzymatic conversion of fucosterol to antheridiol would require four general transformations, and through these the sexually active hormone could be elaborated: oxidation of fucosterol in the B ring at the C_7 position; hydroxylation or oxidation at C_{22} and C_{23}; oxidation at C_{29}, and lactone formation.

ULTRASTRUCTURAL BASIS FOR ANTHERIDIOL INDUCTION

In 1974, two papers recorded observations on some ultrastructural aspects of the effect of antheridiol activity in the E87 strain of *Achlya ambisexualis*. Nolan and Bal (1974) found that electron-dense reaction bodies appeared in dictyosomes and vesicles surrounding unidentified storage bodies (or in the space between the plasmalemma and hyphal wall) in induced, cellulase-exposed preparations. They concluded that synthesis or activation of cellulase (triggered by antheridiol) took place within the dictyosomes, that the enzyme was then transported to the plasmalemma, and there released to the region between the membrane and the wall. Mullins and Ellis (1974) saw that hormone-induced vesicle production and aggregation at loci along the hyphal wall coincided with a visible thinning of the wall. These vesicles appeared about two hours after the hyphae were treated with antheridiol, and, at the time that branching took place, the apex of each branch was thin-walled. Three events then, seem to be coordinated in the early stage of sexual development, namely, cellulase induction, vesicle development (migration?), and thinning of the wall. On the basis of the occurrence of these events, Mullins and Ellis assumed that the cellulase was located in the migrating and coalescing vesicles at the site of the plasmalemma.

INTERTHALLIC REACTIONS WITH ANTHERIDIOL

As early as 1950(b), J. R. Raper had reported experimental attempts to determine if monoecious representatives of the Sarolegniaceae responded to "hormone A". One species that did (in terms of mating) was *Thraustotheca clavata*; Musgrave and his collaborators (1978) have confirmed this response. Barksdale (1960) explored widely the inter- and intraspecific matings among the dioecious watermolds -- and some monoecious ones as well -- propagating 29 strains (isolates) in pairs in all possible combinations. She detected three "degrees" of sex reaction. In some matings, sexual reproduction proceeded through to oospore development. In others, the sexual reaction

terminated when the antheridial branches reached the oogonial initials; no antheridial cells or oospheres developed. Finally, there were matings in which the antheridial branches from one of the juxtaposed pair grew and wrapped about the hyphae of the other, but without those hyphae having formed oogonial initials. The numerous attempts at matings did in fact show that some monoecious (homothallic) species are potentially dioecious in that they participate in matings with dioecious strains. Barksdale's (1960) study indicates that there are at least twelve sexual interaction types in the Achlyas: one male type expressed by *A. flagellata* (=*debaryana*) and *A. caroliniana*; one male type found only in *A. americana, A. conspicua*, and a male strain of *A. ambisexualis*; four male types expressed only by the dioecious (heterothallic) isolates; two female types found only in *A. ambisexualis*, and four female types represented exclusively by *A. bisexualis*. In sum, both dioecious and monoecious species of watermolds exhibit variation in maleness and femaleness. At one extreme (Barksdale, 1960) are the dioecious strains with a strong propensity for interthallic mating; at the other, the monoecious extreme, the species tend toward consistent self-conjugation. Mutational-directed differences in sensitivity to hormone A, Barksdale suggested, might have established heterothallic species from homothallic parental stock. A mutation exceptionally sensitive to the sex hormone would be the one most likely to produce the antheridial initials, and thus be established as a male strain.

Four of seven strains of a species named *Achlya heterosexualis* (identified as *A. klebsiana* B4 by Barksdale, 1960) were shown by Barksdale (1965) to conjugate to some degree with the dioecious species, that is, some strains functioned either as oogonial or antheridial thalli when mated, but only the antheridial branches were cross-induced. Degrees of sensitivity to antheridiol also exists among the strains of *A. heterosexualis*. In analyzing the reactivity of *A. heterosexualis* and the two dioecious species, Barksdale emphasized that taken together the three taxa constituted a distinct gradient with respect to antheridiol. At one extreme was *A. heterosexualis*, generally having low hormone productivity but high sensitivity to it, and at the other, *A. bisexualis*, a species with generous hormone production and low sensitivity.

OOGONIOL

In 1939(a, b), Raper predicted that the female thalli of cross-conjugating species of *Achlya* responded (by forming oogonial initials) to a diffusing chemical substance, hormone B, elaborated by the activated male thallus. Barksdale (1967) thought that this male-secreted hormone might also trigger septum formation to delimit the oogonial initial from its stalk. Using culture liquids assayed for hormones A and B, Barksdale and Lasure (1973) uncovered an apparent correlation between mating phenotypes and response to the two sex incitants. Certain strains of the species they tested, reacting as females, secreted antheridiol but no B hormone. Other strains, functioning as males, produced little antheridiol, but secreted B only when stimulated by exogenous antheridiol. Finally, hermaphroditic strains of *Achlya heterosexualis* were found that could secrete the oogonium-inducing hormone in the absence of exogenous antheridiol.

The structure and biosynthesis of oogoniol has by no means been explored as fully as that of antheridiol. The production of hormone B (oogoniol) was reported by Barksdale and Lasure, in 1974. They tested antheridiol, its three isomers (at C22, C23), 7-deoxy 7-dihydro antheridiol, and fucosterol for their ability to stimulate oogonial production. Only the natural product and the deoxy-dihydro analogue induced the test fungus, *Achlya heterosexualis*, to secrete more oogoniol than the noninduced controls. Fucosterol inhibited secretion of the hormone, and also suppressed oogonial initials in *A. ambisexualis*.

Subsequently, McMorris and collaborators (1975) succeeded in obtaining two crystalline compounds and one noncrystalline one from extracts of a culture liquid supporting *Achlya heterosexualis*. These were designated as oogoniol 1, 2, and 3, and were found to have structures which differed only in the kind of ester group attached at C_3 of the steroid nucleus (Fig. 50). In a later study of oogoniols by means of a ^{13}C nuclear magnetic resonance spectrum analysis, McMorris and his associates (1978) corrected an earlier supposition (McMorris *et al*., 1975) on a portion of the structures of these compounds. They found that the primary hydroxyl was located at the C-29 position rather than at C-26.

McMorris and White (1977) defined the late stages in the biosynthesis of oogoniol by *Achlya heterosexualis* and *A. ambisexualis* (E87). The producing cultures were grown in the presence of labeled sitosterol and its 24-epimer clionasterol; fucosterol was also used. *Achlya heterosexualis* synthesized oogoniol-1 and -2 (radioactive) when supplied with each of these steroids, although fucosterol was incorporated to a much greater extent than the others. The investigators conclude that fucosterol, via two pathways -- either to antheridiol or to the three oogoniols -- was the basic compound from which both the male and female steroids in *Achlya* species were assembled. In 1978, R. H. White and McMorris presented evidence from [CD_3]-methionine experiments that fucosterol is converted to a C-29 aldehyde and then reduced to C-29 hydroxyl in the assembly of oogoniol. Further experimental work by Preus and McMorris (1979) resulted in isolation and identification of the 24(28)-dehydro analogue of oogoniol-1 [3β, 11α, 15β, 29-tetrahydroxostigmasta-5, 24(28)(*E*)-dien-7-one, 3β-isobutyrate] which has a fucosterol skeleton. Moreover, they reported evidence for additional polyhydroxy-7-keto-fucosterol derivatives in culture liquids in which the *Achlya* isolate they used had been grown.

Further insight into the structural requirements for the biological activity of steroids related to oogoniol was provided by E. J. Taylor and Djerassi (1977). Starting with 7-dehydrocholesterol benzoate, they attempted to construct parent oogoniol, a tetraol. They succeeded in producing a compound which, when saponified, yielded chloest-5-ene-3β-triol-7-one that had the nuclear functionality of oogoniol. This compound, however, showed no activity when bioassayed. It appears that identical nuclear structure is not enough to give biological activity; a hydrated side chain is evidently essential (E. J. Taylor and Djerassi, 1977) as well. The 24 (28)-dehydro analogue of oogoniol has a higher activity level than oogoniol-1 when applied to the female strain of *A. ambisexualis*. Preus and McMorris (1979) therefore have suggested

that the dehydroogoniols are the true hormonal excitants in the Achlyas. When these compounds are metabolized to the saturated analogues much of their biological activity is lost.

SUMMARY

There is in some *Achlya* species a capacity to produce at least two hormones (pheromones), antheridiol and oogoniol, and a propensity to react to them. A gradation of these tendencies can exist such that thalli act as strong or total males, as neuters (act as male or female to a strong female or male), or as total females. In the dioecious species, certainly, the known sexual process is triggered when the female phenotype secretes antheridiol to which it is insensitive; it does not produce oogoniol to which it is sensitive. The male phenotype, conversely, forms little or no antheridiol, but is sensitive to this steroid, and under its excitation produces oogoniol, to which it is insensitive. Presumably there are appropriate receptor sites to insure that the thalli receive the proper signal for interacting in a sequential fashion, but these have not been discovered (Bu'Lock, 1976).

The known hormones in the Achlyas trigger a variety of biochemical processes. These processes are linked, even if only coincidentally, to the phenotypic expression of sexual activation, that is, to the appearance of antheridial branches and oogonial initials. Among the hormone-linked events are cellulase induction (and perhaps vesicular transport to sites of activation), protein synthesis, transcription and translation, and glucan metabolism. Still to be uncovered and analyzed are the hormonal mechanisms (if such exist) that function in the latter stages of sex cell morphogenesis and mating: septum formation, oosphere cleavage, and fertilization tube development and orientation. Whether Raper was correct in supposing activity of the yet to be discovered hormones C and D, or whether A or B do in fact elicit further responses beyond those known to be attributable to them is a problem calling for attention.