PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Antheridiol and the Oogoniols, Steroid Hormones Which Control Sexual Reproduction in Achlya [and Discussion]

T. C. McMorris, D. Klambt and G. W. Gooday

Phil. Trans. R. Soc. Lond. B 1978 **284**, 459-470
doi: 10.1098/rstb.1978.0082

Phil. Trans. R. Soc. Lond. B. **284**, 459–470 (1978)
Printed in Great Britain

Antheridiol and the oogoniols, steroid hormones which control sexual reproduction in *Achlya*

By T. C. McMorris
Department of Chemistry, University of California, San Diego, La Jolla, California 92093, *U.S.A.*

[Plate 1]

The sexual reproductive process in *Achlya*, a widely distributed genus of saprophytic aquatic fungi, is initiated and coordinated by the steroid hormones, antheridiol and the oogoniols. The discovery of these hormones, and their isolation and structural identification, are reviewed in this paper. *Achlya* contains a number of sterols, the major one being fucosterol, which serves as the biosynthetic precursor of antheridiol and the oogoniols. The biosynthesis of the hormones is discussed as well as some of their specific functions in the organism.

The function of sterols in plants is a subject which has interested scientists probably from the time steroids were first found to possess important hormonal functions in animals. Sterols such as sitosterol are of very widespread occurrence in plants and the question naturally arises whether sitosterol and other sterols have functions in plants similar to those of cholesterol in animals. Cholesterol is the source of steroid hormones, androgens and oestrogens, which are formed by oxidative reactions of the cholesterol molecule.

It is now evident that in one plant organism at least, a situation similar to that in animals does exist. The aquatic fungus *Achlya* has as its major sterol component the well known fucosterol. This can serve as the biosynthetic precursor of the steroid hormones antheridiol and the oogoniols which control sexual reproduction in *Achlya*.

I should like in this paper to say something about the discovery of these hormones, their isolation and identification and their biosynthesis. I shall then discuss some of the specific functions of antheridiol in the organism.

Discovery of hormones and their subsequent isolation

In a series of classical experiments carried out about 40 years ago, John Raper established that sexual reproduction in this filamentous fungus is initiated by a diffusible substance secreted into the surrounding water by the female mycelium (Raper 1939, 1940). The substance was named hormone A. In response to the hormone the male hyphae produce specialized branches which later develop into antheridia or male sex organs. The antheridial branch is sinuous and narrower than the vegetative hypha and is thus readily distinguished.

Raper also discovered that hormone A induces the male to secrete another hormone (hormone B) which causes the female to produce oogonia or female sex organs. These have a very distinctive spherical shape. With appearance of the young oogonium, the antheridial hyphae grow toward it and envelop it. Crosswalls are formed near the antheridial tips and the resulting delimited cells are the antheridia. At about the same time a crosswall is formed at the base of the

oogonium. Within this cell, protoplasm reorganizes to form between 1 and 20 mononucleate eggs. Fertilization is accomplished by passage of male nuclei through fertilization tubes that extend from the antheridium to each egg or oosphere. Meiosis is believed to take place in the antheridium and the oogonium, and the fertilized egg or oospore is thus diploid (Barksdale 1968). On germination the thick walled resistant oospore develops into the hypha of a new plant.

Early attempts to isolate hormone A were made by Raper & Haagen-Smit (1942). This was perhaps too formidable a task at the time in view of the minute amount of hormone secreted by the fungus and the unavailability of modern chromatographic methods. Nevertheless they were able by solvent partition and selective adsorption to achieve considerable purification of the hormone. In 1966 Professor Raper supplied me with samples of his most purified extracts. Thin layer chromatography showed them to contain antheridiol, 23-deoxyantheridiol and other unidentified components.

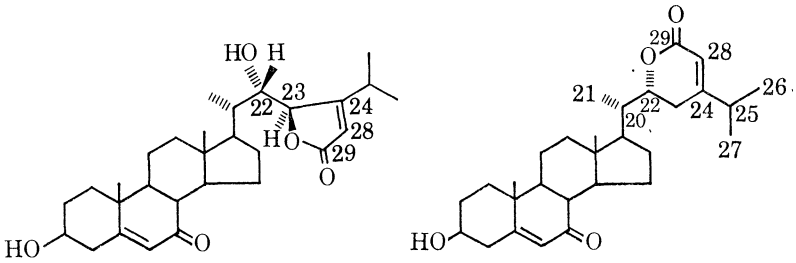

FIGURE 2.

Studies on the isolation of hormone A by Alma Barksdale began in 1956 at the New York Botanical Garden. In 1964 I suggested to her that I attempt isolation of the hormone. She had been growing *Achlya* on a large scale and had developed a sensitive bioassay for detection of the hormone.

Crude extracts of culture liquids of *Achlya bisexualis* were thus chromatographed on silica gel with ethyl acetate as solvent followed by thin layer chromatography with chloroform–methanol. Isolation of the hormone was guided by bioassay. In this way about 2 mg of crystalline hormone were isolated from 85 l of culture liquid in 1965 (McMorris & Barksdale 1967). The pure hormone, which was renamed antheridiol, was found to induce branching in *A. ambisexualis* E87 at a concentration as low as 10 pg/ml.

The structural elucidation of antheridiol was a collaborative effort with Klaus Biemann and Guy Arsenault of the Massachusetts Institute of Technology. They provided high resolution mass spectrometric evidence which, together with other spectral data on antheridiol and its derivatives, enabled us to propose a structure for the hormone (Arsenault *et al.* 1968).

The structure was soon confirmed by a synthesis carried out by Edwards *et al.* (1969) at the Syntex Corporation, California. The stereochemistry of the side chain was not defined though they were able to show that it had the *erythro* rather than *threo* configuration at C-22, C-23.

Culture liquids of female strains of *Achlya* were found to contain another steroid, 23-deoxyantheridiol, which proved to be closely related to antheridiol. The structure was confirmed by synthesis of the C-22 epimer by Green *et al.* (1971). The stereochemistry at C-22 was established by comparison of the circular dichroism curve of 23-deoxyantheridiol with those of lactones of established absolute configuration. This allowed the absolute configuration of antheridiol to be

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

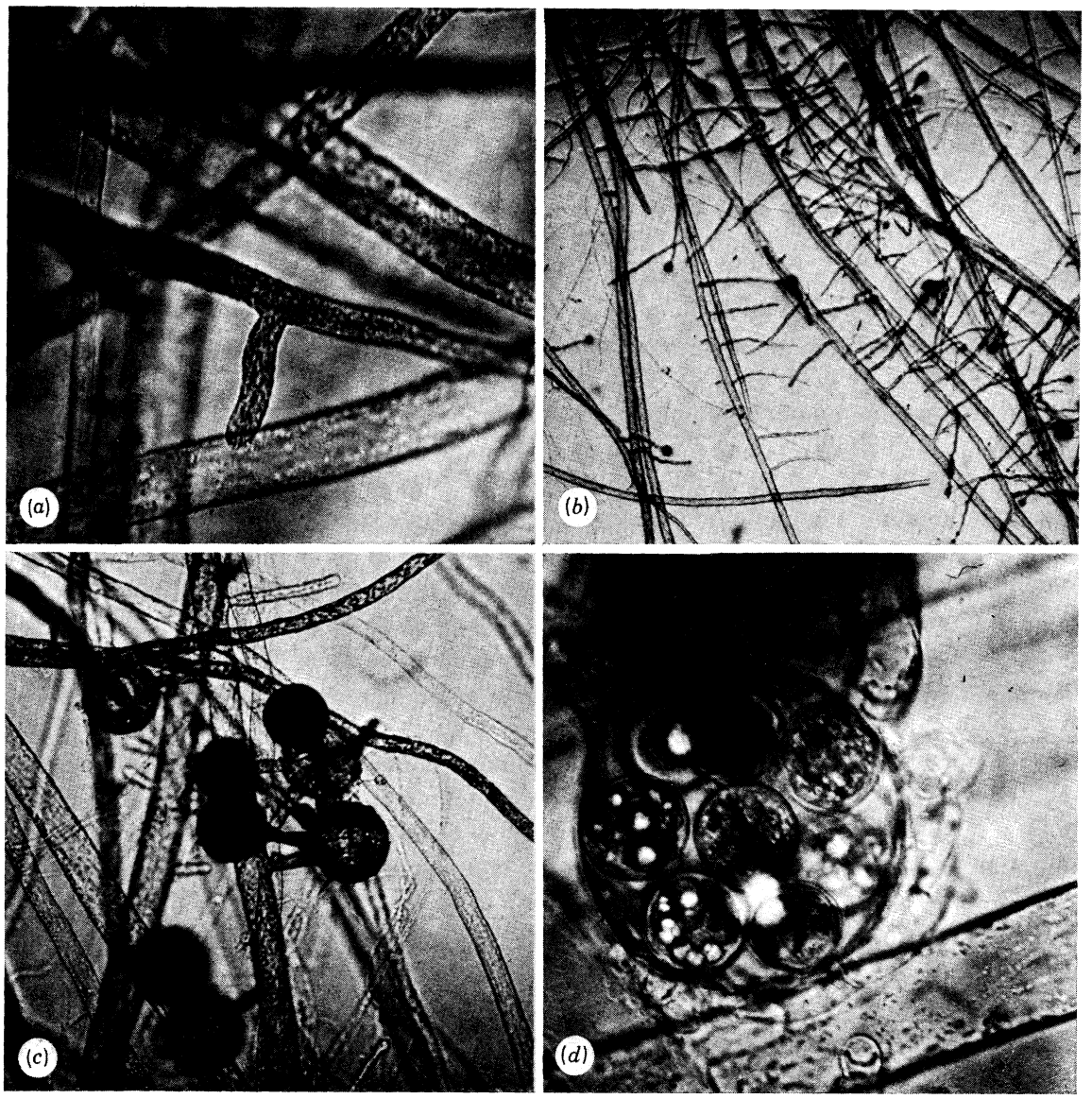

FIGURE 1. Hyphae and sex organs in *Achlya ambisexualis*. (*a*) Vegetative hyphae (magn. × 300); (*b*) hyphae with lateral antheridial branches initiated by antheridiol (magn. × 40); (*c*) oogonial initials initiated by the oogoniols (magn. × 100); (*d*) oogonium containing eggs (magn. × 700).

(*Facing p.* 460)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

assigned as 22*S*, 23*R*. In later synthetic studies of antheridiol by Edwards *et al.* (1972) the stereochemistry was confirmed.

We ourselves have developed a synthesis of antheridiol which gives the hormone in reasonable yield (McMorris & Seshadri 1971; McMorris *et al.* 1972, 1974) and we have used the method to prepare labelled hormone ([22-^{3}H, 23-^{3}H]antheridiol) (McMorris & Arunachalam 1975). We have recently completed a synthesis of 23-deoxyantheridiol also and have thus been able to show that this steroid will induce branching in *A. ambisexualis* E87 though not at as low a concentration as antheridiol (Weihe & McMorris 1978).

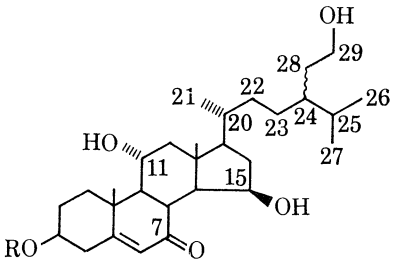

FIGURE 3. The structure of oogoniols: for oogoniol-1, R = $(CH_3)_2CHCO$; for oogoniol-2, R = CH_3CH_2CO; for oogoniol-3, R = CH_3CO; for oogoniol, R = H.

Hormone B, the second hormone discovered by Raper, is secreted by male strains of *Achlya* on stimulation by antheridiol. In our first attempts at isolation of this hormone, antheridiol was added to cultures of the male strain *A. ambisexualis* E87 and the culture liquids later examined for the presence of hormone B. Barksdale had developed an assay for hormone B and it indicated that very small amounts of hormone were being produced. Chromatography of active extracts showed that hormone B was slightly less polar than antheridiol and mass spectral data suggested that it might be a steroid.

Progress on the structural elucidation was very slow because of the small quantities of hormone available. The situation improved somewhat when Barksdale discovered that *A. heterosexualis*, a hermaphroditic strain, secretes hormone B without prior stimulation by exogenous antheridiol (Barksdale & Lasure 1973, 1974). Chromatography of methylene chloride extracts of culture liquids of this strain gave a biologically active crystalline fraction. The yield was roughly 1 mg from 20 l of culture liquid, i.e. about the same order of magnitude as the yield of antheridiol.

The crystalline material was resolved by thin layer chromatography into three steroidal components, and non-steroidal lipid material. Two of the components were eventually obtained as sharply melting compounds and named oogoniol-1 and 2 (McMorris *et al.* 1975). Structural elucidation of the compounds involved mainly interpretation of spectral data. Mass spectra indicated closely related steroidal skeletons differing only in an ester function at C-3. The propionate and isobutyrate moieties were confirmed by hydrolysis of oogoniol-2 and 1 with methanolic potassium carbonate solution and isolation of propionic acid and isobutyric acid as the amido-naphthalene derivatives. Hydrolysis also yielded a tetrahydroxy steroid. (The latter was recently isolated as a natural product and named oogoniol.) Ultraviolet, infrared and n.m.r. spectra revealed the Δ^5-7-ketone system. Location of the hydroxyl groups was established by acetylation and oxidation. Thus oogoniol could be converted to a tri- and a tetra-acetate. Oxidation of the triacetate with Jones reagent gave a product whose infrared spectrum

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

indicated the presence of a cyclopentanone. Circular dichroism and mass spectral measurements on the cyclopentanone indicated that the carbonyl was at C-15. One of the hydroxyl groups in oogoniol was therefore at C-15. The location and stereochemistry of the hydroxyl at C-11 was obtained from comparison of the chemical shifts of the C-18 and C-19 methyl protons in the n.m.r. spectra of the oogoniols and their derivatives, with values calculated for these structures.

Oxidation of oogoniol-2 afforded a triketocarboxylic acid, the carboxyl group being derived from a primary hydroxyl located on the side chain. Initially, we proposed the C-26 position for this hydroxyl because the 220 MHz n.m.r. spectrum showed a clean triplet at 0.84 parts/10^6 which could be assigned to C-29 methyl protons. There were also doublets at 0.95 and 1.01 parts/10^6 assignable to C-27 and C-21 methyl protons. In order to confirm the location of the primary hydroxyl, model 3, 26- and 3, 29-dihydroxystigmast-5-enes were synthesized and their ^{13}C n.m.r. spectra compared with that of oogoniol-1. The resonance line for C-29 in the 3, 29-dihydroxystigmastene occurred at 62.06 parts/10^6, almost identical with the line at 62.01 parts/10^6 in the spectrum of oogoniol-1. The resonance line for C-26 in the 3, 26-dihydroxystigmastene occurred at 66.66 parts/10^6. The primary hydroxyl in oogoniol is therefore at C-29 (McMorris *et al.* 1978). The triplet at 0.84 parts/10^6 in the ^1H n.m.r. spectrum of oogoniol-1 is now assigned to the isopropyl group the methyls of which (C-26 and C-27) are non-equivalent. There is coincidental overlap of the lower arm of one doublet with the upper arm of the other. In the 90 MHz spectrum two doublets instead of a triplet are observed. We now believe that the doublet at 1.01 parts/10^6, which was assigned to C-21 methyl protons, is due to the presence of another steroid, actually 24(28)-dehydro-oogoniol-1. It apparently co-chromatographed and also co-crystallized with oogoniol-1. The C-26 and C-27 methyl protons of this compound would be expected to give a doublet in the region of 1.01 parts/10^6. The C-28 vinyl proton would give a triplet at about 5.3 parts/10^6 and such a signal was actually observed in the n.m.r. spectrum. The intensity varied with different isolates of oogoniol-1. In some cases it is estimated that the dehydro analogue amounted to as much as 25% of the total steroid. The protons at C-29 in 24(28)-dehydro-oogoniol-1 would be expected to give a doublet but this was not discernible in the n.m.r. spectrum presumably because it occurred in the same region as the C-11 proton signal.

The 220 MHz spectrum of oogoniol-2 also showed the presence of a second component, presumably 24(28)-dehydro-oogoniol-2. We had attempted to resolve the problem of the structure of the side chain by preparing a crystal of oogoniol-2 which we submitted to Dr John Clardy at Iowa State University for X-ray crystallographic analysis. He was able to confirm the structure of the tetracyclic nucleus but was unable to determine the structure of the side chain (J. Clardy, personal communication). This is understandable if the crystal consists of two steroids possessing the same nucleus but different side chains.

Two other steroids isolated from culture liquids of *A. heterosexualis* were identified as an acetate of oogoniol (oogoniol-3) and a C-15 ketosteroid corresponding to oogoniol-2. They were obtained as a mixture which could not be separated by thin layer chromatography. The n.m.r. spectrum indicated that a small amount of a C-24, C-28 dehydrosteroid was also present in the mixture.

We have recently obtained biosynthetic evidence which supports the presence of a C-29 hydroxyl in the oogoniols. This is discussed in the next section. The presence of 24(28)-dehydro-oogoniols in the samples of oogoniols, which were found to be biologically active, raises the question as to which component is the true hormone. We suspect that the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

dehydro-oogoniols may be the true hormones but this has not been definitely established (see the section on bioassay for the oogoniols).

Biosynthesis of antheridiol and the oogoniols

Studies on the biosynthesis of antheridiol have been reported by Popplestone & Unrau (1973, 1974). They first showed that fucosterol was the major sterol in *Achlya bisexualis* and that 24-methylene cholesterol and cholesterol were present as well. The latter sterols had earlier been reported to be constituents of *Achlya* (McCorkindale *et al.* 1969). It is interesting to note that the sterol composition in several species of *Achlya* resembles that in brown algae (Ikekawa *et al.* 1968; Patterson 1971) and provides further evidence for grouping the Oomycetes with the algae rather than the true fungi. (For a recent discussion of this relation see Bu'Lock & Osagie 1976.)

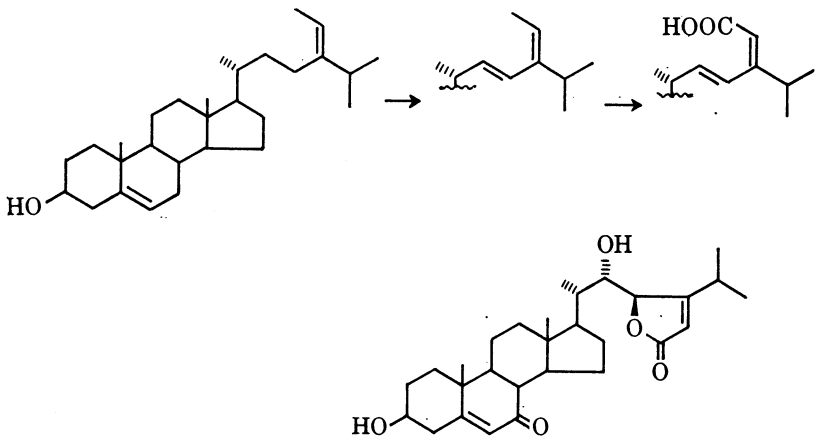

Figure 4. Proposed biosynthetic pathway to antheridiol from fucosterol.

Antheridiol possesses the carbon skeleton of fucosterol and it was logical for Popplestone & Unrau to propose that the latter compound is the precursor of the hormone. The proposition was confirmed by experiments in which radioactive antheridiol was isolated from cultures of the female strain *A. bisexualis*, to which [^{3}H]fucosterol had been added. In the conversion of fucosterol to antheridiol, oxygen functions are introduced at C-22 and C-23 perhaps by hydroxylation of a 22-dehydrofucosterol intermediate, and C-29 is oxidized to carboxyl before lactone formation. Feeding experiments with stigmasta-5,22(E)24(28)-trien-3-ol and 3-hydroxystigmasta-5,22(E)24(28)-trien-29-oic acid showed that these steroids were intermediates in the biosynthesis.

Popplestone & Unrau concluded from their experiments that dehydrogenation at C-22, C-23 precedes oxidation at C-29 of fucosterol in the conversion to antheridiol. These results are summarized in figure 4. Although the biosynthesis of deoxyantheridiol has not been examined, it is likely, from a consideration of the structure, that this steroid is formed by a very similar pathway to that for antheridiol. The trienoic acid mentioned above might well be a common intermediate.

The carbon skeleton found for oogoniol is the same as that of sitosterol (or its C-24 epimer, clionasterol). It seemed to us that one or other of these sterols might be a suitable substrate for conversion to oogoniol by appropriate strains of *Achlya*. We selected *Achlya heterosexualis*

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

B-14 for feeding experiments because it secreted relatively large amounts of the oogoniols and it did not require exogenous antheridiol to induce this secretion (McMorris & White 1977). Sitosterol was found to be converted to oogoniol by *A. heterosexualis* but only to a very small extent. We thought that the stereochemistry at C-24 might be the reason for the poor conversion so clionasterol was tried instead. A similar result was obtained, clionasterol being converted to oogoniol to about the same degree as was sitosterol.

In both experiments, sterols from the mycelium were also examined. The substrates, sitosterol and clionasterol, were isolated and found to have unchanged specific radioactivity indicating that no endogenous sitosterol or clionasterol was present in the mycelium. Cholesterol, 24-methylene cholesterol and fucosterol were isolated and found to be radioactive, the specific activity of fucosterol in both experiments being about the same order of magnitude as that of the oogoniols. One way to interpret this result is that sitosterol (and clionasterol) is converted to a small degree to fucosterol which is then efficiently converted to oogoniol. This possibility was tested by adding [^{3}H]fucosterol to cultures of *A. heterosexualis*. Very efficient incorporation of fucosterol into oogoniol was observed, the specific activity of the latter being about 50 % the specific activity of the fucosterol isolated from the mycelium.

Having established that fucosterol could be efficiently converted to oogoniol, feeding experiments were then carried out with [CD_3]methionine in order to confirm the location of the primary hydroxyl. It is known that fucosterol can be derived from desmosterol and S-adenosylmethionine (Lederer 1969). The carbons 28 and 29 should be bonded to a total of four deuterium atoms when [CD_3]methionine is the substrate. This has been confirmed for fucosterol isolated from the mycelium of *A. heterosexualis* when the fungus was grown in the presence of [CD_3]methionine (White & McMorris 1978). If fucosterol is indeed the precursor of oogoniol, the latter compound would be expected to contain four deuterium atoms provided the primary hydroxyl is at C-26, but only three deuterium atoms if the hydroxyl is at C-29. In the mass spectrum of oogoniol (molec. mass 476) the base peak occurs at *m/e* 458. The mass spectrum of oogoniol isolated from a feeding experiment with [CD_3]methionine (100 μg/ml) showed the base peak at *m/e* 460. From the intensities of the neighbouring peaks it could be calculated that 55 % of the molecules contained two deuterium atoms, 33 % contained one and 12 % were unlabelled. No molecule contained more than two deuterium atoms.

The exact location of the deuterium atoms was determined by carrying out another feeding experiment on a larger scale. The concentration of [CD_3]methionine was 10 μg/ml. Oogoniol-2 was isolated and found to have 60 % of the molecules unlabelled, 32 % with one and 8 % with two deuterium atoms. It was oxidized to a triketoacid which was then methylated with diazomethane. The mass spectrum of the unlabelled ester had strong peaks at *m/e* 482, 467, and 409. The intensity of the peaks at *m/e* 483 and 468 in the spectrum of the ester from the labelling experiment indicated that 22 % of the molecules contained only one deuterium atom (and none contained more than one). This value is almost exactly the amount of deuterium expected if one atom is at C-28 and the other at C-29 in oogoniol-2. The loss of a deuterium atom on oxidation indicates that the primary hydroxyl is located at C-29.

The peak at *m/e* 409 results from loss of CH_3OOCCH_2 by 'β cleavage' at C-24, C-28 of the ion *m/e* 482. The intensity of the *m/e* 410 ion in the spectrum of the labelled ester indicated that there was no deuterium label present in that ion. Thus, the location of a deuterium atom at C-28 was confirmed.

In the conversion of fucosterol to oogoniol it now appears that oxidation occurs at C-29 to

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

give first an alcohol and then an aldehyde or its equivalent. Subsequent reduction of the C-24, C-28 double bond and aldehyde would give the side chain of oogoniol. Hydroxylation at C-11 and C-15 and oxidation at C-7 may occur before or after modification of the side chain of fucosterol. The results of biosynthetic experiments with the oogoniols are summarized in figure 5.

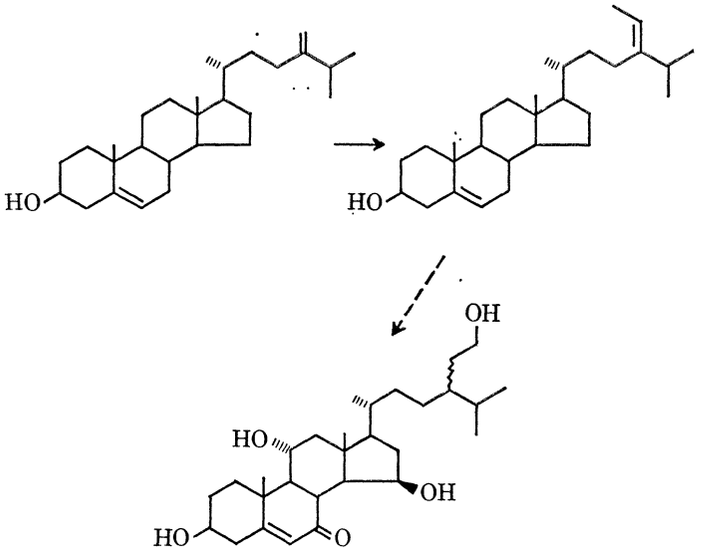

FIGURE 5. Proposed biosynthetic pathway to oogoniol.

BIOASSAY FOR ANTHERIDIOL

In the procedure currently employed, which is a slight modification of Barksdale's method (Barksdale *et al.* 1974), the strong male strain *Achlya ambisexualis* E87 is inoculated from agar slants onto 90 mm Petri dishes containing Barksdale's agar medium. Plugs are cut from the edge of the mycelial mat, just before it has grown out to the side of the dish, and placed in small Petri dishes (30 mm, one plug per dish together with medium for antheridiol assay, 3 ml for each dish). The plugs are allowed to grow for 72 h at 20 °C. The sample to be tested can be dissolved in methanol or acetone but this solution must be diluted with distilled water to give a final concentration of methanol or acetone no greater than 1 % in the solution. Higher concentrations of organic solvent inhibit development of antheridia. About 1 ml of the test solution is added to the Petri dish. Another aliquot of test solution can be diluted with distilled water for tests at higher dilutions. The Petri dishes are kept at room temperature (20 °C) for 2 h then examined for antheridial branches. In Barksdale's assay, if branching has occurred on 25 % or more of the hyphae nearest to the surface, the dilution is recorded as active.

The lowest concentration of antheridiol which will cause branching is approximately 10 pg/ml of solution. The concentration of antheridiol determines both the number of branches initiated and the time that elapses between the addition of hormone and the appearance of branches. The number of branches increases with increasing concentration of hormone until an upper limit is attained. The length of time before branches appear decreases with increasing concentration until a minimum of 40–45 min is reached. Branching also depends on the presence of nutrient, i.e. amino acids (edamin) and glucose. Without these, very little branching occurs even with high concentrations of antheridiol. Indeed Barksdale (1970) has suggested that hormone A′

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

might be an amino acid mixture. Raper had suggested that this factor is secreted by male mycelia, because filtrates from the male strain were found to enhance the effect of antheridiol (Raper 1942). The properties reported for hormone A′ are consistent with those expected for an amino acid mixture.

Antheridiol is almost completely insoluble in water but it is so potent that only minute amounts are needed to produce a biological effect. It is secreted together with a great quantity of lipid material which possibly helps in the transport of the hormone in water. Treatment with dilute acid or base rapidly inactivates antheridiol. The C-3 hydroxyl is readily lost by acid or base catalysed elimination. Also the hydroxybutenolide system on the side chain is very sensitive to base which causes isomerization of the double bond and ring opening.

Modifications in the structure of antheridiol give compounds with sharply reduced activity. Thus the structure lacking the C-7 ketone has about 5 % the activity of antheridiol. Structures with a different configuration at C-22 or C-23 have activities which are 0.1 % or less that of antheridiol. 23-Deoxyantheridiol and the trienoic acid which is believed to be a biosynthetic intermediate (see preceding section), each possesses about 0.1 % the activity of antheridiol. Mammalian sex hormones were completely inactive in the bioassay for antheridiol.

Bioassay for the oogoniols

Plugs of mycelium from the female strain *Achlya ambisexualis* 734 are grown in Petri dishes in the same way as described for *A. ambisexualis* E87. The assay medium for the oogoniols is similar to that for antheridiol (Barksdale & Lasure 1973). The test solution can be added in the same way as for antheridiol or the assay medium can be poured off from the plug and then replaced by test solution containing only the hormone and no nutrient. Oogonia (strictly speaking oogonial initials) are visible after about 12 h and reach a maximum number in 24–48 h. Like antheridia, the number of oogonia increases with increasing concentration. Oogonia have a distinct spherical shape and are easier to count than are antheridia. In a strongly positive reaction there are well over fifty oogonia in any field near the edge of the mycelial mat. The protoplasm in the oogonia may be quite dense compared with that in the hyphae. However, protoplasm does not differentiate to form oospheres as is observed when male and female strains are grown together and conjugation of antheridial hyphae and oogonia occurs. In some cases the oogonia put out lateral branches which develop into additional oogonia.

The lowest concentration at which oogoniol-1 exhibits biological activity was first reported to be 620 ng/ml (McMorris *et al.* 1975). We now know that the samples of oogoniol-1 were not pure but contained small amounts of 24(28)-dehydro-oogoniol-1. Separation of the two compounds was recently achieved by means of high pressure liquid chromatography, and preliminary tests indicate that the dehydro analogue is more active than oogoniol-1 perhaps by a factor of 100. The lowest concentration of dehydro-oogoniol-1 which elicits a reaction in the female strain is approximately 50 ng/ml.

Functions of antheridiol

The biological effects of antheridiol may be summarized as follows: the hormone which is secreted by vegetative female mycelium induces the formation of antheridial hyphae in the male. It also stimulates the male to produce oogoniol which in turn induces formation of oogonial initials on the female mycelium. It is believed that the oogonial initials (which later

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

develop into oogonia) secrete antheridiol which is responsible for the chemotropic growth of the antheridial hyphae to the oogonia. Antheridiol may also be involved in delimitation of the antheridia which occurs after the antheridial hyphae have conjugated with the oogonial initials.

Several studies of the biochemical processes initiated by antheridiol have been reported. Silver & Horgen (1974) have found that antheridiol stimulates the production of RNA during the early stages of male sex organ development. Protein synthesis is also stimulated. The activation of the synthesis of RNA, presumably mRNA, is required for initiation of the antheridial branches. Addition of actinomycin D or cordycepin inhibits the accumulation of the mRNA and also prevents normal morphological differentiation. The rapid decline in the specific activity of the mRNA fraction when actinomycin D is added suggests that the life span of this fraction is very short (less than 1 h). Cordycepin, a drug which is known to affect the post transcriptional addition of polyadenylic acid into mRNA, rapidly inhibited the incorporation of [^{3}H]adenosine into the mRNA fraction. This suggests that the mechanism for the addition of the poly(A) fraction to *Achlya* presumptive mRNA is similar to the mechanism operative in mammalian cells, Silver & Horgen conclude.

In an independent study Horowitz & Russell (1974) also observed inhibition of DNA-dependent RNA synthesis by actinomycin D. Antheridial branch elongation was inhibited to a degree proportional to the concentration of actinomycin D. At the highest concentration tested (25 μg/ml) there was complete inhibition when the actinomycin D was added at the same time as antheridiol. However, at lower concentrations (15 μg/ml) some growth occurred.

Timberlake (1976) has obtained similar results. By means of pulse labelling techniques he showed that the rate of incorporation of [^{3}H]adenosine into RNA is stimulated soon after addition of antheridiol to vegetative cultures. Both poly(A) and poly(A) + RNA synthesis are accelerated. Actinomycin D and *p*-fluorophenylalanine each completely inhibited the appearance of antheridial branches when added at the same time as antheridiol. Addition of actinomycin D 90 min after addition of antheridiol, when branches were appearing, caused only about 25 % inhibition of branching. *p*-Fluorophenylalanine inhibited branch formation for a substantially longer period than actinomycin D. Stimulation of protein synthesis was found to occur at about the same time as stimulation of poly(A) + RNA synthesis. Timberlake concludes that early hormone induced differentiation in *Achlya* is dependent on continued transcription and translation, but continued transcription for a finite period only, since administration of an inhibitor of RNA synthesis after 180 min in the presence of hormone did not prevent development of the normal number of antheridial branch initials. Initial activation of cells by antheridiol occurs very rapidly, within an hour. Entrance of the hormone into the cells is immediately followed by the synthesis of development-specific RNA species and their subsequent translation. By 180 min after the addition of hormone, no further RNA synthesis is required for antheridial branch initiation to occur. Protein synthesis, however, is required virtually throughout development.

Groner *et al.* (1976) have reported a study on the induction of specific proteins in *Achlya ambisexualis* by antheridiol. They detected an induced protein, with a molecular mass of 69000, 1 h after addition of the hormone. The synthesis of this protein precedes the microscopic appearance of antheridial initials. Pulse labelling with L-[^{3}H]leucine showed that synthesis of the 69000 molec. mass protein decreased after about 5 h. Proteins excreted into the culture medium were also examined. Mycelium induced with antheridiol for 1 h excreted

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

protein migrating at 40000 molec. mass and smaller amounts at 29000, 64000 and 88000. Increasing the labelling time to 3 h or more resulted in excretion of a complex pattern of proteins, different from that found inside the mycelium and therefore not attributable to cell lysis.

What are the specific functions of proteins induced by antheridiol? Thomas & Mullins (1967, 1969) were the first to demonstrate that there is increased production of cellulase enzymes accompanying morphological expression of antheridial hyphae. Experiments with cycloheximide and actinomycin D indicated that both translation and transcription are required for the induction of cellulase. Hormone-treated mycelia previously treated with cycloheximide or actinomycin D were still capable of showing a delayed production of antheridial hyphae and cellulase when the inhibitors were removed by washing.

Cellulase is believed to cause a localized softening of the hyphal wall which produces weak spots that are blown out into lateral blebs by turgor pressure. Electron microscope studies by Mullins & Ellis (1974) and also by Nolan & Bal (1974) indicate that the cellulase is contained in vesicles which are localized at points of branch formation. The cell wall is known to contain approximately 15 % cellulose and 85 % of an amorphous non-cellulosic polysaccharide complex. Interestingly, antheridiol does not stimulate cellulase secretion in female mycelia.

Another function of protein induced by antheridiol appears to be the synthesis of the oogoniols, the hormones which induce formation of oogonial initials in female mycelium. In water matings of male and female strains of *Achlya ambisexualis*, Raper observed that antheridial branches appeared within 2 h of the time that the pairings were made. Beginning about 12 h after the appearance of antheridial branches, oogonial initials started to appear along the line of intermingling of the two opposed mycelia. We believe that oogonial initials are induced by the oogoniols secreted by the male after stimulation by antheridiol. Barksdale & Lasure (1973) found that the strong male *A. ambisexualis* E87 will not secrete the oogoniols unless grown together with a female, *A. ambisexualis* 734, or grown in the presence of antheridiol. We have confirmed these results and also demonstrated that the oogoniols are biosynthesized from fucosterol, the major sterol present in the mycelium. Thus [^{3}H]fucosterol, when added to the culture, was found to be gradually taken up by the mycelium over a period of several hours (where it was diluted by endogenous fucosterol) but no oogoniol was produced unless antheridiol was added. In one experiment the addition was made about 2 h after that of the fucosterol. After a further hour, radioactive oogoniols could be detected in the culture liquid and the amount increased steadily for about 6 h then levelled off. It seems that on adding antheridiol the enzymes required for the synthesis of the oogoniols are rapidly induced concurrently with enzymes required for development of antheridial hyphae. As expected, addition of cycloheximide at the same time as antheridiol, completely inhibited production of the oogoniols (McMorris & White 1977).

Very little is known about the biochemical events which underlie chemotropic growth of antheridial hyphae to the oogonial initials. Raper postulated that this directional growth was caused by a hormone distinct from hormone A (antheridiol), but Barksdale (1963) later showed that antheridial hyphae were attracted to particles of plastic previously treated with a solution of hormone A. Thus she concluded that hormone A was responsible for the chemotropic effect. Barksdale's experiment was carried out with a highly purified sample of hormone A. We felt it would be worthwhile to repeat the experiment using synthetic antheridiol so as to remove any possibility that the chemotropic effect might have been caused by another

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

substance present in Barksdale's sample. Silica gel particles (200 mesh) on which antheridiol was adsorbed were found to exhibit a strong chemotropic effect on antheridial hyphae, thus confirming Barksdale's conclusions.

From all the studies that have been reported it is evident that antheridiol plays a very important rôle in the life cycle of *Achlya*. Some of its effects are still far from well understood and will require further investigation. In the case of the oogoniols it will be important to confirm the structures by synthesis and to demonstrate that the synthetic compounds possess biological activity. The functions of the oogoniols remain to be examined particularly at the molecular level. Finally, there may be other hormones which are involved in the delimitation of oogonia and differentiation of the oogonial contents to form oospheres. These processes merit further investigation.

Our investigations were supported by the National Institutes of Health.

References (McMorris)

Arsenault, G. P., Biemann, K., Barksdale, A. W. & McMorris, T. C. 1968 *J. Am. chem. Soc.* **90**, 5635.
Barksdale, A. W. 1963 *Mycologia* **55**, 627.
Barksdale, A. W. 1968 *J. Elisha Mitchell scient. Soc.* **84**, 187.
Barksdale, A. W. 1970 *Mycologia* **62**, 411.
Barksdale, A. W. & Lasure, L. L. 1973 *Bull. Torrey bot. Club* **100**, 199.
Barksdale, A. W. & Lasure, L. L. 1974 *Appl. Microbiol.* **28**, 544.
Barksdale, A. W., McMorris, T. C., Seshadri, R., Arunachalam, T., Edwards, J. A., Sundeen, J. & Green, D. M. 1974 *J. gen. Microbiol.* **82**, 295.
Bu'Lock, J. D. & Osagie, A. U. 1976 *Phytochemistry* **15**, 1249.
Edwards, J. A., Mills, J. S., Sundeen, J. & Fried, J. H. 1969 *J. Am. chem. Soc.* **91**, 1248.
Edwards, J. A., Sundeen, J., Salmond, W., Iwadare, T. & Fried, J. H. 1972 *Tetrahedron Lett.*, 791.
Goad, L. J., Lenton, J. R., Knapp, F. F. & Goodwin, T. W. 1973 *Lipids* **9**, 582.
Green, D. M., Edwards, J. A., Barksdale, A. W. & McMorris, T. C. 1971 *Tetrahedron* **27**, 1199.
Groner, B., Hynes, N., Sippel, A. E. & Schutz, G. 1976 *Nature, Lond.* **261**, 599.
Horowitz, D. K. & Russell, P. J. 1974 *Can. J. Microbiol.* **20**, 977.
Ikekawa, N., Morisaki, N., Tsuda, K. & Yoshida, T. 1968 *Steroids* **12**, 41.
Lederer, E. 1969 *Q. Rev. chem. Soc.* **23**, 453.
McCorkindale, N. J., Hutchinson, S. A., Pusey, B. A., Scott, W. T. & Wheeler, R. 1969 *Phytochemistry* **8**, 861.
McMorris, T. C. & Barksdale, A. W. 1967 *Nature, Lond.* **215**, 320.
McMorris, T. C. & Seshadri, R. 1971 *Chem. Commun.* 1646.
McMorris, T. C., Arunachalam, T. & Seshadri, R. 1972 *Tetrahedron Lett.*, 2673.
McMorris, T. C., Seshadri, R. & Arunachalam, T. 1974 *J. org. Chem.* **39**, 669.
McMorris, T. C. & Arunachalam, T. 1975 *J. labelled Compd.* **11**, 577.
McMorris, T. C., Seshadri, R., Weihe, G. R., Arsenault, G. P. & Barksdale, A. W. 1975 *J. Am. chem. Soc.* **97**, 2544.
McMorris, T. C. & White, R. H. 1977 *Phytochemistry* **16**, 359.
McMorris, T. C., Schow, S. R. & Weihe, G. R. 1978 *Tetrahedron Lett.*, 335.
Mullins, J. T. & Ellis, E. A. 1974 *Proc. natn. Acad. Sci. U.S.A.* **71**, 1347.
Nolan, R. A. & Bal A. K. 1974 *J. Bact.* **117**, 840.
Patterson, G. W. 1971 *Lipids* **6**, 121.
Popplestone, C. R. & Unrau, A. M. 1973 *Phytochemistry* **12**, 1131.
Popplestone, C. R. & Unrau, A. M. 1974 *Can. J. Chem.* **52**, 462.
Raper, J. R. 1939 *Am. J. Bot.* **26**, 639.
Raper, J. R. 1940 *Am. J. Bot.* **27**, 162.
Raper, J. R. & Haagen-Smit, A. J. 1942 *J. biol. Chem.* **143**, 311
Raper, J. R. 1942 *Proc. natn. Acad. Sci. U.S.A.* **28**, 509.
Silver, J. C. & Horgen, P. A. 1974 *Nature, Lond.* **249**, 252.
Thomas, D. des S. & Mullins, J. T. 1967 *Science, N.Y.* **156**, 84.
Thomas, D. des S. & Mullins, J. T. 1969 *Physiol. Pl.* **22**, 347.
Timberlake, W. E. 1976 *Devl. Biol.* **51**, 202.
Weihe, G. R. & McMorris, T. C. 1978 *J. org. Chem.* (In the press.)
White, R. H. & McMorris, T. C. 1978 *Phytochemistry*. (In the press.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Discussion

D. Klämbt (*University of Bonn, Germany*). The biosynthetic pathway of oogoniol was reported for *Achlya heterosexualis*. This organism produced oogoniol without stimulation by antheridiol. *Achlya bisexualis* produces oogoniol in very low concentration after stimulation by antheridiol, as Dr McMorris mentioned. It is possible that antheridiol may act as a precursor for the production of oogoniol?

T. C. McMorris. We have considered that possibility but we think that it is unlikely for a number of reasons. For example, antheridiol containing a tritium label was added to cultures of *Achlya ambisexualis* E87. Oogoniol was subsequently isolated and purified and it was found to possess negligible radioactivity.

G. W. Gooday (*Microbiology Department, University of Aberdeen, U.K.*) Could Dr McMorris tell us something about the relative biological activities of the three esters of oogoniol, and also of their biosyntheses? Are they made via CoA derivatives of acetate, propionate and isobutyrate?

T. C. McMorris. The isobutyrate and propionate esters appear to possess similar biological activities. We have not been able to isolate the acetate in a pure enough form to test its activity. As for their biosynthesis I think it quite possible that they are made via CoA derivatives of the respective acids but we have not carried out experiments to test this.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

FIGURE 1. Hyphae and sex organs in *Achlya ambisexualis*. (*a*) Vegetative hyphae (magn. ×200); (*b*) hyphae with lateral antheridial branches initiated by antheridiol (magn. ×40); (*c*) oogonial initials initiated by the oogoniols (magn. ×100); (*d*) oogonium containing eggs (magn. ×700).