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THE SELFINDUCED METABOLISM OF ANTHERIDIOL IN WATER MOULDS

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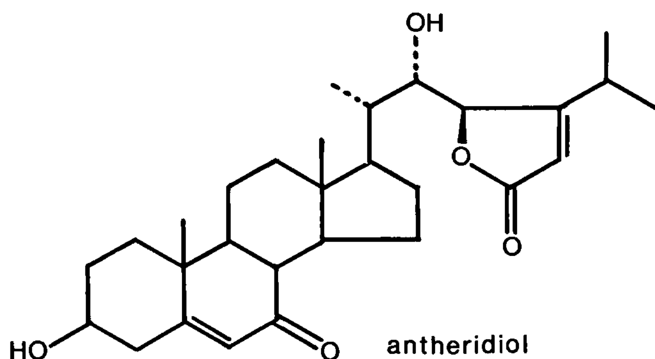
SUMMARY

The self-induced inactivation of antheridiol is a natural hormonal effect specific to *Achlya* species, as is demonstrated by its occurrence in homothallic cultures of *A. americana* in association with the formation of sex organs. We have also shown that progesterone and a biologically inactive isomer of antheridiol do not induce their own metabolism, and that water moulds that do not respond sexually to antheridiol are also not induced to metabolise it.

1. INTRODUCTION

Female *Achlya* strains produce a sterol hormone called antheridiol that induces sex organs on potentially male strains. Antheridiol fed artificially to heterothallic males also induces its own metabolism to biologically inactive derivatives (MUSGRAVE & NIEUWENHUIS 1975). The induced metabolism seems associated with the sexual response in that non-responsive female strains are not induced to metabolise the hormone, and homothallic strains that self-induce sex organs, metabolise added hormone without a lag period, as if the metabolising enzyme had already been induced. Since the induction of male sex organs is known to be a highly specific effect of antheridiol (BARKSDALE et al. 1974) it seems likely that this form of self-induced metabolism is also specific to antheridiol. However, in our antheridiol metabolism studies we used radioactive hormone of such low specific activity that abnormally high concentrations (10^4 times higher than needed to obtain a sexual response) must be administered in order to monitor the hormone's fate. High concentrations of sterols, for example progesterone, are known to induce self-metabolism in some fungi without having a specific hormonal effect (KOEPSELL 1962; PERLMAN et al. 1957; SHIBAHARA et al. 1970). Consequently, we have sought further evidence that self-induced metabolism in *Achlya* strains is antheridiol specific and associated with its hormonal effect. Firstly, we have tried to determine whether hormone-induced inactivation takes place naturally in sexually responding homothallic mycelia, which self-induce sexual activity without the addition of antheridiol. Secondly, we have tested whether progesterone and a relatively inactive isomer of antheridiol also induce self-metabolism, and lastly, we have tested whether antheridiol induces its own metabolism in water moulds other than *Achlya* species, most of which do not respond sexually to antheridiol.



2. MATERIALS AND METHODS

All fungal strains were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, Netherlands: *Achlya ambisexualis* 101.50 (female); *A. americana* 527.67 (homothallic); *A. bisexualis* 100.42 (male); *Aphanomyces cladogamus* 108.29; *Dictyuchus sterilis* 164.38; *Isoachlya unispora* 213.35; *Saprolegnia ferax* 283.38; *S. parasitica* 302.56; *Thraustotheca clavata* 557.67. Stock cultures were maintained on corn meal agar at 4°.

Radioactive antheridiol (22S, 23 R – ^{3}H) spec. act. 15 mCi/mM and radioactive 7-deoxy-antheridiol-3-acetate (22R, 23S – ^{3}H) spec. act. 32 mCi/mM were generously supplied by Dr. T. McMorris and Dr. A. W. Barksdale. Radioactive deoxy-antheridiol was prepared from the acetate as described by McMorris & Arunachalam (1975). Progesterone (1a, 2a(n)–^{3}H) spec.act. 42 Ci/mM was bought from the Radiochemical Centre, Amersham, U.K., and diluted with unlabelled progesterone to spec.act. 15 mCi/mM. All radioactive compounds were chromatographically pure in our TLC systems (see below).

To assay the response to antheridiol, each fungus was grown on 1.5% agar plates containing the medium designed by Barksdale (McMorris & Barksdale 1967). In front of the advancing mycelium 20 × 10^{-9} moles of antheridiol in 5 μl methanol plus 200 μl water was pipetted into a small well. After 24 h incubation at 28° the mycelium was observed for the production of antheridial branches.

For hormone metabolism studies, mycelia were grown in 25 ml PYG medium (Timberlake et al. 1973) in 100 ml flasks in a shaking water bath at 28°. Approximately 2 × 10^{-9} moles of radioactive steroid were added per flask and at various times the cultures harvested onto a Buchner funnel. The medium was separated from the mycelium under vacuum, and the fresh weight per mycelium determined before freezing for storage. The radioactivity in a methanol extract of the mycelia, the insoluble pellet, a dichloromethane extract of the medium and that remaining in the aqueous phase, was determined as described by Musgrave & Nieuwenhuis (1975). The methanol and dichloromethane fractions were chromatographed on layers of silica gel G with the solvent chloroform: methanol (10:1 or 15:1 v/v).

Sections of each chromatogram were scraped into vials containing a scintillation fluid based on xylene and Triton X-100 (ANDERSON & MCCLURE 1973) and the radioactivity assessed in a PACKARD liquid scintillation spectrometer fitted with an absolute activity analyser.

3. RESULTS

3.1. The natural association between antheridiol metabolism and male sex organ formation in a homothallic *Achlya* species.

To test this possible association, we studied the development of both processes in cultures of the homothallic *A. americana*, which at a certain stage of maturity naturally forms sex organs. Therefore at various times after inoculating new cultures, ^3H – antheridiol was added, and the formation of the major metabolite (metabolite A, Rf 0.5 relative to antheridiol) partitioning in the dichloromethane phase was assayed after incubation periods of 20 and 120 min. Since enzyme induction takes approximately 45 min, appreciable activity after only 20 min implies that the enzyme was already present, while activity only after 120 min implies it was induced by the ^3H-antheridiol. The results are presented in *fig. 1A*. Twenty-four-hour-old cultures did not significantly metabolise antheridiol even after 120 min of incubation. Thirty-four-hour-old cultures only metabolised

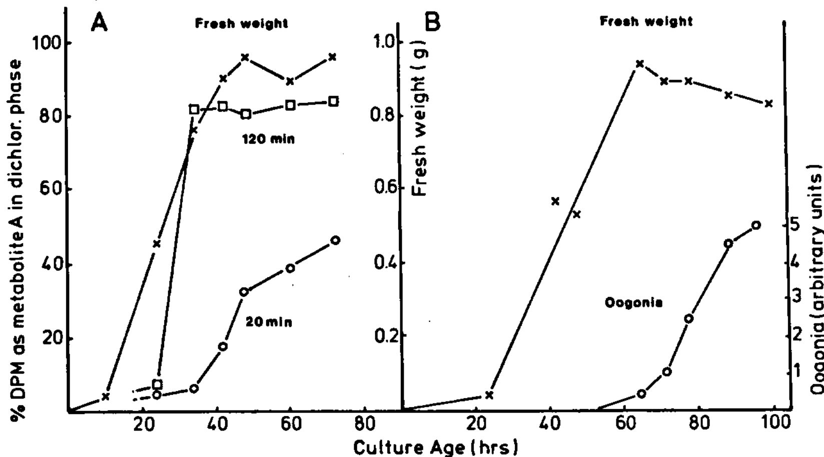


Fig. 1A. The percent radioactivity per *A. americana* shake culture as metabolised antheridiol in the dichloromethane phases, and the average mycelial fresh weights, with age. At the stipulated ages, two cultures were treated with ^3H-antheridiol and harvested 20 or 120 min later. The percent radioactivity as metabolite A was then determined. Since the fresh weights per culture at 24 h were only half those of mature cultures, two mycelia were combined before adding ^3H-antheridiol.

Fig. 1B. The number of oogonia per petri dish on *A. americana* cultures and the mycelial fresh weights. The growth conditions were as described for metabolism studies except that the cultures were kept stationary. Oogonia were initially produced in patches on each mycelium and their numbers could only be arbitrarily assessed, except that 1 unit was at least 10 oogonia per dish and 5 units were approximately 2.5×10^5 oogonia. The data are the averages of four separate cultures.

antheridiol after a lag period of approximately an hour. Apparently at this age the enzyme was not naturally present but could be induced while in older cultures the enzyme appeared to be already present, because in each case, antheridiol was metabolised within the first 20 min. Thus the enzyme occurs naturally in *A. americana* cultures but only when the cultures have reached maximum fresh weight. The question now was whether sex organs developed in this same time period. Sex organs do not develop consistently in shake cultures, but in stationary cultures, under otherwise similar conditions, sex organs readily develop, and the average number of oogonia in four such cultures with time is shown in *fig. 1B*. Oogonia and not antheridial branches were assessed because antheridial branches were invariably associated with oogonia and can in practice only be distinguished from vegetative hyphae by their association with oogonia, and because they were much easier to quantitate. Stationary cultures grew more slowly than shake cultures as can be seen from *fig. 1B*, but the formation of sex organs, just as the presence of the antheridiol-inactivating enzyme in shake cultures, was strongly associated with the attainment of maximum fresh weight. We therefore assume that antheridiol inactivation is associated with the natural production of reproductive organs.

3.2. Metabolism of 7-deoxy isomer of antheridiol and progesterone in *Achlya* strains

The 7-deoxy isomer and its acetate are approximately 10^4 times less active than antheridiol, while progesterone is completely inactive (BARKSDALE et al. 1974).

The metabolism of these three steroids was first studied in 48 h old cultures of *A. bisexualis* (100.42), a typical heterothallic male. Radioactivity administered as progesterone was largely recovered in the dichloromethane (48%) and methanol (38%) extracts of the media and mycelia respectively. On chromatographing these fractions we found that progesterone was slowly converted to two metabolites (Rfs 0.75 and 0.5 relative to progesterone) present in the dichloromethane phase (*fig. 2*). Metabolism was constant and slow, and in no way resembled the dramatic metabolism induced by antheridiol. Progesterone fed to *A. americana* cultures was metabolised at the same slow rate and therefore we conclude that it does not induce its own metabolism in male-acting *Achyla* cultures.

Metabolism of the antheridiol isomer and its acetate were very similar, so only the data for the parent compound will be given. Radioactivity was mainly recovered in the methanol extracts (50%) of the mycelia but with a large part (30%) in the dichloromethane extracts of the media. Chromatography of these phases showed that the isomer was rapidly converted into a compound Rf 0.6 relative to the isomer. Its formation with time in the dichloromethane phase is represented in *fig. 2*. Its formation with time in the methanol extracts was slower but otherwise similar. Metabolism was immediate and without a lag period although antheridiol metabolism took place only after 30 min (*fig. 2*). There was no sign of induced metabolism of the isomer. Cycloheximide treatment (1 μg/ml, a protein synthesis inibitor that blocks antheridiol-induced events, MUSGRAVE & NIEUWENHUIS 1975) had no effect on metabolism. The strong female *A. ambisexualis*

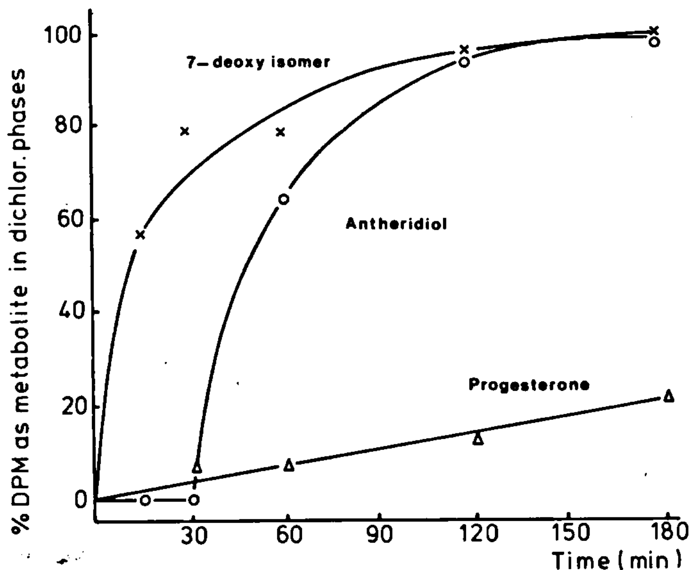


Fig. 2. The percent radioactivity per culture as metabolised steroid in the dichloromethane fractions from *A. bisexualis* (100.42) cultures treated with radioactive antheridiol, a 7-deoxyantheridiol isomer or progesterone, with time.

(101.50) reacted similarly, metabolising the isomer without a lag period, although we have never been able to induce antheridiol metabolism in this strain. Even young cultures of *A. americana* (28 h-old) which were unable to metabolise antheridiol, metabolised the isomer without a lag. We conclude therefore that metabolism of this isomer is non-specific and not related to sexual development.

3.3. Antheridiol metabolism in various water moulds

In general, radioactive antheridiol was added to liquid cultures when they had attained maximum fresh weight. In most cases this was after 48 h, but for example *I. unispora* grew more slowly and therefore was treated after 72 h. In all cases most of the radioactivity was recovered in the dichloromethane and methanol extracts of the media and mycelia respectively. On chromatographing these fractions the radioactivity migrates as antheridiol or as metabolite A. The metabolite's appearance in time in both dichloromethane and methanol fractions was similar and for the dichloromethane fractions is expressed in *fig. 3* for the various fungi tested. In male-acting heterothallic *Achlya* strains the formation of metabolite A always followed a lag period, and an example of this sort of induced metabolism is given for *A. bisexualis* (100.42) cultures in *fig. 1*, as a reference. Only *S. parasitica* and *I. unispora* did not significantly metabolise antheridiol. *T. clavata* rapidly metabolised antheridiol without a lag period and thus behaved as a typical *Achlya*-type homothallic. When 1 μg/ml cycloheximide was added, antheridiol metabolism was unaffected. *D. sterilis*, *A. cladogamus* and *S. ferax* also metabolised antheridiol without a lag period and metabolism was also unaffected by cycloheximide

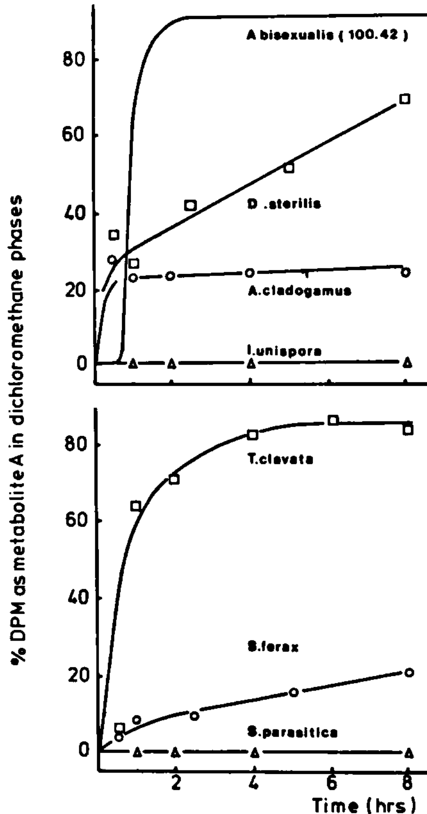


Fig. 3. The percent radioactivity per culture as metabolised antheridiol in the dichloromethane fractions from various water mould cultures, with time. The data for *A. bisexualis* (100.42) are presented for comparison.

treatment. Thus, although fungi other than *Achlya*-types are capable of metabolising antheridiol, none of the species tested showed signs of enzyme induction.

When tested for a morphological response to antheridiol only *T. clavata* reacted positively. It grew as a typical homothallic in plate cultures producing antheridia and oogonia without stimulation from added hormones. In the presence of antheridiol, oogonia formation was suppressed and a mass of antheridial branches developed. Its ability to respond to antheridiol is already known, for SALVIN (1942) reported that it produced antheridial branches when cultured with a female *Achlya* strain. None of the other species exhibited any form of sexual activity.

4. DISCUSSION

There is little doubt that self-induced antheridiol metabolism is a natural phenomenon and is not simply the result of the high concentrations of antheridiol we use.

Thirty-four-hour-old cultures of *A. americana* do not possess the inactivating enzyme although it can be induced by applied antheridiol. Older cultures naturally possess the enzyme. This implies that the enzyme is not present before the cultures are sexually mature, but is naturally induced by antheridiol produced within the homothallic mycelium. We have also shown that the heterothallic male *A. bisexualis* (100.42) can be induced to metabolise antheridiol by as little as 2×10^{-12} moles of antheridiol per culture. When ^{3}H-antheridiol was then added two hours later it was metabolised without a lag period. Thus antheridiol inactivation is probably induced concurrently with sexual development in all male responding *Achlya* strains.

The induction of self-metabolism seems to be specific for antheridiol and associated with its hormonal action, for the biologically inactive sterols tested did not induce their own metabolism. Likewise, water moulds that did not respond sexually to antheridiol were not induced to metabolise antheridiol. However, it is only the induction of metabolism that is specific for antheridiol, because the isomer of antheridiol was rapidly metabolised by all *Achlya* strains tested, and some water moulds non-responsive to antheridiol readily metabolised the hormone.

Despite the close structural similarity between antheridiol and its isomer, many *Achlya* strains were able to specifically metabolise the isomer. It seems possible that this represents a natural safeguard against the production of inactive isomers of antheridiol.

A. americana cultures did not produce sex organs until they had attained maximum fresh weight when we assume the nutrient content of the medium was depleted. High nutrient concentrations are known to inhibit sexual development in *Achlya* (see for example MULLINS & WARREN 1975) to the extent that preformed sex organs will revert to vegetative branches in a strong nutrient solution (BARKSDALE 1970). It seems that the enzyme that inactivates antheridiol may also be sensitive to nutrient concentrations, for 24 h-old cultures were unable to metabolise antheridiol. Either young cultures are unresponsive to antheridiol, or they actually respond but enzyme activity is inhibited by the nutrient concentration. We think the latter can sometimes be the case, for 48 h-old cultures which were able to metabolise antheridiol without a lag period, when transferred to fresh culture medium plus ^{3}H-antheridiol, did not metabolise the hormone. On testing which of the medium constituents was effective, we found that glucose, the major constituent, was without effect, but that both mycological peptone and yeast extract effectively blocked metabolism.

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