PHYSIOLOGY OF SEXUAL REPRODUCTION IN *PYTHIUM SYLVATICUM*.

By

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SUMMARY

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An investigation has been made of the physiology of sexual reproduction in the heterothallic fungus, Pythium sylvaticum. Strains 431a, 431b, 431c, 431d and 431e were obtained from Reading University for use in this project. It was confirmed that this species has a requirement for sterol for sexual reproduction, as is well known in all Phytophthora spp. Pythium sylvaticum has a sexual system similar to that reported in heterothallic Achlya spp. The strains of Pythium sylvaticum ranged in sexual activity from strong male, through strains of intermediate potency to strong female. Strains c and d were strong females while strains e and b were strong males. Strain a varies between male and female depending on the strain with which it was paired. Female strains produce oogonia and male strains, antheridia. When strains c and e were paired on opposite sides of an agar plate oospores formed in the centre of the plate when the strains came into contact. When the paired strains were separated by polycarbonate membrane, the induction of oogonia and antheridia occurred but no oospores were formed. Oospores only formed when the hyphae came into contact. The pores in the membrane (0.2μm) pore size, are too small to allow hyphae to pass through, thus fertilisation can not occur and no oospores form. However, there must be some agent or agents produced by each strain which diffuse through the membrane and initiate and control reproduction in the other strain.

When 72h old cultures of male and female were paired, antheridial branches were produced by the male after 4.5h and oogonial initials by the female after 6h. Pairing induced with uninduced cultures confirmed the female first induces the male to produce antheridial branches. The induced male then induces production of oogonial initials by the female. Attempts to isolate the inducing hormones were made, but proved unsuccessful.

The induction of sexual reproduction without hyphal contact and the sequential production of antheridia and oogonia is very similar to that described in heterothallic Achlya spp. Sexual reproduction is controlled by steroid hormones in Achlya. To test the hypothesis that the sterols required for reproduction by Pythium sylvaticum are metabolized to steroid hormones, sterol metabolism in this organism was studied. Cholesteryl palmitate was added to the medium in which male and female were grown and paired, or in which strains were grown alone. Cholesterol and metabolites more polar than cholesterol were produced, but only minor differences were noted in the metabolites produced by induced and uninduced strains which could not be related to reproductive phenomena.

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ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAMP	adenosine - 3'-5'-cyclic monophosphate
cpm	counts per minute
cps	counts per second
dpm	disintegrations per minute
EGTA	Ethylene glycol tetra-acetic acid
HPLC	high performance liquid chromatography
m/e	mass to charge ratio
MeOH	methanol
mRNA	messenger ribonucleic acid
ODS	octa decylsilane
PPO	2,5 - diphenyloxazole
POPOP	1,4 - Bis-(4-methyl-5-phenyl-2-oxazolyl)benzene
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
sit	sitosterol
SM	modified Schmitthenner's medium
TMS	trimethylsilyl
TLC	thin-layer chromatography
v/v	volume for volume
w/v	weight for volume
♂	male
♀	female

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CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Species of *Pythium* can adopt one of two modes of existence, either as a saprophyte or a parasite of plant tissue. The organisms grow well saprophytically in the soil but a plant which is stressed or damaged, can be attacked by the fungus. Rotting of fruit, roots and stems and damping off in seeds and seedlings can be caused by *Pythium spp.*

This thesis describes observations on physiological and biochemical aspects of sexual reproduction in the heterothallic species *Pythium sylvaticum*, a parasite of seedlings of many important species including apple, cotton and flax. In years prior to 1967 *Pythium* species were considered to be universally homothallic i.e. sexual reproduction occuring in a colony obtained from a single uninucleate spore: the thallus being self fertile. However, Papa *et al* (1967) showed that the species *Pythium sylvaticum* would only reproduce sexually if two compatible strains were present and thus it was heterothallic. Heterothallism has also been reported in other Oomycetes e.g. *Achlya* (Raper 1939), *Phytophthora* (Ashby 1922, Savage *et al* 1968), and *Bremia* (Michelmore and Ingram 1980, 1981, 1981).

Sexual reproduction has been extensively studied in *Achlya* and *Phytophthora* and less so in *Pythium*. Sexual reproduction is

initiated in these species by the induction of morphologically distinct organs. In Achlya and Pythium spp.; heterothallic species, the male organ (the antheridium) is tubular or lobed and occurs on fine branches extending from the main hyphae. The female organ (the oogonium) is spherical, either forming intercalary or terminally, on short lateral branches. These sex organs come into contact and fertilization occurs by the production of a tube which carries the male gametes into the female gametangia. In Phytophthora, the oogonia and antheridia develop in contact with each other. Although, the sexual structures and the final outcome are similar, the sexual relationship in the species Achlya and Pythium differs from that found in Phytophthora. Pythium spp. and Achlya spp. show relative sexuality where strains act as either male or female depending on the strain with which they are paired. Heterothallic Phytophthora spp. exhibit two mating types A_1 and A_2 although the strains range from strong ♂ to strong ♀ within each mating type. (Savage et al 1968).

1.2. SEXUAL REPRODUCTION IN ACHLYA

Two heterothallic species of Achlya, Achlya ambisexualis and Achlya bisexualis have been extensively studied by Raper (1939-1952). These strains show classical relative sexuality, where each strain reacts according to the sexuality of the other mating strain. Strains can be placed in one of four groups depending

on their sexual reaction.

(1) PURE MALE:- will act only as a male producing antheridial branches but no oogonial initials.

(2) PREDOMINANT MALE:- which can act as male or female depending on which it is paired with. If the strain in question is paired with a strong male it will act as female and produce oogonia. If it is paired with a strong female it will adopt male sexual characteristics, producing antheridia and inducing oogonial production in the female. It does, however, tend to act as male more than female.

(3) PREDOMINANT FEMALE:- similar to system found in the predominant male strain but tends to act more as female than male.

(4) PURE FEMALE:- acts only as a female i.e. produces oogonia but no antheridia.

The male strains appear to be sexually stable whereas the female strains may produce a few oogonia and antheridial branches without being induced by a male strain. Members of predominantly male and predominantly female strains may also produce antheridia and oogonia in single culture. Fertilization and development of

oospores does rarely occur in these single cultures but usually only pairings between the different strains are fertile (Raper 1940). Reactions can also occur between homothallic species and male and female strains of heterothallic species (Raper 1950). Matings between heterothallic strains and between heterothallic and homothallic strains can result in various sexual reactions. Strains may be completely compatible i.e. mature oospores are formed or they may be incompatible i.e. no reaction occurs. They may also evoke some response with the formation of sexual organs but no fertilization.

1.3. HORMONE THEORY

When male and female plants are placed together, the development of sexual structures proceeds in several definite steps. Firstly, a large number of thin, gnarled branches with numerous lateral branches (antheridial branches) can be seen on the male hyphae. Oogonial initials then form on the female hyphae. The antheridial branches grow towards the oogonial initials where the antheridia become delimited. After delimitation of the antheridia, oogonial delimitation also occurs. These studies on sexual reproduction in A.bisexualis and A.ambisexualis suggest that the formation of sexual structures and the succeeding events may be controlled by a series of hormones secreted by the male and female. This is further

substantiated by the fact that contact between the hyphae of the two strains appears to be unnecessary for the initiation of sexual reproduction since activation will occur when the male and female are approximately 6mm apart on agar. Fertilization to form oospores does not, however, occur until the strains come into contact.

Raper (1939) proposed that four hormones A,B,C and D were involved in the process. First, hormone A was produced by the female hyphae and induced the male to produce antheridial branches. He suggested that hormone A was produced by the female at all times without prior contact with the male strain. This induced male then released another hormone, Hormone B, which appeared to initiate the formation of oogonial initials on the female hyphae. The antheridial branches of the male plant were thought to grow towards the female in response to the production of a third hormone, Hormone C, by the female.

It appeared from work carried out by Barksdale (1967) that Hormone A and Hormone C were identical. Polystyrene beads were coated with Hormone A and antheridial branches were attracted to the beads and antheridia were delimited. This hormone also induced delimitation of the anteridia which then emitted Hormone D causing delimitation of the oogonia on the female. There seems to have been no further investigation into the existence, function

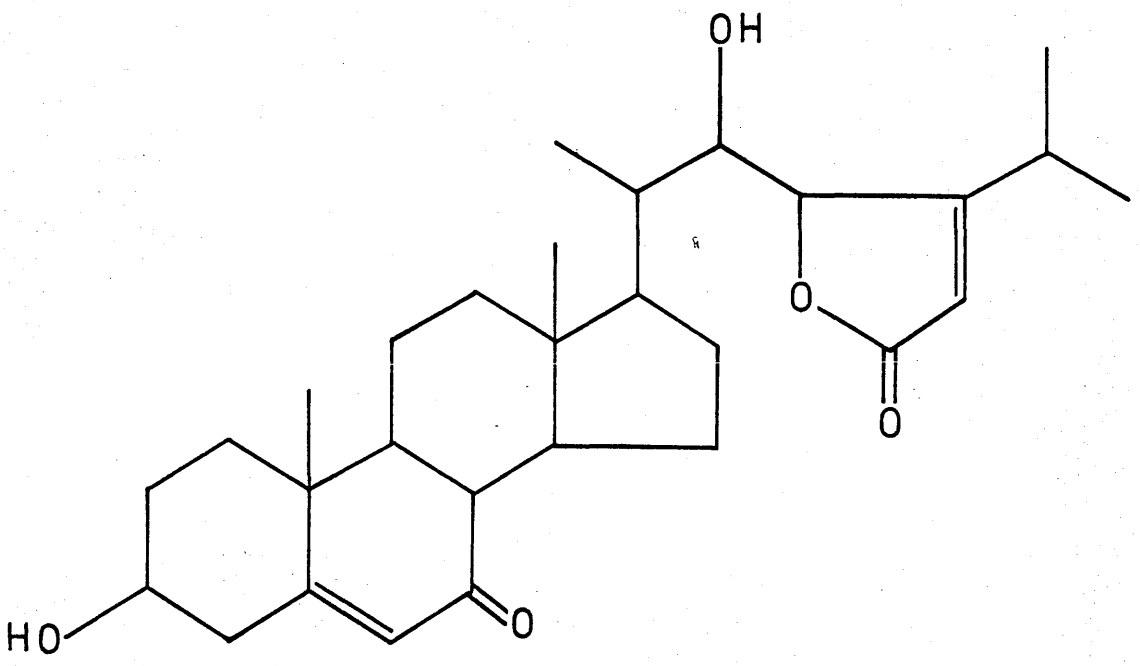
or structure of Hormone D. A fifth hormone (Hormone A^1) is indicated which may be released by the male strain resulting in an increase in the response of the male to Hormone A (Raper 1942). Raper's scheme is a sequential reaction with the later events being blocked by interuption of the initial stages. Predominantly male and predominantly female plants have the ability to act as either male or female suggesting that each strain has the ability to produce all the above hormones. The sexual activity of a strain probably depends on the quantity of hormone produced and the ability of the plant to react to the hormone (Raper 1940).

1.4. HORMONE A ISOLATION AND CHARACTERISATION

Techniques have been developed which enable sufficient quantities of Hormone A to be extracted for use in chemical analysis (Raper and Haagen-Smit 1942). A.bisexualis female was used as a source of Hormone A and A.ambisexualis male to test for the Hormone A in a bioassay system. These species were chosen initially due to their sexual reactions in mating studies. A.bisexualis produced large amounts of Hormone A and A.ambisexualis has been shown to react dramatically to Hormone A by producing numerous antheridial branches. The reaction with A.ambisexualis was easily detected even with low concentrations of the hormone.

Hormone A was found in culture medium in which female strains, but not male strains, had been growing (Barksdale 1963). This confirmed Raper's belief that Hormone A was produced constantly by female strains. The other events in the sequence did not occur, however, unless compatible mating strains were paired. In cultures containing both male and female strains the concentration of Hormone A equalled that of a male strain grown alone. This suggested that the males may have the ability to take up Hormone A from the medium. It has also been observed that the higher the concentration of the hormone the faster it was accumulated. Thus the strains which take up Hormone A readily produced antheridia, those strains which produced oogonia had an inability to utilize Hormone A. Barksdale (1967) found that Hormone A actually appeared to inhibit the formation of oogonia. Although only branches which were induced by Hormone A delimited antheridia, the concentration of nutrients, particularly nitrogen, determined whether vegetative hyphae or antheridia were formed from a Hormone A-induced branch. Both the concentration of Hormone A and that of nutrients determined the number of branches produced (Barksdale 1970). This relationship between nutrient concentration and antheridial formation was very similar to that reported by Raper (1942) on the relationship between antheridial formation and the concentration of Hormone A^1. Culture filtrates from the female plant had a similar effect to Hormone A^1 on antheridial formation.

Figure 1 Structure of antheridiol.


OH
O
O
HO
O

Hormone A was isolated in crystalline form and named antheridiol (McMorris and Barksdale 1967). Culture liquid of A.ambisexualis was extracted and chromatographed with ethyl-acetate as the solvent on silica gel, followed by TLC with Chloroform/Methanol. 1mg of crystalline Hormone A was isolated from 85 l of culture fluid. 10pg ml^{-1} of this pure hormone was sufficient to induce branching in the male strain of A.ambisexualis E87. Antheridiol has since been characterised (McMorris and Barksdale 1967), and the structure is now known (fig. 1).

It appears that antheridiol is a very specific molecule since any modification in the side chain results in a loss of activity. This was illustrated in experiments involving the addition of 53 steroids to strains of Achlya to determine if a response could be induced. 34 were completely inactive including 18 steroids with hormonal activity in mammals and 16 structurally related to antheridiol. The C-22 and C-23 sterioisomers of antheridiol had considerably reduced activity. Another steroid was found in the culture fluid, 23-deoxyantheridiol which is structurally related to antheridiol; this had a reduced effect in initiating antheridial branches (Barksdale et al 1974).

1.5. EFFECT OF HORMONE A

Although it is known that steroid hormones are produced by Achlya, their mode of action has not been completely determined. Radioactive binding studies with a synthetic antheridiol suggested that there may exist within the cell a specific antheridiol binding protein. By comparison with adding other steroids and steroid hormones this specificity for antheridiol was confirmed (Riehl et al 1984).

Quantitative changes in protein synthesis and RNA synthesis have been noted (Riehl et al 1984, Horgen 1977). Before any impact can be made on cell metabolism, the hormone must enter the cell and be transported to the action site. It has been suggested that this system may be similar to that found in mammalian tissues which react to steroid hormones. In mammalian tissues the hormone first enters the cell and binds to a specific protein receptor which carries the hormone to the nucleus. En route the protein is modified. On entering the nucleus the protein-hormone complex binds to a specific activation site where it affects RNA synthesis both quantitatively and qualitatively. The sterol ring system of antheridiol and the oogoniols is similar to that found in steroid hormones found in mammals. It is, therefore, not unreasonable to suggest that the uptake system may be similar, although, as already stated these hormones are specific and not known to have any effect on higher eukaryotes. Once in the cell antheridiol appears to affect both

RNA and protein synthesis. Total cellular RNA production increases at first, as demonstrated by the incorporation of (^{3}H) uridine by the organism, approximately 30 minutes after the addition of the hormone. The new RNA formed is ribosomal RNA (rRNA). After about 3 hours the hormone also stimulates the incorporation of the amino acid (^{3}H) leucine into protein. This occurs just before or on the appearance of antheridial branches in the male. An increase in messenger RNA (mRNA) production is also recorded after 3 hours. The hormone affects both gene activation and gene product accumulation in the form of new proteins. It has been suggested that prior to the formation of antheridial branches a localized softening of the hyphal wall must occur, due to the enzyme cellulase; one of the new gene products has in fact been shown to be the enzyme cellulase (Thomas and Mullins, 1965).

1.6. HORMONE B - ISOLATION AND CHARACTERISATION

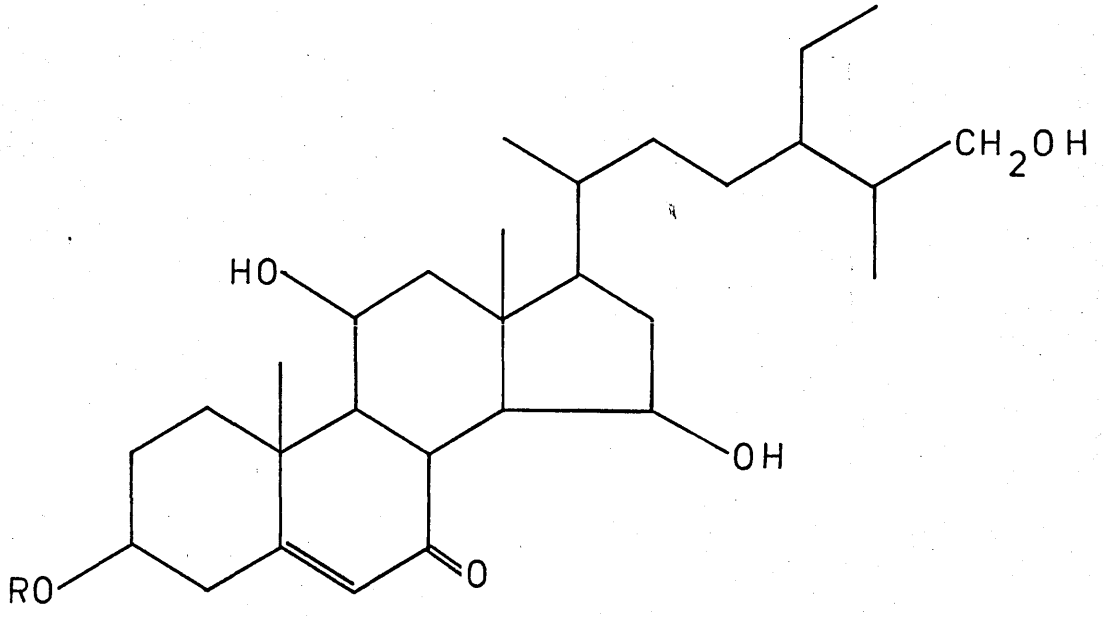
Hormone B which is produced by the induced male strain has also been isolated and characterised. (Barksdale and Lasure 1973, Barksdale and Lasure 1974 and McMorris et al 1975). The hormone was originally isolated from A.heterosexualis, a homothallic species. Hormone B is produced by the male without induction by a female strain, although production is stimulated by antheridiol produced by a female strain. Crystalline compounds have now been isolated with Hormone B activity, renamed Oogoniol

Figure 2 Structure of oogoniols.

In oogoniol -1 R = $(CH_3)_2$ CHC = O

oogoniol -2 R = $CH_3CH_2C{=}O$

oogoniol -3 R = CH_3 C = O


CH2OH
HO
OH
RO
O

-1, -2 and -3 (McMorris *et al* 1975). (Fig. 2)

As can be seen from the structures of the two hormones (antheridiol and oogoniol) the oogoniols differ from antheridiol in not having the lactone ring on the side chain in the substituents at C7, C11 and C15 and in being esterified at C-3. Because of the similarity it was initially suggested that Hormone B was derived from Hormone A but it has since been shown (McMorris and White 1977) that antheridiol and oogoniols are both derived from fucosterol but by different pathways. The mycelium of *Achlya* has been found to contain 24-methyl cholesterol and cholesterol as well as fucosterol (McMorris 1978).

1.7. SEXUAL REPRODUCTION IN PYTHIUM AND PHYTOPHTHORA SPECIES

Sexual reproduction processes in heterothallic *Pythium sp.* have been reported (Papa *et al* 1967) as being very similar to that found in *Achlya*. The sexual pattern closely approximates dimorphism, wherein sexes are segregated into mating thalli. *Pythium sylvaticum* only forms sexual structures when two compatible strains are paired. Oogonia do, however, appear occasionally in old cultures of female strains especially around the inoculum. Pratt and Green (1973) also found that male strains in single culture on grass leaves often produce oogonia.

As already mentioned, sexual reproduction in *Phytophthora sp.*

differs from that found in Achlya spp.. Galindo and Gallegly (1960) observed matings between various isolates of Phytophthora infestans, where results indicated that there were two mating types (compatibility types), A^1 and A^2, each being bisexual. Relative degrees of maleness and femaleness were recorded within each mating type. In some cases a given isolate may act as a male and in others as a female. The grading from strong male to strong female is similar to that already referred to in Achlya. As would be expected matings between males and females of opposite mating types resulted in the formation of oospores. It was also possible, however, to get numerous oospores when two strains which were both relatively strong male or strong ♀ were paired, provided, of course, that they were opposite mating types. These observations clearly illustrate the differences in mating in Phytophthora and Achlya and Pythium since on pairing strong males or females of the latter two genera no reaction occurs.

1.8. STEROL REQUIREMENT FOR SEXUAL REPRODUCTION

Although Pythium and Phytophthora differ in their mode of sexual ity they are both members of the Pythiaceae and as such have other physiological characteristics in common. The one characteristic which was of particular interest in this project was these organisms' inability to synthesize sterols. In

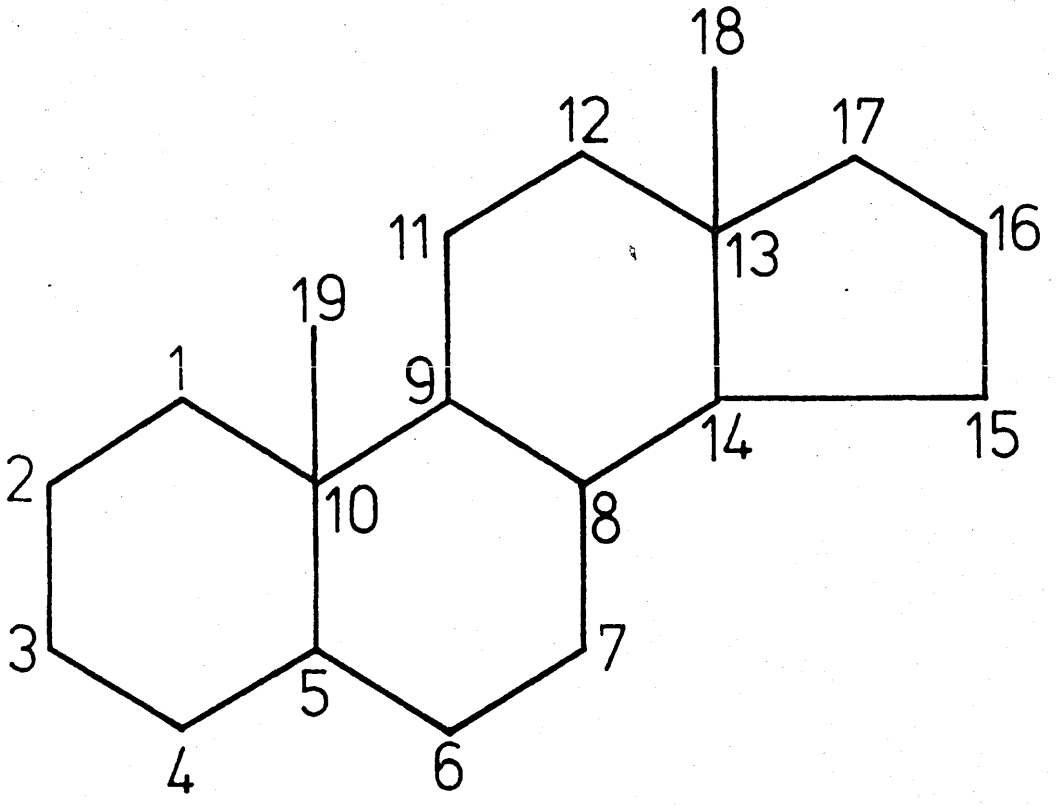
Phytophthora, although vegetative growth occurs without sterol, it was enhanced by the addition of sterol to the medium, (Hendrix 1965, Gonzales and Parks 1981, Schlosser and Gottlieb 1968). Sexual reproduction does not, however, occur without the presence of sterols, (Alhassan and Fergus 1968, Schlösser and Gottlieb 1968). Early workers observed the effects of vegetable oils and other fats on sexual reproduction in Pythium sp.. Haskins et al (1964) identified the most active fraction of sunflower seed oil as a phytosterol, β-sitosterol.

Cholesterol and other sterols of similar structure were also reported as being active in inducing sexual reproduction. Hendrix (1965) also noted that cholesterol induced sexual reproduction in Pythiaceous species, but that it had no effect on sterol producing species e.g. Saprolegniaceae and Mucorales. In their natural habitat, the fungi can utilize the host plant sterols but when grown under laboratory conditions sterol must be added to the medium. Not surprisingly the major plant sterols, sitosterol and stigmasterol are the most effective in inducing sexual reproduction.

1.9. STEROL SYNTHESIS

All steroids originate from the linear triterpene squalene ($C_{30}H_{50}$) which is derived from mevalonate and ultimately from

Figure 3 Numbering of C-atoms in 4-ringed structure common to all sterols.


18
12
17
11
16
13
19
1
9
14
15
2
10
8
3
7
5
4
6

acetate.

That Pythium and Phytophthora species are unable to synthesize sterols was shown by the absence of sterol in mycelia grown on sterol-free medium (Elliott et al 1964), and by the failure to incorporate ^{14}C of acetate with digitonin - precipitated material (i.e. sterol) (Hendrix 1966). Schlosser and Gottlieb (1969), using gas chromatographic techniques, demonstrated the presence of small amounts of squalene but no lanosterol or ergosterol in species of Pythium. On adding ^{14}C acetate to a culture of P.graminicola no squalene was found suggesting a block at, or before the formation of squalene.

Wood and Gottlieb (1978) demonstrated the point at which the block occurs in the sterol biosynthetic pathway. Two organisms were compared, Rhizoctonia solani, a sterol producer, and Phytophthora cinnamomi, which is unable to synthesize sterols. In P.cinnamomi small amounts of squalene were produced but no squalene epoxide or sterol when the organism was grown on sterol free medium. In R.solani, however, both the epoxide and the sterol (ergosterol) were found under the same conditions. When the two strains were fed (^{14}C) acetate, only labelled geraniol, farnesol and squalene were recovered in P.cinnamomi whereas in R.solani, ergosterol was again formed as an end product. Similar results were recorded when the strains were fed (^{14}C) mevalonate.

From these results it appears that squalene is the final compound in the sterol synthesizing pathway of P.cinnamomi. Not only is the enzyme responsible for the conversion of squalene to squalene epoxide missing but also those inducing the cyclisation of squalene epoxide and the conversion of lanosterol to ergosterol.

In animals and ergosterol-synthesizing fungi, the product of squalene cyclization is lanosterol, but in flowering plants it is cycloartenol. In this respect, Oomycetes appear to resemble plants. Bu'Lock and Osagie (1976) reported the conversion of cycloartenol to fucosterol in Saprolegnia ferax. Warner et al (1982) found evidence for a vestigal sterol synthetic pathway, derived from cycloartenol in several Oomycetes including Phytophthora and Pythium spp.

The work followed the earlier observations of Domnas et al (1977) that zoospores of Lagenidium giganteum, an Oomycete which parasitises mosquito larvae, were not produced unless exogenous sterol was supplied. They found that on the addition of mevalonic acid to the growth medium, sterol was synthesized, though only in small amounts. However, when lanosterol was added no sterol was recorded. On feeding the organism cycloartenol, fucosterol and cholesterol were produced. Warner et al (1982) suggested that in members of the Peronosporales exogenous cycloartenol can be metabolized to lanosterol and in some organisms to fucosterol,

Figure 4 Structures of various sterols. (groups added at C-17)

1. Cholesterol, 2. 24-B-methylcholesterol, 3. Sitosterol
4. fucosterol, 5. brassicasterol, 6. desmosterol.
7. Stigmasterol.

OH
1
2
3
4
5
6
7

cholesterol and ergosterol. Nes and Patterson (1981), however, reported that Phytophthora cactorum was unable to transform cycloartenol to lanosterol or other sterols. Although Phytophthora spp. cannot synthesize sterol, they do have the ability to modify sterol which is added to the medium e.g. Δ^7 and $\Delta^{5,7}$ sterols are converted to Δ^5 sterols by Phytophthora cactorum (Knights and Elliott,1976).

1.10. WHICH STEROLS ARE ACTIVE?

Not all sterols are effective in stimulating sexual reproduction. Researchers in this field have reported certain structural requirement for induction (Elliott et al 1966, Elliott 1972, 1979, Nes et al 1980, Nes and Stafford, 1983, 1984). (Fig. 4).

Activity of the sterol molecule is associated with the size of the side chain at C-24, the configuration of this side chain, the position of the double bond in the B-ring and the hydroxyl group at C-3 of A-ring (Fig. 3 and 4).

Δ^5 sterols differing in the structure of their side chain were used as medium supplements for a strain of Phytophthora cactorum and the oospores production measured (Elliott 1979). Oospores production increased with increasing size of the side chain at C-24 i.e. H CH_3 C_2H_5 etc. When this chain was 24α-methyl

it was more active than in the 24 β-methyl configuration. There was, however, little difference in effects of configuration of ethyl groups at C-24.

Sterols with the double bond at C-5 are more active than Δ^7 sterols when added in low concentration. On adding higher concentration no consistent relationship was observed (Elliott 1979). Loss of the hydroxyl group at C-3 results in a loss of activity (Elliott et al 1966).

Good inducers are, therefore, sterols with a double bond at C-5 hydroxyl group on C-3 and a chain of 8-10 carbon atoms on C-17. e.g. cholesterol, sitosterol and campesterol.

Child and Haskins (1971) found the requirement for sterol of P.sylvaticum and P.catenulatum was similar to that in Phytophthora and that only sterols with a structure similar to that of cholesterol were active in inducing sexual reproduction.

The number of oospores formed depended on which strain of P.sylvaticum had access to the sterol (Pratt and Mitchell 1973). Most oospores were formed when sterol was added to both strains. There were, however, more oospores when sterol was added to the female and not to the male than if added to the male and not the female. Fewest were formed when sterol was added to neither strain.

1.11. ROLE OF STEROLS

The role of sterols in sexual reproduction has not as yet been established. Researchers in this field tend to adopt one of two theories:- (1) that the sterol is involved in altering some aspect of the cell membrane, which somehow thereby affects reproduction Or (2) that some conversion of the sterol occurs resulting in the formation of, for example, a hormone.

1.12. STEROLS IN MEMBRANES

The effect of sterols on vegetative growth is thought to be due to their ability to fit well into phospholipid membranes affecting membrane fluidity and permeability, without themselves undergoing structural alteration.

Hyphal membranes are basically a bilayer of lipid interspersed with protein (including glycoprotein) molecules. The lipids found in these membranes are primarily phospholipids, which are composed of a glycerol backbone; two of the OH groups being esterified with longchain fatty acids and the third a phosphorylated alcohol.

Sterols in phospholipid membranes stabilize the bilayer reducing movement of the fatty acyl chains. It is thought (Phillips and

Finer 1974) that when a sterol molecule lies alongside a phospholipid molecule with the polar group of the lipid and the hydroxyl group of the sterol lying side by side the first 8-9 methylene groups of the fatty acid chain on the phospholipid are constrained by the nucleus of the sterol molecule.

Incorporation of sterol into the membrane increases the membranes ability to resist stretching. The strong interaction between the fatty acyl chains and the unsaturated sterol side chain prevents rupture of the membrane. Stabilization of the membrane is affected by the presence of a double bond a C-22 of the sterol molecule (Hossack and Rose 1976).

1.13. STEROL EFFECTS ON GROWTH

The beneficial effects of sterols on growth in *Phytophthora spp.* are believed to be due to their good fit into membranes. In *P.cactorum*, cholesterol, campesterol and sitosterol promote growth. 5α- cholestanol, 5α- cholestan -3-one and epicholestanol showed no significant effect on growth. 5β - cholestanol and coprostanol slightly inhibit growth. Saringosterol, carpesterol and 20-hydroxycholesterol strongly inhibit growth. Sterols which promote growth contain a double bond at C-5, a hydroxyl group at C-3 in the β position and a side chain on C-17 with 8-10 carbon stoms (Nes *et al* 1979).

Hossack and Rose (1976) grew strains of S.cerevisiae under anaerobic conditions to induce the requirement for an unsaturated fatty acid and sterol. They reported that although sterols alter the membrane, cells enriched with sterol did not differ in volume, growth rate, content of free sterol, ester and phospholipid or phospholipid composition. They concluded that sterols have little effect on the regulation of the membrane lipid metabolism but that they probably act as structural components of the membrane.

Nes et al (1979), however, suggested that although the sterol did not increase absolute growth rate during the log phase of growth (in Phytophthora cactorum) the growth rate in the lag phase was stimulated. Varying the structure of the sterol fed to a sterol dependent mutant was also reported by Parks et al (1980) as having a considerable effect on growth in Saccharomyces cerevisiae. Different sterols alter the organisms growth rate over different pH ranges. The pH range over which growth occurs when ergosterol is added to the medium is greater than when cholesterol was added. Parks suggested that this may be a clue as to the aspect of cell physiology most sensitive to changes in the sterol structure. From results with sterol mutants of Saccharomyces cerevisiae, Parks proposed that there was a bulk phase transition occuring in the mitochondrial membrane altering its permeability. The transmembrane potential required for oxidative phosphorylation was destroyed. It appears that the presence of sterols in the

mitochondria of these mutants may prevent this phase transition in the mitochondrial membranes.

In Phytophthora sp. glucose consumption and CO_2 production were monitored in log phase cultures grown with sterols (Parks et al 1982). The uptake of the glucose did not increase; however, the evolution of CO_2 increased 3-fold. Little or no change was observed in the activity of the glycolytic enzymes and it has been suggested that the presence of sterol in the cell affects oxidative phosphorylation. Reduced amounts of cytochrome were noted in cultures grown without sterol.

1.14. HOW DOES EFFECT OF STEROL ON MEMBRANES RELATE TO THEIR EFFECT ON SEXUAL REPRODUCTION?

The sterol as well as providing an architectural function in the membrane also, due to its ability to fit well into phospholipid membranes, affects the physiology of the cell, including the effects on sexual reproduction.

Child et al (1969) noted that when cholesterol was added to cultures of Pythium sp. there was a decreased leakage of nucleotides, nitrogen and protein constituents of the mycelium, but that the leakage of carbohydrates was significantly increased. They suggested that the induction of sexuality and the formation of

spores may partially be associated with enhanced starvation for carbohydrate.

In another Oomycete, Lagenidium giganteum, a parasite of mosquito larvae, which also requires sterol for sexual reproduction, a role has been suggested involving the membrane bound enzyme adenylate cyclase (Kerwin and Washino 1984). It is possible that there is a sterol-induced alteration in membrane fluidity around the enzyme when the sterol associated with it. This association may account for the specific structural requirements of the sterol. If the sterol interacts with this enzyme, it is possible that the enzyme activity may be altered. Adenylate cyclase catalyses the conversion of ATP to cAMP and P_1.

In high concentrations cAMP was found to reduce oosporogenesis; therefore, if the sterol modifies the enzyme activity, it may reduce the conversion to ATP to cAMP thus increasing oospore production.

1.15. EFFECT OF CALCIUM ON OOSPOREGENESIS.

It is important at this point to mention calcium which also has a significant effect on oospore production (Lenney and Klemmer 1966, Yang and Mitchell 1965 and Elliott 1972). On the addition of Ca^{2+} to the medium oospore production is increased. Oospore

production may also be linked to the concentration of cAMP (Kerwin and Washino 1984). Ca^{2+} modifies the activity of the phosphodiesterase which converts cAMP to adenosine 5^1-phosphate (AMP) and P_1. This in effect reduces the concentration of cAMP. Steroid hormones may also affect phosphodiesterase activity indirectly by changing the permeability of the membrane thus increasing the intercellular concentration of Ca^{2+} which may in turn activate the enzyme and reduce the concentration of cAMP. Incorporation of phosphodesterase inhibitors results in a block in metabolism of cAMP in L.giganteum and reduces or eliminates oosporogenesis. Calcium may also contribute to the lowering of cAMP concentration by inhibiting adenylate cyclase. (Pall 1981).

Although sterol is not required for vegetative growth, its presence in the medium does enhance growth. The previous argu ment suggests that the mechanism by which sterols promote sexual reproduction and enhance vegetative growth is similar, i.e. that sterol is incorporated into the cell membrane affecting permeability. If this was the case, it would be expected that the same sterols which affect vegetative growth would stimulate sexual reprodcution. This, however, is not always found. Increased vegetative growth results from the addition of sterols and pentacyclic triterpenoids with wide ranging structures, whereas, in sterol reproduction only sterols with particular structures have this ability.

Also, sterols affect membranes in different ways. The way a sterol affects a membrane is not necessarily an indication of its capacity to induce sexual reproduction. For example, sitosterol and stigmasterol have different effects on the membrane and yet their inducing capacity is very similar, whereas, sitosterol and cholesterol have similar effects on the membrane but sitosterol is better inducer of sexual reproduction than cholesterol (Elliott 1983).

These findings do not rule out that the membrane is somehow involved but they do suggest that there must also be other factors involved.

1.16. STEROL METABOLISM

It has been suggested that the differences in a sterols ability to induce sexual reproduction were not due to differences in uptake or effects on the membrane but possibly to differences in metabolism of the sterol and variation in the activity of the hormones derived from them.

Elliott and Knights (1981) have shown that in Phytophthora cactorum sitosterol and cholesterol are converted to various metabolites. When cultures of this organism were grown in medium supplemented with cholesterol and sitosterol, the cholesterol was selectively

incorporated into the free sterol fraction, whereas, sitosterol was metabolized to a relatively greater extent to esters and polar material. As already stated sitosterol is more effective than cholesterol at promoting sexual reproduction and this may be due to the large amount of polar metabolites formed.

Nes et al (1982) reported that certain sterols as well as triterpenoids stimulate growth, but that only sterols induce significant oospore production. Differences were noted in the metabolism of sterols and triterpenoids. Sterols were converted to both esters and glycosides, whereas, triterpenoids were only esterified. This again suggests that the metabolites produced may be important in sexual reproduction.

When esters such as cholesteryl palmitate, cholesteryl acetate, and cholesteryl oleate were added to medium with cholesterol, the free sterol was initially taken up more quickly but after 24h the uptake of the esters was equal to that of cholesterol (Elliott and Knights 1974, 1981). Cholesteryl palmitate and cholesterol were added together to cultures of Phytophthora cactorum; the cholesteryl palmitate was converted to free sterol and the cholesterol was metabolized to esters of linoleic, linolenic and oleic acids and metabolites more polar than cholesterol.

Polar metabolites have also been noted in Pythium sp. When Pythium periplocum was grown in medium supplemented with cholesterol two metabolites were observed: one was a sterol ester, the other a compound more polar than cholesterol. Similar metabolites were obtained when sitosterol was added instead of cholesterol. The polar metabolites were confined to the mycelium, whereas the ester was found in both the mycelium and the medium (Hendrix et al 1970).

In Pythium sylvaticum (McMorris et al 1977) cholesterol β-D-glucoside -6^1-O- palmitate was identified as the polar metabolite in mated culture of this organism in the presence of cholesterol. Again the equivalent sitosterol ester was observed when sitosterol was substituted for cholesterol. Esters of myristic and stearic acid were also found.

Sterol metabolism to steroid hormones has been shown to occur in the Oomycetes. As already stated sexual reproduction in Achlya sp is controlled by a number of hormones derived from sterol metabolism.

Ko (1978) has shown that agents inducing sexual reproduction are released by heterothallic Phytophthora species when compatible mating types are paired. Homothallic strains have the ability to induce sexual reproduction in some heterothallic strains. He paired strains of Phytophthora on agar discs separated by a

polycarbonate membrane. These membranes prevent the hyphae of the strains coming into contact but allow the transfer of substances through the membrane. In these matings sexual reproduction was induced and self oospores produced, suggesting the presence of a diffusible agent. The agent produced by A_1 isolates of Phytophthora is called Hormone α_1, and that produced by A_2 strains, Hormone α_2. Hormone α_1 appears to induce sexual reproduction in A^2 isolates and α_2 will affect A^1 isolates. A^1 strains appear to form more oospores in culture than A^2. Young cultures of A^2 isolates are better producers and A^1 isolates are more responsive to these agents. It has not been established whether hormones α_1 and α_2 induce sexual structure formation directly or whether they induce the production of other hormones which in turn induce antheridial and oogonial production.

CHAPTER 2

METHODS, MEDIA AND NUTRITION

2.1. GENERAL MATERIALS AND METHODS

1. Organism

The organism used throughout this investigation was *Pythium sylvaticum*. The strains were designated as follows:-

431a-CBS 453.67, ATCC 18196, T, from soil under seedlings of *Prunus persica* (isolated by F.F. Hendrix Jnr.).

431b-CBS 452.67, ATCC 18195, T.

431c-BKW (Moscow) F1513.

431d-CBS 232.68, from soil, Netherlands.

431e-CBS 234.68, from soil, Netherlands.

The cultures were obtained from Dr M.W. Dick, University of Reading, and throughout the text will be referred to as *a*,*b*,*c*,*d* and *e*.

11. Maintenance of cultures

The strains were maintained on slopes of potato carrot agar in loz bottles, and stored at room temperature, covered with sterile paraffin oil.

111. Culture conditions

a) Basic Medium. Throughout modified Schmittenner's medium (Waterhouse, 1967) was used:-

sucrose	2.50g
asparagine	0.27g
KH_2PO_4	0.15g
$MgSO_4.7H_2O$	0.10g
$CaCl_2$	0.10g
trace element solution	1.0mls
phthalic acid	2.00g
distilled water	1 litre

The pH of the medium was adjusted to pH6 with NaOH.

Trace element solution

$Na_2B_4O_7.10H_2O$	88mg
$Cu\ SO_4.5H_2O$	393mg
$Fe_2(SO_4)_3.6H_2O$	910mg
$Mn\ Cl_2.\ 4H_2O$	72mg
$(NH_4)_6\ Mo_7O_{24}.4H_2O$	37mg
$ZnSO_4.\ 7H_2O$	4403mg
EDTA	5g
Water	1 litre

The medium was autocalved at 121°C, 15psi for 20 minutes.

b) Specific Media

(1) Solid Medium. Schmitthenner's medium was used as stated above for all liquid studies. For solid media exercises, Difco agar was added. 20g of powder was added to 1 litre of medium (2% W/V) before autoclaving. In one exercise 16g agarose was substituted for Difco agar.

(11) Sterol addition. It was noted that sexual reproduction did not occur in the absence of sterol. A solution was made up of sitosterol in acetone at a concentration of 1.0mg ml^{-1}. $10ml^{-1}$ of this solution were added to both liquid and solid media, when required, before autoclaving. Tests showed that there was no difference in the inducing capacity of autoclaved and unautoclaved sterol.

c) Inoculum production. 25ml aliquots of Schmitthenner's with agar added were dispersed into 9cm Petri dishes. When set, these plates were inoculated with 3mm blocks of agar containing mycelial growth. After incubation at 24°C, the plates were used for inoculating test plates.

d) Incubation. All plates and flasks throughout this work were incubated at 22-24°C. The period of incubation is stated for

each individual exercise.

IV. Mating Studies

In order to observe sexual reproduction in this heterothallic species, it was necessary to pair the strains. They were paired in either solid of liquid media as follows:-

a) Solid Medium

i) 15ml aliquots of media were dispersed in 9cm Petri dishes and inoculated on both edges of the plate. The inoculum consisted of $\sim$ 3mm plugs, cut using a size 2 cork borer, from the culture plates described in 3c. These plates were incubated at 24^{o}C for 72h or until the advancing hyphae met in the centre of the plate.

ii) In most of the pairing exercises a standard method was involved. 15ml of solid Schmitthenner's medium in 9cm dishes was cut into four equal discs using a large borer $\sim$33mm in diameter and each one inoculated in the centre with a disc of an agar culture of the various strains as already described. These plates were incubated for 72h. The 33mm discs were lifted out and paired, one above the other with a polycarbonate membrane (pore size 0.2mm obtained from Nuclepore Corporation, California) between them. The membrane prevented

the hyphae of the two strains coming into contact with each other. Controls of strains paired with themselves were also set up.

On separating, the surface of the disc in contact with the polycarbonate membrane was examined.

iii) Examination The paired cultures were examined using a Nikon microscope (x10 eyepiece, x10-x40 objective).

The number of oogonia formed in each pairing was noted in three fields in each of 5 discs, making a total of 15 fields. The results were analysed by nested analysis of variance.

b) Liquid medium 20ml aliquots of Schmitthenner's medium were added to 100ml flasks and the flasks autoclaved. The flasks were inoculated in the same way as the agar plates using cork borer discs of various strains. The flasks were, however, incubated for longer, usually 5 days, before pairing. To pair, the contents of the two flasks were combined. The contents of the flask was poured into a glass Petri dish for microscopic examination. Three fields were in each of 5 pairings (15 fields total) and statistical analysis carried out to determine differences between the different pairings.

2.2. TO DETERMINE IF SITOSTEROL IS REQUIRED FOR THE FORMATION OF SEXUAL STRUCTURES (OOGONIAL INITIALS AND ANTHERIDIAL BRANCHES)

Flasks containing 10ml of SM with and without sitosterol were inoculated with discs of agar with inoculum and incubated at 24°C, each strain was inoculated alone and paired with all other strains. After 3-5 days, the contents were emptied into sterile glass Petri dishes and examined for the production of oogonia. Different sterols were also compared, i.e. sitosterol, cholesterol, and cholesteryl palmitate.

Cultures containing sterol produced sexual structures, providing a compatible mating type was present. In cultures with no sterol, few oogonia were formed.

When different sterols were tested for their effect on sexual reproduction, the following results were obtained:-

pairing		a/e		c/e		c/a		d/d	e/e	a/a	c/c
medium	strain examined	a	e	c	e	c	a	d	e	a	c
Schmitthenner's medium (SM) + sitosterol		50	34	174	9	164	37	54	34	36	14
SM + cholesterol		64	38	143	8	139	45	65	20	32	11
SM + cholesteryl palmitate		49	44	206	9	164	53	55	19	59	18

Table 1. Production of oogonia by P.sylvaticum a, c, d and e when paired with different sterols, separated by a polycarbonate membrane. The strains were grown for 72h and paired for 24h. The number of oogonia in each strain was counted on separation. Total number of oogonia in 9 fields given (3 field, 3 plates).

a with e

	Sof S	d.f.	variance	F
Total	491	26		
Between Media	16	2	8.00	0.40
Residual	475	24	19.79	NS

e with a

	S of S	d.f.	variance	F
Total	214	26		
Between Media	6	2	3	0.35
Residual	208	24	8.67	NS

c with e

	S of S	d.f.	variance	F
Total	16.96	26		
Between Media	220	2	110	1.79
Residual	1476	24	61.5	NS

e with c

	S of S	d.f.	variance	F
Total	19	26		
Between Media	0.11	2	0.05	0.063
Residual	18.89	24	0.79	NS

c with a

	S of S	d.f.	variance	F
Total	1934	26		
Between Media	46.7	2	23.33	0.30
Residual	1887.3	24	78.64	NS

a with c

	S of S	d.f.	variance	F
Total	190	26		
Between Media	14.2	2	7.1	0.97
Residual	175.8	24	7.32	NS

d

	S of S	d.f.	variance	F
Total	253	26		
Between Media	8.55	2	4.28	0.42
Residual	244.45	24	10.18	NS

e

	S of S	d.f.	variance	F
Total	93.6	26		
Between Media	15.6	2	7.8	2.4
Residual	78	24	3.25	NS

a

	S of S	d.f.	variance	F
Total	415.6	26		
Between Media	47.15	2	23.58	1.54
Residual	368.45	24	15.35	NS

c

	S of S	d.f.	variance	F
Total	25.52	26		
between Media	2.74	2	1.37	1.44
Residual	22.78	24	0.95	NS

Table 2 . Statistical analysis of the numbers of oogonia produced by strains when grown on media with different sterols added. (See Table 1).

p 5% = 3.40 p 1% = 5.61 NS = Not significant

The results above indicate that there was no significant difference in the number of oogonia formed in the strains tested when different sterols were used (Analysis of variance, Table 2). In Phytophthora cactorum (Elliott and Knights 1974, 1981) , there is evidence to suggest that although free sterol was taken up more quickly than ester initially, after 24h, their uptake was equal. It has been suggested that the esters require to be hydrolysed to the free sterol before uptake. When ester is added to the medium, it is deesterified to the free sterol which is taken up by the mycelium. It is probable that initially, the enzyme which hydrolyses the sterol is produced in the vicinity of the cell membrane. However, since the free sterol is found in the filtrate extracts, the esterase must be released into the medium.

It is useful to incorporate the sterol ester into the medium since it is less readily broken down than the free sterol. The Δ^5 bond in the sterol molecule is important for sexual reproduction. Sterol added in its free form, is readily subject to autoxidation. The acid part of the ester molecule is attached to C-3 of the A ring protecting the double bond at C-5.

2.3. EFFECTS OF GROWING e, c AND a STRAINS OF PYTHIUM SYLVATICUM IN THE PRESENCE AND ABSENCE OF SITOSTEROL IN MATING EXERCISES

Strains were grown on SM with and without sitosterol and paired

after 72h incubation at 24°C separated by a polycarbonate membrane. The pairings were reincubated and examined after 24h.

Strains paired					
c	e	c	c	e	e
+	+	+	+	+	+
+	-	+	-	+	-
-	+	-	-	-	-
-	-				

Table 3 Pairings of 431c and 431e examined.(+) represents strains grown in agar containing sitosterol and (-) those grown on agar with no sterol added.

The exercise was repeated with strains c, e and a. The strains were grown for 72h, and then paired with a polycarbonate membrane between them. After 24 hours the strains were separated. They were examined on separating and at various intervals thereafter.

strains in pairing	no. of oogonia in a	no. of oogonia in c	no. of oogonia in e
c+a+	9	170	
c+a-	6	39	
c-a+	10	201	
c-a-	0	8	
a+e+	67		6
a+e-	24		4
a-e+	87		27
a-e-	0		0
c+e+		219	0
c+e-		75	0
c-e+		335	0
c-e-		19	0
a+a+	18		
a+a-	(a+)22 (a-)4		
a-a-	4		
c+c+		3	
c+c-		(c+)4 (c-)3	
c-c-		1	
e+e+			10
e+e-			(e+)7 (e-)3
e-e-			0

Table 4 Production of oogonia by P.sylvaticum 431c, 431a and 431e when paired with and without sitosterol added to the medium. The strains were grown for 72h and paired, separated by a polycarbonate membrane for 24h. The numbers of oogonia produced by each strain was counted on separation. The figures given are the number of oogonia formed in 15 fields 5 plates, 3 fields per plate. (+) represents strains grown with sitosterol. (-) strains grown in absence of sterol.

	c/e mean sq. (D.f.)
Between all treatments	4080.0 (3)
Residual	110.4 (16)
F	40.6 ($P < .01$)

	t	
1 with 3	3.66	($P < 0.01$)
1/3 with 2	10.38	($P < 0.01$)
2 with 4	1.76	(NS)

	c/a mean sq. (D.f.)
Between all treatments	1817.0 (3)
Residual	52.0 (16)
F	35 ($P < 0.01$)

	t	
1 with 3	1.37	(NS)
1/3 with 2	13.50	($P < 0.01$)
2 with 4	1.37	(NS)

	a/e
	mean sq. (D.f.)
Between all treatments	314.2 (3)
Residual	10.7 (16)
F	29.4 ($P < 0.01$)

	t	
1 with 3	1.92	(NS)
1/3 with 2	9.19	($P < 0.01$)
2 with 4	2.32	(NS)

Table 5 Statistical analysis of results in Table 4.

Treatment 1 = ♂ and ♀ with sterol

Treatment 2 = ♀ with sterol ♂ without sterol

Treatment 3 = ♂ with sterol ♀ without sterol

Treatment 4 = ♂ and ♀ without sterol

NS = not significant.

Analysis of the results statistically showed that when strains c (♀) and a (♂) and strains a (♀) and e (♂) were paired, there was no significant differences in the number of oogonia formed when both partners were grown on sterol containing medium and when only the male had sterol available. However, there was a significant difference between the numbers formed when sterol was added to the male and when sterol was added to the female. There was no significant difference when the sterol was added to the female and when no sterol was available to either culture. The results on pairing c(♀) with e (♂) were slightly different. Again, no difference in the number of oogonia was noted when only the female had sterol available and when neither male nor female had sterol in their growth medium. More oogonia were produced when sterol was added to both strains than when sterol was added to the male only. However, the general trend is that the greatest number of oogonia produced in any combination occurs when the male strain is provided with sterol.

Pratt and Mitchell (1973) counted the numbers of oospores formed when sterol was added to male strains, female strains, both ♂ and ♀ and when no sterol was used. They noted that fewest oospores were formed when no sterol was added. Sterol added to the male side only, had no significant effect on oospores production but sterol added to the female side resulted in a significant increase in oospore formation. A further increase was noted when sterol

was added to both cultures. The method used by these researchers varied slightly from those used in this laboratory. Foil shields with small holes and water agar were used to separate the two cultures. They were able to count the number of oospores formed when the hyphae of ♂ and ♀ came into contact, whereas, in our exercises oogonia were counted, since no contact between the strains occurred and thus no fertilization and no oospores were produced. The differences in procedure, however, do not explain why the results reported by Pratt and Mitchell and our results completely contradict each other.

2.4. CALCIUM REQUIREMENT:

Strains were grown in liquid culture in SM with EGTA, (Ethylene Glycol Tetra Acetic acid) which is a chelating agent for calcium. The mycelia from these cultures was used to inoculate medium. 15ml of medium were added to sterile glass Petri dishes and each dish was inoculated with the above mycelium. The plates were incubated at 24°C and examined after 5 days. 6 different media were involved.

1 SM alone

2 SM + EGTA

3 SM + $CaCl_2$

4 SM + sit. + EGTA

5 SM + sit.

6 SM + $CaCl_2$ + sit.

The following strain combinations were used:-

c alone, e alone, d alone, dc, de, ce, a alone, ac.

d with c, d with e, c with e, a alone and a with c.

strains paired / medium	d/e	c/e	c/a	c	e	d	a
Schmitthenner's medium (SM)	20	0	0	0	0	4	0
SM + EGTA	14	0	0	0	0	0	0
SM + $CaCl_2$	63	0	0	0	0	43	0
SM + EGTA + sitosterol	46	0	0	0	0	8	0
SM + sitosterol	40	4	4	0	0	51	4
SM + $CaCl_2$ + sitosterol	54	75	75	0	0	53	36

Table 6 Production of oogonia by Pythium sylvaticum 431a, 431c, 431d and 431e when paired in different liquid media in 100ml wide necked flasks. The cultures were 72h old when they were mixed and the number of oogonia in each was counted after 24h. The figures given are the number of oogonia formed in 15 fields (5 plates, 3 fields per plate). The results of pairings which produced no oogonia were omitted from the table.

SM = modified Schmitthenner's medium.

EGTA = Ethylene Glycol Tetra Acetic Acid.

The organisms require Ca^{2+} for sexual reproduction. However, trace amounts of calcium appear to be sufficient since strains grown in media to which there has been no $CaCl_2$ and no EGTA had been added do produce a few oogonia. Strains grown in the absence of calcium, i.e. grown throughout the exercise in medium containing EGTA produced no oogonia. It is obvious from the results that different strains have different requirements for sexual reproduction. Strain d, when grown alone produces oogonia without previous induction by another, more male strain. If the strain was initially grown with a limited amount of Ca (i.e. no EGTA was added to the medium) and the resulting mycelial growth used for experimental inoculation, there was no significant difference in the number of oogonia produced in any of the 6 test media. However, if d was grown in the complete absence of calcium, the results show a slightly different pattern. When strain d was grown in SM, SM + EGTA or SM + sit. + EGTA the number of oogonia produced was less than in strains grown in SM + $CaCl_2$, SM +sit. and SM + sit. + $CaCl_2$.

It would appear that the addition of $CaCl_2$ or sitosterol has a similar effect at least in strain d.

Several workers have indicated a requirement for Ca^{++} in vegetative growth (Erwin 1968 and Hendrix and Guttman 1970) in species of Phytophthora and Pythium. Apparently some species have

an absolute requirement, e.g. Phytophthora fragariae, while in other species growth is stimulated by the addition of Ca^{++}, e.g. Pythium graminicola(Lenney and Klemmer 1966).

Sexual reproduction is also reported to be affected by the addition of Ca^{++} to the medium (Lenney and Klemmer 1966, Yang and Mitchell 1965). In Pythium graminicola calcium caused an increase in the number of oogonia and was essential for maturation since, in its absence, antheridia and oospores were not observed (Lenney and Klemmer 1966). Yang and Mitchell (1965), working with Pythium debaryanum, P.ultimum and P.irregulare, also demonstrated that Ca^{++} has a significant effect on sexual reproduction. EGTA, a calcium chelator, counteracts this reaction.

2.5. SUITABLE BUFFERING MEDIUM

It was found that the number of sexual structures formed by paired strains decreased at low pH values. For this reason, it was necessary to further modify the medium by incorporating a suitable buffering agent.

The following media were tested:-

1 SM + $CaCl_2$ + trace elements + sitosterol

2 Medium (1) + 2g phthallic acid (l^{-1})

3 Medium (1) + 5.4g sodium succinate (l^{-1})

4 (Medium (1) - asparagine) + 0.24g $(NH_4)_2 SO_4$ + 5.4g sodium succinate. (l^{-1}).

The strains were combined as follows:- c alone, e alone, d alone, in Petri dishes containing these media. d with c, d with e, c with e, a alone and a with c.

It was found that the medium containing the phthallic acid was the best buffering medium maintaining the pH at 5.6 - 6.8.

In conclusion, the medium which appears to be most suitable for sexual reproduction studies in Pythium sylvaticum contains:-

2.5g sucrose

o.27g asparagine

2g phthallic acid ($C_6H_4(COOH)_2$)

o.15g KH_2PO_4

0.1g $Mg SO_4$

0.1g $CaCl_2$

1ml trace elements

10mg sitosterol in 10ml acetone

1 litre distilled water

This medium was then used throughout this project.

Conclusions

When cultivating this organism for use in sexual reproduction studies, the medium used must observe certain criteria. Sterol, Ca^{2+} and trace elements increase oogonial production when added together or separately to basic Schmitthenner's medium. It is also necessary for the medium to be buffered sufficiently, to maintain a pH of $\sim$ 6.0.

The sterols used in this exercise did not differ in their effect on the organism, however, it did seem more important that sterol was available to the male strain than to the female strain.

CHAPTER 3

EVIDENCE FOR HORMONAL CONTROL OF SEXUAL REPRODUCTION

3.1. INTRODUCTION

Pairing with a compatible strain is necessary for sexual reproduction to be induced in this heterothallic species. The evidence for a hormonal system, similar to that found in Achlya, is reported in this chapter.

3.2. TO TEST IF CONTACT BETWEEN THE STRAINS WAS NECESSARY FOR THE INDUCTION OF SEXUAL REPRODUCTION AND INDICATION OF "MALENESS" AND "FEMALENESS" OF THE ISOLATES

Culture methods were as described in the previous chapter. After inoculation, the plates were incubated in the dark at 24°C for 72h. The strains were paired with and without polycarbonate membranes between them. As a control, the strains were inoculated on opposite edges of a 90mm plate and incubated under the same conditions as the polycarbonate pairings. The two discs were separated and the surfaces in contact with the polycarbonate membrane examined. The number of oogonia in 5 fields on each of three discs were counted and the results given in the table (Table 7), indicate the total number of oogonia noted. On the 90mm plates, the line of contact between the strains was examined for sexual activity.

	a	b	c	d	e
e	186 57	22 17	536 0	278 0	0
d	259 301	0 218	173 160	98	
c	178 479	0 442	0		
b	249 50	0			
a	15				

Table 7 Production of oogonia by five strains of Pythium sylvaticum paired in all combinations

72h old cultures were paired, with a polycarbonate membrane between them, for a further 72h, and the numbers of oogonia in each of the paired strains was then counted. Totals of 15 microscope fields are given. The upper figure of each cell is the number of oogonia produced by the culture indicated at the top of the column in combination with that indicated on the left. The lower figure of each cell is the number of oogonia produced by the strain indicated at the left in combination with that indicated at the top of the column.

Sexual reproduction was induced when the strains were separated by the polycarbonate membrane. The membrane does not allow the hyphae to pass through. When controls were set up with a disc of sterile solidified medium, a polycarbonate membrane and a disc containing cultures of any of the strains, the sterile disc remained uninfected by the fungi, since the hyphae were unable to penetrate the membrane. The polycarbonate membrane evidently allows the passage of substances between strains by virtue of the holes (0.2µm) in it. Antheridial branches and oogonia were formed when the strains were not in contact; however, the oogonia were not fertilized and thus oospores were not formed.

In the 9cm plates oogonia, antheridia and oospores were formed in the zone where male and female strains came into contact. This zone forms a sharp boundary on the male side but more diffuse on the female side. This confirmed reports of earlier workers (Papa et al 1967). The strains met after 36h and differentation occurred approximately 24h later when antheridial branches and oogonial initials were first noted. No contact between the hyphae appeared to occur until the antheridial hyphae touched the oogonial initials. This observation was similar to that noted by Raper (1940) working with A.ambisexualis where he stated that contact between the strains was unnecessary for the induction of sexual reproduction. There was great variation between the strains in the number of oogonia formed by each strain,

according to the strain it was paired with. Further polycarbonate membrane pairings showed that strains e and b produced no oogonia whereas strains c, d and a produced large numbers. Female strains produced oogonia whereas male strains produce antheridia. The number of oogonia formed in each strain was totalled.

Number of oogonia formed in each strain:-

a	b	c	d	e
872	72	1630	957	74

If the number of oogonia formed is taken to be an indication of the strength of mating characteristic, then the five strains may be placed on a scale representing their maleness or femaleness as follows:-

STRONG MALE			STRONG FEMALE
b e	a d		c

Male strains may be characterised by the comparatively small number of oogonia they form & by their ability to induce oogonia in other strains. The number of oogonia induced by each strain was totalled.

Number of oogonia induced by each strain:-

a	b	c	d	e
887	916	338	432	1022

A strain which had the ability to induce large numbers of oogonia can be classed as a strong male/weak female and likewise a strain inducing few oogonia may be classed as weak male/strong female.

STRONG MALE		STRONG FEMALE
e b	a d	c

Statistical analysis (Table 8) indicated that the number of oogonia produced by c and d varied significantly depending on which strain it was paired with. There was, however, no difference in the effects of different strains on a. Few or no oogonia were found when strains were paired with themselves. It also appears from the results that strain d has the ability to produce oogonia spontaneously without prior contact with a male strain. More oogonia are, however, produced when d was paired with a strong male. This suggests that strain d is more female than male. This spontaneous production of oogonia without induction may merit future investigation.

When the paired cultures were separated by a single polycarbonate

strain a

	S of S	d.f.	variance	F.value
other strains	130.23	3	43.41	0.76
dishes within strains	1133.41	12	94.45	
within dishes	1429.34	32	44.67	NS

strain b

	S of S	d.f.	variance	F.value
other strains	140.67	3	46.89	5.46
dishes within strains	103.33	12	8.61	($p < 0.05$)
within dishes	100	32	3.12	

strain c

	S of S	d.f.	variance	F.value
other strains	6359.06	3	2119.69	8.42
dishes within strains	3022.06	12	251.83	
within dishes	2067.33	32	64.60	($p < 0.01$)

strain d

	S of S	d.f.	variance	F.value
other strains	1587.06	3	529.02	10.92
dishes within strains	581.26	12	48.44	
within dishes	750.68	32	23.46	($p < 0.01$)

strain e

	S of S	d.f.	variance	F.value
other strains	180.75	3	60.25	2.76
dishes within strains	261.83	12	21.82	NS
within dishes	81.34	32	2.54	

Table 8 Analysis of variance carried out on plate counts to determine whether there is any significant difference when each strain was paired with the other strains. (see Table 7)

p 5% = 3.49 p 1% = 5.95 for 3 and 12 degrees of freedom. NS = Not siginificant.

membrane only, oogonial induction occurred. However, in an exercise where strains were paired, separated by blocks of uninoculated water agar (solidified with either 2g Difco Bactoagar/100ml water or 0.8g agarose/100ml water) 3.9, 5.8 or 7.9mm thick, no induction of sexual structures occurred, even with the thinnest block of agar, indicating that the agent was only active over short distances. The inducing substances were non-volatile. When strains were paired on discs separated by 6mm of air (one disc on lid and one on base of a sterile glass Petri dish), no sexual induction was noted. Uninoculated discs of agar were also paired in this way with inoculated discs; these uninoculated discs remained sterile. These results suggested that hyphal fragments and the agent responsible for sexual induction were not transferred aerially.

3.3. TO INVESTIGATE THE LENGTH OF INCUBATION TIME REQUIRED FOR OOGONIA FORMATION AND GROWTH OF THE ORGANISM

To determine the time at which cultures were competent to react sexually, 72h old cultures of strains c and e were paired, separated by a polycarbonate membrane. As controls the strains were paired with themselves as well as the opposite strain, i.e. c with e, c with c and e with e. The strains were separated after 24, 36, 48, 72, 96 and 120h. The number of oogonia in strain c was determined. Numerous oogonia were found on separating the discs after 24h, the cultures had therefore,

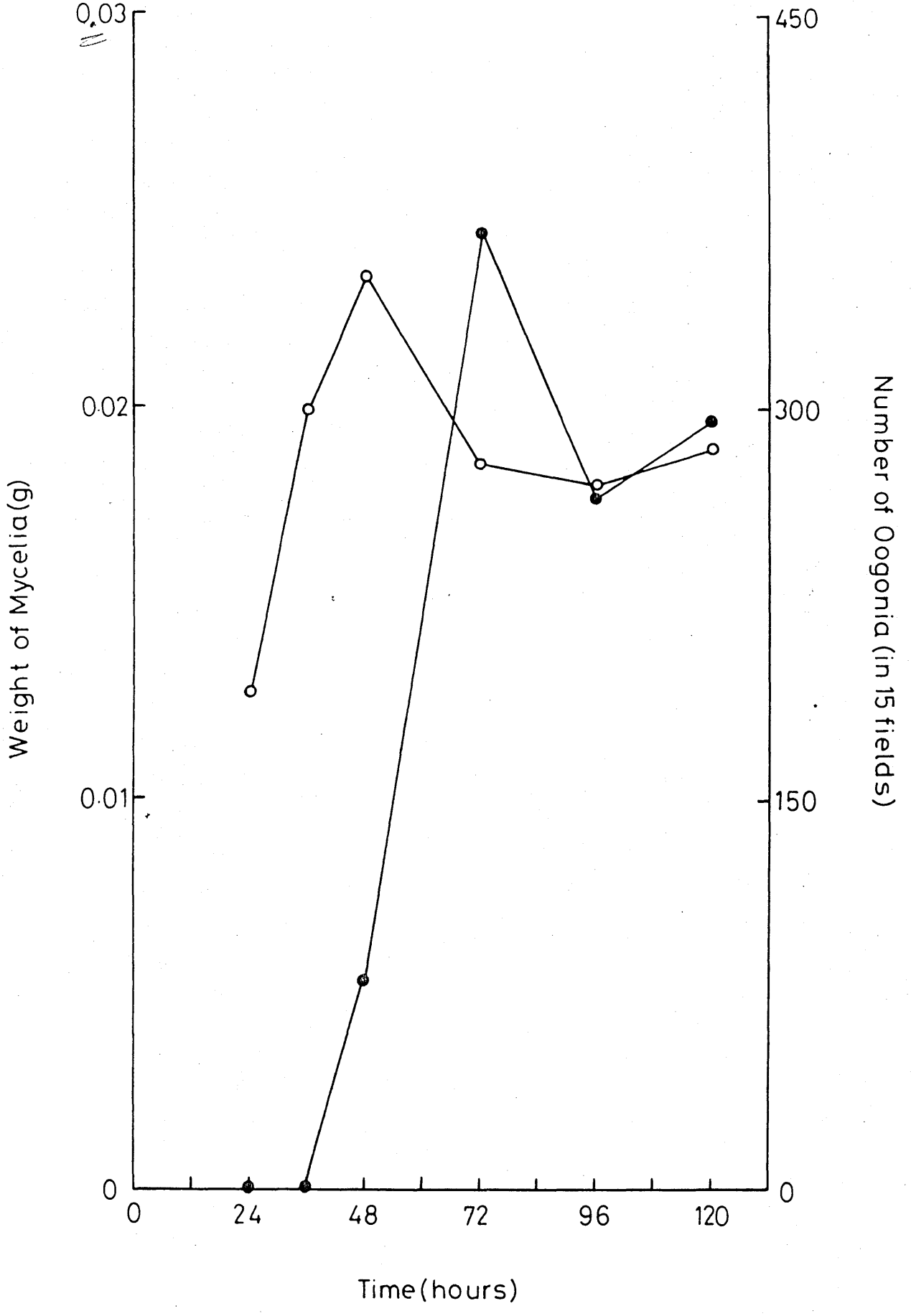
reached competence at 96h: 72h old cultures paired for 24h.

The age of competence was further narrowed down by pairing the male strain e of different ages, i.e. 24, 36, 48, 60, 72 and 144h, with the female strain c 72h old, conversely pairing strain c of different ages with e 72h old. The cultures were paired for 24h and then separated and examined. The agar discs containing growth of c were then placed in 200ml of boiling water for 1 minute. The resulting mixture was filtered through filter paper in a Buchner funnel. The filter papers (previously weighed) were then placed in a hot air oven at 80°C and the dry weight recorded after 3 days. It was found that cultures of both strains must be at least 36h old when paired and thus between 48 and 60h old when able to react sexually. This appears to correspond to the cultures reaching maximum weight (see fig. 6).

It was noted in this exercise that some oogonia were present in the male cultures. This was further investigated by the following experiment.

Discs inoculated with strains a, e, c and b were incubated at 24°C for 72h. The cultures were then paired (a with e, c with a, c with e and b with c) separated by a polycarbonate membrane and reincubated at 24° for 8, 24, 48 and 72h. The test plates which were set up for 8h were examined after separation at this time

Figure 6 Time course of production of dry weight of mycelium and of oogonia by P.sylvaticum 431c when mated with 431c. (see page 65 for details of exercise). O----O, weight of mycelium; ●-----●, numbers of oogonia formed.


0.03
0.02
0.01
0
450
300
150
0
0
24
48
72
96
120
Weight of Mycelia (g)
Number of Oogonia (in 15 fields)
Time (hours)

and then after 16, 40, 64 and 88h (i.e. 24, 48, 72 and 96h after pairing). The 24h plates were examined on separating (24h) and then at 48, 72 and 96h after pairing. The 48 and 72h pairing were similarly examined. Both the male and female discs were checked for sexual activity.

The results (Fig.7a) for the c/e pairing show the number of oogonia produced by the female c increasing until the cultures were separated, after which no more oogonia were induced. There were no oogonia formed in e until the strains were separated; after this, however, the number produced by e rose significantly. In all other exercises using e, the cultures were examined only at separation and no oogonia were noted; since e was therefore considered strong male, no female structures were expected. The results recorded for b/c, a/e and c/a were very similar (fig. 7b-d). One explanation of the results may be that something was being produced by the male which stimulates the female to produce oogonia. Before the female was removed, the male did not produce oogonia suggesting that when the female was present it utilized this substance. However, when the female was removed and was no longer utilizing the inducing factor produced by the male, the male had access to the substance and thus produced oogonia. The female may take up the substance more quickly and thus it will not be available to the male strain, OR the male may have fewer binding sites, OR the binding sites may be less specific in the

Figure 7a Time course of production of oogonia by P.sylvaticum.

72 old cultures of c (female) and e (male) were paired with a polycarbonate membrane between them. The numbers of oogonia in each strain were counted on separating the discs and at regular intervals thereafter, ●——●, strain c (♀), ■——■ , strain e (♂). The vertical dashed line indicates time at which cultures were separated.

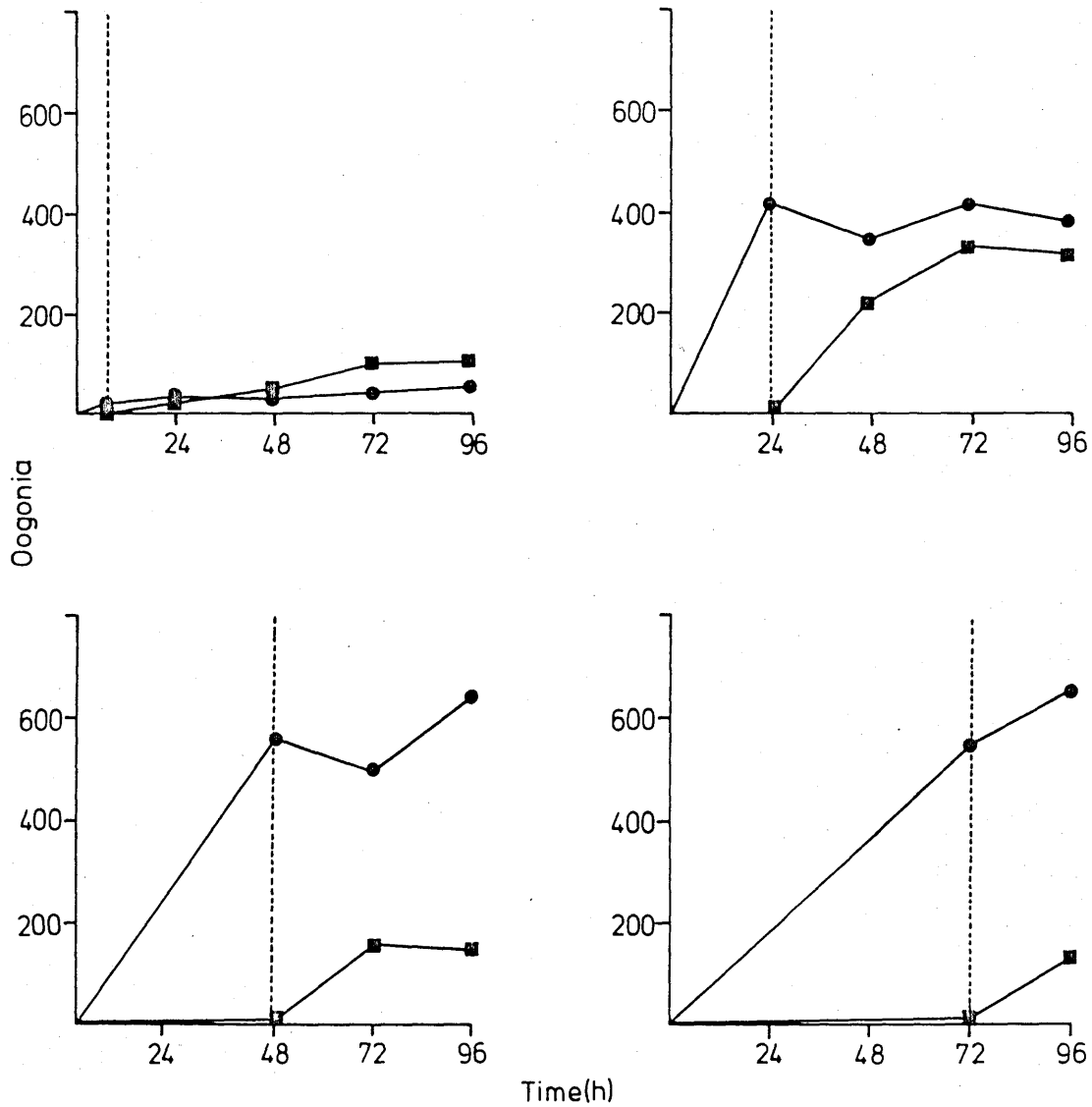

600
400
200
24
48
72
96
Oogonia
Time(h)

Figure 7b A similar experiment to that shown in fig. 7a , with strain a (here functioning as female) and strain e (male). ●——●, strain a (♀); ■—■ strain e (♂).

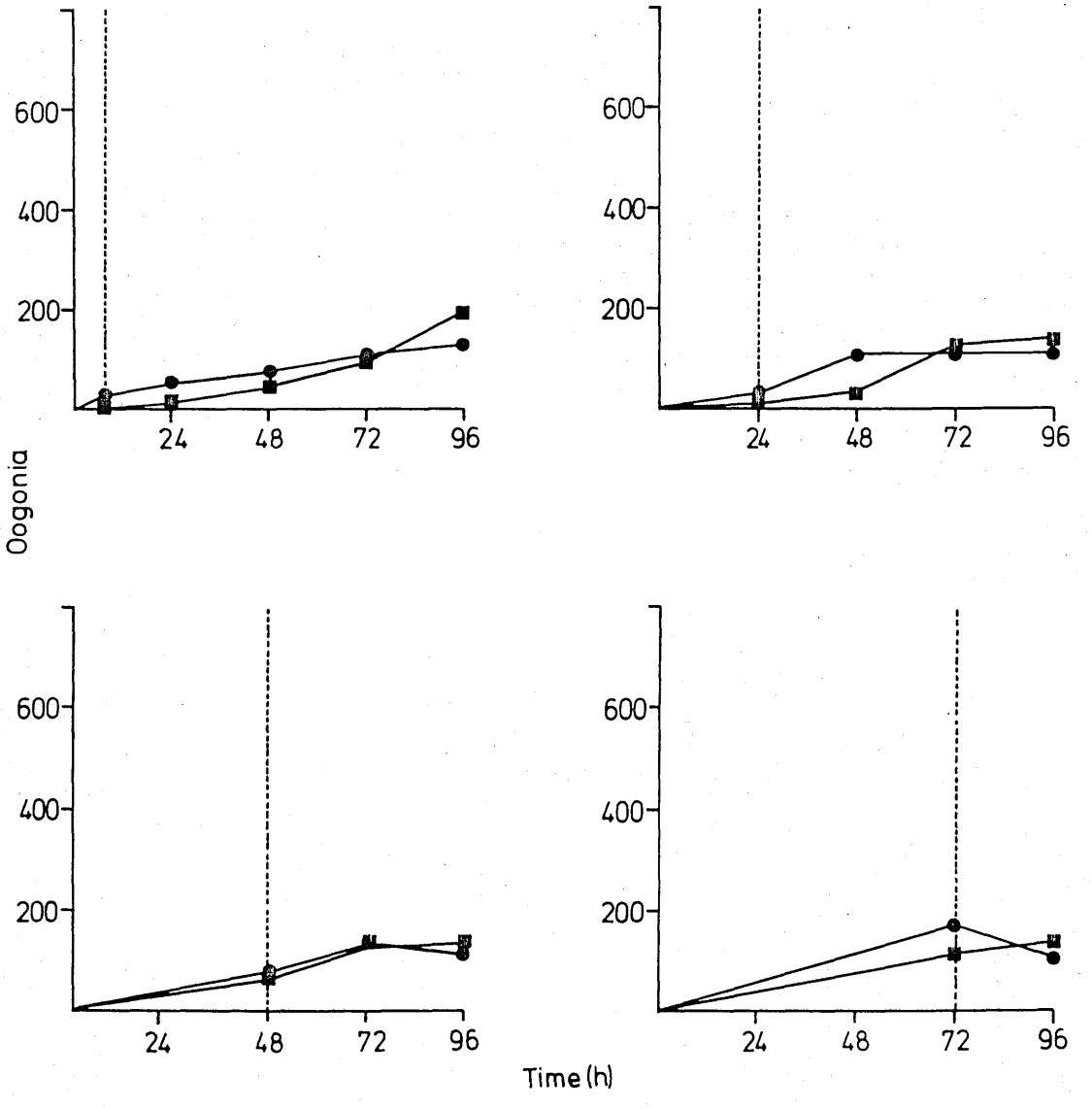

600
400
200
24
48
72
96
Oogonia
Time (h)

Figure 7c A similar experiment to that shown in fig. 7a, with strain c (female) and strain b (male). ●—● , strain c (♀); ■—■ strain b (♂).

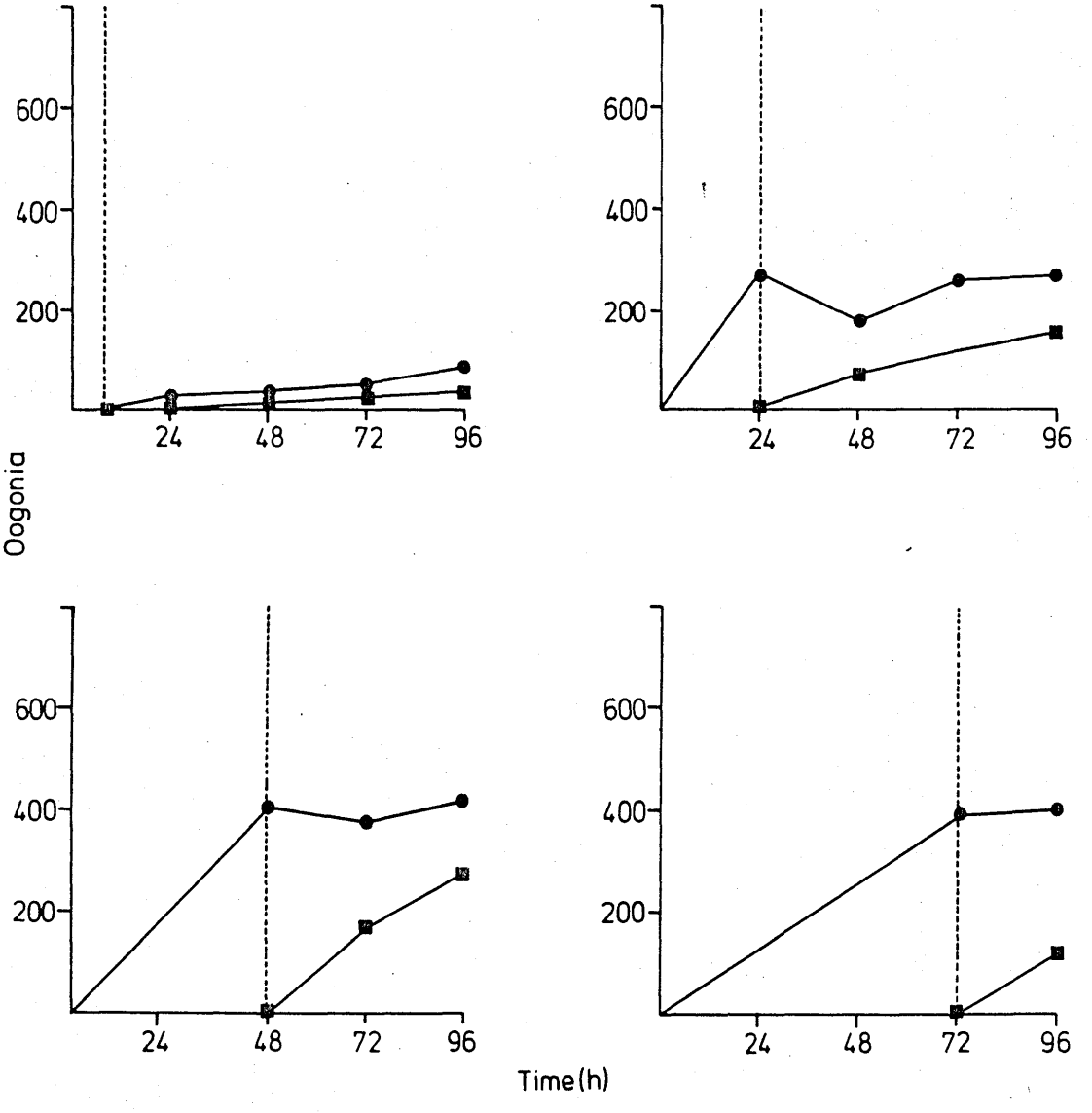
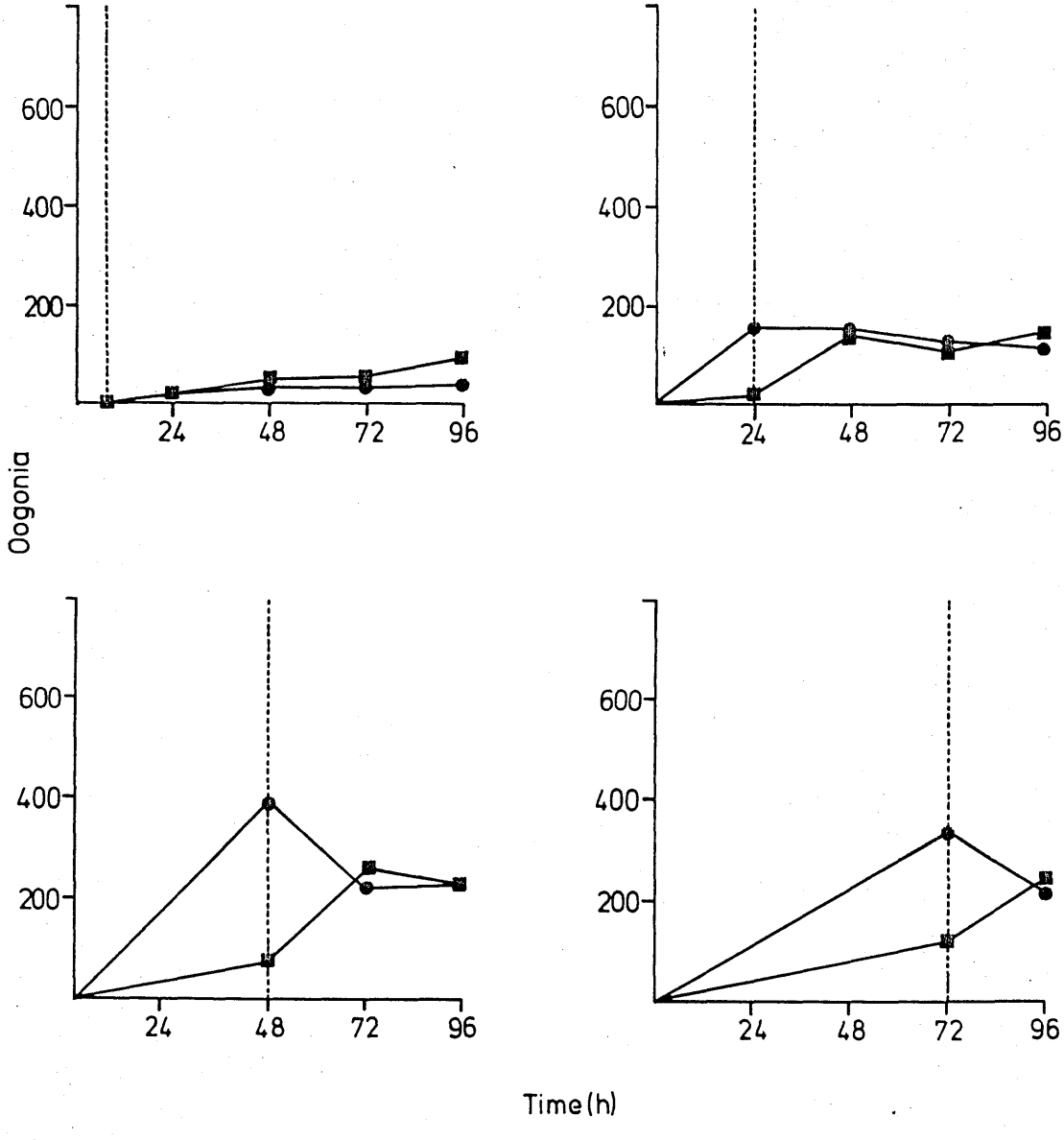

600
400
200
24
48
72
96
Oogonia
Time (h)

Figure 7d A similar experiment to that shown in fig.7a , with strain c (female) and strain a (here functioning as male). ●——●, strain c (♀); ■—■ , strain a (♂).


600
400
200
24
48
72
96
Oogonia
Time (h)

male than in the female. It is obvious, however, that the male strain must be induced by the female before any of this can occur since unpaired male strains or pairings of 2 male strains do not produce oogonia.

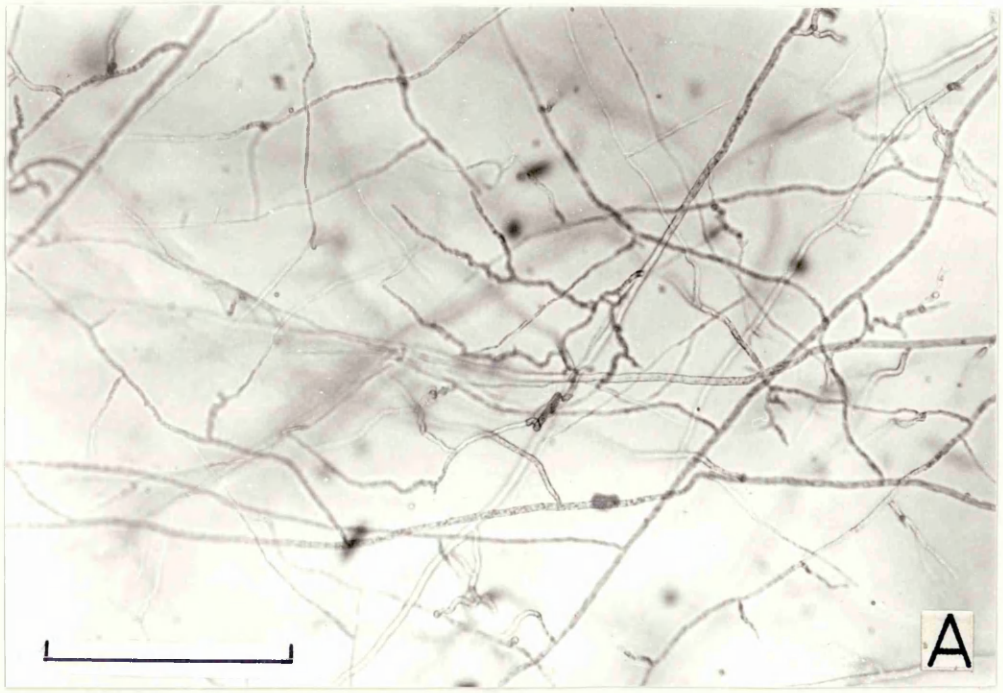
There are similarities between this system and that found in the Achlya sp. hormone system. Contact between strains was not necessary for the induction of sexual reproduction indicating a diffusing agent. As with Achlya, there appears to be a sequence of events resulting in one partner being induced before the other, again indicating a hormone system. With Achlya, however, (on carrying out an exercise similar to the last described above) no oogonia were formed in the male strain after separation suggesting that although the system may be similar in some ways, it differs in others. (C.G. Elliott, unpublished).

3.4. SEQUENCE OF EVENTS IN INDUCING PROCESS

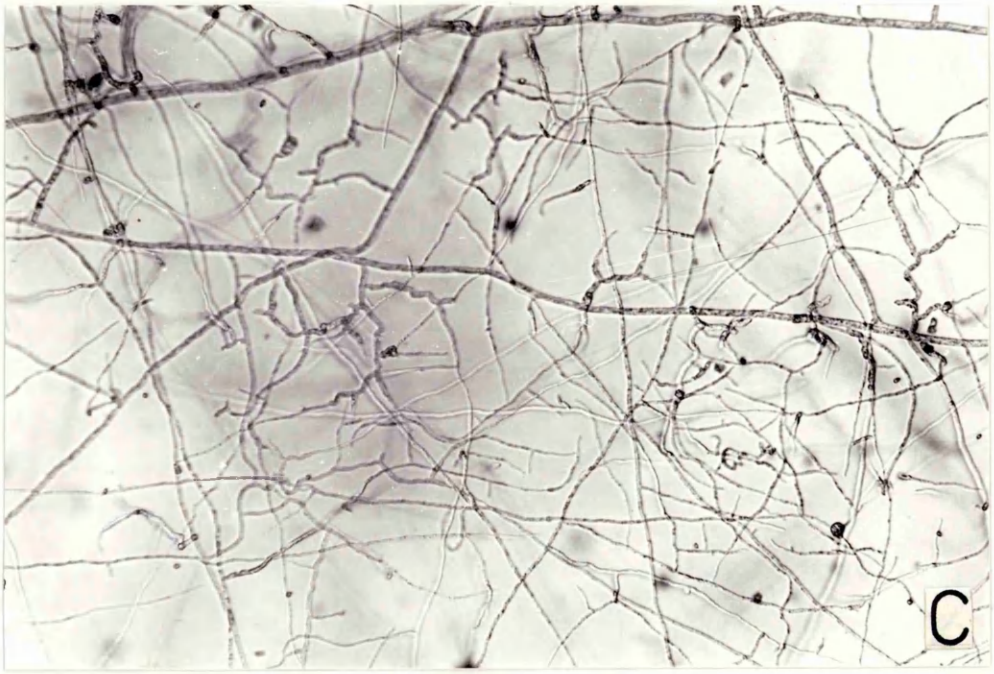
To ascertain whether oogonia or antheridia were formed first, 72h old cultures of c and e were paired. with a polycarbonate membrane between them, and separated at ½ hourly intervals between 4 and 8h. The male and female cultures were both examined and photographs recording the sequence of events taken using a Leitz Ortholux 11 microscope (12.5 x eyepiece, 10 x objective) with a Leitz Orthomat - W microscope camera attached.

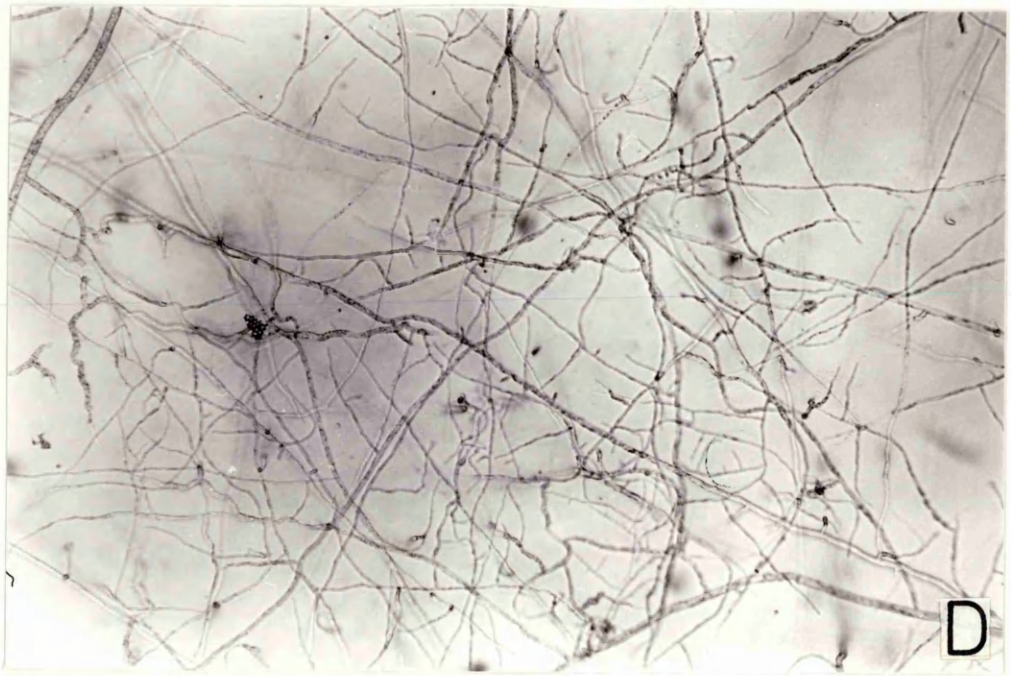
Kodak technical film 2415 (TP135-36) was used. Controls were set up of each strain paired with itself. Four photographs were taken of each plate at the times indicated, two fields in the centre of the plate, where the inoculum was and two nearer the outer edge of the colony. The fields were randomly selected and not simply photographed if they were of interest. It is, therefore, suggested that the contents of the photographs are a reasonable indication of the sequence of events. The photographs (fig. 8a & b) show that the antheridial branches formed in the male strain before oogonial initials in the female strain. The antheridial branches were first noted at about 4½h and the oogonial initials at ~6h. The main hyphae in strains which have not been paired with compatible strains produced lateral branches which taper characteristically to a point. The branches, presumably antheridial branches, in the induced males differ in that they are blunt ended and of more uniform diameter. They also appear to branch at more or less right angles to the main hyphae, eventually forming a grid-like appearance. The oogonia form on off shoots of the pointed lateral branches. They can occur in an intercalary position or terminally. The fact that antheridial branches were noted before oogonial initials may simply indicate that the oogonia take longer to form but that both oogonia and antheridia are induced at the same time. The following exercise was carried out to estimate when oogonia were induced. The effect of induced and uninduced strains on each other was compaired. (Table 9).

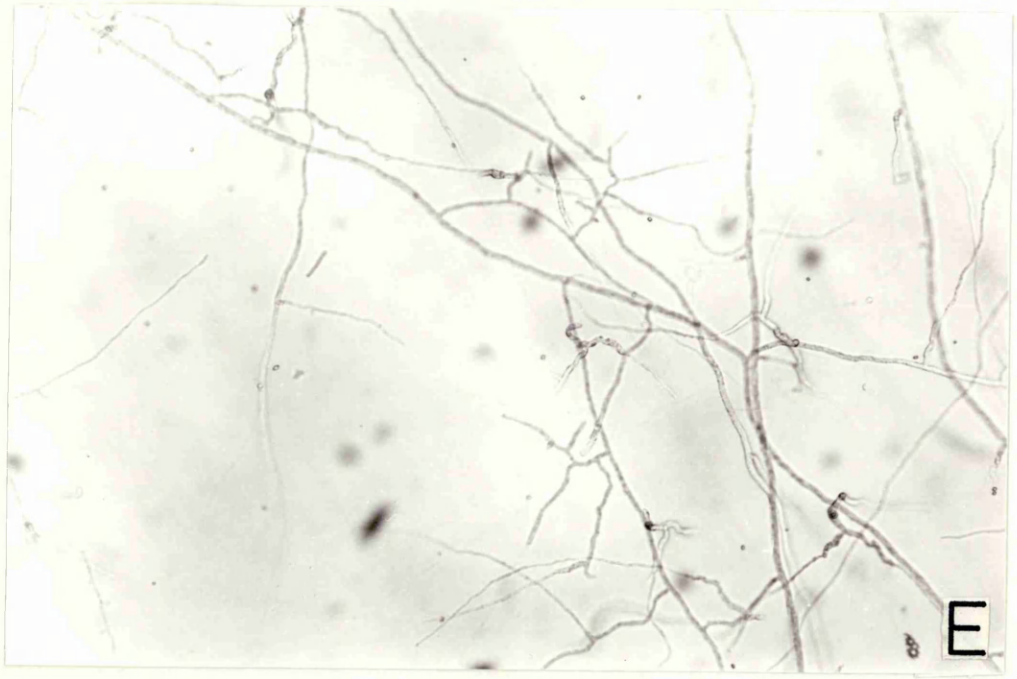
Figure 8a (A,B,C,D,) Development of antheridial hyphae in the male strain (431e) of Pythium sylvaticum when paired with the female strain (431c). (E,F,G,H,). Control cultures (strain e paired with a similar culture of e). Cultures were paired with a polycarbonate membrane between them, separated at intervals, and the surface of the culture in contact with the membrane photographed. (A,E) 4h after the cultures were paired; (B,F,) 5h; (C,G,) 6h; (D,H,) 7h. Scale bar, 0·2mm.

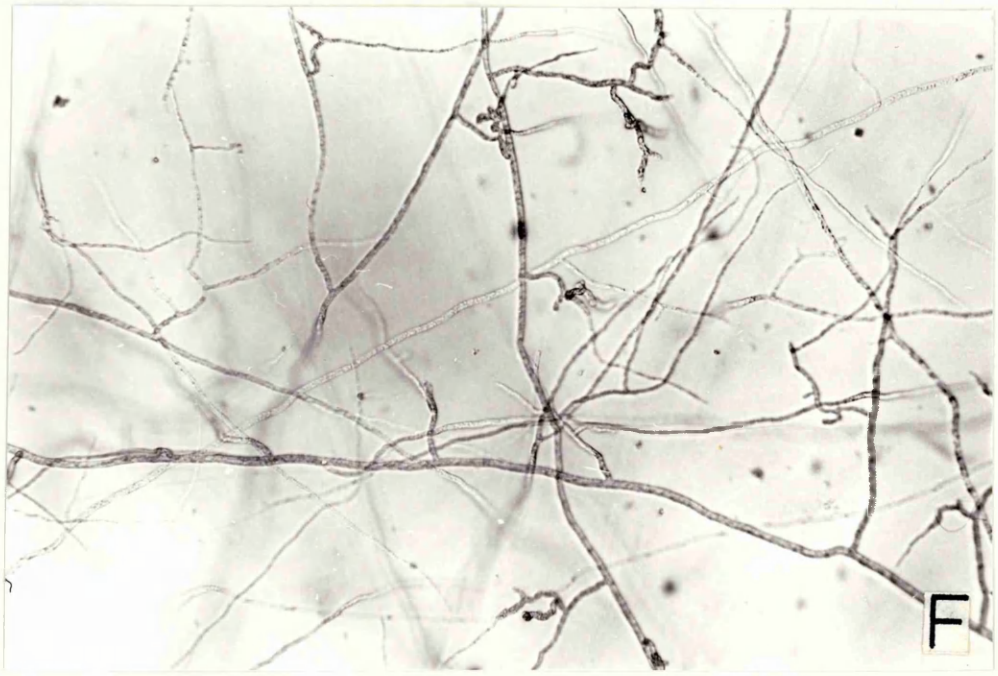

A

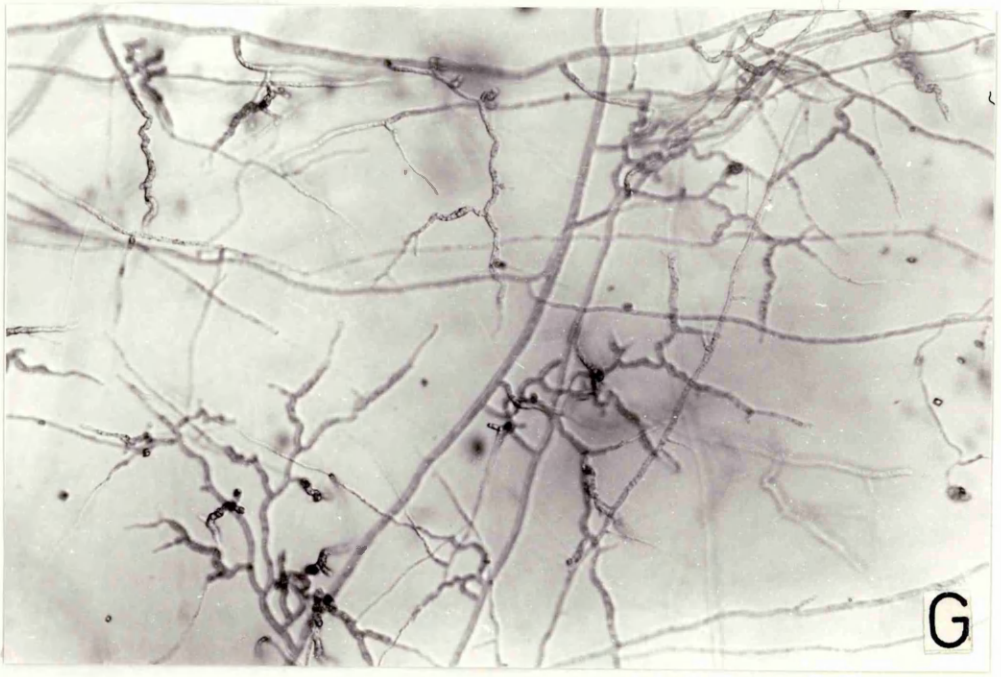

B


C


D


E


F


G

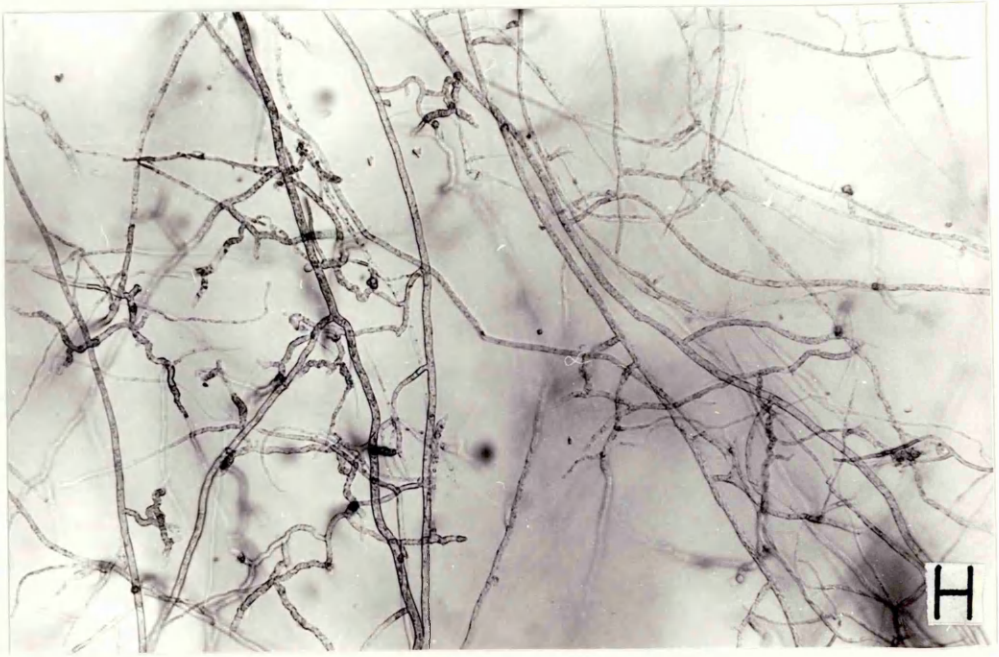
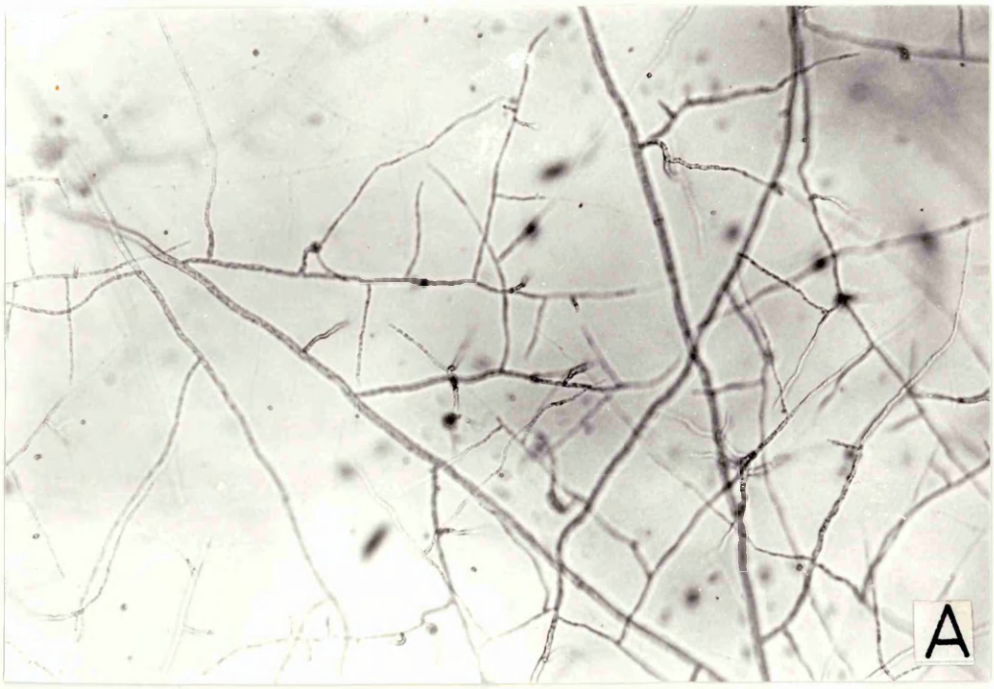
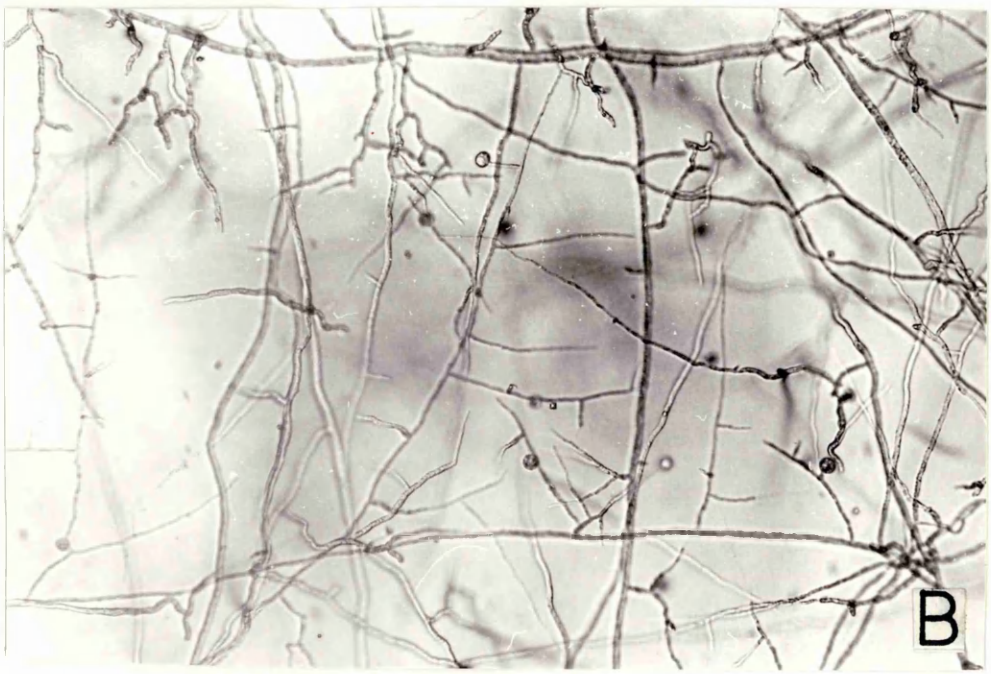

H

Figure 8b (A,B,C,D) Development of oogonia in the female strain (431c) of P.sylvaticum when paired with male strain (431e). (E,F,G,H) control cultures (c paired with a similar c) (A,E) 4h; (B,F) 5h; (C,G)6h; (D,H) 7h.


A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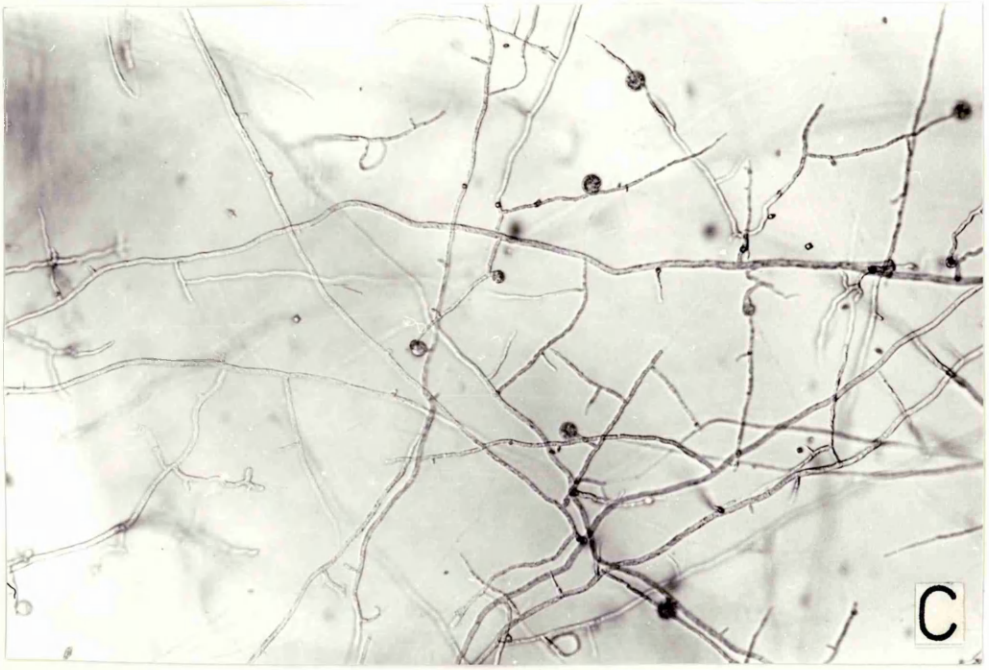
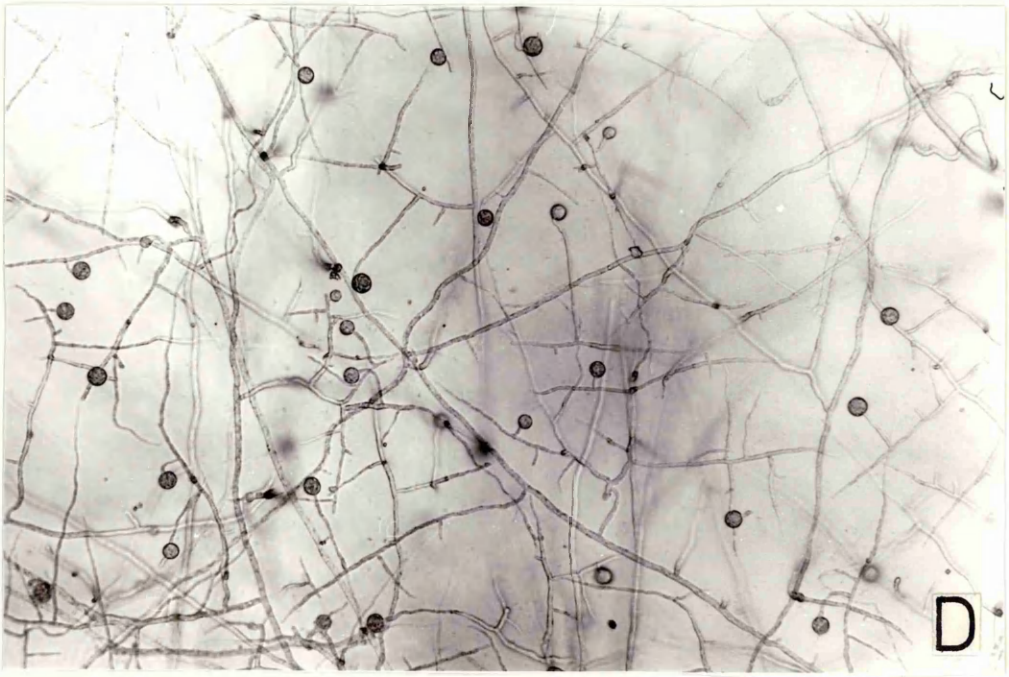

C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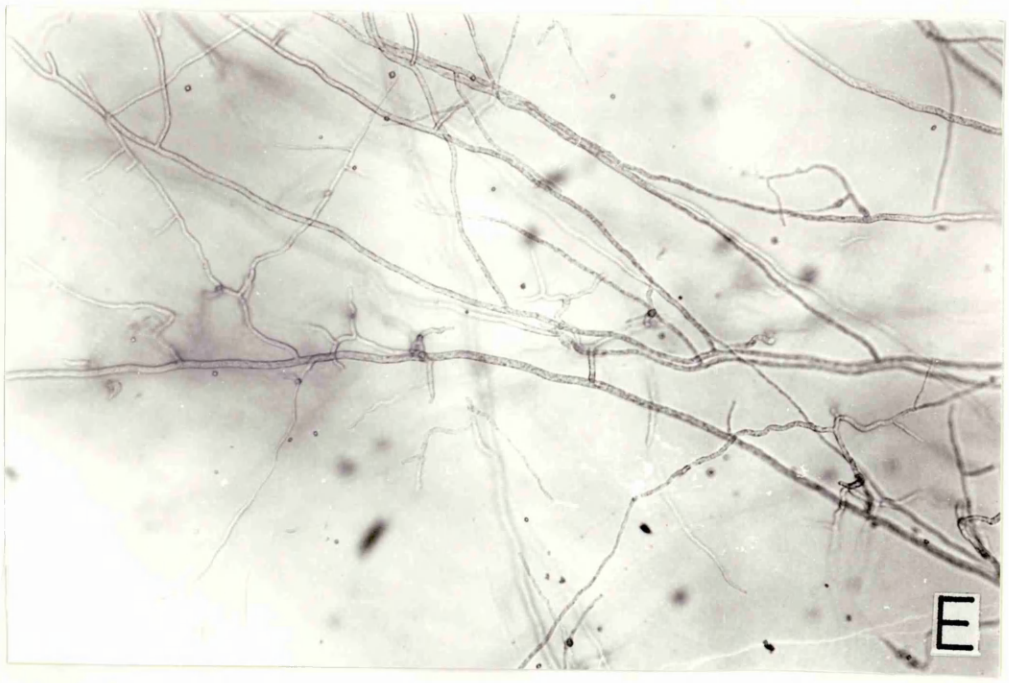
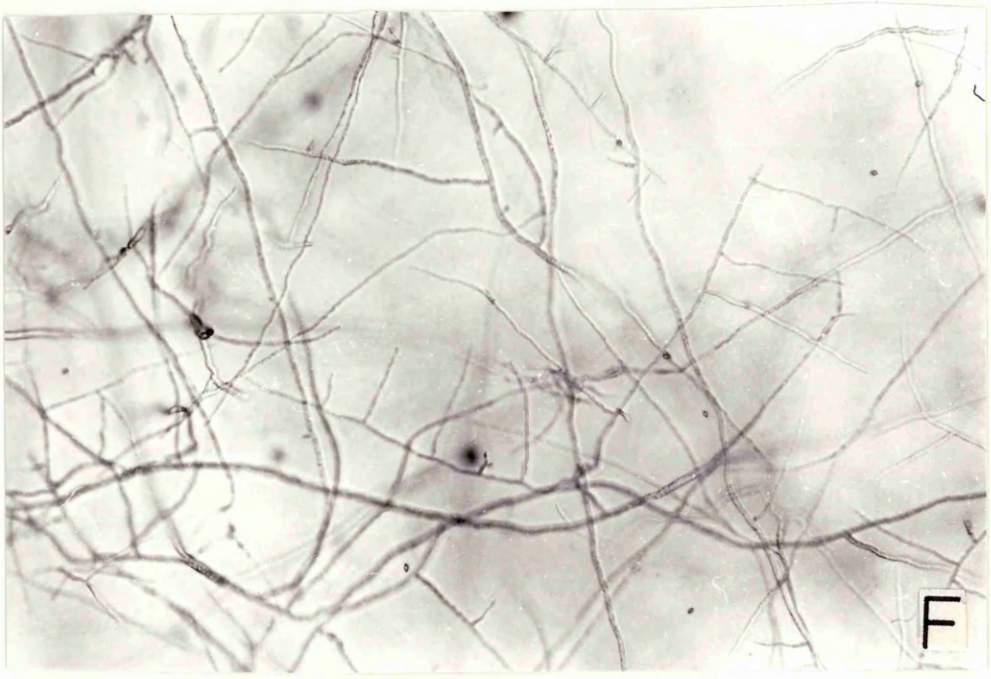
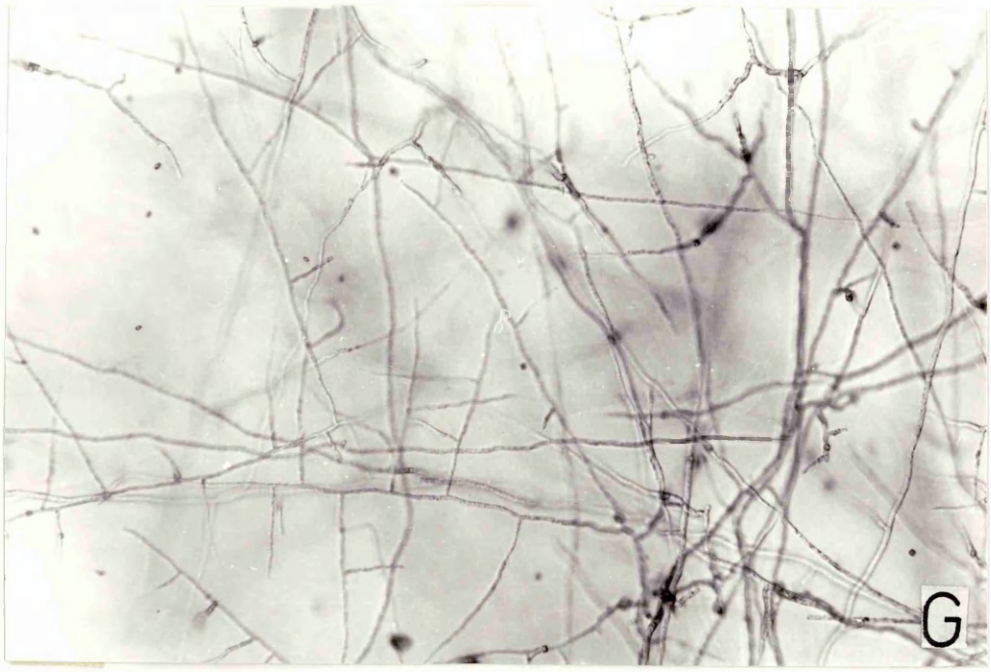
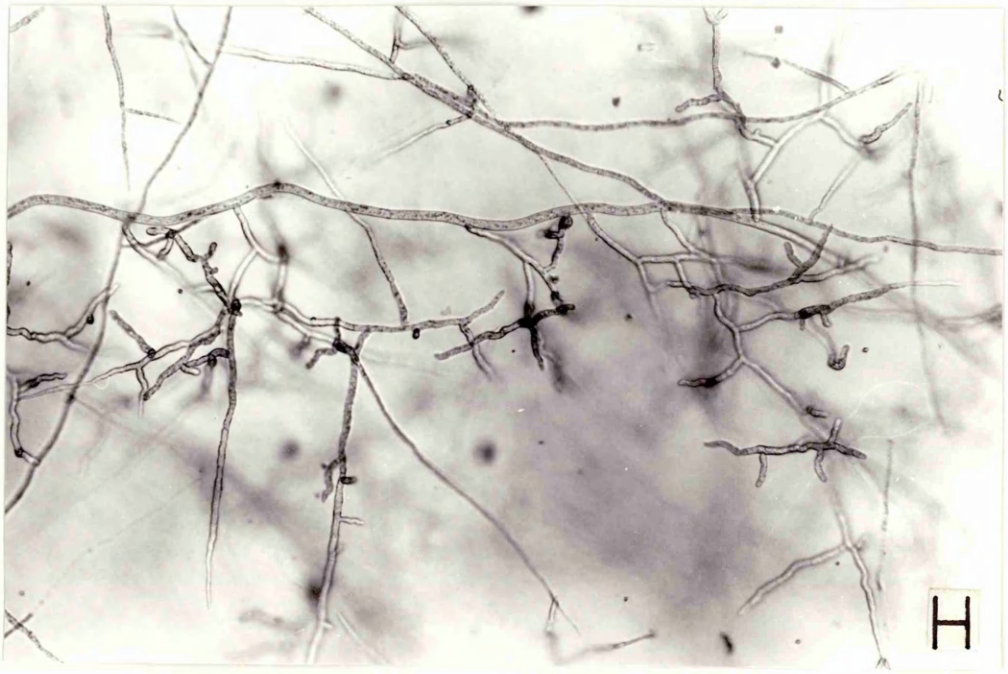

E


F


G


H

Strain c(♀) and e(♂) were incubated for 72h. The strains were then paired as follows:-

σ_u / φ_u incubate 5h σ_i / φ_i examine after 0,1,2,3,4,5,6 and 24h

σ_u / φ_u incubate 5h σ_i / φ_i → σ_i / φ_u ; φ_i / σ_u examine after 0,1.2,3, 4,5,6 and 24h

σ_u / φ_u examine 1,2,3,4,5,6 and 24h after pairing (set up at time 5h of first two treatments).

σ_u examine at 1,2,3,4,5,6 and 24h

φ_u examine at 1,2,3,4,5,6 and 24h

σ_u = uninduced male

σ_i = induced male

φ_u = uninduced female

φ_i = induced female

Time(h)		0	1	2	3	4	5	6	24
strains paired									
σ_i / φ_i	σ	+	+	+	+	+	+	+	+
	φ	-	-	+	+	+	+	+	+
σ_u / φ_u	σ	-	-	-	-	$\pm$	+	+	+
	φ	-	-	-	-	-	-	+	+
σ_i / φ_u	σ	+	+	+	+	+	+	+	+
	φ	-	-	-	-	$\pm$	$\pm$	+	+
σ_u / φ_i	σ	-	-	-	-	$\pm$	+	+	+
	φ	-	-	-	-	-	$\pm$	+	+
σ / σ		-	-	-	-	-	-	-	-
φ / φ		-	-	-	-	-	-	-	-

Table 9 Production of oogonia in female (c) and antheridia in male (e) strains of Pythium sylvaticum. 72h old cultures were paired and examined as illustrated in Fig.

σ_i = induced male, φ_i = induced female.

σ_u = uninduced male, φ_u = uninduced female.

(+) represents the appearance of numerous oogonial initials in the female, or numerous antheridial branches in the male.

($\pm$) represents the appearance of few oogonial initials in the female or few antheridial branches in the male.

(-) represents the absence of oogonial initials in the female or antheridial branches in the male.

As expected, oogonia appeared earlier in the female strain *c* when it was paired with an induced culture of *e* in which antheridial branches were present, than when paired with an uninduced culture of *e*. However, there was no difference in the time taken for *e* to produce antheridial branches when paired with a fresh culture of *c* or with one which had had contact with *e*.

In conclusion, the strains used in this exercise ranged from strong male to strong female. Contact between the hyphae of compatible strains was not necessary for sexual reproduction suggesting that a hormone mechanism may be involved.

The strains were competent for sexual reproduction at between 48 and 60h old when the mycelium had reached its maximum weight. The male strain was induced to produce antheridial branches before the female produced oogonial initials, again suggesting that a hormone may be involved.

The female strain produced oogonia as long as it was in contact with the male. However, on removing the male, oogonial production in the female did not increase. When the male and female were separated, oogonia appeared in the male cultures.

CHAPTER 4

ATTEMPTS TO ISOLATE HORMONE CONTROLLING SEXUAL REPRODUCTION

4.1. INTRODUCTION

It is obvious from the polycarbonate membrane exercises that some agents pass through the membrane and induce the paired strains to reproduce sexually. To trap these substances and devise some bioassay to detect them, would obviously be essential for characterization of these hormones.

4.2. TO DETERMINE IF SUBSTANCE WAS RELEASED INTO MEDIUM IN WHICH STRAINS WERE GROWN.

15ml aliquots of liquid Schmitthenner's medium with sitosterol were placed in 9cm Petri dishes, inoculated with strains of P.sylvaticum and incubated at 24°C for 72h. 20ml of the same medium was placed in 100ml wide necked flasks, inoculated and incubated at 24°C for 72h with the plates. After 72h, the liquid was removed from the Petri dishes and the same amount of filtrate from the culture in the flasks added to the Petri dish. The following combination were set up:-a/c, a/d, a/e, c/d, c/e, d/e, c/a, d/a, e/a, d/c, e/c and e/d (where the strains underlined represent fluid from the flasks). After transferring the fluid from the flasks, the Petri dishes were reincubated at 24C and examined hourly over 8h and then after 24h.

Compatible strains grown together in liquid culture formed oogonia.

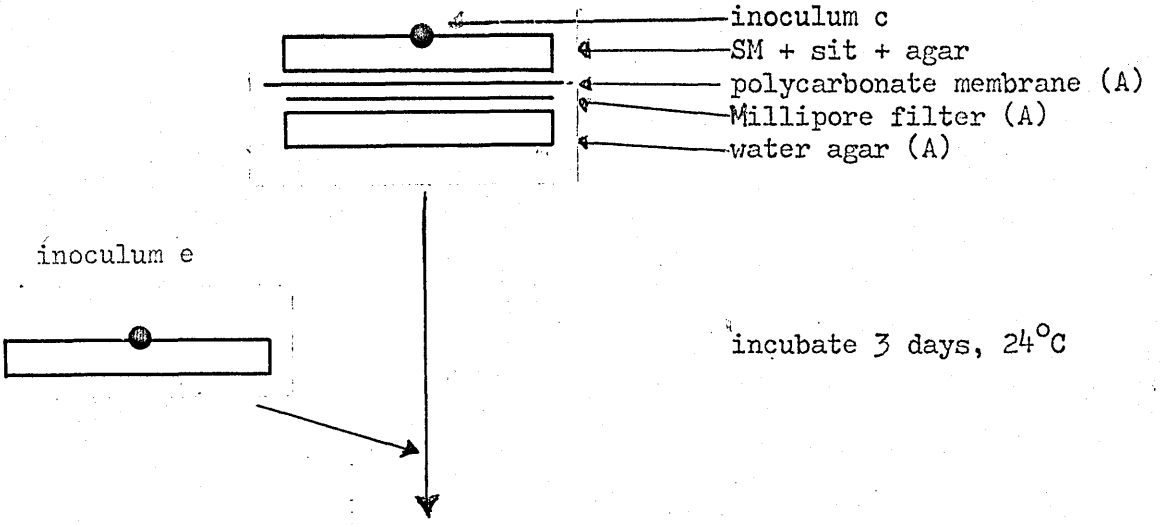
However, in this transfer exercise, no sexual structures were induced in any of the pairings. The "hormone" may not be produced in high enough concentrations to induce the strains when the liquid transfer method was used. It is possible that the concentration is only high enough in the small area directly around the hyphae producing it; thus when strains meet or are separated only by very short distances, the concentration may be high enough to induce sexual reproduction. This hypothesis is supported by the pairing exercise reported in chapter 2, where strains were paired, separated by varying thicknesses of water agar and no induction was recorded unless strains were in very close contact; separated only by a polycarbonate membrane.

As the hormone could not be detected by this method, it was decided to try to trap the hormone in various ways, thus concentrating the agent in a small area. Attempts were made to trap the hormone:-

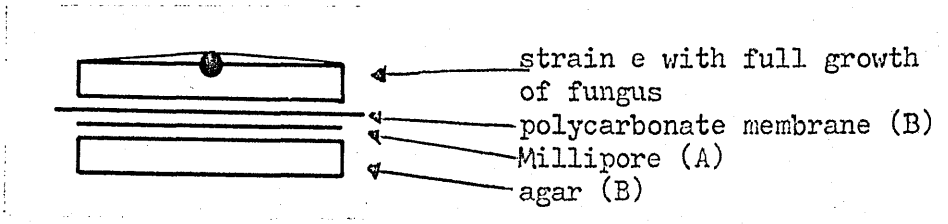
(1) on a Millipore filter (type RAWP).
(2) in agar/agarose
(3) in activated charcoal.

4.3. TRAPPING THE HORMONE ON A MILLIPORE FILTER

Outline of experimental technique



remove Millipore and set up sandwich again as follows:-



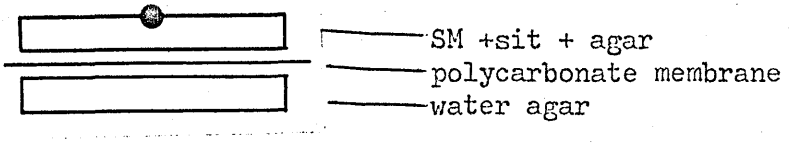
The water agar base and the polycarbonate membrane were changed so that if a response was detected, it could be attributed to the Millipore filter or something contained in the filter

incubate for a further 24h

examine discs (surface in contact with the polycarbonate membrane).

This exercise was also carried out with e in the original pairing and c added after dismantling the first "sandwich".

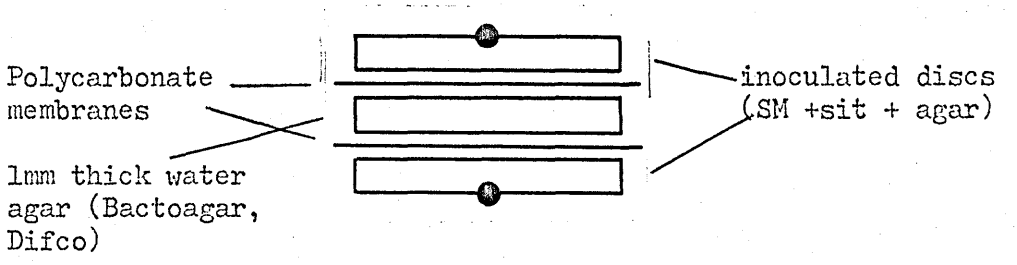
Control



Growth was stimulated by the Millipore filter but no sexual induction was noted. It was considered that the Millipore or something associated with the Millipore was causing this. The Millipores were therefore, washed in either distilled water or ether followed by distilled water to ensure that they were clean and the experiment repeated. The result, however, was similar to that noted with the unwashed filters.

4.4. TRAPPING THE HORMONE IN AGAR/AGAROSE

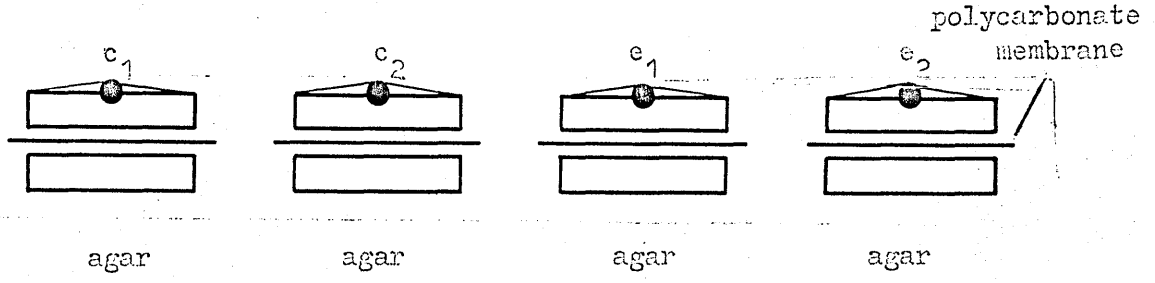
Outline of experimental technique



Strains c/e, c/c or e/e paired

↓ 72h incubation at 24°C

water agar removed and paired



c_1 = original strain c left in contact with separating agar

c_2 = 72h old culture of c

e_1 = original strain e left in contact with the separating agar

e_2 = fresh 72h old culture of e

↓ incubate at 24°C for 24h

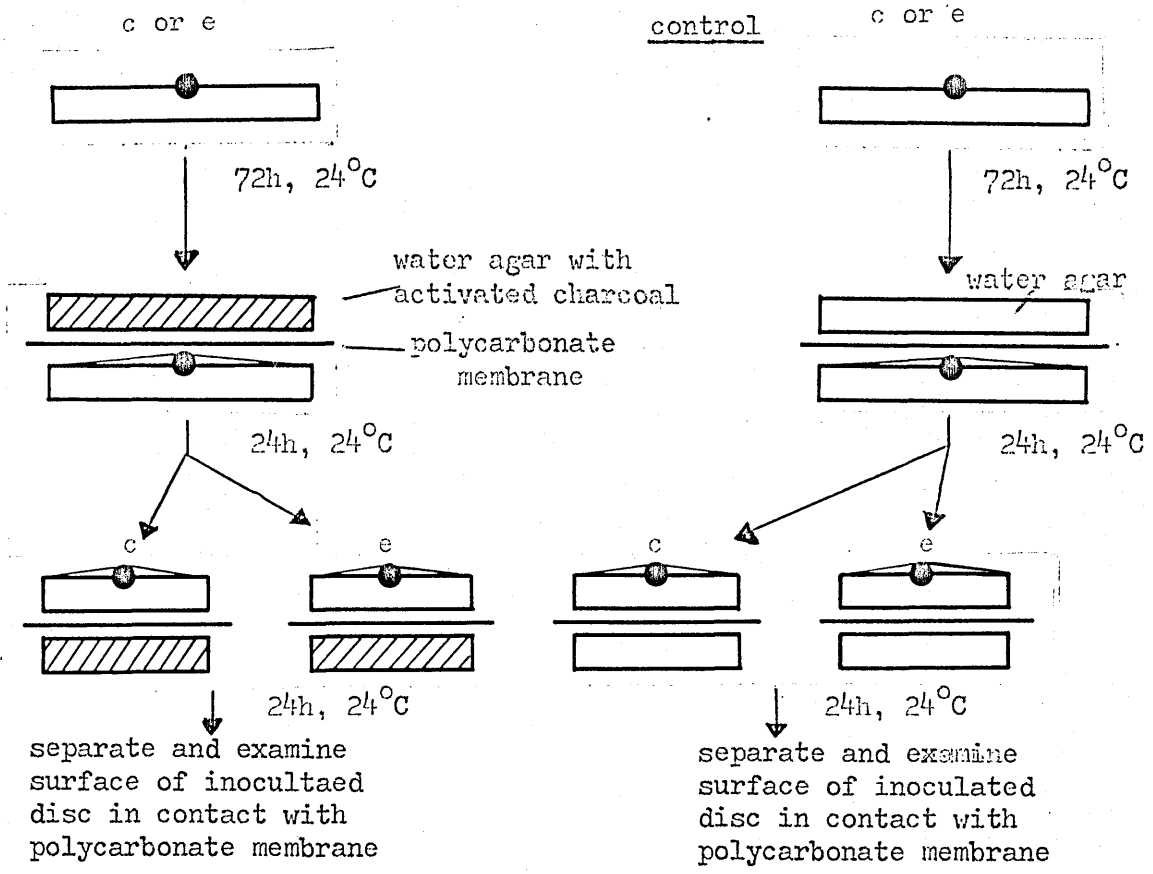
separate discs and examine surface of inoculated disc in contact with polycarbonate membrane.

Controls containing c/c and e/e were set up at the same time.

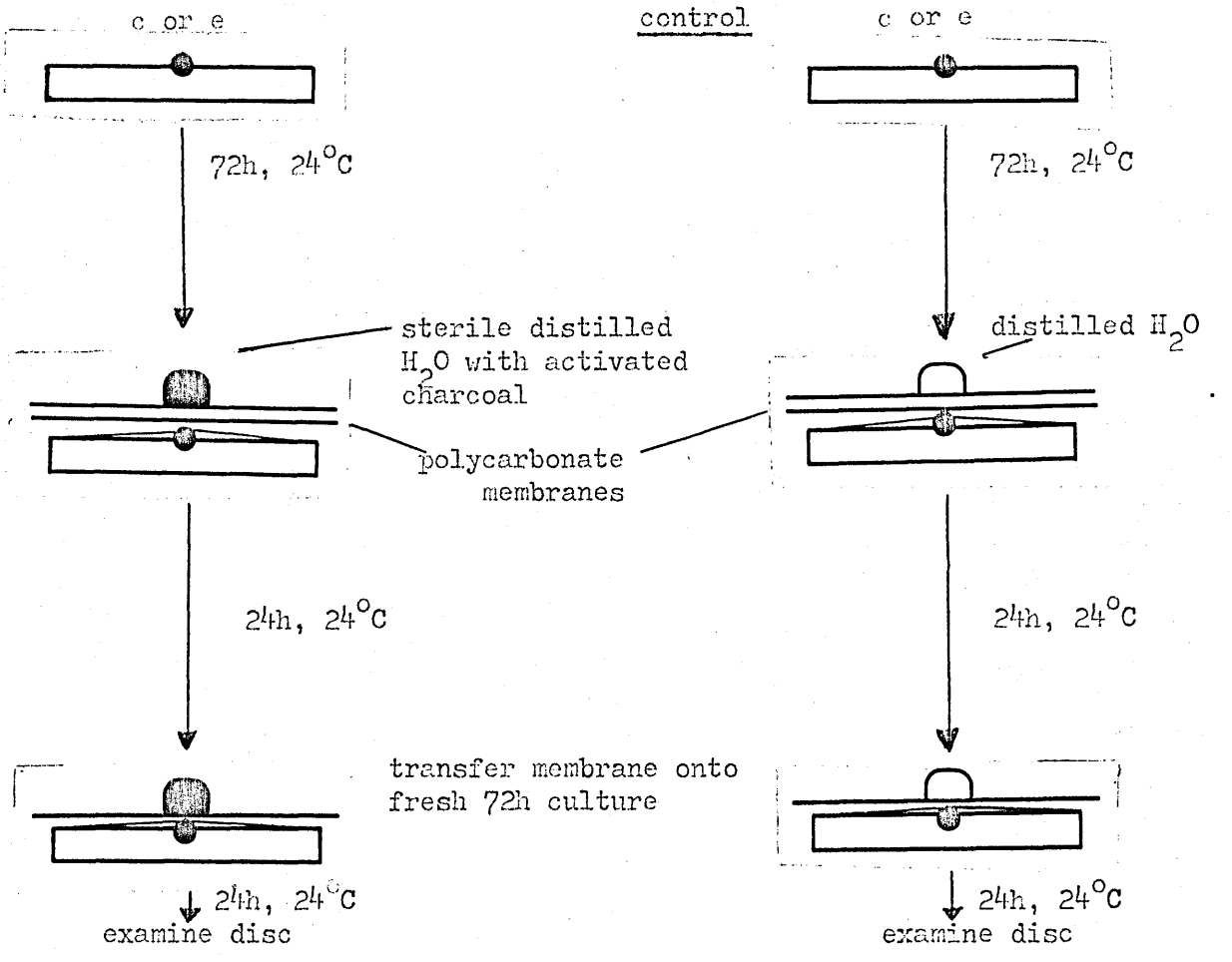
Vegetative growth was slightly stimulated but no sexual reproduction occurred. It was suggested that the inducing agant may bind to the agar and thus be unavailable to the strains. For this reason agarose was substituted for the Difco Bactoagar. However, as before, no sexual induction was observed.

4.5. TRAPPING HORMONE IN ACTIVATED CHARCOAL

Outline of experimental technique



Before use, the charcoal was washed in hexane, acetone and finally in distilled water before use to remove contaminants.



In both these exercises, vegetative growth was stimulated, whether as a result of a substance absorbed by the charcoal, or by the charcoal itself is not clear. Sexual reproduction, however, was not induced.

As attempts to trap the hormone in agar, millipore filter and activated charcoal failed, it was decided to try to extract the hormone from liquid and solid media using various solvents.

4.6. DEVELOPMENT OF EXTRACTION PROCEDURES

1. Growth, pair ing and extraction of agar cultures of strains c and e

Outline of experimental technique

200x5cm plates containing 5ml of SM + agar + sit were inoculated as follows:-

Inoculate plates

- 40 - uninoculated (0)
- 80 - strain c
- 80 - strain e

↓ 24°C, 72h

pair strains (separated by polycarbonate membrane)

- 40 c/e
- 20 c/c
- 20 e/e
- 20 o/o

↓ 24°C, 24h

separate discs and freeze dry.

Discs ready to extract

40 discs of c which had been paired with e

40 discs of e which had been paired with c

40 discs of e paired with e

40 discs of c paired with c

40 discs uninoculated media.

Extraction

Freeze dried agar discs were placed in cellulose extraction thimbles and extracted in a Soxhlet apparatus with 250ml Chloroform/Methanol (2:1 v/v) for 14h. The chloroform/MeOH was dried off using a rotary evaporator and the contents of flask redissolved in small amounts of Chloroform/MeOH (2:1), and transferred to a 2ml vial, dried under Nitrogen and redissolved in 1ml MeOH.

11. Growth, pairing and extraction of liquid cultures of strains c and e

160x100ml wide necked flasks containing 20ml of SM +sit were inoculated as follows:-

Inoculate flasks — 40 - uninoculated (0)

60 - strain e

60 - strain c

↓ 24°C, 5 days

pair strains — 20 c/e

ethyl acetate or chloroform. The extracts were dried under reduced pressure in a rotary evaporator.

2. The filtrate was placed in a 3 litre round bottomed flask. The flask was filled with ethyl acetate and the medium extracted by a method known as continuous ether extraction for 24h.

In some cases the pH of the filtrate was adjusted to 3 before extraction, then readjusted to 8 and re-extracted.

iii Assay of extracts

1. 9cm Petri dishes containing 15ml SM + agar were inoculated with either strain c or strain e on one edge and incubated at 24°C. (1) When the plate was completely covered by fungal mycelium, a cut was made, (11) and $\frac{1}{2}$ the agar was removed. Fresh agar containing the extract was placed in this part of the plate, (111) The plates were then reincubated at 24°C and examined when the hyphae had grown through this fresh medium.

2. Strains c and e were grown separately on agar plates of Schmitthenner's medium. The extract was dropped onto the plates at certain points; one near the centre, one between the centre &edge and one on the edge of the colony. The plates were examined regularly over a 24h period.

3. A water agar block containing the extract was placed in a glass Petri dish with a polycarbonate membrane and the block of Schmitthenner's medium with 72h growth of either strain c or strain e. The sandwich was dismantled after 24h and the surface in contact with the polycarbonate membrane examined.

As controls for these three assays strain c and e were paired either on large Petri dishes or separated by a polycarbonate membrane as described in earlier exercises.

The amount of hyphae was increased by the addition of these extracts to the medium, but there was no sign of sexual reproduction in any of the bioassay systems.

Conclusion

No trace of hormone activity was recorded in any of these experiments. Because of this, and because it is known that sterols are important in sexual reproduction, the next step

in the investigation of sexual reproduction in this organism was to look at sterol metabolism

CHAPTER 5

STEROL METABOLISM

5.1. INTRODUCTION

It was established in chapter 2, that sterol was required for sexual reproduction in Pythium sylvaticum. The role of the sterol in this organism is not known. It may act as a structural component of the cell, or it may be converted by the organism resulting in the formation of metabolites which may in turn act as hormone precursors. Observations of the sterol metabolism in this organism may indicate whether the substances produced are characteristic of induced and uninduced strains.

5.2. MATERIALS AND METHODS

i) Culture conditions

Culturing conditions were as described in chapter 2.

ii) Synthesis of Radioactive Cholesteryl Palmitate

(^{14}C) cholesterol palmitate was synthesized from (4 - ^{14}C) - cholesterol by Dr. V. Math, Mr R McGowan and Dr. C.G. Elliott. The high specific activity cholesterol (50 uCi) in toluene (as supplied) was cooled to $0^{o}C$ and treated with excess dry triethylamine (0.2ml) followed by palmitolyl chloride (0.2ml). The mixture was kept at $0^{o}C$ for 2h. The solvent was removed at room temperature under a current of oxygen free nitrogen. The residue (Et_3NHCl + cholesteryl palmitate + palmitoyl anhydride)

was dissolved in a minimum of dry $CHCl_3$ and applied to 20 x 20 TLC (silica-gel G-L5) plates which were developed in n-Hexane containing 10% diethyl ether. The plates were scanned on a TLC scanner. The second band (away from the baseline) (R.t. 0.5 - 0.6), corresponding to authentic cholesteryl palmitate, was collected and eluted with Et_2O. Solvent was removed under reduced pressure. The product was redissolved in n-Hexane and kept refrigerated until required.

iii) Counting Radioactivity

To determine the radioactivity present in the ^{14}C cholesteryl palmitate the n-Hexane was removed in oxygen-free nitrogen and the ester redissolved in 2 drops of n-Hexane, followed by 21ml acetone. 10µl aliquots of this solution were placed in 3 x 6ml scintillation vials with 2ml Triton scintillant and shaken before counting the radioactivity in an LKB Minibeta liquid scintillation counter. The amount of radioactivity used is stated in individual exercises.

Triton Scintillant

PPO (2,5-diphenyloxazole)	5g
POPOP (1,4-Bis-(4-methyl-5-phenyl-2-oxazolyl) benzene)	0.3g
Triton X-100	200ml
Distilled Toluene	800ml

iv) Addition of Sterol to Medium

Unlabelled Sterol

0.155g of unlabelled cholesteryl palmitate was dispersed in a few drops of n-Hexane and 100ml acetone. 1ml of this solution was added per 100ml of medium before autoclaving.

Labelled Sterol

When working with liquid medium, the sterol was added to each flask before autoclaving. When working with agar, the sterol was added to the medium before autoclaving and then dispensed into Petri dishes.

5.3. EXPERIMENTAL DESIGN

Strains c and e were used in these experiments in both liquid and agar media. After drying, the cultures were extracted and these extracts analysed by HPLC to observe sterol metabolism.

The strains were paired in liquid and in agar cultures to give as wide an indication as possible as to what and where the metabolites were. With liquid cultures both the mycelium and the filtrate could be examined to find out in which phase metabolites were present. It was not possible in liquid culture, however, to determine whether there was any difference between induced male and induced female, since both were together in the same flask and could not be separated. This was why agar cultures

were also included. Induced males and females which had been separated by a polycarbonate membrane, could be analysed separately although it was not possible in this case to determine whether the metabolite was in the mycelium or the agar, since both were extracted together and could not be separated. Thus, these exercises together, showed where the metabolite was found in cultures, and whether induced males and induced females produced different sterol metabolites from males and females grown alone.

i) Agar Cultures

5 x 250ml flasks each containing 200ml Schmitthenner's medium, 3.1mg cholesteryl palmitate dissolved in 2ml acetone, (^{14}C)-cholesteryl palmitate (24 x 10^6 dpm) and 2% Difco Bactoagar were autoclaved and 5ml aliquots of the medium dispersed in 200 x 5cm Petri dishes.

Plates inoculated

80 with strain c

80 with strain e

40 remain uninoculated (0)

↓ incubate 24°C, 72h

Strains paired

Separated by

a polycarbonate

membrane

40 c with e

20 c with c

20 e with e

20 0 with0

↓ reincubate at 24°C (time specified in each exercise)

Agar discs separated and freeze dried and extracted.

Extraction Procedure

The freeze dried discs were placed in cellulose thimbles and extracted in chloroform/methanol (2:1 v/v) for 14h in a Soxhlet apparatus. The chloroform/MeOH was removed under reduced pressure in a rotary evaporator and the extract transferred to a small vial and and redissolved in MeOH ready for use in HPLC, or ether for TLC.

Extracts for analysis

Strain c which has been paired with e ($\frac{c^*}{e}$),

Strain e which has been paired with c ($\frac{e^*}{c}$),

Strain c paired with c (c),

Strain e paired with e (e),

and the uninoculated medium (0).

(ii) Liquid Cultures

20ml aliquots of Schmitthenner's medium with cholesteryl palmitate were dispensed into 160 x 100ml wide necked flasks. The ^{14}C cholesteryl palmitate was then added to each flask(0.39×10^6 dpm per flask).

Flasks inoculated

60 with strain c

60 with strain e

40 reamin uninoculated (0)

↓ incubate 24°C, 5 days

Cultures paired by combining the contents of the flasks

20 c with e

20 e with e

20 c with c

20 0 with 0

↓ reincubate at 24°C (time specified in each exercise)

↓ Pairings filtered through filter paper in a

↓ Buchner funnel and washed. The filter paper containing the mycelium was removed, freeze dried and extracted.

Extraction procedures

Filtrate

The filtrate was extracted in a separating funnel with an equal volume of ethyl acetate. After removing the solvent using a rotary evaporator, the extract was redissolved in MeOH ready for HPLC or ether for TLC.

Mycelium

The freeze dried mycelia were placed in cellulose thimbles and extracted with chloroform/MeOH (2:1 v/v) for 14h in a Soxhlet apparatus. The solvents were removed and the extract redissolved in MeOH for HPLC an ether for TLC.

Extracts for analysis

Filtrate

Filtrate in which c had been paired with e ($\frac{c}{e}$)

c with c

e with e

and the uninoculated medium.

Mycelium

c with e ($\frac{c}{e}$)

c with c (c)

e with e. (e)

The radioactivity of the samples and extracts was recorded throughout the extraction procedure to determine recovery and the efficiency of the technique.

The samples were kept in the freezer until required.

5.4. ANALYSIS

(i) Thin Layer Chromatography (TLC)

For TLC, the dried extracts were redissolved in 2ml ether. This was streaked in lines 2cm long, 4cm from the edge of the plate and 1-2cm apart. Analytical silica gel plates were used. The chromatographs were developed first in 6% ether in petroleum

spirit and then in ethyl acetate/petroleum spirit (40-60 boiling range) (1:1 v/v). The plates were dried and the position of the radioactive bands determined using:-

1. Panax radioactive scanner (fig. 9)
2. Placing the complete TLC plate in contact with photographic film for 4 weeks (Fig. 10).

Unfortunately, TLC did not give a very satisfactory result. 4 radioactive zones more polar than cholesterol were visualised, but the bands were very faint and hazy. For this reason, a more sensitive system was used, namely High-Performance liquid chromatography (HPLC), which has the ability to resolve compounds which are $\frac{1}{2}$min apart going through the column.

Figure 9 Trace obtained when a developed TLC plate, streaked with extract of filtrate in which strain 431c and 431e of P.sylvaticum had been paired, was scanned using a Panax radioactive scanner. The peaks recorded on the trace represent bands of radioactivity on the TLC plate.

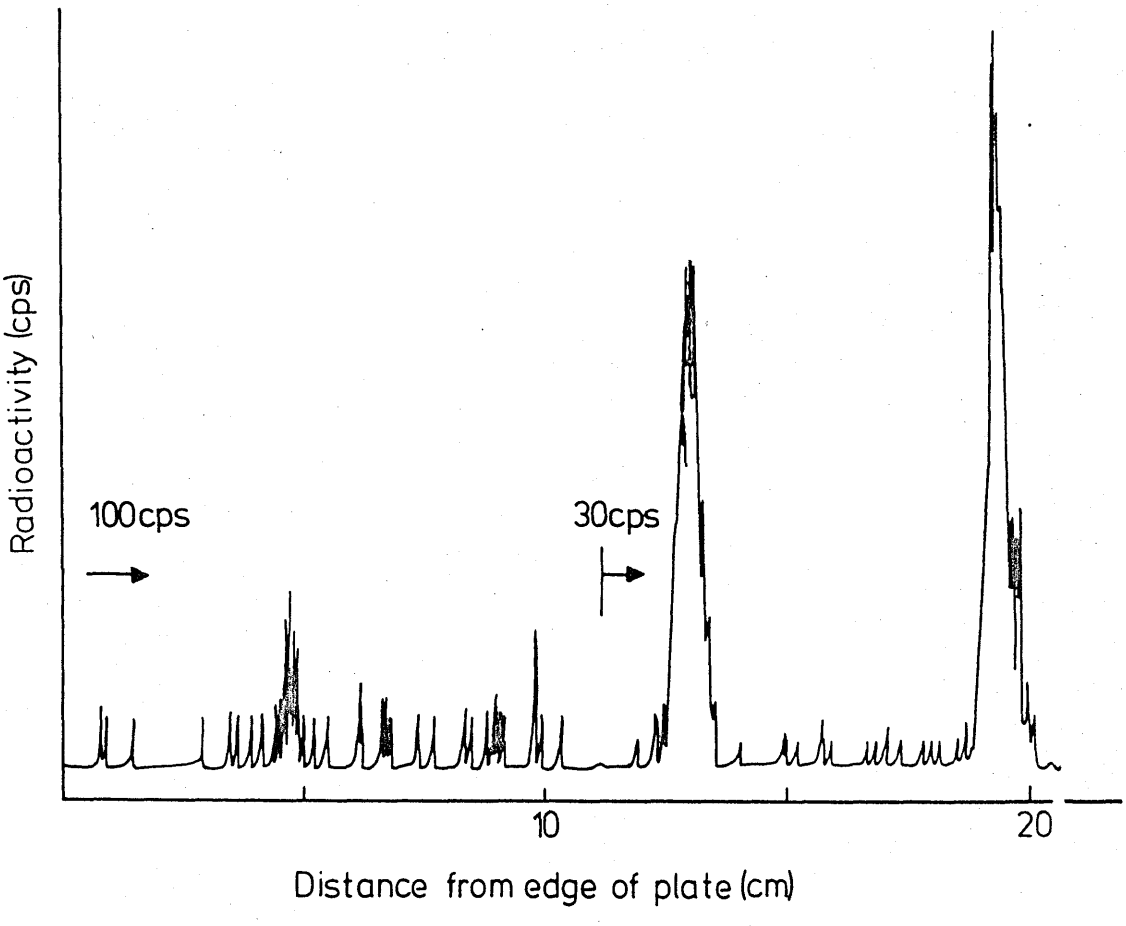

Radioactivity (cps)
100cps
30cps
10
20
Distance from edge of plate (cm)

Figure 10 Extracts of P.sylvaticum c mycelium (1), e mycelium (2), $\frac{c}{e}$ mycelium (3) and e filtrate (4) were streaked on a TLC plate developed and the dried plate placed in contact with photographic film for 4 weeks. The dark bands represent areas of radioactivity.


1
2
3
4

(ii) High Performance Liquid Chromatography (HPLC)

Column packing

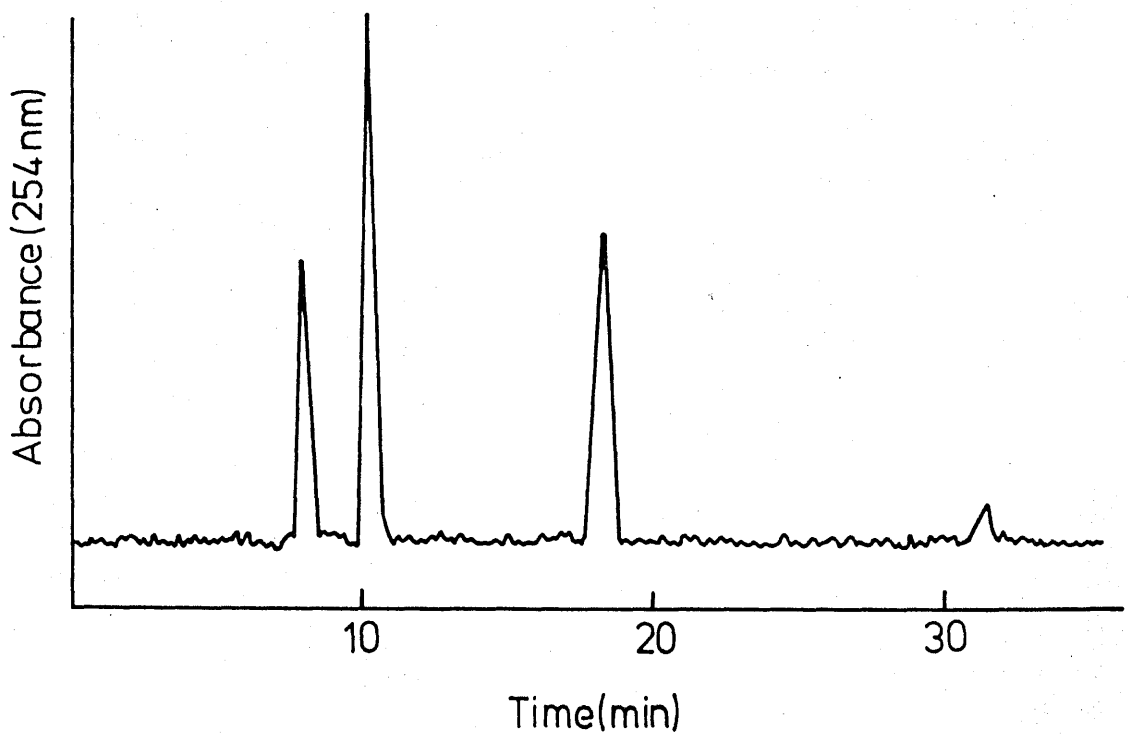
Using a Shandon packing pump.

Column Type	Shandon 5 x 250mm
Packing Material	5um MOS Hypersil
Slurry	6g in 33ml isopropanol
Ultrasonicate	2 minutes
Packing Pressure	100 000 psi
Solvent A	Hexane 100ml
Solvent B	MeOH 40ml
invert column	MeOH 40ml
Solvent C	50% aqueous MeOH 100ml

3 x 30 sec. bursts at 12000 psi

Test conditions 10µl ODS test mixture, UV 254nm, Solvent MeOH in H_2O, flow rate 1ml min^{-1}.

TRACE


Absorbance (254 nm)
10
20
30
Time(min)

Column Type and Conditions for Chromatography

Solvents were delivered by either a Spectra physics 8100 or Spectraphysics 8700. On isocratic runs and for column cleaning, solvents were delivered at a rate of 1ml min^{-1}. However, most analysis was carried out using a gradient programme:-

Time(mins)	Water(%)	Methanol(%)	flow(ml min^{-1})
0	20	80	1
30	0	100	1
35	0	100	2
70	0	100	2
70.1	0	100	1
75	20	80	1

Samples were delivered into a 250µl loop before injection onto the column.

All work was carried out in a reverse phase system. Initially a Shandon 250 x 5mm column was used with 5mm C18 ODS -Hypersil, but it was found that cholesteryl palmitate was retained for some time, even at 100% MeOH. For this reason a packing material less non polar than ODS-Hypersil was used, namely MOS-Hypersil (C8) (mean particle size 5mm). With this packing material compounds were therefore released more quickly when running a gradient from a

polar solvent (20% H_2O, 80% MeOH) to a less polar solvent (100% MeOH). MOS Hypersil was particularly useful for separating molecules with a wide range of polarites.

Injection

The sample to be analysed was dissolved in methanol and depending on the % MeOH present at the beginning of each run on the HPLC, the sample was diluted with H_2O accordingly. The sample could not be injected in 100% MeOH if the gradient of the run started at 80% MeOH as this would give false retention times; the sample should be injected in 80% MeOH. Approximately 100,000dpm were injected in each run. The approximate weight detectable can be estimated using the specific activity of the ^{14}C cholesterol used to synthesize the cholesteryl palmitate.

Specific activity of cholesterol 50mCi $mmol^{-1}$.

$$1\mu Ci = 2.2 \times 10^6 dpm$$
$$\therefore 50mCi = 1.1 \times 10^{11} dpm$$

1.1×10^{11}dpm in 0.386g cholesterol

$\therefore$ 1.0×10^5dpm in 3.509×10^{-7}g cholesterol

$\therefore$ Each injection contains $\sim$ 3.5ng of radioactive cholesterol plus cholesterol derived compounds. Unlabelled material was also present.

5.5. DETECTION

Three systems were used to detect to radioactive compounds separated by the column. The extraction procedure and column conditions were the same for each system only the detector was changed. Liquid and agar cultures were extracted in 3 separate experiments. In two of these exercises the SP8700 with liquid scintillation system was used, and in the third SP8100 and solid scintillant monitor was used. In other cases the solution emerging from the HPLC column was collected in a series of test tubes, using a Gilson Fraction collector (see below) and the radioactivity in each fraction was determined. This system was used to isolate peaks for identification.

(i) Radioactivity Monitor - Liquid Scintillation System

The base of the column was connected to an ICN Coruflow CMF liquid scintillation system wnich mixed 3ml of reverse phase scintillant with 1ml of chromatographed sample before entering the flow cell. The radioactivity was given in counts per minute (cpm). The peaks produced were recorded on a Penheat 2 pen chart recorder.

Reverse phase scintillant

PPO	10g
Distilled Xylene	670ml

Methanol	150ml
Triton-X	330ml

(ii) Radioactivity monitor - Solid Scintillant System

The radioactivity in the chromatographed sample was also detected using a solid scintillant cell, composed of Ytrium silicate.

This machine could be linked to a plotter which automatically printed the trace on completion of the run. The advantage of this system over the liquid scintillation system is that the sample could be collected after it had travelled through the solid scintillant in its pure state i.e. without scintillant. Unfortunately this system was only available for use for a short period of time and the test could only be carried out once. The results produced by this method were the same as with the liquid system, and since the liquid system was repeated, the traces and results shown relate to the liquid and not the solid scintillant studies.

(iii) Fraction collecting - LKB Minibeta Liquid Scintillation System

The sample on elution from the column could also be collected as a series of fractions and the radioactivity in each fraction recorded by counting in a scintillation counter. The column base

was connected to a Gilson Microcol TDC80 fraction collector and 1 min. samples collected in 10ml tubes. 1% of the volume of each tube was then placed in a 6ml scintillation vial 2ml Triton Scintillant and counted in the LKB Minibeta liquid scintillant counter. The samples in each peak of activity were then combined and retained for identification.

Triton Scintillant

PPO	5g
POPOP	0.3g
Triton-X	200ml

to 1 litre with distilled toluene.

5.6. RESULTS

The results for the liquid and agar culture exercises over the three times that the experiment was performed were consistent.

(i) Standards. The cholesterol standard gave a peak at 30 mins. and the cholesteryl palmitate standard at 43 mins. (fig. 11)

(ii) Agar cultures. In the extracts of agar cultures 7 main peaks were apparent in the chromatograms. (fig. 12-15).

Peak no	Retention Time (min)
1	21
2	22
3	24.5
4	25
5	30
6	36
7	43

The control extract of agar which was uninoculated gave the peaks at 30 mins. and 43 mins. but none of the earlier peaks was present. The peak at 43 mins. (peak 7) had 7 times the area of the peak at 30 mins (peak 5). There must be some break down of cholesteryl palmitate to free sterol during incubation, extraction, storing and analysis by HPLC. All 7 peaks appeared in the culture extracts of the 2 and 8h induced male, 2 and 8h induced female and in

uninduced males and females.

Evidently peak 5 of the sample extract was cholesterol and peak 7 was unmetabolized cholesteryl palmitate.

The fact that compounds more polar than cholesterol (peaks 1-4) were not noted in the control indicates sterol metabolism by the organism.

There were no qualitative differences in the peaks produced by the different paired strains. However, there were certain quantitative differences (see fig. 12-15). Larger amounts of polar compounds were present in paired cultures than in uninduced cultures.

Ideally all peaks would be compared with thehighest peak, in this case peak 5, but since this is off scale in nearly all the traces it was necessary to compare the peaks with another "standard" viz. peak 7 (cholesteryl palmitate) although the extent of metabolism of the palmitate will vary from case to case. Comparison of the relative amounts of peaks 1 and 2 and peak 7, indicates peaks 1 and 2 (polar compounds) in the uninduced strains were about half the size of peak 7, whereas in induced cultures 1 and 2 were about twice the size of peak 7. A similar trend was noted on comparing peaks 3 and 4 with peak 7.

Table 12 , shows the percentage area of each peak relative to cholesterol (peak 5), derived from peak area figures obtained from an integrator linked to the HPLC system. Unfortunately, the integrator used did not have the ability to distinguish peaks less than 0.5 - 1 min apart. The area of some peaks were recorded as being much larger than they actually appeared when examining the trace. Therefore, although these results are included here (Table 12), they are not considered a satisfactory quantification of the results recorded on the traces. Larger peaks were recorded when using the liquid scintillant then solid scintillant. Liquid scintillation counting is known to be more efficient than solid scintillant because it is common for the sample to adhere to the solid material.

(iii) Liquid Cultures

Peaks corresponding to peaks 1, 2, 3, 4, 5 and 7 mentioned in (ii) were apparent in filtrate extracts of paired and unpaired strains. Peak 6 was absent. This may be due to inadequate compound added to the column. In the mycelial extracts the 6 peaks found in the filtrate extracts were observed. As with the exercises involving agar cultures, no qualitative differences were observed between induced or between filtrates and mycelium. However, the proportions of the first 4 peaks (again 5 was off scale), differed in mycelial and filtrate extracts. In filtrate extracts peaks 1 and 2 were

AGAR

Culture	Total added	Chloroform/MeOH extract of dried agar discs	In extracted agar discs	% recovery
c/e*	2.12×10^7	4.30×10^6	8.40×10^6	60
c*/e	2.12×10^7	4.07×10^6	9.12×10^6	62
c	2.12×10^7	3.40×10^6	10.04×10^6	63
e	2.12×10^7	3.80×10^6	9.01×10^6	60
o	2.12×10^7	4.2×10^6	8.20×10^6	58

Table 10 Radioactivity of samples at various stages in extraction procedure (in dpm) in agar cultures.

LIQUID

Culture	Total added	filtrate before extraction	filtrate after extraction	Ethyl acetate extract of filtrate	Chloroform/ Methanol extract of mycelium	% recovery	wt. of mycelium (g)
c/e 2h	15.6×10^6	1.87×10^6	0.35×10^6	1.58×10^6	0.15×10^6	11	1.46
c/e 8h	15.6×10^6	1.64×10^6	0.43×10^6	1.21×10^6	0.48×10^6	11	1.47
c	15.6×10^6	2.40×10^6	0.16×10^6	1.97×10^6	1.97×10^6	16	1.45
e	15.6×10^6	2.06×10^6	0.72×10^6	1.40×10^6	1.92×10^6	16	1.47
o	15.6×10^6	5.05×10^6	0.03×10^6	5.48×10^6	-	-	-

Table 11 Radioactivity of samples at various stages during extraction procedure (in dpm) in liquid culture.

PEAKS

	Experiment	1	2	3	4	5	6	7
AGAR								
c alone	Solid Scintillant	-	1.69	5.57	-	100.00	3.26	3.07
e alone	"	1.20	1.18	3.36	0.98	100.00	1.52	3.28
c"/e	"	0.98	0.92	6.35	-	100.00	3.31	4.02
c/e*	"	0.87	1.58	5.18	-	100.00	1.12	1.35
uninoculated medium	"	-	-	-		100.00	-	732.45
LIQUID								
FILTRATE								
c alone	Solid Scintillant	5.68	23.02	8.29	-	100.00	-	10.10
		4.65	21.10	9.84	2.01	100.00	0.23	1.68
	"	4.65	21.66	6.46	1.68	100.00	-	4.38
	Liquid Scimtillant	11.77	73.07	34.23	7.21	100.00	-	1.55
e alone	Solid Scintillant	4.26	20.03	4.51	-	100.00	-	6.68
	"	5.93	23.37	4.79	1.78	100.00	-	9.65
c/e	Solid Scintillant	-	23.10	-	-	100.00	-	2.65
	"	8.71	23.59	4.13	1.19	100.00	-	6.16
	Liquid Scintillant	14.13	81.60	21.95	5.82	100.00	1.63	2.10
		20.32	98.56	26.07	7.79	100.00	-	2.55
uninoculated filtrate	Solid Scintillant	-	-	-	-	100.00	148.50	117.49
	"	-	-	-	-			

MYCELIA

c alone		Not Available						
e alone	Solid Scintillant	9.70	10.41	20.38	5.71	100.00	9.19	4.7
c/e	Solid Scintillant	7.12	13.84	21.51	6.02	100.00	6.81	0.71
		4.68	6.21	13.08	-	100.00	-	30.24

Table 12. Percentage area of each peak relative to cholesterol (peak 5), derived from peak area figures obtained from an integrator linked to the HPLC system.

2 - 3 times larger than peaks 3 and 4, whereas, in mycelial extracts peaks 1 and 2 were about ½ the size of peaks 3 and 4. The filtrate control again produced no polar material only some cholesterol and cholesteryl palmitate. (fig. 15-19).

As can be seen from Tables 10 and 11, the recovery of radioactive material from agar cultures was reasonable, varying between 58 and 63% recovery. The recovery in liquid media was very poor, 11-16%, probably due to adhesion of material to the glass of the large number of small flasks used for growing and combining the cultures.

The differences in the quantity of metabolites observed, cannot be easily explained in relation to sexual reproduction. There was little qualitative difference bewteen metabolite production in induced and uninduced strains; this suggests that control of sexual reproduction may not be related to these sterol metabolites.

Some of the HPLC traces display an eratic baseline before 20mins. It was suggested that some of these initial peaks may be important. However, their size could not be increased by adding more sample in the injection of by increasing the sensitivity of the machine. Also these small peaks did not have a common retention time in each run. This shows that these small peaks were simply background noise.

Figure 11 Reverse phase analysis of known standards. A. cholesterol B.cholesteryl palmitate.

Mobile phase: see page 119

Detector : see page 121

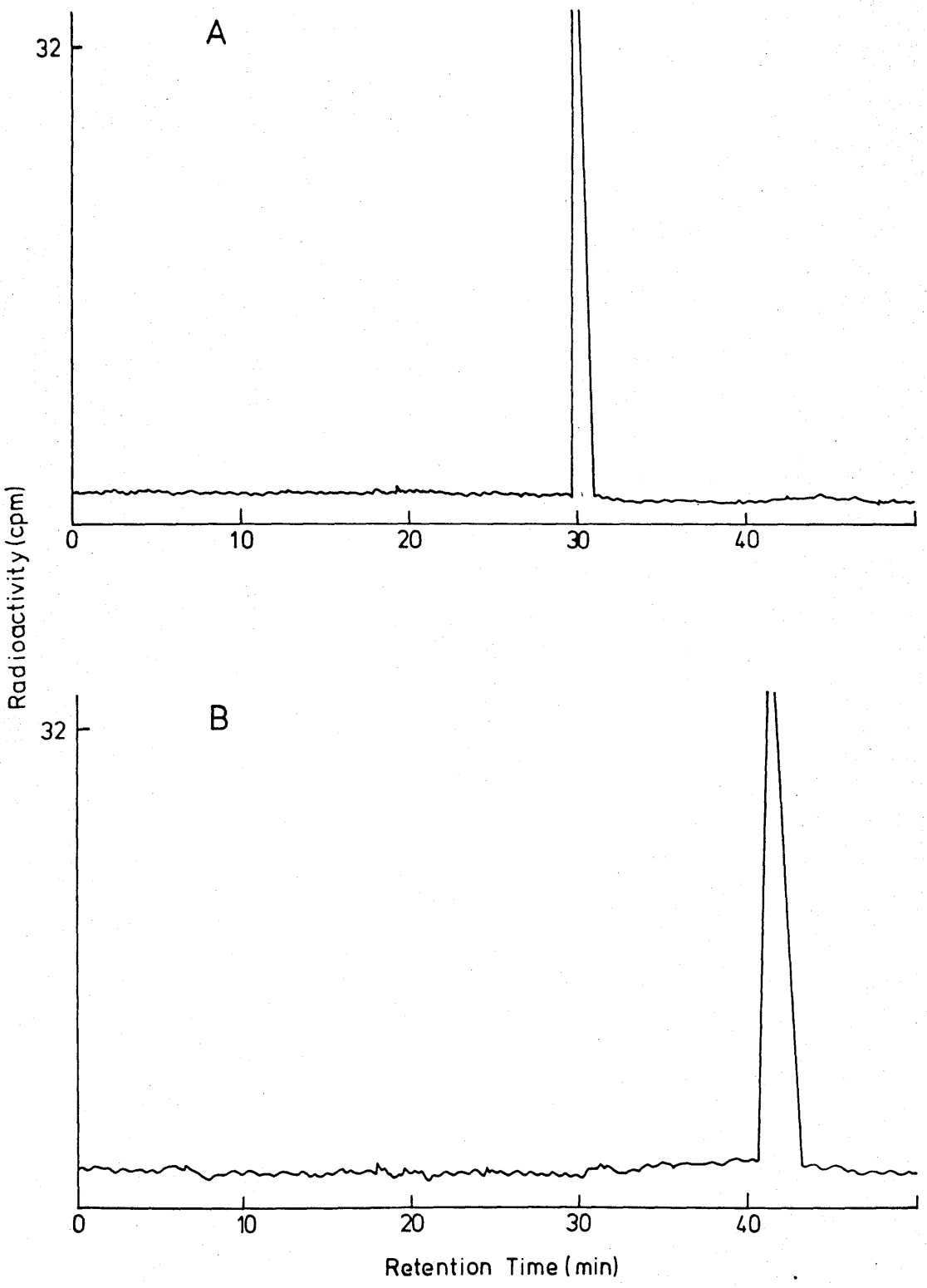

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 12 Reverse phase analysis of sterol metabolites in chloroform/methanol extracts of agar cultures. A. Strain c (♀) B. Strain e (♂) (see page 109 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

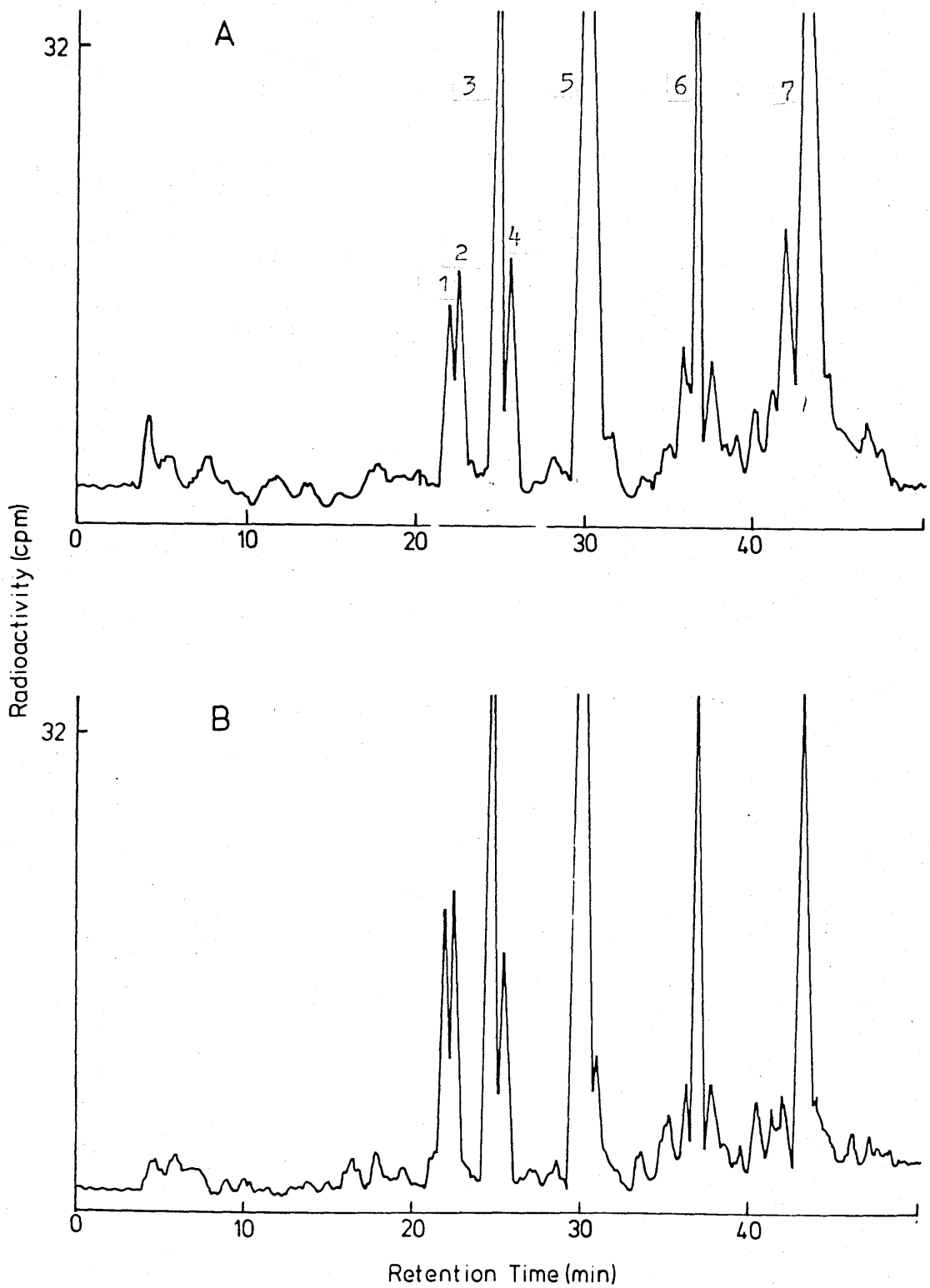

A
B
32
32
1
2
3
4
5
6
7
0
10
20
30
40
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 13 Reverse phase analysis of sterol metabolites in chloroform/methanol extracts of agar cultures. A. Strain e (♂) which had been paired with strain c (♀) for 2h before extraction ($\frac{c^*}{e}$ 2h). B. Strain e which had been paired with strain c for 8h before extraction ($\frac{c^*}{e}$ 2h) (see page 109 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

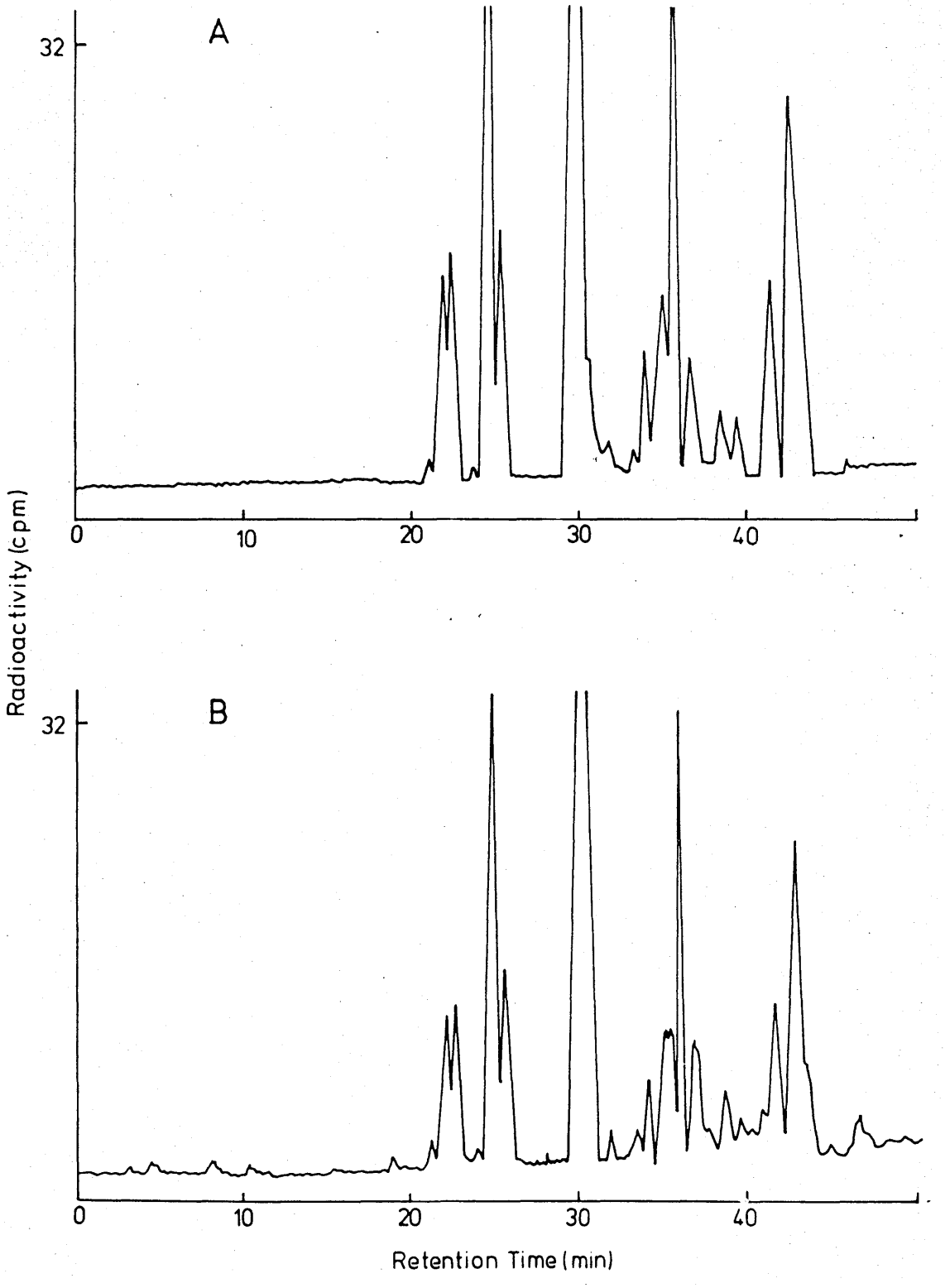

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 14 Reverse phase analysis of sterol metabolites in chloroform/ Methanol extracts of agar cultures.A. Strain c (♀) which had been paired with strain e (♂) for 2h before extraction ($\frac{c}{e*}$ 2h) B. Strain c which had been paired with strain e for 8h before extraction ($\frac{c}{e*}$ 8h) (see page 109 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

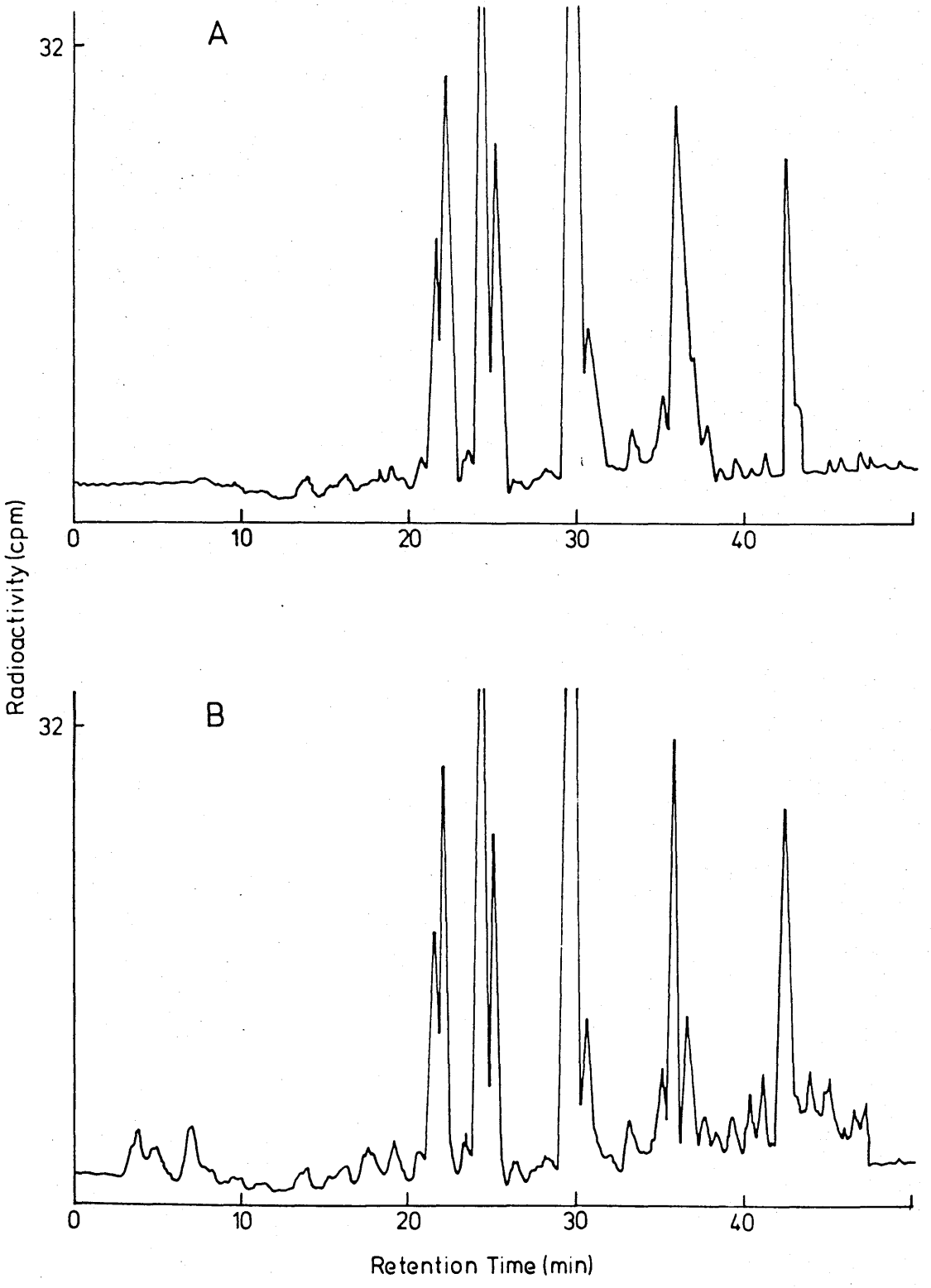

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 15 Reverse phase analysis of controls in agar liquid exercises. (uninoculated media) A. Chloroform/methonal extract of uninoculated agar B. Ethyl acetate extract of uninoculated liquid medium (see page 109 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

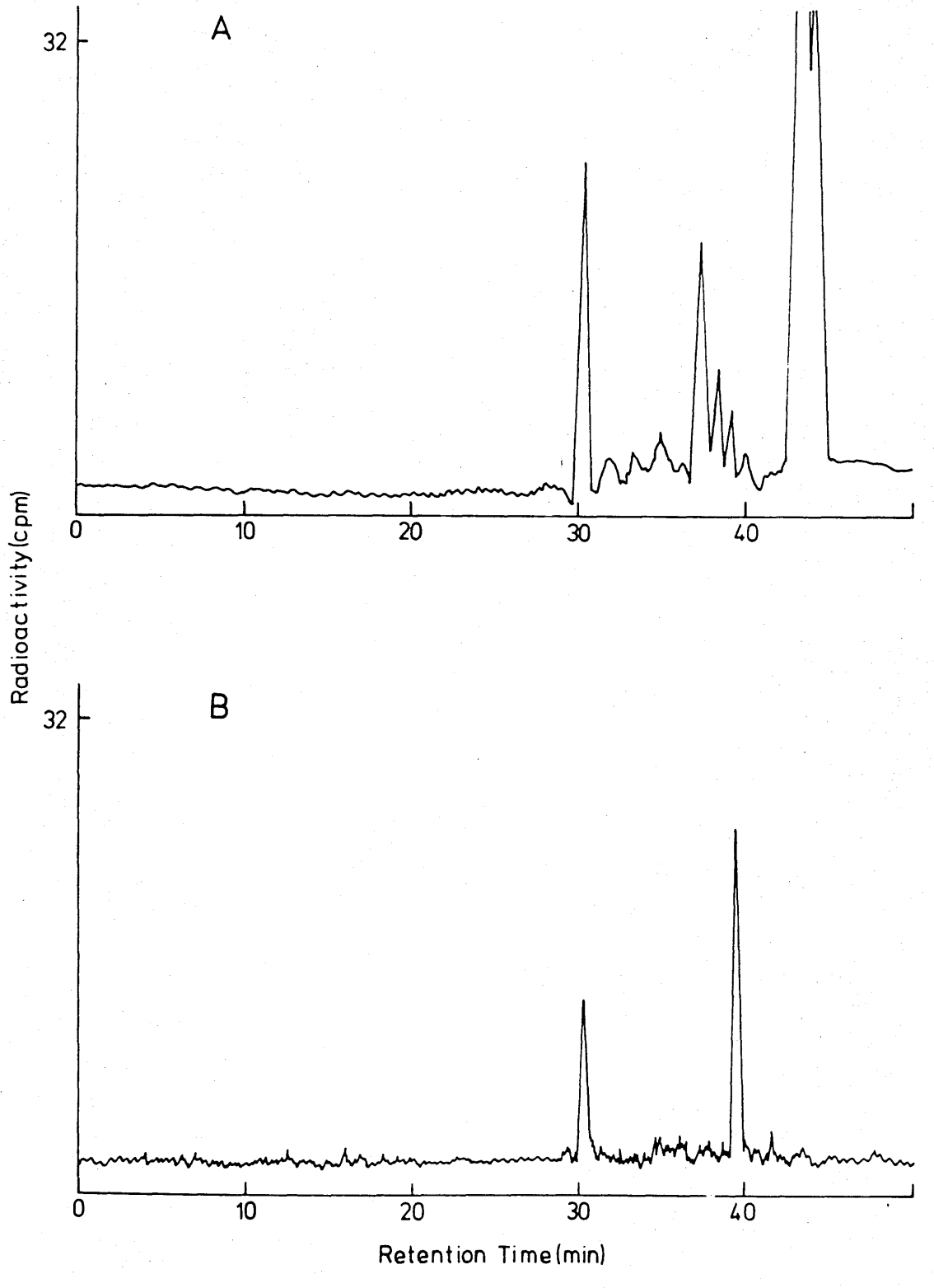

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity(cpm)
Retention Time(min)

Figure 16 Reverse phase analysis of sterol metabolites in ethyl acetate extracts of filtrate. A. Strain c with strain e paired for 2h before extraction ($\frac{c}{e}$ 2h) B. Strain c with strain e paired for 8h before extraction ($\frac{c}{e}$ 8h). (See page 111 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

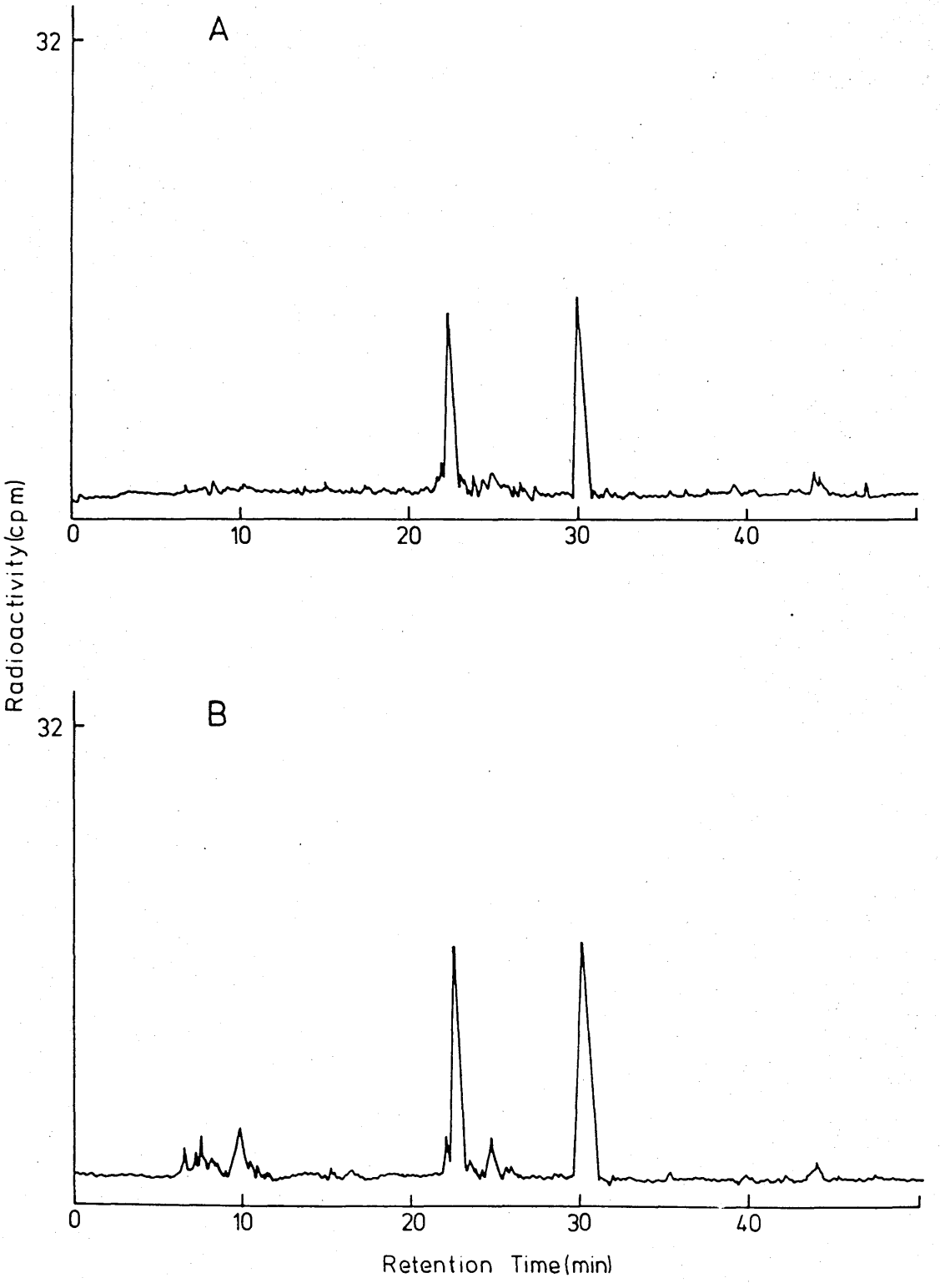

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity(cpm)
Retention Time(min)

Figure 17 Reverse phase analysis of sterol metabolites in ethyl acetate extracts of filtrate.A. Strain c(♀) B. Strain e (♂) (see page 111 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

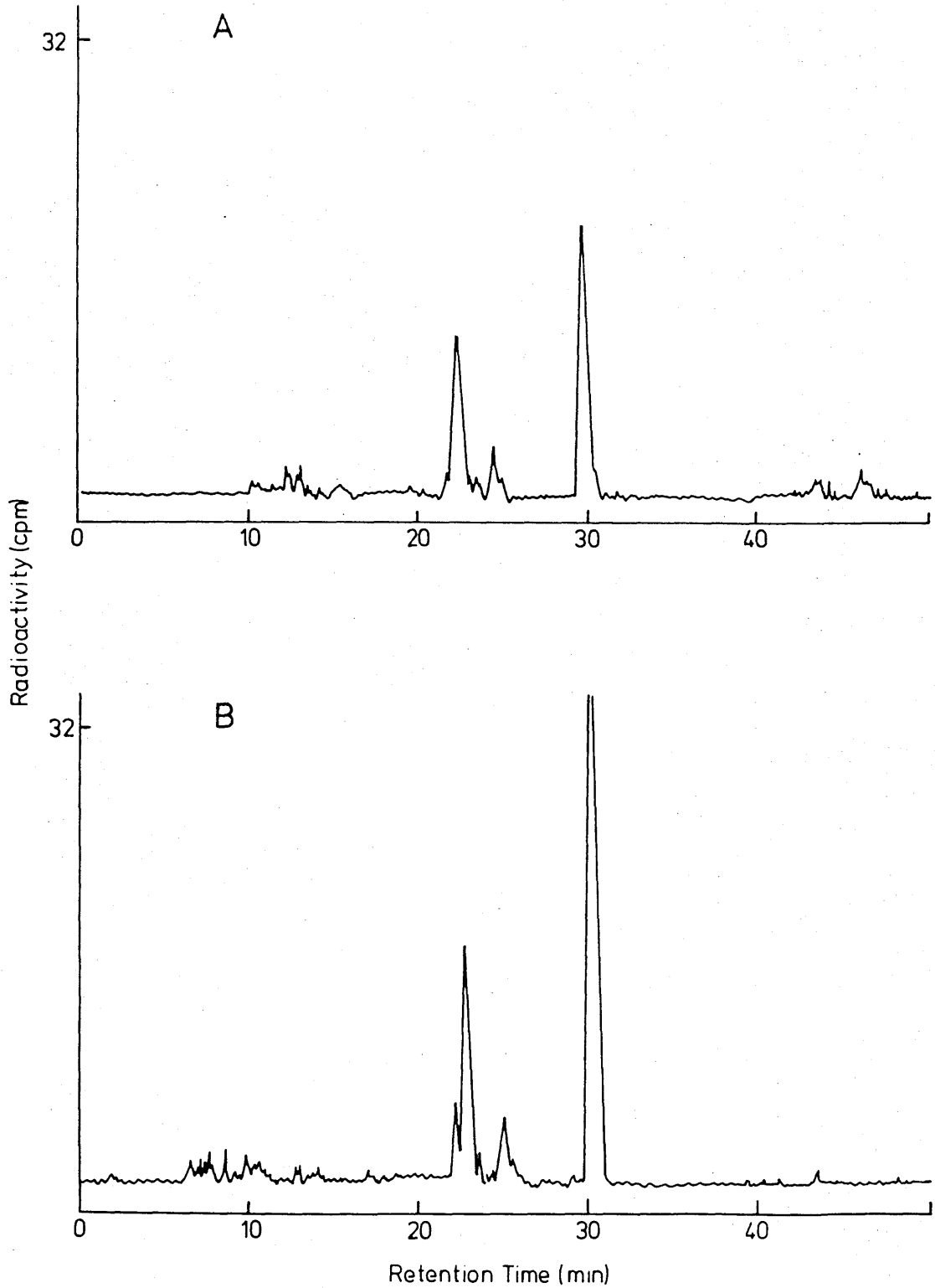

A
32
0
10
20
30
40
B
32
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 18 Reverse phase analysis of sterol metabolites in chloroform/methanol extracts of dried mycelium. A. Strain c with strain e paired for 2h before extraction ($\frac{c}{e}$ 2h) B. Strain c with strain e paired for 8h before extraction ($\frac{c}{e}$ 8h). (see page 111 for details of extraction).

Mobile phase: see page 119

Detector : see page 121

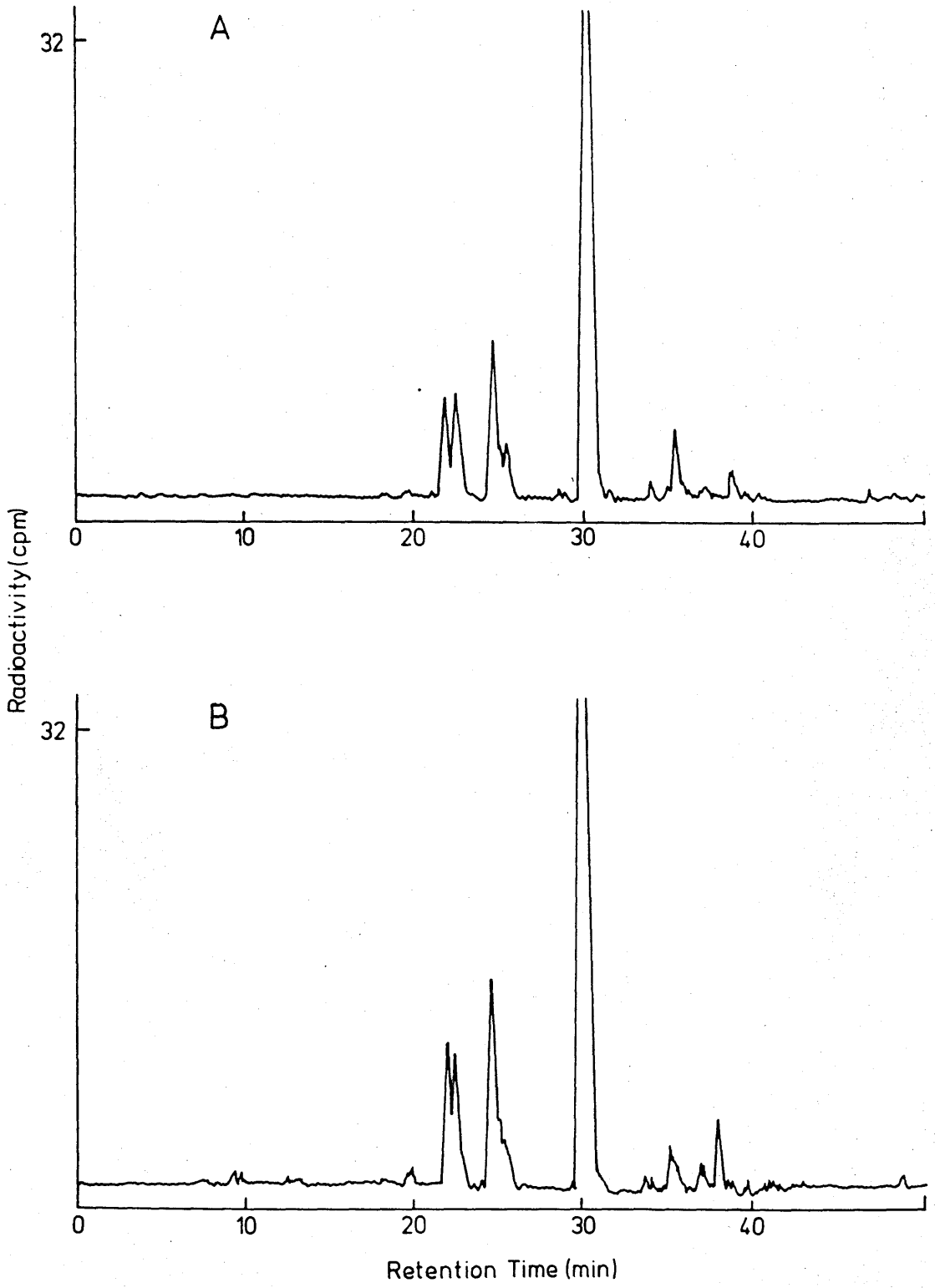
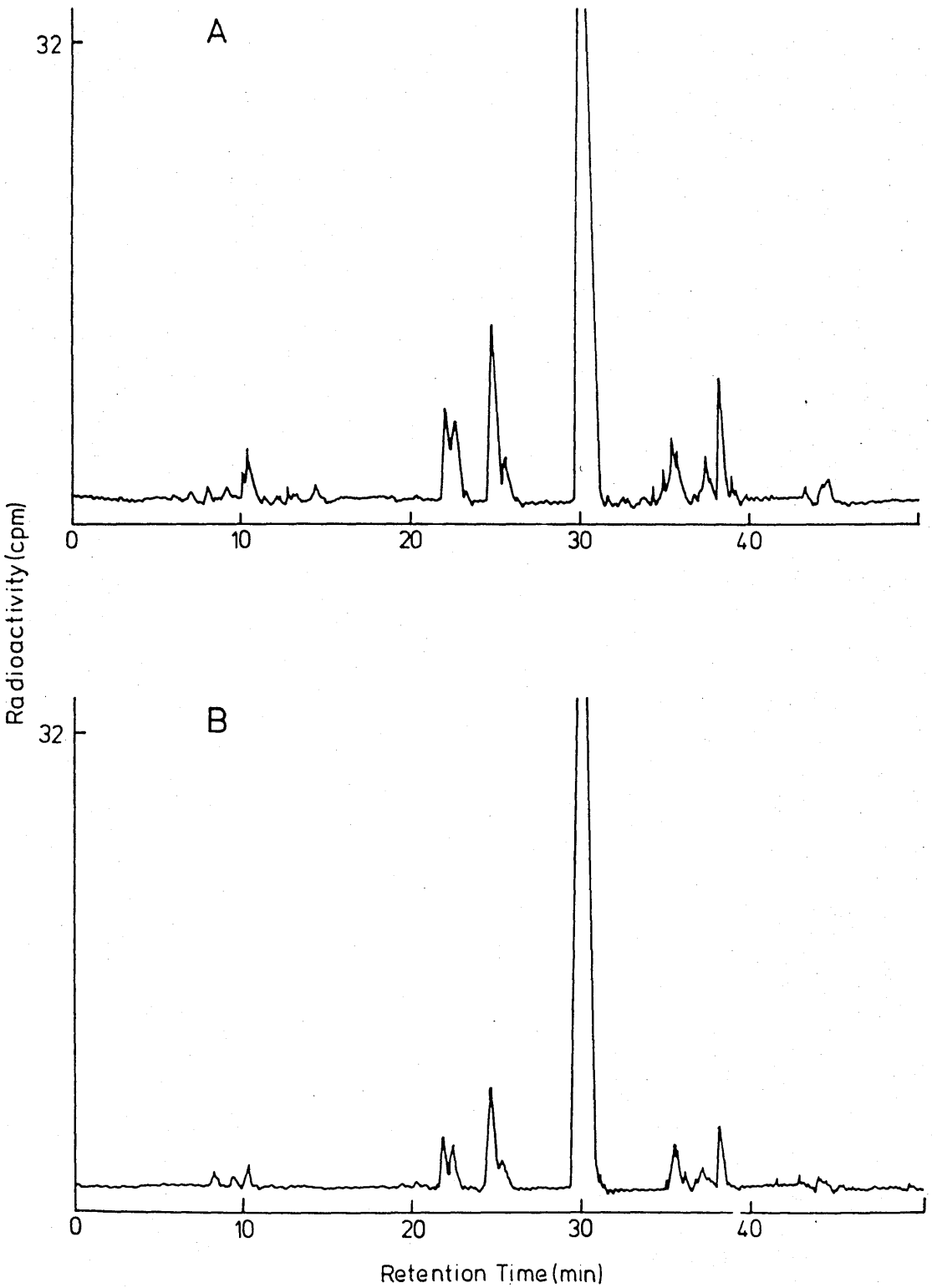

A
B
32
32
0
10
20
30
40
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Figure 19 Reverse phase analysis of sterol metabolites in chloroform/methanol extracts of dried mycelium.A. Strain c (♀) B. strain e (♂) (see page 111 for details of extraction).

Mobile phase: see page 119

Detector : see page 121


A
B
32
32
0
10
20
30
40
0
10
20
30
40
Radioactivity (cpm)
Retention Time (min)

Some of the radioactivity was lost in a solid deposit when the samples were dissolved in MeOH so it is possible that there were still metabolites present which could not be detected by this method of extraction and analysis. However, the polar metabolites produced should all be readily soluble in MeOH. The solid deposit was ultrasonicated to break it up to allow MeOH to penetrate, so it is unlikely that any polar metabolites were still present in this solid. The polar metabolites may not all have been extracted in ethyl acetate and this may explain why the peaks produced in filtrate extracts were so small compared with Chloroform/Methanol extracts. However, since Chloroform does not partition readily with aqueous media and since MeOH is miscible with the filtrate, it was not convenient to use these solvents to extract the liquid.

Considerable work still requires to be carried out in this direction and it is, therefore, not possible to rule out the presence of a sterol metabolite responsible directly or indirectly for sexual reproduction.

5.7. IDENTIFICATION

Attempts were made to identify the compounds present in the extracts by using known standards, mass spectrometry and enzyme studies.

(i) KNOWN STANDARDS

As already mentioned when standards of cholesterol and cholesteryl palmitate were chromatographed in the system used to analyse the samples, cholesterol was retained for 30 mins. and cholesteryl palmitate for 43 mins. These correspond to peaks 5 and 7 respectively. Three other standards which were considered possible products were also chromatographed using the SP8100 and a variable UV monitor (λ 235nm). 7-keto-cholesterol when chromatographed in the gradient described (section 5.4.) had a retention time of 18.5 mins. which did not correspond to any of the sample peaks. Unfortunately, the other two standards, sitosterol glucoside and cholesterol α oxide could not be detected using the UV monitor, even at other wavelengths. The standards were therefore of little use when trying to identify the metabolites.

(ii) ENZYME STUDIES

It was argued that if the compound was a glucoside, the enzyme treatment with a glucosidase should remove the glucose moiety from the cholesterol; thus the glucoside peak should disappear and a new peak appear at the retention time of cholesterol. The glucose was unlabelled so it would not show on the trace. Therefore, part of the sample was chromatographed (HPLC) and a trace obtained. To portions of the remainder, an α or B glucosidase was then

added and the sample incubated at a suitable temperature.

The MeOH was removed from the substrate (sample) and the sample redissolved in 2.8ml and 3.0ul Tween 80. The enzyme was added to the substrate and incubated in a shaking water bath at 37°C for 24h. The substrate/enzyme solution was then extracted in ethyl acetate, dried and redissolved in MeOH and rechromatographed.

To test enzyme activity in Tween 80

As the samples were dissolved in MeOH which would affect the glucosidase activity, the MeOH was removed and the samples re-dissolved in 0.1% Tween 80 in H_2O. It was, however, also necessary to test the effect of Tween 80 on the enzyme. Standard compounds O-Nitrophenyl-β-D-glucopyranoside, & p-Nitrophenyl -α- D-glucopyranoside. which produce a colour change in the presence of an active β or α glucosidase, were used.

The optimum condition for the enzyme α glucosidase was pH6.8 at 37°C and for β glucosidase pH5.0 and 37°C.

The solutions for use in the test for enzyme activity in Tween 80 were set up as follows:-

β glucosidase

Substrate O-Nitrophenyl-β-glucopyranoside (0.00603g in 10ml buffer (pH5.8), 2mM soln).

Enzyme β-glucosidase (5.2 units mg^{-1})
(1mg enzyme added to 0.52ml buffer (pH6.8)).

Tween 80 0.1% Tween 80 in buffer pH6.8.

∝ glucosidase

Substrate p-Nitrophenyl-∝-D-glucopyranoside
(0.00603g in 10ml buffer (pH5.0), 2mM soln).

Enzyme ∝-glucosidase (75units mg^{-1})
(1mg enzyme added to 7.5ml buffer (pH5.0).

Tween 80 0.1% Tween 80 in buffer pH5.0.

To test if the enzyme were active in Tween 80, 5ml of substrate soln., 100µl enzyme solution and 50µl Tween 80 solution were mixed, placed in a cuvette and the absorbance at λ 405nm noted at minute intervals over 15 minutes.

Controls containing 1) substrate, enzyme and buffer pH8 i.e. no Tween 80 and 2) Tween 80 and buffer were incorporated into the exercise.

The absorbance increased steadily over the 15 minutes indicating enzyme activity.

No differences were noted in the traces obtained before and after enzyme treatment of the samples, suggesting unless the conditions are totally unsuitable for enzyme hydrolysis or that the enzymes are not specific for the glucosides present, that the compounds are not glucosides.

This was unexpected since McMorris *et al.* (1977) reported that a glucose metabolite was produced when cholesterol was fed to paired strains of *Pythium sylvaticum*. As will be shown in the mass spectrometry work (see below), glucosides were present in the extract, indicating that the enzymes used were unsuitable for sterol glucoside hydrolysis.

(iii) MASS SPECTROMETRY

Peaks from the HPLC were collected in the fraction collector and dried down under oxygen-free nitrogen. The samples were then re-dissolved in ether and introduced by probe to the electron-impact mass spectrometer.

For the mass spectrometer, the composition of the samples was as follows:- sample (1) constituted peaks 1 and 2 from HPLC, sample (2) contained peaks 3 and 4 from HPLC, sample (3) was peak 5 and

sample (4) contained all the material exuded from the column after peak (5).

The principal ions in the mass spectrum are:-

The molecular ion is the ion formed by the loss of one electron from part of the molecule (M^+).

Isotopic ion. The molecular ion contains the most abundant isotopes of the constituent elements. The parent peak (M^+) is usually accompanied by other peaks of higher mass that are caused by the ions which contain the heavier isotopes.

Fragment ions are formed by the fragmentation of the molecular in in the ion source.

Rearrangement ions are formed by rearrangement or by transposition and are not simply a result of a simple rupture of a bond in the molecule.

Two spectra were obtained for each sample (Fig. 20). An aliquot of the complete sample was added by probe and also part of the sample was silylated by treatment with bis - trimethylsilyl acetamide to give the TMS ether and a spectrum obtained again by introducing the sample by probe to the mass spectrometer.

The mass spectrum of sample (1) showed two kinds of substances. Peaks at m/e 147, 167 and 279 in the underivitised sample represent plasticizer. In the silylated sample peaks were recorded at m/e

Figure 20

A Mass spectrum of sample 1

B Mass spectrum of TMS derivative of sample 1

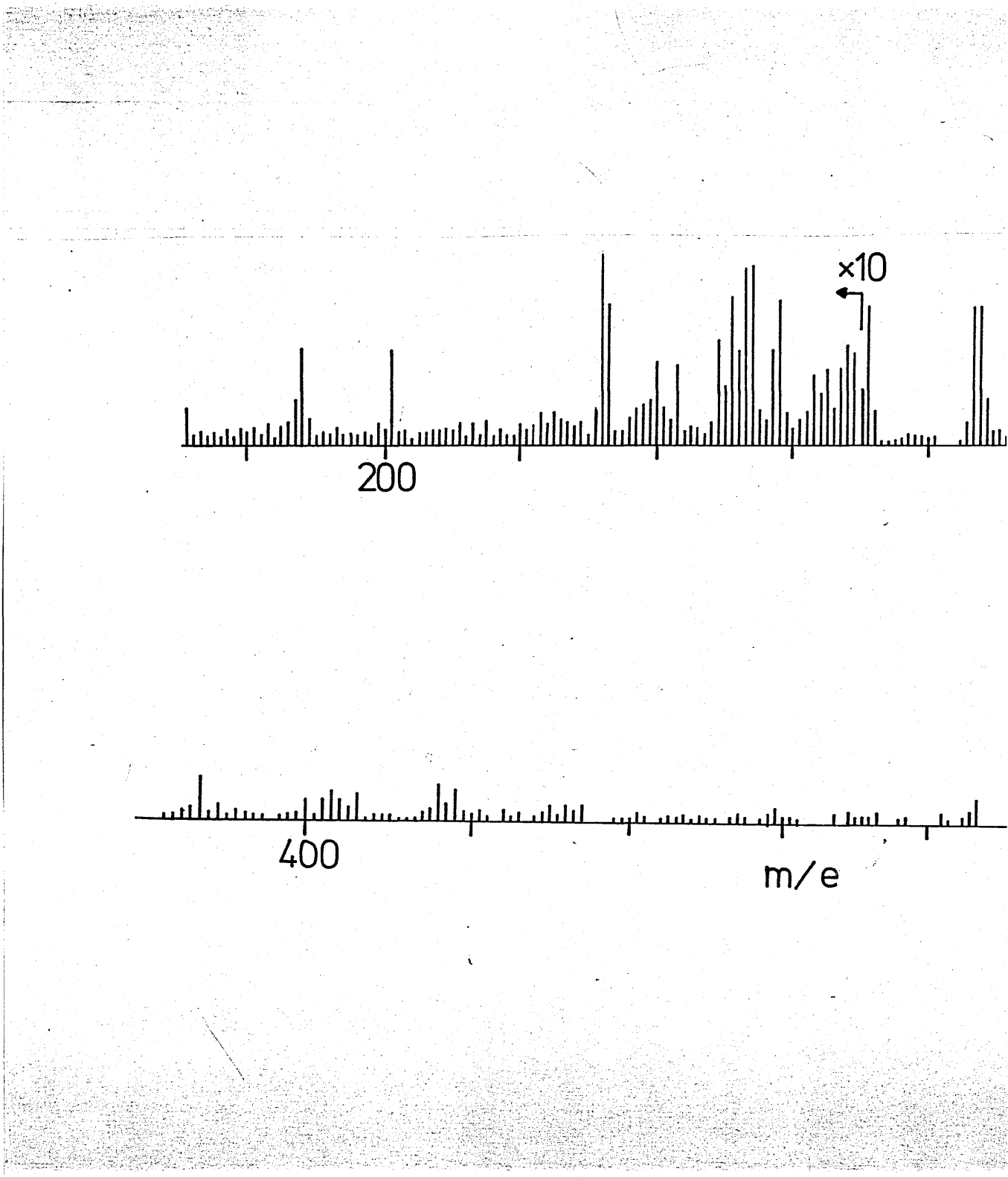
C Mass spectrum of sample 2

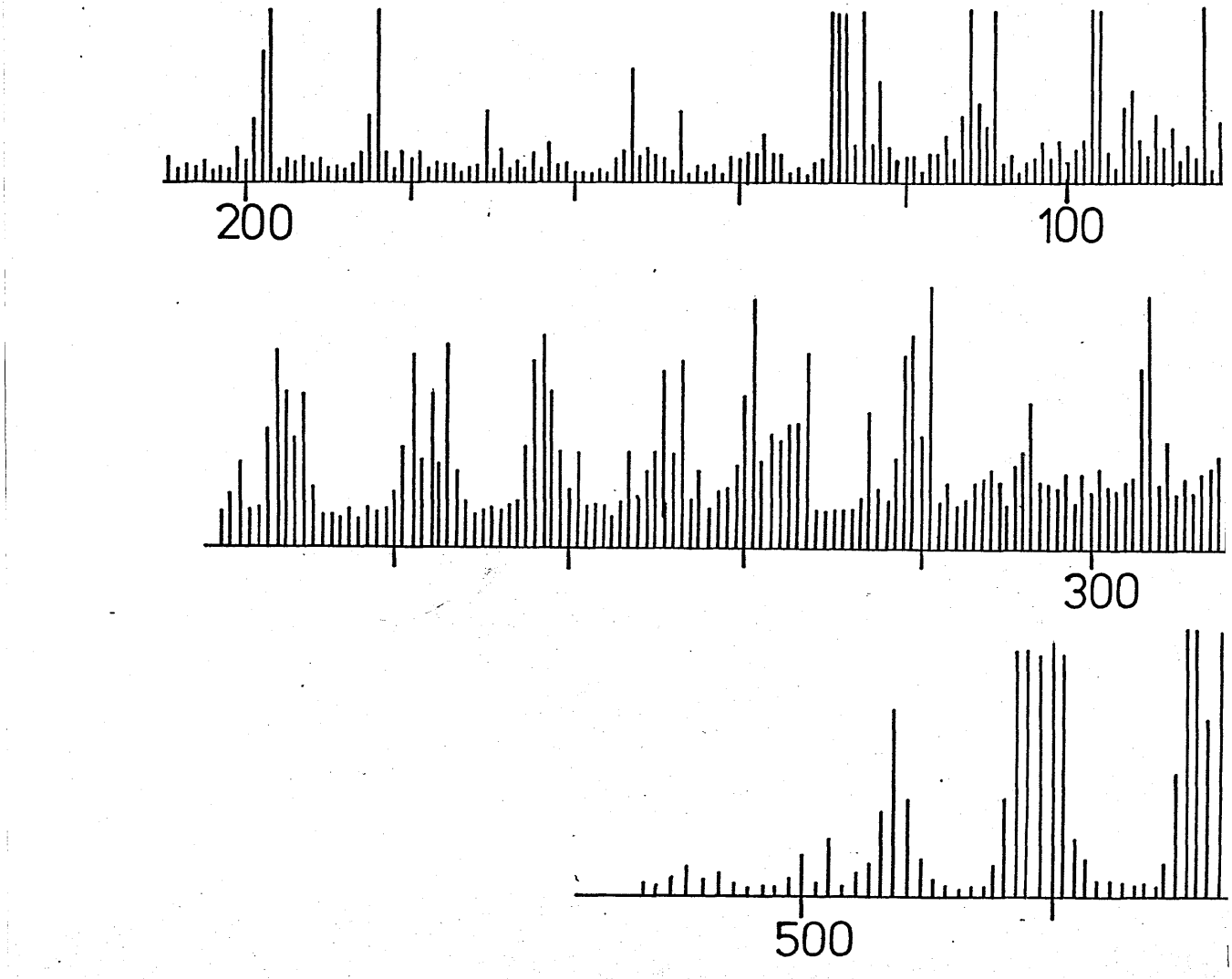
D Mass spectrum of TMS derivative of sample 2

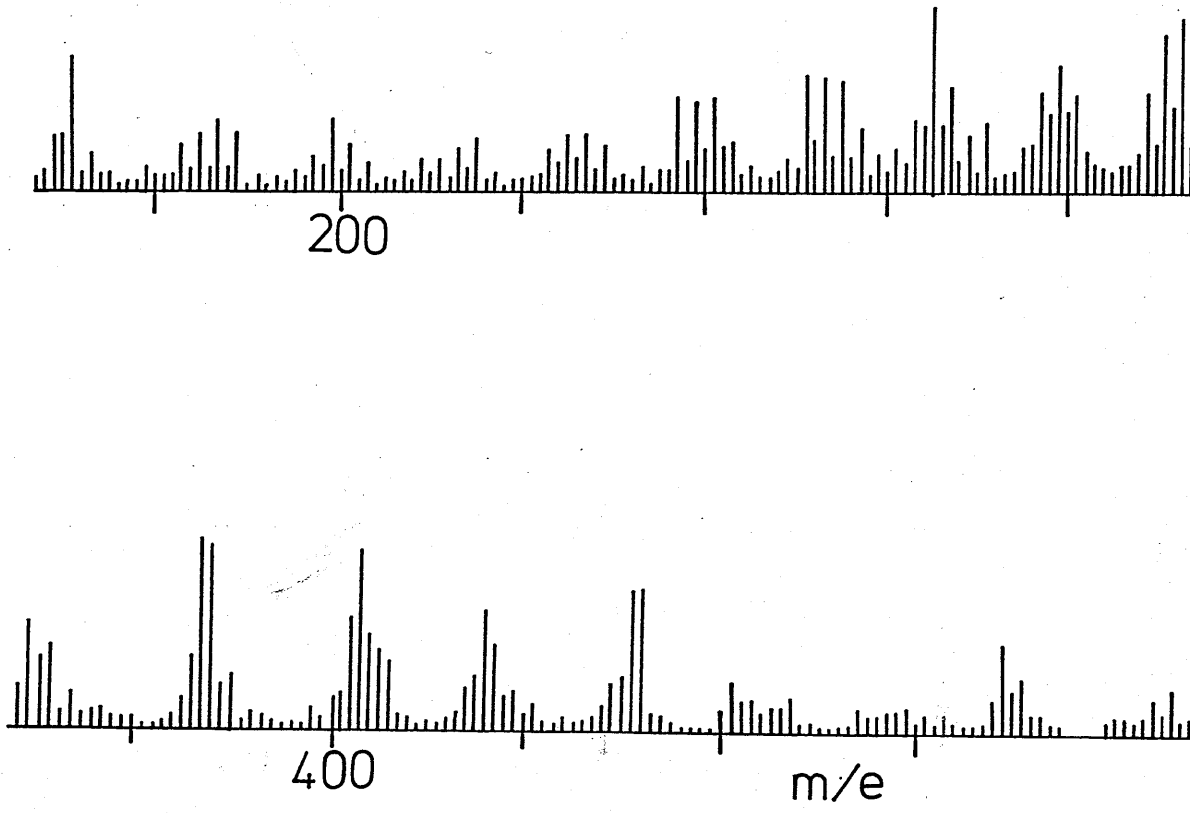
E Mass spectrum of sample 3

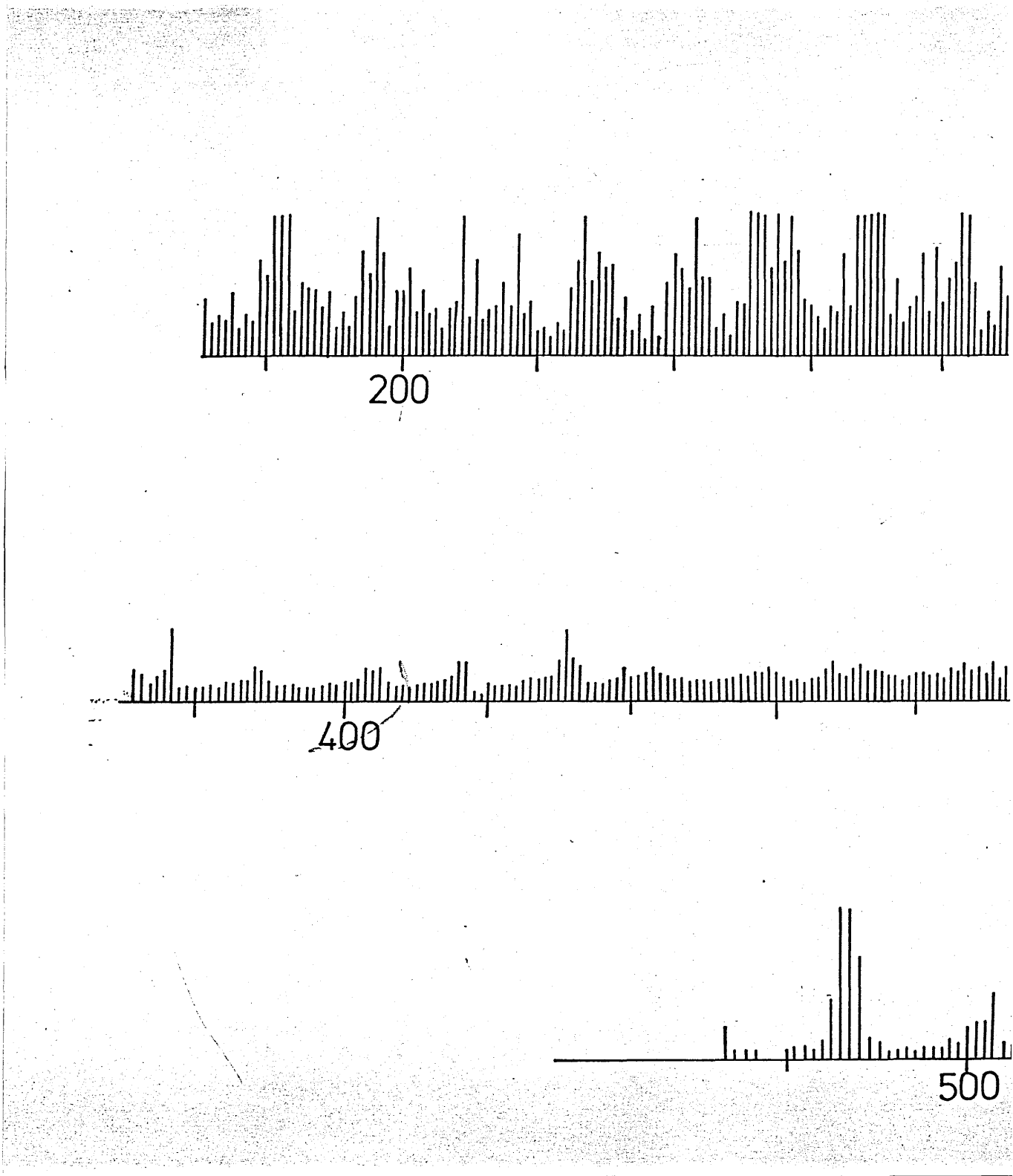
F Mass spectrum of TMS derivative of sample 3

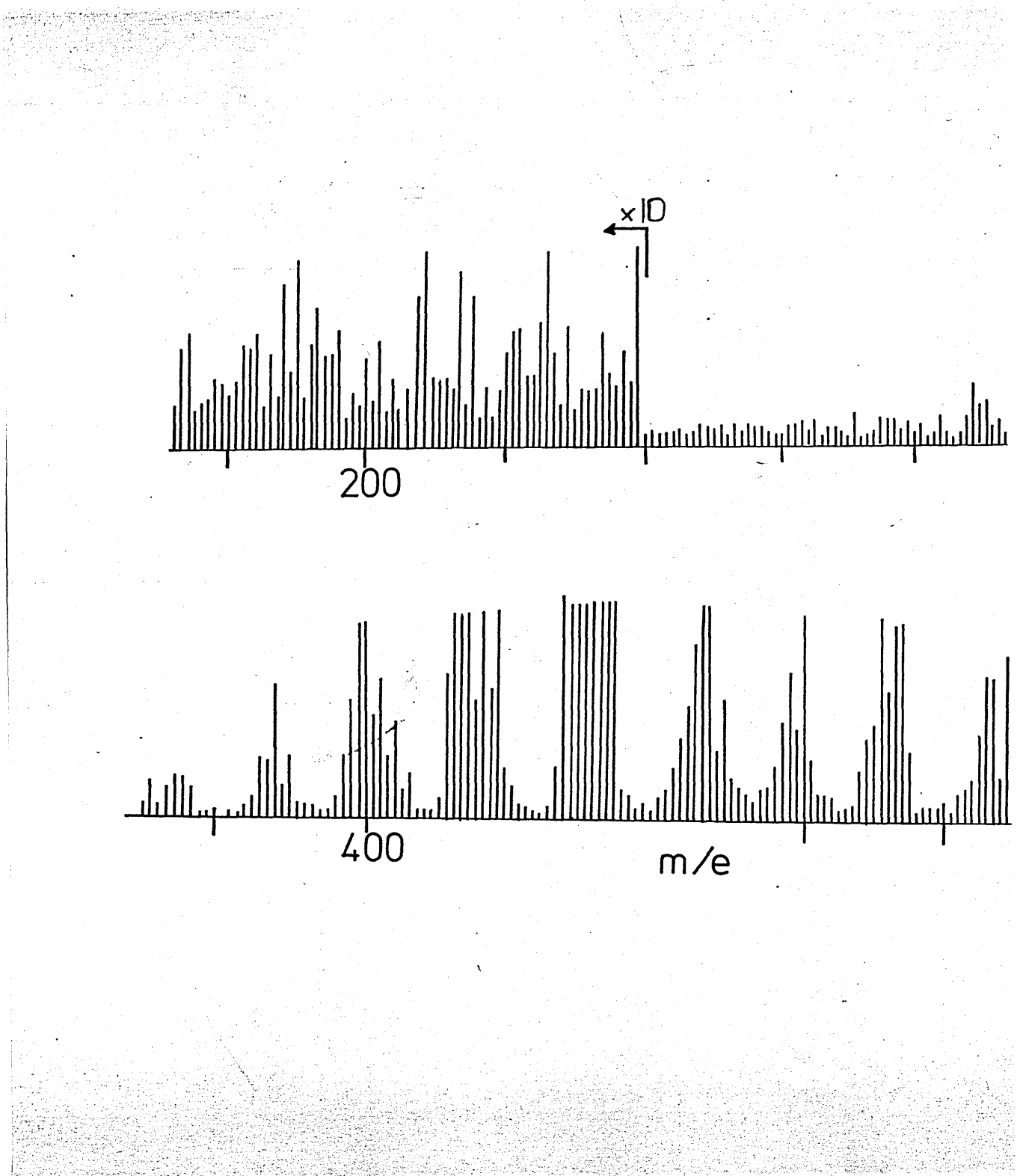
G Mass spectrum of sample 4

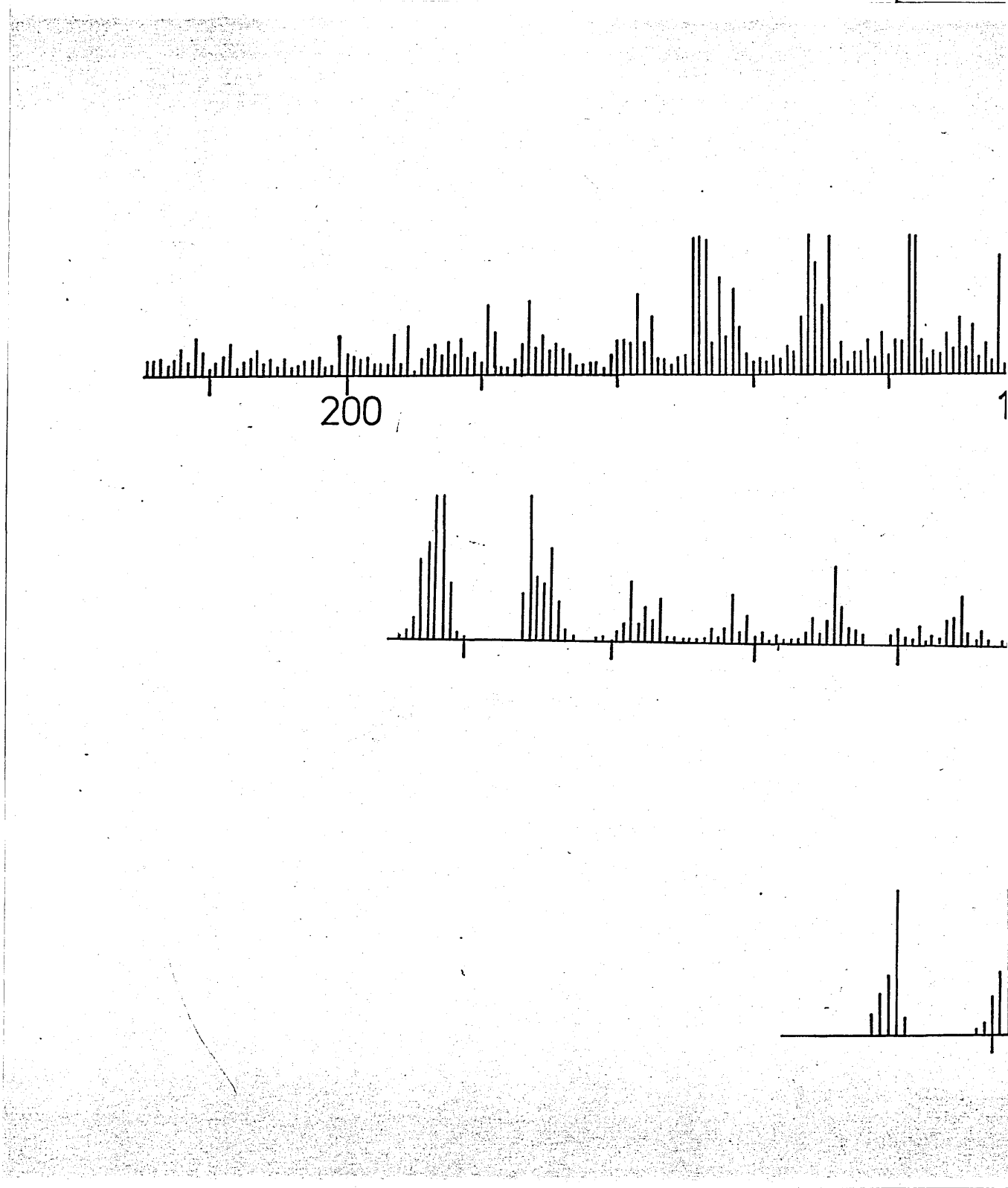

×10
200
400
m/e

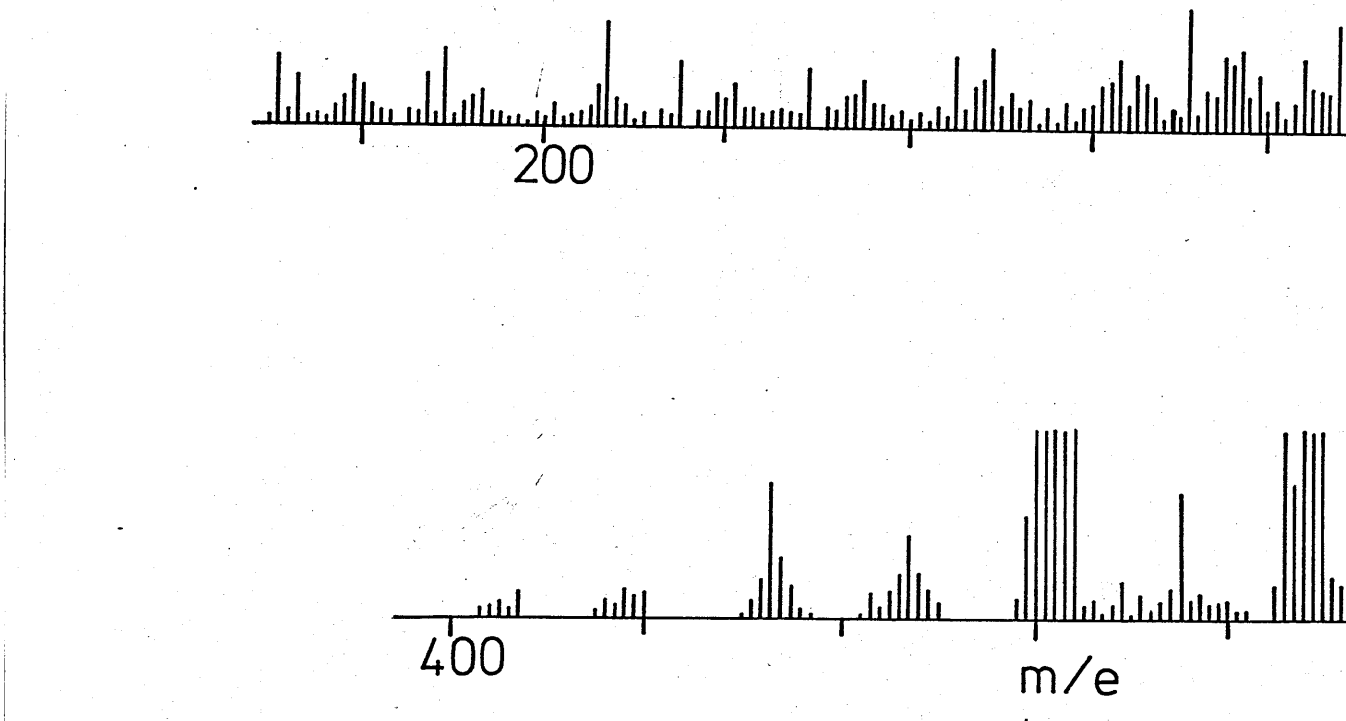

200
100
300
500


200
400
m/e


200
400
500


x10
200
400
m/e


200


200
400
m/e

191, 204 and 217, indicating a hexose sugar. Peaks at m/e 367 and 369 show the presence of cholesterol in the sample. Therefore, sample (1) appears to contain a cholesterol glycoside, probably glucoside. With the pyranose derivative (6 member ring) the ratio of m/e 204 to 217 is 3:1. With the furanoside derivative (5 member ring) the ratio of m/e 204 to 217 is 1:17.
In sample (1) the ratio of m/e 204 to 217 is 5:7. This suggests that the hexose component is a mixture of pyranoside and furanoside derivatives.

The mass spectrum of sample (2) showed that no plastisizer present. The presence of peaks m/e 191, 204 and 217 in the silylated sample indicates a glycoside. The peak heights could not be measured in this sample to indicate whether the hexose was in the 5 or 6 carbon ring formation.

The m/e of the molecular ion (M^+) in samples 1 and 2 could not be determined as the molecule was too large and readily broken down in the mass spectrometer.

The probe analysis of the underivatised sample 3 showed a molecular ion (M^+) of m/e 386. On silylation and reanalysis peaks were produced which correspond to those for cholesterol TMS ether viz. 129, 329, 353, 368, 443, 458. This sample also seems to contain an oxidation product. This compound has an M^+ of m/e 472 which is

explained by the loss of 2H atoms and the addition of 1 oxygen to the molecule cholesterol TMS ether. This is most likely 7-keto cholesterol.

Sample 4 showed peaks at m/e 367 and 369 due to cholesterol and other peaks indicating a mixture of saturated and unsaturated fatty acids; predominant was palmitate, indicated by peak m/e 256.

In conclusion, sample 1 contains cholesteryl glucosides and plastisizer, sample 2 contains cholesterol and glucose moities and is probably cholestryl-6-acyl-glucoside which was reported as a metabolite of Pythium sylvaticum (McMorris and White 1977). Nes et al (1981) reported esterified and glycosylated sterol when (^{14}C) cholesterol was added to mycelial cultures of Phytophthora cactorum. Sample 3 is free cholesterol confirming the identification by HPLC retention time of cholesterol standard and finally sample 4 contains a mixture of esters the most abundant of which is cholesteryl palmitate, the compound which was fed to the organism.

FUTURE RESEARCH

FUTURE RESEARCH

Pythium sylvaticum, strains 431c and 431e, strong female and male respectively, have been used in most of these exercises, because they fall on either side of the spectrum and the response of both is easily monitored. However, 431d obviously merits intensive study as it produces oogonia abundantly in single culture, and has less restrictive nutrient requirements for reproduction.

The attempts made to isolate the hormones have not, so far, met with success. The following indicates the various approaches taken, and notes their evident shortcomings.

(1) Since it appeared that the first step in the sequence is the production of an antheridiol-like hormone by the female strain, the activity of crude culture filtrates of the female strain on the male strain were tested. No activity was observed.

(2) It was supposed that the hormone was present in too low concentrations, and culture filtrates were therefore, extracted with various solvents. No activity was recorded with these concentrated extracts. The extracts were supplied to male strains growing in agar culture. Extracts of mycelium were similarly applied. Some of the extracts promoted growth, but none induced reproduction.

These procedures were adopted on the hypothesis that the compound of interest was a steroid. Other kinds of substance might be considered, and the filtrates could be put through columns of sephadex or ion exchange resins. In most cases the crude filtrate (ph 5.2) was extracted. In some cases the pH of the filtrate was adjusted to 3 before extraction, then readjusted to 8 and re-extracted. More attempts on these lines need to be made.

(3) Attempts to trap the hormone in blocks of agar with and without activated charcoal proved unsuccessful. Agarose was no better than agar. Agar possibly binds or inactivates the hormone and does not seem a very suitable medium for assay.

(4) No attempt has been made to perform the extraction at low temperatures or in the dark. Lack of success may be due to thermo- or photolability. Attempts need to be made to control these features.

(5) The fungus has only been grown in small batch cultures on Schmitthenner's medium. The male and female cultures grown together in such conditions reproduce. Other media might produce greater sexual activity. The female might produce more hormone when grown under conditions of continuous nutrient supply, strong aeration etc.

(6) It is difficult to understand why the filtrates of female cultures have no activity. Lowness of concentration of active

material is the most likely explanation, together with inadequate scale of operation and inappropriate techniques for handling the material. However, it is possible that the material is taken up and metabolically degraded by the fungus as it produces it. The lack of activity in filtrates of the female cultures could also be because the female requires induction by the male in order to produce the antheridiol-like hormone.

It is assumed in the first instance that the hormone is present in the female culture filtrate, but that the lack of success to date in isolating it is due to its being in too low concentration to be detected by the methods so far used. Attention should be paid to all the following aspects: (1) method of culture, (2) the extraction of the filtrate and (3) the bioassay.

1. Culture Hitherto, the fungus has been grown in 100ml conical flasks and the maximum amount of filtrate extracted at any one time was approximately 650ml. The scale of operation should be greatly increased. 10 or 20 litres of filtrate should be produced for extraction, growing the fungus in sufficiently large containers to substantially reduce any possible loss through adherence to the glass. The use of stirred and aerated conditions and of enriched media should also be investigated.

2. Extraction The filtrate should be extracted with ethylacetate

or methylene chloride, the pH being adjusted sequentially to acid, neutral and alkaline conditions. Attention should be paid to keeping the material in the dark, and at as low temperatures as feasible. Antioxidant should be added to the filtrate.

3. Bioassay Since it seems possible that agar media are not very suitable for bioassay, the assay should be performed principally in liquid media. The extracts would thus be added in non-toxic solutions, i.e. in water or dilute ethanol.

Attempts to characterise biologically active material should be made using mass spectrometry. It may be useful to include standards of known compounds.

In conclusion, it is obvious that this project has merely touched the surface of the hormone theory and sterol metabolism in Pythium sylvaticum. A considerable amount of research is still necessary to determine why this organism requires sterol.

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