PHYSIOLOGY OF CORTEXOLONE BIOTRANSFORMATION BY FUNGI

by

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In loving memory of my dear sister Vanessa, who died April 1986

"O death, where is thy sting?
O grave,where is thy victory".
1Co 15v55

"And further, by these, my son be admonished: of making many books there is no end; and much study is a weariness of the flesh." Ecc 12v12.

Our greatest glory consists not in never falling but in rising every time we fall.

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I would also like to thank my family both here and abroad for their perpetual love and encouragement, which made all of this possible.

Mrs. Betty Jones for her typing of this thesis.

Merci Beaucoup.

Abstract

The hydroxylation of cortexolone at the 11-position has been investigated using a range of isolates from filamentous fungi. Fungi obtained both from natural sources and culture collections have been screened for cortexolone and/or progesterone hydroxylation. About 75% of isolates from *Agave* were active modifiers of cortexolone, but 11β-hydroxycortexolone and 11α-hydroxycortexolone were not produced in large amounts relative to other products, and so these organisms were not chosen for further study. In contrast, several organisms obtained from culture collections, whilst hydroxylating the substrate at the 11-position produced fewer by-products. Germinating spores, mycelium and pellets of fungal biomass all showed hydroxylating activity. Cortexolone is transformed to both 11α- and 11β-hydroxylated products, whereas progesterone is preferentially converted by 11α-hydroxylation, presumably due to steric hinderance of 11β-hydroxylases. *Phascolomyces articulosus* and *Absidia spinosa* were discovered to be novel in producing both 11β-hydroxycortexolone and 11α-hydroxycortexolone.

Comparative spore germination and vegetative cell transformation studies have been performed using *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans*. Cortexolone bioconversion was found to correlate with germ tube production in *Absidia spinosa* and *Cunninghamella blakesleeana*. In all three organisms initial 11β-hydroxycortexolone production always preceded 11α-hydroxycortexolone production. The 11β- and 11α- activities occurred during growth, but can function when growth has ceased.

Pellet formation appeared to promote steroid metabolism in conditions of glucose exhaustion. In *Absidia spinosa*, the cessation of growth promoted the secondary transformation of the primary transformation products. Primary transformation enzymes can still be operative in the decline phase, but are superceded by other reactions. 11β-hydroxycortexolone was the major source of secondary transformation products, 11α-hydroxycortexolone being metabolized in decline phase.

Studies during vegetative growth of *Absidia spinosa* revealed several novel findings. Cortexolone and 11β-hydroxycortexolone do not inhibit the fungal growth rate, but energy may be required to remove them from the cell.
Both cortexolone and 11β-hydroxycortexolone can be metabolized in exponential growth. Both 11β- and 11α- hydroxylation products were produced very quickly in response to cortexolone addition. Both 11β- and 11α-activities have closely linked but distinct properties. Both enzymes are active over a broad extracellular pH and temperature range. Cortexolone bioconversion occurred at 35°C, in the absence of growth. The enzymes are differentially affected by antifungal agents. Investigations on the feasibility of immobilizing fungal spores into Chromosorb W beads were successful and are discussed. Further experimentation was not continued as studies with non-immobilized cells seemed to be more appropriate before exploiting the cells on immobilized supports. The preparation of cell-free extracts is described but successful retention of biotransformation activity was not recorded due to the instability of the enzymes involved in steroid bioconversions once cell breakage was performed. 11-Hydroxylating activity could fulfil mainly a detoxification role, providing transformation, rapid excretion or degradation of hydrophobic steroid molecules.

Abbreviations

2-AAF	2-acetylaminofluorene
AMP	adenine monophosphate
C	cortexolone
DNA	deoxyribonucleic acid
DOT	dissolved oxygen tension
DTT	dithiothreitol
t_d	doubling time
EDTA	ethylenediaminetetraacetic acid
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
g	gravity
GSH	glutathione
hr	hours
11aC or 11αC	11α-hydroxy cortexolone
11αPG	11α-hyroxy progesterone
11BC or 11βC	11β-hydroxy cortexolone (cortisol or hydrocortisone)
11βPG	11β-hydroxy progesterone
K_m	rate constant
mag	magnification
μ_{max}	maximum specific growth rate
V_{max}	maximum velocity
MeOH	methanol
3MC	3-methylcolanthrene
mol/g	moles per gram dry fungal weight
mol/l	moles per litre
NAD^+/ NADH	oxidised/reduced nicotinamide adenine dinucleotide
$NADP^+$/NADPH	oxidised/reduced nicotinamide adenine dinucleotide phosphate
$\%CO_2$	percentage carbon dioxide
$\%O_2$	percentage oxygen
PB	phenobarbital
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
SDS	sodium dodecyl sulphate
TCDD	2-,3-,7-,8-, tetrachlorodibenzo(p)dioxin

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CHAPTER 1
INTRODUCTION
MICROBIAL TRANSFORMATION OF STEROIDS

1.1.1 What is microbial transformation?

Microbial transformation, sometimes termed "biotransformation", "bioconversion" or "enzymation" (Yamada and Shimizu 1988), is distinct from fermentation as it incorporates the relatively new concept of biological synthetic chemistry - the use of microorganisms to synthesise or modify chemical compounds. A microbial transformation is the conversion of an exogenously added substance (substrate) to another product(s) by a microorganism. The enzymes involved normally occur in cells and were thought to be responsible for normal chemical processes essential to life. However, most microorganisms can often substitute natural substrate(s) for structurally related exogenous compounds added to the growth medium. This concept will be discussed in this thesis (Chapters 4 and 5). Some microorganisms can occasionally catalyze reactions not commonly found in endogenous metabolism (Yamada and Shimizu 1988).

1.1.2 Microbial transformation compared to fermentation

One can compare the differences between microbial transformation and fermentation from Table 1.1. Commercial fermentation can be seen as a biological method for the production of naturally produced compounds, such as antibiotics (penicillin, streptomycin), amino acids (lysine, tryptophan) nucleic and organic acids (adenosine triphosphate, lactic acid) and solvents such as ethanol (Yamada and Shimizu 1988).

In microbial transformations the reaction is often simple involving one or two steps. The microorganism does not necessarily have to be living as the isolated enzyme can often perform the same function. (Cremonesi *et al* 1975; Carrea 1984). Microbial transformations also have advantages over chemical synthesis.

Table 1.1: General characteristics of microbial transformation and fermentation. (Yamada and Shimuzi 1988)

	Microbial transformation	Fermentation
Microorganism	growing, resting, or treated cells	growing cells
Reaction	simple catalytic reaction (one or several steps)	lifeprocess (multi-step reaction)
Reaction time	short	long
Starting materials	expensive substrates	inexpensive carbon
Product	natural or unnatural	
Product concentration	high	low
Product isolation	easy	tedious

1.1.3 Microbial transformation compared to chemical synthesis

Many of the details of steroid chemistry have been well known for a long time (Samuels and Reich 1952; Shoppee 1964; Fried and Edwards 1972). The differences between microbial transformation and chemical reactions are outlined in Table 1.2. Microorganisms permit stereospecific and regiospecific chemical reactions in a aqueous environment, under physiological conditions, giving a low concentration of product but can produce high percentage yields. If whole cells are used for biotransformations, one can improve yields by strain improvements, modification of substrates and manipulation of media, temperature and period of incubation (Mahato and Mukherjee 1985). Chemical reactions often exhibit a low substrate specificity and can produce a mixture of

isomers. A high concentration of substrate can be employed for product synthesis.

Table 1.2: General characteristics of enzymatic and chemical reactions

	Enzymatic reaction	Chemical reaction
Reaction conditions		
Temperature	physiological	high
Pressure	physiological	high
Source of the reaction energy	change in enzyme conformation (making and breaking of van der Waals bonds, hydrogen bonds, etc).	thermal
Solvent	water	water, organic solvents
Specificity		
Substrate specificity	high	low
Stereospecificity	high	low
Regiospecificity	high	low
Concentration of substrate and/or product	low	high

1.1.4 What kind of molecules can be transformed?

Many different types of molecules can be transformed by micro-organisms. These include non-steroidal as well as steroidal cyclic compounds (Kieslich 1976). For example rotenoid compounds such as rotenone derived from the roots, stems and leaves of many leguminous species, are well characterized inhibitors of NADH dehydrogenase in the mitochondrial respiratory chain and can be modified by various species of *Absidia, Aspergillus, Cunninghamella, Trichothecium, Penicillium* and *Phanerochaete,* mainly via hydroxylations and reductions (Sariaslani *et al* 1983). This molecule is used successfully as a selective pesticide with low environmental hazard, as

well as a natural antitumour agent. Transformation studies are currently being used to obtain a product with a greater efficiency against cancer than the parent compound. Terpenes such as α-pinene, mono- and sesquiterpenes have also been modified by *Aspergillus niger* (Prema *et al* 1962a, 1962b). The biotransformation of dehydroabietic acid has also been studied. It is a fish-toxic diterpene resin which occurs in certain pulp mill effluents, and can be transformed to non-toxic products (Kutney *et al* 1985). It has also been shown that some microflora, i.e. *Eubacterium lentum* in rat intestines, are able to hydrogenate long-chain unsaturated fatty acids (linoleic acid) into transvaccenic acid (Verhulst *et al* 1986).

The interest in this project centres on steroid molecules with the typical perhydrocyclopentanophenanthrene structure (this structure will be referred to in Section 1.2.1). These molecules are amongst the most frequently used for microbial transformations (Sebek and Perlman 1979). The majority of studies on steroid biotransformations have been undertaken in mammals, as they possess a rich and ready source of endogenous monooxygenases (Waxman 1986).

1.1.5 The diversity of steroid biotransformations carried out by microorganisms

Mamoli and Vercellone (Charney and Herzog 1967)were the first to report ring oxidation, isomerization and hydrolysis of 17-keto steroids, by yeast and bacteria. These transformations however were not of commercial interest. The first bioconversion of commercial significance was reported by Peterson and Murray (1952). They successfully introduced oxygen at C-11 (microbial 11α-hydroxylation), thus converting progesterone to 11αPG, using *Rhizopus arrhizus* and *Rhizopus nigricans*. The quantitative yield seen was in excess of 50% and 85-95% respectively after a transformation period of 24-48 hr. The rationale behind this was to transform readily available steroids directly to

adrenal cortical hormones, or to intermediates which could be easily converted to these substances (Peterson and Murray 1952). The list of transformations carried out by fungi is extensive (Table 1.3).

Table 1.3: Types of microbial transformation of steroids (Sebek and Perlman 1979).

A.	Oxidation
	1. Conversion of secondary alcohol to ketone
	2. Introduction of primary hydroxyl on steroid side chain
	3. Introduction of secondary hydroxyl on steroid nucleus
	4. Introduction of tertiary hydroxyl on steroid nucleus
	5. Dehydrogenations of ring A of steroid nucleus in positions 1(2) and 4(5).
	6. Aromatization of ring A of the steroid nucleus
	7. Oxidation of the methylene group to ketone group
	8. Cleavage of side chain to pregnane at C-17 to form ketone
	9. Cleavage of side chain of pregnane at C-17 and opening of D ring to form testololactone
	10. Cleavage of side chain of steroids to form carboxyl group
	11. Cleavage of side chain of pregnane steroids at C-17 to form secondary alcohol
	12. Formation of epoxides
	13. Decarboxylation of acids
B.	Reduction
	1. Reduction of ketone to secondary alcohol
	2. Reduction of aldehyde to primary alcohol
	3. Hydrogenation of double bond at position 1(2) of ring A
	4. Hydrogenation of double bond at positions 4(5) of ring A and at 5(6) of ring B
	5. Elimination of secondary alcohol
	6. Formation of homosteroids of the androstane series from pregnane derivatives
C.	Hydrolysis
	1. Saponification of steroid esters
	1. Acetylation
D.	Esterfication

These reactions have been used to produce a host of medicinally useful compounds (Table 1.4). The bioconversions of most commercial interest, involve 11-hydroxylation at the α and β positions. The 16α-, 17α-, 21-

hydroxylations and 1-dehydrogenation are also of potential practical importance. These will be referred to later (Section 1.6.5)

Table 1.4: Some steroid transformations of commercial importance (Sebek and Perlman 1979)

Reaction	Substrate→product Some industrial producers	Microorganism
11α-Hydroxylation	Progesterone→11α-hydroxyprogesterone (The Upjohn Company)	*Rhizopus nigricans*
11β-Hydroxylation	Compound S→cortisol (Pfizer Inc: Gist-Brocades)	*Curvularia lunata*
16α-Hydroxylation	9α-Fluorocortisol→9α-fluoro-16α-hydroxy-cortisol (E.R.Squibb and Sons) (Lederle Laboratories)	*Streptomyces roseochromogenus* *S.argenteolus*
1-Dehydrogenation	Cortisol→prednisolone (Schering Corporation)	*Arthrobacter simplex*
	Dienediol→trienediol (The Upjohn Company)	*Septomyxa affinis*
1-Dehydrogenation side-chain cleavage and ring D expansion	Progesterone→1-dehydrotestololactone (E.R.Squibb and Sons)	*Cylindrocarpon radicicola*
Side-chain cleavage	β-Sitosterol→androstadienedione 9α-hydroxyandrostenedione and/or androstenedione (G.D.Searle and Company) (The Upjohn Company)	*Mycobacterium* spp

1.2.1 Structure of steroid molecules

The basic structure of all steroids are related (Gower 1979). They consist of a fully reduced phenanthrene ring (perhydrophenanthrene; Fig.1.1), to which is fused a five-membered ring. This tetracyclic hydrocarbon is referred to as the perhydrocyclopentanophenanthrane nucleus (Fig.1.1). The parent hydrocarbon related to this (cholestane; Fig.1.2) is the precursor of cholesterol. This molecule possesses a side-chain eight carbon atoms long, attached at C-17 of ring D. The numbering sequence of the carbon atoms presented in Fig.1.2, is common to all steroid molecules. Cholestane is related to various other hydrocarbons and nearly all steroids can be defined by reference to this compound.

The three dimensional structure of cholestane cannot be truly represented by the full formula or the abbreviated formula. The cyclohexane rings, A,B and C (Fig.1.3) are in the 'chair' form, which contributes to the thickness of the molecule. The alternative 'boat' structure for cyclohexane is less stable, and does not normally occur. Equilibrium favours the chair form. The free valencies of carbon atoms involved in the ring structure exist in two positions, forming axial and equatorial bonds. In the axial 'a' bonds, one bond of each atom is perpendicular to the plane of the molecule. The equatorial 'e' bonds makes an angle of 30° to the plane of the molecule. The presence of 'e' and 'a' bonds can contribute to the shape of the molecule.. The aromatic A ring of estrogens is unsaturated and planar, and this unsaturation also results in the coplanarity of rings A, B and C.

In cholestane the 'angular' methyl groups attached at C-10 and C-13, and the side-chain by convention define the upper (β) side of the molecule (Fig.1.3); the lower side is referred to as the α-side. By convention when describing two-dimensional steroid structure, groups which lie above the plane of the molecule are represented as full lines (——). Groups lying below the plane of the paper are represented as broken lines(-----).

Steroid molecules, other than cholestane, differ primarily in the number, type and location of the substituent functional groups, and in the number and position of the double bonds. If a three-carbon fragment is removed from the cholestane side-chain (i.e by fission between C-24 and C-25), then cholane (C-24) is obtained. The bile acids such as cholic acid are related to cholane (Fig.1.4). Removal of the majority of the cholestane side-chain by cleavage between C-2O and C-22, gives pregnane (C-21) to which the corticosteroids (cortisol, cortisone) and progesterone are related. If the complete side chain is removed by fission between C-17 and C-20, this results in androstane (C-19). Androgens, for example testosterone, are related to androstane. The removal of the C-10 methyl group of androstane, results in oestrane, to which the oestrogens

(oestradiol) are related. These relationships are depicted in Fig.1.4. One example of each form is presented for each group.

1.2.2 A brief description of steroid nomenclature

Steroid nomenclature follows the rules of the International Union of Pure and Applied Chemistry (IUPAC). The systemic name of each steroid is derived from the parent hydrocarbon. The insertion of "en" in the stem name indicates the presence of unsaturated C-C bonds. The number outside the stem indicates the position of unsaturation present in the molecule, but only the lowest carbon number is used. Substituent molecules encountered in steroid hormones are used as prefixes or suffixes e.g. a hydroxyl is referred to as hydroxy-or-ol, if a prefix and suffix respectively. By convention there may be a number of prefixes but only one suffix. The choice of suffix is governed by the groups present, in decreasing order this is acid, lactone, ester, aldehyde, ketone, alcohol, amino, ether. A good example is a pregnane derivative with a double bond at C-4; the systematic name includes 4-pregnane (Fig.1.4). The group substituents are three hydroxyls at C-11β, 17α and 21, with two ketone (oxo) groups at C-3 and C-20. Ketone groups have precedence over hydroxyl groups in

Figure 1.1: Structural Formulae of Phenanthrene and Cyclopentanoperhydrophenanthrene

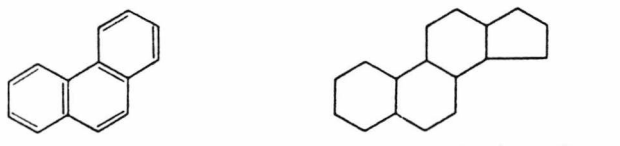


Figure 1.2: Structure of cholestane C-27

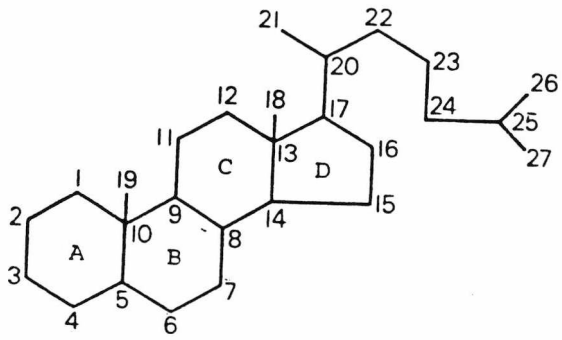
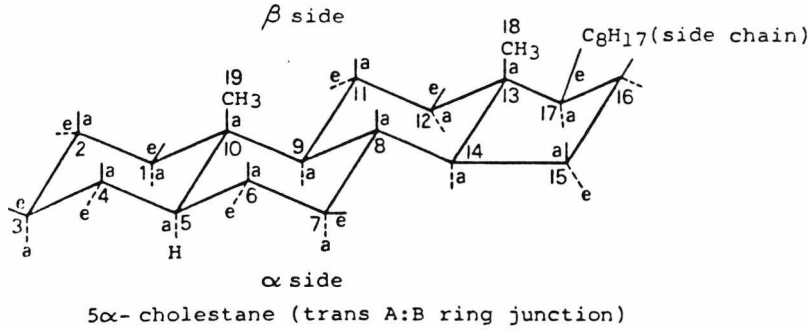
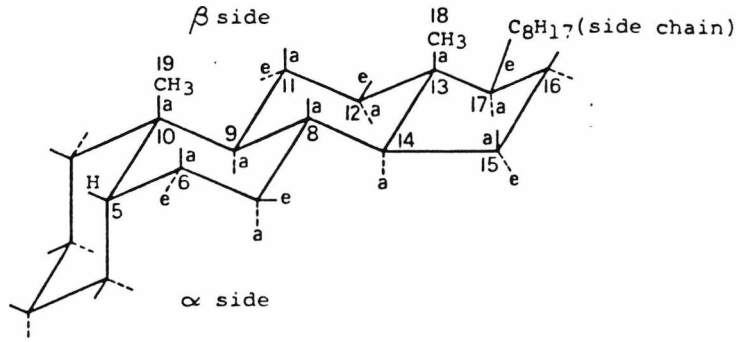


Figure 1.3: The three-dimensional structure of 5α- and 5β-Cholestane

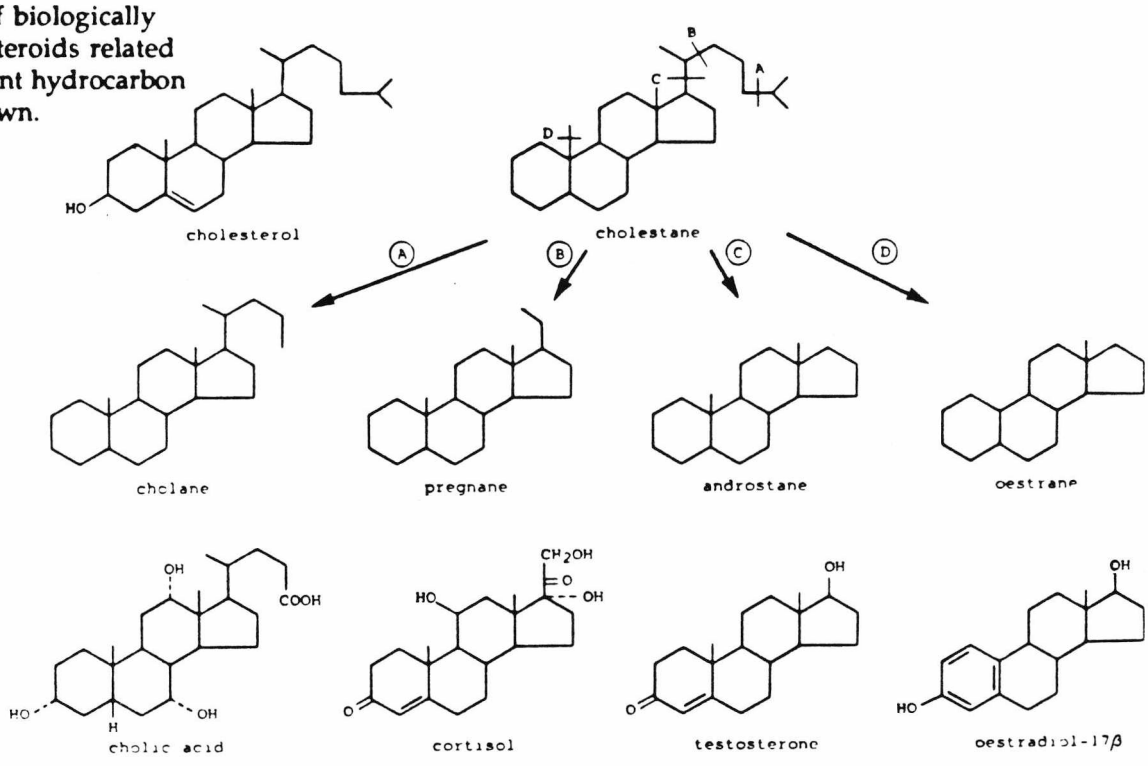


5α- cholestane (trans A:B ring junction)



5β- cholestane (cis A:B ring junction)

Figure 1.4
Structural relationships between Cholestane (C_{27}), Cholane (C_{24}), Pregnane (C_{21}), Androstane (C_{19}) and Oestrane (C_{18}). Examples of biologically important steroids related to each parent hydrocarbon are also shown.



choice of suffix, the systematic name is, 11β, 17α, 21-trihydroxy-4-pregnane-3, 20-dione. Steroid molecules can be derived from numerous sources in plants and animals.

1.2.3 Sources of steroid molecules

Cholesterol is the main sterol in animals. It gives rise to corticosteroids, oestrogens and androgens (Gower 1979). Corticosteroids are generally formed in the adrenal glands. The adrenal cortex synthesizes glucocorticoid hormones such as, cortisol and corticosterone, mineralocorticoids (aldosterone) androgens (androstenedione and dehydroepiandrosterone). The ovaries are the source of

estrogens in pre-menopausal women, these include oestradiol, oestrone and oestriol. The ovaries can also make the androgen testosterone. Male testis tissue can also synthesize oestrogens and testosterone (Waterman *et al* 1986; Jefcoate 1986). In mammals the sites in the cells for steroidogenesis occur at the mitochondrial inner membrane or agranular endoplasmic reticulum (Tamaoki 1973). Bile acids formed in the liver can be changed chemically to steroid molecules.

Plants sterols (phytosterols) are the main commercial source of steroid molecules, as they are abundant and inexpensive raw materials (Sebek and Perlman 1979). Phytosterols can be chemically modified to the desired steroid molecules. Plant sterols such as diosgenin and stigmasterol are good potential sources for the production of pregnenolone and progesterone respectively (Miller 1986). Disogenin can be obtained from the wild Mexican barbasco plant (*Dioscorea composita*). Stigmasterol is produced in great amounts from soya beans (*Glycine max*). Other phytosterols include hecogenin and solasodine from *Agave* and *Solanum* respectively (Miller 1986). Campesterol, β-sitosterol from soya beans have also been used to a lesser extent as steroid starting materials (Wovcha *et al* 1978). Some steroids like cortexolone can be synthesised chemically and they have a variety of uses.

1.2.4 Uses of steroid molecules and cortexolone

Both steroids and sterols are required for growth and reproduction in eukaryotic organisms (Siegel 1981). Steroid molecules act as anti-inflammatory agents against injuries and diseases, such as rheumatoid arthritis (corticosteroids). Some function as progestational agents, sedatives, as well as anabolic (testosterone) and anti-tumour substances (tamoxifen). Some are effective in allergic, dermatological and ocular diseases, in cardiovascular therapy, veterinary products and as oral contraceptives (Sebek

and Perlman 1979). Other steroid molecules such as cortexolone can act as inhibitors of hormone action.

Cortexolone has been used for over twenty years as a useful tool for analysing the mechanism of glucocorticoid hormone action (Gagne *et al* 1988; Stevenson and Taylor 1988). It has been demonstrated that cortexolone could partially block the *in vitro* effects of glucocorticoid agonists and inhibit uridine uptake in rat thymus cells. Munck *et al* (1968) have shown that cortexolone competes with the cortisol receptor and so reduces glucose uptake. Cortexolone is known to act as an antiglucocorticoid in many target tissues (Schmidt *et al* 1987). These workers have shown that cortexolone fails to promote an *in vitro* activation of cytoplasmic receptors, under a variety of experimental conditions. Cortexolone was found to be a potent competitive agonist of triamcindone acetonide. Cortexolone competes for the unactivated receptor binding site, but as it does not change the receptor configuration, no binding to DNA takes place. Given its importance this compound is in great demand. Steroid type molecules have also been detected in fungi, for example as sex hormones (Elliot 1977).

1.3.1 Sterol biosynthesis in fungi

As this project centres on the transformation of steroids in filamentous fungi the general pathway for ergosterol biosynthesis will be described, as this may be important in steroid bioconversions. However, in filamentous fungi there is no direct evidence to prove this.

For the synthesis of endogenous sterol molecules like ergosterol, the basic starting chemical unit is acetate (Fig.1.5), this is converted to mevalonate. Mevalonate is formed from acetyl-CoA via β-hydroxyl-β methyl-glutaryl-CoA. Mevalonate is converted into the isomers 3-isopentenylpyrophosphate (C-5) and dimethylallylpyrophosphate (C-5), by the loss of one carbon, via phosphorylated intermediates. These condense to form geranyl pyrophosphate

(C-10) and then farnesyl pyrophosphate (C-15). Two molecules of farnesyl pyrophosphate condense to form squalene (C-30). Squalene is an intermediate in the synthesis of ergosterol, the major sterol in higher fungi and plants (Siegel 1981). Squalene is also an important precursor of cholesterol and of steroid hormones such as progesterone, testosterone, estradiol and cortisol etc in mammalian systems. (Sebek and Perlman 1979).

Figure 1.5: Common pathway for squalene synthesis in fungi.

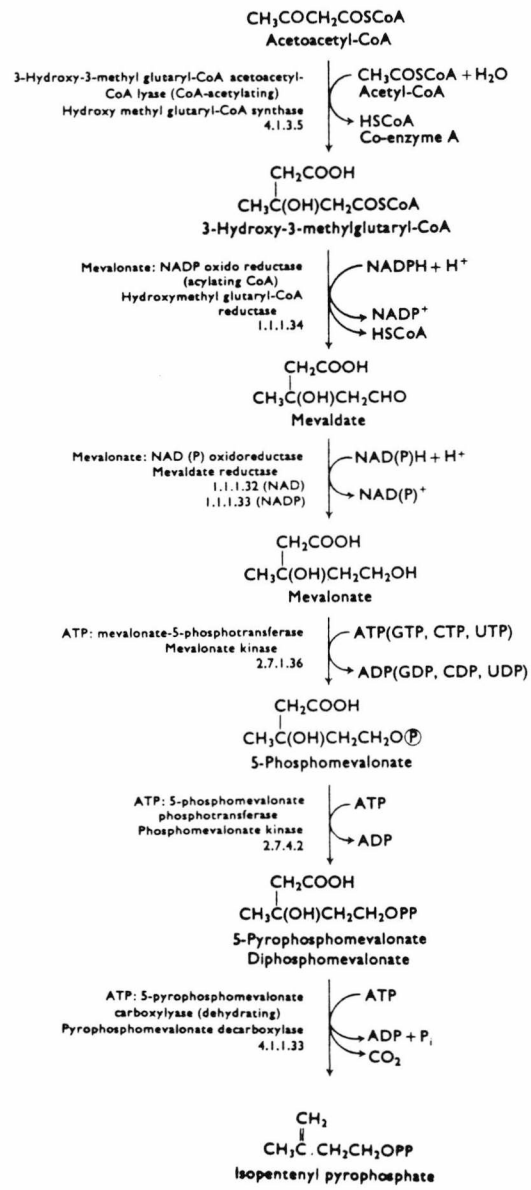


Figure 1.5:

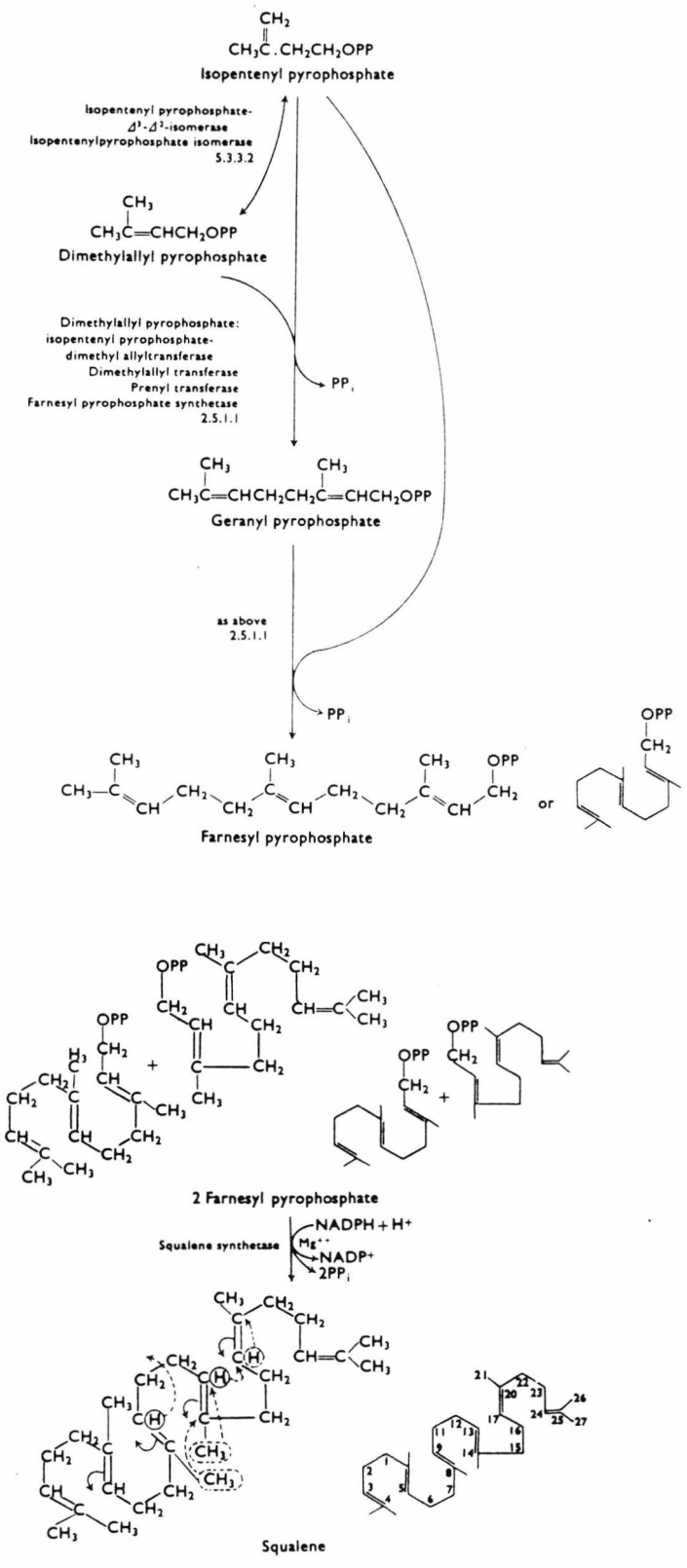
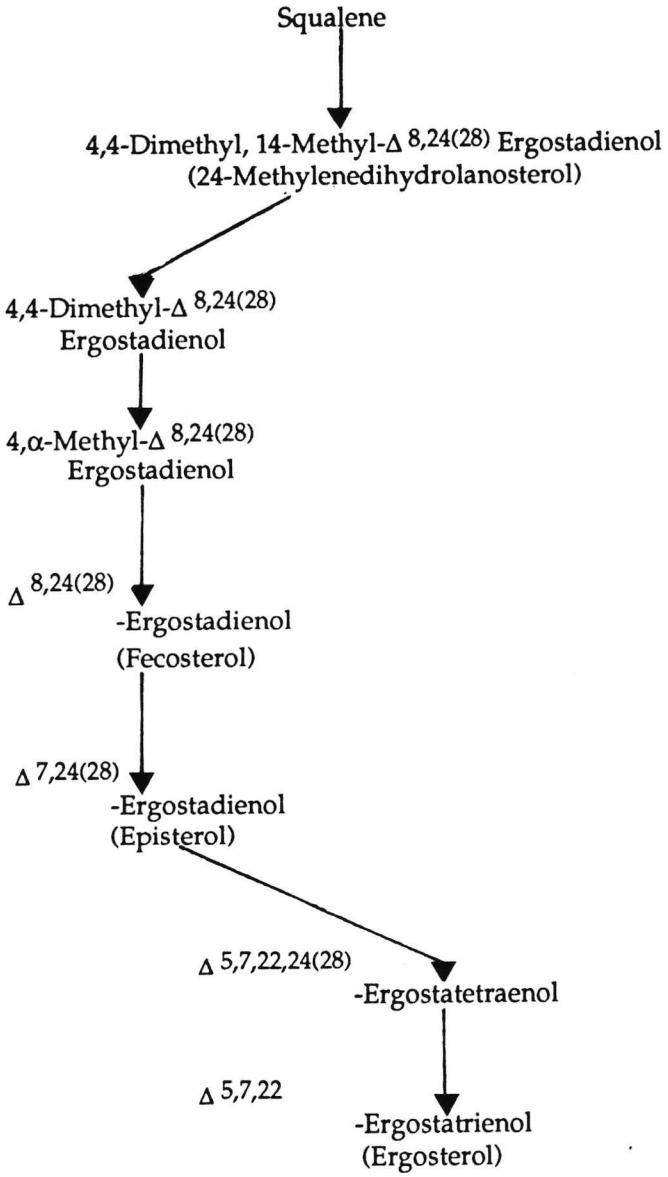

Isopentenyl pyrophosphate
Isopentenyl pyrophosphate-
Δ³-Δ²-isomerase
Isopentenylpyrophosphate isomerase
5.3.3.2
Dimethylallyl pyrophosphate
Dimethylallyl pyrophosphate:
isopentenyl pyrophosphate-
dimethyl allyltransferase
Dimethylallyl transferase
Prenyl transferase
Farnesyl pyrophosphate synthetase
2.5.1.1
PPi
Geranyl pyrophosphate
as above
2.5.1.1
PPi
or
Farnesyl pyrophosphate
2 Farnesyl pyrophosphate
Squalene synthetase
NADPH + H+
Mg++
NADP+
2PPi
Squalene

Figure 1.6: The normal sterol biosynthetic pathway from squalene to ergosterol found in fungi (Siegel 1981).



The cyclization of squalene to the first sterol molecule lanosterol, is the first step in a series of complex reactions leading to the synthesis of ergosterol Fig. 1.5). The general pathway (Fig.1.6) illustrates the main steps involved in ergosterol biosynthesis. The order of some steps in the pathway could vary in different species of fungi. Several steps in the pathway can be inhibited by antifungal agents such as azoles. The demethylation reactions which are known to involve cytochrome P-450 are especially vulnerable (Aoyama *et al* 1987).

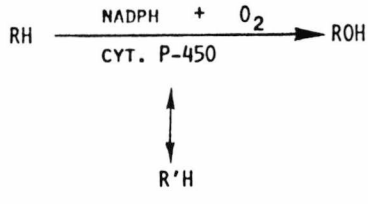
Sterol biosynthesis occurs in the endoplasmic reticulum in fungi and mammals (Siegel 1981; Tamaoki 1973; Jefcoate 1986). A microsomal mixed function monooxygenase system is involved. Carrier proteins which allow interaction of enzymes with sterol molecules are present in all steps of sterol biosyntheses (Siegel 1981). The enzymes responsible for steroid transformations are known to be membrane associated (Broad *et al* 1986). These enzyme complexes have also been reported as being associated with the endoplasmic reticulum of mycelial cells (Broad *et al* 1986; Breskvar *et al* 1977). These mixed function monooxygenases contain cytochromes P-450.

The word cytochrome is Greek in origin and literally means "coloured". The colour is derived from the properties of d electrons of transition elements such as iron (Nebert *et al* 1982). The term "P-450" denotes that the cytochrome contains a pigment which has a major optical absorption peak (Soret maximum) at about 450 nm when it is reduced, and combines with CO (Omura and Sato 1964). Cytochrome P-450 represents a family of haemoproteins that are active towards many substrates (Hostetler *et al* 1987). The estimated molecular weights of the various P-450's range from 48,000 to 60,000 on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE; Waxman 1986).

1.4.1 Monooxygenase activity of cytochrome P-450

Cytochrome P-450's are the terminal oxidases of a variety of biotransformation systems used by most organisms. A monooxygenase is an enzyme that can insert one atom of atmospheric oxygen into its substrates (Fig.1.7), whereas a dioxygenase inserts two molecules of oxygen. The various forms of P-450 represent a large subset of all monooxygenases. The mechanism

Figure 1.7: Cytochrome P-450 monooxygenase activity against a substrate (RH)



of monooxygenation has been reported to be similar in mammals and microorganisms (Holland 1982). A trimolecular complex is formed among substrate, enzyme and molecular oxygen (Nebert and Jaiswal 1987). The P-450 haemoprotein receives two electrons from cofactors NADPH and/or NADH and these electrons are received one at a time usually via reductases (flavoproteins). During catalysis, cytochrome P-450 overcomes the low kinetic reactivity of molecular oxygen, it also oxidises carbon-hydrogen bonds with a variable degree of regio- and stereochemical selectivity according to the overall stoichiometry.

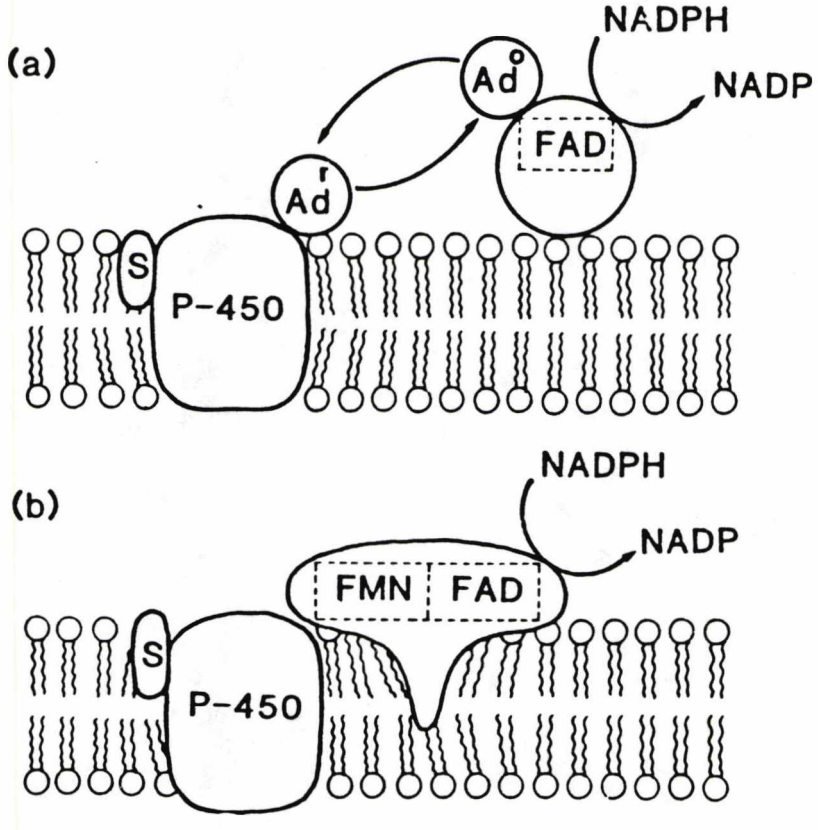
1.4.2 Phase I and Phase II metabolism of steroids

Of the many compounds that exist in the environment, monooxygenases are active against many of these as well as endogenous substrates in the organism (Nebert 1982). Foreign chemicals are termed xenobiotics and are often hydrophobic in nature. Once inside mammalian cells

they are converted to more hydrophilic molecules for excretion. In Phase I metabolism, polar functions like -OH groups are introduced into the substrate, thereby presenting Phase II conjugating enzymes with a substrate in mammals. Phase I could well be the prevalent reaction in microorganisms for exogenous molecules.

Phase II enzymes can use the polar groups to attach other very water-soluble moieties such as glucuronide, sulphate or glycine. The enzymes responsible for conjugation i.e. UDP-glucuronyltransferase may themselves be induced by xenobiotic inducers (Bock *et al* 1987). Phase II enzymes are very prevalent in mammals, and allows polar molecules to be excreted by the organism. These active electrophilic metabolites have the potential to interact with cellular macromolecules, causing toxic reactions such as necrosis or genotoxic effects, which can lead to cancer. The balance between Phase I and II enzymes in mammals determines the accumulation of reactive metabolites (Nebert *et al* 1982).

Figure 1.8: The structure of the cytochrome P-450 11β system in bovine adrenal mitochondria(a) and microsomal systems(b) (Takemori and Kominami 1984)



NADPH/NAD(P)	reduced/oxidised nicotinamide adenine dinucleotide (phosphate)
Ad^r/Ad^o	reduced/oxidised adrenodoxin
FAD	flavine adenine dinucleotide
FMN	flavine mono nucleotide
P-450	cytochrome P-450
S	substrate

1.5.1 Cytochrome P-450's and their involvement with steroid biotransformation

Cytochrome P-450's have been reported in bacteria, yeast, mammals and fungi (Holland 1982; Muller *et al.*1984). The presence of cytochrome P-450 in these various groups will be discussed, as it bears some relevance to the data presented later. A comparison between cytochrome P-450 and steroid bioconversions will also be made to show the similarities of both systems.

1.5.2 Bacterial cytochrome P-450

Many bacterial cytochrome P-450's have been reported (Sugar and Murray 1986; Table 1.5). They appear to be as diverse in number as their eukaryotic counterparts (Waxman 1986). The evidence would suggest that all cytochrome P-450 dependent monooxygenases function by a similar mechanism (Sato *et al* 1978). The monooxygenase system consists of two components in hepatic microsomal systems, with an NADPH-specific FMN and FAD containing P-450 reductase (Ruckpaul and Bernhardt 1984; Fig.1.8). In microorganisms it consists of three proteins a NADH/NADPH specific flavoprotein, containing FAD or FMN (a reductase) and an iron-sulphur protein, for electron transfer with a P-450 terminal oxidase (Muller *et al* 1984; Table 1.6). This would suggest that the enzyme systems in microorganisms in general, resembles the mitochondrial rather than the microsomal P-450 systems (see Table 1.6).

Cytochrome P-450 has been studied extensively in *Bacillus megaterium* (Wilson and Vestling 1965; Berg *et al* 1976; 1979) and *Pseudomonas putida* (Katagiri *et al* 1968; Hayaishi 1969; Tanaka *et al* 1970; Tsai *et al* 1971; Tyson *et al* 1972).

Table 1.5: Bacterial cytochrome P-450 systems (Sugar and Murray 1986).

Bacterium	Function
Acinetobacter caloaceticus (EB104)	ω-Alkane hydroxylase
Bacillus megaterium (ATCC 133680	15β-Hydroxylase of 3-oxo-Δ^4-steroids
B.megaterium (ATCC 14581)	ω-2-Fatty acid hydroxylase-epoxidase
Nocardia sp. (NH1)	*p*-Alkylphenyl ether dealkylase
Pseudomonas incognita	Linalool-10-methyl-hydroxylase
	Linalool-8-methyl-hydroxylase
P.putida (ATCC 17453)	Camphor-5-*exo*-hydroxylase
P.putida (JT810)	*p*-Cymene-7-hydroxylase
P.putida (PL-W)	*p*-Cymene-7-hydroxylase
Rhizobium japonicum (CC705)	Unknown.
Rhodococcus rhodochrous	ω-Alkane hydroxylase (ATCC 19067)
Rhodococcus sp.	Camphor-6-*endo*-hydroxylase

Table 1.6: The occurrence, composition and properties of some selected P-450 systems from different sources (Mohr *et al* 1984).

Species	Electron donor	Electron transfer system: flavoprotein	Electron transfer system: non heme iron protein	Substrate(s)	References
Rat,rabbit (liver microsomes)	NADPH	NADPH P-450 reductase	-	drugs	Lu *et al* (1972)
Bovine(supradrenal gland)(mitochondria)	NADPH	adrenodoxin reductase	adrenodoxin	steroids in position 11β	Omura *et al* (1966)
Pseudomonas putida	NADH	putidaredoxin reductase	putidaredoxin	camphor in position 5-exo	Katagiri *et al* (1968)
Bacillus megaterium	NADPH	megaredoxin reductase	megaredoxin	steroids in position 15β	Berg *et al* (1976)
Lodderomyces elongi-porus (microsomes)[1])	NADPH	NADPH specific reductase	-	alkanes	Riege *et al.* (1981), Müller *et al* (1979)
Candida tropicalis (microsomes)	NADPH	NADPH specific reductase	-	alkanes and fatty acids in β-position	Duppel *et al* (1973)

1) in first publications designated as *Candida guilliermondii*

1.5.3 Yeast cytochrome P-450

The yeast *Saccharomyces cerevisiae* possesses a cytochrome P-450 monooxygenase which has properties in common with the mammalian liver system (Yoshida *et al* 1977). One of the yeast monooxygenases exhibits aryl hydrocarbon hydroxylase activity towards benzo(a)pyrene, forming mono- and dihydroxy- derivatives.

It has been found that the formation of cytochrome P-448/P-450 in *Saccharomyces cerevisiae* occurs under fermentative conditions at high glucose concentrations or under semi-anaerobic conditions (Blatiak *et al* 1985). When the mitochondrial cytochrome aa_3 formation was repressed. The effect of oxygen limitation has also been demonstrated in *Candida maltosa* (Schunck *et al* 1987a; 1987b). Current evidence suggests that the intracellular concentration of cyclic AMP controls the *de novo* synthesis of cytochrome P-448/P-450 in *Saccharomyces cerevisiae*. The concentration of glucose in the medium is thought to determine the level of cyclic AMP, probably in an inverse relationship (Wiseman *et al* 1978; Wiseman 1980).

High cytochrome P-450 levels are obtained when the mitochondrial cytochrome concentration decreases as a result of inhibitors, for example chloramphenicol, an inhibitor of mitochondrial protein synthesis. Cytochrome a and b biosynthesis can be inhibited by chloramphenicol, but cytochrome P-450 could only be detected in the presence of chloramphenicol.

Different forms of cytochrome P-450 have been detected in *Candida tropicalis* and *Saccharomyces uvarum*(Sanglard *et al* 1984; 1986). So far two forms of cytochrome P-450 have been found in yeast microsomes. They were differentiated by CO-reduced difference spectra and inhibition by propiconazole. One form catalyzed 14α-demethylation of lanosterol. This form has been purified and reconstituted (Aoyama *et al* 1987). The other form catalyzed the terminal hydroxylation of aliphatic hydrocarbons.

A 14α-methyl-demethylase activity has also been detected in higher plants (*Zea mays*). This is also a cytochrome P-450 mediated reaction (Rahier and Taton 1986).

1.5.4 Cytochrome P-450 in filamentous fungi

The existence of multiple forms of drug metabolising monooxygenases was first suggested by Conney *et al* (1959) using benzo(a)pyrene. It was found that use of this xenobiotic compound produced differential effects on the microsomal metabolism of many substances (Astrom and Depierre 1986). Benzo(a)pyrene, a polycyclic aromatic hydrocarbon, is a widespread environmental pollutant that is both toxic, mutagenic and carcinogenic to organisms. Its products can interact with critical cellular structures such as DNA (Ghosh *et al* 1983). Benzo(a)pyrene is therefore a good marker for cytochrome P-450 in most organisms. Benzo(a)pyrene hydroxylase systems have been detected and characterized to a limited extent, in *Aspergillus ochraceus* (Ghosh *et al* 1983) and *Cunninghamella elegans* (Cerniglia and Gibson 1979). Cell free extracts have been shown to possess benzo(a)pyrene hydroxylases, which are sensitive to classical P-450 inhibitors, such as metyrapone, SKF-525A and other imidazoles. The hydroxylases were also sensitive to carbon monoxide, but not to cyanide, azide and antimycin A. A CO-reduced difference spectrum also revealed a cytochrome P-450 maximum, characteristic of these systems (Ghosh *et al* 1983). It has been shown that fungi such as *Cunninghamella bainieri*, have a benzo(a)pyrene hydroxylase system which is similar in mechanism to that found in hepatic microsomes, producing similar qualitative products; both were membrane linked (Gibson *et al* 1975; Cerniglia and Gibson 1978). Cytochrome P-450 in *Cunninghamella elegans* has also been shown to be involved in the conversion of naphthalene to 1-naphthol and trans-1,2-dihydroxy-1,2-dihydronaphthalene (Cerniglia and

Gibson 1978). The products formed were conjugated to water soluble sulphates and glucuronides (Cerniglia and Gibson 1979).

1.5.5 Are steroid hydroxylations in filamentous fungi P-450 mediated?

Available information suggests that there are similarities between the fungal and hepatic microsomal P-450 systems with respect to intracellular localization and the structure of the enzyme processes involved (Muller *et al* 1984). The presence and multiplicity of cytochrome P-450 in mammals has been shown using several approaches. These include induction, *in vitro* inhibition, kinetic, developmental and genetic studies. Purification and sequencing of separate enzymes in mammals has also provided evidence of multiple forms of P-450, supported by SDS-PAGE and isozyme specific immunoglobulins (Lu and West 1980).

Table 1.7 A list of selected P-450 mediated reactions in filamentous fungi reported in the literature by various groups.

Microorganism	Physiological function or catalyzed reaction	References
Cunninghamella bainieri	hydroxylation of arylhydrocarbons	Ferris *et al* 1976
Cunninghamella elegans	hydroxylation of naphthalene	Cerniglia an
Gibson		
		1978a.
Penicillium patulum	involvement in patulin biosynthesis: hydroxylation of the methyl group of m-cresol	Murphy *et al* 1974
Rhizopus nigricans	11α-,6β-hydroxylation of progesterone	Breskvar and Hudnik-Plevnik 1977
Aspergillus ochraceus	11α-,6β-hydroxylation of progesterone	Jayanthi *et al* 1982
Curvularia lunata	11β-,14α-hydroxylation of cortexolone	Zuidweg 1968

Research in filamentous fungi has not advanced as far as in mammalian systems. A comparison of some of these characteristics found in mammals will be made with filamentous fungi, to show that steroid hydroxylations are indeed P-450 mediated (see Table 1.7).

1.5.6 Induction of cytochrome P-450

In mammals induction is complex and is rivalled only by the immune response which is capable of being active to many unique foreign compounds (Nebert *et al* 1982). Inducers can also be considered to be depressors which increase the amount of cytochrome P-450 present (Klinger 1987). There are over five dozen inducers, the most commonly used inducers are benzo(a)pyrene (Souhaili-El *et al* 1986), 2-AAF (Darby *et al* 1984) PB (Wrighton *et al* 1985) and 3-MC (Sinclair *et al* 1987).

The different enzymes present in mammalian liver can be divided roughly into two groups (Netter 1987). The first group provides the broad specificity of enzymes responsible for the detoxification of xenobiotics. Their primary function is to convert such compounds to a form that can be easily excreted from the body. The enzymes of this group are also thought to have endogenous functions, usually in the biosynthesis of biologically active agents such as steroids, hormones and prostaglandins. These apparently constitutive forms may be inducible and occur in the liver of rats and mice in several different forms. Each of these enzymes metabolizes a whole range of compounds with a greater specificity for certain compounds than for others, but the enzymes also exhibit different degrees of overlapping substrate specificity. The second group consists of wholely inducible or narrow specificity cytochrome P-450 forms. These enzymes would appear to be responsible for more effective metabolism of foreign compounds entering the organism from the environment. The constitutive cytochrome P-450 forms metabolize the foreign chemicals to a certain degree before the more specific induced enzymes are available.

Two of the most widely studied xenobotics, PB and 3-MC induce different forms of P-450 in mammals (Eisen 1986). It has been shown that PB

induces enzymes with a very wide specificity whereas 3-MC induces a form with a relatively narrow specificity (Eisen 1986).

Induction in mammals can be divided into two types (Netter 1987). One is PB-type induction and the other is induction by polycyclic aromatic hydrocarbons. PB induction of cytochrome P-450 has been shown to be controlled at the level of transcription (Pike *et al* 1985). PB induces a 2Kb mRNA which carries the code for translation of phenobarbital induction. PB increases the transcription of the respective gene, no receptor is thought to be involved in the phenobarbital effect. With the polycyclic aromatic hydrocarbons such as 3-MC and TCDD, the inducer is dependent on the presence of a genomic locus, which can make some animals, such as a certain strain of mice, responsive to this type of induction. These type of molecules act via a receptor which combines with the inducer and is transferred to the nucleus and causes an increase in arylhydrocarbonhydroxylase. Inducers can themselves be substrates.

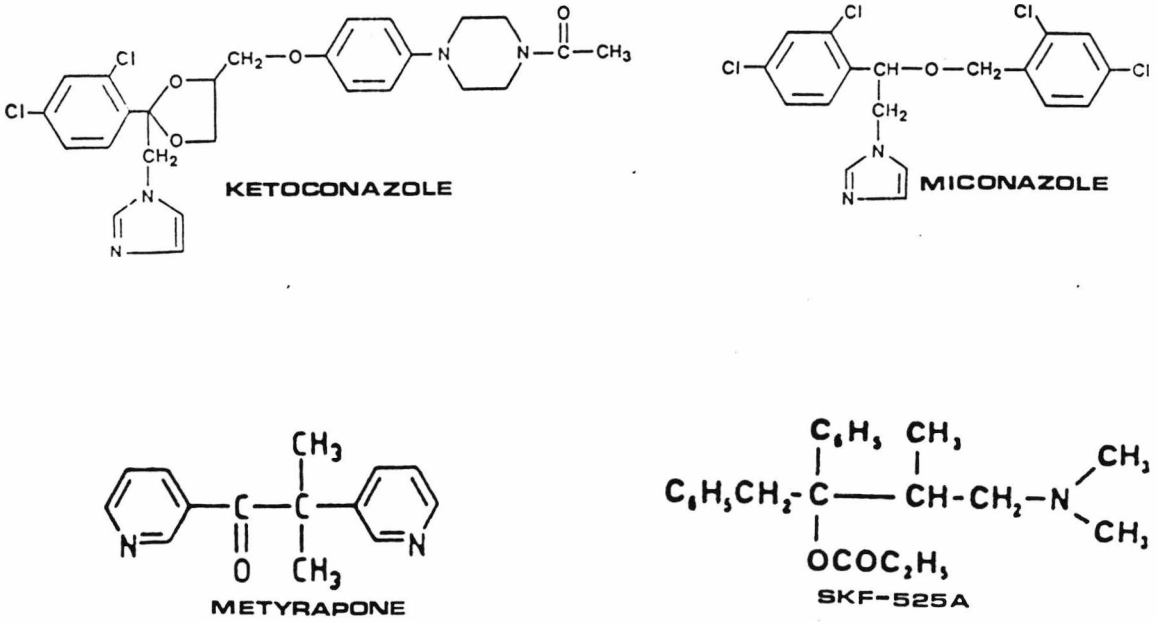
In filamentous fungi the P-450 systems have been shown to be inducible, the substrates themselves have been used as inducers. Lin and Smith (1970a) have used substrates 19-nortestosterone, rac-19-nortestosterone, testosterone and rac-13β-ethyl-17β-hydroxygon-4-ene-3-one to induce different hydroxylations (10β-, 11β-,17α-,14α-) in *Curvularia lunata* NRRL 2380. These various hydroxylations depend on the inducer used. Lin and Smith (1970a) have also showed the differential effect that different inducers can have on the transformation of various substrates. In addition Lin and Smith (1970b) reported that induction occurs. They found that no detectable hydroxylase activity could be demonstrated in a cell free system unless prior induction with 19-nor testosterone was employed in vegetative cells of *Curvularia lunata* NRRL 2380. Similar results have been reported when using vegetative cells from *Streptomycetes, Aspergillus ochraceus, Rhizopus nigricans, Pellicularia filamentosa* and *Cunninghamella elegans* (Jaworski *et al* 1985). Jaworski *et al*

(1985) have also reported that the 11-hydroxylase systems in spores of *Cunninghamella elegans* were inducible. The use of cycloheximide to indicate induction will be discussed in Chapter 5.

1.5.7 Inhibition of cytochrome P-450 in mammals and filamentous fungi

Cytochrome P-450 inhibitors are nitrogen-containing aliphatic and aromatic compounds, such as azoles and imidazoles, that can react with haemoproteins. The effect of inhibitors such as pyridine derivatives and imidazole derivatives (SKF-525 A, metyrapone, miconazole and ketoconazole (Fig.1.9) are well documented in the literature (Testa and Jenner 1981; Pye and Marriott 1982). Well known 'selective' fungal P-450 mediated reactions involve the demethylation of lanosterol or 24-methylene dihydrolanosterol at C-14. These endogenous enzymes (i.e. 14α- demethylase) have a limited ability to metabolize xenobiotics (Coulson *et al* 1984). Inhibitors can also be used to demonstrate different P-450 isozymes. It is well known that metyrapone and SKF-525A can inhibit the metabolism of a variety of microsomes where the rats

Figure 1.9: A selection of antifungal agents used in this study



have been pretreated with PB, but have little effect on metabolism by microsomes from 3-MC treated rats (Testa and Jenner 1981). Ghosh and Samanata (1981) found that the *in vitro* hydroxylation of progesterone by *Aspergillus ochraceus* was stimulated by cyanide and inhibited by metyrapone, suggesting the reaction was P-450 mediated. Carbon monoxide, which can reversibly interact with haemoproteins, is a good detector of P-450. Breskvar and Hudnik-Plevnik (1977) have inhibited 11α-hydroxylation of progesterone in *Rhizopus nigricans* by the use of carbon monoxide. By using spectrophotometric analysis CO-reduced difference spectra located P-450 in a fraction sedimenting at 105,000 g. Carbon monoxide was found to differentially inhibit the hydroxylation of testosterone at several positions, using microsomes. These results provide indirect support for the concept of multiple forms of cytochrome P-450 with different specificities (Lu and West 1980).

Table 1.8 The major forms of cytochrome P-450 purified by a number of research groups from rat hepatic tissue (Astrom and Depierre 1986; Waxman 1986)

Major groups		
Levin *et al*	Guengerich *et al*	Waxman *et al*
a	UT-F	PB-3
b	PB-B	PB-4
c	BNF-B	-
d	BNF/ISF-G	-
e	PB-D	PB-5
f	-	-
g	-	-
h	UT-A	PB-2C
i	-	2d
j	-	-
-	PB-C	PB-1
-	PB/PCN-ε	PB-2a
-	UT-H	-
-	-	PB-2b
-	-	PB-6

The symbols used are examples of agents commonly used to induce the indicated P-450's: UT, untreated; PB, phenobarbital; ISF, isosafrole; BNF, β-naphthoflavone; PCN, 16α-cyanopregnenolone.

The data available to date do not allow a comparison of all the different isozymes.

1.5.8 Multiple forms of cytochrome P-450 in mammals and filamentous fungi

Multiple forms of cytochrome P-450 have been reported in rat hepatic tissue (Waxman 1986). At least 13 distinct P-450 isozymes have been expressed in rat hepatic tissue after exposure to PB and 3-MC (Astrom and Depierre 1986; Table 1.8). In addition many developmental and sexual differences in P-450 catalyzed hydroxylation of steroids also indicate the existence of multiple forms. A number of isozymes have been isolated after purification of the separate forms and their resolution on SDS-gels by their different relative mobilites. Some isolated P-450 enzymes have been reconstituted (Lu *et al* 1972; Gustafsson and Ingelman-Sundberg 1976).

Immunochemical techniques, have been used to identify P-450 isozymes exhibiting close structural homologies (Thomas *et al* 1987). Cross reactivity experiments have been performed using a specific P-450 antibody made from rat liver (P-450p), which cross reacts with samples from rabbit, hamster, gerbil and mouse liver. The use of monoclonal antibodies, has shown immunological relatedness between them (Wrighton *et al* 1985). Multiple forms of P-450 can also be shown where monoclonal antibodies do not cross react with the same P-450 isozymes. There are no reports of cytochrome P-450's from a filamentous fungal source being successfully purified to homogenity since active cell-free extracts were first prepared by Zuidweg *et al* (1962). In contrast NADPH-cytochrome P-450 reductases have been purified from Oomycetes (*Pythium coloratum*), Basidiomycetes (*Ustilago maydis*) and Ascomycetes (*Nectria haematococca, Fusarium solani, Fusarium oxysporum, Cochliobolus heterostrophus, Colletotrichum lindemuthianum, Aspergillus nidulans, Neurospora crassa* and *Saccharomyces cerevisiae*; Scala *et al.* 1988). These reductases were found to be immunologically similar, and possessed

molecular weights which were comparable to rat liver reductases. This supports the theory that the mammalian hepatic system is very similar to the fungal system (Holland 1982). There is no consistent argument to explain all the diverse hydroxylations observed for different substrates in the same organism other than a number of separate isozymes.

Curvularia lunata will transform cortexolone to the resulting hydroxylated 11β- and 14α- major products with 7α- and 11α- minor products (Zuidweg 1968). When the same organism (but different strain) was used with 19-nor testosterone, the majority of products were 11β- and 11α- derivatives with the 10β-hydroxylated products (Lin and Smith 1970a). Multiple forms of cytochrome P-450 are further supported by the knowledge that different microorganisms can hydroxylate every position on the steroid molecule (Charney and Herzog 1967; Kieslich 1980; Laskin and Lechevalier 1984). Even organisms such as *Cunninghamella blakesleeana, Cunninghamella elegans, Absidia orchidis* and *Curvularia lunata* can perform hydroxylation reactions at different positions on the steroid molecule (Table 1.9).

Table 1.9: The number of steroid hydroxylations reported by a selection of filamentous fungi used in this study.

Organism	The number and type of hydroxylations reported (Charney and Herzog 1967; Kieslich 1980; Laskin and Lechevalier, 1984)
Cunninghamella blakesleeana	(9) 1β-,5β-,7β-,9α-,11α-,11β-,14α-,15β-,16β-
Cunninghamella elegans	(8) 1α-,6β-,7α-,7β-,11α-,11β-,14α-,21-
Absidia orchidis	(13) 1β-,2β-,5β-,6α-,6β-,7α-,7β-,9α-,11α-,11β-, 14α-,15β-,21-.
Curvularia lunata	(12) 6β-,7α-,9α-,10β-,11α-,11β-,14α-,15α-,15β-, 16β-,17α-,20β-.

Smith *et al.* (1988) have reported multiple double hydroxylation reactions of progesterone, in *Apiocrea chrysosperma*. These products included reduced 6β-, 9α-, 14α-, 15α-, and 15β- hydroxy-derivatives. It was observed that hydroxylation at position 15 could accompany any other hydroxylation apart

from 6β-hydroxylation and vice-versa. This probably indicates steric hindrance of 15- and 6β-hydroxylases for 6β- and 15- hydroxy-derivatives respectively, suggesting two separate forms.

The variety of hydroxylation reactions described for filamentous fungi are unlikely to be explained by a single multifunctional non-specific enzyme complex. The ultimate proof however for separate isozymes in filamentous fungi, is their purification and characterization.

1.6.1 The physiology of steroid bioconversions by filamentous fungi

Knowledge of the steroid-hydroxylating enzymes from various filamentous fungi is limited, whereas many mammalian cytochrome P-450 hydroxylases have been purified and sequenced (Astrom and Depierre 1986; Waxman 1986).

The vast majority of the published literature has concentrated on work involving whole cells and on increasing steroid transformation by variation of the medium, pH, temperature and oxygen concentration, especially for industrial scale-up (Bihari *et al* 1988). Work on cell-free preparations has lagged behind, due to the difficulty in preparing active cell-free enzymes (Smith *et al* 1989).

1.6.2 Methods used for steroid biotransformation reactions

The methods generally used in screening microorganisms for their steroid hydroxylation ability are:

A The growth of microorganism in liquid media

B Steroid addition in the presence of organic solvent

C Incubation of the organism in the presence of steroid

D Steroid extraction

E Quantitative or qualitative identification of the transformation products (Sebek and Perlman 1979).

To find microorganisms which catalyse the reaction(s) of interest with the highest possible yield, an extensive screening of pure cultures is often undertaken. These organisms are obtained from culture collections or isolated from natural sources. Many microbially catalysed steroid transformations have been published in the literature (Iizuka and Naito 1967; Charney and Herzog 1967; Kieslich 1980; Laskin and Lechevalier 1984).

The microorganisms are generally grown in complex media containing for example yeast extract, or peptone, or corn steep liquor as well as a carbon source (glucose or sucrose) and sometimes phosphate and sulphate nutrients (Sallam *et al* 1971; Abdel-Fattah and Badawi 1975a; Ghosh and Samanta 1981; Kolot 1982; Clark *et al* 1983; Morrin and Ward 1989). The ideal substrate for transformation should be soluble in the fermentation medium and be able to pass into the cell, without being toxic to the microorganism (Smith 1984). Poorly soluble substrates like steroids are dissolved in relatively non-toxic water miscible solvents like methanol, ethanol, acetone, ethyleneglycol and dimethylformamide etc. (Miller 1986). The use of water miscible solvents will be commented on later. Solubility can be enhanced by use of emulsifying agents like Tweens (e.g. Tween 80) or by microionizing substrate particles.

The microorganism is grown for 1-2 days at 30°C prior to steroid addition (Sebek and Perlman 1979) and incubation is continued for 1 or 5 days. Alternatively steroid can be added at the time of inoculation or to resting cell suspensions after cell harvesting. Biotransformations have also been performed using immobilized cells, as immobilized cells often show a higher operational stability than free cells. Other reasons for using immobilised organisms will be discussed in Section 1.8.

During a literature review it was noted that many authors studied the hydroxylase systems without stating the point in growth to which their

measurements referred (i.e. early or late exponential growth, stationary or decline phase). This is important as one should know if any changes in steroid bioconversion that do occur are associated with different stages in growth of the organism. This approach was undertaken in the work described in this thesis.

Fungal biomass is harvested by centrifugation or filtration when using resting cells for bioconversion reactions. The transformation products are usually obtained by avoiding harvesting and extracting directly from the liquid culture (Sebek and Perlman 1979). Both polar and non-polar organic solvents can be used and include: dichloromethane, chloroform or ethyl acetate etc. Steroid molecules are resolved, identified and quantified using thin layer liquid chromatography, high performance liquid chromatography, gas liquid chromatography, nuclear magnetic resonance spectroscopy and mass spectroscopy (Makin 1975).

1.6.3 11-Hydroxylation in filamentous fungi and mammalian adrenal gland

Steroid transformation in filamentous fungi has concentrated on the 11α- and 11β- hydroxylase systems. The hydroxylation of progesterone and cortexolone at the 11-position is very important (see Table 1.4) as 11α PG can be converted to cortisone, an important antiflammatory agent. The 11α-hydroxylation of progesterone almost immediately brought the price of cortisone in 1949 from $200 to $6 per g, and to less than $1 per g by 1979 (Sebek and Perlman 1979). The organisms commonly used for this stereospecific conversion are *Rhizopus nigricans* and *Aspergillus ochraceus* (Hanisch *et al* 1980). Conversion yields are very high (~ 90%) using these organisms. A common side-product is 6β-, 11α-, dihydroxyprogesterone.

The first published 11β-hydroxylation was by *Curvularia lunata* (Shull *et al* 1953); Shull and Kita 1955) and *Cunninhgmella blakesleeana*(Hanson *et al* 1953) using cortexolone as the substrate (Table 1.4). The product 11βC another

anti-inflammatory agent is not produced as efficiently as 11α PG. Conversion yields are usually about 60% for the 11β- hydroxylation of cortexolone. The number of by-products produced in 11β- hydroxylation are more numerous than those encountered in 11α- hydroxylation. Zuidweg (1968) reported that 14α- hydroxylation always accompanied 11β- hydroxylation. Both positions are axial to the plane of the ring system.

The 11β- hydroxylase system in filamentous fungi has been shown to be of similar structure to that found in bovine adrenal (Martsev *et al* 1982; see Fig.1.8). The mechanism and purification of the reductase and adrenodoxin have been determined (Takemori *et al* 1975; Lambeth *et al* 1979; Takemori and Kominami 1984; Suhara *et al* 1971; Suhara *et al* 1972). The P-$450_{11\beta}$ from bovine adrenal was found to be localized on the matrix side of the mitochondrial inner membrane. This differs from filamentous fungi, where 11- hydroxylation has been reported in the microsomal fraction (Martsev *et al* 1982; Ghosh and Samanta 1981; Jayanthi *et al* 1982),

P-$450_{11\beta}$ from bovine adrenal can catalyze the hydroxylation of 3-keto- Δ^4 steroids. It is highly stereospecific for 11β- hydroxylation, but is not totally specific for 11β- hydroxylation. The C-18 and C-19 angular methyl substituents provide C-H bonds, which are in close proximity to the 11β- position, and react with the activated oxygen complex, but the 11β-position is preferred (Sato *et al* 1978). P-$450_{11\beta}$ can perform both 18- and 19- hydroxylations and this has been used to show the similarity between mammalian (bovine) adrenal and fungal systems. It has been shown that C-19 steroids (4-androst-ene-3,17,-dione) can be 11β- and 19- hydroxylated, while C-21 steroids (11-deoxycorticosterone) can be 11β- and 18- hydroxylated in both bovine and rat adrenal and *Pellicularia filamentosa*(Nakamura and Tamaoki 1970; Clark *et al* 1982; Suhara *et al* 1986a). This will be commented on later in this thesis. It has been demonstrated that cortexolone can be converted to 11βC using bovine P-$450_{11\beta}$,

as it is in filamentous fungi (Takemori and Kominami 1984). Suhara *et al* (1986b) have also shown that bovine P-450$_{11\beta}$ can also function as an aromatase, generating adrenal estrogen from androstenedione and its 19- hydroxy and 19-keto derivatives, but this biocataytic activity has not been shown in fungi. The similarities of both systems has also been demonstrated by the fact that copper inhibits P-450$_{11\beta}$ in both rat adrenal (Veltman and Maines 1986), and *Aspergillus ochraceus* (Samanta and Ghosh 1987).

1.6.4 11-Hydroxylation in whole cells and cell free extracts

Experiments in batch fermentation using *Pellicularia filamentosa* , has shown that both 11β- and 19- hydroxylations are probably performed by the same enzyme (Ohta *et al* 1987). This dual hydroxylation has also been demonstrated in purified bovine adrenal by P-450$_{11\beta}$ (Suhara *et al* 1986a). Both hydroxylations have an optimum DOT for initial production at 15% saturation, and this differed from the optimum DOT (30% saturation) for further synthesis. (Clark *et al* 1982). Hanisch *et al* (1980) stated that the requirement of oxygen for the synthesis of the 11α- hydroxylase in *Rhizopus nigricans* is much less, compared to what is required to achieve maximum conversion rates.

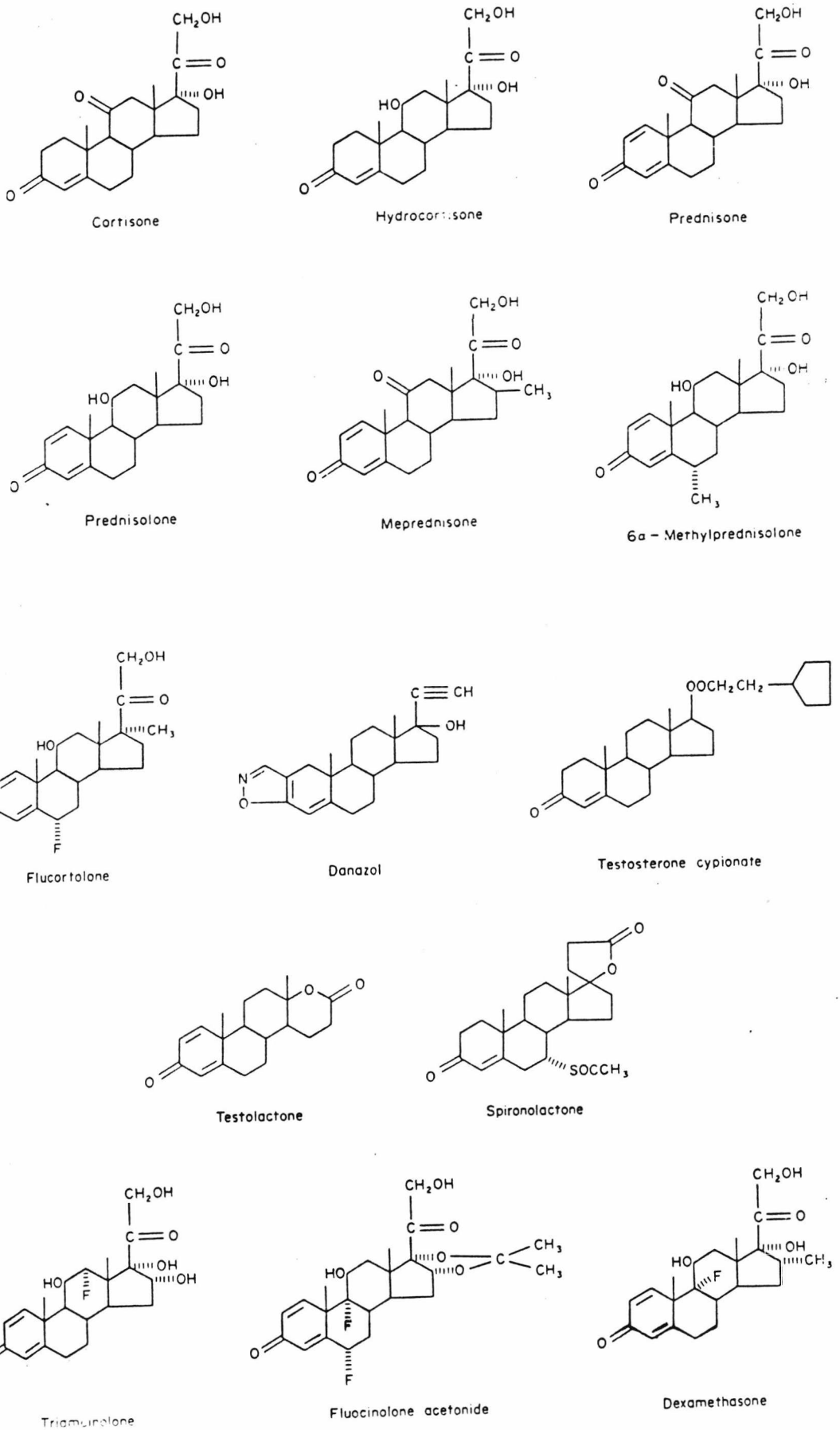
An *Aspergillus ochraceus* mutant has been used in batch fermentations with high substrate concentrations of up to 40 g/l to produce high yields (90%). Mycelial cultures were used in preference to spores as they can tolerate high concentrations of the substrate (Somal and Chopra 1985). The maximum induction of 11α- hydroxylase by *Rhizopus nigricans* has been reported using a progesterone concentration of 0.5 g/l or above (Hanisch *et al* 1980). Samanta *et al* (1978) have reported that *Aspergillus ochraceus* could carry out 11α-hydroxylation of progesterone over a broad pH range (4-8). Maximum conversion was reported at pH 6.5-7.0. They also reported that metal ions such as Zn^{2+} did not affect the specifity of the hydroxylation reaction. A pH range of

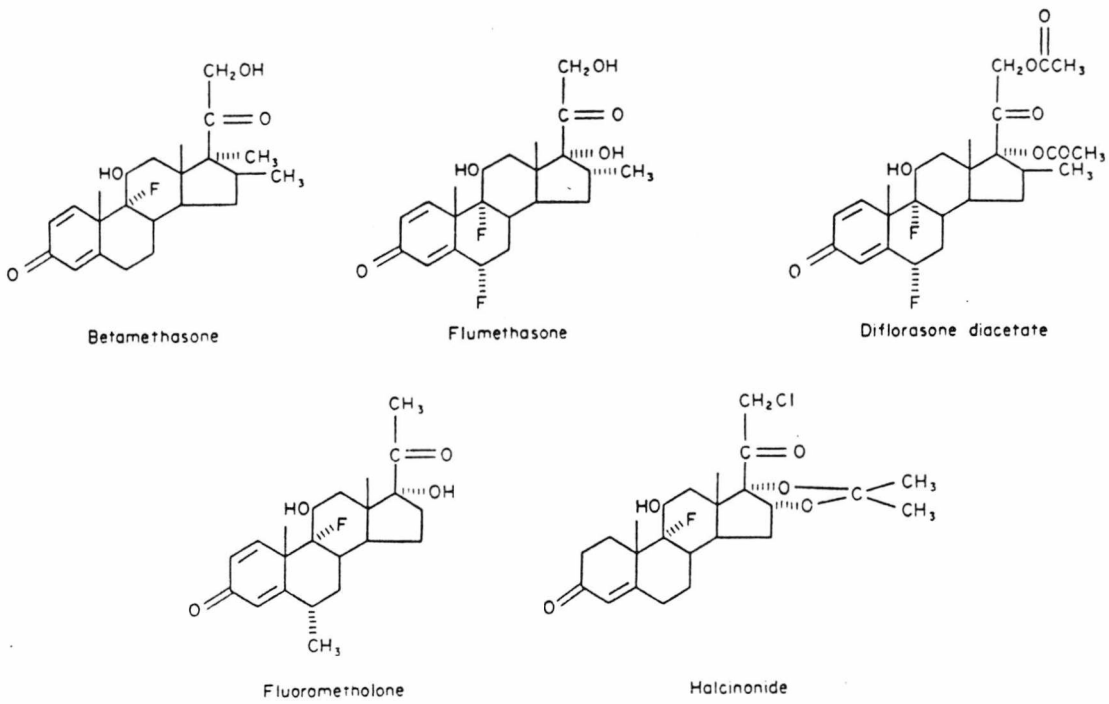
6-7 was also reported by Somal and Chopra (1985). This was supported by Bihari *et al* (1988). Most authors grow filamentous organisms at 28-30°C for transformation purposes. Whole cells of *Curvularia lunata* have been used to calculate an apparent Km for the 11β- hydroxylase (0.34 mM) which was also identical for 10β- and 14α- hydroxylation (Lin and Smith 1970b). It has been demonstrated that the addition of magnesium and phosphate stimulate the conversion of progesterone to 11α PG by *Rhizopus stolonifer* (El-Rafai *et al* 1970). The use of fungal protoplasts, which are free of cell wall material, shows that the system(s) involved in hydroxylation were intracellular and does not appear to be dependent on the cell wall for its activity (Sedlaczek *et al* 1984; Dlugonski 1988). The tough complex cell wall structure has however been reported to impair substrate access to the enzyme.

The preparation of cell-free extracts provides more information on the enzymes responsible for steroid modification. The ultimate aim is of course the purification of individual enzymes for further study. The information which can be gained from cell-free preparations is limited due to the labile nature of the enzymes involved. In fact the enzyme complexes involved in steroid bioconversion are so susceptible to damage that they lose activity very quickly at 4°C (Zuidweg 1962; Zuidweg 1968). *Rhizopus* and *Aspergillus* species have been used for the partial purification of the 11α- hydroxylation system (Breskvar and Hudnik-Plevnik 1981; 1987 and Madyastha *et al* 1984, respectively). They demonstrated that these systems were membrane bound. Their activity was stabilized in the presence of EDTA at pH 8.3, which inhibits proteolytic activity, and DTT or GSH which protects thiol groups (Breskvar and Hudnik-Plevnik 1977; Jayanthi *et al* 1982).

Samanta and Ghosh (1987) have demonstrated that the 11α- hydroxylase enzyme complex of *Aspergillus ochraceus* was associated with phosphatidyl choline. This is not unlike that in hepatic microsomes in mammals (Lu *et al* 1969).

Figure 1.10: Steroid products of commercial importance that have one or more bioconversion steps as part of their synthesis (Miller 1986).





They stated that induction caused enrichment of phospholipid in the membrane. They also showed that both cytochrome P-450 and the NADPH-dependent cytochrome c reductase were induced. NADPH cytochrome c reductase involvement was shown by inhibiting hydroxylation with cytochrome c. The enzyme was inhibited by Cu^{2+}, Ca^{2+} and Co^{2+} ions. Breskvar and Hudnik-Plevnik (1981) have reported the *de novo* synthesis of the 11α- hydroxylase system in *Rhizopus nigricans* and *Rhizopus arrhizus,* and have proposed that this probably occurs with the reductase as well. This is similar to mouse liver microsomes, where low levels of cytochrome P-450 were found in uninduced cells.

The optimal induction time for the 11β- hydroxylase complex was quoted as 1-3 hr in *Curvularia lunata* (Lin and Smith 1970b). The maximum pH reported was pH 8 (Zuidweg 1962; 1968). Zuidweg was one of the first to show that hydroxylation of cortexolone was NADPH-dependent. Cell-free preparations of *Aspergillus ochraceus* producing 11α- hydroxylase had a similar pH maximum (pH 7.7) to the 11β- hydroxylase complex (Madyastha *et al* 1984). These pH optima are not dissimilar to those seen in whole cells. The 6β-hydroxylase system from *Aspergillus niger* has been shown to possess a pH maximum of 6.5. EDTA was found to inhibit its activity, but pyruvate, NADPH and α-ketoglutarate stimulated it (El-Kady 1982). Like *Aspergillus ochraceus, Aspergillus niger* was inhibited by divalent ions such as Co^{2+} and Cd^{2+}. The various publications form a picture of hydroxylase enzymes with closely related activities.

1.6.5 Steroid transformation in aqueous miscible systems

Steroids have been reported to have a low solubility in water, in the order of 10^{-4} - 10^{-5}M (Cremonesi *et al* 1975). The concentration of steroid substrate used in a bioconversion process can exceed its solubility (Chen *et al* 1985). For example when *Arthrobacter simplex* is grown in the presence of hydrocortisone, the solid material dissolves gradually into solution as Δ^1-dehydrogenation proceeds (Chen *et al* 1985). The resultant product (prednisolone) can sometimes crystallize out of solution (pseudocrystallo fermentation). The problem is that a mixture of substrate and product often results in lowering the purity of the fermentation product. This problem can be alleviated, using two-phase immiscible systems.

Immiscible solvents include carbon tetrachloride, benzene, toluene, cyclohexane etc. (Cremonesi *et al* 1975; Buckland *et al* 1975; Carrea 1984). A

disadvantage of aqueous immiscible systems is the damage that organic solvents cause to cells. Biomolecules are often structurally altered when exposed to organic solvents. One also encounters higher surface tensions in these systems than in aqueous-miscible systems (4×10^{-3} N/m). This leads to the concentration of the material in one or other phase (Mattiasson 1983).

Aqueous-immiscible systems can be advantageous if the microorganism performs the desired reaction in the presence of large portions of organic solvent (Buckland *et al* 1975). Sometimes the hydrophobic nature of some enzyme systems is stabilized by the presence of organic solvents. These systems are also dependent on the solubility of the substrate in the aqueous phase and whether the presence of microorganisms puts severe limitations on the conditions that can be used for the transformation process (Buckland *et al* 1975). Most of the current fermentations involving steroid molecules utilize aqueous-miscible systems. Aqueous-miscible systems permit easy sterile transfer of steroid to the medium, it also reduces the surface tension to about 1×10^{-4} N/m, allowing good mixing and even distribution in the system (Mattiasson 1983). For these reasons aqueous-miscible systems were used in this project.

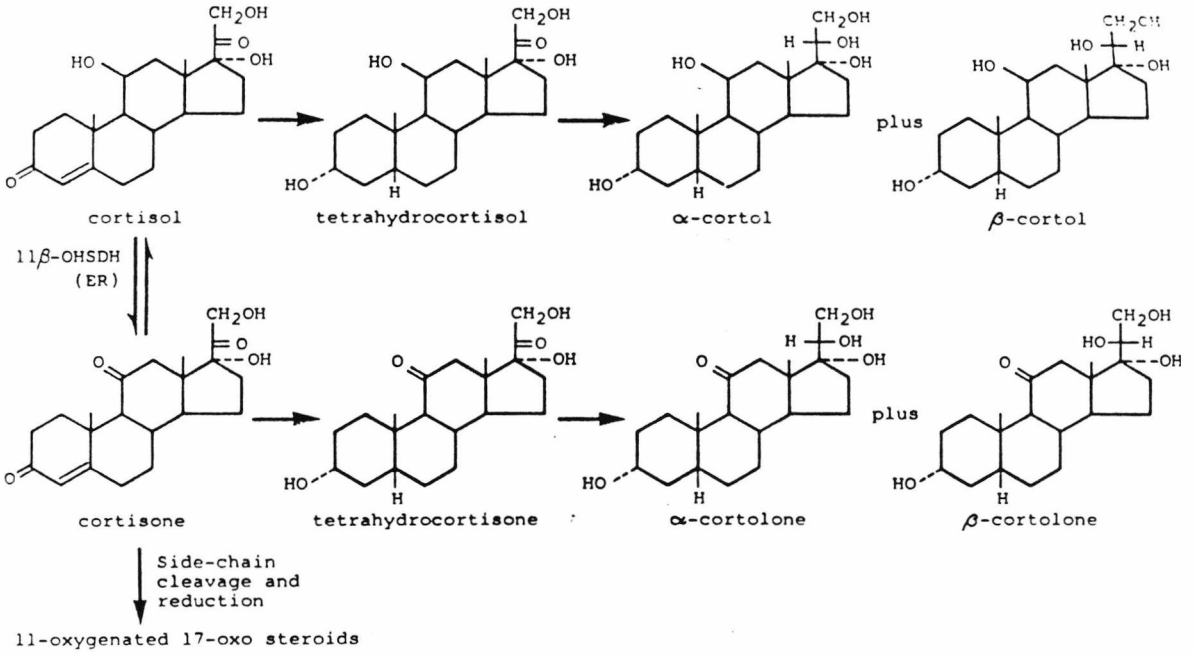
1.7.1 Degradation of steroids by microorganisms

Steroid degradation will be discussed, as the transformation substrate cortexolone was metabolized by some of the fungi used in this study. Steroid molecules can be degraded from the side chain or the A-ring (Mahato 1985). In mammals steroid molecules are made physiologically inactive in the liver and kidneys, by reductive reactions, to render the molecules more polar (Fig. 1.11). The inactivation of cortisol and cortisone leads to water-soluble metabolites which can be conjugated as sulphates or glucuronides, for excretion in the urine (Gower 1979).

The degradation of the C-17 side chain has received considerable attention for economic reasons (Mahato 1985). The starting materials used include cholesterol, sitosterol and campesterol, which occur naturally and are being used to supplement or supplant the commercial processes from diosgenin (Fujimoto *et al* 1982a).

Selective microbiological cleavage of the hydrocarbon sidechain is useful, without degradation of the steroid nucleus. Degradation of the side chain leads to the formation of androst-4-ene-3,17-dione or androsta-1,4-diene, 3,17-dione, which can be used to form other corticosteroids or other medicinally useful steroids, such as estrone and the hypertensive drug spironolactone.

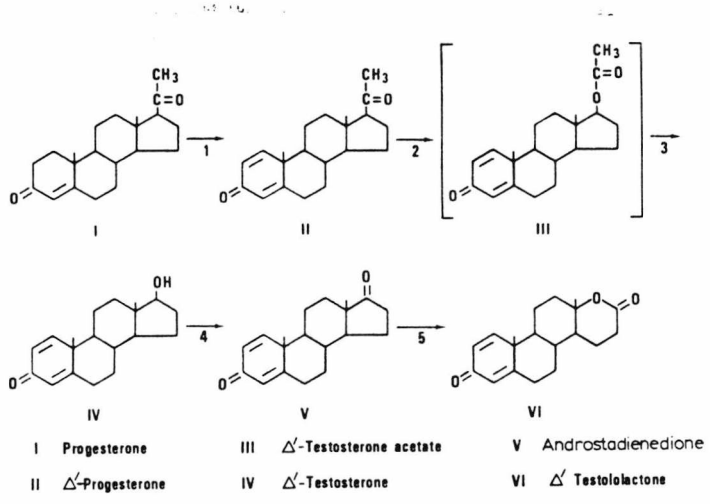
Figure 1.11: Abbreviated catabolic pathways of cortisol and cortisone (Gower 1979).



1.7.2 Progesterone side chain cleavage and sterol side chain degradation

Progesterone side chain cleavage has been reported in bacteria and fungi. Fried *et al* (1953) reported that progesterone could be converted to androsta-1,4-diene, 3,17-dione (7%) and testololactone (70%) by *Streptomyces lavendulae* and *Penicillium chrysogenum* respectively. These workers have also demonstrated that Δ'-testololactone could be formed from progesterone, cortexolone or testosterone in yields of about 50% using *Cylindrocarpon radicicola*. The formation of such compounds led Miller (1972) to propose a sequence for progesterone cleavage by *Septomyxa affinis* (Fig.1.12).

Figure 1.12: The proposed sequence for progesterone side-chain cleavage by *Septomyxa affinis* (Miller 1972).



It can be seen that progesterone can be reduced to Δ'-progesterone. An oxygen atom can then be inserted between carbons 17 and 20 to form Δ'-testosterone acetate. Testosterone is then formed by the cleavage of the acetate group by an esterase. The 17-hydroxysteroid can be oxidised to the 17-ketosteroid-androstadienedione (1,4-androsta-diene,3,17,-dione). Δ'-testololactone is formed by insertion of an oxygen atom between C-17 and C-13 to form a ring D lactone.

Some microorganisms can hydrolyse the ring D lactone, to give testolic acid. Δ'-testololactone has been used to treat breast cancer (Miller 1986). The enzymes involved in steroid side chain cleavage have been reported to be inducible (Miller 1972). Commercially these sequence of reactions are of limited practical importance as a source of steroid hormones.

Turfitt (1948) first reported complete degradation of the steroid structure. He demonstrated that *Proactinomyces erythropolis* could break down cholest-4-en-3-one. Other microorganisms such as *Arthrobacter, Bacillus, Microbacterium, Nocardia, Mycobacterium* and *Streptomyces* can catabolize cholesterol to carbon dioxide and water (Marsheck *et al* 1972; Santer and Ajl 1952).

Side chain degradation of sterols such as cholesterol, cholest-4-ene-3-one, β-sitosterol and campesterol to 3-keto-Δ^4 steroids in microorganisms has been investigated by many researchers (Zaretskaya *et al*, 1968; Schubert *et al.* 1969; Sih *et al.* 1967a; 1967b; 1967c; 1968 and Fujimoto *et al* 1982b).

1.7.3 Degradation of the steroid A ring

Sih and his collaborators were responsible for elucidating A ring degradation using *Nocardia restrictus* (Sih *et al* 1965b; 1965c; Gibson, Wang and Sih *et al* 1966) 3-keto-Δ^4 steroids were found to be degraded to pyruvate, which can enter the tricarboxylic acid cycle (Sih *et al* 1965b). It has also been reported that *Nocardia restrictus* can oxidise progesterone to carbon dioxide and water, via Δ'-progesterone (Sih 1962a).

1.8.1 What is immobilisation?

The word immobilize means "to make immobile", or "to make incapable of movement". Immobilisation studies of whole microbial cells spores and enzymes, has been intensively carried out over the past 15 years, in or on numerous supports or carriers. (Kolot 1981a; Kolot 1981b; Kolot 1982;

Kolot 1983). Part of this project was to assess the potential of using immobilized cells for steroid biotransformations.

1.8.2 Advantages of immobilized cells over normal fermentation

The use of immobilized biocatalysts has been thought to provide potentially more advantages than traditional fermentation processes, using free cells (Ohlson *et al* 1978; Vieth 1979; Kolot 1980), for several reasons:

1. Immobilization of fungal spores allows the fermentation to proceed longer on a homogeneous basis irrespective of fungal morphology.
2. Immobilized microorganisms are easy to handle and provide easy separation of product from the catalyst.
3. They are reusable and suitable for continuous or repeated batch operation, allowing better process control, which would ultimately improve product yield.
4. It is possible in continuous culture conditions to operate the system at high dilution rates, without washout.

1.8.3 Common methods of immobilization

The most widely used immobilization methods can be roughly divided according to whether the cell or biocatalyst is chemically bound by absorption (electrostatic or non-electrostatic interactions) or covalently coupled. The cells or biocatalysts can also be physically retained (Hartmeier 1985). This classification however is not clear cut as physical entrapment can also involve ionic and non-electrostatic interactions (Table 1.10). Filamentous fungi have been used with increased frequency for immobilization. The most popular method is entrapment using polyacrylamide, alginate, agar gels plus photo-crosslinkable resin prepolymers and urethane prepolymers. (Ohlson 1980; Maddox 1981; Sonomoto *et al* 1982; Sonomoto *et al* 1983; Bihari 1984).

Table 1.10: Immobilization techniques, their mechanisms advantages and limitations (Kolot 1981a)

Immobilization techniques	Factors,affecting immobilization, supports, coupling agents	Mechanisms	Advantages/ limitations
Adsorption → Cell Properties	Cell wall comp.	Electrostatic interactions between carrier and cell surface	Simple/ pH depended
	charge		
	Age		
Adsorption → Carrier Properties	Comp.		
	Surface charges		
	Surface area		
	pH		
Entrapment	Agar	Entrapment	/Substrate diffusion /Gel by phosphates
	Alginate		
	Pectate		
	Carrageenan		
	Plastic		
	Acrylamide	Free radical polymerization	/Toxicity
Coupling	Isocyanate	Covalent bond formation	/Toxicity
	Amino silane		
	Glutaraldehyde	" " "	/Toxicity
	Carbodiimide	" " "	/Toxicity

Whole cell immobilization has many advantages over isolated enyzmes as biocatalysts. (Vieth 1979; Hartmeier 1985; Tramper 1985; Ohlson *et al* 1980).

1.8.4 Criteria for a good support (carrier)

For a support to be seriously considered for immobilization purposes it should fulfil a set number of criteria (Kolot 1981a):

1. It should be non-toxic to the cell, and should not affect the cell metabolism. Ideally one should find a support which will increase the cell membrane permeability or enhance cell metabolism.
2. The support should retain high microbial loading. This can be defined as 'the amount of dry weight of cells (g) incorporated per g of carrier'. The retention capacity of the support can be defined as 'the difference between the amount of cells initially added to the support, and the amount which did not react with the support and therefore was washed out'.
3. The support should be stable at high temperatures and pressures.
4. Supports should have a high porosity not just to provide high microbial loadings, but also to allow good oxygen and nutrient diffusion to the cell.
5. Any supports used should be non-toxic and resistant to microbial degradation.
6. Supports used should be inexpensive and easily obtainable.

1.8.5 Properties of Chromosorb W as an immobilizing support

Chromosorb W (Celite) is a specially treated form of Kieselgel (Gbewonyo *et al* 1983). It has been used to entrap *Penicillium chrysogenum*

spores (Gbewonyo *et al* 1983) and has commonly been used as a filter in recovery operations, and for the separation of molecules. Chromosorb W is a porous silica material which is obtained from the fossilized deposits of the shells of diatoms. The main chemical constituent is silica (90% by weight). There are also smaller amounts of Al_2O_3, Fe_2O_3 and CaO present. The metals can permit electrostatic bonds of attraction as the whole network permits physical entrapment. The spherical geometry of Chromosorb W particles, reduces the non-Newtonian viscosity of the liquid culture which occurs with mycelia and pellet formation; and relieves the limitation of gas-liquid mass transfer. Germinating fungal spores are provided with a structure within which the organism may grow, protected from high external shear, and within which even weakly adhesive or flocculent organisms will be retained (Black and Webb 1986).

Chromosorb W is non-toxic to the organism and is resistant to microbial degradation. It is also stable at high temperatures and pressures. The method of immobilization using fungal spores is simple, and Chromosorb W is inexpensive. Mercury intrusion porosimetry, reveals that 70% of the entire bead volume is void space, which can be employed for mycelial growth. The growing fungus can also be protected in the bead network. As part of the initial stages in this investigation, it was decided to assess the potential of Chromosorb W as an immobilization carrier for fungi used in steroid hydroxylation.

1.9.1 The aims of the study

The aim of this project was to select from a screening programme a number of fungi which could perform 11- hydroxylation. Due to the time involved few isolates could be investigated in detail although it was important to characterize which organisms could perform the transformation. The fungi chosen from the survey were to be used to investigate when steroid transformation took place during a batch culture cycle and if steroid

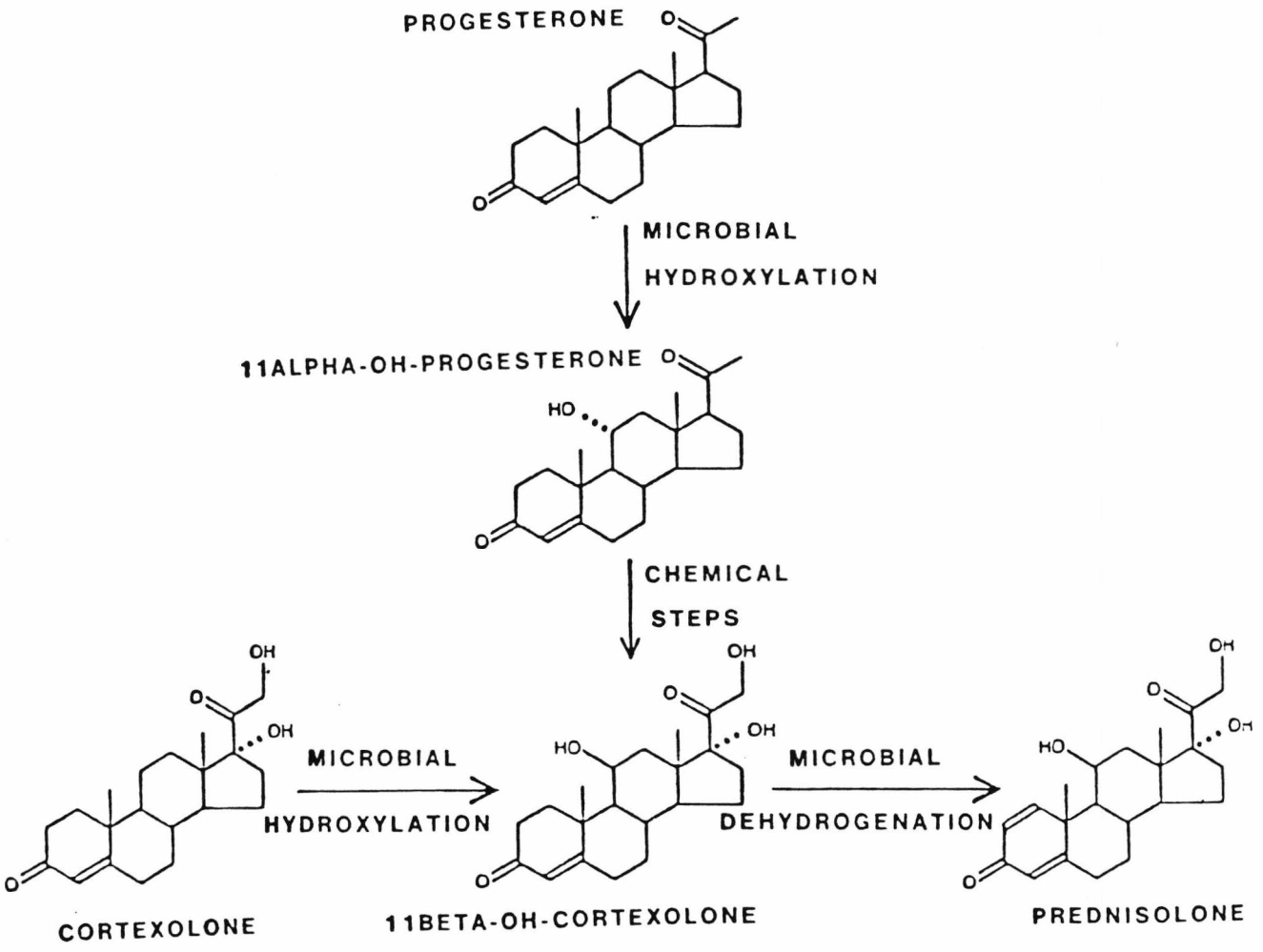
degradation occurred. A single species would then be chosen for further study of the physiological factors that affect the bioconversion, when the organism was exposed to cortexolone. Work with whole cells has mainly centred on qualitative reports of steroid biotransformation of different types and numbers of products formed by different fungi (Kieslich 1980; Smith 1984). It was intended to investigate if possible the biochemical properties of 11-hydroxylation. Such information would give clues as to the nature of the enzymes involved and their relationship to fungal metabolism.

It was also intended to develop and assess a system to immobilize fungal spores in Chromosorb W beads for transformation purposes. Mycelial cultures can grow as a mass which limits mass transfer of substrates and products. The use of a supportive bead network could therefore be useful commercially to provide a homogeneous mix which would hopefully not hinder hydroxylation and in addition permit the easy separation of the transformation products from the cell culture, which can then be rescued. This idea is appealing as downstream processing costs can often be the most significant expense in an industrial process. The formation of discrete support/biomass particles was possible, but problems of restraining microbial growth were experienced. As it was thought that the understanding of the 11-hydroxylase systems in unimmobilized cells was a more important initial step before immobilization, immobilization studies were discontinued. The results are presented in Chapter 7.

Cortexolone was chosen as substrate for most of the studies presented, as it is the direct precursor of the important anti-inflammatory corticosteroid drug, hydrocortisone. The conversion, which involves NADPH and molecular oxygen, proceeds via a cytochrome P-$450_{11\beta}$ monooxygenase (Fig.1.13). Hydrocortisone can be converted to other important corticosteroids such as prednisolone (Takeda *et al* 1959). Cortexolone could be either 11β- or 11α-

hydroxylated by filamentous fungi, whereas progesterone was predominantly hydroxylated to the 11α-product (Laskin and Lechevalier 1984). Cortexolone 11-hydroxylation is possibly the most interesting from a physiological and biochemical point of view, as one can compare both 11α- and 11β - hydroxylations.

Figure 1.13: Schematic presentation of hydrocortisone and prednisolone formation by microbial and chemical routes



CHAPTER 2
MATERIALS AND METHODS

2.1.1 Source of chemicals

All chemicals used were of analytical grade unless otherwise stated. Steroid standards were obtained from Sigma, except for 11αC which was obtained from the steroid reference collection (Queen Mary College, London).

2.1.2 A brief outline of fungal taxonomy

The outline classification of fungi described here, is based on Deacon (1980). The fungi obtained from the various culture collections originate from both the Zygomycotina and the Deuteromycotina

Zygomycotina (class:Zygomycetes)

Order:	Mucorales		
Family:	Mucoraceae;	Cunninghamellaceae;	Thamnidiaceae
Genus:	*Rhizopus;*	*Cunninghamella;*	*Thamnidium*
	Absidia		*Phascolomyces*
	Mucor		*Cokeromyces*
			Mycotypha

Deuteromycotina (subclass:Hyphomycetes)

Order:	Moniliales	
Family:	Moniliaceae;	Dematiaceae
Genus:	*Aspergillus;*	*Curvularia*

Portuguese isolates such as: *Phoma, Penicillium, Cladosporium, Trichoderma* and *Alternaria* species belong to the Deuteromycotina.

2.1.3 List of isolates used

The isolates were obtained from the hecogenin-rich *Agave mexicana*. The organisms were isolated by Professor Baptista Ferreira and Margarida Barata (Botanical Garden, University of Lisbon, Portugal).

Isolates were obtained from Portugal on four occasions (see below). Isolates suffixed 'e' came from surface swabbings. Those suffixed'i' came from surface-sterilised leaf plugs. Isolates U1-U9 came from *Agave* soil rhizosphere.

The first batch isolates was supplied in January 1986 and included:

Number of isolate	Identification of isolate
840601e	*Aureobasidium pullulans*
840612e	*Cladosporium* sp.
8406159e	*Trichoderma* sp
8406205e	*Torula* sp
8406192e	*Diplodia* sp
8406221e	*Phoma* sp
850101i	*Penicillium* sp
850102i	*Cladosporium* sp
850103i	*Cladosporium* sp
850106i	*Penicillium* sp
850108i	*Cladosporium* sp
850109i	*Penicillium* sp
850110i	*Cladosporium* sp
850111i	*Cladosporium* sp

The Second batch was supplied in February 1986

Number of isolate	Identification of isolate
860101e	*Aureobasidium pullulans*
860102e	*Cladosporium chlorocephalum*
860103e	*Cladosporium sp*

860104e	*Cladosporium sp*
860105e	*Cladosporium sp*
860106e	*Aureobasidium pullulans*
860107e	*Aureobasidium pullulans*
860108e	*Cladosporium cladosporioides*
860109e	*Aureobasidium pullulans*
860110e	*Aureobasidium pullulans*
860111e	*Cladosporium sp*
860112e	Not identified
860113e	Not identified
860114e	*Aureobasidium pullulans*
860115e	*Penicillium sp*
860116e	*Alternaria sp*
860117e	*Alternaria sp*
860118e	*Alternaria sp*
860119e	*Phoma glomerata*
860120e	*Phoma sp*
860121e	*Phoma sp*
860122i	*Penicillium sp*
860123i	*Penicillium sp*
860124i	*Penicillium sp*

The third batch was supplied in July 1986

Number of isolate	Identification of isolate
860214e	not identified
860515e	Coelomycete
860216e	not identified
860217e	not identified
860218e	not identified
860219e	not identified
860220e	not identified
860221e	not identified
860222e	*Cladosporium chlorocephalum*
860223e	*Cladosporium chlorocephalum*
860224e	Coelomycete
860225e	*Penicillium sp*

860226e	Coelomycete
860227e	Coelomycete
860228e	Coelomycete
860229e	Coelomycete
860230e	Coelomycete
860231e	*Aureobasidium pullulans*
860232e	Coelomycete
860233e	*Phoma herbarum*
862034e	*Cladosporium chlorocephalum*
860235e	*Cladosporium chlorocephalum*
860236e	*Aureobasidium pullulans*
860237e	*Aureobasidium pullulans*
860201i	*Penicillium sp*
860202i	*Penicillium sp*
860203i	*Penicillium sp*
860204i	*Penicillium sp*
860205i	*Penicillium sp*
860206i	*Penicillium sp*
860207i	not identified
860208i	*Cladosporium chlorocephalum*
860209i	*Penicillium sp*
860210i	*Cladosporium cladosporioides*
860211i	*Penicillium sp*
860212i	*Aureobasidium pullulans*
860213i	*Aureobasidium pullulans*
860214i	*Cladosporium chlorocephalum*

The fourth batch was supplied in August 1986

Number of isolate	Identification of isolate
860301e	*Phoma sp*
860302e	*Alternaria sp*
860304e	*Alternaria sp*
860305e	*Penicillium sp*
860306e	*Aureobasidium pullulans*
860307e	*Cladosporium sp*
860310e	not identified

860311e	*Alternaria sp*
860312e	not identified
860313e	*Alternaria sp*
860314e	not identified
860315e	*Penicillium sp*
860316e	*Aureobasidium pullulans*
860317e	*Alternaria sp*
860318e	*Alternaria sp*
860319e	*Aureobasidium pullulans*
860301i	*Aspergillus sp*
860302i	*Penicillium sp*
860303i	*Phoma sp*
860304i	*Phoma sp*
860305i	*Phoma sp*
860306i	not identified
860307i	*Penicillium sp*
CH11i	*Alternaria sp*
U_1	not identified
U_2	not identified
U_3	not identified
U_4	not identified
U_5	not identified
U_6	not identified
U_7	not identified
U_8	not identified
U_9	not identified

2.1.4 List of filamentous fungi used

The filamentous fungi were obtained from various public culture collections: CMI, CAB International Mycological Institute, Kew, Surrey, England; FSU, Friedrich Schiller Universitat Jena, Freiherr-vom-Stein-Alleez, 53 Weimar, Deutsche Democratische Republik; UKC, University of Kent, Canterbury, Kent, and the UE, University of Edinburgh culture collection, Edinburgh. These include:

Organism	Class	Source and Identification	Number
Absidia glauca(+)	Zygomycotina	CMI	15405
Absidia glauca(-)	Zygomycotina	CMI	15406
Absidia orchidis (coerulea)	Zygomycotina	UE	
Absidia spinosa	Zygomycotina	FSU	P5
Aspergillus niger	Deuteromycotina	CMI	17454
Cokeromyces recurvatus	Zygomycotina	UKC (CMI	12 077585)
Cunninghamella blakesleeana	Zygomycotina	CMI	63877
Cunninghamella echinulata	Zygomycotina	CMI	188395
Cunninghamella elegans	Zygomycotina	CMI	21198
Curvularia lunata parent strain	Deuteromycotina	CLR56+ CMI	83444
Mucor genevensis	Zygomycotina	CMI	89324
Mycotypha africana	Zygomycotina	CMI	139108
Phascolomyces articulosus	Zygomycotina	CMI	81613
Rhizopus stolonifer	Zygomycotina	CMI	57761
Thamnidium elegans	Zygomycotina	CMI	132643

+ This mutant was obtained from Professor G.Holt, (Polytechnic of Central London, London).

2.1.5 Organism maintenance

Organisms were grown on malt extract agar (2% Oxoid malt extract, 2% Oxoid technical agar), in 300ml Erlenmyer flasks, containing 50ml of the solid medium. Flasks were inoculated with spore suspensions prepared from Petri dishes, using sterile distilled water and 0.1% (v/v) Tween 80 to dislodge spores from surface cultures. Cultures were allowed to grow for two weeks before spores were harvested for inoculation of liquid medium.

2.1.6 Growth media used

Medium A

Glucose	(20 g/l)
Mycological peptone	(20 g/l)
$MgSO_4.7H_2O$	(10 g/l)
KH_2PO_4	(10 g/l)
Tween 80	(0.02% V/V)

Components were sterilized separately at 10 psi for 15 min.

Defined medium B

This was a modified version of the Carter and Bull (1969) minimal medium

Glucose	(10 g/l)
NH_4Cl	(2.5 g/l)
KH_2PO_4	(7.5 g/l)
K_2HPO_4	(3.46 g/l)
Polypropylene glycol	(0.1% v/v)

These components were made up separately and sterilized at 10 psi for 15 min.

The trace elements solution contained:

$MgSO_4, 7\ H_2O$	(2.5 g/l)
$CaCl_2, 2\ H_2O$	(0.2 g/l)
$ZnSO_4, 7\ H_2O$	(0.2 g/l)
$MnSO_4, 4\ H_2O$	(0.2 g/l)
$CuSO_4, 5\ H_2O$	(0.05 g/l)
$FeSO_4, 7\ H_2O$	(0.5 g/l)
Na_2SO_4, anhydrous	(0.5 g/l)
EDTA	(0.08% W/V)

The vitamin solution contained:

Biotin	(4 mg/l)
Pyridoxine	(4 mg/l)

Thiamine-HCl	(20 mg/l)
Riboflavin	(10 mg/l)
p-Aminobenzoic Acid	(10 mg/l)
Nicotinic acid	(10 mg/l)
Folic acid	(10 mg/l)
DL-Calcium pantothenate	(0.2 mg/l)
B12	(10 mg/l)

Both the trace elements solution and the vitamin solution were made up at 100 times final strength and stored at -20°C until used. These components were filter sterilized through at 0.2 µm acrodisc filter before use.

2.1.7 Spore preparation

Spores were used as a source of inoculum in shake flask experiments. Spores were removed by addition of medium to each 300ml-Erlenmeyer flask. Glass beads (3mm diameter) were then added, and the flasks gently shaken to dislodge spores. The isolates obtained from Portugal with numbers prefixed 8601 or 8602 were grown in Petri dishes and spores obtained as previously described. The spore suspension was then filtered through two layers of muslin. Spore counts were performed using a Weber haemocytometer.

2.1.8 Incubation mixture

Erlenmeyer flasks (250ml) were cleaned in Decon 90 before use, to ensure that steroid did not adhere to the flask interior. Each flask was autoclaved at 15 p.s.i for 15 min, before use. Growth medium (20ml) was added followed by 10 mg of cortexolone in 500µl of methanol giving a final cortexolone concentration of 0.39 mg/ml (1.13 mM in the medium). Fermentation was initiated by the addition of 5ml of spore suspension, and the mixture incubated at 200 rpm, 30°C for 72 hr, on a Gallenkamp orbital shaker, which was used

throughout this period of study. All shake flask experimentation was performed in triplicate.

2.1.9 Sampling of germinating spores

In experiments using germinating spores 2×1ml samples were aseptically removed from each flask. 1ml was placed in a 1.5ml Eppendorf tube for subsequent glucose and biomass determination. The remaining 1ml was transferred to clean test tubes for steroid extraction.

2.1.10 Microscopic examination of germinating spores

During experiments using germinating spores, small samples were periodically removed from a fourth flask and observed using an Olympus BH light microscope. The percentage of germinating spores was determined by counting the proportion of spores (from 100 spores) with germ tubes exceeding 6μm

2.1.11 Harvesting of mycelia

Fungal mycelia were harvested in two ways for qualitative or quantitative analysis. For qualitative work mycelia were harvested by filtering culture media through two layers of muslin, and washing with deionized water (3x5ml). If it was not possible to filter the organism then mycelia was harvested by centrifugation in a MSE bench centrifuge (3,300g) for 10 min. For quantitative analysis of steroid in media or mycelial fractions, mycelia were harvested by filtering culture medium onto the sintered glass filters of gouch crucibles (No.2), under vacuum. The flasks and mycelia were washed with deionized water (3x5ml), mycelia and diluted filtrate (1ml) was then removed and transferred to clean boiling tubes for steroid extraction.

2.1.12 Steroid extraction

Steroid was removed using solvent extraction. The solvent used was dichloromethane (3×5ml). After each addition of dichloromethane samples were vortexed for 10 seconds then allowed to settle. In aqueous samples the organic layer remains at the bottom of the tube with the immiscible aqueous layer on top. The organic layer was removed, and run down a column (0.5 mm × 50 mm) of anhydrous sodium sulphate to remove any remaining water or aqueous contaminants. Samples were dried in a Gallenkamp vacuum oven overnight at room temperature using a dry-ice solvent trap. Dried samples were resuspended in dichloromethane (3×0.7ml) and transferred to $^1/_2$ dram vials and redried in a Gallenkamp vacuum oven using a liquid nitrogen solvent trap for 4 hr. Dried samples were then used quantatively for high performance liquid chromatography (hplc) analysis and qualitatively for thin layer chromatography (tlc).

2.1.13 High performance liquid chromatography (hplc)

Hplc was used for steroid separation and detection. Steroid molecules absorb light in the ultra-violet region of the spectrum, due to their double bond ketone conjugate which is present in the A ring of the steroid molecule. An LDC Milton Roy detector was used with a calormetric IIIG pump, spectromonitor III variable wavelength detector and C1-10 integrator. The mobile phase used was 97 dichloromethane : 3 methanol (Fisons hplc grade). This was filtered through a sintered Buchner filter (No.4) and degassed under vacuum before use. This solvent system was chosen as the solubility of cortexolone is much greater in this system than in MeOH/water systems. A Partisil 5μ, 25 cm female main column with a Partisil 5μ, 5 cm female guard column was used for separation of the steroid molecules. When not in use, columns were stored in hexane (Fisons hplc grade).

2.1.14 Hplc operating conditions at room temperature

Flow rate = 2ml/min
Wavelength (λ) = 254 nm (this coincides with the λ max of cortexolone in this system (248-250 nm))
Pressure = 1380 - 1430 psi
Chart speed of integrator = 3 mm/min
Minimum area = 500 (arbitary units)

Samples were resuspended in 0.5 or 1ml of running solvent before analysis. 40µl of sample was injected into a 20µl hplc loop using a hplc needle. The concentration of cortexolone, 11α C and 11β C were found to be linear between 0-3 mM in this system. The parahydroxybenzoic acid methyl ester standard was linear from 0-0.5µM. Sample concentrations were determined by reference to standard curves. A control sample in the absence of cortexolone for organisms from culture collections was analysed. No molecules co-migrating with the transformation products was found.

2.1.15 Thin layer chromatography (t.l.c)

Steroid molecules can be separated on a solid phase support of charged silica, by use of a mobile liquid phase, on the basis of their polarity. Several running solvents were tested to find the ideal solvent system for separation of cortexolone and some of its selected hydroxylated derivatives (11β-/11α-). 100ml of solvent mixture was added to a glass tank (30 cm × 27 cm × 9.5 cm) and left to equilibrate for 2-3 hr, using Whatman filterpaper (18.5 cm; No.6) to maintain a saturated atmosphere. Tlc Kieselgel fluorescent (254 nm) plates (20 cm × 20 cm × 0.25 mm; Merck) were prepared before use, by removing 5 mm from the edge of each plate. A pencil line was drawn 20 mm from the bottom of the plate to load standards. The plates were activated by heating at 105°C for 10 min before use. 10 µg of standards were spotted equidistantly onto the plate

with a 10µl Hamilton syringe. Plates were run in the given solvent system until the solvent was 25 mm from the top of the plate. After running, the plates were examined using a fixed wavelength (254 nm), ultra violet light box and the positions of the spots were marked with a pencil. The most appropriate solvent system proved to be dichloromethane:acetone (7:3). Samples obtained from steroid fermentations were resuspended in 100µl of dichloromethane before being spotted onto plates. The atmosphere in the glass tank was saturated for 2-3 hr. Samples (10µl) were spotted equidistantly with 10µg of standard. Plates were run for 45-60 min. It was found that ultra violet illumination of the plates (254 nm) produced a clearer visualization of the spots than iodine staining. The number of steroid molecules seen in each case was the same. Suitable tlc protocols and solvent systems had to be devised during this project. They will be described under the results section. Tlc plates were photographed using a manually operated mounted camera and polaroid 665 film. The plates were exposed to ultra violet light (254 nm) for 1 min in complete darkness for photographs to be taken. Control spore germination experiments in the absence of cortexolone for *Absidia spinosa*, *Cunninghamella elegans and Cunninghamella blakesleeana*, have been performed and are presented in Chapter 4.

2.1.16 Biomass determination

Mycelia harvested from shake flasks were resuspended in 20ml of deionized water. Mycelia were filtered onto preweighed Whatman filter paper (9cm, No.1) by applying a vacuum. Fungal biomass was left to dry at 105°C for 18 hr in a Gallenkamp hot box. Dried biomass was then transferred to a desiccator to cool, before being weighed.

When culture medium (1ml) was removed for analysis, the sample was centrifuged (11,600 g) in a MSE microcentaur for 5 min. The supernatant was removed, and the pellet washed with methanol (1ml), to remove steroid

molecules. The mycelia were then centrifuged (11,600 g for 5 min). The pellet was resuspended in deionized water (1ml), centrifuged (11,600 g, 5 min) and the supernatant removed. The pellet was resuspended in deionized water, and transferred to preweighed aluminium test tube caps. Biomass was determined as previously described.

2.1.17 Glucose determination

Culture medium (1ml) was centrifuged on a MSE microcentaur bench centrifuge (11,600 g, 5 min) and the supernatant removed and stored in 1.5ml Eppendorfs at -20°C. Glucose was determined by the GOD-Perid method using a glucose assay kit (Boehringer Mannheim GmbH Diagnostica).

2.2.1 The use of germinating spores and vegetative cells to study the hydroxylation of cortexolone by selected organisms

The organisms used (*Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans*) were chosen for further study as they were easy to handle, and grew filamentously (*Absidia spinosa*) or as very fine pellets (*Cunninghamella blakesleeana* and *Cunninghamella elegans*) in shake flask culture. *Absidia spinosa* and the Cunninghamellaceae produce more 11βC and 11αC. *Cunninghamella* species are also well documented in the literature for their ability to transform cortexolone.

2.3.1 The effect of altering selective parameters on the hydroxylation of cortexolone by whole cells of *Absidia spinosa*

Absidia spinosa was chosen for further study, as it grows quickly and filamentously in liquid culture. It also exhibited secondary transformation of its primary products in batch culture decline phase. This was not seen in *Cunninghamella elegans* or *Cunninghamella blakesleeana*. The protocol,

unless otherwise stated, involved *Absidia spinosa* grown in a steroid-free medium at 30°C, 200 rpm, for 8 hr on a Gallenkamp orbital shaker. After this time 97% of spores had germinated. 10 mg of cortexolone in 500µl of methanol was added (giving 0.39 mg/ml (1.1 mM) in the medium), and the transformation of cortexolone followed periodically.

2.3.2 11β C transformation in vegetative cells

The experimental procedure used was the same as that previously described in cortexolone batch growth experiments. 11βC, a product of cortexolone transformation, was used as substrate (0.4 mg/ml (1.1 mM) in the medium). This was to determine if an epimerase was present which could convert 11βC to 11αC, and to investigate if further transformation of 11βC was possible.

2.3.3 Cortexolone transformation in decline phase

The protocol followed was the same as that previously described in cortexolone batch growth experiments. *Absidia spinosa* was grown at 200 rpm, 30°C for 48hr on a Gallenkamp orbital shaker before cortexolone addition. This was to investigate if cortexolone could be transformed in decline phase, and what products were produced. It was decided to investigate the effect of cortexolone concentration, pH and temperature, on cortexolone bioconversion by *Absidia spinosa*.

2.3.4 Effect of cortexolone concentration

The concentrations of cortexolone used were 0.098 mg/ml (0.22 mM), 0.916 mg/ml (0.56 mM), 0.39 mg/ml (1.13mM) and 0.8mg/ml (2.26 mM) in the medium.

2.3.5 Effect of pH

Absidia spinosa was grown in 75 mM phosphate buffer pH 6 and 7 and 100 mM phosphate/citrate buffer pH 3,4,5,6 and 7.

2.3.6 Effect of temperature

All shake flasks were maintained at 200 rpm, 30°C for 8 hr in 100 mM phosphate/citrate buffer pH 6, cortexolone was then added and the flasks transferred to the desired temperature (20,25,30,35 and 40°C) for 4 hr.

2.3.7 The effect of antifungal inhibitors on cortexolone hydroxylation

The inhibitors used were: ketoconazole, metyrapone, SKF-525A and miconazole (obtained from Pfizer) at a concentration of 1×10^{-5} M in the medium. Inhibitors were added in 100µl of methanol at 8 hr.

2.3.8 The effect of cycloheximide on cortexolone hydroxylation

Cycloheximide (Sigma) was added in 100µl of methanol at different concentrations in the medium (1000,500,100,10,1,0.1 µg/ml) at 8 hr.

2.3.9 Variation in the time of cortexolone addition and different nitrogen sources on cortexolone hydroxylation in defined medium (B)

It was decided to investigate if cortexolone transformation was similar in defined and complex media, and to compare the product profiles produced. *Absidia spinosa* was inoculated and grown as previously stated. Cortexolone was added 8 or 12 hr after growth had started. *Absidia spinosa* was grown on three different readily available nitrogen sources of equivalent molar concentration (47 mM):NH_4Cl(2.5 g/l), Urea (2.8 g/l) and $NaNO_3$ (3.9 g/l), to

investigate if the variation of the nitrogen sources had any effect on cortexolone transformation.

2.4.1 Growth of *A.spinosa* on defined media in a fermenter

Fermentation in an LH 2000 I series fermenter (3l vessel) was undertaken to investigate the transformation profiles and the ratio of products produced in a controlled system using a defined medium.

2.4.2 Inoculum preparation

Spores (40ml) 10^7 spores/ml) were added to 160ml of medium A in a sterile 2l-Erlenmeyer flask, and shaken at 200 rpm, 30°C for 8 hr. The cells were then centrifuged in a MSE bench centrifuge (3,300 g, 10 min) in four sterile 50ml Nunc centrifuge tubes. The supernatant was decanted and the cells washed with sterile distilled water (4x50ml) containing 0.01% (v/v) Tween 80. The cells were then centrifuged (3,300 g, 10 min) and resuspended in 4×50ml sterile distilled water(and 0.01% (v/v) Tween 80), recentrifuged (3,300 g 10 min), and resuspended in sterile distilled water (400ml) containing 0.01% (v/v) Tween 80. This was used to inoculate the fermenter.

2.4.3 Medium sterilization

The fermenter was sterilized *in situ* using a 1 kw heating rod. The fermentation vessel was sterilized at 121°C for 1 hr with 2l of distilled water. When the temperature inside the vessel had decreased to 105°C, steam was released and air bubbled into the fermenter. When the water had cooled to ambient temperature it was drained and the fermenter filled with defined medium B.

2.4.4 Fermenter operating conditions

A.spinosa was grown at 30°C, 1000 rpm (using two paddles 7 cm apart) at an initial dissolved oxygen tension of 100% air saturation. A constant temperature was maintained by the use of a 250w heater and a chiller which responded to any change in temperature. The pH was maintained at 5.0 using 6M hydrochloric acid and 1M sodium hydroxide.

2.4.5 Sampling

Samples (24ml) were removed periodically for glucose, ammonium choride, phosphate, steroid and biomass determination by drawing medium into a universal vial using a syringe attached to an acro 50 (0.2 μm) filter unit.

2.4.6 Biomass determination

Culture medium (20ml) was centrifuged at 3,300 g, 10 min in a MSE bench centrifuge. The supernatant was stored at -20°C for glucose, ammonium choride and phosphate analysis. The mycelia were washed with 2ml of MeOH for every 10ml of culture medium. The procedure followed thereafter was the same as that described previously (2.1.16).

2.4.7 Ammonium determination

Nitrogen was supplied as NH_4Cl and was assayed using the indophenol method (Fawcett and Scott 1960). Samples were diluted 1 in 9 and absorbances were read at 600 nm. The standard curve was linear from 1-10 μg of nitrogen using NH_4Cl as standard.

2.4.8 Inorganic phosphate determination

The method used was a modified method of Chen *et al* (1956). The phosphate standard curve was constructed by dissolving 87.8 mg of KH_2PO_4 in distilled water. This was made up to 100ml. 5ml of this solution was then

made up to 100ml to give 0.0439 mg/ml KH_2PO_4. This standard solution contains 1µg of phosphorus per 0.1ml. The standard curve was linear from 1-10 µg of phosphorus. The colour reagent was made up by mixing 3M H_2SO_4 (40ml), 2.5% (w/v) ammonium molybdate (40ml), water (120ml) and 1 g of ascorbic acid (total volume 200ml). This reagent was kept at 4°C and prepared fresh before use. Samples were diluted 800 fold so that they contained 1-10 µg of phosphorus, 4ml of colour reagent was added to 4ml of sample and the solution was mixed by vortexing and incubated at 37°C for 2 hr. The solutions were then cooled to room temperature and absorbances read at 820 nm against a distilled water/reagent blank.

2.4.9 Gas analysis

The percentage carbon dioxide (%CO_2) produced in the fermentation was determined using an infra-red gas analyser (Analytical Development Co.Ltd.). The analyser measures the amount of radiation absorbed by a concentration of CO_2 in the sample cell. For an accurate measurement the cell was full when operated at room temperature and pressure. The reference cell was calibrated using gases which were free from infra-red absorbing molecules such as nitrogen or dry air (CO_2 free). The reference cell was standardized with 1.76% CO_2 in nitrogen (Cryo Service Ltd.), at room temperature and pressure. The analyser output meter is calibrated in terms of gas concentration, but this instrument operates as a comparator. Gases from the LH2000 series I fermenter were passed through silica gel to remove moisture and then into the sample cell. The machine calculates the % CO_2 present in air from the CO_2 standard, to give the % change in CO_2 in the fermentation . The percentage oxygen (% O_2) was determined using an O_2 analyser (Taylor Servomex Oxygen Analyser type OA 184). O_2 is measured because it exhibits a unique paramagnetism. O_2 free nitrogen is introduced into the reference channel to set the zero. A known content of air is then passed through the reference channel, the reference

potentiometer is then adjusted so that it reads the correct O_2 content. The same procedure was used to calibrate the sample channel. A known amount of O_2 is then passed through the reference channel and the alternative channel measures the amount of unknown O_2 from the fermenter, to give the % change in O_2 concentration during the fermentation.

2.5.1 Preparation and assay of cell-free extracts from *A. spinosa*

A biochemical approach to the enzymic system(s) involved in cortexolone transformation enables a direct study of these systems. The preparation of cell-free extracts is the first step in an *in vitro* study, and allows the determination of many parameters (K_m,V_{max}, pH optimum, temperature optimum etc). It also permits the investigation of the effect that exogenous compounds such as steroids, can have on enzyme kinetics.

2.5.2 Culture conditions

The inoculation was prepared as previously described. 250ml flasks were incubated for 8 hr (200 rpm, 30°C) in cortexolone free medium. 10 mg of cortexolone in 500µl of methanol was added aseptically to each flask (0.39 mg/ml or 1.13 mM in medium), for 4 hr. In control experiments *A.spinosa* was incubated for 4 hr in the absence of cortexolone.

2.5.3 Harvesting Mycelia

Mycelia were separated from the medium by filtering onto two layers of muslin and washing with 0.9% (w/v) NaCl to remove excess medium and steroid residues. The mycelia were then blotted dry and the wet weight noted, so that biomass yield could be determined.

The mycelia were transferred to a pestle and mortar kept at -20°C overnight. Mycelia were crushed to a fine powder by addition of liquid N_2 and

precooled ballotini beads (2g of ballotini beads for each flask used). When the powder had thawed to a paste it was again ground. The fine paste was transferred to a precooled measuring cylinder at 4°C. 10ml of 0.25 M Sucrose, 10% glycerol, 1 mM EDTA and 1mM DTT in 0.1 M phosphate buffer (pH 7.4, at 4°C) was added for each flask used. The volume was noted. The mycelial fraction was then filtered onto two layers of muslin. The filtrate was centrifuged at 4°C (1090g) in a Beckman J2-21 centrifuge with a JA-20, (8×50) rotor for 20 min. The supernatant was removed and centrifuged at 4°C (10,000g) in a Beckman J2-21 centrifuge with a JA-20, (8×50) rotor for 20 min. The microsomal fraction was harvested by centrifuging the supernatant at 4°C (105,000 g) in a MSE prepspin 50 ultracentrifuge with a 8 × 50 rotor for 1 hr. The supernatant was recentrifuged at 4°C (105,000 g) for 1 hr to maximize microsomal yield. Samples were taken from all fractions for analysis. Mitochondrial and microsomal pellets were transferred into 0.25 M sucrose, 10% glycerol, 1 mM EDTA and 1mM DTT in 0.1 M phosphate buffer (pH 7.4 at 4°C) using a spatula. The pellet was resuspended using a teflon glass Potter-Elvehjem hand held homogeniser. Samples were assayed before aliquoting into 1.5ml tubes and frozen in liquid nitrogen. Frozen samples were stored at -80°C.

2.5.4 Preparation of dextran coated charcoal (DCC)

Dextran coated charcoal (DCC) was used to removed endogenous steroid in some fractions before assaying. 25ml of 0.1 M phosphate buffered sucrose (PBS) pH 7.4 was added to 0.25 g of Nont A charcoal activated (Sigma, 250-350 mesh) and 0.025g dextran (40,000, Pharmacia). This was stirred overnight, centrifuged (2000 rpm, 10 min), and the supernatant decanted. Cooled pellets were resuspended in phosphate buffered saline (100ml). Endogenous steroid was removed by adding 500µl of ice-cold DCC (while stirring) to 500µl samples. Samples were vortexed and left for 15 min on ice. The DCC was then

centrifuged in a MSE microcentaur at full speed. (11,600 g, 5 min). 500µl of supernatant was then removed, taking care not to disturb the charcoal pellet. Aliquots (400µl) were then used for incubations.

2.5.5 Assaying for hydroxylating activity

Fractions were assayed for steroid hydroxylating ability by the use of a NADPH regenerating system. This involved the use of isocitrate dehydrogenase which uses isocitrate as substrate, and $NADP^+$ as cofactor. This system is used as standard when assaying mammalian microsomes for steroid hydroxylating ability. The fractions assayed using this system were; homogenate, post 1090g supernatant, post 10,000g supernatant (post mitochondrial supernatant), 10,000g pellet, 105,000g supernatant (cytosol) and 105,000 g microsomal rich pellet. Each incubation was started by the addition of 500µl of each fraction. These were then vortexed and transferred to a 37°C water bath for 1 hr. At the end of the incubation period the reaction was stopped by the addition of 5ml of dichloromethane, the mixture was vortexed and left to settle. Steroid was removed as previously described.

Concentration of compound in reaction mixture	Volume(µl) + regenerating system	- regenerating system	control
0.5 mM $NADP^+$	25	0	0
5 mM Isocitrate	25	0	0
02iu Isocitrate dehydrogenase	8.1	0	0
5 mM $MgCl_2$	5	0	0
5 mM $MnCl_2$	5	0	0
0.1 mg/ml Cortexolone * +[^{3}H]Cortexolone (0.66µCi)	20	20	0
Fraction	500	500	500
0.1 M phosphate buffer	411.9	480	500

* The [^{3}H] cortexolone (Dupont) used had a specific activity of 47.5 Ci/m mol. Each incubation was performed in duplicate.

2.5.6 [^{3}H] Steroid separation using t.l.c

Radioactive cortexolone was used to detect any steroid conversion that might take place. Hplc was not used as it was found to be not as sensitive as radio assay using [^{3}H] cortexolone for detection of products, because of the small concentrations of products involved. The plates used were plastic backed silica gel plates (20 cm × 20 cm × 0.25 mm from Sigma), which were cut into rectangular blocks (3 × 25 mm) for analysis. Each sample was spotted along a 15 mm line. A 10 mm gap was used to separate each sample. After separation of the steroid molecules, the plates were cut into 25 mm strips. These were then cut every 3mm and added to scintillation vials for counting. The scintillant used was a toluene based Omnifluor (New England Nuclear). Samples were left for at least 3 hr to allow equilibration before being counted on a Beckman LS7800 scintillation counter.

2.5.7 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins in cell-free extracts of *A.spinosa*, which were exposed or not exposed to cortexolone, to observe if any gross differences could be found between the protein profiles obtained from mycelia subjected to these two treatments. The method used was a modification of Laemmli *et al* (1970). 10% acrylamide running gels were routinely used. A 40% stock solution composed of; 40% (w/v) acrylamide, 1% (w/v) bisacrylamide (Sigma), 375 mM Tris-HCl pH 8.8 and 0.1% (w/v) SDS. The gel mixture was degassed in a 250ml Buchner flask under vacuum. 0.2ml of 10% (w/v) ammonium persulphate and 30µl of N, N, N^1, N^1-tetramethylethylenediamine (TEMED) was added for every 40ml of gel mixture. The solution was poured between sealed, grease-free glass plates, separated by 1.5 mm thick teflon spacers, and overlaid with water saturated isobutanol to give a level surface to

the gel. The gel was left to polymerise at room temperature for 30-60 min. When the running gel had set the water saturated isobutanol was decanted before adding the stacking gel mixture, which was composed of 12.5% (w/v) acrylamide. This was obtained from a 40% stock solution composed of 40% (w/v) acrylamide, 1% (w/v) bisacrylamide, 12.5 mM Tris-HCl, pH 6.8 and 0.1% (w/v) SDS. This was degassed and mixed with 0.1ml 10% (w/v) ammonium persulphate and 16µl of TEMED per 20ml of gel mixture. The stacking gel mixture was poured over the running gel. A teflon comb was inserted to allow the formation of sample wells. The stacking gel was left to polymerise at room temperature for 30 min. Gels were transferred to the running apparatus (BRL model V-1G vertical gel electrophoresis system), which was filled with running buffer. This buffer was made up at five times working strength (125 mM Tris, 960 mM glycerine and 0.05% (w/v) SDS). Protein samples containing 2 µg and 40 µg of protein were denatured in five times concentration sample buffer (125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue and 0.4% (w/v) SDS), by boiling in a waterbath for 2 min. All the sample was loaded into the gel wells and gels were separated at 3.5V cm^{-1}. Gels were run until the dye front had just reached the bottom of the gel. The gels were then stained for 30 min in a solution of methanol:water:acetic acid (40:4:15) containing 0.1% (w/v) coomassie blue (PAGE Blue 83; BDH). Gels were destained in methanol:water:acetic acid (10:82.5:7.5). The gels were then stained with silver using a modified method of Merril *et al* (1979). The gel was soaked in 50% methanol overnight in a gel box, and then washed several times in distilled water. The silver stain was prepared by mixing two solutions: solution A containing 0.8 g of silver nitrate dissolved in 4ml distilled water and solution B containing 21ml of 0.36% sodium hydroxide and 1.4ml of 14.8 M ammonium hydroxide.

Solution A was added stepwise into solution B with constant stirring. The mixture was made up to 100ml with water and used immediately. The stain was left for 20 min the gels were then washed for 5 min in distilled water. Gels were developed for 10-15 min in 2.5ml of 1% (w/v) citric acid and 0.25ml of 38% (w/v) formaldehyde made up to 500ml with water (prepared fresh before use). The gel was washed with water and 50% (v/v) methanol or 50% (v/v) methanol plus 10% (v/v) acetic acid to stop any further stain development. The gel was destained using Kodak rapid fix diluted five times in water with 10% (v/v) methanol followed by 50% (v/v) methanol.

2.5.8 Protein determination

Protein concentration was determined using the method of Lowry *et al* (1951). The absorbance of samples was measured at 500 nm, and found to be linear from 10-100 μg of protein, using bovine serum albumin as standard.

2.5.9 CO-reduced difference spectra

This was undertaken to determine if cytochrome-P450 was present in any of the cell-free extracts obtained from *A.spinosa*. Samples from each fraction (1ml) were added to reference and sample plastic cuvettes which contained between 3-8 mg/ml of protein. A base line was established using a split beam spectrophotometer (Applied Photophysics Ltd.). The Photophysics spectrophotometer was computer controlled. Samples were scanned between 350-650 nm using a set programme at medium scan speed. The protocol used for CO-reduced difference spectra was the same as that of Omura and Sato (1964). H_2O_2 was also used in place of CO to detect cytochrome peaks.

2.6.1 Immobilization of *Mucor genevensis* and *Aspergillus niger* in Chromosorb W beads.

Aspergillus niger was used to investigate the feasibility of immobilizing fungal spores on the celite preparation Chromosorb W. *A.niger* was chosen as a model organism as it produces large numbers of distinct black spores that are spherical and ideal for penetrating deep into the bead structure. *A.niger* is a fast growing filamentous fungus that is able to transform steroids such as progesterone to 11αPG. *Mucor genevensis* spores were compared to *A.niger* for immobilization to Chromosorb W beads.

2.6.2 Comparison of a static and shaking incubation phase for immobilization

Two methods of immobilization were compared.

(i) A static incubation of fungal spores in distilled water containing 0.5% (v/v) Tween 80 in the presence of Chromosorb W beads (Gbewonyo *et al* 1983).

(ii) Shaking Chromosorb W beads in the presence of fungal spores in medium A.

The effectiveness of spore entrapment in Chromosorb W beads which had been either sterilized by dry heat or steam was also investigated.

2.6.3 Spore preparation

Sterile distilled water (100ml) or medium containing Tween 80 (0.5% v/v) was added to each 2l-Erlenmeyer flask containing *M.genevensis* or *A.niger*. Glass beads (3mm diameter) were used to dislodge spores by gentle shaking. The spore suspension was then filtered through two layers of muslin to remove any large vegetative hyphae that might be present.

2.6.4 Chromosorb W bead sizing

Chromosorb W beads (Jones Chromotography) were available in four different mesh sizes, 30-60, 60-80, 80-100 and 100-120. Bead size ranges were obtained by sizing them under a light microscope using a graduated eyepiece. Chromosorb W 30-60 mesh size was used for future experiments as this contained the largest bead sizes which were presumed to be more useful for immobilization of fungal spores. There were approximately 1×10^5 beads per gram.

2.6.5 Immobilization of fungal spores in Chromosorb W beads (30-60)

Different sterilization treatments of Chromosorb W beads were investigated to observe which approach was appropriate for immobilization of fungal spores. 250ml flasks containing 1g of Chromosorb W beads were sterilized by either: (1) Steam in a pressure cooker at 10 psi for 15 min; (2) Dry heat at 400°C for 3 hr. Control flasks contained non-heat treated Chromosorb W beads. A comparison was then made between Gbewonyo and Wang's (1983) static incubation phase, and the use of a shaking incubation phase.

In the static method 5ml of spore suspension (10^7 spores/ml) in sterile distilled water containing 0.5% (v/v) Tween 80 was added. The flasks were allowed to stand for different times, the spore suspension decanted, and the beads washed with two volumes of sterile distilled water containing 0.5% (v/v) Tween 80. Sterile medium A (25ml) was added to start the fermentation.

In the shaking incubation treatment 5ml of spore suspension containing 0.5% (v/v) Tween 80 and 20ml of medium A were added to Chromosorb W. All flasks were then incubated on a Gallenkamp orbital shaker (200 rpm, 30°C). Samples were taken periodically by removing whole flasks. Medium was decanted and the beads washed with three volumes of sterile distilled water containing 0.5% (v/v) Tween 80. Free fungal spores that were not entrapped in

the beads were counted using a Weber haemocytometer and the percentage of spores entrapped in the beads calculated.

2.6.6 Observation of *A.niger* growth in Chromosorb W beads by scanning electron microscopy and light microscopy

Samples (1ml) were removed at intervals using sterile 1ml pipettes and transferred to 1.5ml Eppendorfs. The Chromosorb W beads were left to settle. Medium was removed using a Pasteur pipette and the beads were washed with deionized water (3x1ml). Washed samples were prepared for the scanning electron microscope by fixing in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7). Fixed samples were dehydrated in a graded acetone series (25%, 50%, 75%, 100% for 15 min each). Samples in 100% acetone were subjected to three 15 min acetone washes to remove all the water before being transferred to a Polaron E3000 critical point drying apparatus, in cigarette paper full of acetone, for critical point drying. Dried samples were stuck to specimen stubs with double sided sellotape and transferred to a Polaron E500 diode sputtering system, and coated with gold for 5 min at 1.5 kV. Scanning electron microscopy was carried out using a Cambridge Stereoscan 600. Photographs were taken at maximum contrast and reduced gain at a scan speed of 100 frames/second using 35 mm Pan F film. Fixed samples were viewed using a Zeiss microscope and photographs were taken using 35 mm Pan F Film.

2.6.7 Biomass determination of free and immobilized cells of *A.niger*

The flask contents were filtered under vacuum into a Buchner filter, onto preweighed Whatman filterpaper (9 cm, No.1) and washed with deionized water (20ml). Fungal biomass and bead weight were determined as previously described.

2.6.8 Investigation of increasing spore loading and % entrapment in Chromosorb W beads (30-60) using *A.niger*

A.niger was used at four different spore concentrations, 1.2×10^8, 2.4×10^7, 1.1×10^7 and 1.2×10^6 spores/ml, as described previously, flasks were shaken on a Gallenkamp orbital shaker (200 rpm, 30°C) for 3 hr. A sample of culture medium (1ml) was removed and the number of free spores present was determined as described previously. The growth of *A.niger* was observed at 48 hr.

Cortexolone transformation (in mol/g) reflects the amount of cortexolone transformed per gram dry fungal weight. This can be seen from graphs showing the amount of cortexolone remaining per gram dry fungal weight (presented graphically as cortexolone present (mol/g) in this thesis). The amount of 11αC and 11βC produced in relation to biomass is referred to as 11αC and 11βC production and is also expressed as mol/g.

The term `primary transformation' used in this thesis refers to the conversion of cortexolone to its monohydroxylated derivatives. The term `secondary transformation' refers to the conversion of the monohydroxylated derivatives to other products detected using tlc and hplc systems.

2.7.1 Mass spectrometry of some steroid samples

A. spinosa was grown in medium A at 200 rpm, 30°C for 8 hr on a Gallenkamp orbital shaker. 1.1mM cortexolone in 0.5ml MeOH was added and growth continued for 4 hr. Flasks were removed and whole culture medium was extracted three times with dichloromethane (100ml). Steroid molecules were separated twice in dichloromethane : acetone (7:3) on plastic backed fluorescent Kieselgel plates (60). Kieselgel plates were cut into strips and the

silica transferred onto aluminium foil, and then to beakers. Steroid molecules were removed from the silica using dichloromethane : acetone (50:50 (50ml). Samples were filtered through glass wool and dried using nitrogen gas. Samples were resuspended in a few drops of dichloromethane: acetone (50:50) and analysed on a Kratos CONCEPT-32 mass spectrometer using electron impact spectroscopy.

CHAPTER 3

SCREENING OF MICROORGANISMS FOR THEIR ABILITY TO HYDROXYLATE CORTEXOLONE AND PROGESTERONE

RESULTS

3.1.1 The development of a t.l.c system for separation of progestagens and corticosteroids

Fifteen solvent systems were tested for the separation of corticosteroids (Table 3.1). The Rf values that were obtained are presented in Appendix 1. *P.articulosus* was used to compare migration of cortexolone and its transformation products with authentic steroid standards in different solvent systems (Appendix 1). On the basis of these results the solvent systems used could be divided into three groups; A, B and C. Group A solvent mixtures produced a clear, distinct separation of the steroid samples. Group B solvent mixtures separated the steroid samples, but the difference in resolution between spots was relatively small compared to group A. Group C solvents did not resolve all the steroid samples. The best solvent systems were those containing high levels of polar halogenated hydrocarbons, (chloroform, dichloromethane), aromatic toluene and smaller amounts of acetone and ethanol (Table 3.1, Group A). The dichloromethane:acetone mixture (7:3) proved to be the most effective at separating the steroid standards:cortexolone (Rf = 0.59), 11βC (Rf = 0.28), 11αC (Rf = 0.10), progesterone (Rf = 0.88), 11β PG (Rf = 0.70) and 11αPG (Rf = 0.47). This solvent system also allowed clear visualization of the steroid spots under UV light (254 nm).

3.1.2 Recovery of cortexolone from the medium

In trials to determine the efficiency of cortexolone extraction from samples of medium, it was found that cortexolone could be extracted very efficiently using dichloromethane. After 1, 2 and 3 solvent extractions, 84.9% ±

Table 3.1: Comparison of solvent systems for separation of corticosteroids

Group A	
Dichloromethane:Acetone	(7:3)
Chloroform:Acetone	(7:3)
Chloroform:Acetone	(8:2)
Dichloromethane:Chloroform:Acetone	(4:4:3)
Toluene:Ethanol	(95:5)
Dichloromethane:Ethanol	(20:1)
Group B	
Chloroform:n.Propanol	(7:3)
Diethylether:n.Propanol	(7:3)
Toluene:Methanol	(7.5:2.5)
Diethylether:Ethanol	(7:3)
Diethylether:Methanol	(7:3)
Group C	
Chloroform:Ethanol	(7:3)
Chloroform:Methanol	(7:3)
Methanol:Water	(7.5:2.5)
Dichloromethane:Toluene	(7:3)

The above solvent systems were run for 45-60 min, in a solvent saturated glass tank on fluorescent silica gel plates (254nm). The corticosteroid standards used were cortexolone, 11βC and 11αC. Group A solvent mixtures produced a clear, distinct separation of the samples Group B solvent mixtures produced small resolution of the spots, and Group C did not resolve all the steroid samples.

Table 3.2 Cortexolone bioconversion to various products by isolates from *Agave mexicana*.

Number of isolate	Identification of isolate	No. of products detected	Product co-migrating with 11βC	Product co-migrating with 11αC
840601e	Aureobasidium pullulans	3		+
8406205e	Torula Sp	2		
8406221e	Phoma Sp	2		
850101i	Penicillium Sp	2		
850102i	Cladosporium Sp	2		+
85)109i	Penicillium Sp	2		
850111i	Cladosporium Sp	2		+
860102e	Cladosporium chlorocephalum	5	+	+
860106e	Aureobasidium pullulans	3		
8606107e	Aureobasidium pullulans	1		
860108e	Cladosporium cladosporioides	2		
860110e	Aureobasidium pullulans	2		
860112e	Cladosporium sp	1		
860113e	Not identified	4		
860115e	Penicillium sp	4		
860118e	Alternaria sp	2		
860120e	Phoma sp	2		
860112i	Penicillium sp	4		
860123i	Penicillium sp	1		
860124i	Penicillium sp	3		
860215e	Coelomycete sp	2		
860216e	Not identified	1		
860217e	Not identified	4		+
860218e	Not identified	5		+
860219e	Aureobasidium pullulans	6	+	+
860220e	Aureobasidium pullulans	1		
860221e	Not identified	2		+
86022e	Cladosporium chlorocephalum	4		+
860223e	Cladosporium chlorocephalum	6	+	+
860224e	Coelomycete sp	3		
860225e	Penicillium sp	2		

Table 3.2 (Continued)

Number of isolate	Identification of isolate	No. of products detected	Product co-migrating with 11βC	Product co-migrating with 11αC
860226e	Coelomycete sp	5		
860227e	Coelomycete sp	5		+
860228e	Coelomycete sp	1		
860229e	Coelomycete sp	1		
860230e	Coelomycete sp	4		
860231e	Aureobasidium pullulans	2		
860232e	Coelomycete sp	2		
860233e	Phoma herbarum	5		+
860234e	Cladosporium chlorocephalum	3		+
860235e	Cladosporium chlorocephalum	2		
860236e	Aureobasidium pullulans	2		
860237e	Aureobasidium pullulans	3		+
860201i	Penicillium sp	2		
860202i	Penicillium sp	1		
860203i	Penicillium sp	2		
860204i	Penicillium sp	3		
860205i	Penicillium sp	2		
860206i	Penicillium sp	2		
860208i	Cladosporium chlorocephalum	6	+	+
860209i	Penicillium sp	1		
862011i	Penicillium sp	3		+
860213i	Aureobasidium pullulans	2		
860301e	Phoma sp	2		
860302e	Alternaria sp	1		
860304e	Alternaria sp	2		
860305e	Penicillium sp	2		
860306e	Aureobasidium pullulans	2		
860307e	Cladosporium sp	2		
860210e	Not identified	2		
860311e	Alternaria sp	5		+
860312e	Not identified	6	+	+
860314e	Not identified	3		+

Table 3.2 (Continued)

Number of isolate	Identification of isolate	No. of products detected	Product co-migrating with 11βC	Product co-migrating with 11αC
860315e	Penicillium sp	6		+
860316e	Aureobasidium pullulans	2		
860317e	Alternaria sp	4		+
860319e	Aureobasidium pullulans	2		
860301i	Aspergillus sp	3		
860302i	Penicillium sp	4		+
860303i	Phoma sp	4		+
860304i	Phoma sp	5		+
860305i	Phoma sp	5		+
860306i	Not identified	6	+	+
860307i	Penicillium sp	3		+
U2	Not identified	3		+
U3	Not identified	2		
U4	Not identified	1		
U9	Not identified	5	+	+

Isolates were grown at 30°C, 200 r.p.m. in liquid medium A for 72 hr. Cortexolone and its bioconversion products were detected on t.l.c. plates under ultra violet light (254 nm).

Table 3.3: Cortexolone bioconversion by *Agave mexicana* isolates

	Place where isolate was found			Total
	External Surface	Internal Tissues	Rhizosphere Soil	
Number of isolates	67	33	9	109
% that modify cortexolone	76.1	75.8	44.4	73.4%
% 11α Hydroxylating activity	25.4	33.0	22.2	27.5%
% 11β Hydroxylating activity	6.0	6.1	11.1	6.4%
% 11β and 11α Hydroxylating activity	6.0	6.1	11.1	6.4%

Isolates were grown for 72 hr in liquid medium A at 30°C, 200 r.p.m. in the presence of cortexolone (0.39 mg/ml; 1.13 mM) isolates active against cortexolone were separated using t.l.c. and detected by ultra violet light set at 254 nm.

1.9, 96.1% ± 2.5 and 98.4% ± 1.2 respectively of the cortexolone was extracted. Thus three extractions each with 5ml of dichloromethane was adopted as a standard protocol for steroid extraction for every 1ml of medium.

3.1.3 Qualitative screening of fungi obtained from the steroid-rich Portuguese *Agave-mexicana*

Some of the isolates obtained from Portugal were identified by Professor Baptista Ferreira and Margarida Barata (Botanical Garden, University of Lisbon, Portugal) or by Dr.Jeffries and Paul Richmond (University of Kent, Canterbury: Table 3.2). These included *Aureobasidum, Cladosporium, Trichoderma, Torula, Diplodia, Phoma* and *Penicillium* species. Most of the isolates were active modifiers of cortexolone (73.4%: Table 3.3). The isolates that produced a transformation product with a Rf value comparable to the 11βC standard were:-

860102e; 860219e; 860223e; 860208i; 860312e; 860306i and U_9.

The isolates that produced a transformation product with a Rf value comparable to the 11αC standard were:

840601e; 850102i; 850111i; 860102e; 860217e; 860218e; 860219e; 860221e; 860222e; 860223e; 860227e; 860233e; 860234e; 860237e; 860208i; 860210i; 8602211i; 860311e; 860312e; 860314e; 860315e; 860317e; 860302i; 860303i; 860304i; 860305i; 860306i; 860307i; U_2 and U_9.

Finally several isolates appeared to produce transformation products with a Rf value comparable to both 11βC and 11αC, these were:-

860102e; 860219e; 860223e; 860208i; 860312e; 860306i and U_9.

Additional products were formed in all isolates and were present in greater portions than either 11βC or 11αC. This was estimated from the size and density of the spots present on the t.l.c. plates.

The results obtained would suggest that the ability to transform cortexolone was not dependent on whether the organism grows on the leaf surface or interior (Table 3.3). The ratio of isolates that could modify cortexolone was similar in the internal and external categories screened. 76.1% (total 67) of external isolates modified cortexolone and 75.8% (total 33) of internal isolates were able to carry out this biotransformation. 44.4% (total 9) of isolates obtained from *Agave* rhizosphere carried out this modification but only a small number were screened. There does not appear to be a strong correlation between ability to modify externally supplied steroid and the ability to grow in the deeper tissues of *Agave mexicana* (Table 3.3). *Agave mexicana* samples collected in Portugal were shown to have high levels of the sterol hecogenin (Lumley, pers. commun). The frequency of those organisms appearing to exhibit both 11β- and 11α-hydroxylating activity is similar in fungi from the external surface, internal tissues and rhizosphere soil. The 11α-hydroxylating activity was more frequent (27.5%) among the isolates than 11β-hydroxylating activity (6.4%). This was also the case with the external isolates (11α- and 11β-hydroxylating activity was 25.4 and 6.0% respectively), internal (11α- and 11β-hydroxylating activity was 33.0 and 6.1% respectively) and soil isolates (11α- and 11β- activity was 22.2 and 11.1% respectively). The isolates tested that exhibited 11β-hydroxylating activity also possessed 11α-hydroxylating activity (Table 3.3).

3.1.4 Qualitative screening of filamentous fungi for progesterone and cortexolone biotransformation

The range of fungi screened is presented in Table 3.4. All of the *Absidia* species grew filamentously in medium A (Table 3.4). Some fungi grew as coarse pellets (*Cokeromyces recurvatus, Rhizopus stolonifer*) others as very fine pellets (*Cunninghamella elegans, Cunninghamella blakesleeana, Cunninghamella echinulata* and *Phascolomyces articulosus). Curvularia*

Table 3.4: 11α- and 11β- hydroxylating activity of selected fungal species towards progesterone and cortexolone

Organism	11α PG present	11β PG present	11βC present	11αC present	Morpholgy in medium A
C.recurvatus	+	-	-	-	Pellets
M.genevensis	+	-	-	-	Filamentous
R.stolonifer	+	-	-	-	Pellets
M.africana	+	?	-	-	Filamentous
C.elegans	+	-	+	+	Pellets
A.niger	+	-	-	-	Filamentous
T.elegans	+	-	-	-	Filamentous
A.glauca (+)	+	-	+	+	Filamentous
A.glauca (-)	+	-	+	+	Filamentous
A.orchidis	-	-	+	+	Filamentous
A.spinosa	-	-	+	+	Filamentous
P.articulosus	-	-	+	+	Pellets
C.blakesleeana	NT	NT	+	+	Pellets
C.echinulata	NT	NT	+	+	Pellets
C.lunata	NT	NT	+	-	Pellets and Filamentous

All organisms were grown at 30°C, 200 r.p.m. in liquid medium A for 72 hours. Steroid molecules were detected on tlc plates under ultra violiet light at 254 nm.

+ = positive identification

- = negative identification

NT = not tested

? = a trace amount may be present

lunata (CLR56) grew as a mixture of both pellet and mycelium. The remaining fungi tested grew filamentously. It can be seen (Table 3.4) that 11β-/11α-hydroxylation occurs irrespective of fungal morphology.

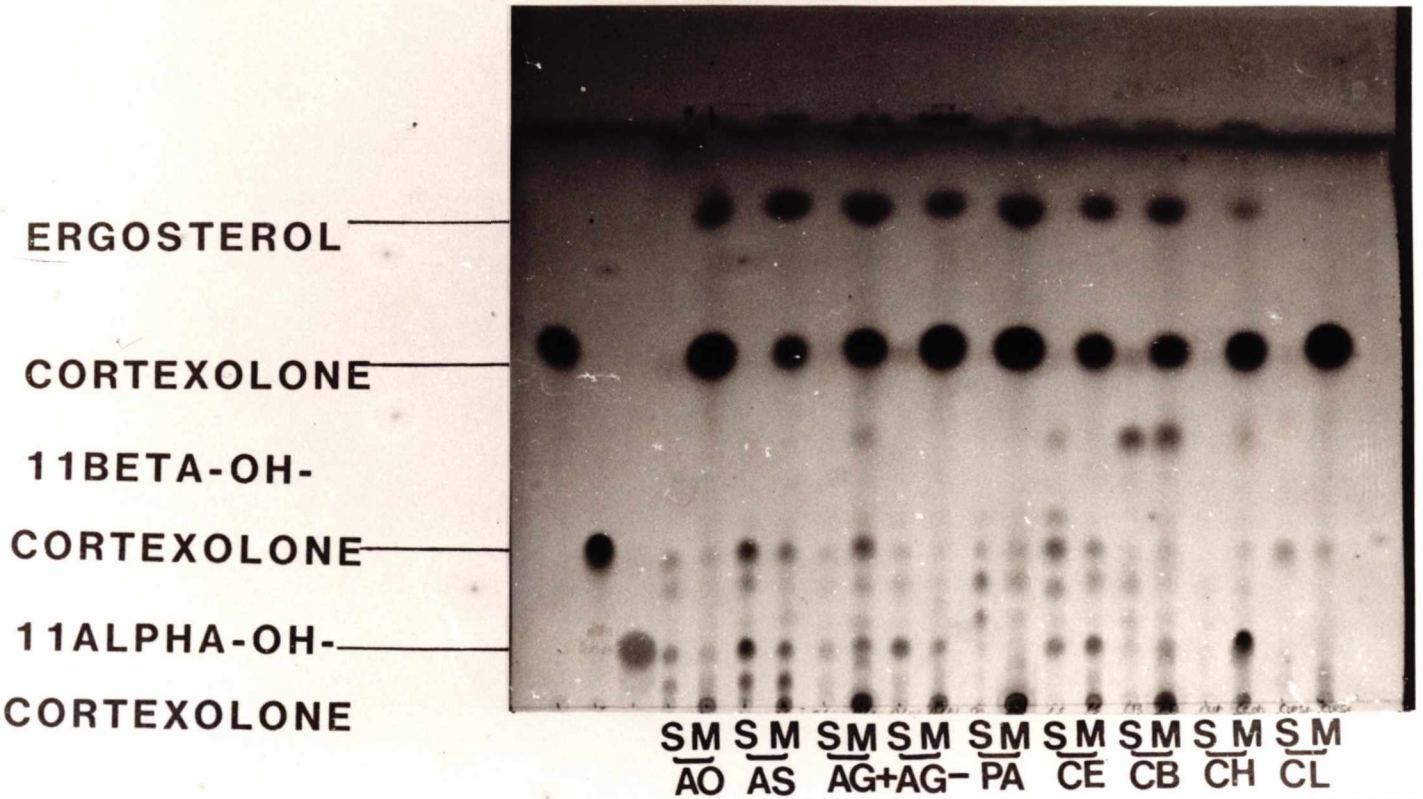
Not all of the fungi tested could produce both 11αPG and 11αC from progesterone and cortexolone respectively (Table 3.4). Only *Cunninghamella elegans, Absidia glauca(+)* and *Absidia glauca(-)* were able to perform both these conversions. They could transform cortexolone to 11βC and 11αC and progesterone to 11αPG but no 11βPG was formed by these organisms.

Some organisms (*Cokeromyces recurvatus, Mucor genevensis, Rhizopus stolonifer, Mycotypha africana, Aspergillus niger* and *Thamnidium elegans*), were only able to convert progesterone to 11αPG but do not form either 11βC or 11αC from cortexolone. Conversely other organisms (*Absidia orchidis, Absidia spinosa* and *Phascolomyces articulosus*) convert cortexolone to 11βC and 11αC but do not form either derivative when progesterone was used. It is apparent that all the organisms able to convert progesterone to 11αPG do not appear to produce the 11β-product. It was also noted that all organisms that produced 11βC can form 11αC except for the mutant strain of *Curvularia lunata* CLR56. This mutant strain was selected for its 11β- hydroxylating activity and possessed no detectable 11α- hydroxylating activity.

Figure 3.1 shows those organisms that modify cortexolone to a variable number of transformation products (Table 3.5). Products were visualized and counted on tlc plates under UV light in complete darkness. Some of these products are present in the media and the mycelial fractions (Fig.3.1). *Cunninghamella elegans* produced the most products (4 or 5) and *Curvularia lunata* the least (2: Table 3.5). All the other *Cunninghamella* and *Absidia* species produce 4 products.

Cortexolone was observed only in the mycelial fractions in all the organisms tested. This does not imply that cortexolone is absent from the medium only that none could be detected using this system. In Fig. 3.1 the

Fig3.1Tlc plate of steroids extracted from a select number of filamentous fungal mycelia and media fractions,and separated using dichloromethane : acetone (7:3)



Organisms were grown at 30°C, 200 r p m in liquid medium A for 72hr. Cortexolone and its bioconversion products were detected on t l c plates under ultra violet light (254nm).
M = mycelium fraction S = medium filtrate
The symbols used represent the organisms tested, these include: AO, Absidia orchidis; AS, Absidia spinosa; AG+, Absidia glauca (+); AG-, Absidia glauca (-); PA, Phascolomyces articulosus; CE, Cunninghamella elegans; CB, Cunninghamella blakesleeana; CH, Cunninghamella echinulata; CL, Curvularia lunata. For each organism a fraction of the medium filtrate (1 ml) was used

Table 3.5: Cortexolone biotransformation products detected from a select number of filamentous fungi

Organism	No.of products visualized on tlc plates
C.blakesleeana	4
C.elegans	5
C.echinulata	4
C.lunata	2
A.spinosa	4
A.orchidis	4
P.articulosus	4
A.glauca (+)	4
A.glauca (–)	4

mycelia had been treated with liquid nitrogen. This allowed the release of all free endogenous sterol as well as cortexolone and its biotransformation products. The only additional compound appearing on the plates from the mycelial fractions appeared to be ergosterol.

3.1.5 Quantitative analysis of cortexolone hydroxylating ability by filamentous fungi

Absidia species were amenable for culturing in medium A under these particular conditions (Table 3.6). The *Absidia* species grew filamentously in medium A (Table 3.4), and produced a higher spore count for inoculation than the other organisms (approximately 10^7 spores/ml: Table 3.6). The Cunninghamellaceae and *Phascolomyces articulosus* grew as fine pellets and were easy to handle. They produced a spore count in the inoculum of approximately 10^5 spores/ml. *Curvularia lunata* grew as large pellets with large filamentous hyphae and was not easy to handle experimentally. This organism produced < 10^4 conidia/ml for inoculum purposes.

By 72 hr the Cunninghamellaceae produced the greatest biomass on average (Table 3.6), whereas *Cunninghamella echinulata* produced 10.78 g/l ± 0.55, *Phascolomyces articulosus* (10.6 g/l ± 0.17), *Absidia orchidis* and *Absidia spinosa* (9.1 g/l ± 0.09 and 9.1 g/l ± 0.17 respectively), *Absidia glauca* (+) and *Absidia glauca* (-) produced 7.5 g/l ± 0.25 and 8.45 g/l ± 0.28 respectively. *Curvularia lunata* produced the least biomass of all the organisms used (6.7 g/l ± 1.41) by this time period (72hr).

The Cunninghamellaceae modify cortexolone more quickly than the other organisms (approximately 92% on average by 72 hr). The other fungi also transformed cortexolone efficiently (approximately 79-84% by 72 hr). It is apparent that these microorganisms are active towards cortexolone over a 72 hr period (Table 3.6). For all organisms tested for 11β- and 11α- hydroxylation of cortexolone, the 11β- form was the major product Table 3.6). The ratio of

Table 3.6: Quantitative assessment of a select number of organisms used for cortexolone biotransformation

Organism	Biomass by 72 hr (g/l)	% loss of Cortexolone by 72 hr	%11βC by 72 hr	% 11αC by 72 hr	Ratio of 11βC:11αC	Handling of the organism	Spore inoculum concentration in 40ml × 10 6
C.blakesleeana	9.77 ±0.11	92.5 ± 1.41	22.8 ± 3.4	1.9 ± 0.9	12:1	GOOD	0.64
C.elegans	9.02 ±0.21	92.7 ± 2.0	23.7 ± 3.7	7.7 ± 0.4	3:1	GOOD	0.78
C.echinulata	10.78 ± 0.55	92.0 ± 0.68	3.3 ± 1.1	0.4 ± 0.2	8:1	GOOD	0.64
C.lunata	6.7 ± 1.41	79.4 ± 0.78	4.7 ± 0.9	0	11β MAJORITY	V.POOR	$<10^{-2}$
P.articulosus	10.6 ± 0.17	84.6 ± 2.05	4.1 ± 0.8	0.08 ± 0.06	50:1	GOOD	0.41
A.spinosa	9.1 ± 0.9	82.27 ± 0.50	8.5 ± 1.4	3.8 ± 0.4	2:1	V.GOOD	12.5
A.orchidis	9.1 ± 0.17	84.23 ± 3.18	7.0 ± 4.6	3.1 ± 0.8	2:1	V.GOOD	17.3
A.glauca (+)	7.5 ± 0.25	80.0 ± 0.85	2.7 ± 0.8	1.3 ± 0.5	2:1	V.GOOD	24.0
A.glauca (-)	8.47 ± 0.28	79.2 ± 1.91	2.1 ± 0.8	0.9 ± 0.5	2:1	V.GOOD	24.0

All organisms were grown at 30°C, 200 rpm for 72 hr in medium A before being harvested . Biomass was left to dry at 105°C for 18 hr before weighing. Cortexolone and its bioconversion products were determined quantitatively using hplc. Spore counts were performed on a haemocytometer. The handling of the organism was judged by growth in liquid culture, and the ease in which biomass was separated from culture medium.

Comparison of cortexolone bioconversion and distribution patterns from mycelia and media fractions, in a select number of filamentous fungi.

fig 3.2 Cortexolone Distribution

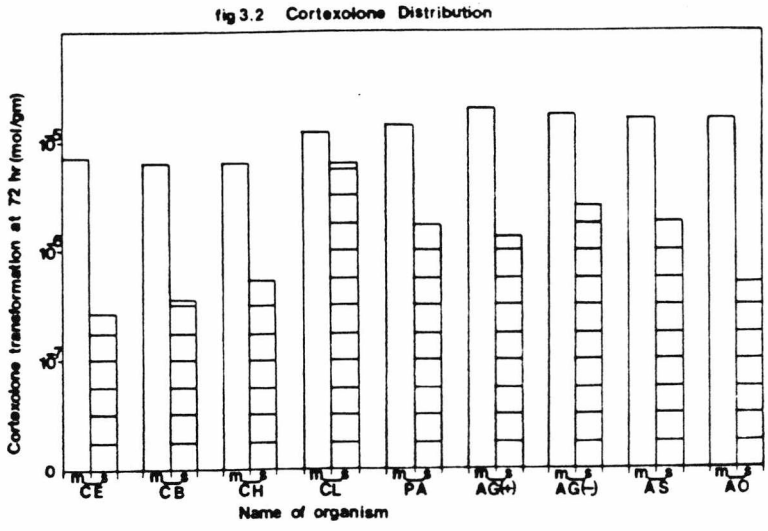


fig 3.3 11βC Distribution

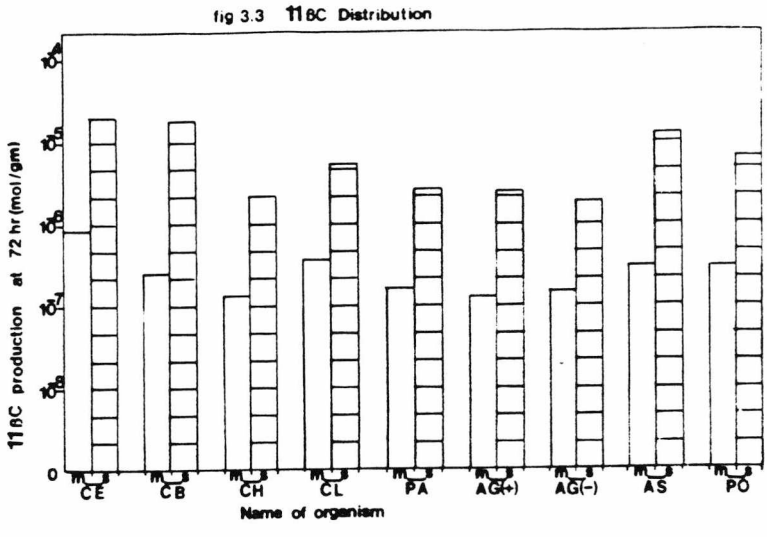
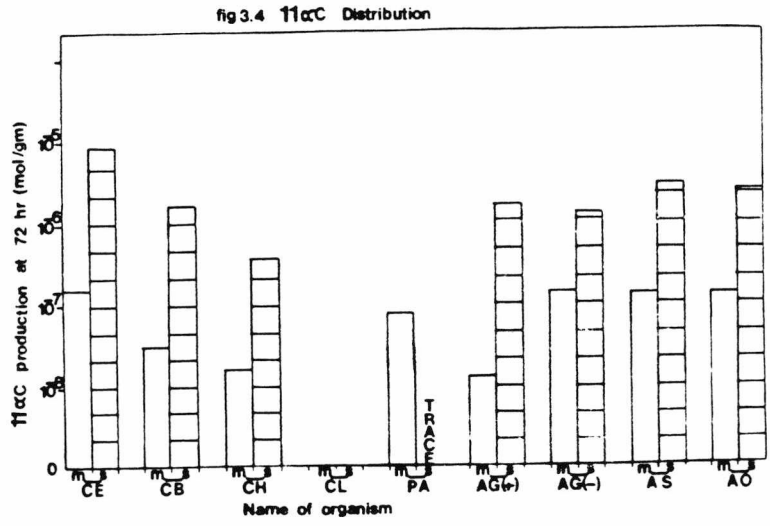
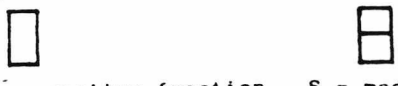


fig 3.4 11αC Distribution



Organisms were grown at 30°C, 200 r.p.m. in liquid medium A for 72hr. Cortexolone and its bioconverstion products were detected and quantified using hplc by their absorbance of light at 254nm.



M = mycelium fraction S = medium filtrate

The symbols used represent the organisms tested, these include: AO, Absidia orchidis; AS, Absidia spinosa; AG+, Absidia glauca (+); AG-, Absidia glauca (-); PA, Phascolomyces rticulosus; CE, Cunninghamella elegans; CB, Cunninghamella blakesleeana; CH, Cunninghamella echinulata; CL, Curvularia lunata.

Table 3.7: A comparison of cortexolone biotransformation patterns from a select number of filamentous fungi.

Organism	cortexolone (mol/gm)	11βC (mol/gm)	11αC (mol/gm)
C.elegans	$9.07.10^{-6} \pm 2.4.10^{-6}$	$2.97.10^{-5} \pm 2.2.10^{-6}$	$9.58.10^{-6} \pm 7.9.10^{-7}$
C.blakesleeana	$8.64.10^{-6} \pm 1.7.10^{-6}$	$2.64.10^{-5} \pm 2.2.10^{-6}$	$2.17.10^{-6} \pm 6.9.10^{-7}$
C.echinulata	$7.92.10^{-6} \pm 5.6.10^{-7}$	$3.46.10^{-6} \pm 6.6.10^{-7}$	$4.19.10^{-7} \pm 1.5.10^{-7}$
C.lunata	$4.07.10^{-5} \pm 7.9.10^{-6}$	$7.92.10^{-6} \pm 3.2.10^{-6}$	0
P.articulosus	$1.66.10^{-5} \pm 2.9.10^{-6}$	$4.32.10^{-6} \pm 7.9.10^{-7}$	$8.64.10^{-8} \pm 1.5.10^{-8}$
A.glauca(+)	$3.07.10^{-5} \pm 2.5.10^{-6}$	$3.99.10^{-6} \pm 8.6.10^{-7}$	$1.94\ 10^{-6} \pm 4.6.10^{-7}$
A.glauca(-)	$2.77.10^{-5} \pm 3.1.10^{-6}$	$2.76.10^{-6} \pm 7.7.10^{-7}$	$1.22.10^{-6} \pm 5.0.10^{-7}$
A.spinosa	$2.20.10^{-5} \pm 1.4\ 10^{-6}$	$1.06.10^{-5} \pm 1.2.10^{-6}$	$4.66.10^{-6} \pm 3.8.10^{-7}$
A.orchidis	$1.94.10^{-5} \pm 4.2.10^{-6}$	$8.64.10^{-6} \pm 3.9.10^{-6}$	$3.79.10^{-6} \pm 3.6.10^{-6}$

All organisms were grown at 30°C, 200 rpm in medium A for 72hr. Cortexolone and its bioconversion products were separated, detected and quantified using hplc by their absorbance of light at 254nm.

11βC:11αC differed between some of the organisms (Table 3.6). For example a ratio of 2:1 was observed in all of the *Absidia* species used under these conditions. A difference was observed within the Cunninghamellaceae. *Cunninghamella blakesleeana* produced a 12:1 ratio of 11βC:11αC, *Cunninghamella elegans*; 3:1 and *Cunninghamella echinulata*; 8:1. *Phascolomyces articulosus* produced fifty times more 11βC than 11αC. In *Curvularia lunata* 11βC was the only product detected.

The majority of cortexolone recovered from all these organisms was found in association with the mycelial fractions (Fig. 3.2). The majority of 11βC and 11αC was found in association with the medium (Figs. 3.3 and 3.4 respectively). Presumably cortexolone, once modified inside the mycelium, is excreted into the medium.

The producers of the most 11βC per unit biomass were *Cunninghamella elegans* and *Cunninghamella blakesleeana* (Table 3.7: 2.97. $10^{-5} \pm 2.2. 10^{-6}$ and 2.64. $10^{-5} \pm 2.2. 10^{-6}$ mol/g respectively). The producers of the least 11βC per unit biomass were *Absidia glauca*(-), *Absidia glauca*(+), *Cunninghamella echinulata* and *Phascolomyces articulosus* (2.76 $10^{-6} \pm 7.7\ 10^{-7}$, 3.99 $10^{-6} \pm 8.6\ 10^{-7}$ 3.46. $10^{-6} \pm 6.6. 10^{-9}$ and 4.32. $10^{-6} \pm 7.9\ 10^{-7}$ mol/g respectively). Among the *Absidia* species used, *Absidia spinosa* proved to be the producer of most 11βC (1.06. $10^{-5} \pm 1.2. 10^{-6}$ mol/g). The producer of most 11αC among the Cunninghamellaceae and the other organisms was *Cunninghamella elegans* (9.58. $10^{-6} \pm 7.9. 10^{-7}$ mol/g). The lowest producer of 11αC among the Cunninghamellaceae was *Cunninghamellae echinulata* (4.19. $10^{-7} \pm 1.5. 10^{-7}$ mol/g). *Absidia spinosa* was the greatest producer of 11αC among the *Absidia* species (4.66. $10^{-6} \pm 3.8. 10^{-7}$ mol/g). *Absidia glauca*(-) and *Absidia glauca*(+) produced the least 11αC among Absidia species (1.22. $10^{-6} \pm 5.0. 10^{-7}$ and 1.94. $10^{-6} \pm 4.6. 10^{-7}$ mol/g respectively). *Phascolomyces articulosus* was the lowest producer of 11αC of all the fungi tested (2.76. $10^{-8} \pm 1.5\ 10^{-8}$ mol/g). *Curvularia lunata* produced no detectable 11αC using this method of analysis. The above

trends between these organisms was in close agreement with the %11αC and %11βC remaining by 72hr (Table 3.6).

The use of liquid nitrogen to macerate the mycelium did not result in increased steroid release from the mycelial fractions. Potassium hydroxide was used for saponification of the fungal cell membrane to release free and bound sterols (Astin and Haslam 1977). This method caused agglutination of the exogenous and endogenous steroid molecules, and produced free ergosterol, but no steroid was detected using h.p.l.c.

Hplc traces for *A.spinosa, C. blakesleeana* and *C. elegans* are presented in Chapter 4 (Figs. 4.19(a); 4.19(b); 4.20 and 4.21). 11βC and 11αC peaks were found to increase by spiking some samples with authentic 11βC and 11αC standards (not shown). Mass spectroscopy of standards co-migrating alongside samples using tlc have been performed (Appendix 2). Comparison of 11βC (sample 1) and 11αC (sample 2), shows monohydroxylated products with expected molecular weights of 362, with similar fragmentation peaks as the standard. Comparison of cortexolone with sample 3 shows a compound with an identical molecular weight (346) with similar fragmentation peaks as the standard. Sample 4 shows the silica gel blank, some of which was present in the samples.

3.1.6 DISCUSSION

108 fungi isolated from the leaf or rhizosphere of *Agave mexicana* in Portugal and 15 filamentous fungi from various culture collections (see Sections 2.1.3 and 2.1.4) were screened for their activity towards cortexolone. No other comparative studies of 11β- and 11α- hydroxylation among different fungi have been published.

Among the *Agave mexicana* isolates, some species have been found both on the surface and in the internal tissues i.e. *Cladosporium sp, Phoma sp, Penicillium sp, Alternaria sp,* and *Aureobasidium pullulans.* This range of species is common on leaf surfaces which implies that the deep-seated isolates

may represent superficial species difficult to access by surface-sterilants rather than species which grow within and metabolize *Agave* tissues. An interesting comparison may be obtained if *Agave* plant pathogens were used such as *Phytopthora arecae, Necriella miltina, Dimerosporium agavectova, Dothiorella sisalanae, Diplodia natalensis* and *Colletotrichum agaves.* These organisms may actively transform cortexolone as they can grow within the sterol rich hecogenin environment (Smith 1984).

The frequency of isolates able to modify cortexolone was very high (approximately 75%). This shows that the majority of organisms i.e. *Aureobasidium pullulans, Cladosporium sp, Penicillum sp,* etc. can modify a foreign compound such as cortexolone to several different products. The difference in the number of products produced from identical species can be explained by strain differences between fungi. Some filamentous fungi can express more diverse activity towards a given substrate than others of the same species (Charney and Herzog 1967). The 11α- hydroxylation of cortexolone occurs more frequently and can occur independently of 11β- hydroxylation in *Agave* isolates. It was also observed that 11β- hydroxylation always appeared to accompany 11α- hydroxylation. This will be discussed later.

No isolate produced a predominant derivative which co-ran with 11βC or 11αC, judged by the size and density of the spots produced. This would imply that random screening of the leaf and rhizosphere of *Agave* without any selection pressure, will not yield a 11β- or 11α- hydroxycortexolone product from the isolates that occurs in greater amounts, relative to the other products. The filamentous fungi used produced both 11βC and 11αC in greater amounts relative to the other products.

In the filamentous fungi used, biotransformation occurred irrespective of fungal morphology. Morrin and Ward (1989) have demonstrated that progesterone can be 11α- hydroxylated by different morphological forms of *Rhizopus arrhizus.* One can therefore follow bioconversions regardless of

whether filamentous fungi change their morphology, as long as the form of growth adopted is consistent. One can also compare filamentous and pellet-promoted bioconversion reactions in different fungi. (cf Chapter 4). This type of comparison has not been reported previously.

When progesterone was used as the substrate, some fungi (Table 3.4) showed a specificity for 11α- hydroxylation rather than 11β- hydroxylation. The addition of cortexolone stimulated both 11α- and 11β- hydroxylations. When progesterone was used as substrate, both 11β- and 11α- hydroxylases have been reported and separated by centrifugation (Adel-Fattah and Badawi 1975a; 1975b) using *Aspergillus niger*. These data would strongly suggest that 11α- hydroxylation is distinct and independent of 11β- hydroxylation. The two activities can be further seen to be distinct, when the *Curvularia lunata* mutant (CLR56) is used. This produces solely 11βC together with another product, probably 14α- hydroxycortexolone (Zuidweg 1968). These two activities are well known to occur together and have been postulated to come from the same enzyme in *Curvularia lunata*. The parent strain is known to produce both 11β- and 11α- hydroxycortexolone (Bill Spice, pers. commun.). These two distinct enzymes apparently performed two separate functions. As with the *Agave* isolates there is a higher frequency of 11α– hydroxylation.

The structural difference between progesterone and cortexolone is that the latter compound has additional hydroxyl groups at C-17 and C-21. The presence of substituents at different positions on the steroid nucleus has been shown to affect conversion rates in *Syncephalastrum racemosum* (Sen and Samanta 1981). Zakelj-Mavric *et al* (1986; 1987) have shown that the presence of certain groups on the D ring can prevent steroid and enzyme binding in the 'normal way'. This may explain the absence of 11β- hydroxylation when progesterone was used as substrate for those species where both 11β- and 11α- hydroxylations of cortexolone were observed. It could also be possible that progesterone may not induce the 11β- hydroxylase. The former is probably the

correct explanation, as Abdel-Fattah and Badawi (1975a; 1975b), have reported 11βPG and 11αPG production from progesterone using cell-free extracts from *Aspergillus niger*.

Filamentous fungi have a higher frequency of 11α- hydroxylation relative to 11β- hydroxylation. Fungi may prefer to utilize the 11α- hydroxylase for microbial transformations. This is understandable as the 11α- product is more polar than the corresponding 11β- product, as can be seen from its migration in t.l.c. and h.p.l.c. systems. This increased polarity would mean it would be more water-soluble, thus aiding its removal from the cell. The production of polar water-soluble products from a more hydrophobic substrate would help to shift the equilibrium towards product formation. The separation of mycelium from medium, to investigate steroid distribution, has revealed that once the products are formed they are excreted into the medium. Only a small proportion of products is found in association with the mycelial fractions. With cortexolone, the opposite is true. It occurs in larger amounts in the mycelia fraction than in the medium, as cortexolone enters the cell before bioconversion. In those fungi with both 11α- and 11β- hydroxylating activity, though 11α- hydroxylation occurs at a higher frequency in the fungi tested, they preferentially produce 11βC (Table 3.6). Possibly the 11β- hydroxylase system carries out its bioconversion at a much faster rate, or is produced in greater amounts. This could be further investigated by the preparation of cell-free extracts, and the separate purification of both activities, and determination of K_m and V_{max} for both enzymes.

Phascolomyces articulosus and *Absidia spinosa* were discovered to be novel producers of 11β- and 11α- hydroxycortexolone.

The Cunninghamellaceae would be an interesting family for further study. Fungi such as *Cunninghamella blakesleeana* and *Cunninghamella elegans* produce large amounts of biomass and are the most efficient transformers of cortexolone. They also produce more of the 11α- and 11β-

monohydroxylate compared to other fungi used in this study. Both fungi are well documented in the literature and produce a variable 11β-/11α- product ratio. The high incidence of 11βC with respect to the 11α- product could indicate that pellet formation may favour 11β- hydroxylation. This observation has not been reported previously. In *Absidia* species the 11β-/11α- product ratio is more consistent. This difference has not been demonstrated previously and there is no known reason for it. *Absidia spinosa* was chosen for further investigation as it not only grew filamentously, but it was also the most prolific producer of 11β- and 11α- hydroxycortexolone among the *Absidia* species tested.

CHAPTER 4

I COMPARATIVE STUDY OF CORTEXOLONE TRANSFORMATION BY GERMINATING SPORES OF *Cunninghamella blakesleeana*, *Cunninghamella elegans* AND *Absidia spinosa*

RESULTS

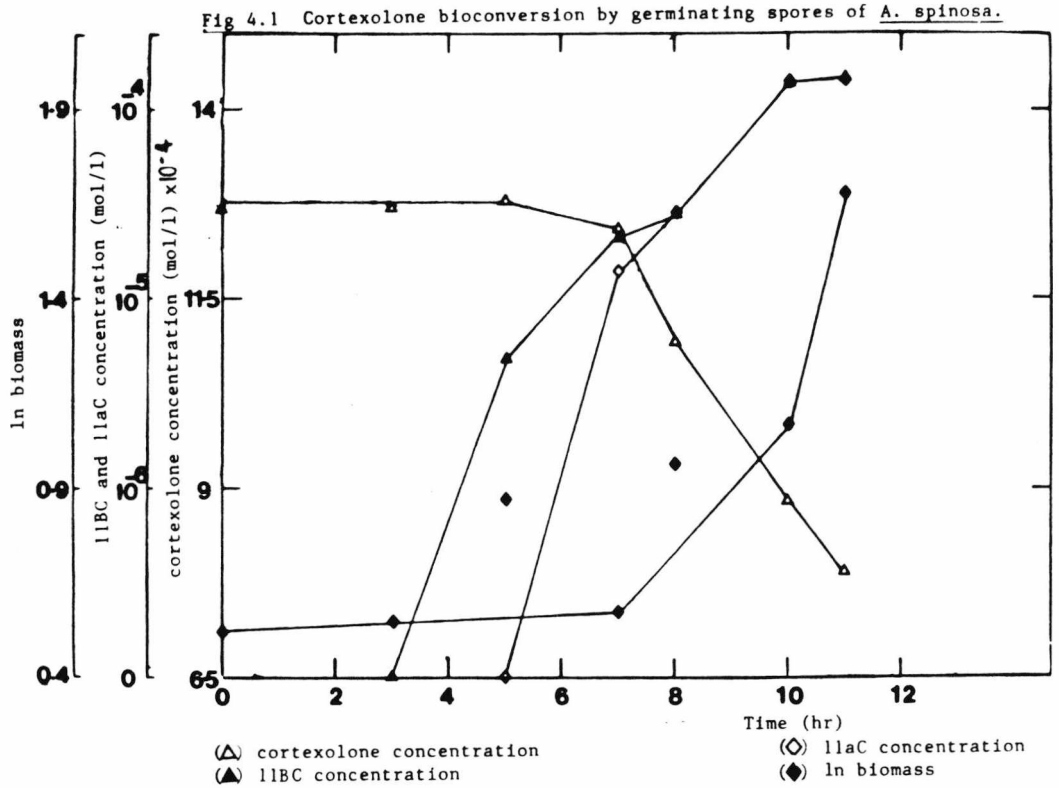
The purpose of this chapter is to compare how the three fungi *A.spinosa*, *C.blakesleeana* and *C.elegans* interact with a exogenously supplied xenobiotic compound (cortexolone) through their life cycle in batch culture, from germinating spores, to growing vegetative cells and in the deline phase of growth.

4.1.1 Cortexolone biotransformation by germinating spores of *Absidia spinosa*

Cortexolone concentration did not change noticeably for the first 5 hr in the medium (Fig.4.1). The level of cortexolone then dropped steadily between 5-7 hr after inoculation, and from 7-11 hr cortexolone concentration appeared to decrease linearly. The growth of *Absidia spinosa* increased inversely with the decrease of cortexolone concentration (Fig.4.1). *Absidia spinosa* grew slowly from 0-7 hr after inoculation. By 7 hr growth increased rapidly as the organism entered exponential growth. At 6 hr, 3% of spores had produced germ tubes exceeding 6μm in length (Fig. 4.2). By 8 hr, 95% of spores had produced germ tubes, this increased to 97% after 10 hr (Fig. 4.2). A fall in glucose concentration by 8 hr (Fig. 4.2) corresponded to an increase in *Absidia spinosa* biomass, and a fall in cortexolone concentration in the medium.

Three major products were detected using h.p.l.c. and could also be seen on t.l.c. plates (Fig. 4.3a). 11βC was detected at 5 hr. Product concentration increases with a corresponding fall in cortexolone concentration. The product,

Fig 4.1 Cortexolone bioconversion by germinating spores of A. spinosa.



Figs 4.1, 4.2 and 4.4

Medium A, containing 1.1mM cortexolone in 2% (v/v) MeOH, was inoculated with A.spinosa spores and incubated at 200 rpm, 30°C for 11hr, and samples removed at intervals. Glucose was determined using a glucose assay kit, and the % germination of spores noted using light microscopy. Cortexolone and its biotransformation products were detected and quantified using hplc by their absorbance of light at 254nm.

Fig 4.2 Glucose utilization and % germination of A. spinosa spores in the presence of cortexolone

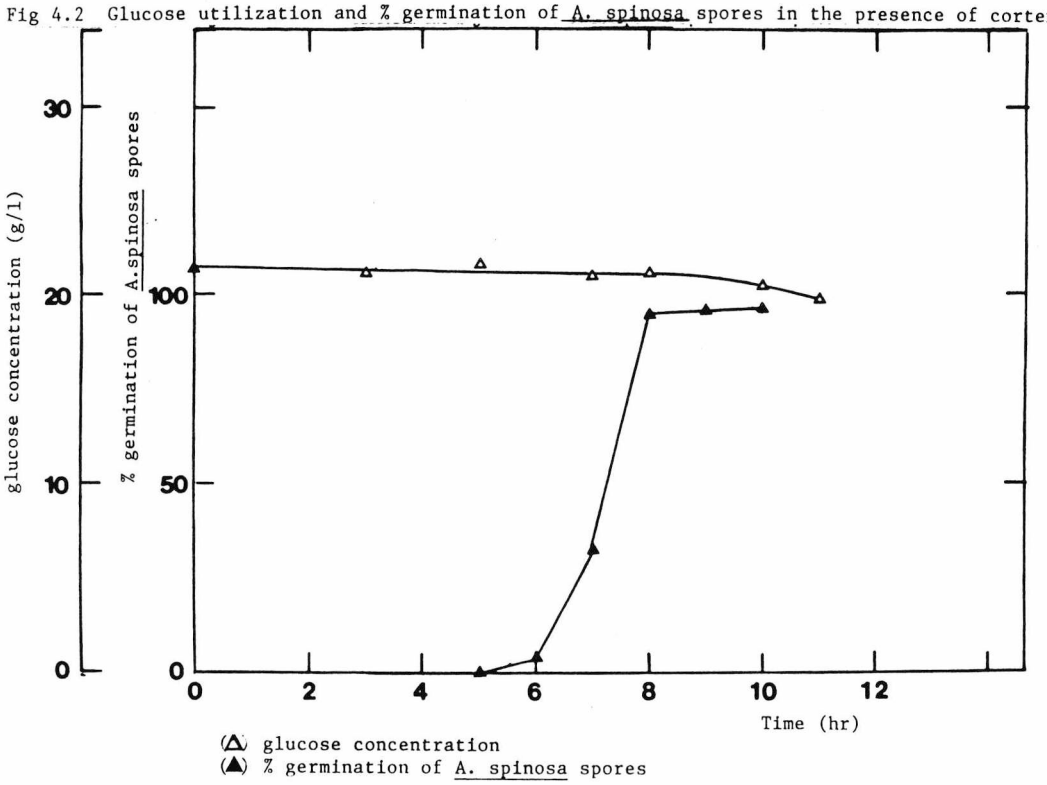
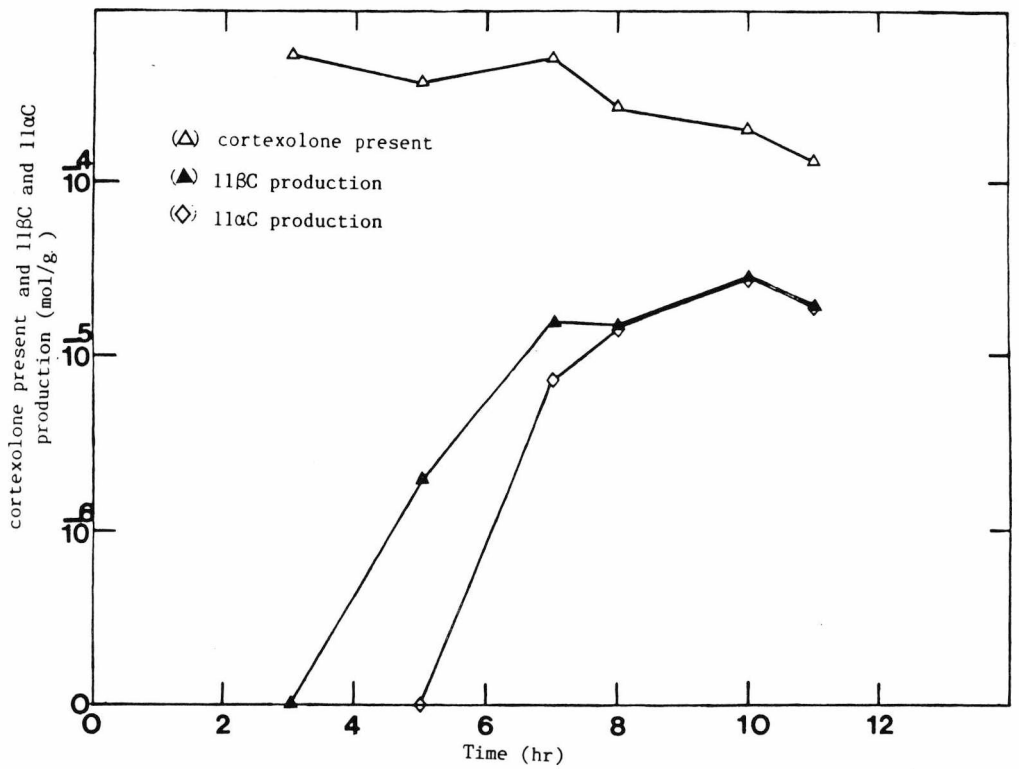


Figure 4.4: Cortexolone bioconversion to 11αC and 11βC by germinating spores of A.spinosa



Tlc plates of steroids extracted from whole culture medium containing germinating spores of A.spinosa

Fig4.3(a) Cortexolone treated

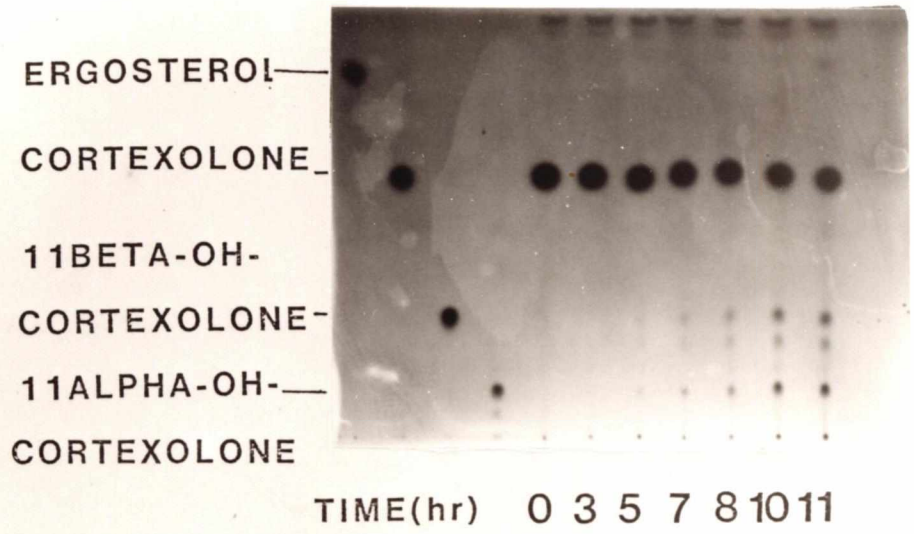


Fig4.3(b) Control

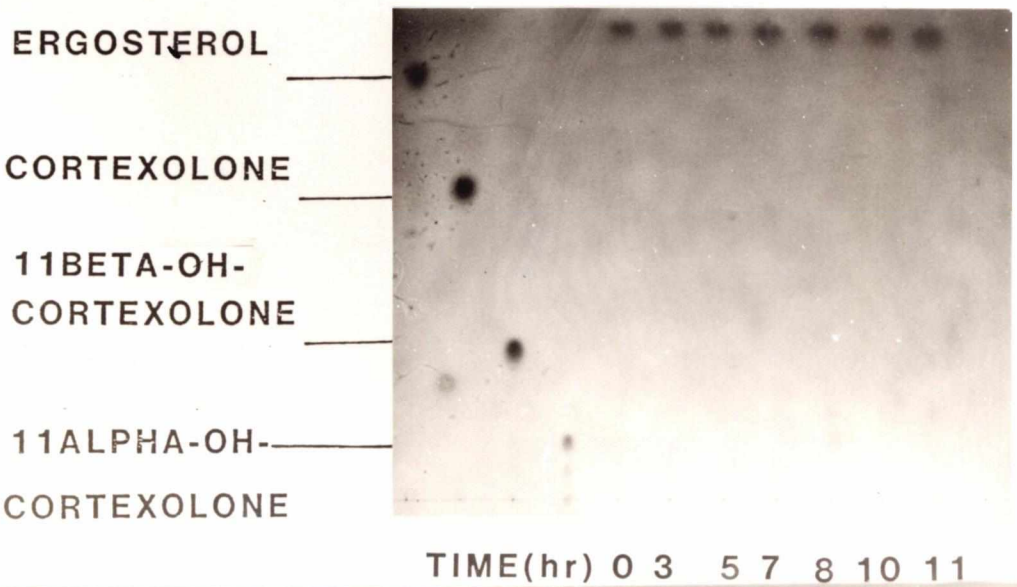
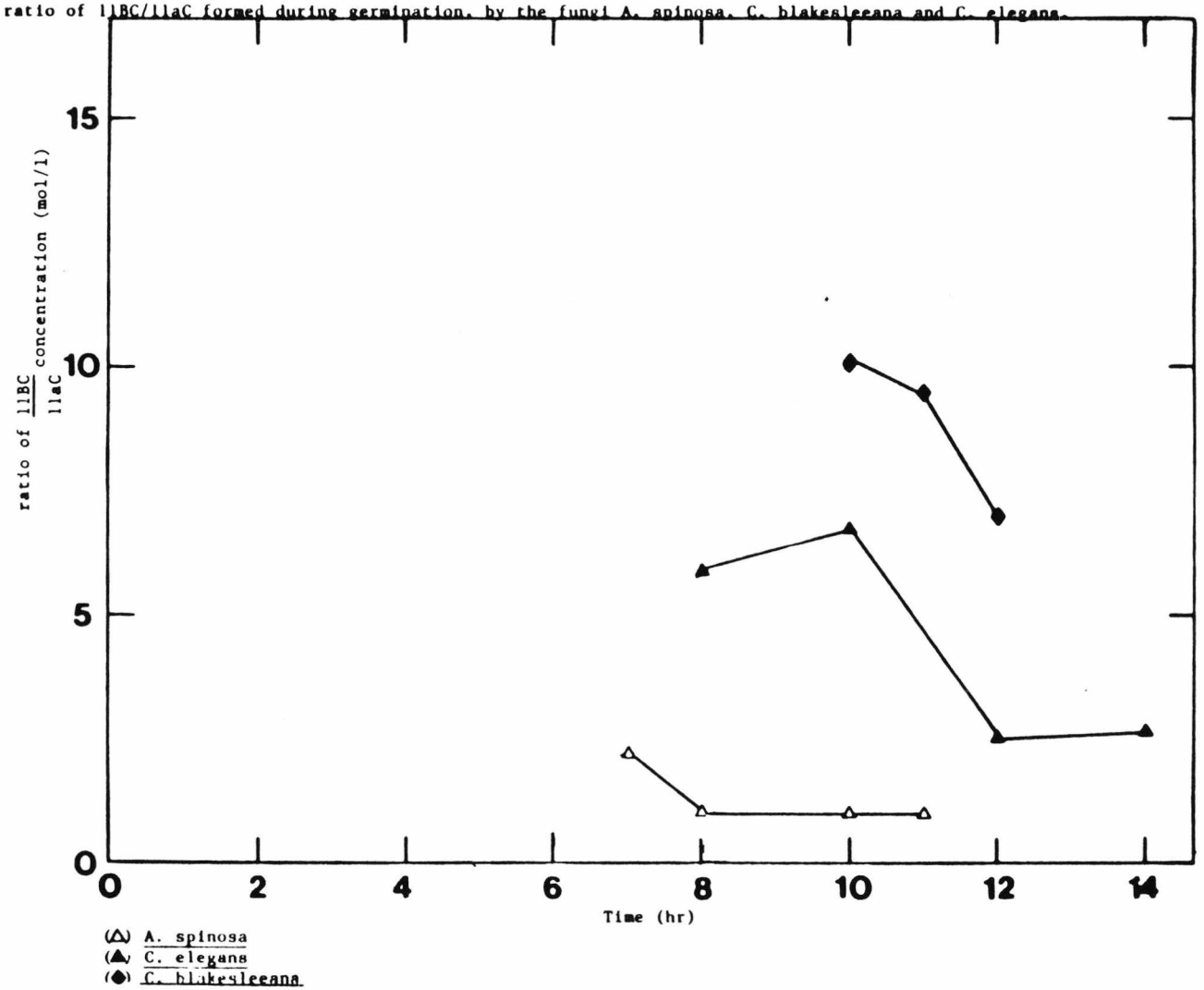


Fig 4.5 The ratio of 11BC/11aC formed during germination, by the fungi A. spinosa, C. blakesleeana and C. elegans.



11αC was detected by 7 hr after inoculation. Figure 4.1 reveals that the fall in cortexolone concentration corresponds to an increase in both 11βC and 11αC. 11βC appeared to precede 11αC in its initial production, by 8 hr, when the majority of spores had produced germ tubes (95%), the two products were produced in equimolar amounts (Fig. 4.4). No transformation spots were detected in control samples of inoculated media when *A.spinosa* was grown in the absence of cortexolone (Fig. 4.3b).

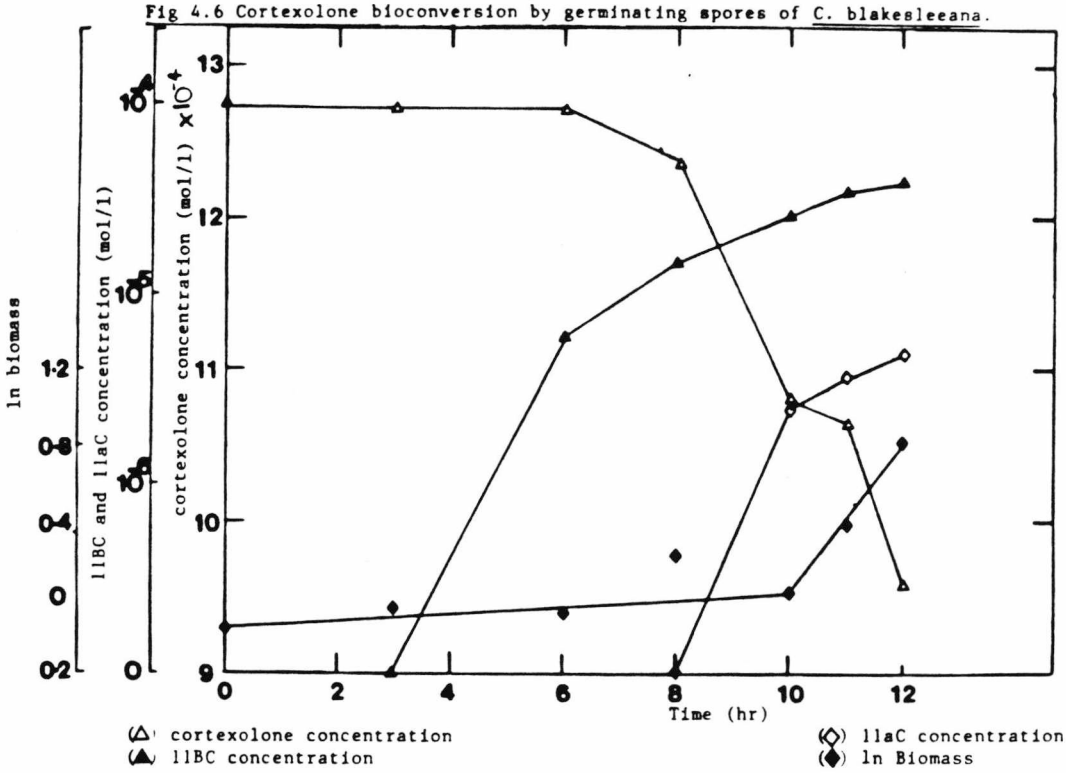
The level of cortexolone declined between 3-11 hr, (Fig.4.4). Cortexolone levels fell to $1.47\ 10^{-4} \pm 1.2\ 10^{-5}$ mol/g by 11 hr. The production of both 11βC and 11αC increased from 0 to a maximum of $4.52\ 10^{-5} \pm 3.5\ 10^{-6}$ mol/g and $4.42\ 10^{-5} \pm 4.9\ 10^{-6}$ mol/g respectively between 0-10 hr (Fig. 4.4). At 10 hr all viable spores had produced germ tubes, and the organism was in early growth. At 11 hr the production of both 11βC and 11αC fell to $2.82\ 10^{-5} \pm 2.2\ 10^{-6}$ mol/g and $2.76\ 10^{-5} \pm 3.8\ 10^{-6}$ mol/g respectively. The ratio of 11βC:11αC declined between 7-8 hr. This shows that 11βC exceeds 11αC in its initial production (2:1 by 1 hr). Between 8-11 hr this remains a constant ratio of 1:1 (Fig. 4.5).

4.1.2 Cortexolone biotransformation by germinating spores of *Cunninghamella blakesleeana*

For the first six hours after inoculation of the medium, the cortexolone concentration did not change noticeably (Fig.4.6). The level of cortexolone concentration then fell steadily from 6-8 hr. Between 8-12 hr there was a sharp linear drop in cortexolone concentration.

Fungal biomass increased slowly between 0-10 hr (Fig. 4.6). In the interval of 10-12 hr, there was sharp increase in biomass, corresponding to the aforementioned drop in cortexolone concentration. 4% of *Cunninghamella blakesleeana* spores had produced germ tubes in excess of 6μm long by 6 hr (Fig.4.7). 95% of spores had germinated by 11 hr (Fig. 4.7). Glucose concentration fell slightly 11 hr after inoculation (Fig. 4.7), as the biomass

Fig 4.6 Cortexolone bioconversion by germinating spores of C. blakesleeana.



Figs 4.6, 4.7 and 4.9

Medium A, containing 1.1m M cortexolone in 2 % (v/v) MeOH was inoculated with C.blakesleeana spores and incubated at 200 rpm, 30 °C for 12 hours, and samples removed at intervals. Glucose was determined using a glucose assay kit, and the % germination of spores noted using light microscopy. Cortexolone and its biotransformation products were detected and quantified by their absorbance of light at 254nm.

Fig 4.7 Glucose utilization and % germination of C. blakesleeana spores in the presence of cortexolone

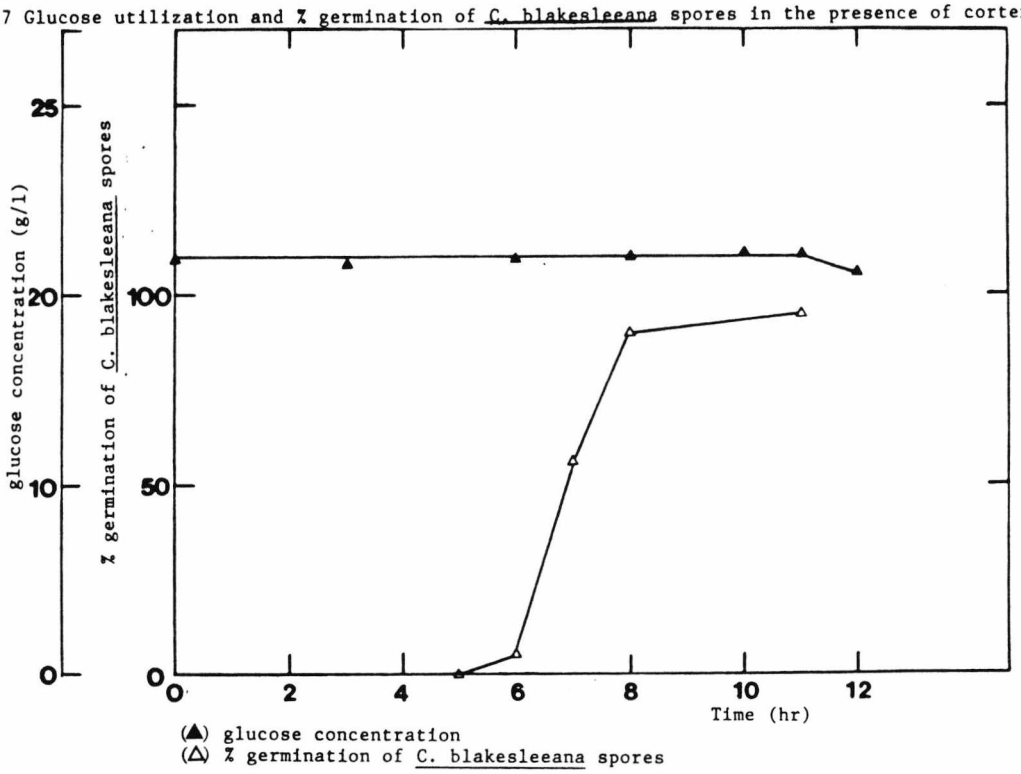
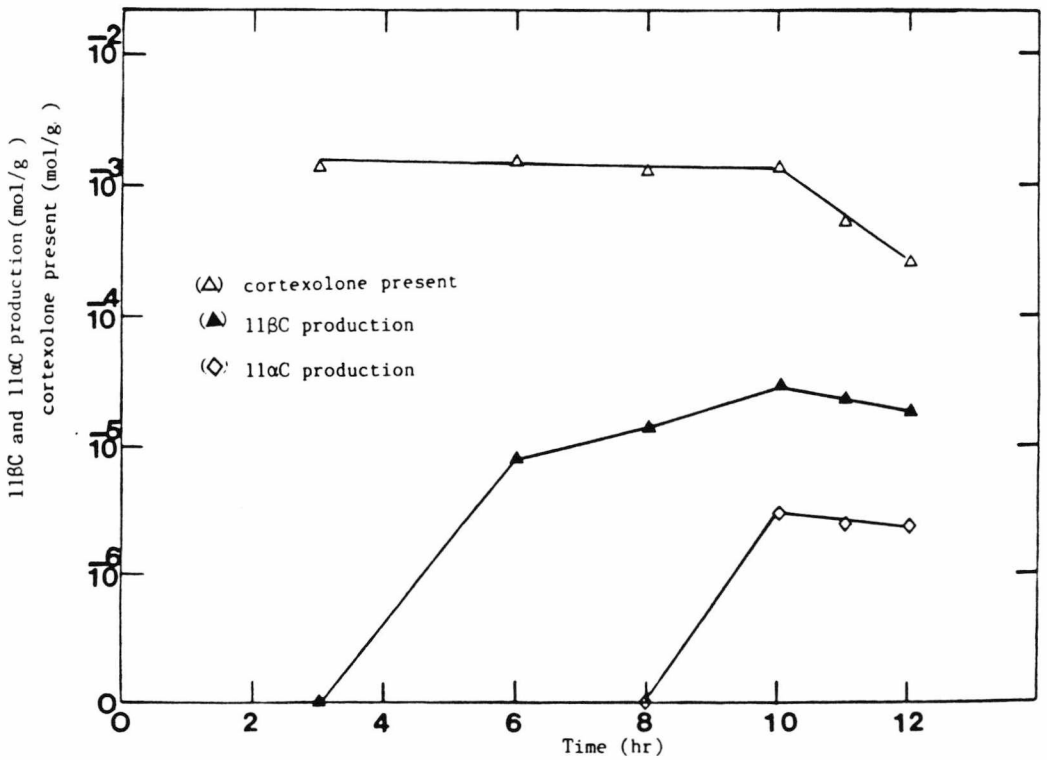


Figure 4.9: Cortexolone bioconversion to 11βC and 11αC by germinating spores of C.blakesleeana



Tlc plates of steroids extracted from whole culture medium containing germinating spores of C.blakesleeana

Fig4.8(a) Cortexolone treated

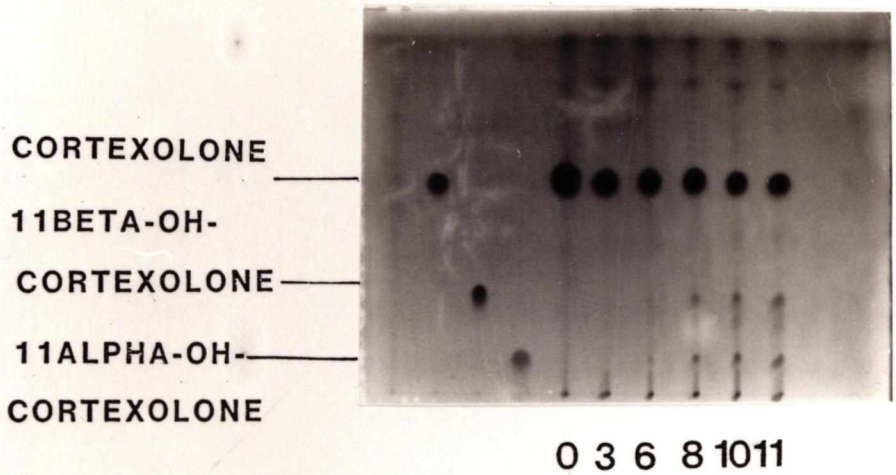
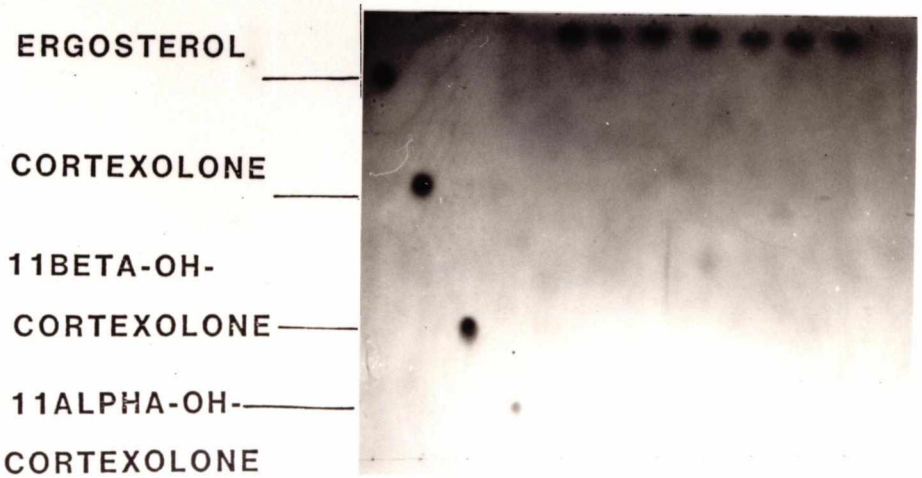


Fig4.8(b) Control



increased. Four or five major products were detected using h.p.l.c. and some of these were also visible on the t.l.c. plate (Fig. 4.8a). The 11βC product preceded 11αC in its production. The formation of 11βC and 11αC was detected 6 hr and 10 hr respectively, after inoculation of the medium (Fig. 4.6). The increase in product formation correlated with a decrease in cortexolone concentration (Fig. 4.6). No transformation spots were detected in control samples of inoculated media when *C.blakesleeana* was grown in the absence of cortexolone (Fig. 4.8b).

Cortexolone levels in relation to biomass decreased between 3-10 hr after inoculation (Fig.4.9). From 10-12 hr there was an increase in cortexolone transformation. As with *Absidia spinosa* this corresponds to an increase in fungal biomass. The cortexolone level was $4.36.\ 10^{-4} \pm 1.3.\ 10^{-4}$ mol/g 12 hr after the start of the fermentation. The production of 11βC and 11αC increased from zero to a maximum of $4.64.\ 10^{-5} \pm 1.5.\ 10^{-5}$mol/g and $4.60\ 10^{-6} \pm 1.8\ 10^{-6}$ mol/g respectively between 0-10 hr. The production of 11βC and 11αC then tailed off as *Cunninghamella blakesleeana* entered early exponential growth ($2.60\ 10^{-5} \pm 7.3\ 10^{-6}$ mol/g and $3.70\ 10^{-6} \pm 7.3\ 10^{-7}$ mol/g respectively 12 hr after the start of the fermentation). The ratio of 11βC:11αC declines from 10 to 7, 10-12 hr into the fermentation (Fig.4.5).

4.1.3 Cortexolone biotransformation by germinating spores of *Cunninghamella elegans*

Following inoculation of the medium, the cortexolone concentration did not change noticeably for the first 8 hr of the fermentation (Fig.4.10). The level of cortexolone then dropped between 8-14 hr (Fig.4.10). The growth of *Cunninghamella elegans* was slow from 0-14 hr. *Cunninghamella elegans* did not produce germ tubes as quickly as *Absidia spinosa* or *Cunninghamella blakesleeana*. *Cunninghamella elegans* did not produce normal germ tubes, but instead produced enlarged distorted hyphae which grew into pellets (not

Fig 4.10 Cortexolone bioconversion by germinating spores of C.elegans

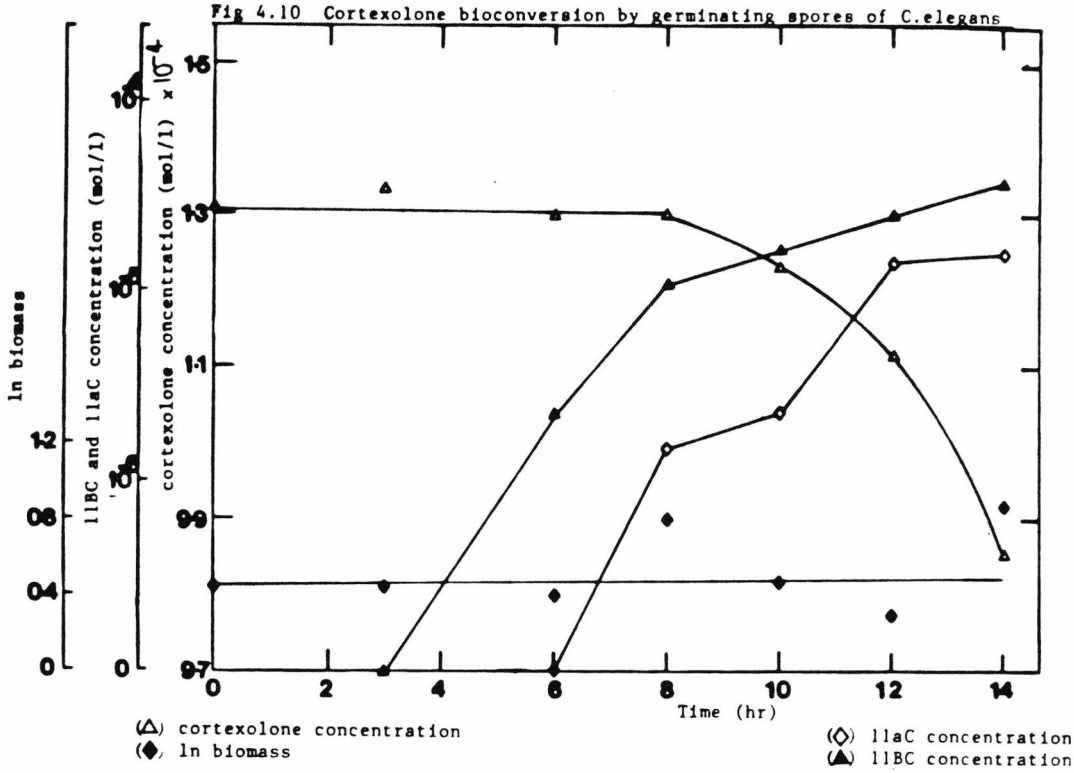
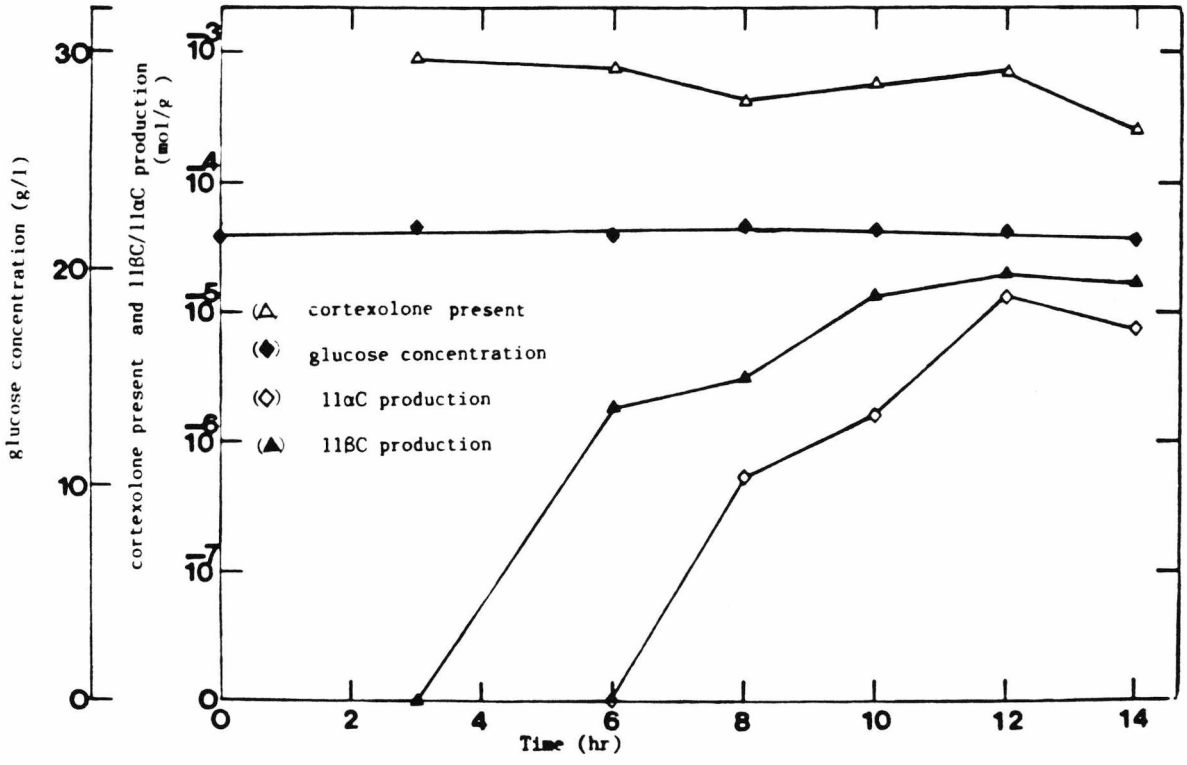


Figure 4.11: Cortexolone bioconversion to 11βC and 11αC and glucose utilization by germinating spores of C.elegans



Figs 4.10 and 4.11

Medium A, containing 1.1mM cortexolone in 2% (v/v) MeOH was inoculated with C.elegans spores and incubated at 200 rpm, 30°C for 14 hours and samples removed at intervals. Glucose was determined using a glucose assay kit, and germinating spores were observed using light microscopy. Cortexolone and its biotransformation products were detected and quantified using hplc by their absorbance of light at 254nm.

Tlc plates of steroids extracted from whole culture medium containing germinating spores of C.elegans

Fig4.12(a) Cortexolone treated

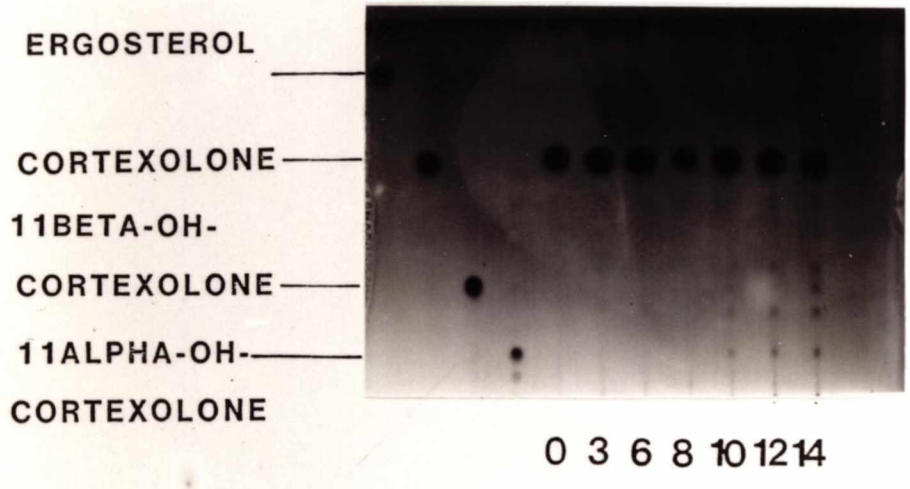
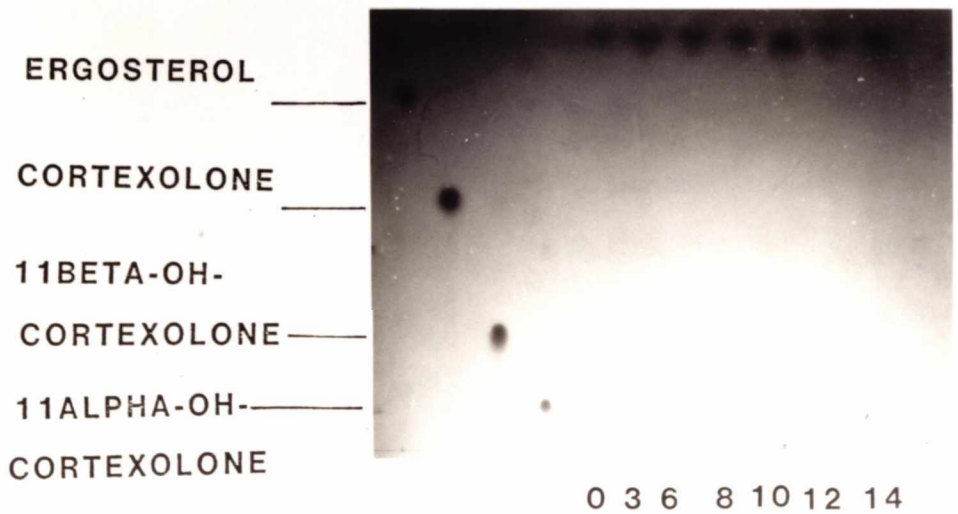


Fig4.12(b) Control



shown). The level of glucose changed very slowly throughout the period of study (Fig.4.11).

Four major products were detected using h.p.l.c. and t.l.c. plates (Fig. 4.12a). From the t.l.c. plates one can see a small decrease in the amount of cortexolone and the appearance of both 11βC and 11αC by 8 hr. Graphical presentation also shows a fall in cortexolone concentration and an increase in both 11βC and 11C by 6 and 8 hr respectively (Fig.4.10). As with *Absidia spinosa* and *Cunninghamella blakesleeana,* 11βC preceded 11αC in its initial production. No transformation spots were detected in control samples of inoculated media, when *C.elegans* was grown in the absence of cortexolone (Fig. 4.12b). The level of cortexolone in relation to biomass (Fig. 4.11) showed a gradual decline from 0-12 hr, followed by a rapid decrease between 12-14 hr. Cortexolone levels had fallen to $8.52\ 10^{-4} \pm 1.4\ 10^{-4}$ mol/g by 12 hr. The production of 11βC and 11αC increased from zero to $3.01\ 10^{-5} \pm 4.6\ 10^{-6}$ mol/g and $1.21^{-5} \pm 3.2\ 10^{-6}$ mol/g respectively 12 hr after inoculation. Product production declined to $1.7\ 10^{-6} \pm 4.5\ 10^{-8}$ mol/g (11βC) and $6.2\ 10^{-7} \pm 2.3\ 10^{-8}$ mol/g (11αC) by 14 hr. The ratio of 11βC:11αC increased from 6:1 by 8 hr to 7:1 by 10 hr. This fell to 3:1 by 12 hr and remained unchanged between 12-14 hr (Fig. 4.5). No spots were detected in control samples of uninoculated media (Appendix 3). The only spots detected in spore germination studies were non-polar molecules which migrated to a position above that of ergosterol.

4.1.4 Comparison of *Absidia spinosa*, *Cunninghamella blakesleeana* and *Cunninghamella elegans* in spore germination studies

All three organisms can modify the exogenously added substrate cortexolone. Cortexolone was not immediately converted to its transformation products by *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans* as there was a lag of between 5-8 hr before products were detected. Glucose depletion corresponded to an increase in biomass for

Absidia spinosa and *Cunninghamella blakesleeana* which produced germ tubes and a hyphal network. This was not as obvious with *Cunninghamella elegans*. Glucose does not have to be appreciably reduced before cortexolone bioconversion begins. Different numbers of products are produced from cortexolone by *Absidia spinosa* (3), *Cunninghamella blakesleeana* (4 or 5) and *Cunninghamella elegans* (4). The initial formation of 11βC precedes 11αC in all three organisms.

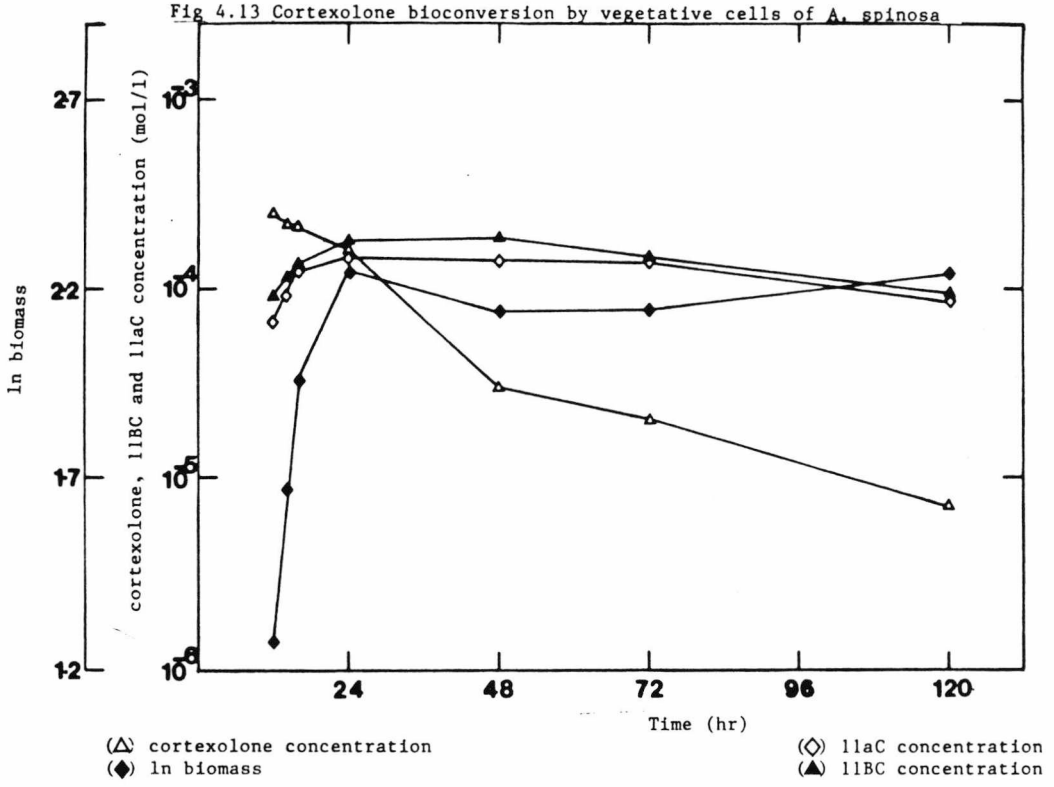
The maximum production of 11βC was similar in *Absidia spinosa* and *Cunninghamella blakesleeana* ($4.5\ 10^{-5} \pm 3.5\ 10^{-6}$ mol/g and $4.6\ 10^{-5} \pm 1.6\ 10^{-5}$ mol/g respectively) but lower in *Cunninghamella elegans* ($3.0\ 10^{-5} \pm 4.6\ 10^{-6}$ mol/g). The maximum production of 11αC was different in all three organisms; *Absidia spinosa* ($4.4\ 10^{-5} \pm 5.0\ 10^{-6}$ mol/g), *Cunninghamella blakesleeana* $4.6\ 10^{-6} \pm 1.8\ 10^{-6}$ mol/g), *Cunninghamella elegans* ($1.2\ 10^{-5} \pm 2.2\ 10^{-6}$ mol/g). In addition the ratio of 11β-:11α- hydroxycortexolone was different for all three organisms used.

II. COMPARISON OF THE GROWTH PATTERNS OF *Absidia spinosa*, *Cunninghamella blakesleeana* AND *Cunninghamella elegans* IN SHAKE FLASK IN THE PRESENCE OF CORTEXOLONE

4.2.1. Growth in liquid culture

Absidia spinosa grew filamentously in liquid culture (medium A). The maximum specific growth rate (μ_{max}) was 0.19 hr^{-1} with a doubling time (t_d) of 3.6 hr. Maximum biomass (9.5 ± 0.2 g/1) was attained after 24 hr growth in this medium (Fig.4.13). Fungal biomass then declined slightly (8.53 ± 0.4 g/l) after 48 hr, 24 hr after glucose exhaustion (this will be referred to later). Growth did not appear to change from 48-72 hr, but rose slightly from 8.53 ± 0.4 g/l to 9.36 ± 0.2 g/l by 120 hr.

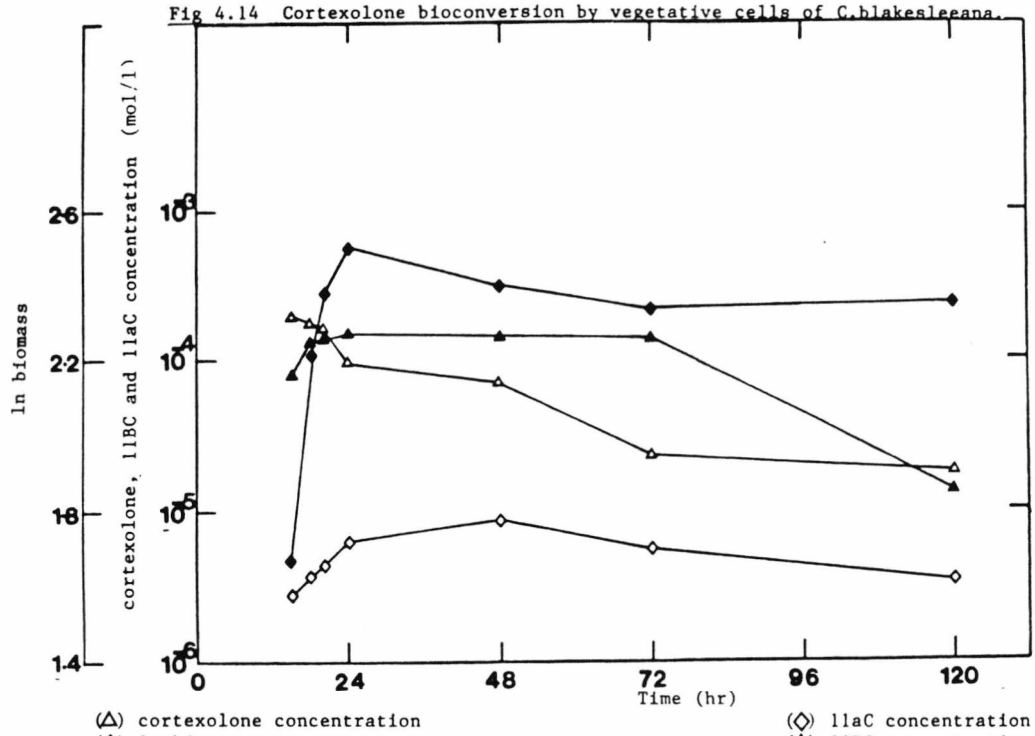
Fig 4.13 Cortexolone bioconversion by vegetative cells of A. spinosa



Figs 4.13, 4.14 and 4.15

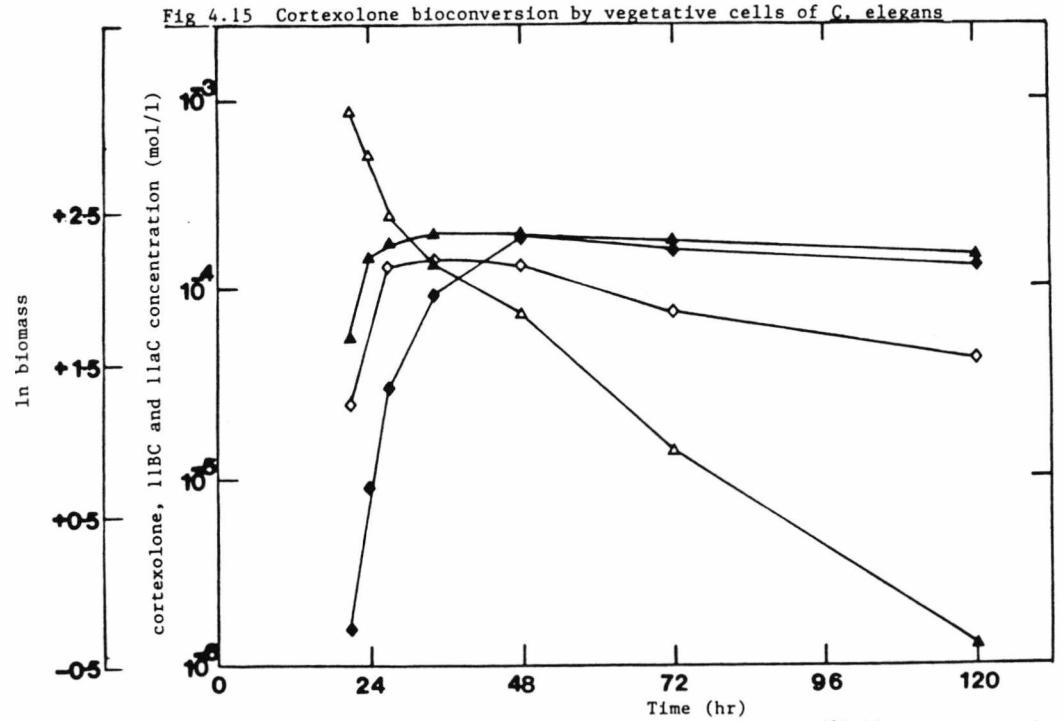
A.spinosa, C.blakesleegana and C.elegans were grown at 200 rpm, 30°C for 120hr, in medium A containing 1.1mM cortexolone in 2 % (v/v) MeOH. Whole flasks were removed at intervals for analysis. Fungal mycelia was separated from culture medium by filtering onto gouch crucibles. Cortexolone was detected and quantified using hplc, by its absorbance of light at 254nm.

Fig 4.14 Cortexolone bioconversion by vegetative cells of C.blakesleeana.



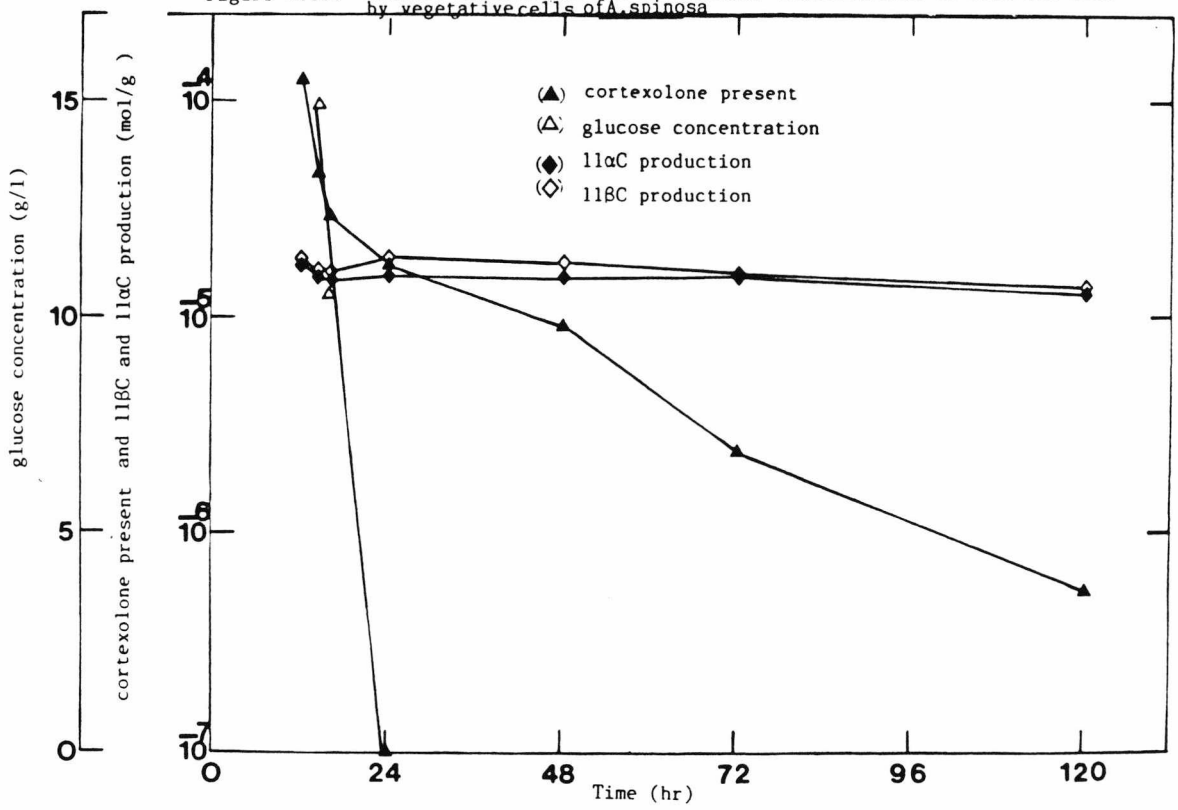
(△) cortexolone concentration
(◆) ln biomass
(◇) 11aC concentration
(▲) 11BC concentration

Fig 4.15 Cortexolone bioconversion by vegetative cells of C. elegans



(△) cortexolone concentration
(◆) ln biomass
(◇) 11aC concentration
(▲) 11BC concentration

Figure 4.16: Glucose utilization and cortexolone bioconversion to 11βC and 11αC by vegetative cells of A.spinosa



Figs 4.16, 4.17 and 4.18

A.spinosa, C.blakesleeana and C.elegans were grown at 200 rpm, 30°C for 120 hr in medium A containing 1.1mM cortexolone in 2% (v/v) MeOH. Whole flasks were removed at intervals for analysis. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm. Glucose concentration was determined using a glucose assay kit.

Figure 4.17:Glucose utilization and cortexolone bioconversion to 11βC and 11αC by vegetative cells of C.blakesleeana

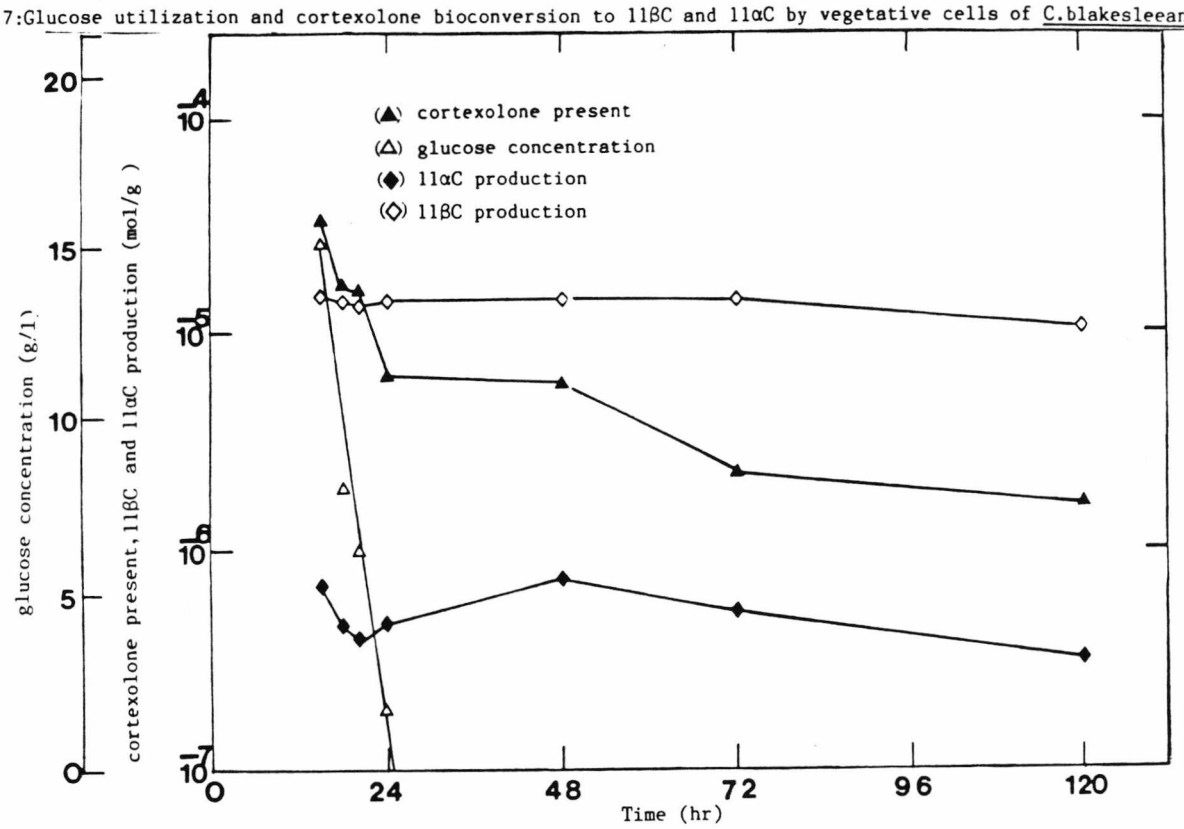
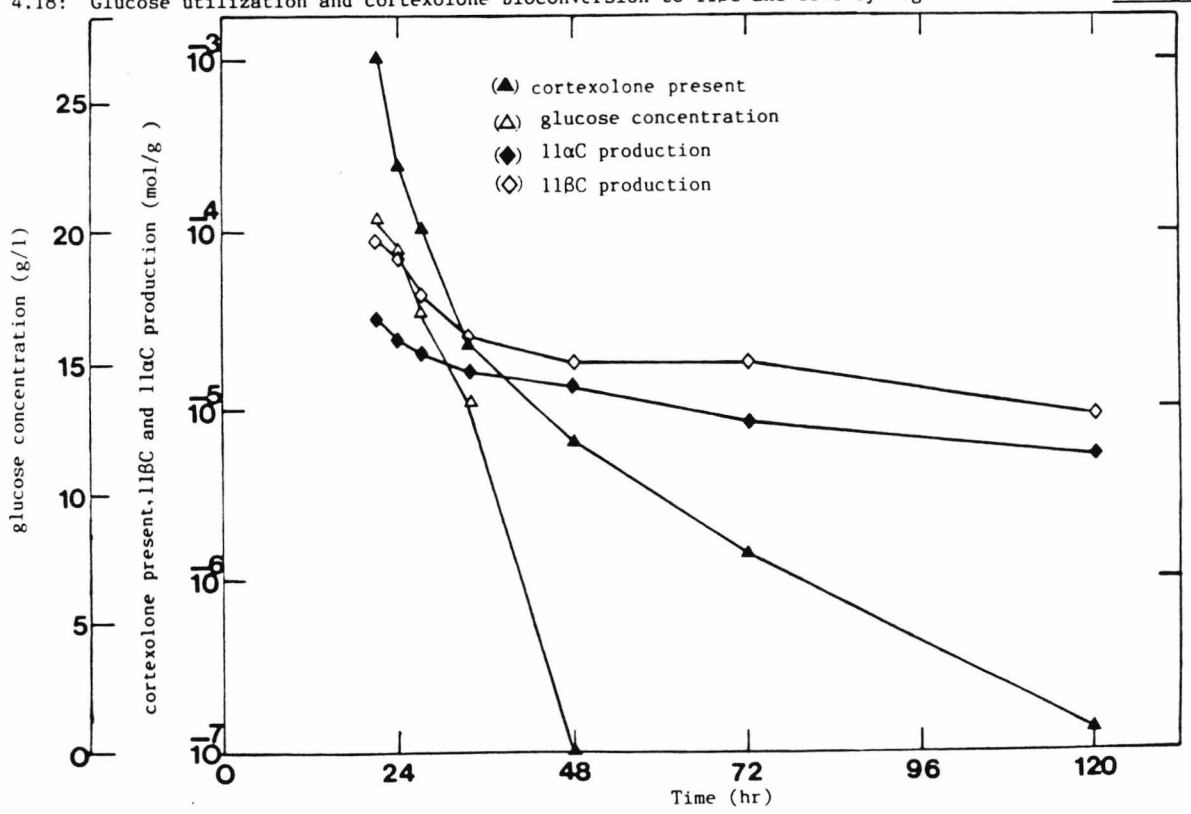


Figure 4.18: Glucose utilization and cortexolone bioconversion to 11βC and 11αC by vegetative cells of C.elegans



Cunninghamella blakesleeana initiated growth by producing a hyphal network, which produced small pellets as growth continued in liquid culture. The maximum specific growth rate (μ_{max}) was 0.18 hr^{-1} (t_d, 3.8 hr). The maximum biomass produced in this medium (Fig.4.14) was 12.15 ± 0.6 g/l at 24 hr. After peak biomass had been reached, growth declined to 10.5 ± 0.9 g/l by 120 hr. *Cunninghamella elegans* grew as fine pellets in medium A, μ_{max} was 0.15 hr^{-1} with a t_d of 4.5 hr. Maximum biomass (10.6 ± 0.9 g/l) was produced 48 hr after inoculation (Fig. 4.15). Fungal biomass then declined slightly to 8.57 ± 0.3 g/l by 120 hr. Of the three organisms tested *Absidia spinosa* possessed the fastest growth rate then *Cunninghamella blakesleeana* and *Cunninghamella elegans* the slowest. *Cunninghamella elegans* produced the greatest total biomass, whereas *Absidia spinosa* produced the lowest total biomass in half the time of *Cunninghamella elegans.*

Glucose was exhausted by *Absidia spinosa* (Fig. 4.16) and *Cunninghamella blakesleena* (Fig. 4.17) by 24 hr respectively. With *Cunninghamella elegans* glucose was exhausted by 48 hr (Fig. 4.18).

4.2.2 Distribution of cortexolone between mycelia, media and filter fractions

When the organisms used were filtered under vacuum to separate the mycelia from the media, it was found that only a small proportion of the transformation substrate, cortexolone, was retained by the filter in two of the organisms used. With *Absidia spinosa* no more than 1.2% of the total cortexolone recovered was retained by the filter (Table 4.1). The amount of cortexolone retained by the filter decreased throughout the fermentation as the concentration of cortexolone decreased (Table 4.1). No cortexolone could be detected on the filter throughout the period of study, using *Cunninghamella blakesleeana* (Table 4.2). The difference between *Absidia spinosa* and *Cunninghamella blakesleeana,* could reflect that the filtration is more efficient

% Distribution of cortexolone between mycelia, media and filter fractions using *A.spinosa*, *C.blakesleeana* and *C.elegans*

Table 4.1: *A.spinosa*

Time (hr)	% Cortexolone mycelium	medium	filter
12	69.0	29.8	1.2
14	76.6	22.3	1.1
16	80.8	18.5	0.7
24	84.1	15.5	0.3
48	83.5	16.4	0
72	85.0	14.8	0.2
120	70.8	29.2	0

Table 4.2: *C.blakesleeana*

Time (hr)	% Cortexolone mycelium	medium	filter
15	87.1	12.9	0
18	77.4	22.6	0
20	96.2	3.8	0
24	96.2	3.8	0
24	98.8	10.2	0
48	85.5	14.5	0
72	75.1	24.9	0
120	75.2	24.8	0

Table 4.3 *C.elegans*

Time (hr)	% Cortexolone mycelium	medium	filter
21	54.2	29.8	16.0
24	61.8	16.7	21.5
27	70.9	27.0	20.1
34	82.6	16.0	1.4
48	73.6	26.3	0.1
72	99.8	0	0.2
120	100	0	0

A.spinosa, *C.blakesleeana* and *C.elegans* were grown at 200 rpm, 30°C for 120 hour, in medium A containing 1.1mM cortexolone in 2% (v/v) MeOH. Whole flasks were removed at intervals for analysis. Fungal mycelia was separated from culture medium by filtering onto gouch crucibles. Cortexolones were detected and quantified using hplc, by its absorbance of light at 254nm.

% Distribution of 11βC and 11αC between mycelia, media and filter fractions, using *A.spinosa*, *C.blakesleeana* and *C.elegans*

Table 4.4 *A.spinsoa*

Time (hr)	% 11βC mycelium	% 11βC medium	% 11βC filter	% 11αC mycelium	% 11αC medium	% 11αC filter
12	0.8	99.2	0	0.6	99.4	0
14	0.9	99.1	0	0.9	99.1	0
16	2.9	97.1	0	0.9	99.1	0
24	1.6	98.4	0	1.2	98.8	0
48	1.0	99	0	0.7	99.3	0
72	1.2	98.8	0	0.7	99.3	0
120	0.8	99.2	0	0.4	99.6	0

Table 4.5: *C.blakesleeana*

Time (hr)	% 11βC mycelium	% 11βC medium	% 11βC filter	% 11αC mycelium	% 11αC medium	% 11αC filter
15	2.7	97.3	0	trace	-100	0
18	2.3	97.7	0	trace	-100	0
20	3.7	96.3	0	trace	-100	0
24	2.2	97.8	0	trace	-100	0
48	1.2	98.8	0	trace	-100	0
72	0.7	99.3	0	trace	-100	0
120	13.9	86.1	0	trace	-100	0

Table 4.6 *C.elegans*

Time (hr)	% 11βC mycelium	% 11βC medium	% 11βC filter	% 11αC mycelium	% 11αC medium	% 11αC filter
21	2.3	97.7	0	2.0	98.0	0
24	1.8	98.2	0	1.9	98.1	0
27	3.5	96.5	0	2.7	97.3	0
34	3.2	96.8	0	2.1	97.9	0
48	2.1	97.9	0	2.4	97.6	0
72	4.1	95.9	0	2.8	97.2	0
120	3.6	96.4	0	3.4	96.6	0

A.spinosa, *C.blakesleeana* and *C.elegans* were grown at 200 rpm, 30° for 120 hr, in medium A containing 1.1mM cortexolone in 2% (v/v) MeoH. Whole flasks were removed at intervals for analysis. Fungal mycelia was separated from culture medium by filtering onto gouch crucibles. 11βC and 11αC were detected and quantified using hplc, by their absorbance of light at 254nm.

for the separation of cell mass and medium when fungal morphology is in the pellet rather than the mycelial form.

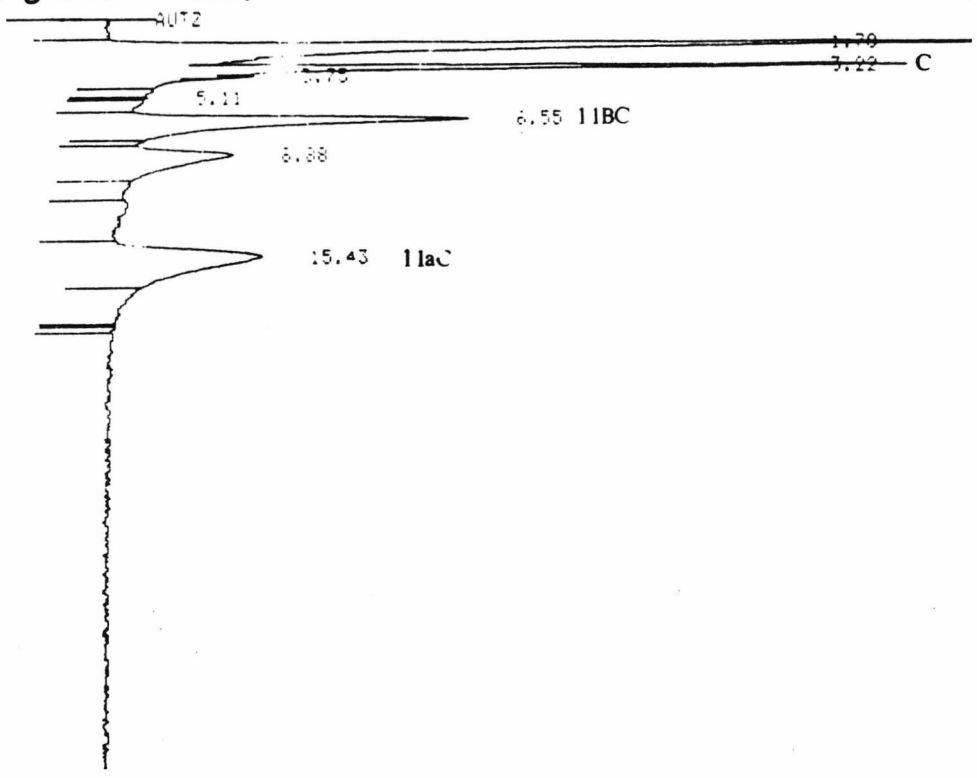
In *Cunninghamella elegans*, which grew more slowly than the other two organisms, the amount of cortexolone detected in association with the filter was much higher (Table 4.3). The possible significance of this will be discussed in Section 4.3.1. Between 16-21.5% of the total cortexolone recovered was retained by the filter between 21-27 hr of growth in this medium (Table 4.3). This dropped to 1.4% by 34 hr and continued to fall throughout the fermentation to 0% by 120 hr. This fall in the amount of cortexolone retained by the filter, can also be followed from Fig. 4.22. No transformation products were detected in association with the filter, throughout the fermentation in all three organisms (Tables 4.4, 4.5 and 4.6).

In the samples from all three organisms, cortexolone was found associated mainly with the mycelium rather than the medium filtrate (Tables 4.1, 4.2 and 4.3). In *Absidia spinosa* the percentage of total cortexolone recovered from the mycelium ranged from 69-85% throughout the fermentation (Table 4.1). Values ranged from 75-96% and 54-100% for *Cunninghamella blakesleeana* (Table 4.2) and *Cunninghamella elegans* (Table 4.3) respectively.

Cortexolone was not exhausted in the medium by either *Absidia spinosa* or *Cunninghamella blakesleeana* (Table 4.1 and 4.2 respectively). No cortexolone was detected in the medium after 72 hr growth using *Cunninghamella elegans* (Table 4.3). Cortexolone bioconversion to its transformation products can be seen on hplc traces for *A.spinosa* (Figs. 4.19a and 4.19b), *C.blakesleeana* (Fig. 4.20) and *C.elegans* (Fig. 4.21). The above trends for cortexolone mycelial and media fractions were also seen on t.l.c. plates for *Absidia spinosa* (Figs. 4.23 and 4.24), *Cunninghamella blakesleeana* (Figs.4.25 and 4.26) and *Cunninghamella elegans* (Figs. 4.27 and 4.28).

Typical hplc traces of cortexolone bioconversion by *A.spinosa*, *C.blakesleeana* and *C.elegans*.

Fig4.19(a) **A.spinosa (t= 12hr)**



RUN TIME 48.28

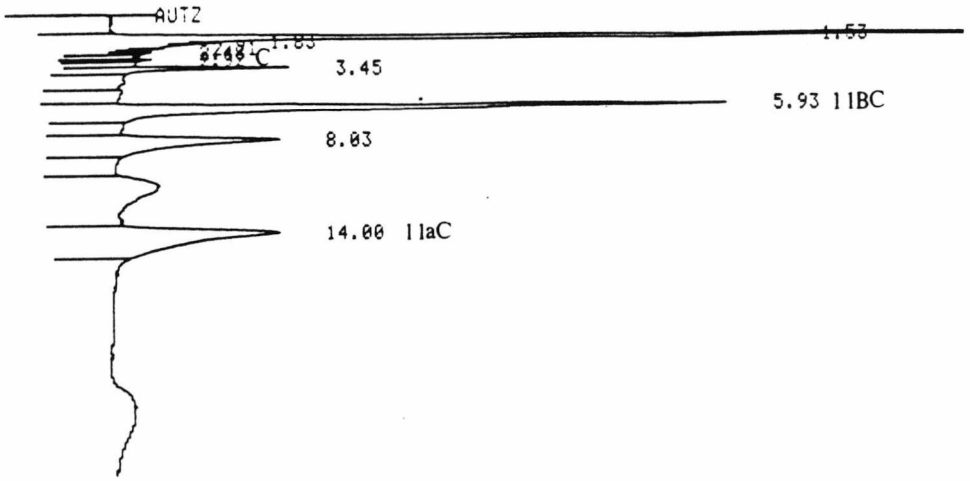
DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
1.78	10843	[illegible]		45.7966085
3.22	6864	[illegible]	SP	14.1468009
3.75	430	[illegible]	S	0.5927811
6.55	3206	[illegible]		17.0268033
8.88	949	[illegible]		7.4468048
15.43	1299	[illegible]		14.9902010
TOTAL		[illegible]		100.0000000

Figures 4.19, 4.20 and 4.21.

A.spinosa, *C.blakesleeana* and *Celegans* were grown at 200 rpm, 30°C for 120 hr, in medium A containing 1.1m M cortexolone in 2% (v/v) MeOH. Whole flasks were removed at intervals for analysis. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254 nm.

Fig4.19(b) A.spinosa(t=40hr)

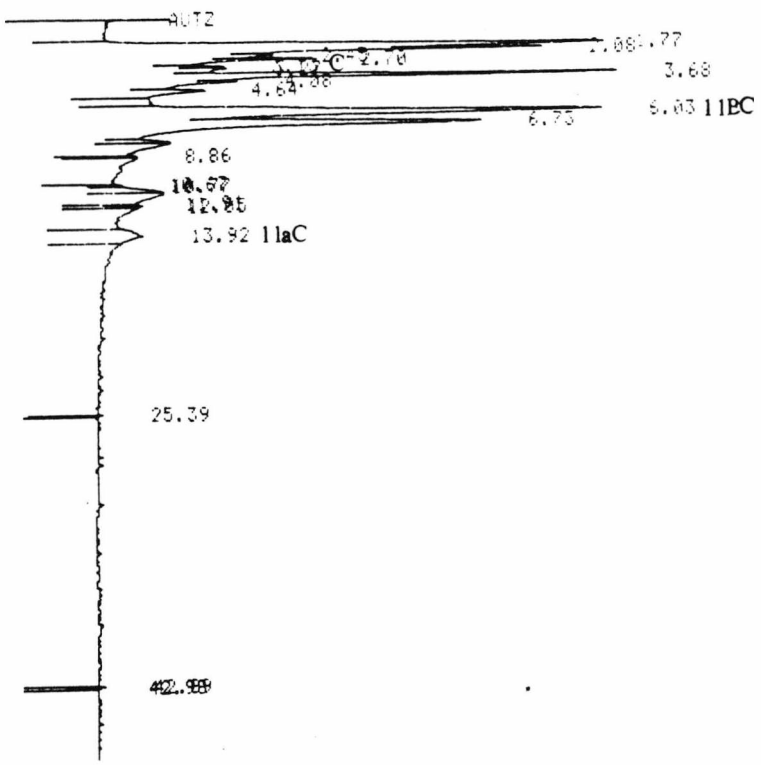


RUN TIME 29.66

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
1.53	177202	1757892		77.1409230
2.48	100	772	S	0.0338773
2.92	167	2031		0.0891256
3.45	1617	28894		1.2679447
5.93	5989	240101		10.5362632
8.03	1551	94807		4.1603804
14.00	1480	154309		6.7714847
TOTAL		2278806		100.0000000

Fig4.20 C.blakesleeana(t=120hr)

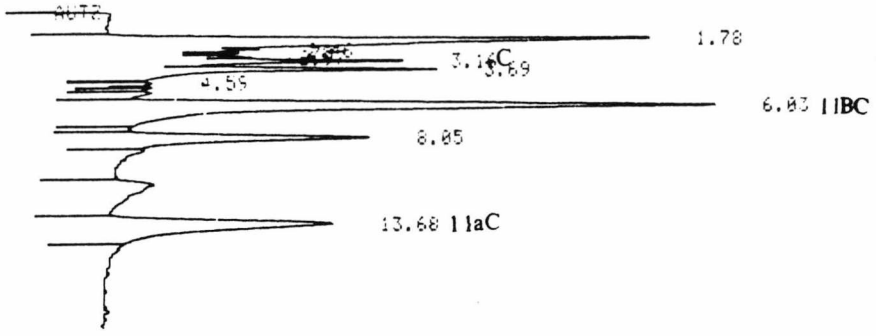


RUN TIME 47.70

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
1.77	4910	172058	P	17.2842530
2.08	4319	111468	P	11.1976260
2.45	1541	39617	P	3.9797641
2.70	2091	55046	P	5.5296993
3.13	983	16062	P	1.6135237
3.27	1019	35572	P	3.5734197
3.68	4930	130717	P	13.1313020
4.08	1009	32387	P	3.2534674
4.64	636	19789		1.9877231
6.03	4415	211262	P	21.2225190
6.75	3315	159397		16.0123800
13.92	254	11549		1.1601659
42.89	80	537	P	0.0539448
TOTAL		995461		100.0000000

Fig 4.21 **C.elegans(t=27hr)**



RUN TIME 20.42'

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
1.78	5385	281456	P	23.6785473
2.46	1372	32714	P	2.7521886
2.72	1135	22896	P	1.9262123
2.97	1227	27054	P	2.2760197
3.16	2940	85483	P	7.1915797
3.69	2941	98529		8.2891236
4.59	64	886		0.0745380
6.03	5776	341588		28.7373785
8.05	2296	114973		9.6725371
13.68	2067	183075		15.4018747
TOTAL		1188654		100.0000000

Tlc plates of steroids extracted from C.elegans mycelia/media and filter fractions and separated using dichloromethane acetone (7:3)(cortexolone as substrate)

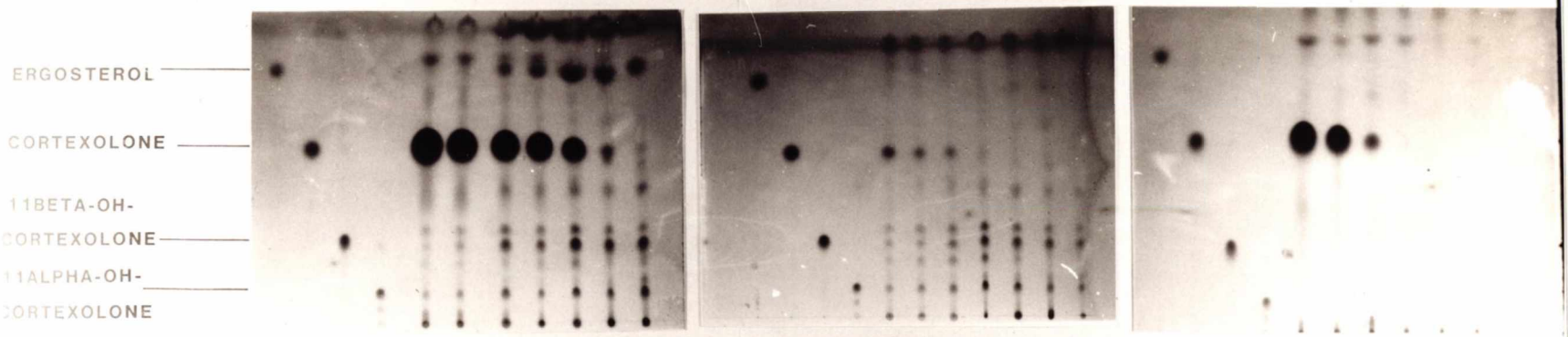


Fig4.27 MYCELIUM

Fig4.28 MEDIUM FILTRATE

Fig4.22 FILTER

Tlc plates of steroids extracted from A.spinosa mycelia and media fractions and separated using dichloromethane:acetone (7:3) (cortexolone as substrate)

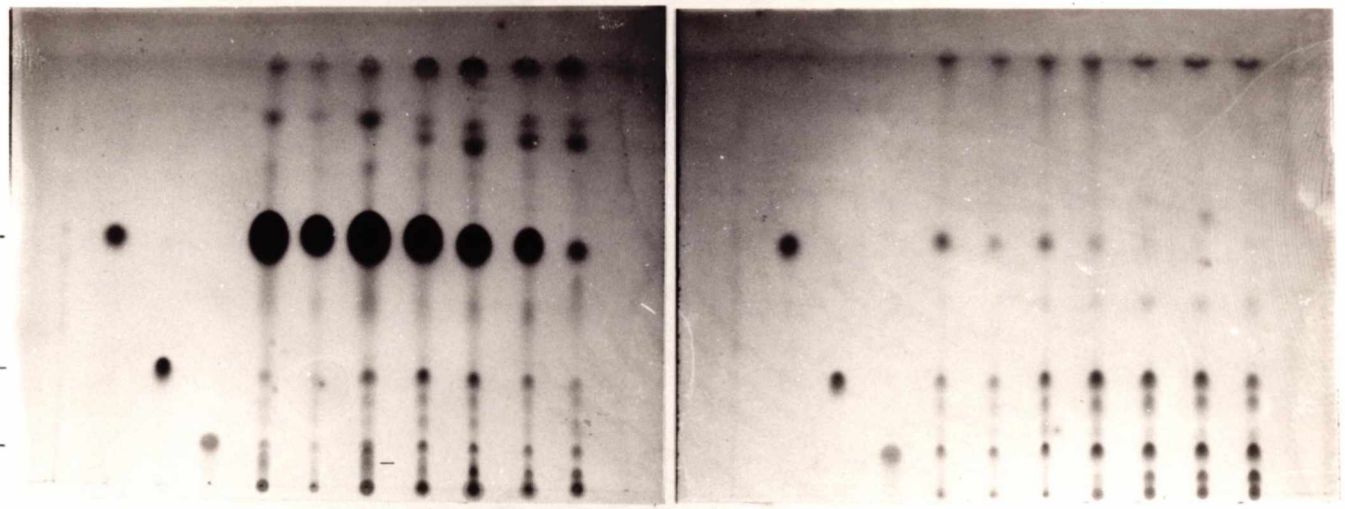


Fig4.23 MYCELIUM

Fig4.24 MEDIUM FILTRATE

Tlc plates of steroids extracted from C. blakesleeana mycelia and media fractions and separated using dichloromethane:acetone (7:3) (cortexolone as substrate)

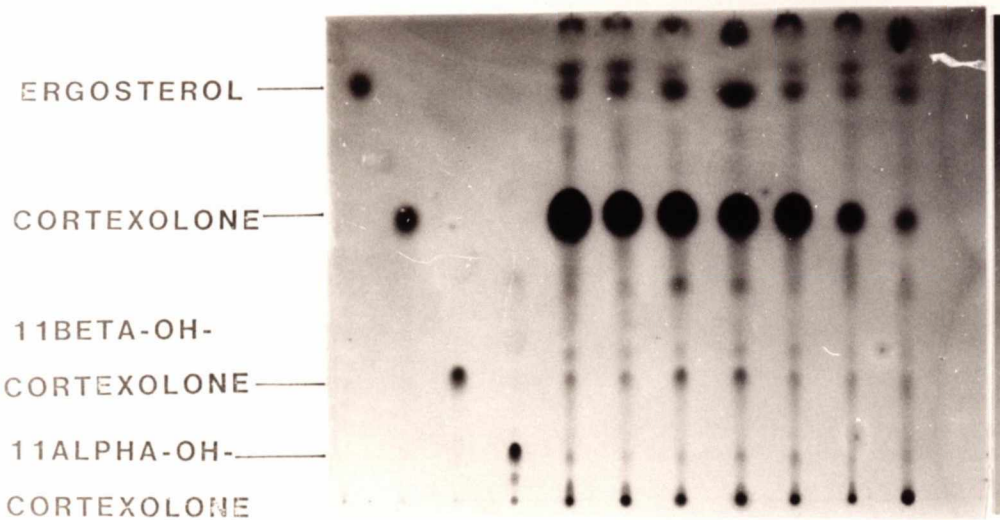


Fig4.25 MYCELIUM

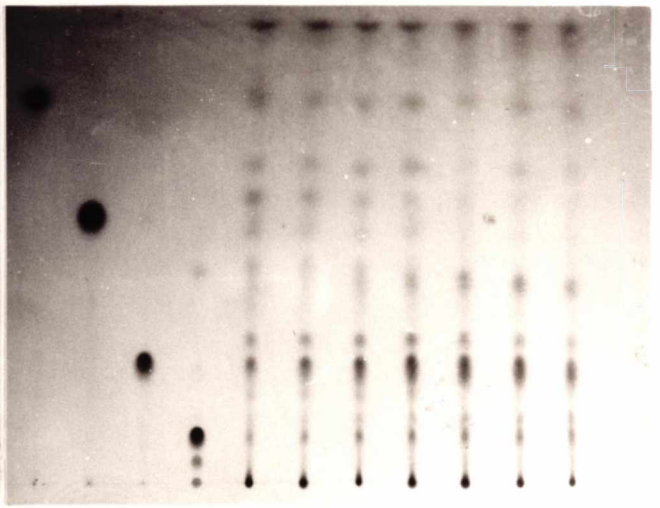


Fig4.26 MEDIUM FILTRATE

4.2.3 Comparison of cortexolone transformation

Changes in cortexolone levels follow the same trend in all three organisms (Figs. 4.16, 4.17 and 4.18). The decrease in transformation substrate in the decline phase by *Cunninghamella elegans* and *Cunninghamella blakesleeana* when products are also declining, shows that steroid metabolism might be taking place - this will be referred to later (Figs. 4.14 and 4.15). With *Absidia spinosa* cortexolone also declines in decline phase due to either metabolism and/or secondary transformation of the primary products formed (Figs. 4.13 and 4.24). The significance of this will be discussed in Section 4.3.1. The fact that cortexolone was not exhausted throughout the period of study could suggest that these systems work quite slowly under these conditions.

4.2.4 Comparison of products produced

T.l.c showed that during exponential growth the concentration of the transformation products (11βC and 11αC) increased in both the mycelial and media fractions as the concentration of cortexolone decreased in *Absidia spinosa*, *Cunninghamella blakesleeana*, and *Cunninghamella elegans* (Figs. 4.23, 4.24, 4.25, 4.26, 4.27 and 4.28 respectively). The concentration of products was greatest when biomass was at its maximum (24 hr, 25 hr and 48 hr for *Absidia spinosa*, *Cunninghamella blakesleeana* and *Cunninghamella elegans* respectively). This coincided with glucose in the medium being exhausted. *Absidia spinosa*, *Cunninghamella blakesleeana* and *Cunninghamella elegans* produced 5, 8 and 6 different transformation products respectively, detected by h.p.l.c and t.l.c of the media fractions. *Cunninghamella blakesleeana* appeared to produce three molecules which are less polar than cortexolone, these could possibly be reduced cortexolone molecules. At least two of the molecules are not found in the mycelial fraction where it might be expected that endogenous

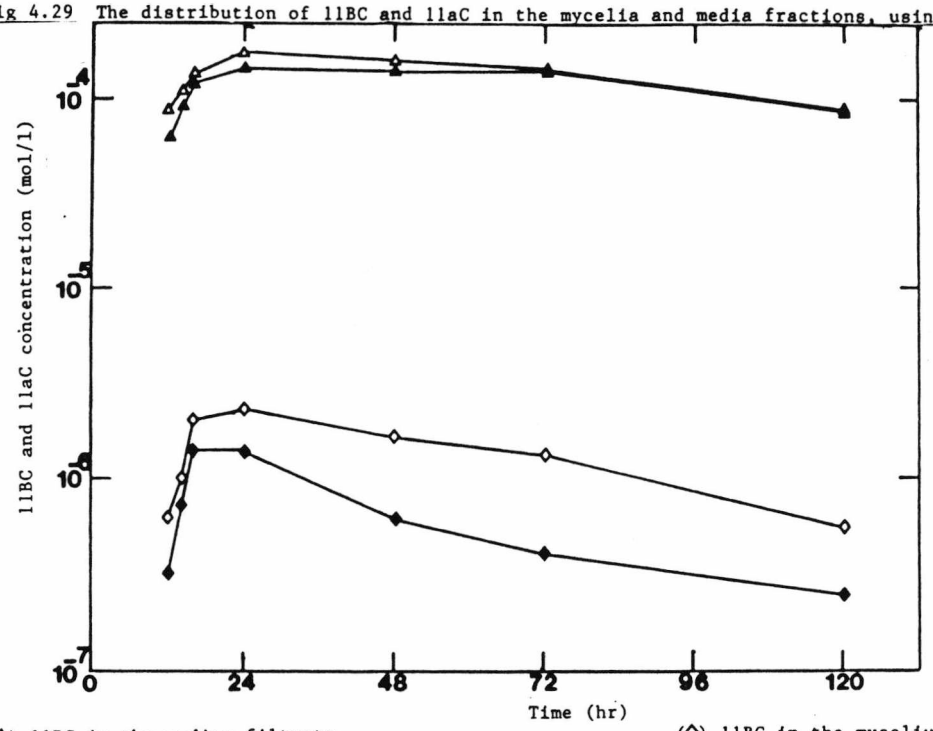
non-polar sterol molecules would occur. Ergosterol could be one of the molecules seen in the mycelium fraction of *Cunninghamella blakesleeana* and *Cunninghamella elegans* (Figs. 4.25 and 4.27 respectively).

4.2.5. Comparison of the distribution of 11βC and 11αC between mycelia and media fractions

No transformation products were found associated with the filter at anytime during the period of study. In all three organisms the majority of 11βC and 11αC was recovered from the medium filtrate:- *Absidia spinosa*, 98.4-99.6% (Table 4.4, Fig.4.29), *Cunninghamella blakesleeana* 86.1-100% (Table 4.5, Fig. 4.30) and *Cunninghamella elegans* 95.9-98.2% (Table 4.6, Fig. 4.31). The percentage of 11βC and 11αC present in association with the mycelium or pellets was found to be much smaller:- *Absidia spinosa* 0.4-2.9% (Table 4.4, Fig. 4.29), *Cunninghamella blakesleeana*, trace-13.9% (Table 4.5, Fig. 4.30) and *Cunninghamella elegans* 1.8-4.1% (Table 4.6, Fig. 4.31). For these three organisms an increase in transformation products in the mycelia led to an increase in the media fractions (Figs. 4.29, 4.30 and 4.31). Similarly it was found that a decrease in 11βC and 11αC after maximum biomass production occurred in both mycelia and media of all three organisms (Figs. 4.29, 4.30 and 4.31). The above trends also occurred with unidentified transformation products produced by all three organisms (results not shown). The transformation of cortexolone to various products occurred irrespective of the morphology of the organisms in liquid culture.

Once maximum growth of *Cunninghamella blakesleeana* and *Cunninghamella elegans* was attained the products were metabolized along with the transformation substrate cortexolone (Figs. 4.14 and 4.15 respectively). In *Absidia spinosa* at least two secondary transformation products were produced by 48 hr (Figs. 4.19b and 4.27). This occurred even when cortexolone and the transformation products were in decline (Figs. 4.16 and 4.27).

Fig 4.29 The distribution of 11BC and 11aC in the mycelia and media fractions, using A.spinosa

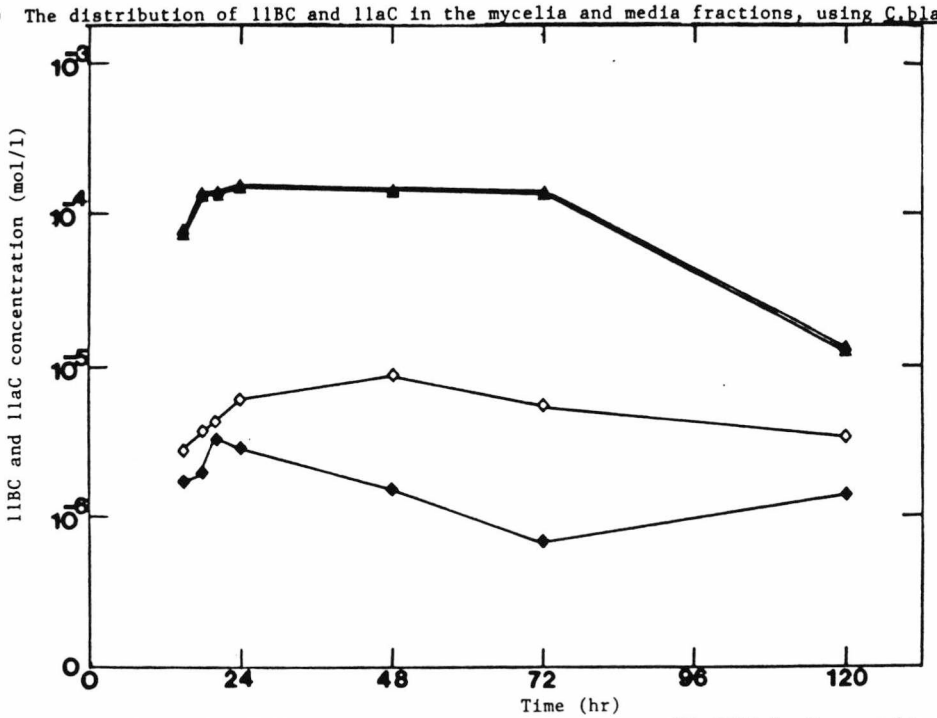


(△) 11BC in the medium filtrate
(▲) 11aC in the medium filtrate
(◇) 11BC in the mycelium
(◆) 11aC in the mycelium

Figs 4.29, 4.30 and 4.31

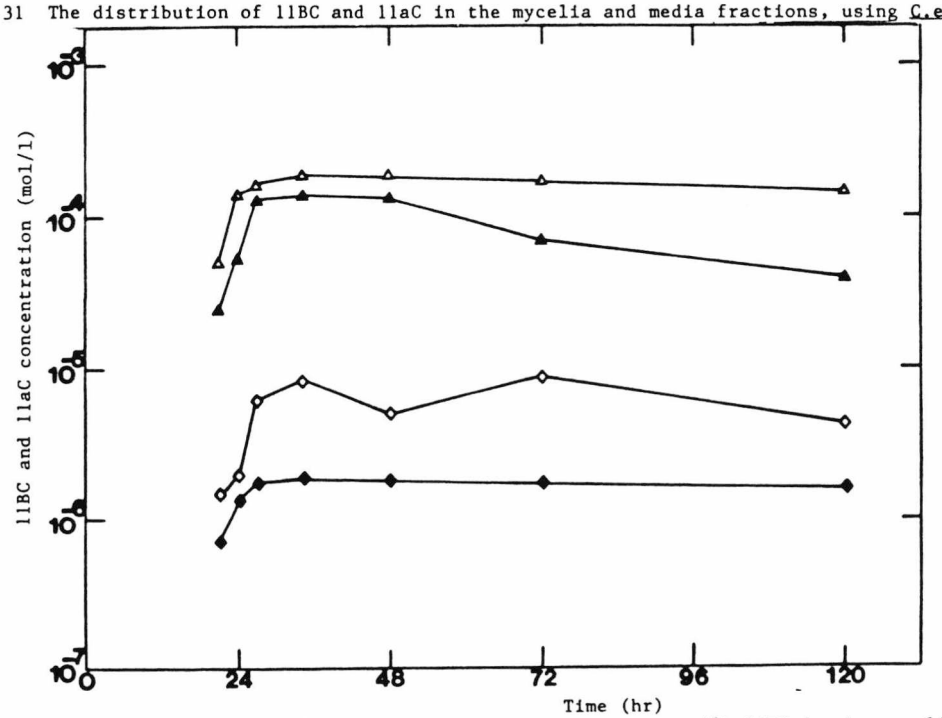
A.spinosa, C.blakesleeana and C.elegans were grown at 200 rpm, 30°C for 120 hrs in medium A containing 1.1mM cortexolone in 2% (v/v) MeOH. Whole flasks were removed at intervals for analysis. Fungal mycelia was separated from culture meduim by filtering onto gouch crucibles. IIBC and IIaC were detected and quantified using hplc, by their absorbance of light at 254nm.

Fig 4.30 The distribution of 11BC and 11aC in the mycelia and media fractions, using C.blakesleeana



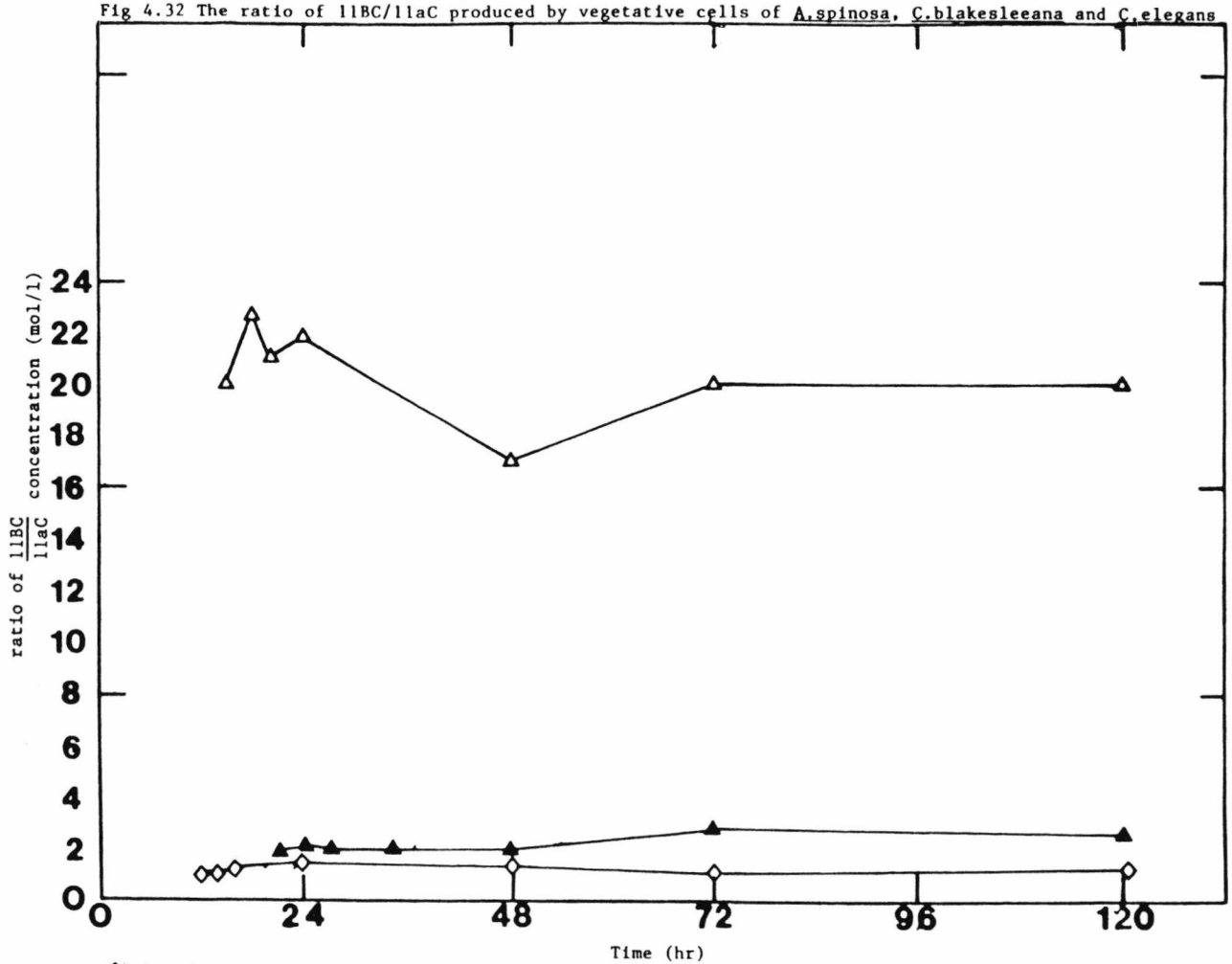
(△) 11BC in the medium filtrate
(▲) 11aC in the medium filtrate
(◇) 11BC in the mycelium
(◆) 11aC in the mycelium

Fig 4.31 The distribution of 11BC and 11aC in the mycelia and media fractions, using C.elegans.



(△) 11BC in the medium filtrate
(▲) 11aC in the medium filtrate
(◇) 11BC in the mycelium
(◆) 11aC in the mycelium

Fig 4.32 The ratio of 11BC/11aC produced by vegetative cells of A.spinosa, C.blakesleeana and C.elegans



4.2.6 Comparison of the production of 11βC and 11αC

The production of both 11βC and 11αC remains unchanged during growth (Figs. 4.16 and 4.17) for *A.spinosa* and *C.blakesleeana*. The amount of product formed per unit biomass decreased in vegetative growth for *C.elegans*. Production of both epimers was greatest during growth when all viable spores had germinated, and only declined in *C.elegans* cultures. The production of both 11βC and 11αC in stationary/decline phase in all three organisms was the same as that seen during growth. The production of 11βC varied between the three organisms. *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans* produced 2.66 $10^{-5} \pm 1.1\ 10^{-6}$, 1.48 $10^{-5} \pm 1.3\ 10^{-7}$ and 8.3 $10^{-5} \pm 3.8\ 10^{-6}$ mol/g of 11βC respectively by 24 hr (Figs. 4.16, 4.17 and 4.18 respectively). This was also the case with 11αC: *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans* produced 1.76 $10^{-5} \pm 8.6\ 10^{-6}$, 6.74 $10^{-7} \pm 1.3\ 10^{-7}$ and 3.77 $10^{-5} \pm 2.1\ 10^{-8}$ mol/g respectively by 24 hr (Figs. 4.16, 4.17 and 4.18 respectively). The ratio of 11βC:11αC was relatively constant throughout the fermentation (Fig. 4.32). In *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans* the average ratio of 11βC:11αC was 1.3 ± 0.12, 20.5 ± 1.3 and 2.2 ± 0.30 respectively (Fig. 4.32). These distinct but closely linked activities will be discussed in Section 4.3.1. The difference in the ratio of 11βC to 11αC in Chapter 3, compared to that encountered in this comparative study, can possibly be attributed to a different batch of mycological peptone.

4.3.1 DISCUSSION

The three organisms tested: *Absidia spinosa, Cunninghamella blakesleeana* and *Cunninghamella elegans* can modify cortexolone to a varying number of transformation products, as germinating spores or as mycelia growing loosely or in pellet form. The initial lag phase seen in cortexolone

transformation in the spore germination experiments occurred in all three organisms. This corresponds to the germinating phase in which morphological and physiological changes occur within the confines of the spore wall (Vezina *et al* 1968), this is associated with spore swelling. No activity towards cortexolone however was seen in the presence of swelling spores, only in the presence of swollen spores and spores which had produced germ tubes. Vezina *et al* (1968) has defined germination itself as the act of germ tube protrusion. The data would suggest that this active growth phase in germination is primarily responsible for cortexolone modification, as more cortexolone is transformed as progressively more spores produced germ tubes. Peak product production was seen when the majority of spores had produced germ tubes.

The production of differing numbers of product from the same substrate (cortexolone) in these three different organisms, is further evidence as to the possible presence of different enzyme systems being active, even in germinating spores. The spectrum of products produced from germinating spores was the same as that seen in mycelia or pellet form when glucose was in excess, this will be commented on later. The fall in cortexolone medium concentration was associated with germ tube elongation in *Absidia spinosa* and *Cunninghamella blakesleeana*.

The difference in cortexolone transformation between *Absidia spinosa*, *Cunninghamella blakesleeana* and *Cunninghamella elegans* could reflect several things. There could for example be differing amounts of enzymes in each organism, although there is no direct evidence to support this. An important control factor is the low solubility of steroids in aqueous medium. This may cause limited access of substrate (cortexolone) to the reaction site (Sedlaczek *et al* 1981). Another governing factor is transport into the cell and the complexity of the spore wall. Cell envelopes can be selective in influencing the permeability of certain exogenous chemicals. (Sedlaczek *et al* 1981). It is known that spore walls of *Cunninghamella* species are multilayered. The fact

that *Cunninghamella elegans* exhibits a lower level of 11β– hydroxylation than *Absidia spinosa* could be partially explained by reduced permeability of the spore walls. This is supported by Hawkers findings (1970) who showed by electron microscopy that Cunninghamellaceae such as *Cunninghamella elegans* have very complex thick spore envelopes. It could initially reduce their permeability for steroid molecules. Some fungi such as *Curvularia lunata* produce spores which appear not to allow steroid transformation outside the vegetative form (Ohlson *et al* 1980). The explanation given was that the hydroxylases were absent. It is probable that it could also be explained by the nature of the spore wall.

It has been shown that chemicals like dilute KOH and EDTA or Helix Pomatia digestive enzymes can increase cortexolone hydroxylation up to four fold as these can loosen the rigid structure of the spore walls, and increase their permeability for the transformation substrate (Jaworski *et al* 1982). The increased permeability of cells for steroids and other chemical compounds is also made possible by treating microorganisms with organic solvents (Buckland *et al* 1975; Jaworski *et al* 1982). The chemical components in the cell wall include phospholipids and esters.

Induction in germinating spores has not been clearly shown as previous authors like Jaworski *et al* (1985) have used cycloheximide on germinating spores as proof of induction. It is well known that this chemical is wide spread in its inhibition of protein synthesis and is toxic to living eukaryotic cells. It is therefore possible that so-called uninduced cycloheximide-treated cells, which show a low loss of cortexolone could be dying. It was observed that as far as cortexolone modification is concerned the change in biomass and glucose concentration are important. This has been supported by the fact that in glucose limited cultures, increased hydroxylation rates were observed with increased glucose consumption rates (Clark *et al* 1983). These initial results suggest that the enzymes involved may be growth associated in some way. All

three organisms shared the same trends in many respects the only difference was the number of products formed and the amount of 11βC and 11αC produced relative to each other.

Both 11β- and 11α- hydroxylations follow the same pattern in growing cultures of *Absidia spinosa*, *Cunninghamella blakesleeana* and *Cunninghamella elegans*. The activities appear to be quite closely linked. Lin and Smith (1970b) demonstrated that a strain of *Curvularia lunata* (NRRL 2380) exhibited 14α-, 11β- and 10β hydroxylase activities, which were closely related even at different temperatures (4-28°C). The observed K_m for each hydroxylase was 0.34 mM.

A number of non-polar compounds were seen in the medium fraction from *Cunninghamella blakesleeana* cultures, these could represent reduced cortexolone products which may or may not be hydroxylated. This would suggest the existence of hydroxysteroid dehydrogenases. This has been detected in *Apiocrea chrysosperma* where progesterone was reduced in the B ring (Smith *et al* 1988). 17 and 20β- hydroxy steroid dehydrogenases were also detected in the filamentous fungus *Cochliobolus lunatus* (Plemenitas *et al* 1988 and Horhold *et al* 1986, respectively). 20β- hydroxysteroid dehydrogenase was found to be separate from the 11β- hydroxylase when cortexolone was used as substrate, no 11α- hydroxylation was reported. Both hydroxysteroid dehydrogenases were found to be microsomal membrane bound and constitutive (Plemenitas *et al* 1988; Horhold *et al* 1986). This implies these enzymes are ideally located for interacting with the hydroxylase systems. Other hydroxysteroid dehydrogenases may be present, but have not been reported.

The growth of vegetative cells was also found to influence cortexolone transformation. When growth ceases then all so-called primary growth transformation ceases, suggesting that active cell growth was required for these primary transformation reactions, this will be discussed later. Harmful aromatic and polycyclic compounds which enter the cell, must be transformed

to more polar molecules which are readily aqueous soluble and therefore once removed do not readily re-enter the cell. This will be discussed further in the next Chapter. The data show that this does occur in all three organisms regardless of morphology. When glucose was exhausted product molecules are able to re-enter the cell in significant amounts. This could suggest that energy dependant processes i.e. active transport of molecules out of the cell or other barriers to steroid re-entry, may breakdown. Alternatively one could say that this observation is a consequence of cell death and decline, which allows these molecules into the cell for energy purposes. Clark *et al* (1983) has postulated that glucose metabolism serves to regenerate NADPH, and hence will indirectly regulate hydroxylase activity. It was proposed that this could be supported by the knowledge that fungal spores which perform steroid hydroxylations, require an energy source (Vezina *et al* 1968). If this hypothesis is true it could account for product decline which occurred after glucose exhaustion as reducing equivalents may no longer be abundant. It may be seen in the Cunninghamellaceae where the steroid carbon skeletons can provide energy when carbon is exhausted. Clark *et al* (1983) demonstrated that the absence of glucose causes 11β- and 11α-hydroxylation to cease in *Pellicularia filamentosa* as was observed in the three organisms used in this study. The data presented show clearly that glucose exhaustion does not halt steroid transformation in *Absidia spinosa*. The aforementioned observation has not been reported previously. *Absidia spinosa* can further transform the primary hydroxylated molecules to secondary products which are even more polar and water soluble than the parent molecules and this act may reduce stress on the organism. The non-growth dependent transformation of cortexolone derivatives may be due to enzymes normally involved in natural growth processes in conditions of glucose exhaustion and this will be expanded on in Chapter 5. The difference seen between the *Cunninghamella* species tested and *Absidia spinosa* may be associated with their morphology. The former grew as fine pellets the latter as

loosely filamentous mycelia. The non-growth dependent secondary transformation of cortexolone derivatives in the decline phase of growth, by *Absidia spinosa*, has not been reported previously, neither has the suggestion that it could be a function of filamentous morphology in liquid culture. These secondary transformations, one would assume, would involve di- and tri-hydroxylations. This was seen in *Apiocrea chrysosperma* (Smith *et al* 1988). This organism was grown for seven days in the presence of progesterone and produced loosely filamentous mycelia. No mention is made of any degradation of steroids, but a catalogue of multiple hydroxylations involving mainly double hydroxylations were observed. The production of secondary transformation products from progesterone was also demonstrated by Somal and Chopra (1985), but it seems they are unaware of the significance of their data. The progesterone concentration was increased from 5 to 40 g/l in a culture broth in which a mutant of the *Aspergillus ochraceus* was growing and monitored transformation over a 120 hr period. Increasing progesterone concentration produced greater amounts of a dihydroxy progesterone, as 11α- hydroxy progesterone decreased. However no growth curve is presented and no correlation between progesterone bioconversion and growth was made. The h.p.l.c traces in this study revealed at least two molecules produced from the primary products, in fact the peaks were so broad one could imagine more than two molecules being produced in the decline phase. As the other products could not be identified, it was not possible to investigate whether they were conjugated to other molecules such as sulphates which would increase their polarity. It is known that *Cunninghamella elegans* (when it grows filamentously) can conjugate benzo(a)pyrene to sulphates (Cerniglia and Gibson 1979). This reaction is observed in higher organisms such as mammals (Kauffman 1987).

Steroid degradation has been well studied and elucidated in bacteria (Sih and Bennett 1962b; Sih *et al* 1966; Jerussi 1965; Abul-Hajl 1972). The pathway for

ring degradation in filamentous fungi has not been investigated with as much rigour, probably due to the difficulties of working with fungi. Steroid degradation has however been reported in some filamentous fungi such as *Aspergillus ochraceus* (Tan and Falardeau 1970). They reported that progesterone could be degraded as well as modified, but the stage of growth where this occurs has not been reported. Degradation is possible via the A or B ring or side-chain. The A ring is preferred by bacterial species and A or B ring degradation is preceded by reduction. The data show that reduced cortexolone derivatives may be possible. Ring A dehydrogenation has been reported in testosterone by the fungus *Phycomyces blakesleeanus* (Smith *et al* 1989). The reduction of the B ring has also been reported in *Apiocrea chrysosperma*. It cannot be said from the data that reduction of the A ring precedes its degradation. It is clear however that the A ring is attacked in these organisms, as the products formed are no longer detected via their double bond ketone conjugate. The possibility of side chain degradation cannot however be eliminated. One wonders if the transformation substrate (cortexolone), can itself be degraded in exponential growth, this will be discussed in Chapter 5.

The product profiles for 11βC and 11αC in *Cunninghamella elegans* and *Cunninghamella blakesleeana* appears to follow the same patterns as cytochrome P-450 levels in *Saccharomyces cerevisiae* (Blatiak *et al* 1980; 1985a; 1985b). These workers have reported that the accumulation of cytochrome P-450 in *Saccharomyces cerevisiae* is associated with growth, and the enzyme levels begin to decline when rapid growth ends. This could account for the decline in cortexolone transformation in *Cunninghamella elegans* and *Cunninghamella blakesleeana*. Blatiak *et al* (1980) have also reported that oxygen, a substrate for the monoxygenase systems, is required for cytochrome P-450 degradation. In *Absidia spinosa* it is possible that the hydroxylation process is more complex in this organism, with the 'switching on' of 'steroid active' enzymes, when exponential growth has ended.

Steroid molecules such as testosterone can be metabolized and components detected in cell protein, cell nucleic acid and tricarboxylic acid intermediates such as succinate, citrate and α-ketoglutarate, as well as carbon dioxide in *Pseudomonas* species (Santer and Ajl 1952). It may be that this could also apply in filamentous fungi. Further experimentation with radio labelled ^{14}C is needed to find the distribution of activity in various products. Aliphatic hydrocarbon chains can be degraded and assimilated by bacteria (Sih *et al* 1968) and filamentous fungi (Wyatt 1984). Carbon side chain degradation has been shown in filamentous fungi, such as *Mucor, Pencillium* and *Aspergillus* etc. (Rahim and Sih 1966). They proposed that side chain degradation of progesterone and 17α- hydroxyprogesterone (Fig. 4.33), by *Cylindrocarpon radiciola* proceeded via two routes:

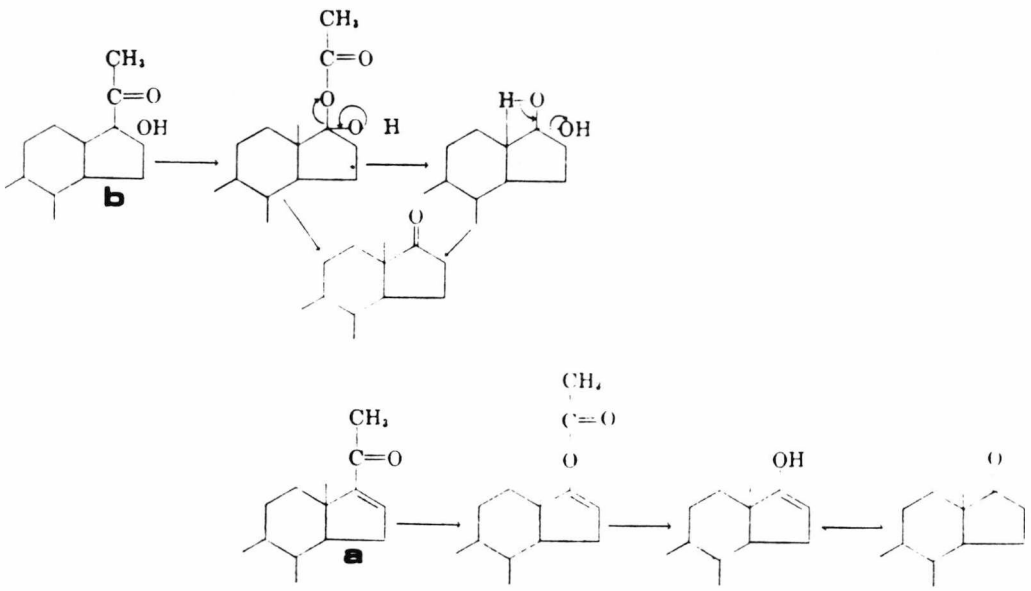
i) Oxygenation of 17α- hydroxy progesterone to yield 17α- hydroxy testosterone acetate, which could then undergo non-enzymatic rearrangement to lose the acetate, and give androst-4-ene-3,17,dione.

ii) the ester could alternatively be hydrolysed by an esterase to yield the unstable intermediate, 17α- hydroxytestosterone. Androst-4-ene-3, 17-dione results from the elimination of water.

Rahim and Sih (1966) also reported this pathway using deoxycorticosterone which possesses the same side chain as cortexolone. It would therefore seem plausible to suggest that the cortexolone side chain could be degraded in this manner. This will be discussed in Chapter 5.

The constant ratio of both 11βC and 11αC (Fig. 4.32) may indicate that the 11β- hydroxylase and the 11α- hydroxylase may have similiar activities. The results obtained using the Cunninghamellaceae show there are species differences in the amounts of 11βC produced relative to the 11αC, and this has not been reported previously. One can point out four significant stages in product formation in all three organisms during growth. These overall

Figure 4.33: Proposed side-chain degradation of progesterone(a) and 17α-hydroxyprogesterone(b)



distinctions were seen in all the organisms tested, and to my knowledge, have not been reported previously:

1) Initial 11β- and 11α- hydroxycortexolone production was associated with germ tube emergence during germination.

2) The maximum production of 11β - and 11α- hydroxycortexolone corresponded with the majority of spores producing extended germ tubes.

3) In *A.spinosa* and *C.blakesleeana* 11βC and 11αC was produced in constant amounts relative to biomass. In *C.elegans* 11βC and 11αC was produced in decreasing amounts relative to biomass. The reason for this is not known.

4) Metabolism of 11β- and 11α- hydroxycortexolone to products no longer detected using this system occurs in the decline phase of growth in *C.blakesleeana* and *C.elegans*, but both epimers were further modified to products detected by this system in *A.spinosa*.

CHAPTER 5

INVESTIGATIONS INTO CORTEXOLONE TRANSFORMATION USING *Absidia Spinosa* BY ALTERATION OF SELECTED PARAMETERS

RESULTS

5.1.1 Controlled batch growth experiments

Absidia spinosa was chosen for further investigation for reasons outlined earlier (Chapter 2, Section 2.3.1). Control batch growth experiments, revealed that in the absence of the transformation substrate cortexolone (1.13 mM), in the presence of MeOH (2% v/v), both μ_{max} and t_d were unaffected (0.19 hr^{-1} and 3.6 hr respectively). These findings are similar to those recorded in earlier experiments in the presence of cortexolone (1.13 mM) and MeOH (2% v/v). In the absence of both cortexolone (1.13 mM) and MeOH (2% v/v), μ_{max} was higher and t_d lower (0.4 hr^{-1} and 1.8 hr. respectively), thus showing that the MeOH inhibited growth. Control flasks produced a greater biomass than MeOH or cortexolone/MeOH treated cells (11.93 ± 0.3; 10.51 ± 0.8; 9.5 ± 0.2 g/l respectively) by 24 hr.

There was no significant difference between the various treatments when glucose was utilized from the medium per unit biomass in *A.spinosa* (Fig. 5.1).

No transformation products were detected in *A.spinosa* control experiments in the absence of cortexolone (see Appendix 4).

5.2.1 The use of 11βC as substrate

When 11βC was used as an alternative substrate to cortexolone, it was found that both μ_{max} and t_d were unaffected (0.19 hr^{-1} and 3.6 hr respectively). Maximum biomass was 9.78 ± 0.1 g/l by 24 hr (Fig. 5.2). After maximum

Fig 5.1 Glucose depletion by growing cells of A.spinosa under various treatments.

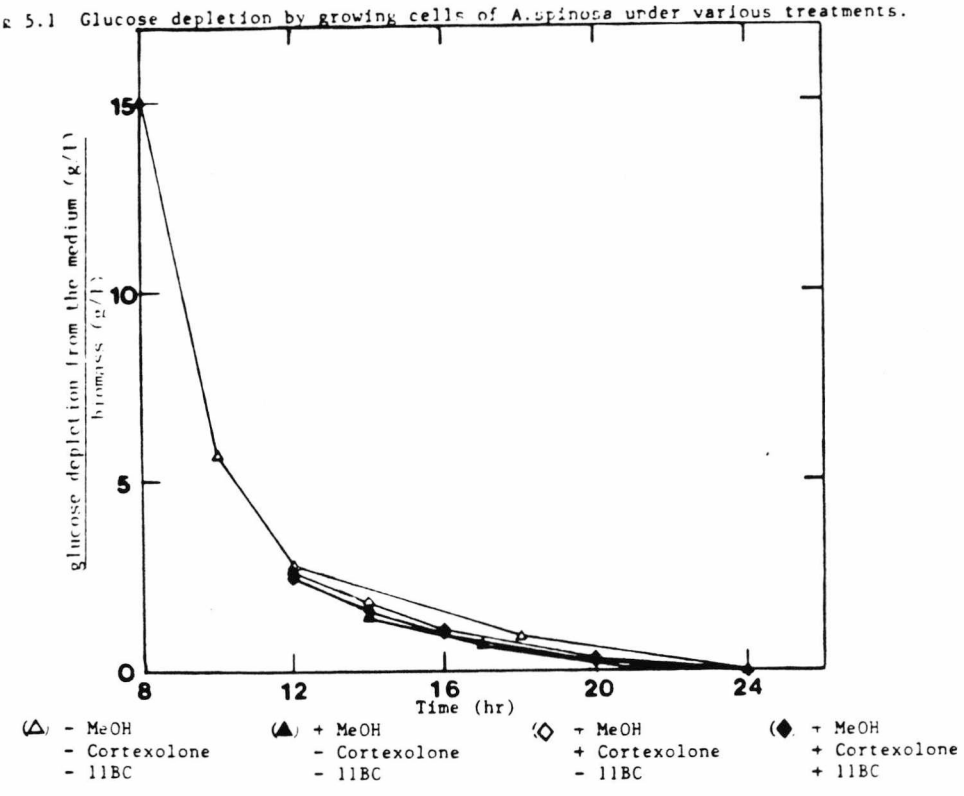
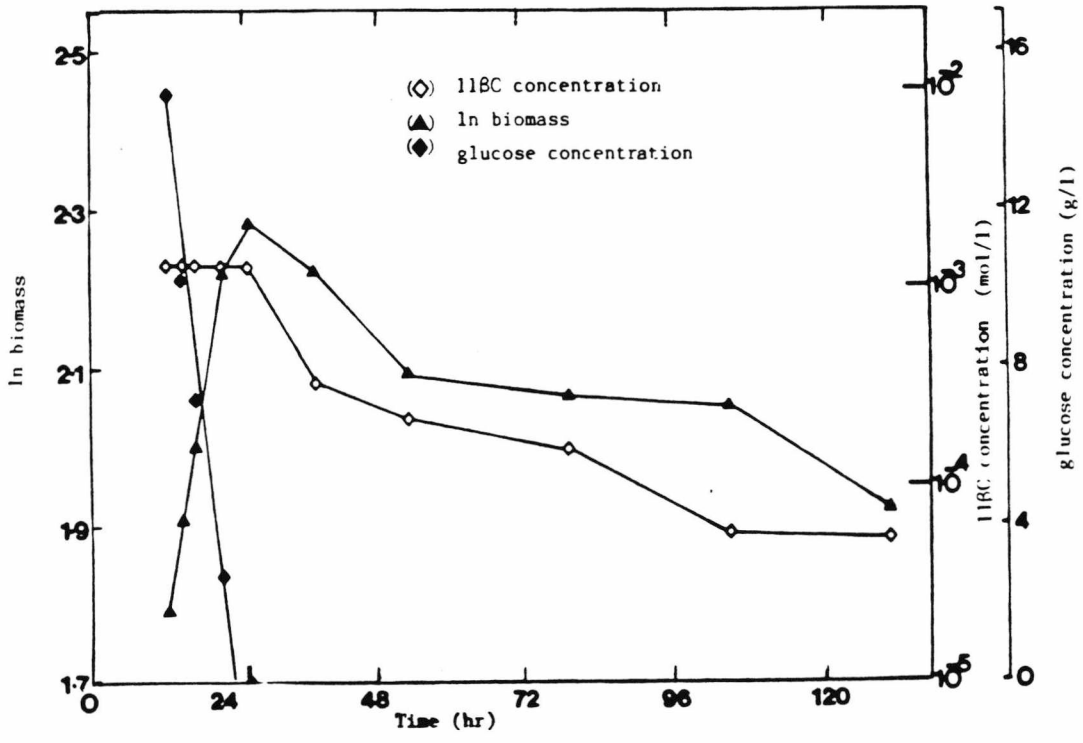


Fig 5.2: 11βC bioconversion and glucose utilization by A.spinosa



Figs 5.1, 5.2

A.spinosa was grown in medium A containing 1.1mM cortexolone or 11βC in 2% (v/v) MeOH at 200 rpm, 30 C for 120hr. Whole flasks were removed at intervals for analysis. Fungal biomass was separated from culture medium by filtering onto gouch crucibles. Cortexolone, 11βC and their bioconversion products were detected and quantified using hplc, by their absorbance at 254nm. Glucose was determined using a glucose assay kit.

Tlc plates of steroids extracted from A.spinosa mycelia and media fractions and separated using dichloromethane:acetone (7:3)(11beta-OH-cortexolone as substrate

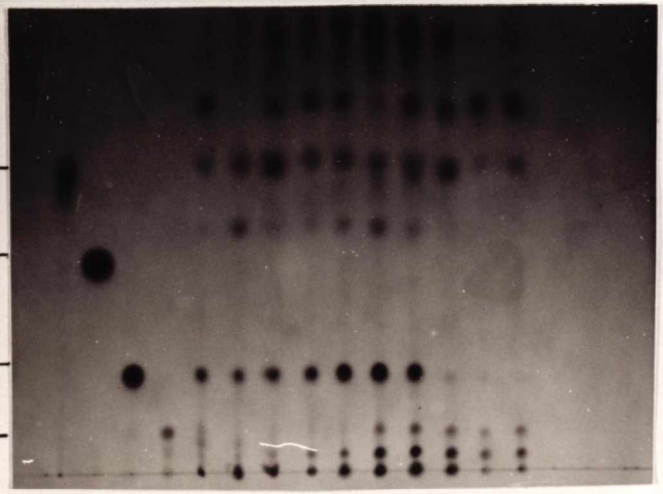


Fig5.3 MYCELIUM

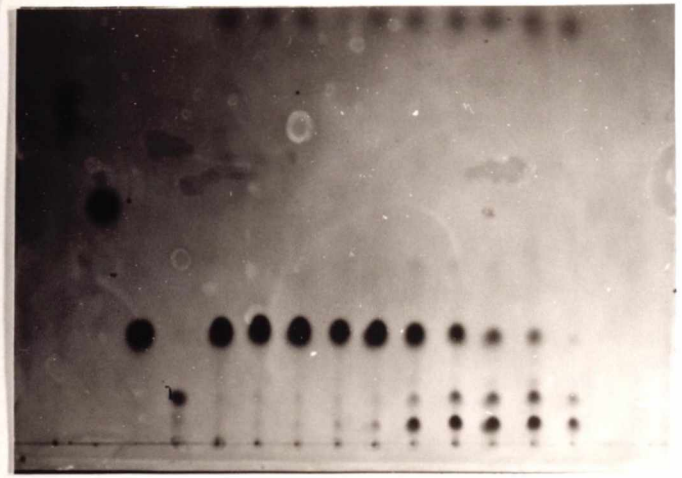


Fig5.4 MEDIUM FILTRATE

Table 5.1: % Distribution of 11βC between mycelia, media and filter fractions using *A.spinosa*

	% 11βC		
Time (hr)	mycelium	medium	filter
12	0.3	99.6	0.1
14	0.4	99.6	0.06
16	0.7	98.1	1.20
20	0.9	99.0	0.02
24	0.5	99.5	0.03
34	1.1	98.9	0.01
48	1.4	98.5	0.1
72	0.4	99.6	0
96	0.6	99.4	0
120	0.5	99.5	0

A.spinosa was grown at 200 rpm, 30° C for 120hr in medium A containing 1.1mM 11βC in 2% (v/v) MeoH. Whole flasks were removed at intervals for analysis. Fungal biomass was separated from culture medium by filtering onto gouch crucibles. Cortexolone was detected and quantified using hplc, by its absorbance of light at 254nm.

biomass was attained, the value declined to 6.83 ± 0.13 g/l by 120 hr (Fig. 5.2). Maximum biomass production corresponded to glucose exhaustion in the medium (Fig.5.2). It can be seen that 98.1-99.6% of the total 11βC recovered was detected in the medium throughout the period of study (Table 5.1). No more than 1.2% of the total 11βC recovered was attached to the filter (Table 5.1). No 11βC was found attached to the filter by 72 hr (Table 5.1). The total concentration of 11βC dropped slightly during logarithmic growth (Fig. 5.2). The transformation of 11βC was initiated in late logarithmic growth (Fig. 5.3). The greatest change in 11βC transformation was seen when growth had ceased and biomass was declining (Fig. 5.2).

At least two major and two minor polar products were detected, with two minor products that were more non-polar than 11βC, (Fig. 5.3). These may be reduced 11βC products which corresponded to secondary products formed in the declining biomass of *Absidia spinosa* (Figs. 5.3 and 5.4), when cortexolone was used as substrate. The secondary products were detected by 20 hr and 34 hr and were seen in both mycelia and media fractions, as 11βC was decreasing (Figs. 5.3 and 5.4).

5.3.1 The growth-independent transformation of cortexolone in decline phase by *Absidia spinosa*

In the presence of cortexolone under conditions of glucose exhaustion *Absidia spinosa* shows a gradual reduction in biomass from 11.4 ± 0.18 to 8.13 ± 0.33 g/l by 48 hr (Fig. 5.5). Cortexolone was still transformed during this period to its primary and secondary transformation products, as can be seen from the mycelia and media tlc plates (Figs. 5.6; 5.7). The distribution of cortexolone between fractions from mycelia, media and filter (Table 5.2) was comparable to that observed in the *Absidia spinosa* batch-growth experiments (Table 4.1).

Fig 5.5 Decline in A.spinosa biomass under conditions of glucose exhaustion in the decline phase of growth.

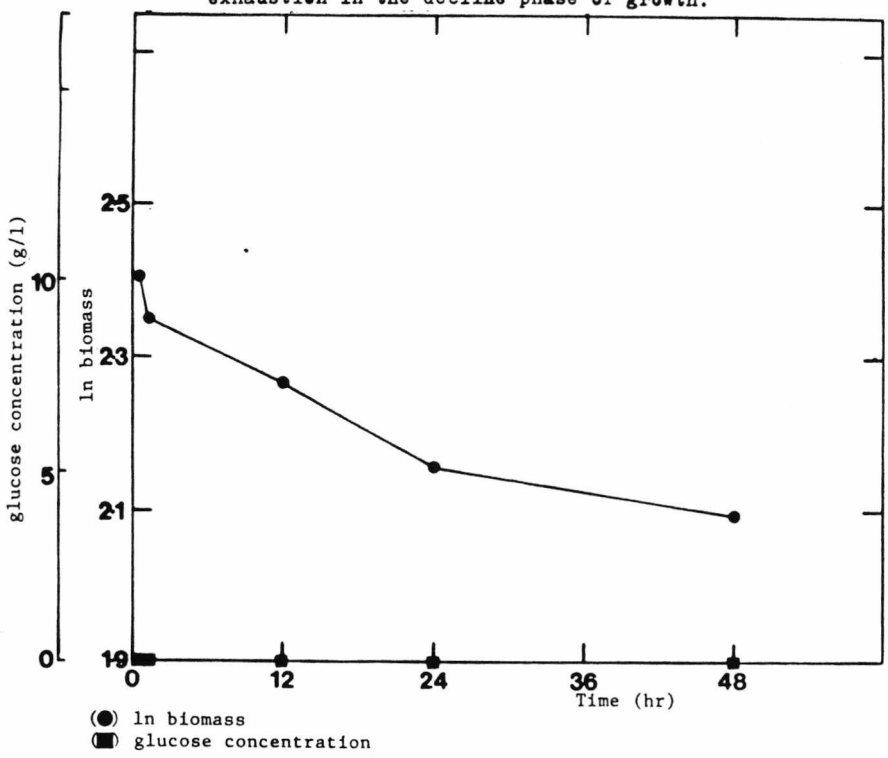


Fig 5.5

A.spinosa was grown in medium A at 200 rpm, 30°C for 48 hr. 1.1mM cortexolone in 2% (v/v) MeOH was then added and growth continued for 48 hr. Whole flasks were removed at intervals for analysis. Fungal biomass was separated from culture medium by filtering onto gouch crucibles. Glucose was determined using a glucose assay kit. Cortexolone and its bioconversion products were detected and quantified using hplc by their absorbance of light at 254nm.

Tlc plates showing cortexolone bioconversion in decline phase by A.spinosa

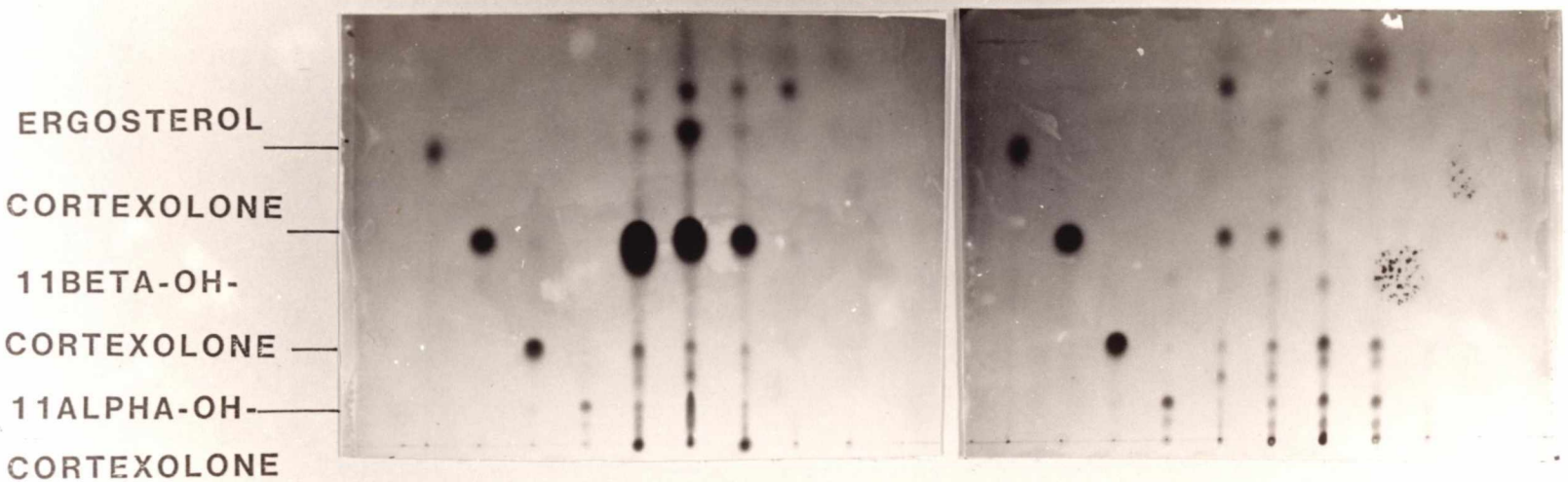


Fig5.6 MYCELIUM

Fig5.7 MEDIUM FILTRATE

Fig 5.8 The distribution of 11BC and 11aC between the mycelia and media fractions of A. spinosa in decline phase

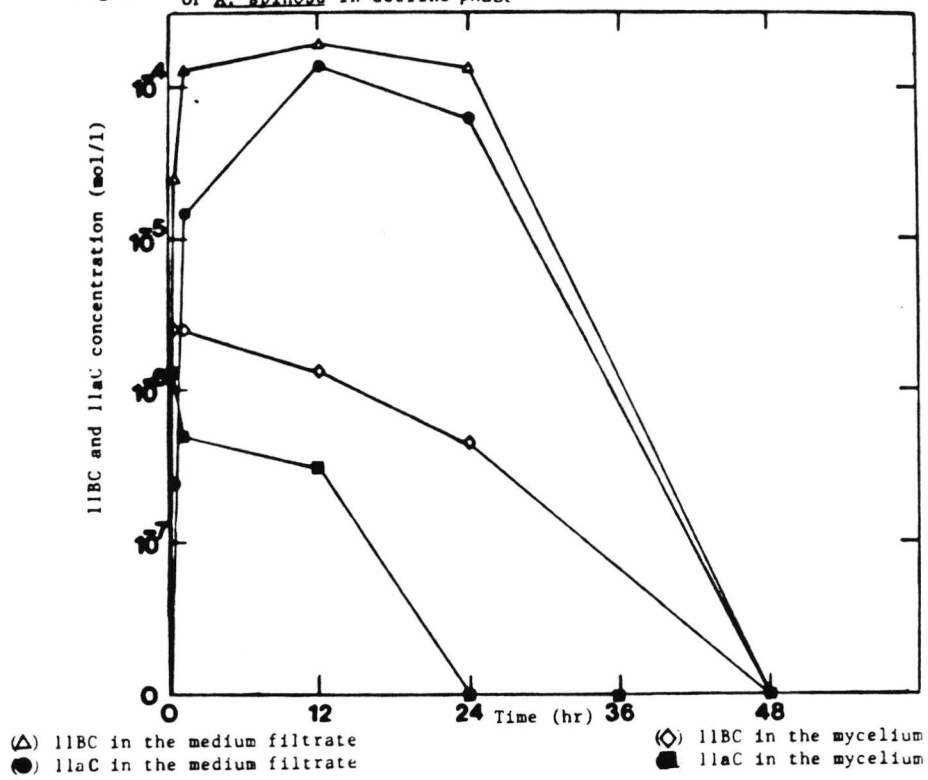
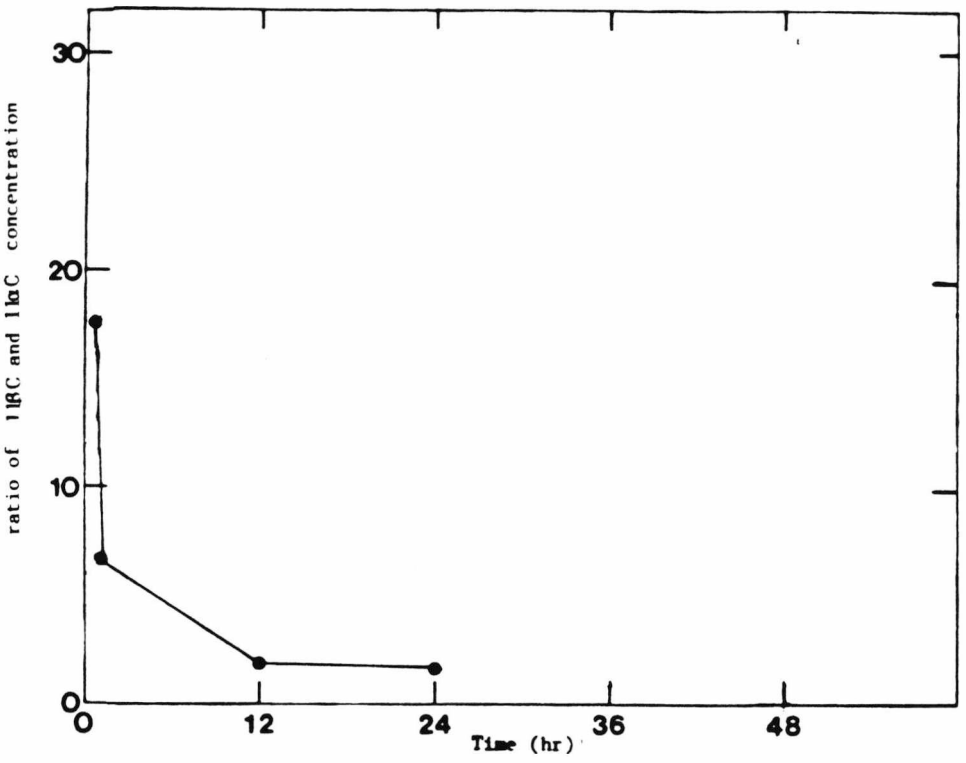


Fig 5.9 The ratio of 11BC/11aC produced in the decline phase by A.spinosa.



Figs 5.8 and 5.9

A.spinosa was grown in medium A at 200 rpm, 30°C for 48hr. 1.1mM cortexolone in 2% (v/v) MeOH was then added and growth continued for 48hr. Whole flasks were removed at intervals for analysis. Fungal biomass was separated from culture medium, by filtering onto gouch crucibles. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm.

% Distribution of cortexolone, 11βC and 11αC between mycelia, media and filter fractions in the decline phase by *A.spinosa*

Table 5.2

	% Cortexolone		
Time (hr)	mycelium	medium	filter
0.5	82.0	17.8	0.2
1	85.4	14.0	0.6
12	87.8	11.1	1.1
24	85.2	13.8	1.0
48	94.2	1.7	4.1

Table 5.3

	% 11βC			11αC		
Time (hr)	mycelium	medium	filter	mycelium	medium	filter
0.5	23.1	76.9	0	100	trace	0
1	3.7	96.3	0	3.8	96.2	0
12	0.4	99.6	0	0.3	99.7	0
24	0.5	99.5	0	0	100	0
48	0	0	0	0	0	0

A.spinosa was grown at 200 rpm, 30°C for 48hr in medium A. 1.1mM cortexolone in 2% (v/v) MeOH was then added and cortexolone bioconversion was allowed to continue for 48 hr. Whole flasks were removed at intervals for analysis. Fungal biomass was separated from culture medium by filtering onto gouch crucibles under vacuum. Cortexolone and its bioconversion products were detected and quantified using hplc by their absorbance of light at 254nm.

The distribution of both 11βC and 11αC between fractions obtained from mycelia, media and filter (Table 5.3; Fig. 5.8) was comparable to that seen in the *Absidia spinosa* batch-growth experiments (Table 4.4; Fig. 4.29). The concentration of both products reached a maximum in the medium by 12 hr, and then decreased (Fig. 5.8). The increase in both 11βC and 11αC between 0.5-12hr in the medium does not correlate with the small fall in both products in the mycelium, over the same period (Fig. 5.8). This could infer that both 11β- and 11α- products were being produced in greater quantities than they were being metabolized or transformed to secondary products. No 11βC or 11αC remained in the growth-independent transformation of cortexolone by 48 hr. This was not the case in the batch-growth experiment where cortexolone remained after 48 hr.

The production of both products was detected after 0.5 hr in the decline phase using hplc. (Fig. 5.8). The ratio of 11βC:11αC reveals that 11βC was initially produced in greater amounts (16.7 fold) than 11αC (Fig. 5.9). This fell to a steady two fold difference by 12 hr and remained at this level for 12 hr, before declining to zero by 48 hr (Fig. 5.9).

5.4.1 The effect of cortexolone concentration on *Absidia spinosa*

It was observed that increasing cortexolone concentration from 0.22 mM to 0.56 mM, increased cortexolone loss without an increase in product concentration (compare Figs. 5.10 and 5.11), but the specific growth rate (μ_{max}) was unaffected (not shown). The concentration of cortexolone began to fall sharply between 1-2 hr after cortexolone addition. When 0.22 mM, 0.50 mM, 1.13 mM and 2.26 mM cortexolone concentrations were used; 0.32%, 3.1%, 38.6% and 75.8% respectively of cortexolone remained at 3 hr. Using 0.22 mM, 0.56 mM, 1.13 mM and 2.26 mM concentrations; 0%, 0%, 28% and 71% cortexolone respectively remained 4 hr after cortexolone addition (Fig. 5.10).

Fig 5.10 BIOTRANSFORMATION OF CORTEXOLONE AT DIFFERENT CONCENTRATIONS BY A.spinosa

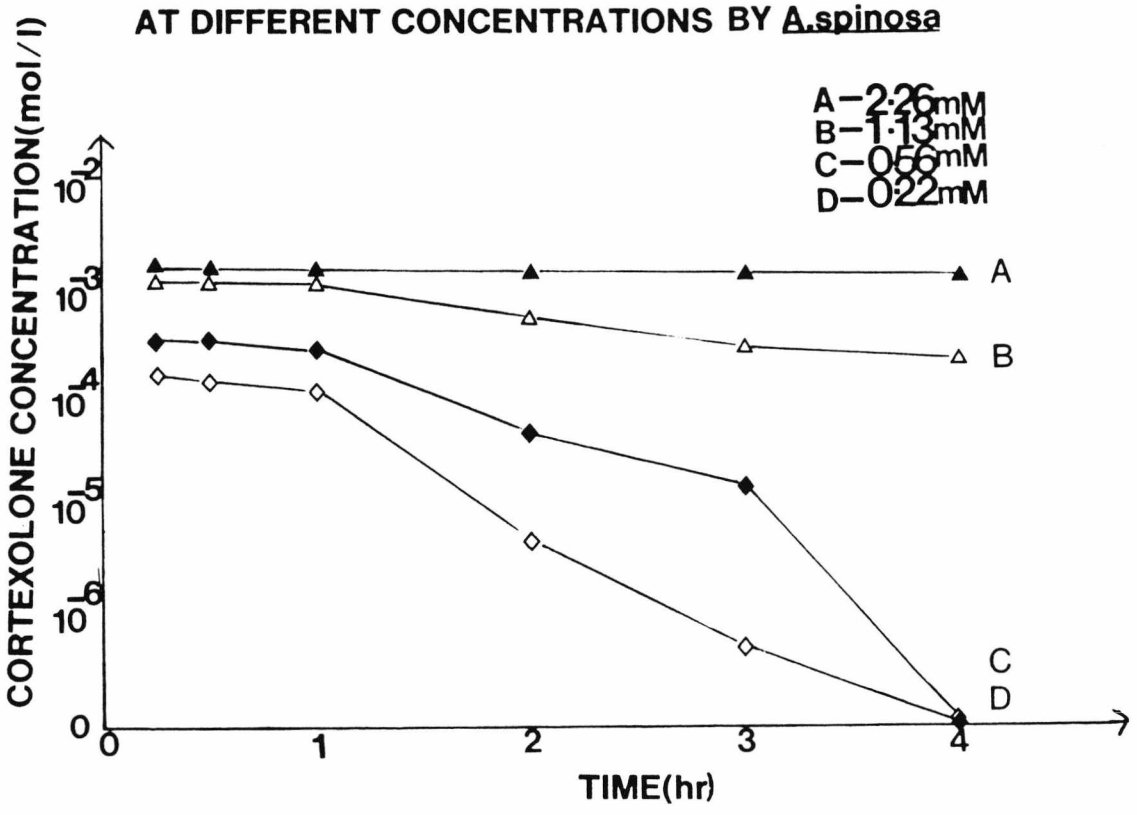


Fig 5.10

A.spinosa was grown at 200 rpm, 30° C for 8hr in medium A. Selected cortexolone concentrations (0.22mM, 0.56mM, 1.13mM and 2.26mM) in 2% (v/v) MeOH (final concentrations) were then added. Samples were removed at intervals for analysis over a 4hr period. Cortexolone was detected and quantified using hplc, by its absorbance of light at 254nm.

Fig 5.11 CONCENTRATION OF 11-BETAHYDROXYCORTEXOLONE AT DIFFERENT CORTEXOLONE CONCENTRATIONS BY A.spinosa

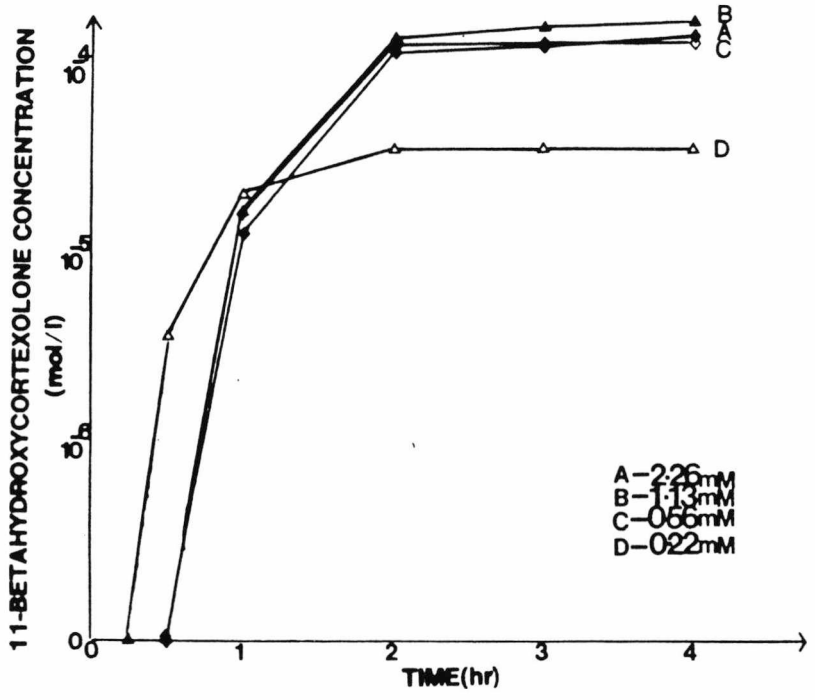
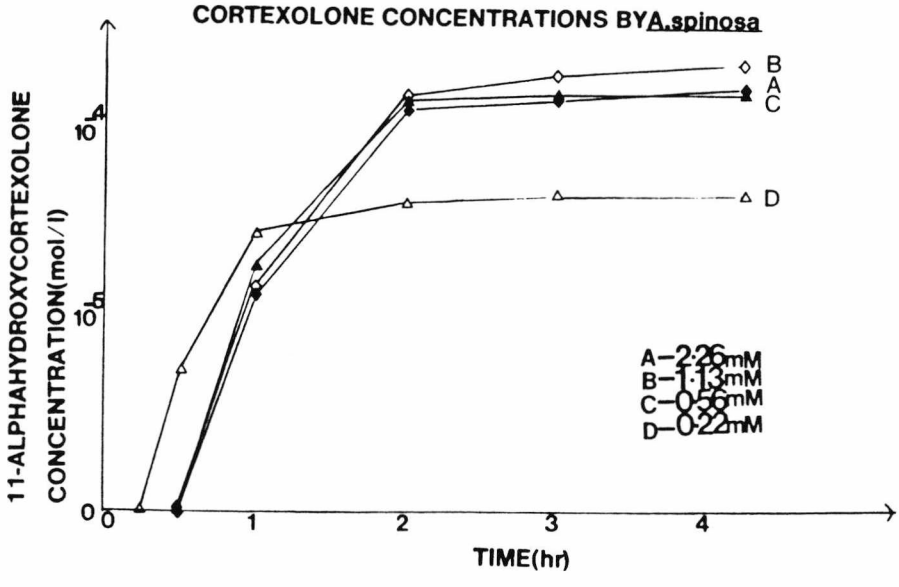


Fig 5.12 CONCENTRATION OF 11-ALPHAHYDROXYCORTEXOLONE AT DIFFERENT CORTEXOLONE CONCENTRATIONS BY A.spinosa



Figs 5.11 and 5.12

A. spinosa was grown at 200 rpm, 30°C for 8 hr in medium A. Selected cortexolone concentrations (0.22mM, 0.56mM, 1.13mM and 2.26mM) in 2% v/v MeOH (final concentrations) were then added. Samples were removed at intervals for analysis over a 4 hr period.
Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm.

No secondary products were seen at any time during the period of study. When using 0.22 mM cortexolone, the concentration of cortexolone remaining continued to fall while the concentration of both 11βC and 11αC did not increase. (Figs. 5.10, 5.11 and 5.12). The production pattern of 11βC and 11αC was similar throughout the period of study (compare Figs. 5.11 and 5.12).

Increasing the cortexolone concentration above 0.22 mM to 2.26 mM does not appear to inhibit the production of both 11βC and 11αC (Figs. 5.11 and 5.12). Initial production of 11βC and 11αC occurred between 0.5-1 hr after cortexolone addition, both products being produced in equal amounts. Using 0.22 mM and 0.50 mM cortexolone. 56% and 60% respectively of the cortexolone finished up as both 11βC and 11αC. Both epimers were no longer produced after 3 hr. Using 1.13 mM and 2.26 mM cortexolone the concentration of both 11βC and 11αC continued to increase 4 hr after cortexolone was added to the medium.

5.5.1 The effect of pH on cortexolone transformation in *Absidia spinosa*

Absidia spinosa produces more biomass at the higher pH values used (Fig. 5.13). There was little difference between cortexolone transformation between pH 4 and 7 (Fig. 5.14). The appearance of 11βC and 11αC occurs between 0.5-1 hr and 1-2 hr respectively (Figs. 5.15 and 5.16). Both forms are produced between pH ranges 3-7 and have similar profiles. The $11\beta^-$ product was detected before the $11\alpha^-$ product, but once produced both epimers occurred in equal amounts throughout the period of study (compare Figs. 5.15 and 5.16). *Absidia spinosa* transformed cortexolone over pH range 3-7. These results were repeated in phosphate buffer at pH 6 and 7 and similar results were observed (results not shown).

Fig 5.13 GROWTH OF A.spinosa AT DIFFERENT pH VALUES,4hr AFTER CORTEXOLONE ADDITION.

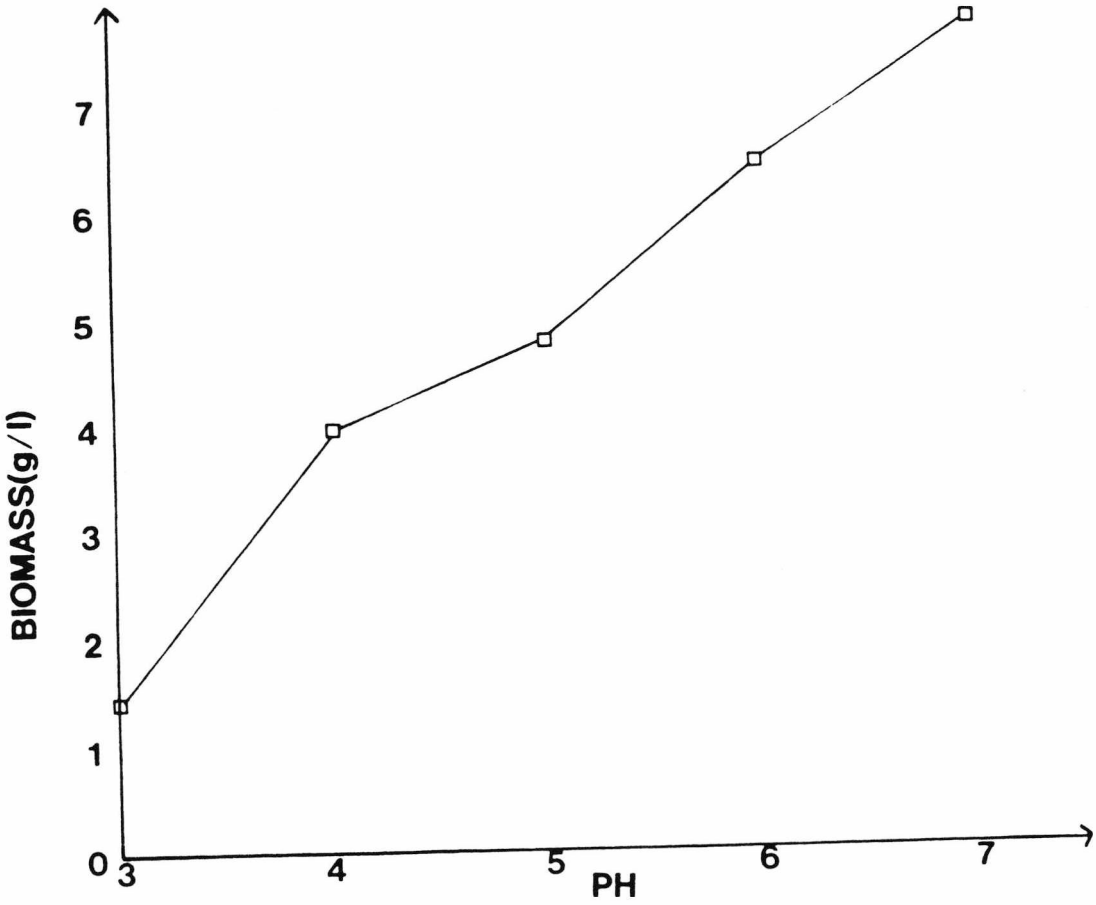


Fig 5.14 CORTEXOLONE BIOCONVERSION AT DIFFERENT pH VALUES BY A.spinosa.

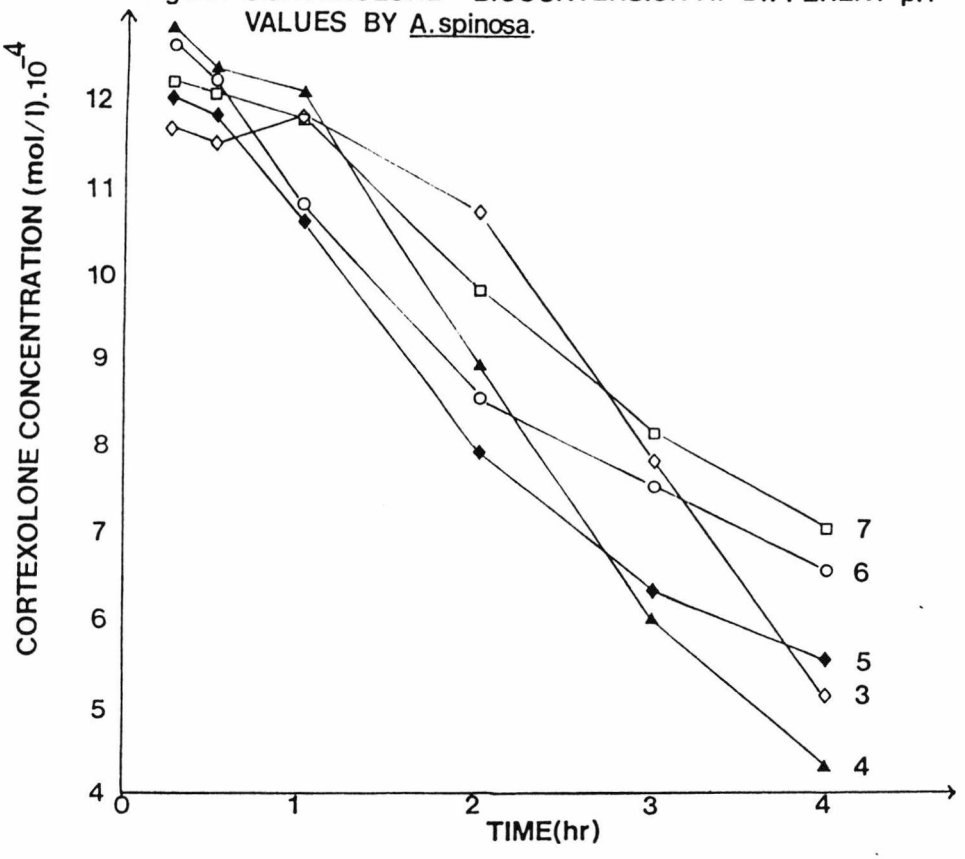


Fig 5.14

A. spinosa was grown at 200 rpm, 30°C for 8 hr in medium A at pH 3, 4, 5, 6 and 7 in 100mM citrate/phosphate buffer. 1.13mM cortexolone in 2% (v/v) MeOH (final concentrations) were then added. Samples were removed at intervals for analysis, over a 4hr period. Cortexolone was detected and quantified using hplc, by its absorbance of light at 254nm.

Fig 5.15 CONCENTRATION OF 11-BETAHYDROXYCORTEXOLONE AT DIFFERENT pH VALUES BY A.spinosa.

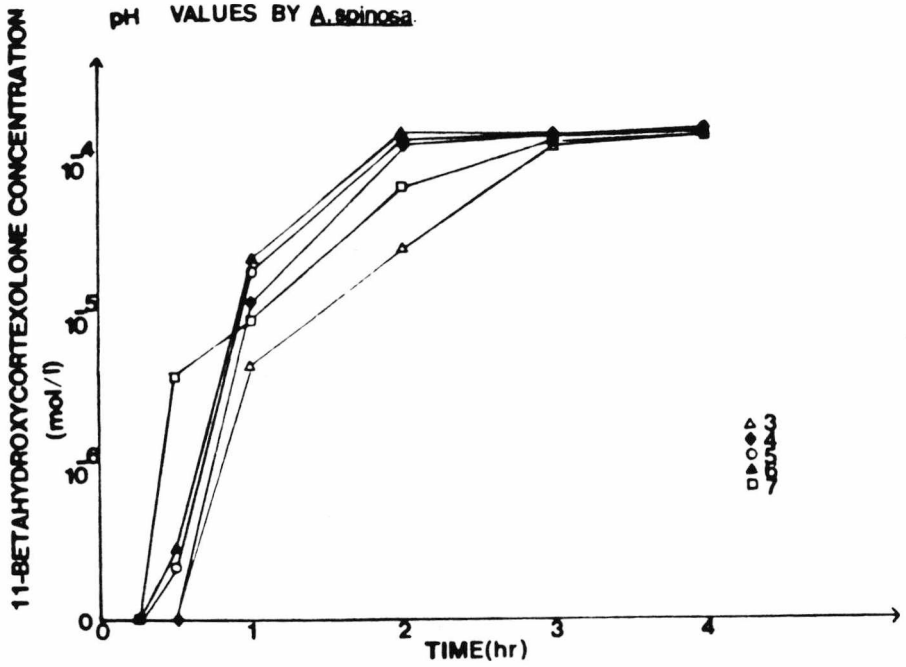
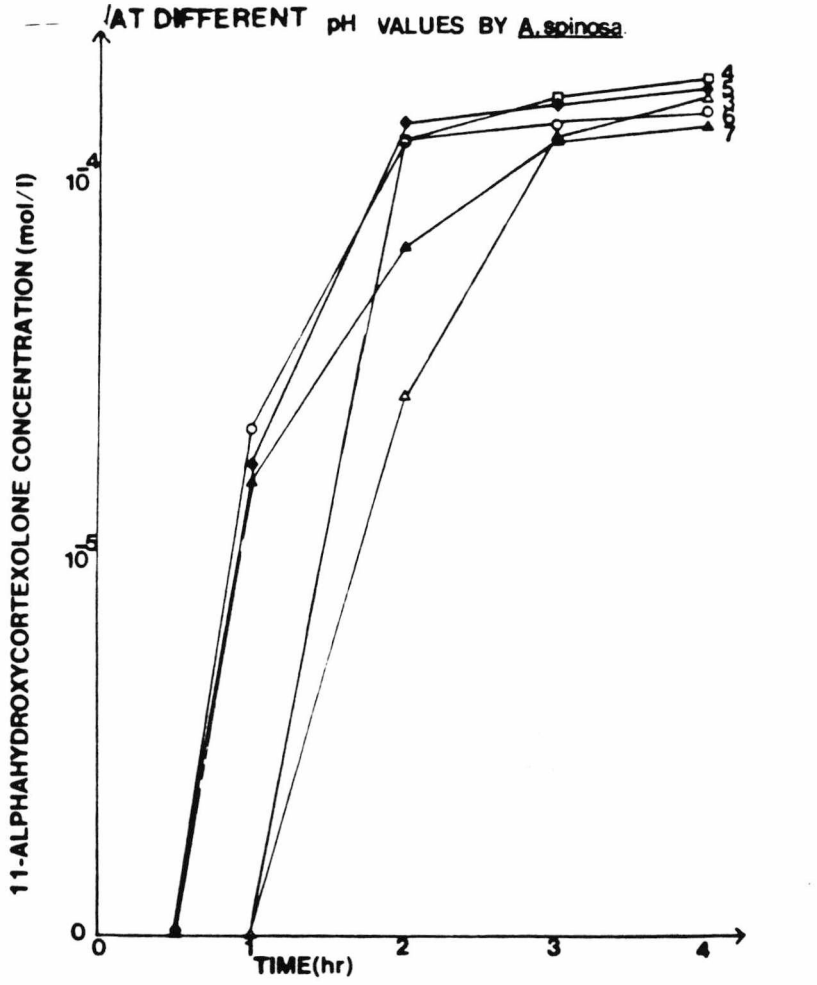


Fig 5.16 CONCENTRATION OF 11-ALPHAHYDROXYCORTEXOLONE AT DIFFERENT pH VALUES BY A.spinosa.



Figs 5.15 and 5.16

A.Spinosa was grown at 200 rpm, 30°C for 8hr in medium A at pH's 3,4,5,6 and 7 in 100mM citrate/phosphate buffer. 1.13mM cortexolone in 2% v/v MeoH (final concentrations) were then added. Samples were removed at intervals for analysis over a 4hr period. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254 nm.

5.6.1 The effect of temperature on cortexolone transformation by *Absidia spinosa*

The growth rate of *Absidia spinosa* was highest between 25-30°C (μ_{max} = 0.18 and 0.16 hr^{-1} respectively). At 20°C μ_{max} was reduced to 0.11 hr^{-1}, whereas no growth occurred at 35°C or 40°C (Table 5.4).

Cortexolone was transformed more effectively at 30°C than at other temperatures (30°C > 25°C > 20°C > 35°C > 40°C'; Fig. 5.17). No cortexolone transformation took place at 40°C (Fig. 5.17). Both 11βC and 11αC are produced between 0.5-1 hr over the temperature range of 20-30°C. (Figs. 5.18 and 5.19).

Neither 11βC or 11αC was formed at 40°C. It was also observed that the ratio of 11βC:11αC once produced occurred in equimolar amounts throughout the period of study. Once again the transformation patterns of 11βC and 11αC at the different temperatures, was similar.

When *Absidia spinosa* was grown at 30°C and pH6, a typical cortexolone bioconversion can be seen (Fig. 5.20). As the amount of cortexolone decreases, three major products are formed. Two of these were 11βC and 11αC. A fourth minor product was also visible.

5.7.1 The effect of antifungal agents on cortexolone transformation by *Absidia spinosa*

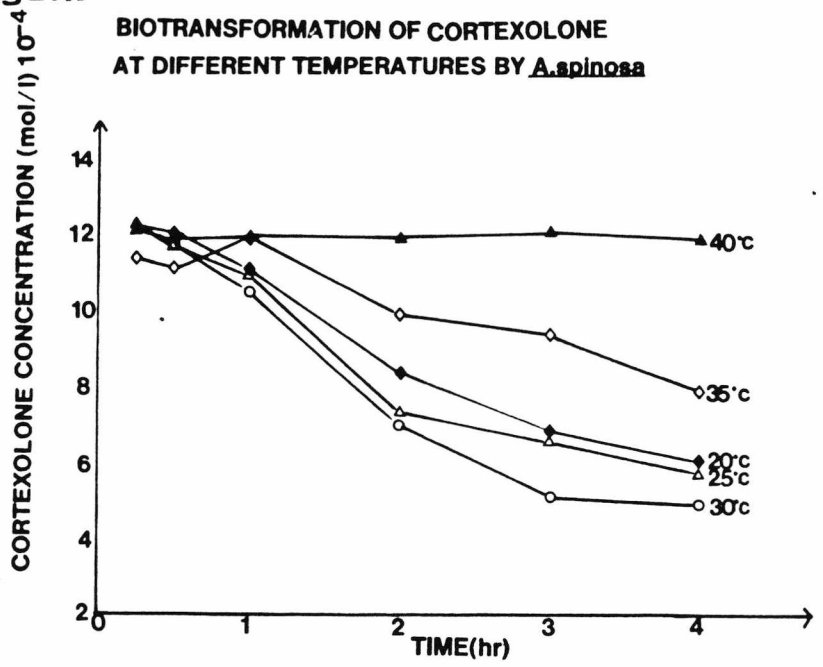
The inhibitors used showed various effects on growth and hydroxylation of cortexolone by *A.spinosa*. These are summarized in Table 5.5.

All the antifungal agents inhibited cortexolone bioconversion to some degree, except metyrapone (Table 5.5). Miconazole was the most potent inhibitor of cortexolone transformation (21.2% relative to the control). Miconazole caused a 60% inhibition of growth and completely inhibited 11α-hydroxylation by 4 hr. 11β-hydroxylation occurred at 3.1% of the control level. Ketoconazole caused a 67% inhibition of growth. With ketoconazole the 11βC

Table 5.4: The maximum specific growth rate of *A.spinosa* at different temperatures

Temperature (°C)	μ_{max} (hr^{-1})
20	0.11
25	0.18
30	0.16
35	0
40	0

Fig5.17



Figs 5.17, 5.18 and 5.19

A.Spinosa was grown at 200rpm, 30° C for 8hr in medium A 1.13mM cortexolone in 2% (v/v) MeOH (final concentrations) were then added and flasks transferred to selected temperatures (20°C, 25°C, 30°C, 35°C and 40°C.) Samples were removed at intervals for analysis over a 4hr period. Cortexolone and its bioconverion products were detected and quantified using hplc, by their absorbance of light at 254nm.

AT DIFFERENT TEMPERATURES BY A.spinosa

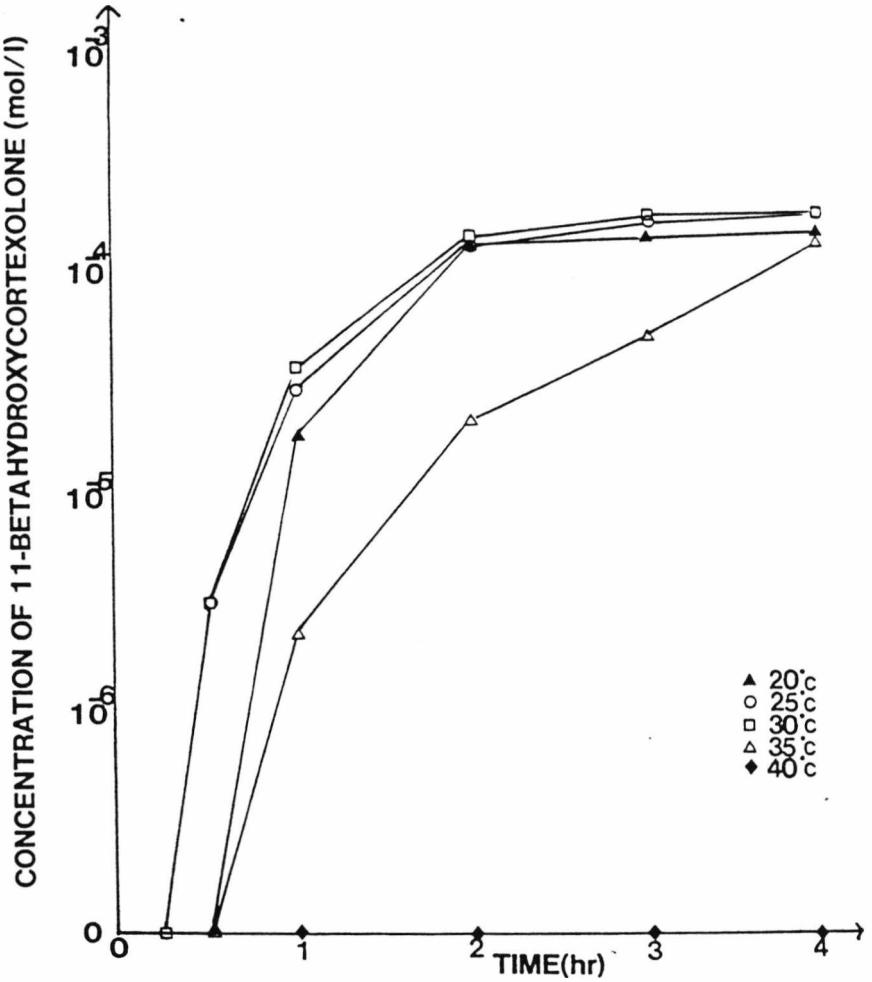


Fig 5.19 CONCENTRATION OF 11-ALPHAHYDROXYCORTEXOLONE AT DIFFERENT TEMPERATURES BY A.spinosa

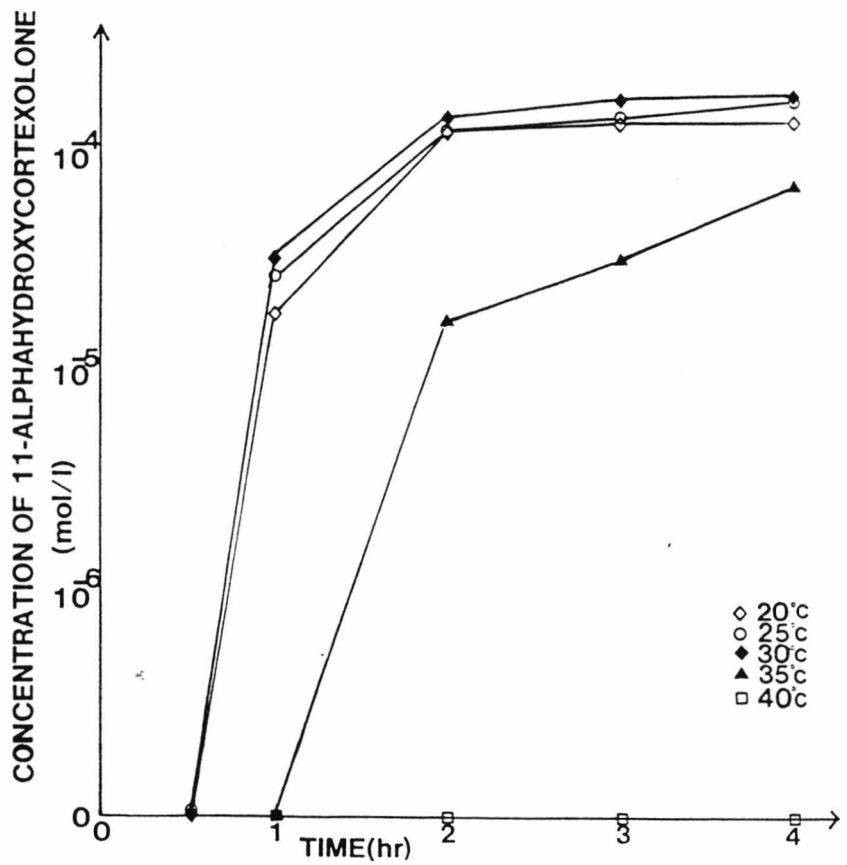


Fig5.20 Typical tlc plate of cortexolone bioconversion by vegetative cells of A.spinosa

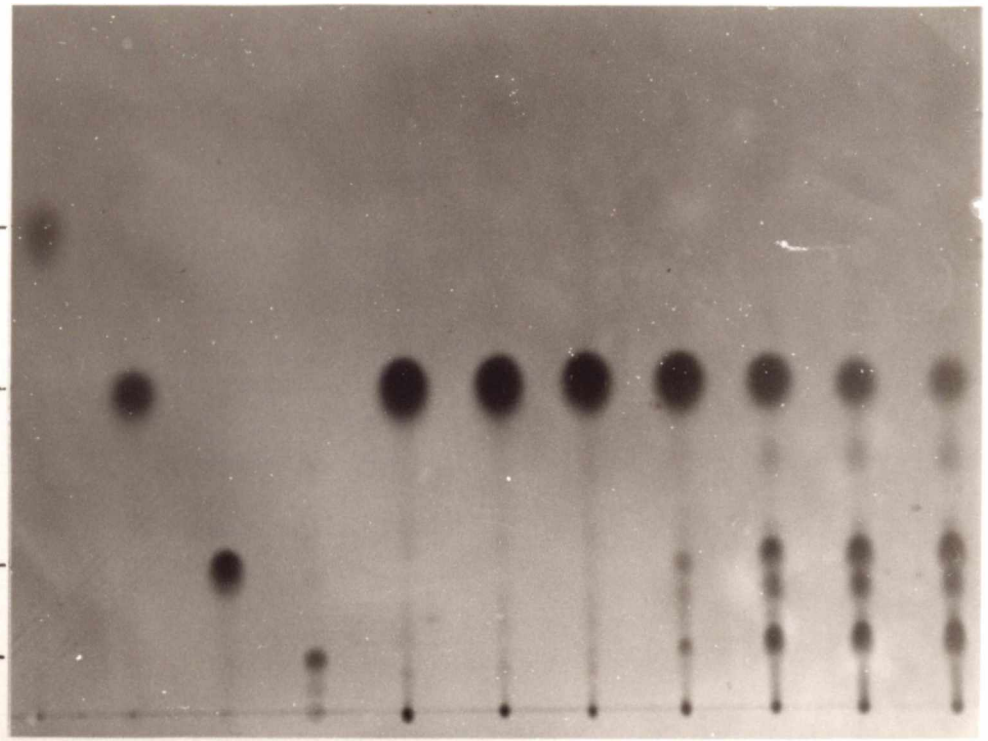


Table 5.5 The effect of antifungal agents on *A.spinosa* growth and 11-hydroxylation.

inhibitor	% inhibition of growth	ratio of 11βC/11αC	% cortexolone remaining	% amount of 11αC	% amount of 11βC
Control	0	1.1	45.2±5.7 (100)*	20.1±1.0 (100)*	21.1±0.5 (100)*
Miconazole	60	11β only	96±0.5 (21.2)*	0 (0)*	0.7±0.3 (3.1)*
Ketoconazole	67	0.66	39.0±1.6 (86.3)*	18.8±2.3 (93.3)*	12.5±1.7 (59.0)*
SKF-525A	13	1.1	39.7±4.5 (87.8)*	16.0±0.4 (79.8)*	18.0±0.3 (85.5)*
Metyrapone	8	1.1.3	47.0±2.1 (104.0)*	16.2±0.8 (80.7)*	21.0±0.7 (99.5)*

* The values in parentheses represent the percentage cortexolone transformed or the percentage 11βC and 11αC produced relative to the control.

formed was 59.0% that of the control, and the 11αC produced was 93.3%. Thus although growth was greatly inhibited 11α-hydroxylation was only slightly affected.

SKF-525A caused a 13% inhibition of growth but did not alter the ratio of 11βC:11αC produced by *Absidia spinosa*. The percentage of 11βC and 11αC produced in relation to the control was 85.5% and 79.8% respectively.

Metyrapone inhibited the growth of *Absidia spinosa* by 8% compared to the control. The ratio of 11βC:11αC encountered was 1:1.3. Metyrapone preferentially inhibited 11α- hydroxylation (80.7%) in preference to 11β-hydroxylation (99.5%) compared to the control. It was observed that *Absidia spinosa* had overcome any inhibition of growth by all antifungal agents used by 24 hr.

5.8.1 Effect of cycloheximide on cortexolone transformation by *Absidia spinosa*

Cycloheximide is a well known inhibitor of protein synthesis (Stryer 1981). It inhibits the peptidyl transferase activity of the 60S ribosomal subunit in eukaryotes.

Absidia spinosa growth was inhibited at even the lowest cycloheximide concentration used (0.1μg/ml; Fig. 5.21). The greatest inhibition on growth (70%) occurred at 10 μg/ml and above (Fig. 5.21). No growth occurred above 10μg/ml. Above 10μg/ml all cortexolone bioconversion was abolished in *Absidia spinosa* (Fig. 5.22).

Fig 5.21 A.spinosa growth in the presence of varying cycloheximide concentrations.

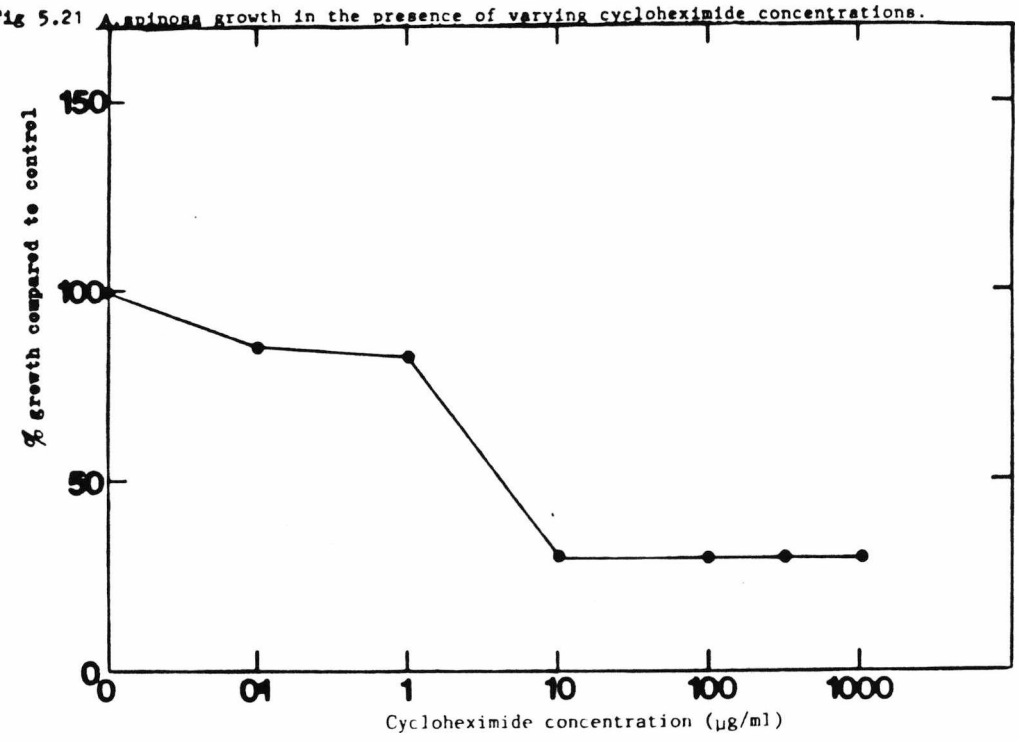
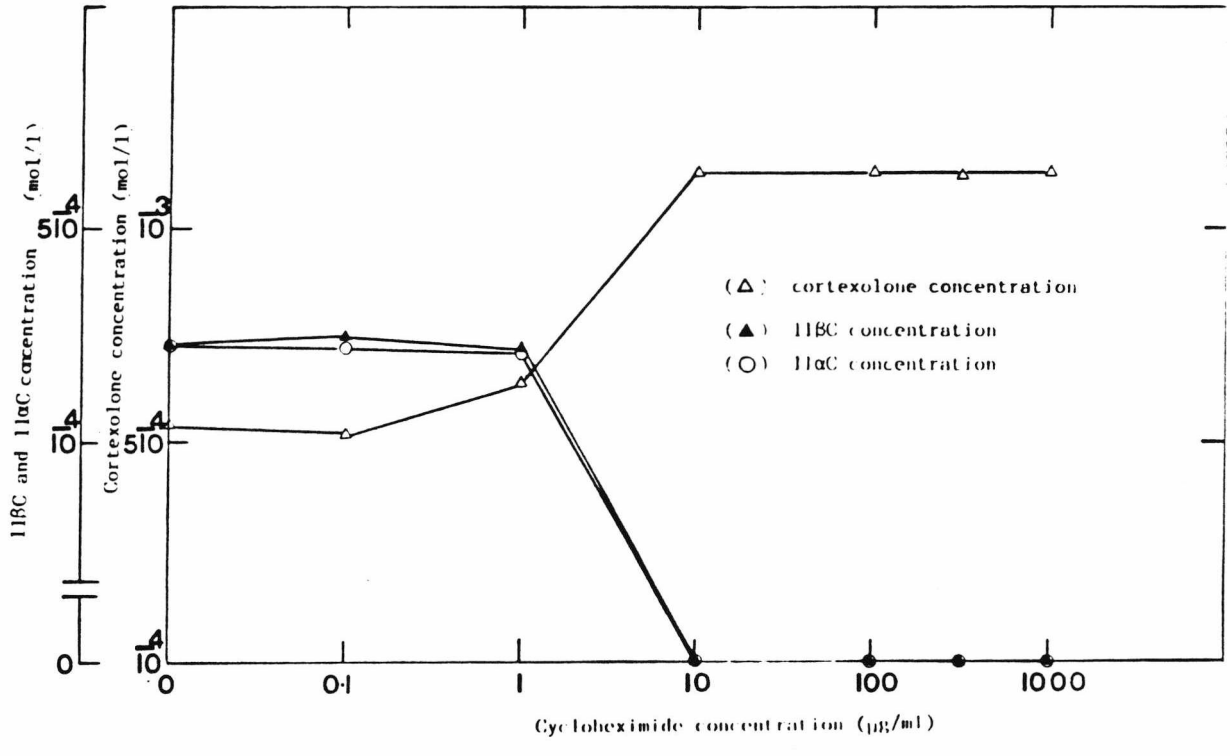


Fig.5.22· Cortexolone bioconversion in the presence of varying cycloheximide concentrations by A.spi



Figs 5.21 and 5.22

A.spinosa was grown in medium A at 200 rpm, 30° C for 8hr. 1.1mM cortexolone in 2 % (v/v) MeOH was then added and growth continued for 4hr. Samples were removed at intervals for analysis during this period. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm.

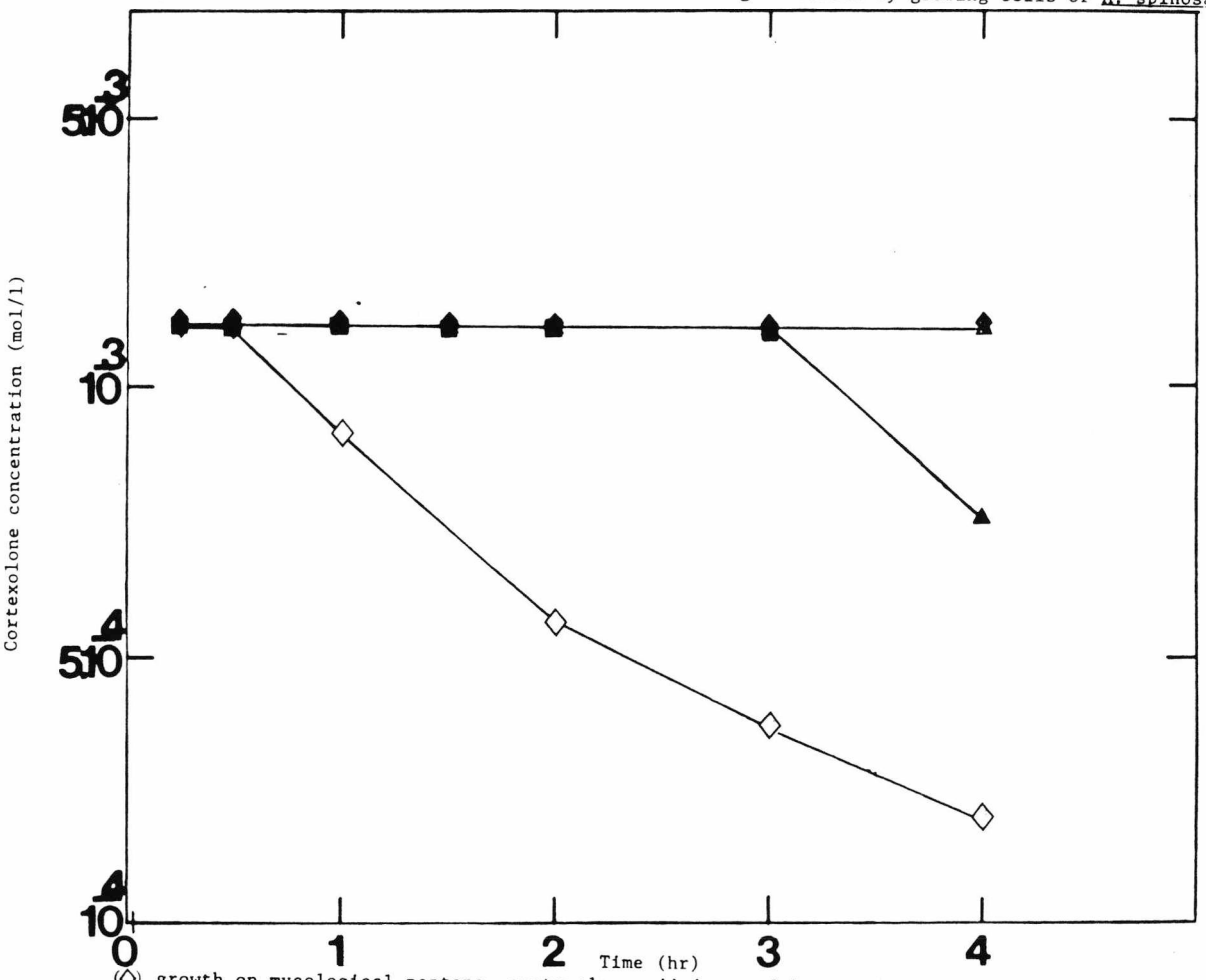
5.9.1 The effect of different nitrogen sources and time of cortexolone addition on cortexolone transformation by *Absidia spinosa*

Absidia spinosa was able to grow on a defined medium (medium B). The percentage germination of spores by 12 hr was lower (3%) than that achieved on a peptone based medium (97%). By varying the nitrogen source used and the time of addition of cortexolone to the medium, differences were noted in cortexolone transformation (Fig. 5.23). Cortexolone modification was more effective in a medium containing ammonium choride (NH_4Cl) than one containing urea or sodium nitrate ($NaNO_3$), (Fig. 5.23). Cortexolone was transformed more effectively in the peptone based medium A than in any other tested (Figs. 5.23). The response time of *Absidia spinosa* in converting cortexolone to detectable amounts of products is dependent on the nitrogen source used. In medium A or medium B with NH_4Cl the response time for 11βC and 11αC was between 0.5-1 hr (Figs. 5.24 and 5.25). In medium B with urea (t = 12), the response time for 11βC and 11αC production after cortexolone addition increased to 1-2 hr for 11βC and 11αC (Fig. 5.26).

$11\beta^-/11\alpha^-$ hydroxylation of cortexolone was initially largest in peptone based medium than any other tested; peptone > NH_4Cl > urea > $NaNO_3$ (compare Figs. 5.24, 5.25 and 5.26). No $11\beta-/11\alpha^-$ hydroxylation took place when $NaNO_3$ was used as sole nitrogen source (Fig. 5.26).

The time of cortexolone addition also affected the amount of 11βC and 11αC produced when NH_4Cl was used as the sole nitrogen source. Cortexolone was initially transformed more effectively when it was added 12 hr into growth instead of 8 hr (Figs. 5.24 and 5.25). Both 11βC and 11αC were produced in equimolar amounts throughout the period of study.

Fig 5.23 Cortexolone bioconversion on different nitrogen sources by growing cells of A. spinos

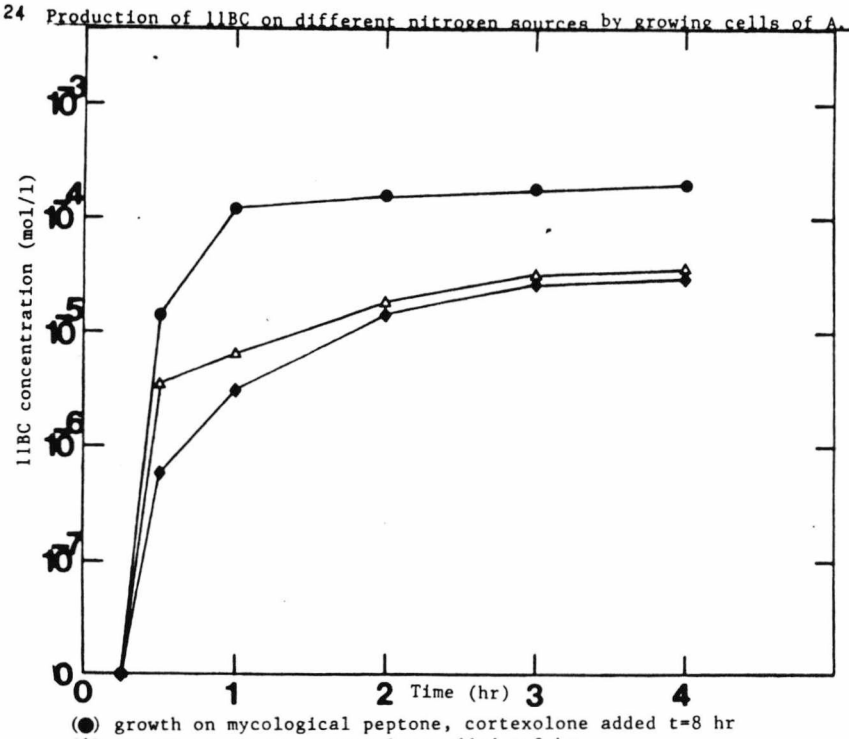


(◇) growth on mycological peptone, cortexolone added at t=8 hr
(◆) growth on NH_4Cl, cortexolone added at t=8 hr
(■) growth on $Na\ NO_3$, cortexolone added at t=12 hr
(▲) growth on NH_4Cl, cortexolone added at t=12 hr

Fig 5.23, 5.33 and 5..34

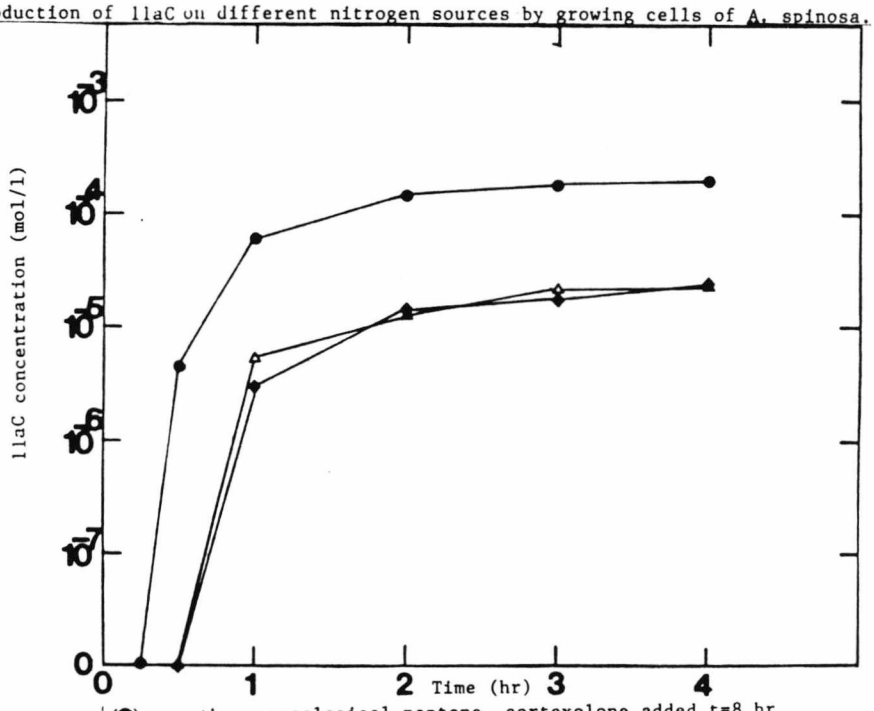
A.spinosa was grown in complex medium A with mycological peptone as sole nitrogen source or in defined medium B with NH_4 11, urea or $NaNO_3$ as sole nitrogen source. The organism was grown at 200 rpm, 30°C for 8 or 12hr. 1.1mM cortexolone in 2% (v/v) MeOH was then added and growth continued for another 4hr. Samples were removed at intervals for analysis during this period. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm.

Fig 5.24 Production of 11BC on different nitrogen sources by growing cells of A. spinosa.



(●) growth on mycological peptone, cortexolone added t=8 hr
(◆) growth on NH_4Cl, cortexolone added t=8 hr
(△) growth on NH_4Cl, cortexolone added t=12 hr

Fig 5.25 Production of 11aC on different nitrogen sources by growing cells of A. spinosa.



(●) growth on mycological peptone, cortexolone added t=8 hr
(◆) growth on NH_4Cl, cortexolone added t=8 hr
(△) growth on NH_4Cl, cortexolone added t=12 hr

Figs 5.24 and 5.25

A.spinosa was grown in complex medium with mycological peptone as sole nitrogen source, or in defined Medium B with NH_4CL, urea or $NaNO_3$ as sole nitrogen source. The organism was grown at 200 rpm, 30°C for 8 or 12hr. 1.1mM cortexolone in 2% (v/v) MeOH was then added and growth continued for another 4hr. Samples were removed at intervals for analysis during this period. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm.

Fig 5.26 The production of 11BC and 11aC using ammonium chloride and urea as nitrogen sources, by growing cells of A.spinosa.

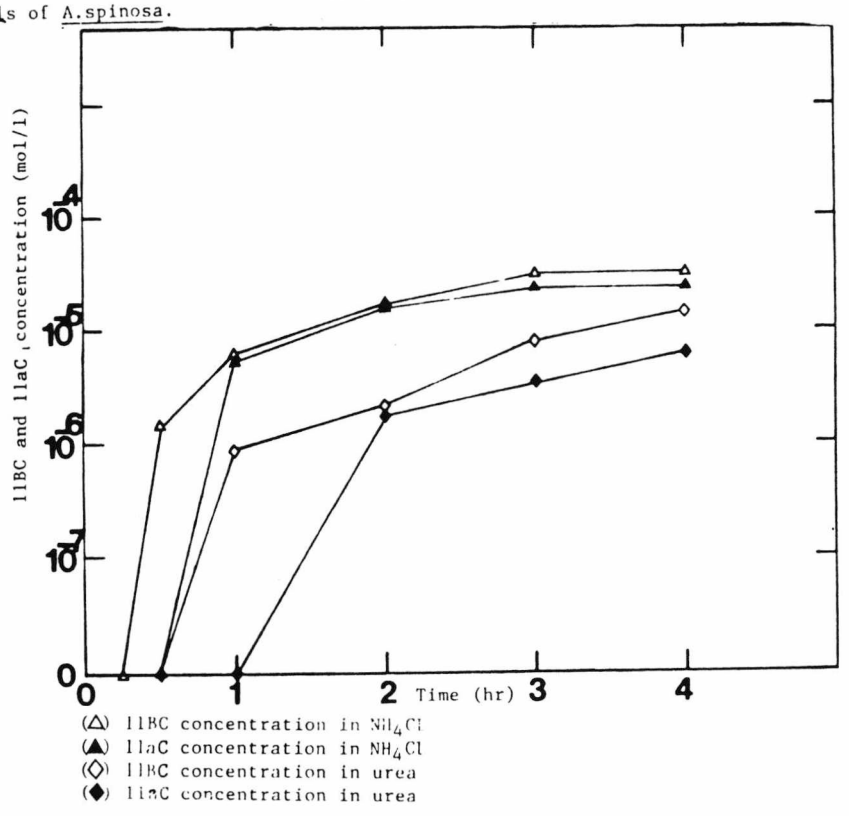


Fig 5.26

A.spinosa was grown in defined medium B with urea or NH_4CL as sole nitrogen source, at 200 rpm, 30°C for 12 hr. 1.1mM cortexolone in 2% (v/v) MeOH wass then added and growth continued for another 4hr. Samples were removed at intervals for analysis during this period. Cortexolone and its bioconversion products were detected and quantified using hplc, by their absorbance of light at 254nm. No bioconversion products were seen when $NaNO_3$ was used as sole nitrogen source.

5.10.1 *Absidia spinosa* batch growth under controlled conditions for cortexolone transformation in defined medium B

When *Absidia spinosa* was grown in the fermenter, foaming problems occurred in the presence of high concentrations (0.1% v/v) of anti-foaming agent, polypropyleneglycol (PPG). By selective omission of various components of the medium, the cause was found to be Tween 80. The problem was solved by a tenfold reduction of the concentration of Tween 80 in the medium to 0.002% (v/v).

A second problem encountered was the low percentage germination (3%) of *Absidia spinosa* spores in the defined medium (B). This was overcome by growing *Absidia spinosa* in peptone rich medium A (97% germination of *Absidia spinosa* spores by 8 hr). The organism was allowed to grow for 12 hr (early exponential phase), by which time all the germinated spores had produced branching hyphal networks. After a series of washes and centrifugations the organism was transferred to sterile water/Tween 80, and used as inoculum for the fermentation.

Exponential growth of *Absidia spinosa* in medium B did not occur directly after inoculation and there was a lag period of 2 hr before biomass detectably increased (Fig. 5.27). μ_{max} was 0.14 hr^{-1} and t_d; 4.95 hr. The amount of growth in medium B with NH_4Cl was 26% lower than the amount of growth in peptone based medium. Maximum biomass levels (2.63 g/l), were reached by 25 hr in the medium with 10 g/l glucose (Fig. 5.27). Biomass declined to 2.07 g/l by 48 hr. At the conclusion of the experiment glucose levels were not exhausted (Fig. 5.28). Glucose concentration remained level for the 2 hr lag period, after which the concentration decreased as *Absidia spinosa* initiated growth. As the growth of *Absidia spinosa* reached a maximum, less glucose was utilized from the medium. Glucose concentration fell to 2.9 g/l by 48 hr (Fig. 5.28).

Fig 5.27 Cortexolone bioconversion in defined medium B in a fermentor by A.spinosa.

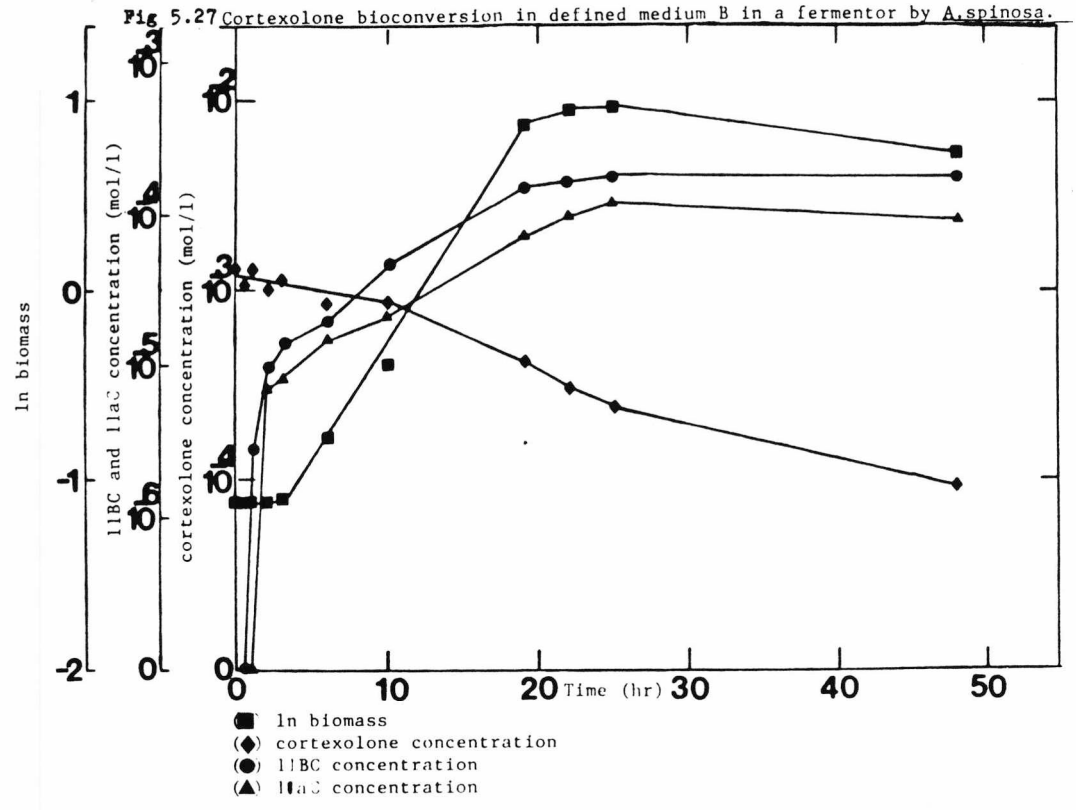
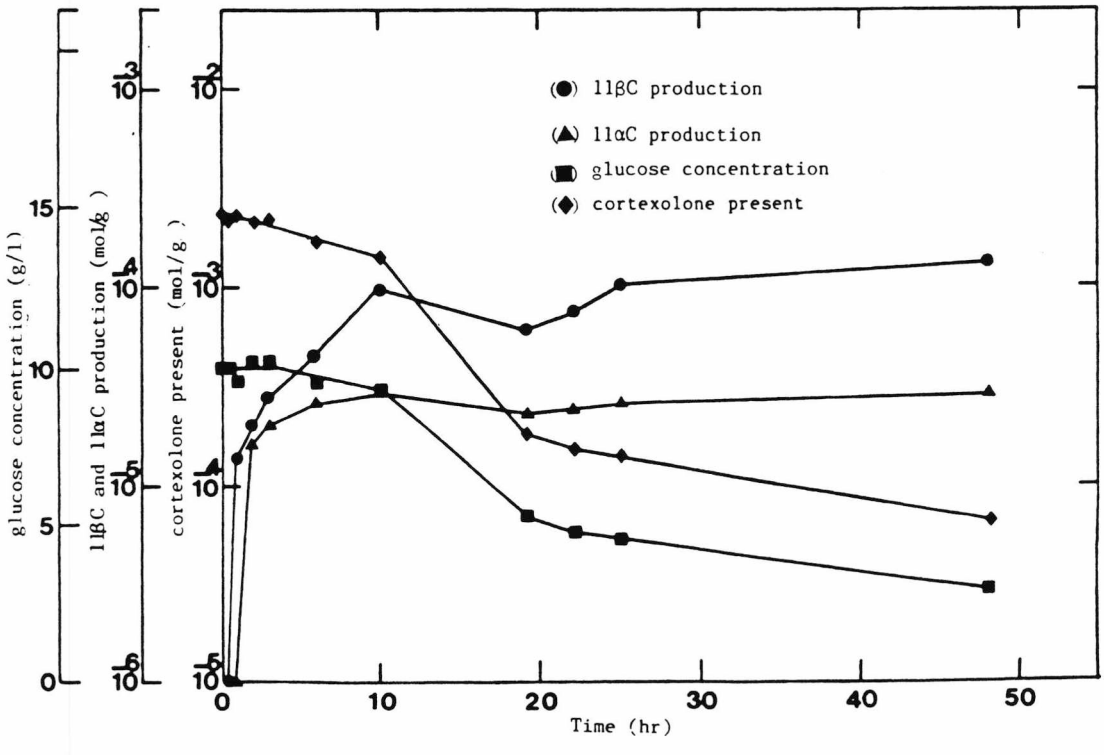


Fig 5.28 Cortexolone bioconversion and glucose utilization in defined medium B in a fermentor by A.spinosa



Figs 5.27 and 5.28

A.spinosa was grown in medium B containing 1.1mM cortexolone in 2% (v/v) MeOH, at 1000 rpm, 30 °C, pH5.0, at a constant dissolved oxygen tension for 48 hr. Samples were removed at intervals for analysis. cortexolone and its bioconversion products were determined using hplc by their absorbance of light at 254nm. Glucose was detected and quantified using a glucose assay kit.

NH_4Cl concentration did not fall for the first 6 hr, but a linear decrease was noted between 6-10 hr (Fig. 5.29). This levelled off as *Absidia spinosa* approached maximum biomass. NH_4Cl was not exhausted at the end of the period of study, and fell to 1.6 g/l by 48 hr. (Fig. 5.29).

The concentration of phosphate remained unchanged at 2.32 g/l for 6 hr after inoculation (Fig. 5.29) before declining linearly to 1.6 g/l by 48 hr. Thus when growth rate was decreasing, carbon, nitrogen and phosphate supplies were not exhausted but remained present in the medium.

Oxygen consumption by *Absidia spinosa* increased during exponential growth (Fig. 5.30) and peaked around 18.5 hr, when *Absidia spinosa* growth was decelerating. In the stationary and decline phases, the amount of oxygen consumed decreased. As expected carbon dioxide production increased in exponential growth (Fig. 5.30), optimum production occurring around 18 hr after inoculation. The amount of carbon dioxide produced increased in the stationary and decline phases.

Cortexolone concentration falls slowly during the lag phase of growth but the amount of cortexolone transformed increases as growth accelerates (Fig. 5.27). The fall in cortexolone concentration in relation to biomass was largest during logarithmic growth (Fig. 5.28). The pattern of cortexolone transformation (Fig. 5.28) was similar to that encountered when mycological peptone was used as nitrogen source.

11βC was detected in the medium 1 hr after inoculation, the 11αC product was detected only in trace amounts (Fig. 5.27). Both 11βC and 11αC concentrations increased in the initial stages of growth when biomass was unchanged (lag phase). The highest change in product concentration was observed during exponential growth (Fig. 5.27). The amount of these primary hydroxylated transformation products produced was reduced as the growth rate decelerated. The concentration of 11αC dropped at 48 hr when growth was in decline (Fig. 5.27). This change was not observed with the 11β- epimer.

Fig 5.29 Phosphate and ammonium chloride utilization in the presence of cortexolone by growing cells of A. spinosa.

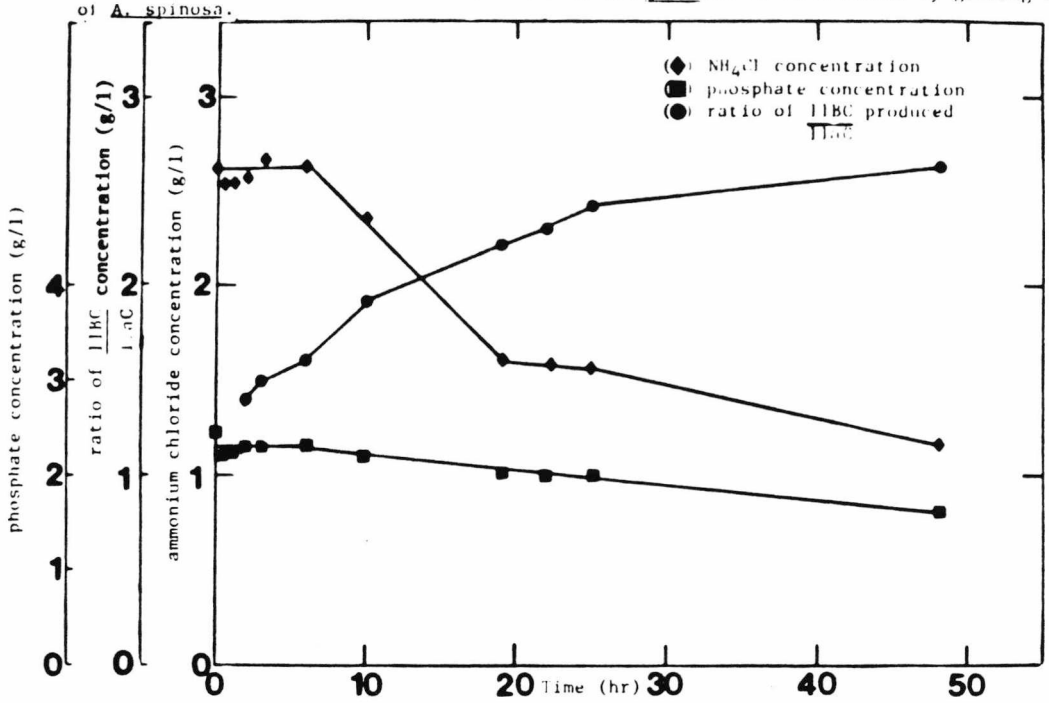
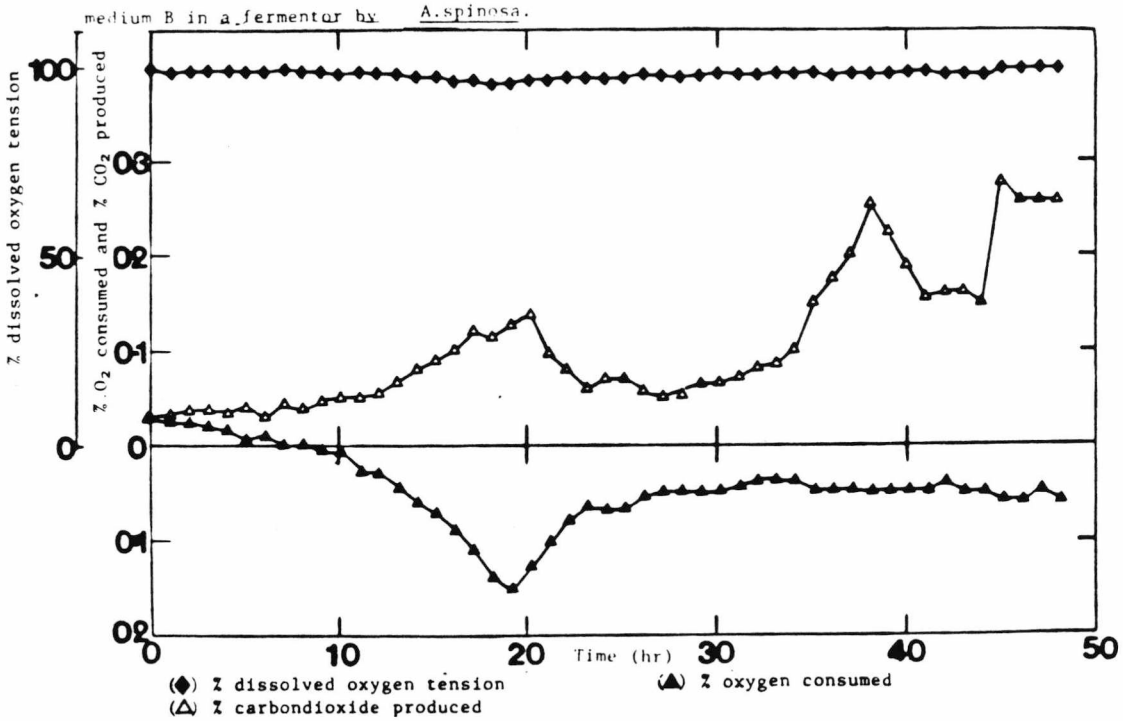


Fig 5.30 Oxygen consumption and carbondioxide production at a constant dissolved oxygen tension in defined medium B in a fermentor by A.spinosa.



Figs 5.29 and 5.30

A.spinosa was grown in medium B containing 1.1mM cortexolone in 2% (v/v) MeOH, at 1000 rpm, 30° C, pH5.0, at a constant dissolved oxygen tension for 48hr. % O_2 consumed and % CO_2 produced were determined at intervals by gas analyzer. Samples were also removed for ammonia and phosphate determination. The ratio of 11BC:11aC produced was also noted.

No secondary transformation products were detected throughout the period of study.

At 25 hr it was discovered that 66.2% of cortexolone had been transformed, 50% being converted to 11βC and 11αC. The ratio of 11βC : 11αC increased throughout the experiment from 1:1.4 by 2 hr to 1:2.6 by 48 hr (Fig. 5.29). The amount of 11βC and 11αC produced in relation to biomass in defined medium B follows the same pattern as that encountered in peptone based medium (Fig. 5.30).

5.11.1 DISCUSSION

Controlled experiments on cortexolone transformation have been performed. Cortexolone itself does not inhibit the growth rate of *Absidia spinosa* at the concentrations used. This will be expanded on later. The MeOH solvent inhibits the growth of *Absidia spinosa*. This may be due to its effect on spore structure and metabolism which has lasting effects throughout exponential growth. MeOH is known to inhibit 11α- hydroxylation of progesterone by *Rhizopus stolonifer* (Sonomoto *et al* 1982), when used at 2% (v/v) and above. As an organic solvent MeOH can 'loosen' the membrane structure and increase cortexolone permeability into the cell thereby stimulating steroid enzyme activity directly. Greater biomass production in the absence of both MeOH and cortexolone is indicative that biomass production is more efficient in the absence of transformation substrate and MeOH. As biomass production was less efficient in cortexolone and 11βC treatments relative to the methanol control, this might indicate that transformation, and elimination from the fungal interior could require energy. A primary transformation product 11βC like cortexolone has no effect on *A.spinosa* growth rate at 1.1mM (μ_{max} and maximum biomass remained unaltered compared to cortexolone treated fungi). This supports the initial findings of Lin and Smith (1970b) that growth processes i.e. membrane

biosynthesis which involves sterol molecules is not interrupted by these molecules in exponential growth or if it is, an alternative pathway which is as efficient as the former one, is being utilized. The data do strongly suggest that the former case is probably the correct interpretation of the events seen. This was supported by the fact that increasing cortexolone concentrations from 0.22 mM to 2.26 mM caused increased cortexolone loss, without affecting μ_{max}

Progesterone can strongly inhibit filamentous fungal growth (Weaver *et al* 1960). Progesterone can also bind to fungal receptor proteins with high affinity (Schar *et al* 1986), whereas 11βC is only very weakly bound, and other steroids like estradiol are not bound in microorganisms (Feller and Trager 1985). The evidence from the literature shows that virtually all steroid molecules can be biotransformed by filamentous fungi (Charney and Herzog 1967; Laskin and Lechevalier 1984; Smith 1984). Is it possible that the ability of progesterone to inhibit fungal cell growth is due to binding to endogenous steroid receptor proteins, which may be involved in growth and reproduction? The fact that cortexolone and 11βC do not inhibit the fungal growth rate may be reflected by their low affinity for steroid binding proteins and possibility its inability to elicit a physiological response in a similar manner to progesterone (Schar *et al* 1986). Cortexolone and 11βC may have a lower affinity for these receptor proteins and therefore do not noticeably interfere with the endogenous metabolism of the organism in exponential growth. This hypothesis brings up consideration of the purpose of steroid monooxygenases in fungi. These systems could generally appear to be primarily for detoxification (via transformation) and utilization of foreign compounds as alternative energy sources. Steroid metabolism is evident in the Cunninghamellaceae and in *Absidia spinosa*. *A.spinosa* shows a limited capacity to degrade both cortexolone and 11βC in exponential growth, this will be discussed later. No secondary transformation of 11βC occurred during exponential growth as no secondary products were detected. A possible

explanation for the lack of conversion of 11βC in this stage of growth could be its polarity. At the concentration used (1.1. mM) 11βC in the presence of MeOH was soluble in the medium. This may be a barrier to its re-entry into cells or aid in its active removal, as is the case with mammalian systems where hydrophobic molecules are made more aqueous soluble for elimination purposes (Astrom and Depierre 1986; Kauffman 1987). This is achieved via conjugation in mammals. There is no evidence from the data that conjugation occurs in this system, but it is known to occur in other filamentous fungi such as *Cunninghamella elegans* (Cerniglia and Gibson 1979). When growing filamentously this organism can conjugate benzo(a)pyrene to sulphates for elimination. This all points to a system in fungi which is similar to that in mammals. The primary purpose of these endogenous enzyme systems could be one of detoxification, by converting hydrophobic molecules encountered in the environment to more polar molecules. 11βC modification did not begin until late exponential phase, despite the fact that small amounts were detected in fungal mycelia. It is apparent under conditions of glucose exhaustion in this system, that expression of enzymes responsible for secondary transformation of the primary product 11βC was seen. These enzymes are probably those responsible for normal cell growth. When growth is decelerating they might "switch" their affinity for exogenous substrates, and become active against them. To my knowledge these findings using this system have not been reported previously. The possible presence of reduced 11βC products as well as polar products is not unexpected as 11βC is known to induce hydroxysteroid dehydrogenases (Feller and Trager 1985). It can be seen that 11βC is one of the main sources of secondary products and no epimerase was present to convert 11βC to 11αC. No 11αC could be obtained to find out whether the reverse is true, but this would be unlikely as 11βC was produced prior to 11αC (Chapter 4, Section 4.1.2).

11β- and11α- hydroxylating activity was still operative in decline phase. The secondary transformation system is also operative, but when cortexolone is not modified in exponential growth, both systems are superceded by metabolic routes. As the products of cortexolone transformation are preferentially metabolized, it is probable that they may stimulate their own metabolic routes when cortexolone is added in the decline phase. It would therefore appear that all three of the fungi tested; *Cunninghamella blakesleeana, Cunninghamella elegans* and *Absidia spinosa* can metabolize cortexolone and its products, proabably to derive energy from carbon skeletons. Nebert and Gonzalez (1987) give a similar hypothesis as to the purpose of these monooxygenase systems. They proposed that cytochrome P-450 in prokaryotes and fungi can use foreign chemicals as an energy source. The data show a difference between the 11β- and 11α- hydroxylating activities. The 11β- product is produced before the other epimer and is produced in much larger amounts (17 fold) in the first 30 min, before decreasing to a constant amount prior to metabolism. As the 11α- activity lags behind the 11β- activity, one wonders if separate DNA transcripts are present. The results obtained might suggest that induction of the enzyme systems may be responsible for the initial increase in both 11βC and 11αC production.

When 0.22mM cortexolone was used, cortexolone concentration continued to fall when the concentration of both 11βC and 11αC did not increase. This indicates that the transformation substrate can be partially metabolized in exponential growth as has been mentioned previously. The metabolized substrate could end up as biomass and/or carbon dioxide and water. An intermediate degradative compound was detected and could be androst-4-ene-3, 17-dione. This compound can be formed by the removal of the hydroxylated acetone side chain and a proton, by a route mentioned previously (see Section 4.3.1). No secondary transformation was seen during exponential growth even when cortexolone was completely converted to its primary

transformation products at 0.22 mM and 0.56 mM. This would again suggest that these reaction routes were not operative in exponential growth in conditions of glucose excess. The primary hydroxylated products like 11βC can stimulate the enzymes involved in their metabolism when growth is decelerating.

Absidia spinosa produces more biomass at higher pH values. Both 11βC and 11αC production occured at all the pH values investigated here. Steroid hydroxylases would appear to be active against cortexolone over a broad pH range. This agrees with Samanta *et al* (1978) who reported that *Aspergillus ochraceus* hydroxylation is active over a broad pH range (pH 4-8). They also showed that the specifity of the hydroxylases were unaffected with variations in pH. The effect of temperature, shows that these enzymes are active over a broad temperature range (20-35°C). This is not unexpected as one would expect the cortexolone bioconversion systems to be at their most active when the fungal cells are growing. The temperatures which produced the highest μ_{max} values were 25°C and 30°C. Cortexolone transformation was also more efficient at these temperatures. At 35°C, when no growth was occurring, cortexolone transformation was still operative, showing the independence of steroid hydroxylating activity from normal growth processes. To my knowledge, this has not been reported previously. The effect of substrate concentration, pH and temperature shows that the enzymes responsible for cortexolone bioconversion have a fast response time of 0.5-1 hr before products are detected (Nebert *et al* 1982). This fast response to steroids has also been reported previously (Breskvar and Hudnik-Plevnik 1981). Blatiak *et al* (1985) have observed that a modulator gene was involved in cytochrome P-450 production in *Saccaharomyces cerevisae*. This phenomenon might explain the concerted production of the primary hydroxylated products seen in *Absidia spinosa*. Induction itself has been suggested by Feller and Trager (1985) to be selective and complex involving different induction mechanisms for each

compound encountered. Induction has been suggested to be *de novo* for the cytochrome P-450 and the NADPH-dependent cytochrome c reductase (Breskvar and Hudnik-Plevnik 1981).

Cortexolone transformation was found to occur in defined medium B with NH_4Cl as the preferred nitrogen source. No secondary transformation products were detected in this medium. Mycological peptone may contain certain compound(s) which stimulate the transformation of cortexolone. Fermentation studies with NH_4Cl reveals that at high O_2 saturation levels (> 96%) the transformation of cortexolone still occurs. A response time of 1 hr was observed before products were detected. No secondary products were seen throughout the fermentation even in the decline phase of growth. This poses the question, "Is glucose limitation necessary for secondary transformation to occur?" It is apparent from the data that all that is required to "halt" primary transformation is the cessation of growth. The enzymes can however be "switched" on in decline phase. The data show growth independent transformation by both primary and secondary transformation systems in complex medium.

In fermentation studies one does not see a constant ratio of 11βC:11αC as was seen in previous experiments (Fig. 4.35). This again shows that though the 11β- and 11α- hydroxylases have closely linked properties, in this system 11β-hydroxylation exhibits a higher activity than 11α- hydroxylation. The data suggest that cortexolone metabolism can go via two possible routes; compulsory primary transformation and partial metabolism. The primary products can undergo secondary transformation under conditions of glucose exhaustion in complex medium. Lin and Smith (1970b) using a defined medium detected, only monohydroxylated derivatives (10β-, 11β- and 11α-) in aerated cultures. No secondary products were detected. It is possible that certain nutrients present in complex media stimulate the secondary transformation system seen in filamentous fungi. Preferential metabolism of

transformation products was also seen if cortexolone was not present in exponential growth. The reason for this is unclear, but these comparative studies, to my knowledge, have not been published to date with *Absidia spinosa*.

The use of antifungal inhibitors was to show the differential effects of these compounds on 11β–/11α- hydroxylation and the growth of *A.spinosa* in liquid culture. It is possible that the effects seen are not the result of direct interaction with the enzymes involved in cortexolone bioconversion, but are an indirect effect. However both 11β- and 11α- hydroxylating activities have previously been shown to be inhibited in fungal microsomes by these agents. (Ghosh and Samanta 1981; Madyastha *et al* 1984). All the inhibitors bind to the haem prosthetic group of cytochrome P-450 (Ortiz de Montellano and Reich 1986), most are fairly non-specific. Miconazole and ketoconazole are potent inhibitors of *Absidia spinosa* growth. They are imidazole antimycotic agents (Woda *et al* 1988) which are known to inhibit 14-demethylase activity in fungi at nM concentrations. (Ortiz de Montellano and Reich 1986; Beach *et al* 1986; Yoshida and Aoyama 1987). The inhibition of 14-demethylase leads to the cessation of ergosterol biosynthesis, and the accumulation of 14α-methylsterols. The use of both inhibitors could suggest that the 11β-and 11α-hydroxylating activities are probably not involved directly in growth. Miconazole inhibits both activities and ketoconazole preferentially inhibits 11β-hydroxylation, but both are potent inhibitors of growth. Both metyrapone and SKF-525A are not as potent inhibitors of growth as miconazole or ketoconazole, the former two inhibitors caused a slight inhibition of fungal growth. Metyrapone preferentially inhibits 11α- hydroxylation. Metyrapone is a selective inhibitor for phenobarbital-inducible forms of cytochrome P-450 (Testa and Jenner 1981; Lin and Franklin 1985). It is a well known inhibitor of adrenal 11β- hydroxylase but has hardly any effect on 11β- hydroxylating activity in whole cells of *Absidia spinosa* . With SKF-525A its lack of potency

on *A.spinosa* growth might be explained by the fact that it is a competitive inhibitor which could act as an alternative substrate and is metabolized.

One would expect the use of potent inhibitors like miconazole and ketoconazole to result in direct cellular damage or cell injury due to 14α-methylsterol accumulation and their abnormal incorporation into membranes and other critical structures (Feldman 1986; Marriot 1980; Pye and Marriot 1982). Observation of fungal biomass revealed that inhibition was overcome by 24 hr. *Absidia* species are well known to be resistant to antifungal inhibitors (Berg *et al* 1986). Weete and Wise (1987) have reported that some fungi such as the Mucorales exhibit a high degree of natural tolerance to sterol biosynthesis inhibitors. It is thought that they may degrade them to microbiologically inactive metabolites, as is seen in the liver system in mammals (Berg *et al* 1986).

The use of cycloheximide was to show that it is toxic to fungal cells and cannot be used to prove induction in living whole cells, as has been shown by Lee *et al* (1971). At 10 μg/ml and above steroid hydroxylation was completely abolished. In retrospect cycloheximide should have been added during cortexolone conversion to see its effect on cortexolone transformation. Other researchers have shown that by 3 hr after cycloheximide addition, steroid transformation was completely abolished (Zakelj-Mavric *et al* 1986; Clark *et al* 1982), due to its inhibitory effect on protein synthesis.

When an organism is exposed to a foreign compound like cortexolone it can deal with it in two ways - by transformation or metabolism. After transformation, steroid is removed from the cell by rapid excretion (Astrom and Depierre 1986). This work shows that steroid metabolism is the main pathway when glucose is exhausted. As some xenobiotics are hydrophobic, the development of systems for excretion is fundamental to life. It is clear that cortexolone can readily enter the cell and is rapidly excreted after it is dealt with by such polar reactions as hydroxylation. The primary products of

bioconversion 11βC and 11αC are prevented from re-entering the cell in great amounts during exponential growth, probably partially due to their polarity, as they are readily aqueous soluble and can be actively excreted. The cortexolone-converting enzymes respond quite quickly, the products were detected generally 0.5 hr after cortexolone addition. One might expect this, as sudden exposure of an organism to a potentially lethal foreign substance would require a rapid response within minutes or hours (Nebert *et al* 1982). Nebert *et al* (1982) have proposed that organisms possess the genetic capacity to form hundreds or thousands of different P-450 species to cope with different compounds at any given time.

CHAPTER 6

INITIAL INVESTIGATION INTO THE BIO-CHEMISTRY OF CORTEXOLONE BIOCONVERSION BY *Absidia Spinosa*

RESULTS

6.1.1 Preparation and analysis of cell-free extracts from *Absidia spinosa*

In initial experiments to determine the most effective preparation protocol, a Polytron homogeniser was unable to break up the fungal cell wall sufficiently to allow protein extraction. The use of a French-press on the other hand achieved cell breakage, but the subsequent assay of all fractions produced no detectable cortexolone hydroxylating activity. This could possibly be due to the harsh pressures applied which could cause localized heating or internal structural damage to membrane associated proteins. Acceptable cell breakage was finally achieved using glass ballotini beads and liquid nitrogen in a pestle and mortar

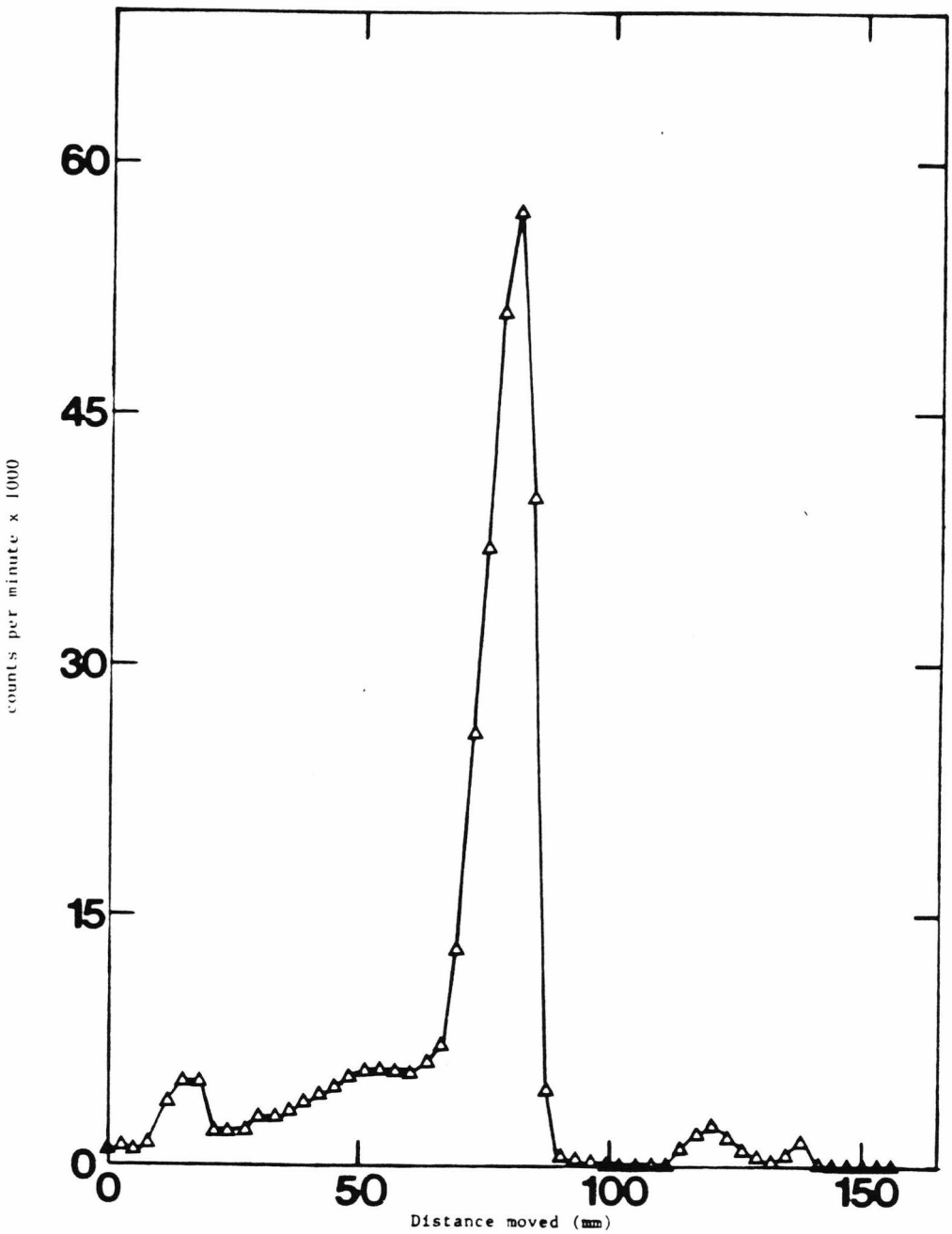
The yields of protein obtained from cortexolone and non-cortexolone treated *Absidia spinosa* cells by this method, were 36 and 16.3 mg of protein per g wet weight of fungal cells respectively. Samples were assayed using a regenerating system immediately at every stage of preparation from homogenate to microsomal and cytosolic fractions, but no activity was detected in any of the fractions used (Figs. 6.1 and 6.2). The use of liquid nitrogen was continued as it was the least severe method for obtaining cell protein. The use of the protease inhibitor PMSF (10μM), cyanide (1mM), dextran coated charcoal, peptone medium (2% w/v), prolonging the incubation time from 1-3 hr and changing the assay temperature from 30°C to 25°C and 37°C produced no detectable activity in any of the fractions used. Cofactors NADPH and NADH

were used in place of a regenerating system but still no activity could be detected.

Table 6.1

Organism	Disruption technique used	Authors
Cunninghamella elegans	Glass beads with a brown tissue homogenizer in the presence of liquid carbon dioxide	Cerniglia & Gibson (1978)
Curvularia lunata	Motor-driven potter-Elvehjem all glass homogenator for 4 min at 1000 rpm	Zuidweg (1968)
Aspergillus niger	Sand with a pestle and mortar at 4°C	El-Kady (1982)
Rhizopus nigricans	Sorvall Omni-mixer (16000 rpm) at 0°C	Sallam *et al* (1971) Breskvar & Hudnik-Plevnik (1977)
Aspergillus ochraceus	Glass powder in a pestle and mortar at 0-4°C	Jayanthi *et al* (1982)
Asperillus ochraceus	Frozen mycelia (whole cells) mixed with alumina and ground with a pestle and mortar	Ghosh & Samanta (1981)

Fig 6.1 Investigation into [^{3}H] cortexolone bioconversion in cell-free preparations of A.spinosa using t.l.c.

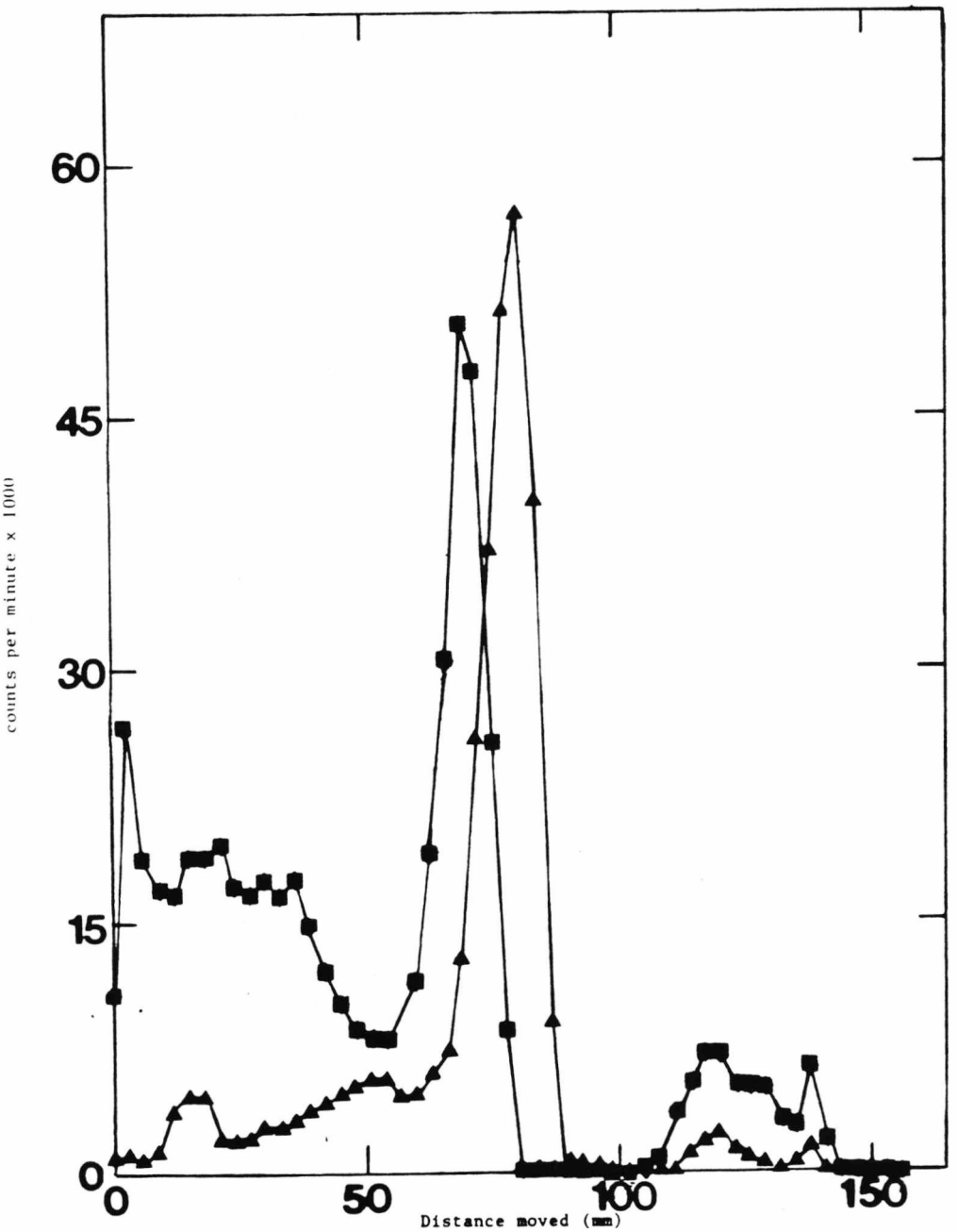


Figs 6.1 and Figs 6.2

A.Spinosa cell - free preparations were incubated with ; 0.5mM NADP+, 5mM cortexolone and 0.66 μCi (3H) cortexolone in 0.1 M phosphate buffer. Samples were extracted using 3 x 5ml dichloromethane and separated in a dichloromethane:acetone (7.3) system. Radioactivity was measured by a Beckman LS7 800 scintillation counter.

(3H) Cortexolone standards were either extracted (0.66μCi or not extracted (0.6 μCi) before separation. The extra peaks seen in all samples are contaminants in the standard used, not transformation products.

Fig 6.2 Separation of [^{3}H] cortexolone standards on t.l.c. plates.



● [^{3}H] cortexolone standard
(▲) [^{3}H] cortexolone extracted from incubation mixture, in the absence of cell-free fractions.

Fig6.3 Coomassie blue staining of A.spinosa cell-free fractions

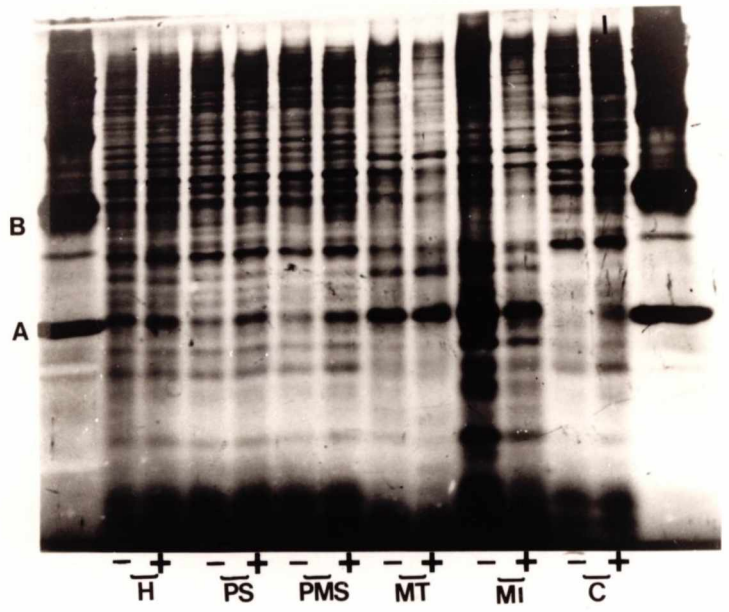
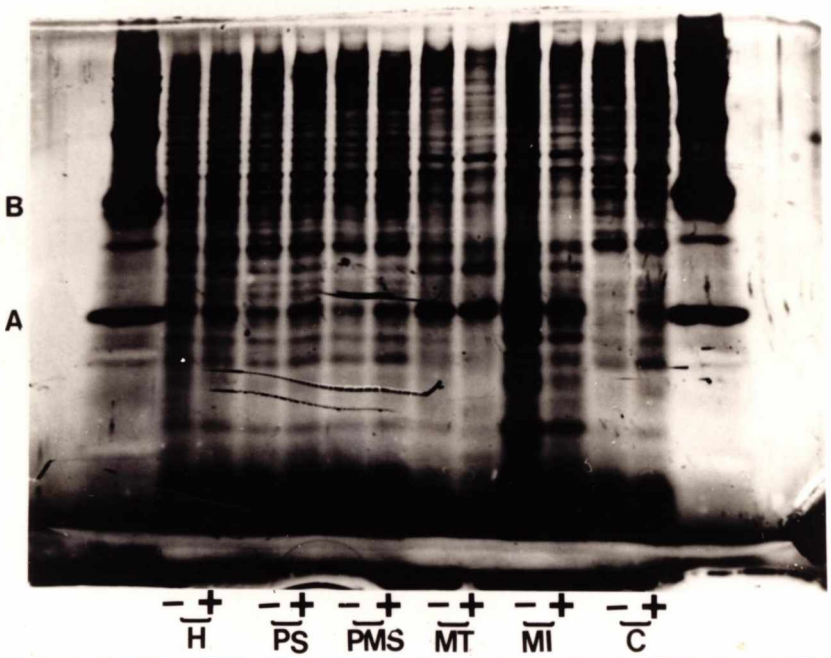


Fig6.4 Silver staining of A.spinosa cell-free fractions



Figs 6.3 and 6.4

A.Spinosa cell-free fractions were separated using sodium dodecyl sulphate - polyacrylamide gel electrophoresis. When coomassie blue staining, 40μg of protein was loaded into gel wells. For silver staining, 2μg of protein was loaded +; steriod treated ; -, non-steroid treated H, homogenate; PS, 1090g supernatant; PMS, postmitochondrial supernatant; MT, mitochondrial pellet ; MI, microsomal pellet; C, cytosol. The MI(-) fractions shown, have been overloaded with protein.

Molecular weight markers; A, triosephosphate isomerase (molecular weight 26,600) B lactate dehydrogenase (molecular weight 36,500).

Oxidised vs reduced difference spectra produced cytochrome peaks at 420 nm and 550 nm (not shown). These peaks were restricted to the mitochondrial fraction. No peaks were found in post mitochondrial supernatant, microsomal or cytosolic fractions suggesting no cytochrome P-450 was present. Coomassie blue staining and silver staining of PAGE preparations from all fractions revealed that there was no gross difference between control and steroid treated fungal cell proteins (Figs. 6.3 and 6.4 respectively). This would imply that induction only occurs at low levels in relation to the total cell protein. It was found that when the mycelium was washed with saline and cell breakage had occurred that cortexolone was present in the homogenate.

6.2.1 DISCUSSION

The preparation of active cell-free extracts from *Absidia spinosa* was not possible. If more time were available, other enzymes which characterize cytochrome P-450 such as benzo(a)pyrene hydroxylase would have been searched for (Dutta *et al* 1983), as well as marker enzymes for various fractions i.e. alkaline phosphatase for microsomal fractions and glucose-6-phosphate dehydrogenase for cytosolic fractions. I do not believe that the use of liquid nitrogen grinding in a pestle and mortar in the presence of fine glass beads was responsible for the loss of activity seen. Liquid nitrogen has been used for some time to obtain active cell-free extracts from filamentous fungi (Smith *et al* 1968). Other authors have reported loss of hydroxylase activity when known methods for cell disruption were applied (Zakelj-Mavric *et al* 1986; El-Kady 1982). Talboys and Dunnill (1985), have suggested that one of the principal reasons for the sensitivity of the enzyme-membrane complex could be shear damage. They proposed that any loss of constituent enzymes from the membrane or distortion of their spatial relationships within the membrane could be detrimental to enzyme activity. The techniques used for cell disruption are not standardized, and several methods exist (Table 6.1). Some of

these varied procedures generate more shear than the use of liquid nitrogen, and still lead to active cell-free extracts. In fact Smith *et al* (1989) used liquid nitrogen to freeze mycelia. The frozen mycelia were then ground with glass-powder in a pestle and mortar. The buffers used were comparable to that used in this project and active cell-free preparations were obtained from *Phycomyces blakesleeanus*. The majority of published literature on steroid hydroxylation refers to the 11α- hydroxylase from *Rhizopus nigricans, Rhizopus arrhizus* and *Aspergillus ochraceus* (Breskvar and Hudnik-Plevnik 1981; Zakelj-Mauric and Belic 1987; Jayanthi *et al* 1982). The literature on other organisms is somewhat limited apart from the 11β- hydroxylase from *Curvularia lunata* (Zuidweg 1962; 1968 and Lin and Smith 1970b). It is probable that the hydroxylase systems are labile (Sonomoto *et al* 1981) and it could be more difficult to prepare active cell-free extracts from some species than from others. It has already been reported that the 11α- hydroxylase system is complex, and may be distributed between the microsomal fraction and cytosol (Cresnar *et al* 1985; Breskvar *et al* 1987). This could be the reason for the lack of hydroxylase activity. The fact that the hydroxylase enzyme complex appears more labile in some species of filamentous fungi could reflect a structural difference between them. It is possible that the components of the system are loosely bound to the membrane and hence easily removed during the preparation of cell-free extracts from filamentous fungi when they are removed from their native hydrophobic environment (Madyastha *et al* 1984; Sonomoto *et al* 1980). Shibahara *et al* (1970) have reported that some enzymes were more labile than others. They obtained active 11α- hydroxylase in cell-free extracts of *Aspergillus ochraceus* but the 17β- hydroxysteroid dehydrogenase which was active in vegetative cells, lost all activity in cell-free preparations. This instability has also been seen in some mammalian systems, such as the aromatase enzyme systems in mammalian placenta (Young *et al.* 1964; Muto and Tan 1986).

It has been proposed that inactive steroid hydroxylases could be due to the presence of oxygen which is known to inactivate cytochrome P-450. (Muto and Tan 1986). Protease inhibitor PMSF and EDTA at pH 8.3 are known to inhibit proteolytic activity in cell-free extracts (Madyastha *et al* 1984). Cyanide inhibits normal respiration and enhances the activity of the enzyme complex (Ghosh and Samanta 1981). DCC removes any endogenous steroid that could compete with cortexolone, but no activity was seen. The peptone medium was used as it was thought that it might contain some elements which would activate the enzyme systems.

The choice of 12 hr as the point in growth to harvest cells exposed to cortexolone and cells not exposed to cortexolone, was made because the enzymes present at this point in growth are near peak activity in relation to fungal biomass. If one harvests the cells at this point in growth one would expect more active material in relation to unwanted mycelial mass. Cells growing exponentially have been used by Zuidweg (1968), to prepare active cell-free preparations from *Curvularia lunata* and therefore were not thought to pose a disadvantage.

If more time were available cells would have been harvested in stationary/decline phase to observe if active hydroxylases were present. Stationary/decline phase in growth was not favoured initially as it had been reported that late on in fermentations, there was a drop in induction levels of the 11α-hydroxylase in *Aspergillus ochraceus* (Ghosh and Samanta 1981). From the results obtained in Sections 4 and 5, it was thought that there could be an alternative reason for a fall in induction levels apart from proteolytic activity, as proposed by Ghosh and Samanta (1981). It was envisaged that perhaps steroid degradation or secondary transformation might account for the drop in levels seen. It was therefore decided that peak activity in exponential growth was the best choice to study the primary transformation of cortexolone in whole cells and cell-free preparations. The absence of a absorbance peak at

450 nm does not necessarily mean that there is no cytochrome P-450 present. A possible explanation is that the amount of cytochrome P-450 could be so low that it was not detected. A more plausible explanation was suggested by Samanta and Ghosh (1987). They obtained a cytochrome P-450 peak with solubilized microsomes. The inability of microsomal pellets to furnish a maximum at 450 nm in the CO-reduced spectra may be due to the presence of some contaminants. A possible candidate was cytochrome oxidase which is believed to interfere with CO-reduced spectra.

No gross changes in the amount of protein could be observed using coomassie blue or silver staining from induced or control samples. This would imply from our knowledge of such systems, that the enzymes responsible for steroid hydroxylations may not be induced in large levels, in relation to total cell protein. Talboys and Dunnill (1985) suggested it may not be possible to greatly increase intracellular levels of these enzyme complexes, because of the physical limitations in the capacity of cells to contain more membrane. The fact that cortexolone was detected inside the mycelium after careful washing is indicative that this must be removed before any accurate analysis can be undertaken. This problem is not tackled by some of the researchers in the field. Smith *et al* (1989), using the techniques of nuclear magnetic resonance spectroscopy and chromatography have corroborated my findings. They detected steroid molecules after washing steroid-induced cells. They suggested that some of the published literature may have presented false positive results, because of the presence of contaminating steroid in cell-free preparations. Contaminants occurred even when very low concentrations of inducer were used, and the fungi had been extensively washed with water and saline. One possible solution to the problem is the use of DCC as this removes hydrophobic steroid type molecules. This interesting possibility could not be explored as no activity was detected in any of the fractions tested.

CHAPTER 7

INITIAL INVESTIGATION OF THE FEASIBILITY OF IMMOBILIZING FUNGAL SPORES INTO CHROMOSORB W BEADS

RESULTS

7.1.1 Immobilization of *A.niger* on 5% Chromosorb W (30-60) beads

Using a stationary incubation phase for immobilization, untreated Chromosorb W (Fig. 7.1) entrapped a similar percentage of spores as steam treated (Fig. 7.1) Chromosorb W (38% and 40% respectively). Chromosorb W sterilised by dry heat entrapped 60% of *A.niger* spores by 2 min. Untreated Chromosorb W reached maximum absorption by 15 min (Fig. 6.1) whereas steam treated Chromosorb W approached maximum entrapment by 20 min (Fig. 7.1).

If one compares a stationary to a shaking incubation phase for immobilisation (Fig. 7.2), it can be seen that there was a difference in the percentage entrapment of spores. In the stationary immobilization method maximum entrapment (38%) occurs by 20 min. If a shaking phase is employed, maximum entrapment (94%) of spores occurred by 2 hr. It was calculated that approximately 270 spores were entrapped by each Chromosorb W bead. It has been calculated (Gbewonyo and Wang, 1981), that 70% of the entire bead volume is void space and that 90% of this may be accessible for microbial growth. The average bead pore diameter quoted was 1-10μm. The range of bead diameter determined by microscopic examination was 250-460μm. The *A.niger* spores used were found to be circular, and 4-5μm in diameter.

Light microscopy and scanning electron microscopy reveal that hyphae (4.3μm wide) emanate from the beads after 6 hr of growth (Figs. 7.3 and 7.4A respectively). The length and number of hyphae continues to increase as growth proceeds (Fig.7.3). It was observed that mycelial fragments and spores

Fig 7.1 A comparison of heat treated, steam treated and untreated 5% chromosorb W for immobilizatio using A. niger

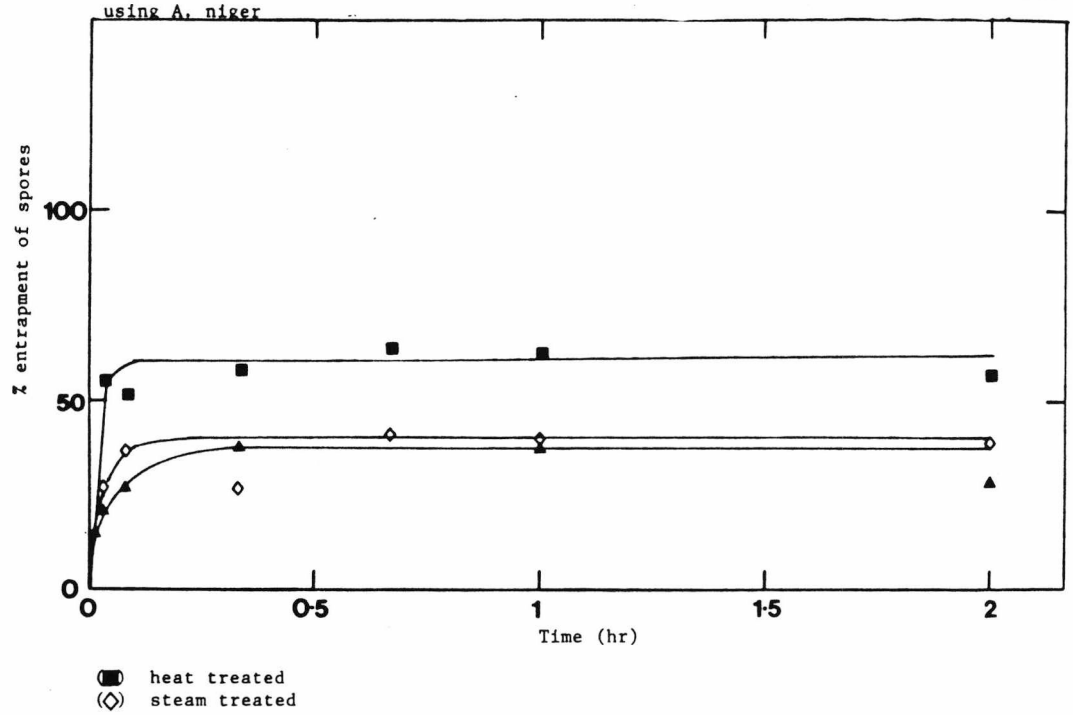
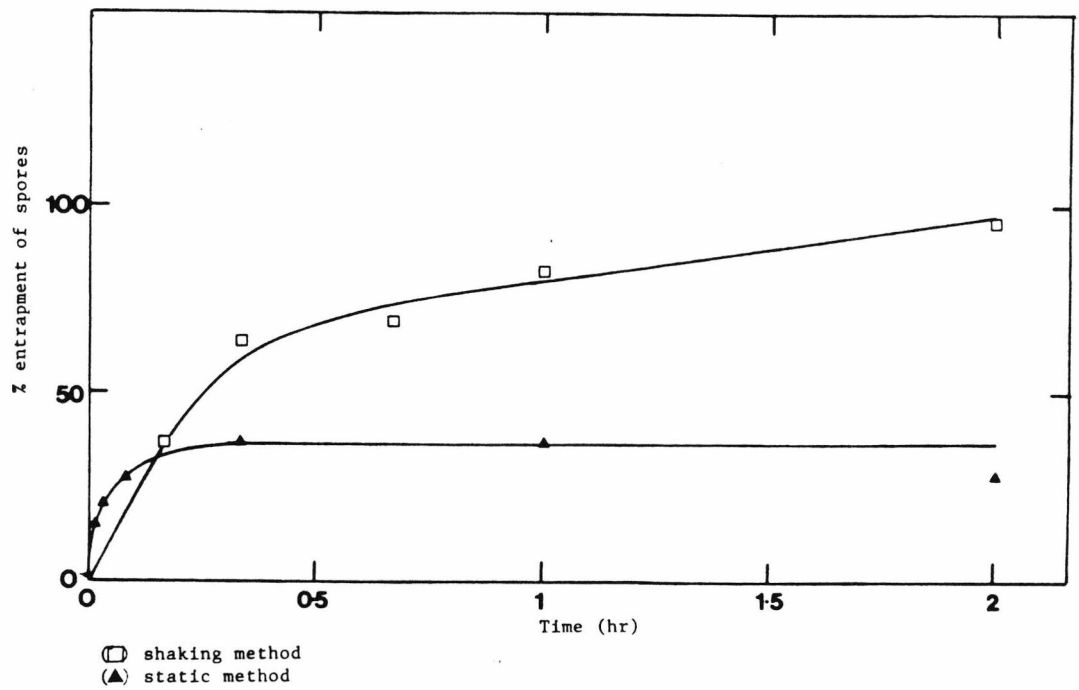


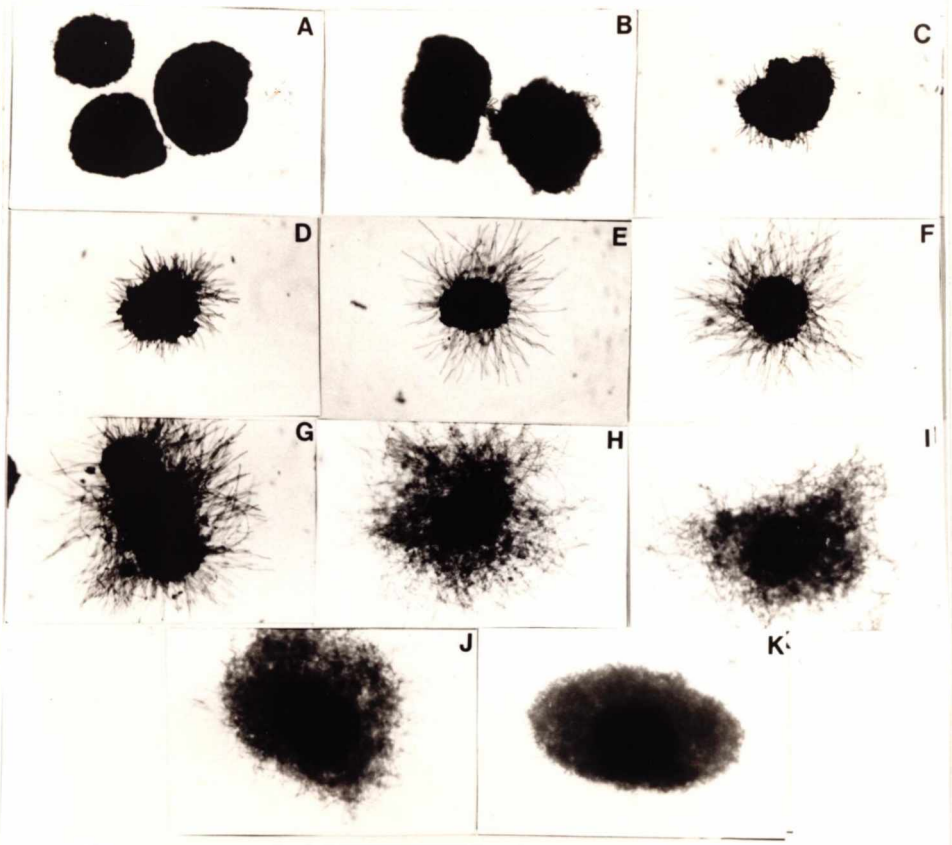
Fig 7.2 A comparison of a shaking and static immobilization method with 5% chromosorb W using A.niger



Figs 7.1 and 7.2

A.niger spores were dislodged using 3mm diameter glass beads. Spores were filtered through two layers of muslin. 1g of chromosorb W beads were then steam sterilized in a pressure cooker at 10 psi for 15 min or dry heat sterilized at 400°C for 3hr. Control flasks contained non-heat treated chromosorb W (using a static immobilization method) In a static immobilization method, 5 ml of spore suspension in sterile distilled water containing 0.5 % (v/v) Tween 80 was added and samples removed at varying time intervals (Gbewonyo and Wang, 1983) In a shaking immobilization method, 25 ml of spore suspension in medium A containing 0.5 % (v/v) Tween 80 was incubated at 200 rpm, 30°C and samples removed at varying time intervals. The medium was decanted and the beads washed with three volumes of sterile distilled water, containing 0.5 % (v/v) Tween 80 free fungal spores were then counted on a haemocytometer.

Fig7.3 Growth of immobilized A.niger spores in chromosorb W beads



A,0hr ;B,3hr ;C,6hr ;D,10hr ;E,17hr ;F,21hr ;G,25hr ;
H,33hr ;I,50hr ;J,73hr ;K,120hr

Fig 7.3

A.niger spores immobilized in 5 % chromosorb W were obtained from a shaking incubation phase. 25ml of medium A was added and flasks incubated at 200 rpm, 30°C. A sample was removed at intervals and fixed in 2% (w/v) glutaraldehyde, before being viewed and photographed under the light microscope (56 x magnification).

Fig7.4 Scanning electron micrographs of A.niger colonized and uncolonized chromosorb W beads

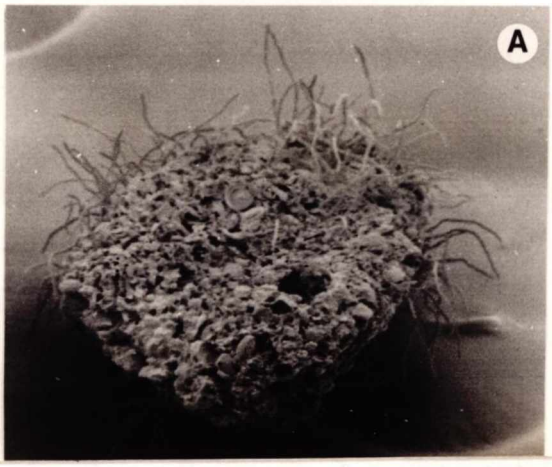


Fig 7.4

A.niger spores immobilized in 5 % chromosorb W were obtained from a shaking incubation phase. 25ml of medium A was added and flasks incubated at 200 rpm, 30°C. A sample was removed at intervals and fixed in 2% (w/v) glutaraldehyde before being prepared for scanning electron microscopy A, 180x mag. (10hr); B, 160 x mag. (uncolonized chromosorb W); C, 160 x mag. (21hr)

Fig 7.5 Growth of A.niger immobilized in 5% chromosorb W and in the free state

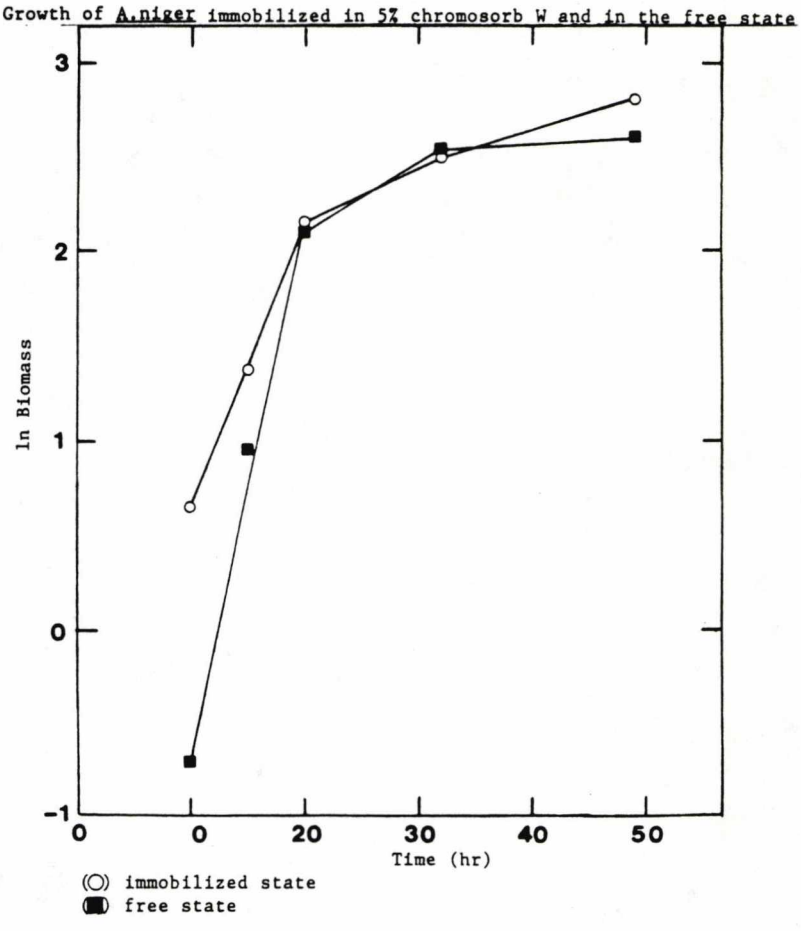


Fig 7.5

A.niger spores were incubated at 200 rpm, 30°C in the presence of 5% chromosorb W for 3 hr. Chromosorb W was steam sterilized at 10 psi for 15 min before use. The medium was decanted and the beads washed with three volumes of sterile distilled water containing 0.5% (v/v) Tween 80. 25ml of medium A was added and flasks incubated at 200 rpm, 30°C for selected times. Flasks were removed at intervals and filtered onto preweighed whatman filter paper under vacuum. Cells were then washed with deionized water. Fungal biomass and bead dry weight were determined.

Fig7.6 Scanning electron micrographs of M.genevensis growing in and around chromosorb W beads

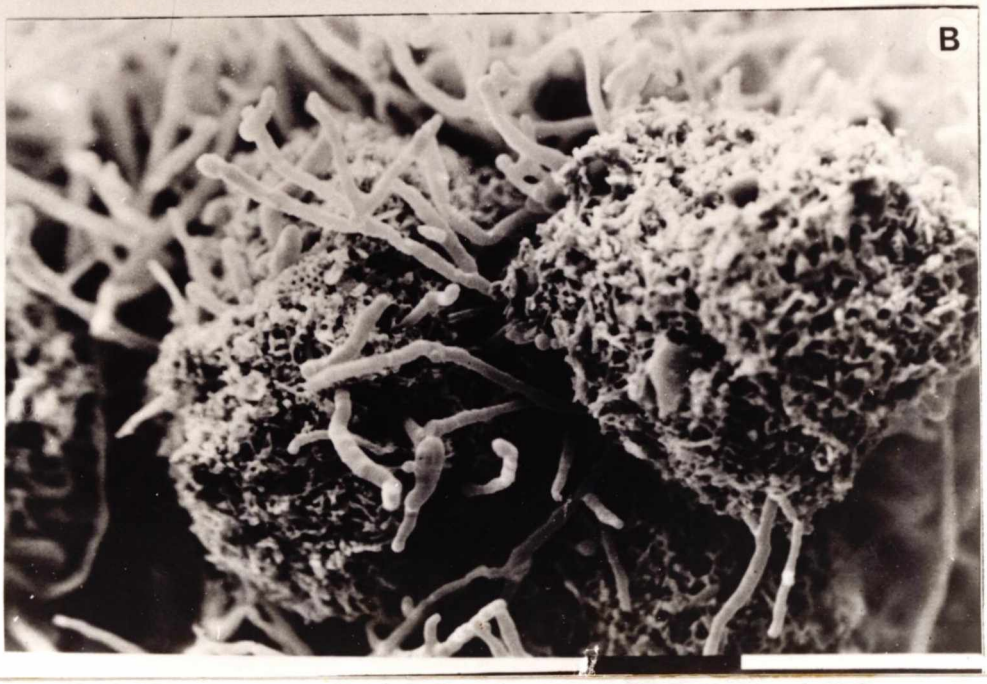


Fig 7.6 .

M.genevensis spores immobilized in 5% chromosorb W were obtained from a shaking incubation phase. 25ml of medium A was added and flasks incubated at 200 rpm, 30° C. Samples were removed at intervals and fixed in 2% (w/v) glutaraldehyde, before being prepared for scanning electron microscopy. A, 218 x mag. (40hr); B, 420 x mag (40hr)

Fig 7.7 % Entrapment of M.genevensis spores in 5% chromosorb W.

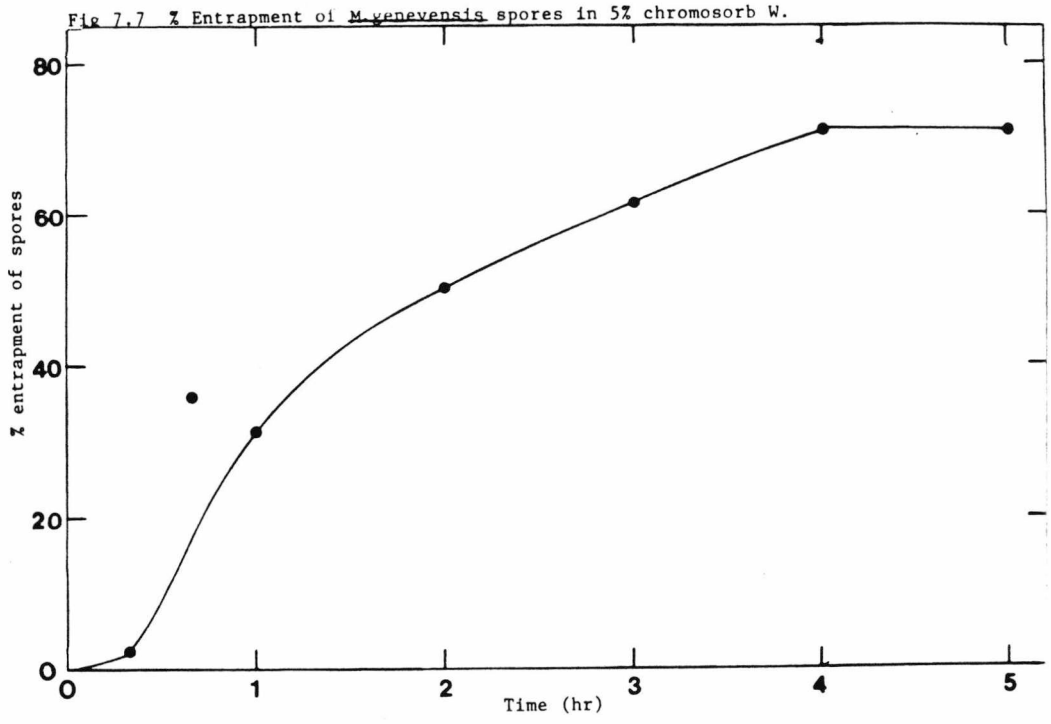


Fig 7.7

M.genevensis spores were dislodged using 3 mm diameter glass beads. Spores were filtered through two layers of muslin. 1g of chromosorb W beads were steam sterilized in a pressure cooker at 10 psi for 15 min. 20ml of medium A was added to 1g of chromosorb W. 5ml of spore suspension was then added and flasks incubated at 200 rpm, 30°C. Samples were then removed at varying time intervals. The medium was decanted and the beads washed with three volumes of sterile distilled water containing 0.5 % (v/v) Tween 80. Free fungal spores were then counted on a haemocytometer.

sources of secondary growth in the system. One can see the difference between uncolonized Chromosorb W beads (Fig. 7.4b) and those colonized by *A.niger* at 21 hr (Fig.7.4c). By 53 hr (Fig.7.3) the hyphae had formed a round ball with the Chromosorb W bead as its centre. This arrangement of hyphae around the bead had become more compact by 120 hr.

In the absence of Chromosorb W beads, *A.niger* exhibited an observed μ_{max} of 0.33 hr^{-1} and a t_d of 2.1 hr (Fig.7.5). In the presence of Chromosorb W beads μ_{max} was reduced to (0.15 hr^{-1}) and t_d increased (4.8 hr). Immobilized *A.niger* had a growth rate which was 45% that of free *A.niger*. Although immobilised *A.niger* grows at a slower rate than free cells (Fig.7.5), it still produced a greater biomass (16.48 ± 0.6 g/l and 13.38 ± 0.00 g/l respectively after 49 hr). The percentage entrapment of *A.niger* spores was approximately 96% for all spore loadings (not shown). Increasing spore loading increased the number of spores entrapped in the bead interior. High spore loadings (> 2.6×10^7 spores) were found to be necessary for extensive bead colonization. Pellet formation and reduced colonization of beads was observed using lower spore loadings, (< 5.2×10^6 spores) by 48 hr. A minimum of around 260 spores/beads are required for good colonization of the Chromosorb W beads.

M.genevensis has ovoid spores (12 × 4μm). It was noted that the initial spore loading (between 10^6 - 10^7 spores), was tenfold lower than *A.niger*. 50% of *M.genevensis* spores were entrapped by 2 hr (Fig. 7.6). Maximum percentage entrapment was found to be 71% by 4 hr. Scanning electron microscopy revealed that *M.genevensis* hyphae (12.7 μm wide) were wider than those of *A.niger* (4.3 μm). Some hyphae were still seen to originate from bead pores (Fig. 7.6a). The growth characteristics of *M.genevensis* was such that it tended to grow in clumps and cover the beads rather than forming separate fungal-bead type colonies as with *A.niger*. This can be seen from the scanning electron micrographs (compare Figs.7.4c and 7.6b respectively).

It was decided that though the initial objective of immobilizing fungal spores into Chromosorb W beads is possible, the easy generation of pockets of secondary growth and the limited knowledge of growth characteristics would make immobilization of fungal spores for steroid transformation implausible, as it was not known how fungal growth was related to steroid transforming ability. The most plausible approach would be to investigate the relationship of fungal growth to steroid hydroxylating ability. Understanding the system from a physiological and even a biochemical point of view would give an overview of the enzyme system(s) in terms of activity and stability. This would provide a better rationale for immobilization. This work was therefore not continued, but the initial results and conclusions are discussed in Section 7.1.2.

7.1.2 DISCUSSION

Spores of *A.niger* can be absorbed by dry or water saturated Chromosorb W. When a stationary incubation phase is utilized, a 20% increase in spore absorption is seen using dry heat sterilization, compared to any other treatment. Spore uptake has been explained in terms of capillary suction (Gbewonyo and Wang 1981; Gbewonyo and Wang 1983). It was proposed that the spores were transported into the bead interior by drag forces generated by convective flow of the liquid into the spore matrix. It was thought that in the course of transport through the bead that spores could be captured at constriction sites within the pore matrix. This physical entrapment is probably supplemented by electrostatic bonds of attraction formed between the metal ions (Si, Al, Fe) in the bead and specific groups on the spore surface. Capillary suction could have been increased in beads which are in a moisture deficient environment. When the method of Gbewonyo and Wang is used, only half the number of spores were absorbed into the bead network compared to *P.chrysogenum*. There are differences between the two methods used however. Gbewonyo and Wang used four times the amount of Chromosorb

W than was used in this work, with higher spore loadings and smaller diameter *P.chrysogenum* spores (2μm) on average compared to *A.niger* (Gbewonyo and Wang 1983).

This work agrees with that of Keshavarz *et al* (1989) who observed 30-40% entrapment of *P.chrysogenum* spores in 5% (w/v) Chromosorb W at 2 hr. The higher percentage entrapment obtained by Gbewonyo and Wang could reflect the increased Chromosorb W concentration (20% (w/v)) used. The fact that the amount of spores entrapped in the beads increased to 94% by 2 hr employing a shaking incubation phase, suggests that spore penetration into the bead interior is not solely due to capillary suction, but passive spore intake is also possible. This implies that the entrapment process is primarily governed by the probability of spores colliding directly with adsorption/entrapment sites in the beads.

By assuming that spores and beads are perfectly spherical and knowing that there are 270 spores/bead, then it can be calculated that the average bead volume was approximately 10^3 - 10^4 times larger than the average total volume of spores associated with it. This suggests that there is more than enough space to accommodate all the spores, and that as 70% of the bead volume is void space (Gbewonyo and Wang 1981), it is probable that if all the spores and beads are in constant association, then most of them could be associated with the microbeads at any given time. This is a reasonable assumption as the average bead pore diameter encountered was 1-10μm (Gbewonyo and Wang 1983).

It was observed using high dilution rates in a bubble column with *Streptomyces cattleya* that the percentage of Chromosorb W coated with biomass was only 45% compared to 75% in shake flasks (Arcuri 1983). Using *A.niger* and *P.chrysogenum* this value was observed to be approximately 100%. This suggests that in shake flasks the incidence of collision is greatly increased as opposed to a bubble column. This points to possible difficulties for *in situ* inoculation of Chromosorb W beads when scaling up in bubble columns.

The type of microbial growth seen for *A.niger* and *P.chrysogenum* showed the same characteristics. Hyphae of both organisms emanated from the bead structure around the entire bead circumference. As with *P.chrysogenum*, *A.niger* produced a greater biomass when associated with Chromosorb W than in the control flasks with no Chromosorb W. Gbewonyo and Wang (1983) have explained this phenomenon as being due to the restructuring of the filamentous morphology of the cells in the spherical particles, and this alleviates gas-liquid mass transfer limitations, which tends to produce higher cell densities for confined fungal cultures. This phenomena has been used to increase penicillin productivity in a 200L tower fermentor (Wang et al 1984). It may also be possible that a change in fungal morphology could lead to a change in fungal metabolism (Sypherd 1978). When the organism is growing in the microbead environment the shear forces it experiences are reduced. When fungal hyphae grow into the medium it is possible for the shear forces together with natural mycelial fragmentation which occurs as growth ceases, remove hyphae, which can then become sources of secondary growth in the late exponential phase. The generation of sporulating structures also produces free cells in the system.

The results show that not all fungal spores are suitable for immobilization. The governing factors would appear to be spore size, shape and charge. The ovoid spores of *M.genevensis* (12 × 4μm) were larger than *A.niger* and this could account for the lower percentage entrapment of *M.genevensis* spores in Chromosorb W, as most pore sizes are between 1-10μm. This may also explain why *M.genevensis* spores would not necessarily penetrate as far into the bead interior as the smaller more spherical *A.niger* spores. *M.genevensis* also produces thicker hyphae than *A.niger* which tended to clump the beads together, suggesting they were probably absorbed/entrapped nearer the bead surface.

The feasibility of immobilization is not in doubt but is dependent on the organisms spore shape, size, charge and growth characteristics of the organism. *M.genevensis* itself is typical of Zygomycotina, having large spores and relatively broad hyphae, which are perhaps less suitable for immobilization, than other fungi, such as *P.chrysogenum* or *A.niger*. The fact that most of the fungi used in this study were Zygomycetes, also suggested that the immobilization studies using Chromosorb W ought to be discontinued.

CHAPTER 8

GENERAL DISCUSSION & SUMMARY

A number of organisms from natural and public sources have been screened for their ability to modify cortexolone and/or progesterone. A high percentage of isolates from *Agave mexicana* (~ 75%) could modify cortexolone to several different products. However, random screening of the leaf and rhizosphere of *Agave* without any selection pressure did not yield a product co-migrating with 11β- or 11α-hydroxycortexolone in greater amounts relative to the other products. Fungi obtained from culture collections were found to produce products co-migrating with both 11β- and 11α-hydroxycortexolone in greater amounts relative to other products and were further studied.

It was found that steroid bioconversion occurred irrespective of fungal morphology in liquid culture. Progesterone was predominantly 11α-hydroxylated even in organisms where 11β- hydroxylation was known to occur. This selective transformation of progesterone could be explained by steric hindrance, due to different functional groups on the D ring.

A survey of all the organisms tested showed that 11α- hydroxylation can occur independently of 11β- hydroxylation, but the latter transformation always accompanied 11α- hydroxylation. The formation of 11αC is understandable as this water soluble compound is more polar than 11βC, thus its removal from the cell could shift the equilibrium of the transformation towards product formation from the more hydrophobic substrate cortexolone. 11βC always occurred in greater or equimolar amounts than 11αC, the reason for this is unknown. *P.articulosus* and *A.spinosa* were discovered to be novel producers of both 11βC and 11αC.

Comparative spore germination and mycelial transformation studies have been undertaken in *A.spinosa*, *C.blakesleeana* and *C.elegans*. When germinating spores were used there was a lag in cortexolone modification with

all three organisms. Cortexolone biotransformation was initiated and correlated quite closely with germ tube production in *A.spinosa* and *C.blakesleeana*. The maximum amount of product produced in relation to biomass, corresponded to the complete germination of all viable *A.spinosa* and *C.blakesleeana* spores. In all three organisms 11βC always preceded 11αC in its initial production in germinating spores or in vegetative cells. Once formed the ratio of both primary transformation substrates was constant. The 11β- and 11α-hydroxylating activities are growth associated in exponential phase, but are also capable of functioning when growth has ceased. A varying number of transformation products were formed from cortexolone by all three organisms in germinating spores and in the vegetative state.

The cessation of growth leads to the "shutting down" of primary transformations. Fungal morphology may direct the fate of steroid molecules in the decline phase. In *C.blakesleeana* and *C.elegans* pellet formation appeared to promote steroid metabolism in the decline phase to products no longer detected using this system. As reduced products could have been formed, it may be possible that fungi may degrade steroids in a similar manner to bacteria, by reduction and hydroxylation of the steroid A or B rings. The purpose of steroid metabolism in conditions of glucose exhaustion could be to derive energy from steroid carbon skeletons.

In filamentously growing *A.spinosa* the cessation of growth did not halt transformation, but changed its direction. Secondary transformation of the primary transformation products was observed in the decline phase. Cortexolone bioconversion in the decline phase showed that both primary and secondary transformation products were detected 0.5-1 hr respectively after cortexolone addition. This could suggest that the enzymes responsible for secondary transformation are those involved in normal growth processes. These enzymes initiate the transformation of steroid molecules in the late exponential phase of growth. 11βC proved to be a major source of the secondary

transformation products. No epimerase was detected to convert 11βC to 11αC. The utilization of "steroidal type" specific enzymes and enzymes normally involved in cellular metabolism for exogenous steroid bioconversions is not unlike that seen in mammals. In the decline phase other reactions such as secondary transformation and metabolism of steroids become predominant and may 'shut down' or supercede primary transformation. The transformation products may promote the metabolic routes involved in their degradation. It would seem that all three fungi, regardless of morphology, are capable of metabolizing steroid molecules.

In *A.spinosa* both cortexolone and 11βC do not appear to inhibit fungal growth rate. Biomass production is however more rapid in the absence of either substrate. The removal of both molecules could possibly derive some energy from the cell.

Studies on *A.spinosa* cells in early exponential growth under varying conditions were undertaken and provided information on the transformation enzymes in whole cells. A fast response time to cortexolone addition (0.5-1 hr) was found. Both 11βC and 11αC produced similar transformation patterns in medium A and B in shake flask experimentation, under different conditions in exponential growth. Both enzymes are active over a broad extracellular pH and temperature range. *A.spinosa* growth rate was highest at 25°C and 30°C. Cortexolone transformation was more efficient at these temperatures. At 35°C, the steroid bioconversion enzymes were still active when growth had ceased. The production of enzymes is related to growth of *A.spinosa*, but they can function independently of it.

Both 11β- and 11α- hydroxylating activities were differentially affected by the use of antifungal agents (ketoconazle and metyrapone). Miconazole was more effective at inhibiting 11-hydroxylation than it was against fungal growth.

At increasing cortexolone concentrations fungal growth rate was not inhibited. Cortexolone has two fates in exponential growth; primary

biotransformation and metabolism. One of the degradative products could be androst-4-ene-3, 17-dione. No secondary transformation products were observed in early or mid exponential growth, even in conditions of cortexolone exhaustion. The use of cycloheximide does not necessarily show induction in fungal cells, as its toxicity abolishes cortexolone bioconversion.

The cortexolone biotransformation system in exponential growth would appear to be involved mainly in a detoxifying role, rather than sterol biosynthesis. It would seem that some enzymes involved in growth processes, which may include sterol biosynthesis, can transform cortexolone products to a number of secondary transformation products when growth ceases. As hydrophobic molecules like cortexolone can enter growing fungal cells, one can imagine such biotransformation systems providing rapid transformation and excretion of foreign compounds out of the cell as well as their metabolism. Steroid polarity may stimulate its active removal from the fungus interior. The primary transformation product, 11βC does not occur in large amounts in *A.spinosa* mycelium during growth as it could be rapidly excreted. As the vast majority of xenobiotics are hydrophobic, the development of such systems are fundamental to fungal life. This "defence system" is needed to ensure the survival of the organism in a chemically diverse environment. Once growth ceases, foreign compounds like steroid molecules can be transformed to secondary products or metabolized for energy purposes.

Investigations into the feasibility of immobilization of fungal spores into Chromosorb W beads was successful, but this line of research was not continued as it was decided that understanding the activity and function of the enzymes in free cells was not only more important, but also a more productive line of research.

The preparation of cell-free extracts was unsuccessful due to the instability of the steroid transforming enzymes once cell breakage occurred. This finding has also been observed by other researchers in the field.

8.1.1. Future Work

The work of this project has involved the screening of a large number of fungi for 11-hydroxylation activity. I have shown that 11-hydroxylation activity is widespread in filamentous fungi. This work has also shown that primary transformation occurs during exponential growth, and that secondary transformation is predominant during the decline phase, the products being excreted into the medium. This work was necessary for biochemical and biotechnological exploitation of the 11-hydroxylase system, as there have been few detailed reports on 11-hydroxylase activity in relation to growth. Subsequent work, attempted to gain more information on the physiological factors which affect 11-hydroxylase activity to gain an insight as to their function in cells. The nature of the 11-hydroxylase system in *A.spinosa* made it difficult to perform an extensive investigation in the time scale of the project. Future work, should be directed more towards the biochemical details of the system involved.

If one could obtain pure radiolabelled ^{14}C cortexolone, it would be interesting to follow degradation inside the cell, and investigate the various stages of degradation. One could investigate if the pathway for steroid degradation in fungi is similar to that in bacteria. It would be possible to see if steroid carbon skeletons end up as cellular components or are completely catabolized via metabolism. A priority for future work in the field of steroid biotransformations must be the preparation of stable cell-free extracts and the purification of individual enzymes. One can then draw a comparison between fungal, mammalian and bacterial systems. Kinetic studies could be performed on purified enzymes and their structures examined. One could then ask and answer many questions. Do fungi control expression of their cytochrome P-450 at the pretranslational level like PB induced enzymes? (Pike *et al* 1985; MacGeoch *et al* 1987). Do fungi have insertion sequences in membranes like

hepatic microsomes in mammals? (Sekaguchi *et al* 1987). Is P-450$_{11\beta}$ in fungi modified at a post-translational level as in bovine adrenal? (Nebert and Gonzalez 1987). Are fungal steroid bioconversion enzymes controlled physiologically by covalent phosphorylation and dephosphorylation reactions as is the case in mammals (Pyerin *et al* 1987).

From a commercial point of view, one can use separate purified enzymes or single enzymes produced from isolated genes to manufacture designer drugs. This could be advantageous , as filamentous fungi can modify every position on the steroid molecule. Many authors have already expressed cytochrome P-450 genes in other organisms (Chen *et al* 1987; Schimizu *et al* 1987). As fungal cloning vectors have been developed in some filamentous fungi, such as *Aspergillus niger, Penicillium chrysogenum* and *Aspergillus nidulans* (Saunders *et al* 1986), it is only a matter of time before their steroid transforming genes will be isolated and successfully expressed in other organisms.

APPENDIX 1

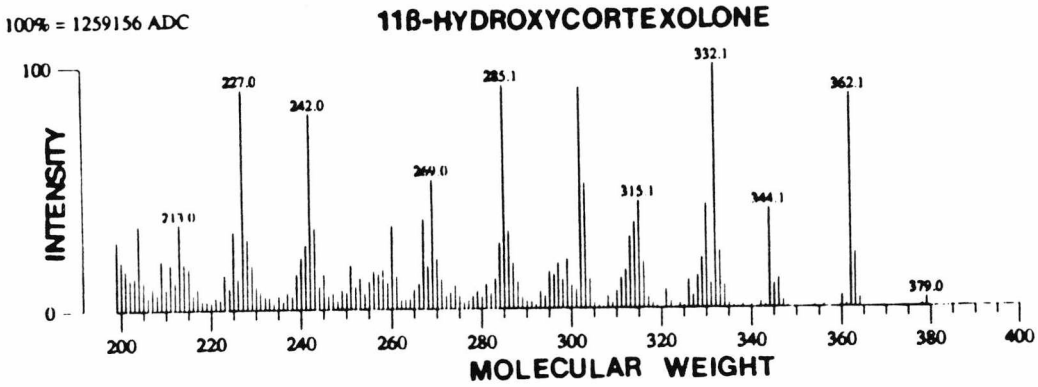
Comparison of solvent systems used for the separation of corticosteroid standards (see Table 3-1)

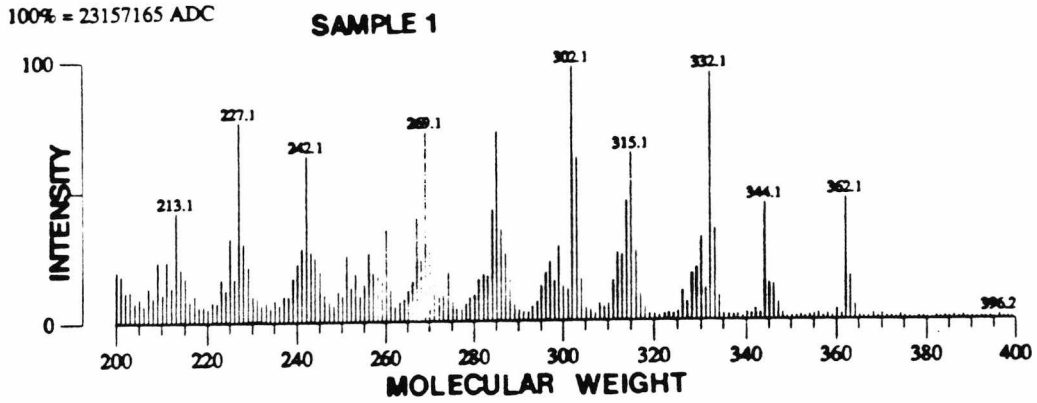
Solvent System	† Rf of steroid standard			† Rf values of steroid molecules extracted from P. articulosus mycelia and media fractions	
	cortexolone	11βC	11αC	number of spots	Rf values of spots
dichloromethane:acetone (7:3)	0.59	0.28	0.10	6	0.80,0.59,0.28,0.21,0.16,0.11
chloroform:acetone (7:3)	0.52	0.23	0.09	6	0.76,0.53,0.25,0.17,0.13,0.09
chloroform:acetone (7:3)	0.49	0.15	0.05	6	0.81,0.49,0.14,0.07,0.05.
dichoromethane:chloroform: acetone (4:4:3)	0.57	0.24	0.08	6	0.82,0.56,0.23,0.15,0.11,0.07
toluene:ethanol (95:5)	0.41	0.11	0.04	6	0.76,0.42,0.17,0.11,0.08,0.04
dichloromethane:ethanol (20:1	0.35	0.23	0.09	6	0.65,0.35,0.23,0.19,0.12,0.09
chlorform:n-propanol (20:1)	0.35	0.23	0.09	3	0.96,0.86,0.72
chloroform:ethanol (7:3)	0.92	0.87	0.83	1	0.88
toluene:methanol (7.5:2.5)	0.49	0.44	0.40	6	0.69,0.50,0.47,0.44,0.42,.0.40
diethylether:n-propanol (7:3)	0.84	0.84	0.70	5	0.93,0.83,0.81,0.70,0.61
diethylether:ethanol (7:3)	0.82	0.82	0.75	5	0.92,0.82,0.81,0.76,0.73
diethlether:methanol (7:3)	0.86	0.86	0.81	4	0.97,0.85,0.81,0.66
methanol:water (7.5:2.5)	0.79	0.79	0.79	1	0.73
chloroform:methanol (7:3)	0.95	0.91	0.89	1	0.91
dichloromethane:toluene (7:3)	0	0	0	1	0

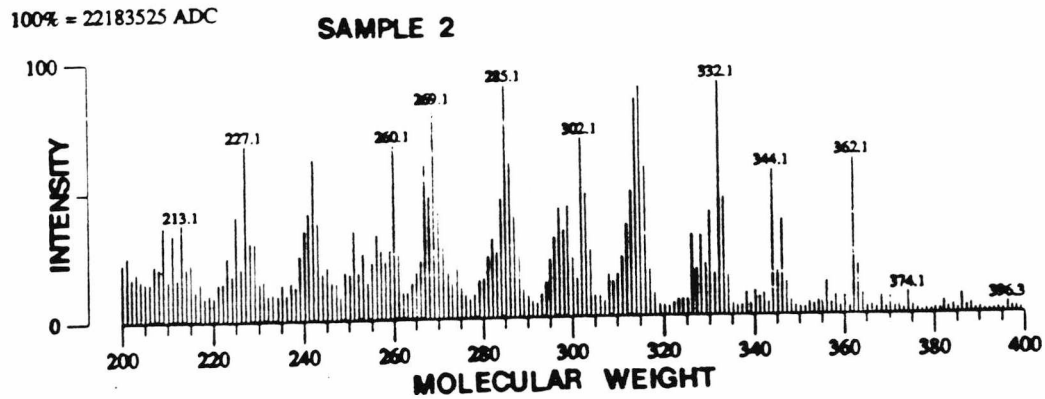
† The Rf values quoted reflect distance moved in relation to the solvent front.

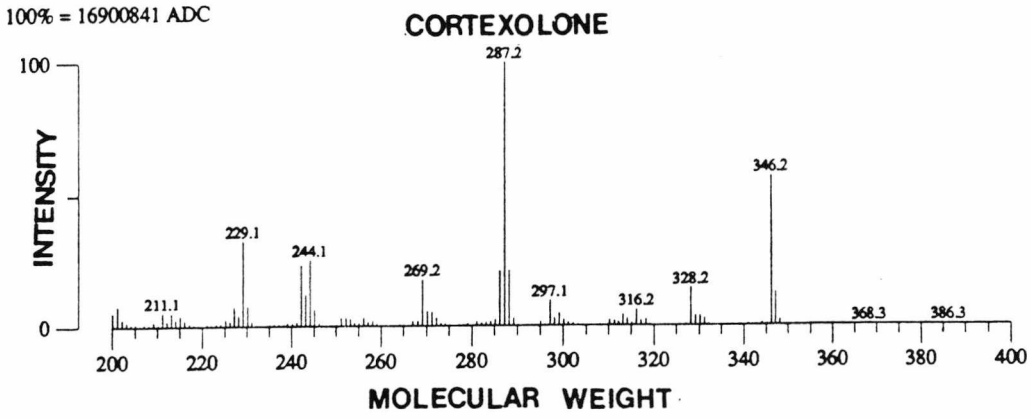
APPENDIX 2

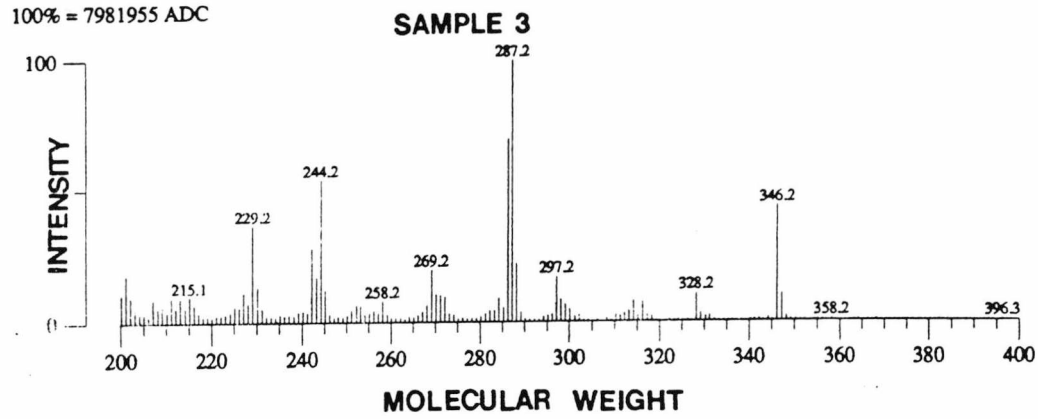
Mass spectroscopy of cortexolone, 11βC and 11αCsamples which had co-migrated alongside authentic cortexolone, 11βC and 11αC standards

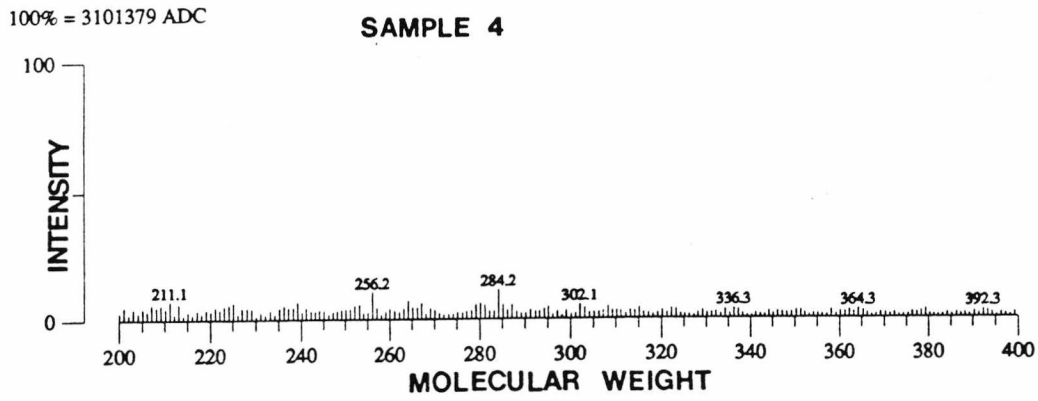








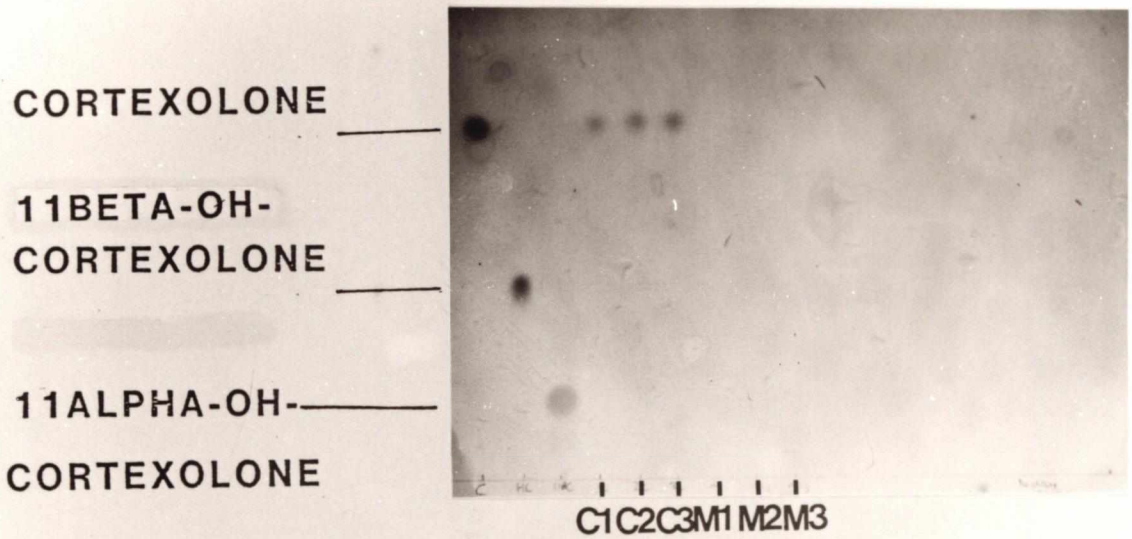




Sample	Molecular weight	Proposed name of sample
1	362.1	11βC
2	362.1	11αC
3	346.2	Cortexolone
4	-	Silica gel

A.spinosa was grown in medium A at 200 rpm, 30°C for 8hr. 1.1mM cortexolone in 0.5 ml MeOH was added and growth continued for 4 hr. Flasks were removed and whole culture medium was extracted three times using dichloromethane. Steroid molecules were separated twice in dichloromethane:acetone (7:3) on plastic backed fluorescent kieselgel plates (60). Steroid molecules were removed from silica using dichloromethane:acetone (50:50). Samples were filtered through glass wool and dried using nitrogen gas. Samples were analysed on a Kratos mass spectrometer using electron impact.

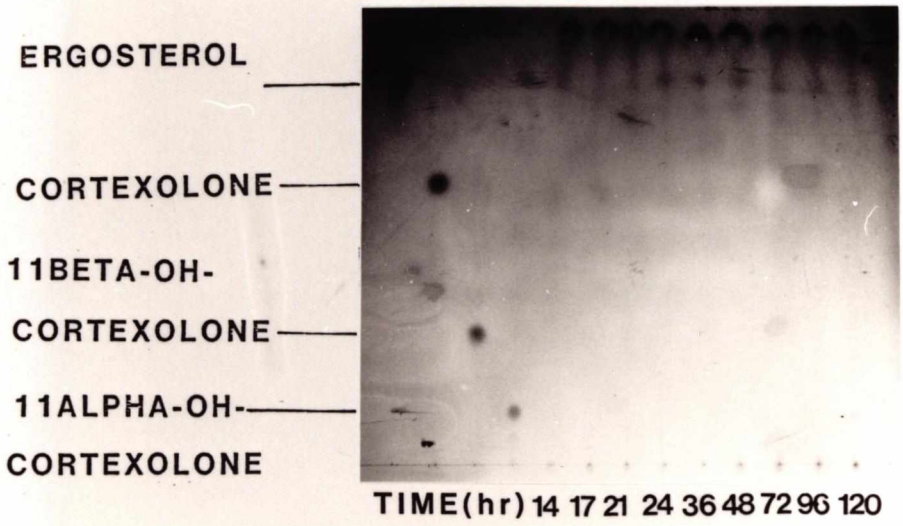
Tlc plate of uninoculated medium extracted with dichloromethane and separated using dichloromethane:acetone(7:3)



M1,M2,M3-medium fraction in triplicate
C1 ,C2 ,C3 - Cortexolone in triplicate extracted from medium

APPENDIX 3

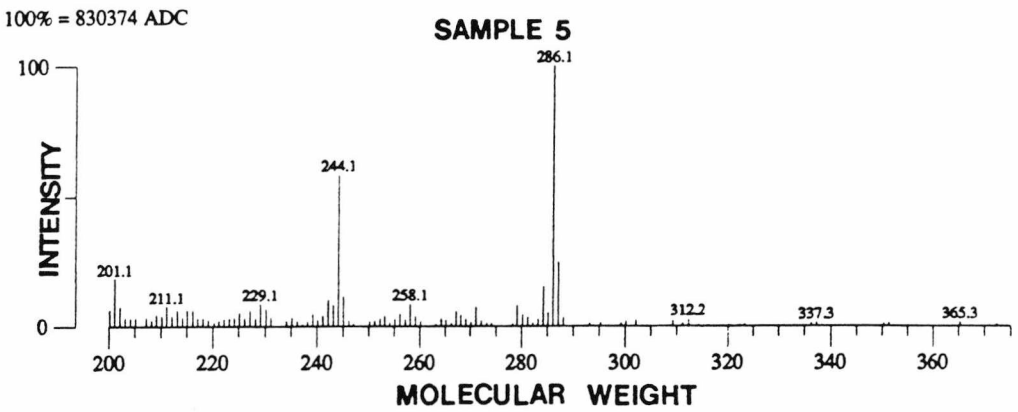
Tlc plate of A.spinosa vegetative cells growing in the absence of cortexolone,extracted with dichloromethane and separated using dichloromethane:acetone(7:3)



APPENDIX 4

APPENDIX 5

Mass spectroscopy of sample 5



Sample 5 has a molecular weight of 286.1 and could be androst-4-ene-3, 17-dione.

A.spinosa was grown in medium A at 200 rpm, 30°C for 8hr. 1.1mM cortexolone in 0.5 ml MeOH was added and growth continued for 4 hr. Flasks were removed and whole culture medium was extracted three times using dichloromethane. Steroid molecules were separated twice in dichloromethane:acetone (7:3) on plastic backed fluorescent kieselgel plates (60). Steroid molecules were removed from silica using dichloromethane:acetone (50:50). Samples were filtered through glass wool and dried using nitrogen gas. Samples were analysed on a Kratos mass spectrometer using electron impact.

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