The 19-Hydroxylation Of Cortexolone By The Fungus *Pellicularia filamentosa*

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THOMAS ALAN CLARK

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ABSTRACT

The microbiological 19-hydroxylation of steroids was studied to investigate the feasibility of a microbial process to produce 19-hydroxysteroids. These are important precursors of the valuable 19-norsteroids. At present, in industrial processes, 19-hydroxylation is performed by chemical synthesis.

Fungi, selected from the genera *Pestalotia* (25 strains) and *Pellicularia* (5 strains), were screened for their steroid-hydroxylating activities. Thus, hydroxylation of the substrates progesterone, 4-androstene-3,17-dione, and cortexolone (17α,21-dihydroxy-4-pregnene-3,20-dione) was studied. Of the organisms tested, only *Pellicularia filamentosa* f.sp. *microsclerotia* IFO 6298 and *Pellicularia filamentosa* f.sp. *sasakii* IFO 5254 were found to perform 19-hydroxylation. Thus, both fungi could produce 19-hydroxycortexolone from cortexolone, with the former organism the more active in this respect. Hydrocortisone (11β-hydroxycortexolone) was also produced by both organisms. Neither organism, however, could similarly hydroxylate progesterone nor 4-androstene-3,17-dione. With these substrates, products other than the 19-hydroxylated derivatives were formed. Thus a degree of substrate specificity was recognised for steroidal-19-hydroxylation by these fungi. None of the *Pestalotia* species tested could 19-hydroxylate any of the three substrates, despite claims in the literature, but instead were very active in 11α-hydroxylation. In particular, many species were able to 11α-hydroxylate progesterone (0.5 g/l concentration) in greater than 90% ($^{w}/_{w}$) yield.

Using *P. filamentosa* f.sp. *microsclerotia* IFO 6298, in batch fermentation, at a cortexolone concentration of 0.5 g/l yields of 19- and 11β-hydroxycortexolone totalled approximately 40% ($^{w}/_{w}$) of

the consumed substrate. The ratio of the two products, typically, was approximately 1.2:1 (19:11β). Only small variations in this ratio were ever observed. The steroid losses which were observed did not proceed via the hydroxy products as intermediates, but via a degradation pathway, from cortexolone, parallel to the hydroxylation reactions.

The 11β- and 19-hydroxylase enzyme-system of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was shown to be inducible by cortexolone. By using the protein synthesis inhibitor, cycloheximide, in fermenter culture the effects of dissolved oxygen tension (DOT) on enzyme induction and enzyme expression were separately investigated. For both hydroxylations, an optimum DOT for induction was shown at 15% of saturation, while the optimum for expression is at 30% of saturation. Thus, maximum rates of hydroxylation were achieved when induction was performed at low DOT, followed by elevation to ensure maximum expression.

The effects of specific glucose consumption rate and specific growth rate were investigated using chemostat cultures, under automatic DOT control (at 30% of saturation). At a constant specific growth rate, the importance of glucose metabolism to the hydroxylation process was demonstrated. Thus, with glucose-limited cultures, decreasing specific hydroxylation rates were observed with decreasing specific glucose consumption rates, possibly as a result of the restricted availability of NADPH, which is required for hydroxylation to occur. Conversely, with nitrogen-limited cultures, it was observed that the hydroxylase system is subject to glucose repression. Thus, with high specific glucose consumption rates, cultures showed low levels of hydroxylation activity. Maximum activity was obtained at a point, which apparently represents a balance between sufficient glucose metabolism to

maintain full expression of activity and a condition of excess glucose uptake resulting in repression of activity. This finding may be of considerable significance for fungal steroid-hydroxylation processes in general, since relief of repression, when it exists, could give several-fold increases in specific hydroxylation rates as observed in this study. Over the range of specific growth rates studied (0.028 h^{-1} to 0.119 h^{-1}) no significant effect on specific hydroxylation rates was observed.

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TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

List Of Figures

PAGE

PAGE

PAGE

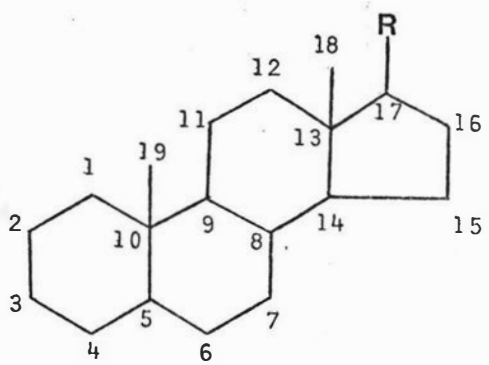
APPENDICES

List Of Tables

STEROID NOMENCLATURE AND STRUCTURE

The trivial names used for steroids are given, followed by the abbreviations (if any) employed, in brackets, and their systematic chemical names (I.U.P.A.C.-I.U.B., 1969).

Numbering of the steroid ring system and side chain groups is as follows:



R	Stem Name
-H	androstane
20 21 $-CH_2-CH_3$	pregnane

Androstenedione (AD) = 4-androstene-3,17-dione

Estrone = 3β-hydroxy-19-nor-1,3,5(10)-androstatrien-17-one

Cortexolone (Co) = 17α,21-dihydroxy-4-pregnene-3,20-dione

Hydrocortisone = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione

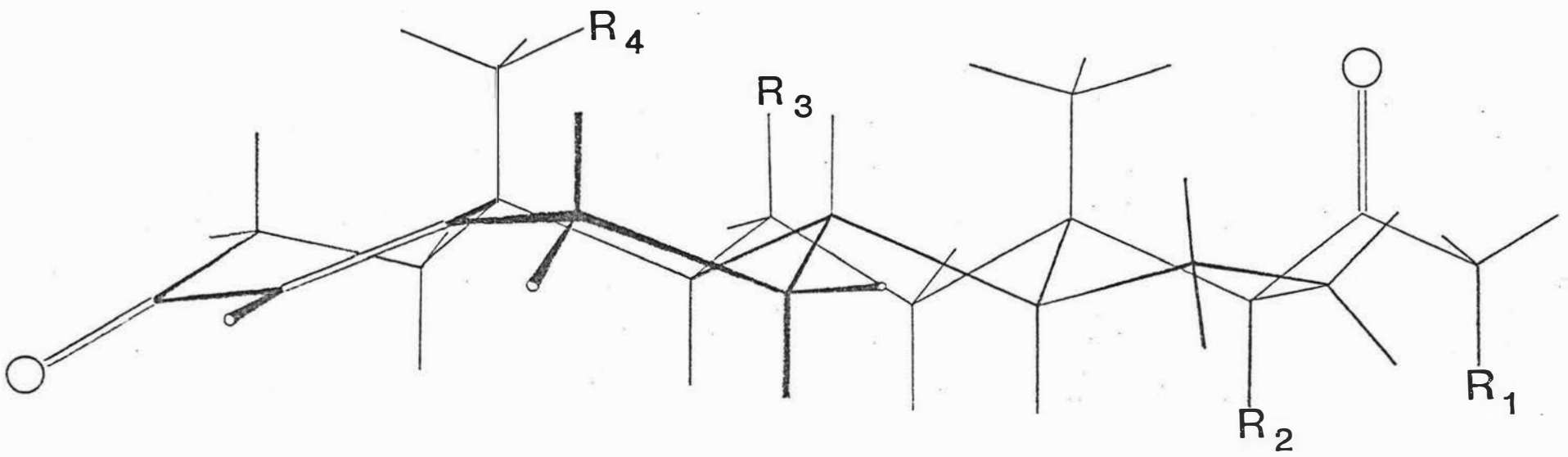
Norethisterone = 17α-ethynyl-17β-hydroxy-19-nor-4-androsten-3-one

Oestradiol = 3β,17β-dihydroxy-19-nor-1,3,5(10)-androstatriene

Progesterone (Pr) = 4-pregnene-3,20-dione

Pregnenolone Acetate = 3β-hydroxy-5-pregnen-20-one acetate

The following figure shows a Dreiding Model of substituted progesterone. The axial and equatorial nature of the various substituent bonds, around the ring system, can be clearly seen.



Dreiding Model of Substituted Progesterone

$R_1 = R_2 = R_4 = OH$, $R_3 = H$ 19-hydroxycortexolone

$R_1 = R_2 = R_3 = OH$, $R_4 = H$ 11β-hydroxycortexolone

DESIGNATION OF STERIOCHEMISTRY

α	(alpha)	designates a bond below the plane of the ring system
β	(beta)	designates a bond above the plane of the ring system
ξ	(xi)	designates a bond of unknown steriochemistry

ABBREVIATIONS

Abbreviations of Units

amu	atomic mass unit
^{o}C	degrees Celsius
d	day
g	gram
h	hour
l	litre
m	meter
M	mole/litre
mM	millimole/litre
m/e	mass:charge ratio
min	minute
mg	milligram
ml	millilitre
nm	nanometer
psi	pounds per square inch
rpm	revolutions per minute
s	second
µF	micro Farad
Ω	Ohm
KΩ	Kiloohm

Other Abbreviations

AcO-	acetyl
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
An.	Acetone
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUFS	Absorbance Units Full Scale
B.P.	Boiling Point
C	Capacitor
cAMP	cyclic AMP
Cyt-P_{450}	Cytochrome-P_{450}
D	Dilution Rate (h^{-1})
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DOT	Dissolved Oxygen Tension
DW	Dry Weight (g/l)
EtOH	Ethanol
HOAC	Acetic Acid
HOBr	Hypobromous Acid
hplc	High Performance Liquid Chromatography
I.D.	Internal Diameter
IFO	Institute for Fermentation, Osaka
IMI	Commonwealth Mycological Institute
IPA	Isopropyl alcohol
I.R.	Infra-red
MDW	Mycelial Dry Weight (g/l)
MeOH	Methanol
MNC	Mycelial Nitrogen Concentration (g/l)

m.p.	Melting Point
MPN	Mycelial Percentage Nitrogen (% w/w)
mRNA	messenger RNA
m.s.	Mass Spectrum
N	Nitrogen
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NBA	N-Bromoacetamide
NMR	Nuclear Magnetic Resonance
PDDCC	Plant Diseases Division Culture Collection (N.Z. D.S.I.R.)
pTsOH	para-Toluene sulphonic acid
Py	Pyridine
q_{19}	Specific Rate of 19-Hydroxylation (mg/g N.h)
$q_{11\beta}$	Specific Rate of 11β-Hydroxylation (mg/g N.h)
q_{gl}	Specific Glucose Consumption Rate (g/g N.h) (g/g DW.h)
R	Resistor
R_f	Tlc mobility of a compound relative to the solvent front mobility
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
R_t	Hplc Retention time (min)
S	Glucose concentration in the fermenter (g/l)
S_o	Feed-medium glucose concentration (g/l)
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
tlc	Thin Layer Chromatography
UV	Ultra-violet
μ	Specific Growth Rate (h^{-1})

CHAPTER 1

INTRODUCTION

The production of steroidal fine chemicals in New Zealand, based on indigenous raw materials, is of considerable interest to both researchers and commercial concerns. The extraction of bile acids from animal gall by New Zealand Pharmaceuticals Ltd. (Garland, 1978) and of solasodine from *Solanum* species by the Fletchers-Diosynth group (Kloosterman, 1979) show that this type of industry can operate commercially in New Zealand.

A potential area of growth in this industry is the further processing of the basic raw materials to higher value steroids prior to export. In this respect, New Zealand faces the problem of whether it can do more efficiently what is already being done by others overseas. Profitability is significantly affected by advantages in process technology (Cullwick, 1981). For example, the partial synthesis of a high value steroid using existing chemical methods is unlikely to be competitive. This is because in New Zealand most of the chemical reagents must be imported and the scale of operation would be much smaller than is the case overseas. Much more suited to the New Zealand situation would be a fermentation-based industry where multi-step chemical syntheses can be replaced by one-step microbiological transformations. Such technology has become established overseas in certain areas of steroid manufacture where a microbiological transformation is more efficient than any known chemical method. Kieslich (1980b) describes examples of these processes.

To obtain a competitive advantage New Zealand industry must develop novel microbiological technology. Research aimed at this long-term goal has been in progress in the Biotechnology Department, at Massey University, for a number of years. The work described in this thesis was undertaken

to investigate the feasibility of a microbiological 19-hydroxylation process. This process, which is at present carried out by chemical synthesis, leads to important precursors of the 19-norsteroids which are used, in particular, as anti-fertility drugs.

CHAPTER 2

LITERATURE SURVEY

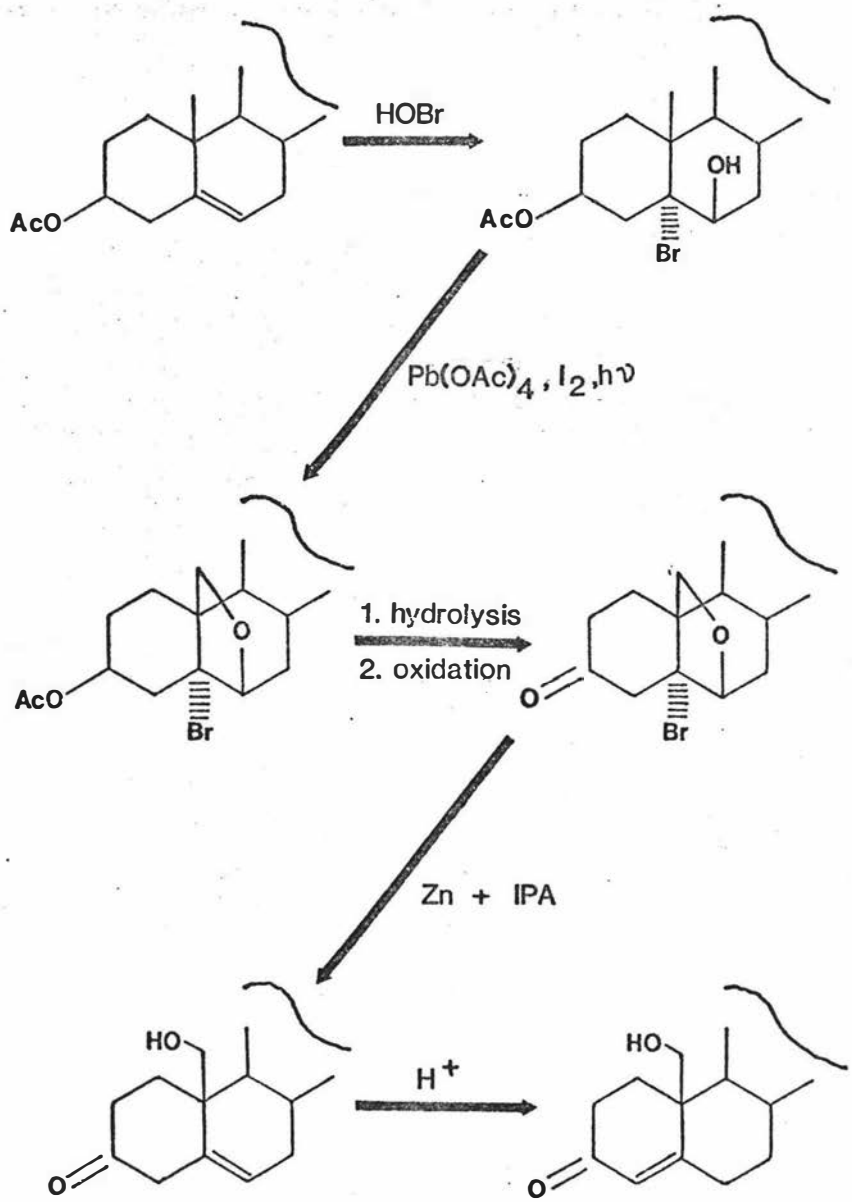
2.1 INTRODUCTION

Since the historic discovery by Peterson and Murray (1952) that a fungal microorganism (*Rhizopus nigricans*) could introduce an 11α-hydroxyl group into progesterone, so providing a new technology for the manufacture of adrenocortical hormones, the impact of microbiology in the field of steroid chemistry has been immense. A voluminous literature on microbiological steroid transformations has accumulated with major review articles and monographs regularly appearing (for example, Vischer and Wettstein, 1958; Charney and Herzog, 1967; Iizuka and Naito, 1967; Marsheck, 1971; Vezina *et al.*, 1971; Hayakawa, 1973; Smith, 1974; Vezina and Rakhit, 1974 and Kieslich, 1980a). In particular, the microbiological hydroxylation of steroids has received intensive study and microorganisms capable of hydroxylating the steroid system at almost every position are now known. Although much of the research has not resulted in the introduction of new microbiological technology, microbial hydroxylations at positions 11α, 11β, and 16α now form important steps in steroid manufacturing processes (Kieslich, 1980b).

The production of 19-hydroxylated steroids is an area of considerable commercial significance, since these compounds are direct precursors of the medically-important 19-norsteroids. The partial synthesis of 19-norsteroids, from readily available raw materials, involves elimination of the C-10 angular methyl group. Functionalisation of this group is a prerequisite for its removal, and hydroxylation is the most common means of achieving this (Heusler and Kalvoda, 1972).

A chemical route for C-19 hydroxylation of steroids has been developed (Bowers *et al.*, 1962), and proceeds via a lead tetraacetate oxidation of 5α-halogen-6β-hydroxy intermediates, which are obtained from the corresponding 5,6-unsaturated steroids

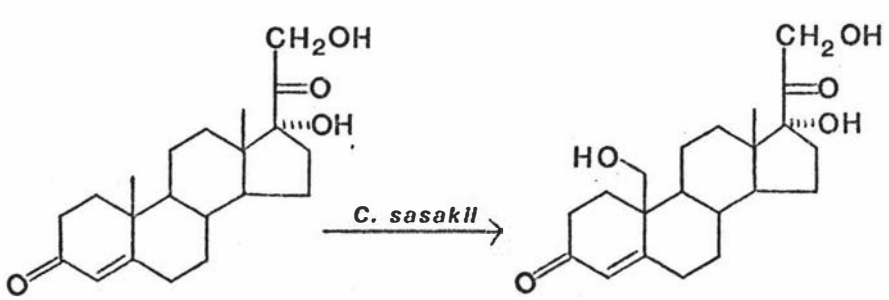
Figure 2.1 A typical synthetic route for the production of 19-hydroxysteroids.



by the addition of hypohalous acid. A typical sequence is shown in Figure 2.1. This route is used commercially to produce 19-hydroxysteroids in 40-50% overall yield (an estimate, based on data from Heusler and Kalvoda, 1972).

Although it is well known that enzymes in animal tissues effect hydroxylation of the C-10 angular methyl group in steroids (Hamberg *et al.*, 1974), it remains a rare steroid transformation among microorganisms. Most of the research concerning a microbiological 19-hydroxylation process was carried out in the late 1950's and early 1960's. Work in this area appears to have almost stopped following the development of the chemical route, although a small number of patents describing this transformation have appeared more recently. Hasegawa and Takahashi (1958) first reported 19-hydroxylation using the Basidiomycete *Corticium sasakii*, which converted cortexolone (Reichstein's Substance 'S') to the corresponding 19-hydroxyl compound (Fig. 2.2). However, hydrocortisone (11β-hydroxy-cortexolone) was the major transformation product observed. Subsequently, this transformation has been reported on a variety of substrates with the fungi *Corticium vagum* and *C. microsclerotia* (Hasegawa *et al.*, 1960), *Hypochnus sasakii* (Shull, 1962a); *Pellicularia filamentosa* f.sp. *microsclerotia* (Takahashi and Hasegawa, 1961b), *Pestalotia* spp. (Hasegawa *et al.*, 1963; Courtieu, 1970), *Calonectria decora* (Chambers *et al.*, 1975), and *Nigrospora sphaerica* (Petzoldt, 1980).

Figure 2.2 The 19-hydroxylation of cortexolone by *Corticium sasakii*.



Interestingly, 19-oxygenated steroids are also available from certain plant species, for example the cardenolide strophanthidin from seeds of the genus *Strophanthus* (Heftmann and Mosettig, 1960). These, however, have apparently never been commercially significant sources of raw materials for 19-norsteroid synthesis.

2.2 MICROBIOLOGICAL 19-HYDROXYLATION OF STEROIDS

Many of the reports of this transformation have appeared in the patent literature with rather a paucity of scientific publications. Information from these two sources are summarised, respectively, in Tables 2.1 and 2.2.

Corticium sasakii, *Hypochnus sasakii*, and *Pellicularia filamentosa* f.sp. *sasakii* are all synonyms for the same organism, as are *Corticium microsclerotia* and *Pellicularia filamentosa* f.sp. *microsclerotia* (Exner, 1953). This narrows considerably the apparent range of fungi capable of hydroxylating steroids at the C-19 position. The initial finding of Hasegawa and Takahashi (1958) was followed by the report that *P. filamentosa* f.sp. *microsclerotia* IFO 6298 produces the 19-hydroxylated compound from cortexolone in greater amount compared to other products (mainly hydrocortisone) than does *P. filamentosa* f.sp. *sasakii* IFO 5254 (Takahashi and Hasegawa, 1961b). Yields of the 11β- and 19-hydroxylated products were given as <10% and approximately 40%, respectively.

Takahashi (1963b) then showed that *P. filamentosa* f.sp. *microsclerotia* IFO 6298 could also 19-hydroxylate 17α, 20α,21- trihydroxy-4-pregnen-3-one, a compound identical to cortexolone except for the presence of a 20α-hydroxyl rather than a 20-ketone group. The 11β-hydroxy product was also produced. Thus a small change in the side chain had little effect on the transformation products. On the other hand, when hydrocortisone was incubated with the organism, no 19-hydroxylation occurred, indicating that the 11β-hydroxyl group blocked the transformation. Takahashi (1964) also incubated

Table 2.1 Patent claims for microbiological 19-hydroxylation of steroids.

Substrate	Organism	Reference
4-androstene-3,17-dione 4-pregnene-3,20-dione 21-hydroxy-4-pregnene-3,20-dione 17α,21-dihydroxy-4-pregnene-3,20-dione 17α,21-dihydroxy-4-pregnene-3,20-dione 21 acetate	*Corticium microsclerotia* IFO 6298	Hasegawa *et al.*, 1960
17α,21-dihydroxy-4-pregnene-3,20-dione	*C. vagum*	Hasegawa *et al.*, 1960
3ξ-hydroxy-4-androsten-17-one 17α-ethyl-17β-hydroxy-4-androsten-3-one 17β-hydroxy-17α-propyl-4-androsten-3-one 17α,21-dihydroxy-4-pregnene-3,20-dione	*Hypochnus sasakii*	Shull, 1962a
11β,17α,21-trihydroxy-4-pregnene-3,20-dione	*Hypochnus sasakii*	Shull, 1962b
17α,21-dihydroxy-4-pregnene-3,20-dione	*Pestalotia* sp.	Hasegawa *et al.*, 1963
17α-ethynyl-17β-hydroxy-4-pregnen-3-one	*Pestalotia* sp.	Courtieu, 1970
6-chloro-17α-hydroxy-1α,2α-methylen-4,6-pregnadiene-3,20-dione 17α-hydroxy-6-methyl-4,6-pregnadiene-3,20-dione	*Nigrospora sphaerica*	Petzoldt, 1980

Table 2.2 References from the scientific literature for microbiological 19-hydroxylation of steroids

Substrate	Organism	Reference
17α,21-dihydroxy-4-pregnene-3,20-dione	*Corticium sasakii*	Hasegawa and Takahashi, 1958; Nishikawa and Hagiwara, 1958; Hagiwara, 1960
17α,21-dihydroxy-4-pregnene-3,20-dione	*Pellicularia filamentosa* f.sp. *sasakii* IFO 5254	Takahashi and Hasegawa, 1961b
17α,21-dihydroxy-4-pregnene-3,20-dione	*Pellicularia filamentosa* f.sp. *microsclerotia* IFO 6298	Takahashi and Hasegawa, 1961b
17α,20α,21-trihydroxy-4-pregnen-3-one		Takahashi, 1963b
5α-androstan-17-one 5α-androst-2-en-17-one 1β,6α-dihydroxy-5α-androstan-17-one 5α-androstane-1,17-dione 5α-androst-2-ene-1,17-dione 6α-hydroxy-5α-androstane-1,17-dione 6α-hydroxy-3α,5-cyclo-5α-androstan-17-one 6α,11α-dihydroxy-3α,5-cyclo-5α-androstan-17-one 3α,5α-cyclo-5α-androstane-6,17-dione	*Calonectria decora*	Chambers *et al.*, 1975
17α,21-dihydroxy-4-pregnene-3,17-dione	*Pestalotia* spp.	Takahashi, 1963b

a variety of C_{19} steroids with *P. filamentosa* f.sp. *microsclerotia* IFO 6298 and examined the products obtained. Substrates were converted as shown in Table 2.3. The most obvious feature of these data is that 19-hydroxylation was not effected with any of these substrates. This suggests that the presence of such side chains as dihydroxyacetonyl and glyceryl at the 17-position may be essential for 19-hydroxylation by this organism. This effect of substrate structure on the positions hydroxylated may result either from the action of different enzymes, or from the same enzyme where the point of hydroxylase attack depends on the nature of binding between steroid and enzyme. Since polar groups in the substrate are probably responsible for the binding (Jones, 1973), the site and structure of these would determine the form of the enzyme-substrate complex. Which of these possibilities is responsible for the apparent substrate specificity of the 19-hydroxylase of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 is unclear. A further interesting feature of the data in Table 2.3 is that the organism did produce strong 11β-hydroxysteroid-dehydrogenase activity. 11β-Hydroxysteroids, if they had no hydroxyl group elsewhere, were easily oxidised to the corresponding 11-ketones. However, this dehydrogenation was considerably inhibited by a hydroxyl group at the 15α-position (δ to the 11β group) and almost completely by a 14α-hydroxyl group (γ to the 11β group) (Table 2.3). Thus, functional groups projecting to the α-side of the steroid nucleus are capable of inhibiting the 11β-hydroxy-dehydrogenation. This is noteworthy since no dehydrogenation ability was described when this organism was transforming cortexolone or 17α,20α,21-trihydroxy-4-pregnen-3-one; both of which compounds possess a 17α-hydroxyl group (γ to the 11β group).

No further work with different substrate structures has been reported for this organism. However, the patent of Hasegawa *et al.* (1960) indicates that *C. microsclerotia* (deposited at IFO and NRRL under accession numbers IFO 6298 and NRRL 2727, respectively) can 19-hydroxylate the range of steroids shown in Table 2.1. Since this is the same isolate

Table 2.3 The transformation of C_{19} steroids by *P. filamentosa* f.sp. *microsclerotia* IFO 6298 (Takahashi, 1964).

Substrate	Functional Group on Nucleus	Main Reactions	Side Reactions
11β-hydroxy-4-androstene-3,17-dione	11β-OH	11β-OH dehydrogenation 14α- hydroxylation	-
11β-hydroxy-1,4-androstadiene-3,17-dione	11β-OH Δ^1		-
4-androstene-3,11,17-trione	11-CO	14α-hydroxylation	-
14α-hydroxy-4-androstene-3,17-dione	14α-OH	11α-hydroxylation	11β-hydroxylation
15α-hydroxy-4-androstene-3,17-dione	15α-OH	11β-hydroxylation	11β-dehydrogenation
9α-hydroxy-4-androstene-3,17-dione	9α-OH	14α-hydroxylation	6β-hydroxylation
1,4-androstadiene-3,17-dione	Δ^1	11β-hydroxylation 11β-OH dehydrogenation 14α-hydroxylation	-

as used by Takahashi (1964) and Takahashi and Hasegawa (1961b), certain of the patent claims seem questionable. For example, although 4-androstene-3,17-dione was not examined by Takahashi (1964), 1,4-androstadiene-3,17-dione was, and 19-hydroxylation was not observed. From the other data reported in Table 2.3, it appears that the Δ^1-double bond does not influence the type of transformation obtained with these C_{19} steroids. It thus seems unlikely that this organism can 19-hydroxylate 4-androstene-3,17-dione. The patent of Hasegawa *et al.* (1960) describing the 19-hydroxylation of cortexolone with *Corticium vagum* also seems dubious on comparison with data from the scientific literature. The name *C. vagum* is regarded as synonymous with *C. solani*, *Rhizoctonia solani*, and *P. filamentosa* f.sp. *solani* (Exner, 1953). In this respect, Takahashi and Hasegawa (1961a), working with 29 strains of *R. solani*, and Takahashi and Hasegawa (1961b), working with *P. filamentosa* f.sp. *solani*, specifically state that in no case was 19-hydroxylation observed on the substrate cortexolone. Another conflict between the patent and scientific literature occurs in the patent of Shull (1962b) who claims that *Hypochnus sasakii* can 19-hydroxylate 11β-hydroxycortexolone (hydrocortisone). In view of the similarity in hydroxylating abilities between *P. filamentosa* f.sp. *sasakii* and *microsclerotia*, with respect to cortexolone (Takahashi and Hasegawa, 1961b), the report that hydrocortisone could not be 19-hydroxylated by *P. filamentosa* f.sp. *microsclerotia* (Takahashi, 1963b) is of some significance. Clearly, any unsubstantiated patent claim must be considered as requiring verification.

References to *Pestalotia* species carrying out 19-hydroxylation of steroids number only three. The patent of Hasegawa *et al.* (1963) stating that a *Pestalotia* sp. can 19-hydroxylate cortexolone, has been verified in the paper of Takahashi (1963b). However, this reference was mentioned only as a footnote and no further evidence was provided to support the claim. Takahashi (1963b) stated that certain *Pestalotia* species can 19-hydroxylate cortexolone, but in low conversion yields. A much more recent patent describes

a *Pestalotia* species capable of 19-hydroxylating 17α-ethynyl-17β-hydroxy-4-pregnen-3-one (Courtieu, 1970) in a process to produce norethisterone. On the basis of such scant information, the extent of steroidal-19-hydroxylating ability within the genus *Pestalotia* is difficult to evaluate. Other steroid transformations have been reported for *Pestalotia* species. Thus, *P. diospyri* (Shirasaka *et al.*, 1961) can produce the 11α-hydroxy derivative from cortexolone, and *P. foedans* is reported to 11α-hydroxylate 17α,21-dihydroxy-16α-ethyl-1,4-pregnadiene-3,20-dione 21-acetate (Oliveto and Weber, 1961) and 17α,21-dihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione (Oliveto *et al.*, 1958). Patent claims have also appeared describing 11α-hydroxylation of progesterone by *P. foedans* and *P. royenae* (Shull *et al.*, 1955), and 16α-hydroxylation of various pregnane derivatives by *P. funerea* (Charney and Herzog, 1967).

The fact that *Calonectria decora* can carry out 19-hydroxylation of steroids (Chambers *et al.*, 1975) was a rather unexpected result emerging during the course of an investigation into the hydroxylating ability of this organism with a variety of different substrates. *C. decora* could 19-hydroxylate a large number of steroids related to 5α-androstan-17-one (Table 2.2). Other transformations occurred consecutively in the sequence shown in Figure 2.3, for 5α-androstan-17-one. Although this finding is of little immediate commercial significance, it is interesting that changes in substrate structure could induce 19-hydroxylation by an organism not normally noted for this ability (Kieslich, 1980a). Chambers *et al.* (1975) showed that the presence of a 1-oxo-group (either present in the substrate, or introduced during the incubation) is essential for the attack on the angular methyl group (C-19) of the substrates studied (17-oxo-5α-androstanes). These results were only part of an extensive study into the pattern of hydroxylation of a range of mono- and di-oxygenated 5α-androstane derivatives by a number of fungi. This work has been reviewed by Kieslich (1980a). The results showed that the direction of hydroxylase attack could be altered by changing the position of the polar keto-

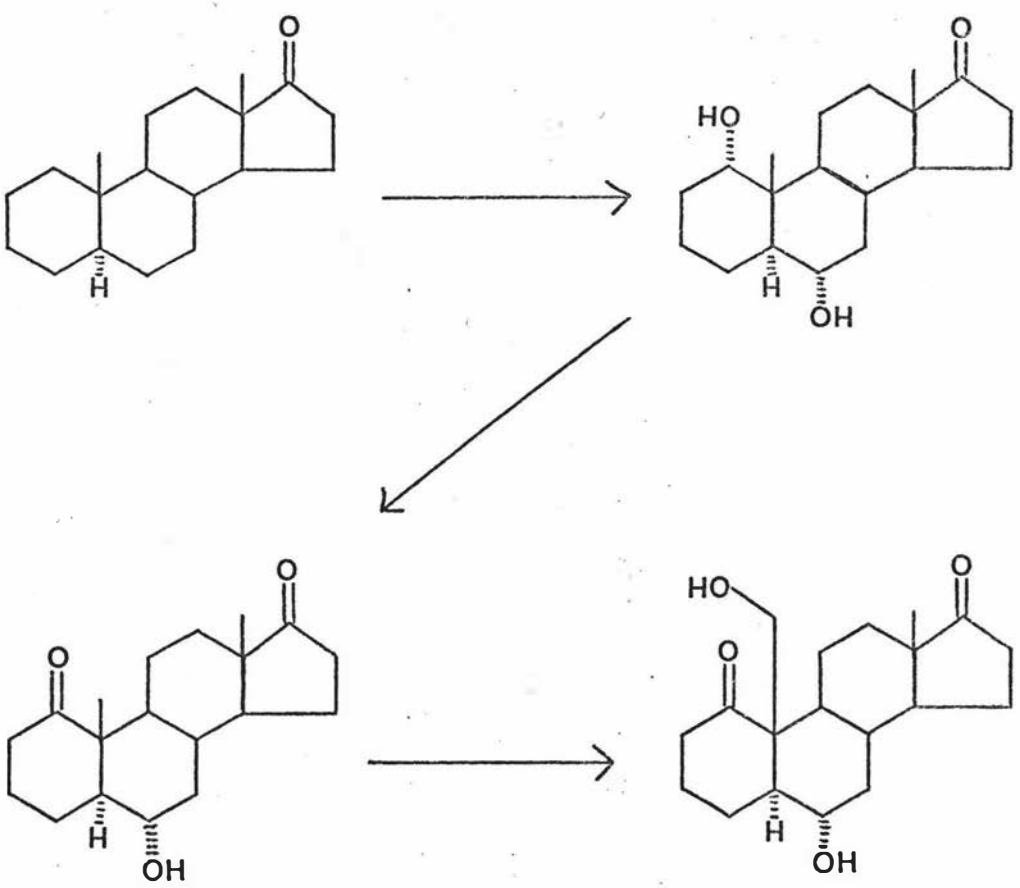


Figure 2.3 The action of *C. decora* on 5α-androstan-17-one (Chambers *et al.*, 1975).

groups. A convincing argument was put forward, in particular for *Calonectria decora*, that the same enzyme was responsible for all the various hydroxylations which were observed with mono-ketone substrates. The point of attack was then dependent on the position of the ketone group, this being the point of binding to the enzyme. Similar, although less extensive, studies into the effects of steroid structure on fungal hydroxylation have been documented (Marsheck, 1971; Kieslich, 1980a).

Finally, the most recent report of steroidal 19-hydroxylation is in a patent by Petzoldt (1980). The fungus *Nigrospora sphaerica* ATCC 12772 is described as being able to 19-hydroxylate the rather extensively modified pregnane compounds listed in Table 2.1.

It has been suggested (Vezina *et al.*, 1971) that microbial aromatization of 19-methyl-steroids may proceed via 19-hydroxy-intermediates, in an analogous mechanism to the biosynthesis of estrone from 4-androstene-3,17-dione by human placental microsomes. In the latter case, 19-hydroxylation precedes estrone formation (Hamberg *et al.*, 1974). In fact, this concept has been applied in the search for 19-hydroxylating microorganisms. Kluepfel and Vezina (1970) used 1,4,7-androstatriene-3,17-dione as the transformation substrate in a microbiological screening programme for organisms capable of hydroxylating carbon-19. Thus, a large range of bacteria and fungi were examined for production of equilin and equilenin (Fig. 2.4). Results indicated that the ability to aromatize was widespread among families, orders and even classes of microorganisms. These authors assumed that the transformation was brought about via 19-hydroxylation of the substrate, followed by spontaneous, non-enzymic rearrangement (reverse aldol reaction) to equilin. However, the same isolates could not aromatize 1,4-androstadiene-3,17-dione into estrone. If the pathway did indeed involve 19-hydroxylation, it would be expected that at least some of the organisms should be able to produce stable 19-hydroxy-products from Δ^4-3-keto-steroids (the spontaneous aromatization will not

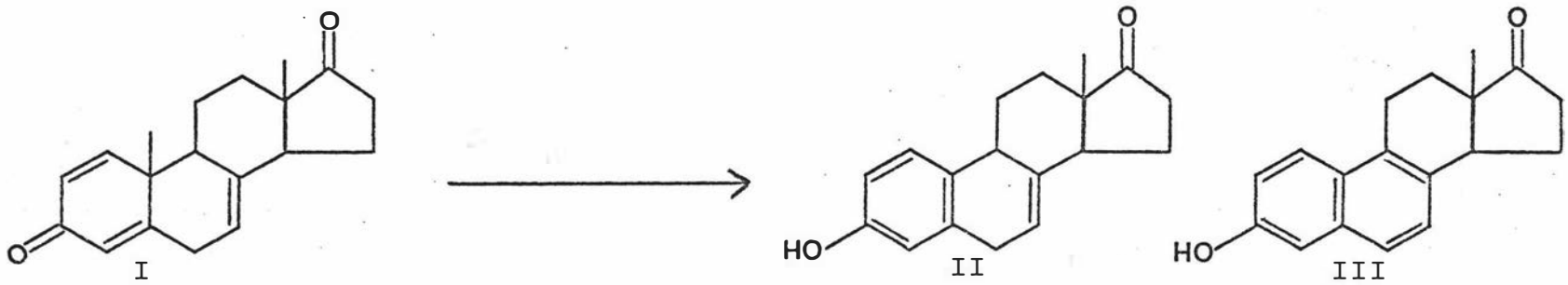


Figure 2.4 The microbial transformation of 1,4,7-androstatriene-3,17-dione (I) to equilin (II) and equilenin (III).

occur in the absence of a Δ^1-double bond). This, apparently, is not the case, since 19-hydroxylating microorganisms have not been reported as a result of this work. The attraction of the 19-hydroxy-intermediate hypothesis was perhaps due to the fact that many microorganisms are able to aromatize the A-ring of 19-nor-, 19-oxo-, and 19-hydroxysteroids (Vezina *et al.*, 1971). However, this occurs via Δ^1-dehydrogenation (and subsequent, spontaneous rearrangement) and hence the organisms reported are all known to be active in this respect. Thus, it is rather natural that aromatization would occur in such circumstances and this should not be used as evidence to suggest that an organism, which can aromatize the A-ring of 19-methyl-steroids, does so via 19-hydroxylation. In this respect, it seems invalid to assume analogy between microbial and mammalian mechanisms. For example, the aromatization of 19-nor-4-androstene-3,17-dione by human placental microsomes involves elimination of the 1β-hydrogen, whereas with *Pseudomonas* sp., *Bacillus sphaericus*, and *Nocardia restrictus*, estrone was obtained via elimination of the 1α-hydrogen (Vezina *et al.*, 1971). Also, Goddard and Hill (1972) and Goddard *et al.* (1975) have shown that a strain of *Escherichia coli* and many *Clostridium* species can produce oestradiol from 4-androstene-3,17-dione. Certainly, in the case of the *Clostridia*, this aromatization was due purely to dehydrogenation reactions, and the involvement of hydroxylation in the elimination of the C-19-methyl group was considered highly unlikely (Goddard *et al.*, 1975).

In conclusion, it is apparent that microbiological 19-hydroxylation is indeed a rare steroid transformation. The most detailed research has been carried out with *P. filamentosa* f.sp. *microsclerotia* IFO 6298, and indicates certain substrate requirements for 19-hydroxylation by this organism. It is difficult to evaluate the various reports with many of the other fungi listed in Tables 2.1 and 2.2. Clearly, despite the efforts which were undoubtedly devoted to this transformation, by industrial laboratories in the late 1950's and early 1960's (Vezina *et al.*, 1971), much of the information gathered has probably not been published.

2.3 MICROBIOLOGICAL STEROID HYDROXYLASES

Steroid hydroxylation in mammalian tissue has received considerable study (Hamberg *et al.*, 1974) and much detail has been established on the mechanism of hydroxylation at the various positions (including the C-19 position). Despite its commercial significance, much less progress has been made in the field of microbiological steroid-hydroxylation. However, the present state of knowledge suggests important similarities between the enzymatic processes from both origins. The available information can be summarized as follows:

(i) Studies involving incubation of steroids with various fungal strains known to effect hydroxylations, in an atmosphere enriched with $O_2{}^{18}$, showed in every case an almost theoretical incorporation of O^{18} into the molecule. Hydroxylations at positions 6β, 7α, 11α, 11β, 12β, 15α, and 21 have thus been examined and it seems reasonable to assume that the direct involvement of molecular oxygen is a common feature of all microbial steroid-hydroxylations (Hayano, 1962).

(ii) A series of experiments designed to determine the participation of hydrogens of steroid methylene groups undergoing hydroxylation, have been carried out. Several groups working with a number of fungal hydroxylation systems have in every instance, found that the incoming hydroxyl group stereospecifically displaced the hydrogen of the position hydroxylated. For example, an incoming 11α-hydroxyl group displaces the 11α-hydrogen (Hayano, 1962).

(iii) In the case of the 11β-hydroxylase of *Curvularia lunata*, in cell-free extract, a specific requirement for the reducing factor NADPH has been demonstrated (Zuidweg, 1968). Although other fungal cell-free hydroxylase systems have been described (Shibahara *et al.*, 1970; Lin and Smith, 1970a; Tan and Falaradeau, 1970; Breskvar and Hudnik-Plevnik, 1977a)

the difficulty of this work seems to have prevented similar definitive studies on the involvement of NADPH. Certainly, the involvement of this cofactor has been demonstrated in almost every case of mammalian steroid-hydroxylation (Hamberg *et al.*, 1974). NADPH-stimulation of hydroxylation has, however, been shown for two bacterial cell-free hydroxylase systems, viz 9α-hydroxylase of *Nocardia restrictus* (Chang and Sih, 1964) and the multiple hydroxylase(s) of *Bacillus megaterium* (Wilson and Vestling, 1965).

These facts place microbial hydroxylases in the class of monooxygenases (Hayaishi, 1974), as with those of mammalian origin.

The majority of the literature on microbial steroid-hydroxylation pertains to hydroxylations at secondary and tertiary carbons of the steroid ring system. Much less data have accumulated on hydroxylations of steroidal methyl groups. As well as the C-19 position, hydroxylations at carbons 18 and 21 have also been reported. Kondo and Tori (1964), Kondo *et al.* (1965), Auret and Holland (1971), and Denny *et al.* (1976) describe steroidal 18-hydroxylation with the fungi *Cercospora melonis*, *Corynespora cassiicola*, *Aspergillus niger*, and *Leptoporus fissilis*, respectively. C-21 hydroxylation by *Aspergillus niger* has also been reported (Charney and Herzog, 1967). The available information indicates that hydroxylations at both methyl and methylene groups are entirely analogous and would occur by the same basic mechanism.

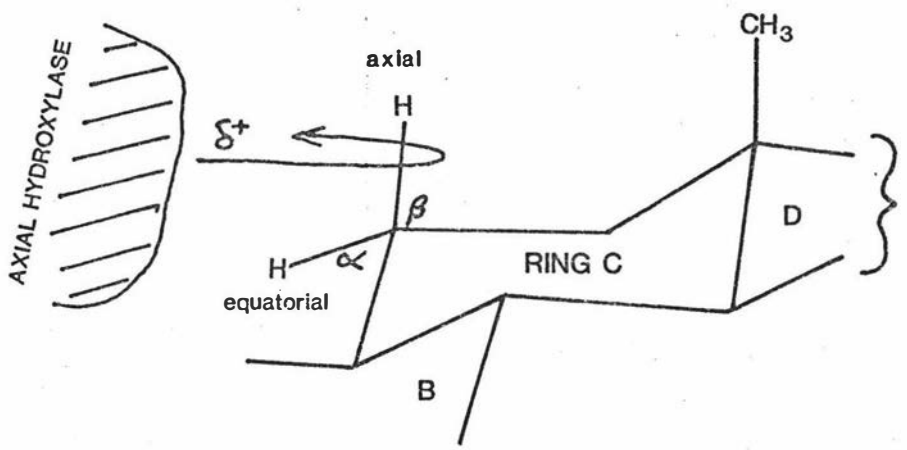
2.3.1 Mechanisms of Hydroxylation

A number of interesting observations involving the relationship between hydroxylation ability and the ability to epoxidise corresponding unsaturated steroids, are reviewed by Hayano (1962). Fungal cultures, able to perform respective

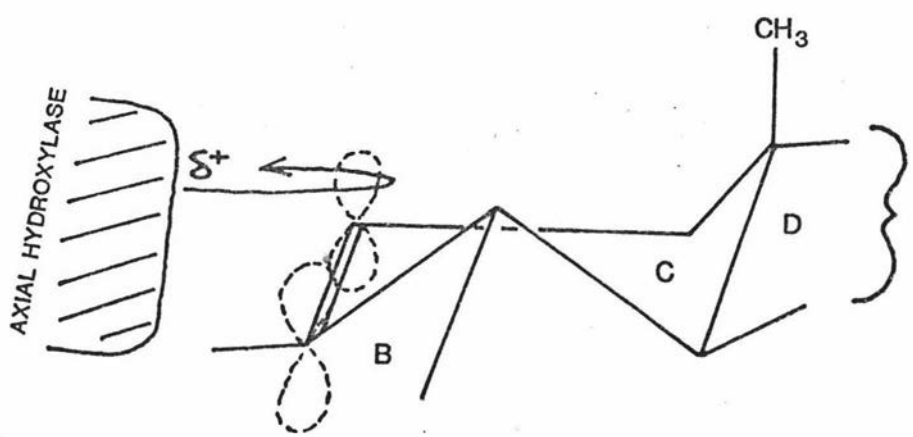
hydroxylations at positions 9α, 11α, 11β, 14α, and 16α, were incubated separately with both an appropriate steroid substrate and its unsaturated analogue (with the double bond at the position of hydroxylase attack). The results can be summarised as follows (Hayano, 1962). An enzyme system capable of introducing an axial hydroxyl function (9α, 11β, 14α) at a specific carbon of a saturated steroid could also effect the introduction of an epoxide grouping "axial" at the same carbon in the corresponding unsaturated substrate. Equatorial hydroxylases (11α, 16α) did not effect a similar conversion. Out of this interesting correlation a hypothesis covering the gross mechanism of steroid hydroxylation has been proposed (Hayano, 1962): *Epoxidation of olefinic steroids may occur because of the spatial resemblance of the double bond π-electron distribution in a given unsaturated substrate to the area of maximum electron density in the related axial carbon-hydrogen bond of the corresponding saturated compound. That is, in the appropraite enzyme-substrate complexes the spatial relationship required for reaction between an "axial"-hydroxylase and an axial carbon-hydrogen bond, in the case of a saturated steroid, is adequately approximated by the "axial"-hydroxylase-π-electron relationship in the unsaturated analogue.* This is shown in Figure 2.5. It follows that a reasonable degree of structural specificity in the oxidising enzyme system would preclude occurrence of the epoxidation phenomenon with "equatorial"-hydroxylases, since equatorial bonds extend outward more in the plane of the cyclohexane ring to which they are attached.

Inherent in this hypothesis is the assumption that hydroxylations proceed by direct electrophilic attack of an OH^+ species. This concept led to a consideration that substrate activation, particularly with respect to positions adjacent to or vinylogous to carbonyl functions, may take place. That is, in reactions at carbons 2, 6, 10 (19-norsteroids), and 16 of steroidal 3,17-diones, and also carbons 17 and 21 of 3,20-diones, the substrate may undergo reaction while in an enolic state. Evidence in support of this, albeit circumstantial,

Figure 2.5 A diagram showing the hypothesised similarity in spatial relationship between enzymic hydroxylation and epoxidation (Hayano, 1962).



Hydroxylation



Epoxidation

is provided by the frequent occurrence of hydroxylations of Δ^4-3,20-diketosteroids at the axial 2β, 6β, 10β (in 19-norsteroids), and 17α-positions, whereas microbial hydroxylations at 2α, 6α, 10α, and 17β are unknown (Charney and Herzog, 1967). Chemical analogies also exist, where electrophilic attack on the corresponding enols give exclusively axial substitution of the electrophile (Holland and Auret, 1975a). The enzyme surface would provide conditions for enolization of hydrogens on methylene or methyl groups adjacent to ketonic oxygens. The resultant high electron density at the positions under attack thus aids the incoming electrophile, envisaged as OH^+. Maximum overlap of π-electrons is expected to occur with axial attack, thus favouring products of this configuration. This model is demonstrated for 6β- and 21-hydroxylations, in Figure 2.6.

The two proposed mechanisms shown in Figure 2.6, have received some detailed study. Holland and Auret (1975a) examined the C-21 hydroxylation of progesterone by *Aspergillus niger* ATCC 9142. By studying the hydroxylation of C-21-deuterium-labelled progesterone they were unable to implicate the involvement of a C-20,21-enol form and it appeared that a mechanism involving the direct insertion of oxygen into a C-21 carbon-hydrogen bond was involved. Thus, a mechanism directly analogous to hydroxylation of non-enolizable carbons best explained their results. However, in studying the 6β-hydroxylating ability of *Rhizopus arrhizus* ATCC 11145 on Δ^4-3-ketonic steroids, Holland and Auret (1975b, 1975c) and Holland and Diakow (1978, 1979a, 1979b) have given convincing evidence that a $\Delta^{3,5}$-enolic intermediate is involved in this hydroxylation.

Thus, it appears that in certain cases the mechanism of hydroxylase action may involve enolization of the substrate. However, the many hydroxylations at carbons where enolization is impossible, implies that these hydroxylases operate by direct insertion of oxygen into a carbon-hydrogen bond. Clearly, the exact mechanistic features of steroid hydroxylase action are still far from completely understood.

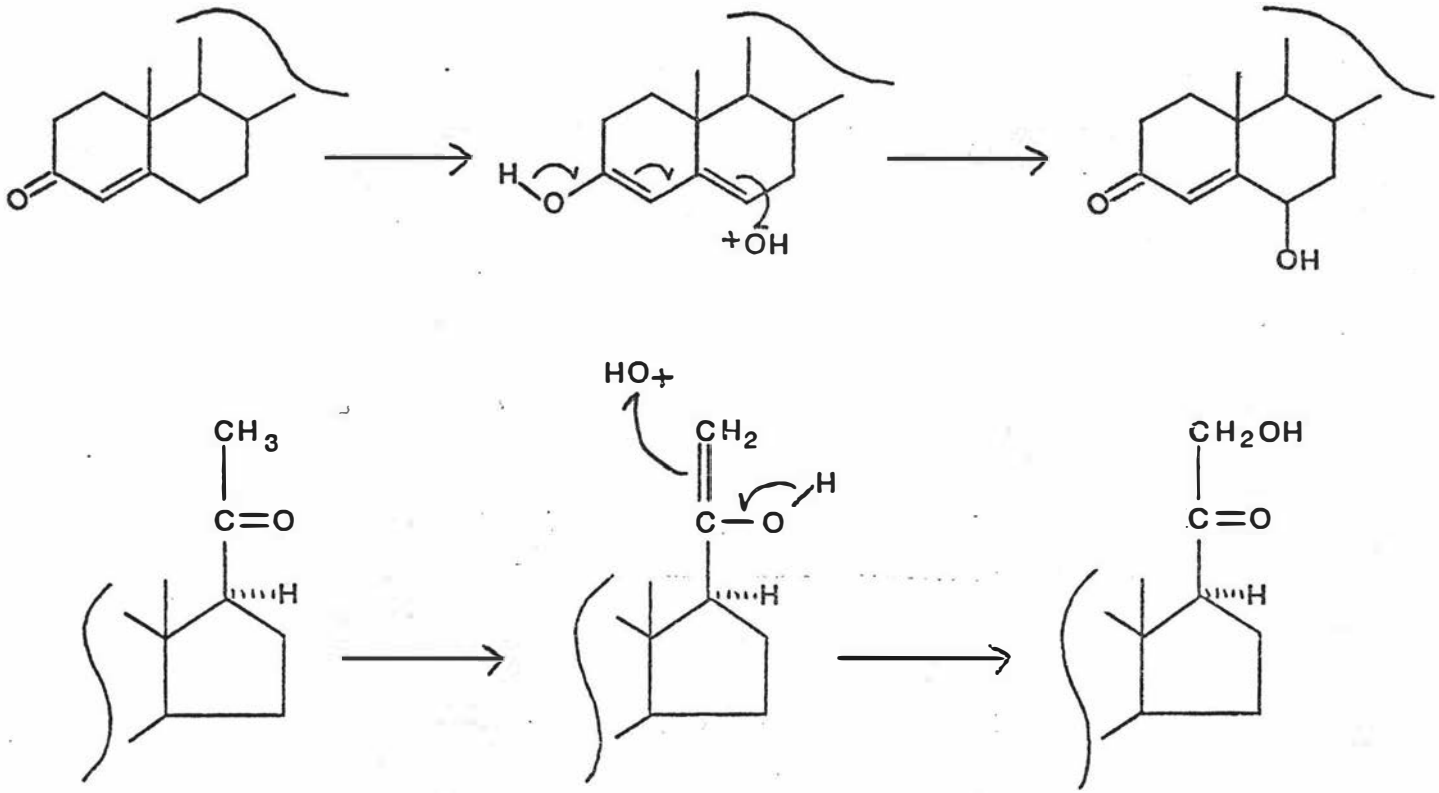


Figure 2.6 A possible mechanism for steroid hydroxylations at 6β and 21 positions.

2.3.2 Hydroxylase Biochemistry

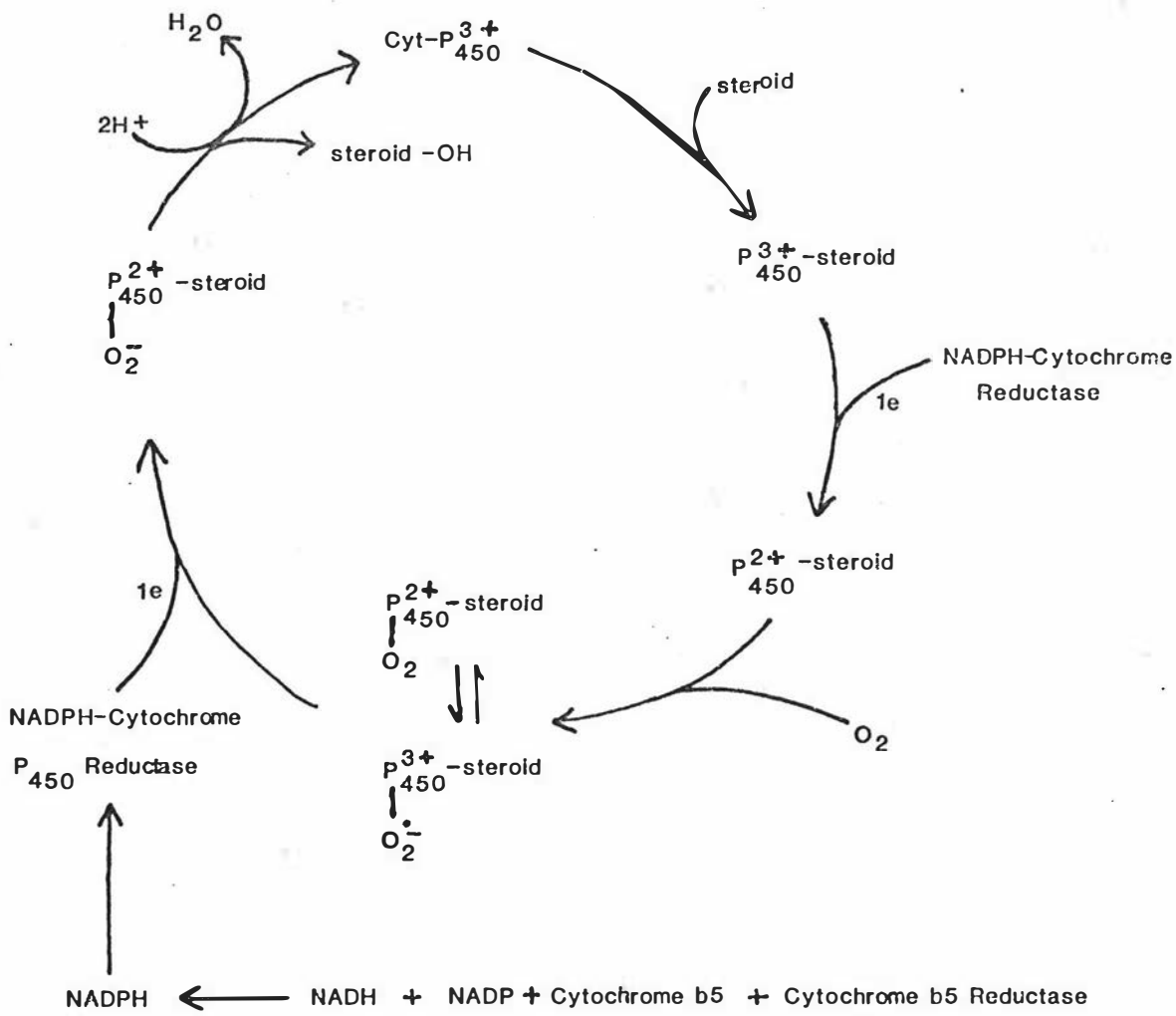
Recently a significant advance in the knowledge of fungal steroid hydroxylases was made by Breskvar and Hudnik-Plevnik (1977a). They convincingly demonstrated the involvement of cytochrome-P450 in the 11α-hydroxylation of progesterone by *Rhizopus nigricans*. Cytochrome-P450 is known to be an essential constituent of various hydroxylating systems in mammalian tissues, higher plants, fungi and bacteria (Boyd and Smellie, 1972). There are many different types of monooxygenases (Hayaishi, 1974), so it may not be valid to suggest that the involvement of cytochrome-P450 will be common to all microbial steroid hydroxylase systems. However, this important finding brings the understanding of microbial steroid hydroxylation much closer to that of hydroxylase systems of animal, plant and microbial origin. Certainly, the involvement of a metallo-enzyme, as in the haemoprotein cytochrome-P450, in the activation of molecular oxygen has long been assumed (Hayano, 1962). It is possible to suggest a pathway for microbial steroid hydroxylation, involving cytochrome-P450 (Rosazza and Smith, 1979) which serves as a useful model (Fig. 2.7). Important features of this model are:

(i) It is a multienzyme complex operating as an electron transport chain.

(ii) Molecular oxygen binds with the reduced Cyt-P^{2+}_{450}-steroid complex which is further reduced to give "activated" oxygen ("ground state" dioxygen is not very reactive (Hayaishi, 1974)).

(iii) Ultimately, one oxygen atom is incorporated into the substrate and the second is reduced to water.

Figure 2.7 A model for steroid hydroxylation via Cytochrome-P_{450}-linked monooxygenases (Rosazza and Smith, 1979).



The intracellular localisation of the steroid hydroxylase-complex in fungi has been the subject of some recent work. Shibahara *et al*. (1970) with the *Aspergillus ochraceus* progesterone-11α-hydroxylase, Zuidweg (1968) with the *Curvularia lunata* cortexolone-11α-hydroxylase, Tan and Falardeau (1970) with the *Aspergillus ochraceus* progesterone-11α-hydroxylase, and Breskvar and Hudnik-Plevnik (1977b) with the *Rhizopus nigricans* progesterone-11α-hydroxylase are all in agreement that the hydroxylase activity resides in the post-mitochondrial supernatant liquid. Breskvar and Hudnik-Plevnik (1977b) localised the activity in the membrane fraction sedimenting at 105,000 x g. As with other known steroid hydroxylase systems the activity appears to be membrane-associated, which may explain the unstable nature of many microbial cell-free hydroxylase preparations (Zuidweg, 1968).

The question of whether the steroid hydroxylases of fungi are inducible or constitutive has received only minor study. Zuidweg (1968) demonstrated that only cell-free preparations from cells of *Curvularia lunata* pre-induced with cortexolone showed any 11β- and 14α-hydroxylase activity. Similarly, Lin and Smith (1970a) showed with whole cell preparations of *C. lunata* that cells without prior induction demonstrated insignificant 19-norsteroid 11β- and 14α-hydroxylase activity. Also, cell-free preparations from non-induced, vegetative mycelia of *Aspergillus ochraceus* yielded inactive extracts, whereas extracts from cells pre-induced with progesterone were active in 11α-hydroxylation (Shibahara *et al*., 1970). Interestingly, Vezina *et al*. (1968) reported that none of the antibiotics that inhibited steroid-transformation by vegetative cells of streptomycetes (Perlman *et al*., 1957) had any effect on the hydroxylase activity of the spores of *A. ochraceus*. Unfortunately, no data were given. However, this report can probably be discounted since all the antibiotics used by Perlman *et al*. (1957) were antibacterial compounds and would not inhibit fungal protein-synthesis. Without protein-synthesis inhibition, inducibility cannot be

proven in this manner. More difficult to explain is the report of Vezina and Singh (1975), that cycloheximide, at levels which caused greater than 90% inhibition of protein synthesis in *A. ochraceus* spores, had little effect on progesterone hydroxylation. If no prior induction by progresterone occurred before cycloheximide addition, this would imply that *de novo* protein synthesis is unnecessary for hydroxylation by spores of this organism. Since the experimental details were not reported it is possible that cycloheximide may not have been added to incubations prior to progesterone, which would negate the claim of a constitutive enzyme-system. On the basis of the above evidence it appears that in most fungi the enzymes involved in steroid hydroxylation are inducible, although there is some doubt as to whether this is so for fungal spores. In the case of the 11α-hydroxylase of *Rhizopus nigricans* mycelium, inducibility has been put beyond all doubt (Breskvar and Hudnik-Plevnik, 1978). During studies on the effect of cycloheximide on the hydroxylation of progesterone, these authors found that only cultures pretreated with inducer-steroid could hydroxylate when hydroxylation was assayed in the presence of cycloheximide. Cycloheximide is a potent inhibitor of 80 S ribosomes (Franklin and Snow, 1981), and these findings show convincingly that hydroxylation follows *de novo* protein synthesis.

The general subject of xenobiotic monooxygenases is receiving considerable attention particularly with the widespread discovery of cytochrome-P450 involvement in many monooxygenases of microbial origin (Smith and Davis, 1980; Rosazza and Smith, 1979). Because of the paucity of detailed studies of fungal steroid-hydroxylase systems it is perhaps instructive to consider analogy with the many mammalian and microbial monooxygenases which have been examined in some depth. Induction can be described as the increased production of monooxygenases. In mammalian cells, the increased production of cytochromes is thought to occur via stimulation of protein synthesis (Smith and Davis, 1980). Various interactions which could ultimately lead to increased protein synthesis are shown in Figure 2.8. Most important of these

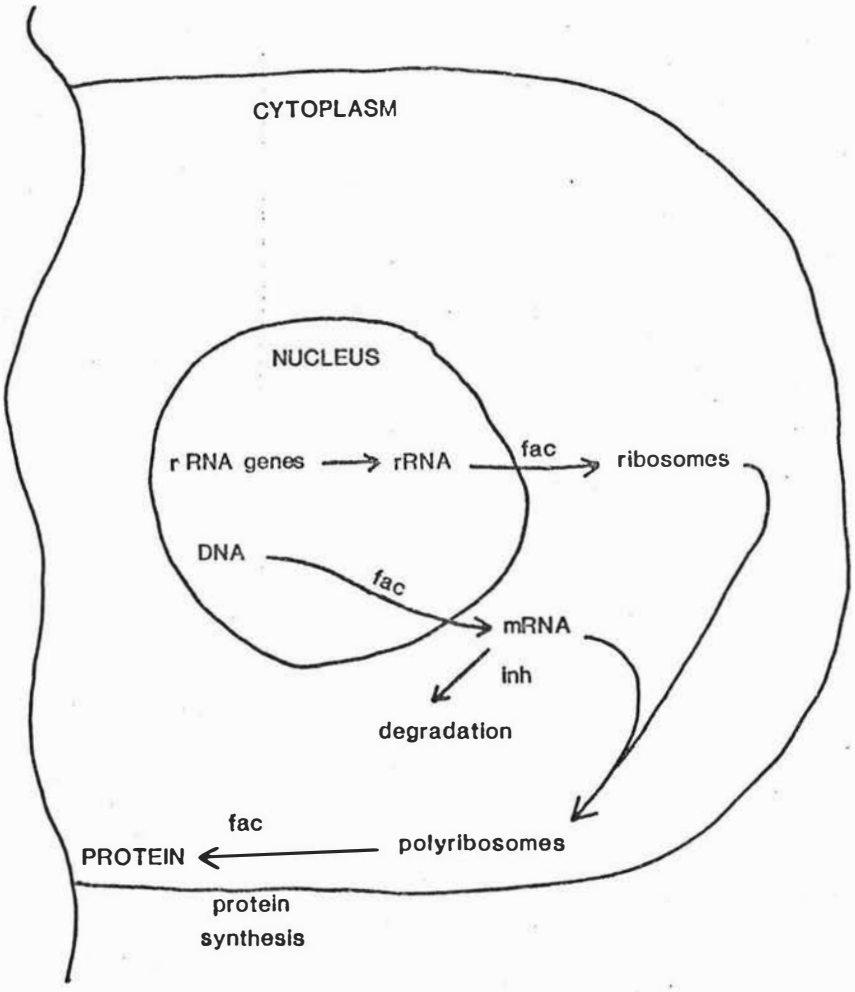
Figure 2.8 Sites within mammalian cells that are reportedly important in the induction of Cytochrome-P_{450} monooxygenases.

Inducers may facilitate (fac) or inhibit (inh) processes as indicated.

Abbreviations:

mRNA messenger RNA
rRNA ribosomal RNA

Source: (Smith and Davis, 1980).



are the binding of inducers such as 3-methylcholanthrene to nuclear DNA. This stimulates the expression of mRNA. Other effects, such as the direct influences of inducers on ribosomal synthesis and stablization (via ribonuclease inhibition), and on protein synthesis reactions, could all lead ultimately to increased protein synthesis and increased levels of monooxygenases. Further, the increased production of a related enzyme, such as NADPH-cytochrome-P450 reductase (using the model of Figure 2.7) which would increase the rate of P450 reduction, could also increase the activity of the monooxygenase system (Smith and Davis, 1980).

P450 monooxygenases studied in fungi have indicated some parallels to induction in mammalian species. In many cases induction by the substrate has been observed. Fewer studies have been performed where the more classical P450 inducers, used routinely in mammalian work, have been examined. In the few cases studied, important similarities have been observed (Smith and Davis, 1980 and references therein). Thus, in the presence of phenobarbital, P450 was induced two-fold (compared to controls) in the alkaloid-producing strain of *Claviceps purpurea*. With *Cunninghamella bainieri*, processing a benzo[a]pyrene hydroxylase system, 10-fold induction of hydroxylase activity was observed when phenanthrene was employed as the inducing agent.

The role of cellular metabolism in the activity of fungal steroid hydroxylases is another area which has received little study. Steroid hydroxylation with starved fungal spores has been shown to require an exogenous source of carbon, and respiratory studies with various sugars generally indicate a relationship between oxygen uptake and steroid hydroxylation (Vezina *et al.*, 1968). Vezina and Singh (1975) reported relatively high activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (the first two enzymes of the hexose monophosphate (HMP) pathway) in cell-free extracts of *Aspergillus ochraceus* spores (an active 11α-hydroxylating organism). The requirement for glucose may be related to the regeneration of NADPH, this

being essential for hydroxylase activity. In many fungi this cofactor is regenerated via the HMP pathway (Berry, 1975; Cochrane, 1976). *Cunninghamella elegans* spores and washed, resting mycelia can carry out cortexolone hydroxylation in distilled water without exogenous glucose (Sedlaczek *et al*., 1981). However, biochemical analysis of intracellular constituents indicated considerable endogenous consumption of storage carbohydrate. Thus it seems reasonable to suggest that whether steroid hydroxylation is performed with either spores or mycelia some metabolism of a carbon source is a requirement for hydroxylation. Sedlaczek *et al*. (1973), working with a number of fungi, found that compounds which blocked electron transport and uncoupling agents of oxidative phosphorylation were able to greatly or totally inhibit 11α-hydroxylation of cortexolone. The authors suggested that energy-yielding reactions are necessary for uptake of steroids by active transport. However, it would seem more likely that inhibition of normal energy-metabolism would result in a cessation of carbon metabolism and hence cofactor regeneration. Similar observations are reported for steroid hydroxylation by fungal spores (Vezina and Singh, 1975).

Sedlaczek *et al*. (1979) demonstrated a dependence of steroid 11α-hydroxylase activity on the redox state of the cellular nicotinamide nucleotide coenzyme pools in *Monosporium olivaceum*. The transformation rate of cortexolone to the 11α-hydroxy derivative was correlated with the ratio of the reduced form of NADP to the total pool size of the cofactor. They stated that a NADPH: (NADP + NADPH) ratio of > 60% was required for rapid steroid hydroxylation. Similarly, Sedlaczek *et al*. (1981) found that this ratio was significantly higher in mycelia of *Cunninghamella elegans* as compared to spores, which correlated with the higher cortexolone 11α- and 11β-hydroxylase activities of mycelia. These findings lend further weight to the importance of NADPH-regeneration-systems, and hence carbon metabolism, in the steroid hydroxylation mechanism.

Jaworski *et al.* (1976a) correlated changes in the cellular content of amino acids, glucose and mannitol of *M. olivaceum*, during growth and starvation, with the cortexolone-11α-hydroxylation activity. They found the highest activity when mycelia contained low amounts of metabolites, which possibly indicates some form of catabolite repression. Further evidence, that this type of metabolic control mechanism may exist, was demonstrated for *Cunninghamella elegans* spores by Sedlaczek *et al.* (1981). Addition of exogenous glucose to cortexolone-hydroxylating spore suspensions resulted in reduced transformation rates, whereas the addition of several amino acids enhanced rates compared to a control.

An investigation of the fungus *M. olivaceum*, by Jaworski *et al.* (1976b), correlated the cellular energy-charge (defined as the ratio ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP])) with cortexolone-hydroxylation activity, during mycelial growth and starvation. An increase in energy-charge was accompanied by a decrease in hydroxylation activity. In starved mycelia, the highest hydroxylation activity was observed when the lowest energy-charge level was reached. Interestingly, ATP or energy-charge regulation of secondary metabolism has been described as a possible control mechanism in many antibiotic fermentations (Drew and Demain, 1977). In these cases, high levels of ATP result in low levels of antibiotic in the fermentation broth.

In an attempt to investigate the effect of growth rate on 11α-hydroxylation of cortexolone by *M. olivaceum*, Sedlaczek *et al.* (1974) obtained mycelia from chemostat cultures operating at two different growth rates. Steroid hydroxylation was then studied under starvation conditions. At a specific growth rate of $\mu = 0.495\ h^{-1}$ the mycelia were 27% autolysed after 48 h starvation, but retained the ability to 11α-hydroxylate. When $\mu = 0.112\ h^{-1}$ the degree of autolysis after 48 h was 51.7% and the steroid hydroxylating ability was lost after 6 h. Macromolecular compositional analyses of the mycelia showed more carbohydrate in the mycelia with

the higher specific growth rate. Thus, it would appear that this effect is one of carbon-source exhaustion rather than growth rate.

It thus appears that cell mechanisms and factors controlling steroid hydroxylation are complex and are still little understood. The possibility of catabolite and/or energy-charge regulation of steroid hydroxylases, could explain many of the changes in hydroxylation activity of cultures growing on different media, which have been observed (Lee *et al.*, 1969; El-Refai *et al.*, 1975; Wettstein, 1955; and Marsheck, 1971).

In conclusion, it is still difficult to generalise about the types and mechanisms of steroid hydroxylases. Although general mechanistic features are now better understood, it is still unclear in many cases of multiple hydroxylations, whether a single non-specific enzyme or a group of specific hydroxylases are involved. In the case of the sequential 11α- and 6β-hydroxylations of progesterone, by *Aspergillus ochraceus*, the two activities have been clearly separated, in cell-free extract (Shibahara *et al.*, 1970). On the other hand, the multiple, monohydroxylase(s) of *C. lunata* have been unable to be similarly separated (Zuidweg, 1968), and various studies have been unable to demonstrate the involvement of more than one enzyme (Lin and Smith, 1970a; 1970b). The possibility has also been suggested (Smith, 1974) that dioxygenases may be responsible for certain dihydroxylations of steroids. Clearly, there is still a need for better understanding of these fundamental areas of microbial steroid-hydroxylation.

2.4 FERMENTATION TECHNOLOGY, APPLIED TO STEROID HYDROXYLATION BY FUNGI

Current trends in microbiological steroid transformation techniques have recently been reviewed (Marsheck, 1971). Although the use of fungal conidiospores (Vezina and Singh, 1975) and immobilized cells (Mosbach and Larsson, 1970) have received considerable interest, the most common technique is the addition of steroid to a batch-grown culture. A slight variant on this method is the washed, resting-cell technique. Mycelia from a batch-grown culture are harvested and suspended in a suitable buffer. Steroid is then added and the transformation is carried out under conditions of aeration and agitation.

The physical and biological properties of steroids can be of considerable significance to transformation processes. Thus, in high concentration the substrate or product of transformation may be toxic or inhibitory to the microorganism. The very ability of steroids to permeate cell membranes, which allows transformations to take place, implies that the organism will in some way be affected by the presence of steroid. In this respect the response of microorganisms to steroids has been reviewed by Buetow and Levedahl (1964). In cases of substrate toxicity, slow substrate feeding can be used to prevent the organism being exposed to high substrate concentrations.

Smith *et al.* (1977) have claimed that only dissolved substrate is available for transformation by microorganisms. There is some evidence, however, which suggests that undissolved steroid can enter microbial cells and be transformed. For example, Zvyagintsev and Zvyagintsev (1969) adsorbed cells of *Mycobacterium globiforme* onto crystals of various steroids and observed the transformations. With some steroids, the transformation rates were greater than in control samples with dispersed substrate. This would suggest that undissolved substrate may be transformed. Nevertheless, it appears to be generally accepted that

enhanced steroid solvation will increase the rate of transformation. In this respect, the low aqueous solubility of most steroids (Kabasakalian *et al*., 1966) is of considerable consequence to the hydroxylation process. Thus, it is common practice to add the steroid to a culture as a solution, in a solvent such as ethanol, acetone, or dimethylformamide. The solvent increases the solubility of the steroid. However, with increasing solvent concentrations, toxicity to the organism becomes an important constraint. The use of two-phase emulsions of aqueous and organic solvents have been described for some steroid transformations (Smith *et al*., 1977). For example, Buckland *et al*. (1975), using whole resting-cells of *Nocardia* species, obtained greater rates of cholesterol oxidation in such a system compared with a single-phase aqueous transformation. Improved steroid solubility appeared to be partly responsible for the effect. The close structural similarity of many substrates and products of steroid transformations can result in interactions, in which the presence of one compound affects the solubility of the other. Examples have been described, in which such interactions can work to decrease the solubility of the substrate, and so gradually stop the transformation as the product concentration rises (Marsheck, 1971).

The physical state of the substrate has also been shown to be important to the rate and yield of some steroid transformation processes (Marsheck, 1971), and various addition techniques have been described. Noteable among these, is the use of micronised substrate or so-called "smooth-particles". For the 11α-hydroxylation of progesterone this technique allowed rapid transformation of high substrate charges (20-50 g/l) with little side-product formation. Also of interest is the phenomenon of "pseudocrystallofermentation" which occurs with certain steroids. In such cases, when substrate is added in solid form (up to 50 g/l) to a culture, and is transformed, crystals of product spontaneously form and effectively force the transformation in the direction of product formation.

Since most fungal steroid-hydroxylases appear to be inducible, a period of enzyme induction will occur following steroid addition to a culture. Fermentation conditions during this period may be critical to the final level of hydroxylase activity. This has shown to be so, with respect to dissolved oxygen tension, for the progesterone 11α-hydroxylase of *Rhizopus nigricans* (Hanisch *et al.*, 1980). Examples, where variation of growth medium composition give improved hydroxylation yields, have also been described (for example, Wettstein, 1955; Marsheck, 1971). It is, however, impossible to generalise from these empirical observations. It is perhaps sufficient to suggest that steroid hydroxylases may be under various forms of regulation, which may be influenced by factors such as growth medium composition. The requirement for oxygen in the hydroxylation process necessitates adequate agitation and aeration. This was demonstrated empirically by Karow and Petsiavas (1956) for the 11α-hydroxylation of cortexolone, and very recently a Michaelis-Menten relationship, between dissolved oxygen tension and hydroxylase activity was shown for the 11α-hydroxylation of progesterone by *Rh. nigricans* (Hanisch *et al.*, 1980).

In conclusion, it is apparent that much of the literature, describing fermentation aspects of steroid hydroxylation processes, is empirical in nature. As such, it is difficult to apply results from one steroid/microorganism system to another.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbiological Media

Potato Dextrose Agar (PDA) was obtained from Oxoid Ltd., London, England. Bacto Yeast Extract, Bacto Malt Extract Agar, and Bacto Malt Extract Broth were supplied by Difco Laboratories, Detroit, Michigan, U.S.A.. Microbiological Agar was obtained from Davis Gelatin Ltd., Christchurch, New Zealand. The compositions of Lactritmel Agar and of the starch and sucrose based media of Hasegawa *et al*. (1960) are detailed in Table 3.1. Total nitrogen and total carbohydrate determinations on the yeast extract powder gave analyses of 10.5% (^{w}/w) and 9.0% (^{w}/w), respectively.

3.1.2 Chromatographic Materials

For thin layer chromatography (tlc) Kieselgel DGF (400 mesh ASTM) was obtained from Riedel-De Haen AG., Seelze-Hannover, Germany. For column adsorption chromatography, silica gel (60-120 mesh ASTM), from BDH Chemicals Ltd. (Palmerston North, New Zealand), was used.

3.1.3 Gases

Oxygen-free nitrogen was supplied by New Zealand Industrial Gases Ltd., Palmerston North, New Zealand.

3.1.4 Chemicals

3.1.4.1 Steroids

The following steroids were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Table 3.1 Media composition

(a) Lactritmel Agar

Honey	7 g
Skim milk powder	14 g
Wheat flour	14 g
Agar	14 g
Water to 1000 ml	

(b) Starch Medium of Hasegawa *et al.* (1960)

Soluble starch	30 g
NH_4NO_3	2.0 g
K_2HPO_4	1.0 g
$FeCl_2.4H_2O$	0.5 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
Vitamin solution	10 ml
Tap water to 1000 ml	

One litre of vitamin solution contains:

biotin	0.2 mg
calcium pantothenate	40 mg
inositol	200 mg
niacinamide	40 mg
p-aminobenzoic acid	20 mg
pyridoxine hydrochloride	40 mg
riboflavin mononucleotide	20 mg
thiamine hydrochloride	40 mg
folic acid	0.2 mg

(c) Sucrose medium of Hasegawa *et al.* (1960)

Sucrose	30 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	1.0 g
Malt extract	25 g
Water to 1000 ml	

4-androstene-3,17-dione
17α,21-dihydroxy-4-pregnene-3,20-dione
11β-hydroxy-4-androstene-3,17-dione
19-hydroxy-4-androstene-3,17-dione
11α-hydroxy-4-pregnene-3,20-dione
11β-hydroxy-4-pregnene-3,20-dione
3β-hydroxy-5-pregnen-20-one acetate
3β-hydroxy-5,16-pregnadien-20-one acetate
4-pregnene-3,20-dione
4,16-pregnadiene-3,20-dione
11β,17α,21-trihydroxy-4-pregnene-3,20-dione

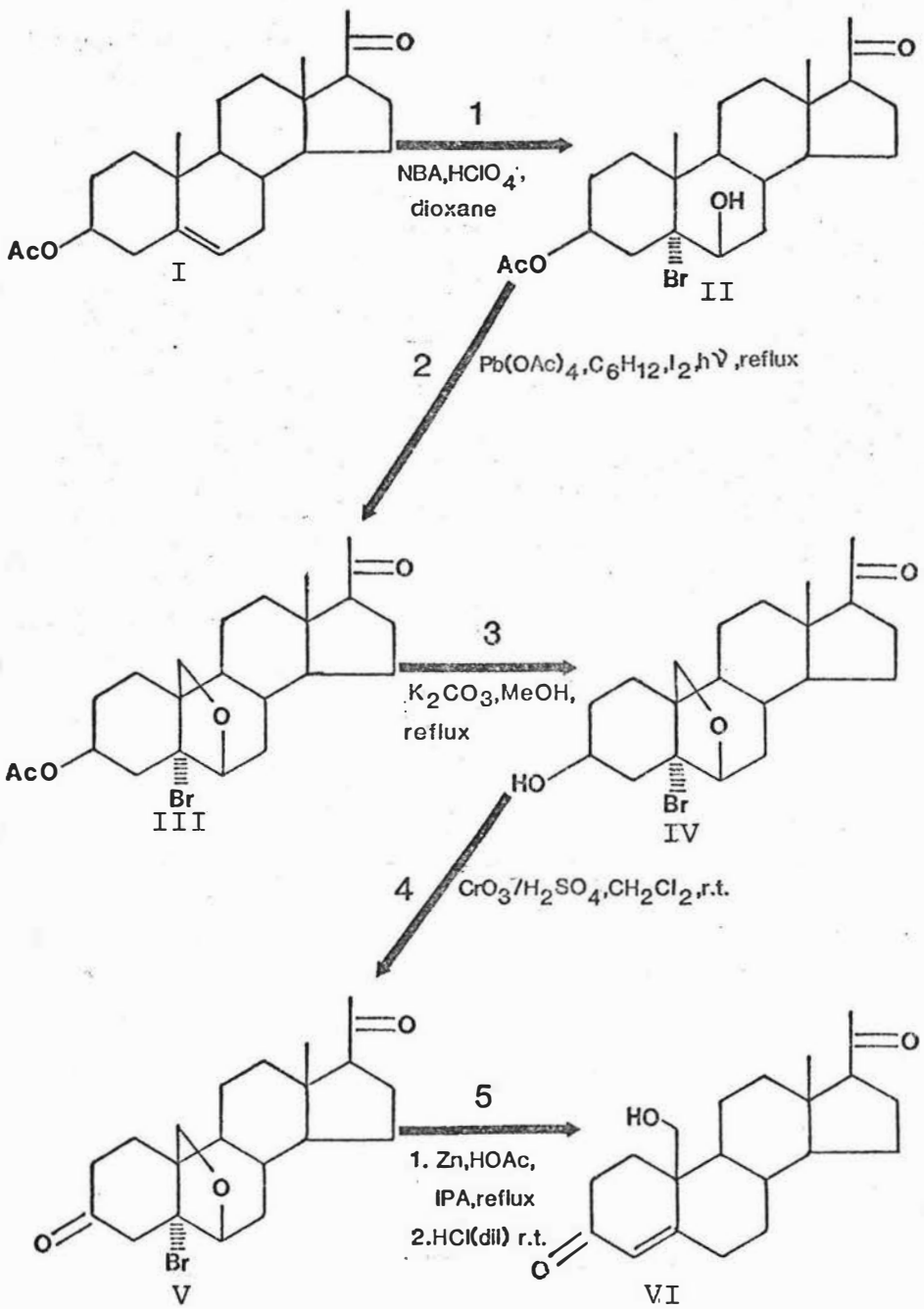
All compounds, required as fermentation substrates or chromatography standards, were checked for purity by melting point and tlc criteria. All were used as received with the exception of hydrocortisone and androstenedione which required recrystallisation from methanol and acetone:petroleum ether (60^{o}-80^{o} BP), respectively.

The following steroids were prepared by partial synthesis as described:

19-Hydroxyprogesterone (19-hydroxy-4-pregnene-3,20-dione)

The partial synthesis of 19-hydroxylated steroids has been studied extensively (Heusler and Kalvoda, 1972). Bowers *et al*. (1962), Kalvoda *et al*. (1963) and Bayunova and Grinenko (1973) describe methods for the partial synthesis of 19-hydroxyprogesterone, although the exact routes used differ. In some cases the methods of achieving the same reaction also differ. The five-step preparation from pregnenolone acetate proved to be very sensitive to the methods used, and several published procedures were tried before a satisfactory route was determined. In particular, the use of benzene as the solvent for the critical 6β-19 cyclisation step, as described by Bowers *et al*. (1962) and Bayunova and Grinenko (1973), gave poor product yields. Its replacement by the saturated hydrocarbon, cyclohexane, resulted in greatly improved yields. The synthetic route, from pregnenolone acetate (I), is shown in Figure 3.1 with the methods used, as follows:

Figure 3.1 Synthesis of 19-hydroxyprogesterone



Step 1: The bromohydrin (II) was prepared by the method of Akhtar and Barton (1964), and the product was recovered by crystallisation (dichloromethane : hexane) prior to Step 2.

Step 2: Cyclisation to give the 6β,19-ether (III) was carried out according to Santaniello *et al*. (1975). The product was purified by crystallisation from dichloromethane: methanol.

Step 3: Mild hydrolysis of the 3β-acetate group was performed by the method of Kalvoda *et al*. (1963).

Steps 4 and 5: Oxidation of the 3β-hydroxyl group with Jones reagent (Fieser and Fieser, 1967a), followed by reductive opening of the 6β,19-ether with zinc in isopropanol/ acetic acid, was carried out according to Bayunova and Grinenko (1973).

The 19-hydroxyprogesterone product (800 mg) was purified by recrystallisation from ethyl acetate and displayed the following properties:

m.p. = 166-170° (cf. 169-170°C, Bowers *et al*., 1962)

λ_{max}^{MeOH} = 241 nm

IR υ_{max}^{KBr} 3420 cm^{-1} (19-hydroxyl), 1700 cm^{-1} (20-keto), 1660 cm^{-1} (3-keto).

The product gave a mass spectrum (Appendix B, Figure B1) identical to that published for 19-hydroxyprogesterone (Heller and Milne, 1978). The pronounced elimination of formaldehyde (30 amu), giving rise to an M-CH_2O ion, results in the base peak at m/e 300. This is highly characteristic of 19-hydroxy steroids which possess the Δ^4-3-one group (see section 3.9.2.). Proton and Carbon-13 NMR spectra (Appendix B, Table B1) demonstrated, unequivocally, the presence of the 19-hydroxyl group.

16α-Hydroxyprogesterone (16α-hydroxy-4-pregnene-3,20-dione)

This was prepared from 3β-hydroxy-5,16-pregnadien-20-one acetate (I) according to the route shown in Figure 3.2. The methods used were as follows:

Step 1: The 16α,17α-epoxide (II) was prepared by reaction with the alkaline hydrogen peroxide, according to Julian *et al*. (1956). The product (0.5 g) was recovered by crystallisation from methanol:water.

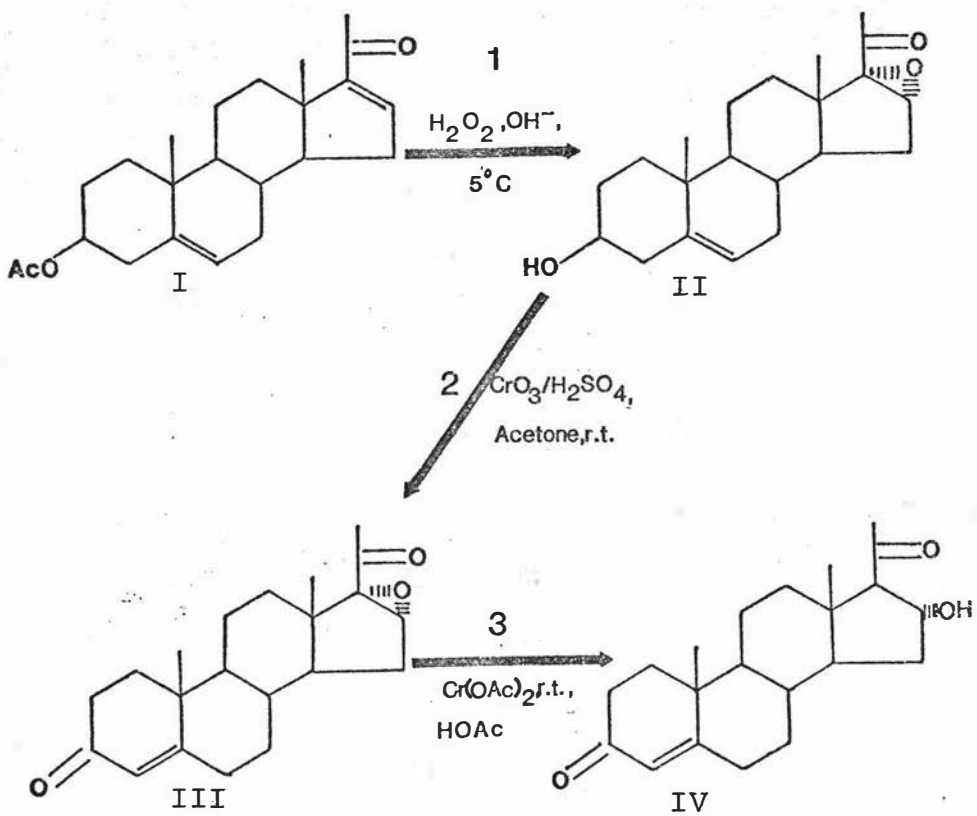
Step 2: The 3β-hydroxyl group was oxidised to the ketone by addition of Jones reagent (Fieser and Fieser, 1967a) to an acetone solution of the compound (II), at room temperature.

Step 3: Reduction of the epoxide to a 16α-hydroxyl group was performed by the method of Cole and Julian (1954), using chromous acetate.

The final product (IV) was separated from unreacted compound III and other side products by preparative hplc under the following conditions: 2.5 ml/min, 80:20 methanol: water, using a Waters Associates Semi-preparative C-18 Reverse-Phase Column (see section 3.4.8 for further details). The product thus obtained was recrystallised from methanol to yield crystals (80 mg m.p. 194-212^{o} (cf. 226-227^{o}C, Cole and Julian, 1954). The product still contained a significant amount of an impurity. A small amount (2 mg) of pure 16α-hydroxyprogesterone (for use as a standard) was obtained by preparative tlc (solvent system 3, section 3.4.7). This was unequivocally identified by mass spectrometry (Appendix B, Figure B2) and by dehydration (Cole and Julian, 1954) to 4,16-pregnadiene-3,20-dione, which showed identical chromatographic behaviour (tlc, solvent systems 2 and 3, section 3.4.7) to authentic 4,16-pregnadiene-3,20-dione. Dehydration, under such conditions, is characteristic of 16α-hydroxyprogesterone (Cole and Julian, 1954).

The base peak in the mass spectrum at m/e 231 is characteristic of both 15- and 16- hydroxyprogesterones (Zaretskii, 1976).

Figure 3.2 Synthesis of 16α-hydroxyprogesterone



The peaks at m/e 244 and 302, which appear in the spectra of 16-hydroxyprogesterones, are absent in spectra of the 15α- and 15β-isomers. The presence of these peaks in Figure B2 (Appendix B) lends proof to the position of the hydroxyl at C-16. The orientation of the group can also be readily verified from the mass spectrum. Zaretskii (1976), gives the relative intensities of the characteristic peaks in the mass spectra of 16α- and 16β-hydroxyprogesterone (m/e 330 (M^+), 312 ($M-H_2O$), 231, 100). By comparison with Figure B2, the 16α-orientation of the hydroxyl group was verified.

11α-Hydroxy-4-androstene-3,17-dione

This was prepared from hydrocortisone (I) according to the route shown in Figure 3.3. The methods used were as follows:

Step 1: Side chain degradation to give the 17-ketone (II) was carried out with sodium bismuthate (after Edwards and Kellie, 1958).

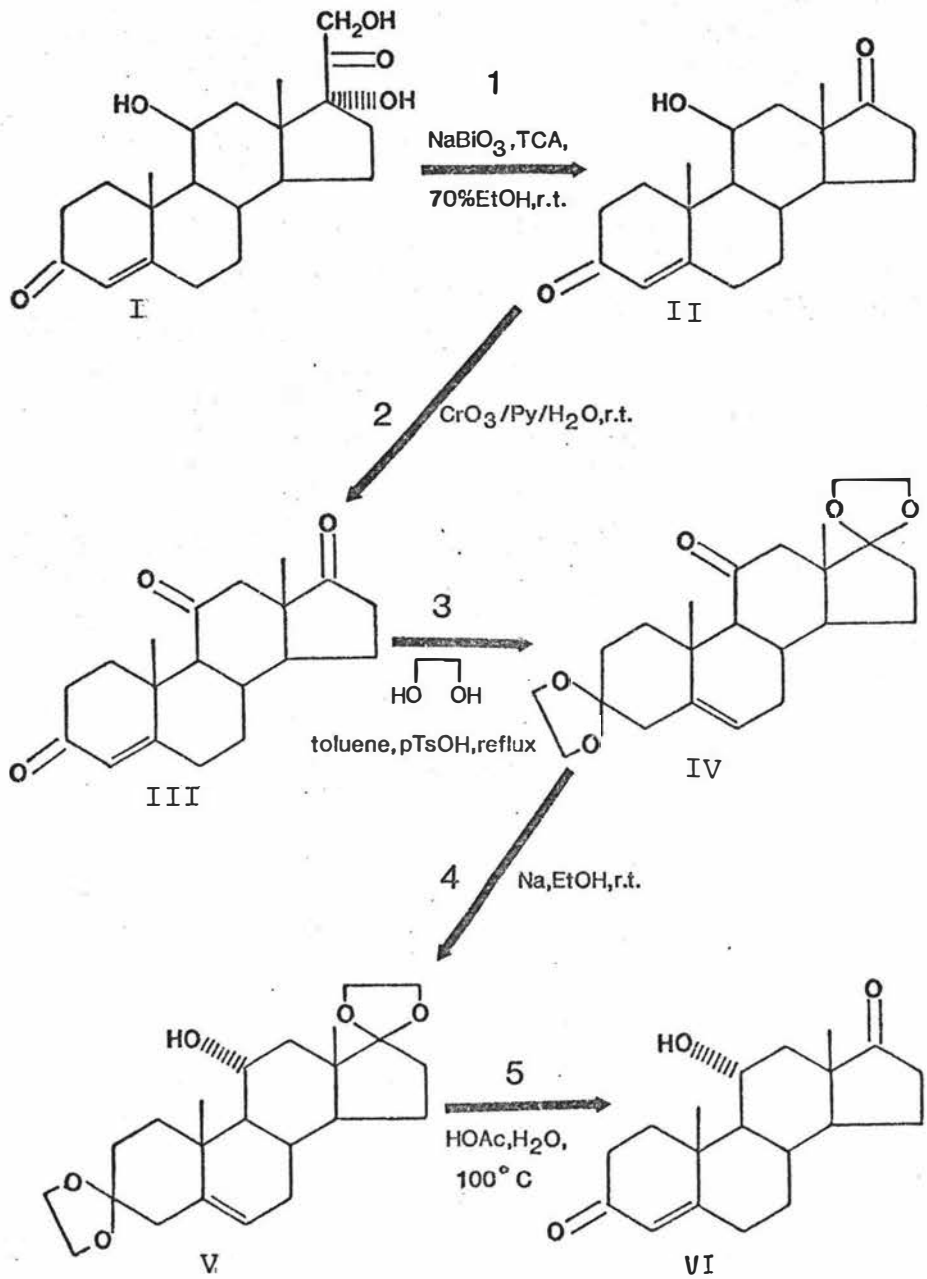
Step 2: Oxidation of the 11β-hydroxyandrostenedione (II) to the trione (III) was performed with Cornforth reagent (Cornforth *et al.*, 1962).

Step 3: Selective protection of the 3- and 17-ketones as ketals (IV) was carried out according to Bernstein *et al.* (1953), with the modification that toluene was used as solvent instead of benzene.

Step 4: Reduction of the 11-ketone with sodium metal in ethanol (scaled up, from the method of Vandenheuvel, 1975) gave predominantly the 11α-hydroxy product.

Step 5: The ketal groups were removed by heating with aqueous acetic acid on a steam bath (1.4 g steroid, 35 ml glacial acetic acid, 14 ml water) for 25 minutes. Final separation of product from unreacted trione (III) was carried out by column chromatography on silica gel, by elution with toluene:diethyl ether (1:1). The product, from pooled

Figure 3.3 Synthesis of 11α-hydroxy-4-androstene-3,17-dione



fractions containing the desired compound, was recrystallised from acetone:petroleum ether to give 11α-hydroxy-4-androstene-3,17-dione (370 mg), m.p. 225-228^{o} (cf. 227-229^{o}, Bernstein *et al.*, 1953), λ_{max}^{MeOH} 241 nm, IR υ_{max}^{KBr} 3450 cm^{-1}, (11-hydroxyl), 1740 cm^{-1} (17-keto), 1665 cm^{-1} (3-keto), which was homogeneous on tlc examination in solvent systems 1 and 2 (refer section 3.4.7).

19-Hydroxycortexolone (17α,19,21-trihydroxy-4-pregnene-3,20-dione)

This compound was obtained by hydroxylation of cortexolone with *Pellicularia filamentosa* f.sp. *microsclerotia* IFO 6298. The organism was grown in fermenter culture (agitation rate, 600 rpm; aeration rate 600 ml/min; for other conditions and procedures refer section 3.5.2) in 1500 ml of a medium containing glucose (25 g/l) and yeast extract (10 g/l) at pH 6.0. After 24 hours growth, cortexolone (700 mg), dissolved in dimethylformamide (15 ml), was added and the fermentation was continued for a further 24 hours.

The products were extracted from the autoclaved culture broth (115^{o}C for 15 min) with ethyl acetate (3 x 500 ml) after removal of mycelium by filtration. The mycelium was extracted with ethanol (50 ml) followed by ethyl acetate (100 ml). The combined extracts were washed with 2.5% (^{w}/v) sodium hydrogen carbonate solution (200 ml) followed by water (200 ml), and dried over anhydrous sodium sulphate. The solvent was removed *in vacuo* at 40^{o}C. The required product, crystallised from the concentrated ethyl acetate extract, was almost uncontaminated with other compounds. It was recrystallised from methanol to yield 19-hydroxy-cortexolone (28 mg), m.p. 230-234 (cf. 234-235^{o}C, Takahashi and Hasegawa, 1961b). The compound gave a positive Porter-Silber reaction (Lisboa, 1969), verifying the presence of the dihydroxyacetonyl sidechain, and was further characterised by mass spectrometry (Appendix B, Figure B3), NMR spectroscopy (Appendix B, Table B2), and degradation to 19-hydroxy-4-

androstene-3,17-dione, which was identified unequivocally by mass spectrometry (Appendix B, Figure B4) and by comparison with the published spectrum (Eggers, 1965).

4-Pregnene-3,11,20-trione and 4-Androstene-3,11,17-trione

These compounds were prepared from the respective 11α-hydroxy compounds by oxidation in Cornforth reagent (Cornforth *et al.*, 1962). After two recrystallisations from acetone, both products were homogeneous by tlc (solvent system 1, refer section 3.4.7), with melting points as follows:

4-Pregnene-3,11,20-trione	170-174°C (cf. 173-175°C, Reichstein and Fuchs, 1943)
4-Androstene-3,11,17-trione	220-222°C (cf. 220-223°C, Herr and Heyl, 1953)

3.1.4.2 Solvents

For steroid synthesis, crystallisation, tlc mobile phases, and solvent extraction procedures, the solvents were usually BDH A.R. grade (BDH Chemicals Ltd., Palmerston North, New Zealand). Analytical grade solvents and distilled water were glass-redistilled for use in hplc and preparative tlc. Laboratory grade chloroform was redistilled for use in the cleaning of glassware.

3.1.4.3 Other Chemicals

Chemicals used in the synthesis of steroids were generally obtained from BDH Chemicals Ltd. or May and Baker Ltd. (Dagenham, England) and were of analytical grade. Lead tetraacetate and chromous acetate, used in the synthesis of 19-hydroxyprogesterone and 16α-hydroxyprogesterone, respectively, were prepared freshly for use, as follows.

Lead Tetraacetate

This was prepared as described by Fieser and Fieser (1967c). The colourless crystals obtained were stored at 5°C, moistened

with acetic acid which contained a trace of acetic anhydride. Prior to use the material was washed on a funnel with acetic acid and dried overnight in a vacuum desiccator containing potassium hydroxide pellets.

Chromium (II) Acetate

The procedure, as described by Fieser and Fieser (1967b), was used. This reagent oxidised very rapidly, especially when moist, and so was used immediately.

Other chemicals and their sources were:

- Phenobarbital and phenanthrene were gifts from the Faculty of Veterinary Science and the Dept. of Chemistry, Biochemistry, and Biophysics, respectively, both of Massey University, New Zealand.
- BDH Chemicals Ltd (Palmerston North, New Zealand). Glucose, 8-hydroxyquinoline, α,α'-bipyridyl, buffer tablets: pH 4.0 and 7.0, formaldehyde solution 40% ($^{w}/v$), sodium bismuthate, chromium trioxide, sodium hydrogen carbonate, hydrochloric acid, sulphuric acid, sodium hydroxide, and various other laboratory chemicals (all of A.R. grade).
- Diversey-Wallace Ltd., (Papatoetoe, New Zealand). Pyroneg$^{(R)}$ detergent.
- Swift Consolidated (NZ) Ltd., (Wellington, New Zealand): Dow-Corning antifoam A.F. emulsion (Food Grade); Ingredients: Dimethylpolysiloxane, silica, stearate emulsifiers, sorbic acid, and water.
- Sigma Chemical Company (St. Louis, Mo., U.S.A.): Cycloheximide.

3.1.5 Organisms

The fungi used in this work were obtained from the following sources:

Institute of Fermentation, Osaka, Japan

Pestalotiopsis funerea IFO 5427
Pestalotia fibricola IFO 6314
Pestalotia cuboniana IFO 6315
Pestalotia microspora IFO 30316
Pestalotiopsis versicolor IFO 6319
Pestalotia diospyri IFO 5282
Pestalotiopsis karstenii IFO 6316
Pestalotia truncata IFO 8584
Corticium practicola IFO 6253
Corticium caeruleum IFO 4974
Pellicularia filamentosa f.sp. *solani* IFO 5289
Pellicularia filamentosa f.sp. *sasakii* IFO 5254
Pellicularia filamentosa f.sp. *microsclerotia* IFO 6298

Commonwealth Mycological Institute, London, U.K.

Pestalotiopsis sydowiana IMI 82405a
Pestalotiopsis populi-nigrae IMI 110878
Pestalotiopsis palmarum IMI 87277
Pestalotiopsis gracilis IMI 89307
Pestalotiopsis cruenta IMI 90778c
Pestalotiopsis mayumbensis IMI 99418
Pestalotiopsis aquatica IMI 87402

American Type Culture Collection, Rockville, Maryland, U.S.A.

Pestalotia microspora ATCC 11816

Victoria University Culture Collection, Wellington, New Zealand.

Pestalotia fineita WU 393
Pestalotia ramulosa WU 145
Pestalotia unicolor WU 144
Pestalotia sp. WU 9/77
Pestalotiopsis sp. WU 215

Plant Diseases Division Culture Collection (Department of Scientific and Industrial Research, Auckland, New Zealand).

Pestalotia vaccins PDDCC 5446
Pestalotiopsis versicolor PDDCC 7
Pestalotiopsis sp. PDDCC 24
Pestalotiopsis sp. PDDCC 8
Pestalotia sp. PDDCC 3062

Maintenance

All organisms, with the exception of the *Corticium* and *Pellicularia* species, were maintained on slopes of Potato Dextrose Agar and subcultured every three months (growth temperature of 25°C; stored at 5°C). The *Corticium* and *Pellicularia* spp. were first maintained on slopes of Malt Extract Agar, but were later changed to slopes of Lactritmel Agar, and subcultured monthly (growth temperature at 25°C; stored at 5°C). The Lactritmel Agar promoted superior mycelial differentiation.

3.2 MEDIA STERILIZATION

Microbiological media were sterilized by autoclaving at 121°C for 15 minutes, except in the case of malt extract agar, malt extract broth, and the glucose/yeast extract medium, which were autoclaved at 115°C for 15 minutes. In some cases, heat labile constituents of a medium (for example, the vitamin solution, Table 3.1) were filter-sterilized through a 0.45 µm membrane filter (Oxoid Ltd., London, England) prior to aseptic addition to the medium.

3.3 CLEANING OF GLASSWARE

All glassware was washed in hot Pyroneg (R) solution, rinsed in tap water, then in distilled water, and hot-air dried. Glassware used for the storage of hplc solvents was treated in chromic acid after the detergent wash, then rinsed thoroughly with distilled water prior to drying. All glassware used in steroid extraction and quantitation proced-

ures was rinsed with chloroform prior to use.

3.4 ANALYTICAL METHODS

3.4.1 Melting Points

These were determined on a Leitz HM-Lux Hot Stage Microscope (Ernst Leitz, Wetzlar GMBH, Germany) and are uncorrected.

3.4.2 pH Measurement

Routine pH measurements were made with a Metrohm pH Meter E520 (Metrohm AG., Herisau, Switzerland) which was regularly calibrated with appropriate buffers.

3.4.3 Mycelial Dry Weight Determination

A measured volume of culture (ca. 20 ml), or whole shake-flask contents, were filtered through Whatman No. 45 filter paper (previously dried at 105^{o}C and preweighed). The biomass was washed twice with distilled water (10 ml per 20 ml of culture) and dried to constant weight at 105^{o}C.

3.4.4 Glucose Analysis

An enzyme electrode method was used to specifically determine the glucose concentration in samples. A YSI Model 27 sugar analyser (Yellow Springs Instruments Ltd., Yellow Springs, Ohio, USA) was used. With regular calibration the relative error in duplicate determinations was less than 1%.

3.4.5 Total Carbohydrate Analysis

Total carbohydrate concentration was not routinely determined but was occasionally used to complement glucose concentration determinations. The anthrone method of Ghosh *et al.* (1960) was used.

3.4.6 Total Nitrogen Determination

This was performed by the Kjeldahl method was follows. A weighed sample of oven dry mycelia (0.01 - 0.03 g N) was transferred to a Kjeldahl digestion flask. Sodium sulphate (2 g), mercuric sulphate solution (5 ml; of 150 g mercuric oxide dissolved in a solution of 180 ml conc. sulphuric acid plus 1320 ml water), and concentrated sulphuric acid (20 ml) were added. The contents were brought to the boil and heated until clear, and then heated for a further 1 hour. The contents were then cooled and transferred quantitatively to a 100 ml volumetric flask. The ammonia content of digested samples (10 ml) was determined by steam distillation in a Markham still after the addition of 15% ($^{w}/v$) sodium hypophosphite (5 ml) and 60% ($^{w}/v$) sodium hydroxide (15 ml). The ammonia was collected in 2% ($^{w}/v$) boric acid (10 ml) containing 2 drops of screened methyl red indicator (2 g methyl red plus 1 g methylene blue dissolved in 1000 ml 96% ($^{v}/v$) ethanol). The ammonia was then titrated with 0.2 M hydrochloric acid to a grey/green end point. Duplicate determinations were performed with each mycelial sample.

3.4.7 Thin Layer Chromatography (tlc)

Thin layers (0.2 mm) of silica gel DGF were prepared on glass plates (5 x 20 cm or 10 x 20 cm). The plates were developed under tank saturation conditions, in one or more of the following three solvent systems (Lisboa, 1969):

I chloroform:ethanol 9:1
II ethyl acetate:hexane:acetic acid 75:20:5
III ethyl acetate:hexane:acetic acid:ethanol 72:13.5:10:4.5

Rf values for chromatograms of a large range of steroids in these solvent systems are given by Lisboa (1969).

The steroids were normally visualised by ultra-violet light (254 nm). To aid identification of transformation products, samples were chromatographed in parallel with

standard compounds. To verify the presence of the dihydroxyacetonyl side chain of corticosteroids the Porter-Silber spray reagent, described by Lisboa, was used. For non-specific compound visualisation, plates were sprayed with methanol:sulphuric acid (1:1) and heated at 160°C for 1 minute.

For preparative thin layer chromatography 20 x 20 cm, 1 mm thick, silica gel DGF plates were prepared. Normally, approximately 20 mg of steroid was applied per plate. The plates were developed as for analytical tlc. The material was visualised under ultra-violet light and the required bands scraped from the plate. Steroid was eluted from the silica by stirring for 10 min with 4:1 chloroform:methanol, followed by filtering to remove the silica gel.

3.4.8 High Performance Liquid Chromatography (hplc)

Quantitative steroid analysis of prepared samples was performed using a Waters Associates Model ALC/GPC 244 liquid chromatograph with a UK6 septumless injector (Waters Associates, Milford, Mass., USA). A μBondapak C-18 reverse-phase column (4.0 mm ID x 250 mm, Bio-sil ODS 10, Bio-Rad Laboratories, Richmond, California, USA) was used for all analyses. The steroids were detected at 254 nm, 0.1 AUFS using a Waters Associates Model 440 ultra-violet absorptiometer and the response was recorded on a CR600 twin-pen, flat-bed chart recorder (J.J. Lloyd Instruments Ltd., Southampton, England). Analyses were conducted at ambient temperature under the following conditions:

(a) For the analysis of progesterone-hydroxylation experiments

1.0 ml/min, 60:40 (methanol:water)

(b) For the analysis of androstenedione-hydroxylation experiments

2.0 ml/min, 40:60 (methanol:water)

(c) For the analysis of cortexolone-hydroxylation experiments

1.8 ml/min, 50:50 (methanol:water)

The mobile phases were filtered and degassed prior to use, through a 0.45 µm filter (Millipore Corporation, Bedford, Mass., USA). Chromatographs of appropriate compounds under each of the above conditions are shown in Figures 3.4, 3.5, and 3.6. Between 10 µl and 50 µl of sample were injected into the chromatograph, the exact amount depending on the steroid concentration in the sample.

Quantitation was normally performed by peak height measurement and reference to standard mixtures of compounds, chromatographed in parallel with the samples. On certain occasions a Varian Aerograph Model 477 Digital Integrator (Varian Aerograph, Walnut Creek, Cal., USA) was available for peak area measurement and served as an alternative quantitation technique.

For preparative hplc, a µBondapak C-18 semi-preparative, reverse-phase column (8 mm ID x 300 mm, Waters Associates, Milford, Mass., USA) was used. Conditions of operation were similar to those for analytical hplc except that detection was by a Waters Associates R401 differential refractometer. Injection volumes of up to 200 µl were used with typical column loadings of 0.1 - 2.0 mg per injection.

3.4.9 Infra-red Spectrophotometry (IR)

A KBr disc containing 1% ($^{w}/w$) steroid, which had been dried at 105^{o}C for 3 hours, was made with a model DMO-1 Beckman Evacuable Minidie (Beckman-Riic Ltd., Glenrothes, Fife, U.K.). The spectra were obtained by scanning such discs at low speed using a Beckman Acculab 8 IR Spectrophotometer (Beckman Instruments Inc., Fullerton, USA).

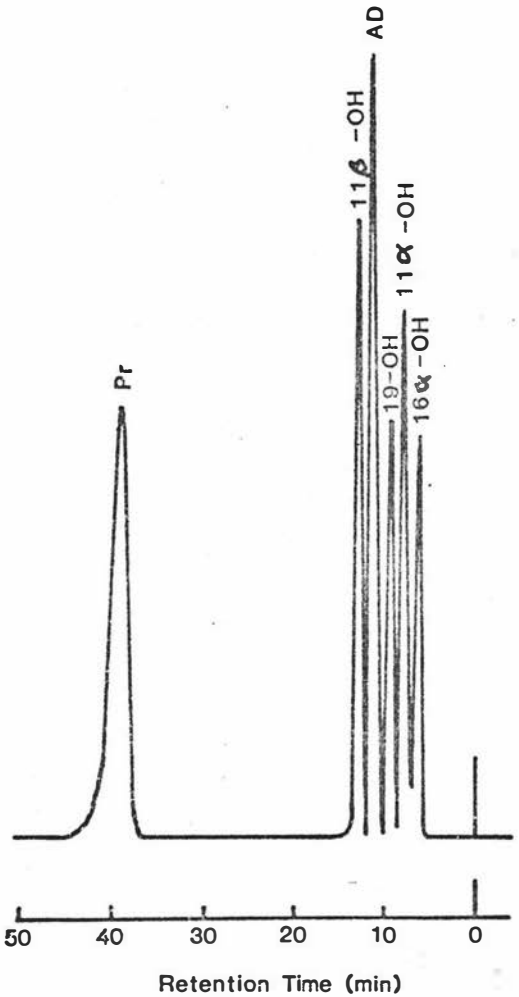


Figure 3.4 The hplc separation of some monohydroxy derivatives of progesterone from progesterone (Pr) and androstenedione (AD). Conditions: (a)

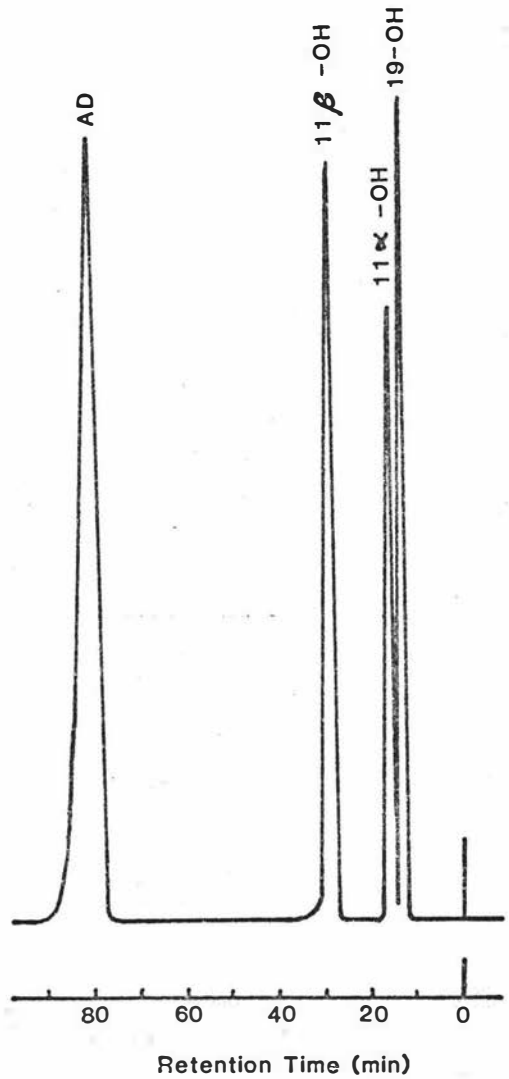


Figure 3.5 The hplc separation of some monohydroxy derivatives of androstenedione from androstenedione (AD). Conditions: (b)

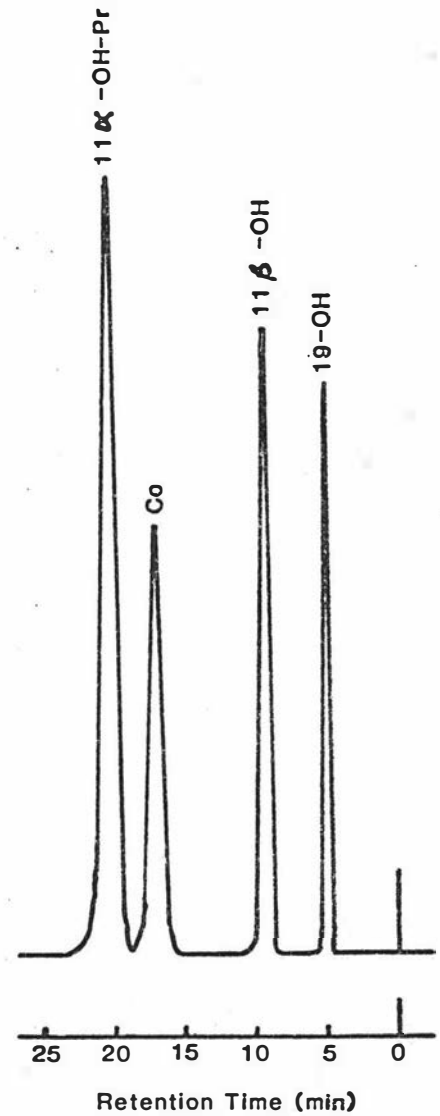


Figure 3.6 The hplc separation of monohydroxy derivatives of cortexolone from cortexolone (Co) and 11α-hydroxyprogesterone (11α-OH-Pr). Conditions: (c)

3.4.10 Ultra-violet Spectrophotometry (UV)

Samples, dissolved in methanol, were scanned using a Varian Techtron Model 634s UV-Visible Spectrophotometer (Varian-Techtron Pty, Ltd., Australia).

3.4.11 Mass Spectrometry (MS)

Mass spectrometric analysis of steroid samples was performed by either the Chemistry Department, Massey University, New Zealand (using a MS 9 double-focussing Mass Spectrometer), or by the Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand (using an AEI MS 30 double-focussing Mass Spectrometer). The samples were run from a direct insertion probe.

3.4.12 Nuclear Magnetic Resonance Spectroscopy (NMR)

Both proton and carbon-13 NMR measurements were performed by the Chemistry Department, Massey University, New Zealand, using a JEOL FX60 Pulse-Fourier Transform Spectrometer. ^{13}C-NMR spectra were accumulated under full proton decoupling conditions. Chemical shifts were expressed in ppm downfield from the internal standard, tetramethylsilane.

3.5 CULTURE CONDITIONS

3.5.1 Shake-Flask Culture

Experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml of medium, except where otherwise stated. Incubation was at 30^{o}C on either an Environ-Shaker Model 3597 (Lab-line Instruments Inc., Illinois, USA) or an Orbital Incubator (Gallenkamp Ltd., London, UK) operating at 180 rpm.

For the screening experiments of Chapter 4, a medium containing glucose (25 g/l) and yeast extract (20 g/l), at pH 6.5, was used. Inoculum preparation was identical for all fungi examined. It consisted of inoculation, from a

maintenance slope, into malt extract broth (10 ml) which was incubated at 25°C, until the culture was well grown. The culture was then transferred into the glucose-yeast extract medium (100 ml), as above, contained in a 250 ml Erlenmeyer flask. This was incubated at 30°C on an orbital shaker at 180 rpm until well grown. The 100 ml culture was finally homogenised in a sterile Waring blender (after Meyrath and Suchanek, 1972) at half-maximum speed, for 10 seconds. This prepared inoculum (2 ml) was transferred into the required number of 250 ml Erlenmeyer flasks containing glucose-yeast extract medium (48 ml). By this procedure replicate cultures of a given fungus could be routinely obtained.

For later work with *Pellicularia filamentosa* f.sp. *microsclerotia* a revised inoculum preparation procedure was used. In a medium containing glucose (25 g/l) and yeast extract (10 g/l), at pH 6.0 (100 ml volume in a 250 ml Erlenmeyer flask) a culture was prepared by inoculation from a maintenance slope and incubation at 30°C and 180 rpm. After 3 days incubation, the culture (5 ml) was added as a standard inoculum to medium (45 ml), composition as above) and incubation was carried out as previously described for 24 hours. Cortexolone, dissolved in dimethylformamide (0.5 g/l and 10 g/l, respective final concentrations, unless otherwise stated), was then added and incubation was continued for the required time.

For the screening experiments (Chapter 4) cultures were incubated until satisfactory growth had been obtained (normally 1-2 days). A standardised incubation time for all the fungi was not possible, since almost all grew at different rates. Steroid was then added as appropriate (0.5 g/l final concentration), dissolved in dimethylformamide (10 g/l final concentration). For experiments involving the transformation of progesterone and androstenedione, flasks were harvested at 8 h and 16 h (two flasks at 16 h). One of the cultures removed at 16 h was examined qualitatively by hplc. This was to ensure that the internal standard, used in product quantitation of the other cultures, did not obscure any transformation products. For screening experiments involving

the transformation of cortexolone, flasks were harvested at 1 and 2 days (for *Pestalotia* spp.) or 5 days (for *Pellicularia* spp.) after steroid addition. No internal standard was used in these experiments.

3.5.2 Fermenter Culture

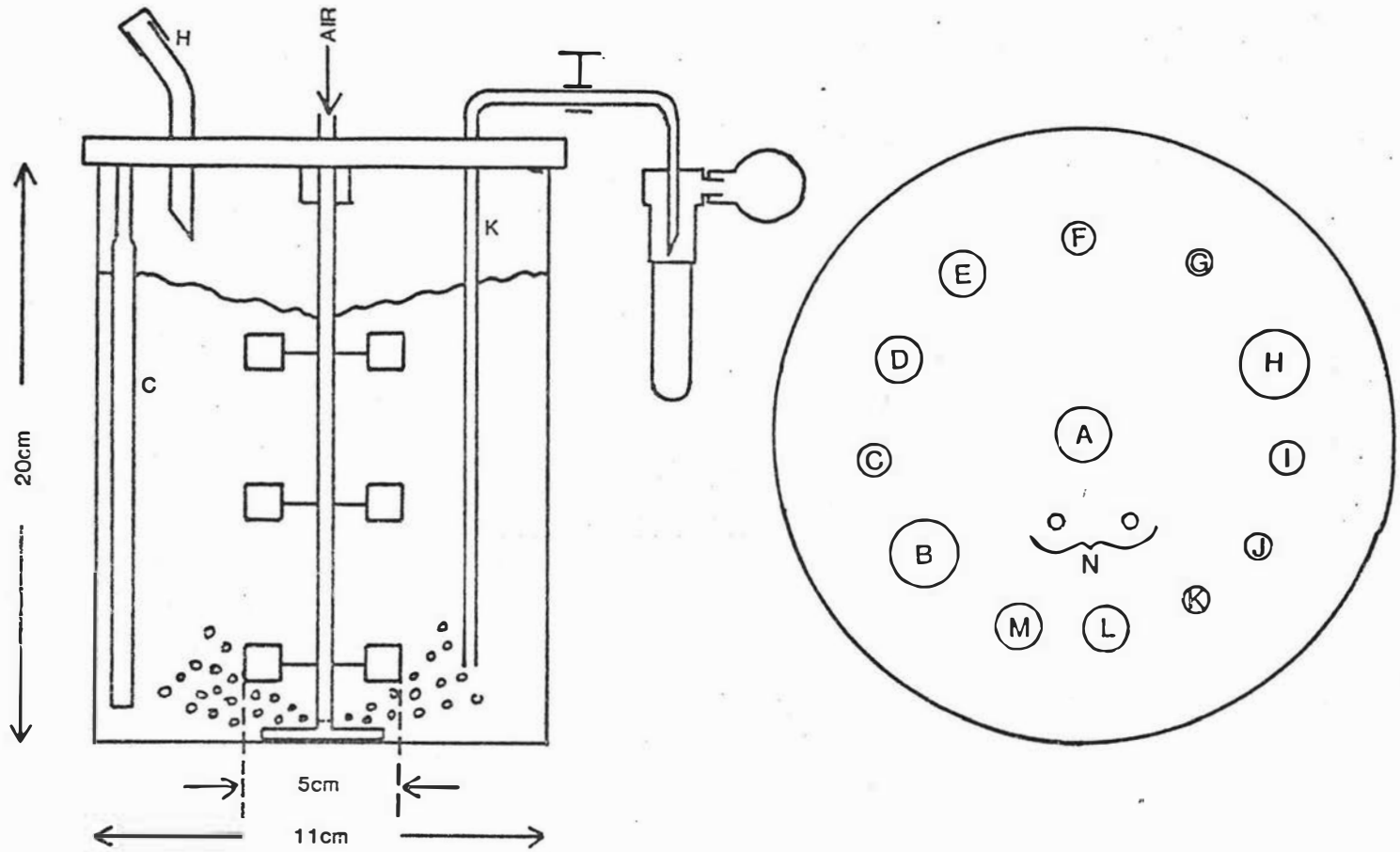
The basic fermenter used was a Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, USA). This was operated in both batch and continuous modes. Figure 3.7 shows a schematic diagram of the fermenter vessel itself. Figures 3.8 and 3.9 show respectively, a schematic diagram and photograph, of the fermenter plus its ancilliary equipment while in continuous operation. Under batch operation, culture overflow and medium feeding facilities were absent.

3.5.2.1 Equipment, Instrumentation, and Control

The fermenter vessel was a 2 litre pyrex jar (standard for the culture apparatus) with a polyethylene-propylene head containing holes for the insertion of sensors and other facilities.

Agitation was provided by an impeller assembly of 3, six-bladed disc-turbine impellers mounted at equally spaced intervals from 5 cm above the vessel base to 5 cm below the culture surface. This was driven by indirect magnetic coupling through the base of the vessel. Agitation speeds from 0 to 1000 rpm (0-2.62 m/s, impeller tip speed) were attainable. A single baffle was used under batch operation to ensure turbulent flow; however, this was omitted for continuous operation.

A solid state temperature controller with a thermistor sensor maintained the fermenter temperature at 30 $\pm$ 0.2^{o}C by means of a heating element operating against a constant cooling water flow. A thermometer was used as a visual check on culture temperature.



A air inlet
B pH probe
C baffle/C.C. overflow tube
D DOT probe
E cooling tube
F addition port
G thermistor tube
H inoculation port
I air outlet
J medium addition
K sample tube
L heating element
M anti-foam addition
N acid/base inlets

Figure 3.7 A schematic diagram showing the fermenter vessel and placement of the various facilities and probes in the vessel head.

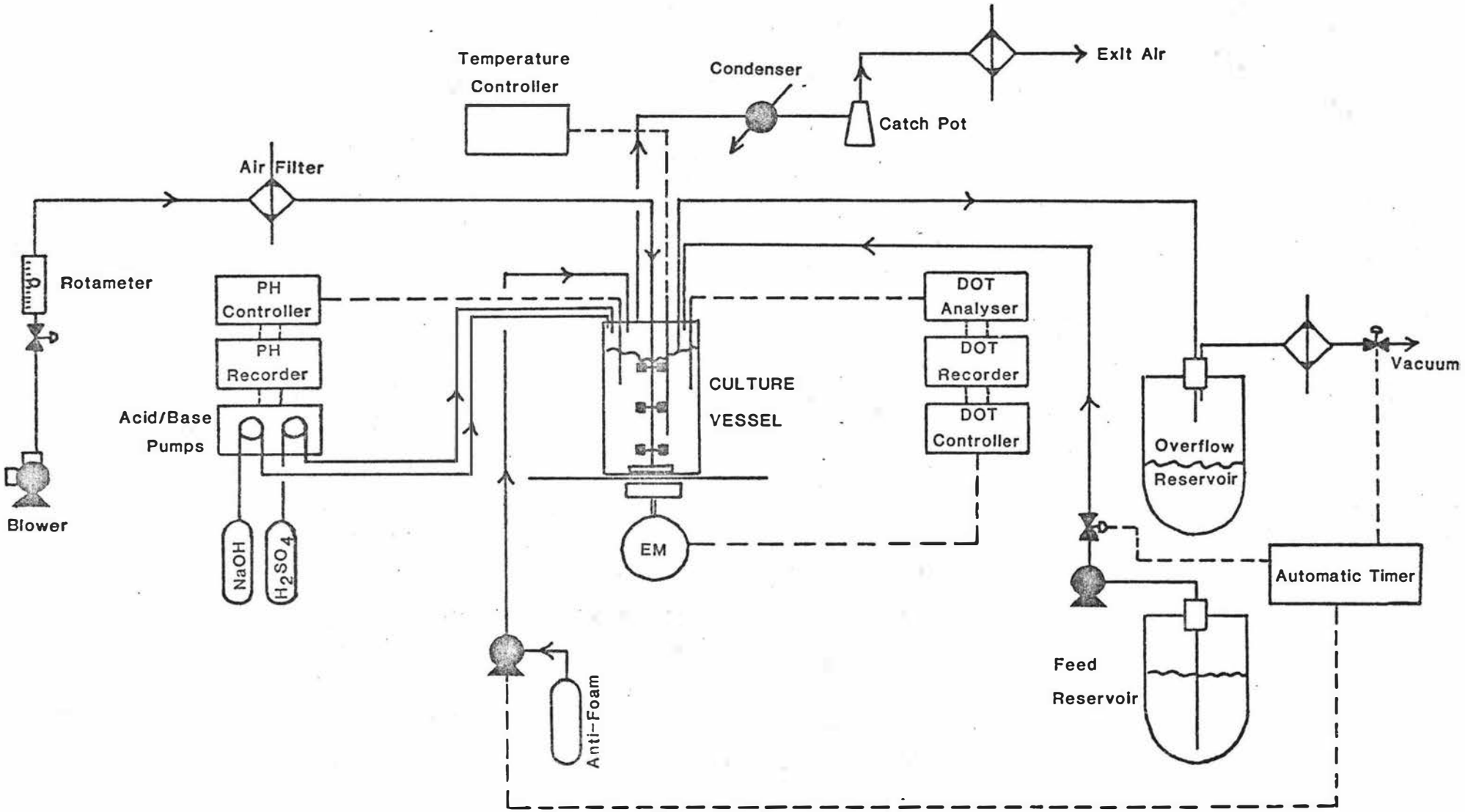


Figure 3.8 A schematic diagram of the fermenter plus its ancilliary equipment (in continuous culture operation).

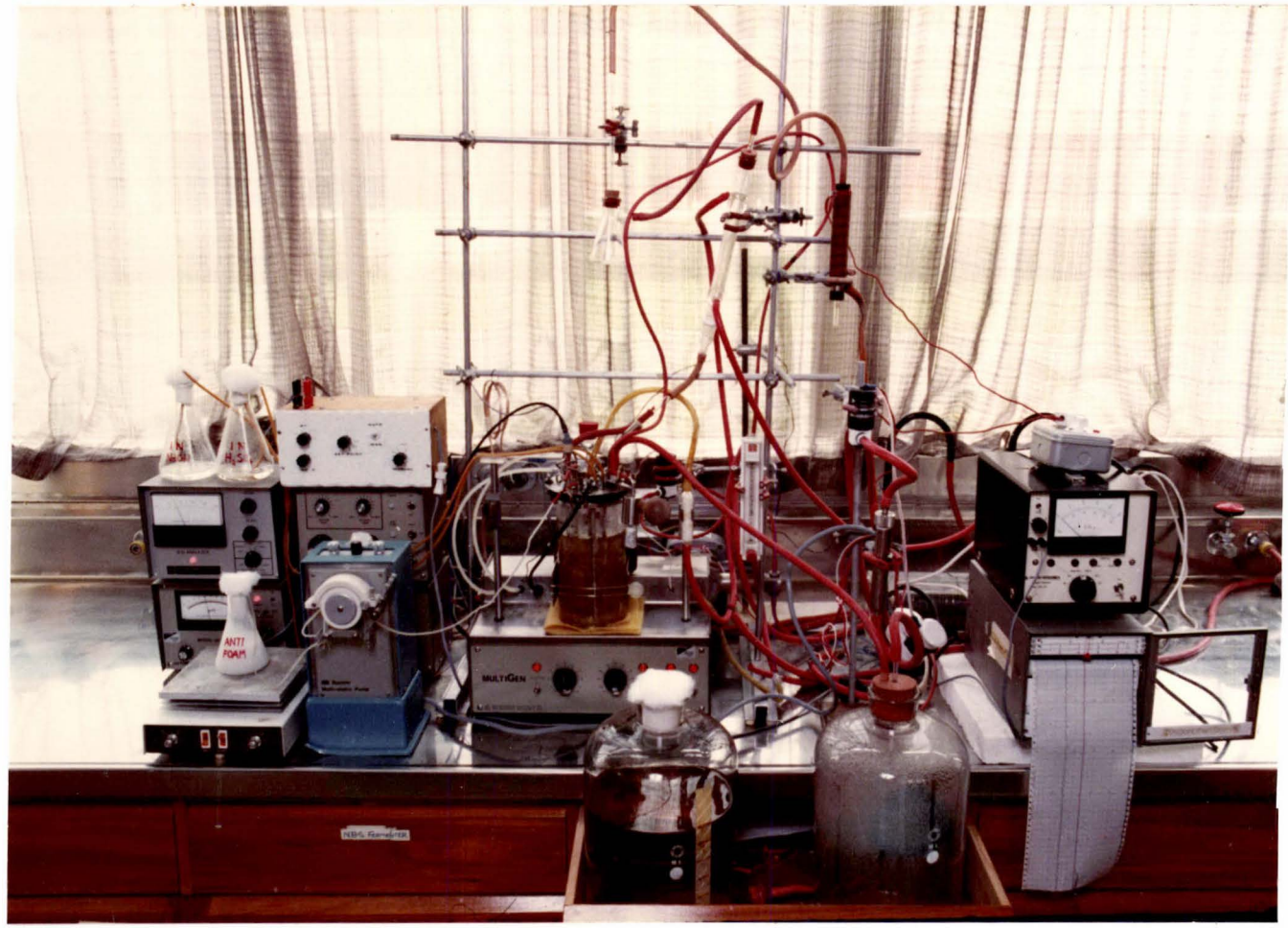


Figure 3.9 The fermenter and ancilliary equipment (in continuous-culture operation)

Air was supplied to the vessel by a blower situated in the Multigen F2000 apparatus. The air flow rate was controlled by a needle valve and measured with a Gap variable-area flow-meter (Platon Flow Control Ltd., Basingstoke, Hampshire, England) over the range 100-1200 ml/min. The metered air was passed through a sterile glasswool-packed filter, before entering the culture vessel through numerous, small holes in the base of the impeller shaft (Figure 3.7). The flow meter was calibrated using a soap bubble meter to an accuracy of $\pm$5 ml/min. However, during fermenter operation the aeration rate could only be read to $\pm$25 ml/min due to fluctuations in the position of the meter float. The entire air flow system in the operating fermenter was checked for leaks under various controlled back-pressures, by measuring the flow rate of air leaving the apparatus against the indicated meter reading. Exhaust air was passed through a water cooled condenser and through a catch-pot to a heated exhaust-filter. This glasswool-packed filter was heated by a thermostaltic tape which raised the exit air temperature to about 60^{o}C, so preventing moisture condensation. A Taylor Servomex Oxygen Analyser, Type OA 272 (Taylor Servomex Ltd., Crowborough, Sussex, U.K.) was used to measure the oxygen concentration in the exit gas from the fermenter. With regular calibration an absolute accuracy of $\pm$ 0.25% (^{v}/v) was possible. In many fermentations less than a 1% (^{v}/v) absolute change in the oxygen content of the air was obtained, making for rather inaccurate oxygen consumption rate measurements. However, in many situations the analyser proved a valuable addition to the equipment.

Culture pH was maintained by an Automatic Mini pH Control System (New Brunswick Scientific Co. Inc.) consisting of a pH40 Controller module, a XpH 42 pump module and a pH40 Recorder module. The culture pH was measured by a XpH-75, Type 761-351B, Ingold combination glass electrode (Ingold Electrodes Inc., Lexington, Mass., USA). This system provided two way pH control by the addition of either 1 M caustic soda or 0.5 M sulphuric acid. A permanent record of culture pH could be printed on the strip chart recorder module. The

pH electrode was calibrated using pH 4.0 and pH 7.0 buffer solutions prior to each fermentation. Occasional soaking in 0.1 M hydrochloric acid, containing pepsin, was required to remove contaminating proteins from the electrode. When not in use, the probe was stored in pH 4.0 buffer solution. The pH of removed fermentation samples was independently measured in order to correct for the drift in pH reading, which was regularly observed. This was apparently an effect of protein contamination on the electrode.

Antifoam emulsion (Dow-Corning Antifoam AF) was added to fermenter cultures to suppress foaming. Initially, a foam-sensing probe was used to actuate antifoam addition. However, this proved unsatisfactory, as the fungal mycelium would attach to the probe and force the system out of control. Regular, timed addition of antifoam was used to overcome this problem. Thus, antifoam emulsion (10 % (^{w}/v)) was added at a controlled rate (0.1 - 0.5 ml/min) for 10 seconds in every 30 minutes. The addition pump (Buchler Multistaltic Pump, Buchler Instruments, New Jersey, USA) was actuated for 10 seconds in every 30 minutes by a cam-timing device. The rate of antifoam addition was adjusted, depending on the degree of foaming which occurred.

A galvanic, dissolved oxygen probe, Type M1016-0208 (New Brunswick Scientific Co. Inc.), was used to measure the culture dissolved oxygen tension. The probe was connected to a model DO-40 Dissolved Oxygen Analyser (New Brunswick Scientific Co. Inc.) which used a 0-10 mV output for chart recorder operation. The probe was calibrated *in situ*, prior to inoculation of the fermenter, by stripping dissolved oxygen from the vessel contents with oxygen-free nitrogen gas (passed through the aeration system) to obtain zero saturation conditions. The vessel contents were then reaerated to give 100% saturation. The fermenter, prior to inoculation, was operated for at least 24 hours to ensure stable dissolved oxygen probe operation. After the completion of a fermentation, the probe calibration was checked to ensure that no significant change had taken place during the experiment.

Dissolved Oxygen Control

During the course of the work, two approaches to dissolved oxygen control were used.

(i) *On-off control of aeration rate*

With this system, when the dissolved oxygen tension (DOT) dropped below the set-point a solenoid valve, connected to the compressed air line, was actuated. This boosted the aeration rate from its normal setting (550 ml/min) to 1200 ml/min. When the DOT rose above the set-point, the valve was switched off. This method was suitable for control over short spaces of time, but required a judicious choice of agitation rate, and regular monitoring. The accuracy of control was $\pm$ 5% of the saturation scale under optimum conditions. However, the system could easily go out of control. The equipment is shown schematically in Figure 3.10.

(ii) *Proportional-integral control of agitation speed*

This more sophisticated approach was necessary to enable precise, automatic DOT control over long periods of time, particularly during fermentations where the final oxygen demand of the culture could not be predicted. The full details of the developed system are given in Appendix A; however, a brief description will be presented here. An analogue circuit was designed to process the DOT signal in such a way as to either increase (if below the DOT set-point) or decrease (if above the set-point) the agitation speed. When at the set-point the speed would remain constant. The mode by which these changes occurred was governed by proportional-integral action. The system is shown schematically in Figure 3.11. This system allowed control to $\pm$ 2% of the saturation scale and could rapidly return the DOT to the set-point following disturbances.

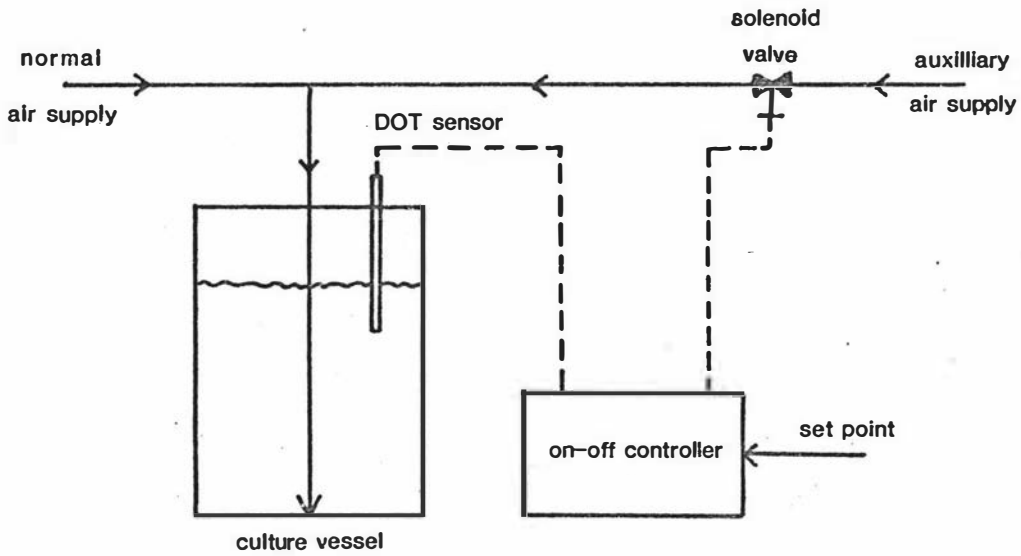


Figure 3.10 A schematic diagram of the DOT control system which utilized on-off control of aeration rate.

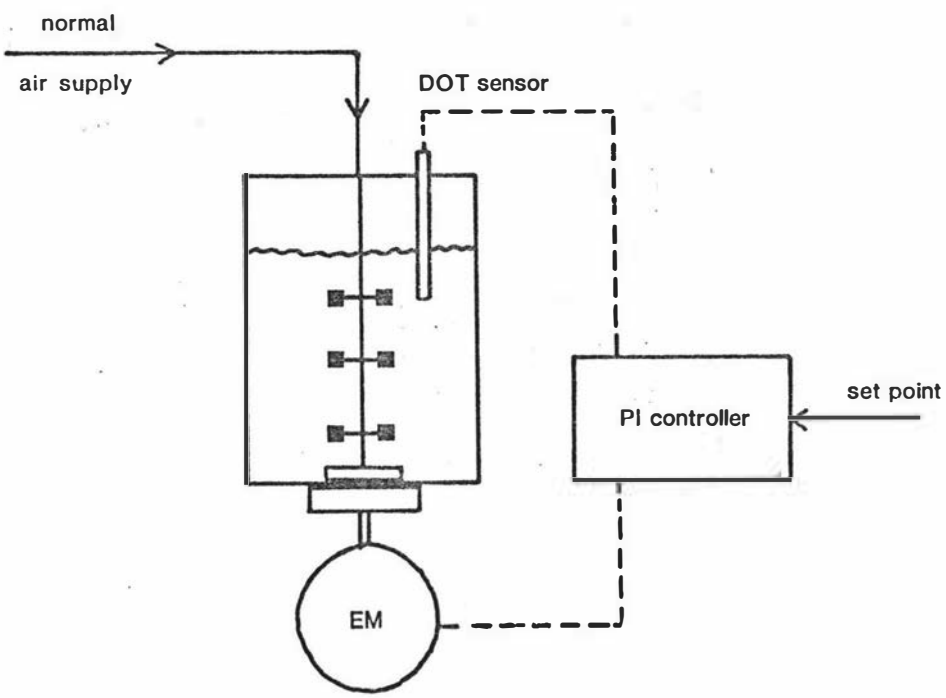


Figure 3.11 A schematic diagram of the DOT control system which utilized proportional-integral control of agitation speed (EM = electric motor).

3.5.2.2 Continuous Culture Operation

For continuous culture of filamentous fungi special modifications to the fermenter vessel are normally required. These are outlined by Righelato and Pirt (1967) and Rowley and Bull (1973) and some of the modifications were adopted for the present work. Thus, the vessel was unbaffled; the heating and cooling tubes and the various probes provided sufficient baffling. A teflon-covered, bar magnet inside the fermenter vessel (secured and moved with a horse-shoe magnet on the outside of the vessel) was used periodically to remove accretions of fungal mycelium from the vessel walls. No previously reported method for culture overflow was found satisfactory in allowing continuous removal of homogeneously mixed vessel contents. Thus, when using a side-arm overflow weir (with air exiting through the same line as the culture) a steady-state biomass level was never achieved. In this case, liquid exited preferentially to the biomass and the culture dry weight continuously increased. The procedure finally adopted was to periodically evacuate the overflow reservoir, thus withdrawing culture through a vertically positioned overflow tube. A schematic diagram of the system is shown in Figure 3.12. The frequency with which the evacuation occurred was adjusted in proportion to the dilution rate, e.g. At $D = 0.064\ h^{-1}$ vacuum was applied for 10 s in every 30 minutes. Culture volume and hence dilution rate varied in a cyclic manner, by approximately 3%, as a result of this procedure. This was considered acceptable within the precision limits of continuous culture practice. In order to prevent the free flow of feed-medium into the vessel while the vacuum was being applied, a solenoid valve was positioned in the feed-line to the vessel. This was operated through a relay switch. The normal valve position was open. When the cam-timer opened the solenoid valve on the vacuum line it also opened the relay switch, so closing the valve on the feed-line. This equipment is also represented in Figure 3.12.

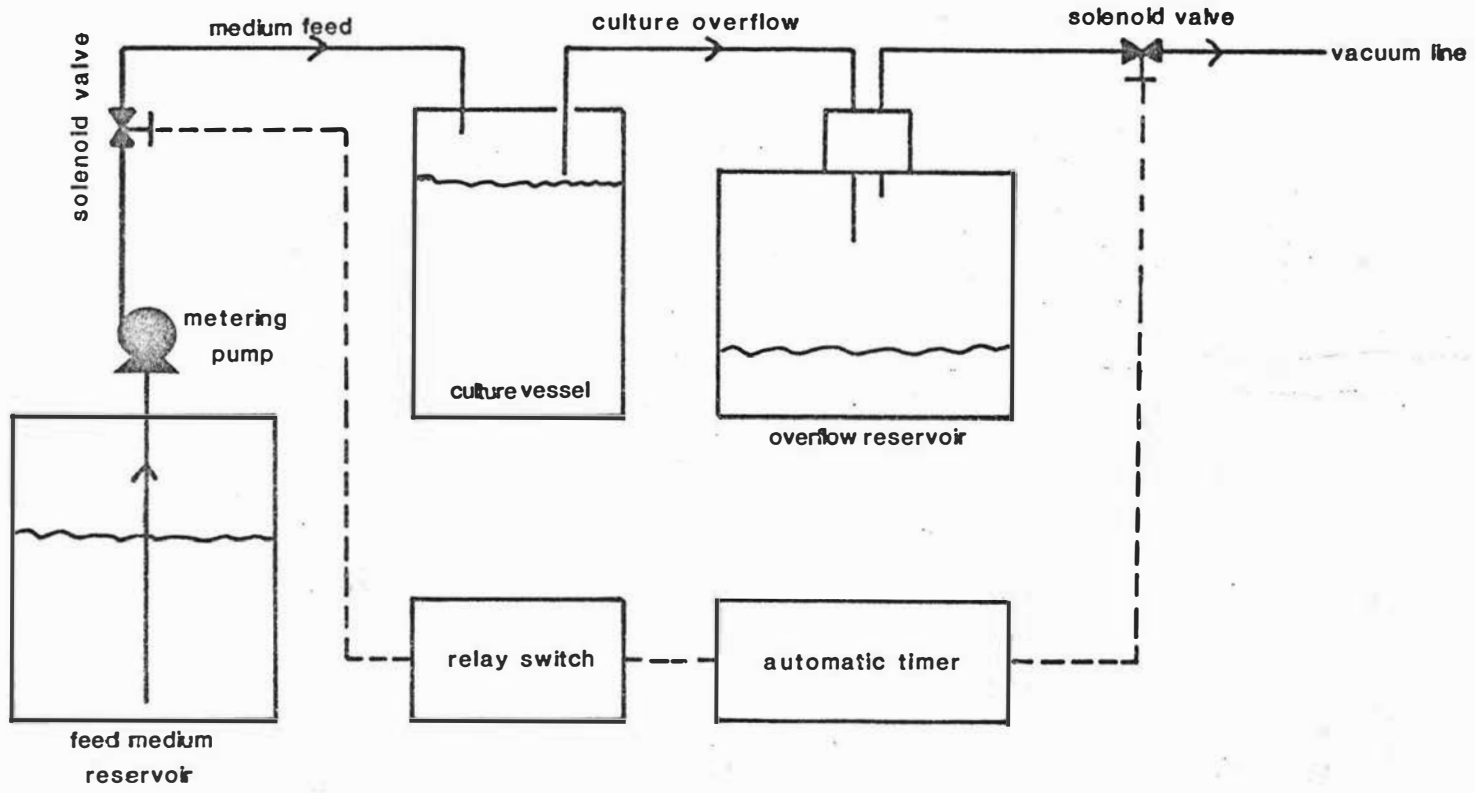


Figure 3.12 A schematic diagram of continuous culture overflow and medium feeding facilities.

3.5.2.3 Sterilization

The fermenter vessel, containing medium but without electrodes, was autoclaved for 20 minutes at 115°C, as were the feed and overflow reservoirs (for continuous operation). Glucose in the feed-medium was autoclaved as a pure solution and later added to the sterile feed-reservoir containing the yeast extract solution.

The dissolved oxygen and pH probes, as well as the solenoid valve in the feed-line, were sterilized by soaking in 5% (^{w}/v) formalin solution followed by rinsing with sterile distilled water. These were then aseptically mounted into their appropriate positions. Acid, base, and antifoam solutions, and related tubing were sterilized by autoclaving for 20 minutes at 115°C. Glasswool-packed air filters were sterilized by dry heat at 160°C for 2 hours.

3.5.2.4 Inoculum Preparation

A standard inoculum of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was prepared by sub-culturing from a maintenance slope into 100 ml of medium, in a 250 ml Erlenmeyer flask, and incubating in shake-culture for 3 days (30°C, 180 rpm). Transfer of 5 ml of this culture into another 95 ml of medium followed by incubation as above provided 100 ml of inoculum. The entire volume of culture (ca. 6-7% (^{v}/v) of the fermenter contents) was added to the fermenter vessel. Inocula of *P. filamentosa* f.sp. *sasakii* IFO 5254 and *Corticium practicola* IFO 6253 were prepared by the same basic procedure. In the case of the former organism an incubation time of 6 days was used.

3.5.2.5 Steroid Addition

Substrate, dissolved in dimethyl formamide, was added at the required time as for shake-flask experiments, except where otherwise stated. In experiments involving semi-continuous feeding of cortexolone, the steroid was added as a fine suspension (made by adding the required amount of steroid, dissolved in dimethylformamide, to 0.1%

($^{w}/v$) Tween 80 solution; proportions of DMF: Tween solution were 15 ml:100 ml). The suspension was pumped into the vessel with a Buchler Multistaltic Pump (Buchler Instruments, New Jersey, USA) operating for 6 minutes in every 30 minutes. The flow-rate was adjusted to approximately 2-5 ml/h, depending on the fermentation requirements.

3.5.2.6 Fermenter Operation

After inoculation, the initial fermenter volume was recorded. The volumes of all subsequent additions and removals from the vessel were also recorded. This allowed a volumetric analysis of the fermentation to be performed, in order to correct for any dilution effects.

Samples (ca. 20 ml) were regularly withdrawn for steroid, mycelial dry weight, and glucose determinations. Prior to sampling, the sample tube was flushed with culture (ca. 5 ml) to remove the resident "dead" volume. The mycelial dry weights of samples so obtained were found to be representative of the actual mixed-culture dry weight.

During continuous culture operation mycelial growth around the vessel walls was periodically dislodged with a captive, teflon-coated magnet (see section 3.5.2.2).

3.6 STEROID EXTRACTION AND ANALYTICAL SAMPLE PREPARATION

For all the following procedures the cultures were inactivated by heating at 100^{o}C for 5 minutes, prior to steroid extraction. By the similar treatment of standard steroids, and by the analysis of cultures with and without such treatment, it was verified that in no case did degradation of steroids occur. This procedure simply ensured containment of the fungi, some of which were potential plant pathogens.

3.6.1 Extraction of Freeze-Dried Cultures

The *Pestalotia* spp. invariably produced highly viscous culture broths which made mycelial removal by filtration, and subsequent solvent extraction, very difficult. For this reason all the fungal cultures examined for their steroid transformation abilities (Chapter 4) were freeze-dried prior to solvent extraction. Each 50 ml culture (in a 250 ml Erlenmeyer flask) was freeze-dried in a cabinet freeze-dryer (Food Technology Dept., Massey University, New Zealand). The dried culture, still in its original 250 ml flask, was then extracted by one of the following procedures, depending on the transformation substrate.

(i) Progesterone and Androstenedione

A known amount (10 mg) of internal standard was added to each culture prior to freeze-drying. For experiments involving the hydroxylation of progesterone, androstenedione was used as internal standard; for androstenedione hydroxylations, 11β-hydroxy-androstenedione was similarly used.

Solvent extraction was carried out as follows:

(a) 1 x methanol:chloroform 1:1 (40 ml)
(b) 1 x chloroform (30 ml)
(c) 1 x chloroform (20 ml)

In steps (b) and (c) of this procedure, the culture, plus solvent, was heated to the solvent boiling point (c a. 60^{o}C). The combined extracts were washed with 2.5% (^{w}/v) sodium hydrogen carbonate (40 ml) and then water (20 ml), prior to drying over anhydrous sodium sulphate, and evaporation to dryness *in vacuo* at 50^{o}C. The dried extract was taken up in methanol (10 ml) for chromatographic examination.

(ii) Cortexolone

The external standard method was used for quantitation of the transformation products from this substrate. Thus, the freeze-dried cultures were extracted as follows:

(a) 1 x methanol:ethyl acetate 1:1 (40 ml)
(b) 1 x ethyl acetate (30 ml)
(c) 1 x ethyl acetate (20 ml)

In steps (b) and (c) the culture, plus solvent, was heated to the solvent boiling point (ca. 70°C). The combined extracts were evaporated to dryness *in vacuo* at 50°C and taken up in methanol (exactly 10 ml) for chromatographic examination.

3.6.2 Solvent Extraction

For later work with the *Pellicularia* spp. (*P. filamentosa* f.sp. *microsclerotia* mainly), involving the hydroxylation of cortexolone, the above freeze-drying procedure was unnecessary. In this case, fermentation samples (ca. 20 ml), or whole shake-flask contents, were extracted as follows. After addition of 11α-hydroxyprogesterone (from 1.0 to 5.0 mg were normally used) as internal standard, the samples were filtered to remove mycelium, and the broth was extracted twice with ethyl acetate (30 ml). The mycelium was washed with ethanol (10 ml) and ethyl acetate (10 ml). The combined extracts were washed with 2.5% ($^{w}/v$) sodium hydrogen carbonate solution (40 ml) and water (20 ml) prior to drying over anhydrous sodium sulphate. After filtering, the solvent was removed at 50°C *in vacuo* and the residue dissolved in methanol (10 ml), prior to chromatographic analysis.

3.7 CHEMICAL METHODS USED FOR COMPOUND IDENTIFICATION

To assist in the identification of steroid transformation products, small scale chemical reactions were performed either on small amounts of preparatively recovered compounds, or on samples of crude culture extracts.

3.7.1 Acetylation

Steroid (typically less than 1 mg) in a ground glass stoppered tube was mixed with pyridine and acetic anhydride 1:1 (0.5 - 1.0 ml of each), under nitrogen, at room temperature, and left overnight. Water (2 ml) and ethyl acetate (5 ml) were then added and the tube shaken vigorously. The ethyl acetate layer was then removed and the solvent evaporated, *in vacuo* at 50°C. The residue was then, either spotted directly onto thin layer chromatography plates or dissolved in methanol (1 ml) for hplc analysis.

3.7.2 Chromium Trioxide Oxidation

Oxidation was performed with chromium trioxide under acidic or basic conditions. Under acidic conditions, the sample steroid (<1 mg), dissolved in acetone (5 ml), was mixed with Jones reagent (Fieser and Fieser, 1967a) and shaken for 1 hour at room temperature. Water (15 ml) was then added and the solution was extracted with chloroform (2 x 10 ml); the chloroform layer was washed with water (10 ml) and dried over anhydrous sodium sulphate. After solvent removal, by evaporation *in vacuo* at 50°C, the residue was ready for chromatographic examination.

Under basic conditions, the steroid sample was dissolved in Cornforth reagent (2 ml) (Cornforth *et al.*, 1962) and left to stand at room temperature for 2 hours. After addition of water (10 ml), the steroid was extracted with chloroform (2 x 10 ml); the chloroform layer was dried over anhydrous sodium sulphate prior to evaporation *in vacuo* (50°C) and chromatography, as above.

3.7.3 Dehydration of 16α-hydroxyprogesterone (Cole and Julian, 1954)

A steroid sample (in a 10 ml ground glass flask) was refluxed for 30 minutes in acetone (5 ml) containing 10% ($^{w}/v$) hydrochloric acid (0.1 ml). After cooling, water (15 ml) was added and the solution was extracted with chloroform (2 x 10 ml). The chloroform layer was washed with water (10 ml) and dried over anhydrous sodium sulphate, prior to evaporation of the solvent *in vacuo* at 50°C. The sample was then ready for chromatographic examination.

3.7.4 Sodium Bismuthate Oxidation

The dihydroxyacetonyl sidechain of corticosteroids can be easily and selectively eliminated by oxidation with sodium bismuthate to give 17-ketonic steroids (Brooks and Norymberski, 1953). The steroid sample (<1 mg) was dissolved in aqueous 50% ($^{v}/v$) acetic acid (2.0 ml) and sodium bismuthate (25 mg) added. The mixture was shaken in the dark for 30 minutes, at room temperature. The suspension was then centrifuged and the supernatant liquid removed and diluted with 3% ($^{w}/v$) sodium sulphite (10 ml). This was then extracted with chloroform (2 x 10 ml) and the chloroform layer was washed with 1 M sodium hydroxide (10 ml) and water (10 ml), then dried over anhydrous sodium sulphate prior to solvent evaporation *in vacuo* (50°C).

3.8 CALCULATIONS

3.8.1 Hplc Data Analysis

Quantitation of steroids by hplc was normally accomplished by peak height measurement using an internal standard in the sample and comparison with a parallel standard of known steroid composition.

From the parallel standard a response factor, F_S, was calculated for each component steroid, S.

$$F_S = \left(\frac{IS}{S}\right)_P \Big/ \left(\frac{IS}{S}\right)_W$$

where: $\left(\frac{IS}{S}\right)_P$ is the ratio of internal standard: steroid peak heights

$\left(\frac{IS}{S}\right)_W$ is the weight ratio of the internal standard: steroid, in the standard.

The response factors were used to calculate the amount of steroids in the sample.

$$S_W = IS_W * \left(\frac{S}{IS}\right)_{PX} * F_S$$

where: S_W is the weight of steroid in the sample (mg).

IS_W is the weight of the internal standard in the sample (mg)

$\left(\frac{S}{IS}\right)_{PX}$ is the ratio of steroid: internal standard peak heights from the sample chromatogram.

F_S is the response factor for the steroid.

3.8.2 Dilution Corrections for Fermenter Transformations

With many of the fermentation experiments significant additions to, and removals from, the vessel occurred. Thus, in order to calculate the actual amount of steroid produced, corrections for these effects were required. This was most obvious when hydroxylations with chemostat cultures were performed.

A mass balance on product, p over the fermenter vessel was as follows (where the initial condition $p(t = 0) = 0$ holds):

Actual product in vessel at time = t = The total amount of product formed from time = 0 up to t

- The amount of product removed from t = 0 up to t

This can be written mathematically as:

$$v(t).p(t) = \int_0^t v(t).q(t)\,dt - \int_0^t F(t).p(t)dt \quad -(1)$$

where: v(t) = fermenter volume at time = t (l)
p(t) = product concentration at time = t (mg/l)
q(t) = rate of formation of product at time = t (mg/l.h)
F(t) = rate of removal of liquid from vessel at time = t (l/h)
t = time (h)

For a continuously growing culture, the simplest case applies, where v(t) and F(t) are constants. Thus (1) becomes

$$p(t) = \int_0^t q(t)dt - D\int_0^t p(t)dt \quad -(2)$$

where: $D = \frac{F}{V}$ = dilution rate

The required quantity is the total concentration of product formed from time = o to t, which is the term

$\int_0^t q(t)dt$, which, by rearrangement of (2) is given as:

$$\int_0^t q(t)dt = p(t) + D\int_0^t p(t)dt \quad -(3)$$

The right-hand-side of (3) is readily evaluated from a plot of p(t) versus t, and a numerical determination of the integral term. Since the chemostat is a constant volume system, the removal of samples (provided these are not too large) has no significant effect on the above analysis.

A more complex situation exists with batch and fed-batch cultures, where the functions F(t) and v(t) may be very complex. That is, additions to and removals from the vessel may occur irregularly and the fermenter volume will hence vary with time. Using equation (1) the term,

$\int_0^t v(t).q(t)\ dt$ is the required 'total product produced up to time t'. For volume removal by *periodic sampling*, the term

$\int_0^t F(t).p(t)\ dt$, becomes the cumulative amount of product removed up to time t. This is calculated from equation (4)

$$\int_0^t F(t).p(t)\ dt = \sum_{i=1}^{n} V_i^s \,.\, p(t_i) \qquad -(4)$$

where: V_i^s = sample volume (plus sample tube "dead" volume) of the i^{th} sample.

$p(t_i)$ = product concentration at the time of the i^{th} sample.

n = the number of samples taken prior to time t.

Thus, by recording fermenter volume, sample volumes, and measuring the product concentration for each sample, the total amount of product versus time could be calculated. These quantities were expressed as concentrations, in terms of the fermenter volume at the time of steroid addition.

3.9 DISCUSSION OF METHODS

3.9.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton-NMR spectra of 19-hydroxysteroids have been described by Takahashi (1963a). Table 3.2 gives the important signals in the NMR spectra of cortexolone, 19-hydroxycortexolone, 4-androstene-3,17-dione, and 19-hydroxy-4-androstene-3,17-dione.

Table 3.2 Important signals[a] in the ^{1}H-NMR spectra of various steroids

Compound	Cortexolone	19-Hydroxy-cortexolone	Androstene-dione	19-Hydroxy-androstenedione
Solvent	Pyridine-d_5	Pyridine-d_5	Chloroform-d	Chloroform-d
18-CH_3	0.74	0.80	0.93	0.90
19-CH_3	1.04		1.23	
19-OH		*		2.92
19-CH_2		4.15(q)		3.96(q)
21-CH_2	4.90(q)	5.04(q)		
4-CH	5.78	6.12	5.73	5.87

* assignment could not be made

q refers to quartet

a chemical shifts (δ) are expressed in ppm relative to the internal standard, tetramethylsilane.

The more powerful technique of ^{13}C-NMR has also been applied extensively to steroids and an extensive review has recently been published (Blunt and Stothers, 1977). Further, Holland *et al.* (1978) have reported the spectral data for some C-19-hydroxysteroids. Table 3.3 gives the spectra for some steroids of interest to this work. The effects of different solvents on chemical shifts can be as much as 2 ppm, but are seldom greater (Blunt and Stothers, 1977).

Table 3.3 Chemical shift data* for the ^{13}C-NMR spectra of selected steroids.

Carbon	Progesterone[a]	11α-Hydroxy-progesterone [a]	Cortexolone[b]	11β-Hydroxy-cortexolone [b]	19-Hydroxy-4-androstene-3,17-dione[a]	4-Androstene-3,17-dione [a]
1	35.6	37.4	35.2	34.0	33.6	35.0
2	33.8	34.1	33.4	33.4	35.0	33.4
3	198.5	199.8	197.4	197.7	199.6	197.9
4	123.7	124.2	123.0	121.4	127.3	124.3
5	169.8	171.0	170.4	172.0	165.6	169.7
6	32.6	33.6	31.9	32.7	33.6	32.0
7	31.8	31.6	30.1	31.3	31.9	31.2
8	35.4	34.9	35.2	31.1	36.1	34.9
9	53.5	58.8	53.0	55.5	54.3	54.4
10	38.5	39.9	38.1	38.8	43.9	38.3
11	21.0	68.5	20.3	66.4	21.0	20.7
12	38.5	50.2	33.5	39.0	31.0	30.6
13	43.7	44.1	47.0	46.2	47.7	47.5
14	55.9	55.2	49.9	51.5	51.5	51.2
15	24.2	24.2	23.2	23.3	21.8	22.0
16	22.8	22.9	32.1	32.9	35.8	35.4
17	63.3	63.0	88.4	88.3	220.0	218.7
18	13.2	14.4	14.5	16.8	13.9	13.7
19	17.3	18.3	17.0	20.4	66.1	17.3
20	208.3	208.3	211.3	211.4	-	-
21	31.3	31.2	65.9	65.8	-	-

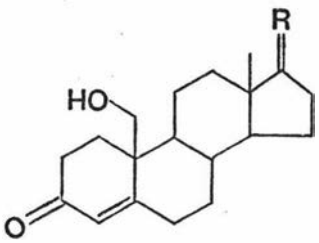
* Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane

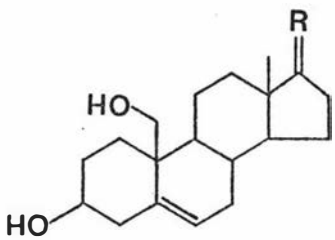
Solvents: [a] chloroform-d

[b] dimethylsulphoxide-d_6

3.9.2 Mass Spectrometry of C-19-Hydroxysteroids

Under electron bombardment, the fragmentation of compounds 1-4 differs markedly from that of hydroxysteroids with a $-CH_2OH$ group in the side-chain at C-17 (Zaretskii, 1976; Eggers, 1965; Kirkien-Konasiewicz *et al.*, 1968).





1 R = H, C_8H_{17} 3 R = H, C_8H_{17}

2 R = O 4 R = O

In the latter case the hydroxymethyl group will be lost as such (31 amu), whereas, in 19-hydroxysteroids, especially those with a Δ^4 or Δ^5 double bond, this group is lost as formaldehyde (30 amu) with back-transfer of a hydrogen atom. With compounds of types 1 and 2 the C-10 hydroxymethyl group is ejected solely as formaldehyde, with the $M-CH_2O$ ion resulting in a base peak in the mass spectra at M-30. The 3,19-dihydroxy analogs (of types 3 and 4) also eliminate a formaldehyde molecule. However, the process is also accompanied by ejection of the entire $19-CH_2OH$ group and the formation of an M-31 ion. In addition, the decomposition of the molecular ions of these dihydroxy compounds is accompanied by intensive dehydration. Ultimately an $M-CH_2O-H_2O$ ion (M-48), whose peak dominates the spectrum, is formed. In most 19-hydroxysteroids, where hydrogen back-transfer cannot occur, the C-10 hydroxymethyl group is ejected as such (31 amu). However, the dominant, base peak is due to an $M-CH_2OH-H_2O$ ion (Heller and Milne, 1978).

The mass spectrum of 2 is markedly different from that of the corresponding 18-hydroxy-4-androstene-3,17-dione

(m/e (%) 302 (100) M^+, 287 (9) M-Me, 284 (10) M-H_2O, and 271 (5) M-CH_2OH), where the dominant peak is due to the molecular ion, and ejection of the C-13 hydroxymethyl group results in only a minor peak at m/e 271 (Auret and Holland, 1971). No elimination of formaldehyde is observed.

Thus the marked elimination of formaldehyde from 19-hydroxysteroids (especially those with the Δ^4-3-one system) is an important diagnostic feature of their mass spectra.

3.9.3 Analytical Methods

Peak height measurement was the normal hplc quantitation technique. Linearity between peak height and the amount of steroid injected was verified for 11α-hydroxyprogesterone, cortexolone, 11β-hydroxycortexolone, and 19-hydroxycortexolone (Fig. 3.13). Chromatography conditions were those given in section 3.4.8., for the cortexolone series. Duplicate injections of the same sample generally gave agreement of analyses to within 1% relative error.

All steroid extraction procedures (section 3.6) could recover appropriate standard steroids, from uninoculated media (0.5 g/l, final concentration), in greater than 90% yield. Analysis of duplicate fermentation samples generally gave agreement to within 2% relative error, although, differences of up to 5% were occasionally obtained.

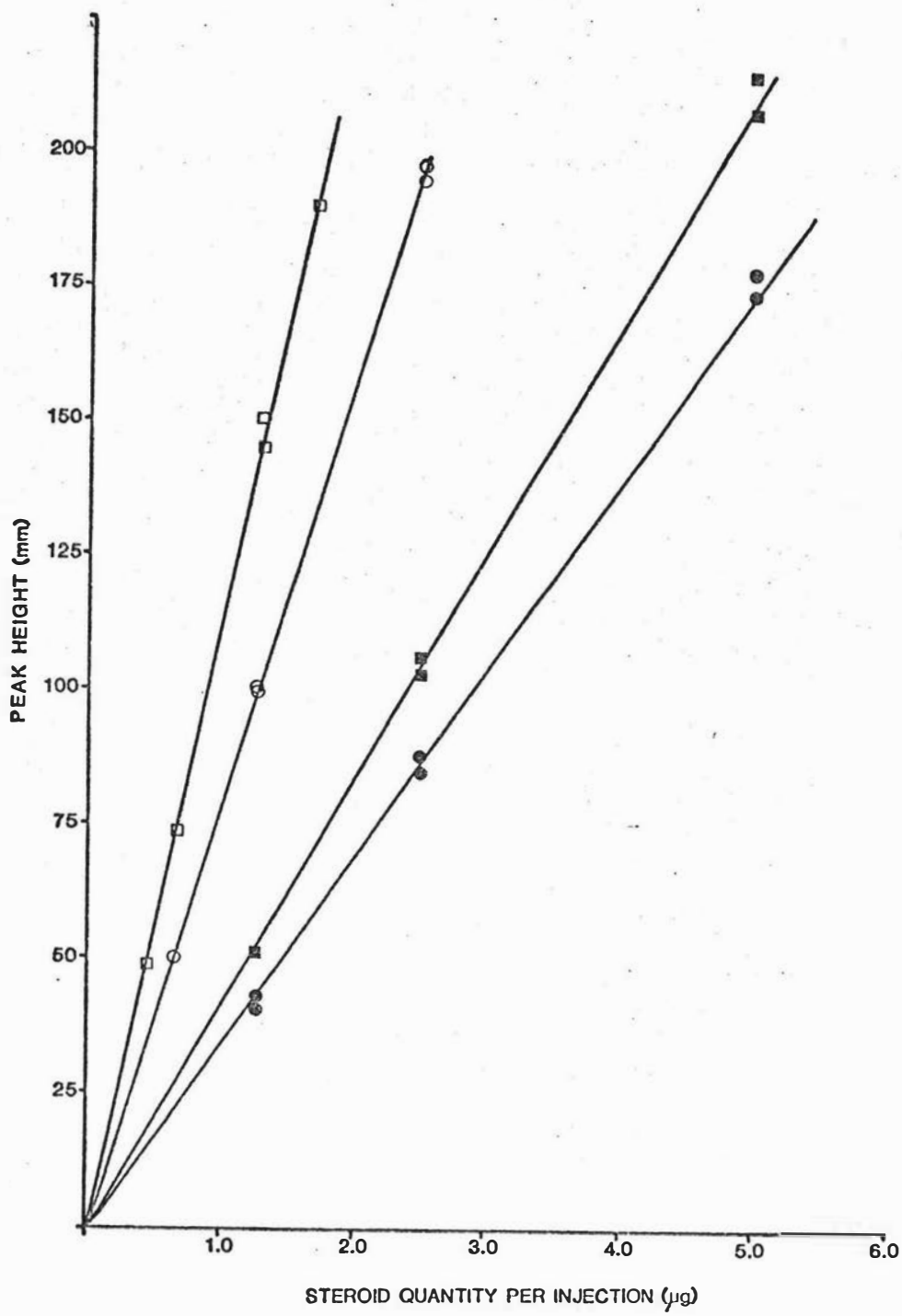


Figure 3.13 Peak heights versus quantity injected, for the hplc analysis of cortexolone (■), 11α-hydroxyprogesterone(●), 11β-hydroxy-cortexolone (○), and 19-hydroxycortexolone (□). (See section 3.4.8 for chromatographic conditions).

CHAPTER 4

EXAMINATION OF SOME STEROID-TRANSFORMING ABILITIES OF THE GENERA *Pestalotia* (*sensu* Guba) AND *Pellicularia* (*sensu* Rodgers)

4.1 INTRODUCTION

As discussed in section 2.2, the 19-hydroxylation of steroids is a rare transformation among microorganisms, and only a very small number of fungi have been implicated in this respect. Clearly, there was a need to obtain fundamental information on this transformation to allow lines of research to be established. This chapter describes a screening programme where a variety of steroids were incubated with selected fungi and the products were examined.

The fungi examined all belong to either of the genera *Pestalotia* (*sensu* Guba) or *Pellicularia* (*sensu* Rodgers). Although *Calonectria decora* and *Nigrospora sphaerica* have also been reported to perform steroidal 19-hydroxylation, these organisms were not examined. In the case of the former organism, the transformation is very substrate specific and is of little practical value (Chambers *et al.*, 1975). The very recent report concerning *Nigrospora sphaerica* (Petzoldt, 1980) was published subsequent to this study.

4.2 TAXONOMY OF THE GENERA *Pellicularia* AND *Pestalotia*

4.2.1 *Pellicularia* (*sensu* Rodgers)

The generic names *Hypochnus*, *Corticium*, and *Pellicularia* have all been used somewhat synonymously to name members of the group *Pellicularia* (*sensu* Rodgers). These fungi all belong to the family Thelephoraceae, order Agaricales, and class Basidiomycetes. Prior to 1943 the name *Corticium* had generally been accepted as the name for

this group. Rodgers (1943), in a critical discussion of the genus *Pellicularia* described its synonymy with certain *Hypochnus* and *Corticium* species. *Rhizoctonia solani* and *R. microsclerotia* were also described as the imperfect forms of *P. filamentosa*.

In an attempt to clarify the situation, Exner (1953) compared four organisms (viz. *C. microsclerotia*, *C. sasakii*, *C. solani*, and an undescribed *Corticium* sp.) and proposed four forms, belonging to the species *Pellicularia filamentosa*, to accomodate them:

P. filamentosa (Pat.) Rodgers

1. Forma specialis *solani* (Kuhn)
   (Synonyms: *Hypochnus solani*, *C. vagum* var *solani*, *C. vagum*, *C. solani*, *C. vagum* subsp. *solani* *Botryobasidium solani*)
2. Forma specialis *microsclerotia* (Matz.)
   (Synonyms: *C. microsclerotia*)
3. Forma specialis *sasakii* (Shirai)
   (Synonyms: *H. sasakii*, *C. sasakii*)
4. Forma specialis *timsii*.

As fungi of this group may be found under any of the various names, a knowledge of the synonymy is of considerable value.

4.2.2 *Pestalotia* (*sensu* Guba)

The taxonomy of this group of Deuteromycetes is rather confused. The genus *Pestalotia* was created in 1839 by deNotaris (Guba, 1961) on the basis of the fungus *Pestalotia pezizoides* (deNot.). The main contributors to the taxonomy of the *Pestalotia* are Guba (1961) and Steyaert (1949). Both base their classifications on conidial morphology, specifically, the number and coloration of cells in the conidium and the number of apical setulae.

Guba (1961) recognises the genus *Pestalotia* as comprising three sub-groups:

(i) Sexoculatae - forms with six celled conidia
(ii) Quinqueloculatae - forms with five celled conidia
(iii) Quadriloculatae - forms with four celled conidia

A form which exhibits only one apical setula is considered to belong to the genus *Monochaetia* (*sensu* Guba), which similarly comprises three groups.

Steyaert (1949), in a major revision of these genera has described the genus *Pestalotia* as comprising only the single, six-celled conidial form *P. pezizoides* (deNot). He has proposed two new genera, i.e. *Truncatella* for 4-celled conidial forms and *Pestalotiopsis* for 5-celled forms, to accommodate the other species from Guba's *Pestalotia* genus. Furthermore, *Monochaetia* has been eliminated and its forms have been distributed in the section Monosetulatae of the above two new genera. The genera *Truncatella* and *Pestalotiopsis* are divided into additional sections; that is, Bisetulatae, Trisetulatae, and Multisetulatae, to describe the number of apiculate setulae.

The controversy over the respective classifications of Guba and Steyaert has not been resolved and organisms may be found deposited in culture collections under any of these names. The significance of this taxonomic confusion from the viewpoint of selecting likely 19-hydroxylating organisms can be summarised as follows:

(i) the group is large and diverse;
(ii) there is no indication of possible relationships between the morphological taxonomy and the physiological abilities with respect to steroid transformation;
(iii) in comparison to the *Pellicularia* group, about which the literature is reasonably specific in describing 19-hydroxylating species, there are no specific references to named *Pestalotia* species with respect to this hydroxylation.

4.3 EXPERIMENTAL METHOD

The recognition of an organism capable of performing the hydroxylation of steroids at C-19 was the main aim of this section of work. A large number of organisms were examined, the majority being *Pestalotia* species (*sensu* Guba), since this group is described poorly in the literature. Three steroid substrates were used since the hydroxylating ability was recognised as possibly being substrate specific. These substrates were progesterone, 4-androstene-3,17-dione, and cortexolone.

Initial compound identification was based on retention time on hplc. In this way, any compound with the same retention time as the 19-hydroxylated standard could be recognised for further identification. This was most readily carried out by mass spectrometry of the preparatively recovered compound (only 1-10 µg was required). A convenient feature of the 19-hydroxysteroids is that all give highly characteristic mass spectra due to fragmentation of the C-10 hydroxymethyl group. The mass spectral characteristics of these compounds have already been described (section 3.9.2).

The identification, where possible, of other hydroxylated products was considered of value, although not of primary concern. Only mono-hydroxylated compounds were considered and only compounds possessing the Δ^4-3-one group were detected by the analytical methods used.

All experiments were performed in shake-flask culture as described in section 3.5.1. Steroid substrate was added at a concentration of 0.5 g/l (25 mg per 50 ml culture). The analyses were performed quantitatively by use of either the internal or external standard method (see section 3.6.1). Where an internal standard was included prior to extraction an additional, replicate culture extract was qualitatively examined by hplc to ensure that the internal standard did not obscure any transformation products (refer to section 3.5.1).

4.4 RESULTS AND DISCUSSION

The results for this section can conveniently be divided into those for each genus/substrate combination.

4.4.1 The Transformation of Progesterone by *Pestalotia* species

Progesterone was incubated with the fungal cultures for both 8 and 16 hours. The quantitative results are presented in Table 4.1, as milligrams of steroid detected at these two times. The initial identification, based on hplc retention time, was verified by other means (*vide infra*).

19-Hydroxyprogesterone was not detected in any of the extracts, thus establishing that these organisms cannot 19-hydroxylate progesterone. 11α-Hydroxylation was characteristic of almost all the fungi and, in some cases, greater than 90% yield of 11α-hydroxyprogesterone was obtained. To verify the initial hplc assignment of structure, a number of fungal extracts were examined by tlc (solvent systems 2 and 3; section 3.4.7.) where, in all cases, the putative compound had the same R_f value as authentic 11α-hydroxyprogesterone. The extracts from *P. funerea* IFO 5427; *Pestalotia* sp. PDDCC 3062, and *P. gracilis* IMI 89307 were subjected to oxidation with Jones reagent (section 3.7.2) and all gave products with same R_f value on tlc (solvent systems 2 and 3) as authentic 11-ketoprogesterone. The hydroxylation of progesterone was repeated on a larger scale with *Pestalotia* sp. PDDCC 3062 and the hydroxylated product was recovered by solvent extraction, and isolated by preparative tlc (solvent system 1). The compound, when recrystallised from dichloromethane:methanol, (24 mg) gave m.p. 162-6°C (cf. 166-7°C, Murray and Peterson, 1952) and its IR spectrum (KBr) was identical to that of authentic 11α-hydroxyprogesterone (Appendix B, Fig. B5). The compound gave a ^{13}C-NMR spectrum (Appendix B, Table B3), which, by comparison with those for progesterone and 11α-hydroxyprogesterone (section 3.9.1), unequivocally showed that C-11 had been hydroxylated.

Table 4.1 The action of *Pestalotia* species on progesterone[a]

Incubation Time (h)	8				16			
Steroid[b] / Organism	11α	16α	19	Pr	11α	16α	19	Pr
P. funerea IFO 5427	1.7	2.7	-	16.7	4.1	1.0	-	1.0
P. versicolor IFO 6319	1.9	-	-	19.8	8.5	-	-	6.8
P. fibricola IFO 6314	2.5	4.7	-	11.7	2.3	3.4	-	11.9
P. microspora ATCC 11816	3.6	-	-	1.3	1.9	-	-	1.6
P. cuboniana IFO 6315	14.6	-	-	4.3	16.1	-	-	1.6
P. microspora IFO 30316	23.4	-	-	1.5	12.6	-	-	1.0
P. karstenii IFO 6316	-	-	-	25.0	-	-	-	25.0
P. diospyri IFO 5282	15.8	-	-	6.6	8.0	-	-	-
Pestalotia sp. PDDCC 7	21.5	-	-	3.5	15.0	-	-	0.5
Pestalotia sp. PDDCC 3062	21.5	-	-	3.0	17.9	-	-	5.3
P. vaccins PDDCC 5446	21.8	-	-	3.0	7.0	-	-	5.1
Pestalotia sp. PDDCC 8	18.5	-	-	4.2	11.5	-	-	1.0
Pestalotia sp. PDDCC 24	17.7	-	-	2.4	9.1	-	-	1.2
P. unicolor WU 144	10.1	-	-	14.0	10.1	-	-	10.3
Pestalotia sp. WU 215	2.7	-	-	22.0	11.5	-	-	13.2
Pestalotia sp. WU 9/77	2.9	-	-	13.1	3.3	-	-	15.3
P. ramulosa WU 145	1.1	-	-	23.0	3.0	-	-	10.0
P. populi-nigrae IMI 110878	22.4	-	-	2.6	15.0	-	-	1.3
P. palmarum IMI 87277	13.3	-	-	4.1	5.0	-	-	2.1
P. sydowiana IMI 82405a	8.1	-	-	16.0	ND	ND	ND	ND
P. gracilis IMI 89307	23.1	-	-	1.0	15.0	-	-	1.4
P. cruenta IMI 90778c	13.0	-	-	2.2	0.7	-	-	-
P. mayumbensis IMI 99418	9.0	-	-	8.5	4.5	-	-	0.7
P. aquatica IMI 87402	-	-	-	20.0	-	-	-	19.0

[a] initial substrate charge was 25 mg/flask

[b] steroid concentrations are given as mg/flask

Abbreviations: 11α = 11α-hydroxyprogesterone
16α = 16α-hydroxyprogesterone
19 = 19-hydroxyprogesterone
Pr = progesterone
ND = not determined

16α-Hydroxyprogesterone was detected in the extracts from *P. funerea* IFO 5427 and *P. fibricola* IFO 6314. Only *P. funerea* has been previously reported to carry out 16α-hydroxylation. To confirm this result the extracts were subjected to conditions under which 16α-hydroxyprogesterone will form the Δ^{16}-ene (section 3.7.3). In both cases, analysis by hplc and tlc (solvent systems 2 and 3) revealed a reaction product with the same retention value as authentic 4,16-pregnadiene-3,20-dione, as well as disappearance of the putative 16α-hydroxy compound. *P. aquatica* IMI 87402 did produce a compound with the same hplc retention time as 16α-hydroxyprogesterone, but which did not undergo dehydration. On the basis of R_f values in tlc systems 1 and 3, the compound was tentatively identified as 15α-hydroxyprogesterone.

4.4.2 The Transformation of 4-Androstene-3,17-dione by *Pestalotia* Species

The experimental procedure used with this substrate was identical to that used for progesterone. The quantitative results, at 8 and 16 h incubation times, are presented in Table 4.2. No organism was found to produce 19-hydroxy-4-androstene-3,17-dione, thus establishing that the *Pestalotia* spp. examined cannot 19-hydroxylate 4-androstene-3,17-dione.

The main product of hydroxylation was 11α-hydroxy-androstenedione, analogous with the results for progesterone. Evidence to substantiate the identity was obtained by tlc (solvent systems 2 and 3) of a number of culture extracts. For two of the more pronounced 11α-hydroxylating cultures (*P. gracilis* IMI 89307 and *P. microspora* IFO 30316), the suspected 11α-hydroxy compounds were recovered by preparative tlc (system 1). These were subjected to oxidation with Jones reagent (section 3.7.2) and the products were examined by tlc (solvent systems 2 and 3). In both cases the products had the same R_f as authentic 11-ketoandrostenedione. By analogy with the behaviour of these fungi towards progesterone, it is not surprising that 11α-hydroxylation was again the predominant transformation. Overall recoveries of steroid, however, were lower than with

Table 4.2 The action of *Pestalotia* species on androstenedione[a]

Incubation Time (h)	8			16		
Steroid[b] / Organism	11α	19	AD	11α	19	AD
P. funerea IFO 5427	-	-	25.0	-	-	23.0
P. versicolor IFO 6319	1.2	-	22.8	1.3	-	6.5
*P. fibricola*IFO 6314	-	-	23.5	-	-	-
P. truncata IFO 8584	0.6	-	15.9	1.1	-	11.8
P. cuboniana IFO 6315	-	-	24.0	-	-	23.6
P. microspora IFO 30316	5.6	-	12.7	18.4	-	5.2
P. karstenii IFO 6316	-	-	10.7	2.6	-	-
Pestalotia sp. PDDCC 7	7.9	-	-	6.4	-	-
Pestalotia sp. PDDCC 3062	-	-	8.9	-	-	-
P. vaccins PDDCC 5446	6.5	-	2.9	4.3	-	-
Pestalotia sp. PDDCC 8	9.7	-	1.0	7.2	-	-
Pestalotia sp. PDDCC 24	9.5	-	-	8.1	-	-
Pestalotia sp. WU 215	-	-	15.4	-	-	0.7
P. unicolor WU 144	0.2	-	15.0	1.7	-	13.4
Pestalotia sp. WU 9/77	-	-	16.8	2.0	-	4.7
P. populi-nigrae IMI 110878	10.1	-	11.5	6.1	-	0.6
P. palmarum IMI 87277	6.8	-	18.3	0.6	-	-
P. sydowiana IMI 82405a	5.5	-	19.2	9.5	-	9.1
P. gracilis IMI 89307	11.7	-	9.5	21.5	-	3.6
P. cruenta IMI 90778c	7.6	-	16.0	2.0	-	-
P. mayumbensis IMI 99418	2.8	-	14.0	2.2	-	12.0
P. aquatica IMI 87402	-	-	25.0	-	-	8.8
P. stictica IMI 89306	2.3	-	10.3	10.0	-	-
P. microspora ATCC 11816	12.4	-	0.5	5.0	-	-

[a] initial substrate charge was 25 mg/flask

[b] steroid concentrations are given as mg/flask

Abbreviations: 11α = 11α-hydroxyandrostenedione
19 = 19-hydroxyandrostenedione
AD = androstenedione

progesterone, indicating that the fungi were either metabolising, or further hydroxylating, androstenedione to undetected compounds. In some cases, small amounts of other compounds were detected by hplc. For example, both *P. funerea* IFO 5427 and *P. fibricola* IFO 6314 produced compounds with a retention time slightly greater than that of 11α-hydroxyandrostenedione. Since both fungi can 16α-hydroxylate progesterone these unknown compounds may be the 16α-hydroxy derivative. As authentic 16α-hydroxyandrostenedione was unavailable, no attempt was made at identification.

4.4.3 The Transformation of Cortexolone by *Pestalotia* Species

The transformation of cortexolone was examined with 8 *Pestalotia* species. Quantitation of products, after incubation times of 1 and 2 days, was by the external standard method (section 3.6.1). The hydroxylation pattern was much more complex with this substrate than with either progesterone or androstenedione. Many of the compounds could not be identified but were quantitated by area integration on the assumption that they possessed the same response factor as known hydroxycortexolones (a reasonable assumption, since the extinction values for the Δ^4-3-one group of steroidal compounds are approximately constant). Hence both the known and unknown compounds are reported in Table 4.3 according to their hplc retention times. Putative 11α-hydroxycortexolone was tentatively identified as follows.

Thin layer chromatograms of the extracts from *Pestalotia* sp. PDDCC 8, *P. karstenii* IFO 6316, *Pestolatia* sp. PDDCC 24, *Pestalotia* sp. PDDCC 7, and *Pestalotia* sp. WU 215 were developed in solvent system 2. The suspected 11α-hydroxycortexolone products gave positive Porter/Silber reactions (see section 3.4.7), verifying the presence of the dihydroxyacetonyl side chain. The extracts of *Pestalotia* sp. PDDCC 24, *Pestalotia* sp. PDDCC 8, and *P. karstenii* IFO 6316, were further subjected to sodium bismuthate oxidation

Table 4.3 The action of *Pestalotia* species on cortexolone[b]

Incubation Period	1 day					2 day				
Compound[a] R_t (min)[c]	x 3.50	x 4.50	x 5.41	11α-OH 6.75	Co	x 3.50	x 4.50	x 5.41	11α-OH 6.75	Co
P. karstenii IFO 6316	-	1.0	-	4.1	-	-	0.6	-	3.5	-
P. truncata IFO 8584	-	1.3	1.45	0.81	4.6	-	1.79	1.96	1.15	3.92
P. vaccins PDDCC 5446	0.17	0.28	0.27	0.45	0.87	0.14	0.42	0.36	0.65	0.11
Pestalotia sp. WU 215	-	0.40	-	1.40	1.90	-	0.70	-	2.80	0.13
Pestalotia sp. PDDCC 7	-	-	-	1.90	0.16	-	-	-	1.30	-
Pestalotia sp. PDDCC 24	-	-	-	3.20	-	-	-	-	1.70	-
Pestalotia sp. PDDCC 7	-	-	-	3.50	-	-	-	-	2.40	-
Pestalotia sp. WU 9/77	-	1.42	0.77	1.34	5.17	-	2.28	1.00	1.71	0.50

[a] x = unknown identity
11α-OH = 11α-hydroxycortexolone
Co = cortexolone

[b] steroid concentrations are given as mg/flask; initial substrate charge was 25 mg/flask.

[c] chromatography conditions are as described in section 3.4.8

(section 3.7.4) to give the corresponding androstane compounds. In all cases the products were chromatographically (hplc and tlc (solvent systems 2 and 3)) identical to authentic 11α-hydroxy-4-androstene-3,17-dione.

The 11α-hydroxy product was present in all culture extracts. In some cases (*Pestalotia* sp. PDDCC 8, *P. karstenii* IFO 6316, *Pestalotia* sp. PDDCC 24, *Pestalotia* sp. PDDCC 7, and *Pestalotia* sp. WU 215) this compound was the major or only monohydroxylated product. On the other hand, *Pestalotia* sp. WU 9/77, *P. truncata* IFO 8584, and *P. vaccins* PDDCC 5446 also produced substantial amounts of compounds with hplc retention times less than that of 11α-hydroxy-cortexolone. One of these unknowns had a retention time (R_t = 4.50 min) very similar to that of 19-hydroxycortexolone. Extracts containing this compound were subjected to sodium bismuthate oxidation. Hplc comparison of the 17-ketonic degradation product with authentic 19-hydroxyandrostenedione revealed that the suspected compound was not, in fact, 19-hydroxycortexolone. Thus, despite the report by Takahashi (1963b), that certain *Pestalotia* species can 19-hydroxylate cortexolone, and the corresponding patent claim of Hasegawa *et al.* (1963), none of the fungi, here examined, produced 19-hydroxycortexolone in detectable quantities. It is also apparent from Table 4.3 that a very low recovery of the original steroid (25 mg/flask) was obtained. Apparently, the substrate and/or products are degraded by these fungi to unidentified compounds.

4.4.4 The Transformation of Cortexolone by *Pellicularia* Species

The steroid hydroxylating abilities of this group of fungi have been studied quite extensively (Charney and Herzog, 1967). Only *P. filamentosa* f.sp. *microsclerotia* IFO 6298 and *P. filamentosa* f.sp. *sasakii* IFO 5254 have been shown to 19-hydroxylate cortexolone. Hence, these two fungi plus three other representatives of the genus (*Corticium practicola* IFO 6253, *C. caeruleum* IFO 4974, and *P. filamentosa*

f.sp. *solani* IFO 5289) were examined. The experimental procedures were as previously described (section 3.5.1) and incubation of cortexolone with the cultures was for 5 days.

4.4.4.1 *P. filamentosa* f.sp. *microsclerotia* IFO 6298

Authentic 19-hydroxycortexolone was obtained from cultures of this organism which had been incubated with cortexolone (section 3.1.4.1). Hence, initial identification of this product was performed without authentic material being available. The culture extract was initially oxidised with sodium bismuthate (section 3.7.4) to give the corresponding androstane compounds, which were then compared with authentic compounds by hplc and tlc (solvent systems 2 and 3). This allowed a tentative identification of the transformation products as 19-, 11β-, and 11α-hydroxycortexolone, plus one unknown compound. This is in agreement with Takahashi and Hasegawa (1961b) who showed the fourth compound to be 6β-hydroxycortexolone. The major transformation products were 19- and 11β-hydroxycortexolone. The other two compounds were estimated as accounting for less than 15% of the total products and were not further characterised.

Putative 19-hydroxyandrostenedione was recovered by preparative tlc (solvent system 1) and a mass spectrum was obtained (Appendix B, Figure B4). The spectrum was identical to that published for 19-hydroxy-4-androstene-3,17-dione (Eggers, 1965), and the elimination of 30 amu as formaldehyde, to give the base peak at m/e 272, is unequivocal evidence for the presence of the 19-hydroxyl group in this compound. The original cortexolone transformation extract was also examined by tlc (solvent system 3) and the developed chromatogram was sprayed with Porter / Silber reagent, to which all the compounds gave a positive reaction. This indicated the presence of the dihydroxyacetonyl side chain (21-aldo-20-keto- and 21-hydroxy-20-oxo-16-dehydrosteroids will also give a positive reaction (Lisboa, 1969)). Finally, a mass spectrum of putative 19-hydroxycortexolone was obtained (Appendix B, Figure B3).

The presence of the molecular ion at m/e 362 verifies the expected molecular weight. The disintegration pattern can be compared to that obtained for cortexolone (Appendix B, Figure B6). Major spectral peaks at m/e M-30, M-59, and M-60 are common to both spectra and probably represent side chain eliminations. The loss of 59 amu from the molecular ion, giving rise to the peaks at m/e 287 and 303 in the spectra of cortexolone and 19-hydroxycortexolone, respectively, almost certainly are due to loss of the C_{20}-C_{21} side chain fragment as $CH_2(OH)\dot{C}O$. In the spectrum of 19-hydroxycortexolone the base peak at m/e 272 (a further 30 amu down from m/e 302) presumably represents the m/e 302 ion minus the C-19-hydroxymethyl group, which was lost as formaldehyde, with back transfer of a hydrogen atom. Both ^1H and ^{13}C-NMR spectra were obtained for this compound to provide further proof of identity (Appendix B, Table B2). These data can be compared with the spectral information given for cortexolone and 19-hydroxycortexolone in Tables 3.2 and 3.3. In the ^1H-NMR spectrum, the disappearance of the 19-CH_3 singlet (one of the dominant signals in the ^1H-NMR spectra of steroids) and the appearance of the 19-CH_2 quartet is proof that hydroxylation has indeed taken place at C-19. The ^{13}C-NMR spectrum also clearly demonstrates the downfield shift of C-19 from ca. 17.5 (in cortexolone) to 65.3, again proving that it has been hydroxylated.

Identity of the putative 11β-hydroxycortexolone (hydrocortisone) from *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was confirmed by its hplc and tlc (solvent systems 2 and 3) behaviour, which was identical to that of authentic material. Furthermore, oxidation with sodium bismuthate gave a compound which behaved identically to 11β-hydroxy-4-androstene-3,17-dione on hplc and tlc (solvent systems 2 and 3).

4.4.4.2 *P. filamentosa* f.sp. *sasakii* IFO 5254

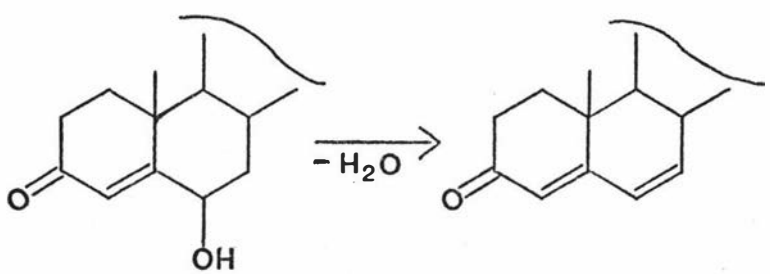
Since authentic 19-hydroxycortexolone had been obtained from *P. filamentosa* f.sp. *microsclerotia* (section 3.1.4.1), initial identification of the products of incubation of *P. filamentosa* f.sp. *sasakii* IFO 5254 with cortexolone was by examination of retention times on hplc. Examination by tlc (solvent systems 2 and 3) further verified the initial identification. There were again, apparently, four transformation products (from hplc retention-time data, the same four products as were obtained with *P. filamentosa* f.sp. *microsclerotia* IFO 6298). The two main products were apparently 11β-hydroxy- and 19-hydroxycortexolone. The minor products accounted for approximately 25% of the total hydroxylated material. Putative 11β-hydroxy- and 19-hydroxycortexolone were recovered from a fermenter-culture of *P. filamentosa* f.sp. *sasakii* IFO 5254 in which cortexolone was incubated (see section 5.2 for details). Solvent extraction of steroidal material was the same as described in section 3.1.4.1 for *P. filamentosa* f.sp. *microsclerotia* The compounds were separated and purified by preparative hplc (methanol:water, 65:35; 2.0 ml/min; other conditions as described in section 3.4.8.) of the ethyl acetate extract. The major product, 11β-hydroxycortexolone, was obtained as slightly yellow crystals (15 mg) from methanol:water, m.p. 200-210^{o} (cf. 204-207^{o}, Takahashi and Hasegawa, 1961b) and its IR spectrum was identical to that of authentic 11β-hydroxycortexolone (Appendix B, Figure B7). Putative 19-hydroxycortexolone was identified by its mass spectrum (Appendix B, Figure B8), which was identical to that of authentic material (ex. *P. filamentosa* f.sp. *microsclerotia*). This characteristic was sufficient to unequivocally identify the compound as 19-hydroxycortexolone.

4.4.4.3 *Corticium practicola* IFO 6253

The products of incubation of cortexolone with this organism were, again, two major and two minor monohydroxylated cortexolone derivatives (based on hplc examination). The major products were neither 11β-hydroxy- nor 19-hydroxy-

cortexolone. The compounds were recovered from a fermenter-culture of *C. practicola* IFO 6253 in which cortexolone was incubated (see section 5.2 for details). Solvent extraction and preparative hplc procedures were as with *P. filamentosa* f.sp. *sasakii* IFO 5254. The compound present in the highest concentration was isolated as an impure gum. The material showed the same chromatographic behaviour (retention time and R_f in hplc and tlc (solvent systems 2 and 3), respectively) as 11α-hydroxycortexolone, obtained from the incubation of cortexolone with certain *Pestalotia* species (section 4.4.3). Sodium bismuthate oxidation of the material gave a compound chromatographically identical (hplc and tlc in solvent systems 2 and 3) to authentic 11α-hydroxy-4-androstene-3,17-dione. Thus, the compound was tentatively identified as 11α-hydroxycortexolone. The second main product was crystallised from methanol to give crystals (30 mg) which were homogeneous by tlc; m.p. 234-8°C, λ_{max}^{MeOH} 233 nm. The mass spectrum (Appendix B, Figure B9) added little in characterising the compound. However, the molecular ion at m/e 362 and subsequent M-18 (m/e 344), M-30 (m/e 232), M-59 (m/e 303), and M-60 (m/e 302) peaks do give some information. The molecular weight verifies the product as a monohydroxy derivative of cortexolone. In the mass spectrum of cortexolone (Appendix B, Figure B6) the pattern of peaks above m/e 280, viz M^+ (m/e 346), M-18 (m/e 328), M-30 (m/e 316), M-59 (m/e 287), and M-60 (m/e 286), show a marked similarity to those in the mass spectrum of the unknown, and so verify the parent compound as cortexolone. The observation of significant metastable peaks at m/e 268 and 273 would support the contention that the peak at m/e 287 is due to the loss of $\dot{C}H_3$ from the ion giving rise to the peak at m/e 302, while the m/e 285 peak is due to loss of H_2O from the ion giving rise to the peak at m/e 303. This loss of H_2O to give the base peak at m/e 285 is almost certainly due to the elimination of the unknown hydroxyl group. Thus, this group must be in a position where loss of water is a highly favourable process.

The ^{13}C-NMR spectrum for the compound (Appendix B, Table B4) gave further information. The peak at δ= 70.94 is due to the hydroxylated carbon (by comparison with the spectrum of cortexolone, Section 3.9.1, Table 3.3). However, the designation of this carbon could only be initially reduced to one of seven possibilities. This was for two reasons; firstly the solvent (DMSO) peaks obscured the peaks of C-1, C-8 and C-10 whilst those of C-2 and C-12 as well as C-6 and C-16 cannot be resolved from each other (see the spectrum for cortexolone). The C-10 position can be immediately eliminated as a possibility. Also, on the basis of available melting point data (Charney and Herzog, 1967) it is possible to eliminate the following compounds: 1β-, 2β-, 7α-, 7β-, 8β-, and 12β-hydroxycortexolone. Thus, the possibilities are considerably narrowed. On the basis of the above information it was deduced that the compound is probably 6β-hydroxycortexolone; cf. m.p. 233-234^{o} uncorr., λ_{max}^{EtOH} 236 nm (Takahashi and Hasegawa, 1961a). In particular, the shift to lower wavelength, of the absorption maximum, is characteristic of steroids with the 6β-hydroxy-Δ^4-3-one grouping (Takahashi and Hasegawa, 1961a). The normal absorption maximum for the Δ^4-3-one group in steroids is 241 nm. Furthermore, the elimination of the 6β-hydroxyl group as water in the mass spectrometer would be energetically favoured, as it extends conjugation in the molecule, i.e.,



This is consistent with the observed spectrum.

4.4.4.4 *Corticium caeruleum* IFO 4974 and *P. filamentosa* f.sp. *solani* IFO 5289.

19-Hydroxycortexolone was not detected in the culture extracts of these two fungi after incubation with cortexolone In fact, *P. filamentosa* f.sp. *solani* IFO 5289 gave no detectable products while *C. caeruleum* IFO 4974 gave a single product which displayed the same chromatographic behaviour as 11β-hydroxycortexolone (hplc and tlc in solvent systems 2 and 3). No further investigations were carried out.

The results from this section are summarised in Table 4.4.

4.4.5 The Transformation of 4-Androstene-3,17-dione and Progesterone by *P. filamentosa* f.sp. *microsclerotia* IFO 6298 and *sasakii* IFO 5254

Hasegawa *et al.* (1960) claimed in their patent that *C. microsclerotia* IFO 6298 can 19-hydroxylate both progesterone and androstenedione. The fungi *P. filamentosa* f.sp. *microsclerotia* and *sasakii*, which can 19-hydroxylate cortexolone, were thus examined with these two substrates in order to verify the claim.

Both organisms transformed progesterone into, apparently, polyhydroxylated products. Thus after only 8 h incubation no monohydroxy products could be detected. To determine if monohydroxy products did appear transiently, early in the course of the transformation, a culture of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was incubated with progesterone for 3 hours. Even at this early stage, with most of the progesterone unconverted, most of the products were much more polar (based on hplc and tlc analyses) than typical monohydroxyprogesterones. However, very low levels of compounds with similar retention times to monohydroxy standards (by hplc) were detected, indicating their presence, albeit transiently. Identities could not be assigned to these compounds. The exact conditions described by Hasegawa *et al.* (1960), for the 19-hydroxylation of progesterone, were also

Table 4.4 The action of various *Pellicularia* (*sensu* Rodgers) species on cortexolone.

Organism	Steroid Concentration [a] (mg/l) 6β	11α	11β	19
P. filamentosa f.sp.				
microsclerotia			58	70
sasakii			47	15
solani	-	-	-	-
Corticium practicola	30	93	-	-
Corticium caeruleum	-	-	40	-

[a] after 5 days incubation with cortexolone (500 mg/l initial concentration)

Abbreviations: 6β = 6β-hydroxycortexolone
11∝ = 11∝ -hydroxycortexolone
11β = 11βhydroxycortexolone
19 = 19-hydroxycortexolone

examined with *P. filamentosa* f.sp. *microsclerotia* IFO 6298. However, similar results to those obtained using the standard screening procedure, were obtained. Most importantly, no 19-hydroxyprogesterone was detected.

When incubated with 4-androstene-3,17-dione for 8 h, both *P. filamentosa* f.sp. *microsclerotia* and *sasakii* appeared to produce the same compounds, but in different relative amounts. Thus, Figure 4.1 shows the hplc chromatograms for the culture extracts of these organisms. By comparison with the standard mixture it is clear that 19-hydroxyandrostenedione was not among the products from either organism. Exact duplication of the conditions for 19-hydroxylation of androstenedione, as described by Hasegawa *et al.* (1960), were performed with *P. filamentosa* f.sp. *microsclerotia* IFO 6298. However, the results were little changed and, again, 19-hydroxyandrostenedione was not detected. Some tentative identification of the compounds obtained from the incubation of androstenedione with these fungi (Fig. 4.1) is possible. Thus, compounds 3 and 4 exhibit the same respective retention times, on hplc, as 11α- and 11β-hydroxyandrostenedione. Tlc (solvent system 2) further verified this conclusion. 11β-Hydroxylation of various 4-androstene-3,17-dione derivatives has already been reported for this organism (Takahashi, 1964). Compound 2 (Fig. 4.1), the major product of *P. filamentosa* f.sp. *microsclerotia*, was recovered for further investigation. Oxidation and acetylation (see section 3.7) of the compound were unsuccessful, suggesting the presence of a tertiary hydroxyl group. Also, based on hplc retention time, the compound was more polar than the standard monohydroxyandrostenediones. The mass spectrum of the compound (Appendix B, Figure B10) exhibited a high intensity molecular ion at m/e 316. This is consistent with an androstenedione derivative bearing one hydroxyl and a ketone group, or two hydroxyl groups and a double bond. As dehydrogenation of the steroid ring system is not a characteristic of this organism (Charney and Herzog, 1967), the former possibility seems more probable. Takahashi (1964) obtained 14α-hydroxy-1,4-androstadiene-3,11,17-trione as the

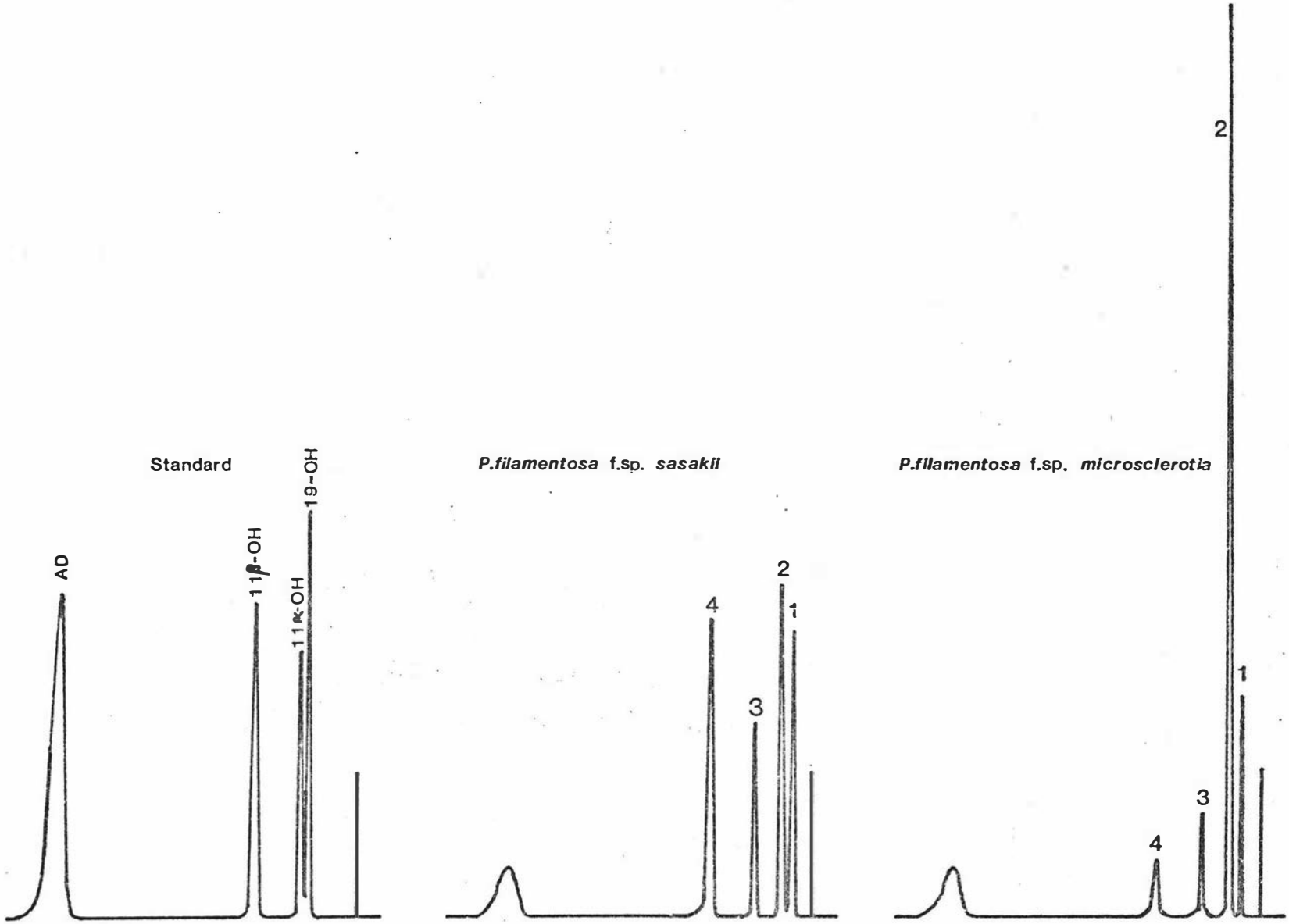


Figure 4.1 Hplc chromatographs of the culture extracts of the indicated fungi (after 8 h incubation with androstenedione) and of a standard mixture containing androstenedione (AD), 11α- (11α-OH), 11β- (11β-OH), and 19- (19-OH) hydroxyandrostenedione. Conditions as described in section 3.4.8.

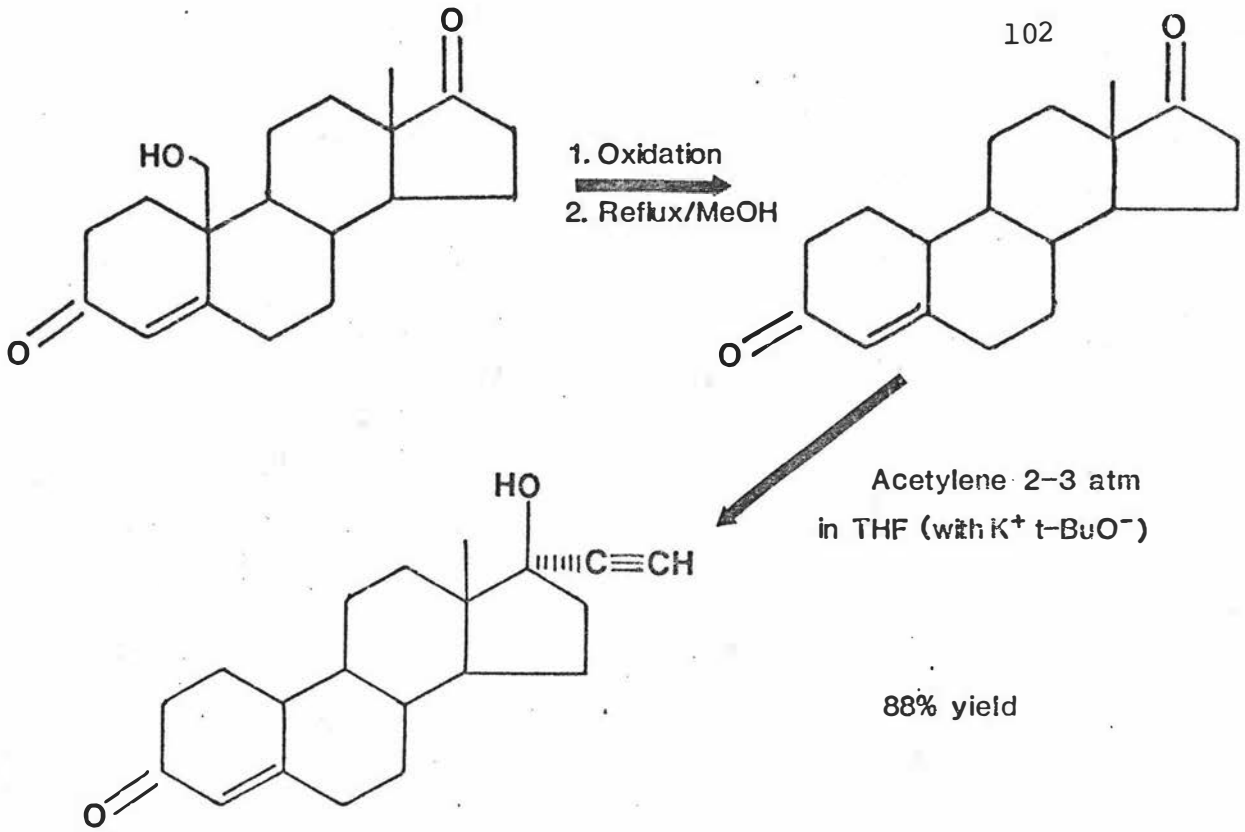
transformation product of 1,4-androstadiene-3,17-dione, with this organism (refer to Table 2.3). The analogous compound, without the Δ^1-double bond, would be consistent with the data available for the unknown product. As discussed in section 2.2, the presence or absence of the Δ^1-double bond seems to be of little consequence in terms of the transformation products of *P. filamentosa* f.sp. *microsclerotia* IFO 6298. Thus the compound was tentatively identified as 14α-hydroxy-4-androstene-3,11,17-trione.

4.5 CONCLUSIONS

The hydroxylation abilities of a large number of *Pestalotia* species and some *Pellicularia* species have been examined under standardised conditions. The steroid substrates androstenedione, progesterone, and cortexolone were used in the study. The reported abilities of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 and *sasakii* IFO 5254 to 19-hydroxylate cortexolone have been verified, with the former organism being the more active in this respect. However, of the *Pestalotia* species examined none showed a similar ability, despite claims in the literature. Instead, the *Pestalotia* group of fungi were very active in 11α-hydroxylation of steroids. This finding may be of value as a chemotaxonomic criterion in clarifying the taxonomy of the genera *Pestalotia* (*sensu* Guba), *Monochaetia* (*sensu* Guba), *Pestalotiopsis* (*sensu* Steyaert), and *Truncatella* (*sensu* Steyaert). In this respect, Chong (1982) has examined the progesterone transformation products of several *Monochaetia* and *Truncatella* species, and has observed significant differences in hydroxylation abilities between the genera, suggesting that there may be a biochemical as well as a morphological difference. Notably, 19-hydroxylation of progesterone was not observed with these organisms, as with the *Pestalotia* species studied here.

Interestingly, neither *P. filamentosa* f.sp. *microsclerotia* IFO 6298 nor *sasakii* IFO 5254 were able to 19-hydroxylate either androstenedione or progesterone, the structures of which differ from cortexolone only in the nature of the side chain at C-17. With progesterone, both organisms apparently produced polyhydroxylated products. The identities of these were not determined, however, and it is possible that one of the positions hydroxylated may have been C-19. Nevertheless, in view of the known ability of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 to 19-hydroxylate both 17α,21-dihydroxy-4-pregnene-3,20-dione (cortexolone) and 17α,20α,21-trihydroxy-4-pregnen-3-one, these results are of some significance. Thus, although many fungi are very substrate non-specific in terms of hydroxylation ability (Charney and Herzog, 1967), this is not the case with this organism. Further studies on the effects of substrate structure may be of considerable value in determining possible applications for 19-hydroxylation by this organism in steroid manufacturing processes. The ease with which very small quantitites of 19-hydroxylated steroids may be detected, using mass spectrometry, would greatly assist such studies.

On the basis of the present data some comments on the commercial implications are possible. A valuable product of a 19-hydroxylation process would be 19-hydroxy-4-androstene-3,17-dione. This can be converted to 17α-ethynyl-19-nortestosterone (19-norethisterone) as shown in Figure 4.2. This compound is a valuable anti-fertility drug (Yamada, 1977). Presently, 19-hydroxyandrostenedione is produced by chemical synthesis from 3β-hydroxy-5-androsten-17-one acetate by a route analogous to that shown in Figure 2.1 (Heusler and Kalvoda, 1972). As an alternative to direct 19-hydroxylation of 4-androstene-3,17-dione, which the present results suggest is not feasible, 19-hydroxylation of cortexolone can lead to the same product via a very selective and efficient side chain degradation reaction using sodium bismuthate. This is shown in Figure 4.3. It is necessary, therefore, to consider whether cortexolone would be prohibitively expensive as a starting material in order



17α -ethynyl-19-nortestosterone

Figure 4.2 The synthesis of 17α-ethynyl-19-nortestosterone (19-norethisterone) from 19-hydroxyandrostenedione

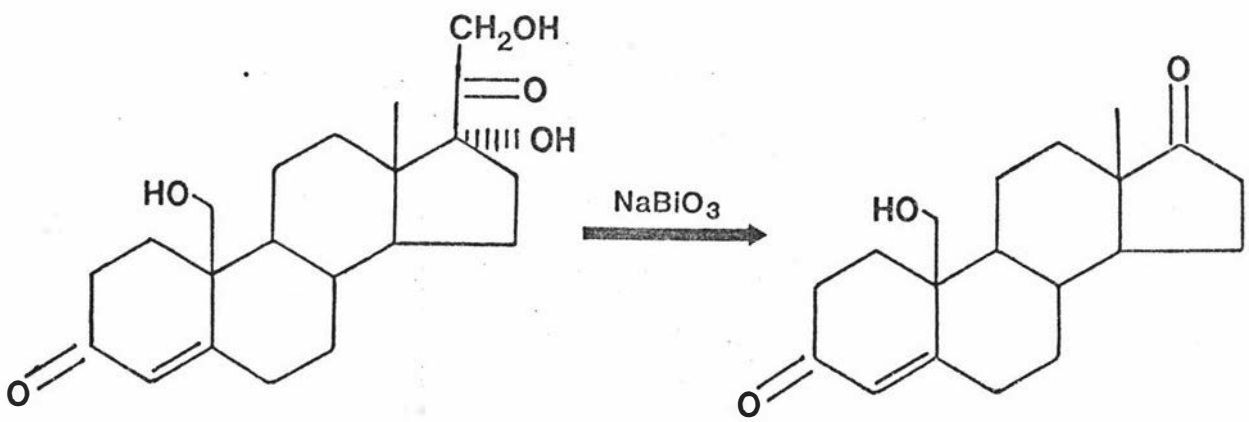


Figure 4.3 The synthesis of 19-hydroxyandrostenedione from 19-hydroxycortexolone.

for such a process to compete with the chemical route. In this respect, the cost of 4-androstene-3,17-dione is a useful basis for comparison.

In comparing the prices of androstenedione and cortexolone as starting materials, it is important to realise that the prices of steroid intermediates, such as these, fluctuate with supply, demand, and quantity. The 1982 market values of androstenedione and cortexolone are approximately $(NZ) 160 and $(NZ) 500 per kilogram, respectively (Kloosterman, 1982). However, cortexolone is apparently no longer a major steroid intermediate (Kloosterman, 1982) and so the above value is much greater than if it were required as a large-scale raw material. Thus, it is conceivable that the microbiological hydroxylation of cortexolone followed by side chain degradation to 19-hydroxyandrostenedione could represent a viable alternative to the chemical route from 3β-hydroxy-5-androsten-17-one acetate, providing that an efficient, high yield microbial hydroxylation process were developed. The prospect of a two product fermentation giving 19-hydroxycortexolone and 11β-hydroxycortexolone (hydrocortisone) could also be feasible, since the latter compound is itself valuable. Experience with culture extracts containing these two compounds has shown that they can be easily separated by crystallisation. Hence, the required separation may not adversely affect the process economics.

This discussion is clearly speculative, and a 19-hydroxylation process based on a lower cost substrate would undoubtedly be more advantageous. In this respect, further investigation into the effects of substrate structure on the 19-hydroxylation of steroids by *P. filamentosa* f.sp. *microsclerotia* may be of great benefit. For example, the possibility of performing 19-hydroxylation on a simple derivative of androstenedione is attractive. However, all further work described in this thesis involves studies on the 11β- and 19-hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia* IFO 6298.

CHAPTER 5

PRELIMINARY STUDIES ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE BY *Pellicularia filamentosa* f.sp. *microsclerotia* IFO 6298

5.1 INTRODUCTION

The previous chapter identified a 19-hydroxylation system suitable for further study. However, preliminary investigations were still required to establish some basic knowledge of the transformation prior to defining specific lines of research. Thus, important parameters such as the product yield and the ratio of 19- to 11β-hydroxy-cortexolone were investigated with respect to their sensitivity to various fermentation variables. Experiments were also designed to gain some insight into the nature of the hydroxylase system of the organism.

5.2 THE TIME COURSE OF CORTEXOLONE HYDROXYLATION IN FERMENTER CULTURE

5.2.1 Introduction

The first experiment was the consideration of how cortexolone hydroxylation proceeds with time. Fermentations were carried out, not only with *P. filamentosa* f.sp. *microsclerotia*, but also with *P. filamentosa* f.sp. *sasakii* IFO 5254 and *Corticium practicola* IFO 6253 to provide comparisons.

5.2.2 Results and Discussion

The fermentations were performed in fermenter culture using a medium of glucose (25 g/l) and yeast extract (10 g/l) at a constant pH of 6.0. Cortexolone was added only when satisfactory growth had been obtained. The time required for this depended on the growth rate of the organism, and hence differed for the three fungi. For *P. filamentosa* f.sp. *microsclerotia* and *C. practicola*, cortexolone (ca. 500 mg/l,

final concentration) dissolved in dimethylformamide (10 g/l, final concentration) was added 24 hours after inoculation. For *P. filamentosa* f.sp. *sasakii*, addition (as above), was made 4 days after inoculation. Dissolved oxygen tension (DOT) was not monitored but agitation and aeration conditions were held constant at 600 rpm and 600 ml/min, respectively. After steroid addition, samples were removed periodically over the subsequent 50 hours. The time courses for the three fermentations are shown in Figures 5.1, 5.2, and 5.3.

A common feature in all cases was the rapid disappearance of cortexolone during the first 5 hours after steroid addition. Almost all of the steroid losses throughout the fermentation could be accounted for in this initial period. This is almost identical to the behaviour of *Curvularia lunata* cultures, when hydroxylating cortexolone (Dulaney and Stapley, 1959). In early work on this 11β-hydroxylation process, yields of 35-40% ($^{w}/w$) were typically reported (Shull and Kita, 1955; Dulaney and Stapley, 1959) which are similar to the combined product yields obtained here. A further feature of all three fermentations was that after autoclaving (115^{o}C for 20 minutes) the entire culture at the completion of the experiment, and solvent extraction of the contents (for product recovery), substantially more cortexolone was detected than was indicated by analytical samples. This may simply have been substrate which had sprayed into the head space of the fermenter and hence was unavailable to the culture. However, it may also reflect "bound" substrate which was not extracted in analytical samples, but only after autoclaving of the culture.

For *P. filamentosa* f.sp. *microsclerotia* the final (11β- + 19-hydroxycortexolone) product yield was approximately 40% ($^{w}/w$), and the ratio of the two main products (1.40:1, 19- to 11β-) remained almost constant through the fermentation. A somewhat lower yield (ca. 25% ($^{w}/w$)) was obtained with *P. filamentosa* f.sp. *sasakii*, and the ratio of 19- to 11β-hydroxycortexolone was considerably different (0.28:1, 19- to 11β-). The transformation of cortexolone by *Corticium*

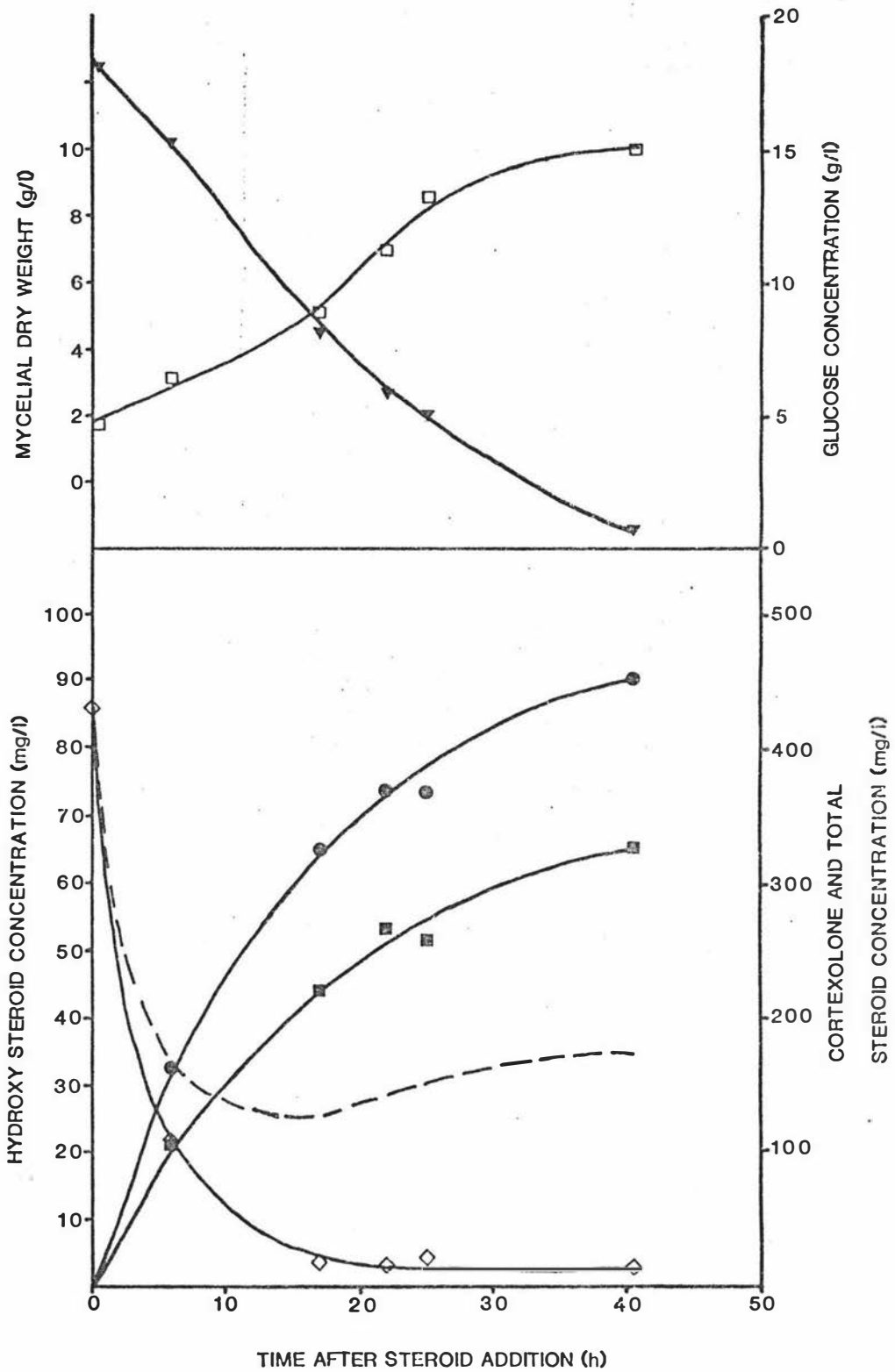


Figure 5.1 The time course for the hydroxylation of cortexolone by a fermenter culture of *P. filamentosa* f.sp. *microsclerotia* IFO 6298. Mycelial dry weight (□), glucose concentration (▼), 19-hydroxy-cortexolone (●), 11ß-hydroxycortexolone (■), cortexolone (◇), total steroid (– –).

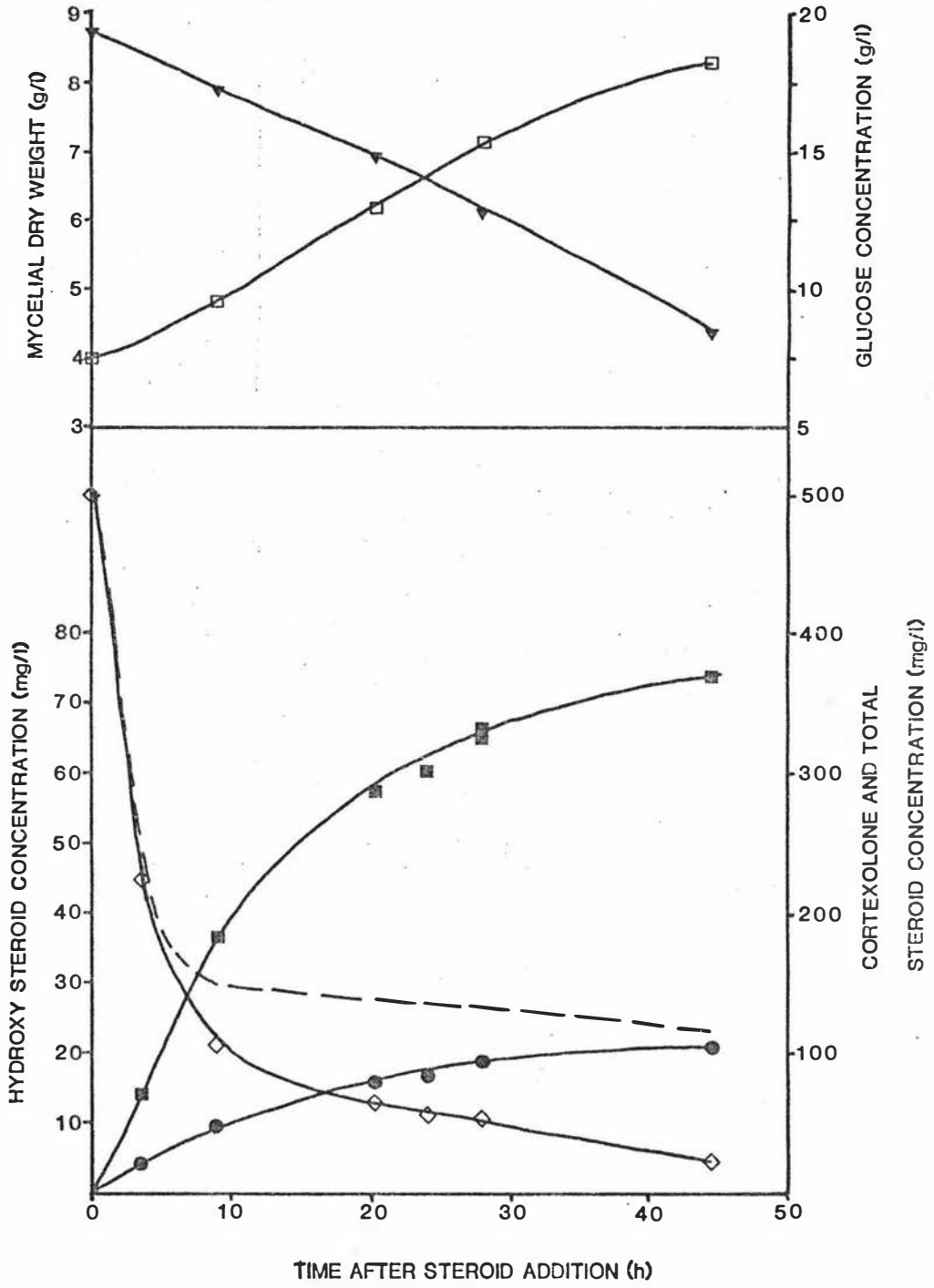


Figure 5.2 The time course for the hydroxylation of cortexolone by a fermenter culture of *P. filamentosa* f.sp. *sasakii* IFO 5254. Legend as for Figure 5.1.

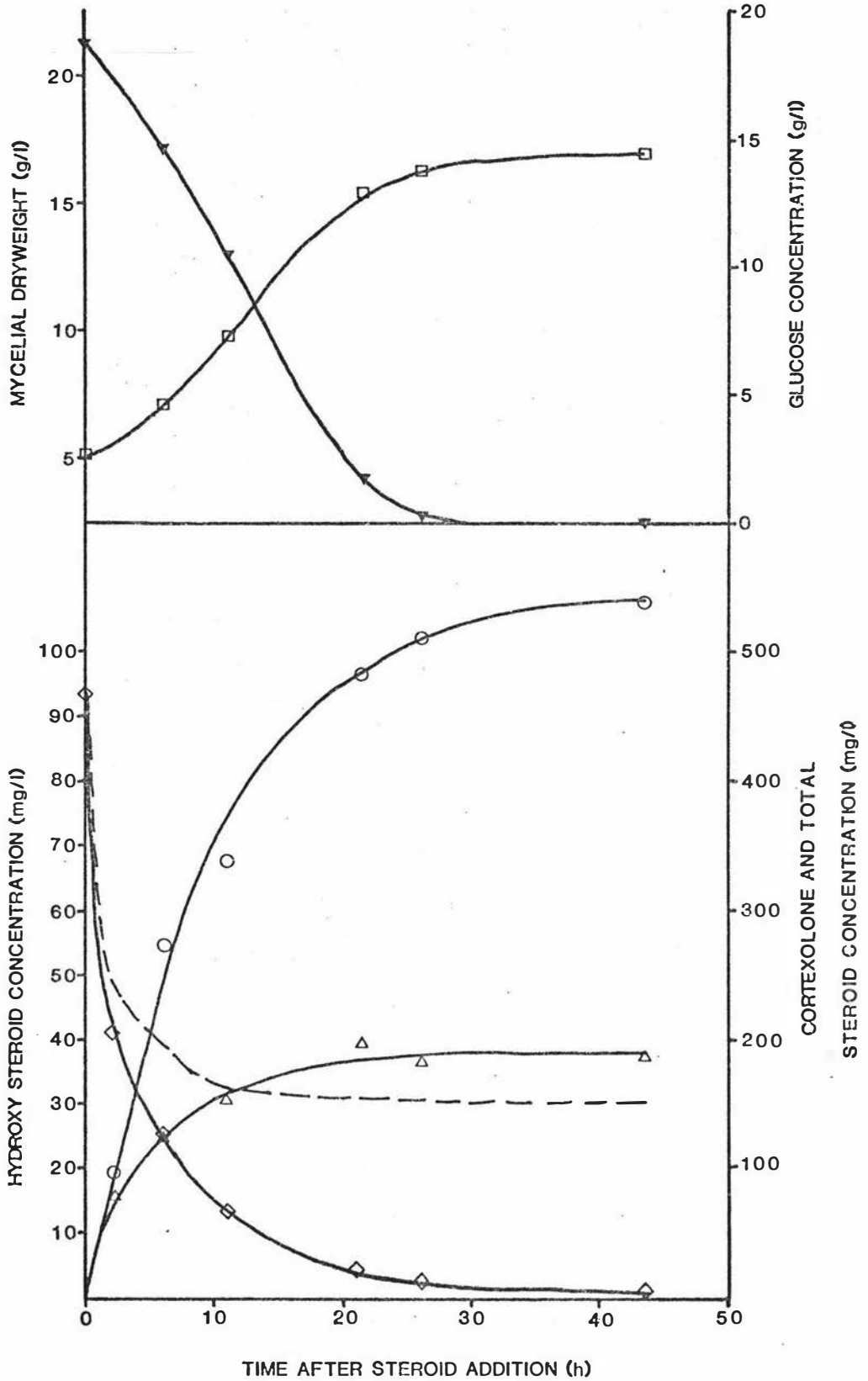


Figure 5.3 The time course forthe hydroxylation of cortexolone by a fermenter culture of *Corticium practicola* IFO 6253. 11α-Hydroxycortexolone (○), 6β-hydroxycortexolone (△); otherwise legend as for Figure 5.1.

practicola provided an interesting comparison. Although different products were formed, their yield (ca. 30% ($^{w}/w$)) and the phenomenon of multiple-monohydroxylation of the substrate was similar to the other organisms.

The difference in the ratios of 19- to 11β-hydroxy-cortexolone, for the two *P. filamentosa* organisms, has interesting implications with respect to whether one or more enzymes are responsible for the two activities. In view of the close taxonomic relationship of these two fungi, and since both produce the same pattern of monohydroxy products (Takahashi and Hasegawa, 1961b), it is not unreasonable to assume the same enzyme system in both organisms. In this respect, it is necessary to consider whether different product ratios are reconcilable with a single enzyme model. This question has been examined in some detail with the multiple-monohydroxylations of steroids by *Curvularia lunata* (Zuidweg, 1968; Lin and Smith, 1970a; Lin and Smith, 1970b). With cortexolone as substrate, this organism produces mainly 11β- and 14α-hydroxy products. Zuidweg (1968), working with cell free extracts, attempted to determine whether these two activities could be ascribed to one single or two separate enzymes. Approaches to this problem included attempts at enzyme purification, differential modification of enzyme activity, and attempts at differential enzyme induction. However, attempts at enzyme purification and fractionation failed to separate the two activities, and incubation of extracts with cortexolone, under a variety of conditions, failed to produce significant fluctuations in the ratio of products. Further, extracts from cultures, induced with a variety of different steroids, did not yield abnormal product ratios upon incubation with cortexolone. Zuidweg (1968) thus concluded that either one single enzyme catalyses the two activities or that two enzymes are under the same control, on a common operon. However, if the latter were true, attempts at differential modification of activity should have been successful, since in similar cases of mammalian steroid hydroxylation at different positions simultaneously, the activities have been distinguished quite readily by the addition of inhibitors (Zuidweg, 1968; Levy *et al.*, 1965).

Lin and Smith (1970a) examined the kinetics of 19-nortestosterone hydroxylation by *Curvularia lunata*. The major products were 10β-, 11β-, and 14α-monohydroxylated compounds. Ratios of the products were not significantly altered by different periods of induction or by different incubation temperatures, and kinetic analysis revealed that all three activities possessed the same apparent Michaelis constant. The most feasible model presented by these authors suggested a single enzyme active site to be responsible for all three activities, but that factors such as differential rate and variant geometry of approach, aberrant binding, and minor misalignment of the steroid substrate molecule result in the formation of different enzyme-substrate complexes which then give rise to different products.

In view of the above findings, with a somewhat analogous system to the *P. filamentosa*/cortexolone system, it would appear that different ratios of products should rule out a single enzyme model.

5.3 THE EFFECT OF GROWTH MEDIUM ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE

5.3.1 Introduction

Takahashi and Hasegawa (1961b) used a starch-based synthetic medium for the growth of *P. filamentosa* f.sp. *microsclerotia*. In the patent of Hasegawa *et al*. (1960) both this medium and a sucrose based medium were used with this organism for the 19-hydroxylation of cortexolone. The present study had found that the organism grew well in a glucose/yeast extract medium and some comparison of hydroxylation abilities on the various media was considered to be useful.

5.3.2 Results and Discussion

The three media were inoculated from a standard inoculum, prepared as described in section 3.5.1, prior to incubation in shake-flask conditions. The cultures were incubated until well grown, at which point the culture pH and mycelial dry weights were determined for replicate cultures. Cortexolone was then added (500 mg/l, final concentration in dimethylformamide, 10 g/l final concentration) and incubation was continued for 20 hours. The cultures were then extracted and steroid levels were determined. The steroid concentrations after 20 h incubation are presented in Table 5.1.

There was little apparent difference in the results obtained from the glucose/yeast extract medium and the starch medium of Takahashi and Hasegawa (1961b). However, the sucrose medium of Hasegawa *et al.* (1960) gave a much lower yield of products (20.4% (^{w}/w)) compared with approximately 40% (^{w}/w) for the other media) and an apparently lower rate of hydroxylation, even when considering the difference in mycelial dry weight. For all further work, therefore, the glucose/yeast extract medium was chosen on the basis of simplicity, and since it more closely approximates a typical industrial medium than does the fully defined starch medium.

5.4 THE EFFECT OF CULTURE pH ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE

5.4.1 Introduction

There is little indication in the literature that culture pH is an important variable in steroid hydroxylation by fungi. Hanisch *et al.* (1980) specifically showed that pH, in the range pH4 to pH6, had no effect on the 11α-hydroxylation of progesterone by *Rhizopus nigricans*. However, it was considered useful to determine if the same holds for *P. filamentosa* f.sp. *microsclerotia*.

Table 5.1 The 11β- and 19-hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia* in different media.

Medium	MDW[ab] (g/l)	pH[b]	Steroid Concentration[c] (mg/l)		
			11β-OH	19-OH	Co
GYE[e]	7.5	6.1	80.5	81.0	93.0
Starch[d]	7.4	4.4	79.0	84.0	105.0
Sucrose[d]	5.6	6.6	31.5	29.0	203.0

a MDW, mycelial dry weight

b determined at time of steroid addition

c determined after 20 h incubation (each result is the average of duplicate experiments).

d media descriptions may be found in Table 3.1

e glucose (25 g/l), yeast extract (10 g/l), pH 6.0.

Experience of fermenter cultures, under constant pH measurement and control, revealed that very little change occurred in culture pH during the course of the fermentation. In view of this, a simple shake-flask approach offered a useful alternative to time-consuming fermenter experiments.

5.4.2 Results and Discussion

The medium, containing glucose (25 g/l) and yeast extract (10 g/l), was prepared as normal, except the initial pH was adjusted to various values using either 1 M hydrochloric acid or 1 M sodium hydroxide. After autoclaving, the culture pH values were determined and the flasks were inoculated in the usual manner. After 24 hours growth, cortexolone was added (500 mg/l final concentration, dissolved in dimethylformamide, 10 g/l final concentration) and incubation was continued for a further 20 hours. The mycelial dry weights of replicate cultures, at each pH value, were determined at the time of steroid addition to ensure the same biomass levels, and hence direct comparison between pH treatments.

The concentrations of steroids 20 hours after cortexolone addition are shown in Table 5.2, along with culture pH values at various times during the experiment. Clearly, over the pH range 4.5 to 7.0, there were no significant differences in steroid levels. Hence, product yields and the 19-:11β-hydroxycortexolone ratios were approximately constant. These findings are in keeping with the literature and show that the 11β- and 19-hydroxylations are not particularly sensitive to culture pH. For all further work a constant culture pH of 6.0 was used.

Table 5.2 The effect of medium pH on the transformation of cortexolone.

pH			Steroid Concentration[a] (mg/l)		
Before Inoculation	24 h (t_o)	44 h (t_{20h})	11β-OH	19-OH	Co
4.50	4.50	4.45	83.1	89.3	41.7
6.00	6.10	6.50	79.9	85.4	50.3
7.00	6.90	7.20	78.8	85.9	41.5

[a] steroid concentrations after 20 h incubation; cortexolone was added at t_o. Each result is the average of duplicate experiments.

5.5 THE EFFECT OF METHOD OF SUBSTRATE ADDITION ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE

5.5.1 Introduction

The manner in which the steroid substrate is added to the fermentation has been shown to influence overall yields and rates of steroid transformations (Marsheck, 1971). Thus it was of interest to determine whether the method of steroid addition affected the present transformation.

5.5.2 Results and Discussion

Replicate, shake-flask cultures of *P. filamentosa* f.sp. *microsclerotia* were prepared. After 24 hours growth cortexolone was added, dissolved in solvents as required, and incubations were continued for a further 16 hours. A comparison was made between the use of no solvent, dimethylformamide, ethanol, and acetone. For the use of ethanol and acetone as solvents, the main constraint was cortexolone solubility. In this respect, the comparison with dimethylformamide was made with 2% (^{v}/v) solvent (final concentration) and 200 mg/l steroid (final concentration). The results are presented in Table 5.3.

The comparison of dimethylformamide, ethanol, and acetone as "carrying solvent" reveals that there is no apparent advantage in any one. However, the superior solvent properties of dimethylformamide for cortexolone make it an obvious choice. In comparing the use of 1% (^{v}/v) dimethylformamide with no solvent (cortexolone final concentration, 500 mg/l) the interpretation of the data must consider whether the ratio of products to losses is affected. Since the ratio in both cases is approximately 0.6:1, it is clear that there is no significant difference in terms of product yield between the two treatments. The fact that hydroxylation has proceeded more rapidly in the presence of 1% dimethylformamide probably reflects the effect of solvent on the steroid solubility and/or increased cell membrane permeability to cortexolone. On this basis, dimethylformamide (10 g/l final concentration) was used as the steroid "carrier" in all further work.

Table 5.3 The effect of different "carrying solvents" on the cortexolone transformation

Solvent [a] (final concentration)	Cortexolone Concentration (mg/l)	Steroid Concentration [b] (mg/l)		
		11β-OH	19-OH	Co
None	500	39.3	51.8	251
1% (v/v) DMF	500	74.3	93.5	53.3
2% (v/v) DMF	200	37.5	43.7	0.0
2% (v/v) EtOH	200	35.4	40.8	0.9
2% (v/v) An.	200	35.0	35.7	1.0

[a] abbreviations are dimethylformamide (DMF), ethanol (EtOH), acetone (An.)

[b] steroid concentrations after 16 h incubation. Each result is the average of duplicate experiments.

5.6 THE INDUCIBILITY OF THE 11β- AND 19-HYDROXYLASE SYSTEM OF *P. filamentosa* f.sp. *microsclerotia*

5.6.1 Introduction

Evidence accumulated to date indicates that most, if not all, steroid hydroxylases of fungal origin are inducible rather than constitutive enzyme systems (see section 2.3.2). If this is so, then control of enzyme induction in a fermentation process can be as important as control of enzyme expression. For this reason, it was important to determine if the 19- and 11β-hydroxylase system of *P. filamentosa* f.sp. *microsclerotia* is inducible by cortexolone. Cycloheximide, a potent inhibitor of 80S ribosomes (Franklin and Snow, 1981), is commonly employed as a protein synthesis inhibitor in eukaryotic cells, and, thus, was used in this investigation.

5.6.2 Results and Discussion

Duplicate, shake-flask cultures (100 ml) of *P. filamentosa* were prepared and, after 24 h growth, cycloheximide (250 µg/ml, final concentration) was added to one of the flasks. After a further 15 minutes incubation, cortexolone (250 mg/l, final concentration) was added to both flasks, and incubation was continued for 6 hours. The cultures were then extracted and analysed for steroid (Table 5.4).

Table 5.4 The effect of cycloheximide on the 11β- and 19-hydroxylation of cortexolone

Treatment	Steroid Concentration[a] (mg/l)			Recovered Steroid[c]
	11β-OH	19-OH	Co	(% yield)
Cycloheximide[b]	0	0	184	74
Control	18.0	19.0	135	69

[a] after 6 h incubation
[b] cycloheximide added 15 min prior to cortexolone
[c] yeild, based on added steroid

In the culture to which cycloheximide was added prior to steroid no hydroxylation was observed. In the control culture, however, levels of 11β- and 19-hydroxy-cortexolone of 18 and 19 mg/l, respectively, were obtained. This is presumptive evidence for the presence of an inducible enzyme. To confirm the result it was necessary to ensure that when the hydroxylase system is present, cycloheximide does not inhibit its expression.

Thus, cycloheximide (250 µg/ml, final concentration) was added to a fermenter culture which was in the process of hydroxylation (cortexolone, 500 mg/l final concentration, was added 5 h prior to cycloheximide to allow induction to occur). Samples were removed from the fermenter over the following 12 h and analysed for steroids. The course of hydroxylation is shown in Figure 5.4. The fermentation shown in Figure 5.1 was performed under similar conditions, except without cycloheximide, and can be used as a control. Clearly, the course of the hydroxylation is initially unaffected by the presence of cycloheximide, and so confirms that the 11β- and 19-hydroxylating enzyme(s) of *P. filamentosa* f.sp. *microsclerotia* are inducible by cortexolone. Furthermore, these enzymes are produced by *de novo* protein synthesis and are not present in the cells prior to addition of the inducer. By comparison with Figure 5.1, it is apparent that in the presence of the protein synthesis inhibitor, hydroxylation continues for only a short time (ca. 4 h). This indicates that continued protein synthesis is essential to maintain the hydroxylation. It would appear, then, that the hydroxylase(s) or some critical component of the enzyme system may be rapidly "turned over" (degraded and resynthesised) under normal cellular behaviour. This is similar to the conclusion drawn by Kennel *et al.* (1981) when studying the differential effect of cycloheximide on penicillin and cephalosporin biosynthesis by *Cephalosporium acremonium*. While penicillin N production was unaffected by cycloheximide addition, cephalosporin C production was markedly inhibited. The authors concluded that the effect on cephalosporin C was due to turnover of a labile bio-synthetic enzyme.

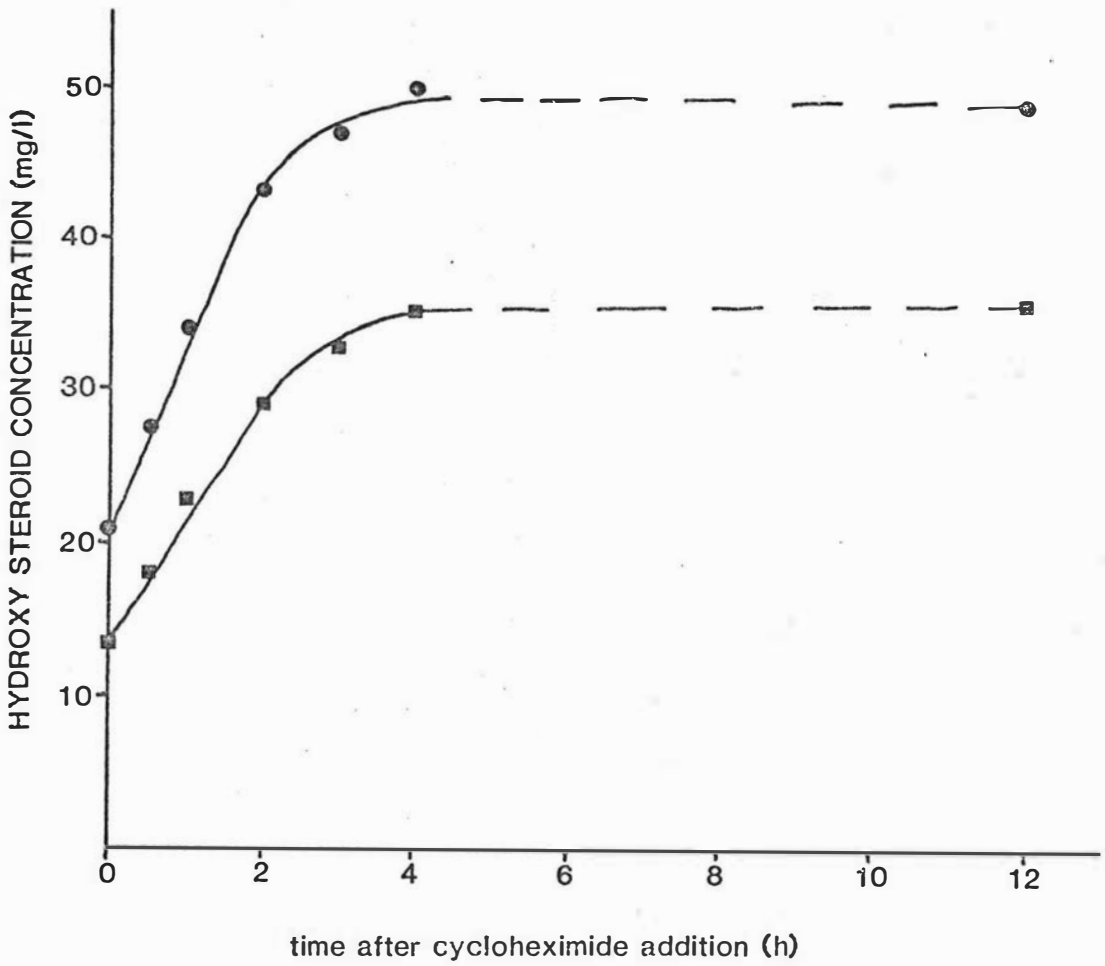


Figure 5.4 The course of cortexolone hydroxylation by a fermenter culture of *P. filamentosa* f.sp. *microsclerotia*, in the presence of cycloheximide (250 µg/ml). See text for experimental details. 19-Hydroxycortexolone (●), 11β-hydroxycortexolone (■).

A further point from these data (Table 5.4) is that even when cycloheximide was added prior to cortexolone, only 74% recovery of steroid was obtained. Thus, substrate losses were occurring even without the formation of hydroxylated products.

5.7 BINDING OF CORTEXOLONE AND 11β-HYDROXYCORTEXOLONE BY MYCELIA

5.7.1 Introduction

The ability of fungal mycelia to bind steroids and sterols has been reported (Buetow and Levedahl, 1964). Sterols such as β-sitosterol, cholesterol and stigmasterol which possess a long lipophilic side-chain and a 3β-hydroxy-5-ene group were particularly susceptible to binding by *Penicillium canescens*, whereas 11β-hydroxycortexolone and testosterone were not bound to any significant extent. If this phenomenon were applicable to the *P. filamentosa*/cortexolone system, then, based on steroid structure, simple binding would not explain the apparent substrate losses which have been observed in both fermenter and shake-flask culture.

Investigation of this phenomenon was complicated by the necessity to avoid any form of steroid metabolism or transformation by the organism during experiments. This can only reliably be achieved by autoclaving the mycelia prior to adding the steroid. This, however, destroys the functional properties of proteins and probably disrupts lipid-membranes, which may both be important for steroid binding by live mycelia. The use of isotopically labelled (^{14}C) cortexolone could be used to determine the fate of steroid added to live cultures. This approach was considered but the cost of obtaining the steroid was prohibitive. Although synthesis of (^{14}C)-C-4 labelled cortexolone is not difficult, the need for special facilities and the required time, precluded this approach.

As a simple method of determining if purely physical interactions between mycelia and steroid could account for losses which have been observed, an experiment was performed involving the incubation of steroids with autoclaved mycelia.

5.7.2 Results and Discussion

Replicate shake-flask cultures of *P. filamentosa* f.sp. *microsclerotia* were prepared, and after 48 h growth (DW = 12.2 ± 0.2 g/l) were autoclaved at 115°C for 15 minutes. Cortexolone and 11β-hydroxycortexolone were then incubated with the cultures. After 24 h, the cultures were extracted and steroid recoveries determined. These are given in Table 5.5 as the percentage of control-recoveries from uninoculated media.

Table 5.5 Recoveries for cortexolone and 11β-hydroxycortexolone after incubation with mycelia.

Steroid	Concentration (mg/l)	Recovery [a] (%)
cortexolone	500	92
	2500	91
11β-hydroxycortexolone	500	100

[a] averages of duplicate experiments

Although, in the case of cortexolone, only approximately 90% recovery was obtained, these losses are insufficient to account for the substantial losses (ca. 60% in 20 h) which have been observed in live cultures. Thus it is possible that the observed losses may result from metabolism of cortexolone by the organism.

5.8 THE EFFECTS OF α,α'-BIPYRIDYL AND 8-HYDROXYQUINOLINE ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE

5.8.1 Introduction

A substantial literature exists describing steroid and sterol degradation by microorganisms (for example, Vezina *et al.* (1971) and Martin (1977)). Bacteria, in particular *Nocardia* and *Arthrobacter* species, have been studied extensively, and it has been shown that attack on the steroid ring system proceeds via 9α-hydroxylation and Δ^1-dehydrogenation reactions. Although little information is available describing fungal steroid-degradation, some fungi are known to possess these abilities (Charney and Herzog, 1967). Also, the presence of degradation products from cortexolone has been described with some fungi (Singh *et al.*, 1965; Casas-Campillo and Esparza, 1968). It thus seemed possible that the steroid losses observed in the present work may be due to degradation of the steroid nucleus by the organism.

The metal-ion-binding compounds 8-hydroxyquinoline and α,α'-bipyridyl have been employed successfully in preventing steroid degradation (Martin, 1977) and many examples exist of their use with bacterial cultures. Therefore, experiments were performed using these compounds with *P. filamentosa* f.sp. *microsclerotia*.

5.8.2 Results and Discussion

Replicate shake-flask cultures (50 ml) of *P. filamentosa* were prepared, and after 24 h growth, the inhibitors (at the required concentrations) were added dissolved in ethanol (8 g/l, final concentration). Simultaneously, cortexolone (500 mg/l) was added. Incubation was continued for 20 h after which time extraction and quantitation of steroids was performed. The dry weight of the solvent-extracted mycelia was also recorded to determine the effects of the inhibitors on growth during the 20 h incubation period. The results are presented in Table 5.6 as total recovered steroid, 11β - + 19-hydroxycortexolone

Table 5.6 The effect of 8-hydroxyquinoline and α,α'-bipyridyl on the 11β- and 19-hydroxylation of cortexolone.

Treatment	DW [a] (g/l)	Total [b] Recovered Steroid (% w/w)	Yield [c] (11β + 19) (% w/w)	Ratio (19:11β)
Control[d]	7.0	58.3	43.6	1.01
α,α'-bipyridyl				
0.2 mM	4.7	62.7	24.5	0.92
1.0 mM	5.5	79.0	16.0	0.98
10 mM	4.7	79.0	0	-
8-hydroxyquinoline				
0.2 mM	7.1	75.3	27.3	1.06
1.0 mM	4.0	70.0	7.5	0.94
10 mM	1.8	81.0	0	-

[a] dry-weight of solvent extracted mycelia

[b] percentage of added cortexolone

[c] percentage of consumed cortexolone

[d] containing ethanol (8 g/l) but without inhibitors

yield, and the product ratio.

From these results it can be seen that increasing levels of both inhibitors did improve the steroid recovery. However, the hydroxylation reactions also became increasingly inhibited. Further, the inhibitors dramatically affected the culture dry weight, suggesting that cessation of growth and, in some cases cell lysis, had occurred.

Since in no case was the product yield improved by the use of either α,α'-bipyridyl or 8-hydroxyquinoline, there was considered to be no benefit in utilizing this approach to minimise steroid losses.

5.9 THE EFFECTS OF PHENOBARBITAL AND PHENANTHRENE ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE

5.9.1 Introduction

Phenobarbital and phenanthrene are typical compounds used to induce cytochrome-P450 in mammalian and fungal cells (Smith and Davis, 1980). Without making any assumption about the nature of the *P. filamentosa* f.sp. *microsclerotia* hydroxylase system, it was of interest to determine the effects of these inducers on the apparent hydroxylation rate of this organism.

5.9.2 Results and Discussion

Replicate shake-flask cultures (50 ml) were prepared, and after 24 h growth cortexolone (500 mg/l, final concentration) was added, together with the inducer compounds in ethanol (8 g/l, final concentration). Incubation was continued for a further 16 h, after which the cultures were extracted and the steroids quantitated. The results are presented in Table 5.7.

Table 5.7 The effect of phenobarbital and phenanthrene on the 11β- and 19-hydroxylation of cortexolone.

Treatment	Inducer Concentration (mM)	Steroid Concentrations [a] (mg/l) 11β-OH	19-OH	Co
Control [b]	0	49.0	44.0	213
Phenobarbital	2	47.5	42.5	215
	5	66.5	59.5	167.5
Phenanthrene	1	30.5	20.5	313
	2	27.5	18.5	318

[a] steroid concentrations after 16 h incubation (each entry is the average of duplicate experiments)

[b] containing ethanol (8 g/l) but without inducers

Phenobarbital, at 2 mM concentration, had no effect on the product levels when compared to the control. However, at 5 mM concentration, a significant (35%) increase in the product levels was observed. Thus, it can be concluded that phenobarbital, at this concentration, either increased the level of induced hydroxylase(s) or enhanced the enzyme(s) activity. Both phenomena would result in an apparent increase in the rate of hydroxylation, as observed.

The effect of phenanthrene was to depress the hydroxylation, as indicated by lower product and higher substrate concentrations compared to the control. Interestingly, however, the ratio of 19- to 11β-hydroxy products was quite significantly altered by this compound (0.67 compared to 0.90 in the control).

Although no definitive conclusions can be drawn, it is interesting that a classical cytochrome-P450 inducer such as phenobarbital is capable of increasing the apparent hydroxylation rate of cortexolone by *P. filamentosa* f.sp. *microsclerotia*.

5.10 THE STABILITY OF HYDROXYLATED CORTEXOLONE IN *P. filamentosa* f.sp. *microsclerotia* CULTURES, AND THE POSSIBILITY OF PRODUCT INHIBITION OF THE HYDROXYLATION PROCESS.

5.10.1 Introduction

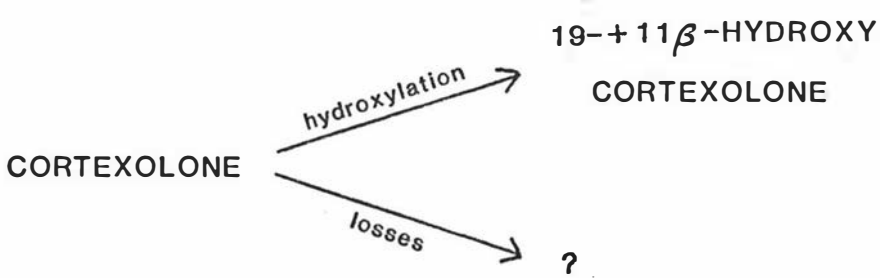
Unaccountable losses of steroid were regularly observed during hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia*. Since this appears not to be caused by mycelial binding (section 5.6), it was of interest to determine whether losses proceeded via the hydroxy products as intermediates in some degradation pathway, or through direct degradation of cortexolone. On the basis of fermenter culture transformations (e.g. Fig. 5.1) it was suspected that the 11β- and 19-hydroxy products were stable. However, further evidence was required. The possibility of product inhibition also warranted some preliminary investigation.

These effects were investigated only in respect of 11β-hydroxycortexolone, since insufficient pure 19-hydroxy-cortexolone was available.

5.10.2 Results and Discussion

Replicate shake-flask cultures (50 ml) were grown for 24 h, after which time the appropriate steroids (each at 400 mg/l, final concentration) were added. Incubation was then continued for a further 24 hours prior to analysis. The results are presented in Table 5.8 as the steroid concentrations after 24 h incubation.

Incubation of 11β-hydroxycortexolone resulted in little appreciable loss and this is in agreement with the observations from fermenter cultures. Thus, this compound is a stable product and is not appreciably metabolised by the fungus. The concentration of 19-hydroxycortexolone has been observed (Fig. 5.1) to remain approximately in constant proportion to that of the 11β-hydroxy product, during the course of fermenter culture. Hence, this result with the latter compound probably also applies to the former. It thus appears that cortexolone is metabolised by two competing pathways as shown:-



Hence, improvement in overall process yield should be able to be achieved by blocking the degradation pathway, either by mutagenesis or chemical inhibition.

The results obtained when cortexolone and 11β-hydroxy-cortexolone were incubated together suggest that the latter

Table 5.8 Steroid concentrations in shake-flask cultures, after 24 h incubation.

Treatment [a]	Steroid Concentration [b] (mg/l)		
	11β-OH	19-OH	Co
11β-hydroxycortexolone	380	-	-
11β-hydroxycortexolone + cortexolone	427	58.5	0
Cortexolone (control)	73.3	78.3	0

[a] each individual steroid was added at 400 mg/l, final concentration

[b] each entry is the average of duplicate experiments.

does cause some inhibition of hydroxylation. Thus, the concentrations of 11β- and 19-hydroxy compounds observed were approximately 50 mg/l (estimated from data in Table 5.8) and 58 mg/l respectively, compared to the control levels of 73 mg/l and 78 mg/l. Alternatively, 11β-hydroxy-cortexolone may act by repressing enzyme synthesis.

5.11 CONCLUSIONS

The experiments described in this chapter have allowed decisions to be made on basic fermentation conditions. Thus, medium constituents and pH, and the mode of steroid addition have been defined. Further, the proven inducible nature of the hydroxylase system may have important consequences in terms of factors which control the induction process.

Typically, the yeild of 19- and 11β-hydroxycortexolone was less than 40% ($^{w}/w$), and thus considerable steroid losses were observed. Investigations into the nature of these losses, and possible techniques to avoid them, have been described. It appears that steroid losses occur via a pathway parallel to the hydroxylation process. Thus, the hydroxy products are stable and are not further metabolised. This is of considerable consequence, since two separate areas of process development arise:

(i) Yield improvement by blocking cortexolone degradation, either by mutagenesis or chemical inhibition.

(ii) Improvements in hydroxylation rate by determining optimum conditions for induction and expression of the hydroxylase(s).

Thus research in each of these areas could proceed almost independently. Development of a mutation programme was outside the scope of this thesis, mainly because of the time required. To find a means of chemically inhibiting cortexolone degradation would involve, largely, an empirical or "black-box" approach. This was not considered an attractive avenue

to explore at this stage. On the other hand, improvements in hydroxylation rate, at such an early stage in a development programme, are probably of greater importance than yield improvements. Furthermore, research into rate improvement would necessarily involve a more mechanistic approach, and so results of consequence to steroid hydroxylation in general, may arise.

Thus, the final two experimental chapters of this thesis describe research aimed at improvements in cortexolone-hydroxylation rates of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 cultures.

CHAPTER 6

THE EFFECT OF DISSOLVED OXYGEN TENSION ON THE INDUCTION AND EXPRESSION OF THE CORTEXOLONE HYDROXYLASE SYSTEM OF *P. filamentosa* f.sp. *microsclerotia*

6.1 INTRODUCTION

Recent studies of microbial mono-oxygenases have indicated the importance of dissolved oxygen tension (DOT) on the induction of these enzyme systems (Mauersberger *et al.*, 1980; Wiseman, 1977; Gmunder *et al.*, 1981). In particular, Hanisch *et al.* (1980) showed that for the 11α-hydroxylation of progesterone by *Rhizopus nigricans* there was a marked optimum DOT for induction of the enzyme. Furthermore, the optimum for expression of activity lay at a much higher DOT. Consequently, maximum production rates of hydroxyprogesterone were achieved when induction was performed at low DOT, followed by elevation of the DOT to enhance expression of the synthesised enzyme.

For these reasons, the effect of DOT on the present hydroxylase system was investigated. It was also considered that if dissolved oxygen tension was an important variable, then knowledge of this would be essential before further experiments could be designed.

6.2 EXPERIMENTAL METHOD

The inducible nature of the *P. filamentosa* f.sp. *microsclerotia* hydroxylase system was demonstrated in preliminary experiments (see section 5.6), so it was now possible to perform experiments where induction of enzyme synthesis could be clearly separated from enzyme expression. All experiments were conducted in batch fermenter cultures (1500 ml working volume) under dissolved oxygen control.

To investigate the effect of DOT on hydroxylase expression, induction was carried out under constant conditions to ensure equal amounts of enzyme synthesis. After addition of cycloheximide (250 µg/ml, final concentration) to prevent further protein synthesis, the DOT was adjusted to the appropriate value, and by measuring the rate of hydroxylation the effect of DOT on expression was investigated. In a similar manner the effect of DOT on induction was investigated by inducing at different DOT levels, followed by addition of cycloheximide to prevent further enzyme synthesis. The amount of enzyme present was then determined by its activity under constant conditions of expression.

After inoculation of the fermenter, growth proceeded for approximately 20 h, during which time the DOT was allowed to stabilize at the level selected for induction of the steroid hydroxylases. DOT was controlled by on-off control of aeration rate, as described in section 3.5.2.1. After a period of stable operation (usually 4-5 h) cortexolone, dissolved in dimethylformamide (10 g/l final concentration), was added to the fermenter. After a further 4 h the DOT was adjusted to that required for hydroxylase expression, cycloheximide being added simultaneously with the change in DOT. The rate of hydroxylation was then measured over the subsequent 2 h period. Results are expressed as specific rates, the culture dry weight being determined at the time of cycloheximide addition.

The agitation speed used for each fermenter experiment depended on the DOT required since the aeration control system could not cope on its own. Thus, agitation speeds between 350 and 500 rpm were used (impellor tip speed 0.92 - 1.30 m/s) with higher speeds for higher DOT levels.

6.3 RESULTS

Initially, experiments were performed to determine the influence of steroid-substrate concentration on the amount of enzyme induced in a 4h period. DOT conditions for induction and expression were chosen as 25% and 30% of saturation, respectively. Cortexolone concentrations of 0.1, 0.5, and 2.5 g/l were used. To ensure the presence of sufficient substrate for full expression of induced activity, an additional 0.7 g/l of steroid was added, immediately after cycloheximide addition, to those fermentations with initial 0.1 and 0.5 g/l cortexolone concentrations. The rate of hydroxylation over the subsequent two hour period after cycloheximide addition was measured, and the results are presented in Table 6.1. It is apparent from these data that, although there is a gradual rise in the level of induced enzyme as steroid concentration increases, even at 0.1 g/l a reasonably similar level of activity was obtained to that at 2.5 g/l

Table 6.1 Effect of cortexolone concentration on enzyme induction.

Cortexolone Concentration (g/l)	Specific Hydroxylation Rate (mg/g DW.h)	
	19-hydroxy	11β-hydroxy
0.1	0.67	0.41
0.5	0.81	0.65
2.5	0.87	0.69

Certainly, the latter seemed sufficient to provide for maximum induction. Hence, for all future work a steroid concentration of 2.5 g/l was used. This also ensured that the hydroxylase system was saturated with substrate during the expression phase of the experiments.

The effect of DOT on the expression of hydroxylase activity was now determined over a range of DOT values. A DOT of 25% of saturation was chosen as the constant level for induction in all experiments. After steroid addition, 4 h was allowed for induction, after which cycloheximide was added and the DOT was simultaneously changed to the required value for enzyme expression. The rate of hydroxylation was measured over the following 2 h period, and the results are presented in Figure 6.1. For both 19- and 11β-hydroxylation, the hydroxylation rates increased with increasing DOT until a maximum rate was observed at 30% of saturation. Thereafter, the rate decreased at 45% of saturation, and maintained this level at 60% of saturation. The decrease in hydroxylation rate as the DOT approached zero was expected since oxygen is a substrate for the hydroxylation. This agrees with the results of Hanisch *et al*. (1980). However, the optimum at 30% of saturation with a decline in rates at 45% and 60% was unexpected.

To investigate this apparent decrease at higher DOT values, two further fermentations were conducted with DOT levels of 50% of saturation during induction. The DOT values during subsequent expression were 30% of saturation in one experiment and 50% in the other. Hydroxylation rates were obtained as detailed in Table 6.2. The ratio of hydroxylation rates between the two fermentations was approximately 1.40 (30%/50% DOT for expression), while the expected ratio from Figure 6.1 was ca. 1.47. Thus, these fermentations verified the decline in rates above 30% DOT.

To determine the effect of DOT during the induction of enzyme activity, 30% of saturation was chosen as the constant DOT for expression. Thus, induction was performed

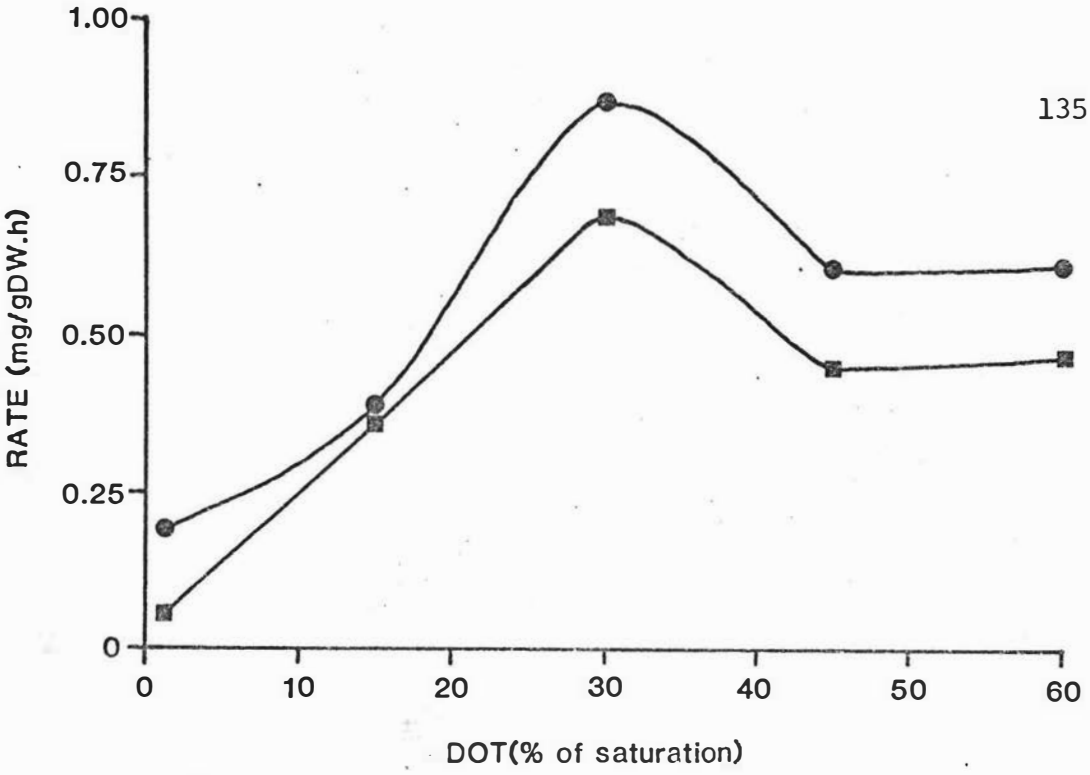


Figure 6.1 The effect of DOT on the specific rate of expression of the cortexolone-11β- (■)and 19- (●) hydroxylation activities of *P. filamentosa* f.sp. *microsclerotia*. See text for details.

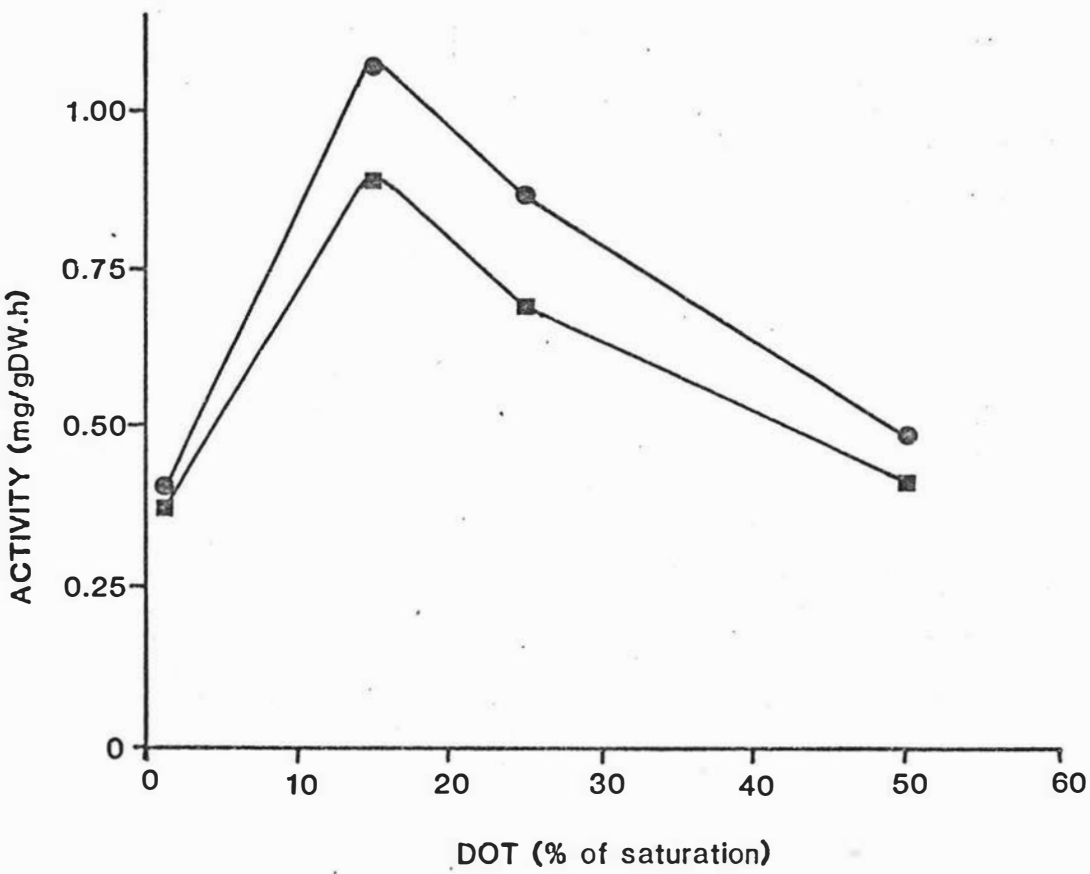


Figure 6.2 The effect of DOT on the induction of the cortexolone-11β- (■)and 19- (●) hydroxlation activities of *P. filamentosa* f.sp. *microsclerotia*. See text for details.

Table 6.2 The effect of DOT on the rates of cortexolone hydroxylation by cultures induced at a DOT value of 50% of saturation.

$[DOT]_{IND}$ (% satn.)	$[DOT]_{EXPN}$ (% satn.)	Specific Hydroxylation Rates (mg/g DW.h) 19-hydroxy	11β-hydroxy
50	30	0.49	0.41
50	50	0.36	0.30

at different DOT levels for 4 h, after which cycloheximide was added and the DOT was raised to 30% for measurement of the expression of activity. The results of these experiments are presented in Figure 6.2, and the activities so determined are a measure of the relative amount of enzyme produced during induction. In this case an optimum for induction was observed at a DOT of 15% of saturation for both 19- and 11β-hydroxylation.

To ensure that the presence of cycloheximide was not causing erroneous results, two further fermentations were conducted without addition of the protein synthesis inhibitor. Figure 6.3 shows the time course for the hydroxylation of cortexolone under a constant DOT of 30% of saturation, while Figure 6.4 shows the same for a fermentation with a DOT of 15% of saturation for the first 4 h (after cortexolone addition), and then 30% DOT, thereafter. The rates of hydroxylation measured during the appropriate period (from 4 to 6 h) are given in Table 6.3 along with the expected rates, derived from Figures 6.1 and 6.2. An acceptable level of agreement was obtained, so verifying the results.

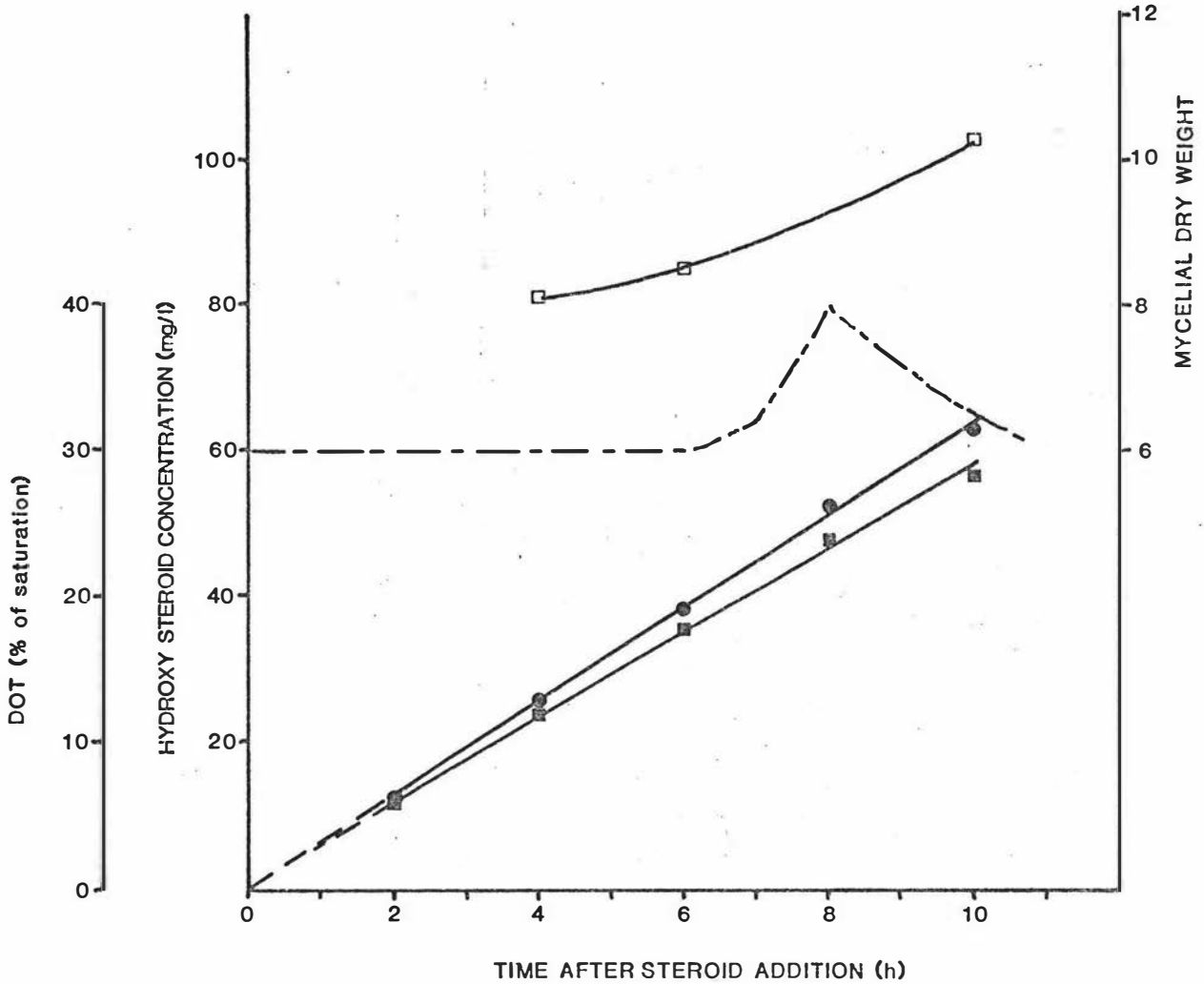


Figure 6.3 The initial time course for the hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia*, under DOT control at 30% of saturation. Mycelial dry weight (□), DOT (—), 19-hydroxycortexolone (●), 11β-hydroxycortexolone (■).

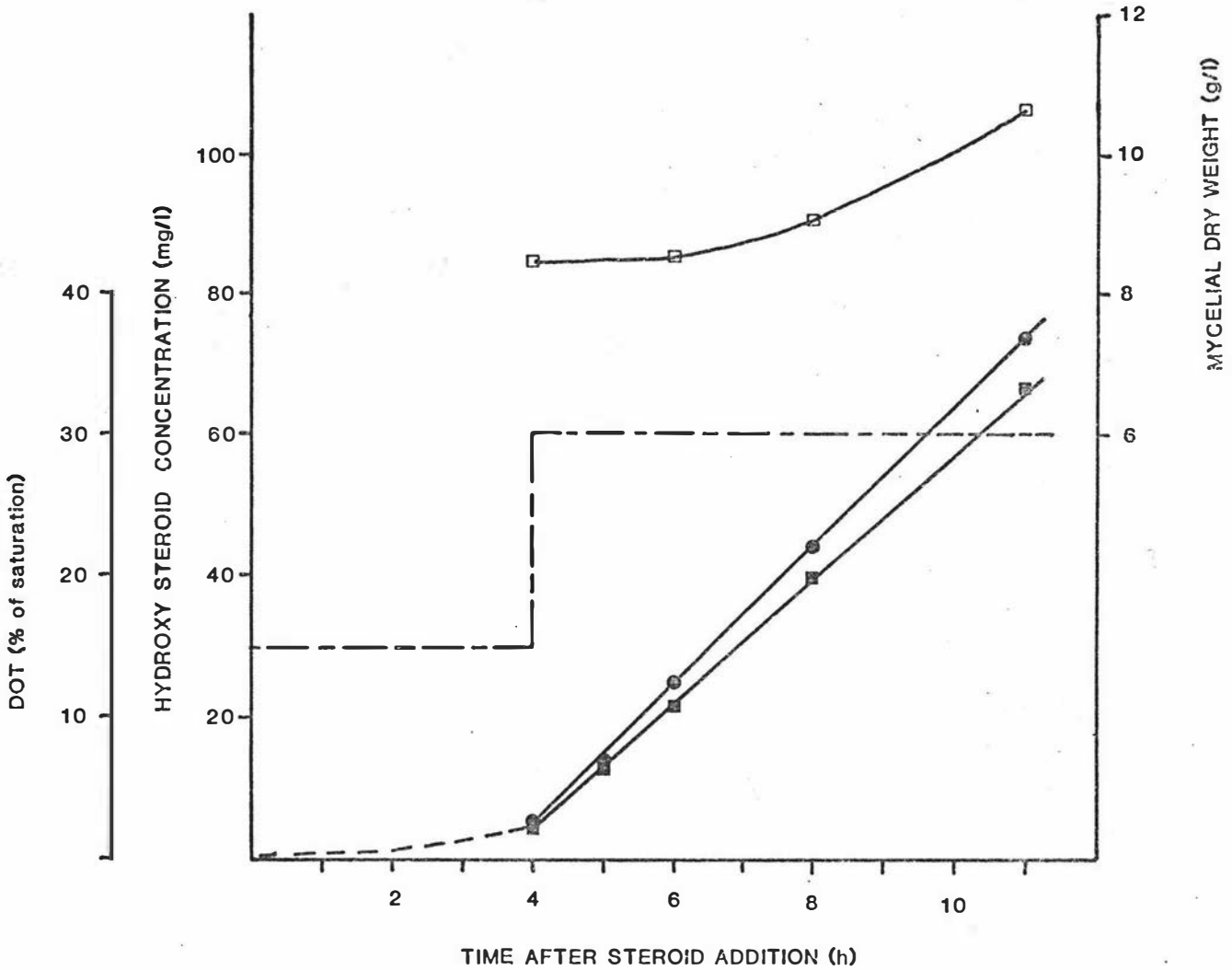


Figure 6.4 The initial time course for the hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia*, under DOT control at 15% of saturation (for the first 4 h), and then, 30% of saturation. Legend as for Figure 6.3.

Table 6.3 Comparison of hydroxylation rates from fermentations performed in the presence and absence of cycloheximide.

$[DOT]_{IND}$ (% satn.)	$[DOT]_{EXPN}$ (% satn.)	Specific Hydroxylation Rate[a] (mg/g DW.h) 19-hydroxy	11β-hydroxy
30	30	0.75 (0.80)	0.71 (0.63)
15	30	1.16 (1.09)	1.01 (0.89)

[a] rates from cycloheximide-treated cultures in parentheses.

Some selected examples of the time course during the 2 h period after cycloheximide addition are presented in Figures 6.5 and 6.6. Figure 6.5 shows two experiments, the results of which are given in Figure 6.1, where DOT during induction was 25% of saturation but with different DOT levels during expression. Figure 6.6 shows two experiments, the results of which can be found in Figure 6.2, which had different DOT levels during induction but constant conditions of expression (30% of saturation). Steroid concentrations are expressed in terms of mycelial dry weight to remove any effect of different dry weights in different fermentations. In examining these figures it can be noted that the specific hydroxysteroid concentrations at zero time are a reflection of the amount of expression occurring during the induction phase. In this respect, lower zero-time levels were obtained when moving from 25%, to 15%, and then to 1% of DOT saturation.

6.4 DISCUSSION

The results obtained in this work are similar to those reported by Hanisch *et al.* (1980) for the 11α-hydroxylase of *Rhizopus nigricans*. These authors demonstrated an optimum DOT for induction at 10% of saturation while the optimum DOT for expression was much higher (using a medium of similar

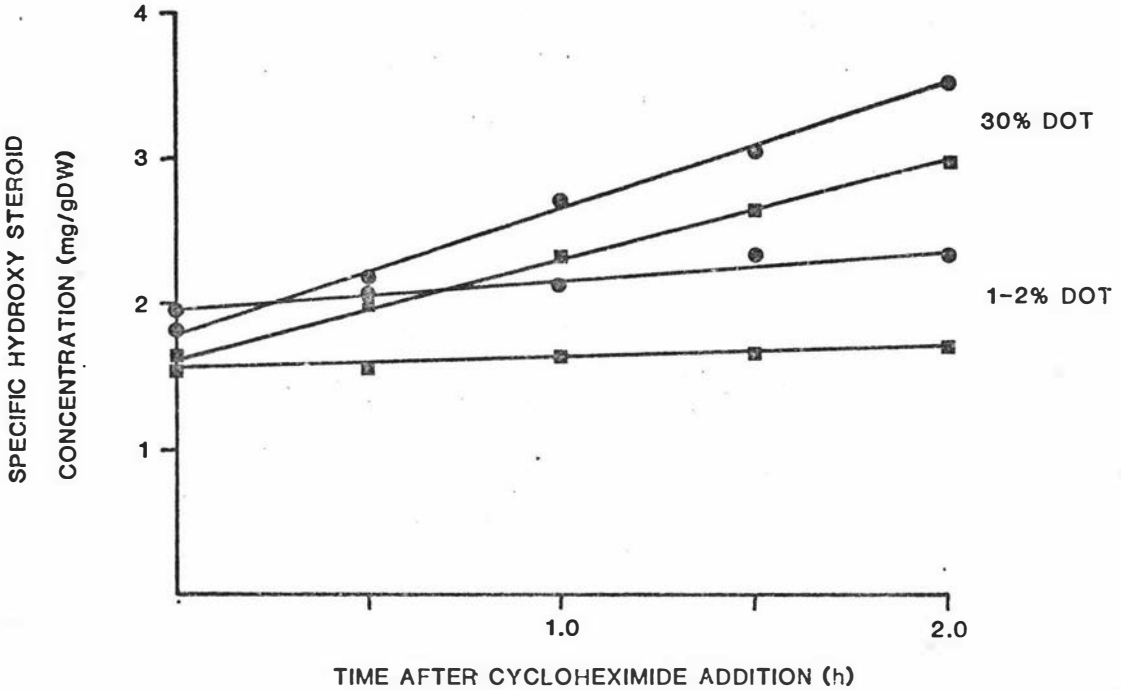


Figure 6.5 Selected examples of the course of hydroxylase expression, under different DOT conditions, during the 2 h period after cycloheximide addition. (DOT during induction was 25% of saturation). 19-Hydroxycortexolone (●), 11β-hydroxycortexolone (■).

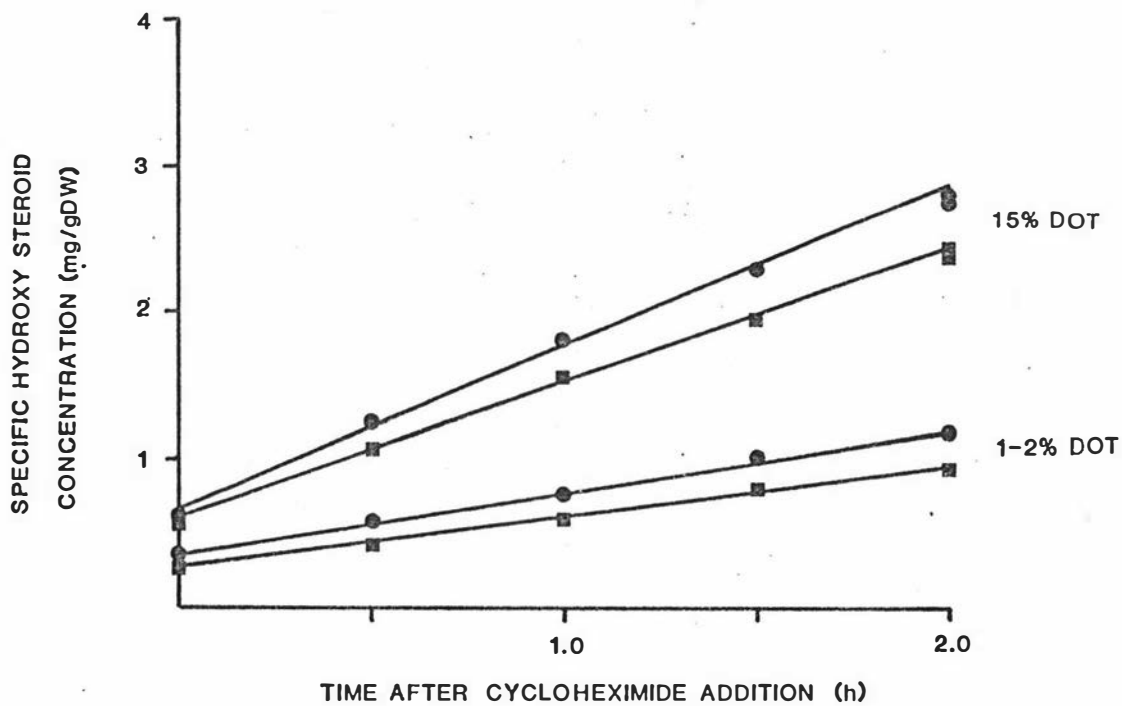


Figure 6.6 Selected examples of the course of hydroxylation, during the 2 h period after cycloheximide addition, following induction under different DOT conditions. (DOT during expression was 30% of saturation). 19-Hydroxycortexolone (●), 11β-hydroxycortexolone (■).

composition to that used in the present work, and operating at 30°C). They did not, however, use a protein synthesis inhibitor to separate induction from expression, but showed that under at least one set of conditions induction was complete after a brief period. Hence, they assumed no further protein synthesis during expression.

The simplest definition of induction is 'the increased production of enzyme' (Smith and Davis, 1980). Thus it includes everything from gene transcription to actual protein synthesis. All enzymes are in an equilibrium state between synthesis and degradation (Dixon and Webb, 1964), so if an enzyme is undergoing rapid turnover then protein synthesis would always be occurring. In this respect, the observation described by Maddox *et al.* (1981), with agar-immobilized cells of *Rhizopus nigricans*, is of some significance. It was observed that when such cells, which were in the process of hydroxylating progesterone, were treated with cycloheximide (250 μg/ml), the final yield of 11α-hydroxy-progesterone was considerably lower than that with untreated cells, and the activity was lost much more rapidly. Since Hanisch *et al.* (1980) had shown that cycloheximide did not directly inhibit the hydroxylation process during the initial period after its addition, this suggests that continued protein synthesis is required to maintain the hydroxylation. Thus, after protein synthesis is stopped, the rate will start to decline as enzyme degradation gradually lowers the amount of hydroxylase enzyme present. In the present work, it was observed that hydroxylation rates were linear for only a few hours after cycloheximide addition (see section 5.6), thus suggesting a similar effect. Thus, without the use of cycloheximide, it would be unwise to assume the separation of induction from expression. Although it has previously been assumed that cycloheximide acts only as an inhibitor of protein synthesis at cytoplasmic ribosomes, increasing evidence is accumulating for multiple sites of action of the drug (Wendelberger-Schieweg *et al.*, 1980. Hence, the possibility that the eventual decline in hydroxylation rates is due to effects other than turnover of

a labile enzyme must be accepted. This possibility does not, however, affect the validity of the present results, since hydroxylation rates, measured for the first two hours after cycloheximide addition, were verified by experiments where cycloheximide was absent.

Since the mRNAs for many intracellular enzymes have very short half-lives (Dixon and Webb, 1964), continued protein synthesis also implies continued gene transcription. This is significant, since the effect of DOT on induction may exert itself at any rate-limiting step from transcription to protein synthesis. A further means of verifying that the eventual decline in rates is due to enzyme degradation would be to block transcription rather than protein synthesis. Thus, Actinomycin-D could be added to a hydroxylating culture. If the mRNAs for the hydroxylase system are short-lived, then protein synthesis would soon stop, and enzyme degradation could explain any observed decline in hydroxylation rates.

Hanisch *et al.* (1980), when discussing the low DOT optimum for enzyme induction, explained their data on the basis that the 11α-hydroxylase is known to be a cytochrome-P_{450} system and low DOT optima have been described for various cytochromes in other microbial systems. It is not known whether the 19- and 11β-hydroxylases of *Pellicularia filamentosa* f.sp. *microsclerotia* are cytochrome-P_{450} systems, but the present findings would support such a hypothesis. An interesting comparison can be made with the inducible, cytochrome-P_{450} system of the yeast *Candida tropicalis* (Gmunder *et al.*, 1981). When growing on alkanes, such as hexadecane, two electron transport chains are involved. The first is the mitochondrial respiratory chain with cytochrome oxidase and the second is the microsomal electron transport chain with cytochrome-P_{450}. The primary hydroxylation of hexadecane by cytochrome-P_{450} was shown to be the rate limiting step in hexadecane uptake and subsequent oxidation to palmitate. When grown under conditions of oxygen limitation, greatly increased levels of both cytochrome

-P_{450} and cytochrome oxidase were observed. This was explained as the result of oxygen being the growth limiting factor, such that both the microsomal and mitochondrial electron transport pathways were limited by the oxygen binding rate of the oxidases. Hence, the cells' response was to increase the levels of these cytochromes in order to compensate. An analogous argument can be applied to the present work. If steroid hydroxylation by fungi is seen as a detoxification mechanism (in that the steroids become more water soluble and so can be excluded from the cell), then under oxygen-limited conditions the rate of such hydroxylation becomes limited by the oxygen supply. Hence, the cells' response is to increase the level of the hydroxylase in an attempt to compensate. Since both Hanisch *et al.* (1980) and the present work have demonstrated low DOT optima for induction of steroid hydroxylases, such an explanation appears feasible.

With regard to the rate of hydroxylase expression, Hanisch *et al.* (1980) observed a Michaelis-Menten relationship with DOT. Although the present results show the effect of oxygen limitation on enzyme expression at low DOT, the optimum at 30% of saturation was unexpected. Attempts were made, therefore, to determine the critical dissolved oxygen tension for this organism, since this may provide additional data to explain the above effect. Two approaches were used. In the first case, the oxygen consumption rate of a batch culture (under constant agitation, 500 rpm, and aeration, 500 ml/min) was followed as the DOT declined during growth. The DOT value at which the oxygen consumption rate started to decline was then taken as $(DOT)_{crit}$. The second approach was to follow the unsteady-state decline in DOT for a fermenter culture after stopping aeration. Two experiments were performed, one with air in the fermenter head space, the other with nitrogen gas. The reason for this, was to eliminate the possibility of headspace re-aeration being responsible for any deviation from zero-order kinetics. Both these experiments are depicted in Figure 6.7. From the first method (Figure 6.7a), a $(DOT)_{crit}$ of ca. 33% was

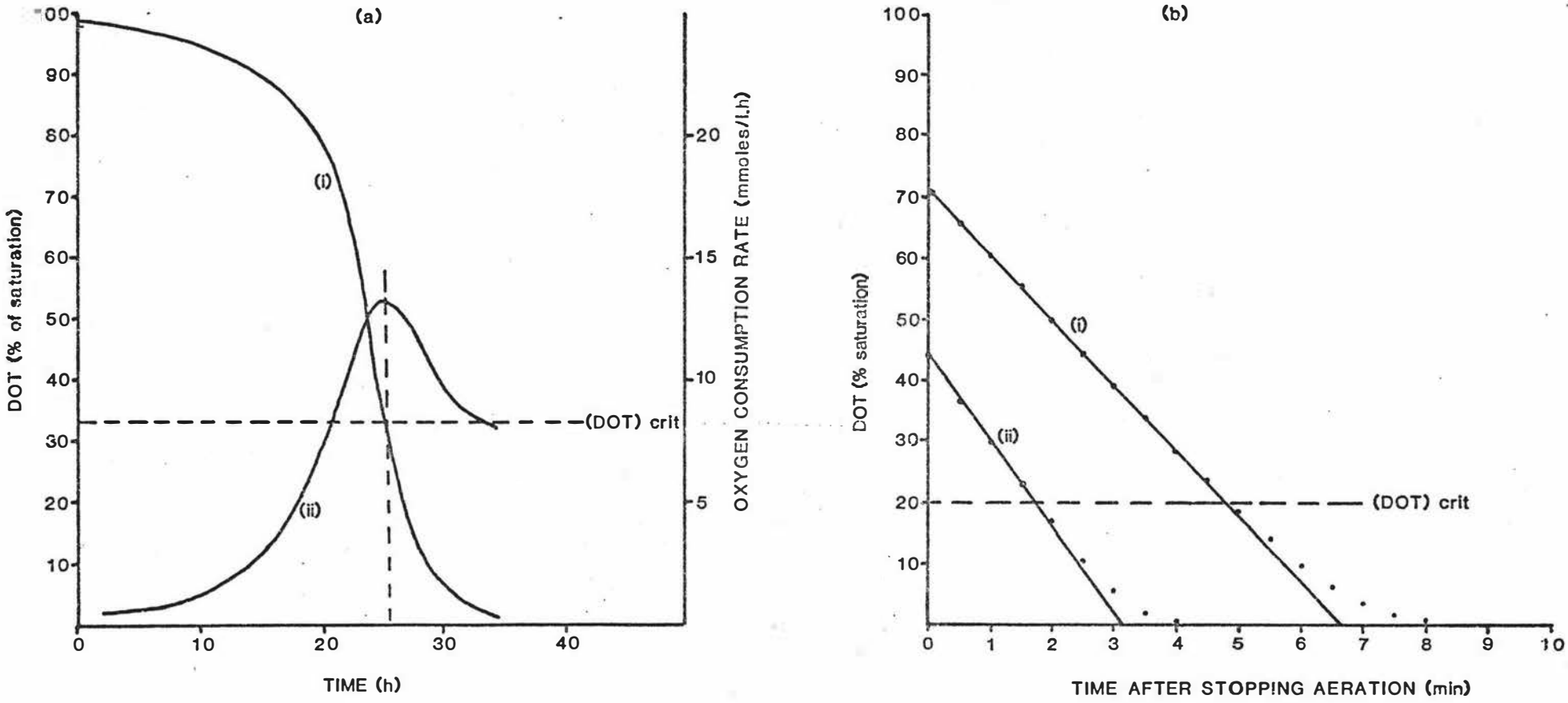


Figure 6.7 The determination of a $(DOT)_{crit}$ value for *P. filamentosa* f.sp. *microsclerotia*. (a) By following the oxygen consumption rate (ii) as DOT (i) declined during growth. (b) By measuring oxygen-exhaustion curves, with (i) air in the fermenter head-space and (ii) nitrogen in the fermenter head-space.

obtained, while from the second (Figure 6.7b) a value of ca. 20% of saturation was observed. In the first method the culture was 25 h old at the time when $(DOT)_{crit}$ was evaluated and the culture dry weight was approximately 10 g/l. In the second method the culture was only 8 h old with a dry weight of only 1 g/l. The discrepancy in $(DOT)_{crit}$ estimates could thus be explained on the basis that the dissolved oxygen electrode measures only the bulk liquid DOT while the organism responds to the actual DOT in its immediate environment. With the 25 h culture, oxygen diffusion from the bulk liquid into the mycelial mat would be much more limiting than with the dilute, 8 h culture. Thus, in the former case, a higher apparent $(DOT)_{crit}$ would be expected.

The $(DOT)_{crit}$ estimate of ca. 33% was measured under fermentation conditions very close to those used in the present study. Thus, the coincidence of this value with the optimum DOT for expression of hydroxylase activity may represent some mechanistic relationship. It is possible that above this critical DOT the metabolism of the organism changes in such a way as to lower the rate of hydroxylase reactions. This could be due to competition for oxygen with other metabolic pathways.

For most unicellular organisms critical DOT values in the range 2-10 mm Hg have been determined; these correspond to only 1-6% of the air-saturation value of 160 mm Hg (Harrison, 1972). Similar studies with filamentous fungi are complicated by their tendency to form mycelial pellets or mats. The diffusion path for oxygen from culture supernatant to the cells inside mycelial mats may be very significant. It is not surprising, therefore, that *in situ* measurements of $(DOT)_{crit}$ will result in much higher apparent values than with single celled organisms or with dilute suspensions in a Warburg apparatus; and that mass transfer conditions and the size of mycelial aggregates will influence the measured value (Finn and Fiechter, 1979). Wang and Fewkes (1977) working with fermenter cultures of *Streptomyces niveus* showed that the relationship between DOT and oxygen consumption

rate was dependent on the agitator speed. The significant observation was made that the 'pseudo critical dissolved oxygen tension' decreased with increasing shear:flow ratio (impeller speed:impeller diameter) for various turbine impellers. It is apparent, therefore, that the measured DOT optima from the present work may similarly be dependent on the mass transfer conditions used. *P. filamentosa* did not, however, form mycelial pellets and so the resistance to oxygen transfer due to such large aggregates would not be manifest. Slightly different impeller speeds were used at different DOT levels and the possible contribution of this to experimental error must be accepted. Nevertheless, the overall patterns in Figures 6.1 and 6.2 are clear. Certainly, the drop in rate of hydroxylase expression as the DOT increased above 30% of saturation cannot be explained purely on the basis of mass transfer or impeller shear effects. The impellor tip speeds used were only 1.0 and 1.3 m/s for 30% and 60% DOT, respectively. These speeds are well below those reported as causing shear damage to even delicate fungal mycelia (Hanisch *et al.*, 1980). Furthermore, if this were the reason for the decline in rates above 30% DOT, successive decreases would be expected at 45% and 60% DOT. This is not the case.

6.5 CONCLUSIONS

The data show that increased rates of hydroxylation of cortexolone can be achieved by inducing at low DOT, followed by elevation of the DOT to enhance enzyme expression. The results apply equally well to both the 19- and 11β-hydroxylations, since little significant variation in the ratio of one to the other was observed during the study. Hence, their relative production rates cannot be manipulated by variation of DOT alone.

CHAPTER 7

THE EFFECT OF GLUCOSE CONSUMPTION RATE AND GROWTH RATE ON THE 11β- AND 19-HYDROXYLATION OF CORTEXOLONE BY *P. filamentosa* f.sp. *microsclerotia*

7.1 INTRODUCTION

The involvement of NADPH in steroid hydroxylation implies that it is an energy requiring process, since this cofactor must be regenerated. In many microorganisms, NADPH is produced by the hexose-monophosphate (HMP) pathway, as glucose is metabolised to ribulose-5-phosphate. Hence, it can be postulated that anything which restricts the rate of glucose metabolism will slow the rate of hydroxylation. As discussed in section 2.3.2 , much evidence has accumulated to support this hypothesis. There is, however, a lack of definitive results. In fact, in much of the literature concerning steroid hydroxylation it is unclear how the age or state of metabolic activity of a fungal culture affects the hydroxylation.

Hanisch *et al.* (1980) reported that on addition of progesterone to a *Rhizopus nigricans* fermentation, there was an increased oxygen consumption rate that could not be explained on the basis of oxygen incorporation into the steroid molecule alone. Although the data were not given, it can be postulated that this increase was due to increased glucose metabolism. Vezina and Singh (1975) also reported a slight enhancement of respiration upon addition of progesterone to spores of *Aspergillus ochraceus* able to 11α-hydroxylate this substrate. There are many examples of fungal metabolism where an increased demand for NADPH, due to biosynthesis, results in an increased utilization of the hexose-monophosphate pathway (Berry, 1975). The hypothesis that steroid hydroxylation is linked to glucose metabolism is further supported by the knowledge that fungal spores performing steroid hydroxylations have a definite requirement for an energy source (Vezina *et al.*, 1968).

Mosbach and Larsson (1970) and Maddox *et al*. (1981) have performed steroid hydroxylations using immobilized mycelia. In both cases, attempts to reuse the immobilized cells resulted in a gradual loss of activity. Maddox *et al*. (1981) suggested that this effect might have been due to lack of mycelial growth in the immobilized system. It was of interest therefore to investigate the effect of growth rate on steroid hydroxylation. In general, the HMP pathway is more active in rapidly growing cells, in which the biosynthetic demand for NADPH is high (Berry, 1975). Thus, it is conceivable that hydroxylase activity may be influenced by growth rate via the supply of NADPH. Optimal specific growth rates have been described in certain antibiotic fermentations (Queener and Swartz, 1979) where production rates are greatly influenced by this parameter. In these cases low growth rates are normally required to trigger production of the secondary metabolites. Gmunder *et al*. (1981) studied the influence of dilution rate on hexadecane assimilation by *Candida tropicalis* in chemostat culture. They measured the specific levels of enzymes and cytochromes involved in the assimilation and metabolism of hexadecane (the first and rate limiting step involves hydroxylation by cytochrome-P_{450}). Cytochrome-P_{450}, and cytochromes a, b and c increased almost linearly with dilution rate. Further examples of the influence of dilution rate on enzyme synthesis in chemostat culture have been described, although the mechanism by which gene action is controlled, is still unclear (Melling, 1970).

7.2 BATCH AND FED-BATCH EXPERIMENTS

A series of batch fermentations was performed to investigate the requirement for glucose metabolism in the hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia*. The fermentations were performed, basically, as described in section 3.5.2. DOT was controlled via the aeration rate (section 3.5.2.1), but during the course of the fermentations the agitation speed required periodic increments to assist the control system, and typically varied between 400 and 800 rpm.

The fermentation described in section 6.3 (Fig. 6.3), where both induction and expression of hydroxylase activity were performed at a DOT of 30% of saturation, was continued past the initial period (for rate measurement) and the complete data are shown in Figure 7.1. It is apparent from this figure that hydroxylation virtually ceased when glucose was exhausted (despite the presence of sufficient steroid substrate). Although DOT was not maintained in-control, its effect would not account for the rapid cessation of hydroxylation.

A further fermentation was performed in which glucose was intermittently fed to the culture to prevent its exhaustion. The time-course for this fermentation is shown in Figure 7.2. (The early period of this fermentation is that described in section 6.3 (Fig. 6.4), where induction and expression of hydroxylase activity were performed at DOT values of 15% and 30% of saturation, respectively). The data clearly show that hydroxylation of cortexolone was maintained by continued glucose consumption, although the rate of transformation decreased from its initial value. DOT was again controlled poorly during much of this fermentation.

To verify the effect that hydroxylation is apparently dependent on glucose consumption, a further fermentation was performed. In this case glucose was allowed to become exhausted and then, about 7 hours later, glucose was fed to the culture. Steroid substrate was fed continuously (30 mg/l.h) to the culture to ensure a satisfactory supply throughout the experiment. The time-course, given in Figure 7.3, shows that the hydroxylation rate did indeed slow almost to zero as glucose was exhausted; it then resumed upon glucose addition, although at a lower rate.

A final fed-batch fermentation was performed where cortexolone (30 mg/l.h) and glucose (0.65 g/l.h) were continuously fed to a batch culture and DOT was to be controlled at 30% of saturation. The results are shown in Figure 7.4. As a result of poor DOT control and an eventual

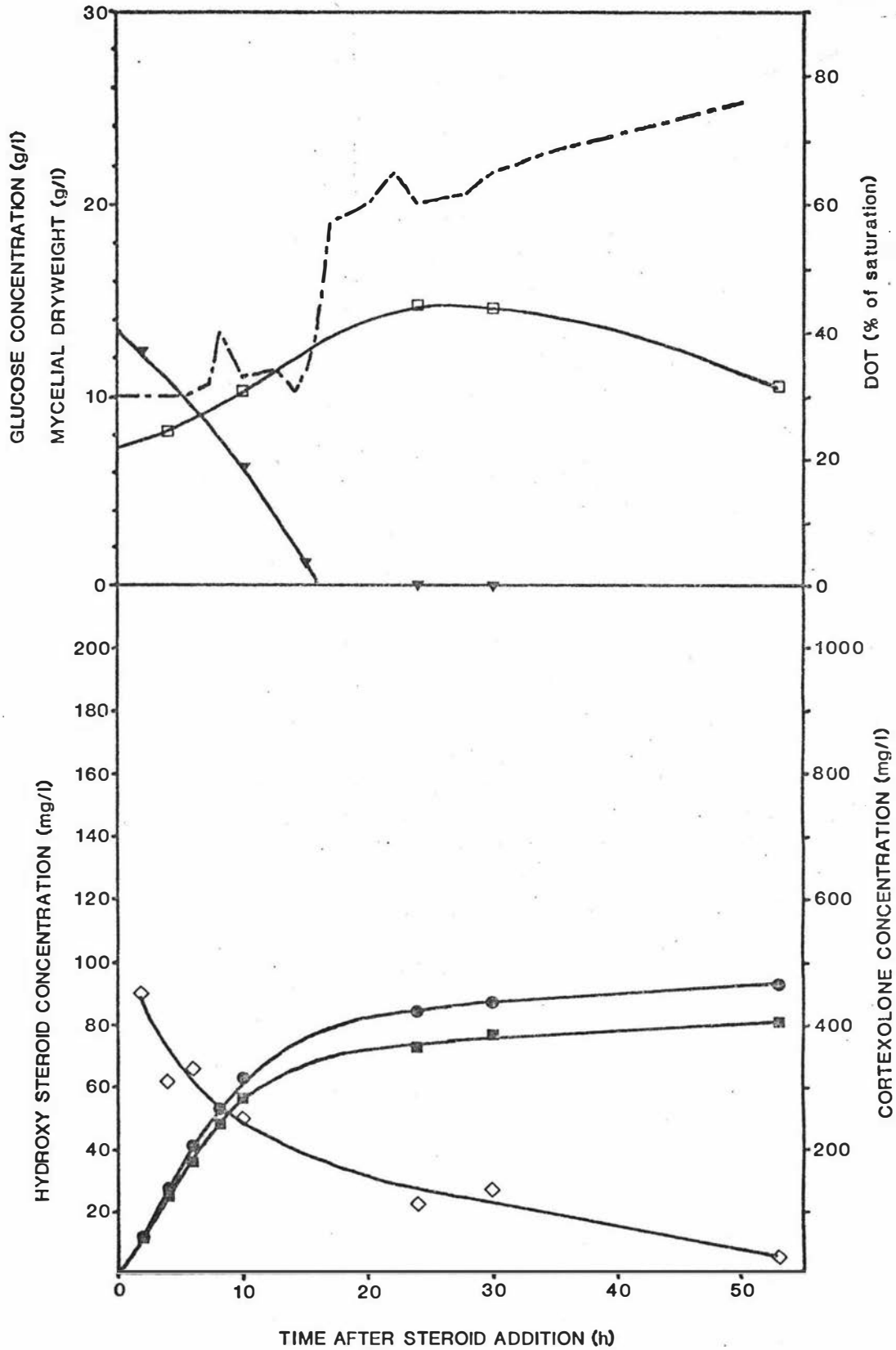


Figure 7.1 The course of cortexolone hydroxylation by a fermenter culture (initial cortexolone concentration, 2.5 g/l). Mycelial dry weight (□), glucose (▼), DOT (– —), 19-hydroxycortexolone (●), 11β-hydroxycortexolone (■), cortexolone (◇).

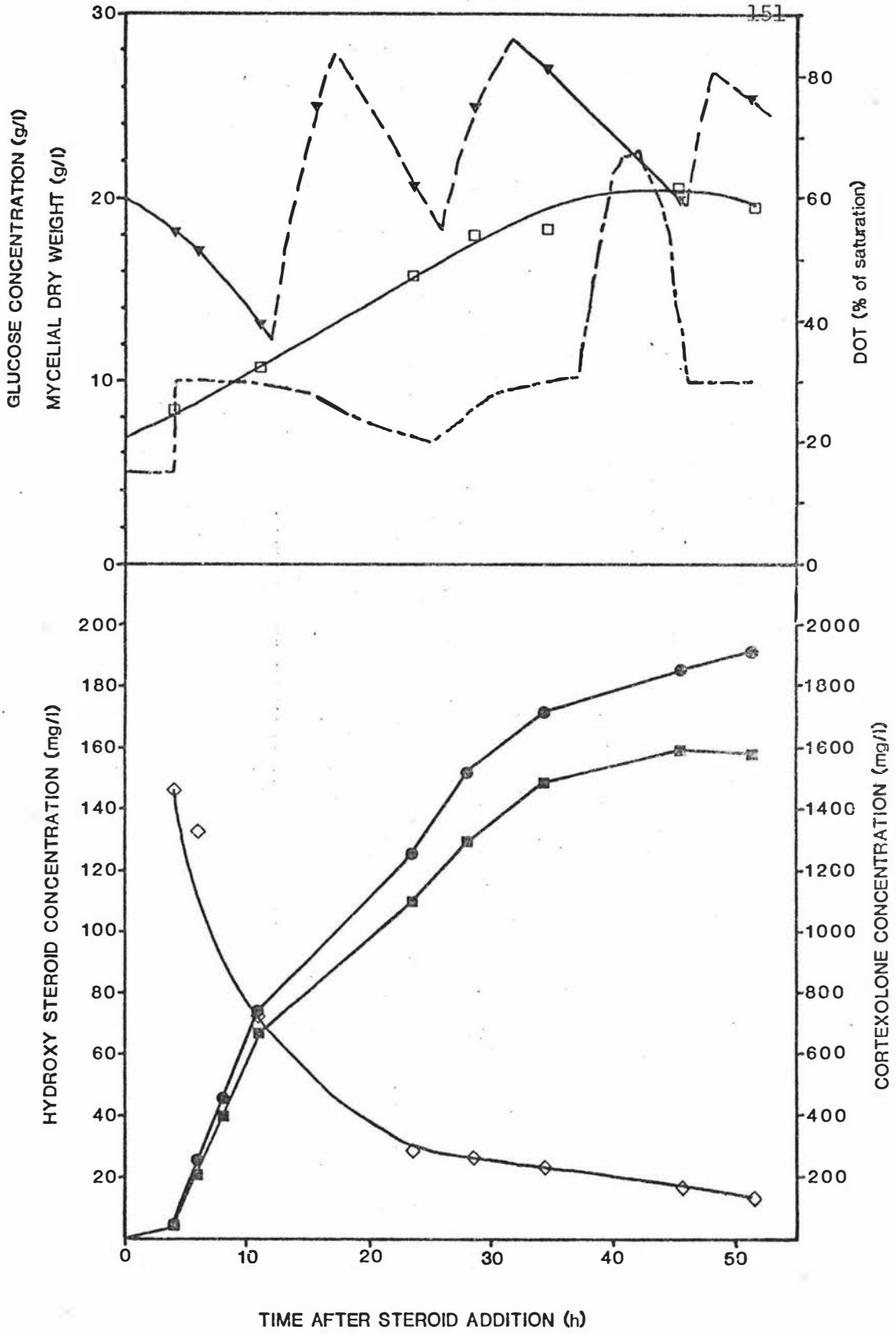


Figure 7.2 The course of cortexolone hydroxylation by a fermenter culture which was periodically fed with glucose. Legend as for Figure 7.1.

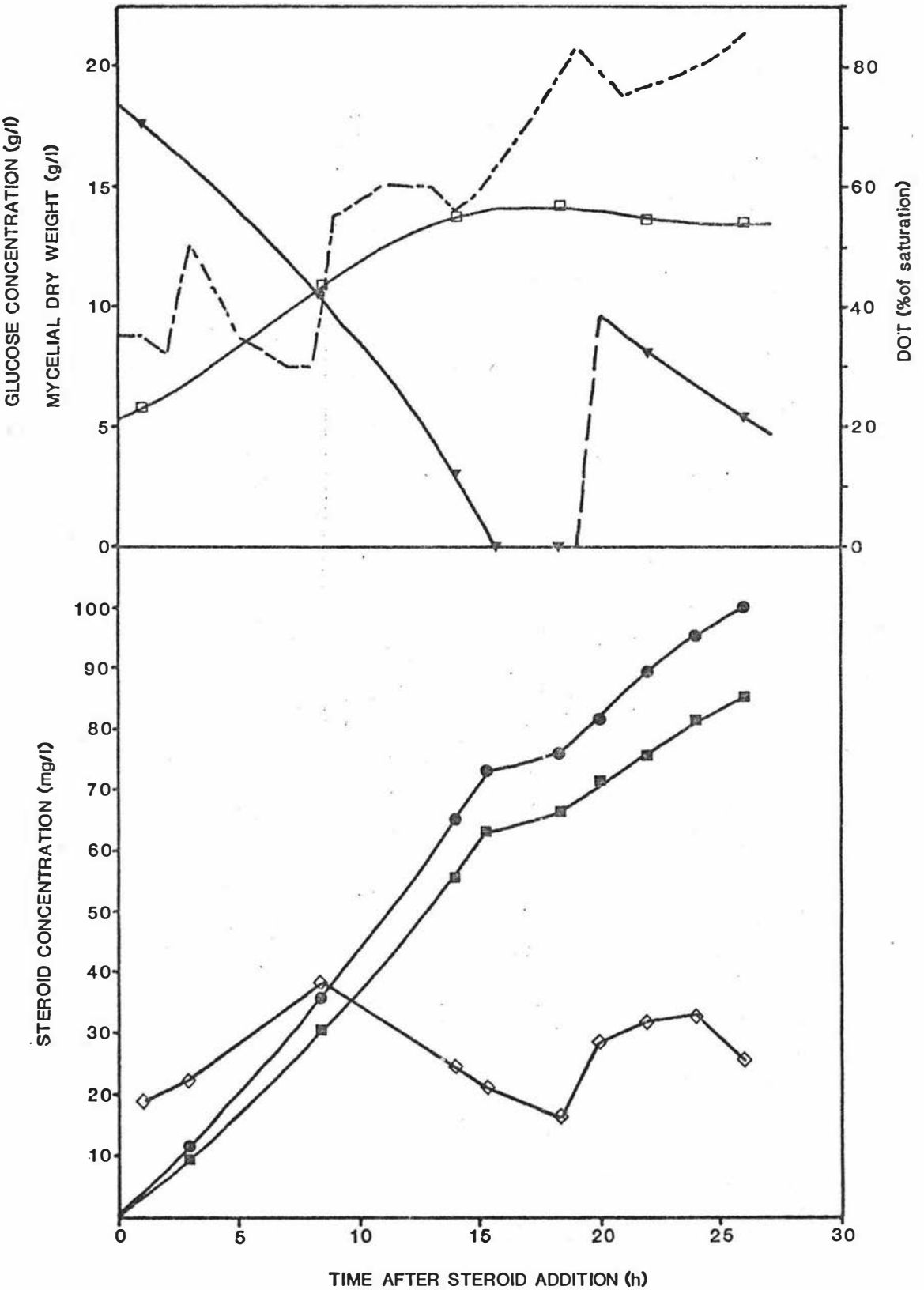


Figure 7.3 The effect of glucose-exhaustion, and subsequent glucose-feeding, on the course of cortexolone hydroxylation. Cortexolone was continuously fed to the culture (30 mg/l.h). Legend as for Figure 7.1.

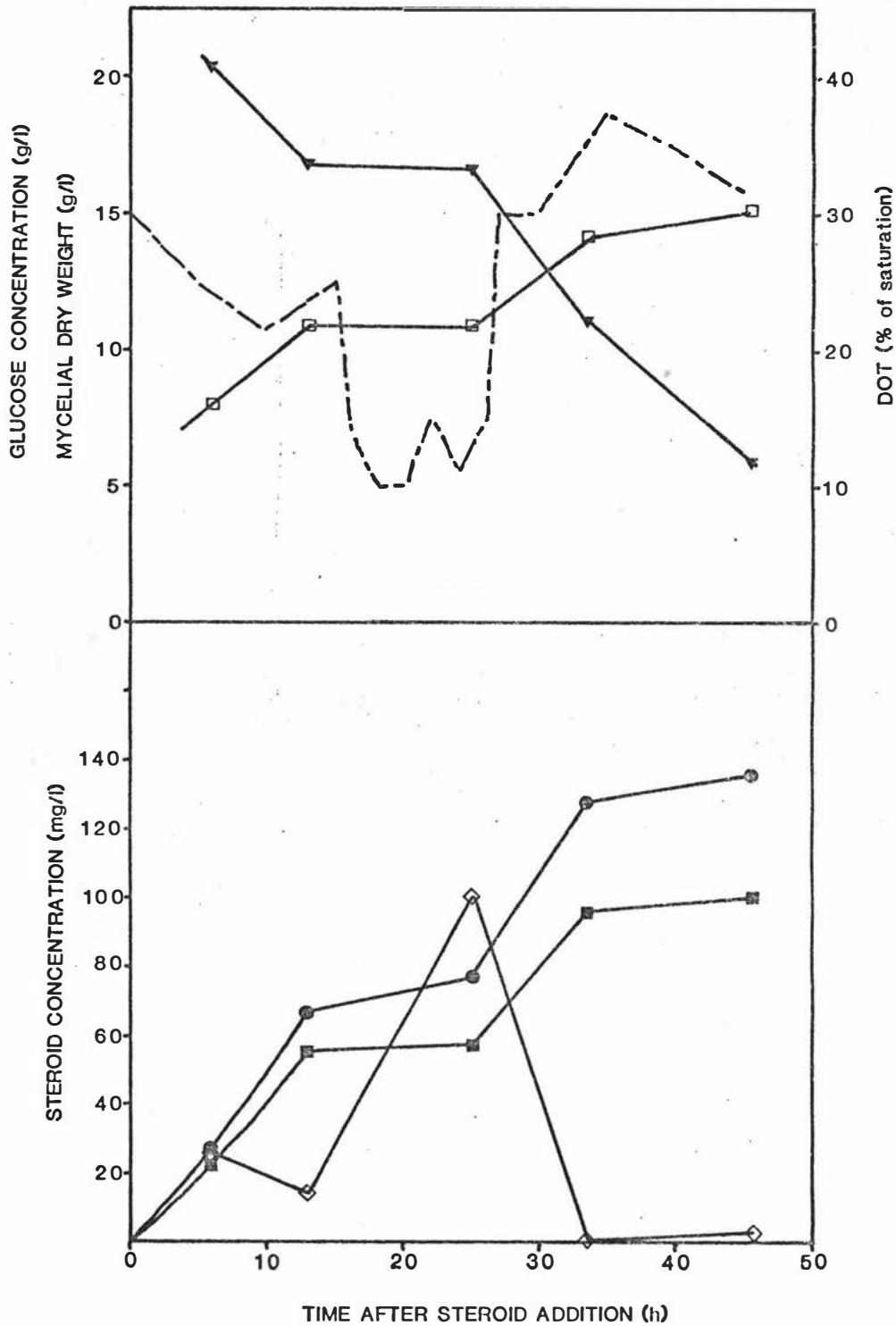


Figure 7.4 The course of cortexolone hydroxylation by a culture which was continuously fed with glucose and cortexolone. Legend as for Figure 7.1. See text for details.

breakdown in steroid feeding (after approximately 33 h) the initial aim of presenting the most favourable conditions for a high yield transformation was not achieved. However, the results do reinforce the previous findings with respect to the DOT effect. Thus, when the culture DOT declined to approximately 10% of saturation, the hydroxylation rate greatly declined and the substrate level increased. Upon resumption of DOT control at ca. 30% of saturation, the hydroxylation recommenced and eventually ceased only when the steroid substrate was exhausted.

These results demonstrated that further investigation of the effect of glucose metabolism on the hydroxylation process would be useful. However, faults with the present experimental system were apparent:

(i) The DOT control system was unsatisfactory for prolonged fermentations and an improved system was required.

(ii) In batch mode, the culture growth rate and glucose consumption rate are continually changing and hence the organism is not in a constant metabolic state. Thus it would be difficult to meaningfully interpret hydroxylation rate measurements with respect to these factors.

The chemostat technique provides for controlled conditions with the organism in a constant metabolic state. Thus, the final series of experiments utilized this method. The problem of DOT control was solved by building a controller which automatically adjusted agitation speed as a means of controlling DOT. This is fully described in Appendix A. The device was successful and DOT control ceased to be a problem.

7.3 CHEMOSTAT FERMENTATIONS

7.3.1 Introduction

Under chemostat operation a fungal culture can be maintained in a constant metabolic state, thus allowing well-

defined transformation experiments to be performed. The hydroxylation reactions were carried out batch-wise by adding cortexolone to a steady-state, chemostat culture and measuring the rate of formation of products.

To determine the effects of growth rate and glucose consumption rate independently, fermentations were required not only at different dilution rates, but also with varying feed-medium glucose concentrations. By maintaining a constant yeast extract concentration in the feed-medium while varying the glucose level, it was possible to manipulate the specific glucose consumption rate of the culture.

7.3.2 Experimental Method

The basic glucose/yeast extract medium (pH 6.0) was used, as in earlier batch work. The yeast extract concentration was maintained at 2.0 g/l in all the experiments. The glucose concentration was varied between 0 and 8.9 g/l, initial glucose levels being determined after autoclaving.

A new culture was established for each steady-state. This not only avoided problems associated with strain degeneration in chemostat, but, more importantly, prior to steroid addition the culture was entirely uninduced, as with batch fermentations.

Steady-state was assumed when successive samples, taken over at least one residence time, showed no significant variation. Because of the highly heterogeneous nature of filamentous fungal cultures it was anticipated that some internal feed-back of biomass may occur. The relationship between the dilution rate (D) and the organism's specific growth rate (μ), in such a situation, is

$$\mu = A.D \quad \text{(Pirt, 1975)}$$

where A is the biomass-concentration factor. In many of the chemostat experiments, the culture was nitrogen-limited. In these situations the concentration factor was calculated from the ratio of

$$\frac{\text{nitrogen concentration in feed}}{\text{mycelial nitrogen concentration}}$$

The degree of concentration was only slight, and this simple calculation allowed the true specific growth rate to be accurately obtained.

To commence the hydroxylation, cortexolone (0.74 g/l) was added, dissolved in dimethylformamide. Samples were taken regularly over the following 10 hour period. The effect of dilution, on product concentrations, was corrected for as described in section 3.8.2. Hydroxylation rates were thus calculated from the linear portion of plots of corrected steroid concentration versus time, when induction of activity was complete.

The dissolved oxygen tension was accurately controlled at 30% of saturation (using agitation rate control, as described in section 3.5.2.1) during all of the continuous culture experiments, so removing this as a possible variable.

7.3.3 Results and Discussion

Initially, three fermentations were performed, at different dilution rates, with a feed-medium glucose concentration (S_o) of 8.9 g/l. The steady-state culture parameters and the specific hydroxylation rates obtained are shown in Figure 7.5. These data, plus some other calculated parameters are reproduced in Table 7.1. Although the specific hydroxylation rates (q_{19} and $q_{11\beta}$) increased slightly with dilution rate (D), the glucose consumption rate (q_{gl}) varied almost in proportion to this. Thus, with both growth rate and glucose consumption rate varying simultaneously no clear interpretation is possible. An interesting feature of these data is that as dilution rate increased, the mycelial percentage nitrogen (MPN) also increased, while the mycelial dry weight (MDW) decreased. The nitrogen concentration of the feed medium was constant at 0.21 g/l. In comparing this with the mycelial nitrogen concentration (MNC) data of Table 7.1, it is clear that the cultures were nitrogen limited at

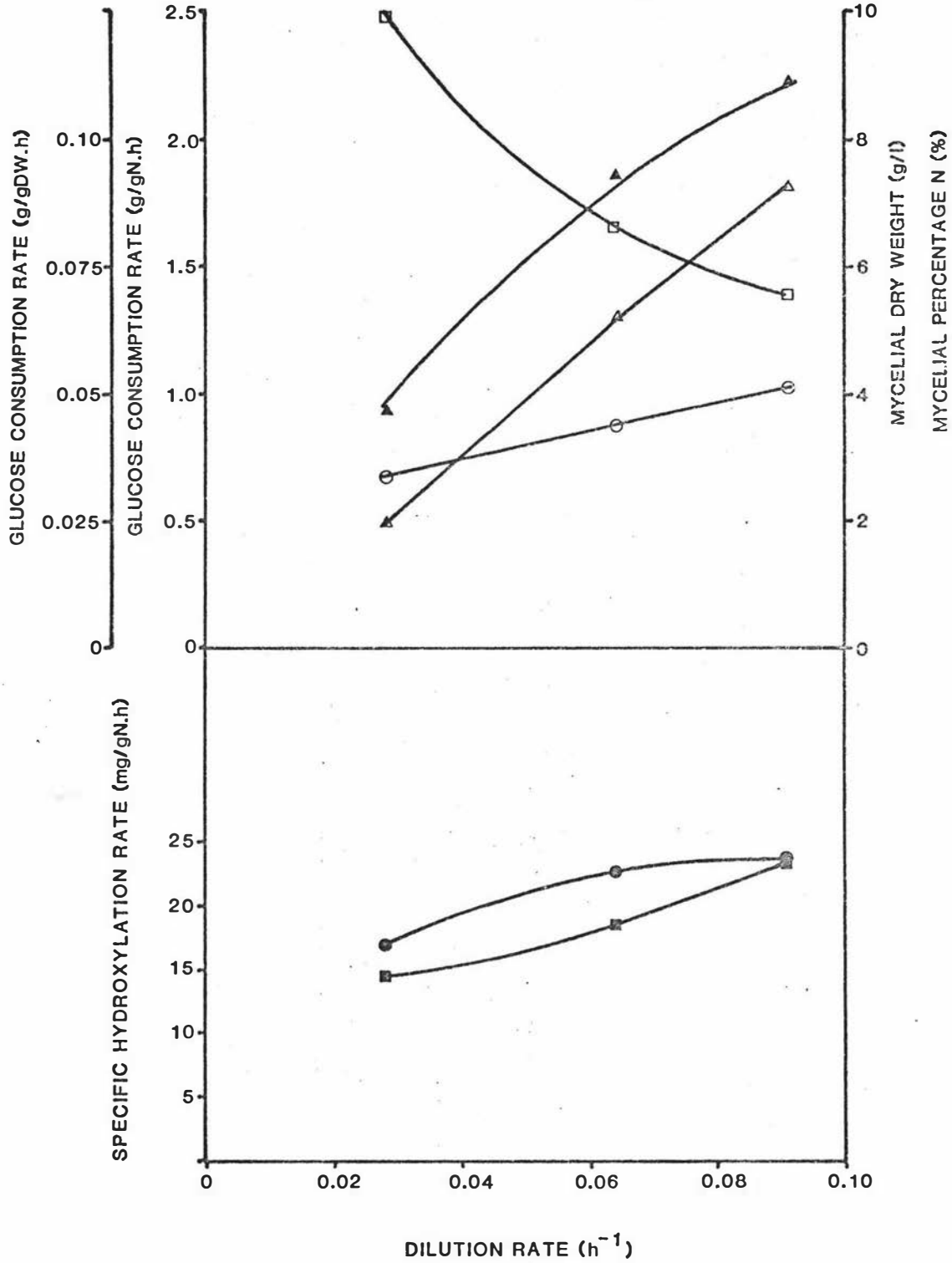


Figure 7.5 Effect of dilution rate on the specific rates of cortexolone hydroxylation. Specific rate of 19-hydroxylation (●), specific rate of 11β-hydroxylation (■), mycelial dry weight (□), mycelial percentage nitrogen (O), specific glucose consumption rate; g/g DW.h (△), g/gN.h (▲).

Table 7.1 The complete experimental data set for Figure 7.5

D (h^{-1})	μ^a (h^{-1})	So (g/l)	S (g/l)	MDW (g/l)	MPN %($^w/_w$)	MNC (g/l)	q_{gl} (g/g N.h)	(g/g DW.h)	$q_{11\beta}$ (mg/g N.h)	q_{19} (mg/g N.h)
0.028	0.022	8.9	0.0	9.90	2.7	0.267	0.93	0.025	14.5	17.0
0.064	0.058	8.9	2.2	6.60	3.5	0.231	1.86	0.065	18.4	22.9
0.091	0.083	8.9	3.3	5.60	4.1	0.230	2.23	0.091	23.5	23.7

[a] for explanation of why $\mu \neq D$, refer to section 7.3.2

all dilution rates. It can be concluded, therefore, that the change in MDW is the result of changing levels of carbon compounds stored in the mycelium. For this reason it was clear that MDW poorly represented the amount of active biomass in the fermentation. Hence, the mycelial nitrogen concentration was chosen instead, and specific hydroxylation rates are expressed in terms of mg/g N.h.

In order to investigate the effect of glucose consumption rate independently of growth rate, fermentations were performed at a constant dilution rate of 0.064 h^{-1}. The feed medium glucose concentration (S_o) was successively lowered from 8.9 to 0 g/l, with the results depicted in Figure 7.6. (The complete data set can be found in Table 7.2). The glucose consumption rate (q_{gl}), expressed in terms of mycelial nitrogen, decreased almost in proportion to S_o. However q_{gl}, expressed in terms of mycelial dry weight, showed a Michaelis-Menten relationship with S_o. The reason for this difference was the increased incorporation of carbon storage materials into the mycelia, as is indicated by the declining MPN as S_o increased. The specific 19-hydroxylation rate (q_{19}) is replotted in Figure 7.7 against q_{gl} (both in terms of N and DW). Clearly, an optimum was obtained. Expressed in terms of mycelial dry weight, the observed maximum rate of 19-hydroxylation (ca. 51 mg/g N.h) is approximately 3.9 mg/g DW.h. Comparing this with the rate obtained in batch culture with DOT-control at 30% of saturation, ca. 0.8 mg/g DW.h, a substantial improvement in hydroxylation rate has been achieved.

The decline in hydroxylation rate as q_{gl} approaches zero verifies the finding from batch fermentation (section 7.2) that hydroxylation is dependent on glucose metabolism. At $q_{gl} = 0$, some hydroxylation still proceeds since the organism is using the yeast extract as a carbon and energy source, and thus some carbon metabolism is occurring. These results do not, however, differentiate between whether the effect of q_{gl} is on induction or expression of the hydroxylase system. That is, the decline in hydroxylation rate as q_{gl} approaches zero may be due to a low level of induction rather

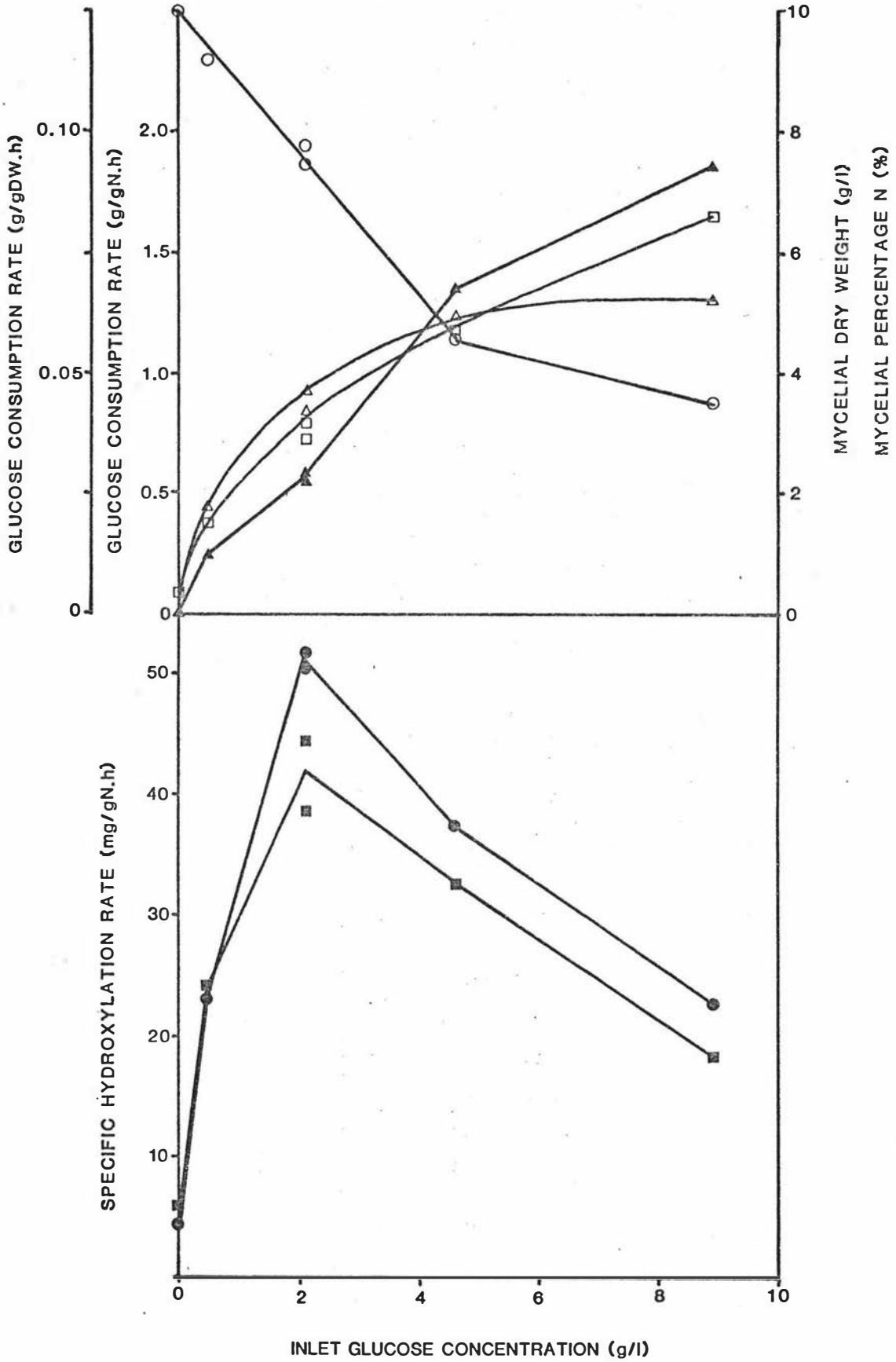
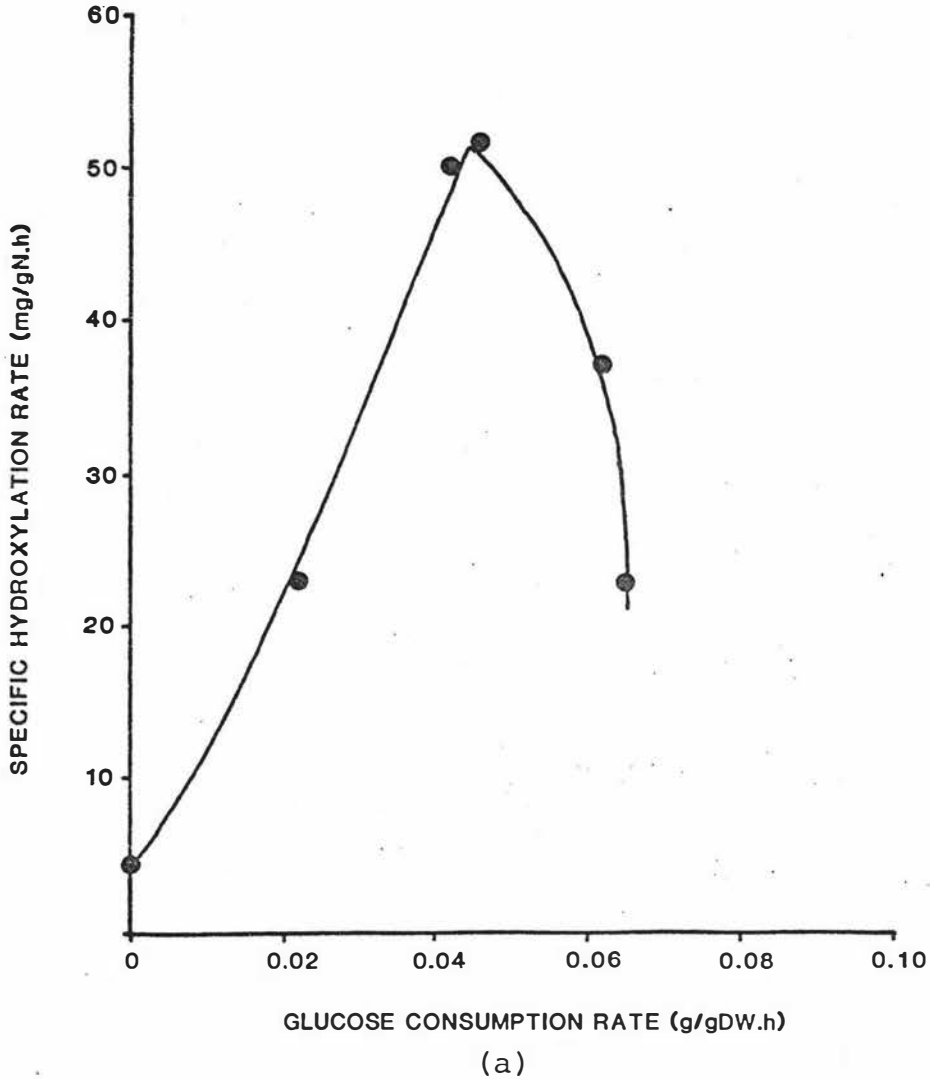


Figure 7.6 Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at D = 0.064 h^{-1}. Legend as for Figure 7.5

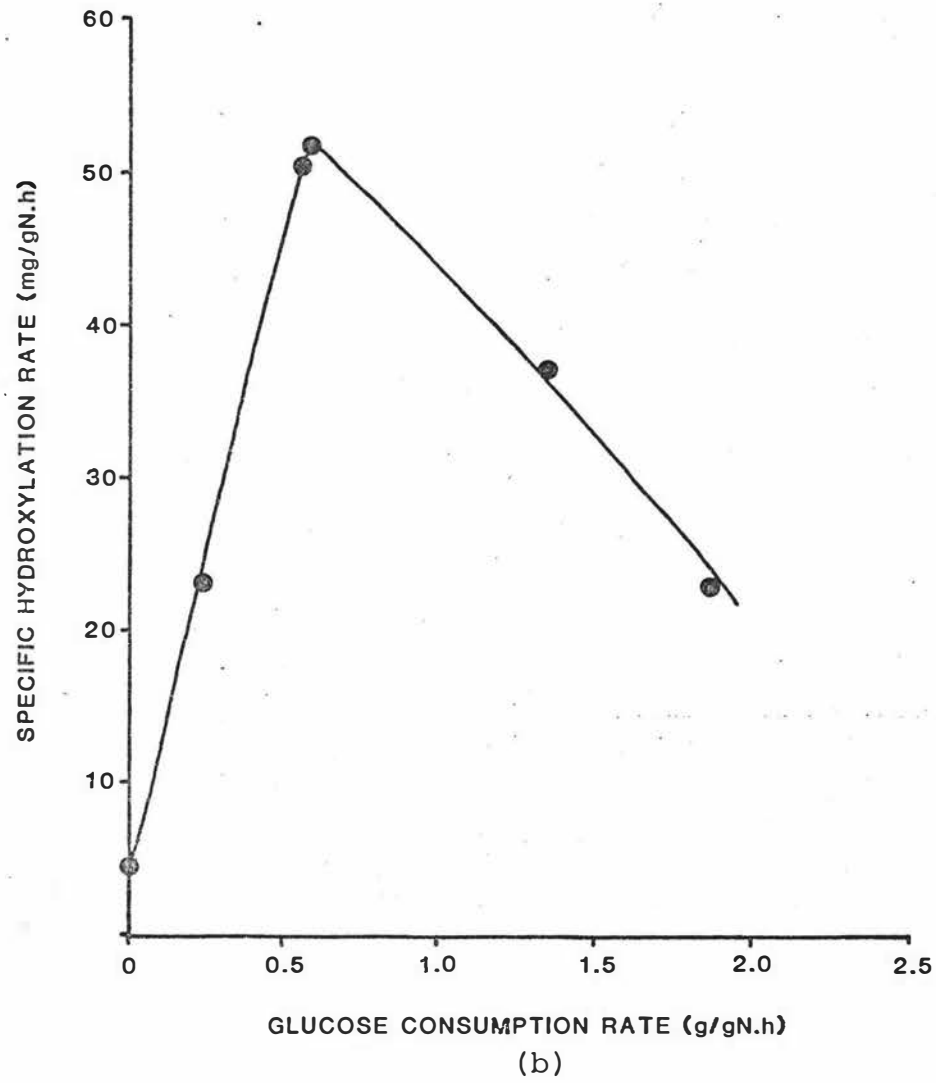
Table 7.2 The complete experimental data set for Figure 7.6.

D (h^{-1})	μ^a (h^{-1})	So (g/l)	S (g/l)	MDW (g/l)	MPN %($^w/_w$)	MNC (g/l)	q_{gl} (g/g N.h)	q_{gl} (g/g DW.h)	$q_{11\beta}$ (mg/g N.h)	q_{19} (mg/g N.h)
0.064	-	0	0	0.37	10.0	0.037	0	0	5.4	4.7
0.064	-	0.46	0.0	1.54	9.2	0.142	0.24	0.022	24.1	23.2
0.064	0.056	2.10	0.0	3.20	7.45	0.238	0.56	0.042	38.7	50.4
0.064	0.059	2.10	0.0	2.90	7.80	0.226	0.59	0.046	44.5	51.8
0.064	0.063	4.60	0.0	4.70	4.57	0.215	1.35	0.062	32.6	37.2
0.064	0.058	8.90	2.2	6.60	3.50	0.230	1.86	0.065	18.4	22.9

[a] for explanation of why $\mu \neq D$, refer to section 7.3.2; μ could only be calculated for N-limited cultures, i.e., when MNC > 0.21 g/l.



(a)



(b)

Figure 7.7 Plots of q_{19} versus q_{gl}, expressed:
(a) in terms of mycelial dry weight, and
(b) in terms of mycelial nitrogen

than purely due to poor expression of the hydroxylase system. However, the results from batch experiments (section 7.2) clearly indicate that a fully induced culture, which is actively hydroxylating, will cease doing so when glucose is exhausted.

The decrease in hydroxylation rates as q_{gl} increased beyond the optimum point indicates that either glucose repression or inhibition of hydroxylation was occurring. To clarify this, a fermentation was established under the conditions required for the optimum point for hydroxylations, of Figure 7.6. However, 8 hours after cortexolone addition exogenous glucose was added to increase the glucose consumption rate. Further steroid substrate (0.2 g/l) was also added at this point to maintain its presence in excess. Samples were taken during the entire experiment and the measured time course is shown in Figure 7.8. The hydroxylation rate declined only slightly during the 4 hours subsequent to the elevation of q_{gl}. If the glucose-effect on hydroxylation was due to direct inhibition of the enzyme system a rapid drop in the hydroxylation rate would have been expected. Hence, it appears that glucose-repression of enzyme synthesis is manifest in the regulation of the steroid hydroxylase(s) of *P. filamentosa* f.sp. *microsclerotia*. If these enzymes undergo rapid "turn-over", for which some evidence has been described (section 5.6), then the slight decline in hydroxylation rates after exogenous glucose addition can be explained on the basis of a glucose-repression model. The phenomenon of catabolite repression has been shown to act at the level of gene transcription (Demain *et al.*, 1979). As most mRNAs for intracellular enzymes have very short half-lives (Dixon and Webb, 1964; Terui, 1972), this implies that if continual protein synthesis is occurring, then continual transcription must also occur. Thus, for a rapidly degraded and resynthesised enzyme, a change in the rate of transcription would manifest itself relatively quickly as a change in enzyme level, and hence activity. In respect of this, it can be difficult to resolve the effects of enzyme repression and inhibition for such rapidly "turned over" enzymes (Drew and Demain, 1977).

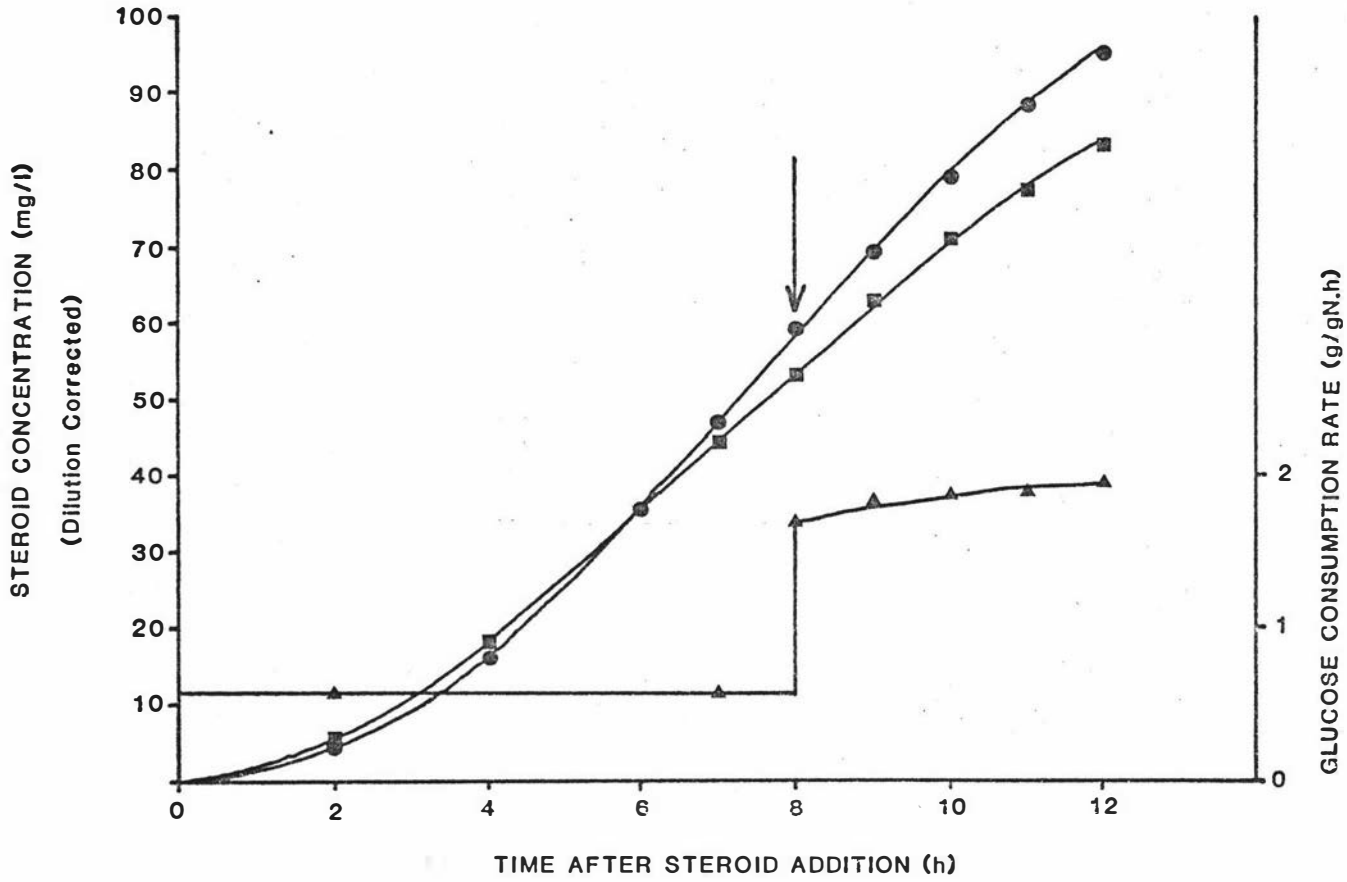


Figure 7.8 Time course of cortexolone hydroxylation, with a "step" change in glucose consumption rate at 8 h. (See text for experimental details). Concentration of 19-hydroxycortexolone (●), concentration of 11β-hydroxycortexolone (■), specific glucose consumption rate (▲). Arrow indicates time of exogenous glucose addition.

The view has been expressed that the degree of catabolite regulation seems to depend upon the rate of metabolism of appropriate substrates, rather than simply their concentrations in the growth medium. Hence, it involves the flux of substrate into the cell (Drew and Demain, 1977; Gray and Bhuwapathanapun, 1980). It may also involve the energy state of the cell, since a high rate of catabolism implies a high cellular energy-charge. Many examples have been described where high cellular levels of ATP or energy-charge (defined as the ratio ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP])) cause repression of certain enzymes, typically those involved in secondary metabolism (Drew and Demain, 1977). At the present time the mechanism of energy-charge regulation is not well understood. The phenomenon of carbon-catabolite repression in certain bacteria has been shown to act according to the scheme in Figure 7.9 (Pastan and Perlman, 1970). It involves the binding of a complex between cyclic adenosine-3',5'-monophosphate (cAMP) and a cAMP receptor protein (CRP) to the promotor site of an operon; this binding stimulates the initiation of transcription by RNA-polymerase. When glucose is present it inhibits adenylate cyclase, the enzyme that converts ATP to cAMP, thus decreasing the concentration of cAMP and inhibiting the transcription by RNA-polymerase of operons subject to this control. Although glucose has been most extensively studied as an effector of this form of regulation, other rapidly used carbon sources are also active (Demain *et al.*, 1979). This phenomenon has been described in a wide variety of bacteria, yeast, and moulds; however a generalised mechanism involving cAMP cannot, as yet, be assumed in all cases. The results presented here do not define the mechanism of repression. However, the observation of this phenomenon, which does not appear to have been previously described for a microbial steroid-hydroxylation process, is of considerable interest. For an industrial process the removal of repression, if present, may offer substantial improvements in hydroxylase titre and hence hydroxylation rate.

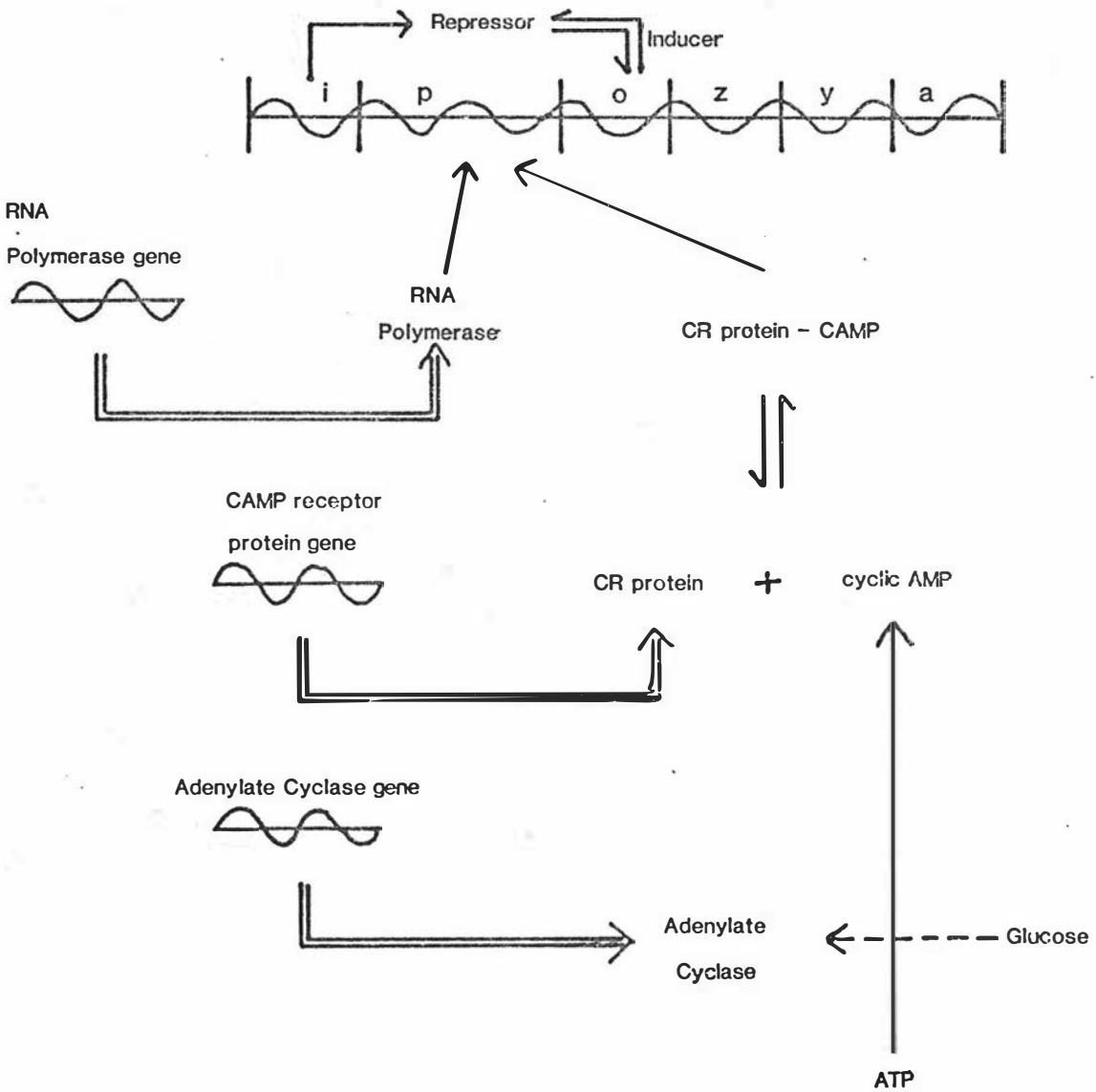


Figure 7.9 Scheme depicting the action of cyclic AMP on the lac operon (after Pastan and Perlman, 1970). See text for explanatory details.

Further fermentations, conducted under similar conditions with different S_o levels, were performed at dilution rates of 0.028 h^{-1} and 0.119 h^{-1}. The results are presented in Figures 7.10 and 7.11, with the full data sets in Tables 7.3 and 7.4, respectively. Similar relationships between hydroxylation rate and q_{gl} were observed.

By comparing the data from Tables 7.2, 7.3, and 7.4, it is apparent that in all three cases there is an optimum glucose consumption rate for hydroxylation (although in the case of the data in Table 7.4 (D = 0.119 h^{-1}) further experiments would be required to determine its exact position). However, the optimum q_{gl} varies with dilution (growth) rate as shown in Figure 7.12. Thus, to determine if the specific growth rate does have an independent effect on hydroxylation, it would not be valid to simply compare results at different dilution rates at which the q_{gl} values are the same. This is because an organism's energy requirements are normally proportional to dilution (growth) rate (neglecting any maintenance energy). Thus, the consumed glucose can be envisaged as comprising two components (under non-growth-limiting conditions) i.e.,

(i) that proportion which is metabolised directly to provide energy and carbon for growth,

and (ii) the remaining proportion which is converted to other products (in *P. filamentosa* f.sp. *microsclerotia* storage material appears to be a major product)

Therefore, two cultures, each at different growth rates, will have different distributions between these two components given the same glucose consumption rate. If the degree of glucose-repression is related to the excess glucose uptake, then the same q_{gl} value, for cultures at different growth rates, will not exert the same repressive influence. Thus, a culture parameter is required which is independent of growth rate, but which describes the degree of glucose-repression, in order to compare fermentations at different growth rates.

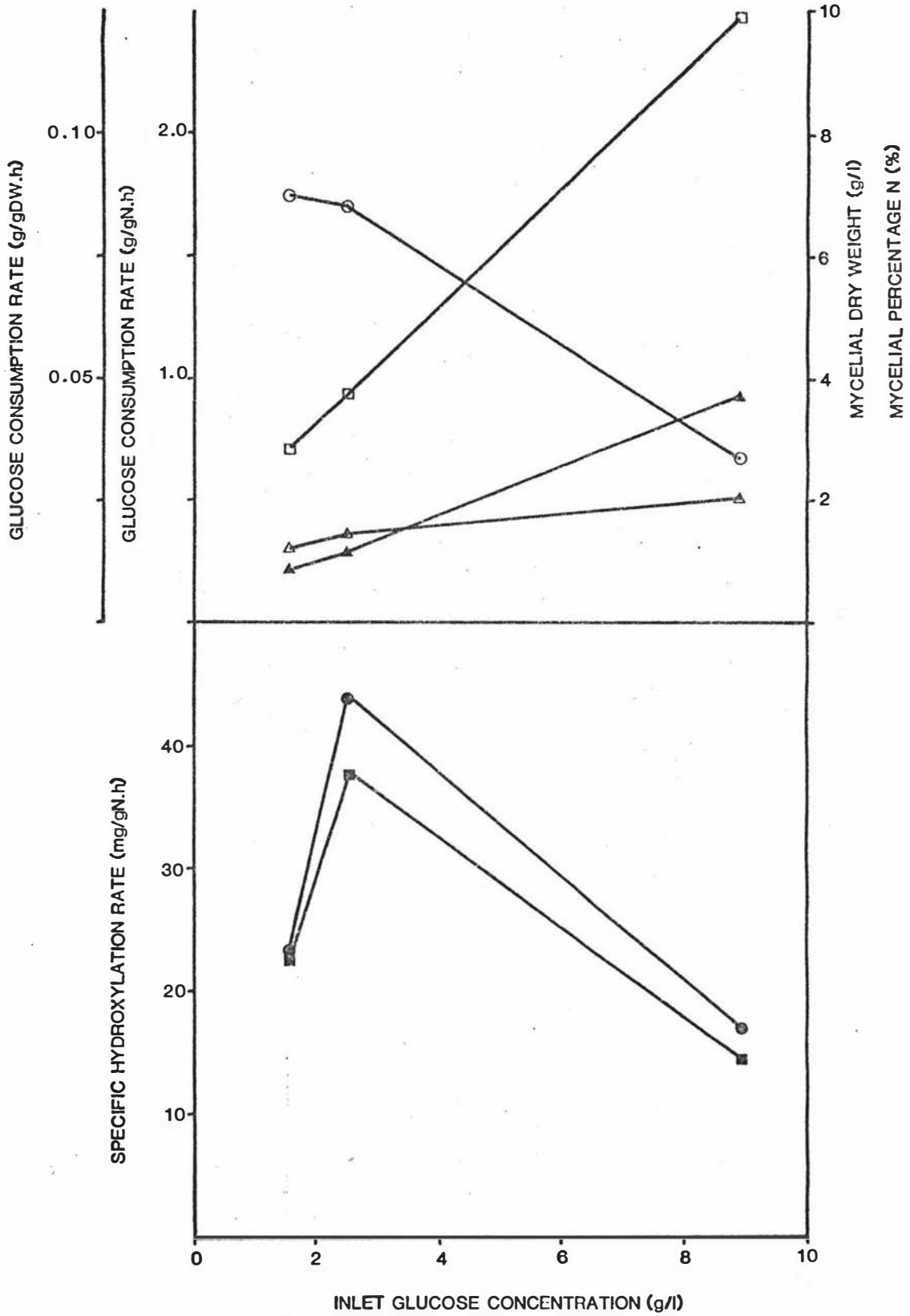


Figure 7.10 Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at D = 0.028 h^{-1}. Legend as for Figure 7.5.

Table 7.3 The complete experimental data set for Figure 7.10

D (h^{-1})	μ [a] (h^{-1})	So (g/l)	S (g/l)	MDW (g/l)	MPN %($^{w}/_{w}$)	MNC (g/l)	q_{gl} (g/g N.h)	q_{gl} (g/g DW.h)	$q_{11\beta}$ (mg/g N.h)	q_{19} (mg/g N.h)
0.028	-	1.58	0.1	2.80	7.0	0.196	0.21	0.015	22.6	23.3
0.028	0.023	2.50	0.0	3.76	6.8	0.256	0.28	0.018	37.7	43.9
0.028	0.022	8.90	0.0	9.90	2.7	0.267	0.93	0.025	14.5	17.0

[a] for explanation of why $\mu \neq D$, refer to section 7.3.2; μ could only be calculated for N-limited cultures, i.e., when MNC $\geq$ 0.21 g/l.

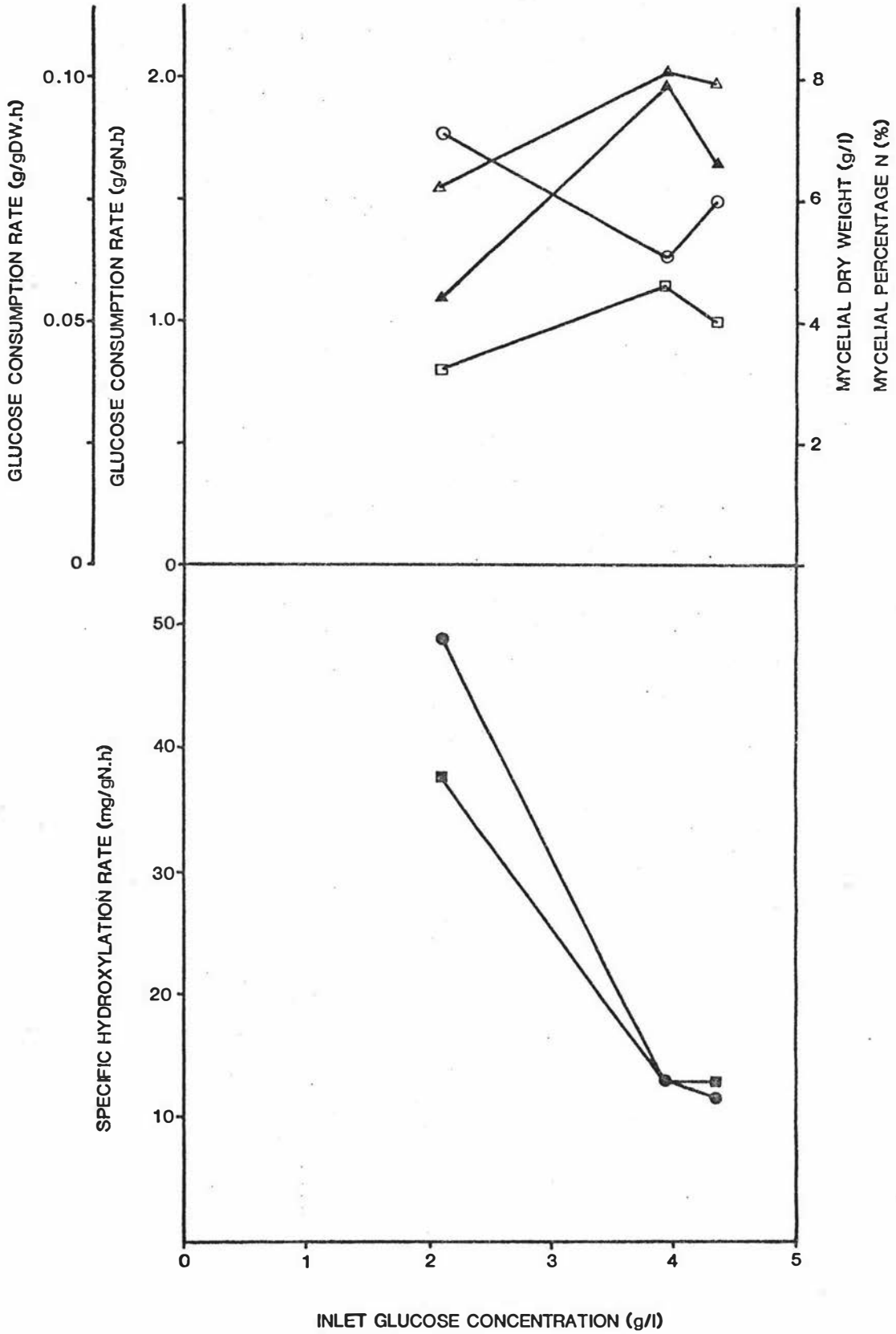


Figure 7.11 Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at $D = 0.119\ h^{-1}$. Legend as for Figure 7.5.

Table 7.4 The complete experimental data set for Figure 7.11

D (h^{-1})	μ^a (h^{-1})	So (g/l)	S (g/l)	MDW (g/l)	MPN %($^w/_w$)	MNC (g/l)	q_{gl} (g/g N.h)	q_{gl} (g/g DW.h)	$q_{11\beta}$ (mg/g N.h)	q_{19} (mg/g N.H)
0.119	0.110	2.10	0.0	3.2	7.1	0.227	1.10	0.078	37.7	48.9
0.119	0.106	3.93	0.0	4.6	5.1	0.235	1.97	0.101	12.8	12.8
0.119	0.104	4.35	1.0	4.0	6.0	0.240	1.65	0.099	12.8	11.7

[a] for explanation of why $\mu \neq D$, refer to section 7.3.2.

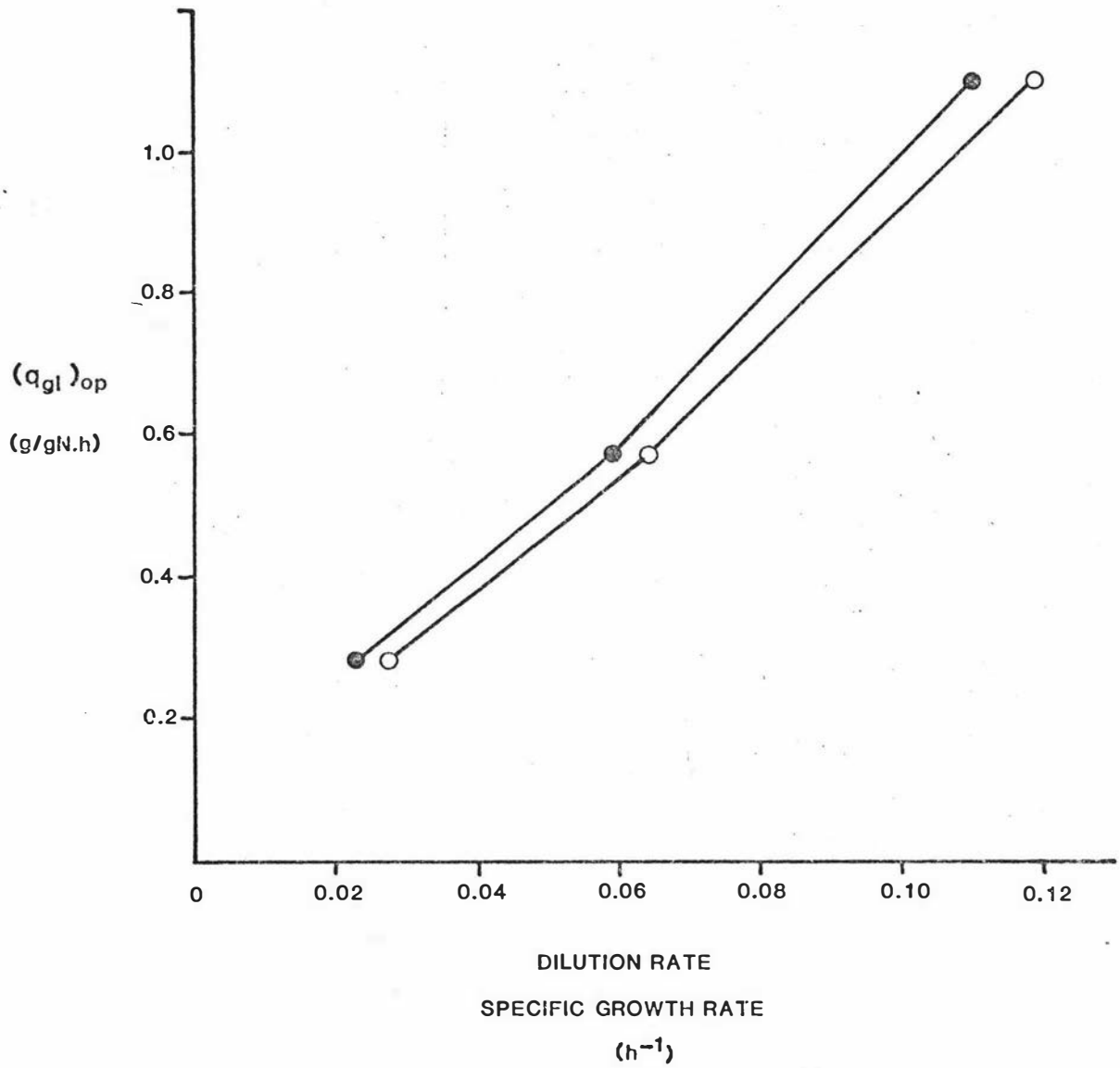


Figure 7.12 Specific glucose consumption rate for optimum hydroxylation rates, $(q_{gl})_{op}$, versus dilution rate (O) and specific growth rate (●).

Plots of mycelial nitrogen concentration (MNC) and mycelial percentage nitrogen (MPN) versus S_o, for all the experimental data are shown in Figure 7.13. From the shape of the MNC versus S_o curve it can be deduced that the cultures change from a state of carbon-limitation to nitrogen-limitation at approximately S_o = 2 g/l. The spread of data points on the "nitrogen-limited" portion of the curve is largely due to differing amounts of internal-feedback of biomass (see section 7.3.2) at the different dilution rates studied, and if the appropriate corrections were made all points would fall at MNC = 0.21 g/l (the nitrogen concentration of the feed medium). Significantly, the highest hydroxylation rates observed at all three dilution rates coincide with approximately the same S_o value (ca. 2.10 g/l). Thus, the point of change in the growth-limiting nutrient (carbon limitation to nitrogen limitation) is approximately the same point at which maximum hydroxylation rates were observed. Furthermore, the mycelial percentage nitrogen (MPN) appears to be dependent only on S_o and independent of dilution rate (Fig. 7.13). It would appear, therefore, that MPN may be a useful parameter to describe the amount of excess carbon material in the mycelia, and so the degree of glucose repression. If this parameter is indeed independent of growth rate, then it would serve as a means of comparing experiments at different dilution rates. Figure 7.14 shows plots of MPN versus q_{gl} for all the fermentations where a state of nitrogen-limitation existed. The differences in the slopes of the lines, depending on whether q_{gl} is expressed in terms of mycelial nitrogen (Fig. 7.14(a)) or mycelial dry weight (Fig. 7.14(b)), reflects the fact that much of the additional glucose uptake results in direct incorporation of carbon storage materials into the mycelial dry weight. Considering Figure 7.14(a), the convergence of the lines (for each dilution rate) to approximately 9.2% ($^{w}/_{w}$), by extrapolating to q_{gl} = 0, is of considerable significance. Thus, by comparing the MPN under conditions where the organism is starved of glucose, and hence when there would presumably be no carbonaceous storage material in the mycelia, the convergence to approximately the same value at all dilution rates indicates

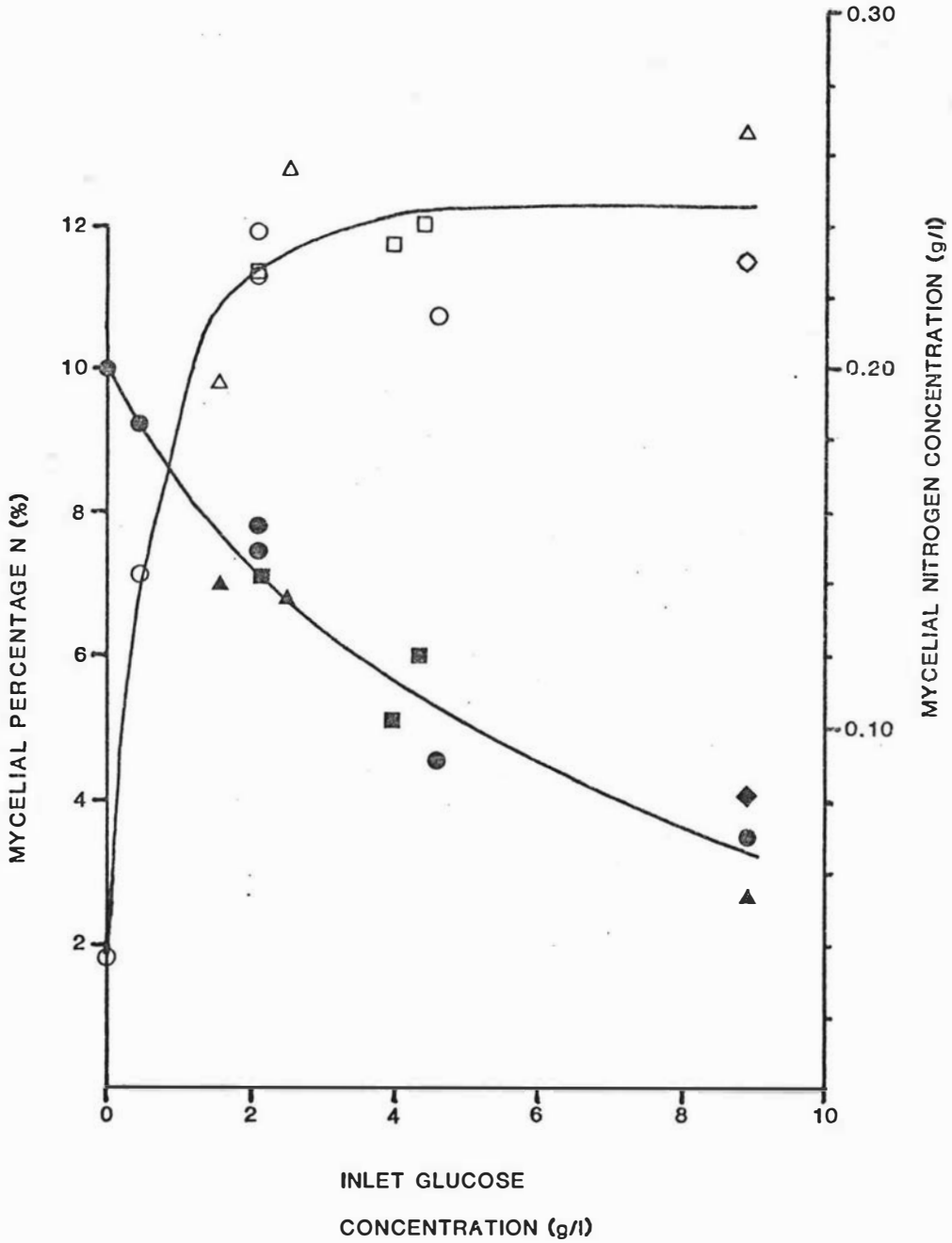


Figure 7.13 Mycelial nitrogen concentration (open symbols) and mycelial percentage nitrogen (solid symbols) versus feed-medium glucose concentration. Symbol shape refers to dilution rate, as follows. D = 0.028 h^{-1} (△ , ▲); 0.064 h^{-1} (○,●); 0.091 h^{-1} (◇ , ◆); 0.119 h^{-1} (□ , ■).

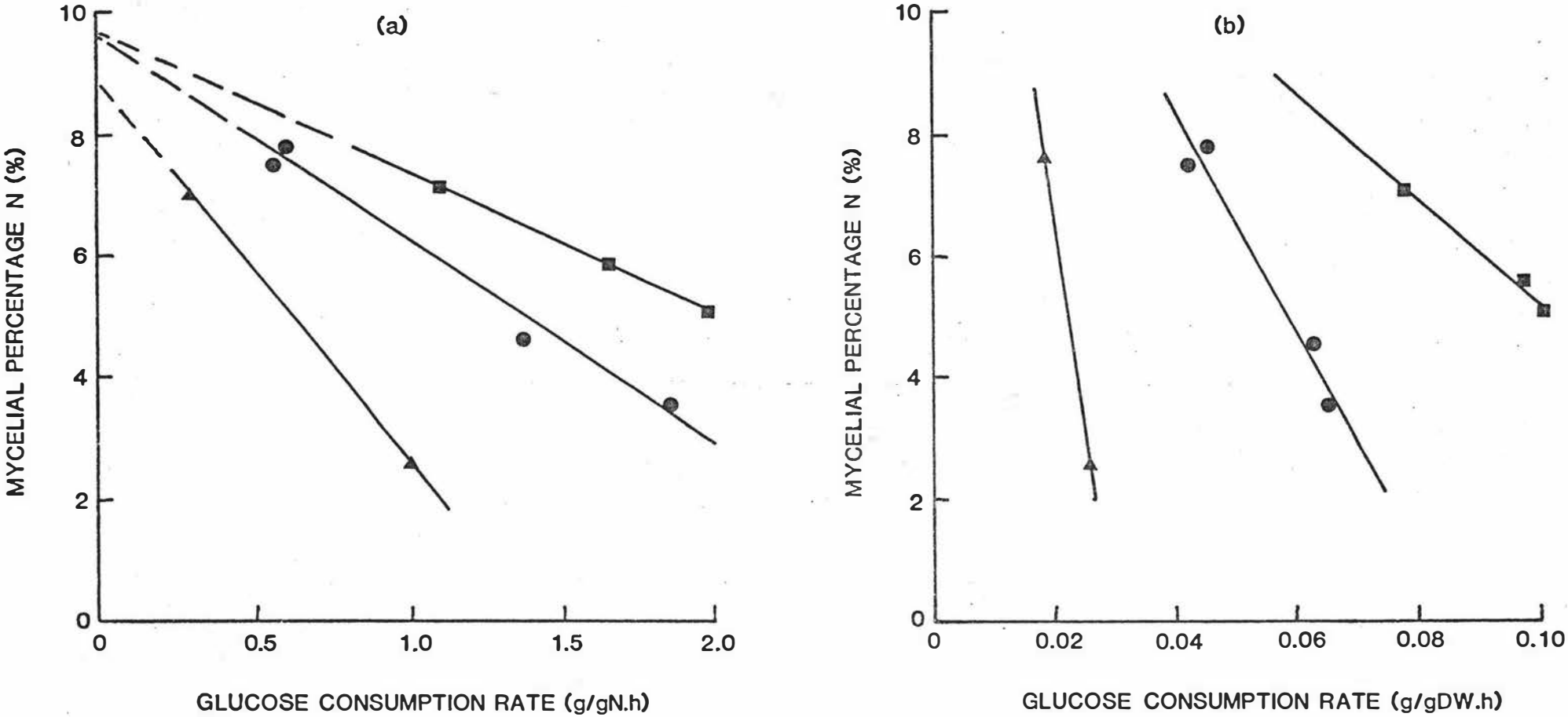


Figure 7.14 Mycelial percentage nitrogen versus specific glucose consumption rate, (a) in terms of mycelial nitrogen, and (b) in terms of mycelial dry weight. Respective symbols refer to dilution rate, as follows: D = 0.028 h^{-1} (▲); 0.064 h^{-1} (●); 0.119 h^{-1} (■).

that the "basic" nitrogen content of the mycelia is approximately independent of growth rate. This is not unexpected. The nitrogen content of the mycelia will be distributed in two main components; protein nitrogen and nucleic acid nitrogen (RNA mainly). Cell wall chitin also contains nitrogen but is not a major contributor (Solomons, 1975). The RNA content of the mycelia is the only component which will vary significantly with dilution rate (Solomons, 1975; Kim and Lebeault, 1981). However, it has been shown with *Penicillium cyclopium* growing at moderately low dilution rates ($< 0.1\ h^{-1}$), that the RNA-nitrogen was less than 15% of the mycelial-total-nitrogen and varied only slightly with dilution rate (Kim and Lebeault, 1981). Thus, it may be assumed that the "basic" mycelial percentage nitrogen of *P. filamentosa* f.sp. *microsclerotia* mycelia should not have varied greatly with dilution rate, as Figures 7.13 and 7.14(a) indicate.

Therefore, on the basis that MPN is a measure of the amount of excess glucose uptake by the organism and, indirectly, of the degree of catabolite repression, it was possible to independently analyse the results for the effect of glucose repression and growth rate. Thus, a multiple-linear-regression analysis of the dependence of the rate of 19-hydroxylation on the growth rate and MPN was performed (Appendix C). The results showed that while MPN was highly significant, growth rate was not significant (at the 95% confidence level). Neglecting growth rate, Figure 7.14 gives a plot of q_{19} versus MPN for all the nitrogen-limited fermentations. With the exception of the two experiments at $D = 0.119\ h^{-1}$ (where q_{gl} was relatively high), the data fall almost onto a straight line. Apparently, at the extreme conditions of the deviant data points, this simple model does not apply. Perhaps increased mycelial-RNA is significant at this growth rate. Since it is the protein-nitrogen which is assumed to measure the mycelial activity, an increase in RNA-nitrogen will decrease the protein-nitrogen content of the mycelia compared with mycelia at lower growth rates. Thus the mycelial-total-nitrogen data at $D = 0.119\ h^{-1}$ may over-estimate, in relative terms, the protein component of the mycelia compared to mycelia at lower dilution rates. If protein-nitrogen rather than

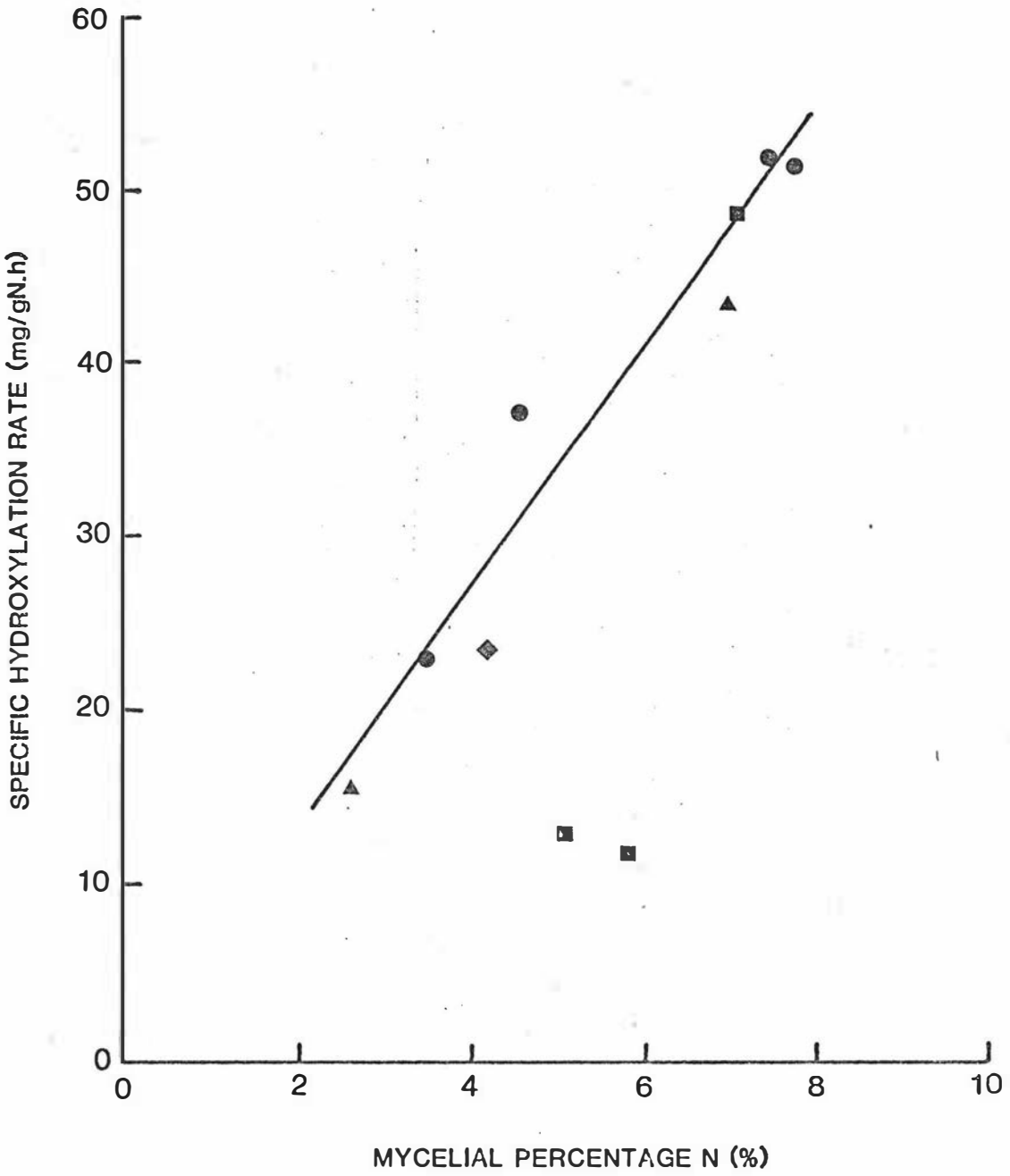


Figure 7.15 Specific rate of 19-hydroxylation versus mycelial percentage nitrogen. Respective symbols refer to dilution rate, as follows: D = 0.028 h^{-1} (▲); 0.064 h^{-1} (●); 0.091 h^{-1} (◆); 0.119 h^{-1} (■).

total-nitrogen content of the mycelia had been measured, the deviant data points of Figure 7.14 may fall closer to the line than is presently the case.

7.3.4 Conclusions

Much of the analysis of the present data has been given in terms of the specific rate of 19-hydroxylation (q_{19}), for reasons of simplicity. Although slight variations in the ratio of the two hydroxylation activities were observed, the specific rate of 11β-hydroxylation ($q_{11\beta}$) remained in approximately constant proportion with q_{19}. Thus the conclusions apply equally to both hydroxylations.

The results of this study indicate that the specific growth rate of the culture, at least under nitrogen-limited conditions, is not of major importance in the hydroxylation of cortexolone by *P. filamentosa* f.sp. *microsclerotia*. Some form of glucose-repression is, however, of considerable significance and a careful choice of medium composition is vital to achieve maximum hydroxylation activity. The optimum conditions for hydroxylation appear to reflect a balance between sufficient metabolism of a readily-available carbon source (in this case glucose) and a state of repressed activity when excess uptake occurs. With the medium used in this study, the optimum point at a given growth rate, coincides with the q_{gl} value at which the culture is only just nitrogen-limited. The MPN can be used as a "marker" for this point. Under carbon-limited conditions the specific hydroxylation rates declined as q_{gl} approached zero, probably because of insufficient carbon metabolism to maintain full expression of the hydroxylase enzyme(s) (possibly, the use of the HMP pathway is restricted under these conditions).

It should be possible to apply these findings to batch fermentation. The main difference between batch and continuous culture is that in the latter the growth of the organism is always limited by the availability of a nutrient, whereas, in the former the organism grows at μ_{max} until some

nutrient becomes limiting. Thus, to confidently apply these results, a fed-batch culture technique should be used, where a medium, composed of glucose (2.10 g/l) and yeast-extract (2.0 g/l), is slowly fed to the culture at such a rate that growth will always be nitrogen-limited. The exact rate of feeding should not be important, since specific growth rate (at least, within the range studied) is apparently not an important factor, under N-limiting conditions. This approach should maintain the culture at the point of maximum hydroxylation rate.

In many cases of steroid transformation, variations in medium composition have been found to influence the process. Ryu and Lee (1975), studying the Δ^1-dehydrogenation of 9α-fluoro-11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione by *Arthrobacter simplex* in a two-stage chemostat process, found that high medium concentration repressed enzyme productivity. Further, Marsheck (1971) described a number of situations where variation of a particular medium component greatly affected steroid transformations. The results of the present study clearly show that catabolite repression of steroid hydroxylation does take place, and that optimal fermentation conditions can be described. It is probable that this phenomenon will occur in many steroid hydroxylation processes. A search for conditions where repression is minimal could result in several-fold enhancement of hydroxylation rates in such situations.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

The feasibility of a microbiological process for the 19-hydroxylation of steroids has been investigated. The commercial attraction for such a process is considerable, since 19-hydroxylated steroids are precursors of the valuable 19-norsteroids. However, since a chemical route to such compounds is presently used, a microbial process must offer significant advantages in order to displace it. In the context of New Zealand industry, one immediate advantage of such a process is that expensive, imported chemical reagents are not required.

The starting point for this project was an examination of fungi implicated in the literature as mediating steroidal 19-hydroxylation. Since the ability to 19-hydroxylate steroids was recognised as possibly being substrate specific, the transformation of several substrates was examined (viz, 4-androstene-3,17-dione, progesterone, and cortexolone). Both *P. filamentosa* f.sp. *microsclerotia* IFO 6298 and *sasakii* IFO 5254 were able to 19-hydroxylate cortexolone, but not the other two substrates. Despite claims in the literature, none of the *Pestalotia* species which were examined could perform this transformation on any of the substrates. *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was finally chosen as the most suitable organism for further study. In transforming cortexolone, this organism produced a mixture of mainly 11β-hydroxy- and 19-hydroxycortexolone. Both these compounds are potentially valuable.

Although yield improvements would be necessary in the development of this process, the present study concentrated on improving the cortexolone-hydroxylation rate of *P. filamentosa* cultures. Thus, the effects of dissolved oxygen

tension on the induction and expression of the inducible hydroxylase system were investigated. Optimal conditions for each phase were described and, noteably, the optimum for induction of activity (DOT, 15% of saturation) occurred at a relatively low DOT level. This is similar to the optimum conditions described for the induction of many cytochromes in microbial cells, in particular, cytochrome-P450 which mediates hydroxylation of many compounds, including steroids. The optimum DOT for expression of activity was at 30% of saturation.

The effects of glucose consumption rate and growth rate were investigated using chemostat cultures of the organism. The importance of glucose metabolism to the hydroxylation process was demonstrated. Thus, with glucose-limited cultures, decreased hydroxylation rates were observed with decreasing glucose consumption rates, possibly as a result of the restricted availability of NADPH, which is required for hydroxylation to occur. Conversely, with nitrogen-limited cultures, it was observed that the hydroxylase system is subject to glucose repression. This finding is of considerable significance not only to this transformation, but, if similar phenomena hold, to steroid hydroxylation processes in general. The relief of repression, where it exists, could give several-fold increases in hydroxylation rates, which, on the industrial scale, would mean more efficient use of available fermenter-space. Interestingly, the specific growth rate of the culture, at least under nitrogen-limited conditions, is apparently unimportant in terms of the rate of cortexolone hydroxylation. Thus, transformations using fed-batch cultures, where specific growth rate will vary, should still yield optimal rates of hydroxylation providing the glucose consumption rate of the culture is controlled.

The investigations described in this thesis have generally, only involved measurements of initial hydroxylation rates. Thus, factors which may be important at high product concentrations, such as product inhibition, could be ignored. The possibility of product inhibition of hydroxylation was

suggested by a preliminary experiment (see section 5.10) and warrants further investigation. Also, evidence has been presented which suggests that the hydroxylase enzyme-system, or some critical component of it, may be rapidly "turned over". This implies that transcription and protein synthesis are continually occurring as hydroxylation proceeds. Thus, conditions which promote optimal induction of activity may be necessary throughout the transformation to maintain the same enzyme titre. This would be significant when optimal conditions for expression of the induced enzyme differ from those conditions for optimal induction. Such is the case in respect of DOT.

The question of whether one or two enzymes are responsible for the 11β- and 19-hydroxylating activities of the organism has been discussed briefly in section 5.2. Variation in the product concentration ratio either during an experiment, or between different experiments, is inconsistent with a model suggesting a single enzyme. In this respect, some variation has been noted during the course of this study. Thus, the evidence obtained suggests that the two activities are the result of separate enzymes. However, conditions have not been described which enable significant manipulation of the ratio of products. This would represent a useful goal for further research.

For development of this process in the longer term, improvement in hydroxylation yield would be necessary. The unaccountable losses of steroid which have been observed, appear to proceed via metabolism of cortexolone in a pathway parallel to the hydroxylation process. Thus, the hydroxy-products are themselves stable. The use of chemical inhibition or mutagenesis to block this degradation pathway has been suggested. In similar development work on the 11β-hydroxylation of cortexolone by *Curvularia lunata*, described by Dulaney and Stapley (1959), mutagenesis was the only approach which gave any improvement in yield. The situation in this case was similar to the present work. That is, 11β-hydroxycortexolone was a stable product, but cortexolone losses were occurring,

apparently, via a parallel degradation pathway. Certainly, mutation, as a means of strain improvement, has long been an essential part of microbiological process development in industry.

The substrate specificity of *P. filamentosa* f.sp. *microsclerotia* IFO 6298, in terms of 19-hydroxylation, has been discussed. While minor changes in the C-17-side chain do not affect the organism's ability to 19-hydroxylate steroids (see Table 2.2), compounds possessing quite different side chains (i.e. progesterone and androstenedione) were unable to be similarly transformed. There could be great advantages in further studies of substrate structure effects, since the scope of a 19-hydroxylation process in an industrial context could be more thoroughly evaluated.

To summarise, further work is necessary before definite conclusions can be drawn as to the potential of a microbiological 19-hydroxylation process. Since this transformation is very rare among microorganisms it is likely that *P. filamentosa* f.sp. *microsclerotia* IFO 6298 is the most suitable organism available, and more screening work would be unlikely to return further benefit. While processes based on substrates other than cortexolone would be attractive, the process investigated in this thesis does have commercial application. In respect of microbiological steroid-hydroxylation in general, the results of this study have considerable significance. Thus, the importance of the dissolved oxygen tension in the culture medium, as first demonstrated by Hanisch *et al.* (1980) has been further reinforced by the present study. Also, the findings in respect of glucose repression and the general importance of the organism's metabolic state may be of considerable significance to other steroid hydroxylation processes.

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APPENDIX A

THE DISSOLVED OXYGEN TENSION CONTROLLER

A.1 Introduction

Many different methods have been described for achieving control of dissolved oxygen tension (DOT) in fermenter culture (for example, Flynn and Lilly, 1967; Vincent, 1974). In particular, variation of agitation speed (often in combination with aeration-rate control) seems to be the most popular control method, with several recent publications describing its application (Yano *et al*, 1978; Yano *et al*, 1979; Kobayashi *et al*, 1980; Landwall, 1980). Commercial controllers, which utilize this control mechanism, are also available. There is, however, little information regarding the technical details of achieving this type of control. Furthermore, no literature is available regarding the control dynamics or how to design for a robust and rapidly converging system.

The DOT-controller described was developed to provide high precision control requiring a minimum of manual adjustment.

A.2 The Equipment

The controller was designed to utilize the 0-10 mV output from the DO-40 Dissolved Oxygen Analyser (section 3.5.2.1) as the process input, with the final control element being the speed of the DC-motor (standard for the NBS F-2000 Culture Apparatus) which drives the agitator. A block diagram of the control loop is shown in Figure A1.

The probe signal is processed by the DO-40 analyser to give a meter indication of the DOT and a 0-10 mV (fsd) output for chart recorder operation. The instrumentation amplifier provides high common-mode rejection and a high input impedance. The pre-amplified signal is passed through a second order low-

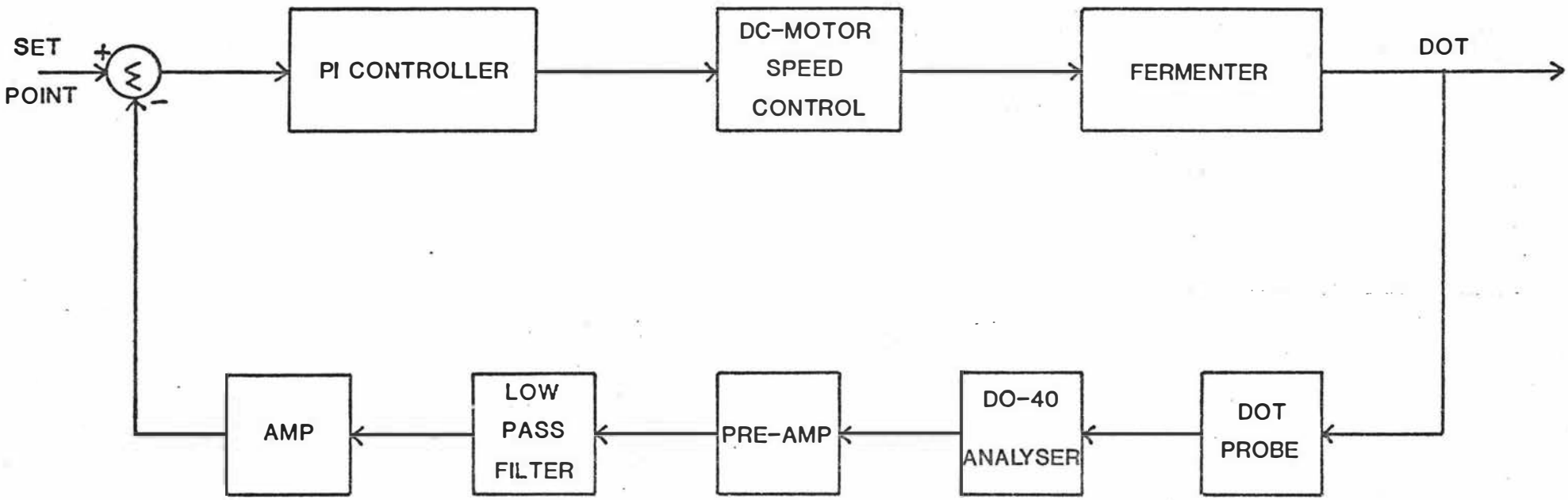


Figure A1 The control loop.

pass filter before being amplified to provide a signal in the range -5V to + 5V. This signal, representing the measured culture DOT level is subtracted from a voltage representing the required set-point value for measured DOT. The resulting error signal is passed to a Proportional-Integral (PI) controller. A final output stage conditions the control signal to provide the correct polarity and voltage range. This output stage could be altered to suit any signal requirements. A full circuit diagram is shown in Figure A2. The control-board power supply (V^+ and V^-) was provided by conditioning the voltage output from a $\pm$ 10 V, centre-tapped transformer (Trotman Electronics Ltd., Auckland, New Zealand). The circuit diagram for this is given in Figure A3.

The final output voltage, controls the DC - motor speed via a thyristor motor-speed control unit (supplied by the Department of Industrial Management and Engineering, Massey University, New Zealand). This unit had the useful facility that a minimum motor-speed could be set, and hence, a minimum fermenter agitation rate ensured. The final output stage of the controller circuit was adjusted such that the agitator speed changed from 0 to 1000 rpm as the output from the PI-controller changed from -0.5 to +0.5 V. The calibration characteristic is shown in Figure A4. When automatic control was not required, manual speed control was available.

The PI-controller circuit is reproduced in Figure A5.

The transfer function for this circuit is:

$$\frac{Vout}{V_{in}} = -\left[\frac{R_2}{R_1} + \frac{1}{R_1 C} \cdot \frac{1}{S}\right]$$

$$= K_c\left[1 + \frac{1}{T_I S}\right] \qquad (1)$$

where, Proportional Gain, $K_c = \frac{R_2}{R_1}$ (2)

Integral Time Constant, $T_I = R_2 C$ (3)

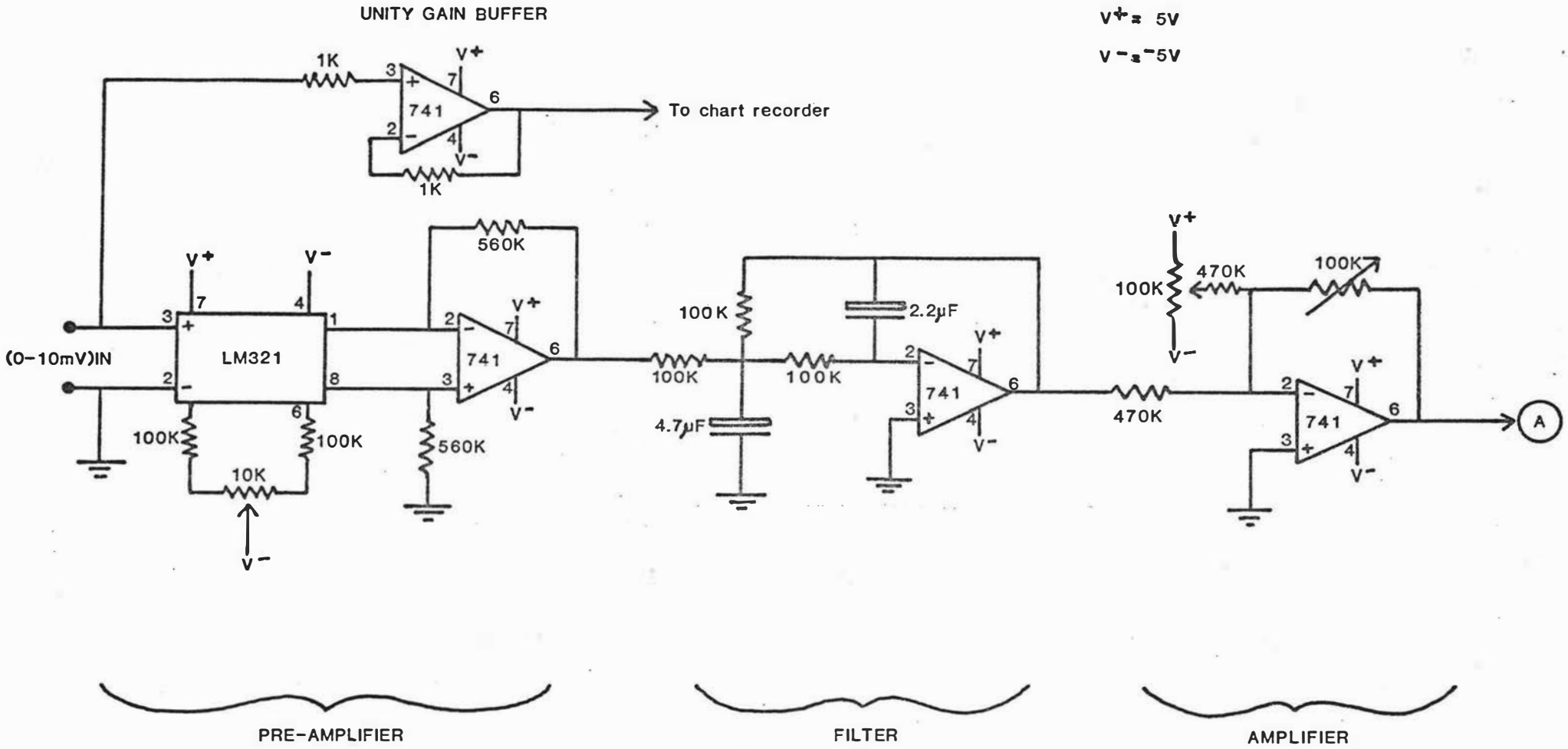


Figure A2 A circuit diagram of the equipment.

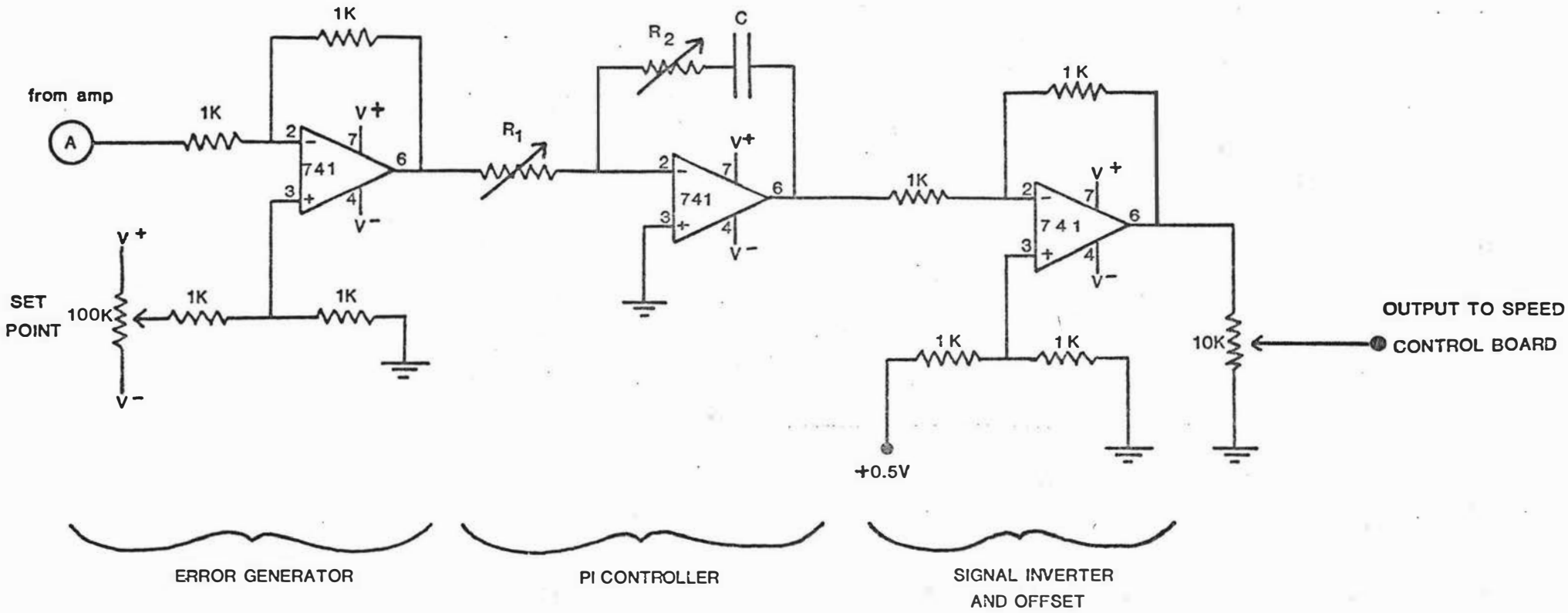


Figure A2 (Continued)

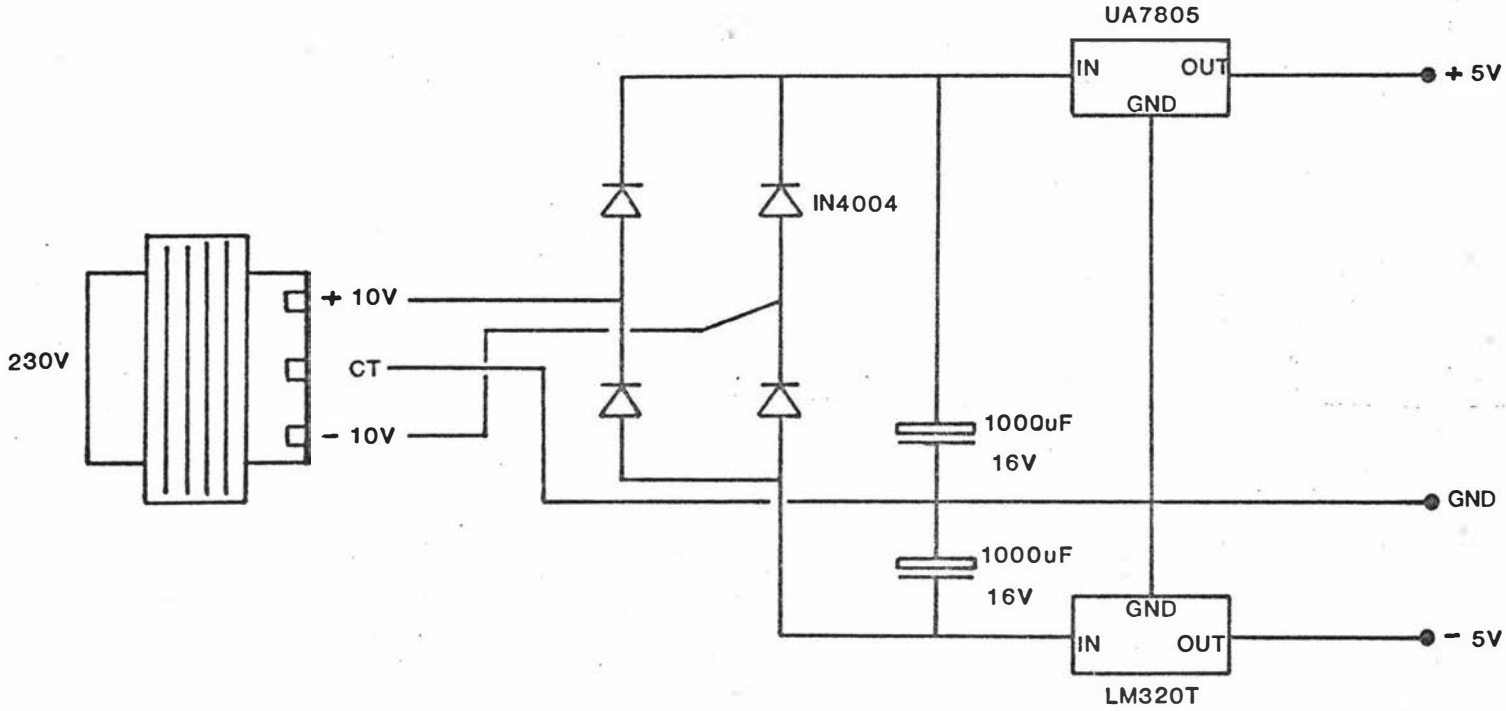
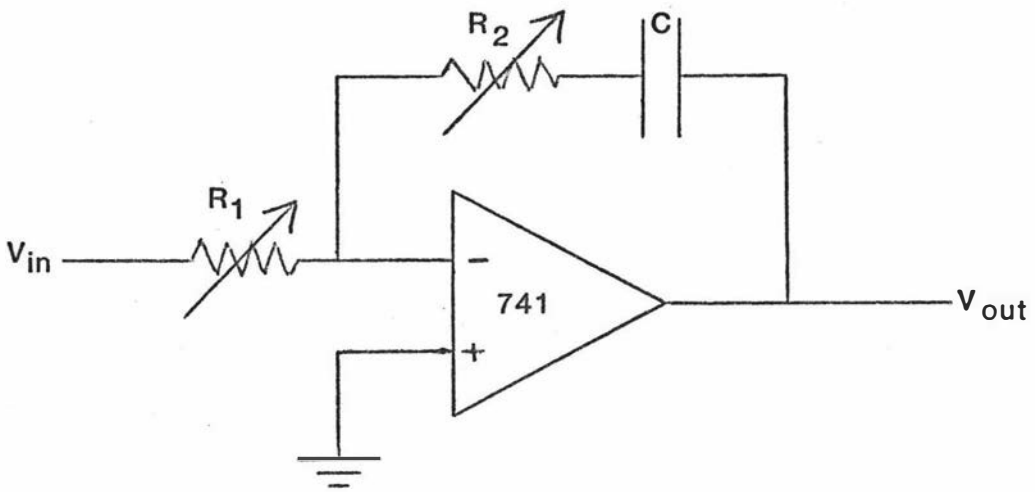


Figure A3 Circuit diagram of control-board power supply.

Figure A5 The PI-controller circuit.



(Coughanowr and Koppel, 1965).

In the time domain, the transfer function (equation (1)), becomes:

$$V_{out} = K_C \left[Vin + \int \frac{Vin}{T_I} \right] dt \qquad (4)$$

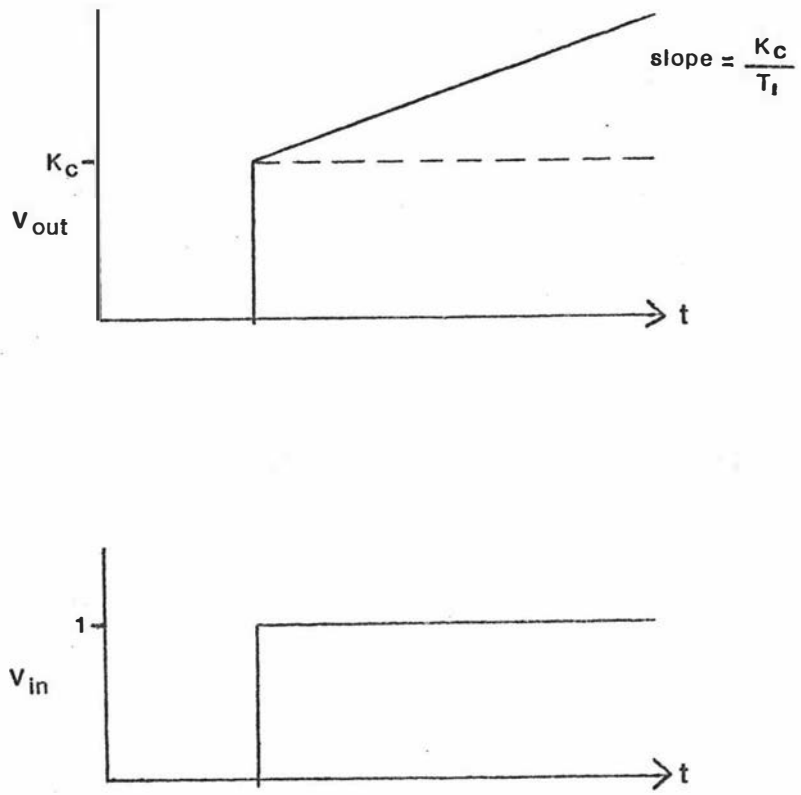
If the input to this circuit suddenly changes from 0 to 1 (step input), the output will behave as shown in Figure A6.

The inclusion of an integral term in proportional controller action ensures that there is no controlled-variable off-set, as occurs with proportional control.

A.3 Controller Tuning

It was necessary to obtain settings for the circuit variables R_1, R_2, and C, such that, adequate transient behaviour of DOT was obtained. This can be done analytically, by characterising the various stages in the control loop (Fig. A1). However, the stages involved in this relationship between agitation speed and the measured culture DOT, tend to be highly non-linear, as described below.

Figure A6 The "step-response" behaviour of the PI-controller.



The transient behaviour of DOT is governed by equation (5).

$$\frac{dD}{dt} = \frac{K\ (D^* - D)}{H} - Q_{O_2} \qquad (5)$$

where, D is the culture DOT (atm)

D* is the oxygen partial pressure in the gas phase (atm)

K is the volumetric oxygen transfer coefficient (s^{-1})

Q_{O_2} is the volumetric oxygen consumption rate of the culture (mmoles/l.s)

H is the Henry's Law Constant for the solution at the prevailing temperature (atm.l/mmole)

By varying agitation speed, it is the coefficient K which is manipulated. The dependence of K on agitation speed N typically

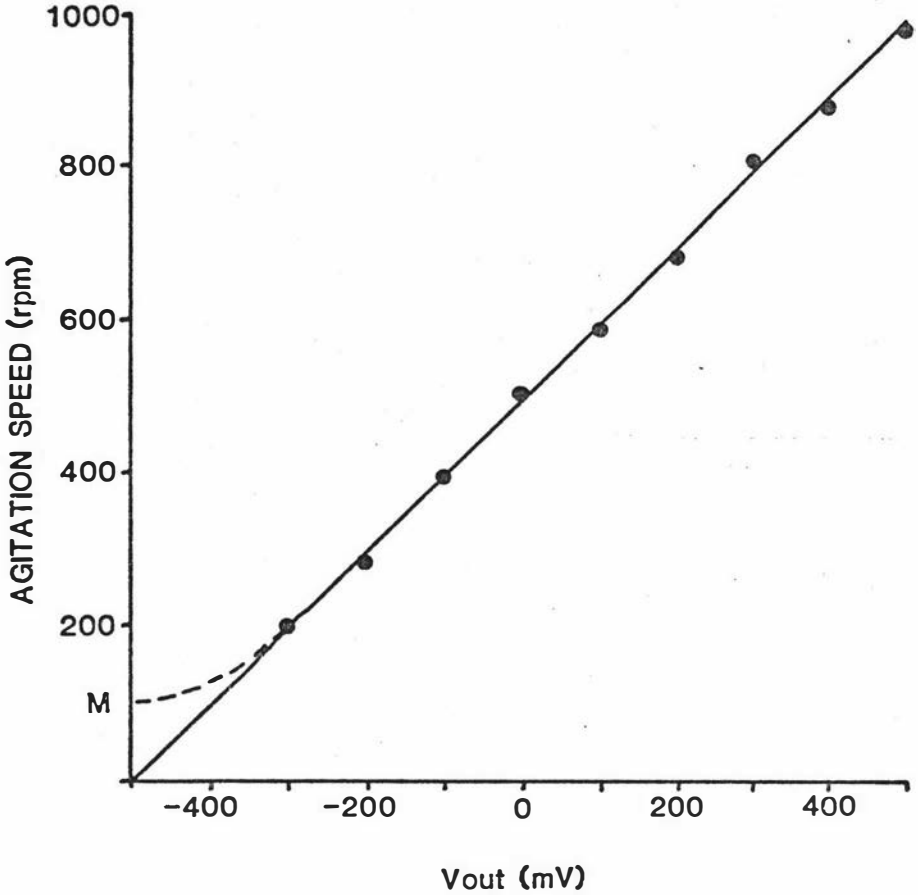


Figure A4 The calibration characteristic of the thyristor motor-drive unit i.e., the relationship between agitator speed and the voltage output of the PI-controller. M indicates a typical minimum-speed setting.

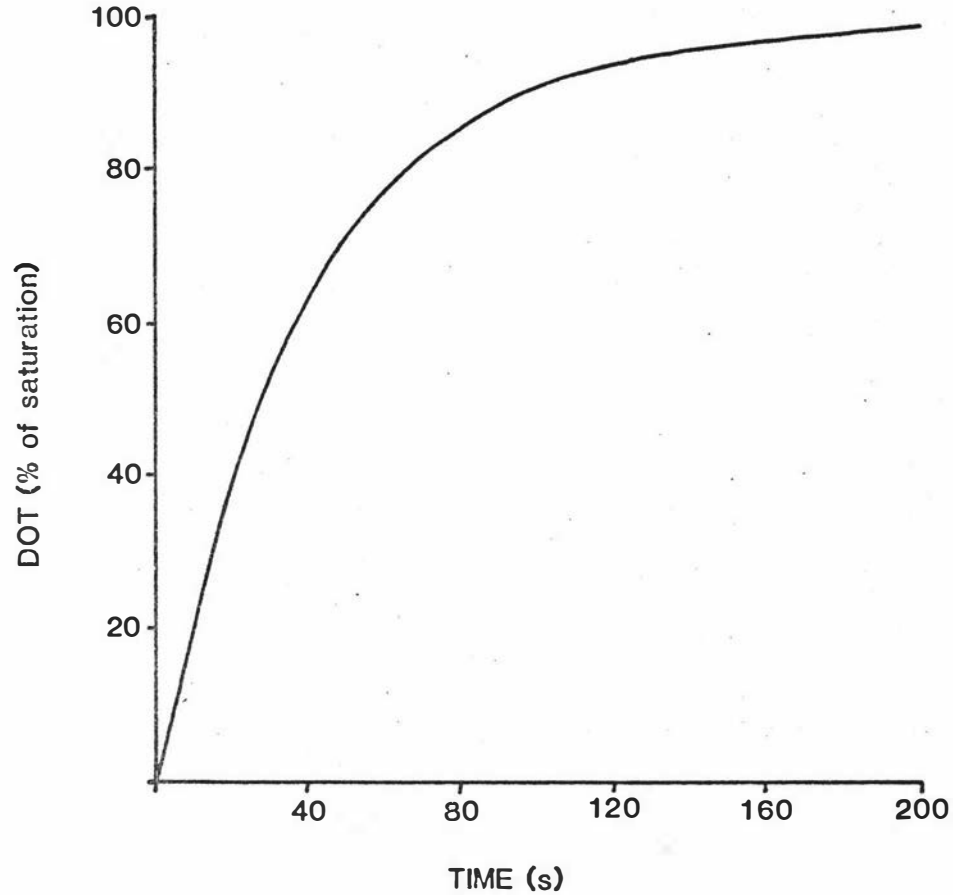


Figure A7 The response of the DOT probe to a step change from deoxygenated water to air-saturated water.

obeys a power law, as in equation (6) (Aiba *et al.*, 1973).

$$K = \text{constant} \cdot N^{P} \qquad (6)$$

Dissolved oxygen probes, used in fermenter studies, do not respond instantaneously to changes in DOT (Lee and Tsao, 1979). The probe used in the present study was no exception and a 90% response to a step change required approximately 90 seconds. The response to a sudden change from deoxygenated water to air-saturated water (at 30°C) is shown in Figure A7. This step response curve can be described as follows (Lee and Tsao, 1979),

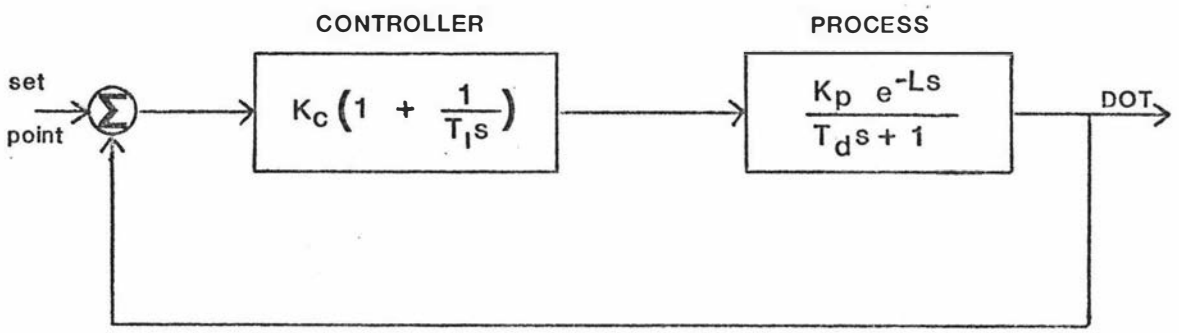
$$\frac{D - D_o}{D_s - D_o} = 1 - e^{-kt} \qquad (7)$$

where, D_o is the initial DOT (atm)
D_s is the final DOT (atm)
k is the probe constant (s^{-1})

This probe characteristic constitutes a measurement lag in the control system.

The difficulties of a purely analytical approach to controller tuning are inherent in the non-linearity of equations (5) and (6). Hence, a simplified approach was used, in which the system was assumed to behave as a first-order process with the probe response described as a "dead-time" lag. This enabled the values for K_C and T_I to be derived from the process reaction curve method of Cohen and Coon (1953). The closed-loop system was thus represented as shown in Figure A8. The process variables K_p, T_d, and L were derived from a process reaction curve. This curve was obtained by subjecting a fermentation to a step-change in agitation speed and measuring the DOT reaction curve.

Figure A8 The closed-loop block diagram.



Example

The process reaction curve of Figure A9 was obtained by subjecting a fermenter culture to a step-change in agitation speed of 200 rpm (ΔP). By the construction method shown in Figure A9, the terms ΔR, L, and T_d were obtained. The calculation of K_C and T_I are according to Cohen and Coon (1953), as follows:

ΔP = 200 rpm = 200 mV (equivalent PI controller output)

ΔR = 40% of satn. = 28 mV (equivalent PI controller input)

$$K_{vp} = \frac{\Delta R}{\Delta P} = \frac{28\ mV}{200\ mV} = 0.14$$

$$K_C = \frac{1}{K_{vp}} \cdot \frac{T_d}{L}\left(\frac{9}{10} + \frac{L}{12T_d}\right)$$

$$= \frac{1}{0.14} \cdot \frac{160}{25}\left(\frac{9}{10} + \frac{25}{12(160)}\right)$$

$$= \underline{42}$$

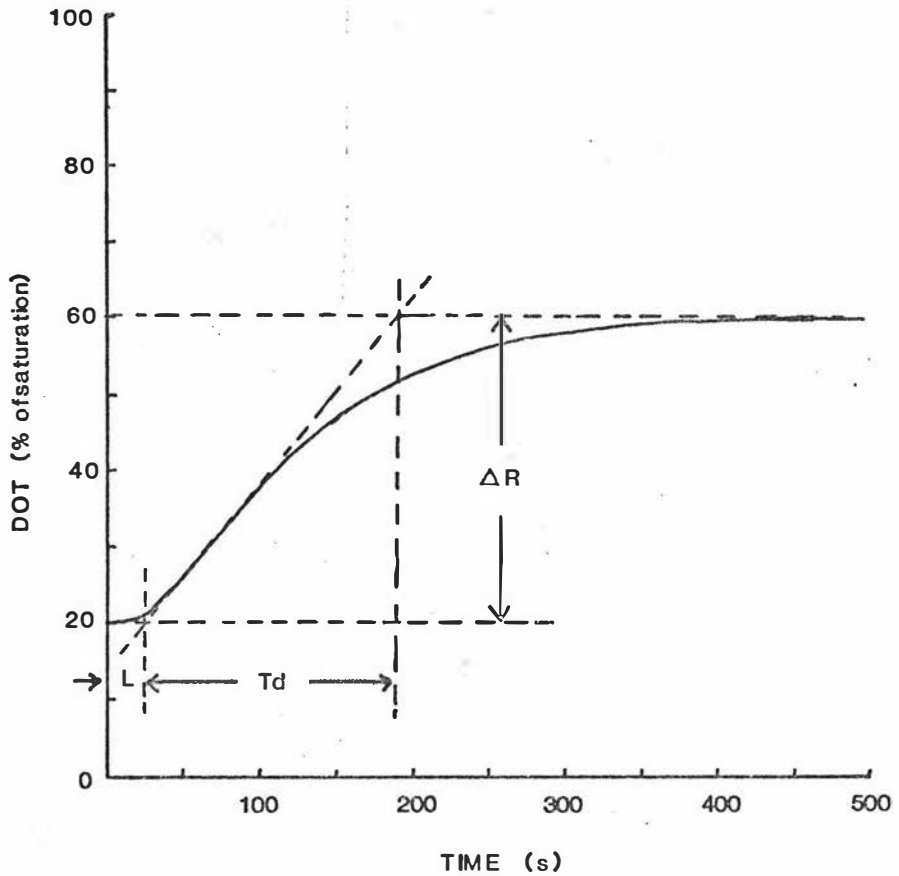


Figure A9. A typical process reaction curve, showing the methods for determining ΔR, T_d, and L.

$$T_I = L\left(\frac{30 + 3L/T_d}{9 + 20L/T_d}\right)$$

$$= 25\left(\frac{30 + 3(25)/160}{9 + 20(25)/160}\right)$$

$$= \underline{63s}$$

Thus, by applying equations (2) and (3) the PI-controller parameters R_1, R_2, and C can be determined.

$$\text{Proportional Gain, } K_C = \frac{R_2}{R_1} = 42$$

$$\text{Integral Time Constant, } T_I = R_2C = 63s$$

Choosing C = $\underline{1000\ \mu F}$, gives:

$$R_2 = \frac{63}{10^{-3}} = \underline{63\ K\Omega}$$

$$R_1 = \frac{63 \times 10^3}{42} = \underline{1.5\ K\Omega}$$

Further refinement of controller parameters is possible using trial and error, or alternatively from examination of closed-loop system poles. From the process reaction curve, the process may be represented by

$$\frac{0.14\ e^{-25s}}{160s + 1}$$

The controller derived using the method of Cohen and Coon (1953) is

$$42\left(1 + \frac{1}{63s}\right)$$

and the open-loop transfer function is

$$\frac{36.75 \times 10^{-3} \quad (s + 15.87 \times 10^{-3})}{s\ (s + 6.25 \times 10^{-3})} \quad e^{-25s}$$

Using the methods described by Ogata (1970), the root locus diagram of Figure A10 was derived. This may be used to determine adjustments to the controller gain K_c, to position the closed-loop poles at a satisfactory operating point. A family of loci for different values of reset gain may be plotted.

Typical performance characteristics are shown in Figure A11. Figure A11(a) shows the response of DOT to step changes in controller setpoint. The Cohen-Coon settings should provide for a damped, oscilliatory response, with a decay ratio of ¼. These settings, applied to the real system, have resulted in a somewhat over-damped response. Figure A11(b) shows the controller performance under typical process disturbances, the most noteable being antifoam addition.

The above approach to controller design involved an approximation to a rather complex system. The various non-linearities involved in the control-loop, require that controller tuning should be performed under conditions close to those at which the system will operate, to ensure the "best" control settings. In practice, however, the same control settings proved adequate for a range of fermentations, with different oxygen demands. Thus, the system appears to be very robust, although no attempts were made to investigate the limits of stability.

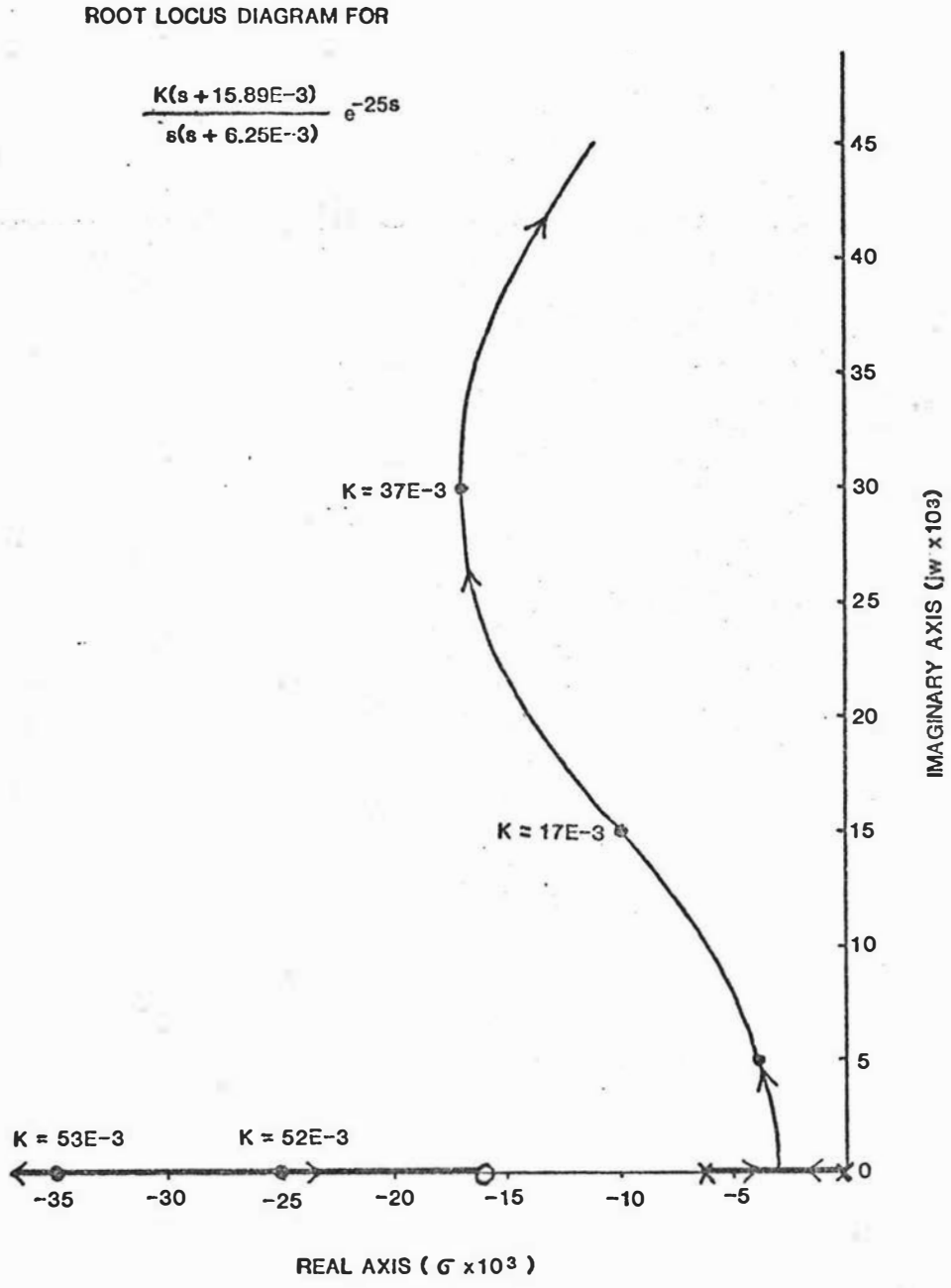
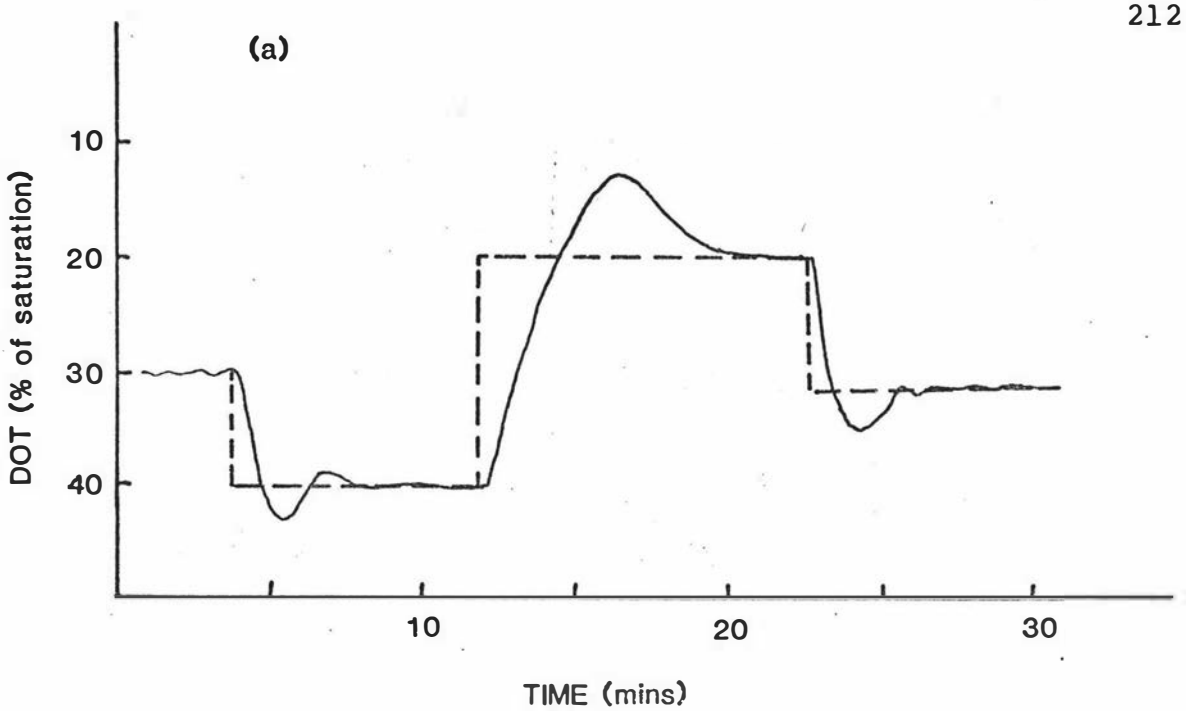


Figure A10 The root locus diagram for the system's open-loop transfer function.



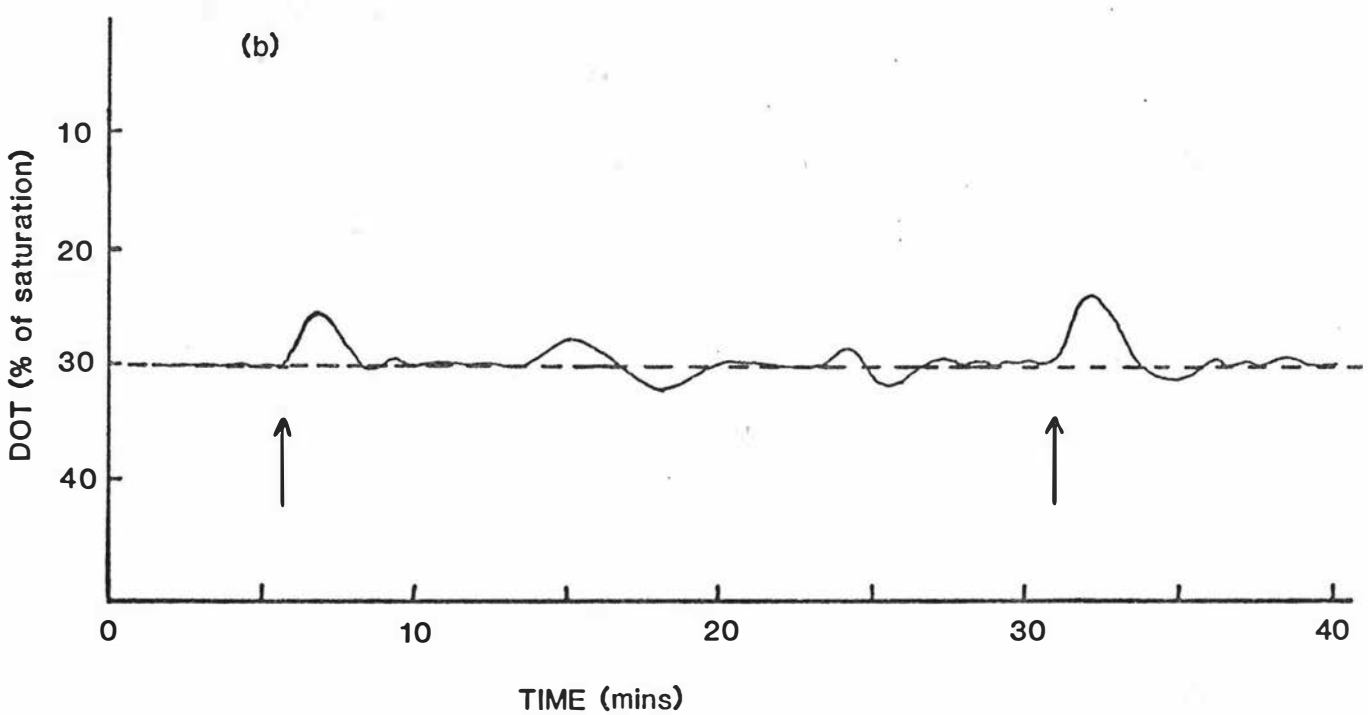


Figure A11 Typical performance characteristics of the control system. (a) The DOT response to step changes in set-point value. (b) A typical DOT-trace during fermenter operation. Arrows indicate when antifoam addition had taken place.

APPENDIX B Chemical Spectra

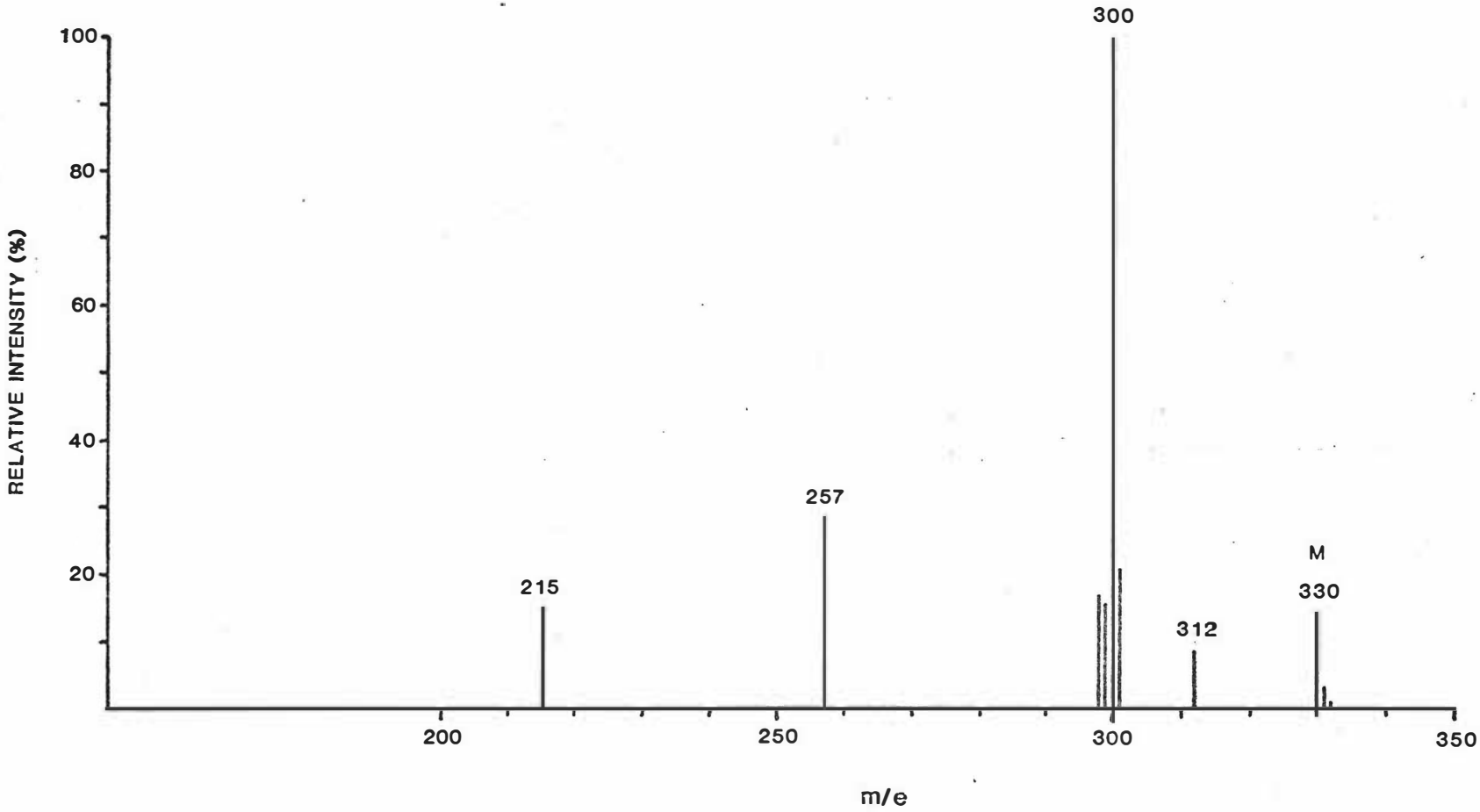


Figure B1 Mass spectrum of synthesised 19-hydroxyprogesterone. (Instrument: MS 30).

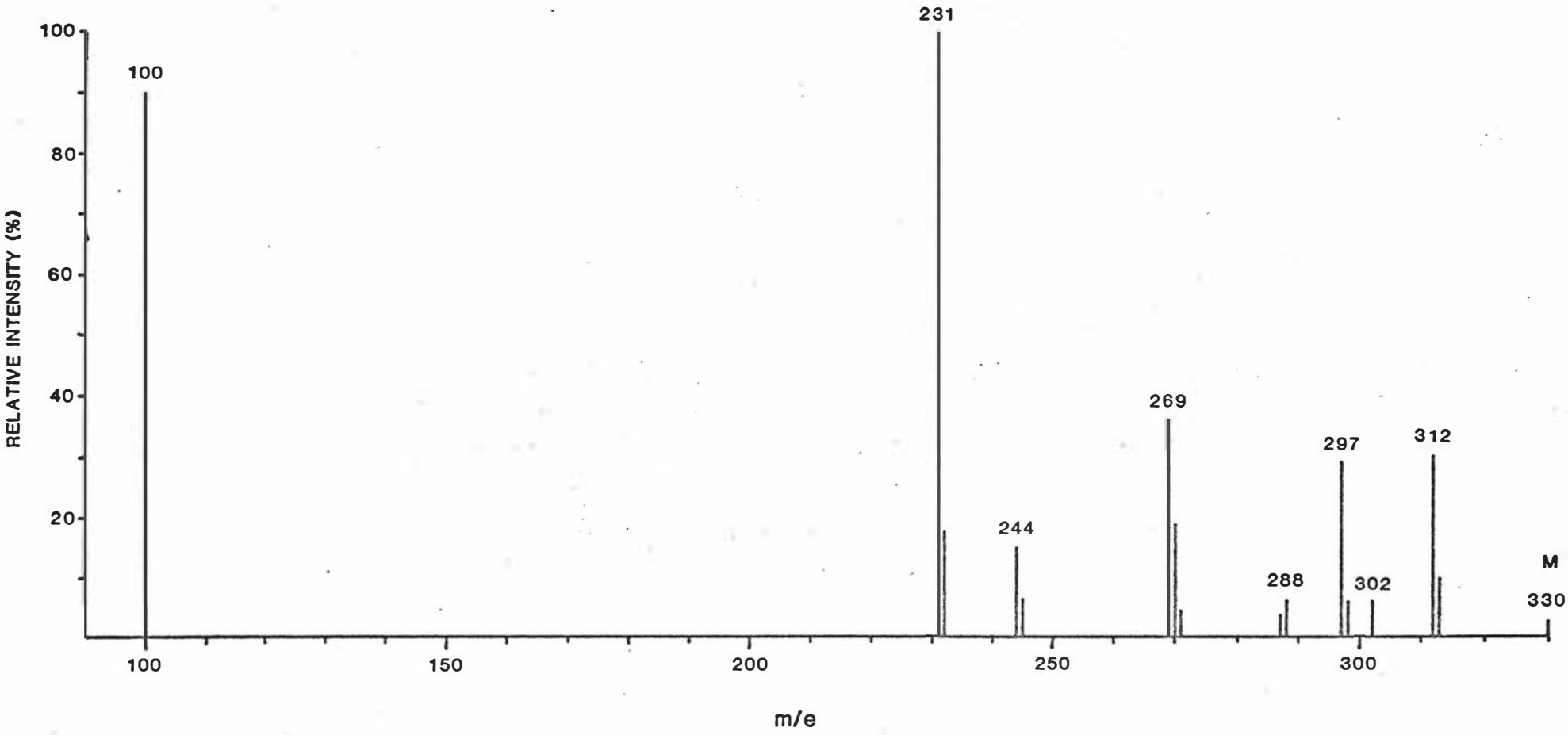


Figure B2 Mass spectrum of synthesised 16α-hydroxyprogesterone. (Instrument: MS 30)

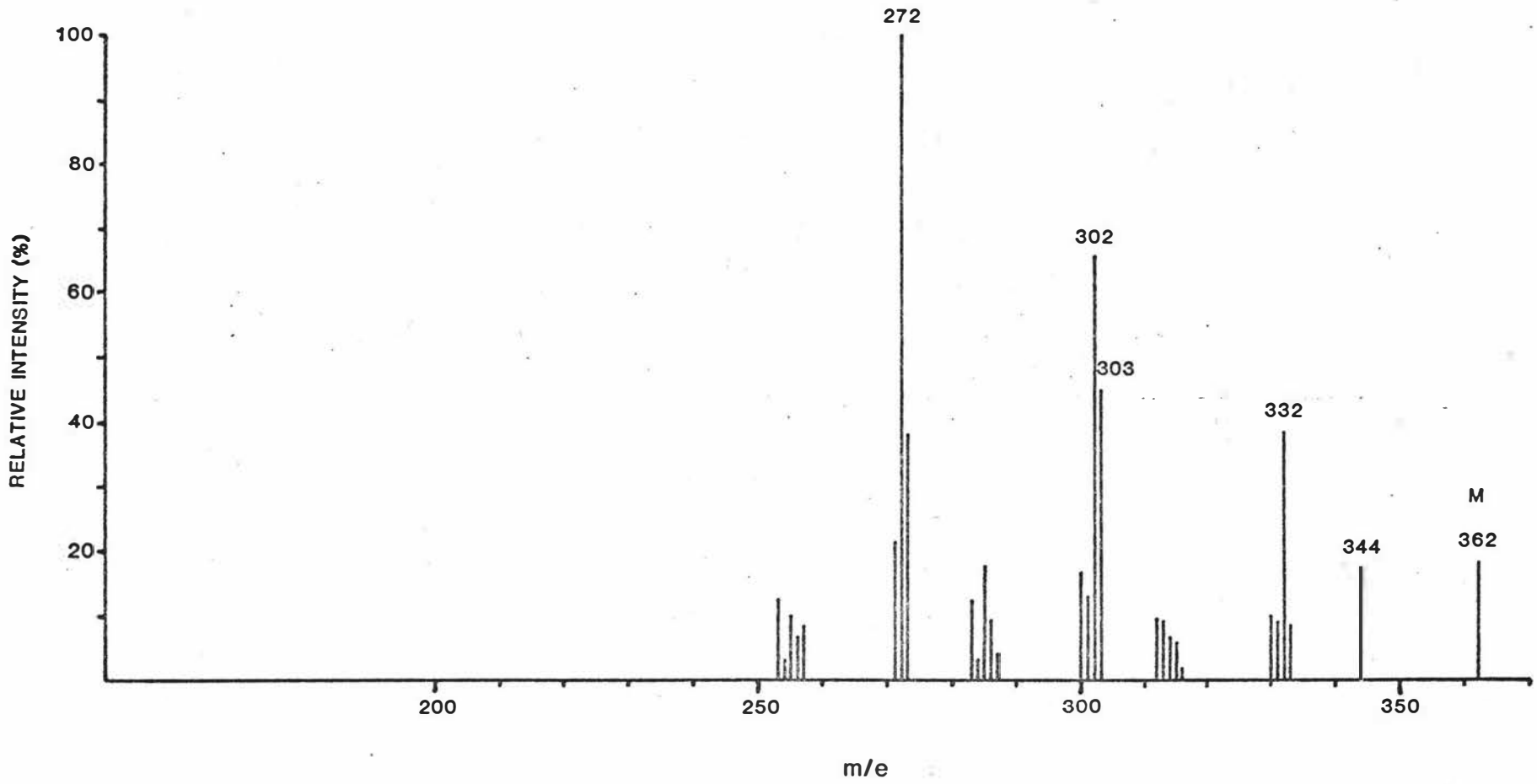


Figure B3 Mass spectrum of 19-hydroxycortexolone; product of the action of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 on cortexolone. (Instrument: MS 9).

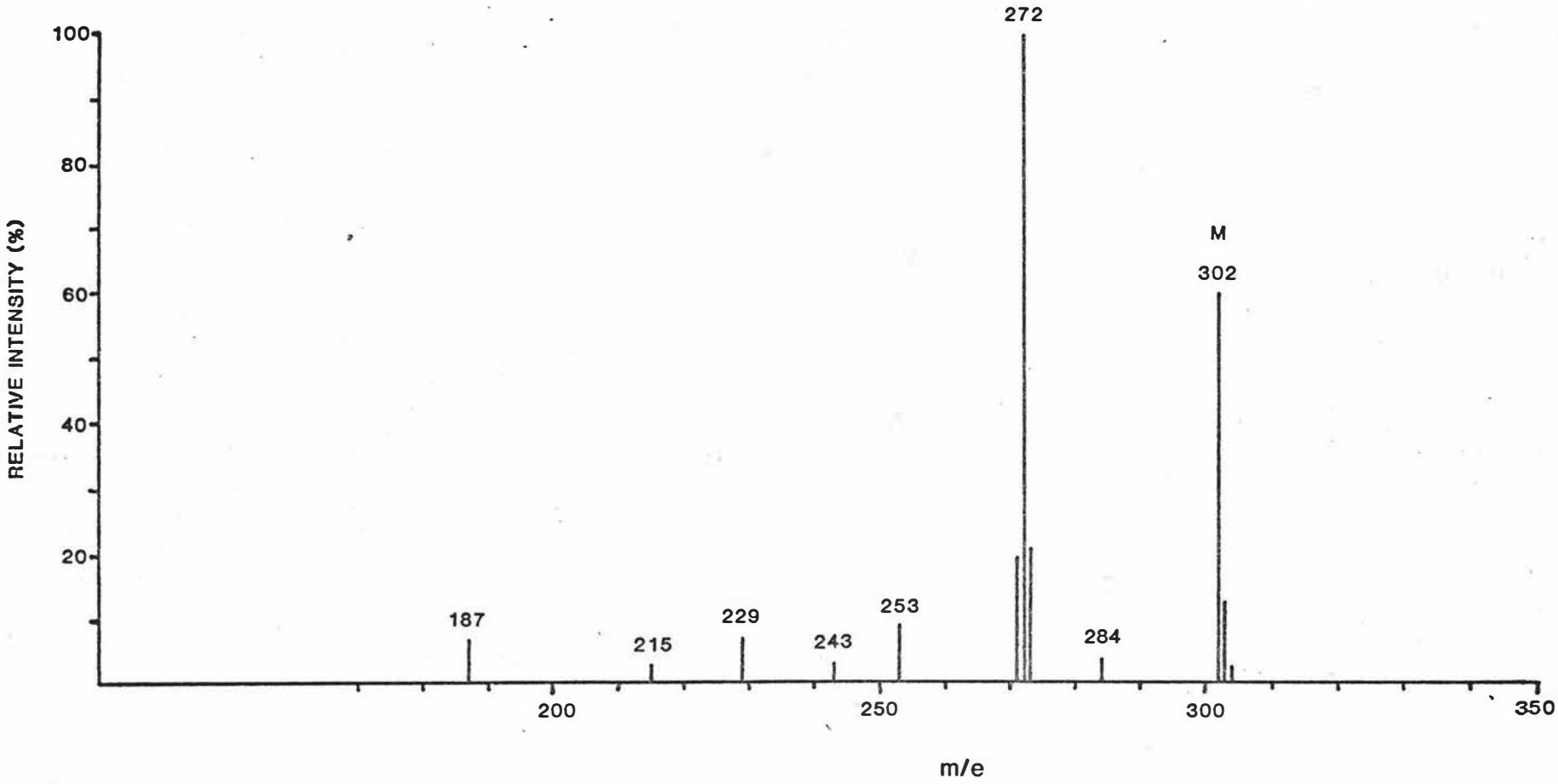


Figure B4 Mass spectrum of 19-hydroxy-4-androstene-3,17-dione; prepared by sodium bismuthate degradation of 19-hydroxycortexolone (product of the action of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 on cortexolone). Instrument: MS 9.

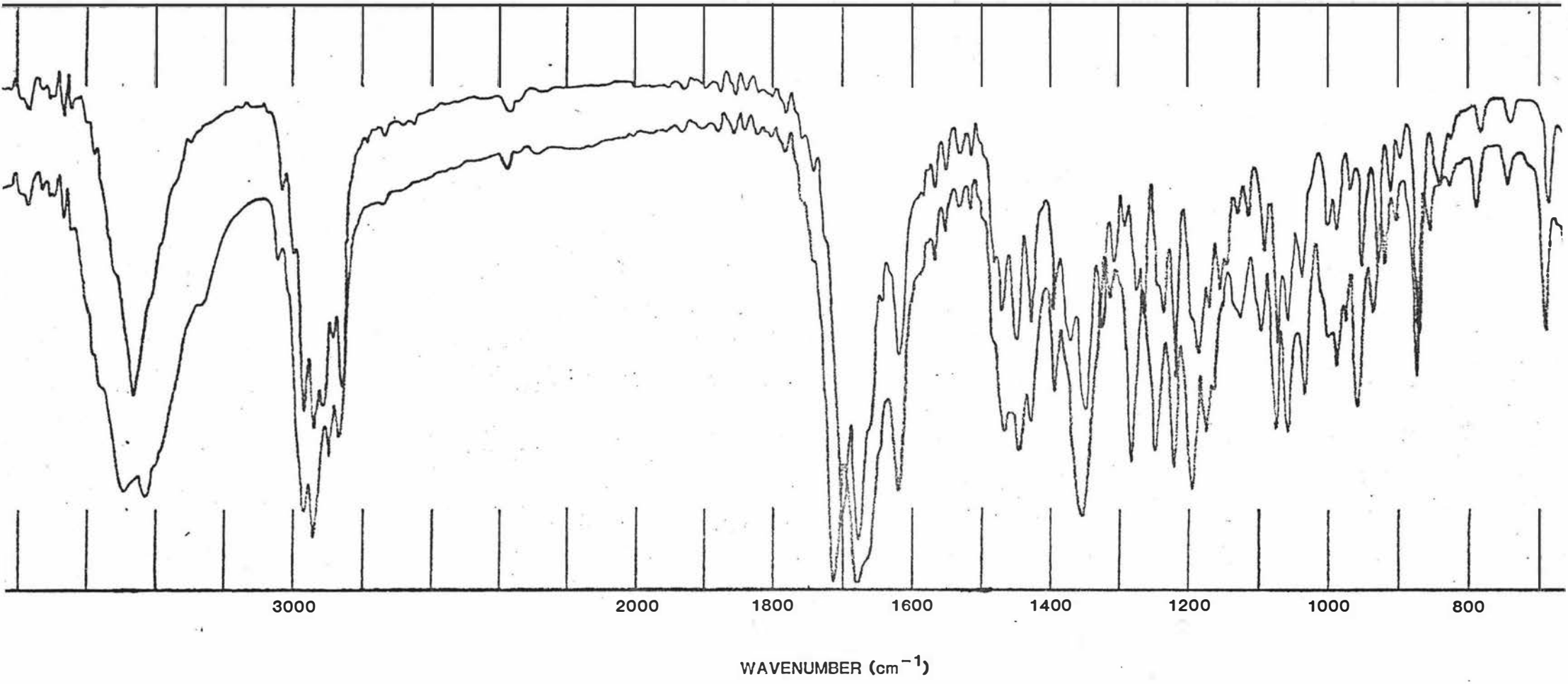


Figure B5 IR spectral characterisation of the progesterone hydroxylation product of *Pestalotia* sp. PDDCC 3062. Authentic 11α-hydroxyprogesterone : upper line Microbial product : lower line

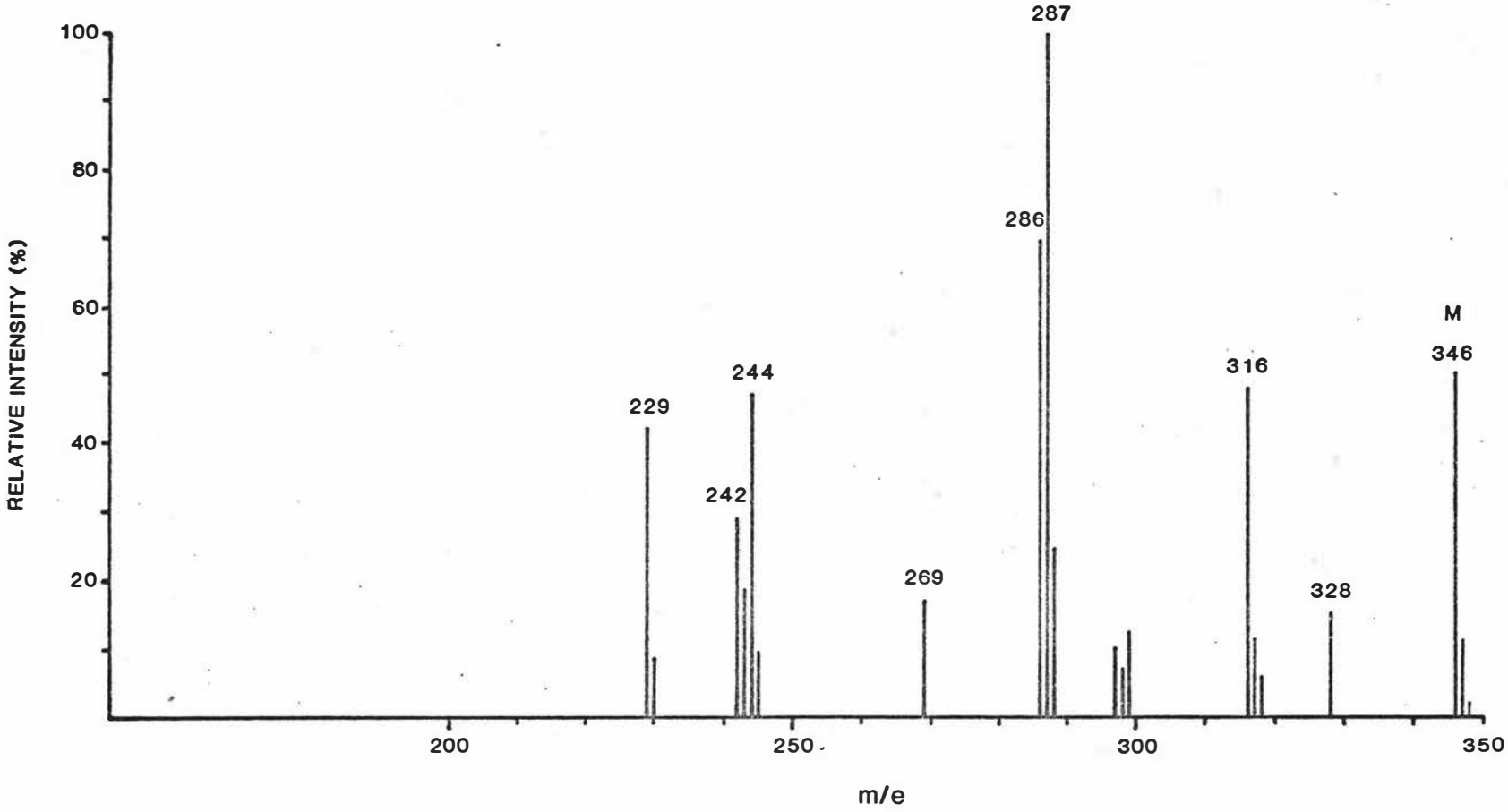


Figure B6. Mass spectrum of cortexolone. (Instrument: MS 9).

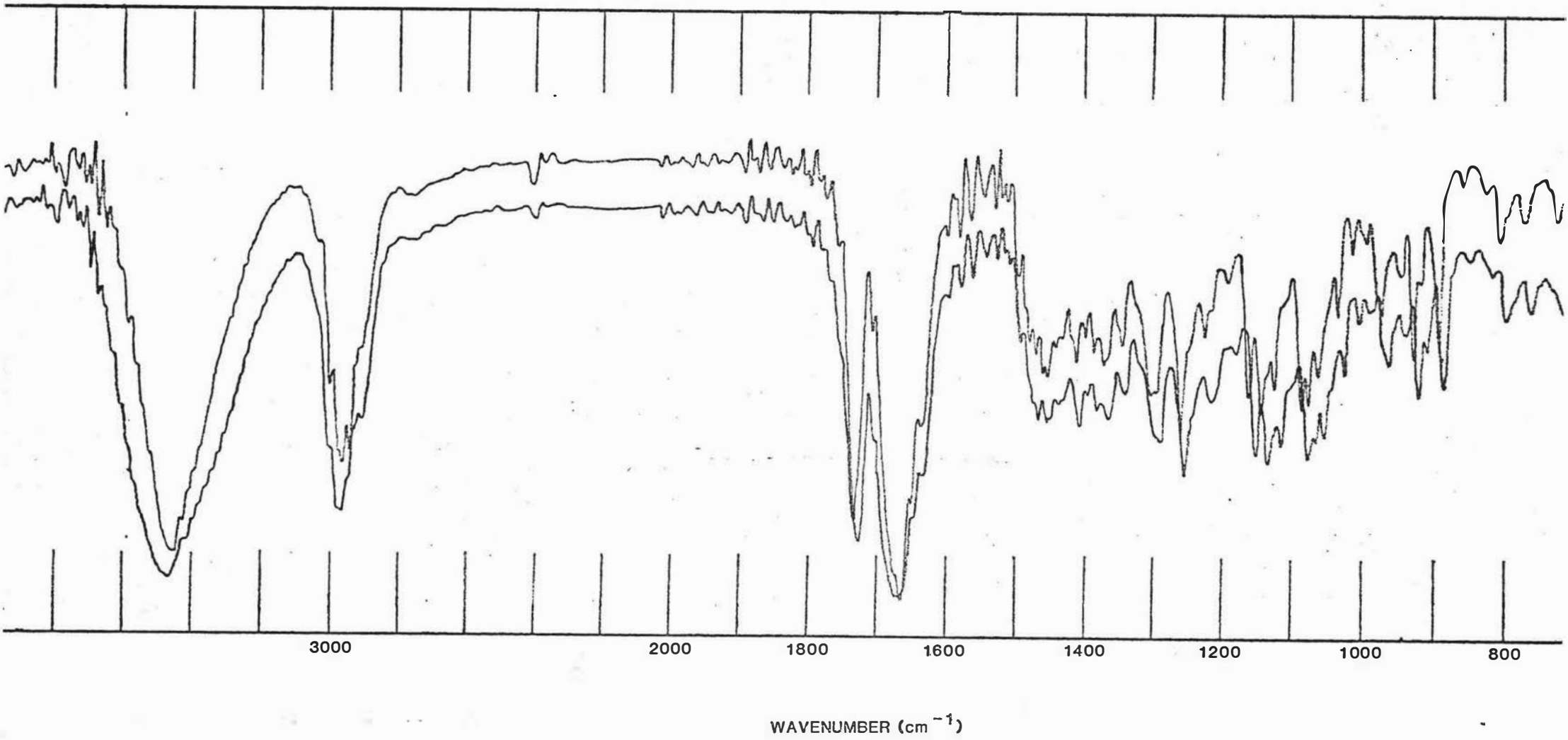


Figure B7 IR spectral characterisation of putative 11β-hydroxycortexolone; product of the action of *P. filamentosa* f.sp. *sasakii* IFO 5254 on cortexolone.
Authentic 11β-hydroxycortexolone: upper line
Microbial product : lower line

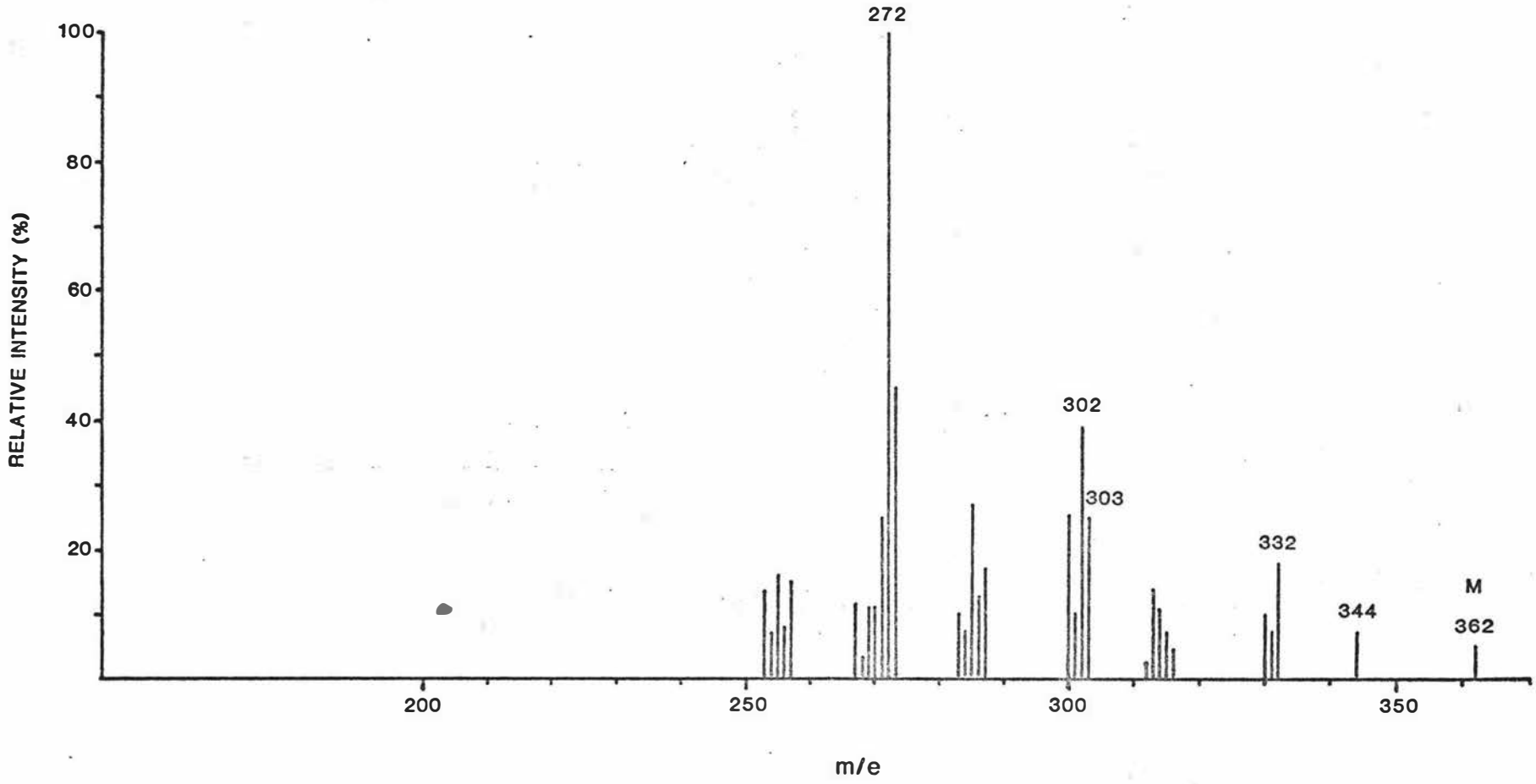


Figure B8 Mass spectrum of 19-hydroxycortexolone; product of the action of *P. filamentosa* f.sp. *sasakii* IFO 5254 on cortexolone. (Instrument: MS 30).

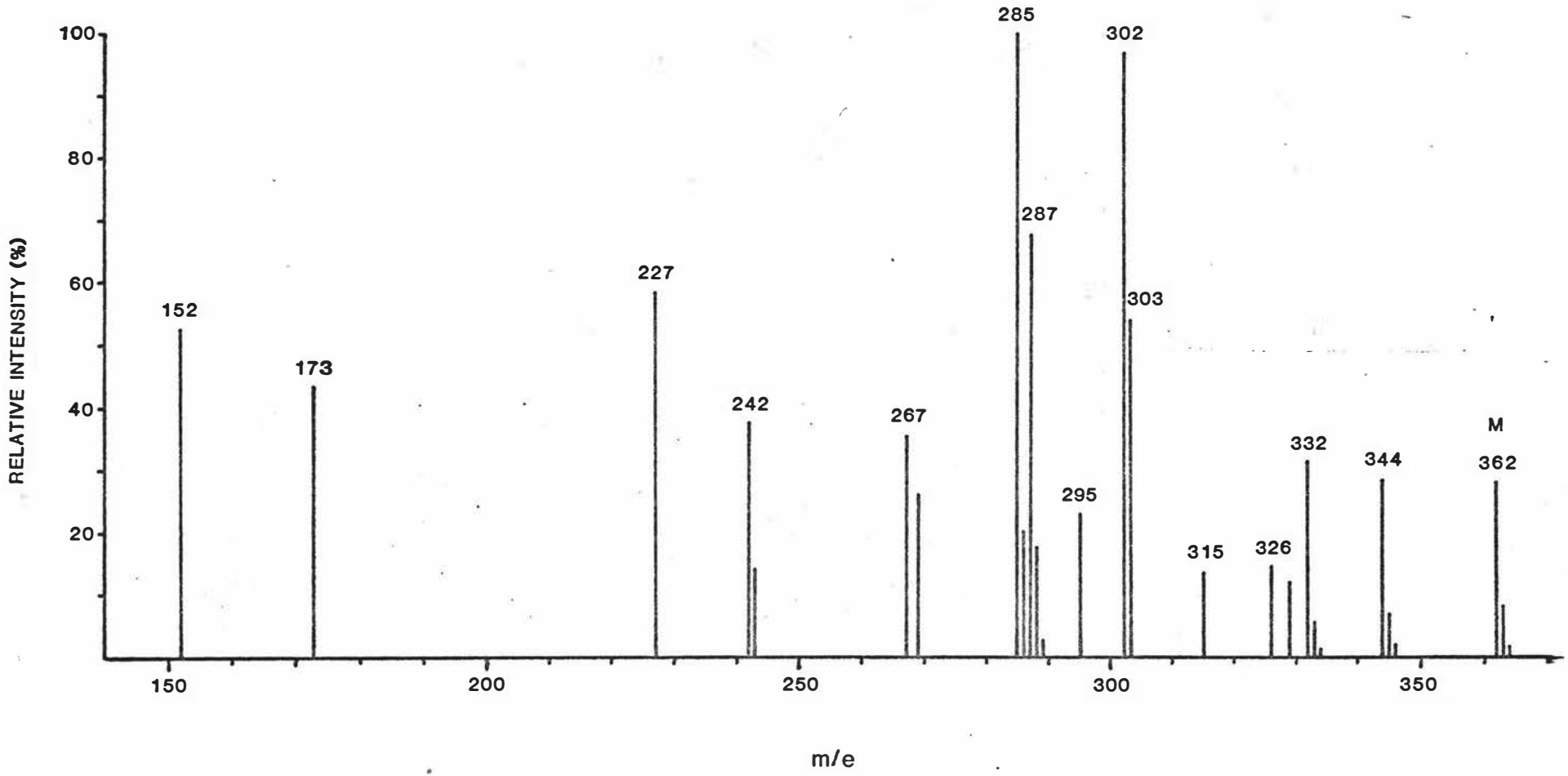


Figure B9 Mass spectrum of putative 6β-hydroxycortexolone; product of the action of *C. practicola* IFO 6253 on cortexolone. (Instrument: MS 9).

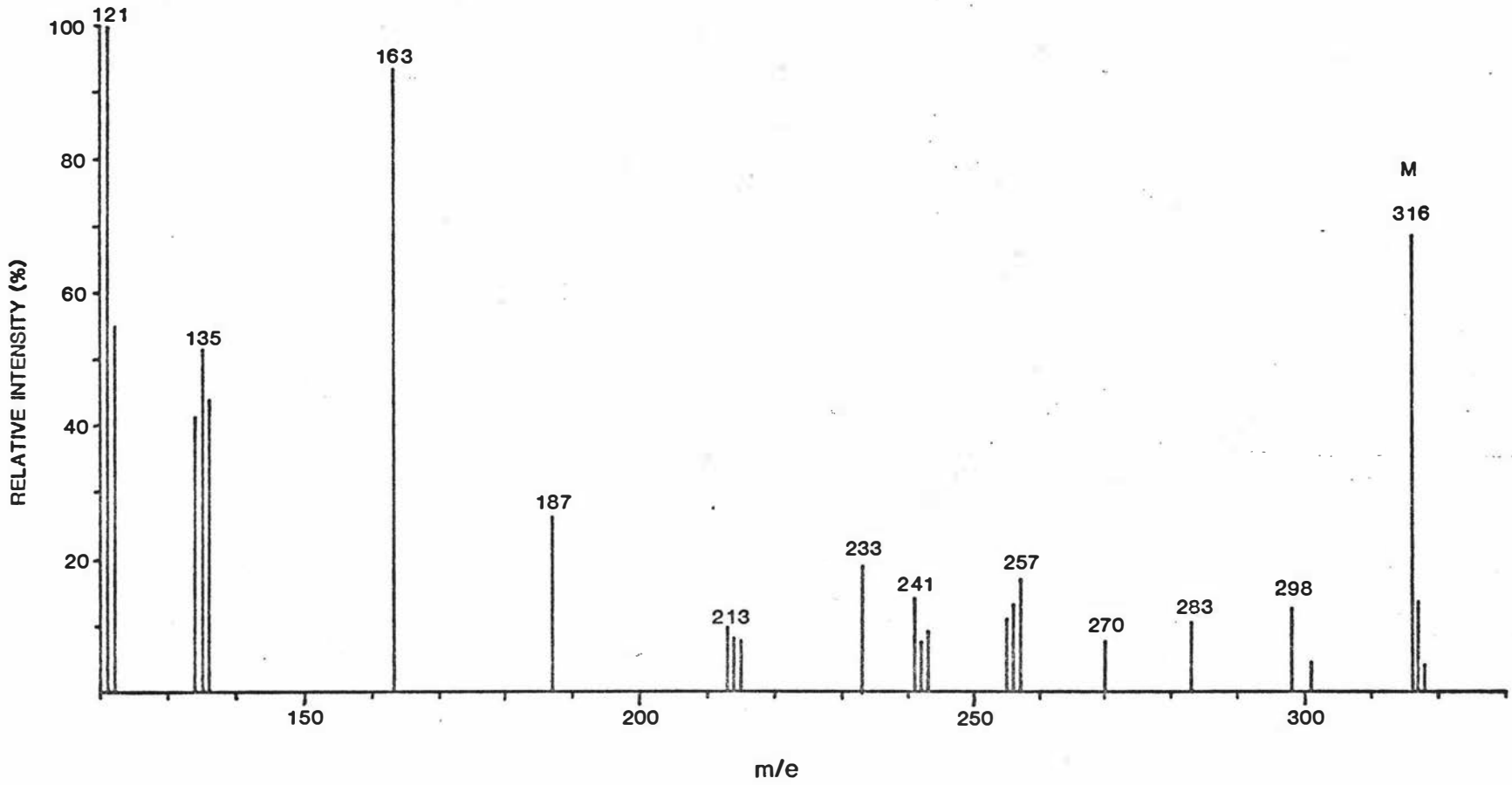


Figure B10 Mass spectrum of putative 14α-hydroxy-4-androstene-3,11,17-trione; product of the action of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 on androstenedione. (Instrument: MS 30).

Table B1 Proton and Carbon-13 Nuclear Magnetic Resonance spectra of progesterone and synthesised 19-hydroxy-progesterone

	^{1}H-NMR		^{13}C-NMR			
	Progesterone	19-Hydroxy-progesterone	Progesterone		19-Hydroxy-progesterone	
18-CH_3	0.678	0.661	C20	208.59	C20	208.75
19-CH_3	1.192	-	3	198.85	3	199.50
19-CH_2	-	4.000(q)	5	170.44	5	166.50
21-CH_3	2.120	2.107	4	124.02	4	126.80
4-CH	5.732	5.930	17	63.63	19	65.90
			14	56.16	17	63.47
			9	53.89	14	56.65
			13	43.99	9	54.05
			10+12	38.80	13	43.99
			1+8	35.87	10+12	39.12
			2	34.09	1	36.36
			6	32.79	8	35.06
			7	32.14	2+6	33.60
			21	31.33	7	32.30
			16	24.51	21	31.33
			15	23.05	16	24.35
			11	21.26	15	23.05
			19	17.53	11	21.75
			18	13.31	18	13.47

(a) Solvents: $CDCl_3$

(b) Chemical shifts (δ) expressed in ppm downfield from the internal standard tetramethylsilane

(c) q refers to quartet

(a) Solvents: $CDCl_3$

(b) Chemical shifts (δ) expressed in ppm downfield from the internal standard tetramethylsilane

(c) Assignments made by comparison with published data (Blunt and Stothers, 1977).

Table B2 Proton and Carbon-13 Nuclear Magnetic Resonance spectra of 19-hydroxycortexolone (product of the action of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 on cortexolone).

	^{1}H-NMR	^{13}C-NMR	
18-CH_3	0.800	C20	213.13
19-CH_3	-	3	199.18
19-CH_2	*	5	168.17
21-CH_2	4.156(q)	4	123.53
4-CH	6.099	17	89.60
		21	67.69
		19	65.26
		9	54.22
		14	51.46
		13	48.37
		10	44.48
		1+8	36.69
		12	35.71
		2	34.74
		16	33.93
		6	32.95
		7	31.63
		15	24.02
		11	21.75
		18	15.42

* obscured by solvent peaks

(a) Solvent: pyridine-d_5

(b) Chemical shifts (δ) expressed in ppm downfield from the internal standard tetramethylsilane.

(c) q refers to quartet

(a) Solvent: pyridine-d_5

(b) Chemical shifts (δ) expressed in ppm downfield from the internal standard tetramethylsilane

(c) Assignments made by comparison with published data (Blunt & Stothers, 1977).

Table B3 Carbon-13 Nuclear Magnetic Resonance spectrum of 11α-hydroxyprogesterone (product of the action of *Pestalotia* sp. PDDCC 3062 on progesterone).

	δ (ppm)
	$CDCl_3$ (solvent)
C20	208.75
3	200.15
5	170.77
4	124.50
11	68.83
17	62.98
9	58.92
14	55.19
12	50.32
13	44.15
10	39.93
2	37.50
8	34.90
1	34.09
6	33.44
21+7	31.33
16	24.19
15	22.89
19	18.18
18	14.45

(a) Chemical shifts (δ) expressed in ppm downfield from the internal standard tetramethylsilane.

(b) Assignments made by comparison with published data (Blunt and Stothers, 1977).

Table B4 Carbon-13 Nuclear Magnetic Resonance Spectrum of putative 6β-hydroxycortexolone (product of the action of *Corticium practicola* IFO 6253 on cortexolone).

	δ (ppm)
	DMSO (solvent)
C20	211.8
3	199.0
5	168.8
4	125.0
17	88.3
*	70.9
21	65.9
9	52.8
14	49.7
13	46.9
2+12	33.8
6+16	30.0
7	29.4
15	23.4
11	20.1
19	19.0
18	14.5

(a) Chemical shifts (δ) expressed in ppm downfield from tetramethylsilane.

(b) Assignments made by comparison with published data (Blunt and Stothers, 1977). Signals for C-1, C-8, C-10 obscured by solvent peaks.

* signal for hydroxylated carbon

APPENDIX C

MULTIPLE LINEAR REGRESSION ANALYSIS OF SPECIFIC RATE OF 19-HYDROXYLATION VERSUS SPECIFIC GROWTH RATE AND MYCELIAL PERCENTAGE NITROGEN

q_{19} (mg/g N.h)	μ (h^{-1})	MPN (%)
50.4	0.056	7.45
51.8	0.059	7.80
37.2	0.063	4.57
22.9	0.058	3.50
23.7	0.083	4.10
43.9	0.023	6.80
17.0	0.022	2.70
48.9	0.110	7.10
12.8	0.106	5.10
11.7	0.104	6.00

(i) Regression of q_{19} versus μ, MPN

$$q_{19} = -174\ \mu + 7.91\ \text{MPN}$$

Variable	Coefficient	St. Dev. of Coef.	t-ratio
μ	-174.49	94.32	-1.85
MPN	7.914	1.225	6.46

with 8 degrees of freedom

(ii) Analysis of Variance

Due to	DOF	Sum of Squares	Mean Squares
Regression	2	11823.95	5911.97
Residual	8	777.51	97.19
Total	10	12601.46	

(iii) Analysis of the significance of each variable in the model, at the 95% confidence level, can be performed by comparing the t-ratios with the standard $t_{0.95}$ value for 8 dof, which is 2.306. Thus μ is not significant at this level, while MPN is highly so.

(iv) By eliminating the final two rows of values from the data (the experiments at D = 0.119 h^{-1} with "high" q_{gl} values), an even clearer regression is obtained.

$$q_{19} = + 10.9\ \mu + 6.62\ MPN$$

Variable	Coefficient	St. Dev. of Coef.	t-ratio
μ	10.86	39.09	0.28
MPN	6.6206	0.4381	15.11

with 6 degrees of freedom.

(iv) Analysis of Variance

Due to	DOF	Sum of Squares	Mean Squares
Regression	2	12237.79	6118.90
Residual	6	62.94	10.49
Total	8	12300.73	

(vi) The appropriate standard $t_{0.95}$ value is 2.447, and by comparison with the t-ratios of μ and MPN, the same conclusions as in (iii) are even more strongly emphasised.

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The Effect of Dissolved Oxygen Tension on 11β- and 19-Hydroxylation of Reichstein's Substance S by *Pellicularia filamentosa*

T. A. Clark, R. Chong, and I. S. Maddox

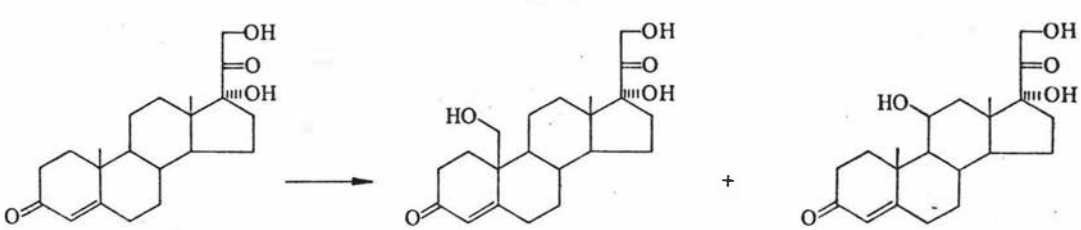
Biotechnology Department, Massey University, Palmerston North, New Zealand

Summary. The 11β- and 19-hydroxylation enzyme(s) of *Pellicularia filamentosa* IFO 6298 have been shown to be inducible by Reichstein's Substance S. By using the protein synthesis inhibitor, cycloheximide, in fermenter culture the effects of dissolved oxygen tension (DOT) on enzyme induction and enzyme expression have been separately investigated. For both hydroxylations, an optimum DOT for induction has been shown at 15% of saturation, while the optimum for expression is at 30% of saturation. The results have been verified in the absence of cycloheximide. Thus, maximum rates of hydroxylation are achieved when induction is performed at low DOT, followed by elevation to ensure maximum expression.

Introduction

The microbiological 19-hydroxylation of steroids has received little study despite the importance of 19-hydroxysteroids as intermediates for the production of 19-norsteroids. This is probably because there exists a chemical route to 19-hydroxysteroids (Heusler and Kalvoda 1972).

Pellicularia filamentosa acts on Reichstein's Substance S to produce the 19-hydroxy compound 17α,19,21-trihydroxypregn-4-en-3,20-dione, and the 11β-hydroxy compound hydrocortisone (Fig. 1) (Takahashi and Hasegawa 1961). In fermentations where hydrocortisone is produced by direct 11β-hydroxylation of Reichstein's Substance S, e.g. by *Curvularia lunata,* the yield may be affected by competing reactions such as 14α-hydroxylation, giving rise to non-useful byproducts (Charney and Herzog 1967). We have been studying the hydroxylation of Reichstein's Substance S by *P. filamentosa* because the possibility of directing the system to produce either the 19- or 11β-hydroxy compound as major product is attractive. A study aimed at manipulating the 19:11β product ratio is in progress. During this study, we became interested in improving the rate of hydroxylation. Recent studies on microbial monooxygenases have indicated the importance of dissolved oxygen tension (DOT) on the induction of these enzyme systems (Mauersberger et al. 1980; Wiseman 1977). In particular, Hanisch et al. (1980) showed that for the 11α-hydroxylation of progesterone by *Rhizopus nigricans* there was a marked optimum DOT for induction of the enzyme. Furthermore, the optimum for expression of



Compound S — 19–hydroxy Compound S — hydrocortisone

Fig. 1. Hydroxylation of Reichstein's Substance S by *Pellicularia filamentosa* IFO 6298

Offprint requests to: I. S. Maddox

0171-1741/82/0014/0131/$01.00

activity lay at a much higher DOT. Consequently, maximum production rates of hydroxyprogesterone were achieved when induction was performed at low DOT, followed by elevation of the DOT to enhance expression of the synthesized enzyme.

In this paper, we describe an investigation into the effect of DOT on the induction and expression of the *Pellicularia filamentosa* hydroxylase system.

Materials and Methods

Organism. Pellicularia filamentosa f.sp *microsclerotia* IFO 6298, obtained from the Institute of Fermentation, Osaka, Japan, was maintained on Yeast Extract Agar (Difco, Detroit, USA) and subcultured monthly.

Chemicals. All solvents were of analytical reagent grade and were obtained from B.D.H. Ltd. (Palmerston North, New Zealand). Reichstein's Substance S (17α,21-hydroxypregn-4-en-3,20-dione), hydrocortisone (11β,17α 21-trihydroxypregn-4-en-3,20-dione), 19-hydroxyandrost-4-en-3,17-dione, 11β-hydroxyandrost-4-en-3,17-dione, 11α-hydroxyprogesterone and cycloheximide were obtained from the Sigma Chemical Co. (St. Louis, USA). Hydrocortisone was crystallised from methanol prior to use as a standard for high-performance liquid chromatography (HPLC).

17α,19,21,Trihydroxypregn-4-en-3,20-dione was prepared by hydroxylation of Reichstein's Substance S using *P. filamentosa* in a fermenter. The medium, fermenter and inoculum preparation were as described below. The dissolved oxygen tension was not controlled; the agitation rate was 600 rpm and the aeration rate 600 ml/min. Reichstein's Substance S (0.5 g/l) was added 24 h after inoculation, and the fermentation was continued for a further 20 h. Extraction of the whole culture with ethyl acetate, followed by concentration of the extract yielded crystals of the 19-hydroxy compound m.p. 220–230 °C. Recrystallisation from methanol gave a product m.p. 230–234 °C, uncorrected (cf. 234–235 °C, Takahashi and Hasegawa 1961), which was used as a HPLC standard.

Product Identification. The 17α,19,21-trihydroxypregn-4-en-3,20-dione obtained from a fermentation by the procedure described above was identified by melting point (*vide supra*), mass spectrometry on a MS9 double focussing mass spectrometer (M^+ m/e 362), and degradation with sodium bismuthate (Brooks and Norymberski 1953) to 19-hydroxyandrost-4-en-3,17-dione. Comparison of the degradation product with an authentic sample using mass spectrometry, high-performance liquid chromatography (40:60, methanol: water, *vide infra* for other operating conditions) and thin layer chromatography further confirmed the identity of the fermentation product.

Hydrocortisone was isolated from the ethyl acetate mother liquor remaining after removal of 17α,19,21-trihydroxypregn-4-en-3,20-dione crystals. The concentrated mother liquor was subjected to preparative thin layer chromatography using ethyl acetate: hexane: acetic, acid, 75:20:5. Work-up of the hydrocortisone band gave crystals which behaved identically to authentic hydrocortisone when subjected to thin layer chromatography, HPLC. (50:50 methanol: water) and mass spectrometry. Identity was further confirmed on degradation with sodium bismuthate to 11β-hydroxyandrost-4-en-3,17-dione.

Thin layer chromatography (t.l.c.) was performed with silica gel GF_{254} (Merck) plates. For analytical t.l.c., 0.25 mm thick plates were run in three solvent systems; chloroform: ethanol 9:1, ethyl acetate: hexane: acetic acid 75:20:5, and ethyl acetate: hexane: acetic acid: ethanol 72:13.5:10:4.5. Preparative t.l.c. was carried out using 1 mm thick plates in the ethyl acetate: hexane acetic acid system.

Mass spectrometric data were obtained from a MS9 double focussing mass spectrometer through the courtesy of Professor R. Hodges, Dept. of Chemistry, Biochemistry and Biophysics, Massey University.

Cultivation. The medium used contained glucose (25 g/l) and yeast extract (10 g/l, Difco Laboratories), adjusted to pH 6.0. For shake-flask experiments, 100 ml of medium were dispensed in 250-ml Erlenmeyer flasks and sterilized at 121 °C for 15 min. Incubation was at 30 °C on an Orbital Incubator (Gallenkamp Ltd., London, UK) at 180 rpm. Inocula were prepared by subculturing from a slope into a shake-flask culture; after 3 days incubation, 5 ml of this culture were used as a standard inoculum. After 2 days incubation, Reichstein's Substance S (final concentration 0.25 g/l), dissolved in dimethylformamide (final concentration 10 g/l) was added.

Fermenter experiments were performed in a Multigen Bench-Top Fermenter, Model F-2000 (New Brunswick Scientific Co., New Brunswick, USA), using a 2-l glass vessel with a working volume of 1,500 ml. During the fermentation, pH was measured using an Ingold combined glass electrode (Type 761-351B), and controlled at pH 6.0 by addition of either 1M NaOH or 1 M H_2SO_4 using a Model pH-40 pH controller (New Brunswick Scientific Co.) coupled to a Model XpH-42 pump module (New Brunswick Scientific Co.). Temperature was controlled at 30° ± 0.2 °C. Dissolved oxygen tension (DOT) was measured with a Dissolved Oxygen Probe, Model M1016-0208 (New Brunswick, Scientific Co.). The flow rate of air sparged into the vessel was set at 550 ± 50 ml/min. In order to control the DOT the flow rate could be adjusted to 1,200 ml/min using an on-off controller coupled to a solenoid valve which was connected to the compressed air line. This allowed control to within ± 5% of the percentage saturation scale. Agitation speed was between 350 and 500 rpm (impeller tip speed 0.92–1.30 m/s) depending on the DOT required for the particular experiment. The fermenter vessel was baffled, and agitation was provided by 3 six-bladed, disc turbine impellers mounted at equally-spaced intervals from 5 cm above the vessel base to 5 cm below the medium surface. The assembled vessel, containing medium but without electrodes, was sterilized in an autoclave at 115 °C for 20 min. The electrodes were soaked in 5% formalin solution and rinsed with sterile distilled water before being aseptically mounted in the vessel. A standard inoculum was prepared by sub-culturing from a slope into 100 ml of medium and incubating in shake-flask for 3 days. Transfer of 5 ml of this culture into another 100 ml of medium followed by incubation for 3 days provided 100 ml of inoculum. After inoculation, fermentation was allowed to proceed for approximately 20 h, during which time the DOT was allowed to stabilize at the level selected for induction of the steroid hydroxylases. After several hours (usually 4–5 h) of stable operation, Reichstein's Substance S, dissolved in dimethylformamide, was added to the fermenter. After a further 4 h the DOT was adjusted to that required for hydroxylase expression. For experiments involving cycloheximide, the protein synthesis inhibitor was added to a final concentration of 250 μg/ml simultaneously with the change in DOT.

Analytical Procedures. Fermentation samples (20 ml), or whole shakeflask contents, were withdrawn as required. After addition of 2.5 mg of 11α-hydroxyprogesterone as internal standard, the samples were filtered to remove mycelium, and the broth was extracted twice with ethyl acetate (30 ml). The mycelium was

washed with ethanol (10 ml) and ethyl acetate (10 ml). The combined extracts were washed with $NaHCO_3$ (40 ml, 25 g/l) and water (20 ml) prior to drying over anhydrous Na_2SO_4. After filtering, the solvent was removed at 50 °C in vacuo and the residue was dissolved in methanol prior to HPLC analysis. Using this extraction procedure, the various compounds (0.5 g/l final concentration) could be recovered from the liquid medium with 94–96% efficiency. Recoveries were also checked with autoclaved mycelia in shake-flasks (100 ml culture). After adding the steroid (0.5 g/l final concentration), and shaking for 2 days at 30 °C, recoveries of 86–90% were obtained.

HPLC analysis was performed on a Waters Associates Model ALC/GPC 244 liquid chromatograph using a BioRad Laboratories ODS-10 reverse phase column (250 mm x 4 mm). The mobile phase was methanol:water, 50:50, operated at a flow rate of 1.5 ml/min. Approximately 10 µl of sample was applied to the column and detection was by a Waters Associates Model 440 absorbance detector at 254 nm, 0.05 AUFS. Peak heights were measured, and the compounds quantitated by reference to a standard mixture run in parallel with the sample injections. Analysis of duplicate fermentation samples gave agreement to within 2% of each other.

Hydroxylation Rates and Activities. For fermentations involving cycloheximide, the progress of the hydroxylation was followed by extraction and analysis of samples withdrawn from the fermenter every 30 min for a period of 2 h after cycloheximide addition. The rates were measured as µ moles of product produced/g dry weight mycelium/h, the dry weights being determined at the time of cycloheximide addition. In experiments which sought to determine optimum conditions for enzyme induction, the rates obtained under constant conditions of enzyme expression were used to describe relative amounts of enzyme activity during the induction period.

For fermentations not involving cycloheximide, samples were withdrawn from the fermenter at 30 min intervals after a 4 h induction period, and rates calculated as above.

Results and Discussion

Initially, experiments were performed to demonstrate the inducible nature of the 19- and 11β-hydroxylases. Duplicate shake-flask cultures were grown for 48 h, at which time cycloheximide was added to one flask to a final concentration of 250 µg/ml. After a further 15 min incubation, Reichstein's Substance S (final concentration 0.25 g/l) was added to both flasks and incubation was continued for 6 h. Where cycloheximide was added prior to steroid no hydroxylation was observed. In the absence of cycloheximide, however, levels of 0.019 g/l and 0.018 g/l were obtained for the 19-hydroxy compound and hydrocortisone, respectively. To demonstrate that cycloheximide has no direct effect on the enzymatic reactions, fermenter cultures which were actively hydroxylating were treated with the protein synthesis inhibitor. When compared to appropriate control fermentations, no enzyme inhibition was observed during the 2 h period following its addition. Hence, both the 19- and 11β-hydroxylases were inducible by Reichstein's Substance S. (For convenience, we are assuming that different enzymes are responsible for the two hydroxylations, but our data do not rule out the possibility that one enzyme is responsible for both activities.)

Having established the inducible nature of the hydroxylases it was then possible to perform experiments where induction of enzyme synthesis could be clearly separated from enzyme expression. All experiments were now conducted in a fermenter. Thus, to investigate the effect of DOT on hydroxylase expression, induction was carried out under constant conditions to ensure equal amounts of enzyme synthesis. After addition of cycloheximide the DOT was adjusted to the appropriate value, and by measuring the rate of hydroxylation the effect of DOT on expression was investigated. In a similar manner the effect of DOT on induction was investigated by inducing at different DOT levels, followed by addition of cycloheximide to prevent further enzyme synthesis. The amount of enzyme present was then determined by its activity under constant conditions of expression.

In all experiments both the 19-hydroxy and 11β-hydroxy products were observed. The ratio of the two compounds was invariably constant, at 1.0:0.84 19-:11β, and so the rates were always related in the same proportion. Hence, only the data for the 19-hydroxylation are presented here; those for the 11β-hydroxylation can be calculated from this ratio.

To investigate the effect of the DOT on hydroxylase expression, induction was performed at a DOT of 25% saturation. After 4 h induction in the presence of 2.3 g/l Reichstein's Substance S, cycloheximide was added and the DOT adjusted as appropriate. The rate of hydroxylation was then monitored. The results are expressed in Fig. 2. Although similar levels of activity could be induced using a Reichstein's Substance S concentration as low as 0.093 g/l, the high substrate concentration was used to ensure that the hydroxylase was saturated with steroid. The results show that there is an apparent optimum for enzyme expression at a DOT of 30% of saturation.

To further investigate the apparent decrease in hydroxylation rate as the DOT changes from 30% to 60% of saturation, fermentations were conducted where induction was at 50% of saturation. Following addition of cycloheximide, the hydroxylase was expressed at a DOT of either 30% or 50% of saturation. In the former case, a rate of 1.4 µmoles/g dry wt./h was observed, compared with 1.0 µmoles/g dry wt./h for the latter. This decrease in rate with increased DOT is in proportion to the expected rates shown in Fig. 2, and thus confirms the effect.

Fermentations were now performed to investigate the effect of DOT on enzyme induction. Induction was carried out at the appropriate DOT for 4 h in the presence of 2.3 g/l Reichstein's Substance S. Cycloheximide was then added and the DOT was adjusted to a standard level of 30% of saturation. Rates of hydroxylase expression were then monitored over a 2 h period. The results, shown in Fig. 3, are plotted as the activity during expression, and

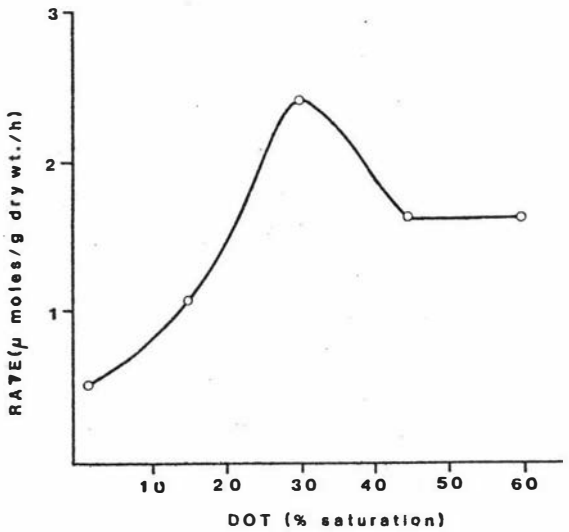


Fig. 2. Effect of DOT on specific rate of 19-hydroxylase expression (see text for experimental details)

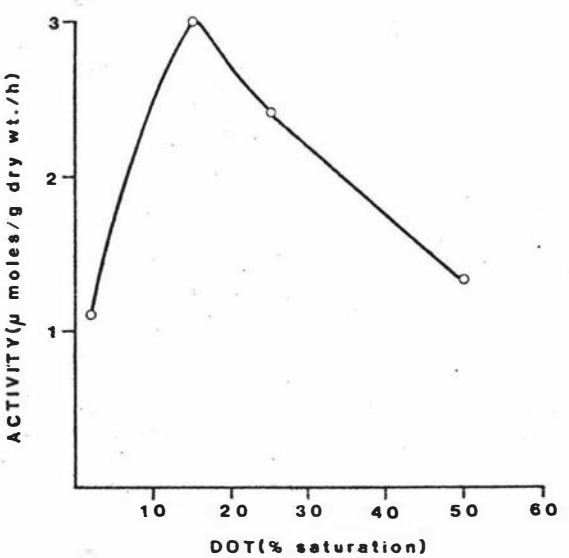


Fig. 3. Effect of DOT on the amount of 19-hydroxylase synthesized (see text for experimental details)

reflect the amount of enzyme present. An optimum DOT for induction was observed at 15% of saturation.

The data shown in Figs. 2 and 3 clearly suggest that maximum production of hydroxylated product is obtained when induction and expression are carried out at DOT values of 15% and 30% of saturation, respectively. To verify that the presence of cycloheximide was not producing erroneous data, a fermentation was performed under optimum conditions, but in the absence of cycloheximide. Thus, after 4 h induction at a DOT of 15% of saturation, the DOT was adjusted to 30% of saturation and the hydroxylation rate was measured over the subsequent 2 h period.

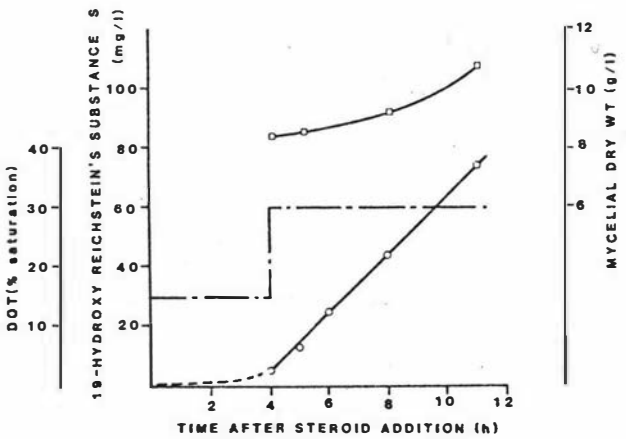


Fig. 4. Course of 19-hydroxylation of Reichstein's Substance S. o——o, 19-Hydroxy Reichstein's Substance S; □——□, mycelial dry weight; – · –, DOT

A rate of 3.2 μmoles/g dry wt/h was observed, compared to 3.0 μmoles/g dry wt./h in the presence of cycloheximide. The course of this hydroxylation is shown in Fig. 4. A further fermentation was now performed in the absence of cycloheximide where the DOT for both induction and expression was 30% of saturation. A hydroxylation rate of 2.1 μmoles/g dry wt./h was observed (cf. 2.2 μmoles/g dry wt./h interpolated from Fig. 3). Thus, the optimum conditions were verified.

The results obtained in this work are similar to those reported by Hanisch et al. (1980) for the 11α-hydroxylase of *Rhizopus nigricans*. These authors demonstrated an optimum DOT for induction at 10% of saturation, while the optimum DOT for expression was much higher. They did not, however, use a protein synthesis inhibitor to separate induction from expression, but showed that under at least one set of conditions induction was complete after a brief period. Hence they assumed no further protein synthesis during expression. We do not require this assumption as protein synthesis had been halted after the induction stage. However, it must be noted that after addition of cycloheximide hydroxylation rates were linear for only a few hours. This implies that continual protein synthesis is necessary to maintain rates, and is similar to the observation described by Maddox et al. (1981) using immobilized cells of *R. nigricans*.

Hanisch et al. (1980) when discussing the low DOT for optimum enzyme induction, explained their data on the basis that the 11α-hydroxylase is known to be a cytochrome P_{450} system and low DOT optima have been described for various cytochromes in other microbial systems. It is not known whether the 19- and 11β-hydroxylases of *Pellicularia filamentosa* are cytochrome P_{450} systems, but the present findings would support this hypothesis.

With regard to the rate of hydroxylase expression, Hanisch et al. (1980) observed a Michaelis-Menten relationship with DOT. While the present results show the effect of oxygen limitation on rates at low DOT, the optimum at 30% of saturation deviates from Hanisch's findings. During growth studies with *P. filamentosa* we have observed a decrease in oxygen consumption rate below a DOT of 30–35% of saturation. It is possible that above this critical DOT the metabolism of the organism changes in such a way as to lower the rate of hydroxylase reactions, perhaps due to competition for oxygen with other metabolic pathways. In this respect it must be noted that the DOT within the mycelial mat will be lower than the measured bulk DOT, and that oxygen diffusion within the mat will limit the rate of oxygen transfer. Although higher impeller tip speeds were used to achieve the higher DOT levels, the actual speeds used were only 1.0 and 1.3 m/sec for 30% and 60% DOT, respectively. These speeds are well below those reported as causing shear damage to even delicate fungal mycelia (Hanisch et al. 1980). Furthermore, if this were the reason for the decline in rates above 30% DOT, successive decreases would be expected at 45% and 60% DOT. This is not the case.

In conclusion, the data show that increased rates of hydroxylation of Reichstein's Substance S can be achieved by inducing at low DOT followed by the elevation of the DOT to enhance enzyme expression. The results apply equally well to both the 19- and 11β-hydroxylations, so that their relative production rates cannot be manipulated by variation of DOT alone.

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