STUDY OF THE EFFECTS OF ANTIFUNGAL AGENTS UPON FUNGI OF MEDICAL IMPORTANCE

By

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DECLARATION

The major part of the *in vitro* study of antifungals was done by the author. Where indicated in the text special facilities and some assistance were provided by Mr. E. Forbes.

Animal experiments were performed jointly with Professor J. C. Gentles and part of the work encompassed in this section has been accepted for publication:-

Odds, F. C., Milne, L. J. R., Gentles, J. C. and Ball, E. Hazel, (1980). The *in vitro* and *in vivo* activity of a new imidazole antifungal, ketoconazole. Journal of Antimicrobial Chemotherapy, 6, 97-104.

Results of other animal experimental work not embodied in this thesis are published in:-

English, Mary P., Gentles, J. C. and Ball, E. Hazel, (1979). Experimental infection of guinea pigs with atypical and dysgonic strains of *Microsporum canis*. Mycopathologia 67 (3), 179-181.

SUMMARY

A cellophane square technique has been used to determine Minimum Inhibitory Concentrations and morphological changes induced by exposure of various medically important fungi to antifungal agents. The procedure allows earlier assessment of fungistatic and fungicidal activity than can be achieved by conventional techniques.

Fungistatic activity of the imidazoles has been shown not to be affected by the phase of growth of the fungi, but fungicidal activity of miconazole and econazole was found to be more pronounced against the actively growing phases of both yeasts and filamentous fungi. Ketoconazole differed by having significantly higher M. I. C. levels against the test fungi, but was more effective in killing stationary phase cells of Trichophyton mentagrophytes. However, fungicidal activity of ketoconazole against both actively growing and stationary phase cells of Candida albicans was low compared to the other two imidazoles.

A feature which was peculiar to the yeasts C. guilliermondii and C. tropicalis was observed on sub-fungistatic concentrations of miconazole, econazole and ketoconazole. These yeasts were particularly sensitive to low concentrations of the imidazoles, but at higher concentrations there was some relief of toxicity resulting in partial recovery before growth was finally inhibited by fungistatic concentrations. Since these two yeasts also differed from others in their response to thiamin, the presence of trace

amounts of this vitamin in the medium may account for the phenomenon. Alternatively the imidazoles may affect a number of different metabolic pathways within the fungal cell.

Resistance to the antifungal agents did not arise spontaneously in any of the fungi during the course of this study. However, physiological adaptation which resulted in an approximate two-fold increase in the amount of growth of C. guilliermondii on econazole was observed although M. I. C. levels remained unaltered, and increased resistance to the imidazoles was induced in Aspergillus nidulans with U. V. light as the mutagenic agent. The potential for resistance to the imidazoles to arise spontaneously in these fungi therefore exists.

Selected antifungals were also examined for their in vivo activity. When administered orally, ketoconazole was found to be highly effective in curing ringworm infections in guinea pigs. Topical application of miconazole was shown to be as effective as an established treatment for ringworm, (tolnaftate), against experimentally induced dermatophytosis in guinea pigs.

GENERAL INTRODUCTION

Fungi were first recognised as pathogens in the 19th Century when Agostino Bassi demonstrated that muscardine disease of silkworms was caused by the fungus now known as Beauveria bassiana. During the next ten years the fungi associated with favus and thrush in man were described. However, it was left inconclusive whether such fungi were the result or the cause of disease. The work of Pasteur and Koch clearly defined the role of bacteria in disease and, later, protozoa and viruses were also recognised as pathogens, but fungi were virtually ignored.

A preliminary survey (Duncan, 1945) showed mycotic infections to be common in Britain, and more than sixty species of fungi were claimed as pathogenic for man and animals (Ainsworth, 1950).

Effective prophylactic and control measures reduce the number of deaths due to bacterial and viral infections. No control measures are known for several of the mycoses which may be fatal, and although a number of the most widespread mycotic infections are seldom fatal, they may cause considerable distress to the patient and are often difficult to cure.

Within the past few decades it has been realised that the division between pathogenic and non-pathogenic fungi is not clearly defined. Opportunistic fungal infections occur when predisposing factors that lead to lowered host resistance allow normally harmless commensal or saprophytic organisms to become pathogenic (e.g. Candida spp. and Aspergillus spp.). Such infections have become an increasing problem in recent years with the growing use of immunosuppressive therapy, valve replacement surgery and the increased survival of patients with congenital and acquired immunodeficiencies.

Chemotherapy of the systemic mycoses is restricted to only a few agents, and topical treatment of superficial infections is of limited success because of the problem of penetration of the agents to the site of fungal activity. As a result treatment failures in all forms of mycoses are not uncommon and a search is continuing for new, effective, non-toxic agents.

The requirements for successful chemotherapy are that the target site on the pathogen must be essential for growth, and the host must be resistant to the effects of the drug.

In the case of bacterial pathogens there are vast differences in the physiology of the prokaryotic organism and the eukaryotic host, and consequently there are numerous targets for drug action. Fungi, however, are eukaryotic organisms and the differences between them and their hosts are much fewer.

Major differences exist between the cell envelopes of animals and fungi. A cell wall is absent in animals while in fungi it is a complex structure containing polysaccharides, protein and lipids. The membrane sterol in animals is cholesterol while in fungi it is ergosterol. It is, therefore, to be expected that the cell envelope would provide the ideal target site for antifungals.

The first antifungal antibiotic to be isolated and found suitable for therapeutic use was nystatin in 1949 (Hazen & Brown, 1950). It contains a number of conjugated carbon-carbon double bonds in a polyhydroxylic lactone ring and was designated a "polyene". Nystatin is used primarily in the treatment of non-systemic Candida infections and is administered orally or topically. Oral administration, as used in the treatment of intestinal candidosis, does not lead to significant serum levels of the drug since

absorption of nystatin from the gastro-intestinal tract of man is slight.

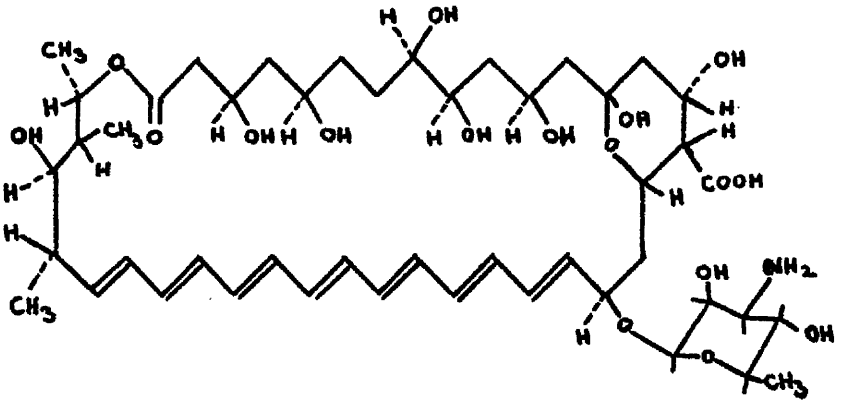
Numerous other polyene antibiotics have been described, but only a few have received much attention as useful antifungal agents because of the high degree of toxicity inherent in all of them. Only one, Amphotericin B, has led to a significant advance in the treatment of systemic fungal infections (Utz & Andriole, 1960).

Work by Gold et al., (1956); Gottlieb et al., (1958) and Gale, (1963) and summarised by Lampen, (1966) has shown that polyenes interact with membranes of sensitive organisms causing leakage of internal constituents and ultimately cell death. The nature of the components lost depends on the nature of the polyene; the smaller the polyene molecule, the greater the membrane damage.

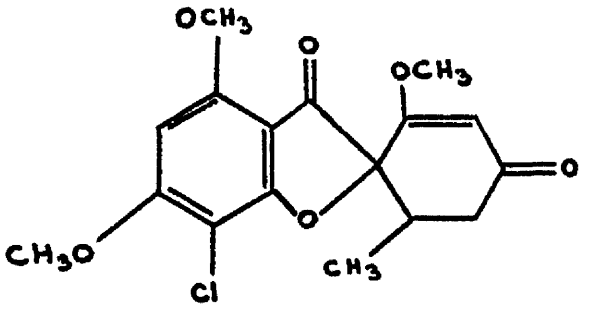
Another antifungal antibiotic, griseofulvin, first isolated by Oxford et al., in 1939, and again independently by Brian et al., (1946), was found to be useful in the treatment of animal ringworm infections when administered orally (Gentles, 1958). The compound causes the mycelium of susceptible fungi to become stunted and in lower concentrations stimulates the development of regular helical curling of the hyphae. Gull & Trinci, (1973; 1974), suggest that griseofulvin inhibits mitosis in fungi by binding to microtubule-associated proteins, although other workers dispute this theory (Grisham et al., 1973).

Attention has recently been focussed on synthetic antifungal compounds.

Tolnaftate was selected from some 3,000 naphthiomates, synthesised during a ten year search for new fungicidal agents (Noguchi et al., 1962). The compound has high activity against dermatophytes and certain other mycelial fungi, but has no effect upon the growth of C. albicans.



AMPHOTERICIN B



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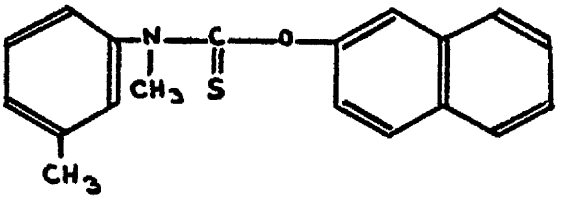
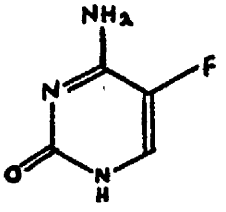


TOLNAFTATE



5-FLUOROCYTOSINE

FIGURE 1 Structural formulae of some antifungals

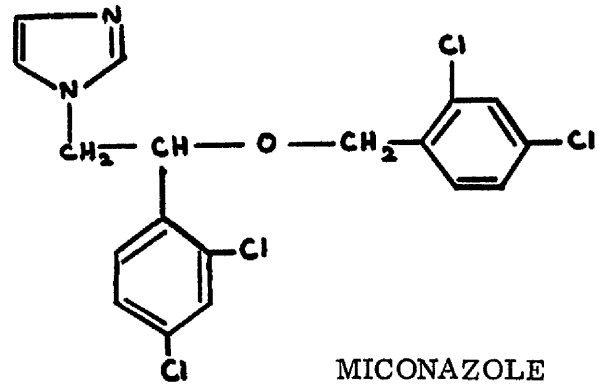
5-Fluorocytosine has been introduced as a treatment for certain systemic fungal infections. It has the advantage of being relatively non-toxic. However problems are encountered because of the development of resistant strains. (Such problems have not occurred with the polyenes or griseofulvin) This fluoro-pyrimidine is thought to act by interfering with RNA synthesis (Giege & Weil, 1970).

Imidazole derivatives have been used in the treatment of protozoal and helminth infections for almost 30 years (Holt, 1976). In 1969 two quite distinct imidazoles with marked antifungal activity entered laboratory and clinical trials; these were clotrimazole and miconazole. Two more are now on the market: chlormidazole and econazole, and a third, ketoconazole, is currently undergoing intensive investigation (see Fig. 2 for structural formulae).

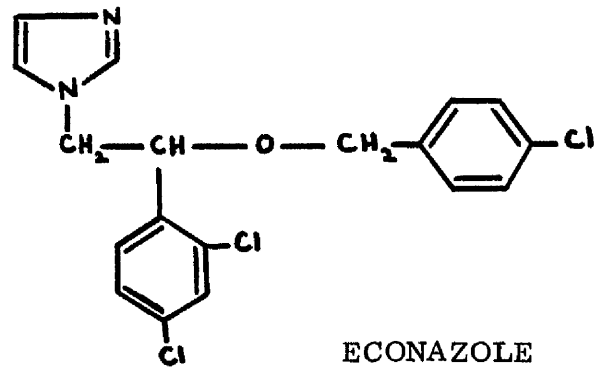
IMIDAZOLES WITH ANTIFUNGAL ACTIVITY

Buchel et al., (1972) made a deliberate search during the last decade for imidazoles with antifungal activity. They concentrated on tritylimidazole derivatives, substituted in either one, two or all three benzene rings and found that many of these compounds had considerable antifungal activity both in vitro and in vivo. A tritylimidazole with chlorine substituted in one benzene ring was eventually selected for further study and given the name clotrimazole.

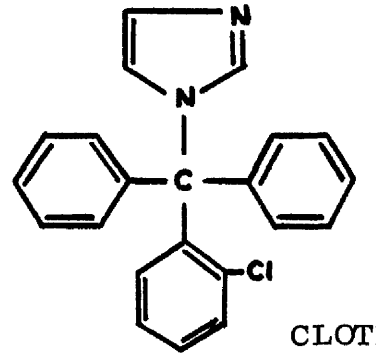
Godefroi et al., (1969) studied the antifungal properties of derivatives of 1 - phenethyl imidazole. One of these compounds, miconazole nitrate, was reported by Van Cutsem & Thienpont (1972) to be active against the dermatophytes, yeasts, dimorphic fungi, aspergilli and the mycetoma - causing agents as well as against gram-positive bacteria.



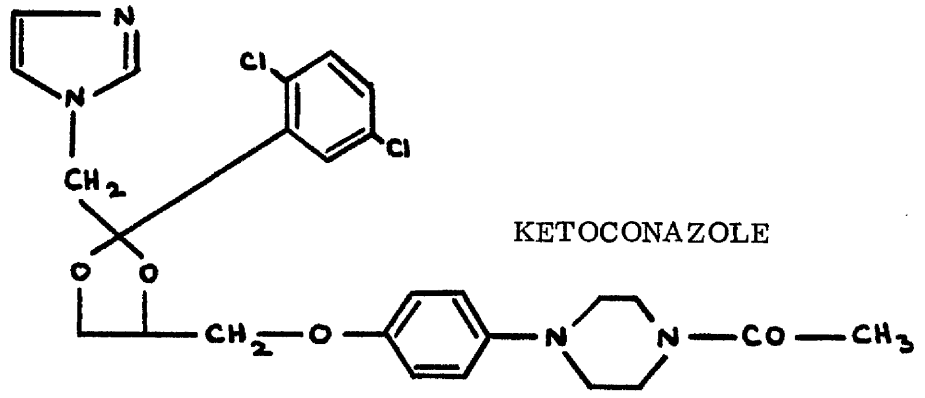
MICONAZOLE



ECONAZOLE



CLOTRIMAZOLE



KETOCONAZOLE

FIGURE 2 Structural formulae of imidazoles

Econazole is another 1 - phenethyl derivative, differing from miconazole only in containing one less chlorine atom. The spectrum of activity of miconazole and econazole is similar, although there are differences in the sensitivity of some of the fungi (Schar et al., 1976).

Clotrimazole, miconazole and econazole have all been used systemically with varying success against deep mycoses, but all suffer from certain defects and their most important use at present is as topical preparations for the treatment of dermatophyte and superficial yeast infections (Holt, 1976).

The chlorbenzyl imidazole, chlormidazole, is available solely as a cream, tincture or powder for topical use. Its properties are described by Seeliger, (1958) and reviewed by Holt in 1976. It shows no advantages over the other imidazoles and although frequently used in certain European countries, it is not in general use in the U.K.

A new imidazole derivative, ketoconazole, is active when administered orally in the treatment of experimental dermatophytosis in guinea pigs and experimental systemic candidosis in rabbits (Odds et al., 1980). It is also active in vitro against several fungi causing deep mycoses, specifically Coccidioides immitis, Blastomyces dermatitidis, Histoplasma capsulatum and Cryptococcus neoformans (Dixon et al., 1978) and when administered orally is active against C. immitis infections in mice (Levine & Cobb, 1978).

MECHANISM OF ACTION OF THE IMIDAZOLES

The mechanism of the antifungal activity of the imidazoles has not yet been fully elucidated.

Using C. albicans as the test organism Iwata et al., (1973a) suggested that fungicidal concentrations of clotrimazole damaged the permeability barrier, possibly through reaction with the cell membrane. Leakage of

intracellular phosphorus compounds and potassium ions then led to inhibition of macromolecular synthesis.

Electron microscopic studies by the same workers, (Iwata et al., 1973b), revealed that exposure to fungicidal levels of clotrimazole induced unusual development and eventual destruction of certain membranous structures. The yeast cell membrane was seen to invaginate to form extensive intracytoplasmic membranes in the vicinity of vacuoles, and the nuclear and mitochondrial inner membranes appeared to deteriorate. The terminal event appeared to be complete loss of the nuclear membrane (Yamaguchi et al., 1973).

Similar studies with clotrimazole were made by Puccinelli et al., (1977) on the mycelial fungus Microsporum canis. Deterioration of the plasma membrane was again noted, but these workers were unable to decide whether it was induced directly by the clotrimazole or if it was a consequence of generalized cell damage.

Electron microscopic examination of C. albicans cells after exposure to different concentrations of miconazole also revealed degradation of the intracytoplasmic membrane systems (De Nollin & Borgers, 1974). This effect is also reported in C. albicans and in Trichophyton rubrum after treatment with econazole (Preusser, 1976 and Preusser & Zimmerman, 1975).

These changes are now thought to be the morphological translation of the biochemical effects of the imidazoles on phospholipids and on ergosterol biosynthesis, recently described by Yamaguchi, (1978) and Van den Bossche et al., (1978).

Using a new method of preparation to overcome the problem of inadequate penetration of fixatives into yeast cells, De Nollin et al., (1975, 1977) studied

cytochemical changes in the topographic distribution of a number of enzymes in cells exposed to fungistatic and fungicidal levels of miconazole. Decreased cytochrome oxidase and peroxidase activity was demonstrated and it was postulated that the differences in sensitivity of actively growing and stationary phase cells to the fungicidal effect of the imidazoles may be due to differences in the level of these enzymes in the cells before they are exposed to the drug. Actively growing cells which do not have an adequate supply of cytochrome oxidase and peroxidase are unable to synthesise the enzymes in the presence of the drug and are, therefore, unable to break down hydrogen peroxide. The cells therefore die.

Dickinson (1977) examined the effects of miconazole on isolated rat liver mitochondria and found that it caused an uncoupling and at higher concentrations an inhibition of oxidative phosphorylation.

Another biochemical study by Van den Bossche (1974) showed that miconazole inhibited the transport of adenine, guanine and hypoxanthine into C. albicans cells but did not inhibit transport of glucose, glycine or leucine. He also showed by studies on the distribution of labelled miconazole in the Candida cell that in log-phase cells most of the radioactivity becomes localized in the fraction containing cell walls and plasmalemma and that in stationary-phase cells the highest radioactivity is present in the microsome fraction. These differences in distribution of the drug may reflect the differences in sensitivity of actively growing and stationary phase cells to the fungicidal activity of the imidazoles.

The ability of miconazole to cause leakage of potassium and sodium ions was demonstrated by Swamy et al., (1974) in yeast cells, and also, unexpectedly, in sheep erythrocytes (Swamy et al., 1976 a). These

workers also found a lysosome-labilizing action of the drug (1976b).

Other properties of the imidazoles also have a bearing on their activity both in vitro and in vivo.

The antifungal activity of both miconazole and clotrimazole is blocked from full expression in complex media but not in synthetic media (Hoeprich & Finn, 1972). Hoeprich & Huston (1976) suggested that possibly the lipids in the undefined media could cause antagonism through sequestration, and the findings of Yamaguchi (1977, 1978) and Kuroda et al., (1978) support this view. Binding of the drugs to lipids, or to plasma proteins as demonstrated by Chappel (1975), would be expected to interfere with their in vivo activity.

Certain other imidazole derivatives, in particular Tetramisole and Levamisole, possess immuno-modulating properties. It is uncertain if the antifungal imidazoles affect the immune response of the host but it has been suggested by Harkness & Renz (1974) that clotrimazole-induced stimulation of host resistance factors, such as leukocyte myeloperoxidase, may play a role in drug action in vivo. Conversely, Thong & Rowan-Kelly (1978) report that miconazole had an inhibitory effect on mitogen-induced lymphocyte proliferative responses, and since recovery from severe mycotic infections may need at least minimal immune responses, this immuno-suppressive property of miconazole may be undesirable.

Despite the lack of understanding of the exact mode of action of the imidazole group of antifungals, these drugs are being used to treat a wide range of fungal infections in man and animals.

OBJECT OF RESEARCH

There is a need for more effective agents for treatment of human and animal mycotic infections. Of the drugs available, many are suitable only for topical application, and those administered systemically all have certain limitations, for example, narrow spectrum of activity, high degree of toxicity, or the occurrence of natural or induced resistance. Recently, imidazole compounds have been, and are still being, intensively investigated with a view to filling this need for a broad spectrum drug which can be easily and safely administered.

Reports of *in vitro* activity of those drugs introduced for clinical use show a wide variation in results because of different investigational conditions. The composition of the medium, the duration of incubation, the phase of growth, the size of the inoculum and the test fungi have all clearly influenced the results obtained by various workers.

The first object of this study was to establish a procedure for comparing the fungistatic and fungicidal activity of certain imidazoles against different growth phases and different morphological forms of medically important fungi and to compare their *in vitro* activity with that of other antifungals.

Although the development of resistance to the imidazoles has not been a significant problem in therapy of mycotic infection, a second object of this research was to study the possible development of resistance to the imidazoles *in vitro*.

Thirdly, since *in vitro* activity, although important, may give little indication of the clinical efficacy of a drug, tests were made on guinea pigs experimentally infected with ringworm to ascertain the potential of certain of the imidazoles in the treatment of dermatophytosis.

EXPERIMENTAL SECTION 1

IN VITRO ACTIVITY OF ANTIFUNGALS

INTRODUCTION

In vitro sensitivity testing is routinely employed to determine the activity of a drug against the etiologic agent of a disease. It is desirable that such tests should be standardised in order to obtain consistent results within and between laboratories.

The techniques employed for testing the imidazoles clearly need to be critically assessed as considerable variations in Minimum Inhibitory Concentrations (M. I. C.) are to be found in the literature. For example, the following M. I. C. levels of miconazole have been quoted by different workers for C. albicans isolates: 0. 5μg/ml (Holt, 1972), 2μg/ml (Milne, 1978), 16μg/ml (Shadomy et al., 1977) and recently Holbrook & Kippax (1979) reported sensitivities ranging from 8 to 32μg/ml.

Certain authors have considered that the growth medium is mainly responsible for the variation in results.

Plempel & Buchel (1975), stated that peptone interfered with the activity of the imidazoles and Hoeprich & Huston, (1976) advocated the use of defined media for testing the in vitro activity of miconazole. Conversely, Swamy et al., (1974) reported that growth inhibition by miconazole was less in synthetic media, where divalent cations, notably Mg^{++} and Ca^{++} were present, than in glucose peptone medium.

Antagonism to clotrimazole and miconazole has been observed with phospholipids containing an unsaturated acyl group and also with acylglycerides, the ester portion of which consists of unsaturated fatty acids (Yamaguchi, 1977; 1978 and Kuroda et al., 1978). In addition polyoxyethylene surfactants were found to antagonise the anti-Candida activity of clotrimazole (Iwata & Yamaguchi, 1977).

However, the contents of the medium are not the only factor that influences the results of M. I. C. tests. It is generally accepted that M. I. C. levels vary according to the size of the inoculum, and recently Langsadl & Jedlikova (1979) have produced quantitative evidence of this with the imidazoles. They demonstrate that the M. I. C. of miconazole against 12 laboratory strains of C. albicans varied between 0. 39 and 12. 5μg/ml for an inoculum size of 2×10^8 CFU/ml, and between 0. 025 and 1. 56μg/ml for an inoculum size of 1.6×10^4 CFU/ml. Inoculum size is also recognised to be important when attempting to measure fungicidal activity of the imidazoles (Yamaguchi & Iwata, 1979).

The duration of incubation needs to be considered and must be varied according to the rate of growth of the test fungus. In the tube dilution test, results for the fast-growing yeasts can be determined after 48 hours, whereas the slower-growing dermatophytes require 7 - 10 days incubation.

It should be appreciated also that the end-point is not always sharply defined. For example, a marked drop in the amount of growth relative to controls may be observed with C. albicans on minimum inhibitory concentrations of the imidazoles, but some multiplication of the inoculum takes place even above this although it becomes progressively less obvious as the concentration of drug increases. A modification of the definition of M. I. C. is therefore required when interpreting results of tests with imidazole antifungals.

The evidence to date concerning the possibility of resistance arising during the course of treatment with imidazoles indicates that it is not likely to be a problem. There is only one report of Candida developing resistance to miconazole following 6 months therapy (Holt & Azmi, 1978).

Investigations of the imidazoles with the gradient plate method (Szybalski & Bryson, 1952), have failed to demonstrate the development of resistance (Schweisfurth, 1974 and Holt, 1976). However, some attention was given in this study to the possibility of resistance arising spontaneously or following treatment with a mutagenic agent.

PRELIMINARY INVESTIGATIONS

Before embarking upon a detailed study of the fungicidal and fungistatic activity of the imidazoles it was first necessary to find a procedure which would give consistent results and which could be used for all drugs and all fungi under test.

Preliminary tests showed glucose peptone medium to be satisfactory for assessing the antifungal activity of all the agents under examination and recent publications, (Borgers et al., 1977; Shadomy et al., 1977; Odds et al., 1980) have also indicated that it is widely accepted as a suitable medium for testing imidazoles. Since this medium readily supports the growth of both yeasts and filamentous fungi it was ideally suited for the purposes of this study.

Using broth and agar dilution tests and inocula of standard size, it was found that consistent results could be obtained for fungistatic activity of various imidazoles, however certain initial problems were encountered in attempts to determine fungicidal activity.

(1) When transferring fungi which had been exposed to fungicidal concentrations of the imidazoles to drug-free medium to test for viability, it became apparent that fungistatic concentrations of the drugs were being carried over and this interfered with determination of viability. This was clearly a factor which was not taken into consideration by other workers (Janssen Pharmaceutica, 1971) in their attempt to determine fungicidal activity of miconazole against filamentous fungi contained in agar blocks. Since miconazole is a charged molecule the possibility was examined that it was binding to the agar by electrostatic forces, however since non-ionic agarose also retained fungistatic concentrations of the drug this technique had to be abandoned.

The insoluble imidazoles could not be removed by centrifugation and washing procedures, and transfer of fungi on cellophane squares from drug-containing to drug-free media served only to illustrate the efficiency with which cellophane absorbs the imidazoles !

Eventually a method was found which overcame this problem of carry-over - that of providing an inoculum containing sufficient viable units to allow dilution to reduce the drug concentration to below fungistatic levels before testing for viability.

(2) The intravenous preparation of miconazole has the advantage of remaining in micro-suspension when diluted in distilled water, and for this reason it was used in early studies. It was found to be quite satisfactory for the determination of M. I. C. s, but fungicidal activity could not be demonstrated.

When dimethyl formamide was used as solvent fungicidal activity was achieved and it seemed, therefore, possible that the solubilising agent present in the i. v. preparation, Cremophor E L (polyethoxylated castor oil), was antagonising the activity of miconazole. This was confirmed by addition of appropriate dilutions of Cremophor E L to tubes of glucose peptone broth containing miconazole dissolved in dimethyl formamide and the finding is substantiated by the reports of antagonism by unsaturated fatty acids and polyoxyethylene surfactants (Yamaguchi, 1977, 1978, Kuroda et al., 1978 and Iwata & Yamaguchi, 1977).

Consequently, dimethyl formamide was used as solvent for the non-water-soluble imidazoles throughout the study of in vitro activity reported here.

MATERIALS AND METHODS

MATERIALS

TEST ORGANISMS

The following fungi were either isolated or received as cultures from clinical material and identified in this laboratory.

Yeasts:

	Source	Date
Candida albicans (BP 3496 B)	faeces	13.11.78
C. parapsilosis (BP 3418)	skin	1.9.78
C. tropicalis (BP 3437)	faeces	18.9.78
C. guilliermondii (BP 3439)	faeces	20.9.78
C. pseudotropicalis (BP 3344)	faeces	19.7.78

Filamentous fungi:

Aspergillus fumigatus (BP 3524)	sputum	30.11.78
Microsporum canis (MC 6) *	scalp	30.4.75
Trichophyton mentagrophytes var. interdigitale (DO 9941C) *	toe nail	30.9.77
T. rubrum (SD 801)	toe nail	25.7.78

* Filamentous fungi, especially dermatophytes, deteriorate if sub-cultured repeatedly over a prolonged time. For this reason T. mentagrophytes var. interdigitale (DO 9941C) and M. canis (MC 6) were stored on silica gel (Gentles & Scott, 1979) soon after isolation. The studies with T. mentagrophytes var. interdigitale extended over many months. Sub-cultures of this fungus were made every two weeks on glucose peptone agar for a maximum of 3 months before being replaced from the silica gel stock. M. canis was freshly cultured from silica gel shortly before use and sub-

culturing was kept to a minimum during the few weeks taken to perform the experiments described.

The Aspergillus nidulans mutants used in the investigations undertaken jointly with Mr. E. Forbes are available in the Genetics Department, Glasgow University.

Mutant	Glasgow Number
an B8	026
bi - 1	051
pro A 1	0157
pyro - 4	0177
ribo A 1	0189

MEDIA

Glucose - peptone medium

Composition/litre

Glucose	40 g
Peptone (for Bacteriological work) (Fisons)	10 g
Agar (for solid medium)	20 g

Sterilised by autoclaving at 121°C for 20 min.

Czapek-Dox + Tween 80 agar

Composition/litre

Czapek-Dox Agar (Oxoid)	45.4 g
Tween 80	10 ml

Sterilised by autoclaving at 115°C for 20 min.

The formulae for the Czapek-Dox medium and minimal medium, and the methods of preparation of the supplements used in the experiments performed in collaboration with Mr. E. Forbes are described by Cove, (1966) modified after Pontecorvo et al., (1953):-

Czapek-Dox medium

Composition/litre

Sodium nitrate	6 g
Potassium chloride	0.52 g
Magnesium sulphate	0.52 g
Potassium dihydrogen orthophosphate	1.02 g
Trace elements	1 ml

Trace elements

Composition/litre

Sodium tetraborate	0.04 g
Copper or cupric sulphate	0.4 g
Ferric orthophosphate	0.8 g
Manganese sulphate	0.8 g
Sodium molybdate	0.8 g
Zinc sulphate	0.8 g

Minimal medium

Composition/litre

Czapek-Dox medium	1 l
Glucose	10 g
Agar	12 g
pH adjusted to 6.5	

Sterilized by autoclaving at 121^{o}C for 15 min.

Ammonium was supplied as ammonium tartrate (10 m mol).

Vitamins were added at a concentration of 0.05μg/ml.

CELLOPHANE SQUARES

Cellophane squares for use in the study of morphological changes were prepared as follows:-

One cm. squares of non-moisture-proof cellophane ("Crystal" jam pot covers) were sterilised by autoclaving between moist filter paper at 121°C for 20 min.

DRUGS

Antifungal drugs were obtained as follows:-

MICONAZOLE and KETOCONAZOLE from Janssen Pharmaceutical Limited, Janssen House, Marlow, Bucks.

ECONAZOLE from Cilag-Chemie Limited,

CH-8201, Schaffhausen, Switzerland

CLOTRIMAZOLE from Bayer Pharmaceuticals Limited, Haywards Heath, Sussex.

TOLNAFTATE and GRISEOFULVIN from Glaxo Laboratories Limited, Greenford, Middlesex.

The solubilising agent, Cremophor E L, (polyethoxylated castor oil), used in the preparation of i.v. miconazole, was provided by Janssen Pharmaceutical Limited.

Stock solutions containing 10 mg/ml were prepared in the appropriate solvent, (dimethyl formamide for miconazole, econazole and clotrimazole; sterile distilled water for ketoconazole; acetone for tolnaftate and griseofulvin), further dilutions were made in sterile distilled water immediately before use.

The intravenous preparation of miconazole, supplied by Janssen Pharmaceutical Limited, contained 10 mg/ml maintained in micro-suspension by 0.1 ml of Cremophor E L/ml and with various other additions, details of which were not revealed by the manufacturer. It was used in early experiments but found not to be satisfactory for measurements of fungicidal activity.

METHODS

PREPARATION OF ACTIVE AND STATIONARY GROWTH PHASES

(i) Yeasts

The actively growing phase of Candida species was obtained by inoculating 0.1 ml of a suspension containing approximately 10^6 cells/ml into glucose peptone broth and incubating for 18 h at 28°C. A dilution of 1 : 100 of this culture in fresh broth was then incubated at 28°C for a further 2 h period. This procedure gave counts in the region of 10^6 cells/ml.

Stationary phase cells were obtained from a 10 day old culture on glucose peptone agar, and suspensions of approximately equal turbidity to the actively growing cell cultures were prepared in glucose peptone broth.

Morphological study Volumes of 0.01 ml of the suspensions were placed on the surface of agar medium containing the drugs and left to stand for 10 - 15 min to allow the liquid to be absorbed into the agar before covering the areas with cellophane squares.

Fungicidal study Cell suspensions were dispensed in 4.5 ml amounts in sterile test tubes immediately before addition of drug.

(ii) Filamentous fungi

Morphological study Actively growing hyphal cells were prepared using a modification of a technique employing cellophane squares, (Carmichael, 1956; Chabbert, 1957; Kondo et al., 1959).

Cellophane squares were placed on drug-free glucose peptone agar and left for approximately 10 min to absorb moisture and adhere firmly to the surface of the medium.

An inoculum of 0.01 ml of a suspension containing approximately 10^5 freshly harvested spores, (from 10 day old cultures), per ml of distilled water was placed on each square and the dishes incubated at 28°C for an appropriate length of time to achieve maximum germination, (approaching 100%), and allow adequate but not excessive hyphal development. For dermatophytes this was 20 h and for A. fumigatus 16 h incubation (Fig. 3 (a) - (d))

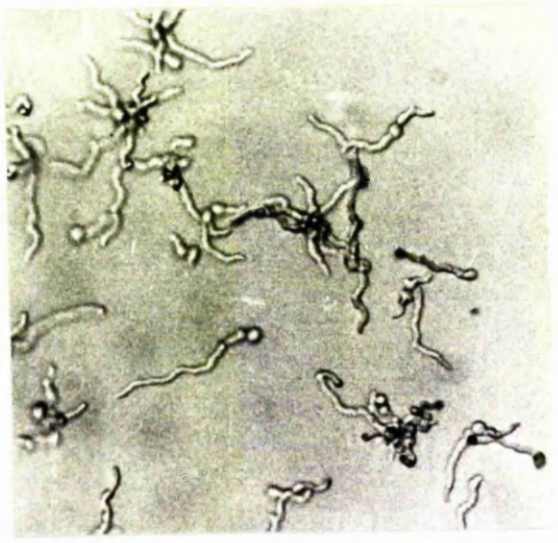
Fungicidal study T. mentagrophytes var. interdigitale (DO 9941 C) was exposed to drug activity in liquid medium.

Actively growing hyphal cells were obtained by inoculating microconidia from a 10 day old culture grown on glucose peptone agar into glucose peptone broth to give a final concentration of approximately 8.0×10^4 microconidia/ml. The broth cultures were incubated at 28°C for 18 h. It was necessary to adjust the numbers of spores to achieve the optimum size of inoculum. If too many spores were present then germination of a significant proportion of the spores was inhibited. However sufficient concentration of spores was required so that at a later stage, after exposure to the drug, it was possible to dilute to below a fungistatic level and still maintain a suitable concentration of spores.

Freshly harvested spores from 10 day old cultures were considered to be the stationary phase of filamentous fungi. Suspensions containing the same numbers of spores as used in the preparation of actively growing hyphae were prepared immediately before the tests were begun.

TESTS FOR FUNGISTATIC ACTIVITY

Agar dilution technique Stock solutions of drugs were appropriately diluted in distilled water and used in the preparation of Petri dishes of glucose peptone agar containing drug concentrations ranging from 0.1 - 100μg/ml. The agar



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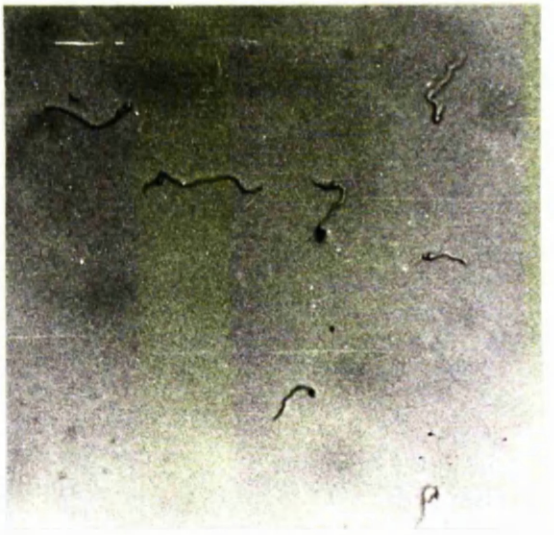

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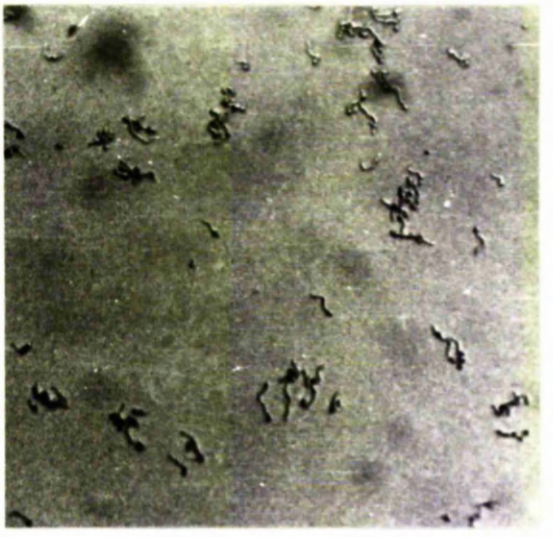


d

FIGURE 3 Germinated spores at stage of development used in fungistatic investigations. (a) A. fumigatus (b) T. mentagrophytes, (c) M. canis and (d) T. rubrum x 150

plates were prepared in advance and stored at +4°C for 1 week to allow the surface to dry.

Broth dilution technique Broth dilution tests were made by inoculating the fungi into glucose peptone broth containing the same range of drug concentrations as above.

(i) Yeasts

Volumes of 0.01 ml of suspensions containing approximately 10^6 yeast cells/ml were placed on the surface of the solid media or into broths containing the drugs. The Petri dishes and tubes were incubated at 28°C for 3 days and minimum inhibitory concentrations recorded.

Quantitative estimation of inhibition of growth

The broth dilution procedure was used to obtain a quantitative estimation of inhibition of growth of yeasts by taking optical density readings after 18 h incubation at 37°C using a CE 343 Single Sample Spectrophotometer set at a wavelength of 440 nm.

(ii) Filamentous fungi

Cellophane squares supporting germinated spores at optimum stage of development (p_{21}) were removed from the agar medium, blotted to remove excess water (the germinated spores remained attached to the cellophane), and placed fungus-side downwards onto solid media containing the selected drugs.

Preliminary investigations showed that the drugs diffused freely through the cellophane and M.I.C. results were the same whether the squares were placed fungus-side-down or fungus-side-up. Placing the squares fungus-side-down allowed easier direct microscopy of the fungi in situ, the cellophane square

acting as a coverslip.

In the parallel study of ungerminated spores, freshly harvested spores were placed directly onto the surface of the drug-containing medium.

The Petri dishes were incubated at 28°C. Observations were made after 1, 2, 3, 6 and 10 days and minimum inhibitory concentrations recorded.

TESTS FOR FUNGICIDAL ACTIVITY

(i) Yeasts

Tests were carried out on actively growing and stationary phase yeast cells. The cell suspensions were prepared as described on p 20. Viable counts were made immediately before addition of the drug. These were adjusted to take into account the dilution factor caused by the subsequent addition of the drug.

Addition of 0.5 ml amounts of 1,000μg/ml concentrations of the drugs to 4.5 ml amounts of the prepared cell suspensions gave final concentrations of 100μg/ml.

The tubes were incubated at 28°C.

Sampling

(a) Comparison of the fungicidal effect of 3 imidazoles on C. albicans

For the actively growing cells 0.1 ml samples were withdrawn at hourly intervals up to 3 h, and for stationary phase cells at daily intervals up to 14 days.

(b) Comparison of the effect of econazole on four different Candida species

Samples of 0.1 ml were withdrawn from the actively growing cell suspensions at 10 min intervals up to 1 h, and after 3, 5, 7 and 9 h intervals, and from the stationary phase cell cultures at 1, 2, 3, 6 and 7 days.

All tubes were returned to the 28°C incubator immediately after sampling.

The samples were diluted to 10^{-2} and 10^{-3} and 0.2 ml spread on the surface of glucose peptone agar in Petri dishes. This procedure ensured dilution of the drugs to below fungistatic levels.

The Petri dishes were incubated at 28°C for 3 days.

(ii) Filamentous fungi

Tests were carried out on actively growing hyphae and freshly harvested microconidia of T. mentagrophytes var. interdigitale (DO 9941 C). The preparation of the suspensions of the different growth phases is described on p 21.

A volume of 0.5 ml of 1,000μg/ml concentrations of each drug was added to 4.5 ml of the spore suspensions in glucose peptone broth to give final concentrations of 100μg/ml.

Samples of 0.1 ml were withdrawn immediately before and approximately 1 min after addition of drug and after 1, 2, 3, 5 and 7 h and after 1, 2, 4, 7 and 16 days incubation at 28°C.

The samples were diluted 1 : 100 in sterile distilled water and 0.2 ml spread on the surface of drug-free glucose peptone agar.

The Petri dishes were incubated at 28°C for 1 week.

MORPHOLOGICAL CHANGES

All fungi were examined in situ on solid medium under x 100 magnification and a photographic record made of the changes in cell morphology.

N.B. The inferior quality of the photographs of yeast cells (Figs. 8 - 12) suggests a deficiency in the method employed for their examination.

However, although it was not possible to photograph the cells at a higher magnification because of the thickness of the medium, the changes in morphology were nevertheless clearly evident on direct microscopic examination.

(i) Yeasts

A comparison was made of the changes in cell morphology of actively growing and stationary phase cells of C. albicans exposed to 100μg/ml miconazole, econazole and ketoconazole at 28°C after 3 h and 24 h.

Non-chlamydospore-forming yeasts in contact with 100μg/ml econazole were examined microscopically after 24 h.

(ii) Filamentous fungi

Changes in the morphology of actively growing hyphae of filamentous fungi were recorded after 48 h at 28°C in sub-fungistatic concentrations, concentrations approaching the M. I. C. and concentrations greater than the M. I. C., of the 4 imidazoles examined. A comparison was made of the morphological effects caused by tolnaftate and griseofulvin.

PRODUCTION OF CHLAMYDOSPORES BY C. ALBICANS

Chlamydospores production was induced in C. albicans by preparing a cut-streak of the yeast in Czapek-Dox/Tween 80 agar. The Petri dish was incubated for 24 h at 28°C before examining the cells under the x 100 magnification through the base of the Petri dish.

INVESTIGATION OF "NORMAL" AND "UNUSUAL" YEASTS

In collaboration with Mr. E. Forbes of the Genetics Department, Glasgow University, an attempt was made to find a reason for the difference in behaviour, when exposed to sub-fungistatic concentrations of the

imidazoles, between C. guilliermondii and C. tropicalis ("unusual" yeasts) and C. albicans and C. parapsilosis ("normal" yeasts).

(i) An auxanographic technique (Pontecorvo et al., 1953) was used to detect differences in nutritional requirements. The minimal medium used in the test was Czapek-Dox medium plus 1% glucose and 1.2% agar, pH 6.5. The growth factors examined were ammonium, biotin, thiamin and pyridoxine.

(ii) Econazole nitrate was tested for the presence of contaminating growth factors. This was done using mutants of A. nidulans which had requirements for thiamin (an B8), biotin (bi - 1), proline (pro A1), pyridoxine (pyro - 4) and riboflavin (ribo A1). Spores of the mutants were prepared as a "lawn" in minimal medium and several crystals of econazole nitrate were placed in the centre of each Petri dish.

(iii) To investigate the possibility that the "unusual" yeasts were degrading the econazole to utilizable metabolites, or alternatively being stimulated by the presence of the drug to produce antagonistic substances which might interfere with low concentrations of the drug, C. guilliermondii was grown in 10μg/ml econazole in glucose peptone broth for 40 h at 37^{o}C. The medium was then sterilized by seitz-filtration and the filtrate diluted 1 : 10 in fresh glucose peptone broth to 1μg/ml econazole, thereby ensuring an adequate supply of nutrients before re-inoculation with C. guilliermondii. Control tubes of glucose peptone broth with 1μg/ml econazole were inoculated with C. guilliermondii at the same time and incubated at 37^{o}C for 2 days.

(iv) A range of growth factors was examined for ability to relieve the toxicity of econazole. C. guilliermondii was seeded into molten minimal medium agar supplemented with biotin and ammonium and containing 7.5μg/ml

econazole, poured into Petri dishes and allowed to set.

The following amino acids were tested by placing approximately 1 mg of the crystals onto the surface of the agar:- alanine, proline, arginine, methionine, phenylalanine, histidine, glutamic acid, ornithine, aspartic acid, threonine, cystine, glycine, valine, serine, tryptophan, tyrosine, cysteine, homoserine, hydroxyproline and isoleucine, together with the crude mixture of amino acids contained in peptone and casein hydrolysate.

The vitamins thiamin, biotin, riboflavin, nicotinamide and pyridoxine were tested by placing approximately 1μg of each on the surface of the seeded agar.

The Petri dishes were incubated at 37°C for 24 h.

(v) Several of the growth factors found to relieve the toxicity of econazole for C. guilliermondii were tested for their effect on the two "normal" and two "unusual" yeasts in liquid minimal medium plus ammonium and biotin containing 20μg/ml econazole. Approximately 20μg/ml of serine, histidine and thiamin were added to appropriate tubes. The same concentration of growth factors were added to control cultures without econazole. All tubes were incubated at 28°C for 5 days.

SUB-CULTURE OF C. GUILLIERMONDII AND C. TROPICALIS FROM SUB-FUNGISTATIC LEVELS OF THE IMIDAZOLES

(a) Econazole

Cells of C. guilliermondii and C. tropicalis were removed from the surface of the agar medium containing 0.5, 1, 5, 10 and 15μg/ml econazole, and from a control plate, after 3 days incubation at 28°C. Suspensions of approximately equal turbidity were prepared in distilled water. A volume of

0.01 ml of each suspension was placed directly onto the surface of a series of the same concentrations of econazole in glucose peptone agar, and onto drug-free medium. The dishes were incubated at 28°C for 3 days.

After passage on drug-free medium the cells were again harvested, suspended in distilled water and placed on medium containing a series of concentrations of econazole.

An attempt was made to standardise the inocula by preparing suspensions of approximately the same turbidity as before.

(b) Miconazole and ketoconazole

The same procedure was followed for C. guilliermondii growing on sub-fungistatic concentrations of miconazole and ketoconazole.

RESISTANCE TO THE IMIDAZOLES

(i) Variation in sensitivity within a population

The Petri dishes used for the determination of M.I.C. by agar dilution were examined microscopically for the presence of cells capable of slow growth on concentrations of the drugs which completely inhibited growth of the remainder of the population.

(ii) Effect of repeated sub-culture

The least sensitive members of a population of T. mentagrophytes var. interdigitale which were capable of slow growth on glucose peptone agar containing 10μg/ml ketoconazole were repeatedly sub-cultured at two-week intervals onto fresh drug-containing medium in order to select for further adaptation.

(iii) Artificial induction of resistant mutants

Mr. Forbes of the Genetics Department, Glasgow University, kindly agreed to collaborate in trying to artificially induce resistance in A. nidulans with ultra-violet light as the mutagenic agent. Conidia of a biotin-requiring mutant of A. nidulans (bi - 1) were kept in suspension by constant agitation while they were irradiated for 13 min with a germicidal wavelength of ultra-violet light (254 nm) from a distance of 45 cm. It is known that approximately 5% of conidia survive this treatment, and of these approximately 1% are mutants.

(a) Isolation of mutants

Selection for resistant mutants was carried out by plating the treated conidia on Czapek-Dox medium supplemented with amino acids and vitamins and containing a sub-fungistatic concentration of econazole.

Colonies which grew more rapidly than controls were sub-cultured onto sub-fungistatic concentrations of miconazole, econazole and ketoconazole in the same medium as before.

(b) Test for relief of toxicity

Conidia of the ECO - 1 mutant were inoculated into molten Czapek-Dox agar supplemented with amino acids and vitamins containing a fungistatic concentration of miconazole. Several amino acids were tested for their ability to relieve toxicity by placing approximately 1 mg of the purified compounds on the surface of the solidified medium.

(c) Uptake of l - phenylalanine

Since l - phenylalanine gave a wide zone of relieve of toxicity of miconazole, uptake of this amino acid by the ECO - 1 mutant was investigated with ^{14}C l - phenylalanine. The procedure was the same as that described

by Hackette et al., (1970) in their study of uptake of ^{14}C methylammonium ion.

(d) Comparison of M. I. C. for biotin - requiring and ECO - 1 mutants of A. nidulans

The mutants were passaged on drug-free glucose peptone agar before determining the M. I. C. of miconazole, econazole and ketoconazole by the broth dilution test.

RESULTS

FUNGISTATIC ACTIVITY

(i) Yeasts

Minimum Inhibitory Concentrations

The M. I. C. s of the imidazoles for different Candida species are listed in Table I. Active and stationary phase cells were inhibited by the same concentration of each imidazole. Direct microscopic examination of the inocula placed on solid media revealed a degree of initial cell multiplication after both growth phases were exposed to fungistatic concentrations of the imidazoles. Identical results were obtained using broth and agar dilution tests.

An anomalous feature, first observed in tests on solid media containing econazole, was the behaviour of two of the yeasts, C. guilliermondii and C. tropicalis. They showed a marked inhibition of growth at 0.5 and 1μg/ml. Growth then increased again at 5 and 10μg/ml before decreasing at 15μg/ml to give an M. I. C. of 20μg/ml.

The other species, C. parapsilosis, C. pseudotropicalis and C. albicans behaved as expected, i. e. the amount of growth steadily decreased with increase in concentration of the drug until the M. I. C. was reached.

C. guilliermondii responded in a similar manner to increasing concentrations of miconazole and ketoconazole, although in keeping with lower sensitivity of the fungus to ketoconazole the peak of partial recovery was less apparent and appeared at higher concentrations (50 - 100μg/ml).

Quantitative estimation of inhibition of growth

The optical density readings were a measure of the amount of light travelling through the cell suspensions and were therefore inversely

	M.I.C. (μg/ml)		
ORGANISM	Econazole	Miconazole	Ketoconazole
C. albicans (BP 3496B)	15	15	50
C. parapsilosis (BP 3418)	10	N.T.	50
C. pseudotropicalis (BP 3344)	5	N.T.	N.T.
C. tropicalis (BP 3437)	20	N.T.	> 100
C. guilliermondii (BP 3439)	20	20	> 100

N.T. = not tested

TABLE I M.I.C. OF IMIDAZOLES FOR CANDIDA SPECIES

proportional to the amount of growth. A graph (Fig. 4) was prepared from the reciprocals of the O.D. readings.

(ii) Filamentous fungi

Minimum Inhibitory Concentrations are listed in Table II.

As already observed for yeasts, microscopic examination of the inocula revealed that growth continued for a short time after the cells came into contact with fungistatic concentrations of the imidazoles.

Direct microscopic examination of hyphae had a distinct advantage over conventional techniques in that it allowed accurate readings of M.I.C. levels to be made in a shorter time. This was particularly evident with the slower growing dermatophytes. It was found that results recorded after 3 days were unchanged after 7 - 10 days.

The M.I.C. results obtained for actively growing hyphae and for spores were identical except for A. fumigatus exposed to miconazole and econazole. Slightly lower concentrations of these two imidazoles were required to inhibit germination of A. fumigatus spores than were required to inhibit growth of the hyphae.

The drug which was most effective against dermatophytes was tolnaftate, (0.1 - 0.5μg/ml), however its activity against A. fumigatus was negligible. The imidazoles showed a wider spectrum of activity than both tolnaftate and griseofulvin, and of these, econazole was the most effective (1 - 5μg/ml). Miconazole and clotrimazole followed closely behind econazole (5 - 15μg/ml), but ketoconazole gave consistently higher M.I.C.s (up to 50μg/ml) indicating a correspondingly lower level of in vitro activity although its spectrum of activity was similar to the other imidazoles.

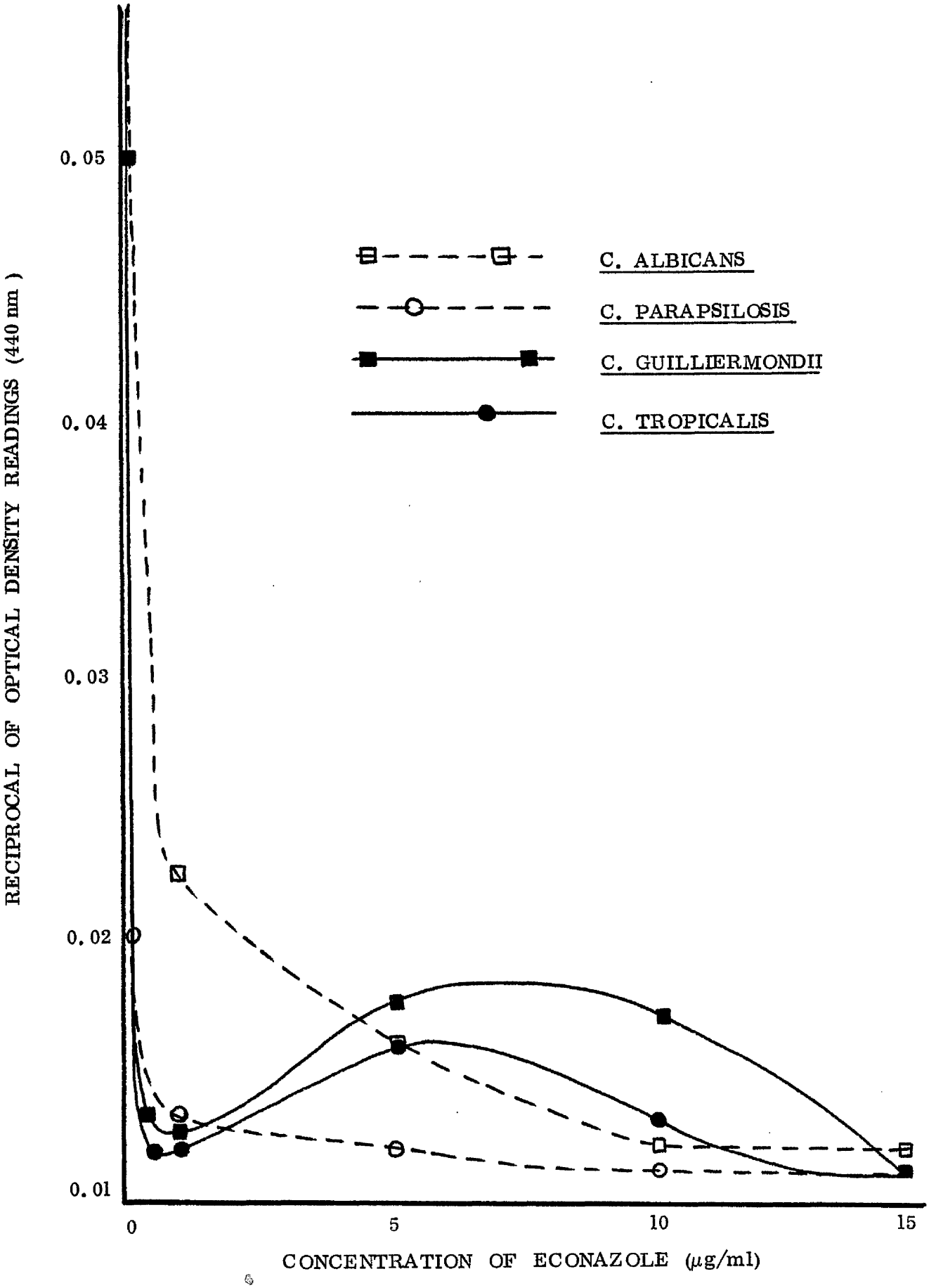


FIGURE 4 Effect of increasing concentrations of econazole upon growth of "normal" and "unusual" yeasts.

Test Organism	Miconazole	Econazole	Ketoconazole	Clotrimazole	Tolnaftate	Griseofulvin
A. fumigatus	15 (10)	5 (1)	50	15	>100 *	>100 *
T. mentagrophytes var. interdigitale	5	1	15	5	0.5	10
Microsporum canis	10	1	50	5	0.5	5
T. rubrum	5	5	50	5	0.1	5

* Highest concentration tested

Brackets ( ) indicate M. I. C. for freshly harvested spores where this differs from M. I. C. for germinated spores.

TABLE II MINIMUM INHIBITORY CONCENTRATIONS (μg/ml) OF SELECTED ANTIFUNGALS AGAINST GERMINATED AND UNGERMINATED SPORES OF MYCELIAL FUNGI

In general, the dermatophytes appeared to be more sensitive than A. fumigatus to the antifungal agents tested.

FUNGICIDAL ACTIVITY

(i) Yeasts

(a) Comparison of the effect of 3 imidazoles on C. albicans

Fig. 5 illustrates the rate of killing of C. albicans cells following exposure to 100μg/ml of the drugs.

With econazole and miconazole the actively growing cells were rapidly killed by the drugs within 1 h. Stationary phase cells were apparently unaffected up to 24 h, but by 3 days a fungicidal effect was evident. Killing was complete in 9 days with econazole, while miconazole required slightly longer (12 days).

For ketoconazole, 100μg/ml was not totally fungicidal for either actively growing or stationary phase cells of C. albicans within 14 days.

(b) The effect of econazole on four different Candida species

The fungicidal effect of econazole on active and stationary phase cells of four Candida species is illustrated in graph form in Fig. 6.

C. guilliermondii and C. tropicalis were more sensitive than C. albicans and C. parapsilosis to the fungicidal action of econazole in both the actively growing and stationary phases.

In all cases viability of actively growing cells dropped rapidly within the first 10 min after addition of the drug. Killing was complete within 40 min for C. guilliermondii and C. tropicalis, and within 3 h for C. albicans and C. parapsilosis.

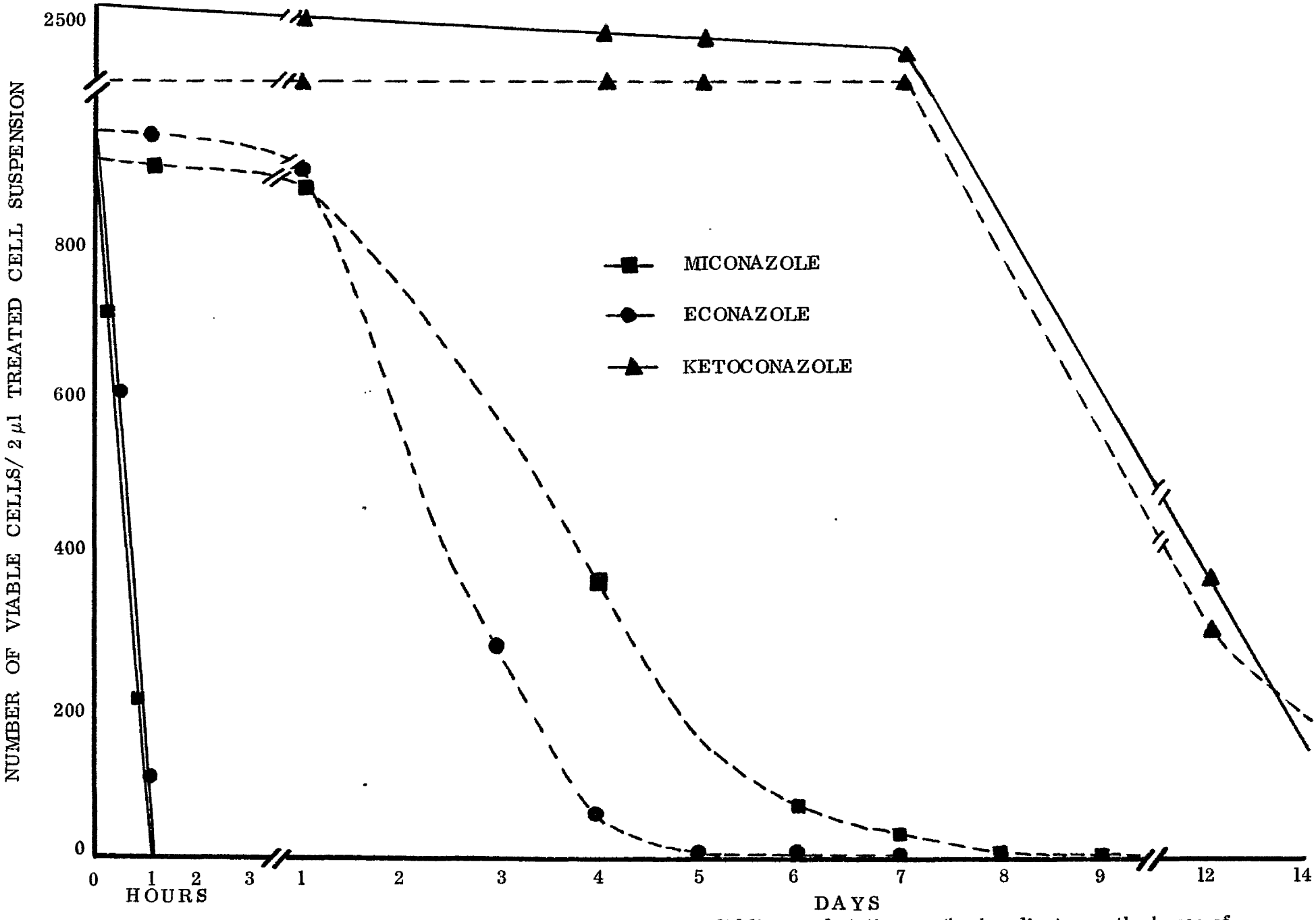


FIGURE 5 Fungicidal effect of the imidazoles on the active (solid line) and stationary (broken line) growth phases of C. albicans (BP 3496 B)

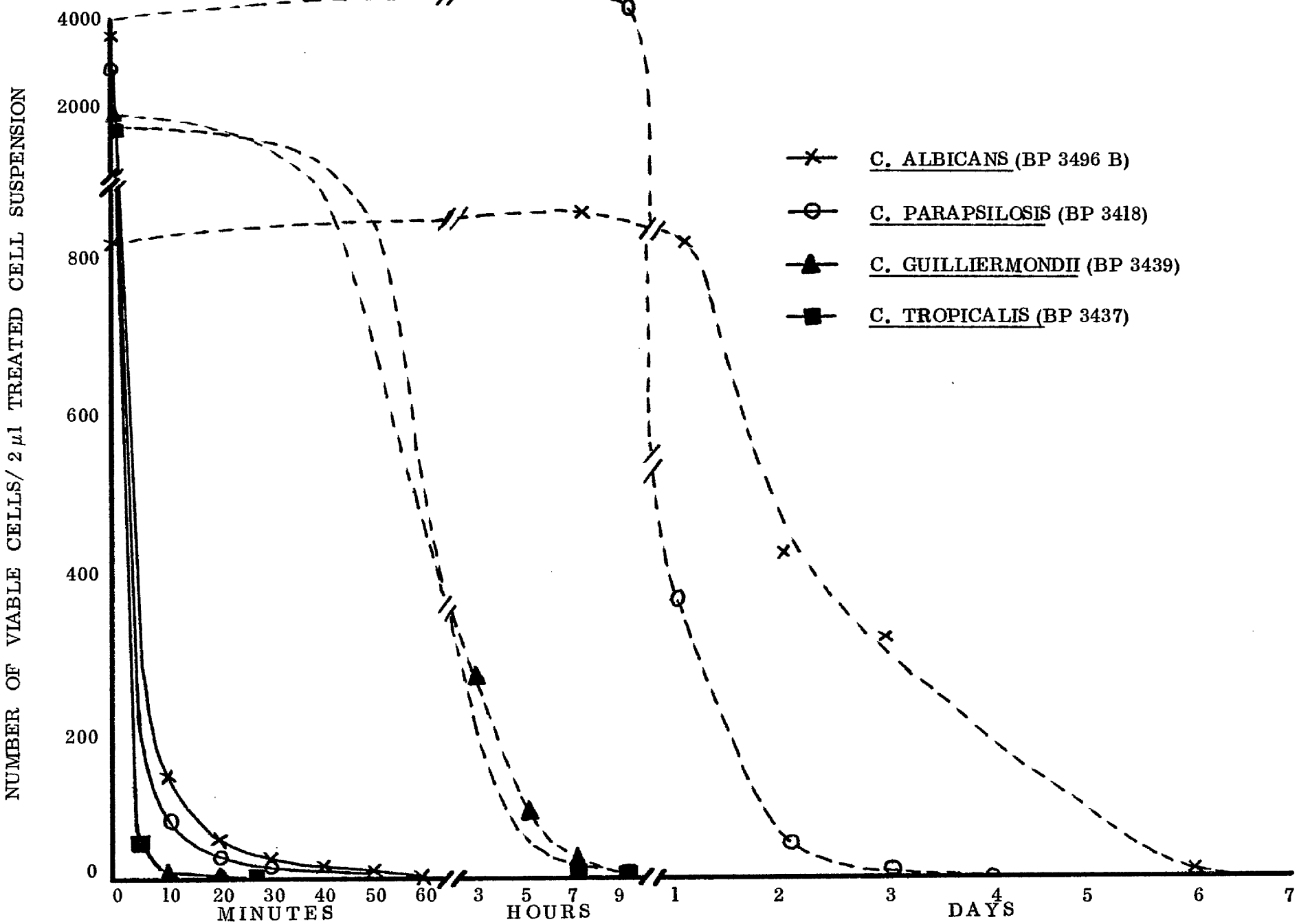


FIGURE 6 Fungicidal effect of econazole on the active (solid line) and stationary (broken line) growth phases of four Candida species.

The viability of stationary phase cells of all 4 species remained unaltered after 1 h at 28°C, however after 3 h the viability of C. guilliermondii began to diminish and killing was complete for both C. guilliermondii and C. tropicalis in 24 - 48 h. Viability of C. parapsilosis was significantly reduced within the first 24 h but some cells of this non-chlamydospore - forming species were still viable after 3 days.

The behaviour of C. albicans conformed to that of the previous experiment, stationary phase cells remained fully viable up to 24 h in the presence of 100μg/ml econazole, after which the viability gradually diminished until total killing was achieved by 7 days.

(ii) Filamentous fungi

The decrease in viability of active and stationary growth phases of T. mentagrophytes exposed to 100μg/ml of the imidazoles is shown in Fig. 7.

As with the yeasts, the fungicidal activity of miconazole and econazole was greatly influenced by the growth phase of the fungi.

Actively growing hyphae showed a dramatic drop in viability immediately after addition of the drugs and killing was complete within 3 - 5 h.

Spores were very much more resistant to the fungicidal activity of these drugs, viability only gradually diminishing over a period of 1 week.

Ketoconazole differs from the other imidazoles in failing to kill actively growing hyphae within the 3 - 5 h observed for miconazole and econazole. However, it was considerably more effective than the other two drugs in its action against ungerminated spores. Only a very small proportion of viable cells remained after 2 days, and total killing of both actively growing hyphae and spores was achieved within 4 days.

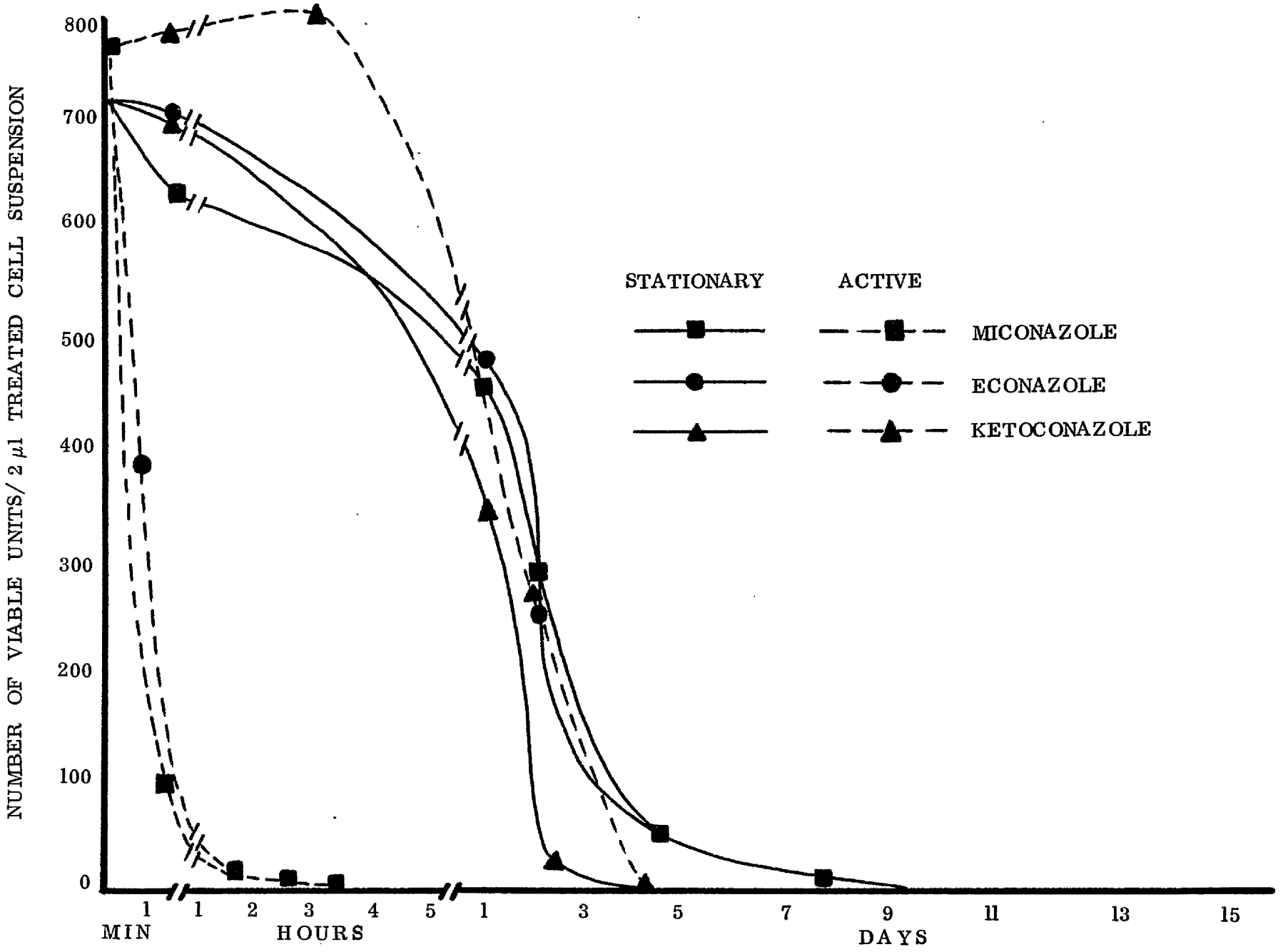


FIGURE 7 Fungicidal activity of 3 imidazole drugs for T. mentagrophytes

MORPHOLOGICAL CHANGES

(i) Yeasts

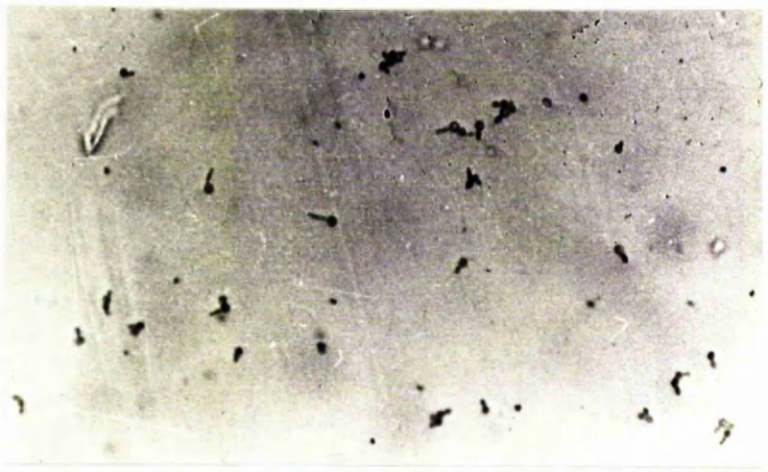
Actively growing cells of C. albicans which had been in contact with econazole and miconazole for 3 h produced a few buds before being killed. A proportion of these buds were more elongate than normal cells but no other changes were noted (Fig. 8 a & b).

The actively growing cells in contact with ketoconazole multiplied a greater number of times to produce short chains of spherical cells (Fig. 8 c).

No further multiplication took place between 3 and 24 h, but morphological changes were apparent. On all 3 drugs the cells appeared to shrink. (Fig. 9 a, b & c).

Stationary phase cells of C. albicans recommenced multiplication on all 3 drugs during the first 3 h of contact although this development was less on econazole than on the other 2 imidazoles. In all cases the cells appeared morphologically normal at a magnification of x 400 (Fig. 10 a, b & c). However, the inoculum of stationary phase cells continued to multiply after the 3 h period and in addition there were changes in their morphology. By 24 h they had increased in size and became refractile ("chlamydospore-like") (Fig. 11 a, b & c).

Non-chlamydospore-forming yeasts also continued to multiply for several hours after exposure to fungistatic concentrations of the imidazoles but, in contrast to C. albicans, they retained their normal appearance when exposed to 100μg/ml econazole for 24 h. Stationary phase cells neither became enlarged in size nor refractile in appearance (Fig. 12 a). Actively growing cells did not shrink (Fig. 12 b).



a

b

c

FIGURE 8 Appearance of actively growing cells of C. albicans after 3 h in contact with (a) econazole (b) miconazole and (c) ketoconazole x 150

a

b

c

FIGURE 9 Appearance of actively growing cells of C. albicans after 24 h in contact with (a) econazole (b) miconazole and (c) ketoconazole x 150

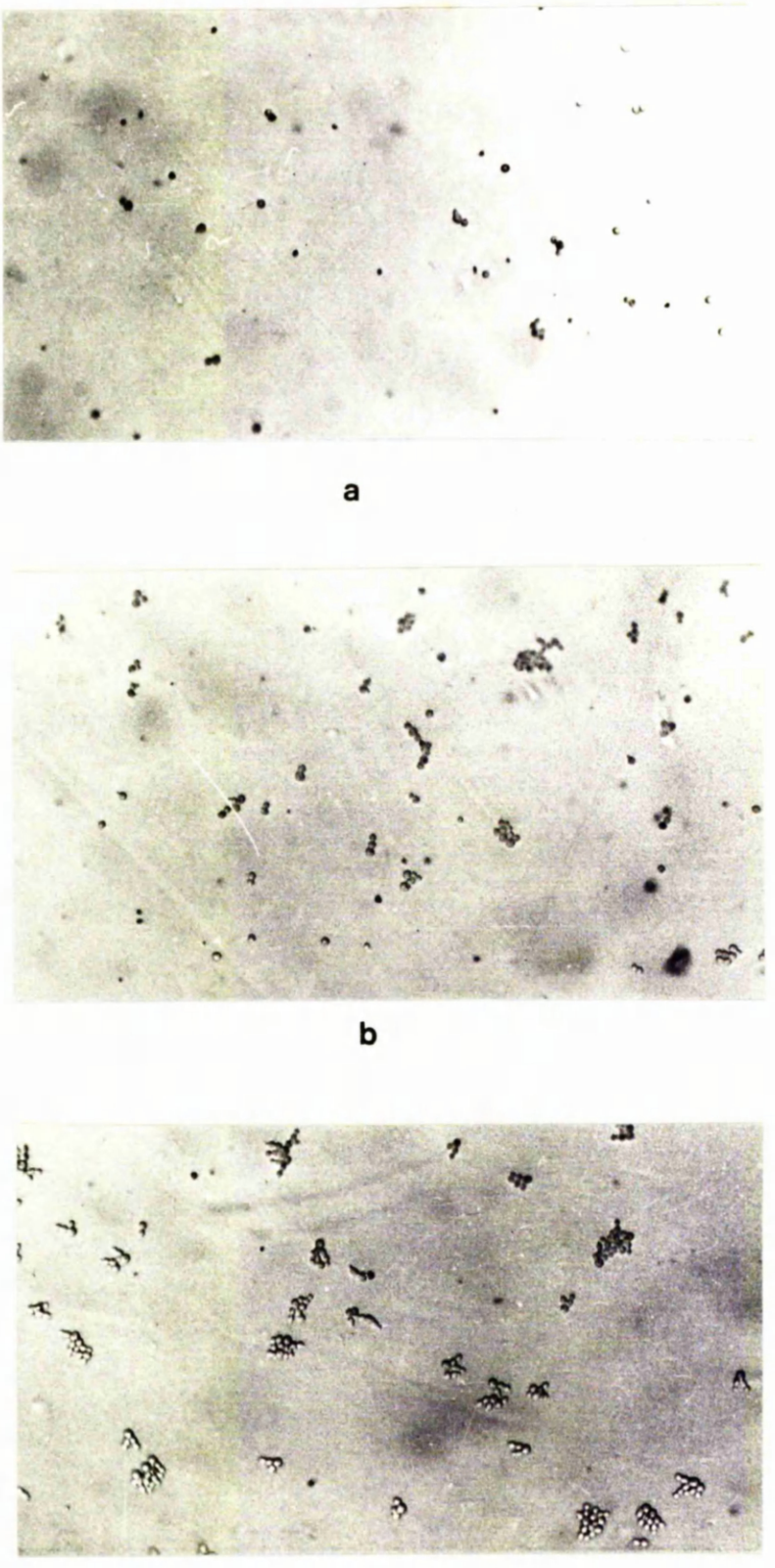
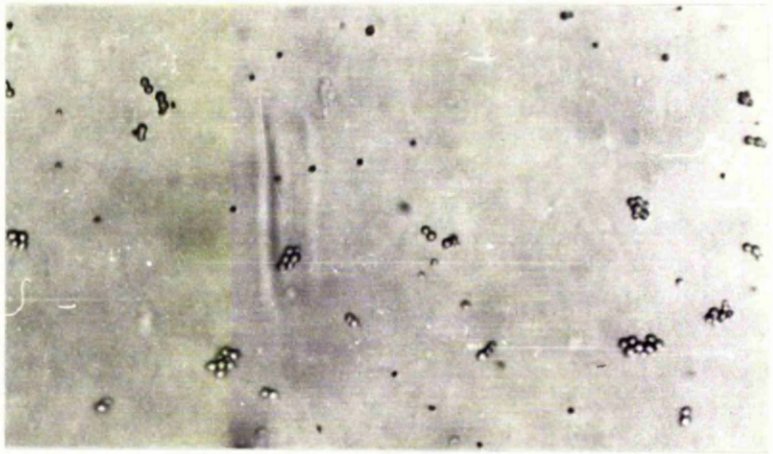
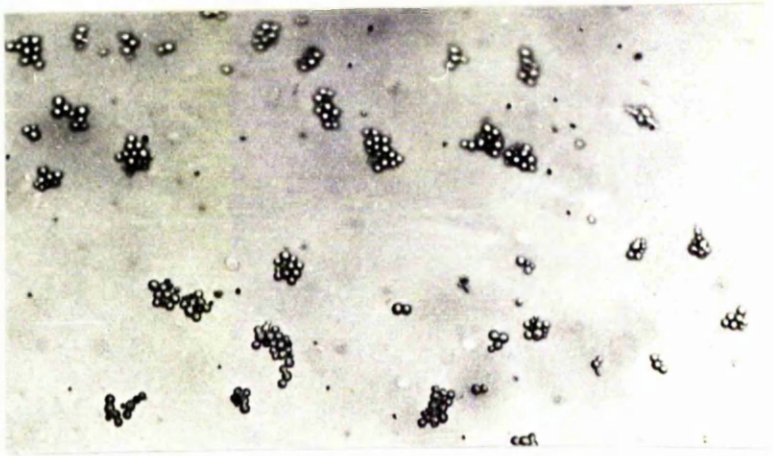


FIGURE 10 Appearance of stationary phase cells of C. albicans after 3 h in contact with (a) econazole (b) miconazole and (c) ketoconazole x 150



a



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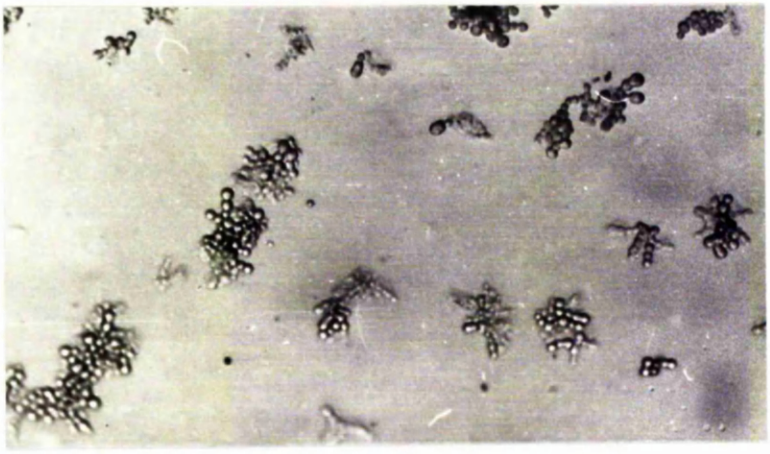
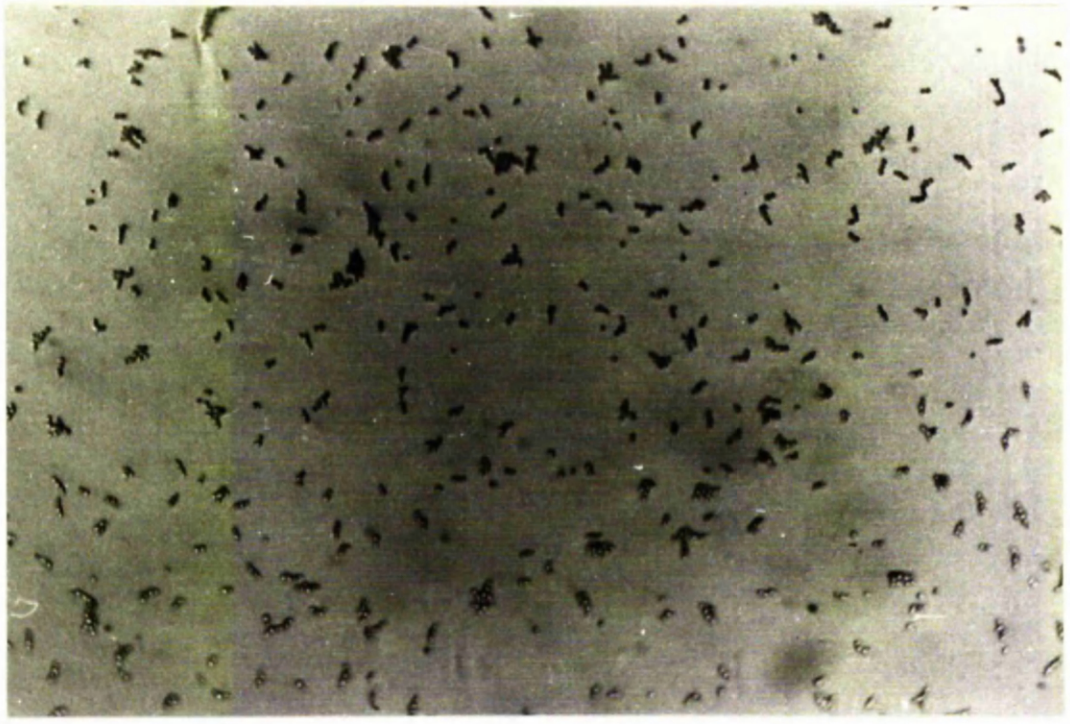


c

FIGURE 11 Appearance of stationary phase cells of C. albicans after 24 h contact with (a) econazole (b) miconazole and (c) ketoconazole x 150



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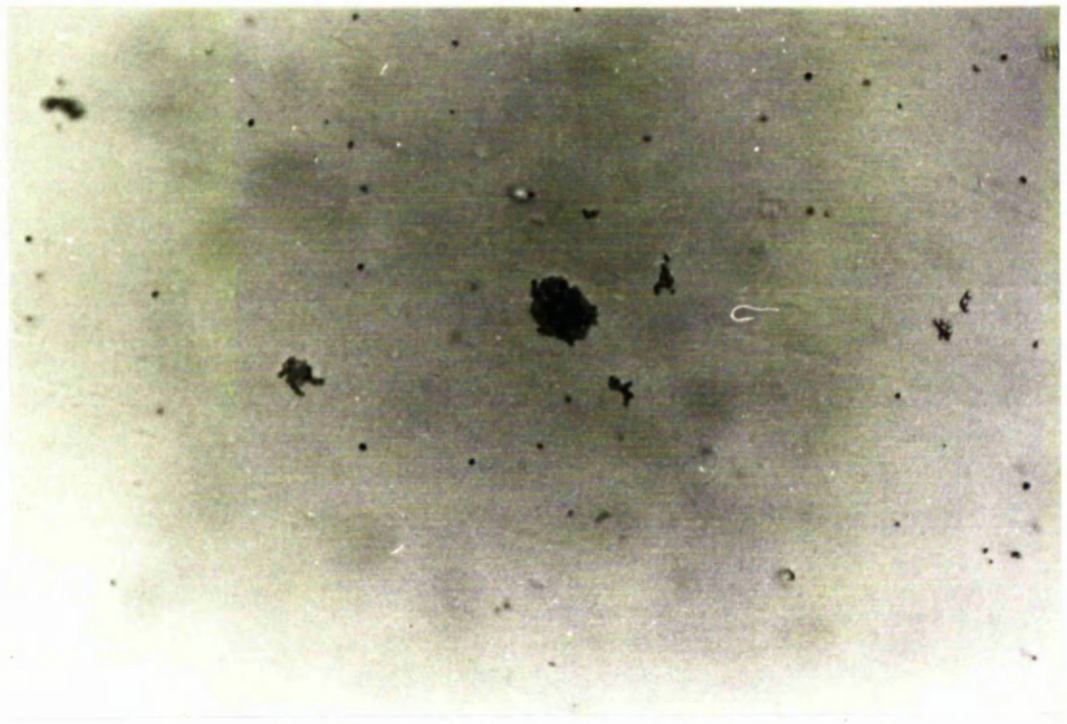


b

FIGURE 12 Appearance of the non-chlamydospore-forming yeast, C. parapsilosis, after 24 h in contact with 100μg/ml econazole: (a) stationary phase cells (b) actively growing cells x 150

(ii) Filamentous fungi

(a) Morphological effects of the imidazoles

The following description summarizes the general trend in the behaviour of the filamentous fungi on sub-fungistatic concentrations, concentrations approaching the M. I. C. and concentrations greater than the M. I. C. (The M. I. C. for each drug against the various fungi are listed in Table II).

The normal appearance of hyphae growing for 24 h on glucose peptone agar at 28°C is shown in Fig. 13 (a) - (d).

When exposed to low concentrations of the imidazoles the fungal hyphae were stunted, but no modification in morphology occurred (Figs. 14 (a), 16(a), 17 (a)).

On concentrations approaching the M. I. C., thickened refractile cells developed, and A. fumigatus and M. canis also produced rounded chlamydospores. The hyphae continued to grow slowly until colonies became easily visible in 7 - 10 days (Figs. 14 (b), 15 (a), 16 (b) , 17 (b)).

On minimum inhibitory concentrations and above limited initial growth allowed the development in the dermatophytes of thickened refractile areas along the length of the hyphae, but as the time of exposure and/or the concentration of the drugs increased these areas diminished in size and became replaced by "empty" shrunken cells which were found to be non-viable (Figs. 14 (c), 15 (b), 16 (c), 17 (c)).

(b) Morphological effects of tolnaftate and griseofulvin

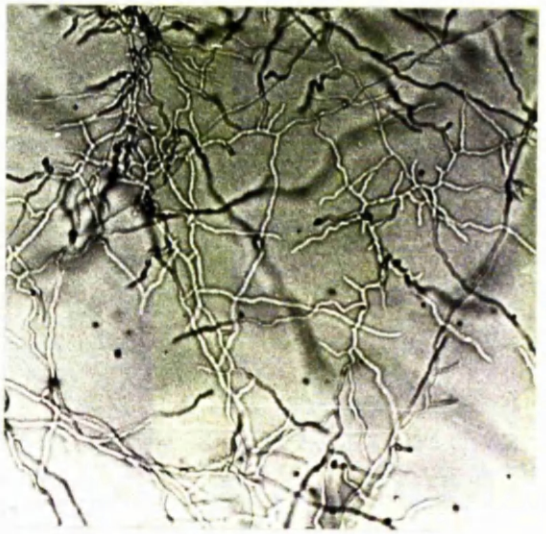
Tolnaftate and griseofulvin also caused the production of swollen and refractile areas along the hyphae (Figs. 18 (a) & (b), 19 (a)).

On low concentrations of griseofulvin "curling" of the hyphae was apparent (Fig. 19 (b)). This "curling" effect is peculiar to griseofulvin and was not caused by any of the other antifungals tested. Comparable concentrations, (i.e. lower than the M.I.C.), of the imidazoles and tolnaftate merely caused stunting of growth.

CHLAMYDOSPORE PRODUCTION BY C. ALBICANS

The terminal chlamydospores produced by C. albicans after 24 h at 28°C on Czapek-Dox/Tween 80 medium are shown in Fig. 20.

a



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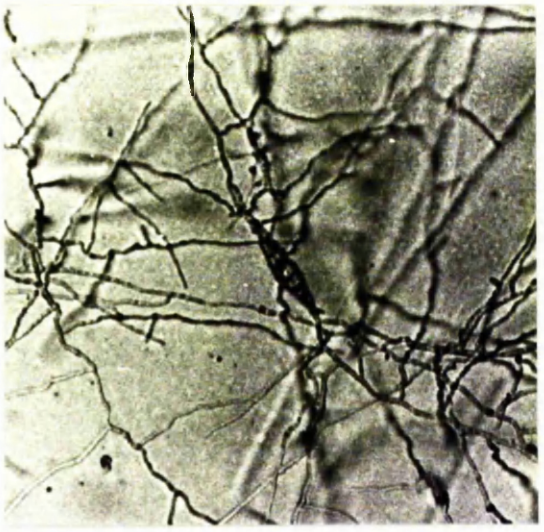

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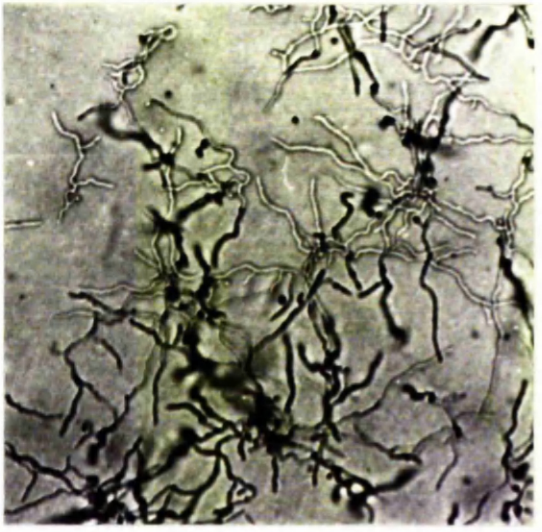
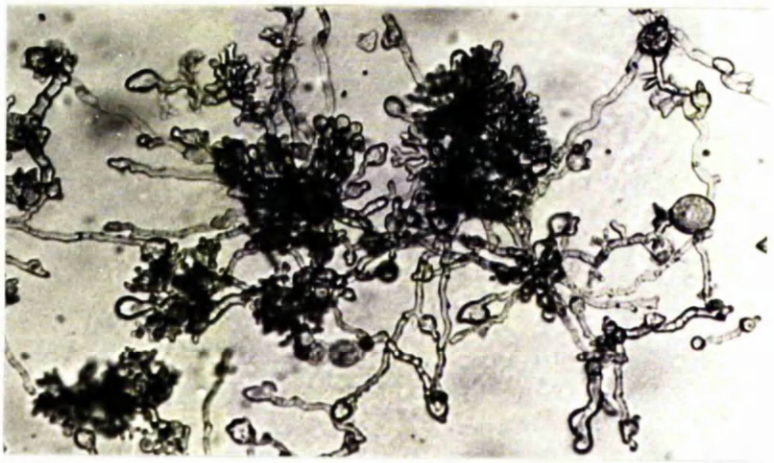


d

FIGURE 13 Normal hyphal development after 24 h on drug-free glucose peptone agar (a) A. fumigatus (b) T. mentagrophytes (c) M. canis and (d) T. rubrum x 150

a



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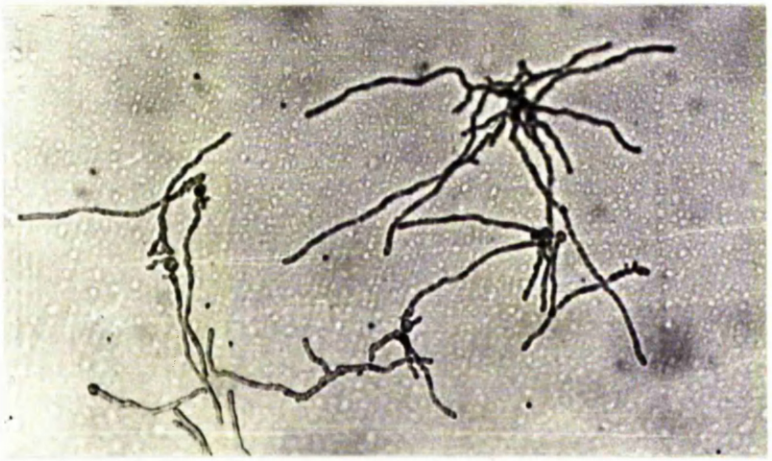
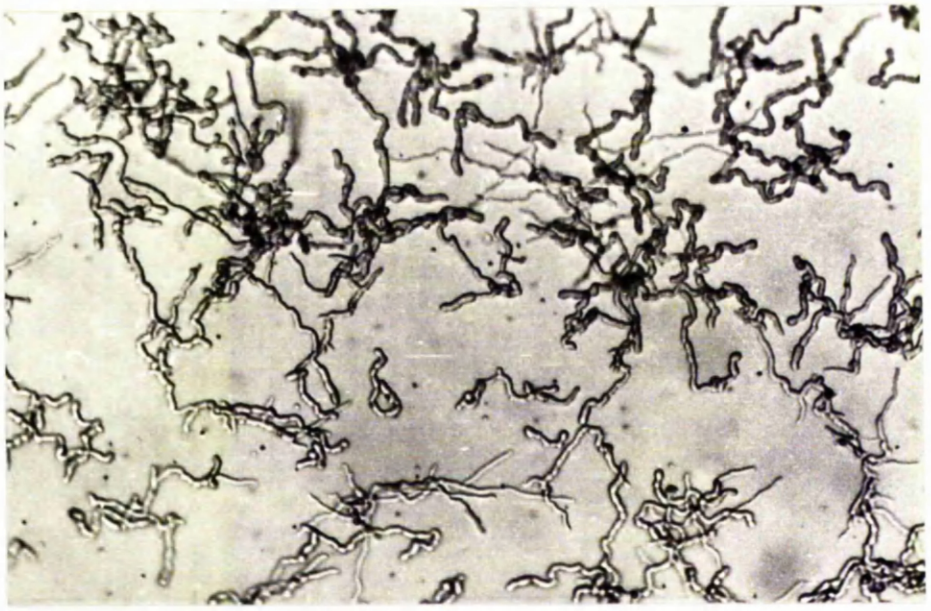
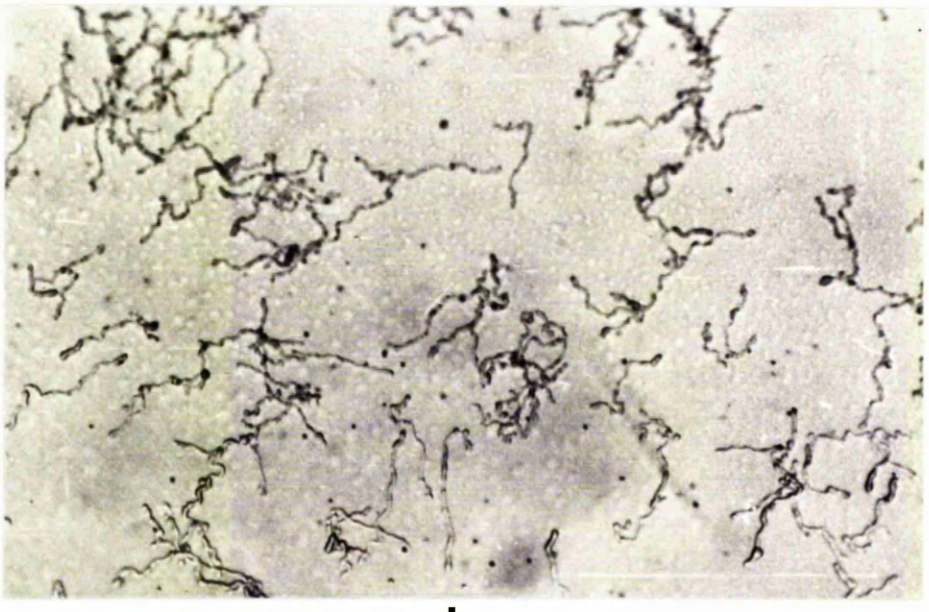


c

FIGURE 14 The development of germinated spores of A. fumigatus during 48 h at 28°C in (a) A sub-fungistatic concentration of miconazole (5μg/ml) (b) A concentration of miconazole approaching the M. I. C. (10μg/ml) and (c) A concentration of miconazole greater than the M. I. C. (50μg/ml) x 150



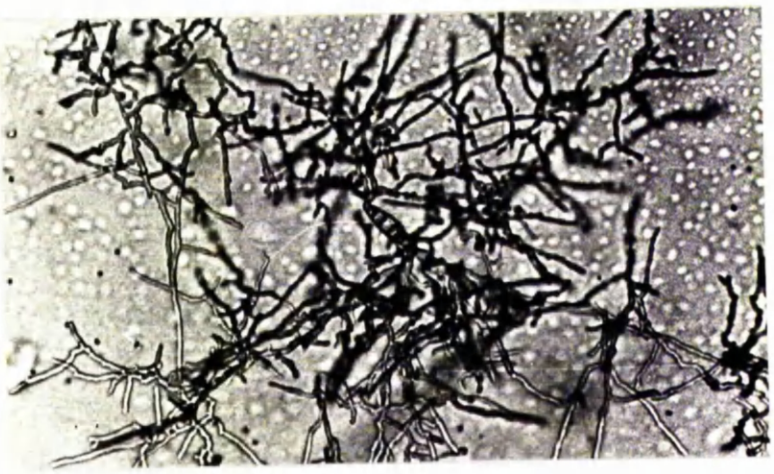
a



b

FIGURE 15 The development of germinated spores of T. mentagrophytes var. interdigitale during 48 h at 28 C in (a) A concentration of econazole approaching the M. I. C. (0. 5μg/ml)* and (b) A fungistatic concentration of econazole (1μg/ml) x 150

* lowest concentration photographed



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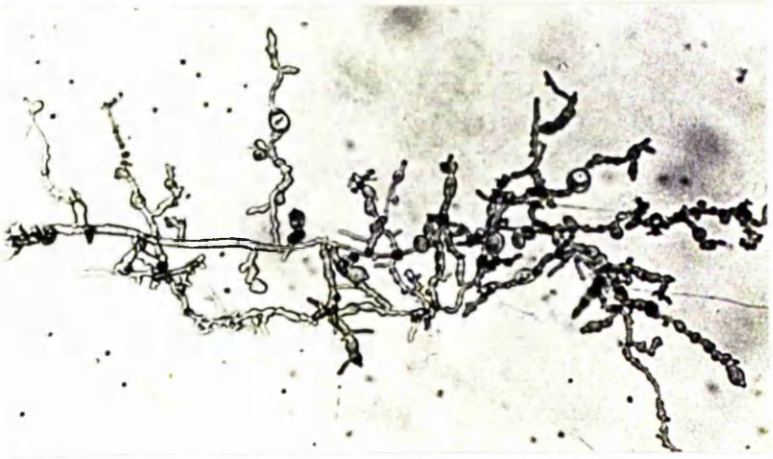

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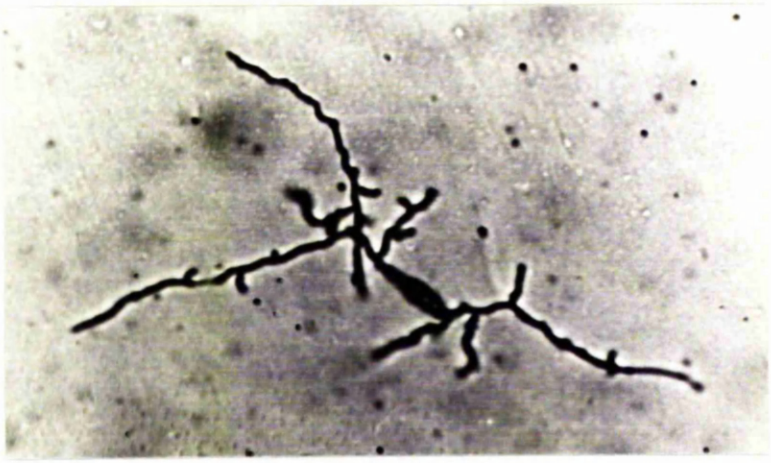
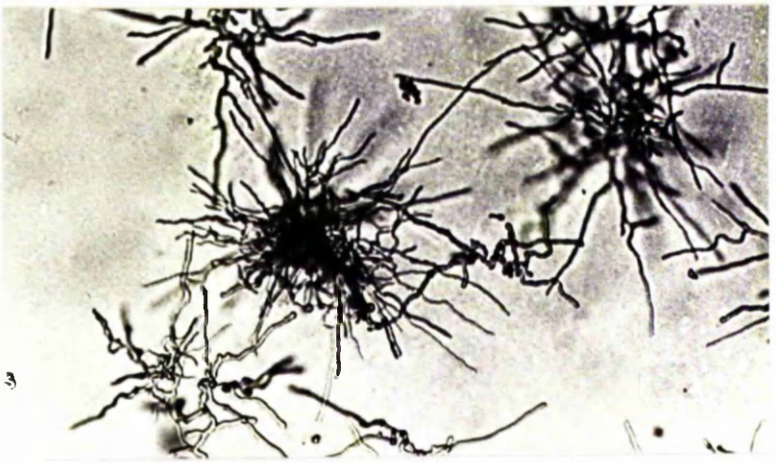


c

FIGURE 16 The development of germinated spores of M. canis during 48 h at 28°C in (a) A sub-fungistatic concentration of ketoconazole (10μg/ml) (b) A concentration of ketoconazole approaching the M. I. C. (20μg/ml) and (c) A fungistatic concentration of ketoconazole (50μg/ml) x 150



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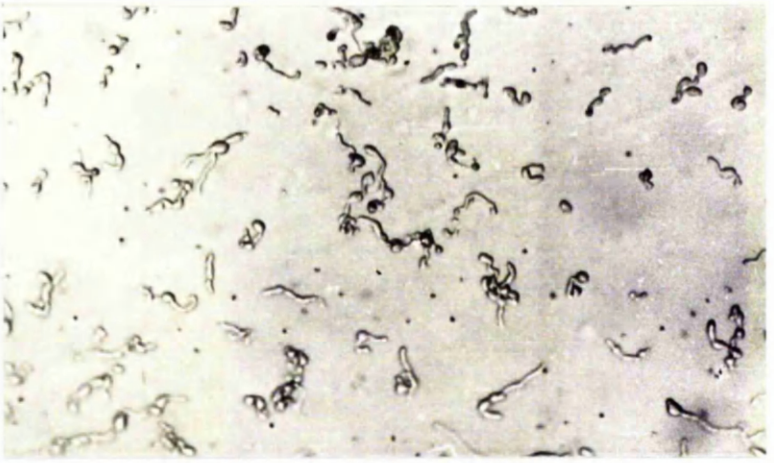

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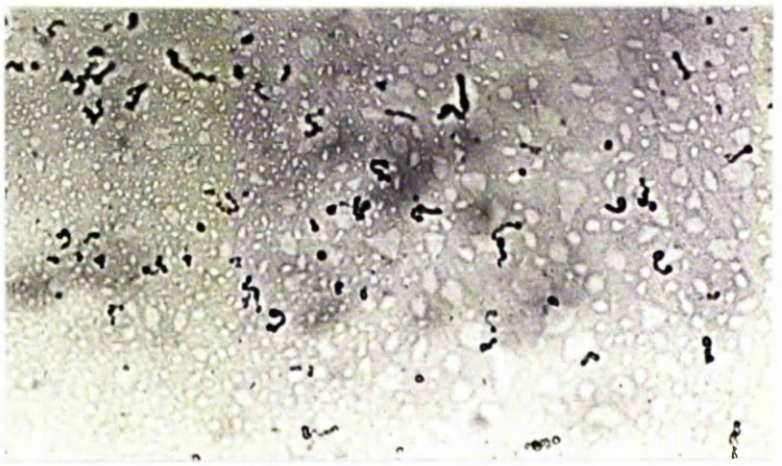
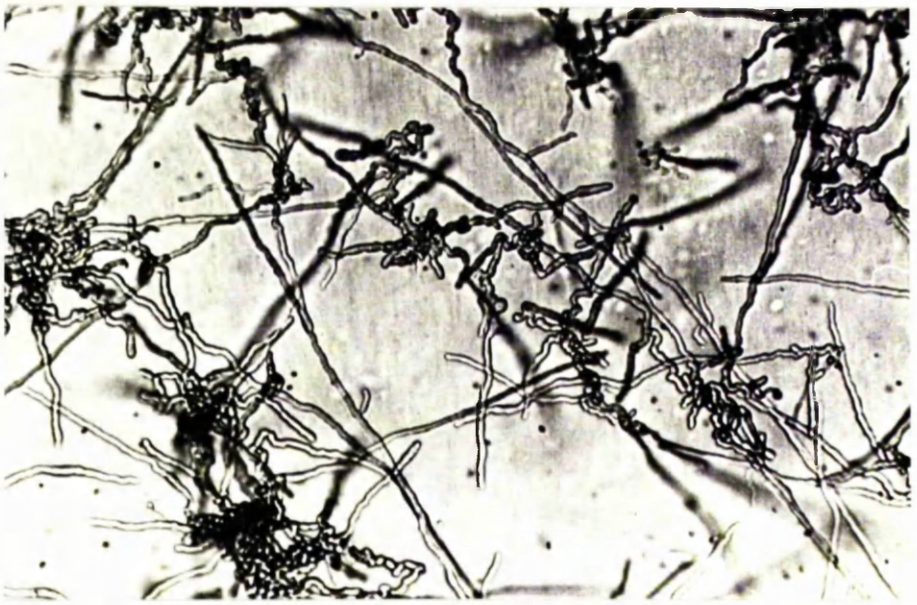


c

FIGURE 17 The development of germinated spores of T. rubrum during 48 h at 28°C in (a) A sub-fungistatic concentration of clotrimazole (0.5μg/ml) (b) A concentration of econazole approaching the M.I.C. (1μg/ml)* and (c) A fungistatic concentration of clotrimazole (5μg/ml) x 150

* The equivalent clotrimazole plate was unreliable due to "clumping" of precipitated drug.



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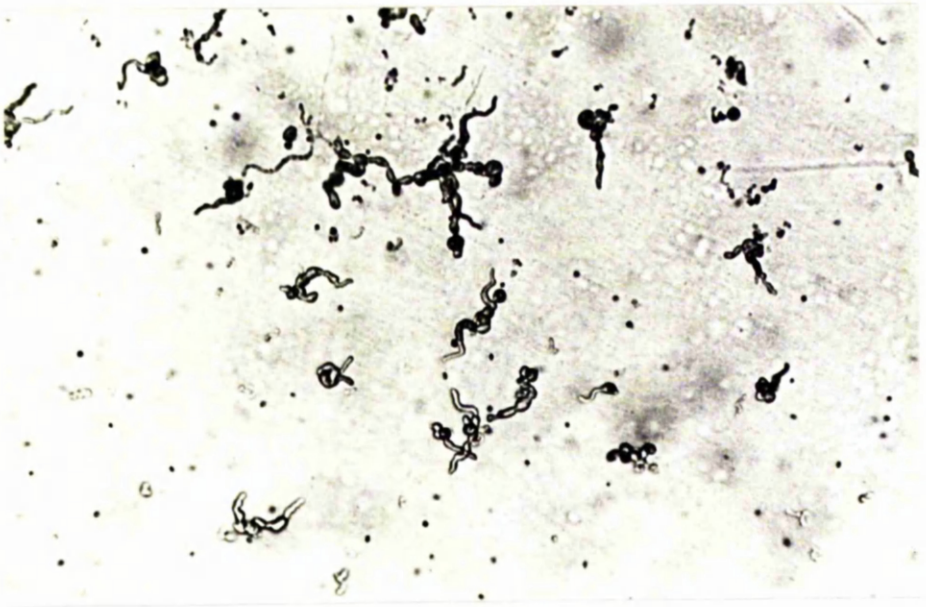
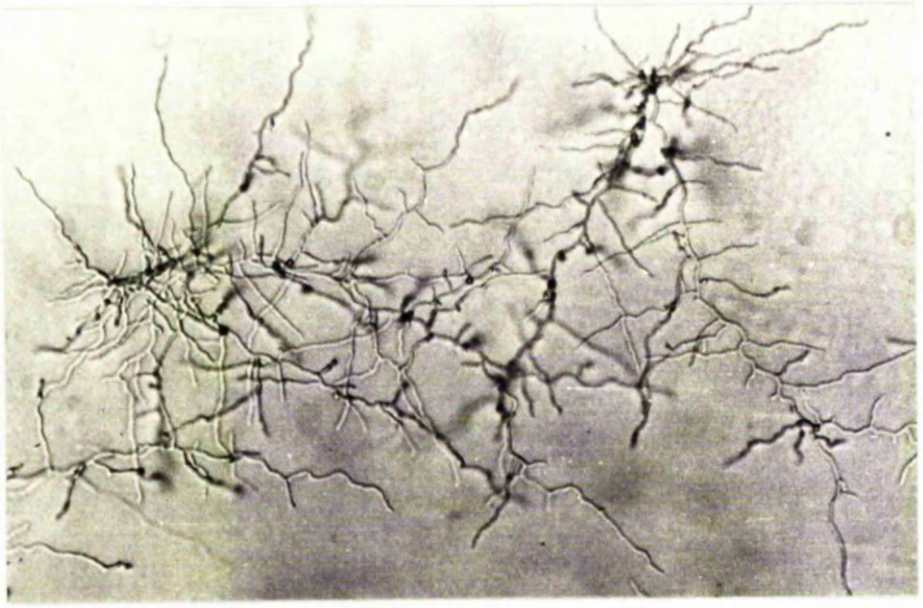


b

FIGURE 18 Morphological effects caused by 0.1μg/ml tolnaftate after 48 h (a) T. mentagrophytes var. interdigitale (b) T. rubrum x 150

a



b

FIGURE 19 Morphological effects of griseofulvin on T. mentagrophytes var interdigitale after 48 h (a) A fungistatic concentration (10μg/ml) and (b) "Curling" on a sub-fungistatic concentration (0.1μg/ml) x 150

FIGURE 20 Terminal chlamydospores of C. albicans. Formed after 24 h at 28°C on Czapec-Dox/Tween 80 medium x 150

DIFFERENCES BETWEEN 'NORMAL' AND 'UNUSUAL' YEASTS

(i) The auxanographic test used to detect differences in nutritional requirements showed that C. albicans and C. parapsilosis were stimulated to a greater extent by biotin and thiamin than were C. guilliermondii and C. tropicalis.

(ii) No contamination of the econazole nitrate with biotin or thiamin was detected.

(iii) Culture filtrates of the cells growing in the presence of sub-fungistatic concentrations of econazole failed to stimulate the growth of freshly inoculated yeasts.

(iv) Several amino acids, (histidine, serine, aspartic acid, cystein, glycine and homoserine), plus peptone and casein hydrolysate and the vitamins biotin and thiamin were found to stimulate the growth of the yeasts in the presence of sub-fungistatic levels of econazole.

(v) Only thiamin showed an ability to preferentially relieve the toxicity of econazole for the two "unusual" yeasts. C. albicans and C. parapsilosis were not affected by the addition of 20μg/ml thiamin to minimal medium supplemented with ammonia and biotin, whereas C. tropicalis and C. guilliermondii were inhibited by addition of the same concentration of this vitamin to the medium. In medium containing 20μg/ml econazole, thiamin relieved the toxicity of econazole for C. guilliermondii and C. tropicalis but not for C. albicans and C. parapsilosis.

SUB-CULTURE OF C. GUILLIERMONDII AND C. TROPICALIS FROM SUB-FUNGISTATIC LEVELS OF THE IMIDAZOLES

(a) Econazole

Sub-cultures of C. guilliermondii and C. tropicalis, taken from the Petri dishes used to determine the M. I. C. (p22) after 3 days, showed different sensitivities depending on whether they were inoculated directly onto fresh medium containing econazole, or whether they were first passaged on drug-free medium.

Both species behaved in the same manner.

Table III compares the results obtained with C. guilliermondii following sub-culture directly (Fig. 22 a), and after passage on drug-free medium (Fig. 22 b), onto medium containing a range of concentrations of econazole.

Fig. 21 illustrates in histogram form the results obtained by sub-culturing directly and after passage onto equivalent concentrations.

Cells taken from 0.5 and 1μg/ml concentrations appeared better adapted, after passage on drug-free medium, to growth at these concentrations than previously unexposed cultures, although the M. I. C. remained unaffected.

(b) Miconazole and ketoconazole

As with econazole a second exposure to miconazole and ketoconazole led to increased sensitivity of C. guilliermondii when made directly, and to decreased sensitivity when transferred via a drug-free medium.

Conc. of Econazole (μg/ml)		Control	0.5	1.0	5.0	10.0	15.0
	Original Sensitivity	++++	+	+	++	++	+
0.5	Direct sub-culture	++++	+	+	++	++	-
	After passage	++++	++	+	++	++	+
1.0	Direct sub-culture	++++	+	+	+	-	-
	After passage	++++	++	++	++	++	+
5.0	Direct sub-culture	++++	+	+	+	-	-
	After passage	++++	++	++	++	++	+
10.0	Direct sub-culture	++++	+	+	+	-	-
	After passage	++++	+	+	++	++	+
15.0	Direct sub-culture	++++	+	+	++	+	-
	After passage	++++	+	+	++	++	+

TABLE III RESPONSE OF C. GUILLIERMONDII TO ECONAZOLE FOLLOWING DIRECT SUB-CULTURE FROM MEDIUM CONTAINING THE DRUG, AND AFTER PASSAGE ON DRUG FREE MEDIUM

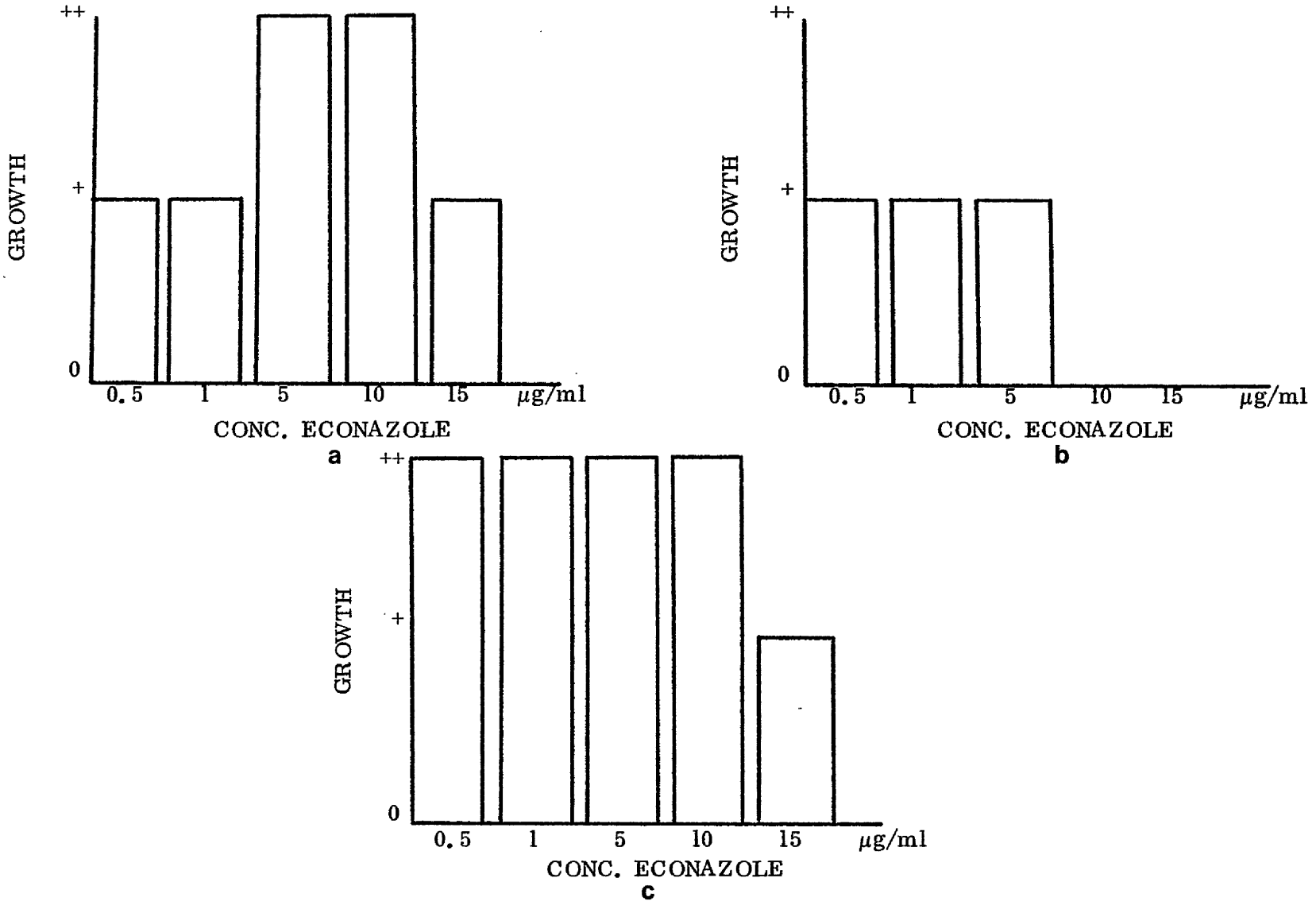
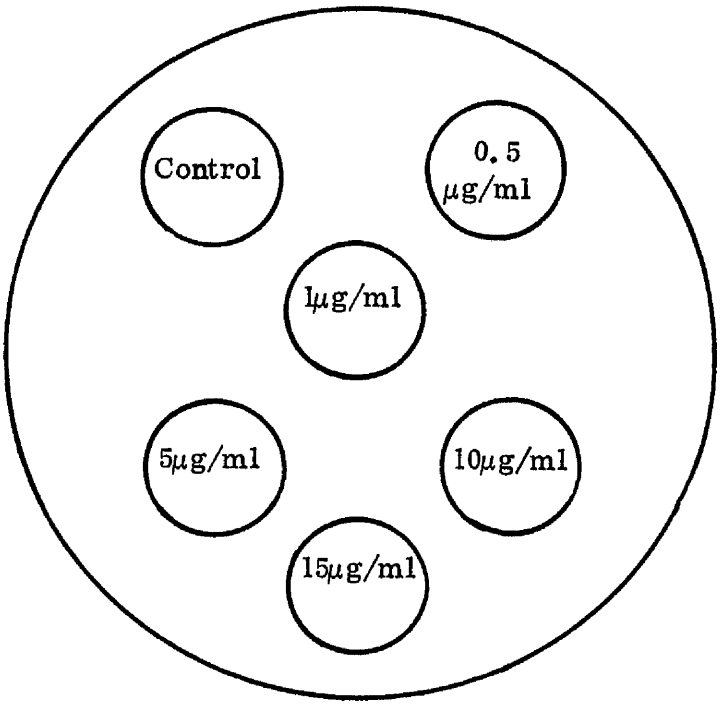
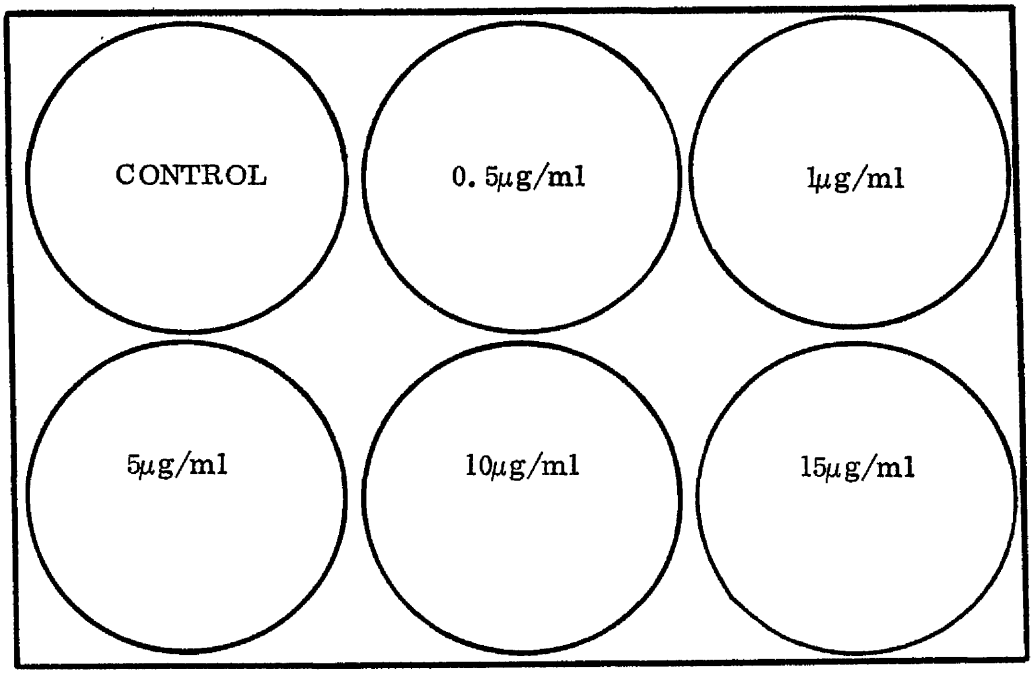


FIGURE 21 Response of C. guilliermondii to econazole. a) Growth on 1st exposure to 0.5, 1, 5, 10 and 15µg/ml. b) Growth on direct transfer to equivalent concentrations c) Growth following passage on drug-free medium.

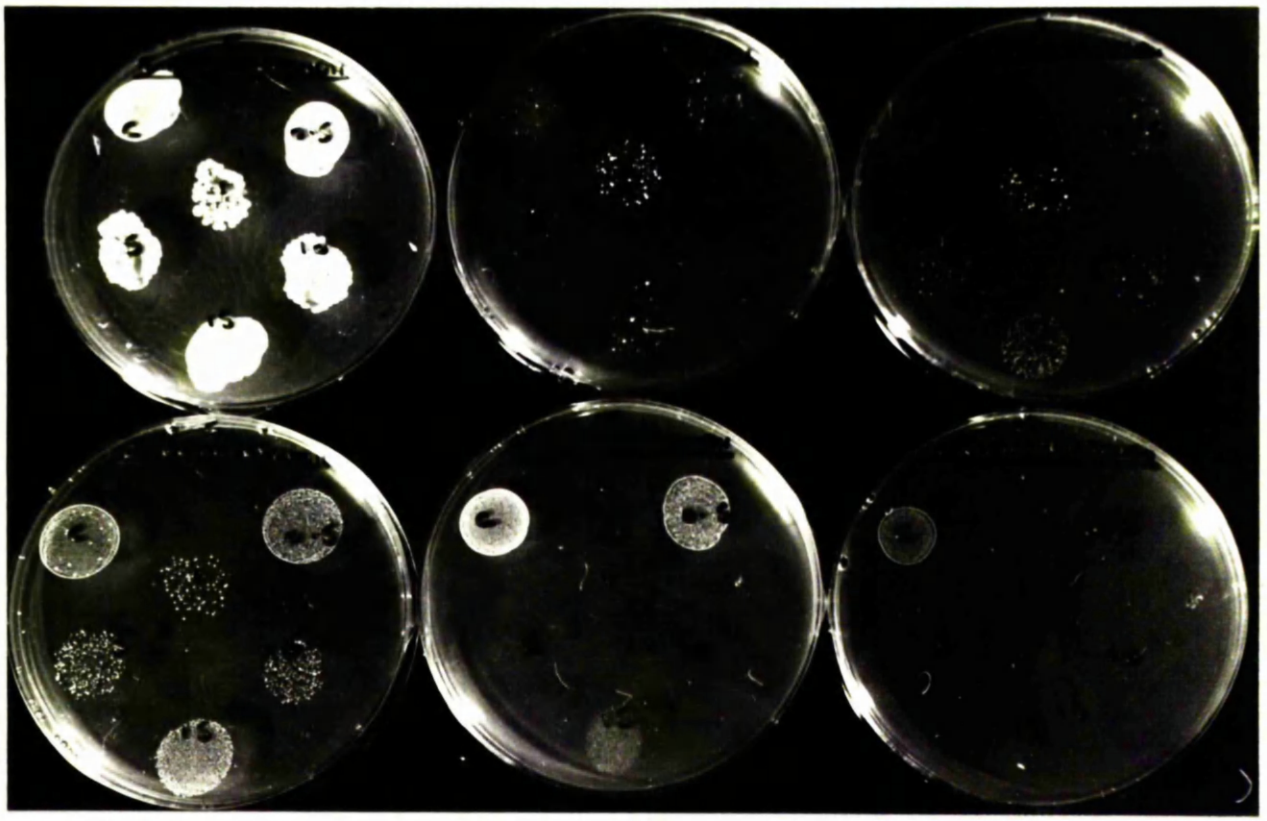


Key to positions of inocula on each Petri dish. Concentrations denote those in medium from which each inoculum was derived.



Concentration of drug in each Petri dish

Diagrams to illustrate position of various inocula and concentrations of drug in each of the Petri dishes in Fig. 22.



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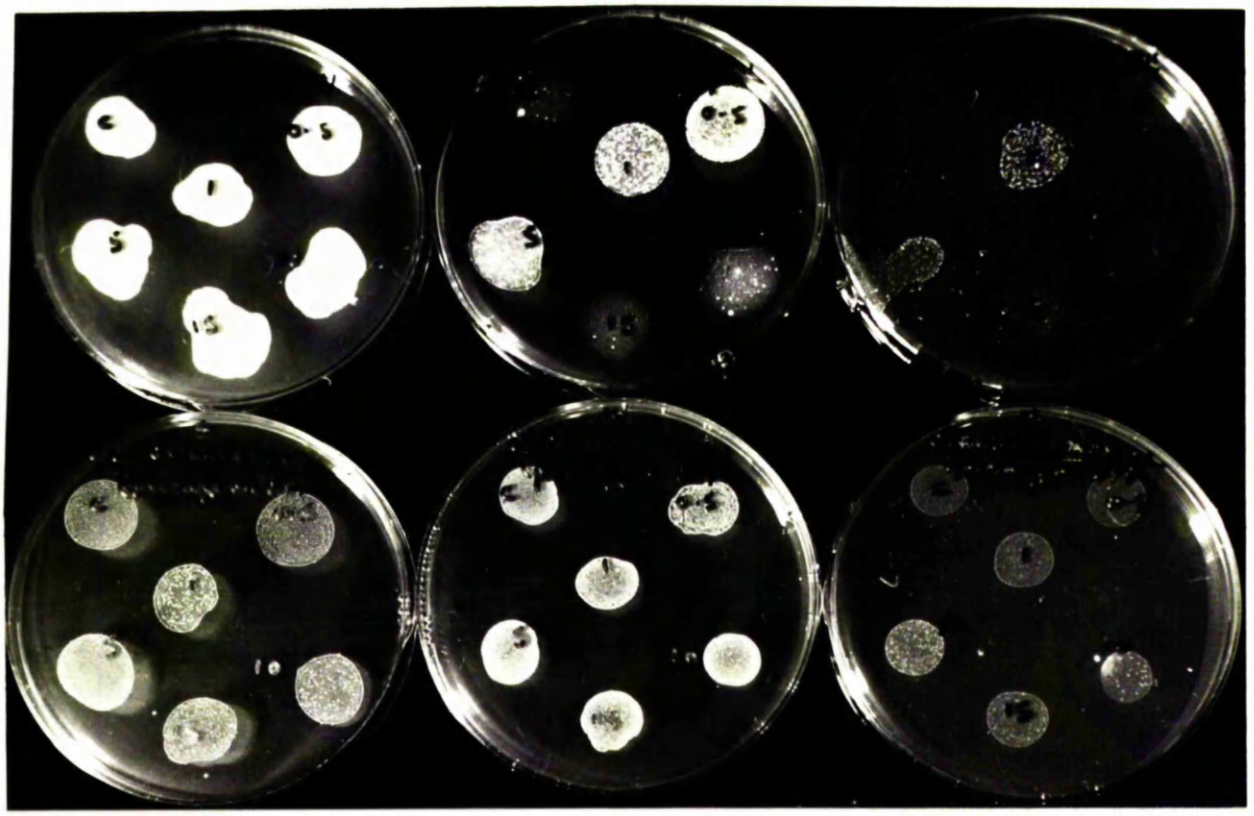


b

FIGURE 22 Growth of C. guilliermondii sub-cultured from sub-fungistatic concentrations of econazole (control, 0.5, 1, 5, 10 and 15μg/ml). (a) Transferred directly onto medium containing the same concentrations as before (b) Transferred via drug-free medium in which grown for 3 days at 28°C (See previous page for key to illustrations.)

RESISTANCE TO THE IMIDAZOLES

(i) Variation in sensitivity within a population

Within a population of fungal cells or spores a small number seem able to grow slowly on concentrations which completely inhibit the rest of the population. Fig. 23 (a) shows the development of a micro-colony of C. albicans after 6 days on 100μg/ml ketoconazole. Fig. 23 (b) illustrates a similar result with the filamentous fungus T. rubrum after 6 days on 15μg/ml ketoconazole.

(ii) Effect of repeated sub-culture

Repeated sub-culture of T. mentagrophytes var. interdigitale on sub-fungistatic concentrations of ketoconazole failed to detect any further adaptation of the least sensitive members of the population.

(iii) Artificially induced resistance in A. nidulans

(a) Isolation of mutants

The U.V. irradiated conidia of A. nidulans (bi - 1) yielded seven mutant colonies which grew more rapidly than controls on medium containing a sub-fungistatic concentration of econazole. Sub-culture of these mutants onto sub-fungistatic concentrations of miconazole, econazole and ketoconazole showed that all 7 isolates possessed a degree of resistance to all 3 imidazoles.

One of these mutants was chosen for further study. It was larger and lighter in colour than the other resistant colonies and was designated ECO - 1.

(b) Relief of toxicity

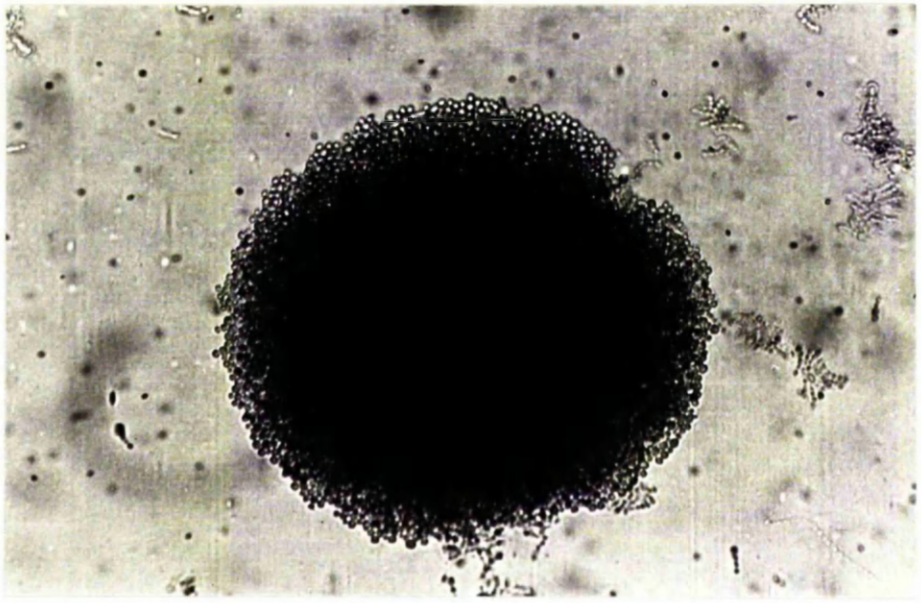
It was found that for the resistant mutant l-phenylalanine gave a wide zone of relief of toxicity from fungistatic levels of miconazole.

(c) Uptake of l.-phenylalanine

Using ^{14}C l.-phenylalanine it was shown that uptake of this amino acid was reduced by a half in the resistant mutant compared to the parent strain (Fig. 24).

(d) Comparison of M. I. C. s for biotin - requiring and ECO - 1 mutants of A. nidulans

ECO - 1 was consistently more resistant to the effect of the imidazoles. The resistance to the three imidazoles varied - compared with the original biotin requiring mutant, the ECO - 1 mutant was twice as resistant to miconazole, 7 times more resistant to econazole and 4 times more resistant to ketoconazole (Table IV).



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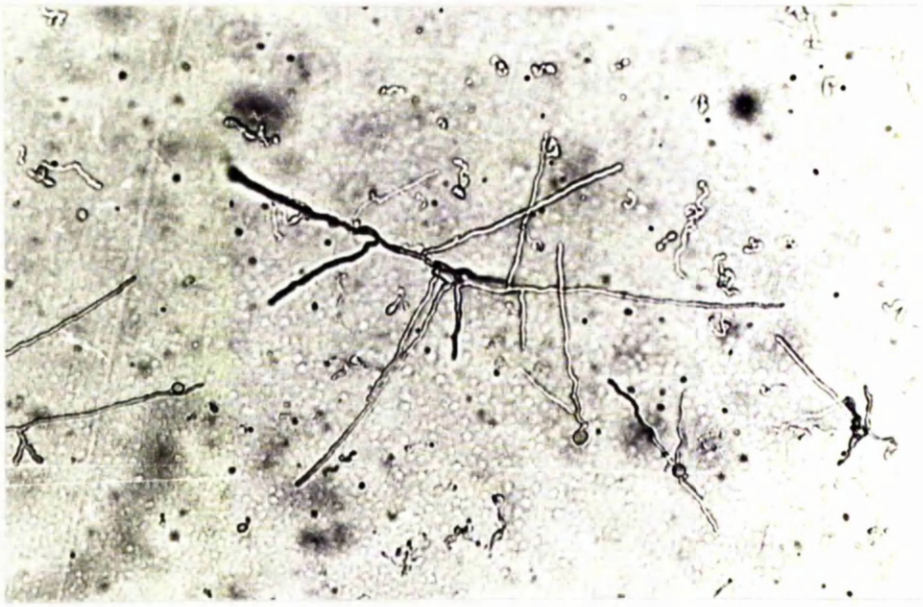


b

FIGURE 23 Resistant colonies developing after 6 days on ketoconazole (a) C. albicans on 100μg/ml (b) T. rubrum on 15μg/ml x 150

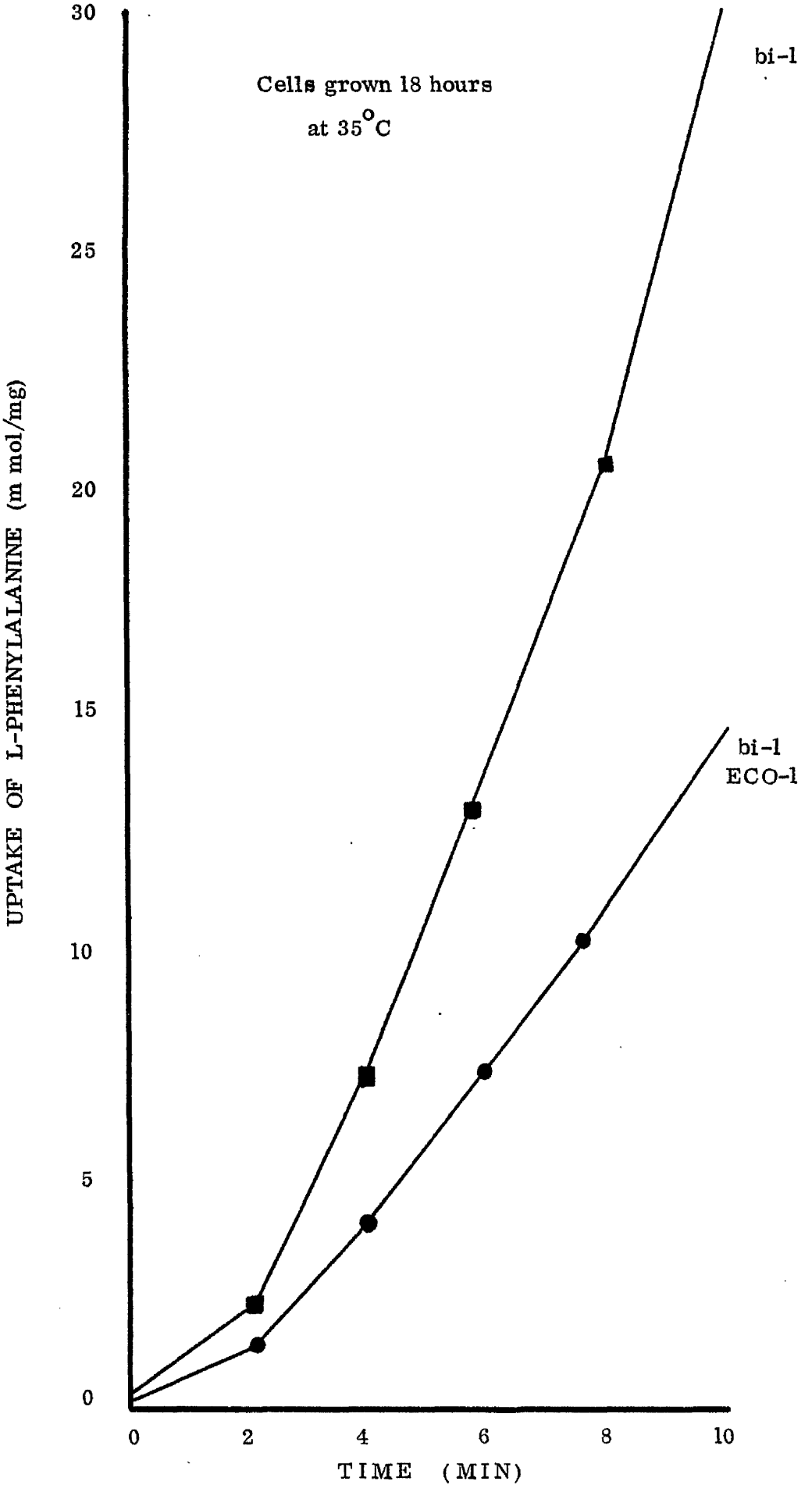


FIGURE 24 l-Phenylalanine uptake by cells of biotin-requiring parent and econazole-resistant mutant of A. nidulans

	MUTANT	M.I.C.
MICONAZOLE	bi - 1	7.5 μg/ml
	ECO - 1	15
ECONAZOLE	bi - 1	1
	ECO - 1	7.5
KETOCONAZOLE	bi - 1	1
	ECO - 1	4

TABLE IV COMPARISON OF M.I.C.s FOR BIOTIN-REQUIRING AND ECO - 1 MUTANTS OF A. NIDULANS

DISCUSSION

The procedures used for these investigations gave reproducible results which were broadly in agreement with those of other workers.

Provided inocula were of a standard size, measurements of fungistatic activity were the same whether made in liquid or solid media, and only for A. fumigatus was there a different drug response between active and stationary phases. Conidial germination of A. fumigatus was, perhaps surprisingly, inhibited by slightly lower concentrations of miconazole and econazole than was required to inhibit growth of the hyphae (Table II). The cellophane square technique has the advantage of allowing estimation of fungistatic activity much sooner than by conventional broth dilution tests.

In in vitro tests the established drugs tolnaftate and griseofulvin compared favourably with the imidazoles in their activity against dermatophytes. However, the spectrum of antifungal activity of these drugs is limited - they are not active against A. fumigatus or yeasts.

The imidazoles have a much broader spectrum of activity. All the organisms tested were sensitive to a greater or lesser extent to all four azole derivatives examined. Econazole was the most active of these compounds, closely followed by miconazole and clotrimazole. Ketoconazole gave consistently higher M.I.C. levels which at first sight might indicate that it is a less useful compound. However, as the in vivo activity of antifungals is greatly influenced by the reaction of the host and the ability of the antifungal to reach the site of infection, it is inadvisable to predict the in vivo efficacy of these drugs solely from in vitro results.

Direct microscopic examination of the inocula exposed to fungistatic concentrations of the imidazoles revealed a degree of initial cell multiplication for both active and stationary phase yeast cells, and for

actively growing hyphae of filamentous fungi. Whether or not metabolic changes occurred in spores of mycelial fungi is not known, but their morphology remained unaltered and the increase in size usually observed in spores prior to germination was not noted. Since these investigations were made, this feature of a short growth period before inhibition has been described by other workers (Plempel, 1979 and Haller, 1979). A similar delay in growth inhibition for several generations is well documented for the antibacterial drug sulphonamide (Davis et al., 1968). Throughout this study the definition of M. I. C. was taken to mean "that concentration of drug required to inhibit continued growth".

The fact that growth continues for a short time after exposure to the imidazoles, and morphological changes occur in response to the adverse conditions created by the drugs, suggests that the cells may possess a store of enzymes/substrates/products/energy reserves, which protects them from an immediate effect of the drugs.

The nature of the mechanisms involved is unclear. A proportion of the new cells formed by C. albicans in the presence of miconazole and econazole were malformed, and short chains of cells were produced by this yeast exposed to ketoconazole. Under normal conditions C. albicans buds in a random fashion to produce rounded clumps of spherical or short oval cells. The abnormal growth may be the result of direct interference with the formation of the new cell wall, e. g. by interfering with ergosterol synthesis as suggested by Van den Bossche, (1978), but it could also be a consequence of disruption of one or more of the many other metabolic processes involved in survival and growth of the cell.

The fungicidal action of 100μg/ml miconazole and econazole against actively growing C. albicans was rapid, the majority of cells were killed within 1 hour. Similar results have been obtained by other workers such as Iwata et al., (1973 a) who reported that 20 - 50μg/ml clotrimazole caused almost total loss of viability of C. albicans within 2 hours, and Swamy et al., (1974) who found a total cidal effect within 4 hours when cells of C. albicans were exposed to 10μg/ml miconazole.

Stationary phase cells of C. albicans were, however, markedly more resistant to the fungicidal effect of the imidazoles and the question arose as to whether the ability of this yeast to form chlamydospores was influencing the results of tests.

Chlamydospore formation is a characteristic feature which is used in the laboratory to separate C. albicans from other Candida species.

The terminal chlamydospores (Fig. 20) produced by C. albicans in Czapek-Dox/Tween 80 and certain other media, are not morphologically indentical to the "chlamydospore-like" cells observed in this study. Nevertheless it is possible that the same mechanism may be involved and the response of C. albicans to the drugs may therefore be different from those yeasts which do not naturally form chlamydospores.

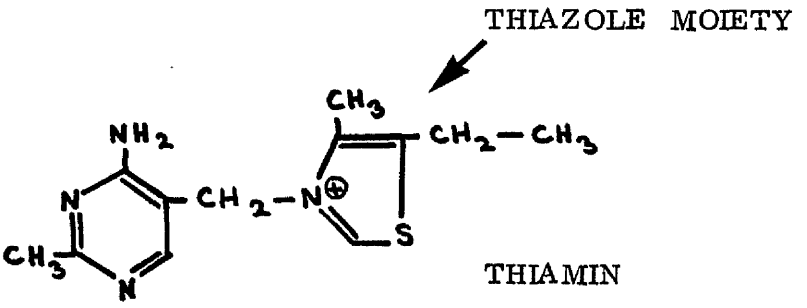
Examination of the fungicidal effect of econazole on 3 non-chlamydospore-forming Candida species showed that actively growing cells of C. guilliermondii and C. tropicalis were markedly more sensitive to the drug than were C. albicans and C. parapsilosis. This sensitivity was even more strikingly exhibited when stationary phase C. guilliermondii and C. tropicalis were exposed to the drug and, to a lesser extent, also by C. parapsilosis. The

viability of stationary phase C. parapsilosis began to diminish within 24 h, while that of C. albicans declined more slowly, total killing not being achieved until after 7 days.

No "chlamydospore-like" structures were produced in response to the drug by the 3 non-chlamydospore - forming yeasts, so it is probable that the ability of C. albicans to form these structures contributes to the survival of this species. However, the wide variation in results between different non-chlamydospore - forming species indicates that chlamydospore formation is not the only factor influencing sensitivity to fungicidal activity and the "unusual" behaviour of C. guilliermondii and C. tropicalis first observed on sub-fungistatic concentrations of the imidazoles in agar medium may be relevant. This behaviour, also quantified by optical density measurements in broth culture, manifests itself as a low, broad peak or "hump" in the graph of growth versus concentration of drug (Fig. 4). The phenomenon has not been previously described for the imidazoles, nor does there appear to be any reports of a similar response with other antifungal or antibacterial agents.

Tests to try to establish the reason for the differences between "normal" and "unusual" yeasts suggest that thiamin may be involved since this vitamin preferentially relieves the toxicity of econazole for C. guilliermondii and C. tropicalis but not for C. albicans and C. parapsilosis.

Thiamin contains a thiazole group which is similar to the imidazole group of econazole and it is possible that the two compounds may compete for the same uptake site or enzyme system within the cell (Fig. 25).



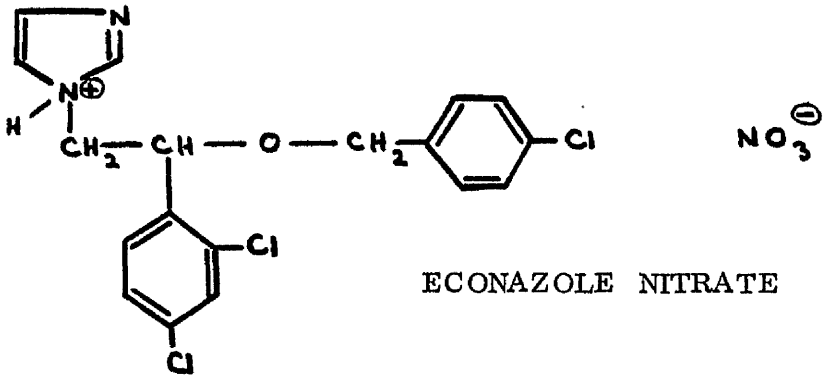


FIGURE 25 Structural formulae of thiamin and econazole nitrate

Growth of a strain of A. nidulans with a requirement for thiamin (an B8) indicated that the glucose peptone medium contained in the region of 1μg/ml thiazole, (the breakdown product of thiamin after sterilization), (Mr. E. Forbes, personal communication), and one explanation, therefore, for the behaviour of C. guilliermondii and C. tropicalis in the presence of low levels of imidazole drugs is of antagonism between the two molecules. Since thiamin at 20μg/ml inhibited growth of C. guilliermondii and C. tropicalis another explanation is that the effect of low concentrations of the imidazoles on these fungi is cumulative to thiamin in the medium, (by interfering with the same mechanism), and causes the significant reduction in growth observed at low levels of the imidazoles. At higher levels of the drugs an alternative pathway may be stimulated which overcomes the block in the main pathway and there is an increase in growth to give the peak of partial recovery. This may also account for the higher M. I. C. s of the imidazoles for these two yeasts when compared with C. albicans and C. parapsilosis (Table I). At still higher levels, (the M. I. C. and above), both pathways are blocked and growth ceases.

This explanation may also be applied to the behaviour of C. guilliermondii exposed to low concentrations of imidazoles and then transferred directly to fresh medium containing the drugs. The cumulative effect of double exposure results in an increased sensitivity to low levels of the drug. However, if the fungi are first passaged on drug-free medium, this cumulative effect is lost although the alternative pathway remains stimulated and the capacity of the cells to grow on equivalent low concentrations is enhanced.

The study of the morphological changes in sensitive fungi caused by the antifungal drugs suggests that since "resistant" structures are produced by filamentous fungi in response to concentrations just below the M. I. C. of all the drugs examined, that these are non-specific changes and simply a reaction by the fungi to adverse environmental conditions, and not a manifestation of interference with any particular metabolic activity. However, all four imidazoles cause similar changes in morphology on equivalent concentrations, (stunting on low concentrations; development of "resistant" structures at the M. I. C. ; and shrinkage on higher concentrations), and it might be expected that they have a similar mode of action.

The shrinkage of actively growing hyphae exposed to concentrations of the imidazoles greater than the M. I. C. indicated that killing was probably being achieved and this was confirmed by tests for fungicidal activity. These tests clearly showed that the imidazoles are as effective against filamentous fungi as against the yeasts and that the activity of miconazole and econazole is much greater against actively growing hyphae than against stationary phase spores.

The rapidity with which reduction in viability of the actively growing hyphae took place suggests that the drugs are either being taken up extremely rapidly by these cells or are binding to some component of the hyphal cell wall or membrane which is not present, or possibly only present in very small amounts, in the spores.

Ketoconazole differs from the other imidazoles. It fails to kill actively growing cells of either yeasts or mycelial fungi as rapidly as miconazole and econazole and its fungicidal activity against stationary phase yeasts is also correspondingly lower. However, it is considerably more effective than the

other two drugs against ungerminated spores of T. mentagrophytes, (the time required to achieve killing was approximately half that required for miconazole and econazole).

This ability of ketoconazole to exert a relatively efficient fungicidal effect on ungerminated spores may be related to its water solubility which might be expected to allow easier penetration of the drug through the spore wall. Whatever the reason, this fungicidal activity is a property which may compensate for the lower fungistatic activity of ketoconazole compared to other imidazoles.

Although some variation in sensitivity to the imidazoles exists within a population of fungal cells, repeated sub-culture on drug-containing medium of the least sensitive members of a population of T. mentagrophytes failed to induce further adaptation.

The increased ability to grow on sub-fungistatic concentrations of the imidazoles observed with C. guilliermondii after drug exposure and passage on drug-free medium was not accompanied by a corresponding increase in M. I. C. and does not represent an increase in resistance. However, the production of resistant mutants of A. nidulans by means of a mutagenic agent indicates that modifications to the genetic material of this fungus can confer a degree of resistance to the imidazoles, and the mechanism therefore exists for resistance to arise spontaneously.

Since there was a reduced ability of the resistant mutant to take up phenylalanine the possibility exists that resistance may result from a similar reduction in uptake of the imidazole molecule, but other explanations, such as an enhanced ability to detoxify the drug once it has entered the cell, are also possible.

EXPERIMENTAL SECTION 2

IN VIVO ACTIVITY OF ANTIFUNGALS

INTRODUCTION

It is not possible confidently to extrapolate from in vitro sensitivity measurements to use of these drugs in therapy of fungal infections in man. The only reliable measure of therapeutic efficacy comes from clinical trials.

Fungi are able to alter their morphology according to environmental conditions. They may form structures which are more resistant and have a lower metabolic activity than the normal vegetative form. Within the tissues of a host such structures may be formed and will be more resistant to antifungal agents than the actively growing phase. When therapy is discontinued, these resistant forms will be capable of developing to initiate further infection.

The accessibility of the fungus to the drug is also of great importance. With dermatophytes, the fungal elements are protected by their growth deep in the keratin and for an antifungal agent to be effective, sufficiently high levels must be achieved at the site of fungal activity.

Numerous reports on the efficacy of topical application of the imidazoles are described in the literature and reviewed by Sawyer et al., (1975 a, b) and Raab (1977). Although they indicate a high activity of clotrimazole, miconazole and econazole, treatment failures are not uncommon (Gentles, Jones & Roberts, 1975).

Topical therapy has many limitations and systemic treatment via the oral route is the method of choice. However, this form of therapy is influenced by a number of factors such as toxicity and side-effects of the therapeutic agents, absorption from the gastro-intestinal tract, and induction of host enzymes which may degrade the active molecule.

The value of animal experiments is limited by species - dependent differences between the experimental animal and man. When griseofulvin was first investigated for use in treatment of ringworm, clinical trials showed it to be of little value when applied topically (Williams et al., 1959). Later, when the drug had been shown to act by the systemic route, Frey & Geleick (1960) reported that griseofulvin was effective when applied topically to experimental guinea pig trichophytosis. Clearly, the animal model had severe deficiencies in this particular instance. However, animal experiments can be of value by providing a useful step between in vitro studies and clinical trials in man. Griseofulvin was first shown to be effective by the oral route in treatment of ringworm in guinea pigs (Gentles, 1958) and found equally effective by the same route in man (Williams et al., 1959).

Griseofulvin was the first antifungal to be discovered which fulfilled the criteria laid down by Wilson in 1955: "The ideal antifungal drug even for the superficial mycoses would seem to be one which could be safely administered internally in amounts sufficient to endow the cells eventually destined to produce keratin with power to resist fungi completely, this power persisting as they become keratinized, and the drug thus exerting its effect from within outward".

During recent years several other antifungals, including a number of imidazoles, have been investigated for their activity via the oral route against experimental guinea pig dermatophytosis.

Clotrimazole and Janssen 34,000 were found to be ineffective, (Gentles & Jones, unpublished data). The induction in the host of an enzyme capable of inactivating clotrimazole is now accepted as the reason for the

failure of this drug (Shadomy, 1970).

A new imidazole, tioconazole, supplied by Pfizer Limited was also tested in this laboratory (Gentles & Ball, unpublished data) and found not to be active by the oral route even though it achieved higher serum levels than did miconazole which is poorly absorbed from the gastro-intestinal tract.

One compound, Bayer 4364 was found to modify the course of infection sufficiently to be considered effective in curing established guinea pig ringworm (Gentles & Jones, unpublished data) but was later reported to cause exfoliative dermatitis.

The water soluble imidazole, ketoconazole, produces relatively high drug levels in body fluids after oral administration to man and animals (Janssen Pharmaceutica, 1978). An experiment was designed to test the in vivo activity of ketoconazole when administered orally to ringworm infected guinea pigs. The results reported here have been accepted for publication in a joint paper describing the in vitro and in vivo activity of this new imidazole (Odds, Milne, Gentles & Ball, 1980).

The guinea pig model of dermatophytosis was also used to compare the efficacy of topically applied miconazole with tolnaftate, already established as a useful drug for topical treatment of ringworm.

MATERIALS AND METHODS

MATERIALS

FUNGI

T. mentagrophytes var. mentagrophytes and M. canis originally obtained from clinical material and preserved on silica gel (Gentles & Scott, 1979) were used to prepare inocula from 10 day old cultures on glucose peptone agar.

EXPERIMENTAL INFECTION OF ANIMALS

The hair was clipped and shaved from the shoulder area of guinea pigs weighing between 350 g and 400 g and the erythematous site was inoculated with the test fungi.

For the study of the therapeutic efficacy of ketoconazole 10 guinea pigs were infected with T. mentagrophytes and 10 with M. canis, and for the comparison of topically applied miconazole and tolnaftate 6 guinea pigs were infected with T. mentagrophytes.

DRUGS

Ketoconazole for oral administration was incorporated in a pellet of ground-up diet bound together with flour and water.

Two per cent miconazole was supplied by Janssen Pharmaceutical Ltd., as a cream (Daktarin) for topical application. The placebo preparation (control) was the vehicle for the drug.

Tolnaftate was supplied by Glaxo Laboratories as a 1% cream (Tinaderm).

METHODS

THERAPEUTIC REGIMEN

Orally administered ketoconazole. Five of each batch of animals were treated with a daily dose of 40 - 50 mg/kg ketoconazole administered orally. Control animals were left untreated. With T. mentagrophytes - infected animals, treatment was started 3 days after infection, by which time fungal invasion of keratin is known to be established (Fig. 26). For animals infected with M. canis, treatment was started 8 days after infection, when the lesions were fluorescent under a Wood's light. Treated and control animals were sacrificed at intervals and material from the infected sites was examined histologically. The animals were examined each day and observations were recorded.

Topically applied miconazole and tolnaftate. Treatment was commenced 3 days after infection when invasion of tissue by T. mentagrophytes was established. (Earlier treatment may indicate only the result of in vitro activity against the fungal inoculum.) The preparations were applied twice daily, (a.m. and p.m.). One guinea pig from each drug treatment, (miconazole and tolnaftate) and a control animal (placebo), was sacrificed after 3 days and the remainder after 5 days treatment and sections of skin were prepared for histological examination. The stain used to demonstrate the presence of fungal elements in the tissue was periodic acid-Schiff (P.A.S.).

PERIODIC ACID-SCHIFF STAIN

1. Bring sections to water.
2. Oxidize for 10 min in 1% aqueous periodic acid.
3. Wash in running water for 5 min and rinse in distilled water.
4. Place in Schiff's reagent for 10 - 20 min.
5. Rinse three times in 1% aqueous sodium metabisulphite, freshly prepared,and wash in running water for 10 min.
6. Counterstain in haematoxylin.
7. Wash and blue in Scott's tap water substitute (0.35% sodium bicarbonate, 2% magnesium sulphate).
8. Dehydrate, clear and mount.

RESULTS

THERAPEUTIC EFFICACY OF ORAL KETOCONAZOLE IN EXPERIMENTAL GUINEA PIG DERMATOPHYTOSIS

In guinea pigs infected with T. mentagrophytes, a marked clinical improvement of the lesions was noted by comparison with controls after 3 days' oral administration of ketoconazole. Histological examination showed that there was almost complete eradication of fungi from infected sites after 6 days' treatment (Table V).

The earliest signs of resolution of infection were apparent in the lowest regions of the hair follicles, and eventually, in treated animals, only the distal portion of the hairs remained affected (Fig. 28) while in the equivalent control animals almost all hair follicles were heavily invaded (Fig. 27).

In M. canis- infected animals, treated from the eighth day after infection, similar histological changes occurred (Table V). Clinically there was a reduction in the inflammatory response and in the extent of hair loss from the infected area (Fig. 29 a, left) by comparison with the untreated animals in which inflammation eventually caused complete hair loss at the site of infection (Fig. 29 a, right). In animals infected with T. mentagrophytes, treated with ketoconazole from the third day of infection, the inflammatory response was barely detectable and the major clinical features were scaling and erythema: in untreated animals the lesions became highly inflammatory (Fig. 29 b) and ran a natural course of 22 - 25 days when they resolved with total loss of hair at the site of infection.

Animal infected with	No. of Treatments	Histological Examination [3] Treated	Untreated
T. mentagrophytes [1]	3	+	++++
	4	+	+++
	5	±	++++
	6	-	++++
M. canis [2]	3	++	++++
	6	+	+++
	9	-	++++

1. Treatment commenced 3 days after infection
2. Treatment commenced 8 days after infection
3. Scores ranged from + = a few follicles infected distally to
   ++++ = a majority of hair follicles infected at all levels

TABLE V THERAPEUTIC EFFICACY OF KETOCONAZOLE IN DERMATOPHYTE INFECTIONS IN GUINEA PIGS

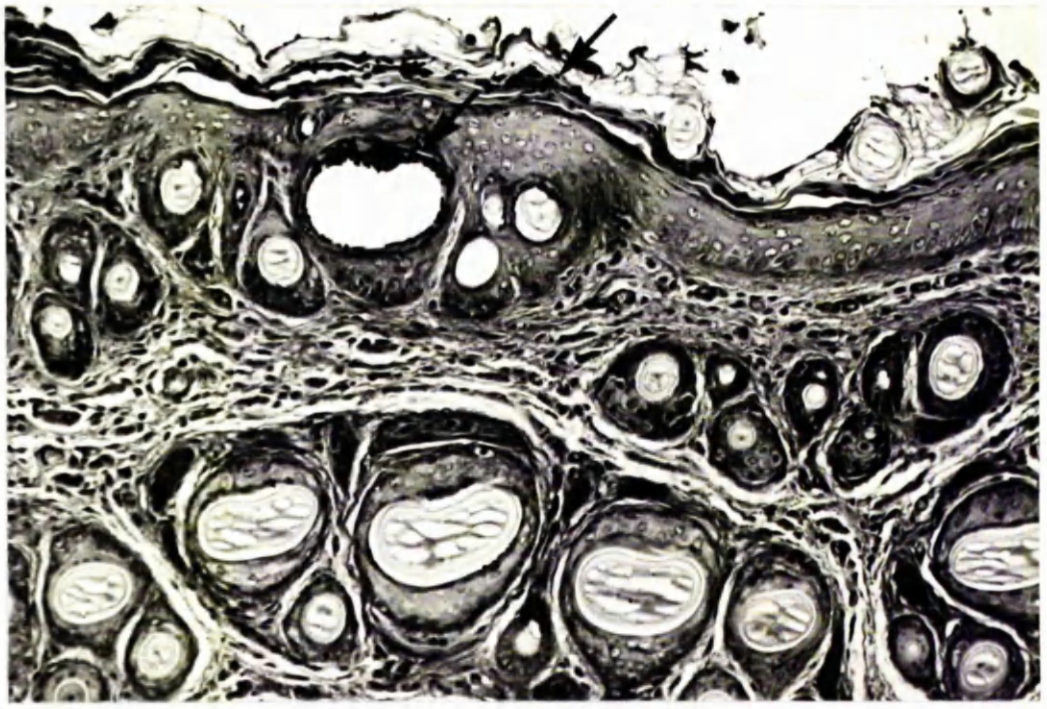


FIGURE 26 Section of guinea pig skin 3 days after infection with T. mentagrophytes. Fungal hyphae (arrows) in base of horny layer and in upper parts of hair follicles. P.A.S. stain x 80

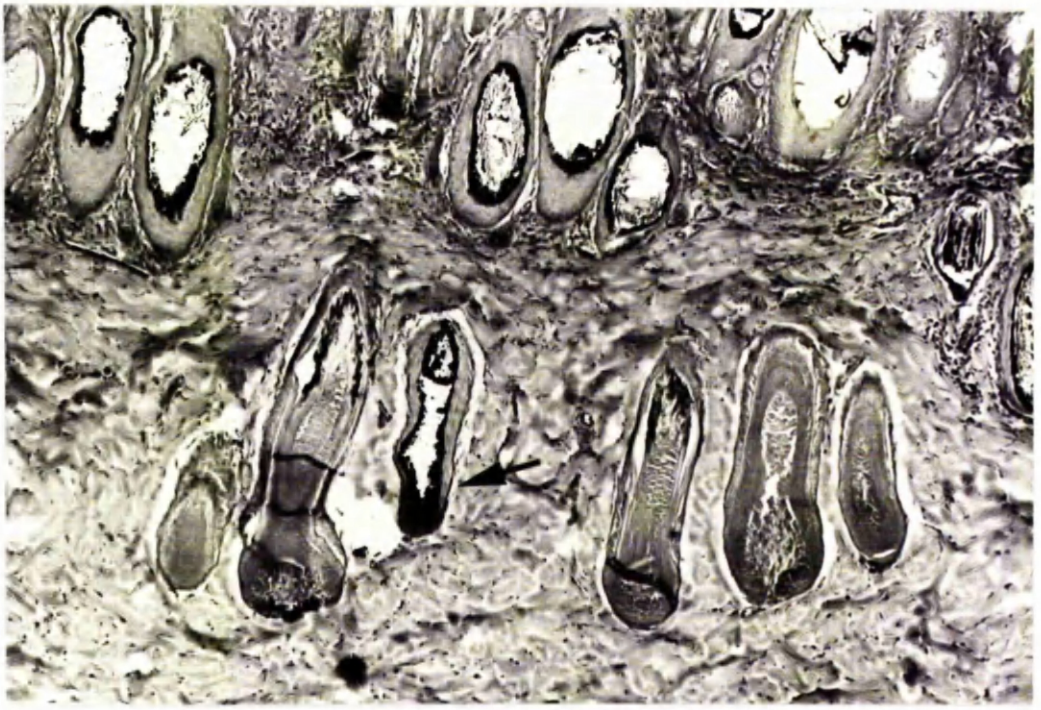
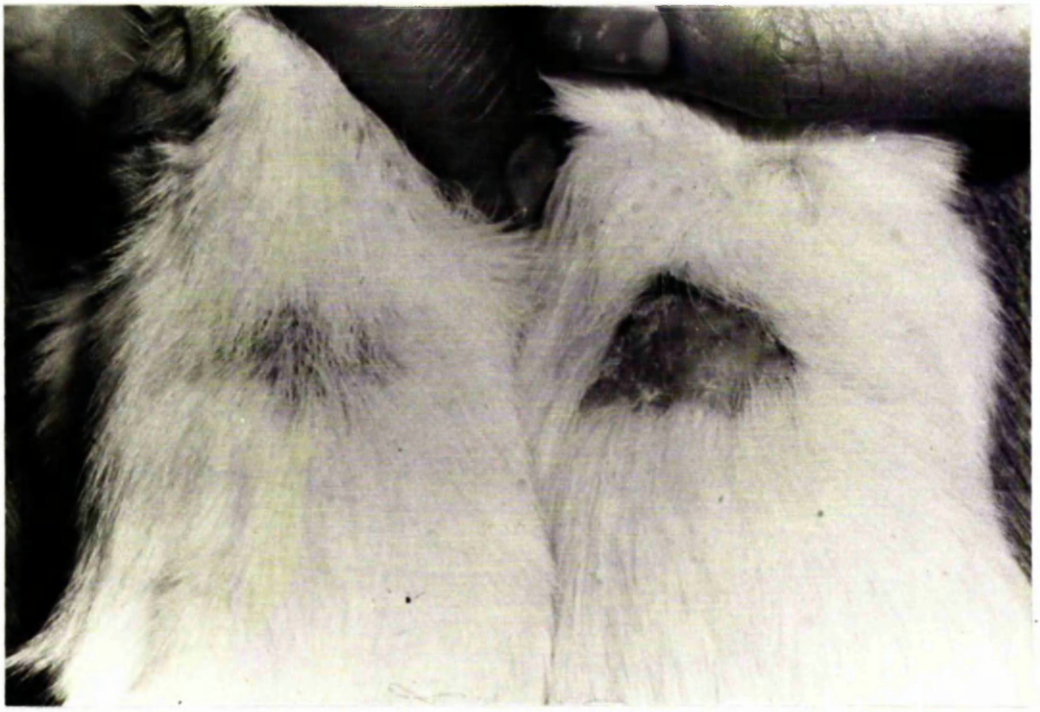


FIGURE 27 Section of guinea pig skin 7 days after infection with T. mentagrophytes. Almost all hair follicles are deeply invaded (arrow). P.A.S. stain x 80

FIGURE 28 Section of guinea pig skin 7 days after infection with T. mentagrophytes and 4 daily treatments with ketoconazole, 40 - 50 mg/kg. A few fungal hyphae are present only at distal end of hair (arrow). P.A.S. stain x 80



a

b

FIGURE 29 Experimental guinea pig dermatophytosis (a) Comparison of treated (left) and control (right) following successful therapy (b) Inflammatory response in untreated animal 14 days after infection

COMPARISON OF THE EFFICACY OF TOPICALLY APPLIED MICONAZOLE AND TOLNAFTATE

Both antifungals showed a beneficial effect by reducing the inflammatory response and minimizing hair loss at the inoculation site. There was no significant difference clinically between the animals treated with miconazole and tolnaftate. Histologically, after 3 days treatment, both antifungals produced a marked reduction in the amount of fungus present compared with controls. After 5 days treatment the amount of fungus was further reduced although neither treatment had succeeded in completely removing all traces of the invading fungus.

DISCUSSION

The results of oral treatment of guinea pig dermatophytosis with ketoconazole clearly demonstrate the potential of this new imidazole in the treatment of dermatophyte infections in man.

The rapidity of the therapeutic effect reflects the results of investigations which have shown rapid absorption and distribution of ketoconazole in experimental animals and humans, (Janssen Pharmaceutica, 1978).

There is every indication that ketoconazole behaves in vivo in a similar manner to griseofulvin. Invasion of new keratin is apparently inhibited and the rate of clearance of infection is dependant on the rate of keratin production.

The broad spectrum of in vitro antifungal activity and the demonstration of the efficacy of this drug in the treatment of rabbits infected experimentally with C. albicans (Odds et al., 1980) adds further support to the view that it may prove to be a promising new drug for the treatment of both superficial and systemic mycoses.

In the comparison of topically applied miconazole and tolnaftate no significant differences were detected in the efficacy of the two drugs under the conditions of this experiment. There was no doubt of the beneficial effect of treatment but, the deficiencies (see p 50) of the guinea pig as an animal model for estimation of topically applied drugs must be taken into account.

GENERAL DISCUSSION

Imidazole drugs have made considerable impact upon the therapy of fungal infections as is evident from the number introduced for clinical use within recent years. They all have a broad spectrum of activity, but each has its own individual characteristics. Some of these are beneficial, others detrimental, e.g. clotrimazole is absorbed from the gastro-intestinal tract, but is subsequently degraded by liver enzymes; miconazole and econazole are not absorbed in sufficient quantity to give active blood levels and for systemic use must therefore be administered intravenously. Ketoconazole is readily absorbed when given orally, and serum levels remain relatively constant throughout the treatment period. Although side effects are known to occur with some of the imidazoles, they are not as serious as with certain other systemically administered antifungals such as the polyene antibiotics.

The antifungal potency of imidazoles as determined by M. I. C. measurements also differs, although many of the variations reported can be accepted only when comparable tests have been made under standard conditions. This is exemplified by the considerable variations in M. I. C. values which are to be found in the literature and can only be attributed to differences in procedures used by the various investigators. In this study the use of a standard procedure allowed direct comparisons to be made.

If measurements of fungistatic activity require to be carefully evaluated before acceptance, the reports of fungicidal activity require even more critical scrutiny. The reasons for this are clear from some of the problems encountered during this study and apply to other drugs as well as imidazole derivatives. Surprisingly, some investigators have omitted to take into account the effect of "carry - over" of drug from the exposure medium to

the viability test medium. This is a basic problem with all water insoluble drugs and not only, as shown here, with imidazoles. It is this factor that probably accounts for the report by Weinstein et al., (1964) that tolnaftate is fungicidal for T. mentagrophytes. The procedure described by these workers is likely to have allowed carry - over of fungistatic drug levels and in addition it is possible that the drug solvent levels used may have affected their results. In 1978, Kessler et al., using a technique similar to the procedure adopted in this study, could find no fungicidal effect for tolnaftate against T. mentagrophytes. Kessler's results may be considered more reliable than those of Weinstein. However, the duration of the experiment may have been too short to measure fungicidal activity and sampling over a longer period may have revealed that tolnaftate is also fungicidal. There is clearly a need for establishment of criteria, e.g. drug concentration and exposure time, for defining fungicidal activity.

Yet another factor of importance is the physiological state of the fungus which may be related to age and morphological form of the culture. The concentration of the population exposed to the drug must also be considered. Yamaguchi & Iwata (1979) emphasised the importance of making measurements of fungicidal activity on similar cell populations at the same cellular concentrations. They report that with increased drug concentrations miconazole exhibits a distinct fungicidal action but that the effects of clotrimazole remain fungistatic. However, the results of this study, while clearly demonstrating the fungicidal activity of miconazole, also indicate by the morphological changes induced by clotrimazole that this imidazole is fungicidal.

The demonstration of differences in activity of the imidazoles for active and stationary growth phases is not surprising, and has previously been recorded by other workers. The differences are not, however, as extreme as has been suggested by Plempel (1976) for clotrimazole who stated that no sporicidal effect could be expected because its mode of action required active metabolism of the cells. Van den Bossche, (1974) and Van den Bossche et al., (1975) describe a marked fungicidal activity of miconazole for actively metabolising cells of C. albicans and not for resting cells. From the results of this study it can be concluded that the imidazoles investigated, (miconazole, econazole and ketoconazole), are fungicidal for both actively metabolising and stationary phase cells, although the times required to achieve killing may vary considerably.

It should be noted that ketoconazole, which has a lower fungicidal activity than miconazole and econazole for actively growing cells of both T. mentagrophytes and C. albicans, was much more effective at killing stationary phase cells of T. mentagrophytes. This property gives further support to the accumulating evidence that ketoconazole may prove to be a significant advance on other imidazoles.

As far as the mode of action of the imidazoles is concerned, firm conclusions are not yet possible and the results of in vitro studies suggest a multiplicity of effects.

The findings of Yamaguchi, (1977, 1978) and Kuroda et al., (1978), that unsaturated fatty acids and phospholipids antagonize the activity of the imidazoles, and the observation by Van den Bossche, (1974) that labelled miconazole becomes localized in the cell walls and plasmalemma of

actively growing cells suggests that the imidazoles are binding to some lipid component of the cell wall or membrane. Binding to lipids probably also explains the antagonistic effect of cremophor observed in this study. Such an hypothesis is further supported by the very rapid effect of miconazole and econazole on actively growing hyphae of T. mentagrophytes.

The consequences of this binding may be either to directly interfere with the integrity of the cell membrane to allow leakage of cell components and therefore indirectly disrupt the metabolic processes within the cell, or to block uptake sites for essential metabolites required for biosynthetic processes.

The marked differences in behaviour of active and stationary phase cells to the fungicidal activity of the imidazoles may reflect differences in the rate of uptake of the drug. Either the lower rate of metabolic activity or the decreased permeability of the cell wall of stationary phase cells may be responsible for reducing drug activity. Alternatively stationary phase cells may possess a store of enzymes which can detoxify the drug, or cell metabolites produced in response to the drug such as the raised hydrogen peroxide levels demonstrated by De Nollin et al., (1977).

Van den Bossche (1974) reported that miconazole did not affect amino acid uptake by C. albicans. However, Yamaguchi & Iwata (1979), working with the same fungus, found that both miconazole and clotrimazole at minimum inhibitory concentrations inhibited uptake of leucine. The findings, during these investigations, of competition for uptake sites in C. guilliermondii between thiamin and econazole, and in the resistant mutant of A. nidulans for phenylalanine are in agreement with those of

Yamaguchi & Iwata, i.e. that inhibition of amino acid uptake is one effect of imidazoles on sensitive fungi.

Competition for uptake sites may also explain the remarkable phenomenon of partial recovery shown by certain yeasts in the presence of the imidazoles. Experiments showed that thiamin provided relief of imidazole toxicity for these yeasts. However, the requirement for thiamin by many fungi is well known, (Hawker, 1950), and a more complex mechanism of drug action may eventually be discovered. Whatever the explanation, a practical problem of the phenomenon would arise in the use of these yeasts for investigations such as serum level estimations.

The behaviour pattern of fungi on repeated exposure to imidazoles must also be considered when attempting to explain their mode of action. Increased sensitivity results when fungi are sub-cultured directly from drug-containing to drug-containing medium indicating a cumulative effect of the drugs possibly as a result of binding to the lipids in the cell membrane. This cumulative effect can be overcome by allowing the fungi to develop on drug-free medium for a short period of time and the M.I.C. returns to normal. However, the previous exposure to sub-fungistatic concentrations of the drugs allows the fungi to become better adapted to growth on these same concentrations. One explanation of this behaviour is that low levels of the imidazoles stimulate an alternative metabolic pathway to overcome blocking of the main pathway. This alternative pathway remains active at sub-fungistatic concentrations but minimum inhibitory concentrations are capable of blocking both pathways. Exactly which pathways are involved is unknown, although ergosterol biosynthesis, catalase and peroxidase

activity and oxidative phosphorylation have all been suggested as possible target sites for the imidazoles, (Van den Bossche et al., 1978, De Nollin et al., 1975, 1977 and Dickinson, 1977).

The changes in fungal morphology brought about by the imidazoles are not unique. Tolnaftate and griseofulvin also cause swollen and refractile areas in hyphae of susceptible mycelial fungi. This common reaction of fungi to adverse environmental conditions created by drugs, highlights the adaptability of fungal cells to overcome changes in their environment and emphasises the problems that may be encountered in the treatment of infections. In this respect the ability of stationary phase cells of C. albicans to produce "chlamydospore-like" structures in response to fungistatic concentrations of the imidazoles must be taken into account in any attempt to extrapolate the results obtained using this species to other sensitive fungi.

The production of chlamydospore-like cells by C. albicans in the presence of drugs has previously been described in the literature. Lee (1973) reports "chlamydospore-like" cells induced by 5-fluorocytosine and the formation of "enlarged bubble-shaped cells" produced by C. albicans in the presence of 10μg/ml econazole has been described by Preusser (1976). Photomicrographic studies by Preusser revealed that these cells contained great quantities of lipid bearing substances.

Despite a knowledge of the ability of C. albicans to form "resistant" chlamydospores much of the published work on imidazole activity, (Iwata et al., 1973 a, b; De Nollin & Borgers, 1974; Van den Bossche, 1974 and Swamy et al., 1974), has been done solely with this species.

The comparative study of fungicidal activity of econazole against C. albicans and non-chlamydospore-forming yeasts indicates that chlamydospore production may be one factor accounting for the ability of stationary phase C. albicans cells to resist fungicidal action for a time. However, the considerable variation in the time required to kill stationary phase cells of different non-chlamydospore-forming yeasts points to the involvement of other factors. For example, the extreme sensitivity of C. guilliermondii and C. tropicalis may be connected with the "unusual" behaviour of these yeasts on sub-fungistatic concentrations of the imidazoles.

Development of drug resistance has presented considerable problems in the treatment of bacterial infections but so far has not proved to be particularly troublesome in the treatment of the mycoses. Nevertheless, different species of fungi vary in their sensitivity to antifungals, and in addition certain isolates of susceptible species which are naturally more resistant are occasionally encountered. Such resistance is, however, generally of a low order such as 2 - 5 times greater than in normal strains and hardly comparable with the increased drug resistance developed by some bacteria. A notable exception is 5-fluorocytosine, and problems of resistance arising durring the course of treatment with this drug are well documented, (Shadomy, 1969). There is a single report by Holt & Azmi (1978) of the emergence of resistance to miconazole of C. albicans during prolonged therapy, but this has not so far presented a problem in practice.

Variation in sensitivity to the imidazoles within a population of fungal cells as observed in this study has recently been discussed by Plempel (1979). He reports the "slow regrowth of initially inhibited colonies" in agar

diffusion and agar dilution tests with imidazole antimycotics when the incubation time is prolonged. This effect is usually explained as being the result of selection of variants resistant to the drugs. However, as far as the imidazoles are concerned this is contradicted by the fact that the fungi do not have increased M. I. C. values when the test is repeated.

Selection of the least sensitive members of the population of C. albicans during therapy would have been an obvious explanation for the rise in M. I. C. levels of the isolate described by Holt & Azmi (1978) but there is no support for this from in vitro investigations.

However, the demonstration of 2 - to 7 - fold resistance of an artificially induced mutant of A. nidulans indicates that a mechanism exists for resistance to develop naturally. Whether this will prove to be the case in treatment of fungal infections, can only be assessed after long-term usage of the drugs.

The in vitro inhibitory effect on dermatophytes of the antifungals tolnaftate, miconazole and econazole are similar, and so any differences in efficacy of these agents in topical treatment of fungal infections will depend on their ability to penetrate so that an adequate concentration of the drug reaches the infecting fungus.

In vivo studies using guinea pigs experimentally infected with ringworm provide valuable information concerning the clinical potential of these drugs. However, experimental infection with T. mentagrophytes in guinea pigs runs a much more rapid course than dermatophyte infection in man. Inoculation of guinea pigs involves scarification of the skin which renders the stratum corneum more permeable to topically applied agents. The

subsequent development of a heavy fungal infection in the host also contributes to the breakdown of the keratin barrier and aids penetration by the antifungal agent. Treatment trials must be made within the short period between establishment of infection and onset of an inflammatory response i. e. between approximately 3 days and 8 days, during which time the horny layer is disrupted and permeable.

In man, treatment of fungal infection does not generally take place until some time after infection, and although the horny layer in the central area of the lesion may be disrupted and allow penetration of topically applied agents, the surrounding area will still be intact although hyphal development may have extended into the lower layers of the epidermis. It has been shown that fungus is present up to 6 cms away from the marginal zone of lesions, (Knudsen, 1975).

The results of the comparison of tolnaftate and miconazole indicate that both are equally effective in curing experimental guinea pig dermatophytosis, and despite the deficiencies of the guinea pig model these results are consistent with the findings of various clinical trials, reviewed by Clayton (1979), that no significant differences have been found between these agents in the treatment of dermatophyte infections in man. However, a mycological cure rate of only 60% was achieved with miconazole in topical treatment of tinea pedis in sportsmen (Gentles et al., 1975), as compared with the 100% success obtained in this study using guinea pigs.

The guinea pig is a more reliable model for testing orally administered drugs since it is the route of transport and the effect of the antifungal at the site of fungal infection which is being examined. The physical state of the stratum corneum and the extent of the lesion is of no consequence. This has

proved to be the case for griseofulvin and there is no reason to believe that it will not be also for ketoconazole.

The findings that orally administered ketoconazole is effective against ringworm infections in guinea pigs and Candida infections in rabbits (Odds et al., 1980) therefore represents a considerable step forward in antifungal therapy by combining broad spectrum antifungal activity with acceptable pharmaco-kinetic properties.

There can be little doubt that the imidazole group of antifungals, while not without certain disadvantages, appear to possess many of the properties desirable for use in the treatment of mycotic infections in man and the synthesis of new imidazole derivatives is continuing at various centres. It is hoped that these investigations may be successful in finding other compounds with even greater activity and lower toxicity which will further advance the therapy of the mycoses.

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