P A T H O G E N I C I T Y A N D D I M O R P H I S M

i n C A N D I D A A L B I C A N S

by

Alan Cockayne B.Sc

Submitted for the degree of Doctor of Philosophy

University of Leicester, October 1982

Contents

Page No

Statement

This thesis, submitted for the degree of Doctor of Philosophy, is based on original work carried out by the author in Department of Microbiology at the University of Leicester in the period October, 1979 and September 1982. None of the work has been submitted for another degree in this or any other university.

Signed Alan Cochayne

Date 29/10/82.

List of Tables

List of Figures

Acknowledgements

I would like to thank several people without whom this work could not have been completed.

My supervisor, Dr Frank Odds for all his patience, help and encouragement during the last three years.

Dr Fiona Macdonald for her advice and encouragement.

Mrs Anne Abbott and other members of the department for technical assistance.

Mrs Rachel Johnson for typing this manuscript.

Finally I would like to express my gratitude to the Medical Research Council from whom I received a grant.

SUMMARY.

The aims of this study were to extend existing knowledge of the interactions of the growth environment and morphological development in C.albicans, to investigate polysaccharide composition in relation to cell shape and age and to consider the significance of dimorphism in relation to the pathogenesis of candidosis.

It was impossible to identify any single environmental factor responsible for the control of the complex morphological development of C.albicans. Many factors appeared to initiate germ tube formation but subsequent growth of the fungus into branched hyphae or unbranched hyphae with secondary blastospores was dependent and influenced by the atmosphere of incubation.

The imidazole antifungal, ketoconazole, at low concentrations, was found to inhibit hyphal branching in C.albicans completely. The antifungal had no effect on germ tube initiation or blastospore budding. Some inhibition of cell growth was, however, observed under conditions that favoured mycelium production or blastospore budding.

The polysaccharide composition of C.albicans was found to be morphology- and age related and was shown to be affected by the composition of the growth medium. Chitin content of C.albicans increased during germ tube initiation and hypha formation. Blastospore budding and secondary blastospore production were associated with a fall in chitin content. Although the activity of the enzyme chitin synthase correlated well with chitin content in germ tubes and blastospores, no correlation was found between enzyme activity and hyphal branching.

Phagocytosis and intracellular killing of standardized biomasses of blastospores, germ tubes and hyphae by human polymorphs and mouse peritoneal macrophages were investigated in an objective radiolabel assay. Blastospores, germ tubes and hyphae were phagocytosed to a similar extent by human polymorphs. Blastospores were, however, less efficiently killed than the filamentous forms and initial resistance to killing did not appear to be related to intracellular germ tube production. C.albicans antiserum did not significantly affect the efficiency of phagocytosis and killing by human polymorphs. Serum antibody and complement, however, appeared to be involved in these processes. Mouse peritoneal macrophages were less efficient than human polymorphs at phagocytosing and killing C.albicans.

CHAPTER 1

Introduction

Throughout the past few decades recognition of the importance of fungal infections has increased substantially. Many such infections occur in compromised hosts whose ability to resist infection is impaired (Gentles & La Touche, 1969). Among the opportunistic fungal pathogens are members of the genus Candida, causing diseases collectively referred to as candidosis. Seven species of Candida, C. albicans, C. tropicalis, C. parapsilosis, C. pseudotropicalis, C. krusei and C. guilliermondii are recognised as human pathogens, and some Torulopsis and Rhodotorula species occasionally cause similar infections (Odds, 1979).

Candida albicans is unique among these species in being dimorphic; it is able to change its morphology in response to environmental stimuli. This ability may be correlated with the isolation of C. albicans as the most common pathogen among the species listed above. (Odds, 1979). The relationship between dimorphism and pathogenicity has sometimes been extended to assume differential virulence of one morphological form over another (Whittle & Gresham, 1960). In most other dimorphic pathogenic fungi, notably Coccidioides immitis, Histoplasma capsulatum, Blastomyces dermatitidis and Paracoccidioides brasiliensis, a clear cut correlation between the morphology of the fungus in tissues and its morphology outside the body or in vitro has been established (Rippon, 1974), but as yet the available data relating to Candida albicans dimorphism and pathogenicity do not allow such conclusions to be drawn.

Study of the factors controlling dimorphism in C. albicans and the significance of dimorphism as a virulence factor may further clarify the mechanisms of pathogenesis in candidosis.

1.1 Infections caused by Candida species

Historical

Descriptions of some of the more common forms of candidosis were recorded as early as the third century B.C. Hippocrates in his 'Epidemics' describes two cases of thrush in patients suffering from underlying disease (Hippocrates, 377 B.C.). Thrush was mentioned in the diaries of Samuel Pepys (1665). The causative organism in a case of oral thrush was first described by Langenbeck (1839) who held the isolate to be responsible for the patient's enteric fever. Bennett (1844) isolated a "cryptogamic plant", later thought to be the causative organism of thrush, from the sputum and lungs of a man suffering from pneumothorax. Bennett described the morphology of the organism in necrotic tissues and showed it to consist of "long branched tubes and round or oval globules", a description which clearly fits that of a dimorphic organism. Berg (1846) in his monograph "Thrush in Children", showed conclusively that thrush was caused by a fungus and again noted the importance of predisposing factors in the incidence of the disease. Since those early reports Candida species have regularly been isolated from infected tissue.

Factors predisposing to candidosis

C. albicans and some other Candida species are members of the normal microbial flora of the human body and may be found in the gut and on the mucous membranes. Average isolation rates of C. albicans from normal healthy individuals have been calculated as 6% from the vagina, 10% from the mouth and 18% from faeces (Odds, 1979). The majority of infections caused by C. albicans appear to be of endogenous origin, a reduction in the resistance of the host to infection being followed by an overgrowth of the host's own fungal flora.

The earliest descriptions of thrush indicated that the disease was commonly associated with some underlying deficiency in the host. Bennett (1844), writing of the presence of fungi in pulmonary disease commented that "their presence is indicative of great depression of the vital powers and important nutritive functions of the economy". Today a large number of predisposing factors which in some way impair the body's resistance to fungal infection are recognised and have been comprehensively reviewed (Gentles and La Touche, 1969). The main categories of predisposing factors relating to candidosis are shown in Table 1:1taken from Odds, 1979. The dividing line between commensal and pathogenic status of the fungus may be fine, and relatively minor disturbances of the normal physiological state of a host may be sufficient to initiate an infection. Alternatively candidosis may occur in hosts already severely debilitated by disease or procedures associated with medication. In general terms the severity of the candidosis relates directly to the severity of the underlying disorder (Winner & Hurley, 1964).

Classes of candidosis

Candida infections of almost every organ of the body have been recorded, varying from very common minor superficial lesions to systemic infection which, although rare, is frequently fatal.

Superficial infections may be divided into cutaneous types, affecting the skin and nails, and mucocutaneous types, primarily affecting the mucous membranes of the mouth, gut and genital tract. These infections tend to be easily treated since the infected site is readily accessible to topical therapy. One exception to this rule is chronic mucocutaneous candidosis (CMC) which is usually associated with one or more underlying deficiencies in lymphocyte function and sometimes associated with susceptibility to a wide range of infectious diseases (Odds, 1979). The first manifestation of CMC is usually oral thrush which may spread to the larynx, oesophagus and face: the face may become totally covered by hyperkeratotic granulomatous

Table 1:1

Factors that predispose the host to candidosis

General classification of localized and systemic factors that predispose humans to candidosis

Classification of predisposing factors	Explanation	Examples
natural factors	1. infectious, idiopathic, congenital or other debilitating diseases and disorders	microbial infections, endocrine dysfunctions, defects in cell-mediated immunity
	2. digressions from normal physiological status	pregnancy, infancy
dietary factors	excess or deficiency of foodstuffs that may alter the composition of the endogenous microbial flora	carbohydrate-rich diets, vitamin deficiencies
mechanical factors	1. trauma	burns
	2. local occlusion or maceration of tissues	wearing dentures, thumb-sucking
iatrogenic medical factors	1. treatment with drugs that alter the composition of the endogenous microbial flora or suppress host defences against infection	antibiotics, corticosteroids
	2. surgical procedures, or introduction of mechanical devices and prostheses into vessels or tissues	bowel resections, heart valve replacements, indwelling catheters

From Odds, 1979.

tissue (Holt et al, 1972). Topical treatment of CMC has been shown to be ineffective and the use of systemic antifungals or reconstitution of the host's immune system by transfer of lymphocytes and other extracts from compatable normal donors are the methods of choice in treatment (Odds, 1979).

Systemic Candida infections are relatively rare and do not present with any characteristic symptoms, so that diagnosis is usually difficult. Infection may occur in any of the deep organs and may be localized or disseminated being spread via the blood stream. Treatment of systemic candidosis is by systemic antifungals such as amphotericin B, 5-fluorocytosine, ketoconazole and miconazole but the severity of the underlying disease in cases of systemic candidosis indicates a poor prognosis and because it is difficult to diagnose, systemic candidosis is most commonly diagnosed post mortem (Taschdjian, Seelig & Kozinn, 1973).

1.2 Pathogenesis of Candidosis

C. albicans is a commensal of the human body and is normally held in check by the host's innate and aquired immune defence mechanisms. In common with other pathogenic microorganisms, C. albicans possesses a number of potential virulence factors which may be important in the pathogenesis of candidosis. Some of these factors are common to all the pathogenic Candida species (Odds, 1981) and as yet no single factor has been shown to be solely responsible for the greater virulence of C. albicans, evidenced by its higher isolation rate from infections,over the other Candida species. It is, however, conceivable that a combination of virulence factors, some unique to this species and others common to all Candida species, may enable C. albicans to initiate infection and persist in the host more readily than the other species.

Toxins

Microbial toxins may be divided into two broad categories; endotoxins which are associated with the microbial cell wall and exotoxins which are

liberated into the microbes' growth environment (Mims, 1982). The role of toxins in the pathogenesis of candidosis is unclear.

Henrici, during studies of experimental candidosis in animals, concluded that some of the pathological changes noted could be due to endotoxin-like substances in the cell wall of C. albicans (Henrici, 1940). This view was supported by Salvin (1952) who showed that formalin killed blastospores of several Candida species and soluble supernatants of mechanically disintegrated C. albicans blastospores were lethal to mice on intraperitoneal injection. Isenberg, Allerhand, Berkman & Goldberg (1963) found that phenol and ethanol-extracts of virulent, but not avirulent C. albicans strains, were lethal for mice and produced skin necrosis in rabbits. Other investigations have, however, been unable to demonstrate such effects using similar cell fractions (Chattaway, Odds & Barlow, 1971; Holder & Nathan, 1973).

Cutler et al.(1972) clarified the evidence relating to endotoxin production. They showed that it was the cell surface glycoproteins of C. albicans which were responsible for the pathological changes seen in animal models. The glycoproteins were pyrogenic in rabbits and lethal in mice pre-treated with actinomycin D.

Glycoproteins of C. albicans are recognised as major antigenic determinants of the cell surface and possesses a range of attributes which may be important in the pathogenesis of candidosis. Davies & Denning (1972) found that cell wall extracts of C. albicans inhibited phagocytosis of the fungus by human neutrophils. Reiss, Stone & Hasenclever (1974) isolated a mannan, a phosphoglucomannan and a glucomannan from the cell walls of C. albicans blastospores and showed that these fractions were capable of eliciting delayed type hypersensitivity reactions in an animal model. Trnovec et al.(1977) studied the effect of various cell wall fractions of C. albicans on the phagocytic activity of the reticuloendothelial system in mice and noted a decrease followed by an increase in phagocytosis when mice

were inoculated with various cell wall fractions including a surface glycoprotein.

C. albicans (Sohnle, Frank & Kirkpatrick, 1976) and C. albicans and other related species (Ray & Wuepper, 1976) have been shown to activate the alternative complement pathway in animal models. Ray et al. (1979) isolated a mannan-rich cell wall glycoprotein from C. albicans and found that this glycoprotein was capable of activating the alternative complement pathway but was non-pyrogenic in rabbits. This component of the C. albicans cell wall was shown to be chemotactic for human neutrophils as demonstrated earlier by Denning & Davies (1973). Recently a role for the sugar moieties of cell wall glycoproteins of C. albicans in the attachment of the fungus to host surfaces has been proposed. Maisch & Calderone (1981) reported that surface mannan was important in the attachment of C. albicans to fibrin clots in vitro. A similar role for surface mannan in attachment of C. albicans to human buccal epithelial cells has also been shown (Sandin et al. 1982). Other Candida species are able to attach to mucosal epithelial cells in vitro and it is possible that glycoproteins are also important in the attachment of these species (King, Lee & Morris, 1980).

A different type of toxin, isolated from the cytoplasm of a single C. albicans strain has been reported by Iwata & Uchida (1969). This protein was reported to be fatal for mice after intravenous inoculation but subsequent studies by other authors (e.g. Chattaway et al. 1971) have failed to confirm the initial reports. The importance of such a strain-specific toxin in the pathogenesis of candidosis is difficult to evaluate.

Enzymes

Many pathogenic bacteria are known to produce a wide range of extracellular enzymes which have been postulated to be important in tissue invasion. Their role in the pathogenesis of infection is however equivocal (Mims, 1982). C. albicans has been shown to produce a number of enzymes, some of which have been claimed to be important potential virulence factors.

An inducible proteinase, secreted into the culture medium when C. albicans is grown on media containing protein as sole nitrogen source was demonstrated by Staib (1965) purified by Remold et al. (1968) and characterised extensively as a carboxyl proteinase by Macdonald and Odds (1980) and Rüchel (1981). Staib (1969) showed that the ability of C. albicans to secrete proteinase was strain dependent and that only proteinase-producing strains caused extensive peritonitis and infection of all viscera when injected into mice. Budtz-Jorgensen (1971) found that the ability to secrete proteinase was more often a property of C. albicans strains than strains of other species although he was unable to find a correlation between proteinase production in vitro and the severity of denture stomatitis lesions from which the fungi were isolated. Macdonald and Odds (1980) demonstrated C. albicans proteinase in infected tissues by an indirect immunofluorescent antibody assay. Recent studies with this proteinase agree with the findings of Staib (1969). Results suggest that the ability to produce the enzyme correlates with virulence since a proteinase-deficient mutant of C. albicans was less pathogenic in a mouse model (Macdonald & Odds, 1982). Other Candida species produce some proteinase but in smaller amounts than C. albicans (F. Macdonald. PhD Thesis, University of Leicester, 1979).

Alkaline and acid phosphatases have been shown to be present in whole cells or extracts of C. albicans (Kurup, 1963; Montes & Wilborn, 1970; Chattaway et al. 1971). Similar enzymes have been shown to be distributed throughout the pathogenic Candida species (Odds & Trujillo-Gonzales, 1974). The ubiquitous presence of these enzymes in species other than C. albicans suggests that although they may be contributory to the pathogenesis of candidosis their role as major virulence factors has yet to be proved.

A third group of enzymes, the phospholipases, have been considered in relation to the pathogenesis of candidosis. Phospholipases A and C were reported in C. albicans, grown on media containing suitable substrates, by Costa et al. (1968). Pugh & Cawson (1975) detected phospholipase A and

lysophospholipase in C. albicans but could not detect phospholipase C as reported by Costa et al. (1968). Pugh and Cawson (1975) showed that the distribution of the enzyme activity was related to the age of the Candida cells. In younger blastospores activity was confined to buds; in older cells activity was localized at the periphery of the cell. To date there is no unequivocal evidence that the phospholipases are significant virulence factors.

Dimorphism

Many early descriptions of the thrush fungus in clinical material drew attention to the dimorphic nature of the organism. Bennett (1844) described two morphological forms of the fungus. The blastospore, mycelial and possibly the chlamydospore form were described by Grawitz (1877) and Audrey (1877) showed conclusively that the different morphological forms belonged to the same organism.

Although C. albicans is usually described as dimorphic, which implies the existence of two different forms within the same species, at least four distinct morphological forms of C. albicans are recognised in vitro. It is possible that intermediate morphological forms may also exist. These morphological forms may be described as follows: (based on Odds, 1979) and are illustrated in Fig 1:1

1. Blastospore: A unicellular form of the fungus characterised by a specific mode of mitotic division referred to as budding. Budding involves the growth of new cellular material from a selected site on the blastospore surface. After bud enlargement has occurred nuclear division takes place and a septum is laid down between mother and daughter cells, which may then separate.

2. Hypha: A long microscopic tube comprising multiple fungal cell units divided by septa. Hyphae may arise as branches from existing hyphae or by outgrowth from blastospores. This latter type of growth is known as germ tube production. Blastospores may lay down new cell wall material in the form of a cylinder or 'germ tube' which grows continuously by extension. Mitotic cell division occurs within the hypha and septa are laid down behind a continuously extending tip.

3. Pseudohypha: Pseudohyphae arise by a modified budding process from a parent blastospore. Unlike conventional budding each generation of buds remains attached to the parent and the buds tend to be narrower and more elongated than the parent blastospore. Pseudohyphae may be distinguished from true hyphae by the presence of constrictions at septal junctions.

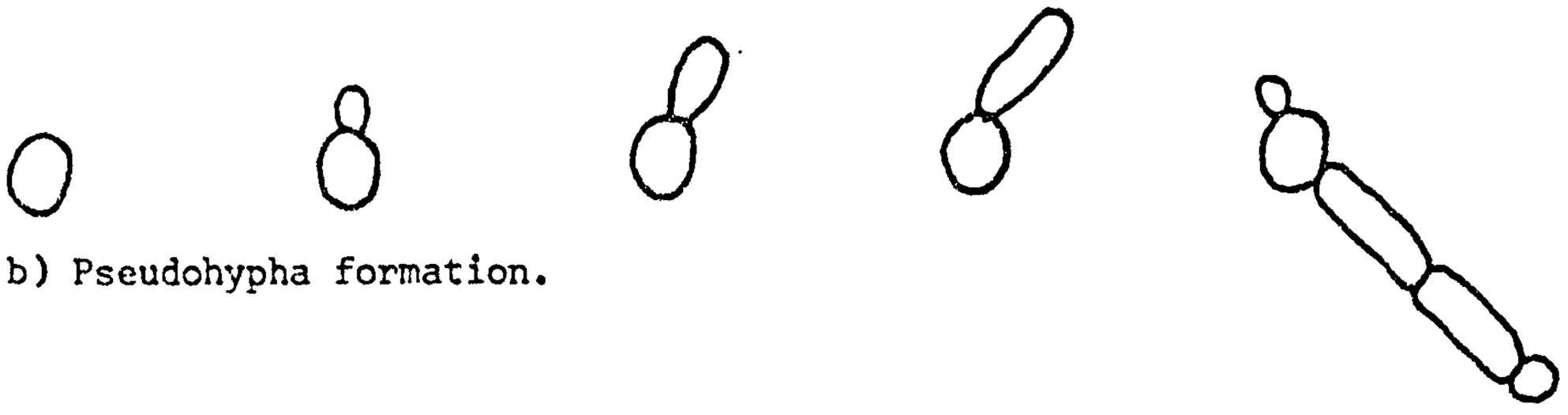
4. Chlamydospores: These are thick walled cells which may represent a dormant phase of the fungus. They have rarely been reported in infected tissue and are of interest only as a form of the pathogen in vitro and in its identification.

Postulated significance of dimorphism of C. albicans in the pathogenesis of candidosis.

Among the pathogenic Candida species, C. albicans is unique in its ability to produce true mycelium reproducibly and it has been suggested that this ability may contribute to the pathogenicity of this species. Stanley and Hurley (1967) reported limited germ tube production by strains of C. tropicalis and Martin (1979) also reported germ tube production by this

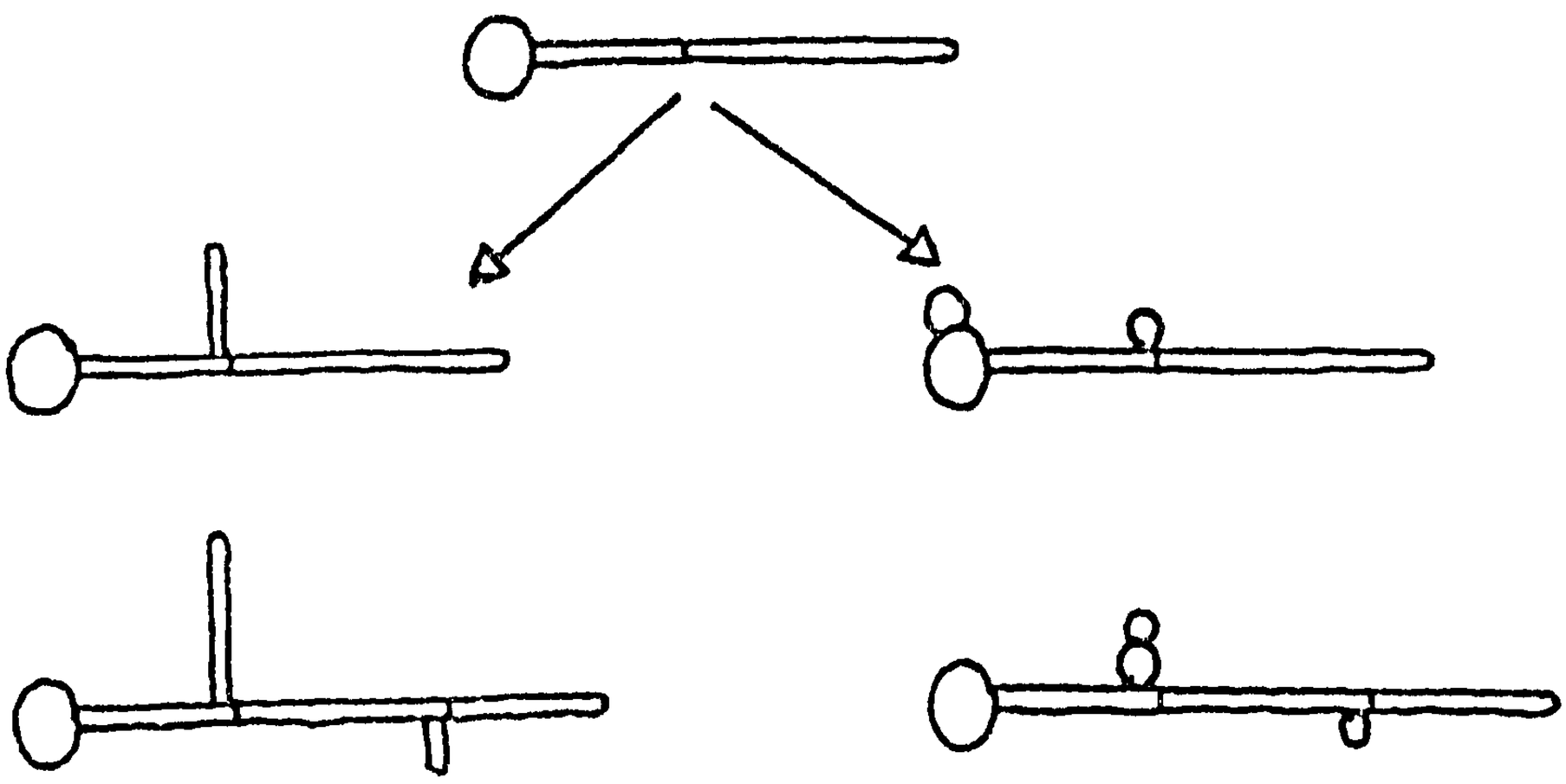
Fig.1.1 Morphological development of C.albicans.

a) Blastospore budding.

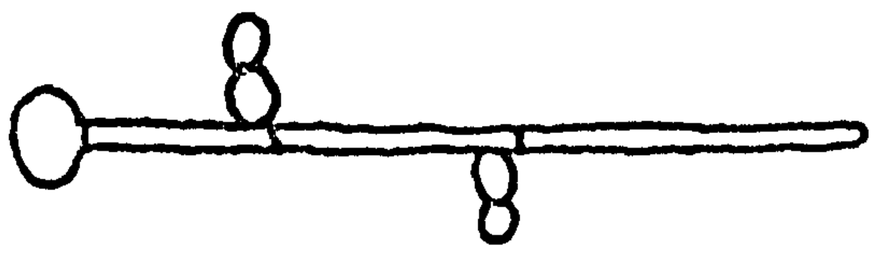


b) Pseudohypha formation.

c) Germ tube initiation.



e) Mycelial branching.



f) Secondary blastospore formation.

species although the property was lost on subsequent subculture. Warwood and Blazenc (1977) reported that 85% of 33 strains of C. tropicalis tested were unable to produce germ tubes in serum at 37 °C. Possibly because of the variation in the ability of C. tropicalis strains to produce true mycelium, this property has only been extensively studied in C. albicans.

The ability of C. albicans to change its cellular morphology may be postulated to confer several advantages in the causation of infection. Blastospores may be more readily spread throughout a host by the bloodstream than larger filamentous forms. Hyphae or pseudohyphae may be more able than blastospores to penetrate directly through tissues and the ability of clumps of hyphae or pseudohyphae to produce emboli may be important. Hyphae may be less readily ingested and killed by macrophages and polymorphs and blastospores may escape killing inside phagocytic cells by producing hyphae that grow out of the phagocytic cells. Finally, long hyphae may offer a larger surface area than unicellular blastospores, for attachment of the fungus to host tissues. In addition variation in enzyme levels and in the composition of the cell surface of the various morphological forms could relate to differential virulence of one form or another.

Consideration of these factors has led many investigators to conclude that the presence of C. albicans hyphae in tissues is indicative of active infection and to the implication that C. albicans hyphae are more pathogenic than blastospores (e.g. Balish & Philips, 1966; Kozinn & Taschdjian, 1966; Jones & Russell, 1974). Microscopic study of the prevalence of the various cell morphologies in C. albicans lesions in vivo, does not however, support these conclusions. In the majority of lesions of C. albicans infection, blastospores, pseudohyphae and true hyphae are usually found together (Rogers, 1966). In addition, the few reports of lesions where blastospores were found in the absence of mycelium (Hauser & Rothman, 1950; Pluss & Kadas, 1954) are matched by cases where hyphae were present in the absence of blastospores (Wolter, 1962; Rebora et al. 1973).

Studies of experimental candidosis in which animals were inoculated with one morphological form or another have yielded contradictory results. One of the major problems with such experiments is the rapid interconversion of morphological forms that occurs *in vivo* making interpretation of results difficult. In addition standardisation of inocula of different morphological forms may also present problems.

Simonetti & Strippoli (1973) concluded that the blastospore form of *C. albicans* was more pathogenic for mice than the germ tube form of the fungus. However the inocula used differed in several other parameters as well as in morphology. Blastospores and germ tubes were grown on different media and for different lengths of time and therefore differed in growth phase. In addition cell biomass was standardised on the basis of optical density measurements. Since blastospores have a smaller surface area per cell unit than germ tubes, germ tube inocula would contain a smaller number of cell units than blastospore inocula of similar optical density. Mardon, Gunn & Robinette (1975) similarly considered *C. albicans* blastospores to be more pathogenic than pseudohyphae. Blastospores and pseudohyphae were grown to the same growth phase in the same medium incubated under different gaseous atmospheres. In these experiments inocula were standardised on the basis of turbidity measurements; no significant differences were noted between turbidity or dry weight measurements when samples of blastospore or pseudohyphal inocula were compared. When assessed by viable count methods 6 and 9 h old filamentous inocula gave lower counts than comparable blastospore suspensions although the accuracy of viable count methods applied to filamentous forms must be in doubt. In neither of these studies was there any histological examination of tissues from dead mice, the morphology of the fungus in infected tissues was not therefore determined and the relative contribution of fungal morphology and biomass of the fungus to the effects seen is therefore difficult to assess. Evans (1981) grew blastospores and hyphae, presumably pseudohyphae, under conditions identical to those of Mardon, Gunn & Robinette (1975). Inocula were standardised

in one of two ways; by turbidity to produce an equal cell mass or by dilution of blastospore and pseudohyphal suspensions to give the same viable counts. In the first method blastospore suspensions contained between 3 and 4 times more viable units than similar pseudohyphal suspensions. In the second method pseudohyphal suspensions contained between 3 and 4 times the cell mass as determined by dry weight determinations. When inocula prepared by both these methods were injected into mice blastospores again were apparently more pathogenic than pseudohyphae. Histological examination of tissues taken from infected mice showed the presence of both blastospores and hyphae, irrespective of the morphology of the inoculum.

Earlier studies (Rogers, 1966) also reported the presence of blastospores, hyphae and pseudohyphae in Candida lesions in vivo. Extrapolation of results obtained in experimental infections with inocula of one morphological form to the situation in true Candida infections may therefore not be valid. Although such experiments, using adequately standardised inocula, may indicate whether blastospores or filamentous forms of C. albicans differ in their ability to initiate systemic infection they do not allow study of the dissemination of the fungus once infection is established, when the presence of various morphologies of C. albicans could be important in overcoming host defence mechanisms. In addition such studies do not take into account the ability of C. albicans to change morphology in vivo, which may in itself be an important virulence attribute. It is also conceivable that in infections of mucosal surfaces the significance of the morphology of C. albicans may differ from that found in systemic candidosis.

Three other key studies of human candidosis, however, suggest that mycelium production may be a factor in the pathogenesis of candidosis. Taschdjian & Kozinn (1957) showed that in young babies from when C. albicans was isolated, direct microscopy of smears showed only blastospores until the clinical lesions of thrush became apparent, at which point hyphae were seen to be present. Similarly the presence of hyphae in the stools of infants

with presumptive Candida diarrhoeae was significantly higher than in the stools of unaffected normal infants (Kozinn & Taschdjian, 1962). A high correlation between the isolation of hyphae and the presence of denture stomatitis was noted in a survey by Budtz-Jorgensen et al. (1975).

1.3 Studies of C. albicans dimorphism in vitro

The possible association between dimorphism and pathogenicity of C. albicans has led to much study of the phenomenon in vitro. Investigators have tended either to look for environmental factors which control dimorphic shifts or for biochemical differences between the morphological forms which may be related to dimorphism.

Environmental factors involved in dimorphic shifts

Audrey (1877) was one of the first workers to recognize that changes in the growth environment could affect the morphology of C. albicans. The wide range of growth conditions and growth media shown to favour the production of one morphological form over another has been reviewed by Odds, (1979). (Table 1:2) Results of experiments designed to study the importance of individual parameters of growth conditions on the morphology of C. albicans have been contradictory. This variation in results obtained by different investigators may reflect differences in the strains of C. albicans used, incubation conditions, terminology used to describe different morphological forms of C. albicans and in the methods used to assess and quantify the morphology of a culture.

In vitro, budding blastospores have been considered the most readily obtainable growth form. Germ tube production and elongation to produce hyphae have been considered transient stages in development since under most growth conditions reversion to a blastospore form of growth occurs on prolonged incubation. This pattern of germ tube production followed by reversion to blastospore morphology was first recognised by Roux & Linossier in 1890. Evans et al. (1975) investigated this developmental process

Table 1:2

Some environmental factors that have been considered to affect C. albicans morphology in vitro

1. Factors that favour filamentation or suppress blastospore formation:	Temperature $\geqslant$ 35 °C
	Temperature $<$ 35 °C
	pH $\geqslant$ 7.0
	pH $<$ 7.0
	Low oxygen tension
	Controlled ratio CO_2:O_2
	Inoculum $\leqslant$ 10^6 yeasts/ml
	Liquid growth medium
	Impoverished growth media
	Nonfermentable carbon source
	Polysaccharide carbon source
	Low sulphydryl content
	Suboptimal concentration of yeast extract
	Nitrate as nitrogen source
	N-acetyl glucosamine
	Albumin
	Amino acids
	L-α-amino-N-butyric acid
	Biotin (concentration $<$ 1 µg/1)
	Cobalt salts
	B-indole acetic acid
	Iron (Fe^{II}) salts
	Maltose
	Phosphate
	Zinc salts (concentration 3-5 µM)
2. Factors that favour blastospore formation or suppress filamentation:	Temperature $<$ 35 °C
	pH $<$ 7.0
	Inoculum $>$ 10^6 yeasts/ml
	Ammonium salts
	Biotin (concentration $>$ 1 µg/1)
	Cysteine or other thiol-containing compound
	Lactate
	2-phenyl ethanol
	Phosphate
	Polyene antifungals

From Odds, 1979

further and quantified the morphology of the fungus as it changed with time. These workers showed that temperature of incubation, pH of the growth medium and the concentration of the blastospore inoculum were critical factors in determining the morphology of C. albicans. An initial concentration of 10^6 C.albicans blastospores ml^{-1}, growth temperatures of 37 °C to 40 °C and medium pH of 7.0 - 7.4 favoured germ tube production, whereas lower temperatures and pH favoured blastospore production. These findings were in agreement with those of earlier investigations e.g. Landau et al. (1965) who found that C. albicans hyphal or blastospore cultures could be produced in the same growth medium simply by altering the incubation temperature. Subsequent studies have re-emphasized the importance of inoculum concentration in the control of germ tube production. e.g. Hazen & Cutler (1979). Other workers, however, have reported mycelium production at lower temperatures e.g. Yamaguchi, Kanda & Osumi, (1974) and it is therefore possible that the morphology of C. albicans is affected by a combination of factors including the strain of the organism used, medium composition and environmental factors such as temperature and pH.

Germ tube production has been readily achieved in vitro with a range of incubation conditions and growth media but maintenance of C. albicans in a purely hyphal form for any substantial length of time has proved difficult. Serum has been shown, under suitable incubation conditions, to stimulate germ tube formation and branching mycelium production in C. albicans. The production of germ tubes in serum has been used in the identification of C. albicans isolates in clinical laboratories.(Taschdjian, Burchill & Kozinn 1960; Mackenzie, 1962). Serum however, has the disadvantage of being a very complex undefined medium and its use in the elucidation of the role of environmental factors in the control of C. albicans dimorphism in vitro is therefore limited. Lee, Buckley & Campbell (1975) were among the first workers to describe a chemically defined medium which allowed extended growth of C. albicans in a mycelial form. Their amino acid-based medium was formulated on the basis of a C. albicans amino peptidase profile determined

in earlier studies (Lee et al. 1975). Several investigators have used tissue culture media for the production of mycelial cultures of C. albicans e.g. Landau et al. 1965; Dabrowa et al. 1970; Diamond, Krezesicki & Jao, 1978; Borgers & Van Den Bossche, 1979; Schwartz & Larsh, 1980. These media, although complex, are chemically defined and their composition may more closely represent tissue conditions in vivo than other conventional mycological media.

Two compounds whose effect as inducers of hypha formation in C. albicans have been the subject of several investigations are N-acetyl-D-glucosamine and cysteine.

Simmonetti, Strippoli & Cassone (1974) and Shepherd et al. (1980) reported the induction of germ tubes in C. albicans by N-acetyl-D-glucosamine. This amino sugar is the monomer of chitin, a major cell wall component of C. albicans, that has been shown to be present in larger quantities in C. albicans germ tubes than blastospores (Chattaway et al. 1968). Simonetti, Strippoli & Cassone (1974) suggested that induction of germ tube formation by N-acetyl-D-glucosamine could be related to the control of chitin synthesis in C. albicans. This hypothesis was supported by Braun & Calderone (1978) who found that incorporation of labelled N-acetyl-D-glucosamine into chitin was ten times higher in germ tubes than in blastospores. Activities of enzymes involved in the metabolism of this hexosamine have been studied to elucidate the mechanism of dimorphism (see below).

Nickerson (1949, 1951) showed that germ tube production could be inhibited by including reducing compounds such as cysteine in the growth medium. Cysteine was thought to stimulate the activity of an enzyme, protein disulphide reductase, which was reported to be involved in blastospore budding in C. albicans (Nickerson & Falcone, 1956). Subsequent studies failed to show cysteine inhibition of germ tube production by C. albicans (e.g. Shepherd & Sullivan, 1976; Wain, Price & Cawson, 1975). More recent studies have, however, found that cysteine (Niimi et al. 1980) and other reducing compounds such as dithiothreitol (Chattaway et al. 1981) do inhibit

germ tube production. It is difficult to explain these contradictory results but variations in experimental conditions and in the strains of the fungus used in these experiments could possibly account for the results seen.

Many investigators e.g. Lee, Campbell & Buckley, 1975; Land et al. 1975 Chattaway et al. 1976, have used amino acids or peptide hydrolysates to induce germ tube production in C. albicans. CAMP has been shown to mediate the effect of extracellular compounds, including peptides on metabolic processes in a number of organisms (Chattaway et al. 1981) and could be controlling dimorphism in C. albicans (see below).

Other aspects of germ tube and mycelium production in vitro

Dimorphism of C. albicans in vitro involves more than just the shape of the fungal cells. Mackenzie (1964) showed that, while blastospore buds are virtually always generated from the apical (polar) sites of blastospores, germ tubes appear to be initiated from all sites on the cell surface.

Moreover study of the ability of C. albicans blastospores to produce germ tubes at different phases of the growth cycle has indicated that the metabolic status of the blastospore and its position in the fungal cell cycle may be important in morphogenesis. Strippoli & Simmonetti (1971) and Chaffin & Sogin (1976) showed that the ability of C. albicans to produce germ tubes was restricted to blastospores in the late logarithmic and stationary phases of growth. These workers suggested that the asynchrony of blastospores within an inoculum may affect the results obtained in studies of morphogenesis and so used inocula of similarly sized blastospores. Chaffin & Sogin (1976) reported that fractions obtained from a population of blastospores in the late stationary phase of growth varied to some extent in their ability to produce germ tubes and in the rate of germ tube production; small blastospores in particular showed a slower and more limited production of germ tubes. However over 90% of the blastospores in the

unfractionated control blastospore inocula also produced germ tubes suggesting that in this case little advantage was to be gained by using synchronised inocula.

Mitchell & Soll (1979) supported the importance of blastospore growth phase in germ tube production when it was shown that once a C. albicans blastospore produced a bud, it, and its progeny lost the capacity to produce germ tubes until they re-entered the stationary phase of growth. Mattia & Cassone (1979) however suggested that germ tube production was not restricted solely to blastospores in the late logarithmic or stationary phases of growth. Although blastospores growing in a yeast extract/glucose medium were unable to produce germ tubes when transferred into fresh medium at 37 °C until they reached stationary phase, transfer into media containing inducers of germ tube production such as N-acetyl-D-glucosamine or serum enabled blastospores at any phase of growth to produce germ tubes. The role of blastospore volume in relation to morphogenesis was further investigated by Chaffin & Wheeler (1981). It was shown that in a population of stationary phase blastospores the larger cells budded first and remained larger throughout the incubation period. Under conditions that favoured germ tube production, larger cells similarly produced germ tubes first but by the time 50% of the inoculum had produced germ tubes there was little difference in the cell volume of blastospores which had or had not produced germ tubes. Bell & Chaffin (1980) studied the effect of nutrient limitation and cell cycle stage on the ability of C. albicans to produce germ tubes and concluded that a combination of growth phase and the metabolic state of the blastospore was important in the control of germ tube production since neither factor alone explained their observations. Shepherd et al. (1980x)also considered the metabolic status of the blastospore important in germ tube production and used starved blastospores, obtained by aeration in distilled water for 24 h, as their inocula.

Clearly then variation in the methods used to prepare inocula for studies of morphogenesis in vitro may affect the results obtained. The

importance of variation between strains of C. albicans in relation to this phenomenon has been emphasised by Manning & Mitchell (1980a). Although their semi-synchronized inocula produced germ tubes more quickly, in synchrony and with a smaller proportion of pseudomycelial cells than an actively budding inoculum, the extent of mycelium production and the rate at which reversion to blastospore formation occurred was still dependent on the strain of fungus used.

Evans, Odds & Holland (1975) reported that once a blastospore had produced a germ tube it was committed to a mycelial form of development and was then resistant to environmental factors which favoured blastospore production. Evans et al. (1975a) showed that in the same growth medium, incubation at 40 °C favoured germ tube production, and incubation at 30 °C blastospore budding. Evans, Odds & Holland (1975) reported, using this system, that blastospores grown at 30 °C could be induced to produce germ tubes only if they were switched to 40 °C early in the growth cycle, presumably while the blastospore was still in the stationary phase of growth. Switching mycelial cultures from 40 °C to 30 °C at hourly intervals during incubation gave no interruption of filamentous growth. Experiments where blastospores growing at 40 °C were switched to 30 °C, at 5 minute intervals following inoculation of the medium, showed that blastospores became committed to germ tube production within 1 h of inoculation. These workers concluded that once the filamentous 'cycle' of development was initiated, the 'cycle' was resistant to changes in the environment.

Mitchell & Soll (1979) similarly showed that altering the pH or temperature of a pseudohyphal culture of C. albicans did not instantly inhibit elongation, suggesting commitment to a mycelial 'cycle' which was possibly temperature and pH dependent. Chaffin & Wheeler (1981) found that the amount of time required at 37 °C to commit blastospores to germ tube formation was much less than that required to affect the cell morphology of mycelial forms by switching the incubation temperature to 25 °C, re-

emphasising the resistance of the mycelial 'cycle' to environmental factors once initiation has occurred.

This resistance of the filamentous growth form to changes in the environment suggests that morphological development may be genetically predetermined once initiated, and that environmental factors such as temperature may be acting on some kind of switching mechanism within the fungal cell. The nature of this metabolic switch and its significance in understanding the mechanism of dimorphism has led to much study of the comparative biochemistry of the morphological forms of C. albicans in an attempt to isolate metabolic changes which may be related to dimorphism.

Biochemical differences between morphological forms in vitro

Biochemical experiments on C. albicans dimorphism have generally involved one of two approaches. In the first, concentrations of various components of hyphae and blastospores have been measured in attempts to establish which molecules are most significant in determining dimorphic shifts. In the second, the activities of enzymes and concentrations of metabolites involved in the biosynthesis of cell componants have been measured, in attempts to explain the interactions between C. albicans and its growth environment and the molecular control of dimorphism. In nearly all such studies, the conditions used to grow hyphae and blastospores for analysis and the test strain of C. albicans have differed from laboratory to laboratory, making correlation and extrapolation of observations difficult.

Yamaguchi (1974); Wain et al. (1976); Wain & Polak (1979) and Shepherd et al. (1980x) have shown that during germ tube production there is no change in the DNA content of C. albicans, suggesting that DNA synthesis is not required for germ tube production and supporting the earlier hypothesis of Nickerson (1948; 1963) who suggested that germ tube production may involve an uncoupling of cell growth from cell division. The timing of DNA synthesis in blastospores and germ tubes has been shown to be different (Wain et al.

1976). In blastospores DNA synthesis coincided with bud formation whereas in germ tubes, emergence of the germ tube preceded DNA synthesis. Soll, Stasi & Bedell (1978) examined the timing of nuclear division during hypha formation in C. albicans. They showed that there was a minimum hyphal length and a minimum incubation time required to synthesise nuclear DNA. Nuclear migration in the developing hypha was also studied and appeared to be independent of DNA replication.

Dabrowa et al. (1970) reported that both the RNA and protein concentrations of C. albicans blastospores increased during germ tube production. Wain et al. (1976) similarly reported an increase in the content of RNA during germ tube production and showed that the increase was exponential in elongating hyphae. Shepherd et al. (1980) reported that the RNA content of C. albicans more than doubled during germ tube synthesis and showed that a range of metabolic inhibitors of RNA and protein synthesis effectively blocked germ tube synthesis.

Dabrowa et al. (1970) investigated water soluble proteins isolated from C. albicans and reported 6 electrophoresis bands common to both morphological forms, four bands seen in blastospores but not filaments and one band seen in filaments which was absent from blastospores. Evans et al. (1973) using crossed counter-immunoelectrophoresis, revealed a mycelial specific component in cytoplasmic extracts of C. albicans. Syverson, Buckley & Campbell (1975) showed each growth form to have six soluble cytoplasmic components unique to its morphology. Manning & Mitchell (1980) radiolabelled proteins synthesised by C. albicans during the production of mycelia and during bud formation. Although mycelial forms of one strain of C. albicans showed ten electrophoresis spots not present in the blastospore form of that strain, these proteins were also present in a second strain of C. albicans grown at the same incubation temperature but which lacked the ability to produce germ tubes under these incubation conditions. These workers concluded therefore that these proteins were not mycelium specific and were insufficient for induction or maintenance of germ tube production

and suggested that although the cytoplasmic protein content of C. albicans was not extensively altered during morphogenesis, proteins specific to one morphological form or other could serve a regulatory function. Brown & Chaffin (1981) pointed out that the technique of radio-labelling proteins throughout the whole period of germ tube synthesis would not necessarily reveal proteins synthesised during the early period of germ tube synthesis which carried out their function and were then broken down. When Brown & Chaffin radio-labelled proteins at intervals during bud and germ tube production by C. albicans, five proteins specific to the blastospore form of the fungus were detected. Although no mycelium specific proteins were found, two proteins showed a net reduction in synthesis during mycelium production. It was therefore suggested that changes in morphology may be the result of changes in gene expression within the fungal cell.

Some study of the lipid composition of blastospore and mycelial forms of C. albicans has been made. Marriott (1975x) reported differences in the lipid composition of isolated plasma membranes from mycelial or blastospore forms of C. albicans. Mycelial plasma membranes contained significantly less sterol, both free and esterified, and proportionately more triglyceride and free fatty acids. Ergosterol was the main free sterol from both blastospore and mycelial plasma membranes and was present in equal amounts in both forms. Ballman & Chaffin (1979) looked at lipid synthesis in whole C. albicans cells during bud formation or germ tube synthesis. The pattern of synthesis seen was similar in both types of culture and no significant differences were observed in the content of neutral phospholipid or phospholipid components between blastospore and germ tube forms. Sundaram, Sullivan & Shepherd (1981) reported that the total lipid content of starved blastospores decreased during bud and germ tube synthesis and that the major components present were sterols and phospholipids.

It has been suggested that the activity of the imidazole antifungals is due to interference with sterol biosynthesis in C. albicans (Van den Bossche et al. 1978). These workers showed that miconazole inhibited

ergosterol biosynthesis in C. albicans. Similar results were obtained by Marriott (1980) although this study failed to indicate any correlation between MIC values for C. albicans and the concentration of the antifungals, miconazole, ketoconazole or tioconazole, required to inhibit sterol biosynthesis.

These compounds may however be useful in the study of the mechanism by which dimorphism is controlled in C. albicans, since previous investigations have shown that very low concentrations of miconazole may inhibit germ tube synthesis in C. albicans without drastically affecting the growth rate of the fungus (Van den Bossche, Willemsens & Van Cutsem, 1975; Borgers et al. 1979).

Although study of the changes in the content of nucleic acids, lipids and proteins during germ tube and mycelium production in C. albicans may be related to dimorphism, it is very difficult to establish that differences seen are specifically related to dimorphism and not to other metabolic processes associated with the growth of the fungus. Other workers have therefore examined polysaccharide components and the control of their synthesis which may be more directly related to changes in C. albicans morphology. Differences in polysaccharide composition have been shown to exist between blastospores and hyphae and the polysaccharides concerned are located in the cell wall. Changes in the shape of the fungus must be related to changes in the composition or arrangement of the rigid cell wall, and may result from the action of enzymes synthesized de novo in response to environmental stimuli or by modification of enzyme systems already present in the C. albicans cell. The most common emphasis in studies of dimorphism in C. albicans has therefore been on cell wall structure, especially polysaccharide structure and its regulation, and this topic will therefore be discussed in detail in the following section.

1.4 Candida albicans cell wall

The cell wall is the major barrier between the environment and the fungal cytoplasm and as such plays a major role in the maintenance of the integrity of the cell. The wall contributes to selective permeability towards solutes and is therefore involved in the uptake of nutrients required for growth and the release of waste metabolic products. In addition the rigidity of the cell wall protects the plasmalemma from physical shock. This rigidity means that the shape of the cell is governed by the shape of the cell wall and that changes in morphology must be accompanied by changes or rearrangements in the fabric of the existing wall. Since the cell wall is the site of interaction of the fungus and its environment, including host tissues, changes in the cell wall structure may also have implications in the pathogenesis of candidosis.

Chemical composition of the C. albicans cell wall

Study of the cell wall requires its isolation from the closely associated plasmalemma and the remainder of the cell cytoplasm. Two main methods have been used to isolate fungal cell walls. Intact cell walls may be isolated from other cell components after mechanical disruption of the fungus or components of the cell wall may be chemically extracted from the intact fungus. Both methods have advantages and disadvantages.

Although mechanical disruption of yeast cells yields larger amounts of cell wall material than chemical extraction (Northcote & Horne, 1952; Bacon et al. 1969) such procedures are more prone to cause gratuitous solubilization of some cell wall components. (Kidby & Davies, 1970; Cassone, Kerridge & Gale, 1979). Even simple washing of C. albicans in warm water may lead to loss of mannan from the cell wall (Sikl, Masler & Bauer, 1964). On the other hand, chemical extraction of intact cells has the disadvantage that the assay procedure used may not distinguish between cell wall components and those of the cell's metabolic pools. Although these procedures may provide pure compounds suitable for chemical analysis, they do not permit analysis of the

spatial arrangement of the various components of the wall *in vivo*.

The possibility that the composition of the growth medium used to grow the fungus could affect the make up of the cell wall has also to be considered. In *S. cerevisiae* the glucan content can be affected by the composition of the growth medium used (McMurrough & Rose, 1967), and although Chattaway *et al.* (1968) found little difference in the composition of isolated *C. albicans* cell walls prepared under different cultural conditions, indirect evidence suggesting that cultural conditions can affect the cell wall composition of *C. albicans* blastospores was provided by McCourtie & Douglas (1981). These workers found that *C. albicans* blastospores grown on different carbon sources differed in their ability to attach to acrylic surfaces *in vitro*. Electron micrographs of blastospores grown in media which favoured attachment showed the presence of an extra outer cell wall layer, not present in control blastospores.

Published studies of the chemical composition of *C. albicans* cell walls should be considered in the context of these limitations.

Kessler & Nickerson, (1959) isolated cell walls of mechanically disrupted *C. albicans* blastospores and obtained two alkali-soluble fractions and one alkali-insoluble fraction, which were then analysed. The major components were shown to be glucose, mannose, glucosamine, protein and free and bound lipid. Some variation in the overall composition was noted between different *C. albicans* strains, but it was clear that glucans, mannans and chitin were the predominant cell wall polysaccharides. Chattaway *et al.* (1968) carried out similar analyses but also looked at differences in cell wall composition between stationary phase blastospores and hyphal forms of *C. albicans*. It was shown that hyphal walls contained approximately four times as much glucosamine, presumably derived from chitin, than blastospore walls but concentrations of other components were similar in the two forms. The results of a third study, involving whole cells of *C.albicans* blastospores and hyphae were in general agreement with the findings of the earlier studies (Schwartz & Larsh, 1980). Thus chitin is implicated as a potentially significant

macromolecule in determining the morphological differences between blastospores and hyphae.

Chemical analysis of blastospore cell wall polysaccharides has shown that the glucan is a highly branched β 1-6 and β 1-3 linked molecule (Bishop, Blank & Gardner, 1960) and the mannan contains mainly α 1-6 and α 1-2 linkages (Bishop, Blank & Gardner, 1960; Sikl, Masler & Bauer, 1964) with some α 1-3 linkages (Yu et al., 1967). N-acetyl-D-glucosamine is the monomeric constituent of cell wall chitin (with β 1-4 linkages) and it may also be involved in linkages between C. albicans mannan and proteins in the cell wall. (see below).

Arrangement of polymers in the C. albicans cell wall

Electron microscope examinations of C. albicans blastospores have shown three (Borgers & De Nollin, 1974), five (Cassone, 1973) and even eight (Poulain et al., 1978) layers in the cell wall. Differences in the reported number of layers probably arise from differing fixation and staining techniques employed. The number of cell wall layers can also vary with cell age (see below). Although the different layers may each contain a predominant macromolecular component, evidence from recent studies shows that the major cell wall polysaccharides are present in more than one stratum.

Venezia & Lachapelle (1973), using ferritin-conjugated anti-C. albicans whole cell antibody, were able to detect label on the internal and the external surfaces of cell walls isolated from blastospores and hyphae. Pre-absorbtion of the antiserum with an alkali-ethanol precipitated cell wall extract of the fungus, prior to ferritin conjugation, abolished the reactivity of the labelled antiserum towards isolated C. albicans cell walls. These workers concluded that the reactivity of the antiserum was with mannan in the cell wall and that this component was therefore widely distributed throughout the cell wall. It is possible, however, that some of the label was bound to mannan that had been released and re-deposited at new cell wall sites during cell wall isolation. Deep and superficial locations for mannan were also indicated by the mannan-specific lectin binding studies of Cassone, Mattia & Boldrini (1978).

In S. cerevisiae, a yeast whose cell wall structure has been more thoroughly investigated than that of C. albicans, mannoprotein complexes are known to be located at the outer surface of the yeast cell wall (Mundkur, 1960; Horisberger & Vonlanthen, 1977).

Poulain et al. (1978) attempted to determine the chemical composition of individual layers of cell walls of stationary-phase C. albicans by cytochemical staining of ultrathin sections. Their results suggested that the majority of the mannan and β 1-6 glucan was present in the outer layers of the cell wall, but that a deeper layer also contained these components. Chitin and β 1-3 glucan were found in layers closer to the plasmalemma. The reactivity of the innermost layer indicated the presence of polysaccharides of low polymerization and it was postulated that this layer may be involved in the assembly and incorporation of polysaccharides into the outer layers of the cell wall. Tronchin et al. (1981) confirmed that chitin was located primarily in the inner cell wall layers of C. albicans blastospores, although some chitin was also demonstrable in the outer layers of the cell wall.

These observations match those for S. cerevisiae, in which glucan has been found in most cell wall strata (Arnold, 1973; Fleet & Manners, 1976). Glucan was also the major component of a fibrillar network laid down initially in the process of wall regeneration from S. cerevisiae spheroplasts (Necas, 1971), although similar fibrils have also been shown to contain chitin (Kreger & Kopecka, 1973). The arrangement and location of chitin in C. albicans cell walls may differ somewhat from that in S. cerevisiae, partly because there is more chitin in C. albicans walls (Chattaway et al. 1968) and partly because Poulain et al. (1978) and Tronchin et al. (1981) were able to demonstrate chitin in several cell wall layers. In S. cerevisiae chitin was long thought to be located exclusively in bud and birth scars (Bacon et al. 1966; Beran & Rehocek, 1969), but it has since been shown to occur in small quantities throughout the lateral walls of budding yeasts (Horisberger & Vonlanthen, 1977).

Close links between glucan and chitin were suggested by the work of Domanski & Miller (1968) who showed that the release of N-acetyl-D-glucosamine

from C. albicans was enhanced in the presence of glucanase. Recent work by Sietsma & Wessels (1981) has shown a similar close link between glucan and chitin in the cell wall of S. cerevisiae.

Proteins in the C. albicans cell wall are almost certainly bound to polysaccharides. The only cell wall enzyme to be isolated and purified from C. albicans proved to be a mannoprotein (Odds & Hierholzer, 1973) and linkages between proteins and mannan have been demonstrated in Saccharomyces species. (Kidby & Davies, 1970; Phaff, 1971). The linkage of protein to yeast mannan is thought to involve N-acetyl-glucosamine linkages between mannan and side chain amino groups in the cell wall proteins. (Phaff, 1971)

The location and attachment of cell wall lipids is unknown.

Differences in the composition of the C. albicans cell wall between blastospore and mycelial forms of the fungus have been noted in several investigations using electron microscopy. (Cassone, Simmonetti & Strippoli, 1973; Yamaguchi, Kanda & Osumi, 1974). Hyphal and germ tube cell walls have been shown to be composed of fewer layers than those of blastospores (Scherwitz, Martin & Ueberberg, 1978) and these findings are in agreement with those of other workers who have suggested that the mycelial cell wall is thinner than that of blastospores e.g. Chattaway et al. 1976a.

Biosynthesis of cell wall polysaccharides

In comparison to the great deal of information available on the synthesis of the components of the cell wall in S. cerevisiae relatively little is known about cell wall synthesis in C. albicans.

In S. cerevisiae the bud tip has been shown to be the major site of mannan synthesis (Tkacz & Lampen, 1972) and the site of ^{3}H mannose incorporation varies with the age of the bud (Farkas et al. 1974). In very young buds incorporation occurred at all parts on the cell wall. As the bud aged tip growth predominated and later still incorporation at the sides of the bud occurred. This pattern of incorporation, with the majority of growth at the

tip and less at the sides of the bud could favour production of an ovoid shaped cell.

In S. cerevisiae the pattern of glucan synthesis appears to be similar to that for mannan. Glucan synthesis has been reported in cell free systems (Balint et al 1976; Lopez-Romero & Ruiz-Herrera 1977; and Larriba, Morales & Ruiz-Herrera, 1981) and autoradiographic studies have shown that the pattern of ^{3}H glucose incorporation into the cell wall is similar to that described for mannan (Biely, Kovarik & Bauer, 1973a). However glucose has been shown to be a precursor for glucan and mannan synthesis and autoradiographic studies using labelled glucose may not adequately distinguish labelling of these two polymers.

Chitin synthesis has been studied extensively in S. cerevisiae and has been reviewed by Cabib, (1975). In contrast to mannan and glucan synthesis which have been shown to occur throughout the cell cycle (Sierra, Sentandreu & Villaneuva, 1973) incorporation of precursors into chitin in synchronised cultures is a step wise process reflecting the deposition of this component principally in the primary septum (Cabib & Farkas, 1971). The chitin synthase system has been found to consist of three components isolated from one another in the yeast cell; chitin synthase zymogen bound to the plasmalemma, a proteolytic activating factor in cell vacuoles and a protein inhibitor found free in the cytoplasm. Studies suggest that the total amount of chitin synthase present in the cell varies little with the phase of growth but that the extent of activation is highest during the exponential phase of growth (Cabib & Farkas, 1971; Hasilak 1974).

In C. albicans mannan synthase activity has been shown in plasma membrane fractions isolated from C. albicans blastospores and germ tubes (Marriott 1977). Little difference was found in the activity of this enzyme between the two forms. Further study of blastospore protoplasts showed that mannose incorporation by mannan synthase was markedly less in intact protoplasts than in membranes isolated after protoplast disruption, suggesting that the majority of the enzyme activity was located on the inside of the plasmalemma

(Marriott, 1977). It is also conceivable that in intact protoplasts, a soluble inhibitor of mannan synthase activity analagous to the chitin synthase inhibitor, could affect the activity of the membrane-bound enzymes. This type of inhibition of mannan synthase activity in lysates of S. cerevisiae spheroplasts was noted by Harrington & Douglas (1980) who found that the inhibition was relieved when washed spheroplast membrane preparations were used as a source of mannan synthase.

Virtually nothing is known of glucan synthesis in C. albicans but chitin synthesis has been thoroughly investigated. Braun & Calderone (1978) reported chitin synthase activity in C. albicans. They noted the location of the enzyme on the inner side of the plasmalemma and that activity of the zymogen isolated from both blastospores and hyphae was increased six to seven fold by trypsin treatment. A vacuolar fraction obtained from blastospores, but not from hyphae, stimulated enzyme activity. Incorporation of N-acetyl-D-glucosamine into an acid-alkali insoluble fraction was ten times higher in hyphae than blastospores; this was partly attributed to the greater uptake of this substrate by hyphae and also to their higher chitin synthase activity. These workers showed by auto radiography that ^{3}H N-acetyl-D-glucosamine was incorporated into blastospores at the site of bud formation and into hyphae principally at the growing apex.

A cytoplasmic inhibitor of the zymogen activating factor has also been described in C. albicans (Braun & Calderone, 1979). Chiew, Shepherd & Sullivan (1980) reported a five fold increase in the chitin synthase activity of germ tubes compared with blastospores. These findings correlated with those of Chiew, Sullivan & Shepherd (1980) who showed that the chitin content of germ tubes was four to five times that of blastospores, an observation made earlier by Chattaway et al. (1968).

Studies of chitin and mannan synthase in C. albicans have however, tended to concentrate on enzyme activities only during bud or germ tube production by stationary phase blastospores or on the activity of enzymes

isolated at a single, early phase in the C. albicans growth cycle. No study of the activity of these enzymes in relation to hyphal branching or secondary blastospore production in older hyphae has been made. Since chitin content, in particular, appears to correlate with morphology in C. albicans e.g. Chattaway et al. (1968), changes in the activity of chitin synthase could play a key role in these processes.

Polysaccharide synthases, including chitin and mannan synthases are involved in the synthesis of complex polymers derived from simpler monomeric cell wall precursors. The possibility that dimorphism in C. albicans may be controlled through the activity of enzymes and co-factors involved in the production of substrates for cell wall synthesis has also been investigated.

Chattaway et al. (1973) investigated the enzymes involved in carbohydrate synthesis and breakdown in blastospore and mycelial forms of C. albicans since earlier work (Chattaway et al. 1968), suggested that the carbohydrate-based cell wall of C. albicans changed composition during morphogenesis. At the peak of mycelium production (4h) phosphofructokinase activity was at its lowest level, making fructose-6-phosphate available for conversion to glucosamine-6-phosphate or mannose-6-phosphate, precursors of cell wall polysaccharides, by aminotransferase or phosphomannose isomerase respectively. The activity of these two enzymes was shown to be maximal at this time. Hexose monophosphate activity was lowest at this time, making substrate available for polysaccharide synthesis. This pathway is known to generate NADPH and thence a flow of electrons which could be used to activate the protein disulphide reductase described by Nickerson & Falcone (1956). Activation of this enzyme, according to the hypothesis of these workers would favour blastospore production.

Increased HMP and phosphofructokinase activity correlated with reversion to a blastospore mode of growth (Chattaway et al. 1973). Intracellular levels of ATP were at all times higher in mycelia and since ATP inhibits the activity of phosphofructokinase, these workers suggested that this mechanism may act as a control point in governing the morphology of the fungus.

The roles of respiration and of cyclic nucleotides in the control of dimorphism have been investigated in several laboratories. Yamaguchi (1974) and Land et al. (1975a) reported that during germ tube production by C. albicans an inhibition of respiration occurred. Land et al. (1975a) also showed that the inhibition of respiration correlated with an increase in cAMP levels. Blastospores in this metabolic state were capable of producing germ tubes under conditions which did not usually favour morphogenesis. Niimi et al. (1980) found that stationary phase blastospores contained ten times as much cAMP as cGMP and that germ tube production followed a rise in cAMP levels. The level of cGMP remained constant. Cysteine, a thiol compound, was shown to suppress germ tube production and also reversed the increase in cAMP levels during germ tube production.

Chattaway, Wheeler & O'Reilly (1981) showed a similar rise in cAMP levels during germ tube production. The rise in cAMP level occurred during the first hour of incubation and required an incubation temperature of 37 °C. 4h incubation at 37 °C was required to attain maximal germ tube production. If the temperature of incubation was reduced from 37 °C to 30 °C after 1h incubation, germ tube production was reduced although the level of cAMP remained constant. The production of germ tubes and the rise in cAMP were stimulated by theophylline, an inhibitor of the enzyme, phosphodiesterase which converts cAMP to cATP. Dithiothreitol was shown to inhibit the rise in cAMP levels and the production of germ tubes. Since ATP may be converted to cAMP enzymically these results are consistent with the earlier findings of Chattaway et al. (1973) and a possible role of these compounds in the control of morphogenesis in C. albicans is suggested.

N-acetyl-D-glucosamine metabolism in C. albicans has been studied both because of the stimulatory effect of this compound on hypha formation and also because it is the monomer of chitin, an important cell wall component. Singh & Datta (1979) reported that C. albicans blastospores had two N-acetyl-D-glucosamine permease systems. One was constitutive and showed a low level of activity, the second system was inducible by N-acetyl-D-glucosamine and showed

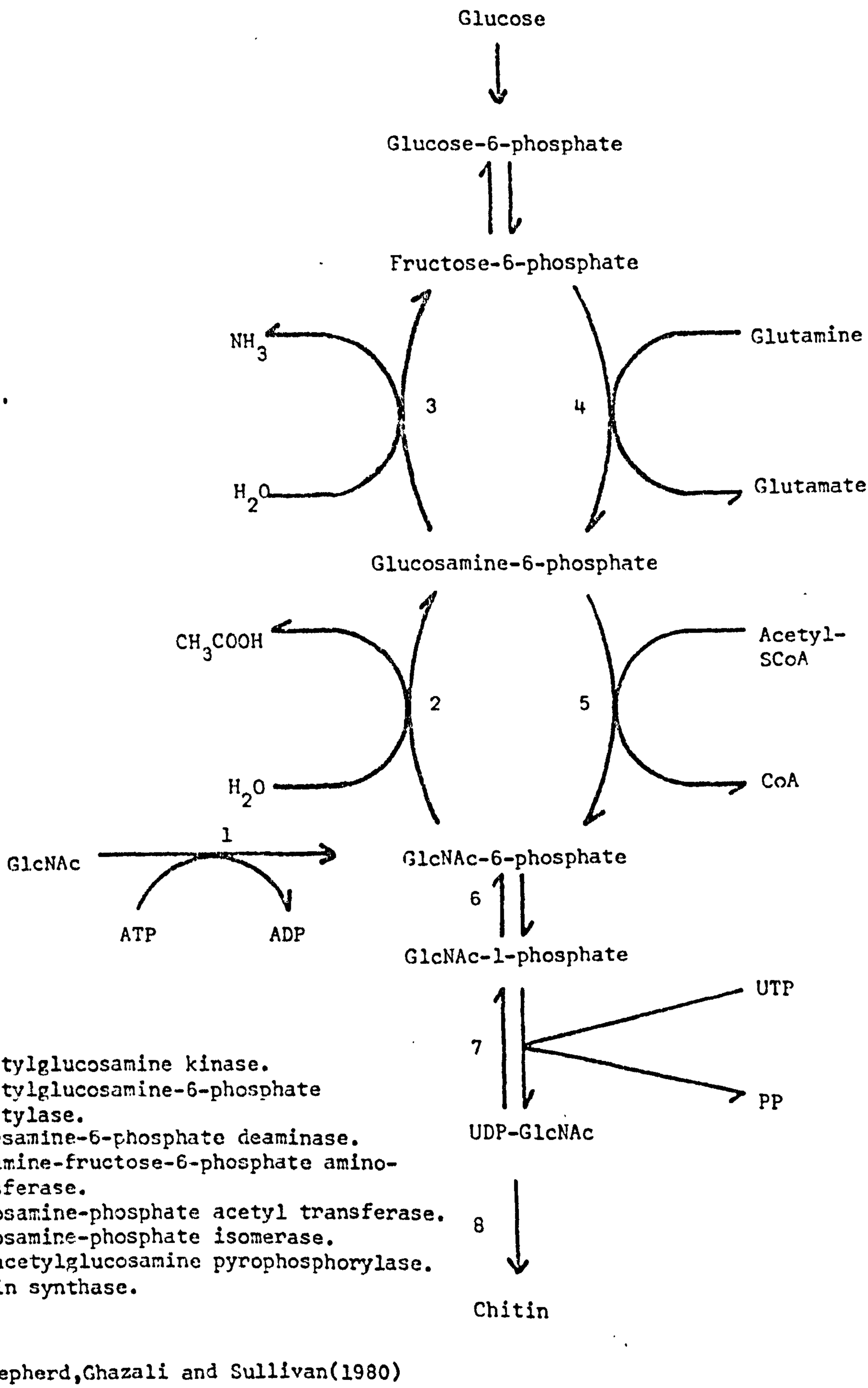
high activity in the presence of the inducer. Once taken up by C. albicans, N-acetyl-D-glucosamine may be metabolised to yield glucose which could either be used to generate energy or may be incorporated into new cell wall material as chitin (Fig1;2). Both pathways share a common intermediate N-acetyl-glucosamine-6-phosphate, which is derived from N-acetyl-D-glucosamine by the enzyme N-acetyl-D-glucosamine-kinase (Shepherd, Ghazali & Sullivan, 1980), so this enzyme could be a controlling factor in C. albicans. However, the enzyme was found to be induced in C. albicans both under conditions that favoured germ tube production and also under conditions where blastospore budding occurred, so its role in the control of cell wall synthesis and dimorphism in C. albicans was therefore put in doubt (Singh & Datta, 1979; Shepherd, Ghazali & Sullivan, 1980). Nevertheless Braun & Calderone (1978) have reported both higher rates of N-acetyl-D-glucosamine incorporation and chitin synthase activity in C. albicans germ tubes than blastospores and it may be postulated that N-acetyl-D-glucosamine kinase may still be important in providing substrate for cell wall synthesis under conditions that favour germ tube production.

Changes in the C. albicans cell wall in relation to growth and dimorphism

Changes in the composition of the cell wall must occur to enable the cell to grow and reproduce whether by budding or by germ tube production. These processes also involve septum formation.

Changes in the arrangement of cell wall layers in relation to dimorphism have been noted in several investigations using electron microscopy. Cassone, Simmonetti & Strippoli (1973) showed that during germ tube formation an electron transparent layer grew out through the blastospore wall to produce an early germ tube. Emergence of the germ tube was facilitated by degradation of the remainder of the overlying cell wall. The degraded regions were re-synthesised but differences in relation to the proportions of the various components in blastospore and germ tube walls were apparent. The deeper layers of the blastospore wall appeared to comprise the emerging germ tube and bud in a study by Scherwitz, Martin & Ueberberg (1978).

Fig.1.2 Pathway for chitin synthesis.



Enzymes.

1. N-acetylglucosamine kinase.
2. N-acetylglucosamine-6-phosphate deacetylase.
3. Glucosamine-6-phosphate deaminase.
4. Glutamine-fructose-6-phosphate amino-transferase.
5. Glucosamine-phosphate acetyl transferase.
6. Glucosamine-phosphate isomerase.
7. UDP-acetylglucosamine pyrophosphorylase.
8. Chitin synthase.

From Shepherd,Ghazali and Sullivan(1980)

Septum formation in C.albicans and S. cerevisiae has been shown to follow a similar pattern (Shannon & Rothman, 1971; Cabib, 1975). An electron lucent primary septum grows inwards from the neck of the bud and at the same time new membrane material is formed. An electron dense secondary septum is then laid down by both mother and daughter on either side of the primary septum. When the two cells separate, the primary septum is retained by the mother and forms a bud scar. The primary septum has been shown to be rich in chitin and Cabib (1975) suggests that the secondary septum that forms a birth scar on the daughter cell surface may have a similar composition to the remainder of the cell wall.

The septum in C. albicans hyphae is also composed of chitin and is formed by a similar process to that reported in the blastospore form (Gow et al, 1980). Gow et al. (1980) also confirmed the finding of Scherwitz, Martin & Ueberberg, (1978) that the hyphal septum had a central pore 25 nm in diameter which was too small to permit the passage of nuclei or mitochondria but permitted cytoplasmic continuity throughout the mycelium.

In addition to the obvious morphogenetic changes which occur in C. albicans there is evidence that the cell wall of C. albicans blastospores and hyphae may undergo more subtle changes in composition or arrangement as the cell ages. Chattaway, Shenolikar & Barlow (1974) found that blastospores and germ tubes treated with dithiothreitol showed similar patterns of release of saccharides, peptides and acid phosphatase in four hour and eighteen hour old cells. However, after 18h incubation blastospores released proportionately larger amounts of a carbohydrate fraction than hyphae and found that cells 18h old released less material than 4h old cells. Similar results were obtained when release of a glycopeptide fraction was studied. Further studies of the effect of a range of cell wall lytic enzymes also indicated differences between blastospores and hyphae and it was concluded that the bonding association between the major components of the cell wall and the spatial distribution of the macromolecules varied appreciably between the two dimorphic forms and with the age of the fungus (Chattaway et al. 1976a).

Hammond & Kliger (1974) and Cassone *et al*. (1979) have shown that the cell wall of *C. albicans* blastospores thickens during the stationary phase of growth and cytochemical staining of *S. cerevisiae* has shown that it is the innermost layers that appear to increase in thickness.(Linnemans, Boer & Elbers, 1977). Electron microscopy has also shown that the ultrastructure of the *C. albicans* cell wall alters with the age of the fungus. Poulain *et al*. (1978) found that older *C. albicans* blastospores lacked a number of layers present in younger blastospores. They considered the difference in wall structure to be due to loss of labile outer layers presumably as a result of age related changes in the strength of bonding to deeper layers of the wall. Cassone *et al*. (1979) reported that in exponential and early stationary phase blastospores the cell wall of *C. albicans* consists of a number of easily recognizable layers of varying electron density. After prolonged incubation (72h) no electron dense layers were visible in the cell wall. Loss of cell wall material during washing of fungal cells has however been reported (Sikl, Masler & Bauer, 1964) and it is possible that this phenomenon could account for some of the variation noted in the composition of cell walls of different ages. Cassone, Kerridge & Gale (1979) investigated the relationship between cell wall structure and resistance to polyene antifungals in blastospores of polyene-resistant *C. albicans* strains. Walls of exponential phase blastospores and blastospores starved for 24h showed a typical multi-layered appearance. After 72h starvation resistance to amphotericin-methyl ester was sixty times that seen in control exponential phase blastospores and electron-dense glucan and mannan layers of the cell wall were absent from starved blastospores. After 144h starvation wall thickness had increased substantially. Twenty four hours starvation led to a decrease in the glucan content of isolated cell walls although the ratio of glucan to mannan remained constant. Sensitivity to amphotericin-methyl ester and a normal wall structure could be regained by incubating starved blastospores for 1h in fresh medium, indicating the reversible nature of the changes seen. Earlier work on the gross chemical composition of cell walls isolated from exponentially growing and stationary phase *C. albicans* blastospores, failed to show any significant differences between

these two types of blastospore (Kerridge et al. 1976). It is possible that if the changes occurring in the cell wall were simply rearrangements of the existing components then differences in gross chemical composition would not be apparent. It is also again conceivable that during isolation of cell walls some of the fabric of one type of blastospore was lost and differences were therefore not detected in later assays.

Control of C. albicans dimorphism in vivo

As indicated in earlier sections, study of the cell wall compositions of blastospore and hyphal forms of C. albicans and of the enzymes involved in cell wall synthesis has revealed differences which may correlate with the morphology of the fungus. In particular the higher content of chitin in the mycelial form of C. albicans correlates with the reports of higher chitin synthase activity in hyphae. Chitin also forms the major part of bud scars and septa in C. albicans and overall these findings suggest a central role for chitin in the morphogenesis of this fungus.

Studies with other fungi suggest that although the shape of the cell wall determines the shape of the cell, the shape that the cell wall assumes is controlled by some internal mechanism since protoplasts, which are spherical, are able to regenerate and form normal ovoid cells (Necas, 1971). The most recent hypotheses of the control of yeast cell wall structure are based on the concept of regulation of cell wall synthesis in S. cerevisiae through changes in the activities of pre-existing polysaccharide synthases (Farkas, 1979). Cabib, (1975) was the first to consider this possibility when he put forward a model for septum formation in S. cerevisiae which involved both temporally and spatially controlled activation of membrane-bound chitin synthase. Farkas (1979) extended this concept to consider yeasts and filamentous fungi. His hypothesis suggested that once cell wall synthesis was initiated the shape assumed by newly synthesized wall material was controlled by the concentration of cytoplasmic inhibitors of polysaccharide synthases at the growing apex of the cell. If the concentration of cytoplasmic inhibitors of synthase enzymes was high at the apex of this "primary growth

zone" rapid inactivation of the active synthase enzyme would occur leading to a gradient of decreasing activity away from the apex. The highest level of synthase activity would be present at the apex and resulting growth would be in the form of a tube or hypha. If however, the concentration of synthase inhibitors was low at the apex, all active synthase would remain active and spherical growth of the bud would occur.

The data for C. albicans in general agree with this hypothesis. At least two cell wall polysaccharide synthase enzymes are located on the plasmalemma (Marriott, 1975; Braun & Calderone, 1978) and an activator and inhibitor of chitin synthase have also been reported (Braun & Calderone, 1979). In addition autoradiographic study has shown chitin to be synthesised predominantly at the bud tip or apex of growing hyphae (Braun & Calderone, 1978). A similar but alternative hypothesis of the control of cell wall shape of C. albicans, which takes into account the effect of environmental factors on C. albicans dimorphism has also been suggested (Odds, 1979). According to this theory, cell wall synthesis involves two stages; a soft, primary cell wall is first laid down and is later rigidified. Differences in cell wall shape are determined by the rate at which this rigidification process occurs. Under conditions favouring germ tube synthesis, cell wall rigidification would be rapid, with the result that continued growth occurred only at the apex and new growth appeared in the form of a cylinder. At lower incubation temperatures the immature wall remains soft for longer periods and allows the cells to swell to a blastospore shape under cytoplasmic turgor pressure. According to this hypothesis further material could be added to the cell wall once the original wall was rigidified by insertions of new cell wall material behind the growing bud or hyphal apex. This latter point could account for the suggestions that the structure and arrangement of the cell wall changes as the cell ages (Chattaway, Shenolikar & Barlow, 1974; Chattaway et al. 1976a).

The hypotheses explain how the latter stages of cell wall formation may be controlled but do not account for all the observations that have been made for dimorphic changes in C. albicans.

C. albicans morphology is known to be affected by a very wide range of environmental factors. Some of these factors may be postulated to have a direct effect in the morphology of C. albicans. The chitin content of C. albicans hyphae is known to be higher than that of the blastospore form and the induction of germ tube synthesis by N-acetyl-D-glucosamine could simply be related to an increase in the supply of substrate available for chitin synthesis. However although the activity of the enzymes involved in N-acetyl-D-glucosamine metabolism increase in activity in the presence of this compound, germ tube synthesis only occurs at the relevant incubation temperature suggesting the complex nature of the mediation of even apparently obvious morphogenetic effects.

Other workers have suggested that cyclic nucleotides may be important in mediating the effects of culture medium components particularly peptides, on C. albicans morphology and such compounds could conceivably control metabolic branch points significant in cell wall synthesis.(Chattaway et al. 1981). But for the majority of environmental factors known to influence dimorphism no explanation of the mechanism of action has been prepared.

Other studies have shown that C. albicans germ tube production is restricted to blastospores in the late logarithmic or stationary phases of growth (e.g. Strippoli & Simonetti, 1971), yet C. albicans blastospores can generate daughter blastospores at all phases of growth, suggesting differences in the mechanisms by which these two processes are controlled. Since stationary phase blastospores are able to produce germ tubes or buds under suitable growth conditions, changes in the ability of blastospores to produce germ tubes presumably occur after growth has started. Germ tube synthesis, as evidenced by substrate incorporation studies (Braun & Calderone, 1978; Chiew, Sullivan & Shepherd, 1980) requires larger quantities of precursors than blastospore budding and it is possible that in actively growing blastospores, substrate for cell wall synthesis may be metabolised to produce energy for other growth processes. This argument assumes that some pre-set level of cell wall substrate is required before germ tube synthesis is initiated and is supported

by the findings of Mattia & Cassone (1979) who reported that germ tube synthesis could be induced in C. albicans blastospores at any phase of the growth cycle if the inducing medium contained N-acetyl-D-glucosamine.

Alternatively such data could be interpreted to suggest genetic control over cell wall synthesis and morphogenesis in C. albicans. At the phase of the cell cycle corresponding to late logarithmic or stationary phase, blastospores may be stimulated by environmental factors to produce blastospore buds or germ tubes. Once growth is re-initiated and blastospores enter logarithmic phase, predetermined, genetically controlled events could occur which allowed bud formation but which prevented germ tube synthesis. Such effects could be mediated through enzyme pathways controlling the supply of substrate to polysaccharide synthases or through changes in the activities of synthase activators and inhibitors and could be relieved by the addition of exogenous cell wall precursors such as N-acetyl-D-glucosamine.

Further support for genetic control of dimorphism in C. albicans comes from studies that have shown that once blastospore budding or germ tube production is initiated, the fungus is committed to that mode of development and becomes resistant to changes in the environment (e.g. Evans et al. 1975a). Environmental stimuli could be envisaged to 'trigger' blastospores, in the correct metabolic and genetic state, to initiate a predetermined course of events leading to germ tube or bud synthesis.

Evidence has also accumulated to suggest differences between budding and germ tube production in the selection of the site, on the parent blastospore wall, of initiation of new cell wall synthesis during these events. Mackenzie (1964) reported that buds in C. albicans were almost invariably produced at the pole of the mother cell, whereas germ tubes may be produced at any point on the blastospore surface. These findings have been confirmed by other investigators (Joshi, Gavin & Wheeler, 1973). Gooday (1977) suggests that autolytic enzymes such as chitinase may release substrate at certain sites on the parent cell wall and that new cell wall synthesis could then be initiated at these sites. Although chitin may be found in lateral

cell walls of C. albicans blastospores, the majority of chitin is found in septa and bud scars (Tronchin et al. 1981). The concentration of substrate released by chitinase at these sites could initiate localised synthase activity. This could explain the distribution of buds at the poles of mother cells, adjacent to earlier bud scars and for the observation that in hyphae secondary blastospores and hyphal branches are formed adjacent to septa (Evans et al. 1975a). In new buds, progeny would be produced adjacent to the parent birth scar.

It is difficult to envisage the initiation of germ tube synthesis in these terms since the distribution of germ tubes on the surface of blastospores appears random (Mackenzie, 1964). Chitin is present in lateral cell walls of C. albicans (Tronchin et al. 1981) and the activity of chitinase at random sites on the cell wall could initiate germ tube synthesis. Such activation would require temporary inactivation of the cytoplasmic inhibitors of chitin synthase at these sites and since germ tube production has been shown to be controlled by environmental factors some metabolic mediation of such factors is conceivable. The more random nature of germ tube initiation is also suggested by the observation that blastospores may initiate two or three germ tubes within a short time, (Shepherd et al, 1980) whereas blastospores invariably complete growth of one bud before they initiate another.

Clearly then the processes involved in the regulation of cell wall synthesis in relation to dimorphism are complex, involving interaction between genetic, metabolic and environmental factors which as yet, are poorly understood. Many observations have yet to be explained, for example the variation in dimorphic development of different strains of C. albicans e.g. Manning & Mitchell, 1981 and the lack of filamentous development in several other pathogenic Candida species. The available evidence suggests however that further study of the enzymes involved in polysaccharide synthesis and their regulation in relation to dimorphism may explain some of these observations.

1.5 Dimorphism as a virulence factor in vitro

Although it has proved difficult to establish unequivocally the relative virulence of the various morphological forms of C. albicans in vivo, studies of dimorphism in vitro in relation to some aspects of virulence have indicated the possible significance of dimorphism in the pathogenesis of candidosis. In particular, some authors have postulated a role for C. albicans hyphae in attachment of the fungus to mammalian cells, in cytotoxic effects and in resistance to phagocytosis by leukocytes.

One of the prerequisites for initiation of many microbial infections is the ability of a pathogen to attach to host tissues (Mims, 1982). Kimura & Pearsall (1978), King, Lee & Morris (1980) and Sandin et al. (1982) have reported adherence of C. albicans to human epithelial cells in vitro. Kimura & Pearsall (1980) found that C. albicans germ tubes adhered in larger numbers than blastospores to epithelial cells and that in mixtures of the two morphological forms germ tubes attached in larger numbers, suggesting some kind of selective attachment mechanism. However, since germ tubes have a larger surface area per unit volume than blastospores, greater attachment of the former is perhaps not surprising. Moreover, several published photographs showing germ tubes attaching to epithelial cells suggest that it is the blastospore part of the fungus which is responsible for the attachment. Nevertheless, King, Lee & Morris (1980) showed that C. albicans blastospores adhered better to human mucosal cells than other Candida species and that variations in their ability to attach to epithelial cells could be partly responsible for the observed differential virulence of Candida species. Maisch & Calderone (1981) examined the ability of Candida species to adhere to fibrin clots in vitro. C. albicans blastospores were shown to attach in larger numbers than blastospores of other Candida species. Clearly there are differences in the ability of different Candida species to attach to mammalian cells, but the extent to which this relates to hypha formation is uncertain.

The cytopathic effect of various Candida species on cultured mouse epithelial cells could be related directly to the ability of the test species

to produce hyphae (Stanley & Hurley, 1967; Hurley & Stanley, 1969). Partridge, Athor & Winner (1971) found that C. albicans was the most pathogenic of six Candida species tested in a chick embryo pathogenicity assay. A possible role of hypha formation in the pathogenesis of this species was again suggested.

One further aspect of the pathogenesis of candidosis in which dimorphism may be involved, the interaction of the fungus and phagocytic cells, has also been investigated in vitro.

Interaction of C. albicans and Phagocytic cells in vitro

One of the major defence mechanisms of a host is the system of circulating and fixed phagocytic leukocytes which are important in removing foreign particulate matter from the bloodstream and other tissues. Louria & Brayton (1964) showed that C. albicans was able to withstand intracellular killing within human polymorphonuclear leukocytes (PMN s) and was able to grow out of the PMN's by producing germ tubes. C. tropicalis and C. guilliermondii, which were unable to produce germ tubes, were unable to escape intracellular killing in the same way. Stanley & Hurley (1969) showed that C. albicans and other Candida species were readily phagocytosed by mouse peritoneal macrophages in vitro but were not killed by these cells. The macrophages containing phagocytosed fungi died, and the death rate was apparently related to the ability of the species to produce filamentous forms.

More recent studies have supported the view that germ tube production is an important attribute of C. albicans in the resistance to intracellular killing by phagocytic cells. De Brabander et al. (1980) reported that inhibition of germ tube production by treatment of C. albicans blastospores with ketoconazole increased the efficiency of killing of C. albicans by guinea pig neutrophils and peritoneal macrophages. The concentration of ketoconazole used in these experiments did, however, markedly reduce the growth rate of the C. albicans strains used and this may have affected the results obtained.

Since these early experiments many studies of the interaction of C. albicans and phagocytic cells have been carried out, the majority of them

concerned with the phagocytosis of blastospores. The ability of different types of phagocytic cells from different animals and anatomical sites to phagocytose and kill ingested fungi, the role of serum factors and cell mediated immunity on these processes and the mechanisms by which ingested fungi are killed have all been investigated.

Differences in phagocytosis and killing of C. albicans by phagocytic cells from different sources

Lehrer & Cline (1969a) were the first workers to suggest that different types of phagocytic leukocytes varied in their ability to phagocytose and kill C. albicans blastospores. Human neutrophils were shown to be more efficient at phagocytosis and killing than human monocytes. Stanley & Hurley (1969) also showed that mouse peritoneal macrophages, although capable of phagocytosing C. albicans and other Candida species, lacked the ability to kill the ingested fungi. Several recent studies support this view (Arai, Mikami & Yokoyama, 1977; Kagaya & Fukazawa, 1981). However other reports have suggested that monocytes and tissue macrophages, from a variety of sources, are capable of killing phagocytosed Candida (Lehrer, 1975; Lehrer et al. 1980; Maiti, 1980).

Variations in the results obtained by different workers may reflect differences in the source of the leukocytes used, particularly in the animal chosen and the site from which the phagocytic cells were obtained. Different animal species vary in their susceptibility to experimental C. albicans infection (Odds, 1979) and variation in the results of assays for phagocytosis and killing in vitro, may reflect such differences. Lehrer et al (1980) have shown that macrophages obtained from different anatomical sites may vary in their phagocytic and killing ability. Rabbit alveolar macrophages were better at killing ingested C. albicans blastospores than were rabbit peritoneal macrophages.

There have been two main approaches to the measurement of phagocytic effects. In the first, Candida and phagocytic cells are allowed to interact

in suspension under continuous agitation. In the second method phagocytic cells, principally macrophages, are allowed to adhere to a solid surface (usually glass coverslips) before a suspension of the fungus is placed on top of the phagocytic monolayer. The ratio of Candida to phagocytic cells is known to affect the efficiency of phagocytosis (Walters & Papadimitriou 1978) and variations in the composition of the phagocytic monolayers obtained could affect the ratio and the reproducibility of assays performed using this method.

In addition the system used to assess the extent of phagocytosis and killing in vitro may affect the results obtained. Phagocytosis has previously been assessed in one of two ways. In the first, the proportion of phagocytic cells that have ingested the fungus is estimated microscopically; in the second the proportion of a known quantity of the fungus that has been ingested by phagocytic cells is determined. Assessment of ingestion in the second case relies on visual or viable counts or on some form of radiometric assay. The results of both these methods of assessment are usually expressed as "percentage phagocytosis" but clearly they represent two different parameters and comparison between the two is impossible. With high Candida : Phagocytic cell ratios the first assay method would produce high values for phagocytosis whereas the second would produce lower results.

Intracellular killing has been assessed by radiometric means, staining of non-viable Candida with methylene blue or by plate counts of viable fungi. Table 1:3 lists the methods used and the results obtained by various workers. The variations in Candida : phagocytic cell ratios, times of incubation and methods used to assess phagocytosis and killing make interlaboratory comparison of the results obtained difficult if not impossible. It is also clear that even when workers have used almost identical assay conditions results may not be in close agreement e.g. Yamamura & Valdimarsson, 1977 and Ferrante & Thong, 1979, suggesting a substantial lack of reproducibility of the system.

Legend

Ratio = Ratio of Candida blastospores to phagocytic cells

c/s = assays where phagocytic cells are tested as a monolayer } Incubation time given in h

susp = assays where phagocytic cells are tested in suspension } Incubation time given in h

V = phagocytosis assessed visually by counting the % of a given quantity of blastospores phagocytosed

V* = phagocytosis assessed visually by counting the number of blastospores per 100 phagocytic cells

V† = phagocytosis assessed visually by counting the percentage of phagocytic cells containing blastospores

ME = killing assessed by methylene blue uptake

PC = killing assessed by viable plate counts

GC = killing assessed visually by counting percentage of ingested blastospores appearing as ghosts

Label = Phagocytosis and killing assessed by radiometric assay

P = Phagocytosis

K = killing

Table 1:3

C. albicans blastospore phagocytosis and intracellular killing assays

Ref	Phagocytic Cells	Ratio	Incubation	Assay	Results % P	Results % K
113.	Human monocytes	2:1	c/s 0.5 h	V/MB	790	4
	Human neutrophils	1:1	susp 1 h		790	29
240.	Human monocytes	1:1	susp 1 h	V	96	50
	Human granulocytes	"	" "		96	58
233.	Human neutrophils	10:1	susp 0.5 h	Label	56	48
1.	Rabbit alveolar macrophages	10:1	susp	V/MB	37	0
	Guinea Pig neutrophils				37	44
239.	Rabbit peritoneal macrophages	2:1	susp 1 h	V*/MB	86*	0.45
	Rabbit alveolar macrophages				65*	0.42
185.	Human neutrophils	1:1	susp 1 h	V?/MB	92	10
65.	Human neutrophils	10:1	susp 0.5 h	Label	47-90	–
115.	Rabbit alveolar macrophages	5:1	susp 1 h	V†/	–	30
	Rabbit peritoneal macrophages	"			–	11
184.	Mouse peritoneal macrophages	0.2-2:1	c/s 3 h	V/PC	–	<15%
206.	Mouse peritoneal macrophages	1:1?	c/s 1-4 h	V/–	?	?
144.	Mouse peritoneal macrophages	2:1	c/s 1 h	V†/–	85	–
	Mouse neutrophils				84	–
174.	Human neutrophils	10:1	c/s 0.25 h	V†/PC	36	20-40
	Mouse neutrophils				45	10-20
86.	Mouse peritoneal macrophages	1:1	P susp 1 h	V/MB	95	5
	Mouse neutrophils	"	K c/s "		95	9
241.	Mouse peritoneal macrophages	1:1-10:1	c/s 0.5-1h	V†/GC	91	5
238.	Mouse peritoneal macrophages	10:1	c/s 0.5 h	V†	60-83	–

The role of serum factors in phagocytosis and killing

Although phagocytic cells may phagocytose particulate antigens in the absence of serum, the efficiency of phagocytosis is increased by opsonisation of the antigen by antibody or complement (Roitt, 1974). The requirement for opsonisation of C. albicans prior to phagocytosis was first demonstrated by Lehrer & Cline (1969a). These investigators showed that serum was required for the phagocytosis of C. albicans by human neutrophils and reported reduced phagocytosis in complement-depleted human serum. The extent of killing of the ingested fungus was similar in control and complement-depleted serum and it was therefore concluded that complement was not required for the intracellular killing of the fungus. Ferrante & Thong (1979) concluded that both heat labile (complement) and heat stable (antibody) opsonins were required for optimal phagocytosis of C. albicans blastospores by human PMN s. In experiments where individual stages of the complement cascade were selectively inhibited, Morrison & Cutler (1980) reported maximal phagocytosis, by mouse peritoneal macrophages and PMN s in mouse serum containing an intact complement system. They assessed phagocytosis in terms of proportions of Candida-containing phagocytic cells. Peritoneal macrophages showed an intermediate level of phagocytosis in the presence of sera in which complement component C_3 was intact but in which the alternative complement pathway was inactive. This serum supported phagocytosis by mouse PMN s no better than when serum was omitted from the test system. Serum depleted of C_3 failed to support phagocytosis by mouse peritoneal macrophages or PMN s.

Other workers have suggested, contrary to the findings of Lehrer & Cline (1969a), that complement plays a major role, not only in phagocytosis but also in the killing of ingested fungi. Yamamura & Valdimarsson (1977) showed that C_3 was necessary for the intracellular killing of C. albicans by human PMN s. Bridges et al. (1980) considered complement to be important only in relation to killing since human PMN's phagocytosed C. albicans equally well in the presence of C_3-depleted and control human serum. In the earlier study of Lehrer & Cline (1969a) intracellular killing was assessed by staining

non-viable Candida blastospores with methylene blue, whereas the latter studies measured killing by radiometric means. It seems likely that the radiometric assay may be more sensitive and less subjective than methylene blue uptake as a measure of intracellular killing, and differences in the assay systems used could explain the variation in results obtained.

Importance of aquired immunity in phagocytosis and killing of C. albicans

Grappel & Calderone (1976) reported that specific anti-Candida antibody inhibited growth and germ tube production by C. albicans blastospores. These workers suggested that inhibition of filamentation by specific antibody could be important in prevention of dissemination of C. albicans throughout a host. If specific antibody were able to prevent germ tube production by phagocytosed blastospores C. albicans could be more readily killed by the phagocytic cell since its ability to escape killing by producing germ tubes would be impaired.

Reports concerning the effect of specific anti-Candida antibody on phagocytosis and killing vary in their conclusions. Morrison & Cutler (1980) and Kagaya & Fukazawa (1981) did not consider immune mouse or rabbit serum to support phagocytosis and killing of C. albicans to any greater extent than control serum. In contrast Arai, Mikami & Yokoyama, (1977) showed that immunization of rabbits with C. albicans increased the rate of phagocytosis by peritoneal macrophages and attributed this increase to an opsonisation effect by serum antibody. No specific evidence that this increase in phagocytosis was antibody mediated was presented and the possibility that the increase in phagocytic activity could be cell-mediated was not considered. Maiti (1981), however, found an increase in phagocytosis by mouse peritoneal macrophages in the presence of immune mouse serum.

Several investigators have examined the effect of immunizing animals with C. albicans prior to obtaining phagocytic cells for assay. Immunity aquired after immunization may be antibody and/or cell-mediated, involving T lymphocytes. T lymphocytes may be stimulated by antigen or non-specific mitogens, in vivo or in vitro to produce lymphokines. Lymphokines have a range of

activities including activation of macrophages resulting in an enhanced phagocytic ability (Roitt, 1974). Although cell-mediated immunity may be playing a role in enhancing the activity of phagocytic cells obtained from immunized animals, some investigations have failed to differentiate between effects due to humoral or cell-mediated immunity or to provide specific evidence that one system is involved to the exclusion of the other, e.g. Arai, Mikami & Yokoyama, 1977. It is not perhaps surprising then that the results of investigations of phagocytosis and killing of phagocytic cells obtained from immune and normal animals have produced variable results.

Arai, Mikami & Yokoyama, (1977) reported that immunization had no effect on the degree of phagocytosis and killing by C. albicans by guinea pig neutrophils. The efficiency of phagocytosis but not killing by rabbit macrophages was however increased by immunization. Evron (1980), however, found little difference in the rate of phagocytosis of C. albicans by peritoneal macrophages immunized or infected with C. albicans but found that the rate of outgrowth of ingested blastospores was lower in peritoneal macrophages obtained from infected animals. Sasada & Johnston (1980) used BCG or LPS to stimulate a cell-mediated response in mice. Peritoneal macrophages obtained from treated animals killed C. albicans blastospores better than resident macrophages. Immunization of rabbits with Freunds complete adjuvant enhanced the ability of peritoneal macrophages to kill ingested C. albicans blastospores, the increase being more marked in these cells than in alveolar macrophages.

Other investigators have used lymphokines, produced by exposure of mixed lymphocyte populations to C. albicans or non specific mitogens in vitro, to activate macrophages obtained from normal animals.

Activated mouse peritoneal macrophages, obtained by stimulation of normal macrophages by phytohaemagglutinin-induced lymphokines in vitro, gave higher phagocytic and candidicidal activities than normal cells (Maiti 1981). Exposure of macrophages to lymphokines derived from lymphocytes stimulated by C.albicans blastospores gave the best results. Similarly Kagaya, Shinoda & Fukazawa

(1981) reported that stimulation of mouse peritoneal macrophages in vitro, by lymphokines obtained by concanavalin A treatment of lymphocytes, enhanced the killing ability of mouse macrophages.

Morrison & Cutler (1981), however, found that phagocytic cells from normal and nude athymic mice behaved similarly in their assay system, suggesting that innate immunity may be important in phagocytosis and killing of C. albicans. Results of experiments with animal models have suggested that innate immunity may be important in preventing systemic Candida infections and presumably blood and tissue phagocytic cells may play a part in this process (Rogers & Balish, 1976; Lee & Balish, 1981).

Killing Mechanisms

Once phagocytosed, intracellular fungi are exposed to a number of enzymatic killing mechanisms. Clearly any factor that allows the fungus to avoid these systems and escape killing may be an important attribute of fungal virulence. Lehrer (1969) reported the presence of a halide-dependent myeloperoxidase in human PMN s which was capable of killing several Candida species and other fungi. Human monocytes contained less of this enzyme. Neutrophils and monocytes from a patient suffering from disseminated candidosis, although able to phagocytose C. albicans, were unable to kill the fungus and this finding correlated with a deficiency of this myeloperoxidase enzyme in the leukocytes (Lehrer & Cline, 1969b). Further studies showed that C. albicans was less readily killed than other Candida species by human neutrophils and studies with myeloperoxidase-deficient neutrophils suggested the presence of a second, myeloperoxidase-independent killing mechanism (Lehrer, 1972). Similar myeloperoxidase linked and independent killing mechanisms have been reported for human monocytes (Lehrer, 1975). The killing of C. albicans by rabbit alveolar and peritoneal macrophages was unaffected by several myeloperoxidase inhibitors and again a second fungicidal mechanism was implicated (Lehrer et al. 1980). Sasada & Johnston (1980) concluded that efficient mouse macrophage candidicidal activity depended on the

generation of oxygen metabolites by the phagocytic cell. C. parapsilosis stimulated a stranger oxidative metabolic burst than C. albicans in these cells and was killed more efficiently than the more pathogenic species. Kagaya & Fukazawa (1981) showed that lymphokine-activated macrophages possessed marked intracellular killing activity and they detected increased superoxide anion and singlet oxygen levels in the absence of increased myeloperoxidase production. They concluded that an oxidative mechanism plays a major role in the killing of C. albicans by mouse macrophages.

Peterson & Calderone (1978) have also shown that lysosomal extracts of rabbit alveolar macrophages are capable of killing C. albicans blastospores and selectively inhibiting the uptake of certain amino acids, and that this ability may lead to nutrient starvation and eventual death of the fungus within phagosomes.

Lehrer (1972) reported that C. albicans was less readily killed than other Candida species by human neutrophils. More recently Richardson & Smith (1981) have shown differences in the extent to which blastospores of attenuated and virulent strains of C. albicans are killed by human and mouse PMN s. Although blastospores of virulent and attenuated strains of C. albicans were phagocytosed to the same extent, blastospores of virulent strains of C. albicans were less readily killed than blastospores of attenuated strains. These strains were shown to vary in their ability to produce germ tubes in serum and in their virulence for mice (Richardson & Smith, 1981b) but it is not clear from the results presented if the strains tested varied in other phenotypic characters which could be important in resistance to intracellular killing by PMN s, such as growth rate. Richardson & Smith also noted that the progeny of blastospores of attenuated strains of C. albicans that survived intracellular killing in PMN s were then more resistant to intracellular killing than the parent strain, suggesting heterogeneity within the blastospore population. A correlation between germ tube production, virulence for mice and the ability to resist intracellular killing inside PMN s is therefore implied from these results.

Studies of the phagocytosis and killing of C. albicans germ tubes and hyphae

The majority of the studies of phagocytosis and killing of C. albicans have been concerned with blastospores but these studies have often suggested that the ability of C. albicans to produce germ tubes inside phagocytic cells may be a mechanism that allows the fungus to escape intracellular killing. However very few studies have compared rates of phagocytosis and killing of pre-formed germ tubes or hyphae, which are usually found together with blastospores in lesions in vivo (Rogers, 1966).

Technically, assays for phagocytosis and killing of blastospores are simpler than those for germ tubes and hyphae. Quantification of fungal biomass for use in these assays and assessment of the extent of phagocytosis by visual means are two major problems.

Scherwitz & Martin (1979) concluded that pre-formed germ tubes were less readily phagocytosed and killed by human PMN s. However since the biomass of the fungus may change during germ tube production, their results could be attributed to an increase in biomass, and not to a change in cell morphology. In addition the visual means of assessment of phagocytosis used in this study must be subjective since the size of the preformed germ tubes used would preclude total phagocytosis by PMN s Diamond, Krzesicki & Jao, (1978) showed that human PMN s were able to attach to C. albicans pseudohyphae and damage them in the absence of serum. The degree of damage was however greater in the presence of serum. These workers used an objective radiolabel assay to assess phagocytosis and killing of C. albicans blastospores and hyphae but they did not compare the extent of phagocytosis and killing of blastospores and hyphae of similar biomass. Davies & Denning (1972) reported that C. albicans hyphae up to two hundred μm long were killed in the presence of whole human blood, presumably by the action of phagocytic cells. The assessment of killing in this study was based on viable counts, a procedure which has been shown to be unreliable when applied to C. albicans hyphae in the presence of serum (Chilgren, Hong & Quie, 1968). More recently Cutler & Poor (1981) studied the phagocytic killing of C. albicans blastospores and

hyphae inside semi-permeable plastic chambers implanted in the peritoneal cavities of mice. Using a viable counting assay system these investigators showed that C. albicans hyphae were more resistant to killing by mouse neutrophils than were blastospores, but failed to take into account the relative biomass of the fungal suspensions used to assess phagocytic killing.

Chemotaxis of phagocytic cells towards C. albicans

Some microbial pathogens produce substances during growth which may be chemotactic to phagocytic cells. In addition chemotactic factors may be liberated during interaction of antigen and complement in vivo (Roitt, 1974; Mims, 1982). Chemotactic substances may attract phagocytic cells to the site of infection and may therefore be important in preventing the spread of the pathogen. Denning & Davies (1973) found that C. albicans blastospores and hyphae were chemotactic for human PMN's and that when a similar biomass of blastospores and hyphae was tested, blastospores attracted a significantly larger number of PMN's than hyphae. A mannan-rich cell wall fraction isolated from C. albicans blastospores was shown to be the major chemotactic component of the fungus. The chemotactic properties of the mannan-rich glycoprotein of C. albicans have also been demonstrated by other workers (e.g. Ray et al. 1979). Denning & Davies (1973) showed that PMN's did not migrate towards C. albicans in the absence of serum and concluded that complement was important in chemotaxis. Cutler (1977), however, showed that C. albicans blastospores were chemotactic for guinea pig neutrophils in the absence of serum and concluded that serum proteins, other than complement, were the important components involved in the chemotactic response. Diamond et al.(1980) isolated a product from C. albicans hyphae which affected the chemotactic response of human neutrophils. Although the C. albicans cell product was not itself chemotactic, neutrophils pre-incubated with the product showed little or no chemotaxis towards zymosan-activated serum. The presence of a neutrophil-chemotaxis inhibitor in C. albicans hyphae could explain the difference in chemotactic response of human PMN's towards blastospores and hyphae noted by Denning & Davies (1973).

1:6 Objectives of the present study

Although dimorphic shifts in C. albicans have been extensively studied, the mechanisms by which such morphological changes are initiated and controlled are still largely unknown.

Many investigations of dimorphism in vitro, have concentrated on the initial aspects of germ tube synthesis and blastospore budding and little attention has been given to the factors that control later stages in development such as secondary blastospore production and hyphal branching. This is due, in part, to the difficulty encountered by many workers in maintaining C. albicans in a purely mycelial form for any length of time.

Since the shape of the C. albicans cell is governed by the rigid polysaccharide-based cell wall, many workers have investigated the composition and synthesis of the component polymers in attempts to identify differences between blastospore and hyphal forms of C. albicans that could be related to morphology. Such studies have indicated differences in cell wall composition between C. albicans blastospores and hyphae but have concentrated on the composition of cells isolated at a single, sometimes unspecified time. Indirect evidence does however suggest that the cell wall composition of C. albicans blastospores and hyphae may change with the age of the cells. Since cell wall components, in particular cell wall mannan, have a wide range of biological properties, possibly related to virulence of C. albicans, changes in the cell wall composition of C. albicans cells, in relation to the age and morphology of the fungus may be important in the pathogenesis of candidosis.

Although experiments designed to test the relative pathogenicity of the various morphological forms of C. albicans, in vivo, have given contradictory results, studies of the interaction of C. albicans and host tissues in vitro suggest that dimorphism may be important in this respect. In particular the ability of C. albicans to produce germ tubes inside phagocytic cells suggests that this process may be important in enabling

the fungus to escape killing by host defence mechanisms and to persist in host tissues. Little objective study of the phagocytosis and intracellular killing of pre-formed germ tubes and hyphae has, however, been made. Differences in the extent of phagocytosis and/or intracellular killing of C. albicans blastospores, germ tubes and hyphae, all of which have been found together in C. albicans lesions in vivo, could indicate differential virulence of one form over another.

The objectives of the present study were therefore:

1. To devise morphological models in which C. albicans cells of a desired morphology could be reproducibly obtained.
2. To investigate, in these models, the environmental factors involved in the control of the initiation and subsequent development of morphology in C. albicans.
3. Investigation of the relationship between morphology and age of C. albicans cells and their content of the polysaccharide components of the cell wall and the enzymes involved in cell wall synthesis.
4. Investigation of the interaction of C. albicans blastospores, germ tubes and hyphae with phagocytic cells in vitro, to determine if any correlation between cell morphology and possible differential virulence in vivo could be determined.

CHAPTER 2

The effect of environmental factors on C. albicans dimorphism in vitro

2:1 Introduction

Since the morphology of C. albicans has been shown to be affected by a range of environmental parameters including culture medium composition and incubation conditions, and has also been shown to be a strain-dependent phenomenon, the first objective of a study of dimorphism of C. albicans in vitro must be to define these parameters to enable reproducible production of the desired morphological form. To enable comparison of the morphology of C. albicans under different growth conditions, a method of quantifying the morphology of C. albicans cultures must also be devised.

In studies of hyphal formation by C. albicans in vitro, investigators have used one of two model systems. In the first the hyphae that develop initially from blastospores later revert to a predominantly blastospore mode of growth by production of lateral buds (Evans et al. 1975a, Evans et al. 1975). In the second model the fungus develops lateral hyphal branches and the mycelium shows little or no tendency to reversion to the blastospore form (Lee et al. 1975; Schwartz & Larsh, 1980). The difficulties in achieving the second model reproducibly are exemplified by the number of reports of different growth media that support it (Yamaguchi, 1974; Lee et al. 1975; Schwartz & Larsh, 1980; Manning & Mitchell, 1981) and by suggestions of unrepeatability, usually attributed to strain variations (Manning & Mitchell, 1981).

Several authors have used tissue culture media to grow the mycelial form of C. albicans. Such media are more likely to resemble environments in vivo than peptone broths and minimal synthetic media, allowing establishment of a better correlation between cellular morphology and pathogenicity. Notably, Borgers et al. (1979) used a tissue culture medium incubated statically under 5% CO_2 in air to achieve a fully hyphal growth of C. albicans.

Although the environmental factors involved in the initiation of germ tube formation in these model systems has been extensively studied, little consideration has been given to the effect of these conditions on the subsequent development of hyphae, in relation to hyphal branching and production of secondary blastospores. Comparison of the morphology of C. albicans under varying environmental conditions in the model systems described by Evans et al. (1975) and Borgers et al. (1979) could indicate the significance of individual environmental factors in the control of these later morphogenetic changes.

Earlier studies have shown that low O_2 tension (Skinner, 1947) or a high CO_2:O_2 ratio (Mardon, Balish and Philips, 1969) favoured mycelium production by C. albicans. The extent to which a culture is shaken, would presumably affect the gaseous composition of the growth medium since shaken cultures would be more aerated than static cultures. In addition, the composition of the growth medium itself is known to affect C. albicans morphology. Although EMEM as used by Borgers et al.(1979) is a chemically defined medium, its composition is complex and the relative importance of individual components of the medium in controlling C. albicans morphology is difficult to assess. Other workers have used simpler chemically defined media to produce C. albicans mycelia (e.g. Marriott, 1975) and such an approach may permit better correlations between medium composition, environmental conditions and C. albicans morphology to be established.

In another approach to the elucidation of the control mechanism of dimorphism in C. albicans, other workers have studied the effect of metabolic inhibitors on the morphology of the fungus in an attempt to identify compounds that affect fungal morphology but not fungal growth (e.g. Shepherd et al. 1980x). This latter consideration is important since changes in growth rate may indirectly or directly affect the morphology of the fungus. Nickerson (1948 &1963) postulated that mycelium production involved an uncoupling of cell division (i.e. cell separation) from cell growth. Recent studies have shown that germ tube production in C. albicans

does not require DNA synthesis, thus supporting this hypothesis (Yamaguchi, 1974; Wain et al. 1976). Compounds which could affect C. albicans morphology in the absence of inhibition of growth could therefore be useful in the study of dimorphism. If dimorphism were an important factor in the pathogenesis of candidosis such compounds could be conceivably useful in the treatment of C. albicans infections. One such group of compounds that have been investigated in this respect are the imidazole antifungals.

Van den Bossche (1974) showed that low concentrations of miconazole (10^{-7} or 10^{-8} M, 48 or 4.8 $ngml^{-1}$) selectively inhibited the uptake of a range of metabolites by C. albicans in the absence of any significant inhibition of fungal growth. Although glucose uptake was not inhibited, uptake of glutamine, which may be involved in cell wall chitin synthesis, was reduced. De Nollin & Borgers (1974) showed that similar concentrations of miconazole affected the ultrastructure of C. albicans and suggested that miconazole was affecting the Candida cell wall and plasmalemma, both of which may be important in determining fungal shape. The minimal effect of low concentrations of miconazole on the growth rate of C. albicans was re-emphasized by Van den Bossche, Willemsens & Van Cutsem (1975) who found that doubling times of C. albicans in the presence of 10^{-7} or 10^{-8}M miconazole were only 11 and 6 minutes longer, respectively, than in control cultures. Borgers et al. (1979) reported that another imidazole derivative, ketoconazole, completely inhibited germ tube production in C. albicans at a concentration of 10^{-8}M (5.3 ng ml^{-1}). Growth rates of control and ketoconazole-treated cultures were not however reported to confirm that the effect described was specific to morphology as opposed to growth inhibition.

This chapter describes the establishment of a series of morphological models in which C. albicans cells of a desired morphology could be reproducibly produced and a method by which C. albicans morphology could be quantified. The effect of a range of incubation conditions and of medium composition on C. albicans morphology was also investigated. The effects of ketoconazole, on the morphology and growth of C. albicans were also invest-

igated in the context of environmental effects on morphology since an antifungal that specifically inhibits development of C. albicans in its mycelial form(s) could be useful for experimental generation of different morphological forms without manipulation of the growth environment.

2:2 Methods

C. albicans - maintenance and preparation of inocula

All isolates were originally obtained from clinical specimens.

The fungi were maintained at room temperature on Sabourauds dextrose agar (Oxoid Ltd).

Blastospore form inocula were prepared in Sabourauds dextrose broth (SAB): mycological peptone (Oxoid), 10g 1^{-1}, D-glucose (BDH Ltd) 40g 1^{-1} in H_2O, incubated over night at 30 ^{o}C in an orbital shaker set at 160 rev:min^{-1} The fungi were harvested by centrifugation at 2500g for 5 min, resuspended and washed twice in sterile water. Blastospore concentrations were estimated by haemocytometer counts and C. albicans was added to growth media in all experiments to give an initial concentration of 10^6 blastospores ml^{-1}.

Media for study of morphogenesis

Modified Sabourauds broth (MSAB), mycological peptone 10g 1^{-1}, glucose 2g 1^{-1} in H_2O (Evans et al. 1975) and Eagles minimal essential medium (EMEM; with Earle's salts and L-glutamine, Gibco Bio Cult Ltd) were the basic media used. The former is reported to stimulate hyphal formation with reversion to blastospore production at 40 ^{o}C, and the latter branched hyphal growth of C. albicans. MSAB is also known to support blastospore growth at the lower incubation temperature of 30^{o}C. As media were incubated under different gaseous atmospheres during these studies, they were buffered appropriately to ensure all cultures had an initial pH of 7.3 to 7.4 at the time of

inoculation, after temperature equilibration in the relevent incubation atmosphere. The buffer systems and incubation conditions used are shown below in Table 2:1. Preliminary experiments showed that C. albicans is unable to use Tris or HEPES buffer salts as growth substrates. $NaHCO_3$ could not be used to buffer MSAB as this compound would not alter the pH of this medium to a sufficient extent.

Table:2.1. Buffer systems and incubation conditions used for study of the effects of environmental factors on the morphology of isolate 73/055.

Medium	Buffer system	pH before equilibration	Incubation conditions
MSAB	None	7.4	Air 30 or 40 °C
"	0.05M Tris/HCl	7.4	Air or O_2 rich 37 °C
"	0.05M Tris/HCl	7.85	5% CO_2 in air 37 °C
"	0.05M Tris/HCl	7.65	Anaerobically 37 °C
EMEM	0.04M $NaHCO_3$	variable	5% CO_2 in air 37 °C
"	0.03M $NaHCO_3$	variable	5% CO_2 in air 30 °C
"	0.02M $NaHCO_3$	variable	Anaerobically 37 °C
"	20mM HEPES	7.4	Air 30 or 37 °C
"	20mM HEPES	7.4	O_2 rich 37 °C

A simple salts based medium, EMEM salts, was also prepared. It comprised (g l^{-1}) L-glutamine, 0.292; D-glucose, 1.000; $CaCl_2$ $2H_2O$, 0.265; KCl, 0.400; $MgSO_4$ $7H_2O$, 0.200: NaCl, 6.800; $NaH_2PO_4.2H_2O$, 0.158 (µg l^{-1}) biotin, 20; nicotinic acid, 400; pyridoxal hydrochloride, 400; riboflavin, 200 and thiamine hydrochloride, 400. EMEM salts was buffered with 0.04M $NaHCO_3$ (37 °C) or 0.03M $NaHCO_3$ (30 °C) and was incubated in 5% CO_2 in air.

Media for static incubation in air or under 5% CO_2 in air, were dispensed in 2 ml volumes in the wells of sterile plastic repli dishes (Sterilin Ltd) or in 10 ml volumes in 50 ml conical flasks when cultures were incubated anaerobically or in an oxygen-enriched atmosphere. Media for shaken incubation in air were dispensed in 50 ml volumes in 250 ml

conical flasks or were dispensed in smaller volumes as described above when shaken in 5% CO_2 in air.

Anaerobic cultures, oxygen-enriched cultures and shaken cultures under 5% CO_2 in air were incubated in polycarbonate gas jars (BBL Ltd). Anaerobic incubation conditions were generated with Gas pak systems (BBL Ltd) which gave an incubation atmosphere of 65% H_2, 12% CO_2 and 23% N_2. Oxygen-enriched atmospheres were created by repeated flushing of gas jars with pure oxygen. For shaken incubation an atmosphere of 5% CO_2 in air was obtained by allowing the gaseous contents of a gas jar to equilibrate with that of the incubator used for the incubation of static cultures under 5% CO_2 in air. After equilibration for 10-20 min, the gas jar was sealed as quickly as possible while still inside the incubator and the gas jar and contents were then incubated in an orbital shaker at 160 rev. min^{-1}. Media in these experiments were allowed to equilibrate overnight to ensure that the gaseous composition of the growth medium was as close as possible to that of the incubation atmosphere.

For static cultures individual wells of repli-dishes were sampled once only to ensure that each sample came from an undisturbed culture. Growth in static cultures was homogenized by addition of a piece of sterile steel wire (0.8 X 0.2 cm) which was used as a magnetic stirrer follower. After 2-4 h incubation mycelial clumps tended to form in shaken cultures. To obtain homogenous samples for assay, 1 ml lots were first gently homogenized in a ground glass tissue homogenizer.

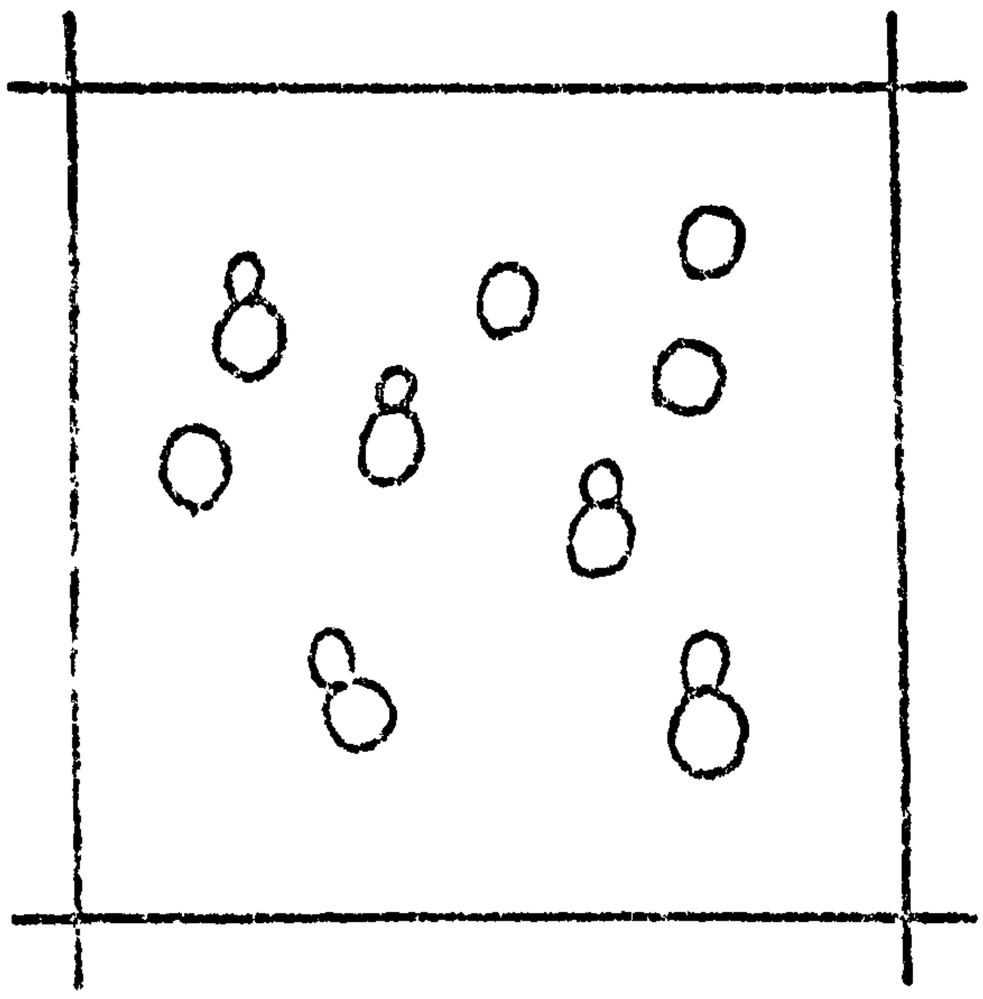
The morphology of C. albicans cells was quantified by a method based on that of Chattaway et al. (1973). Mycelium production was quantified microscopically by assessing mycelial cell units as a percentage of the total cell population of mycelial and blastospore cell units. Hence 50% mycelium production in young cultures indicates that all the blastospores in an inoculum have produced germ tubes. This method takes into account secondary blastospore and branch formation on prolonged incubation but,

unlike the originally described method, does not consider the length of individual mycelia in the final percentage mycelium figure. In this way it is theoretically possible to achieve percentage mycelium figures well above 50% though above 50% the proportional count does not increase in stoichiometric proportion with increases in hyphal length. e.g. a culture containing long hyphae, each with a single hyphal branch would be counted as 67% mycelium even though it would appear subjectively to be predominantly mycelial under the microscope. A further measure of the hyphal nature of cultures can be made by counting the number of branch points per mycelium. These methods of assessing proportions of germ tubes and hyphal strands in cultures are illustrated in Fig 2:1

Growth of cultures was estimated by ATP photometry as described by Odds (1980). 0.25 ml volumes of C. albicans culture were added to 2.5 ml volumes of Tris/EDTA/$MgSO_4$/Acetic acid buffer (Tris; 0.05M, NaEDTA; 0.002M, $MgSO_4$.005M.pH 7.75) preheated to 100 ^{o}C in a boiling water bath in test tubes. Samples were heated at 100 ^{o}C for 4 min then cooled in ice-water. The concentration of ATP released from C. albicans by this procedure was assayed photometrically by measurement of the light emitted from substrate by an ATP-dependent luciferase enzyme. ATP concentrations were determined by reference to an ATP standard after subtraction of readings obtained for the buffer blank. Growth curves were plotted and where indicated growth rates were calculated by linear regression analysis of the data.

The effect of ketoconazole dihydrochloride (a gift of Janssen Pharmaceuticals Ltd) on the morphology and growth of C. albicans was also investigated. The antifungal was dissolved in sterile distilled water prior to addition to media.

Fig.2.1 Quantification of C.albicans morphology.

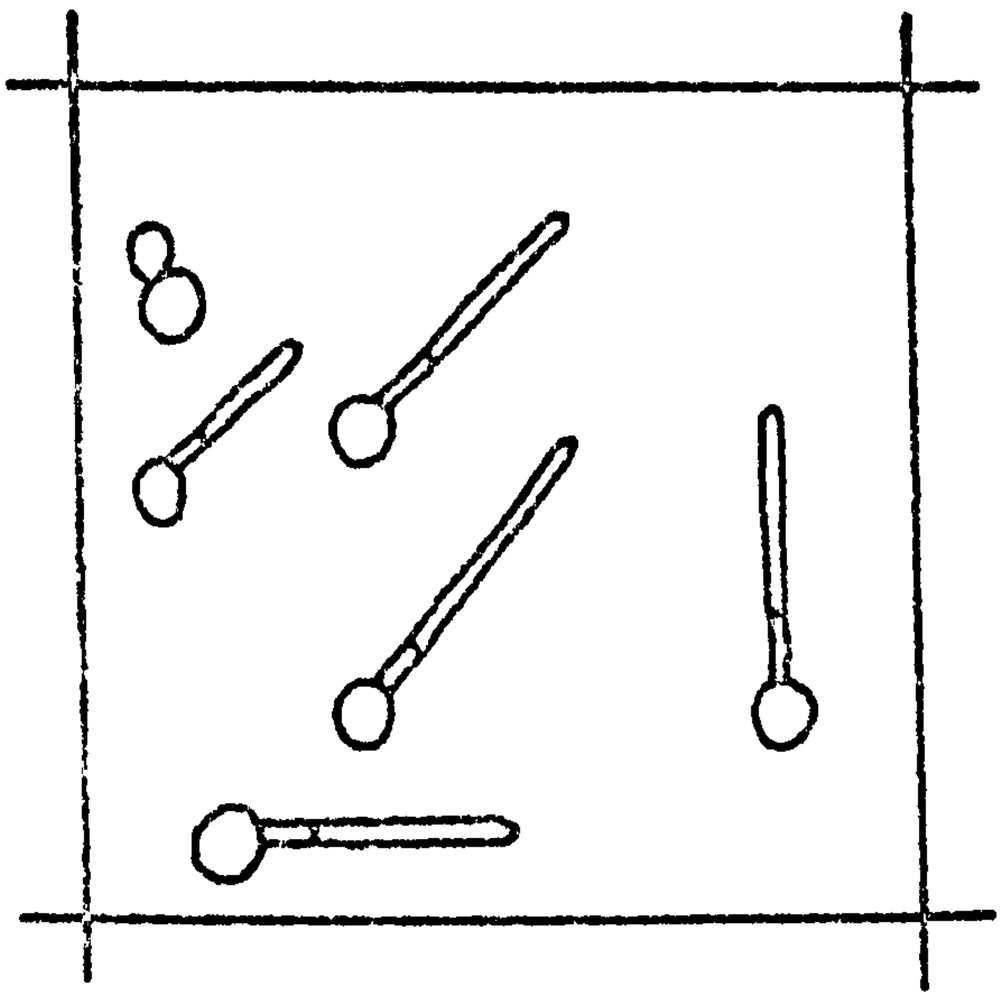


a) Blastospore culture.

14 Blastospore units +
0 Hyphal units.

$$= \frac{\text{0 Hyphal units}}{\text{14 Total cell units}}$$

= 0% Mycelium.

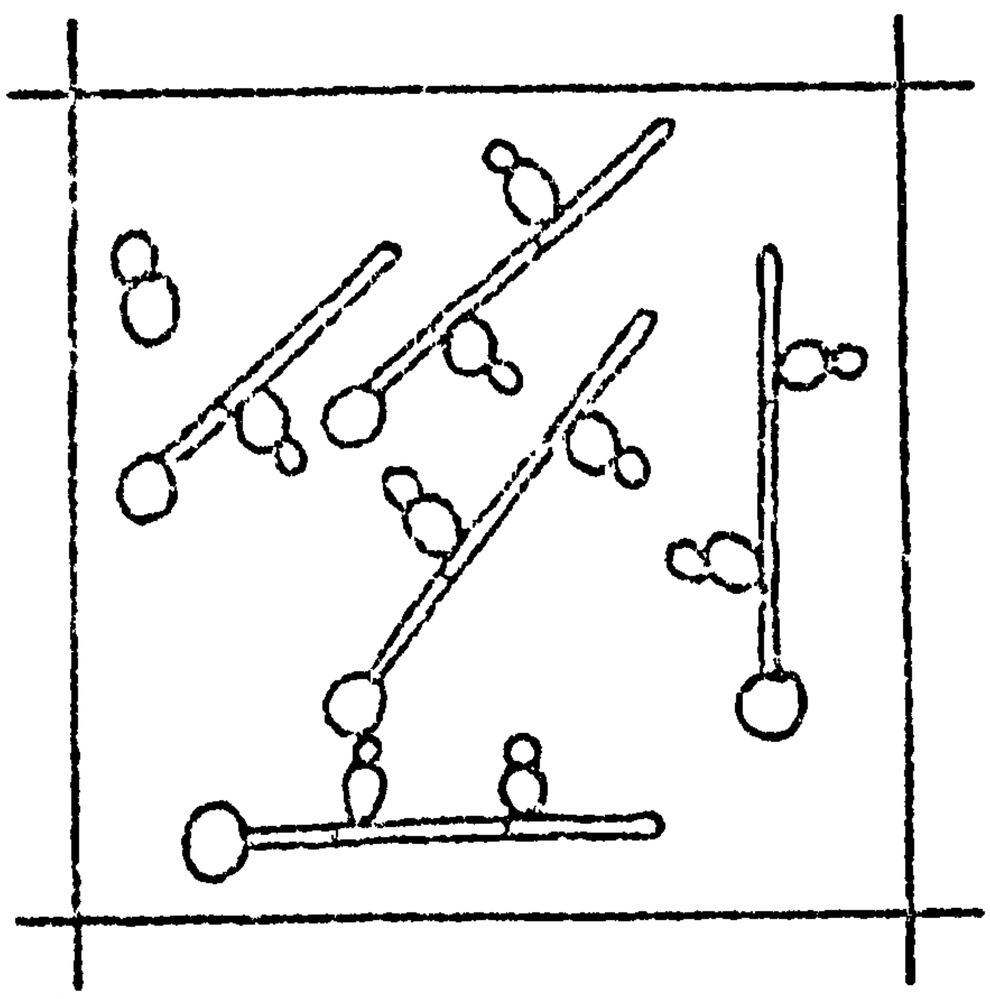


b) Germ tube culture.

7 Blastospore units +
5 Hyphal units.

$$= \frac{\text{5 Hyphal units}}{\text{12 Total cell units}}$$

= 42% Mycelium.

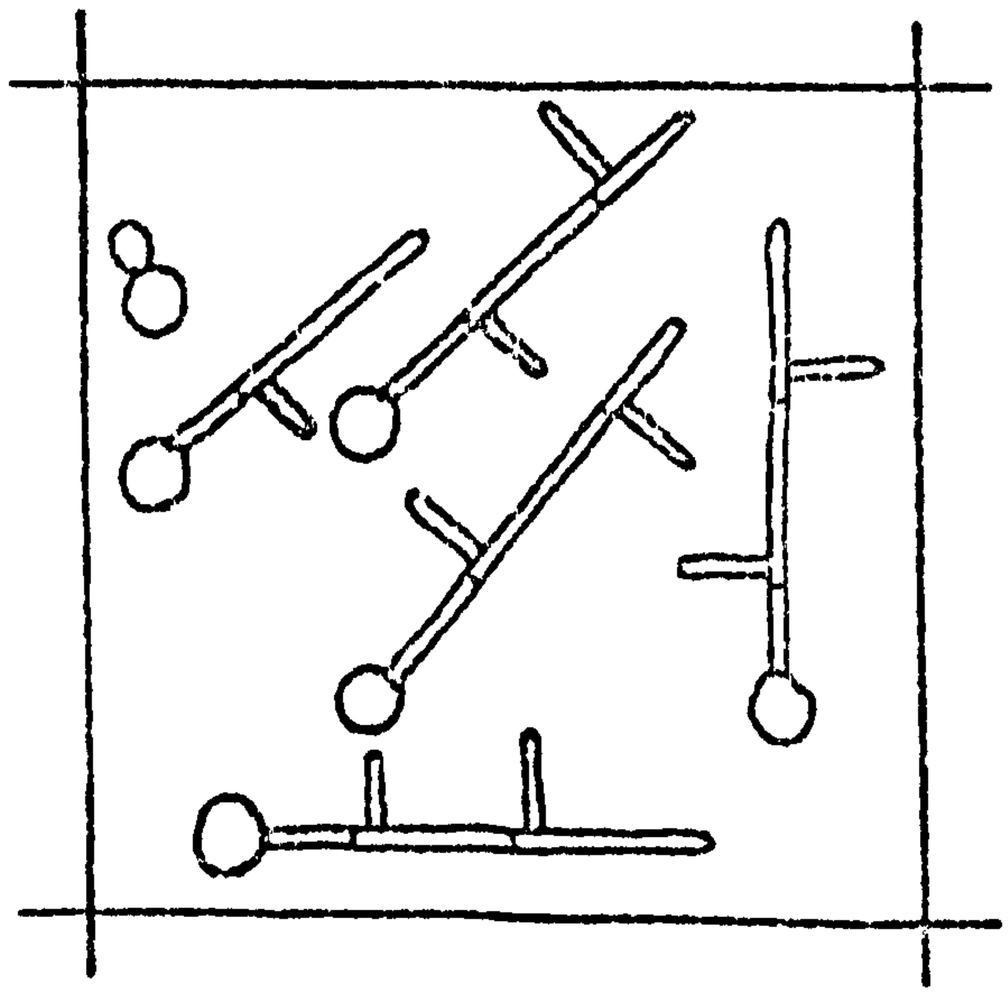


c) Culture producing secondary blastospores.

25 Blastospore units +
5 Hyphal units.

$$= \frac{\text{5 Hyphal units}}{\text{30 Total cell units}}$$

= 17% Mycelium.



d) Culture producing mycelial branches.

7 Blastospore units +
14 Hyphal units.

$$= \frac{\text{14 Hyphal units}}{\text{21 Total cell units}}$$

= 67% Mycelium.

2:3 Results

Preliminary selection of C. albicans isolates and establishment of morphological midels. Morphological development of C. albicans isolates in MSAB incubated in shaken culture in air

MSAB cultures growing at 30 or 40 °C were sampled at hourly intervals after inoculation and the morphology of the cultures determined. All data represent the means of at least two separate determinations.

At 40 °C in air the morphology of the culture was shown to be isolate and time-dependent (Fig 2:2). All isolates showed some germ tube production with a mycelial peak occurring between 2 and 3 h. Germ tube production varied from <5% for isolate 73/002 to over 50% for isolate 73/055. In the majority of isolates germ tube production was followed by secondary blastospore formation, indicated by a decline in the percentage mycelium figures for the cultures. However, isolates 73/055, 81/057 and 81/003 produced some lateral hyphal branches in addition to secondary blastospores. Percentage mycelium figures in cultures of these isolates therefore remained at a higher value than for other isolates.

At 30 °C all isolates grew in the form of budding blastospores

Morphological development of C. albicans isolates in EMEM incubated statically under 5% CO_2 in air

For all isolates tested at 37 °C, virtually all inoculum blastospores had produced germ tubes by 2 h (Fig 2:3). In some instances blastospores were seen to produce 2 or 3 germ tubes. Development after germ tube production was shown to be isolate dependent. Isolates 81/003 and 73/055 produced lateral hyphal branches after 4 h incubation at 37 °C. Hyphae continued to elongate and cultures remained in a hyphal form after 24 h incubation (Fig 2:3). Isolates 81/005 and 81/057 produced some lateral hyphal branches after 4 h incubation but secondary blastospores were also produced and by 24 h these cultures showed a predominantly blastospore

Fig.2.2 Variation in % mycelium production by isolates of C.albicans in MSAB at 40°C shaken in air.

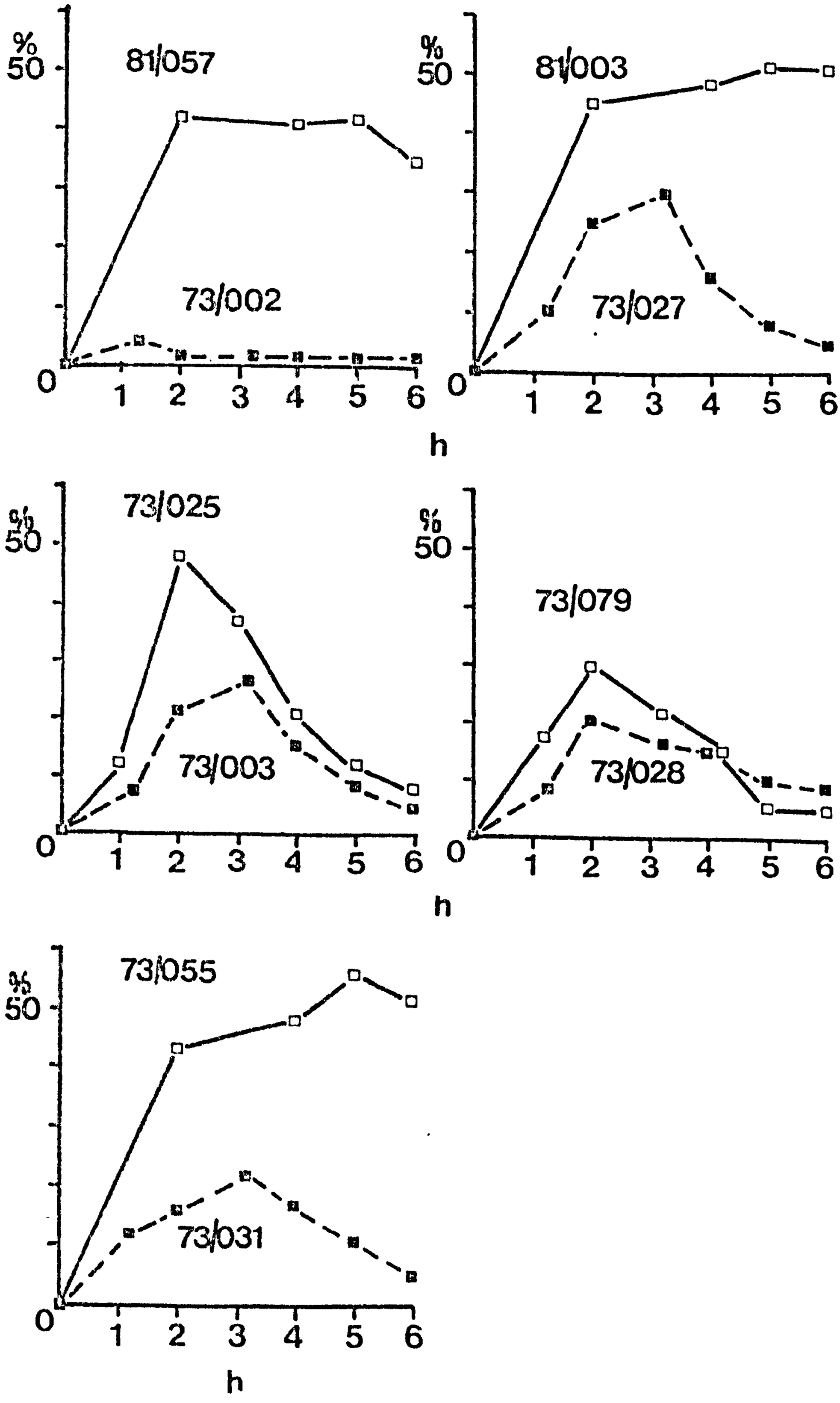
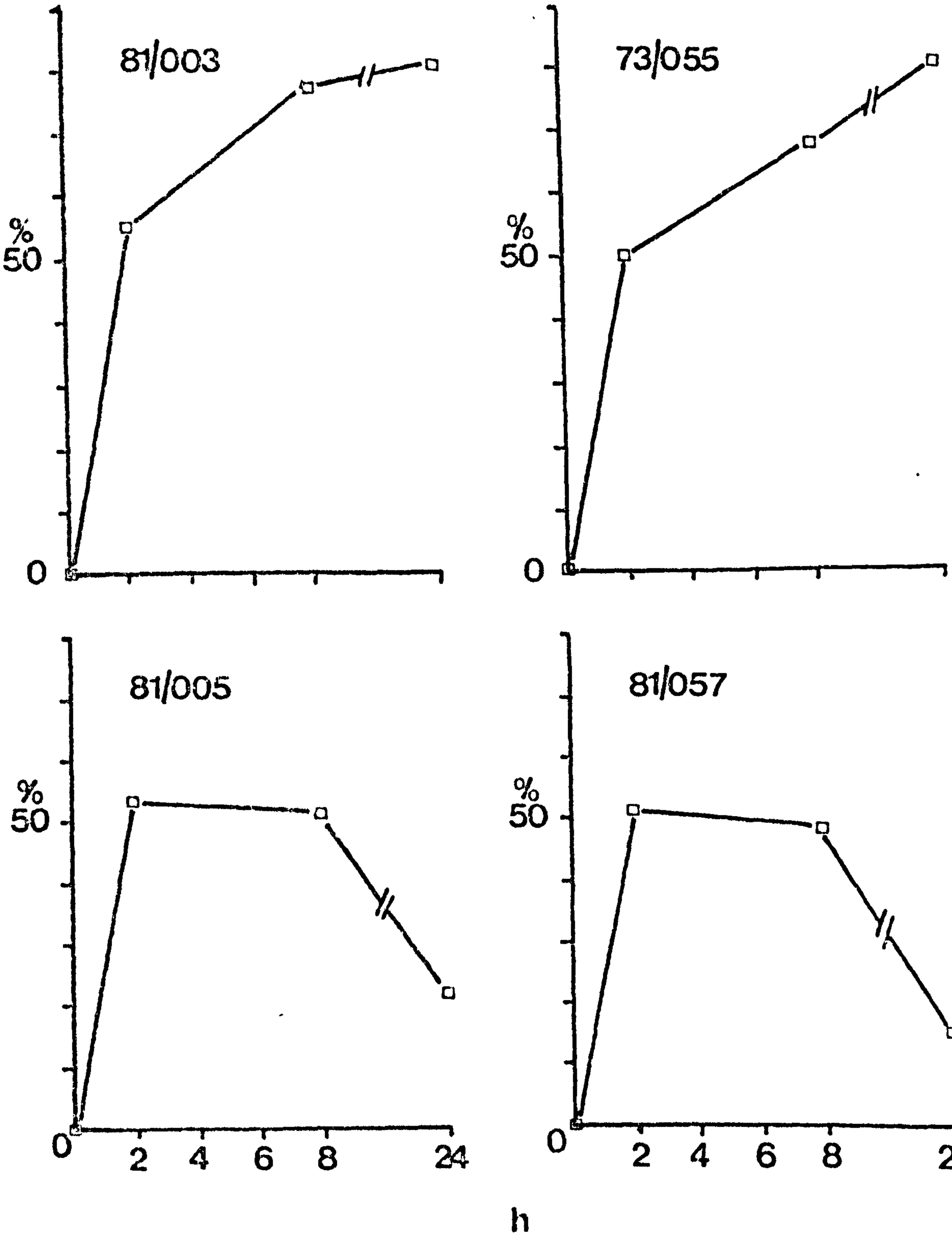


Fig.2.3 Variation in % mycelium production by isolates of C.albicans in EMEM at 37°C incubated statically under 5% CO_2 in air.



morphology (Fig 2:3, Fig 2:4). Further C. albicans isolates were tested for their ability to produce a stable mycelial culture on prolonged incubation but out of 12 isolates tested only isolates 73/055 and 81/003 were shown to have this property.

When C. albicans isolates 73/055 and 81/057 were incubated at 30 °C under 5% CO_2 in air, a different pattern of development from that seen in MSAB cultures at 30 °C in air was noted. Isolate 81/057 showed limited germ tube production under these incubation conditions and the culture remained in a predominantly blastospore form (Fig 2:5). Isolate 73/055 however, showed an initial pattern of development at 30 °C to that seen in EMEM at 37 °C. The majority of blastospores produced germ tubes by 2 h and lateral branch formation with limited secondary blastospore formation occurred. The culture remained predominantly hyphal at 24 h (Fig 2:5).

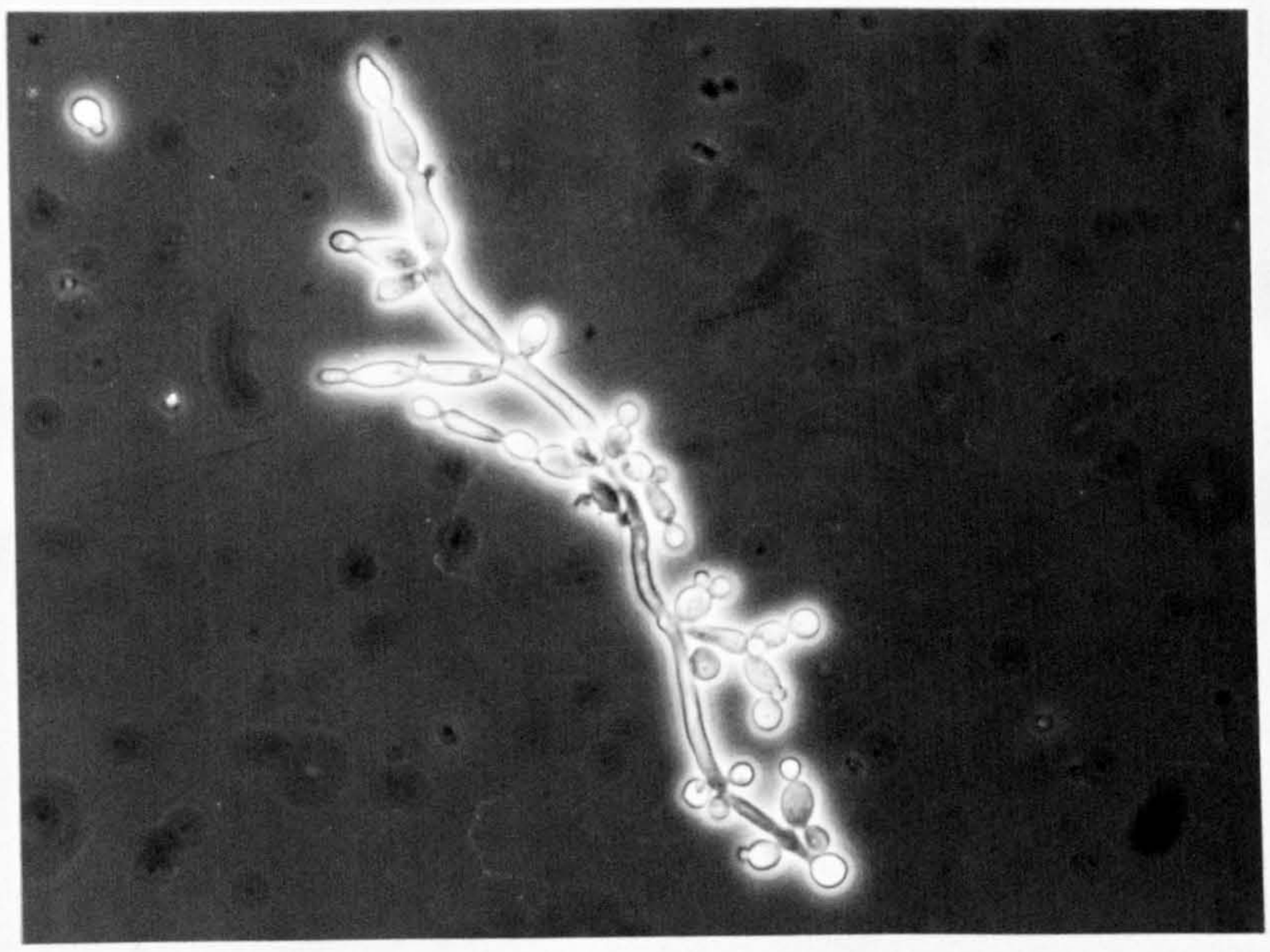
It was found that isolate 73/055 could be grown in a predominantly blastospore form by incubating EMEM cultures, buffered with 20 mM HEPES, statically in air at 30 °C, although some limited mycelium production was noted in older cultures (Fig 2:5).

Selection of isolates for use in subsequent experiments

The morphogenetic phenomena to be investigated included blastospore budding, production of germ tubes, secondary blastospores and lateral hyphal branches. Clearly none of the C. albicans isolates tested was suitable for the study of all these phenomena under the culture conditions used. Isolate 73/025 was therefore selected as useful for study of the blastospore-germ tube-secondary blastospore "cycle" since germ tube production in this isolate was maximal at 2 h and lateral hyphal branches were not produced in MSAB at 40 °C, while an exclusively blastospore growth form was produced in the same medium at 30 °C.

Isolate 73/055 was selected to study the production of hyphae with lateral branches in EMEM under 5% CO_2 in air at 37 °C. This isolate

Fig.2.4 Microscopic appearance of cultures of isolate 81/057 incubated statically for 24h in EMEM at 37 C under 5% CO_2 in air.

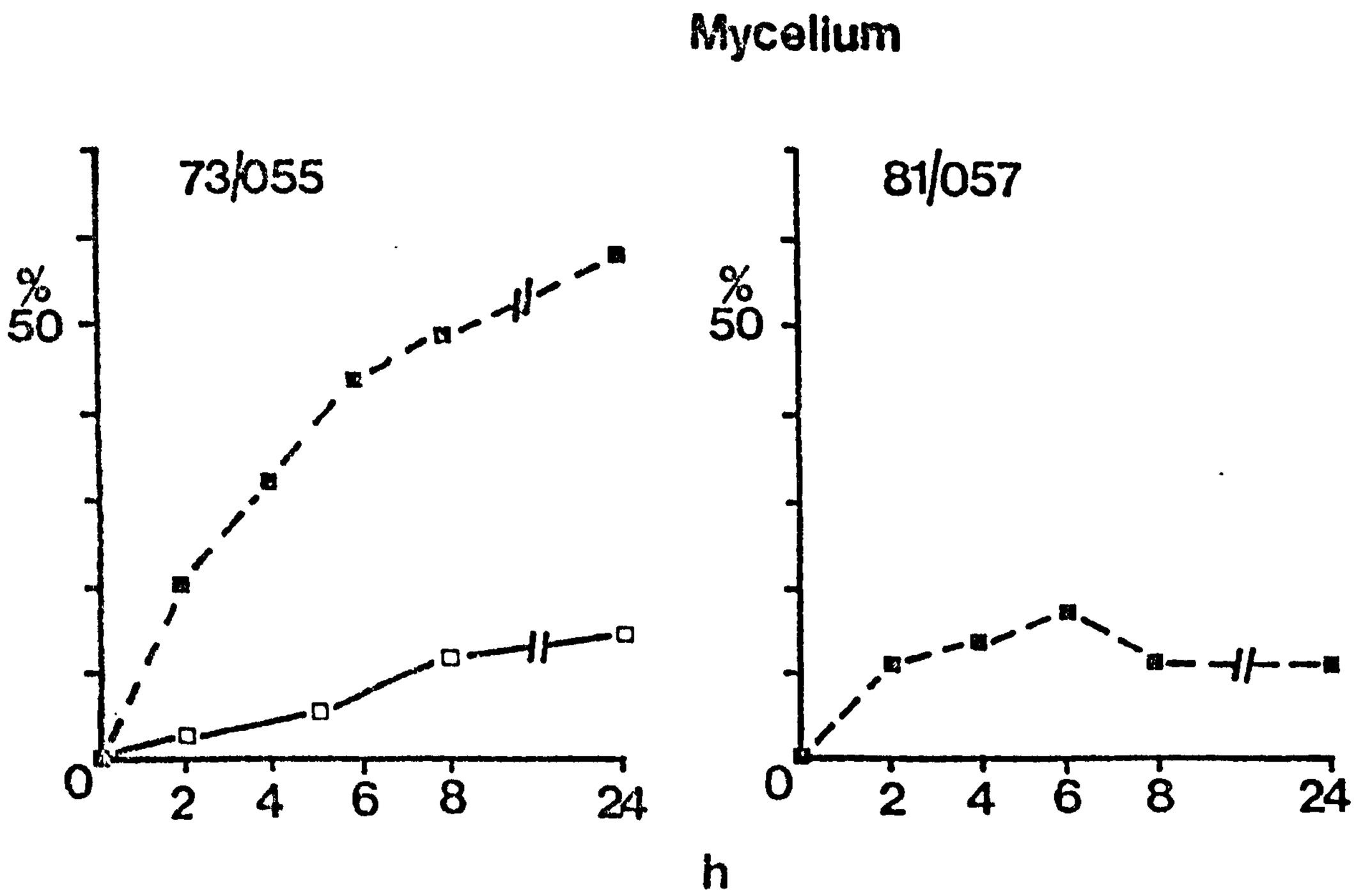


Hypha formation was followed by secondary blastospore production and by 24h cultures were predominantly blastospore in nature.

Fig.2.5 Mycelium production by isolates 73/055 and 81/057 grown statically in EMEM at 30°C.

■ = cultures incubated statically under 5% CO_2 in air.

□ = culture incubated statically in air.



Data represent the mean of two separate determinations.

readily produced stable mycelial cultures with little or no reversion to secondary blastospore production under these growth conditions. It was however impossible to obtain pure blastospore form cultures by incubating this isolate at 30 °C in EMEM under 5% CO_2 in air and a modification of the incubation conditions was required before cultures consisting predominantly of blastospores was obtained. The growth characteristics of isolates 73/025 and 73/055 in the MSAB and EMEM models are summarized in Fig 2:6. Isolate 73/055 was regarded as the most generally useful test isolate since in MSAB at 37 °C it gave a typical "germ tube-hypha-secondary blastospore" growth cycle (see below; Fig 2:7).

Effect of incubation conditions on morphology of C. albicans 73/055

Cultures were sampled at intervals after inoculation and the morphology, ATP content and pH of cultures were determined.

In MSAB, EMEM and EMEM salts under all the incubation conditions tested at 37 °C the growth and morphology of C. albicans was initially the same; hyphal germ tubes grew from virtually all cells in the blastospore inoculum. The effects of differences in media and atmospheres of incubation only became fully apparent in later stages of growth. Under all conditions of culture the pH remained within 0.2 units of the initial pH throughout the 24 h growth period.

Growth rates of C. albicans were higher in MSAB than EMEM and for both media, cultures shaken in air gave the highest growth yields at 24 h (Table 2:2). Growth rates for each medium were broadly similar in air and under 5% CO_2 (Table 2:2). In MSAB and EMEM cultures shaken in air developed lateral secondary blastospores by 8 h and the proportion of hyphae in the cultures fell substantially by 24 h (Fig 2:7). The reversion to budding growth was reflected for shaken cultures in their failure to develop more than occasional hyphal branches (Fig 2:7).

Fig.2.6 Summary of growth characteristics of isolates 73/055 and 73/025 in EMEM and MSAB respectively.

Isolate 73/055 was grown under static incubation conditions at 37°C under 5%CO_2 in air or at 30°C in air.

Isolate 73/025 was grown at 30°C or 40°C with shaking in air.

Data represent the mean of three separate determinations.

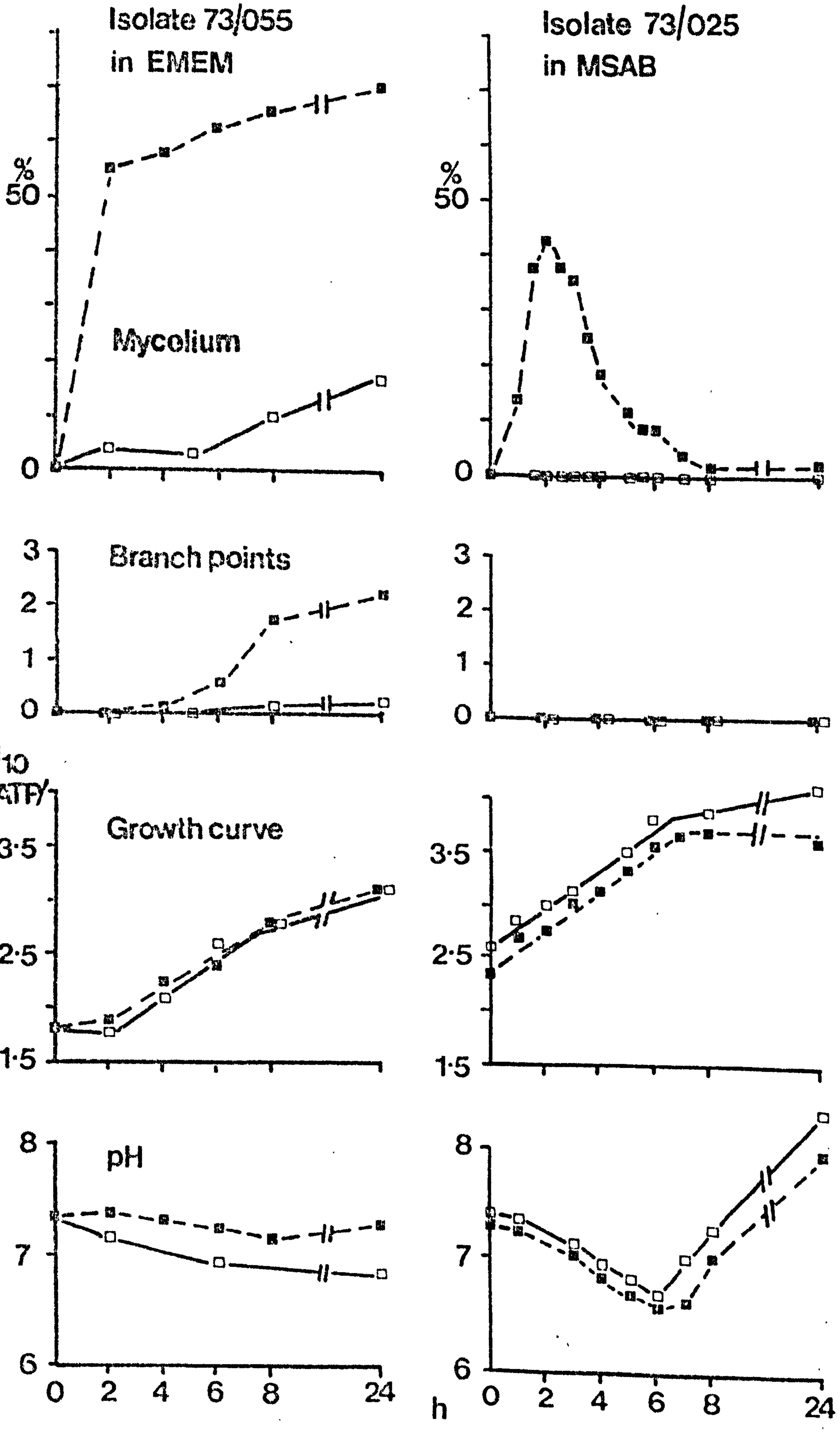

Isolate 73/055
in EMEM
Isolate 73/025
in MSAB
%
50
0
Mycolium
3
2
1
0
Branch points
log10
ng ATP/
ml
3·5
2·5
1·5
Growth curve
8
7
6
pH
0
2
4
6
8
24
h

Table.2.2 Effect of incubation conditions on growth characteristics of isolate 73/055 in EMEM or MSAB at 37 C.

Incubation conditions	%M-8h	%M-24h	Branch points per mycelium 8h	Branch points per mycelium 24h	Growth yield ng ATP ml^{-1} 24h	Doubling time h
EMEM						
O_2 enriched-static	47	56	0.6	0.4	1201	2.21
Air-shaken	40	23	0.3	0.2	2035	1.58
Air-static	65	65	1.7	2.4	1583	1.88
CO_2-shaken	70	58	1.2	0.4	1049	1.95
CO_2-static	65	70	1.9	2.7	1393	1.78
Anaerobic-static	42	42	0.0	0.2	251	3.81
MSAB						
O_2 enriched-static	23	25	0.9	0.6	5355	1.44
Air-shaken	28	5	0.1	0.1	7664	1.52
Air-static	12	5	1.0	0.8	4619	1.44
CO_2-shaken	37	12	0.8	0.5	4040	0.45
CO_2-static	50	17	0.9	1.4	4605	1.36
Anaerobic-static	39	33	0.0	0.0	545	4.3

Data represent the mean of three separate determination.

%M= percentage mycelium.

Fig.2.7 Effect of incubation conditions on the morphology of isolate 73/055 in EMEM and MSAB at 37°C.

■ = Static incubation under 5%CO_2 in air.

□ = Static incubation in air.

▲ = Shaken incubation in air.

Data represent the mean of three separate determinations.

EMEM

MSAB

%
50
0
Mycelium

%
50
0

3
2
1
0
Branch points

3
2
1
0

4
$\log_{10}$
ngATP/
ml
3
2
1
0
2
4
6
8
24
Growth curve

4
3
2
1
0
2
4
6
8
24
h

In unshaken cultures, hyphal branches were formed in all these media by 8 h. Reversion to blastospore budding was only apparent in MSAB and the rate of appearance of blastospores was higher in MSAB cultures incubated in air than under 5% CO_2 in air (Fig 2:7). The degree of hyphal branching was twice as great in EMEM as in MSAB and in MSAB more branches were seen at 24 h in static cultures under CO_2 than in static cultures in air (Fig 2:7). See Figs. 2:8, 2:9.

Although hyphal branch and mycelium production was optimal for isolate 73/055 grown in EMEM incubated statically at 37 °C under 5% CO_2 in air, EMEM cultures incubated statically at 37 °C in air, similarly produced a stable mycelial morphology. In later experiments (Chapter 4), that involved the interaction of C. albicans with phagocytic cells a HEPES buffered medium was used, both for the production of C. albicans germ tubes and hyphae and in the preparation of phagocytic cells. This ensured that phagocytic cells were not exposed to extreme variations in pH as may occur during the preparation of $NaHCO_3$ buffered media and was also technically simpler.

Since the morphological development of C. albicans 73/055 seemed to be similar for static MSAB and EMEM cultures in air and under 5% CO_2 in air, the effect on morphology of altering the CO_2:O_2 ratio more dramatically was investigated by incubating these media anaerobically or in an oxygen-enriched atmosphere.

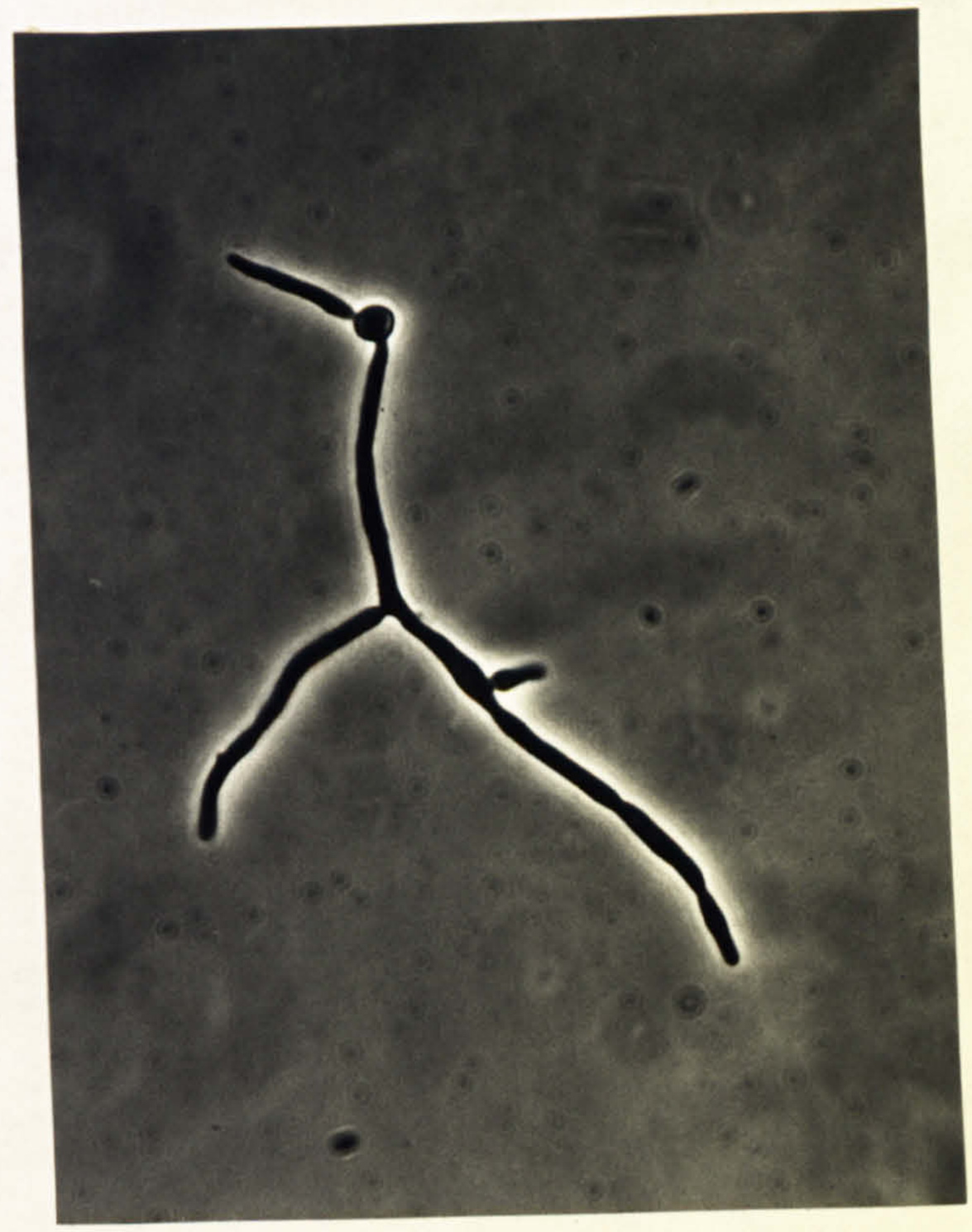
Growth rates and 24 h yields were markedly reduced in both media incubated statically under anaerobic conditions (Table 2:2). In MSAB the pattern of secondary blastospore production was similar to that seen in MSAB cultures in air (Fig 2:10) and cultures contained predominantly blastospores by 24 h. In EMEM, hyphal development after germ tube production was limited with only occasional secondary blastospores produced. Mycelial branches were absent (Fig 2:10). (See Fig 2.11)

Morphological development in an oxygen-enriched atmosphere, however, followed a pattern in both media similar to that for cultures incubated

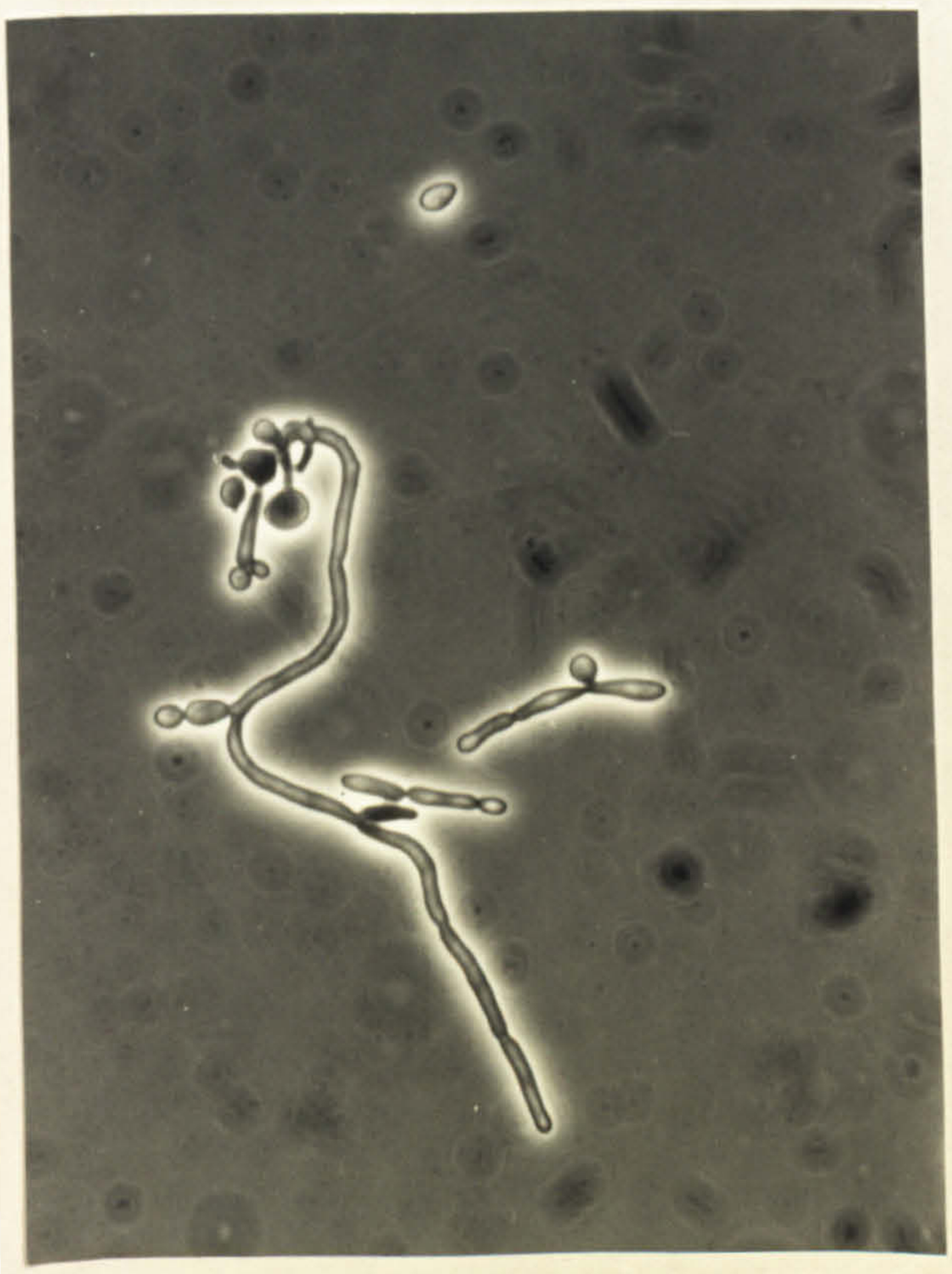
Fig.2.8. Effect of incubation conditions on the microscopic appearance of cultures of isolate 73/055 in EMEM at 37°C.

a) Static under 5% CO_2 in air. 8h.

b) Static in air.8h.



c) Shaken in air. 8h.



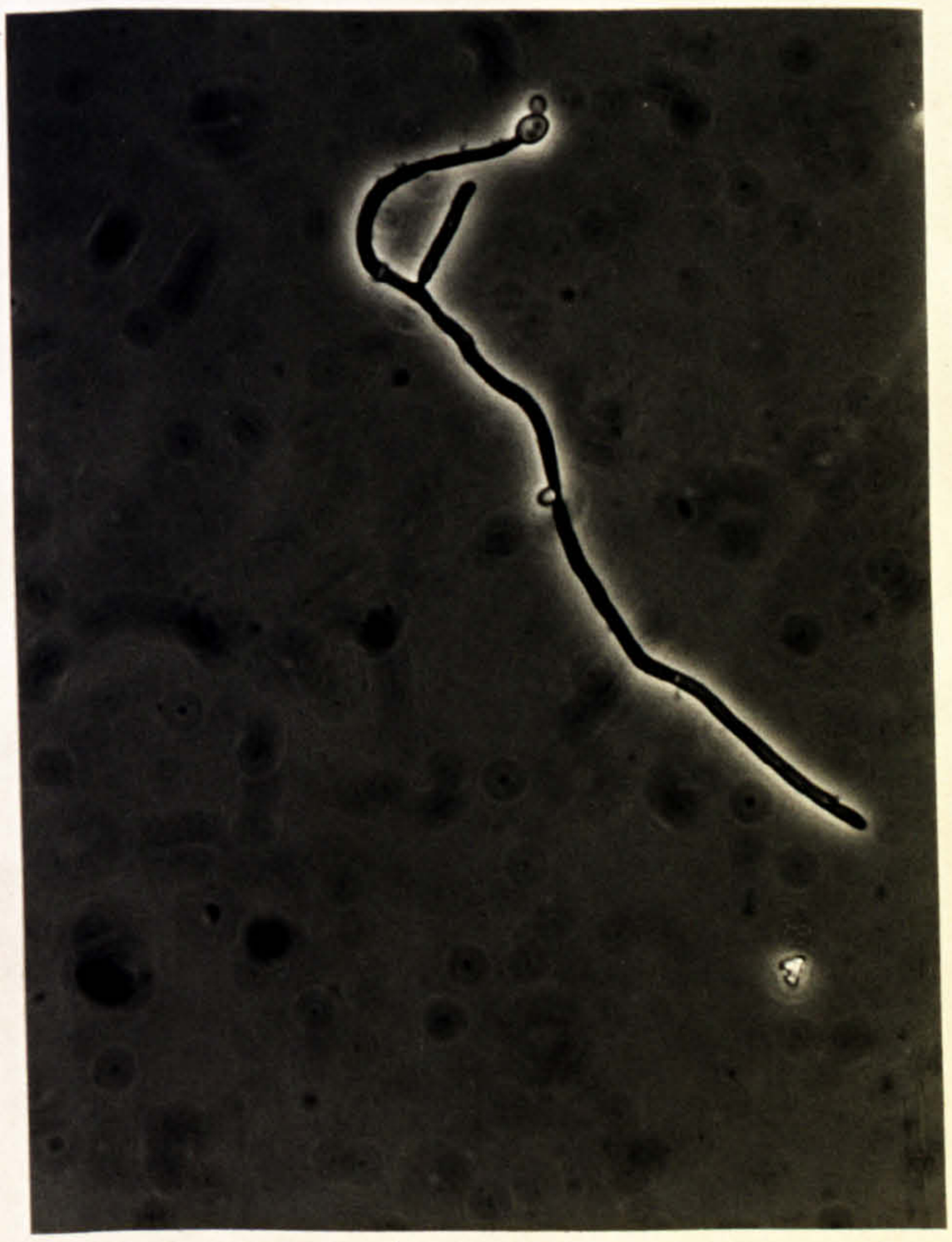
Hyphae in cultures a) and b) were highly branched and secondary blastospores were absent.

In culture c) hyphal branching was reduced and secondary blastospores were produced in abundance.

Fig.2.9. Effect of incubation conditions on the microscopic appearance of cultures of isolate 73/055 in MSAB at 37°C.

a) Static under 5% CO_2 in air. 6h.

b) Static in air. 6h.



c) Shaken in air. 6h.

Hyphae in cultures a) and b) produced both branches and secondary blastospores.

In culture c) hyphal branching was reduced and more secondary blastospores were produced. Hyphae were also shorter.

Fig.2.10 Effect of incubation conditions on morphology of isolate 73/055 in EMEM and MSAB at 37°C.

△ = Shaken incubation under 5%CO_2 in air.

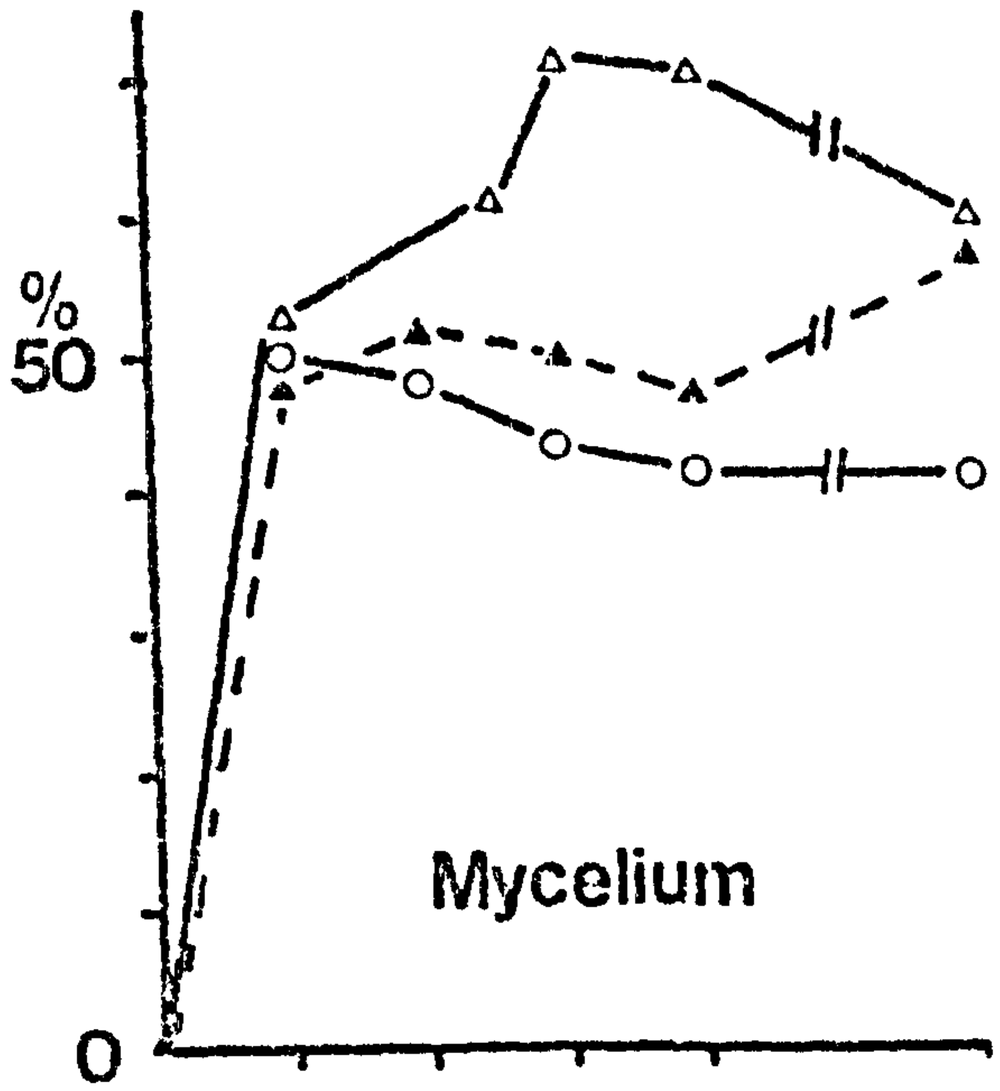
○ = Static incubation under anaerobic conditions.

▲ = Static incubation in an oxygen enriched atmosphere.

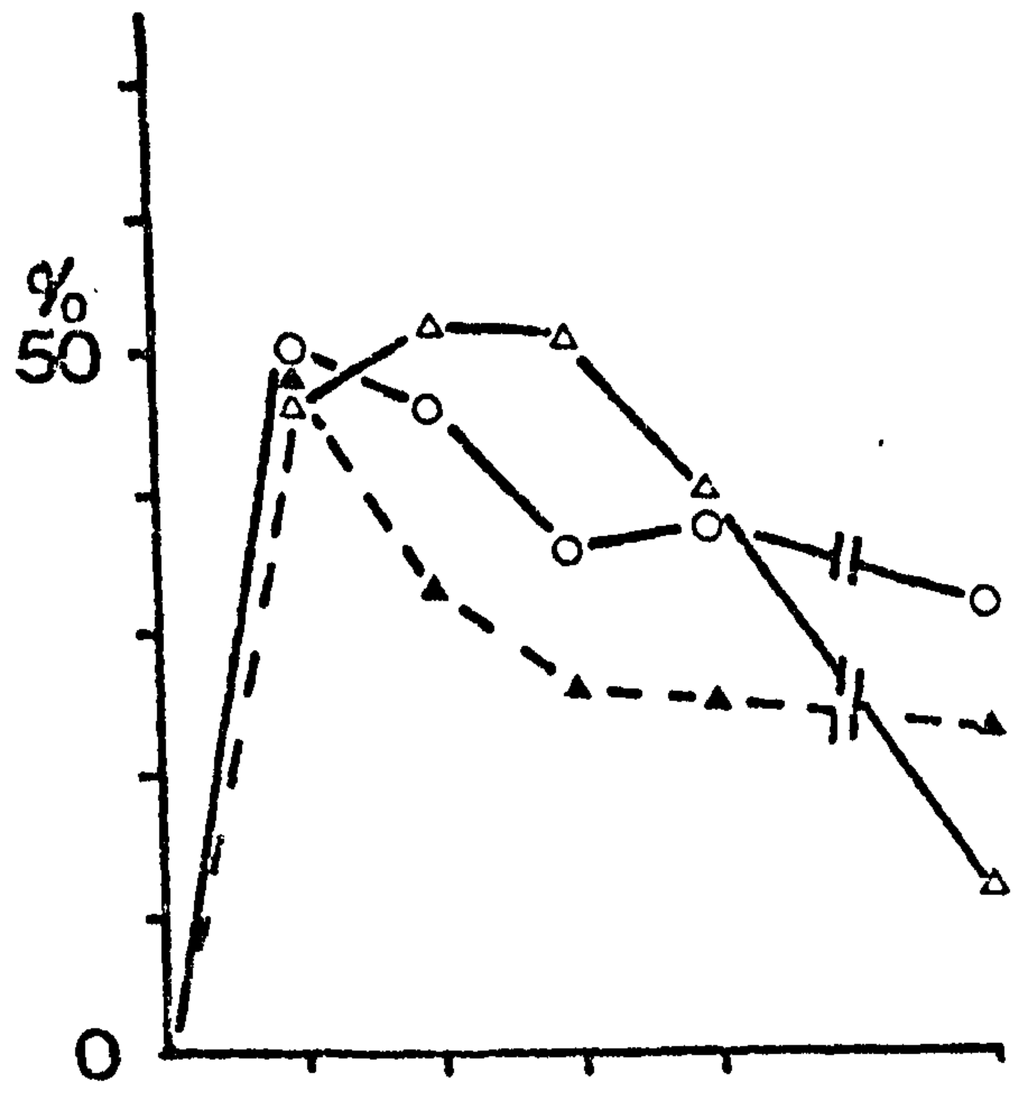
Data represent the mean of three separate determinations.

EMEM

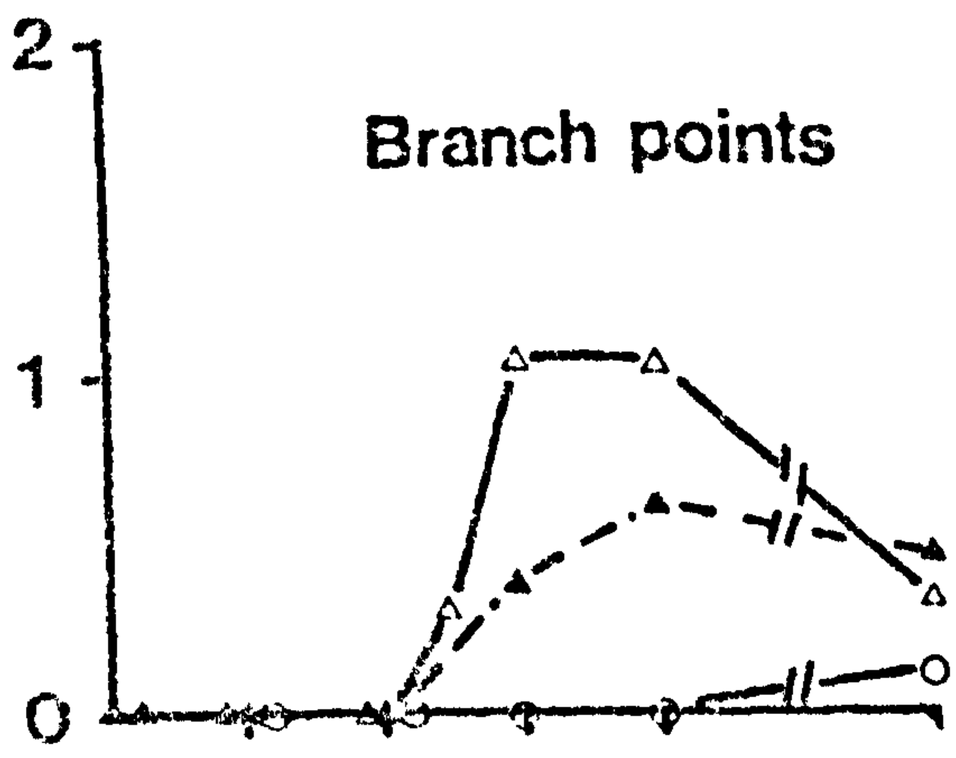
MSAB

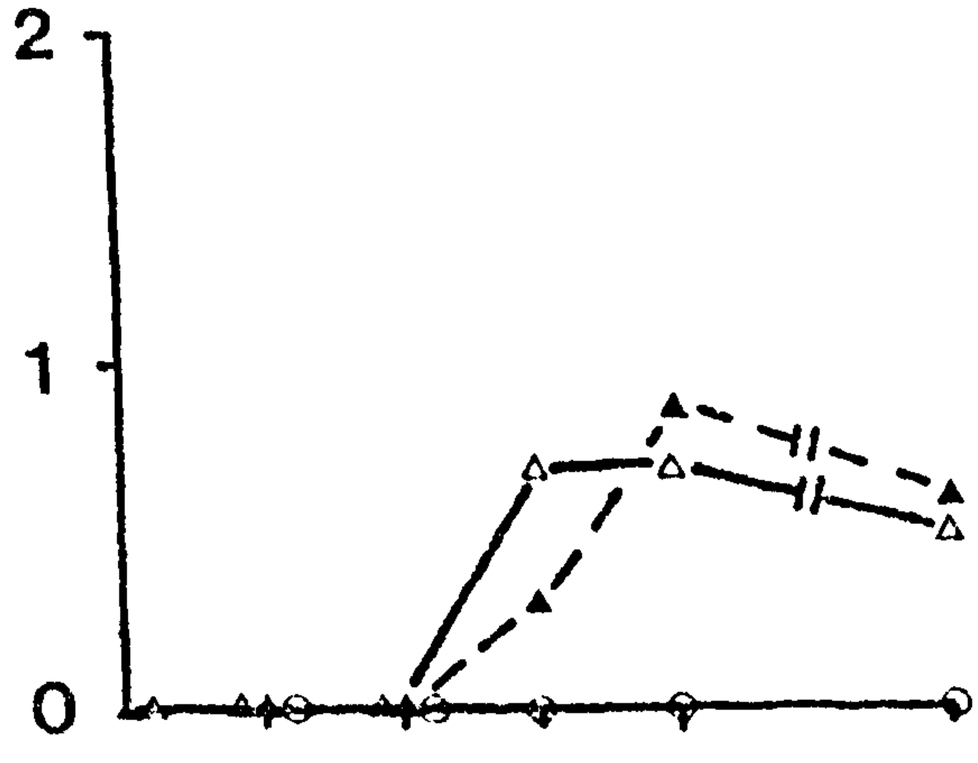


Mycelium

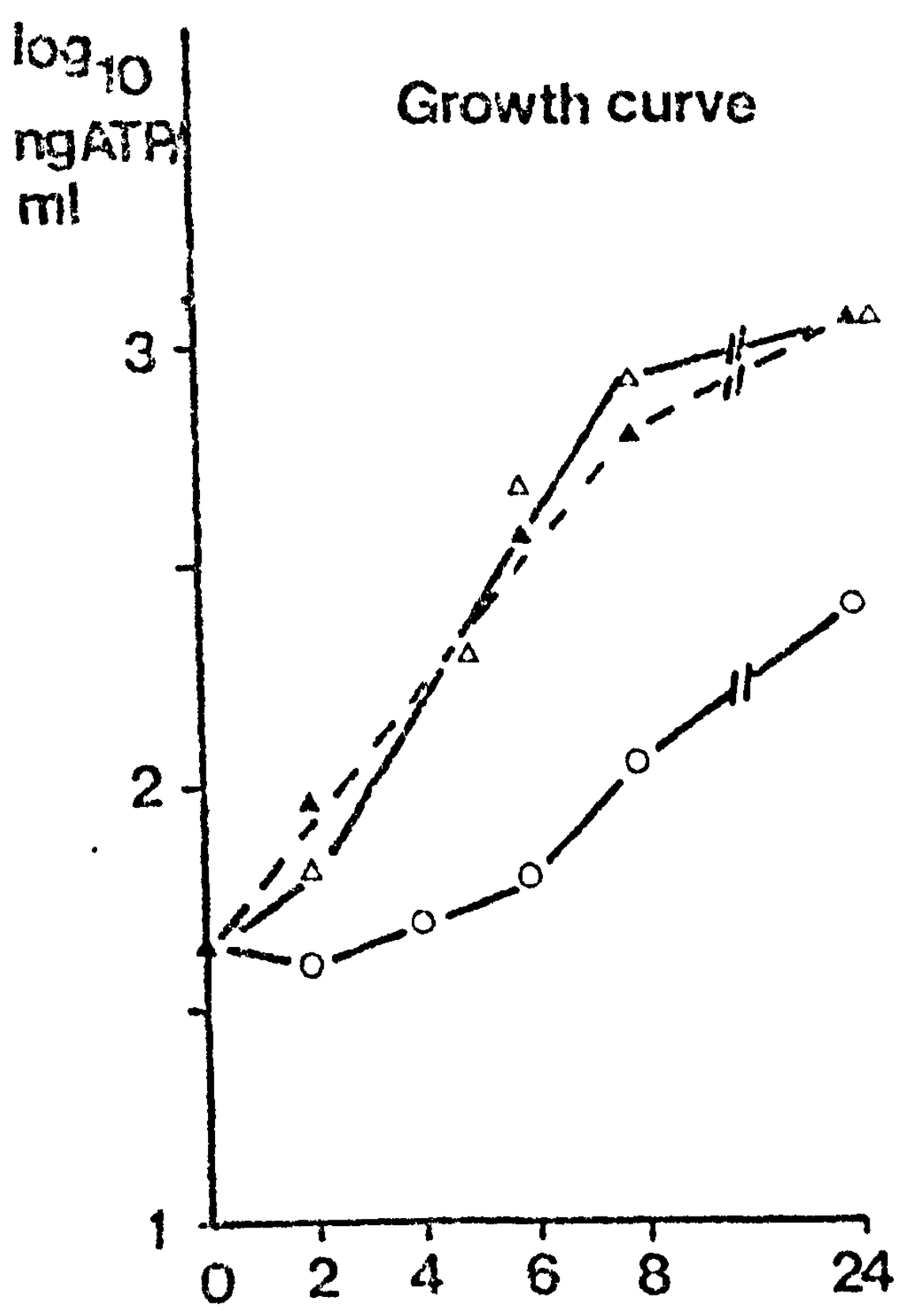


Branch points





Growth curve



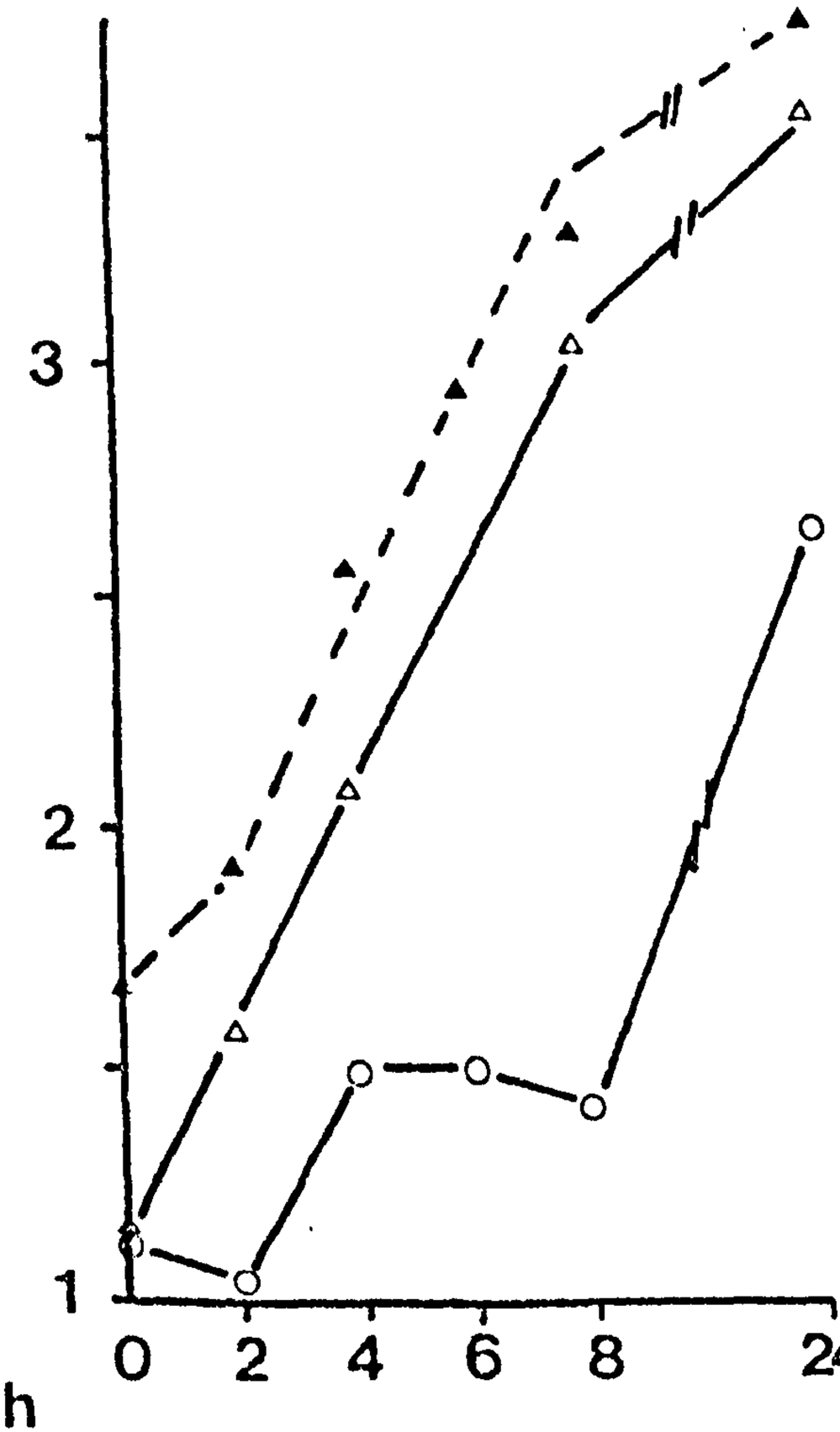
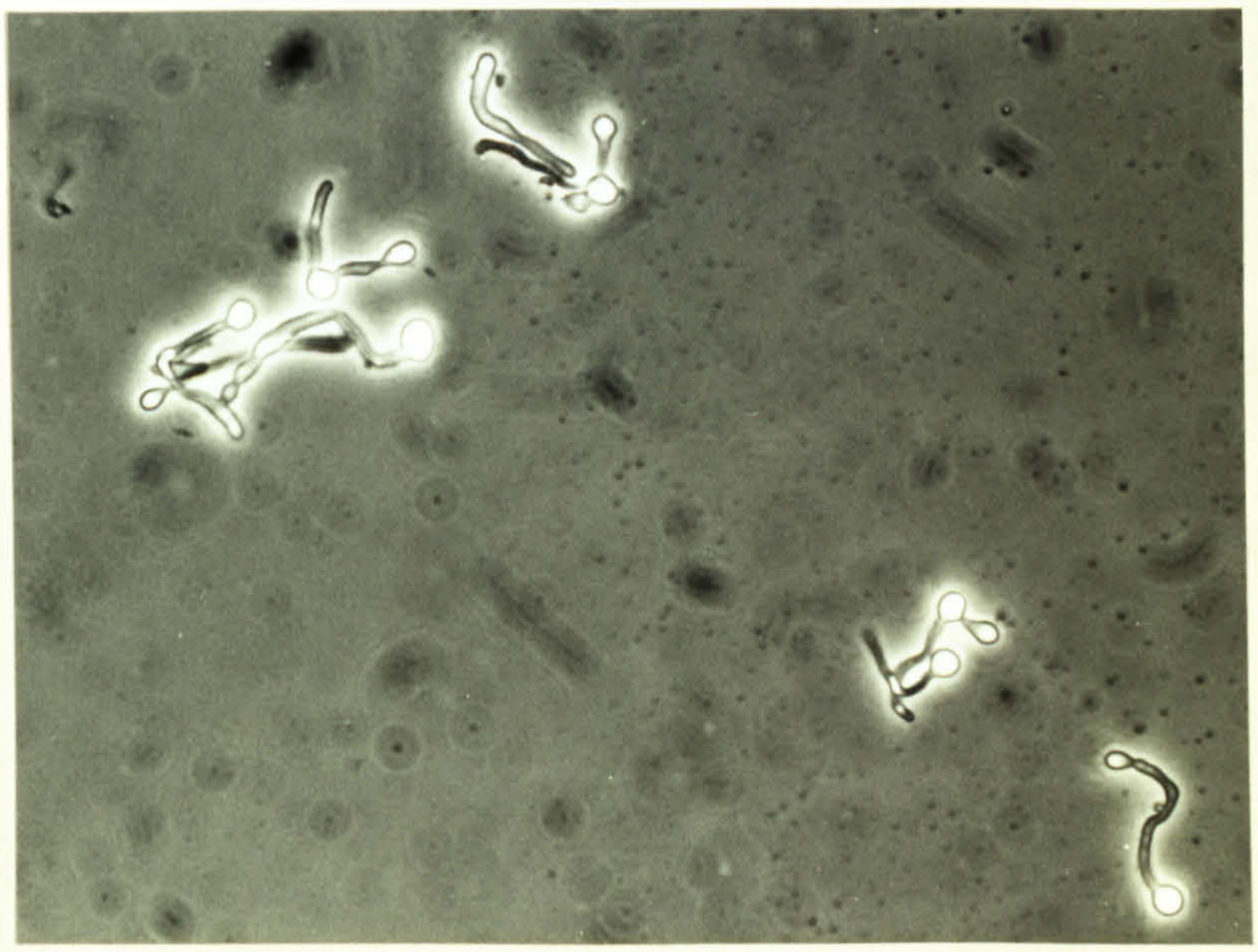


Fig. 2.11 Microscopic appearance of cultures of isolate 73/055 incubated statically under anaerobic conditions in EMEM for 8h at 37°C.



After 8h incubation, hyphae were short, pleomorphic and hyphal branches were absent. Hyphae were also notably phase bright.

statically in air. In MSAB, germ tube production was followed by secondary blastospore production; in EMEM hyphal branches were formed and cultures remained mycelial for up to 24 h (Fig 2:10).

When MSAB and EMEM cultures were shaken under 5% CO_2 in air MSAB cultures reverted to secondary blastospore production and were predominantly blastospore in nature by 24 h (Figs 2:10). In contrast EMEM cultures remained mycelial after 24 h incubation at 37 °C.

Effect of temperature on morphology of C. albicans in EMEM and EMEM salts under 5% CO_2 in air

Incubation of C. albicans under CO_2 at the lower temperature of 30 °C did not lead to budding blastospore growth of 73/055 in EMEM. At 30 °C C. albicans grew slightly more slowly (doubling time = 2.00 h) than at 37 °C but hyphae and hyphal branches remained the predominant morphological forms (Fig 2:12). Hyphae were, however, more pleomorphic when produced at the lower incubation temperature (Fig 2:13). In EMEM blastospore forms of 73/055 were obtainable at 30 °C only in aerobic cultures and even under these incubation conditions some limited mycelial development was apparent (Fig 2:12).

In EMEM salts at 37 °C under 5% CO_2 in air, 73/055 produced branched hyphae and stable mycelial cultures similar to those seen in EMEM. Hyphae in EMEM salts were, however, more pleomorphic than in EMEM. At 30 °C in EMEM salts under CO_2 germ tube and hypha production were markedly reduced and secondary blastospores were produced.(Figs 2:14, 2:15). The predominant morphological form in such cultures was always the blastospore form.

As the relatively simple EMEM salts medium supported mycelial growth of 73/055 at 37 °C to a similar extent as EMEM, the effect on morphology of altering the composition of this simple medium was investigated (Fig 2:16). The results suggested that the nature of the carbon and nitrogen sources provided as substrate may influence the extent and rate of germ tube and

Fig 2.12 Growth characteristics of isolate 73/055 incubated statically in EMEM under 5% CO_2 in air or in air at 30°C.

■ = 37°C

▲ = 30°C

□ = 30°C in EMEM+20mM HEPES in air

Data represent the mean of three separate determinations

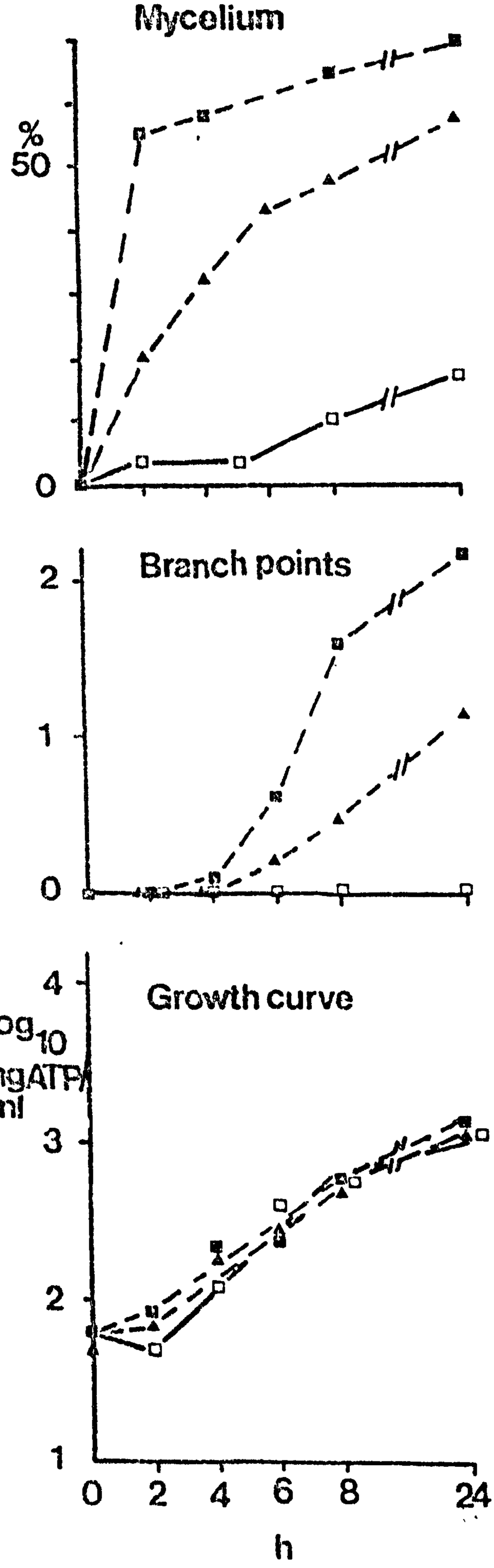
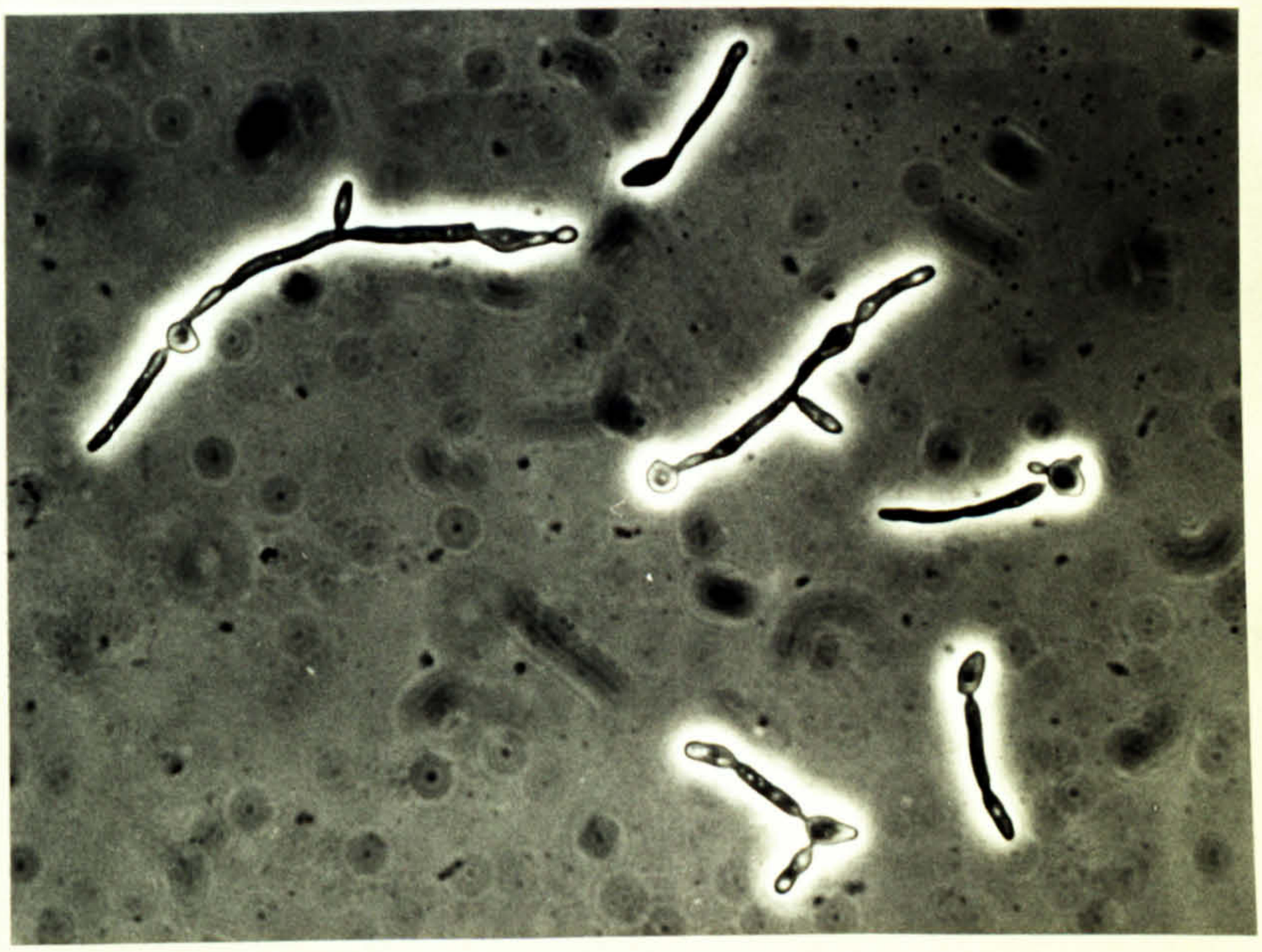

Mycelium
%
50
0
Branch points
2
1
0
Growth curve
log10 ngATP/ml
4
3
2
1
0
2
4
6
8
24
h

Fig.2.13. Microscopic appearance of hyphae of isolate 73/055 incubated statically for 6h in EMEM at 30°C under 5% CO_2 in air.



After 6h incubation at 30°C hyphae were shorter and more pleomorphic than hyphae of a similar age grown in EMEM at 37°C under 5% CO_2 in air. (See Fig.2.15 b).).

Fig.2.14 Growth characteristics of isolate 73/055 incubated statically in EMEM salts at 30° and 37°C under 5% CO_2 in air.

■ = 37°C

□ = 30°C

Data represent the mean of three separate determinations

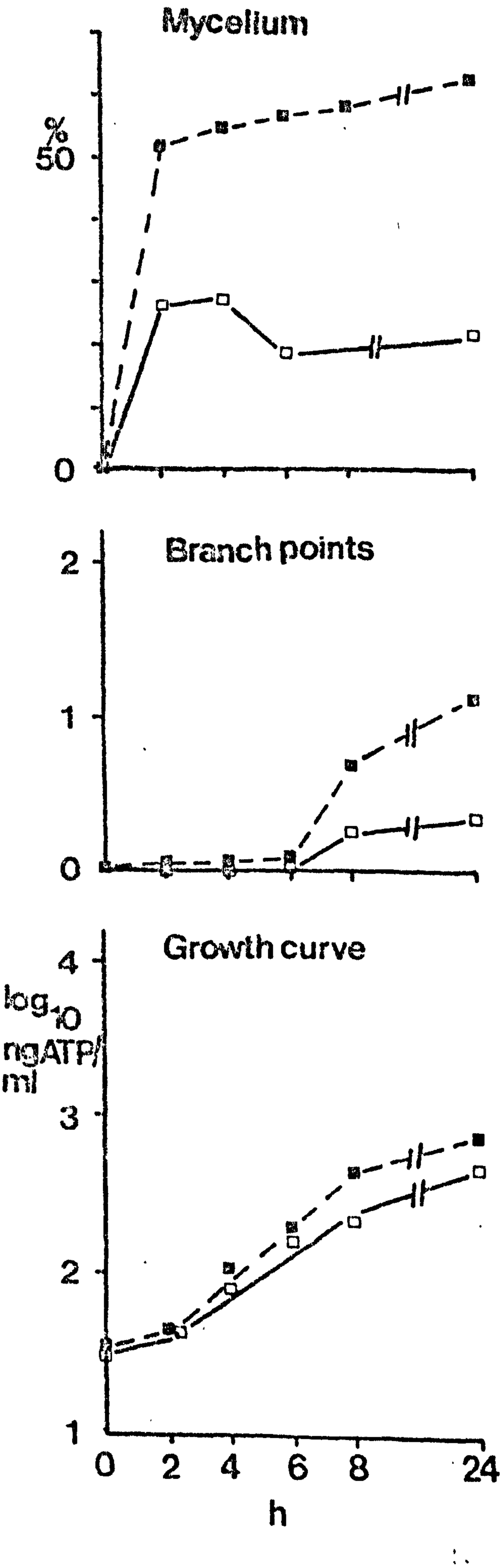
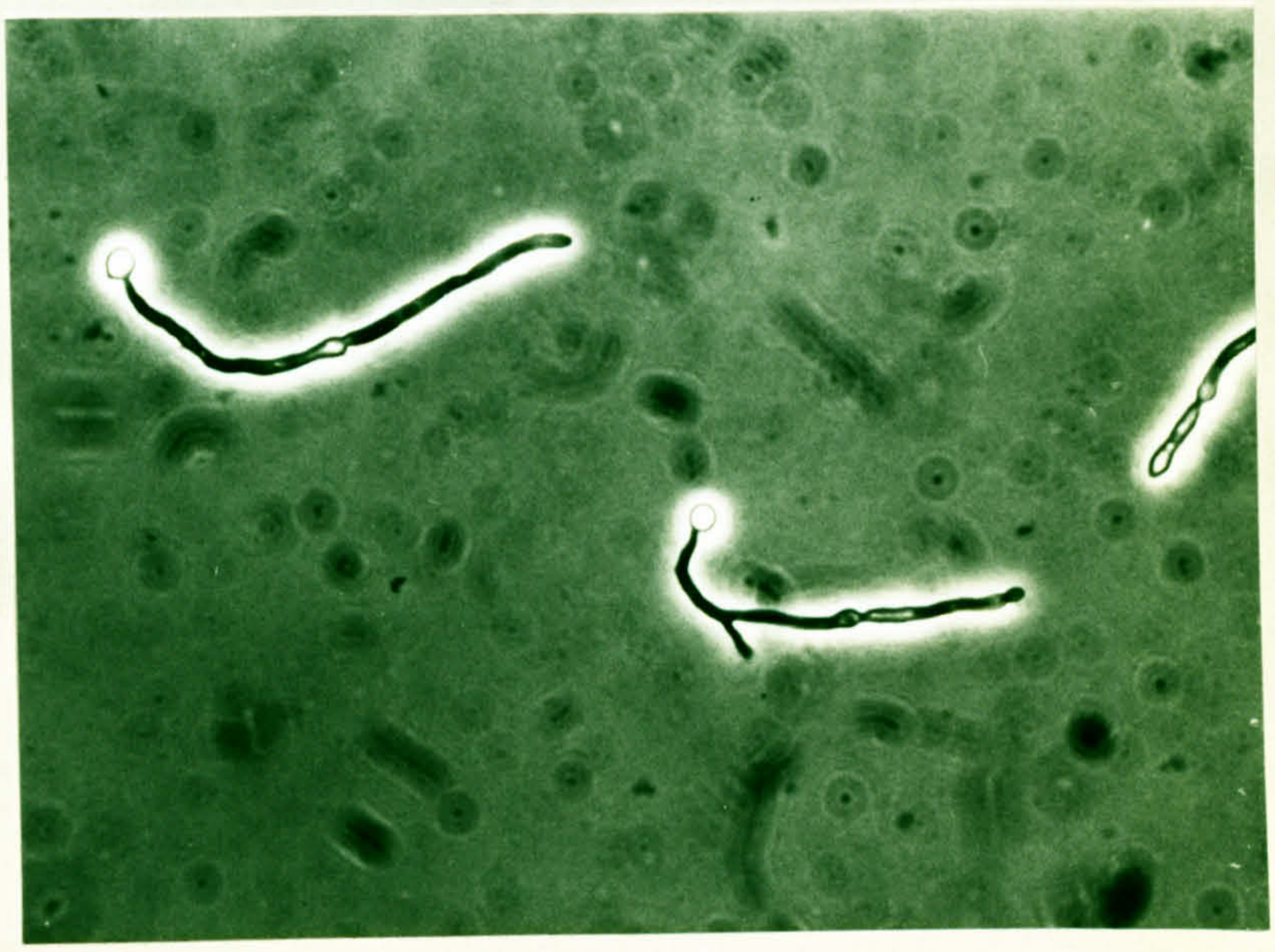
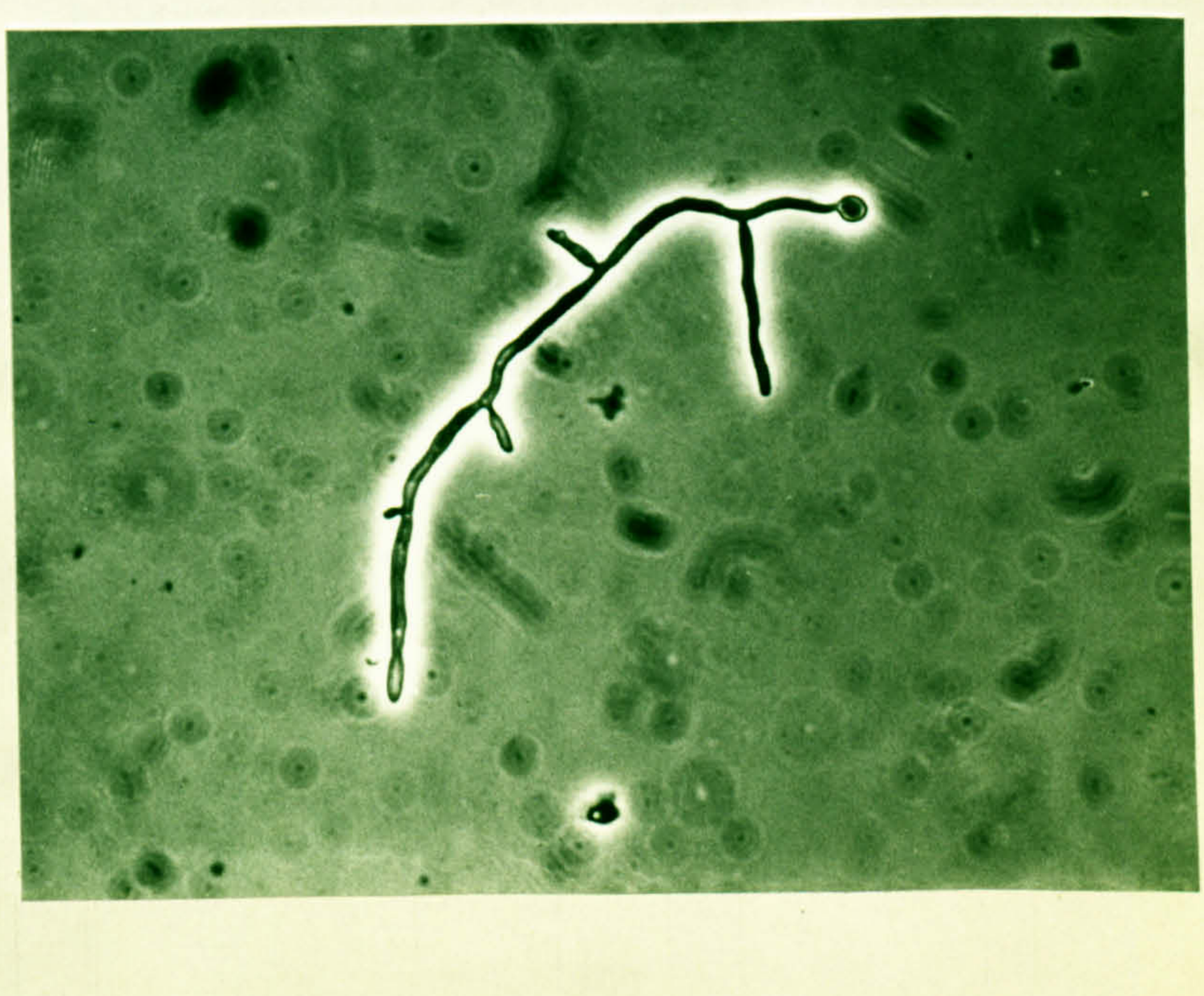

Mycelium
%
50
0
Branch points
2
1
0
Growth curve
4
log10 ngATP/ml
3
2
1
0
2
4
6
8
24
h

Fig. 2.15 Microscopic appearance of hyphae of isolate 73/055 incubated statically for 6h in EMEM salts at 37°C under 5% CO_2 in air.

a) Hyphae grown for 6h in EMEM salts at 37°C.



b) Hyphae grown for 6h in EMEM at 37°C



After 6h incubation in EMEM salts hyphae were noticeably phase dark and pleomorphic. Hyphae were also shorter than those from EMEM cultures and produced fewer branches.

Fig.2.16 Effect of carbon and nitrogen source on morphological development of isolate 73/055 in EMEM salts incubated statically under 5% CO_2 in air at 37°C.

■= EMEM salts + 0.05M glucose + 0.02M glutamine

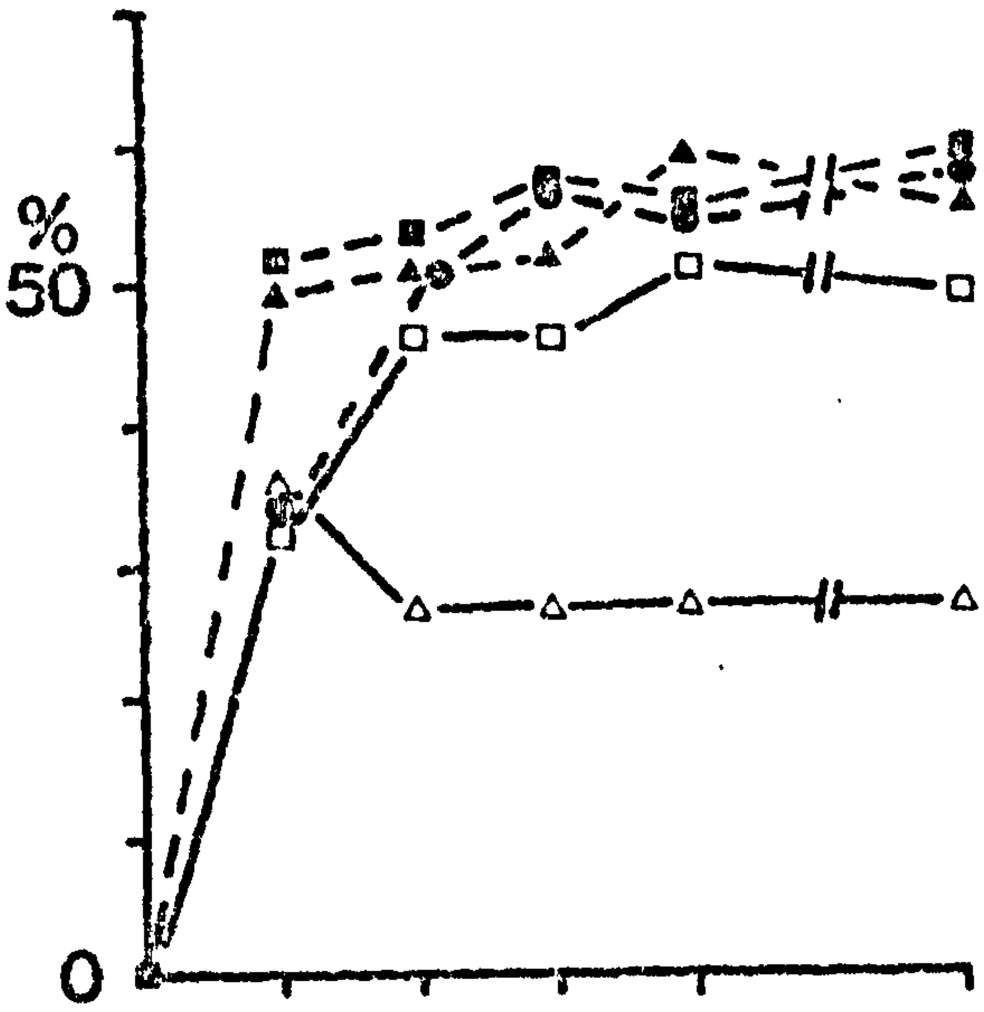
□= EMEM salts + 0.02M glutamine

△= EMEM salts + 0.05M glucose + 0.25M NH_4Cl

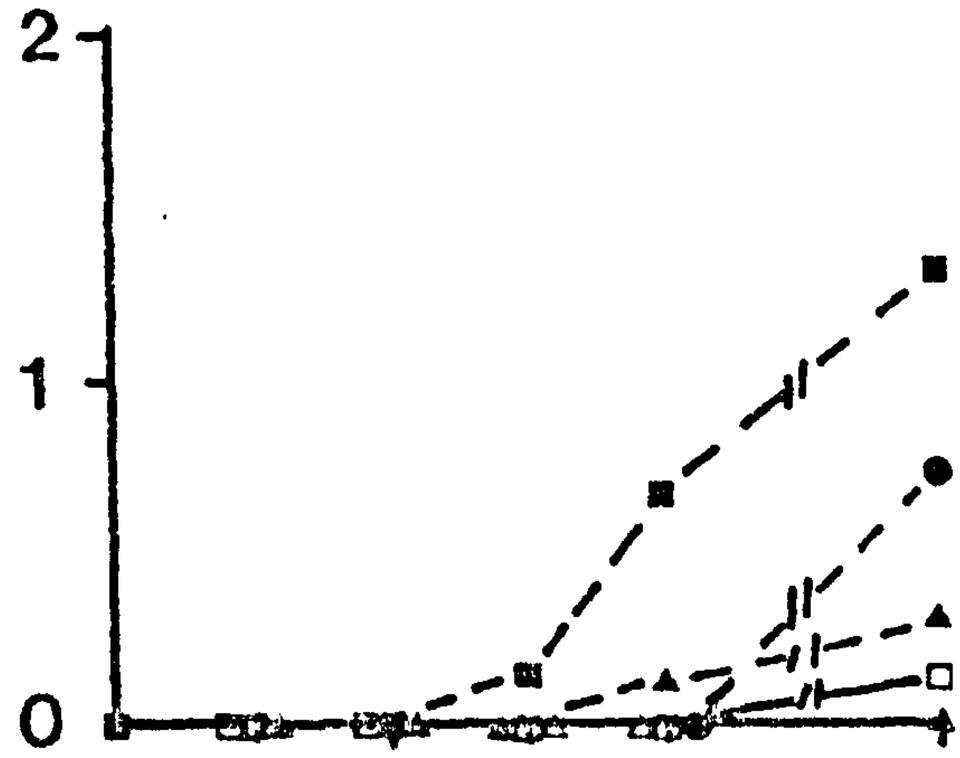
●= EMEM salts + 0.05M glucose

▲= EMEM salts + 0.04 M N-acetyl-D-glucosamine

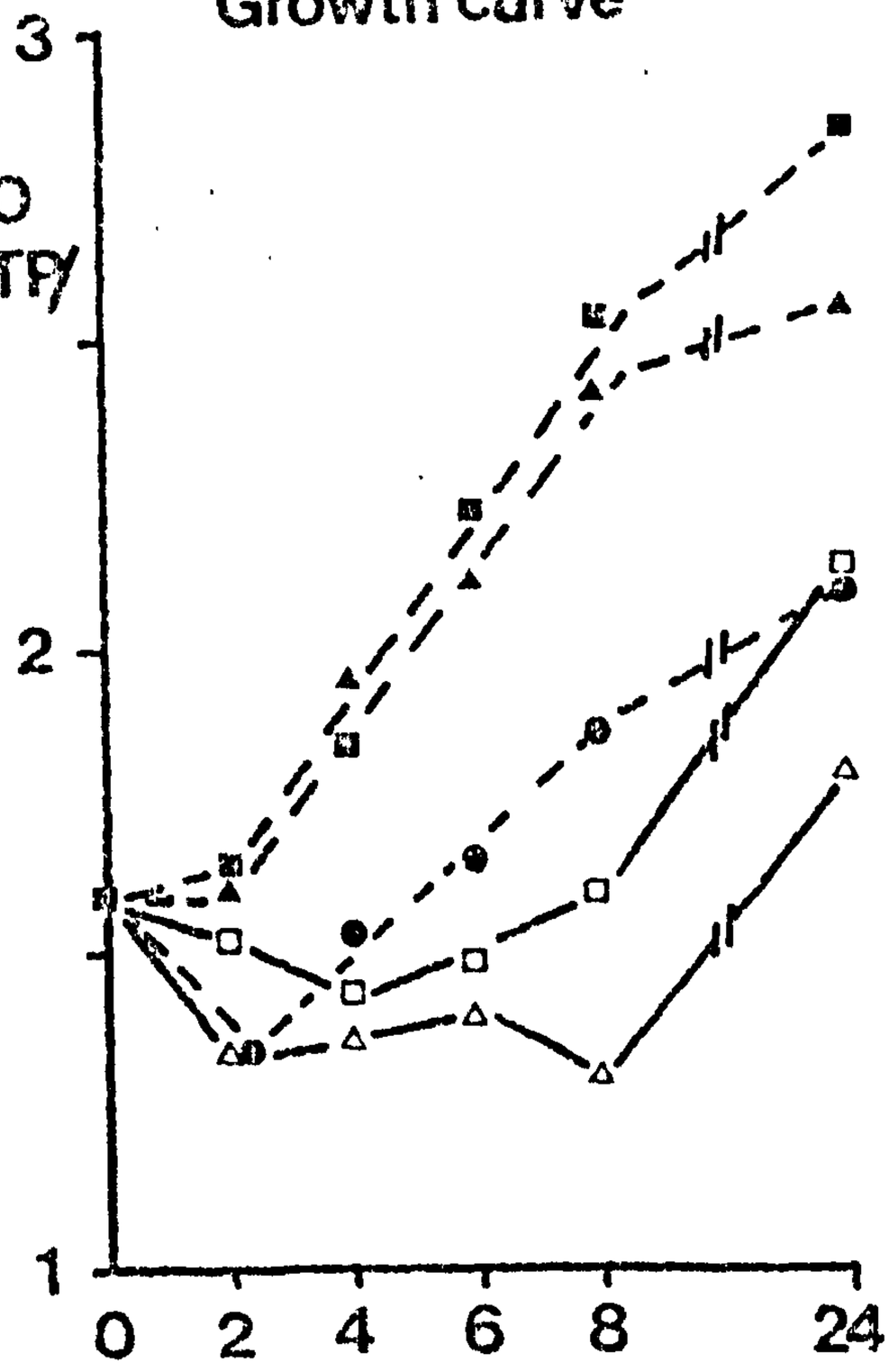
Mycelium



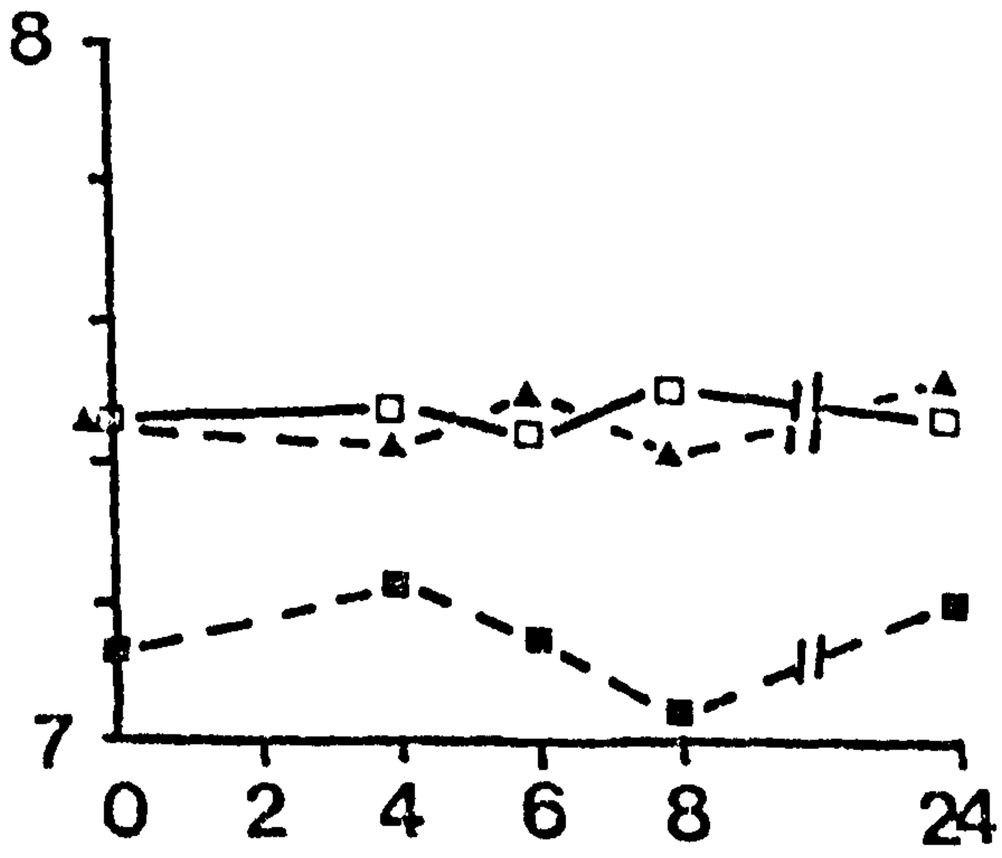
Branch points



Growth curve



pH



h

hypha production. Germ tube production occurred most rapidly in the presence of glucose and either of the nitrogen sources NH_4Cl or glutamine (Fig 2:16). Cultures remained fully hyphal in the absence of glutamine but not in the absence of glucose (Fig 2:16), indicating that in this medium, glucose appeared to be the more important factor controlling hyphal development. Although maximal mycelium production was achieved in the absence of glutamine, maximal hyphal branching occurred when both glucose and glutamine were present in the medium. When C. albicans was supplied with a single combined carbon-nitrogen source, N-acetyl-D-glucosamine, the rate of germ tube production was slower than in the complete EMEM salts medium and although cultures remained hyphal, the extent of mycelial branching was less in the presence of N-acetyl-D-glucosamine (Fig 2:16). Qualitative differences were also apparent between hyphae produced in these two media. After 24 h incubation hyphae grown in EMEM salts were markedly pleomorphic. In the presence of N-acetyl-D-glucosamine, however, hyphae were still parallel sided and had a more regular appearance after 24 h incubation. Hyphae in these cultures also appeared thinner than those seen in EMEM salts after 24 h incubation (Fig 2:17).

When C. albicans 73/055 was incubated in EMEM salts in the absence of added glucose or nitrogen sources, blastospores were the predominant morphological form at 37 °C (Fig 2:16), and there was only minimal growth of the fungus after an initial decrease in ATP concentration.

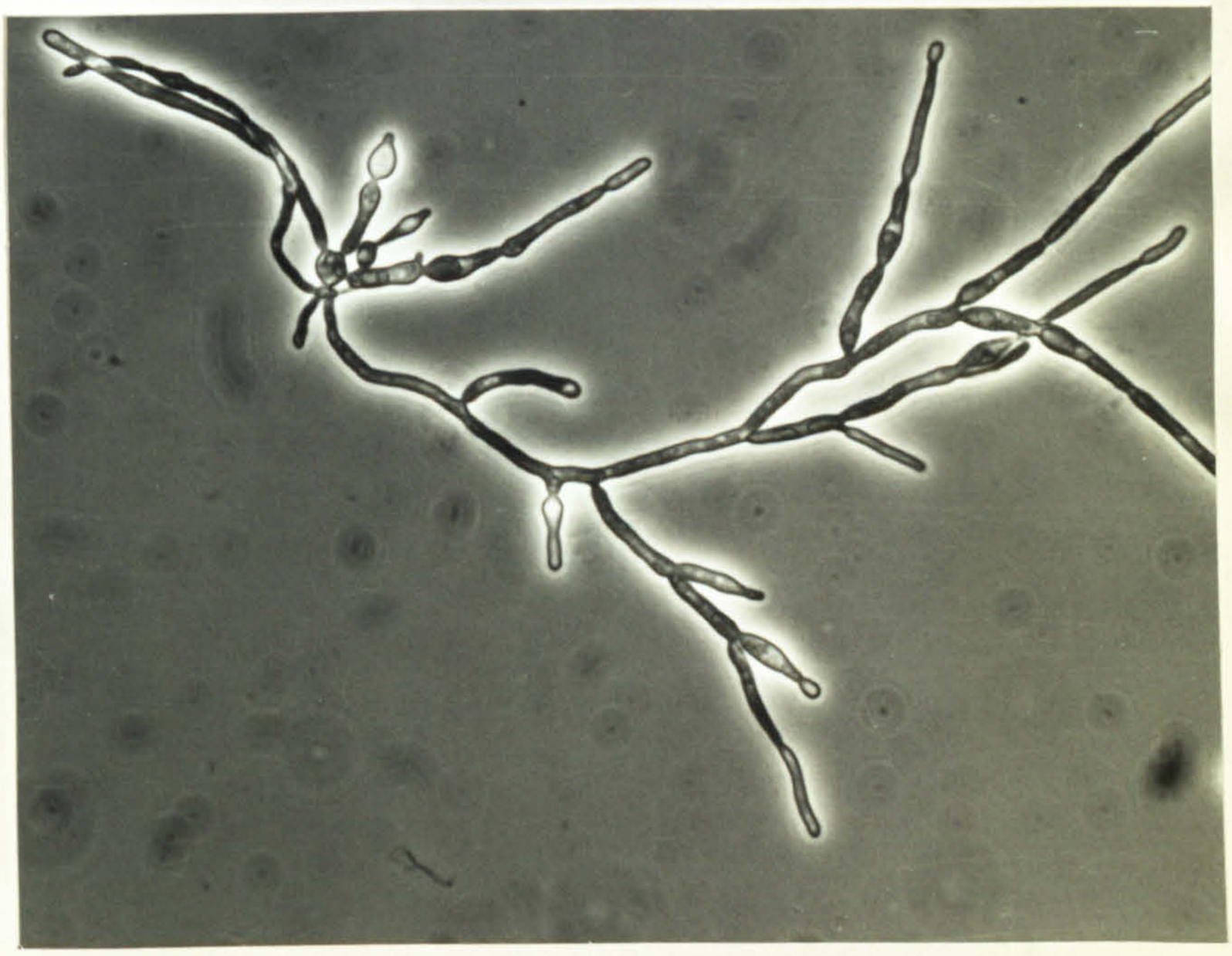
Effect of ketoconazole on morphology and growth of C. albicans

MSAB shaken in air at 40 °C

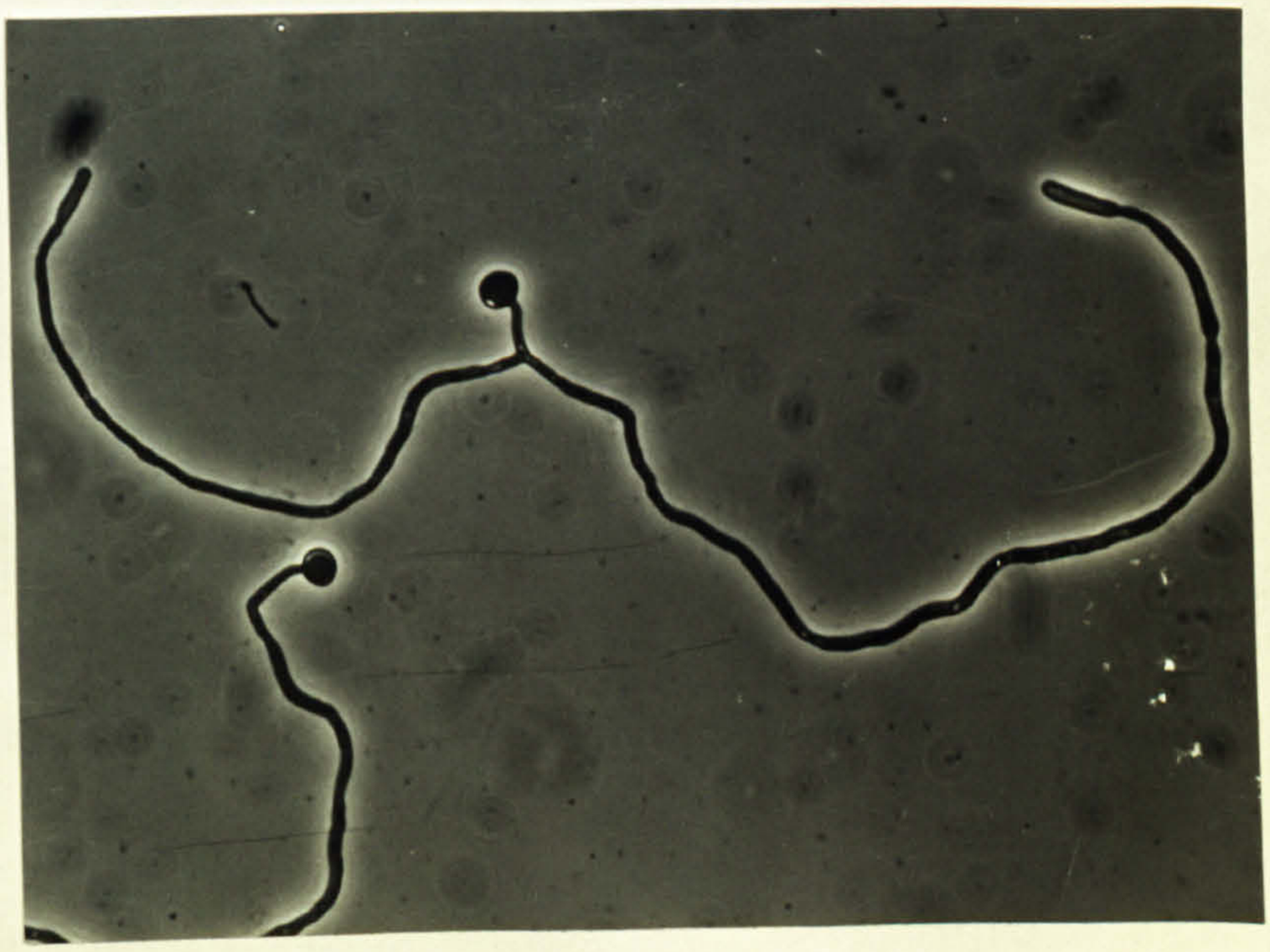
Cultures of isolate 73/025 grown in the presence of ketoconazole in the concentration range 10^{-5} - 10^{-7}M showed similar morphology to that of the control cultures, germ tube initiation being followed by secondary blastospore production (Fig 2:18). Ketoconazole slightly reduced the

Fig. 2.17. Effect of medium composition on the microscopic appearance of 24h old hyphae of isolate 73/055 incubated statically under 5% CO_2 in air at 37°C.

a) Hyphae grown in EMEM salts + glucose + glutamine.

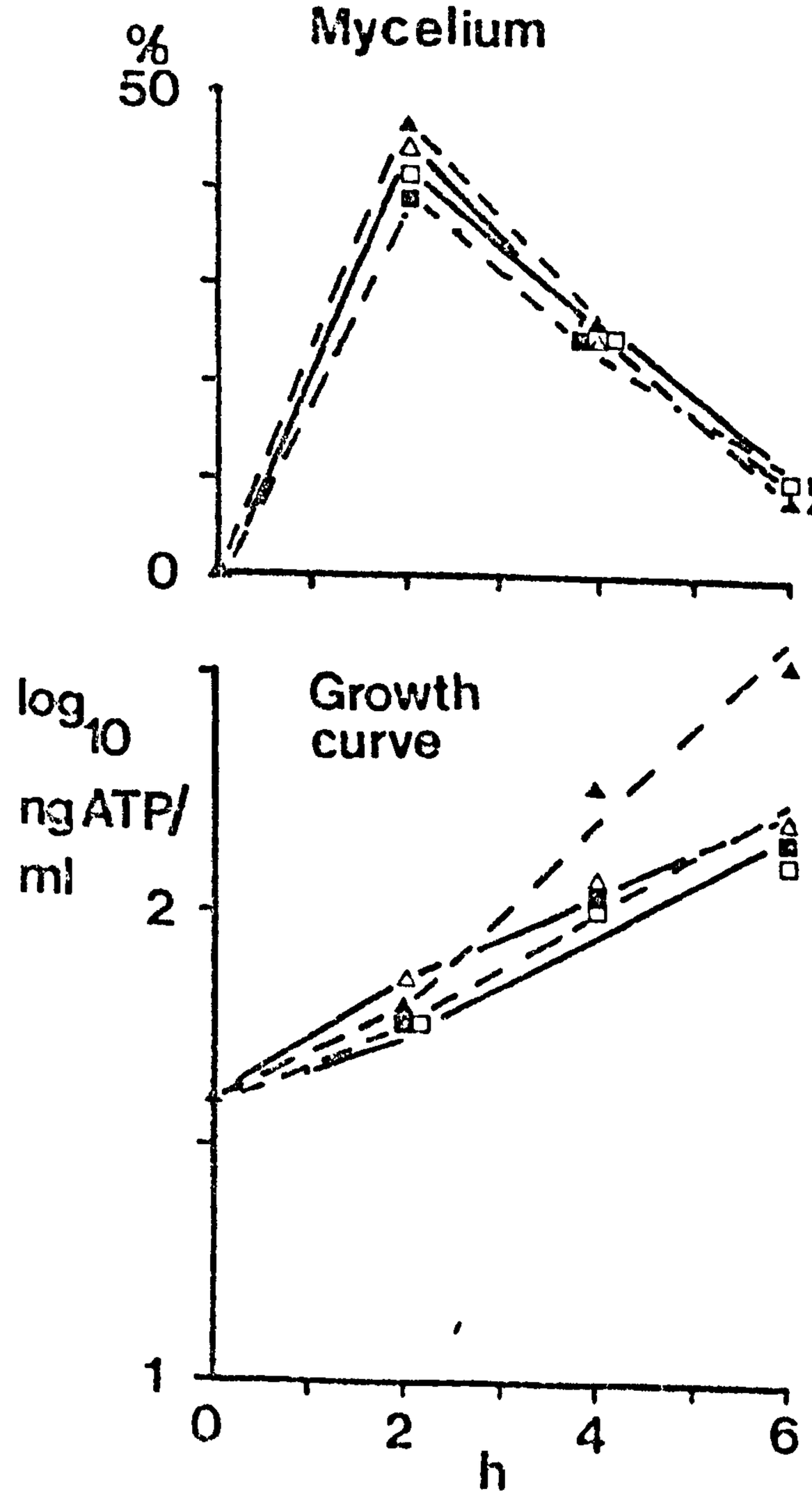


b) Hyphae grown in EMEM salts + N-acetyl-D-glucosamine.



Hyphae grown in EMEM salts + glucose + glutamine were more pleomorphic, more highly branched and, in general, thicker than those grown in EMEM salts + N-acetyl-D-glucosamine.

Fig.2.18 Effect of ketoconazole on mycelium production and growth of isolate 73/025 in MSAB shaken in air at 40°C.



▲ = control

■ = + 10^{-5}M ketoconazole

□ = + 10^{-6}M ketoconazole

△ = + 10^{-7}M ketoconazole

growth rate of 73/025 in MSAB cultures shaken in air at 40 °C (Fig 2:18).

EMEM under CO_2 in air at 37 °C

When added to EMEM cultures at the time of inoculation, ketoconazole in the concentration range 10^{-5} - 10^{-8}M had no effect on the extent of germ tube initiation by isolate 73/055 (Fig 2:19). Subsequent development of hyphae was however affected. Ketoconazole treated hyphae did not produce lateral branches and were shorter than comparable control hyphae. In addition ketoconazole treated hyphae were more pleomorphic and were in some cases distorted, particularly at their apices (Figs 2:20, 2:21). These hyphae were also noticeably phase bright under phase contrast microscopy compared with phase dark control hyphae. (See Fig 2:21). The growth rate of ketoconazole-treated cultures was substantially slower than control cultures (Fig 2:20).

Blastospores of isolate 73/055 grown in EMEM & 20mM HEPES at 30 °C in the presence of 10^{-7}M ketoconazole were similarly phase bright after 6-8 h incubation and grew at a slower rate than blastospores grown in the absence of the drug (Figs 2:22, 2:23).

To determine the effects of ketoconazole added to established hyphal cultures, the compound was added to a concentration of 10^{-7}M to C. albicans in EMEM under 5% CO_2 in air after 2 h, 4 h and 6 h. The effects of ketoconazole on growth and morphology were less pronounced the later it was added to the cultures and there appeared to be a delay of approximately 2 h before any effects could be seen (Figs 2:24, 2:25. Table 2.3). As the cell biomass of cultures increased with age it was possible that the less pronounced effect of ketoconazole in older cultures was due to an effective decrease in the concentration of ketoconazole added per cell unit. In a further series of experiments therefore the concentration of ketoconazole added per unit biomass, at different times after incubation was kept constant by increasing the amount of ketoconazole added as the cell biomass,

Fig.2.19. Effect of ketoconazole on mycelium production and growth of isolate 73/055 in EMEM incubated statically under 5% CO_2 in air at 37°C.

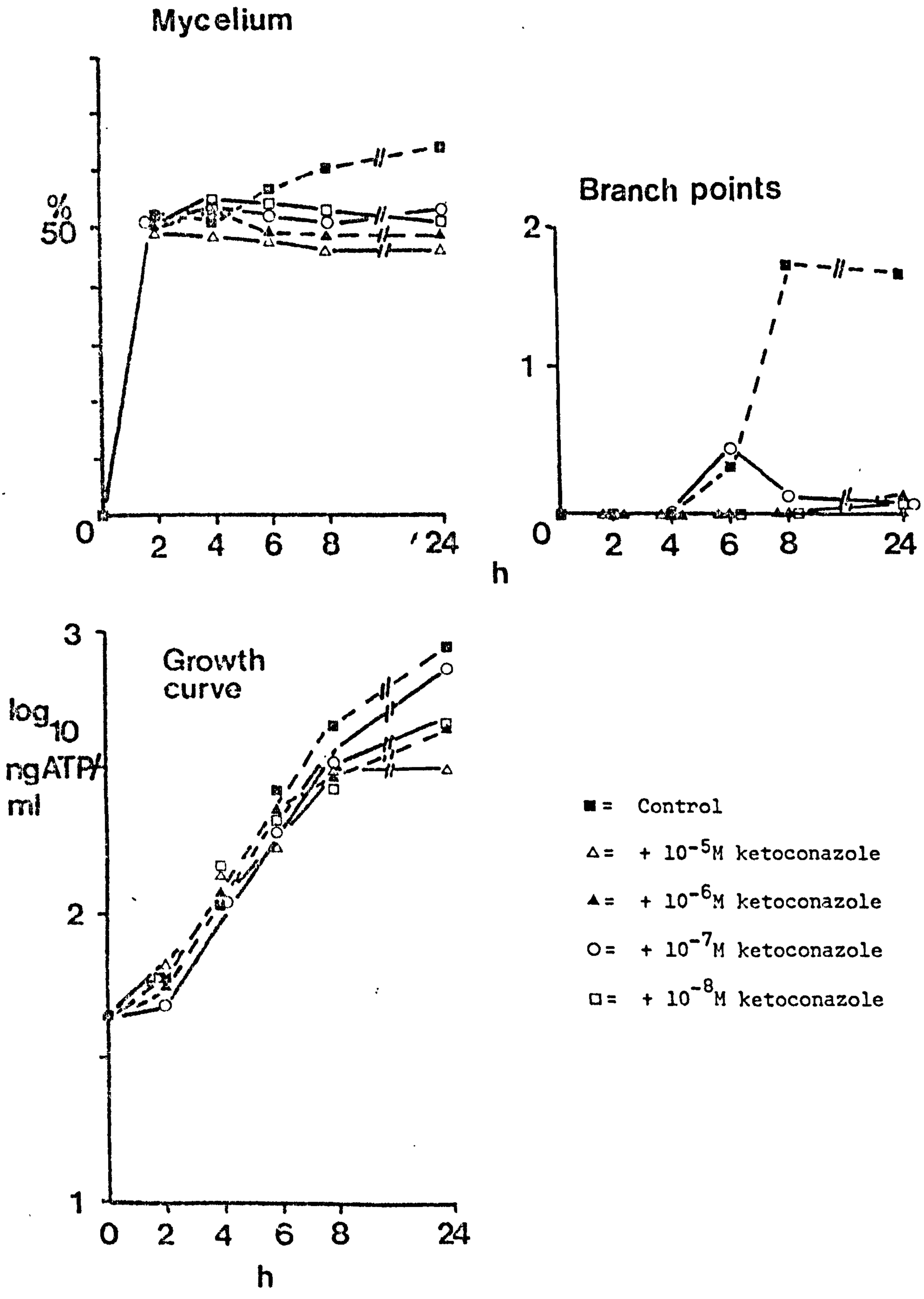
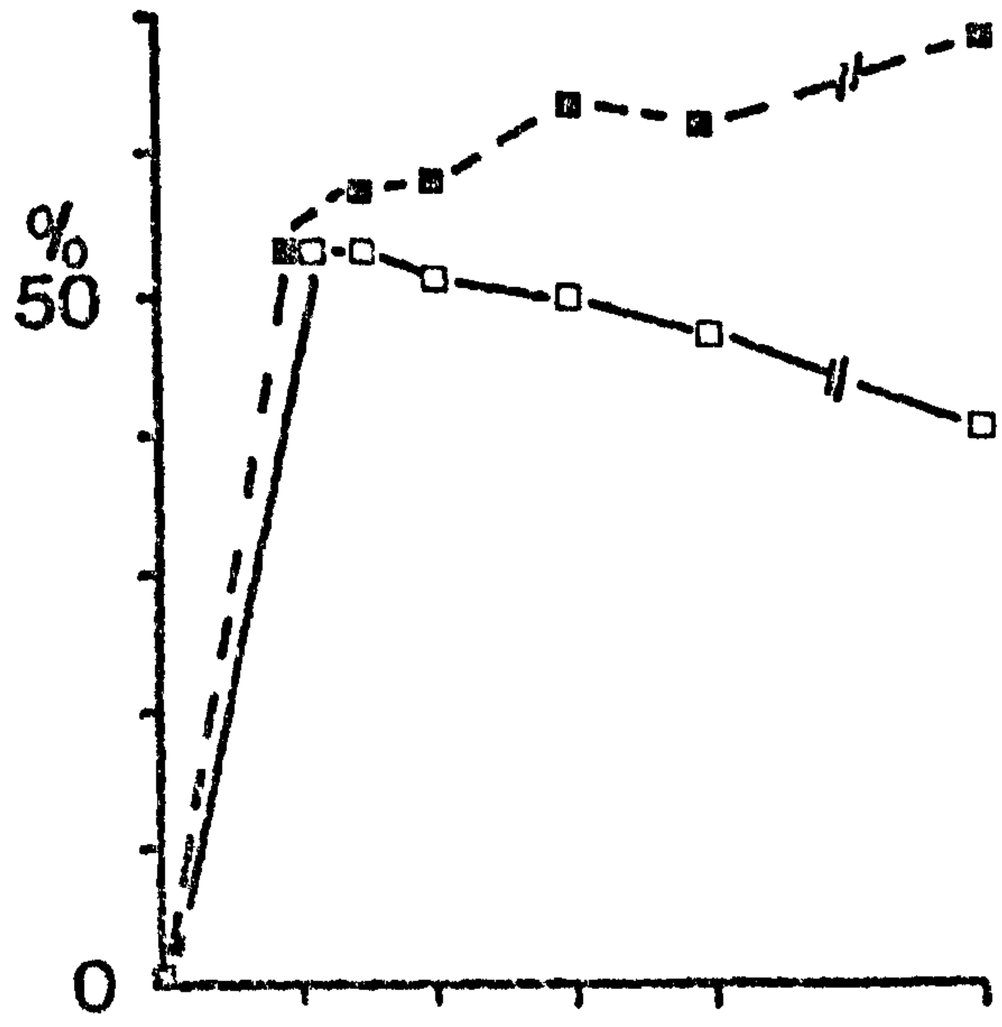


Fig.2.20 Effect of 10^{-7}M ketoconazole on mycelium production and growth of isolate 73/055 in EMEM incubated statically at 37°C under 5%CO_2.in air.

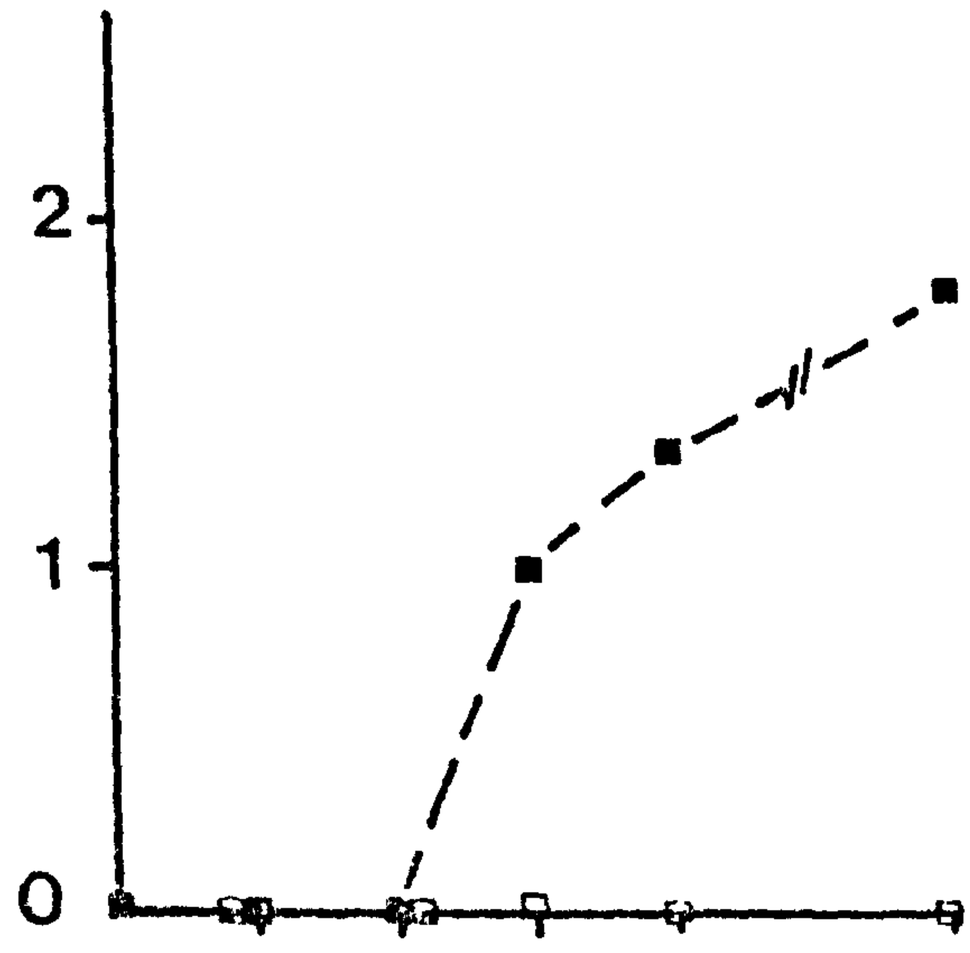
■ = control

□ = 10^{-7}M ketoconazole

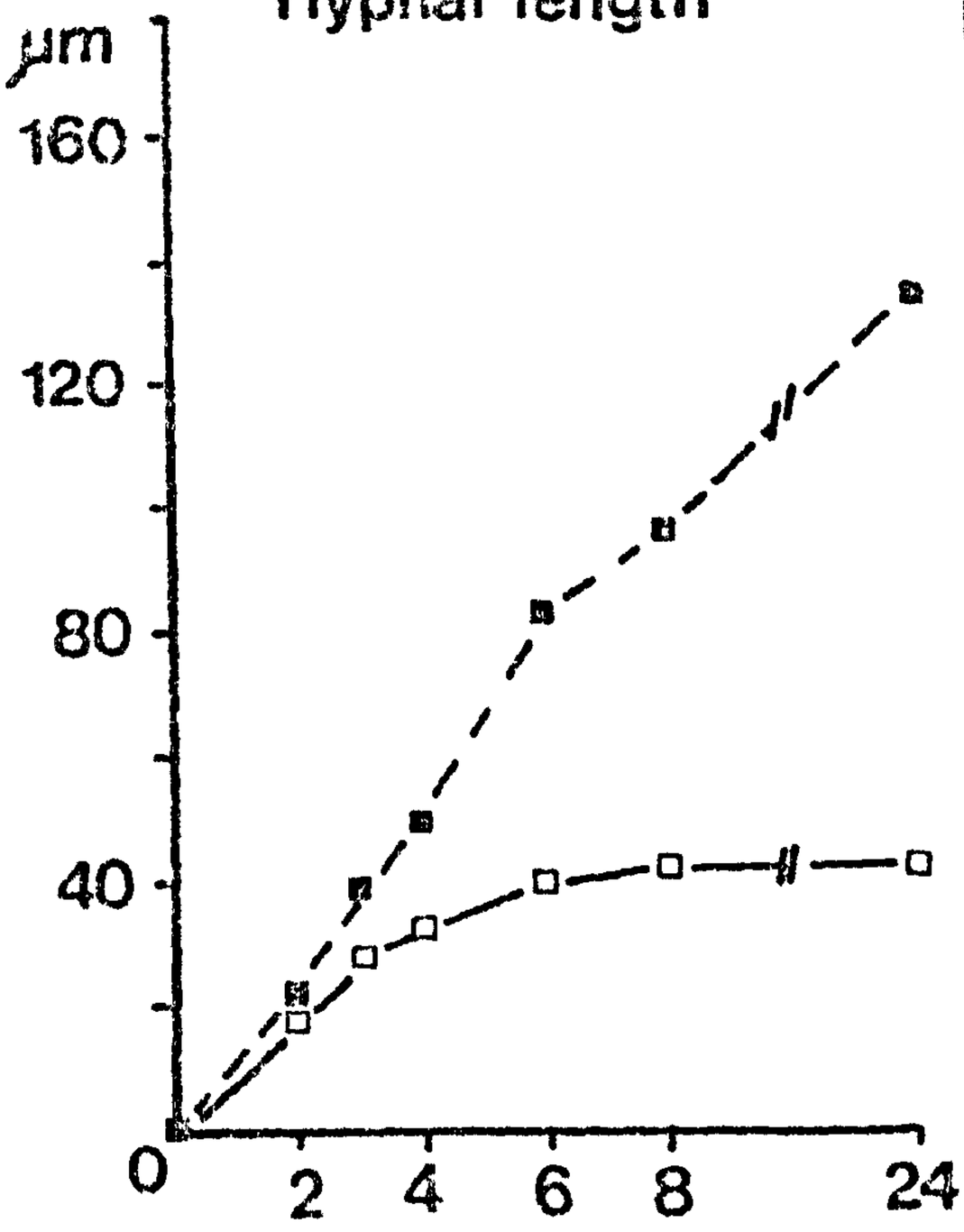
Mycelium



Branch points



Hyphal length



Growth curve

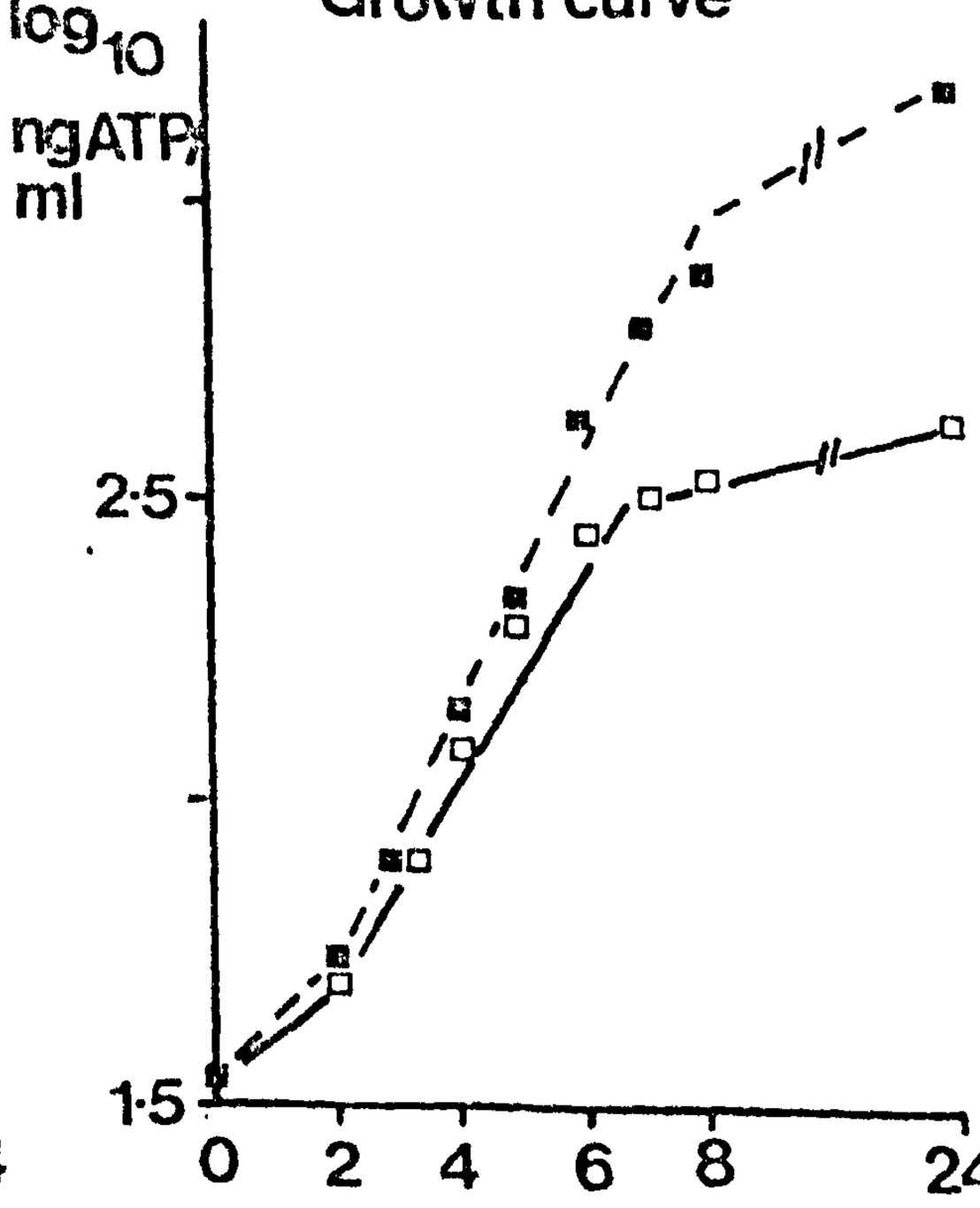
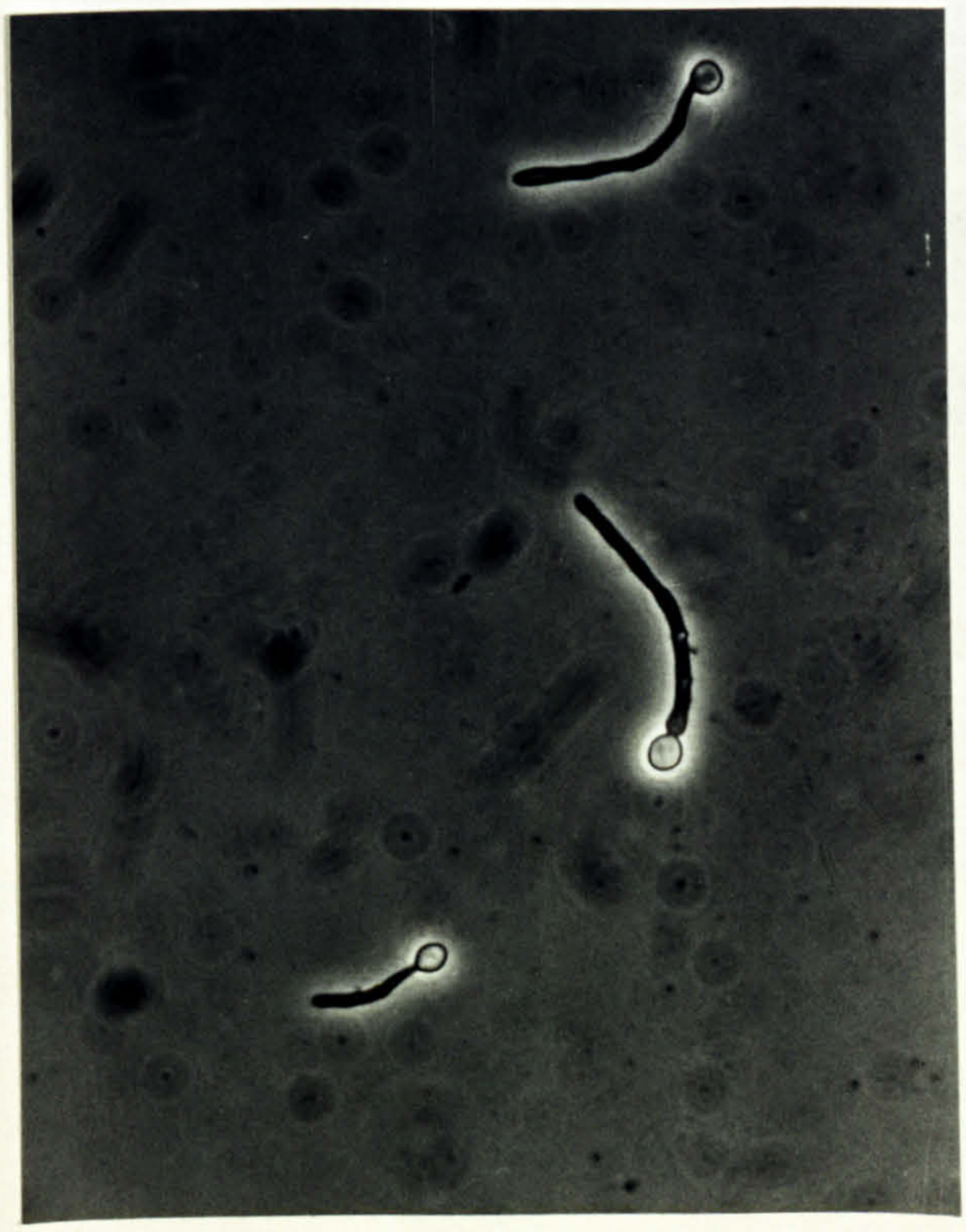


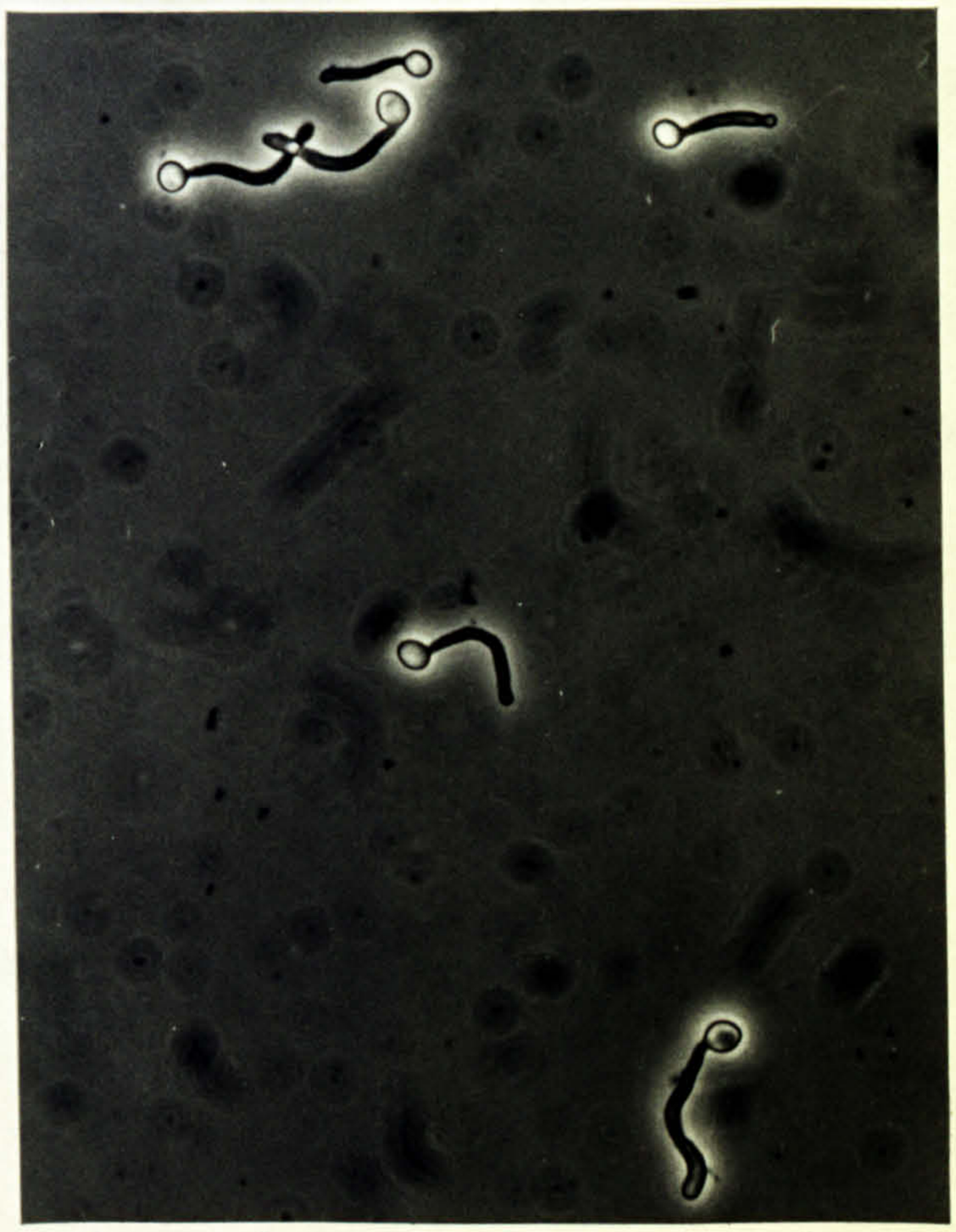
Fig.2.21. Effect of 10^{-7}M ketoconazole on the microscopic appearance of hyphae of isolate 73/055 incubated statically for various periods of time in EMEM at 37°C under 5% CO_2 in air.

a) Control 2h.



Germ tubes, phase dark.

b) +10^{-7}M ketoconazole 2h.

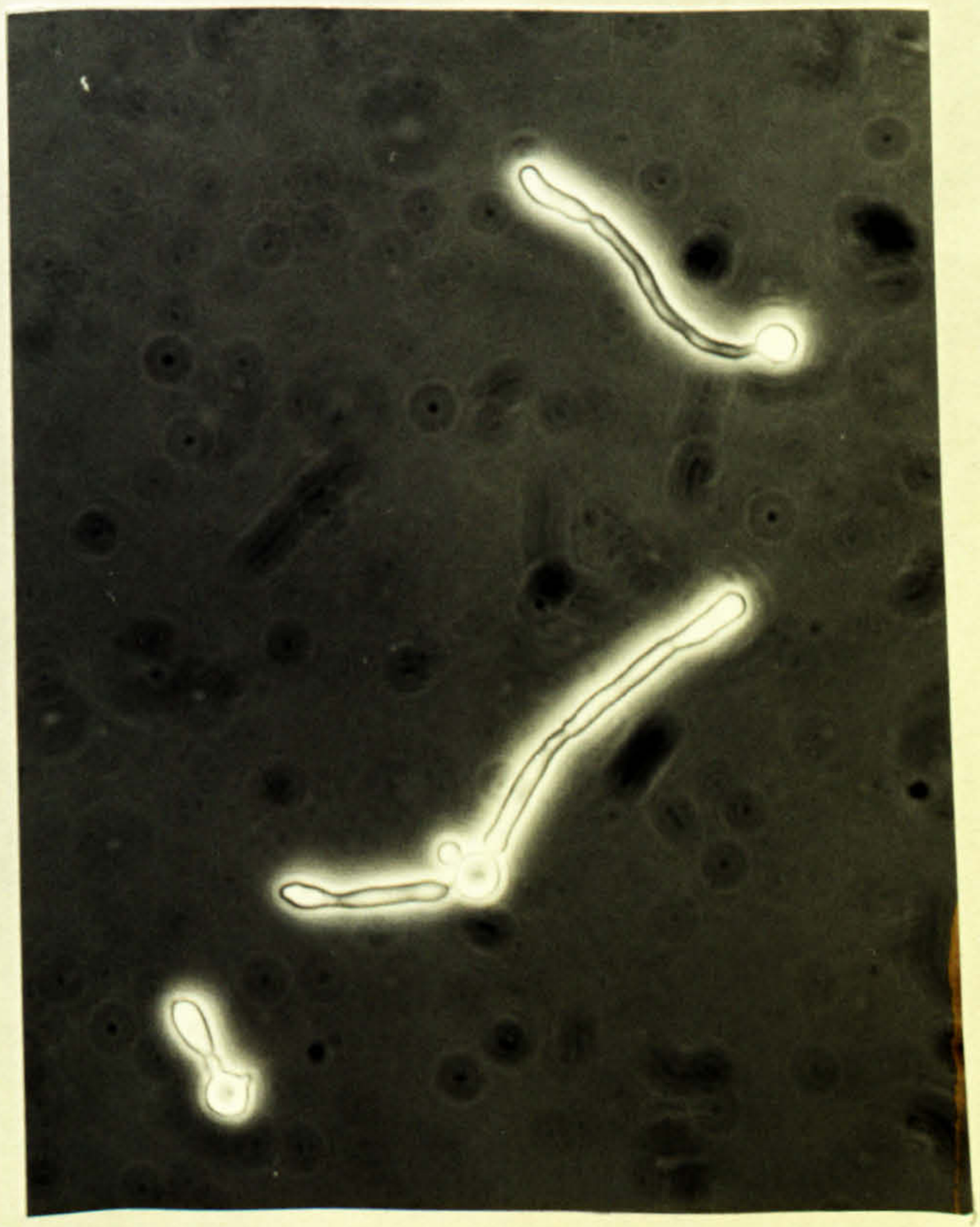


Germ tubes, phase dark but shorter than a).

c) Control 8h.

Hyphae, phase dark and branched.

d) +10^{-7}M ketoconazole 8h.



Hyphae, phase bright, unbranched and shorter than c). Hyphae pleomorphic particularly at

Fig.2.22. Effect of 10^{-7}M ketoconazole on the growth rate of isolate 73/055 incubated statically at 30°C in air.

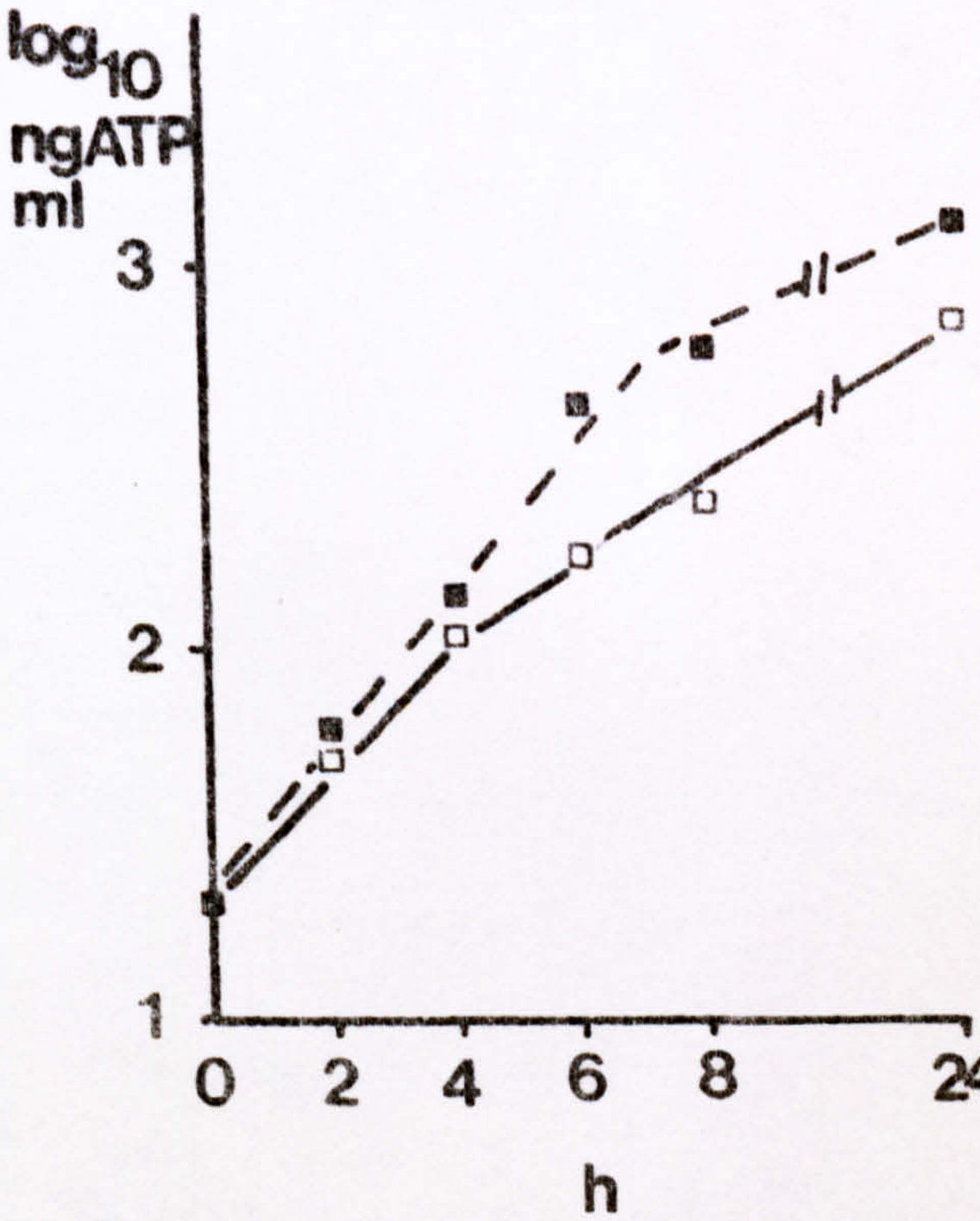
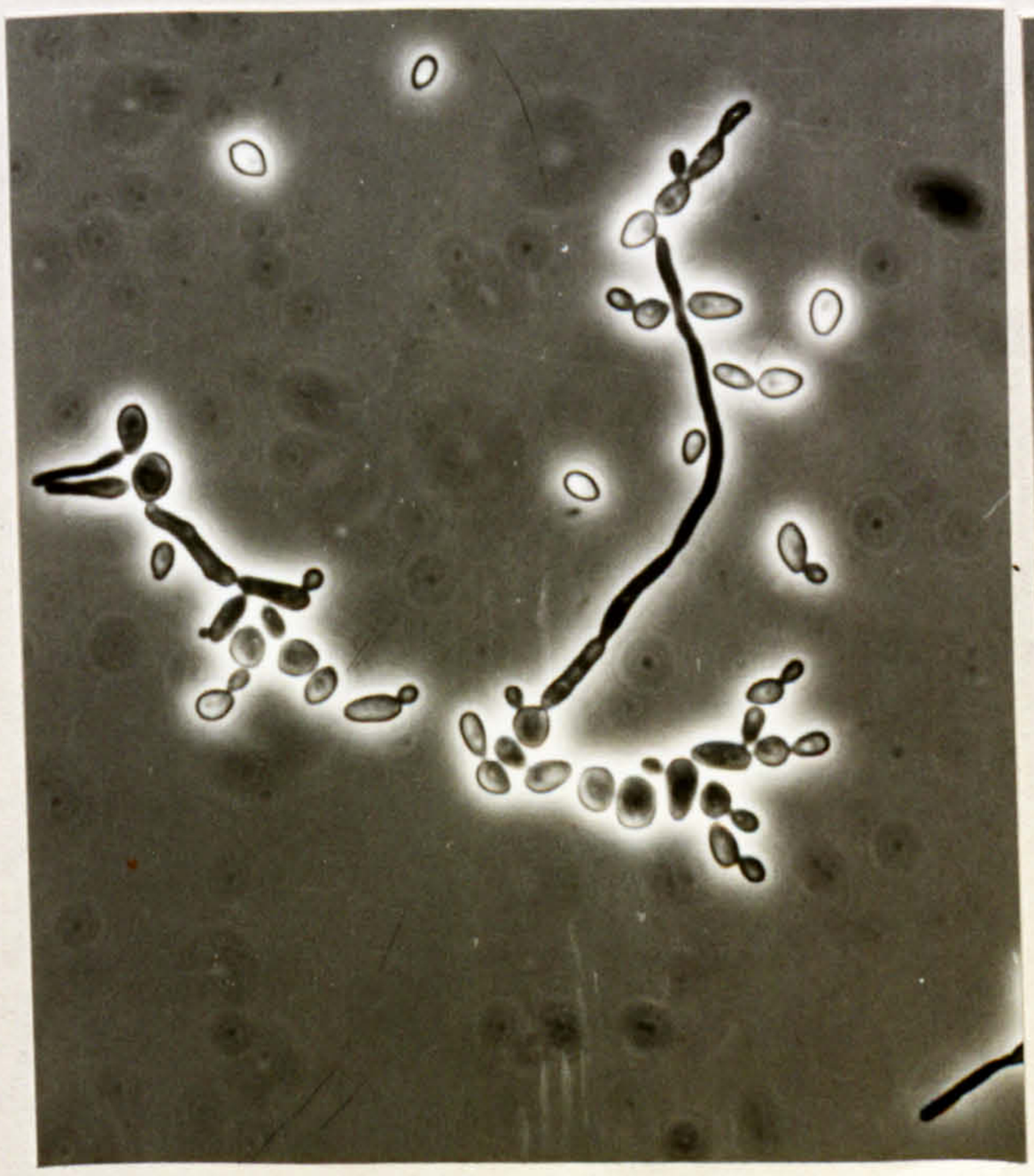


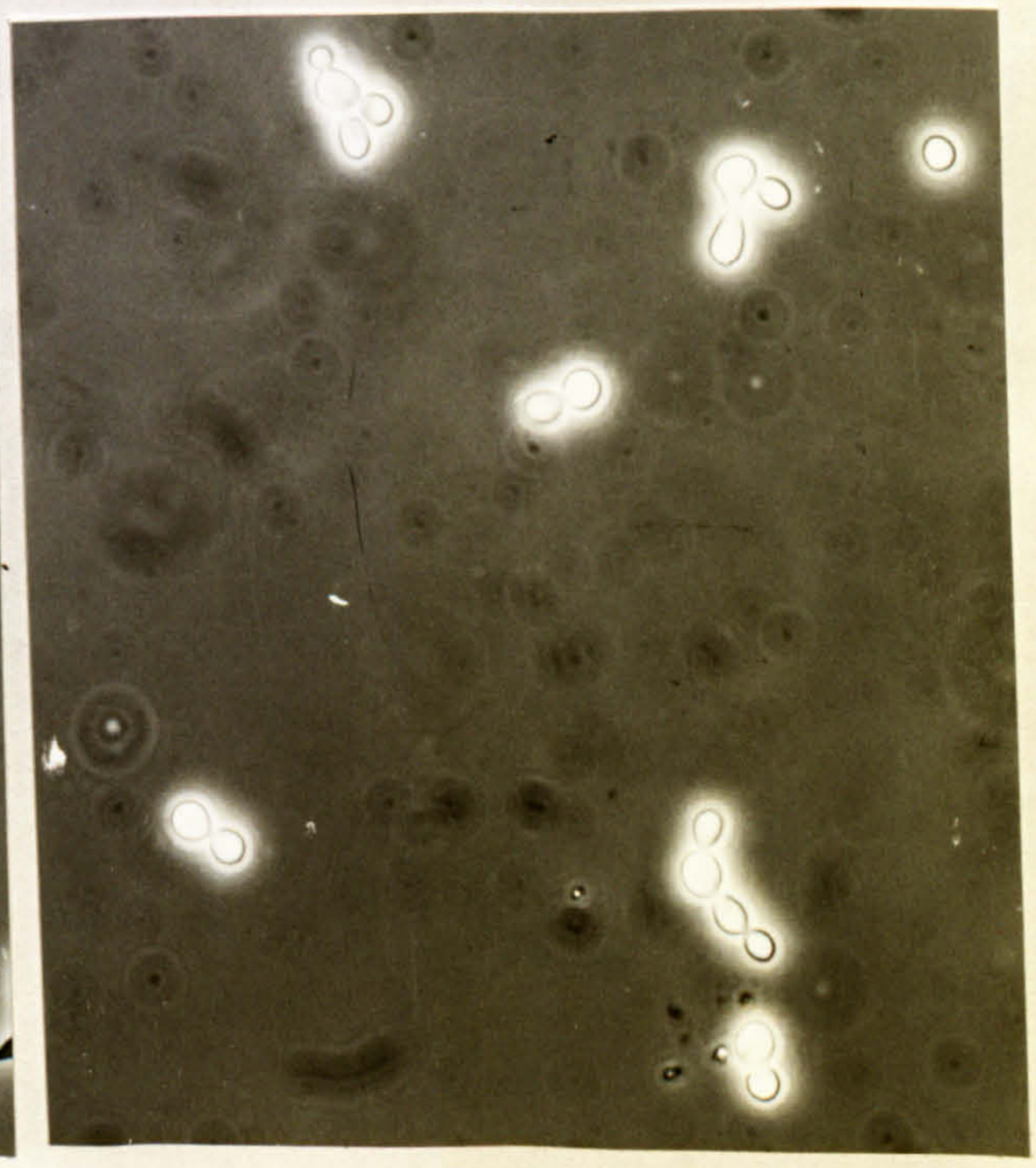
Fig.2.23. Effect of 10^{-7}M ketoconazole on the microscopic appearance of blastospores of isolate 73/055 incubated statically for 8h in EMEM at 30°C in air.

a) Control.

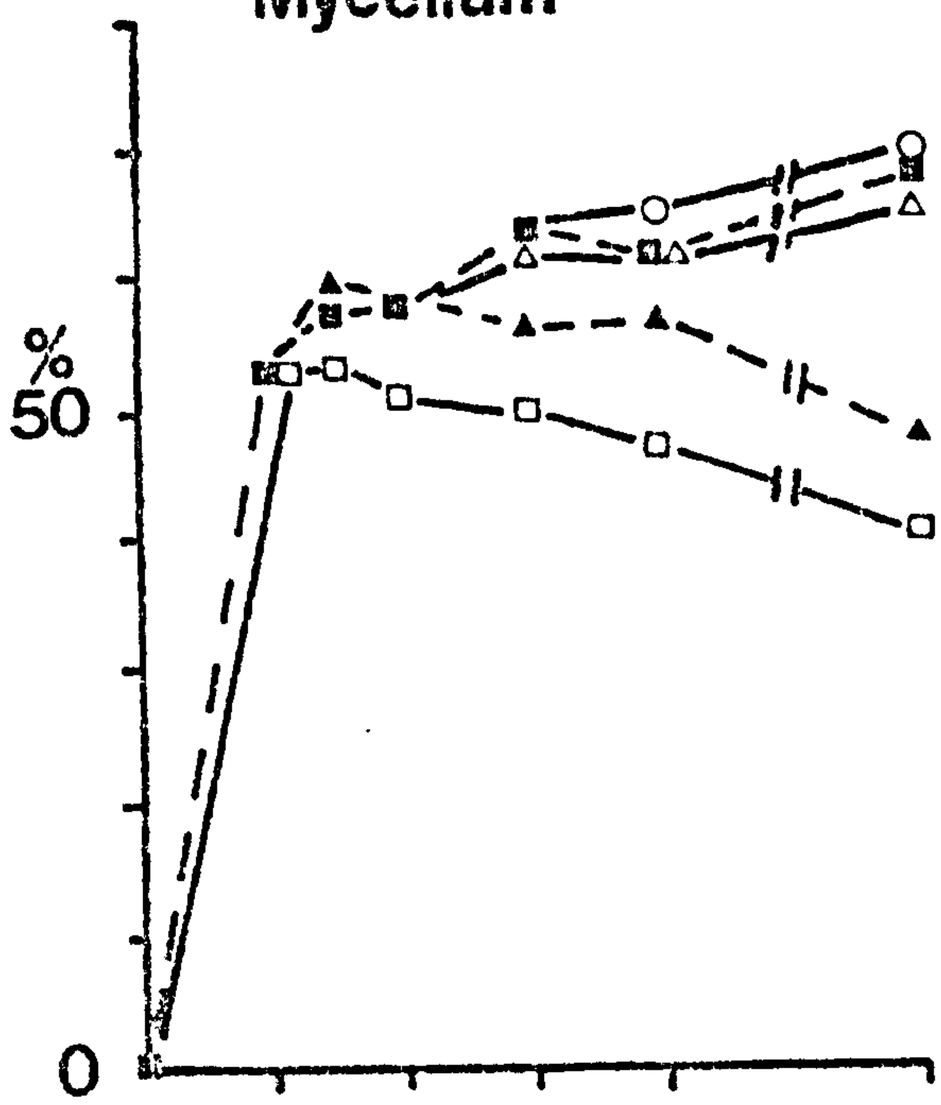


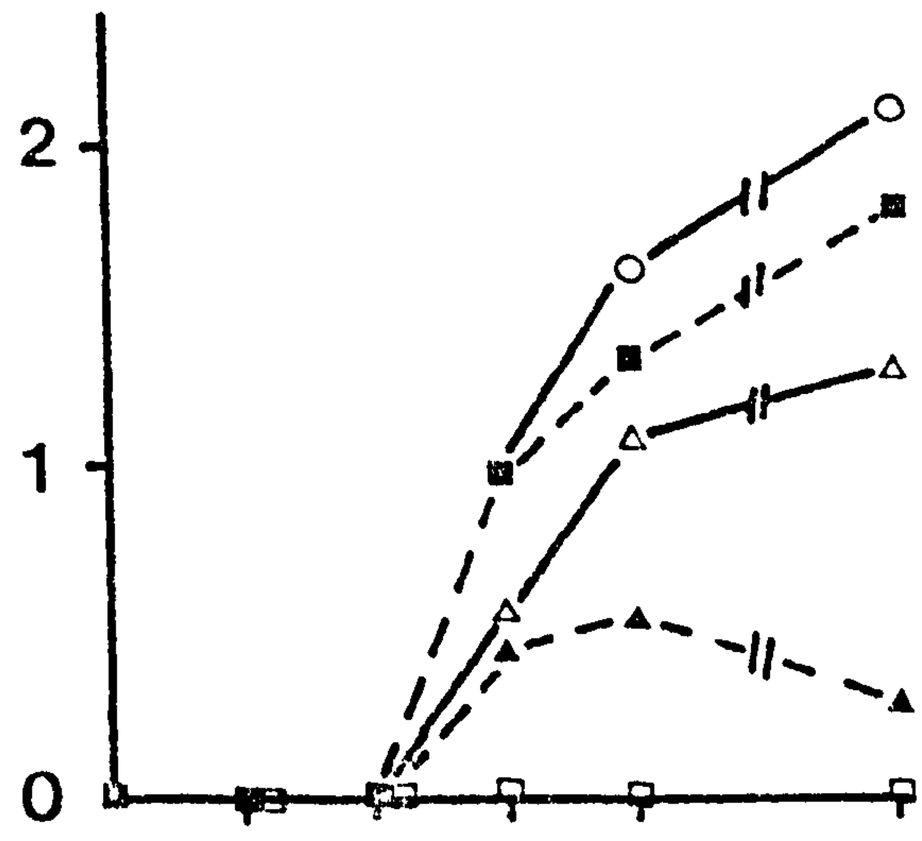
Blastospores phase dark. Some hypha production.

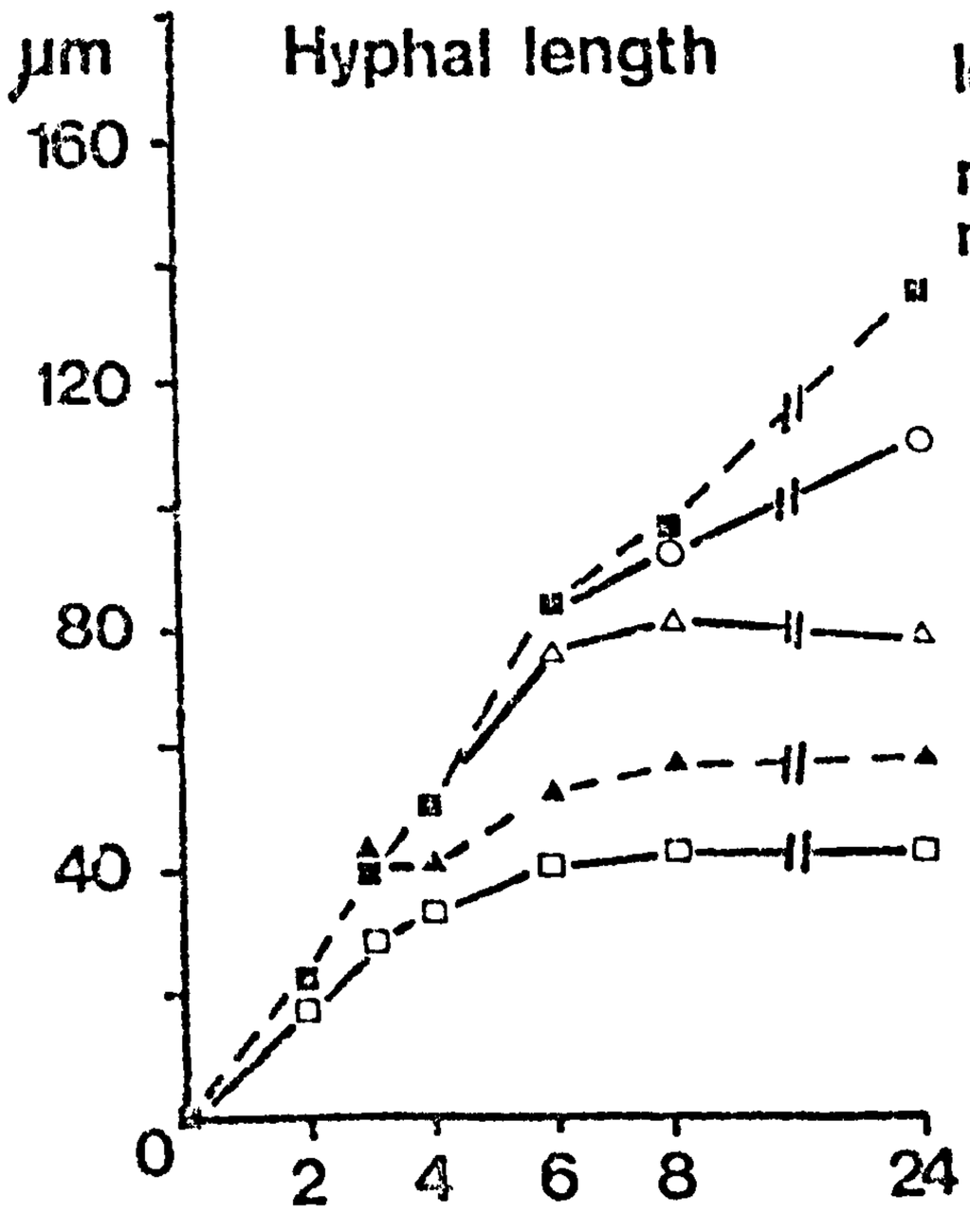
b) + 10-7M ketoconazole.

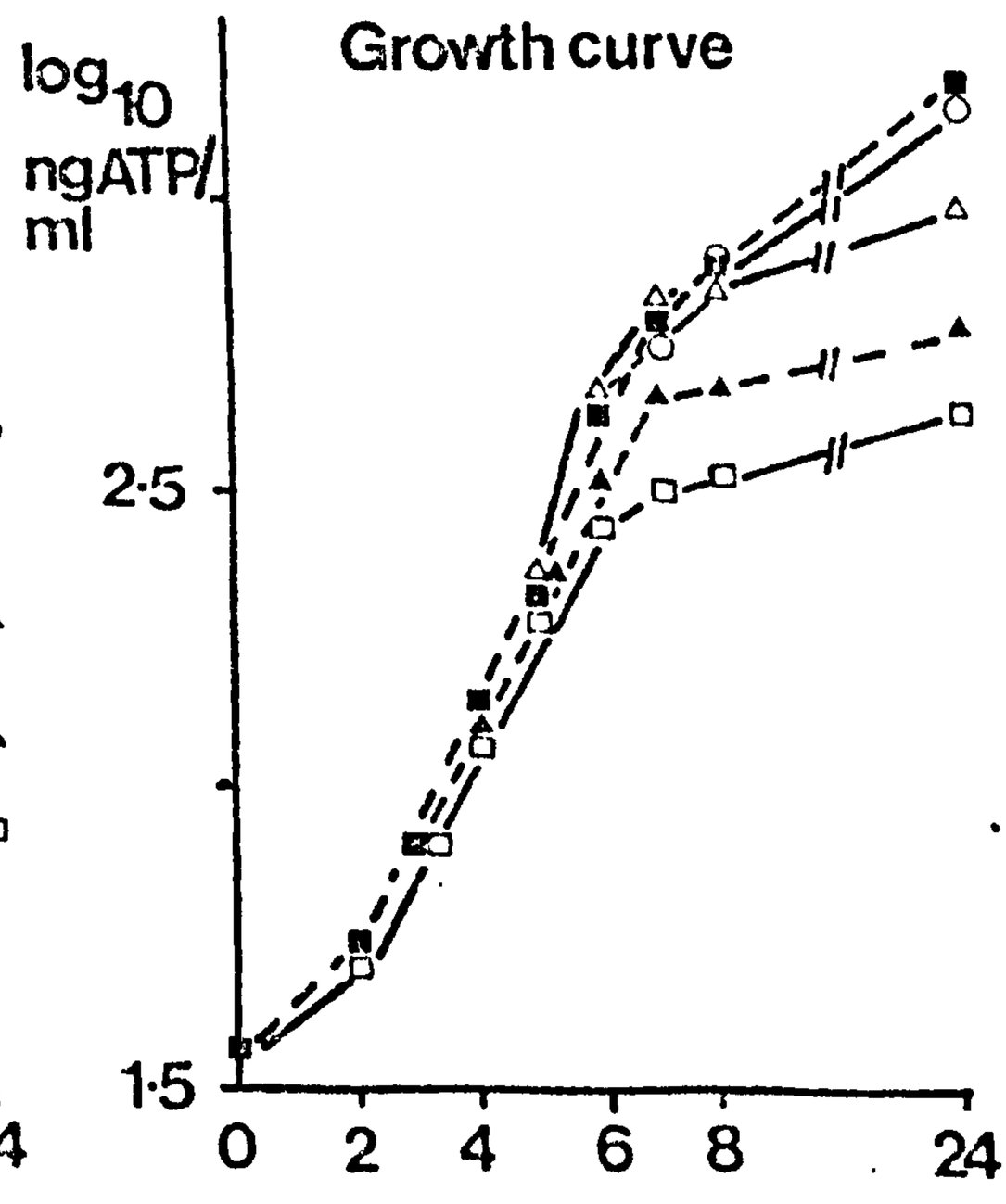


Blastospores notably phase bright. No hyphae produced.









h

Fig.2.24 Effect of addition of 10^{-7}M ketoconazole to cultures of isolate 73/055 incubated statically at 37°C under 5% CO_2 in air, at different times after inoculation.

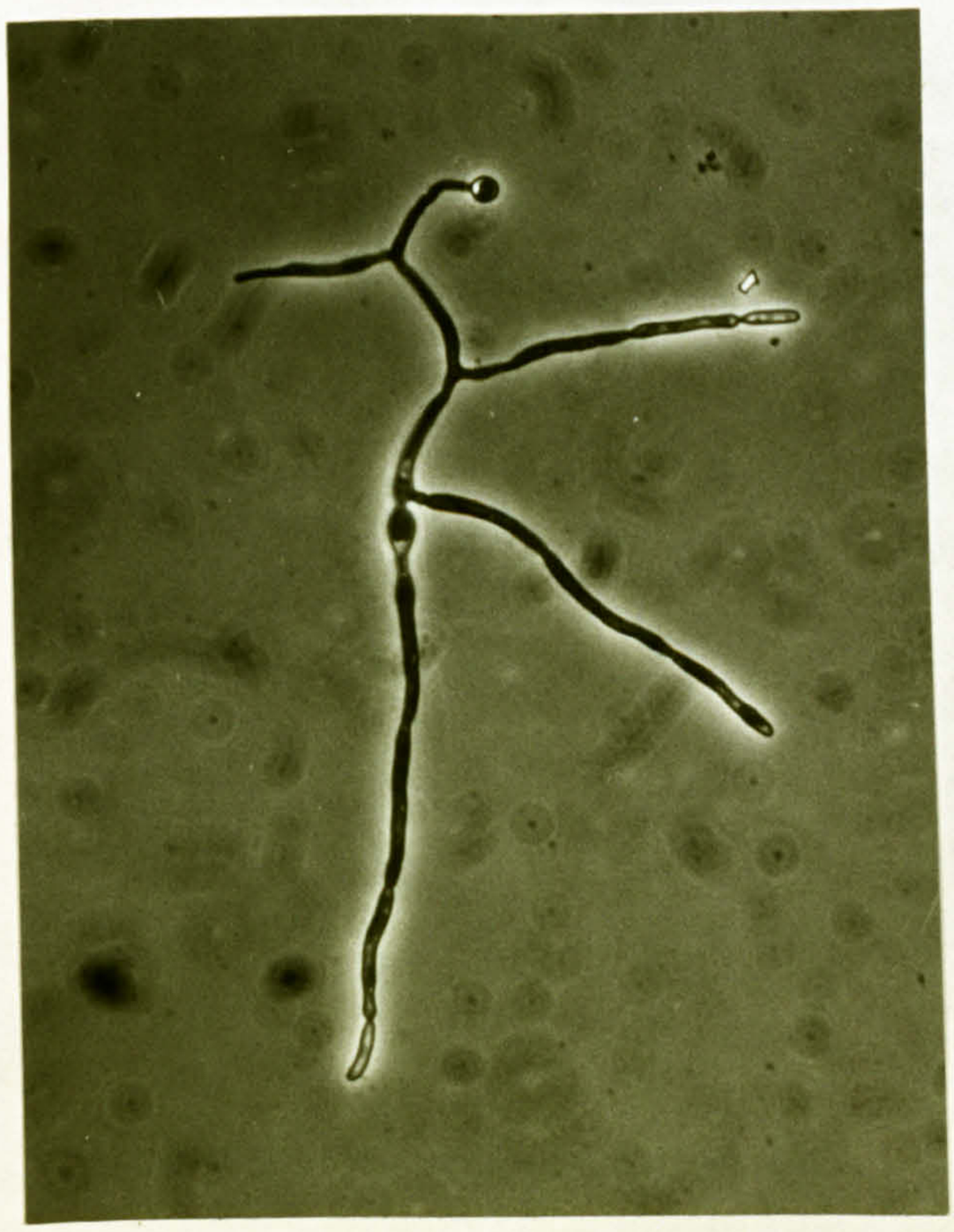
■ = Control
□ = ketoconazole added at t=0h
▲ = ketoconazole added at t=2h
△ = ketoconazole added at t=4h
○ = ketoconazole added at t=6h

Table.2.3 Doubling time(h)

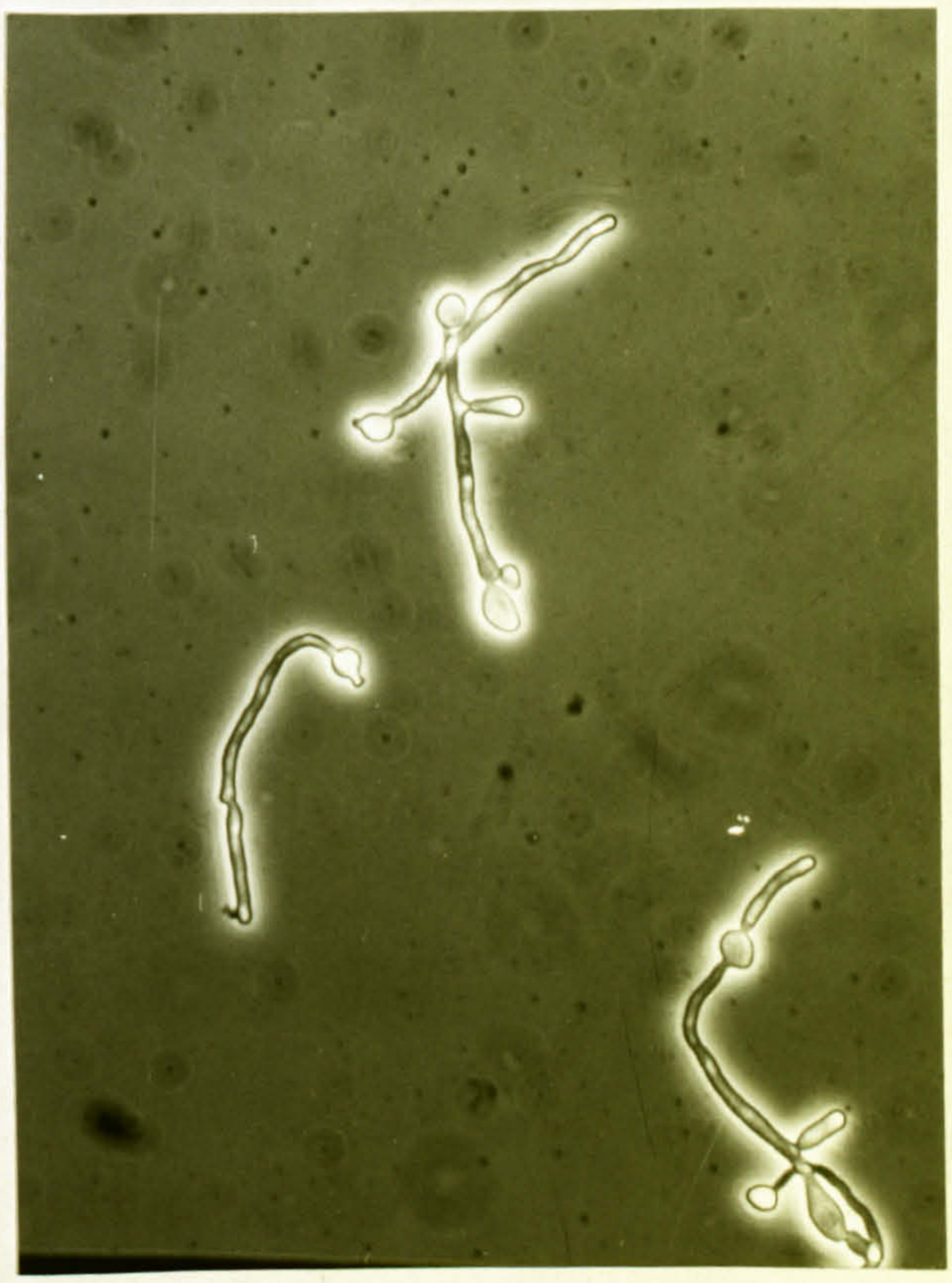
	1.78
	2.89
	1.91
	1.91
	1.85

Fig.2.25. Effect of the addition of 10^{-7}M ketoconazole at different times after inoculation, on the microscopic appearance of hyphae of isolate 73/055 incubated statically for 24h in EMEM under 5% CO_2 in air at 37°C.

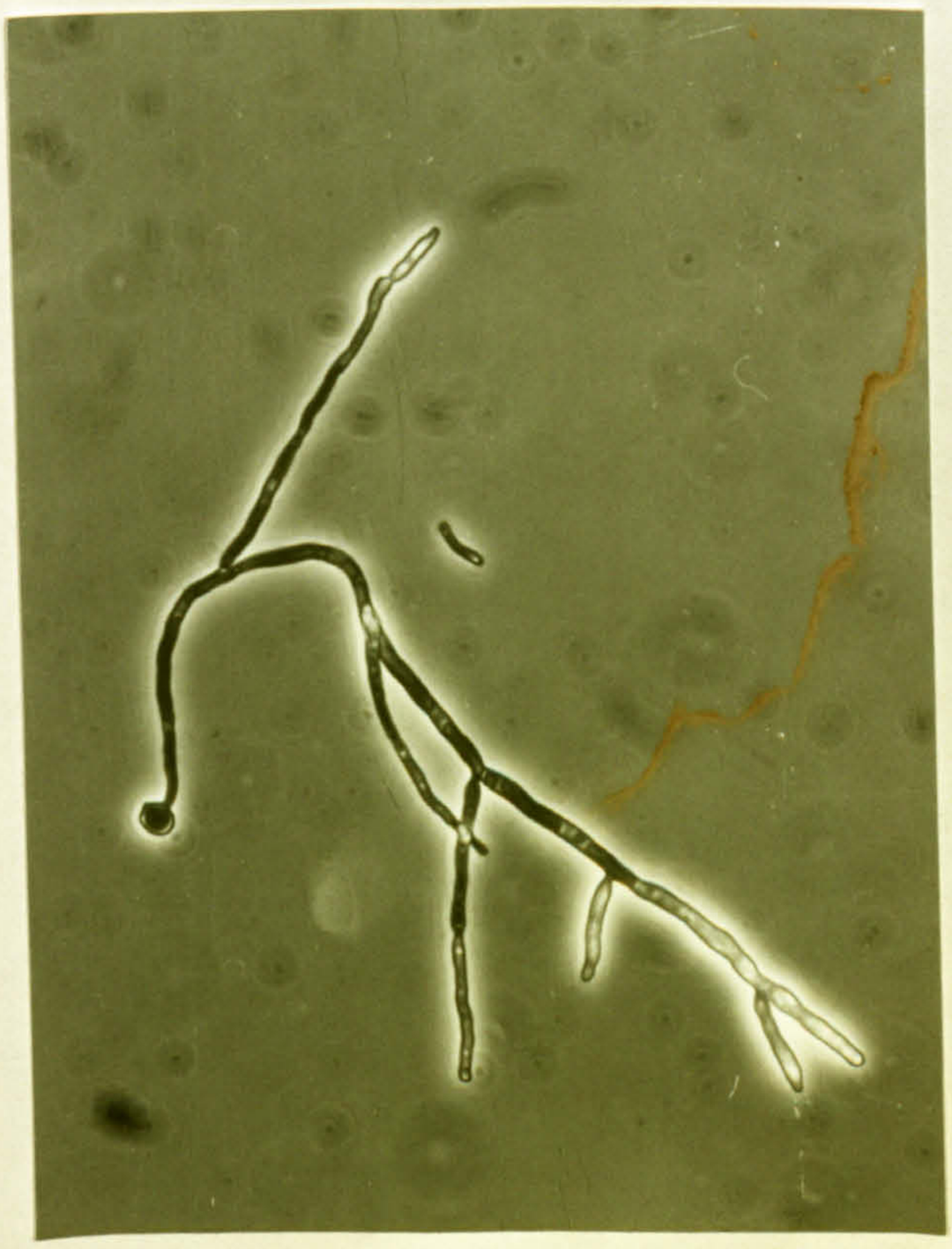
a) Control.



b) Ketoconazole added at 2h.



c) Ketoconazole added at 6h.



Hyphae in the control were highly branched and phase dark. Hyphae in culture b) were short, pleomorphic, unbranched and phase bright. Hyphae in culture c) were of similar length to those in the control and were also highly branched. These hyphae were, however, phase bright at their apices.

as measured by ATP photometry, increased. The results obtained in this series of experiments were, however, similar to those obtained above (Fig 2:26).

As ketoconazole appeared to be most effective when included in the medium at the same time as the C. albicans inoculum, experiments were performed in which inoculum blastospores in SAB broth, were pretreated with ketoconazole at 10^{-7}M or at a constant amount in proportion to cell biomass, for 3 h before the inoculum cultures were harvested and washed. Inoculum blastospores were treated both in the log and stationary phases of their growth. Such pretreatment caused no effects on subsequent growth and morphology of C. albicans in EMEM under 5% CO_2 in air at 37 °C (Figs 2:27, 2:28).

2:4 Discussion

Isolate variation in morphology

The preliminary experiments described in this chapter revealed a considerable degree of isolate variation in morphological development of C. albicans irrespective of the growth medium used. Isolates 73/055 and 73/025 were selected for future study. 73/025 was chosen for use in experiments where morphology was to be controlled by adjustment of incubation temperature of MSAB. However, maintenance of this isolate under laboratory conditions proved difficult and over a period of months the isolate began to produce pleomorphic inocula in SAB broth at 30 °C. This made accurate estimation of germ tube production difficult and reproducible production of cultures of a desired morphology impossible.

C. albicans 73/055 consistently gave homogenous blastospore inocula and highly branched hyphal growth in EMEM under CO_2 in air at 37 °C. At this same temperature in MSAB, 73/055 developed secondary blastospores instead of hyphal branches, which made the isolate valuable for comparison

Fig.2.26 Effect of 10^{-7} M ketoconazole on mycelium production of isolate 73/055 in EMEM when added to cultures at different times after inoculation. Ketoconazole was added at a constant antifungal : cell biomass ratio.

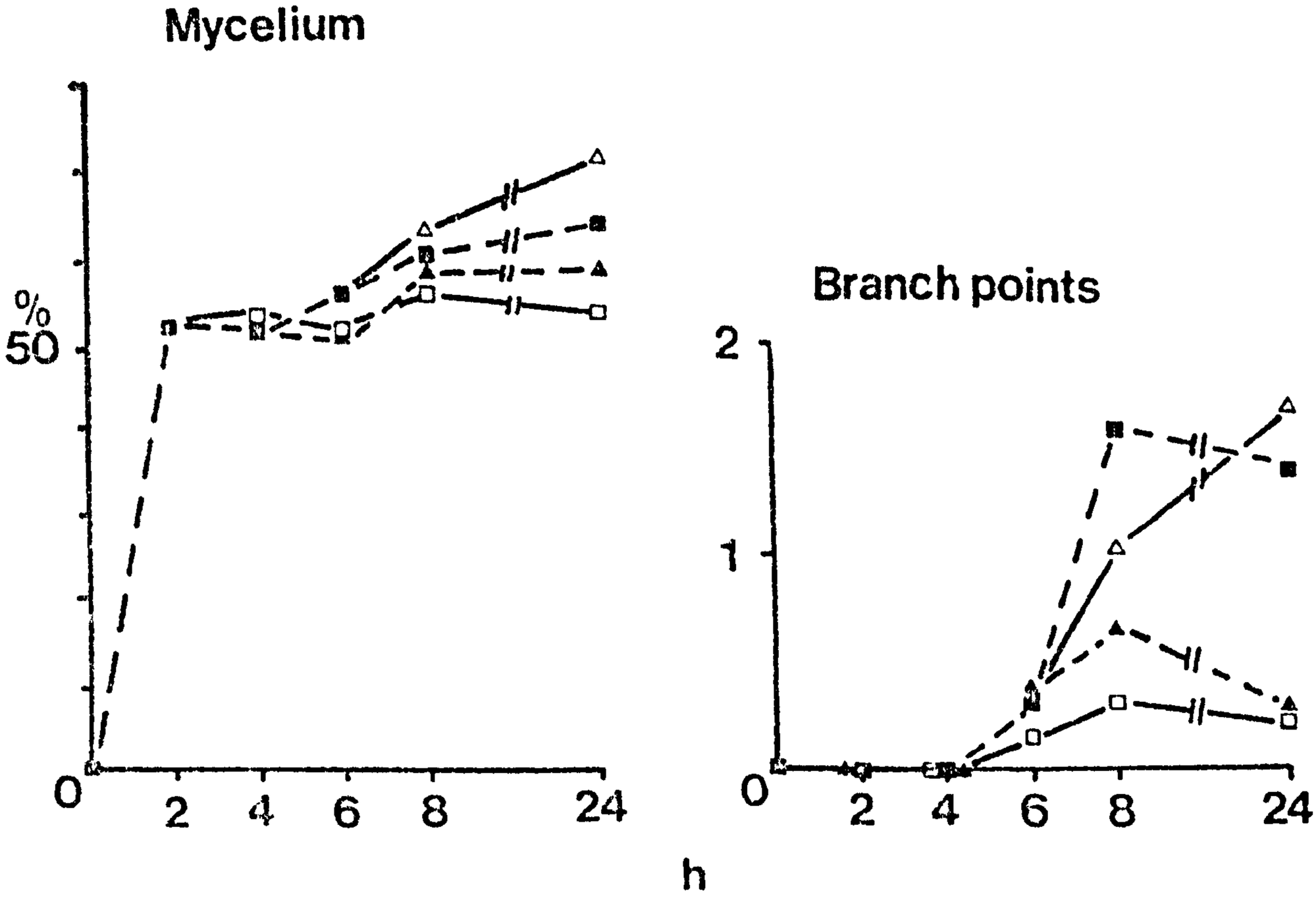


Fig.2.27 Effect of 10^{-7}M ketoconazole on mycelium production and growth rate of isolate 73/055 in EMEM at 37°C when added to blastospores in SAB broth during the preparation of inocula.

A = Ketoconazole added during the logarithmic phase of growth. SAB broth was inoculated to a concentration of 10^6 stationary phase 73/055 blastospores ml^{-1}. Cultures were incubated for 6h at 30°C shaken at 160 rev min^{-1}. Ketoconazole was then added to a final concentration of 10^{-7}M and cultures were incubated for 3h. Blastospores were harvested by centrifugation at 2500g for 5min, washed twice in distilled water and resuspended to their original volume in stale SAB broth removed from a duplicate control culture. Cultures were reincubated at 30°C for a further 9h.

B = Ketoconazole was added during stationary phase of growth. 73/055 blastospores were grown for 15h at 30°C in SAB broth shaken at 160 rev min^{-1}. Ketoconazole was then added to a final concentration of 10^{-7}M and cultures were incubated for a further 3h at 30°C.

Blastospores exposed to ketoconazole during the logarithmic or stationary phases of growth were harvested after 18h incubation at 30 C, washed twice in distilled water and added to EMEM to an initial concentration of 10^6 blastospores ml^{-1}.

▲= Control

△= 10^{-7}M ketoconazole added at t= 0

Data represent the mean of two separate determinations.

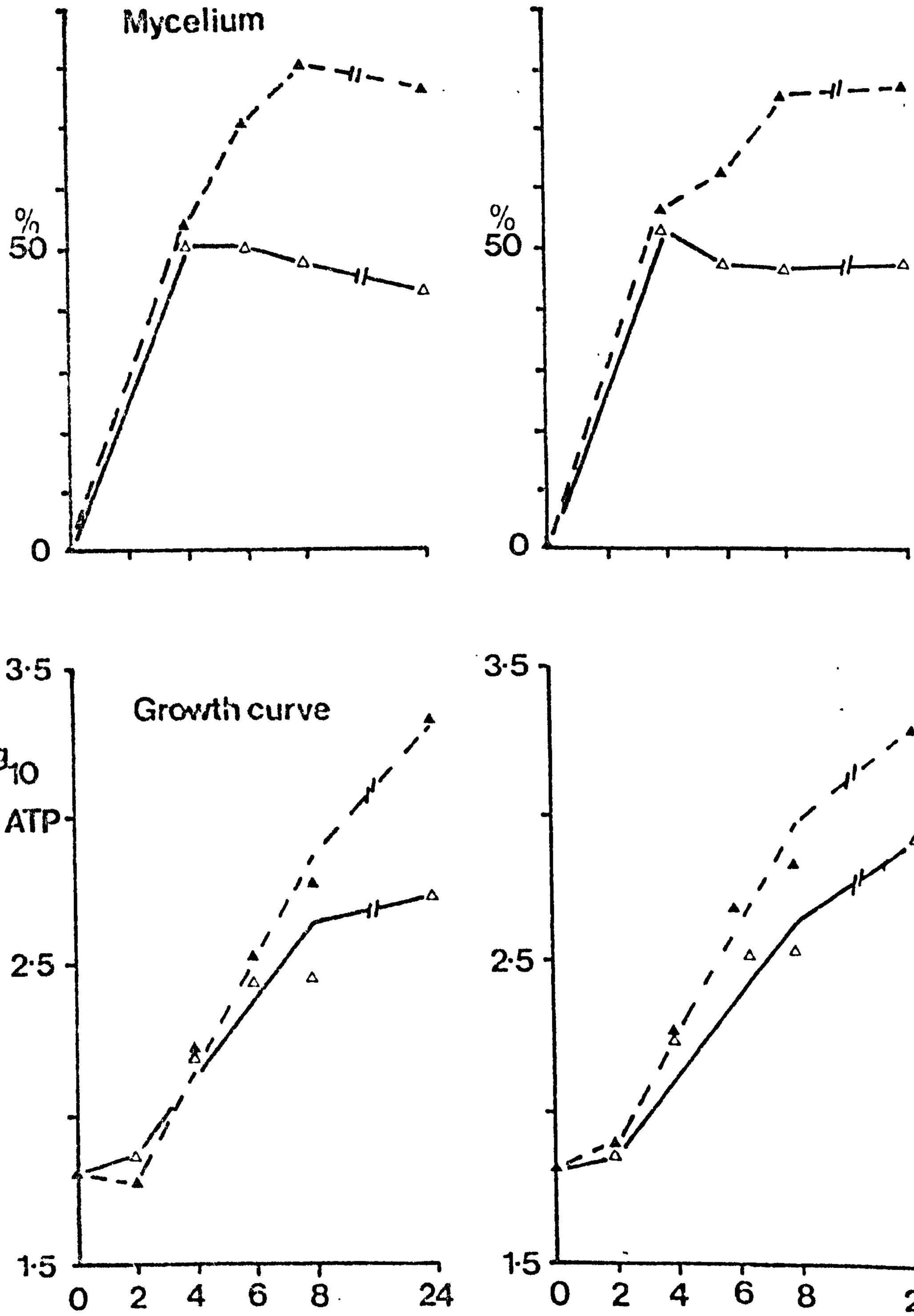

A
B
Mycelium
%
50
0
Growth curve
log10
ng ATP
ml
3·5
2·5
1·5
0
2
4
6
8
24
h

Fig.2.28 Effect of 10^{-7}M ketoconazole on mycelium production by isolate 73/055 in EMEM at 37°C when added to blastospores in SAB broth during the preparation of inocula at a constant antifungal:cell biomass ratio.

The experiments were performed as described for Fig.2.27 except that ketoconazole was added at a constant antifungal: cell biomass ratio.

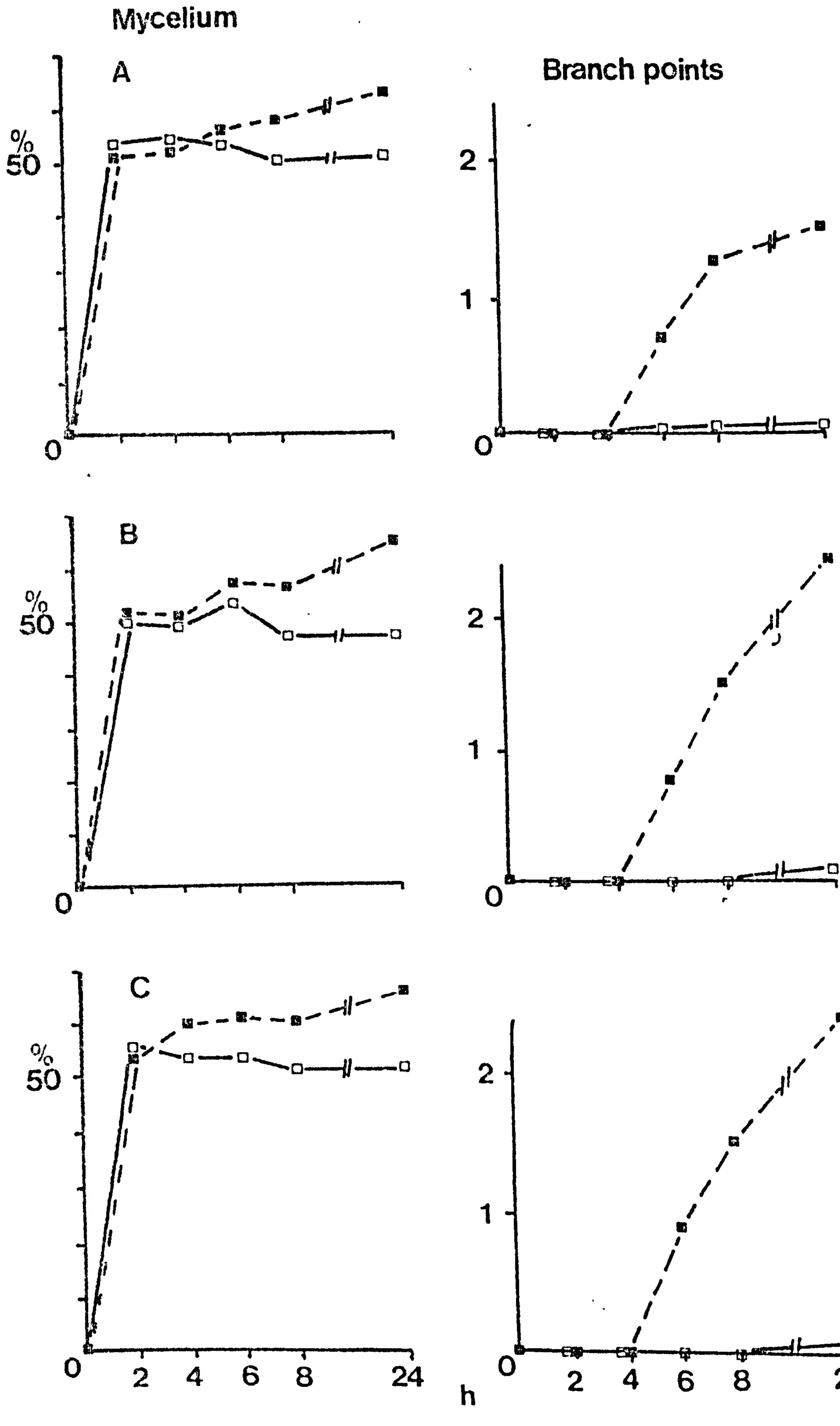
A = control

B = ketoconazole added during logarithmic phase of growth

C = Ketoconazole added during stationary phase of growth

▲ = control

△ = 10^{-7}M ketoconazole added at t=0h


Mycelium
A
%
50
0
Branch points
2
1
0
B
%
50
0
2
1
0
C
%
50
0
2
4
6
8
24
h
2
1
0
2
4
6
8
24

of the effects of the chemical composition of the two media.

Although traditionally the majority of systems for growing C. albicans have used shaken culture media, static incubation appears to have some advantages over this method when the morphology of the fungus is being investigated. In shaken cultures C. albicans hyphae rapidly clump, making homogenization of samples necessary before morphology can be quantified. This procedure may disrupt the organisation of mycelia and although the morphology of cultures may still be quantified in such homogenates, information about the arrangement of components of mycelia may be lost. This applies particularly to hyphal branches which were found to be easily separated from mycelia by the homogenization procedure described above. Since C. albicans hyphae do not appear to clump significantly under static incubation conditions these problems are avoided.

Interaction between environmental conditions and morphological development of C. albicans

The results of experiments in which the physical and chemical nature of C. albicans growth environments was altered emphasize the complex nature of the processes that are actually involved in "dimorphism". None of the environmental factors investigated could be shown to be solely responsible for the observed changes in morphology. The results do, however, suggest that the combination of factors necessary to initiate germ tube production in C. albicans may differ from those that control subsequent development.

The stages involved in C. albicans dimorphism appear to be initiation of new growth (restricted to apical sites in budding cells, unrestricted in cells forming germ tubes; Mackenzie, 1964), development of ovoid or cylindrical outgrowths, and formation of hyphal branches or reversion to blastospore forms in the subsequent generations. The first two of these stages were inseparable in the present experiments, but broad differences could be detected in environmental effects on initiation of germ tube or blastospore growth in the first generation and on subsequent branching or reversion to budding.

The extent to which germ tubes were initiated in C. albicans 73/025 and 73/055 was little affected by the chemical composition of the growth medium when the inoubation temperature was 37 °C or 40 °C but it was reduced at 30 °C. In EMEM salts medium at 37 °C germ tube initiation was independent of the nature of the carbon and nitrogen sources, so long as such sources were supplied (Fig 2:16), and ketoconazole, whether added to EMEM cultures at the time of inoculation or preincubated with inocula did not affect subsequent initiation of germ tubes.

By contrast, the chemical composition of the growth medium, including the nature of carbon and nitrogen sources, the atmosphere of incubation, the presence of ketoconazole and the extent of agitation of cultures all appeared to play a part in the subsequent maintenance of a stable, branched mycelial form. In general, branch formation was higher in static cultures than in shaken cultures (Table 2:2), in EMEM than in MSAB, and under CO_2 in air than in air alone (Table 2:2. Fig 2:8) and with glutamine as nitrogen source rather than with NH_4Cl. At very low concentrations ketoconazole inhibited branch formation in EMEM cultures without greatly retarding growth of C. albicans (Figs 2:19, 2:20) and caused formation of pleomorphic, phase-bright pseudohyphae and budding cells. In all experiments the effects of these environmental factors appeared to act in combination: thus, MSAB cultures shaken in air at 30 °C were likely to produce only blastospores of C. albicans 73/055, while static EMEM cultures under 5% CO_2 in air were likely to produce only stable, branched hyphal forms.

Low oxygen tension (Johnson et al. 1954) and a controlled CO_2:02 ratio (Mardon, Balish & Philips, 1969) have been shown to favour mycelium production in C. albicans and the results of the present study can be considered in these terms. Static incubation in air could allow the establishment of an oxygen gradient of decreasing concentration through the growth medium. In addition, normal energy metabolism of substrates by C. albicans could lead to CO_2 production. In the absence of agitation localised increases in CO_2

concentration around C. albicans cells could occur. Shaken incubation would disrupt both localized and general concentration gradients within the medium and increase the availability of oxygen, leading to secondary blastospore formation. Other workers have previously reported that mycelial morphology of C. albicans correlates with reduced activity of the enzymes involved in the production of substrate associated with processes other than cell wall synthesis and that an increase in the activity of these enzymes correlated with blastospore budding (Chattaway et al. 1973). It is possible that the increased growth rate associated with shaken incubation of EMEM in air could also lead to diversion of substrate away from cell wall synthesis resulting in the production of secondary blastospores.

The observation that in anaerobic culture growth and further development of hyphae in EMEM, after germ tube production, was limited, supports the view that it is the ratio of CO_2:O_2 rather than the total absence of O_2 which is important in the control of hyphal development. Although growth of 73/055 in MSAB was slower under anaerobic conditions than in air secondary blastospore production still occurred, suggesting that this process was not affected by a lack of oxygen in the incubation atmosphere. However, the observation that isolate 73/055 still produced fully mycelial cultures in an oxygen-enriched atmosphere incubated statically in EMEM, supports the suggestion that a combination of factors is important in controlling C. albicans morphology.

Shaken incubation in EMEM and MSAB in air also leads to the formation of hyphal clumps, even after a short period of incubation. In such clumps, the effective cell concentration is raised. Several workers have previously reported the significance of cell concentration in relation to germ tube production (Evans et al.1975; Hazen & Cutler, 1979). It is possible that in shaken cultures, the close proximity of C. albicans hyphae influences morphology and in some way leads to secondary blastospore production. Hazen & Cutler (1979) suggested that some kind of cell-cell interaction

could influence germ tube production by C. albicans and the results presented in this study could reflect a similar effect. In static cultures in EMEM, C. albicans grows as a mat on the base of the culture vessel and clumps are formed only in older cultures. Studies have shown that blastospores become committed to germ tube production shortly after being placed in growth media capable of inducing germ tube formation (Evans, Odds & Holland, 1975). Since the ability of C. albicans to produce hyphal branches or secondary blastospores appears to be determined in young cells (hyphal branches and secondary blastospores usually develop in cultures within 4-5 h of inoculation) a similar process of early commitment to secondary blastospore production induced by cell-cell contact could be envisaged. This could explain the observation that secondary blastospores are not produced in older static cultures despite the presence of hyphal clumps.

The question whether the results obtained in the present study may be due to environmental influences on genetic or metabolic events in C. albicans should also be considered. Germ tube production may readily be induced by a wide range of environmental conditions but subsequent development appears to be sensitive to other environmental conditions. This may suggest that the control of the initiation of germ tube production and later development may not necessarily be closely linked in the C. albicans cell. In addition if the maintenance of mycelial form and reversion to secondary blastospore production were controlled by separate genetic pathways, sensitive to different degrees of environmental stimulation, subsequent development of hyphae may depend on the nature and strength of the environmental stimulus. The mechanism by which such genetic control would be mediated is unknown but could possibly be through control of the enzyme systems involved in the synthesis of cell wall material or in the supply of substrates for cell wall synthesis. Alternatively environmental influences could directly affect metabolism of cell wall substrates and enzyme systems to produce the morphological effects described in this chapter. Published evidence in favour of the "metabolic" hypothesis comes from the studies of Manning &

Mitchell (1981)and Brown & Chaffin (1981) who could find no evidence for de novo synthesis of proteins related to morphological development of C. albicans, suggesting that morphological changes depend on regulation of existing enzyme systems rather than activation of different sets of genes.

The results obtained with ketoconazole in the present study differ from those obtained by Borgers et al. (1979) who reported that at a concentration of 10^{-7}M, ketoconazole completely inhibited germ tube production by C. albicans. These workers, however, used a blastospore inoculum that had been standardized by sequential transfer through four different growth media over a period of 136 h. It is possible that blastospores prepared in this way are more sensitive to the effects of ketoconazole than was seen in the present study where effects were not observed until after germ tubes had been produced.

Although ketoconazole was shown to have some morphogenetic effect on C. albicans blastospores, the most obvious effects were seen under incubation conditions that favoured germ tube production and in particular in cultures which produced branched hyphae. Hyphal branching was completely inhibited in isolate 73/055 in the presence of 10^{-7}M ketoconazole. The lack of inhibition of germ tube production and the apparent lag period before the effects of ketoconozole were observed could suggest that ketokonazole is active only against actively growing C. albicans cells. Earlier experiments in this study have shown that cell biomass may not change during germ tube synthesis in the present study. After 2 h incubation, however, C. albicans hyphae are actively growing and would therefore be sensitive to the activity of ketoconazole. The lack of activity of ketoconazole against stationary phase blastospores could also be explained by lack of penetration of this compound into the C. albicans cell until growth begins. A slow rate of penetration into the C. albicans cell could also explain the 2 h lag period between addition of ketoconazole and the appearance of any morphogenetic effect and could explain the lack of effect of increasing ketoconazole

concentration on germ tube initiation. It is possible that the lack of effect of ketoconazole added to inoculum blastospores during growth in SAB was due to a combination of factors. Van den Bossche, Willemsens & Van Cutsem (1975) reported that miconazole was most effective at inhibiting growth of C. albicans in rich media which favoured rapid cell growth. The growth rate of 73/055 in SAB broth at 30 °C is likely to be less than in EMEM at 37 °C under 5% CO_2 in air and the rate of uptake of ketoconazole under these growth conditions may be too slow to have had any effect on C. albicans blastospores exposed to the compound for only 2-3 h.

In addition to the obvious morphogenetic effects ketoconazole at 10^{-7}M significantly inhibited the growth of the fungus, an observation that had been made indirectly by Borgers et al. (1979).

The mechanism by which ketoconazole exerts its effect on C. albicans is for the most part unknown. Van den Bossche et al. (1978) reported that miconazole inhibited ergosterol biosynthesis in C. albicans - a finding that has been confirmed for ketoconazole by Marriott (1980). Since ergosterol is a component of the C. albicans plasma membrane and this is the site of synthesis of the cell wall polysaccharides glucan, mannan and chitin, changes in the sterol composition of the plasmamembrane could affect cell wall synthesis. Pesti, Campbell and Peberdy (1981) reported that chitin synthase activity in total membrane fractions isolated from two ergosterol-deficient mutants of C. albicans was significantly higher than that of the parent strain. Chiew, Sullivan & Shepherd (1982) supported these findings when they found that ergosterol inhibited both chitin synthase and germ tube production in C. albicans. The pleomorphic nature of ketoconazole-treated hyphae, particularly at the growing tips, suggests that some changes in cell wall structure could be taking place.

The results of the present series of experiments allow manipulation of the growth of C. albicans isolates in a variety of morphological forms. Examination of cell wall composition at critical stages of cell wall

development of these forms should aid in determination of the cellular loci responsible for differation of cell shape.

CHAPTER 3

Changes in cell polysaccharide composition and chitin synthase activity in relation to C. albicans morphology

3:1 Introduction

Chattaway et al (1968) and Schwartz & Larsh (1980) have reported differences in the polysaccharide content of isolated cell walls or whole cells of C. albicans blastospores and hyphae. While the proportional concentrations of glucan and mannan were similar in both forms of the fungus, chitin was three to four times as abundant in hyphae as in blastospores. These studies have determined polysaccharide composition of C. albicans in cells isolated at a single phase in the growth cycle. Indirect evidence suggests that the composition and arrangement of the C. albicans cell wall changes with time in both blastospores and hyphae (Chattaway, Shenolikar & Barlow, 1974; Chattaway et al. 1976b) and electron microscopy has shown the arrangement of the cell wall layers of C. albicans blastospores to change as cells age (Cassone, Kerridge & Gale, 1979). Although Kerridge et al. (1976) have investigated the gross chemical composition of logarithmic and stationary phase C. albicans blastospore cell walls, the cell wall composition of C. albicans hyphae of different ages has not been described. This omission is unfortunate because although the published data implicate chitin as the most significant polysaccharide in the context of C. albicans dimorphism, there is no direct evidence to show that other polysaccharides are not present in different concentrations early in the initiation and development of blastospores and hyphae. Components of the C. albicans cell wall, particularly the mannan, are known to have a range of biological properties of potential importance in pathogenicity and age - or morphology - related changes in the composition of the C. albicans cell wall could be important in the pathogenesis of candidosis.

Although the activity of the enzyme chitin synthase has been investigated in relation to dimorphism, studies have concentrated on the activity of this enzyme only during the initial period of blastospore bud or germ tube synthesis (Braun & Calderone, 1978; Chiew, Shepherd & Sullivan, 1980). Subsequent development of secondary blastospores or hyphal branches could be controlled by variation in the concentration or activity of chitin synthase and such variations could explain the differences observed in the ability of different strains of C. albicans to remain in a pure mycelial form.

The content of the major cell wall polysaccharides of C. albicans blastospores and hyphae, grown for different lengths of time in the MSAB and EMEM models were therefore investigated. The limitations of mechanical and chemical extraction methods for analysis of wall polysaccharides were discussed in the general introduction (Chapter 1). Since large numbers of polysaccharide assays were to be carried out it was considered impractical to isolate cell walls from all samples of C. albicans of differing ages and morphologies and consequently assays were performed on chemical extracts of whole cells. The procedure chosen has limitations of precision because it involves sequential extraction of cells, but it should allow useful comparison of replicate data from experiments done under carefully standardized conditions.

In addition the activity of the enzyme chitin synthase was determined in C. albicans blastospores and hyphae of different ages grown in MSAB and in hyphae grown in EMEM under conditions that favoured stable mycelium production and hyphal branching.

3:2 Methods

Changes in cell wall composition

C. albicans isolates 73/025 and 73/055 were grown in MSAB or EMEM under incubation conditions that favoured blastospore budding or hypha formation

as described in Chapter 2. MSAB cultures were grown in 1ℓ volumes in 2ℓ flasks, EMEM cultures were grown in 100 mℓ volumes in 14 cm diameter vented petri dishes. At intervals after inoculation the morphology of cultures was determined and samples of cultures were harvested by filtration through membrane filters (0.45 μm pore size; Sartorius Ltd). The isolated fungal cells were washed thoroughly in distilled water and diluted to 50 mg wet weight $m\ell^{-1}$ in distilled H_2O. Dry weight determinations of the fungal suspensions were performed by filtering known volumes of the fungal suspensions through dried, pre-weighed membrane filters (0.45 μm pore size). Membranes were dried to constant weight in a hot air oven at 80-100 °C and re-weighed. Results were expressed as mg dry weight $m\ell^{-1}$ fungal suspension.

Determination of polysaccharide content of C. albicans whole cells

Total carbohydrate, mannan and glucan were determined essentially by the method of Trevelyan & Harrison (1956) as described by Herbert, Phipps & Strange (1971). This method involves the sequential extraction of components of fungal cells by sequential treatment of the fungus with acids and alkali. Fractions of non-cell wall polysaccharides e.g. glycogen and trehalose obtained during the extraction process were discarded and not assayed because the manipulation of the large numbers of samples obtained from each culture made it technically impractical to assay all fractions. The extraction method used, as modified from Trevelyan and Harrison (1956) is shown below:

1) Fungal suspensions were adjusted to 50 mg wet weight $m\ell^{-1}$. 10 μℓ of suspension was diluted to 1 mℓ with H_2O, stored at -20 °C, and used for the determination of total carbohydrate.

   Extraction of cell wall mannan and glucan was performed on 150-300 mg wet weight of fungus in 12 mℓ glass conical centrifuge tubes. Each extraction was performed in duplicate.

2) To remove trehalose and other low molecular weight sugars, equal volumes of fungal suspension and 1 M ice-cold-trichloroacetic acid were mixed and allowed to stand at 4 °C overnight or were shaken at 0 °C for 1 h. Suspensions were centrifuged at 2500 g for 5 min and the supernatants discarded. The cell residues were resuspended in 10 mℓ H_2O to wash the residues. Suspensions were centrifuged again and the supernatants discarded.

3) To extract mannan, 2 mℓ of 0.25 M Na_2CO_3 was added per 50 mg wet weight cell debris and the tubes and their contents were weighed. Tubes and contents were then heated in a boiling water bath for 45 min then cooled to room temperature. Tubes were re-weighed and the weights adjusted to the original values with 0.25 M Na_2CO_3. Tubes were then centrifuged and the supernatants removed and retained. Mannan was precipitated at 4 °C overnight by the addition of 0.1 mℓ 10 M KOH and 0.5 mℓ fresh Fehlings solution per mℓ supernatant. Precipitated mannan was washed in 5 mℓ H_2O and redissolved in a minimal volume of 1 M H_2SO_4. The cell residues were washed in 10 mℓ H_2O, centrifuged, and the supernatants discarded.

4) To remove acid-soluble glycogen, 2 mℓ of 0.5 M perchloric acid was added per 50 mg wet weight cell debris. Tubes and contents were heated in a boiling water bath for 30 min. Tubes and contents were then centrifuged and the supernatants discarded. The cell debris was washed in 10 mℓ H_2O and repelleted by centrifugation.

The cell debris, which should contain predominantly glucan, was resuspended in a minimal volume of 2 M NaOH.

Chitin, as glucosamine, was isolated by the method of Wieckowski (1968). To a volume of fungal suspension, in screw capped 12 mℓ glass tubes, was added an equal volume of 12 M HCl. Tubes were sealed and placed in a hot air oven at 100 °C for 18 h then cooled to room temperature. Contents of

tubes were washed into 100 mℓ glass beakers with H_2O and evaporated to dryness three times over a steam bath. The resulting residue was re-dissolved in a minimal known volume of H_2O.

Mannan, glucan and chitin fractions were diluted in H_2O to produce a concentration estimated likely to be in the range 0-200 ug $mℓ^{-1}$ and were stored in 1 mℓ volumes at -20 °C until assayed.

Total carbohydrate, mannan and glucan fractions were assayed with the anthrone reagent as described by Spiro (1966). To 1 mℓ of sample, pre-cooled in ice, was added 5 mℓ anthrone reagent (720 mℓ conc H_2SO_4, 280 mℓ H_2O, 10 g thiourea and 500 mg anthrone). Sample and anthrone reagent were mixed by vortexing, heated in a boiling water bath for 15 min then cooled in ice. The samples were allowed to equilibrate at room temperature for 15 min then OD.620 nm measurements were made with a Beckman Model 35 spectrophotometer and carbohydrate concentrations were calculated from standard curves prepared each time assays were performed. Total carbohydrate and glucan concentrations were determined by reference to a glucose standard and mannan by reference to a mannose standard.

Chitin, as glucosamine, was assayed by the method of Wieckowski (1968). To 1 mℓ of sample was added 1 mℓ of Wieckowski reagent B (0.2 mℓ acetylacetone shaken with 10 mℓ 1 N Na_2CO_3). Tubes and contents were heated in a boiling water bath for 30 min, cooled and the contents treated with 5 mℓ absolute ethanol and 1 mℓ Ehrlich's reagent (0.8 g p-dimethylaminobenzaldehyde in 60 mℓ conc HCl). Tubes were heated in a water bath at 37 °C for 30 min then cooled to room temperature and OD. readings at 526 nm made. Glucosamine concentrations were determined by reference to a glucosamine standard curve prepared with each set of assays.

All results are expressed as μg carbohydrate per mg dry weight of fungus.

Determination of chitin synthase activity

Isolate 73/055 was grown in MSAB at 30 or 40 °C to produce budding blastospores or germ tubes and hyphae respectively. Isolate 73/055 was also grown in EMEM incubated statically at 37 °C.

At intervals after inoculation morphology of cultures was determined and samples of cultures harvested as described earlier. Isolated fungal material was washed and resuspended in a minimal volume of Tris/$MgCl_2$/HCl buffer (Tris/Mg; 100 mM Tris pH 7.5 containing 20 mM $MgCl_2$ in H_2O) in 12 mℓ conical glass centrifuge tubes.

To obtain a crude "chitin synthase enzyme" preparation, C. albicans cells were disrupted by ultrasonication in the presence of 2 mm diameter glass beads. Approximately half the sample volume of glass beads was added to each fungal sample and cells were sonicated for 15 min at a previously determined optimal setting with a Dawe Soniprobe Type 1130A. Samples were cooled in ice-water during disruption. This process gave approximately 80-90% cell breakage as determined microscopically. Unbroken cells and cell wall debris were removed by centrifugation of the tube contents at 2500 g for 5 min and the crude enzyme preparation was stored on ice until used.

The protein concentration of the enzyme preparation was determined by measurements of OD. at 280 nm with reference to a standard curve prepared for an earlier sample. The presence of vacuoles and membrane material in the enzyme preparation prevented accurate determination of protein by direct comparison of OD. 280 nm with a standard curve for a protein solution. A standard curve was therefore prepared by comparing the protein concentrations of dilutions of samples of an enzyme preparation, determined by the method of Lowry et al. (1951) with the results of OD. measurements at 280 nm of similar enzyme dilutions. The corrected protein standard curve provided a more accurate measure of the protein concentration of enzyme preparations and permitted the use of OD. determinations at 280 nm which are non-destructive and much quicker than the method of Lowry et al. 1951.

Chitin synthase was assayed by the method of Archer (1977). The assay was performed in a final volume of 25 µl in 0.5 ml volume Sarstedt plastic vials and the assay mixture contained the following: 10 µl of enzyme preparation in Tris/Mg buffer: 5 µl of a solution of UDP-N-acetyl-D-[U-14C]-glucosamine specific activity 300 m Ci.mmol (Amersham International Ltd, Bucks) in water-total activity 10 nCi: 5 µl of an aqueous solution containing 80 mM-N-acetyl-D-glucosamine, 1 mM UDP-N-acetyl-D-glucosamine: and 5 µl Tris/Mg buffer. This mixture was incubated at 30 °C in a water bath for a previously determined optimal time of 30 min.

Reactions were terminated by addition of 50 µl ice cold trichloroacetic acid (100 $g l^{-1}$) and after 1 h, at 0 °C, radioactive acid precipitable material was collected on membrane filters (0.22 µm pore size). Filters were washed with 20 ml H_2O and dried in a hot air oven overnight at 80 °C. Radiactivity was determined in a Packard Tri-Carb scintillation counter with Fisofluor 3 as scintillant (Fisons Ltd). Control tubes were included in each set of assays.

To determine if the activity of the enzyme preparations could be increased by limited proteolysis, samples of enzyme were treated with trypsin. 0.1 µg of trypsin (BDH Ltd) in 2 µl Tris/Mg buffer was added to 10 µl enzyme preparation containing 50 µg enzyme protein. This mixture was incubated for 5 min at 30 °C and the reaction terminated by the addition of 0.2 µg trypsin inhibitor (BDH Ltd) in 3 µl H_2O. In enzyme preparations where the protein content was less than 50 µg per 10 µl the volumes of trypsin and trypsin inhibitor were adjusted accordingly and the final volume of trypsin-treated samples, made up to 15 µl with Tris/Mg buffer. After trypsin treatment, the remainder of the assay was performed as described above.

The specific activity of chitin synthase was calculated by comparing the observed incorporation values with the theoretical maximum incorporation and was expressed in the units n mol UDP-N-acetyl-D-glucosamine incorporated min^{-1} µg $protein^{-1}$.

3:3 Results

Changes in carbohydrate concentrations related to C. albicans morphology

Reproducibility of assays

Although the patterns of changes in the content of cell polysaccharides in relation to morphology and age of C. albicans cells were reproducible in terms of the Trevelyan and Harrison (1956) method used, some variability in the absolute values of the concentrations of these components was found when assays were repeated (coefficients of variation at different sample times were in the range 2-52% with a mean of 19%.).

Changes in the total carbohydrate and polysaccharide content of C. albicans in MSAB or EMEM, related to morphology and age of cells

Total carbohydrate, mannan and glucan concentrations were higher in stationary phase (inoculum) blastospores of isolate 73/055 than 73/025, however the differences were within the ranges of experimental variation. The chitin contents of blastospores of both isolates were similar (Table 3:1).

Isolates 73/025 and 73/055 in MSAB

At 30 °C the total carbohydrate content of C. albicans 73/025 rose above its original value by 4 h, fell sharply again by 6 h and then gradually declined up to 24 h (Fig 3:1). At 40 °C the total carbohydrate concentrations were consistently higher than at 30 °C and a different initial pattern of variation in levels was observed. Total carbohydrate content rose sharply by 2 h, remained high at 4 h then fell in the same way as was noted at 30 °C At both 30 and 40 °C, although cultures were predominantly blastospore in nature by 24 h, total carbohydrate concentrations and those of glucan and mannan remained less than in the original inoculum.

At 30 °C glucan content increased over the first 2 h incubation and then fell gradually over the remainder of the incubation period, as

Table 3.1 Carbohydrate contents of blastospores of isolates 73/025 and 73/055 grown for 18h at 30°C in SAB broth.

	73/025	73/055
Total carbohydrate	311±39	388±19
Mannan	72±19	118±32
Glucan	105±15	157±29
Chitin	42±3	41±17

Figures represent the mean ± standard deviation from six replicate assays and are expressed as µg per mg dry weight of whole C.albicans cells.

Fig.3.1 Morphology and age related changes in the polysaccharide composition of isolate 73/025 grown in shaken culture in MSAB at 30°or 40°C in air.

Polysaccharide contents are expressed as µg per mg dry weight of fungus.

Data represent the mean of five separate determinations ± standard error of the mean.

30°C

40°C

%

Mycelium

50

0

Total carbohydrate

500

400

300

200

µg per mg dry weight of fungus.

Mannan

100

0

Glucan

200

100

0

Chitin

50

0

2 4 6 8 10 24

0 2 4 6 8 10 24

h

blastospores multiplied by budding (Fig 3:1). At 40 °C a similar increase was noted over the first 2 h of incubation but concentrations remained at this level for a further 2 h before gradually falling by 24 h. The higher glucan concentrations correlated with initiation of germ tube production but the peak of glucan concentration continued after the peak in mycelium production (Fig 3:1). The mannan content of C. albicans 73/025 at both 30 and 40 °C showed an initial increase over the first 2 h of incubation then gradually fell over the remainder of the incubation period and no morphology-related changes in content were apparent (Fig 3:1).

The chitin content of C. albicans blastospores gradually decreased during 24 h incubation at 30 °C. At 40 °C however, chitin content correlated closely with changes in the morphology of the fungus. The peak of mycelium production and chitin content occurred after 2 h incubation. As secondary blastospore formation occurred and the mycelial content of cultures dropped, the amount of chitin present in C. albicans fell, but levels always remained at or above those in the original inoculum (Fig 3:1).

A limited investigation of the polysaccharide composition of isolate 73/055 grown in MSAB at 30 or 40 °C was also made. Samples were taken from cultures after 6 h incubation and the morphology and polysaccharide concentrations determined (Table 3:2). At 30 °C in MSAB isolate 73/055 also grew as budding blastospores and the similarity in the values obtained for concentrations of polysaccharides of this isolate and 73/025 under these incubation conditions correlated well with cell morphology. At 40 °C comparison between polysaccharide concentrations in isolates 73/025 and 73/055 is more difficult since the morphology of these two isolates differs under these incubation conditions (Table 3:2, Fig 2:2 Chapter 2). At 6 h isolate 73/055 was still mycelial whereas isolate 73/025 had reverted to secondary blastospore production and cultures were predominantly in this form. Levels of total carbohydrate, glucan and mannan were all higher in isolate 73/025 at 6 h than in isolate 73/055. The concentration of chitin

Table 3.2 Comparison of carbohydrate concentrations of isolate 73/025 grown for 6h in MSAB at 30°C or 40°C, with those of 73/055 grown in MSAB or EMEM for 6h at 30°C or 37°C.

	73/025			73/055					
	MSAB			MSAB			EMEM		
		*Value	%M		*Value	%M		*Value	%M
Total carbohydrate	30°C	240	0	30°C	304	0	30°C	410	4
	40°C	318	9	40°C	241	53	37°C	425	63
Mannan	30°C	72	0	30°C	64	0	30°C	135	4
	40°C	71	9	40°C	44	53	37°C	120	63
Glucan	30°C	112	0	30°C	106	0	30°C	130	4
	40°C	131	9	40°C	111	53	37°C	148	63
Chitin	30°C	24	0	30°C	24	0	30°C	17	4
	40°C	46	9	40°C	53	53	37°C	64	63

%M = percentage mycelium

* Results are expressed as µg per mg dry weight of whole cells.

Data are expressed as the mean of two separate determinations.

was however higher in isolate 73/055 correlating with the more mycelial nature of the culture. The similarities at both temperatures of the data for the two isolates in MSAB are seen more clearly when the comparable data for 73/055 in EMEM are inspected (Table 3:2). The data suggest more variation in polysaccharide composition resulting from the growth medium than from the fungal isolate.

Isolate 73/055 in EMEM

At 30 °C total carbohydrate concentrations over the first 4 h of incubation behaved in a similar way to those in isolate 73/025 at 30 °C. An initial decrease in concentration at 2 h was followed by a sharp rise by 4 h (Fig 3:2). However, total carbohydrate content gradually increased over the remainder of the incubation period in contrast to isolate 73/025 in MSAB at 30 °C where concentrations had declined substantially by 24 h. At 37 °C total carbohydrate concentrations remained virtually constant during 24 h incubation (Fig 3:2). The glucan content of the cells varied in a similar manner at 30 and 37 °C; after an initial rise in concentration at 2 h, the glucan content of cells gradually fell and remained at the same level as in the inoculum blastospores (Fig 3:2).

At 37 °C the mannan content showed a marked increase at 2 h then fell over the remainder of the incubation period. There was no obvious change in mannan concentration at 30 °C apart from a modest increase in the 2 h and 5 h samples.

The chitin content of C. albicans 73/055 cells fell markedly over the first 2 h of incubation at 30 °C and rose only slightly over the remainder of the incubation period. At 37 °C there was a rise in chitin content that correlated with germ tube and hypha formation and the chitin content remained higher than that of the inoculum for up to 8 h.

After 6 h incubation the content of total carbohydrate and mannan was higher in cultures of isolate 73/055 grown in EMEM than in MSAB (Table 3:2).

Fig.3.2 Morphology and age related changes in the polysaccharide composition of isolate 73/055 grown in static culture in EMEM at 30°C in air or at 37°C under 5% CO_2 in air.

Polysaccharide contents are expressed as µg per mg dry weight of fungus.

Data represent the mean of four separate determinations ± standard error of the mean.

30°C

37°C

%
50
0

Mycelium

500
400
300
200

Total carbohydrate

µg per mg dry weight of fungus.

100
0

Mannan

200
100
0

Glucan

Chitin

0

0 2 4 6 8 10 24 h

0 2 4 6 8 10 24

Glucan and chitin concentrations were similar in EMEM and MSAB cultures after 6 h incubation.

It was impossible to compare the polysaccharide concentrations of isolate 73/025 grown in EMEM due to the difficulties encountered in maintaining this isolate under laboratory conditions, as described in Chapter 2.

Effect of ketoconazole on polysaccharide composition of isolate 73/055 in EMEM at 37 °C under 5% CO_2 in air

The pleomorphic nature of ketoconazole-treated hyphae and their phase bright appearance under the phase contrast microscope (Chapter 2) suggested that ketoconazole could be affecting the composition or structure of the hyphal cell wall. The total carbohydrate, glucan, mannan and chitin contents of ketoconazole treated hyphae were therefore investigated. In addition the effect of ketoconazole on the uptake of labelled glucose and N-acetyl-D-glucosamine, possible substrates for cell wall synthesis, was also investigated.

Although some variations in the absolute values of glucan, mannan and chitin contents between control and ketoconazole treated 73/055 were apparent (Fig 3:3) these variations were not significant when tested statistically by the Mann-Whitney U Test (p values >0.01). Similar p values were obtained when the contents of individual cell polysaccharides were expressed as a proportion of the sum of the contents of glucan, mannan and chitin (Fig 3:4). The effect of ketoconazole on labelled glucose and N-acetyl-D-glucosamine uptake by isolate 73/055 differed. Over the first 2 h incubation ketoconazole treated cells took up more glucose than control cells (Fig 3:4). After 5 and 8 h incubation, however, glucose uptake was similar in both control and ketoconazole treated cultures. N-acetyl-D-glucosamine uptake was markedly reduced in ketoconazole treated cultures after 5 and 8 h incubation (Fig 3:4).

Fig.3.3 Effect of 10^{-7}M ketoconazole on the polysaccharide composition of isolate 73/055 grown in static culture in EMEM at 37°C under 5% CO_2 in air.

■ = control

□ = 10^{-7}M ketoconazole

Data represent the mean of three separate determinations.

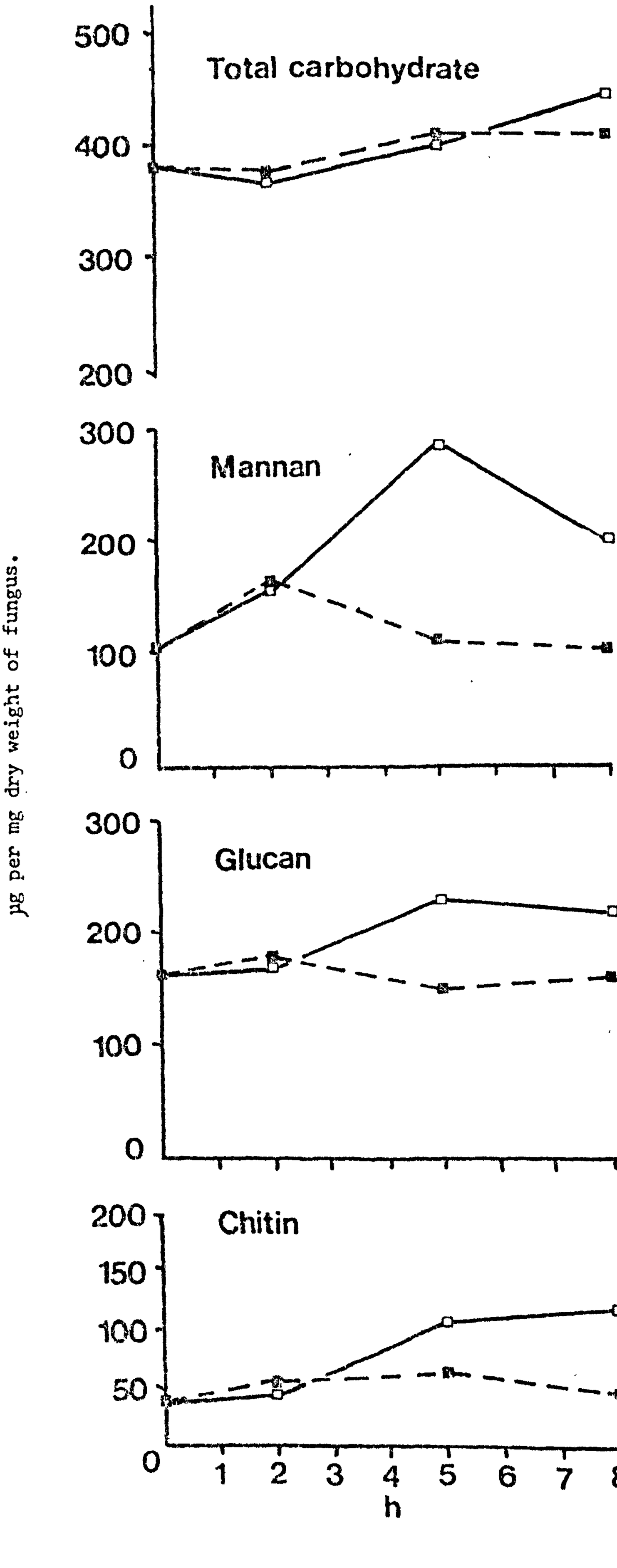

Total carbohydrate
500
400
300
200
Mannan
300
200
100
0
Glucan
300
200
100
0
Chitin
200
150
100
50
0
1
2
3
4
5
6
7
8
h
µg per mg dry weight of fungus.

Fig.3.4

A Effect of 10^{-7}M ketoconazole on uptake of labelled glucose and N-acetyl-D-glucosamine by isolate 73/055 incubated statically in EMEM at 37°C under 5% CO_2 in air.

0.33uCi D- U-^{14}C glucose (specific activity 269.7 mCi/mmol, Amersham International Ltd) or 0.33uCi N-acetyl-D- 1-^{3}H glucosamine (specific activity 2.94 Ci/mmol, Amersham International Ltd) was added to 100ml EMEM or EMEM + 10^{-7}M ketoconazole. Media were inoculated to 10^{6} blastospores/ml and samples removed at intervals after inoculation. Cells were harvested by centrifugation at 2500g for 5min, washed twice and resuspended in distilled water. Cell-associated radioactivity was determined by scintillation counting of duplicate 0.1ml volumes of fungal suspension in 3ml Fisofluor 1. Dry weight determinations were performed on samples of the fungal suspensions and label uptake was expressed as c.p.m. taken up per mg dry weight of fungus.

B Effect of 10^{-7}M ketoconazole on the proportions of individual polysaccharide components of isolate 73/055 expressed as a percentage of the sum of the cell content of mannan + glucan + chitin.

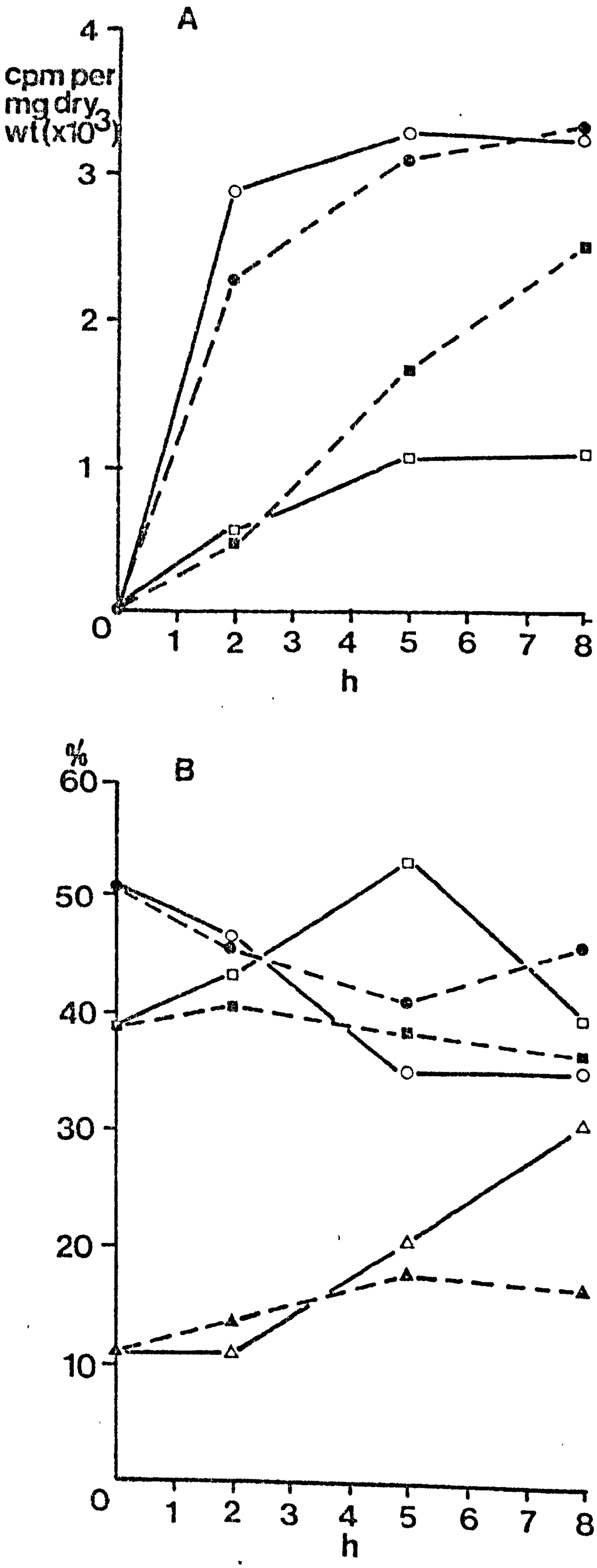
Closed symbols = control

Open symbols = 10^{-7}M ketoconazole added at t=0

O= glucan

□= mannan

△= chitin


A
cpm per mg dry wt (x10³)
4
3
2
1
0
1 2 3 4 5 6 7 8
h
B
%
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8
h

Changes in chitin synthase activity in relation to C. albicans morphology

Reproducibility of assays

Some variation was observed in the specific activity values obtained in duplicate assays. Values varied on average of 5.8 specific activity units between replicate assays. The mean coefficient of variation was 15%. Variation between the results of duplicate assays was largest in enzyme preparations obtained from stationary phase blastospores in which the level of chitin synthase activity was very low.

Isolate 73/055 grown in MSAB at 30 and 40 °C

Enzyme activity remained almost constant throughout 24 h incubation at 30 °C (Fig 3:5). A slight increase in activity be 6 h was followed by a fall to below levels in the blastospore inoculum by 24 h. Enzyme preparations varied slightly in their response to trypsinization but trypsinization in general had little effect on enzyme activity.

At 40 °C enzyme activity was higher than that measured at 30 °C at all times after inoculation of the medium. Germ tube formation at 2 h was accompanied by a sharp rise in chitin synthase activity (Fig 3:5). Enzyme activity increased gradually up to 24 h with a small drop in activity at 6 h. The maintenance of this high level of activity correlated with the mycelial nature of the cultures. Trypsin treatment had little effect on enzyme activity at 2 h but at 4, 6 and 24 h trypsinization increased the activity approximately 2 fold (Fig 3:5)

Isolate 73/055 grown statically in EMEM at 37 °C

In isolate 73/055 levels of chitin synthase activity varied in a similar manner in EMEM as was seen at 40 °C in MSAB. Enzyme activity however reached a peak at 4 h dropped slightly by 6 h and then rose a little at 8 and 24 h (Fig 3:6). Trypsinization had little effect on enzyme activity at 4 h but

Fig.3.5 Chitin synthase activity in blastospores and hyphae of isolate 73/055 grown for different lengths of time in MSAB in air.

Blastospores were produced at 30°C and hyphae at 40°C. Chitin synthase activity was determined at intervals during growth and is expressed as n mol labelled UDP-N-acetyl-D-glucosamine incorporated min^{-1} ug $protein^{-1}$.

O = control

● = enzyme samples trypsinised before assay.

Data represent the mean of three separate determinations.

30°C

40°C

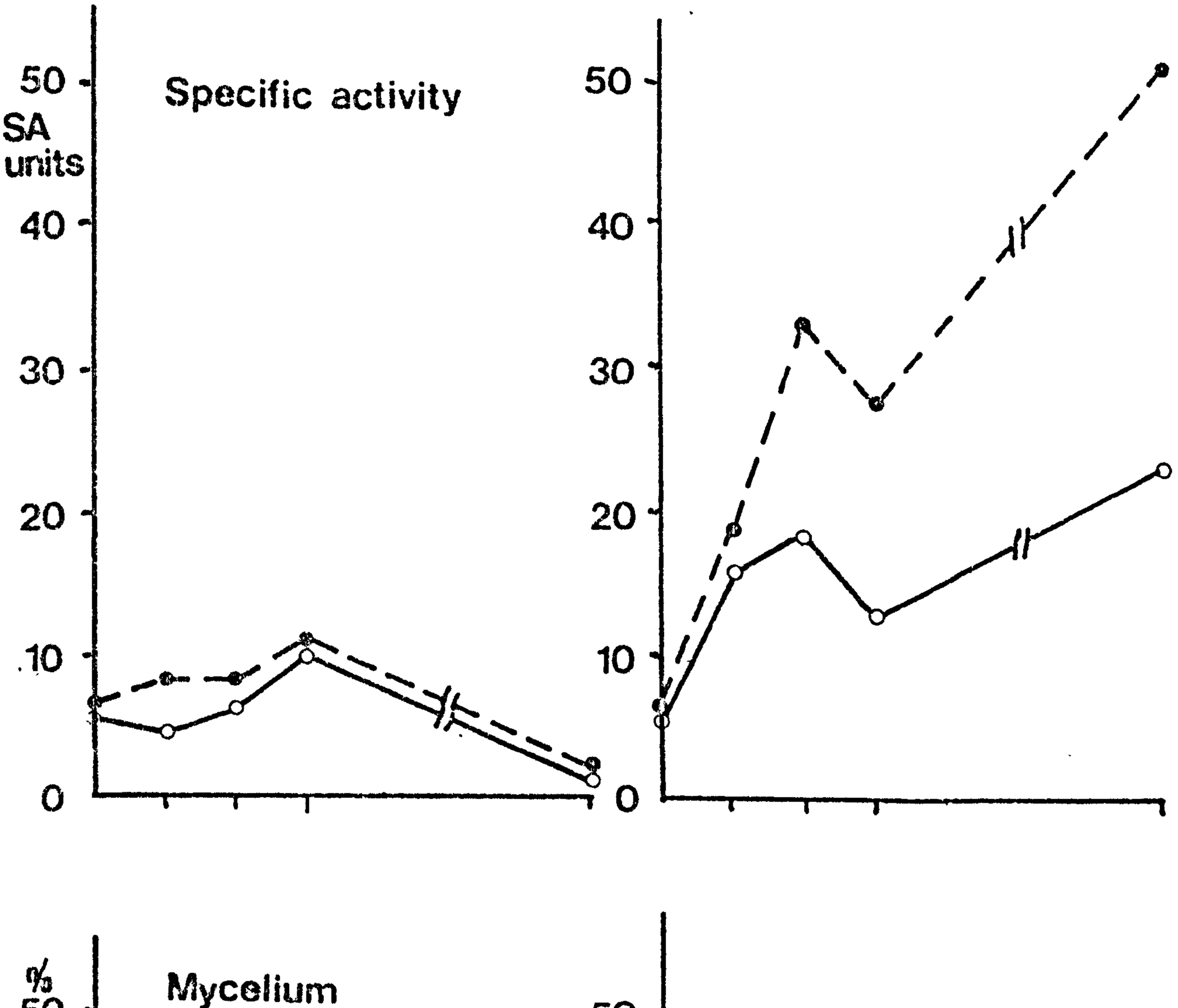

Specific activity
SA units
50
40
30
20
10
0
Mycelium
%
50
0
2
4
6
24
h

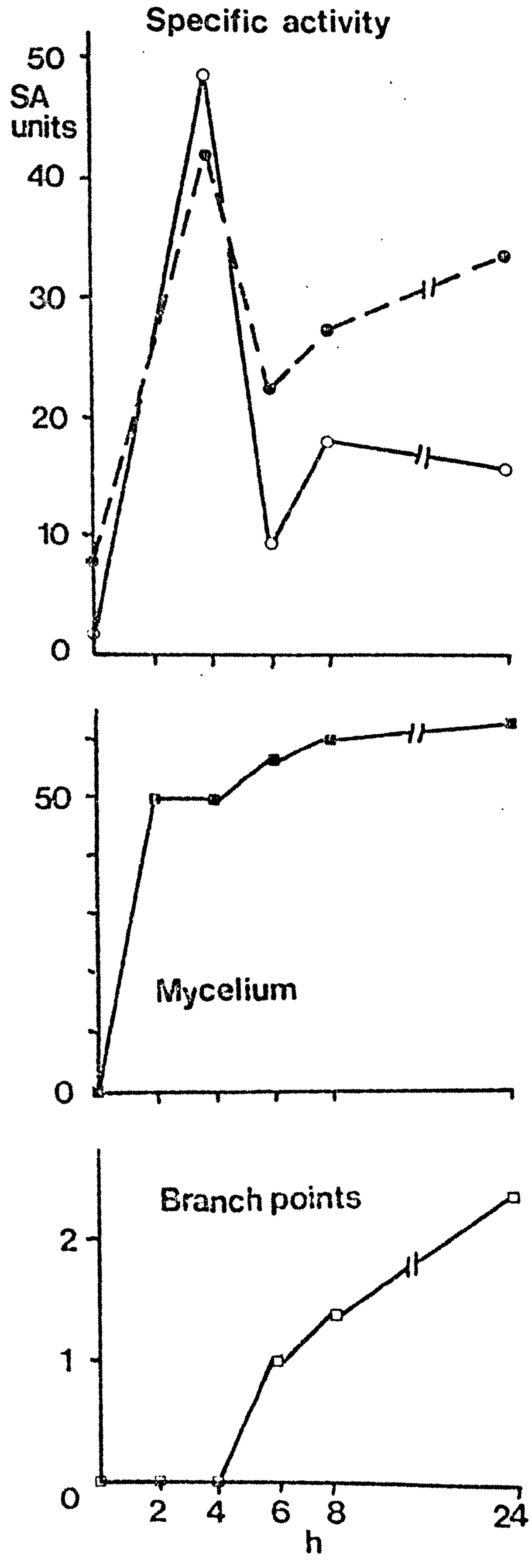
Fig.3.6 Chitin synthase activity in hyphae of isolate 73/055 grown for different lengths of time in EMEM incubated statically at 37°C.

Specific activity is expressed as n mol labelled UDP-N-acetyl-D-glucosamine incorporated min^{-1} μg $protein^{-1}$.

Data represent the mean of three separate determinations.

o = control

● = trypsinized


Specific activity
50
SA
units
40
30
20
10
0
50
Mycelium
0
Branch points
2
1
0
2
4
6
8
24
h

doubled enzyme activity at 6 and 24 h. Trypsin treatment had less effect on enzyme activity at 8 h. Cells of isolate 73/055 remained mycelial during 24 h incubation (Fig 3:6) and over 80% of mycelia had produced hyphal branches by this time.

3:4 Discussion

Changes in carbohydrate concentrations related to C. albicans morphology

Reproducibility of assays

The Trevelyan and Harrison (1956) method of sequential extractions of cell polysaccharides from whole fungal cells was chosen for this work because it was considered impractical to isolate cell walls of C. albicans from all samples to be investigated. As already indicated variation was noted when assays were repeated.

These problems may possibly be attributed to variation in the efficiency of the extraction procedures used and to the problems of multiplication of errors during the sequential extraction procedures. In general extraction procedures requiring the least manipulation of samples provided the most reproducible results, although total carbohydrate contents were found to vary appreciably in some duplicate assays (coefficient of variation 4-39%, mean 15%). This may be due to the fact that samples of cells, particularly for this assay, varied in concentration. Since the final assay procedure, using anthrone reagent, involves hydrolysis of polysaccharides to their monomers, the efficiency of hydrolysis and the final values obtained could have been affected by the amount of polysaccharide material to be hydrolyzed. This argument could also explain the variability noted in some mannan and glucan preparations and the observation that in a small number of assays, the sum of the values obtained for individual polysaccharides exceeded that obtained for total carbohydrate content. The possibility that mannan and glucan fractions were contaminated with other cell polysaccharides must also be considered. The Trevelyan and Harrison method provides crude mannan and

glucan extracts for analysis. Although steps were taken to remove contaminating cell polysaccharides such as glycogen from these extracts it is conceivable that small amounts of cell polysaccharides other than mannan and glucan were present. In preliminary experiments, hydrolysis of mannan and glucan rich extracts gave only mannose or glucose respectively when assayed by thin-layer chromatography. These findings suggest the mannan preparations were pure but do not preclude the possibility of some contamination of glucan extracts with other glucose-based cell components.

In addition, although this method provides crude mannan and glucan-rich extracts for assay, the exact composition of these preparations, in terms of polymerization and the site of origin of their components within the cell is uncertain. Although the majority of mannan extracted may be derived from the cell wall, a proportion may also have been derived from the cell cytoplasm. Glucan preparations presumably represent cell wall glucan since these extracts consisted of cell residues from which mannan and other polysaccharides such as glycogen had previously been extracted. Although the majority of N-acetyl-D-glucosamine in the C. albicans cell is considered to be in the form of chitin, it is possible that this compound may be found in other cell sites. Phaff (1971) showed that in S. cerevisiae N-acetyl-D-glucosamine was involved in the linkage of mannan to other cell wall components and it is possible that the analytical figures obtained for chitin content in the present study could also include N-acetyl-D-glucosamine derived from compounds other than cell wall chitin. A similar problem relating to the origin and composition of total carbohydrate content of C. albicans cells must also be considered. Changes in total carbohydrate values reflect variation both in the concentrations of pool metabolites and cell and cell wall polysaccharides, and interpretation of these data is therefore difficult. The results obtained in the present study should therefore be interpreted with regard to the limitations of the methods used in the extraction and assay of the various cell components.

Comparison of the results obtained in the present study with those of earlier published investigations is also difficult because of the variation in growth media, incubation times and extraction and assay methods used by other workers. The results of some previous published studies are presented in Table 3:3. These results illustrate the variation in the absolute values of polysaccharide concentrations previously reported. If instead of comparing absolute values, the concentrations of individual components are expressed as a proportion of the sum of the concentrations of glucan, mannan and glucosamine (or chitin) inter-laboratory agreement between previously published results is much closer (Table 3:3). (It is possible to express the values obtained for individual components as a proportion of the value obtained for total carbohydrate content. This procedure has the disadvantage that changes in total carbohydrate content may also reflect changes in the cell content of polysaccharides other than mannan and glucan and for this reason data were calculated as described above). This method of expressing results also overcomes some of the problems associated with variations in incubation conditions and incubation times previously described. When data obtained in the present study are compared with those of earlier investigations, obtained under similar growth conditions, results, particularly for MSAB cultures, compare favourably with those of previous studies (Table 3:3).

The results obtained in the present study indicate that polysaccharide concentrations of C. albicans vary with the isolate under test, the growth medium and the age of cells as well as the morphology of the fungus: however, since the isolates tested varied in their morphological development and since morphological development is also a function of cell age and growth medium, these properties are probably inter-related.

The higher total carbohydrate and mannan concentrations in isolate 73/055 grown at 30 °C in EMEM as compared with the same isolate in MSAB (Table 3:2) is very difficult to explain. The two media have similar glucose

Table.3.3 Carbohydrate composition of C.albicans cell walls.

a)Isolated cell walls

	1)						2)				3)	
	Y		M		306		RM806		582		30	40
	A	B	A	B	A	B	A	B	A	B	B	B
Glucose	253	57	304	56	643	68	584	62	688	73	48	49
Mannose	175	40	170	32	283	30	331	35	237	25	37	31
Glucosamine	15	3	65	12	14	1	22	2	12	1	15	20

A = Absolute values obtained, µg per mg dry weight.

B = Content of individual polysaccharides expressed as a percentage of the sum of the contents of glucan + mannan + glucosamine(or chitin).

1)Chattaway et al.(1968) Blastospore(Y) or mycelial(M) form C.albicans grown in a neopeptone-starch medium at 30 or 40°C respectively.Cells harvested at 18h.

2)Kessler and Nickerson(1959). Blastospore form of three isolates, 806, RM806 and 582 grown in a glucose-salts medium at 28°C. Cells harvested at 48h.

3)Results of the present study. Isolate 73/025 grown in MSAB at 30°C (30) or 40°C (40) in air. Cells harvested at 24h.

b) Cell walls extracted by chemical means from whole cells.

	4)				5)	6)	
	Y		M		6406	30	37
	A	B	A	B	A	B	B
Glucan	63	68	74	69	48	47	48
Mannan	22	24	17	16	53	45	35
Chitin	7	8	16	15	NT	8	17
Total carbohydrate	310		512		238		

4)Schwartz and Larsh(1980). Blastospore(Y) or mycelial(M) form C.albicans grown in a modified Auto-Pow EMEM at 30 and 40°C respectively. Cells were harvested "during the period of balanced growth".

5)Cassone, Kerridge and Gale(1979). Blastospore form grown in yeast-nitrogen base-glucose medium at 37°C. Cells harvested at 18h.

6)Results of the present study. Blastospore(30) or mycelial(37) form of isolate 73/055 grown in EMEM at 30 and 37°C respectively. Cells harvested at 8h.

concentrations and pH (EMEM; glucose = 1 g l^{-1}, pH 7.4: MSAB; glucose = 2 g l^{-1}, pH 7.4). The greatest differences between the two media therefore reside in the nature and composition of their minor carbon sources, nitrogen sources, vitamins and inorganic ions, whose effect on cell polysaccharide composition is impossible to determine without further experimentation. The differences in total carbohydrate content of 73/055 grown in the two media may reflect differences in content of carbohydrates other than cell wall polysaccharides; but the differences in mannan content defy rational explanation at present. It is notable that in both media at 30 °C the mannan and glucan and chitin content represents a similar percentage of the total carbohydrate figure and that each of these components constitutes approximately the same proportion of the "total cell wall polysaccharide" (mannan + glucan + chitin).

The possibility that glucose concentration in the growth medium could affect polysaccharide content of blastospores is, however, supported by the observation that in blastospores grown in MSAB, total carbohydrate and glucan concentrations, after 24 h incubation were below those in the inoculum blastospores (Fig 3:1). The glucose concentration of SAB broth in which the inoculum was prepared was 40 g l^{-1} compared with 2 g l^{-1} in MSAB and it is conceivable that such a large variation in glucose concentration could affect polysaccharide content. Presumably the increased content of other low molecular weight carbon sources in EMEM could explain the higher content of total carbohydrate in 24 h old EMEM grown blastospores.

Variations in the proportions of glucan, mannan and chitin in relation to the morphology and age of C. albicans cells are shown in Fig 3:7. Since variation in the proportions of individual components was apparent in duplicate assays the significance of changes in the proportions of glucan, mannan and chitin were determined statistically by the Mann-Whitney U Test. Differences between proportions of polysaccharides were considered significant only at $p < 0.01$.

Fig.3.7 Proportions of individual polysaccharide components expressed as a percentage of the sum of the cell content of mannan + glucan + chitin.

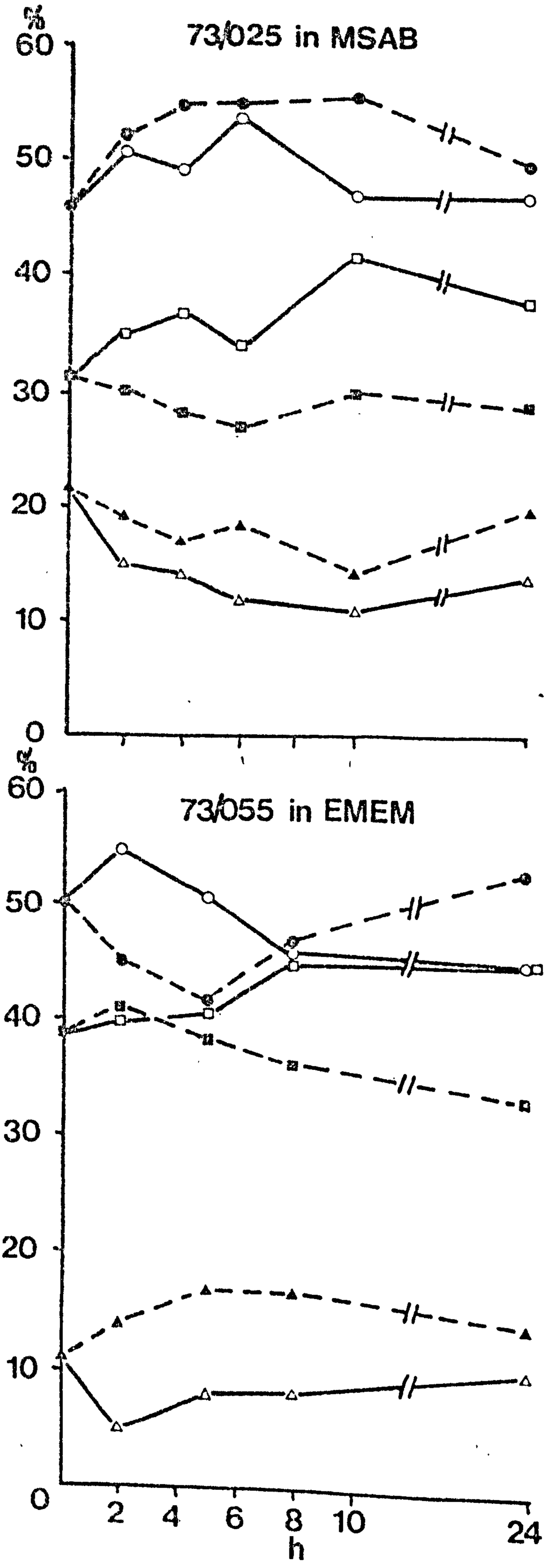
Isolate 73/025 in MSAB at 30°C (open symbols) and 40°C (closed symbols).

Isolate 73/055 in EMEM at 30°C (open symbols) and 37°C (closed symbols).

○ = glucan

□ = mannan

△ = chitin


%
73/025 in MSAB
60
50
40
30
20
10
0
%
73/055 in EMEM
60
50
40
30
20
10
0
2
4
6
8
10
24
h

The chitin content of hyphae of isolate 73/025 were significantly higher after 2 and 6 h incubation than that of blastospores of similar age (Fig 3:7). No significant difference was noted in the proportion of chitin in C. albicans cells grown for 10 and 24 h in MSAB when the morphology of cultures grown at 30 and 40 °C was similar (Fig 3:1). Similar significant differences were seen between the chitin contents of blastospores and hyphae of isolate 73/055 grown in EMEM (Fig 3:7). Differences in chitin content between these cells were apparent at 2, 5 and 8 h but not at 24 h even though at this time cultures were still mycelial in nature (Fig 3:2).

No significant differences in the proportions of glucan and mannan in relation to morphology of isolates 73/025 or 73/055 were found.

Variations in the proportions of glucan, mannan and chitin in relation to the age of C. albicans blastospores and hyphae are also apparent in Fig 3:7 . In 73/025 blastospores grown in MSAB at 30 °C the chitin content of 6 and 10 h old blastospores differed significantly from that of the inoculum blastospores. 2 h old blastospores of isolate 73/055 similarly had a lower proportion of chitin than those of the inoculum. At 37 °C in EMEM the proportion of chitin and glucan present in C. albicans hyphae differed significantly from that of the inoculum blastospores (Fig 3:7).

Since the polysaccharide concentrations and the proportions of individual polysaccharide components have been shown to vary with the growth medium, with the age of cells and therefore with their morphology, it is possible to establish patterns that correlate with particular aspects of morphological development.

Early development of germ tubes is associated with increases in cell chitin content. Under conditions that favour secondary blastospore production the chitin content of cells gradually falls as the proportion of secondary blastospores in the culture increases. The observation that in MSAB even after 24 h incubation at 40 °C the chitin content of 73/025 cells was higher than in cultures of blastospores of the same isolate grown at

30 °C in MSAB could indicate that secondary blastospores differ in chitin content from blastospores produced directly by budding from the parent blastospore. Although not performed in the present study, analysis of secondary blastospores isolated from the parent hyphae could indicate if this were the case. A high chitin content also correlates with the maintenance of hyphal form of isolate 73/055 in EMEM at 37 °C. The chitin content of these hyphae did however fall by 24 h incubation. The decrease in chitin content of hyphae grown in EMEM at 37 °C correlates with a similar decrease in mannan content (Fig 3:2). Phaff (1971) reported that N-acetyl-D-glucosamine may be involved in linkages between mannan and other cell wall components in S. cerevisiae. As the method used in the present study for the extraction and assay of chitin does not differentiate between monomeric or polymeric forms of N-acetyl-D-glucosamine, it is possible that the apparent fall in chitin content observed may, in part, be related also to the fall in mannan content. It is also possible that branches produced by older hyphae may differ in polysaccharide composition from the remainder of the cell.

Blastospore budding also involves changes in chitin content. Budding was associated with a marked drop in chitin content after 2 h incubation in EMEM and a gradual fall in content over 24 h incubation in MSAB at 30 °C. The apparent fall in chitin content in blastospores may be due to an increase in the cell content of other cell polysaccharides, in particular mannan and glucan (Figs 3:1, 3:2) resulting in a proportionately smaller amount of chitin in these cells (Fig 3:7).

Although ketoconazole treatment of isolate 73/055 in EMEM at 37 °C affected the morphology of the hyphae, no significant differences in the glucan, mannan or chitin contents of these cells were found (Fig 3:3). It is possible that the differences in optical properties observed could be due to an accumulation of lipid material in ketoconazole treated hyphae as reported by Borgers et al. (1979). The pleomorphic nature of hyphal apices could reflect changes in the arrangement rather than the composition of the

cell wall and such changes could also possibly account for the observed changes in the amount of labelled substrate uptake in control and treated cells. The difference in the amounts of N-acetyl-D-glucosamine taken up by ketoconazole-treated and untreated hyphae may be a reflection of the fact that ketoconazole effectively inhibits morphological development of C. albicans after germ tube initiation. Glucose may be used as substrate for processes other than cell wall synthesis and may therefore still be taken up by ketoconazole treated cells. In contrast the main use of N-acetyl-D-glucosamine is presumably in cell wall chitin synthesis and the inhibition of morphological development, leading to reduced cell wall synthesis, could explain the reduction in uptake of this substrate.

The importance of cell chitin content in relation to dimorphism is further supported by the increase in chitin synthase activity associated with germ tube formation (Fig 3:5) and the low level of enzyme activity found in budding blastospores. Variation in the analytical figures obtained was considered acceptable and was largest in enzyme preparations from blastospores. Incorporation of labelled substrate was lowest in these samples and the variation between duplicates was therefore proportionately larger than was found in enzyme preparations from hyphae, which generally gave higher incorporation figures. Comparison of the results of the present study with those of earlier investigations is again difficult since other workers have used different methods for the preparation and assay of chitin synthase. Braun & Calderone (1978) compared enzyme activity in washed protoplast membrane preparations obtained from 4 h old C. albicans blastospores (grown in SAB broth) or hyphae (grown in a salts-serum medium). These workers reported the specific activity of enzyme preparations as n moles $[^{14}C]$ UDP-N-acetyl-D-glucosamine incorporated per mg protein, but these are unacceptable units for specific activity because they include no time component. The activities quoted by Braun & Calderone were 0.27 units for blastospores and 0.47 units for hyphae. Trypsin treatment increased the specific activity of enzyme preparations six to seven fold. Results

of the present study for 4 h blastospore and hyphal cells grown in MSAB, recalculated as n moles [^{14}C] UDP-N-acetyl-D-glucosamine incorporated per mg protein, assuming an assay incubation time of 30 min (Braun & Calderone stated only that reactions were terminated "after incubation for various intervals up to 1 h") were 0.2 units for blastospores and 0.5 units for hyphae. These figures agree well with those of Braun & Calderone(1978). However, trypsin treatment did not affect activity in blastospores and only doubled it in hyphae, so the large trypsin activation of chitin synthase reported by Braun and Calderone (1978) was not confirmed in the present study. It is difficult to compare the results of trypsinization in these two studies since Braun and Calderone (1978) do not indicate in their methods the ratio of enzyme protein to trypsin used. These workers used higher quantities of trypsin (0.6 µg) and longer incubation times (15 min) in their trypsinization procedure and this could explain the differences in results obtained. The possibility that the presence of other cytoplasmic proteins in enzyme preparations used in the present study could have reduced the efficiency of trypsin activation of chitin synthase appears to be ruled out since in preliminary experiments in the present study, increasing the ratio of trypsin to enzyme protein did not increase the efficiency of trypsinization.

In addition to variation in activity with cell morphology, chitin synthase activity also varied with the age of blastospores and hyphae, correlating with the changes in chitin content observed in the present study. It is not however possible to correlate changes in chitin synthase activity with hyphal branch formation in isolate 73/055 in EMEM at 37 °C (Fig 3:6). It is conceivable that chitin synthase could be involved in the regulation of hyphal branch formation (and presumably secondary blastospore formation) in C. albicans but that the assay technique used in the present study was not sophisticated enough to detect these changes. The enzyme preparations used represent the average enzyme activity within C. albicans cells and it is possible that regulation of morphology involves localized

changes in chitin synthase activity which would not be detectable by this method.

The results presented in this study indicate that the polysaccharide composition of C. albicans definitely relates to the morphology of the fungus. Chitin in particular plays a major role in controlling changes in cell shape throughout the growth cycle although as indicated earlier, it is difficult to determine whether the assay method used in the present study is detecting polymerized N-acetyl-D-glucosamine in the form of chitin or free N-acetyl-D-glucosamine involved in the linkage of other cell wall polymers. Although changes in chitin synthase activity are apparent during dimorphic shifts in C. albicans further investigation of the other synthase enzymes involved in cell walls synthesis could indicate additional mechanisms by which cell shape changes with age in C. albicans. Such age and morphology-related changes may have implications in the pathogenesis of candidosis and aspects of this process in which morphology and polysaccharide composition of C. albicans cells may be important will be investigated further in the following chapter.

CHAPTER 4

Interactions of C. albicans blastospores, germ tubes and hyphae with phagocytic cells

4.1 Introduction

Although phagocytosis and intracellular killing of C. albicans blastospores have been extensively studied, little quantitative investigation has been made of the interaction between phagocytic cells and C. albicans germ tubes and hyphae. This situation has arisen partly because of the difficulties inherent in manipulating filamentous forms of C. albicans in vitro and also because of problems of quantifying and standardizing the biomass of different morphological forms of C. albicans for use in assays of phagocytosis and intracellular killing. In addition, quantitation of phagocytosis and killing has proved difficult since the size of germ tubes and hyphae precludes their total phagocytosis by single phagocytic cells, making assessment of phagocytosis by visual means, a subjective process.

ATP photometry has been used for the estimation of viable biomass in a number of biological systems (De Luca, 1978). Odds (1980) applied this method to C.albicans and found that viable biomass was independent of cell morphology and it therefore offers an ideal method for standardized quantitation of suspensions of C. albicans blastospores, germ tubes and hyphae. In addition the development of radiometric assays for the quantification of phagocytosis and intracellular killing (Yamamura, Boler & Valdimarsson, 1977) has provided a more objective method of assessing these processes and can be applied to all morphological forms of C. albicans.

Phagocytosis and intracellular killing may be enhanced by the opsonization of microbes by serum components including serum antibody and complement (Roitt, 1974). Opsonization may also activate the classical or

alternate complement pathways leading to the generation of chemotactic complement components which may be important in attracting phagocytic cells towards the microbe (Klebanoff & Clark, 1978; Mims, 1982).

Chemotaxis of human PMNs towards C. albicans has previously been reported (Denning & Davies, 1973). These workers found that the chemotactic component of C. albicans blastospores was a mannan-rich cell wall fraction and concluded that complement was the important serum component in relation to chemotaxis of C. albicans. This observation was supported by other workers (e.g. Ray et al. 1979) who reported that a purified mannan from C. albicans blastospores activated the alternate complement pathway. Cutler (1977), however, reported chemotaxis of guinea pig neutrophils towards C. albicans in the absence of complement and the possibility that other components were therefore important in chemotaxis was suggested. Denning & Davies (1973) also reported that human PMNs differed in their chemotactic responses towards C. albicans blastospores and hyphae but these workers did not investigate the chemotactic properties of C. albicans germ tubes. Variation in the chemotactic properties of different morphological forms of C. albicans could be significant in the pathogenesis of candidosis since the fungus must first be located by phagocytic cells before phagocytosis and intracellular killing can take place.

The phagocytosis and intracellular killing of C. albicans blastospores, germ tubes and branched hyphae, in fungal suspensions standardized in terms of ATP concentration, was therefore determined with a radiometric assay. The ability of human PMNs and mouse peritoneal macrophages to phagocytose and kill C. albicans was compared and the effect of a range of serum factors on these processes was investigated.

The chemotactic response of human PMNs towards C. albicans blastospores, germ tubes and hyphae and culture filtrates of the fungus was also investigated.

4.2 Methods

Preparation of C. albicans for use in phagocytosis, killing and chemotaxis assays

Isolate 73/055 was used throughout these experiments.

Stationary phase blastospores were prepared in SAB broth incubated overnight at 30 °C shaken at 160 rev min^{-1}. Germ tubes and hyphae were prepared in EMEM + 20mM HEPES pH 7.4 incubated statically in air at 37 °C for 2 h and 6 h respectively. Blastospores in the logarithmic phase of growth were prepared in EMEM + 20mM HEPES pH 7.4 incubated statically in air at 30 °C for 2 or 6 h. After incubation fungal cells were harvested by centrifugation at 2500 g for 5 min and the culture filtrates retained. Fungal cells were washed twice in distilled H_2O and resuspended in EMEM + 20mM HEPES pH 7.2. The ATP concentration of suspensions was determined as described in Chapter 2 and suspensions were stored on ice until used.

The pH of culture filtrates was adjusted to 7.2 with 1 M HCl. Culture filtrates were then filter sterilized by passage through a 0.22 µm pore diameter filter and were stored at -20 °C until used.

Source and preparation of phagocytic cells

Human PMNs

10 mℓ volumes of venous blood obtained from volunteer donors was mixed with heparin (20 international units heparin $mℓ^{-1}$ blood) and 1 mℓ dextran solution (6% w/v dextran, Mol. Wt. 176,800 in 0.15M NaCl) and were allowed to stand at 37 °C for 30 min. The leukocyte-rich supernatant was then removed. Leukocytes were washed twice in 10 mℓ EMEM + 20mM HEPES pH 7.2 and were resuspended in this medium to final concentration of approximately 10^7 $mℓ^{-1}$. Over 90% of the leukocytes in these preparations were PMNs as assessed microscopically.

Mouse Peritoneal Macrophages

Swiss white mice were sacrificed by cervical dislocation. A macrophage-rich exudate was obtained from these mice by peritoneal lavage with 2 X 5 mℓ volumes of EMEM + 20mM HEPES containing 5 international units heparin $mℓ^{-1}$. Leukocytes were washed and resuspended as above. Over 90% of the leukocytes in these preparations were macrophages.

Preparation of antigens

Particulate antigens (PA)

Blastospores were prepared by growth in SAB broth incubated at 37 °C for 48 h. Germ tubes (at 2 h) and hyphae (at 6 h) were prepared in EMEM + 20mM HEPES pH 7.4 as described in Chapter 2. Blastospores, germ tubes and hyphae were harvested by filtration through 0.45 µm cellulose nitrate filters and were washed with 50 mℓ sterile H_2O. C. albicans preparations were suspended in phosphate buffered saline (PBS) and the ATP concentrations of suspensions determined by bioluminescence photometry. All suspensions were then diluted to 1000 ng ATP $mℓ^{-1}$ in PBS. Known volumes of C. albicans preparations were killed by exposure to formaldehyde (Fisons Ltd) (final concentration 0.4%) overnight at 4 °C. Suspensions were then washed 3 times in 20 mℓ sterile PBS and were resuspended in their original volume. The effectiveness of formaldehyde treatment was tested by plating a large loopful of the preparation onto SAB agar and incubating at 37 °C overnight. All preparations were shown to be non-viable.

Cytoplasmic antigen extract (CE)

Samples of viable blastospore suspensions in PBS were disrupted by three passages through a pre-cooled French pressure cell. Samples of germ tube and hyphal suspensions were mixed with one quarter their volume of 2 mm diameter glass beads and were disrupted by ultra sonication with a Dawe

Soniprobe Type 1130A. Suspensions were sonicated at a previously determined optimal setting for 15 min and were cooled in ice-water during this procedure.

These methods resulted in disruption of over 90% of the C. albicans cell units. Crude supernatants were harvested by centrifugation at 2500 g for 5 min and remaining cell wall debris was then removed from the supernatants by further centrifugation at 21,000 g for 60 min.

The protein concentrations of the supernatants or CEs were determined by the method of Lowry et al. (1951) with BSA as a standard. CEs were diluted to 2 mg protein $m\ell^{-1}$ and were stored in 0.5 mℓ volumes at -20 °C.

Sources and preparation of sera

Human serum was obtained from 20 mℓ volumes of clotted venous blood, donated by healthy volunteers. Some samples of human serum were heated in a water bath to 56 °C for 30 min, a procedure designed to inactivate the C_3 component of the complement cascade. Foetal calf serum, virus and mycoplasma screened was purchased from Gibco Biocult Ltd. Rabbit serum was obtained from 20 mℓ volumes of clotted venous blood. Anti-Candida albicans antisera were raised against a mixture of PA and CE antigens of blastospores, germ tubes or hyphae of isolate 73/055 in female New Zealand White rabbits. Rabbits were immunized on three consecutive days of the week over a three week period with increasing doses of antigen. On the first day of the first week of immunization each rabbit was injected intravenously with 62.5 µg CE protein and 2×10^3 ng ATP equivalent PA. In addition each rabbit was injected subcutaneously with this dosage of antigen in 0.5 mℓ Freunds incomplete adjuvant (Difco · Ltd). The intravenous injections only were repeated on days two and three. This procedure was repeated in the second and third weeks of immunization with increased amounts of antigen; 125 µg CE and 4×10^3 ng ATP PA in week two and 250 µg CE and 8×10^3 ng ATP PA in week three. Blood was obtained from animals three weeks after the last immunization.

All sera, rabbit, human and foetal calf, were tested for the presence of anti-C. albicans antibody by counter-immunoelectrophoresis (CIE) against the homologous cytoplasmic extract by the method of Odds, Evans & Holland (1975). Human sera, foetal calf serum and normal rabbit serum were negative for anti-C. albicans antibody. Rabbit sera raised against blastospores, germ tubes or hyphae all showed an anti-C. albicans precipitin titre of 1:32.

All sera were stored in 1 mℓ volumes at -20 °C.

Phagocytosis and killing assays

The method used was modified from Bridges et al. (1980). This method involves comparison of ^{3}H-uridine uptake by C. albicans in the presence and absence of phagocytic cells. Phagocytosed C. albicans cells are unable to take up ^{3}H-uridine and the reduction in label uptake in the presence of phagocytic cells, compared with control, is proportional to the extent of phagocytosis. Intracellular killing may be measured in a similar manner by comparing ^{3}H-uridine uptake by viable C. albicans released from phagocytic cells by sodium deoxycholate treatment, with that of the control.

PMNs or macrophages were diluted to 8 X 10^{5} leukocytes mℓ$^{-1}$ in EMEM + 20mM HEPES pH 7.2. Blastospores, germ tubes or hyphal suspensions were diluted in this medium such that after the addition of 10% v/v serum, the desired ATP concentration was obtained. C. albicans suspensions were then preincubated at 37 °C for 10 min. 0.25 mℓ volumes of leukocytes and C. albicans suspensions were mixed in 2 mℓ plastic vials (Sarstedt Ltd) Vials were capped and incubated for 1 h with continuous end over end rotation (30 rev min^{-1}), for 1 h at 37 °C. Each assay was performed in duplicate and additional vials containing mixtures of C. albicans and leukocytes were also included to enable phagocytosis to be assessed visually.

To estimate the extent to which C. albicans blastospores were phagocytosed, 0.2 mℓ C. albicans/leukocyte mixture from each vial was pipetted into the well of a microtiter plate (Nunc Ltd) containing 0.1 μ Ci [6-^{3}H] uridine (Amersham International Ltd, specific activity 20 Ci m mol^{-1}) in 10 μℓ EMEM. To measure intracellular killing of blastospores, a further 0.2 mℓ sample of C. albicans/leukocyte mixture was added to microtiter plate wells containing a mixture of 0.2 μ Ci ^{3}H-uridine in 50 μℓ EMEM, 10 μg DNaseI in 25 μℓ EMEM and 312 μg sodium deoxycholate in 25 μℓ H_2O. (This sodium deoxycholate concentration is half that originally used by Bridges et al. (1980). It was found that at concentrations above 312 μg, sodium deoxycholate significantly inhibited ^{3}H-uridine uptake by C. albicans 73/055 resulting in low uridine uptake figures and proportionately larger variation between duplicates. Microtiter plates were incubated at 37°C for 1 h.

Samples in which germ tubes and hyphae were coincubated with leukocytes tended to form clumps during end over end rotation, which made accurate pipetting of volumes for measurement of uridine uptake impossible. Uridine uptake was therefore measured for germ tubes and hyphae, on the entire 0.5 mℓ contents of vials. For phagocytosis measurement, 0.1 μ Ci ^{3}H-uridine in 10 μℓ EMEM was added to the vials and for intracellular killing measurement a mixture of 0.2 μ Ci ^{3}H-uridine in 10 μℓ EMEM, 20 μg DNaseI in 50 μℓ EMEM and 625 μg sodium deoxycholate in 50 μℓ H_2O, was added to duplicate vials. After addition of the ^{3}H-uridine the test mixtures were reincubated at 37 °C for 1 h, then 150 μℓ of each blastospore sample or the entire contents of vials containing germ tubes and hyphae were pipetted onto a pre-wetted 25 mm diameter GFB filter (Whatman Ltd), washed with approximately 20 mℓ H_2O and dried overnight at 80 °C. Filter associated radioactivity was counted in 3 mℓ Fisofluor I (Fisons Ltd) in a Packard PL Tri-Carb scintillation counter.

Two sets of controls were included with each set of assays: in the first experiments 0.25 mℓ C. albicans suspension and 0.25 mℓ EMEM +

20 mM HEPES pH 7.2 were mixed and assayed. In a number of early experiments it was found that the c.p.m. values obtained from such controls were lower than those recorded for tests, making quantification of the extent of phagocytosis and killing impossible. Investigation of this effect suggested that the leukocytes themselves were not taking up ^{3}H-uridine but that leukocyte components were in some way possibly interfering with the removal of extracellular label during the washing of filters. In later experiments therefore, a second set of controls was included with all assays. 0.25 ml of C. albicans and leukocyte suspensions were incubated separately for 1 h at 37 °C. After 1 h appropriate volumes of C. albicans suspensions were mixed with ^{3}H-uridine, the appropriate volume of leukocyte suspension was then added and mixtures were incubated at 37 °C for the required time.

It was found that in the majority of experiments control c.p.m. values were similar for both types of control. In some experiments however, control suspensions containing leukocytes gave higher c.p.m. than controls containing C. albicans alone and in this situation the higher c.p.m. figures were used in the calculation of results. + ?o

Phagocytosis and intracellular killing were quantified by comparing ^{3}H-uridine uptake by C. albicans in the presence and absence of leukocytes according to the following formula:

$$\%\ \text{Phagocytosis/Intracellular killing} = 100 \times \frac{\text{c.p.m. control} - \text{c.p.m. test}}{\text{c.p.m. control}}$$

The proportion of ingested C. albicans cells killed inside phagocytic cells was calculated according to the following formula:

$$\frac{\%\ \text{C. albicans cells killed}}{\%\ \text{C. albicans cells phagocytosed}} \times 100 = \%$$

Chemotaxis

The method used was modified from that of Denning & Davies (1973) and was performed with modified Boyden chambers (Hurley, 1963). The chambers were washed in distilled H_2O and air dried at 37 ^{o}C prior to use.

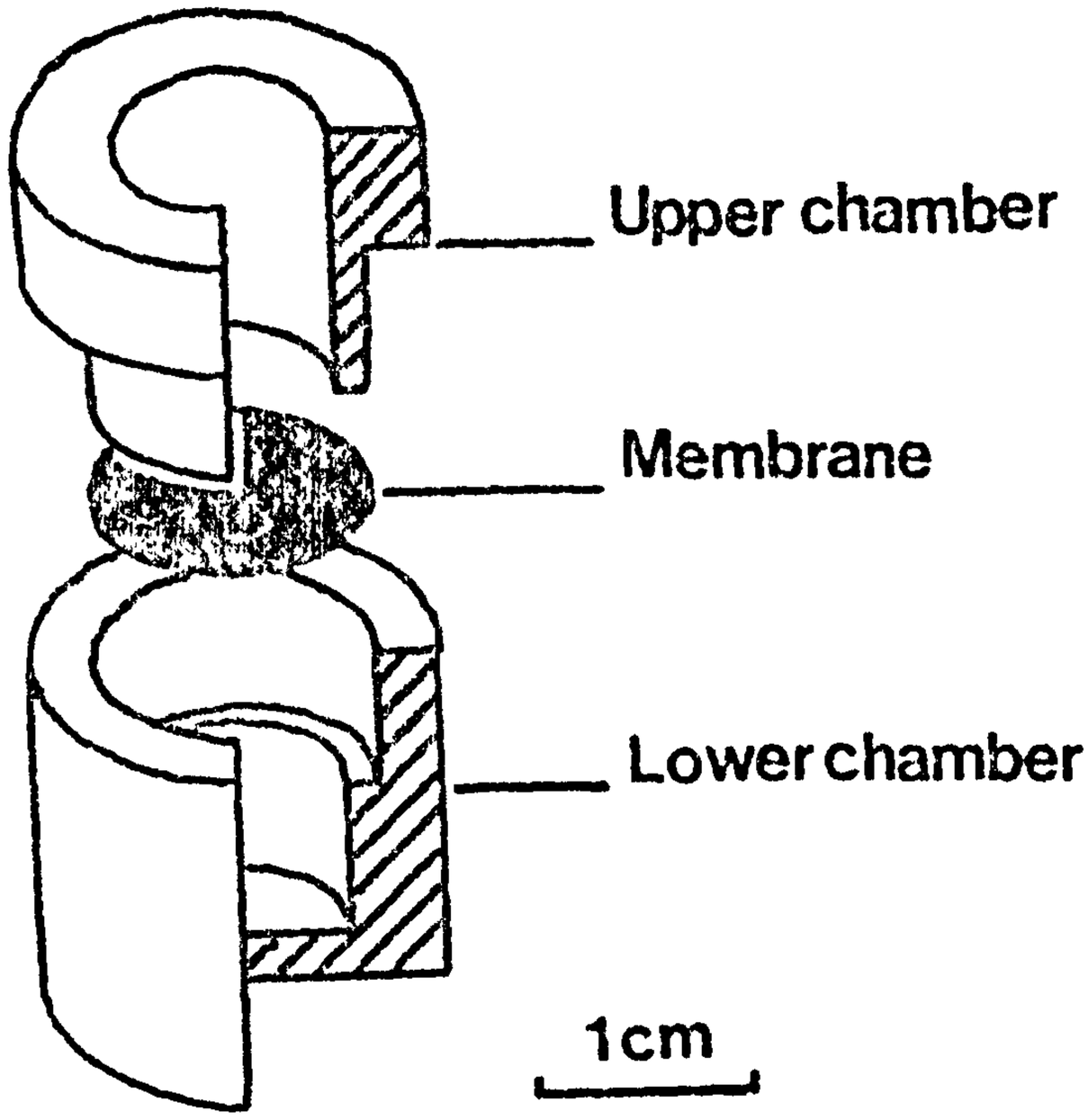
Fungal suspensions to be tested were diluted to the desired ATP concentration by addition of EMEM + 20 mM HEPES pH 7.2 and 10% v/v foetal calf serum. Fungal suspensions were pre-warmed to 37 ^{o}C before use. 10% v/v foetal calf serum was also added to culture filtrates prior to testing.

Human PMNs were diluted by the addition of EMEM + 20 mM HEPES and 10% v/v foetal calf serum to a concentration of 10^6 ml^{-1} and were also prewarmed prior to use.

The lower chamber of the apparatus (Fig 4:1) was filled to the shoulder with the fungal suspension or culture filtrate to be tested. A 13 mm diameter cellulose nitrate filter, pore diameter 3 µm, was then carefully lowered into position so that the lower surface of the filter was in contact with the meniscus of the fluid in the lower chamber, care being taken to avoid the trapping of air bubbles below the filter. The top half of the chamber, smeared with white petroleum jelly, was then fixed into place and 0.5 ml of the PMN suspension was placed in the upper chamber. Chambers were incubated at 37 ^{o}C in a humid atmosphere for 3 h. Each suspension was tested in duplicate and migration of PMNs towards EMEM + 10% v/v foetal calf serum was determined as a control in each set of assays.

After 3 h incubation the upper section of each apparatus was removed and the membrane fixed in absolute ethanol for 5 min. Filters were then placed in H_2O for 3 min, stained in HARRIS's haematoxylin for 2 min and rinsed in H_2O for a further 3 min. Filters were then 'blued' in very dilute NaOH solution (pH 8.0) for 10 min and were mounted, bottom side upwards, on microscope slides in a drop of H_2O. Counts were made of the numbers of PMNs migrating through to the lower surface of the membrane in ten randomly

Fig.4.1 Modified Boyden chamber.



From Denning and Davies (1973)

selected microscope fields (delineated by an eyepiece graticule). Results were expressed as the mean number of PMNs migrating per microscope field.

Results were subjected to statistical analysis by the Mann-Whitney U Test and differences were considered significant at $p < 0.01$.

Results

1) Phagocytosis and intracellular killing

Preliminary experiments were designed to determine the optimal ratio of *C. albicans* cells to PMNs for use in subsequent assays and were performed with 73/055 stationary phase blastospores. The ratio of *C. albicans* to PMNs was varied by altering the concentration of blastospores over the range 50 to 200 ng ATP equivalents per assay. These results indicated that the optimal ratio of *C. albicans* blastospores to PMNs was 75 ng ATP equivalent to 2×10^5 PMNs, an approximate *C. albicans*:PMN cell ratio of 6:1 (Fig 4:2). Subsequent assays were therefore performed at this optimal ratio.

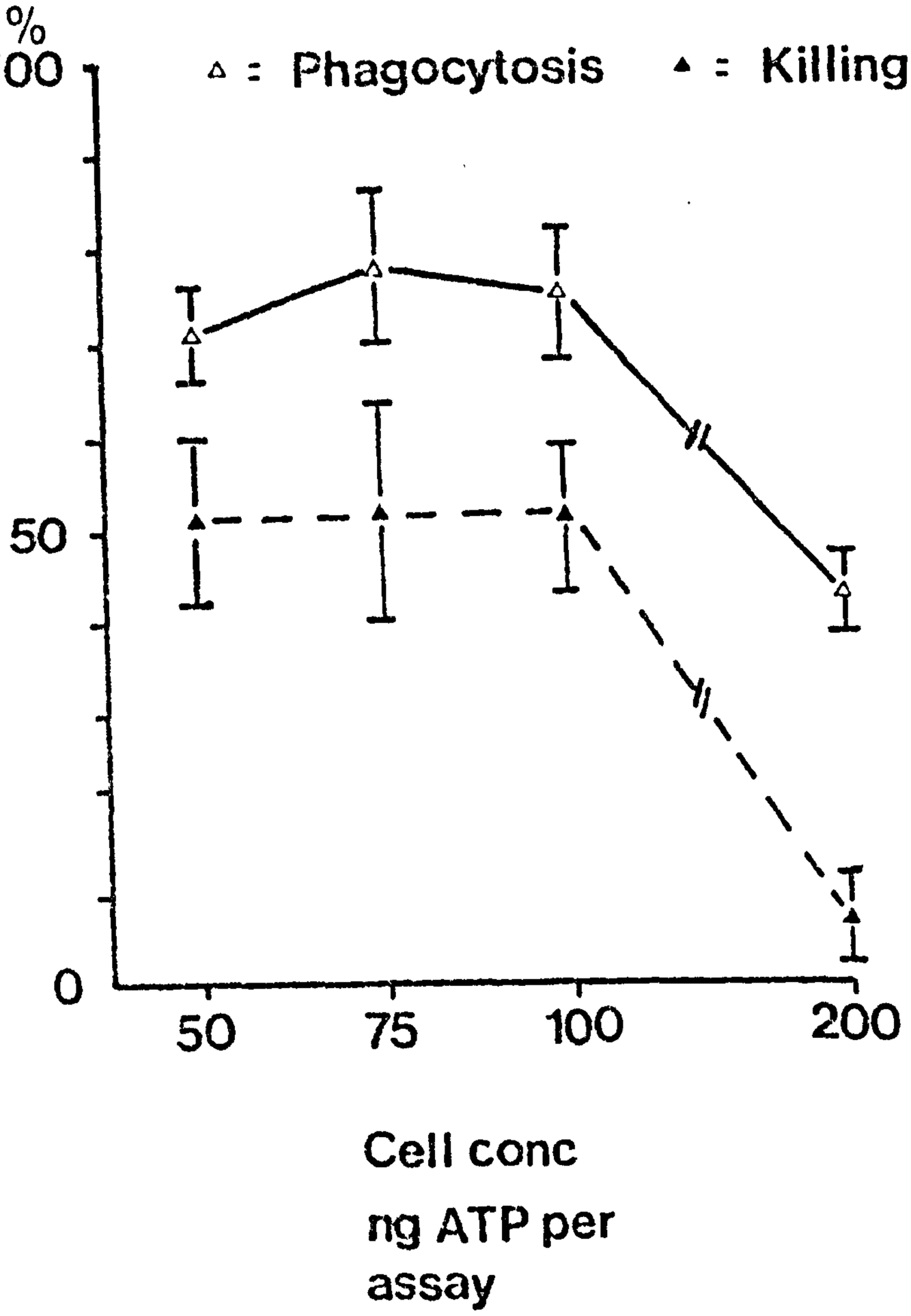
Some variation was noted in the values for phagocytosis and killing by PMNs obtained from different individuals and for the same individual tested at different times but in general the reproducibility of the assay system was as good as that of any previously published data and was therefore considered acceptable (Table 4:1).

Phagocytosis of 73/055 germ tubes and hyphae

Similar experiments were performed with *C. albicans* germ tubes (2 h old - average length 20 µm) and hyphae (6 h old - average length 90 µm).

At low *C. albicans*:PMN ratios (50 - 100 ng ATP equivalent per assay), there were no significant differences in the extent of phagocytosis and killing of 73/055 blastospores, germ tubes or hyphae, although some variation in the results obtained with the different morphological forms were apparent

Fig.4.2 Effect of blastospore: human PMN ratio on the efficiency of phagocytosis and killing of 73/055 blastospores by human PMNs.



Assays were performed in the presence of 5% v/v control human serum and data represent the mean of four separate determinations ± standard deviation.

Table 4.1 Variability between results of phagocytosis and intracellular killing assays with PMNs from different donors and from the same donor tested at different times.

a) Different donors		b) One donor tested at different times	
%Phagocytosis	%Killing	%Phagocytosis	%Killing
85	61	65	52
78	58	69	NT
76	52	85	45
76	50	75	44
76	44	64	51
71	42	81	NT
62	30	76	25
		64	21
$\bar{x}=75\pm7$	$\bar{x}=48\pm10$	$\bar{x}=72\pm8$	$\bar{x}=40\pm13$

Assays tested at 75ng ATP equivalent C.albicans : 2×10^5 PMNs

NT = Not tested

(Fig 4:3). At 200 ng ATP equivalent per assay although germ tubes and hyphae were apparently phagocytosed and killed less well than blastospores, the small number of replicate assays performed meant that statistical analysis of the data was impossible.

In some experiments with germ tubes and hyphae values obtained for percentage phagocytosis were lower than those for percentage killing, although the difference between the values was generally only a few percent and reflects the experimental error already noted in these determinations. The ability of mouse peritoneal macrophages to phagocytose and kill C. albicans 73/055 blastospores, germ tubes and hyphae was also investigated to determine if these phagocytic cells differed, in respect of these functions, from human PMNs. Preliminary experiments (Fig 4:4) indicated that mouse peritoneal macrophages were significantly less efficient both in phagocytosing and in the killing of C. albicans blastospores than were human PMNs. The optimum ratio of C. albicans to phagocytic cells was also lower for macrophages at 50 ng ATP equivalent per 2 X 10^5 macrophages. When tested at this ratio, percentage phagocytosis and killing figures for germ tubes and hyphae were similarly significantly lower than were seen with human PMNs (Table 4:2).

The significance of germ tube production in relation to the ability of C. albicans to resist phagocytosis and/or intracellular killing by human PMNs was investigated fruther. As blastospores and preformed germ tubes were phagocytosed and killed to a similar extent the production of germ tubes by phagocytosed blastospores or the continued elongation of short germ tubes completely phagocytosed by PMNs, leading to rupture of the PMN membrane, could be more relevant in relation to resistance to intracellular killing.

The ability of 73/055 blastospores to escape intracellular killing by producing germ tubes inside PMNs was investigated by microscopy. Phagocytosis and killing assays were performed as described and in addition, after mixtures of C. albicans blastospores and PMNs had been agitated for 1 h, samples were

Fig.4.3 Effect of C.albicans:PMN ratio on the phagocytosis and killing of blastospores, germ tubes and hyphae by human PMNs.

Assays were performed in the presence of 5% v/v control human serum.

Data represent the mean of four separate determinations ±SD

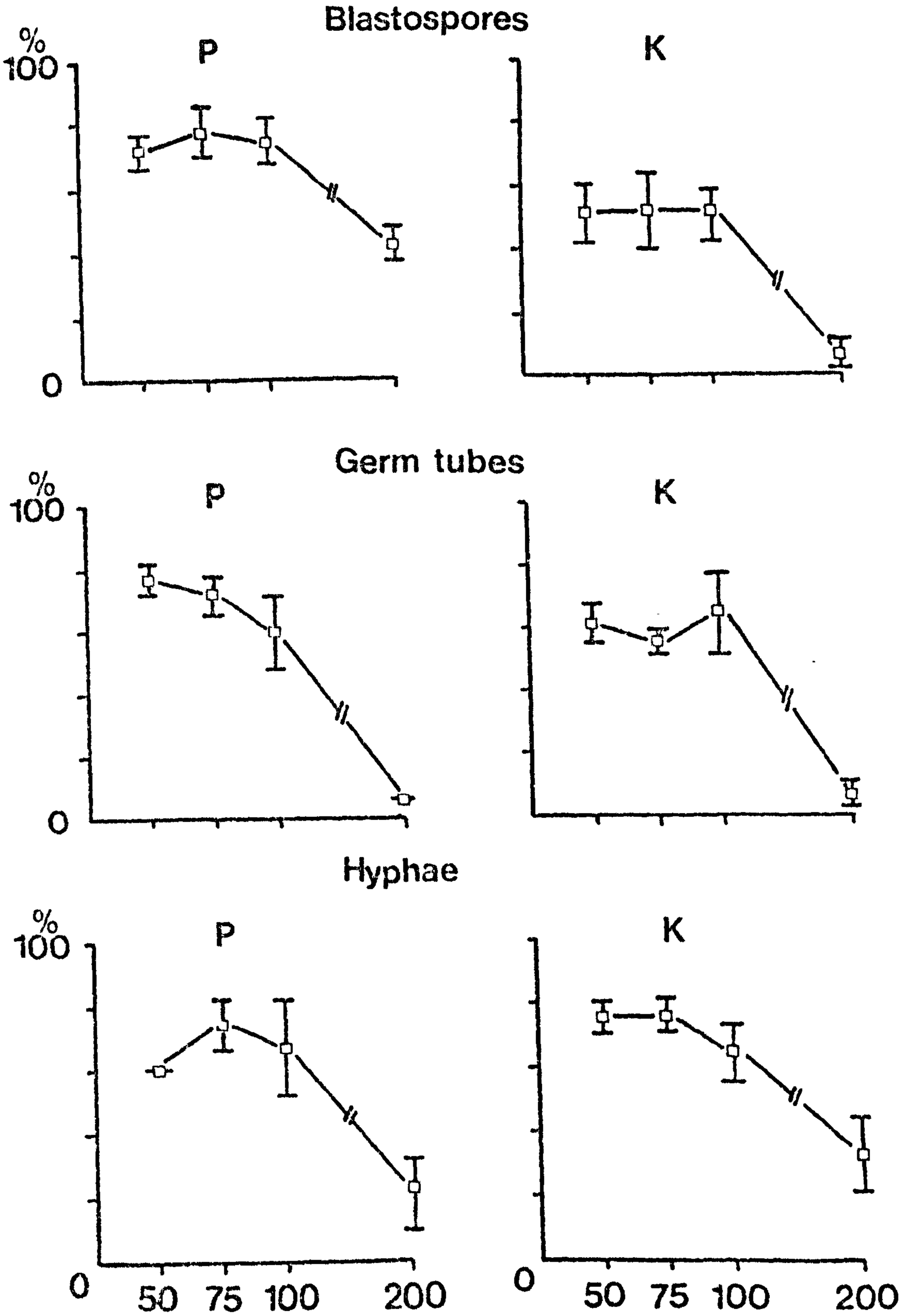
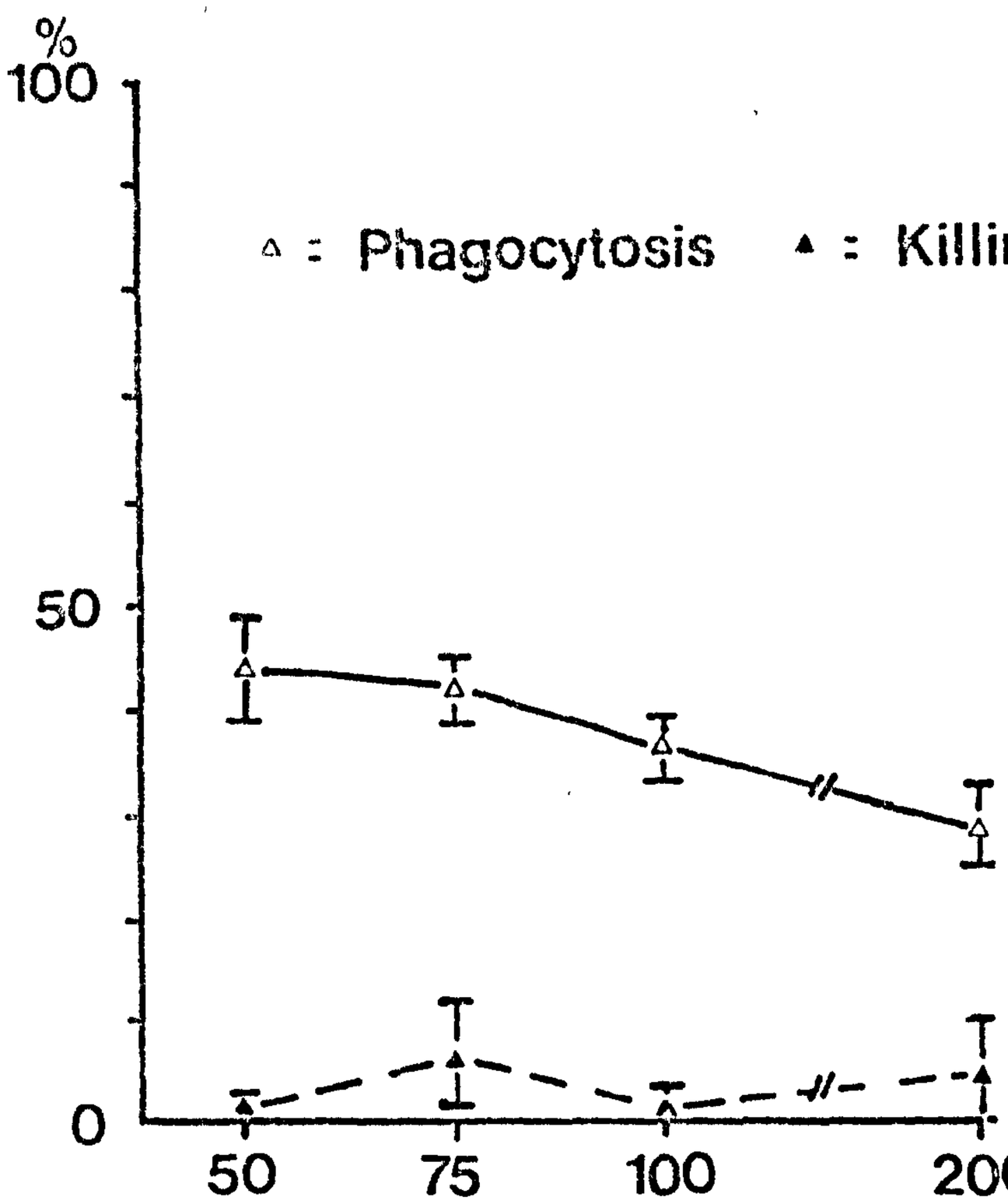

Blastospores
%
100
0
P
K
Germ tubes
%
100
0
P
K
Hyphae
%
100
0
P
K
0
50
75
100
200
0
50
75
100
200
Cell conc
ng ATP per
assay

Fig.4.4 Effect of blastospore: mouse peritoneal macrophage ratio on the efficiency of phagocytosis and killing of 73/055 blastospores by mouse peritoneal macrophages.



Assays were performed in the presence of 5% v/v control human serum and data represent the mean of four separate determinations $\pm$ standard deviation.

Table 4.2 Phagocytosis and intracellular killing of 73/055 blastospores, germ tubes and hyphae by mouse peritoneal macrophages.

	%Phagocytosis	%Killing
Blastospores	39±7	2±4
Germ tubes	27±10	18±12
Hyphae	33±3	0±0

Candida : Macrophage ratio 50ng ATP equivalent : $2x10^5$ macrophages.

Assays were performed in the presence of 5% v/v control human serum.

Data represent the mean of three separate determinations ±SD

removed and examined microscopically. The number of phagocytosed C. albicans blastospores per 100 PMNs and the number of these blastospores producing germ tubes were counted. Similar assays were performed where mixtures of blastospores and PMNs were incubated for 2 h before measurements were made to determine if prolonged incubation increased the extent of germ tube production by phagocytosed blastospores. After 1 h incubation no ingested blastospores were seen to be producing germ tubes and 96% of the blastospores ingested had been killed as determined radiometrically (Table 4:3). After 2 h incubation, however, the percentage of blastospores phagocytosed had increased to 86%. Only 90% of these ingested blastospores were killed by the PMNs and 11% of the blastospores observed inside PMNs had produced germ tubes and were therefore presumably viable.

The possibility that the metabolic status of the blastospores used in the assays could affect their ability to produce germ tubes and their ability to resist intracellular killing was investigated by performing similar assays to those described above with blastospores grown in EMEM + 20 mM HEPES pH 7.4 for 6 h at 30 °C in air. When tested for their ability to produce germ tubes in EMEM at 37 °C, 40% of these blastospores produced germ tubes compared with 49% of control stationary phase blastospores. When these logarithmic phase blastospores were used in phagocytosis and killing assays they were phagocytosed to a similar extent as stationary phase blastospores after 1 and 2 h incubation. Percentage killing figures were slightly higher for logarithmic phase blastospores and the percentage of ingested blastospores producing germ tubes after 2 h incubation was slightly lower than for stationary phase blastospores (Table 4:3). As this experiment was performed once only with PMNs obtained from two donors no statistical analysis of data was possible.

The significance of pre-formed germ tube length in relation to phagocytosis and killing of 73/055 was further investigated. C. albicans 73/055 blastospores were incubated in EMEM at 37 °C in air, for periods of time ranging from 30 to 120 min. Germ tubes obtained in this way differed

Table.4.3 Effect of blastospore age on phagocytosis and killing of 73/055 blastospores by human PMNs and on the production of germ tubes by phagocytosed blastospores.

Stationary phase blastospores

%Phagocytosis		%Killing		%Phagocytosed blastospores killed		% of ingested blastospores with germ tubes	
1h	2h	1h	2h	1h	2h	1h	2h
71	86	68	78	96	90	0	11

Logarithmic phase blastospores

%Phagocytosis		%Killing		%Phagocytosed blastospores killed		% of ingested blastospores with germ tubes	
1h	2h	1h	2h	1h	2h	1h	2h
71	84	77	81	100	96	0	7

Logarithmic phase blastospores were grown for 6h in EMEM at 30 C. Assays were performed at a ratio of 75ng ATP equivalent C.albicans:$2x10^5$ PMNs in the presence of 5% v/v control human serum.C.albicans/PMN mixtures were incubated for 1 or 2h before label uptake was measured.The percentage of ingested blastospores producing germ tubes was assessed microscopically. Data represent the mean of two separate determinations.

in length and were used in the phagocytosis and killing assays system. Germ tube suspensions prepared in this way were not standardised by ATP measurements as over the first 90 min incubation, preliminary experiments suggested that the ATP content of the suspensions remained virtually constant (see below). Statistical analysis of the results presented in Fig 4:5 indicated that germ tubes of average length 2.5 and 10 µm were phagocytosed and killed as efficiently as C. albicans blastospores. Germ tubes 15 µm long were phagocytosed to a similar extent as blastospores ($p < 0.02$) but were killed less efficiently than blastospores ($p < 0.01$). Germ tubes with an average length of 25 µm were also phagocytosed and killed to a lesser extent than blastospores but by 2 h incubation in EMEM the biomass of C. albicans germ tubes in suspensions used in the assay procedure was also higher than was present in similar blastospore assays. As the biomass present in these suspensions had almost doubled by 2 h incubation the effect of addition of 2 times the usual number of PMNs i.e. 4×10^5 per 75 ng ATP equivalent C. albicans, was tested in some experiments. Percentage phagocytosis figures obtained in these experiments were similar to those for blastospores (81%) but the percentage killing figures were lower (27%) than for blastospores ($\bar{x}$ of 2 separate determinations) (Fig 4:5)

In earlier experiments (Chapter 2) ketoconazole at a concentration of 10^{-7}M was shown to inhibit hyphal development in C. albicans. As this compound also slightly reduced the growth rate of C. albicans hyphae, the possibility that ketoconazole treatment of C. albicans could enhance the phagocytic and/or killing activity of human PMNs was investigated. Germ tubes grown for 2 h in the presence of 10^{-7}M ketoconazole were phagocytosed and killed to a similar extent as control germ tubes (Table 4:4). Hyphae grown in the presence of ketoconazole for 6 h were, however, phagocytosed and killed to a greater extent than control hyphae ($p < 0.01$) (Table 4:4).

Fig.4.5 Effect of germ tube length on efficiency of phagocytosis and killing of 73/055 by human PMNs.

EMEM + 10% v/v control human serum was inoculated to a concentration of $4x10^6$ 73/055 blastospores ml^{-1}. 0.25ml volumes were incubated for different lengths of time at 37°C in a water bath to produce germ tubes of different lengths. After incubation for the relevant time, 0.25ml volumes of germ tube suspensions were mixed with $2x10^5$ human PMNs in 0.25ml EMEM. Phagocytosis and killing assays were then performed as described in the methods section.

Data represent the mean of six separate determinations ±SD

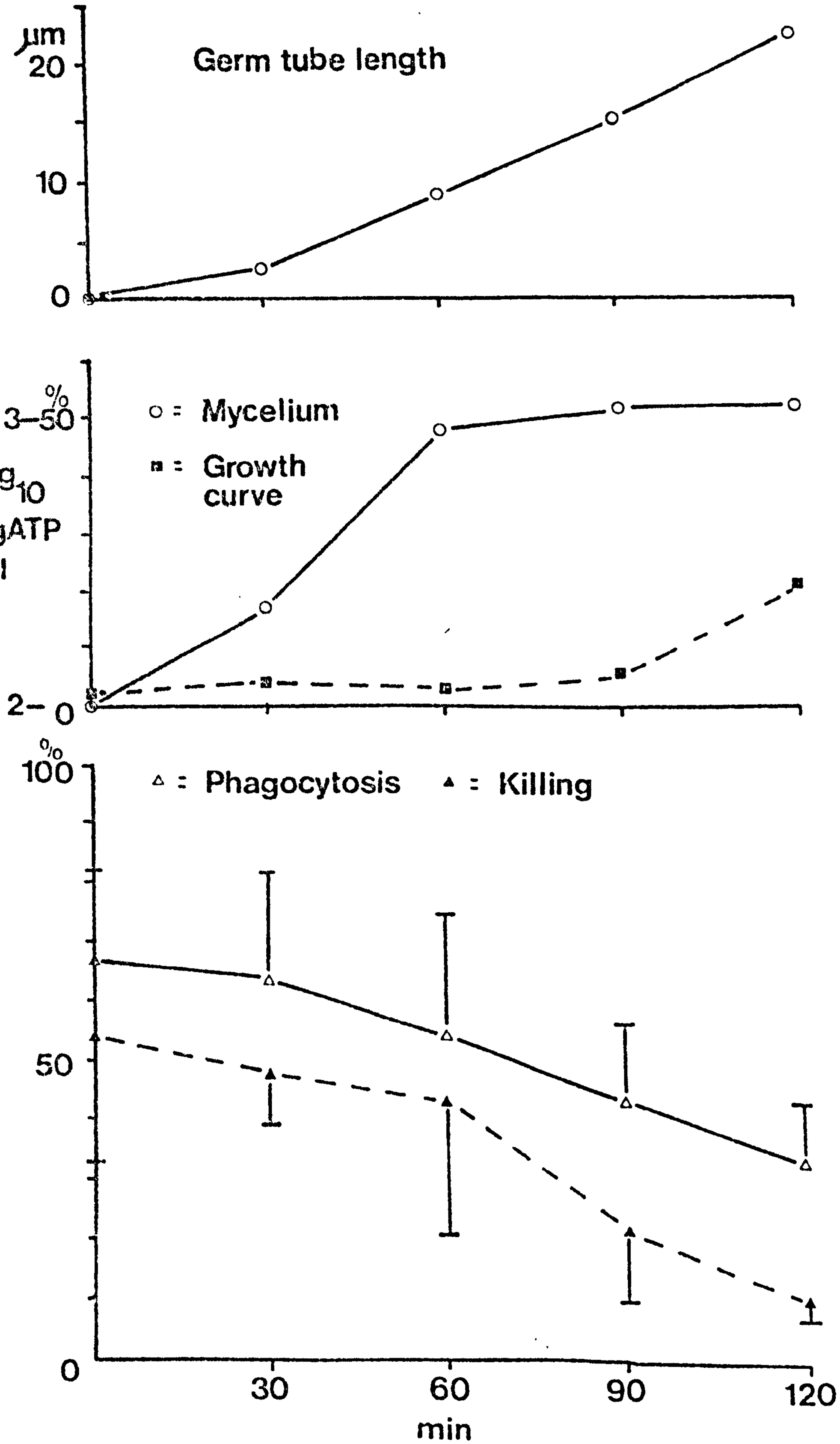

µm
20
10
0
Germ tube length
%
3–50
log10
ngATP
ml
2–0
○ = Mycelium
▪ = Growth curve
%
100
50
0
△ = Phagocytosis
▲ = Killing
30
60
90
120
min

Table.4.4 Effect of ketoconazole treatment on phagocytosis and killing of 73/055 germ tubes and hyphae by human PMNs.

	%Phagocytosis		%Killing	
	Germ tubes	Hyphae	Germ tubes	Hyphae
Control	59±18	38±16	64±9	40±14
Test	62±20	85±8	54±12	81±4

Germ tubes and hyphae were exposed to 10^{-7}M ketoconazole in EMEM at 37 C for 2 or 6h respectively. Control and test cultures were then harvested and assays were performed at a C.albicans:PMN ratio of 75ng ATP equivalent C.albicans:$2x10^5$ PMNs in the presence of 5% v/v control human serum. Data represent the mean of six separate determinations ± standard deviation.

Effect of serum factors on phagocytosis and intracellular killing of 73/055 blastospores germ tubes and hyphae

Phagocytosis of blastospores, germ tubes and hyphae was significantly lower in the absence of control human serum than in the control (Table 4:5). Removal of serum complement from human control serum by heat treatment had little effect on the phagocytosis of blastospores but significantly reduced the phagocytosis of germ tubes and hyphae. Phagocytosis in the presence of foetal calf serum was not significantly different to that in the control for germ tubes and hyphae but was significantly lower when blastospores were tested in this assay system (Table 4:5).

Omission of serum from the test system had no significant effect on the extent of killing of blastospores or germ tubes but significantly reduced the extent of killing of hyphae (Table 4:5). Blastospores and hyphae were killed significantly less in the presence of heat treated serum than in the control but the extent of killing of germ tubes was not significantly affected (Table 4:5). In the presence of foetal calf serum blastospores and hyphae were killed significantly less well than in the control but the extent of killing of germ tubes was not significantly affected. (Table 4:5).

Although the ranges of values obtained for phagocytosis and killing of C. albicans 73/055 blastospores, germ tubes and hyphae were in general higher in the presence of rabbit antisera raised against the homologous morphological form, no statistically significant differences were observed between test and control experiments (Table 4:6). Percentage phagocytosis and killing figures obtained in these assays with rabbit serum were generally lower than those found in earlier experiments when human serum was used and variations in the values obtained with PMNs from different donors were proportionately larger (Table 4:6) and may account for the lack of statistical significance between test and control experiments.

The lack of effect of anti-C. albicans antiserum on the extent of phagocytosis and killing of blastospores was supported, however, by further

Table.4.5 Effect of serum factors on phagocytosis and killing of 73/055 blastospores,germ tubes and hyphae.

%Phagocytosis

Serum	Blastospores	Germ tubes	Hyphae
Control	68±13	60±13	68±13
None	27±21	21±20	36±13
Heat treated	57±7	30±5	13±3
Foetal calf	40±12	45±31	22±14

%Killing

Serum	Blastospores	Germ tubes	Hyphae
Control	41±13	42±14	63±18
None	29±13	18±29	10±8
Heat treated	15±12	26±15	31±12
Foetal calf	14±19	24±8	27±4

Assays were performed at a ratio of 75ng ATP equivalent C.albicans:$2x10^5$ PMNs in the presence of 5% v/v serum.
Data represent the mean of four separate determinations ± standard deviation.

Table.4.6 Effect of antiserum raised against C.albicans morphological forms on phagocytosis and killing of 73/055 blastospores, germ tubes and hyphae by human PMNs.

%Phagocytosis

Serum	Blastospores		Germ tubes		Hyphae	
	Mean	Range	Mean	Range	Mean	Range
Control	57±22	42-83	21±4	18-23	33±6	25-41
Blastospore	69±13	60-84	20±11	15-44	37±9	27-49
Germ tube	70±16	59-89	42±17	16-62	54±17	36-74
Hypha	74±11	67-87	32±12	15-47	38±18	11-51

%Killing

Serum	Blastospores		Germ tubes		Hyphae	
	Mean	Range	Mean	Range	Mean	Range
Control	38±32	18-75	40±21	12-58	39±15	21-54
Blastospore	47±24	53-77	27±20	14-58	41±20	23-68
Germ tube	65±19	52-87	45±10	25-56	68±14	49-81
Hypha	38±29	35-70	40±17	15-54	58±9	47-64

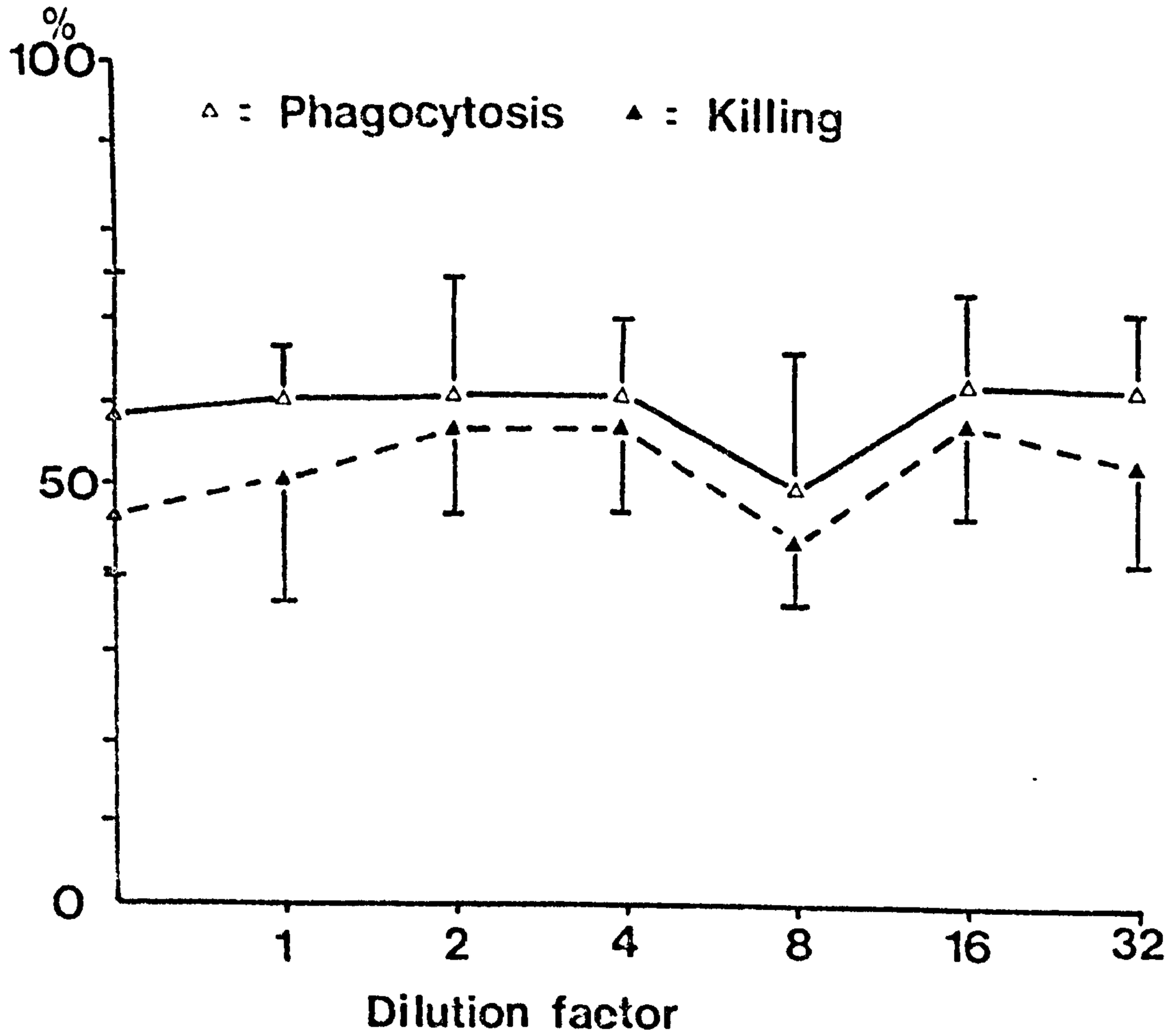
Assays were performed at a ratio of 75ng ATP equivalent C.albicans:$2x10^5$ PMNs in the presence of 5% v/v rabbit serum.
Data represent the mean of four separate determinations ± standard deviation and the range of values obtained.

experiments in which the anti-C. albicans antibody titre of the rabbit serum was reduced, either by dilution with control rabbit serum or by removal of antibody specific to blastospores by absorbtion of the serum with the same combined particulate-cytoplasmic C. albicans blastospore antigen against which the antiserum was originally raised. Dilution of anti-blastospore serum with control rabbit serum had no significant effect on the extent of phagocytosis and killing of 73/055 blastospores by human PMNs (Fig 4:6). In addition, although absorbtion of anti-blastospore serum with homologous antigen reduced the number and intensity of precipitin lines detected by CIE, and some reduction in both phagocytosis and killing were apparent, these differences were not statistically significant (Fig 4:7).

2) Chemotaxis

At a cell concentration equivalent to 800 ng ATP ml^{-1} C. albicans 73/055 blastospores and hyphae were equally chemotactic for human PMNs (Table 4:7). Germ tubes were significantly less chemotactic for PMNs than blastospores ($p < 0.01$) but the difference between the chemotactic response of PMNs towards germ tubes and hyphae was smaller ($p < 0.02$). When the culture filtrates obtained from cultures of 73/055 blastospores, germ tubes and hyphae, grown in EMEM for varying periods of time were tested for their chemotactic properties a similar pattern of results was obtained. Culture filtrates obtained from cultures of blastospores and hyphae grown for 6 h in EMEM were equally chemotactic, although the number of PMNs migrating towards the filtrates was markedly less than that migrating towards the fungus (Table 4:8). The chemotactic response towards culture filtrates of C. albicans blastospore and germ tubes grown in EMEM for 2 h was similar (Table 4:8) and was less than that towards culture filtrates of 6 h blastospore and hyphal cultures.

Fig.4.6 Effect of dilution of anti-C.albicans blastospore antiserum on the efficiency of phagocytosis and killing of 73/055 blastospores by human PMNs.



Rabbit anti-blastospore antiserum, CIE titre 1:32, was diluted by addition of control rabbit serum. Serum diluted in the range 1:1 - 1:32 was then used as the serum component in phagocytosis and killing assays of 73/055 blastospores. Assays were performed at a ratio of 75ng ATP equivalent C.albicans:2×10^5 PMNs.

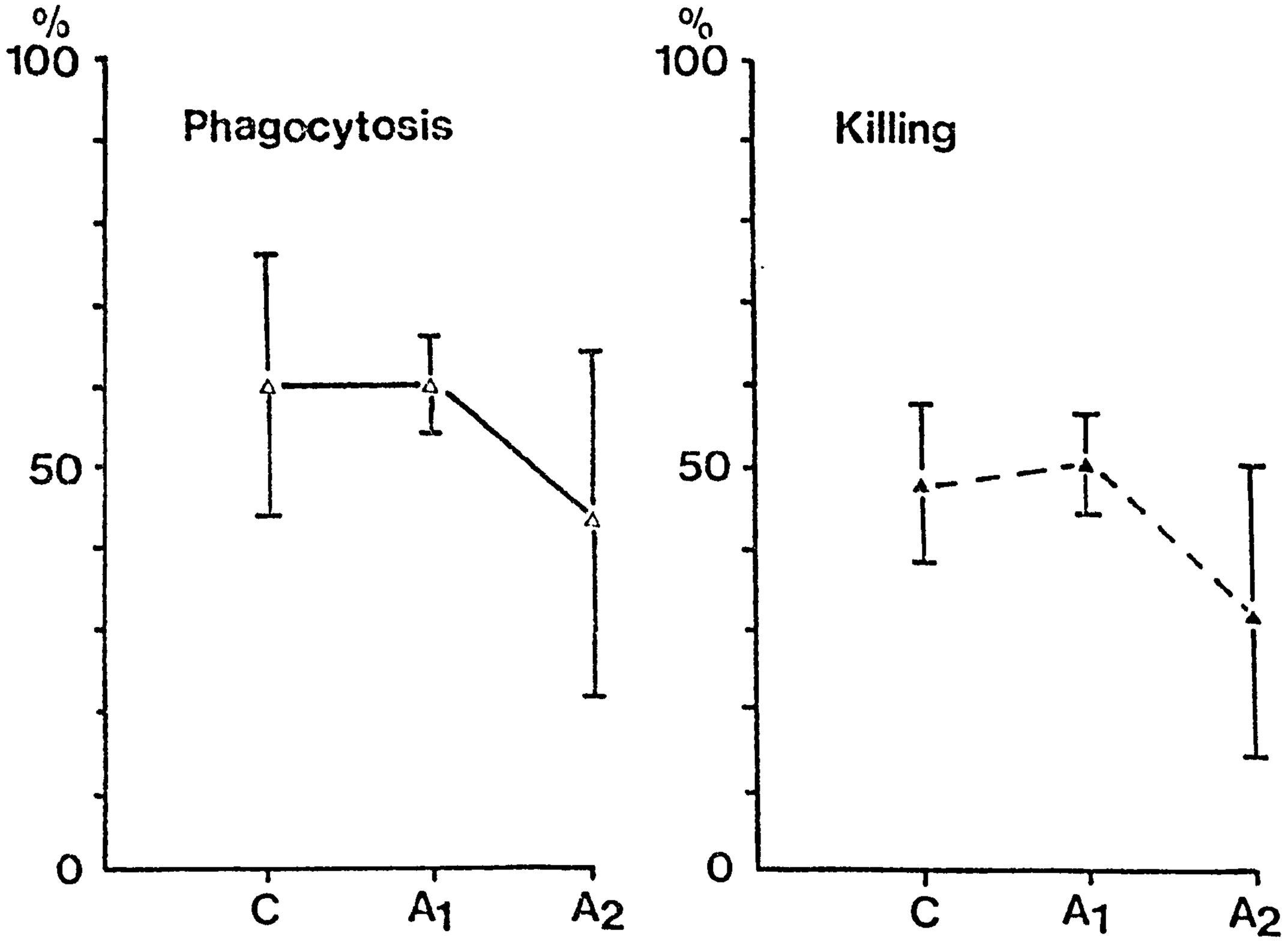


Fig.4.7 Effect of absorbtion of anti-C.albicans blastospore antiserum on the extent of phagocytosis and killing of 73/055 blastospores by human PMNs.

1ml of serum was mixed with 75µg CE and 2000ng ATP equivalent PA against which the antiserum was raised. The mixture was incubated at 37°C for 30min in a water bath. The mixture was then centrifuged at 2500 g for 5min and 0.5ml supernatant removed. The supernatant was reabsorbed as described above. After the second absorbtion the mixture was centrifuged and the supernatant removed.

Absorbed and control sera were diluted overall 1:4 in PBS before use in phagocytosis and killing assays. Serum was included in these assays at 20% v/v and assays were performed at a ratio of 75ng ATP equivalent C.albicans:2×10^5 PMNs.
Data are expressed as the mean of six separate determinations ± standard deviation.

Table.4.7 Chemotataxis of human PMNs towards 73/055 blastospores,germ tubes and hyphae.

Control	Blastospores	Germ tubes	Hyphae
3±2	43±9	26±2	47±6

Data represent mean values of four seperate determinations ± standard deviation and show the number of PMNs migrating per microscope field towards C.albicans. All forms were tested at 800ng ATP equivalent per ml in the presence of 10% v/v foetal calf serum.Control =number of PMNs migrating per field towards EMEM + 10% foetal calf serum.

Table.4.8 Chemotaxis of human PMNs towards 73/055 culture filtrates.

Control	Blastospore 2h	Blastospore 6h	Germ tubes 2h	Hyphae 6h
3	2	13	5	13

Data represent mean values of three separate determinations and show the number of PMNs migrating per microscope field towards filtrates obtained from C.albicans EMEM cultures after different periods of incubation.

4.4 Discussion

The radiometric assay for the quantification of phagocytosis and intracellular killing used in the present study, in general, provided reproducible results particularly when applied to blastospores. When germ tubes and hyphae were tested with this system the results obtained were more variable. In particular, in some assays, values obtained for phagocytosis were less than those for intracellular killing although in the majority of these cases the difference between the two values was only a few percent. As the size of germ tubes and hyphae precludes their total phagocytosis by a single PMN it is conceivable that values recorded for phagocytosis and killing with this assay system, may in fact be measuring similar parameters. If this were the case, the variation between values for phagocytosis and killing is well within the normal range of variation observed between duplicate tubes in these assays. The possibility that the high percentage killing figures for germ tubes and hyphae were due to the presence of undisrupted PMNs in assay mixtures was also considered but visual examination of samples taken from these mixtures showed that all the PMNs originally present had been lysed by the addition of sodium deoxycholate.

It was noticeable that during incubation of Candida/PMN mixtures, germ tubes and hyphae readily formed clumps in the presence of serum. It is possible that, in some cases, this process could reduce the surface area of the fungus available for interaction with the PMNs and could lead to variation in the results recorded.

The variation in the results obtained could also, in part, be attributable to variation in the phagocytic and intracellular killing abilities of PMNs from different donors and in slight day to day variation in the efficiency of these processes in PMNs obtained from the same donor.

Values for phagocytosis and killing of blastospores in the present study are well within the range of results presented by other workers

(Table 1:4, Chapter 1) who used human PMNs in their assays. In contrast to the findings of other workers, e.g. Bridges et al. (1980), in none of the assays performed in the present study, were all the C. albicans blastospores present in assay mixtures phagocytosed. This observation was confirmed visually when samples of these Candida:PMN mixtures were observed microscopically.

As reported by previous workers, e.g. Bridges et al. (1980) the efficiency of phagocytosis and killing was affected by the ratio of C. albicans blastospores to PMNs. At low blastospore:PMN ratios the efficiency of phagocytosis is governed by the frequency with which blastospores and PMNs collide during incubation. As the blastospore:PMN ratio increases the frequency of these collisions increases until an optimal ratio is reached. At blastospore/PMN ratios above this value although the frequency of contact between fungus and leukocyte increases the PMNs are unable to phagocytose any more blastospores and the proportion of the fungus phagocytosed falls. At blastospore:PMN ratios up to 8:1 (up to 100 ng ATP equivalents per assay) the proportion of ingested blastospores killed remained roughly the same.

At 200 ng ATP equivalent per assay (16:1, C. albicans:PMNs) the proportion of ingested blastospores killed fell. Presumably the intracellular killing mechanisms of the PMNs were unable to cope with the increased blastospore biomass and were effectively swamped.

The results of the present study also suggest that when standardised on the basis of viable biomass, C. albicans blastospores germ tubes and hyphae are phagocytosed to a similar extent, particularly at low Candida:PMN ratios. The morphological forms do, however, differ in their resistance to intracellular killing by PMNs. Blastospores were less effectively killed by human PMNs than were germ tubes and hyphae at all C. albicans:PMN ratios tested and the proportion of ingested C. albicans cells killed was also consistently higher for germ tubes and hyphae than blastospores (Table 4:9).

Table.4.9 Proportions of ingested 73/055 blastospores,germ tubes and hyphae killed by human PMNs.

ngATP per assay	Blastospores%	Germ tubes%	Hyphae%
50	65	79	100+
75	50	80	100+
100	64	100+	98
200	14	100+	100+

Data represent mean values and were calculated from the data shown in Fig.4.3.

The increased sensitivity of germ tubes and hyphae to intracellular killing may be due to a number of factors. Although these filamentous forms may not be completely phagocytosed it is possible that localised damage at one site on the cell surface of germ tubes and hyphae may be sufficient to lead to the death of that fungal element. The continuity between different sections of C. albicans germ tubes and hyphae, afforded by the septal pore (Scherwits, Martin & Ueberberg, 1978, Gow et al. 1980) could contribute to this action. The thickness of germ tube and hyphal cell walls of C. albicans differs from that of the wall of blastospores and cell wall structure could be important in resistance to intracellular killing. In particular the hyphal tip may be particularly sensitive to the action of hydrolytic enzymes as this is probably the weakest site on the hyphal cell wall. Finally variation in the composition of the cell wall surface between blastospores and germ tubes and hyphae of C. albicans could affect the intensity of the metabolic burst involved in the killing of ingested viable particulate matter as has been suggested to account for the variation in the extent of killing of C. albicans and other Candida species (Sasada & Johnston, 1980).

Although previous workers have suggested that the ability of C. albicans blastospores to resist intracellular killing is due to their ability to produce germ tubes and then to grow out of phagocytic cells (Stanley & Hurley, 1969) the results of the present study suggest that processes other than germ tube production may be involved in resistance to intracellular killing. An average of 50% of blastospores ingested by PMNs were killed after 1 h incubation as assessed radiometrically (Table 4:9 75 ng ATP equivalent: 2 X 10^5 PMN). However, when the proportion of ingested blastospores producing germ tubes was assessed microscopically at this time, none of the ingested blastospores was seen to be producing germ tubes, an observation that had been made in several earlier experiments in this study. Increasing the incubation time of the phagocytosis and intracellular killing assays did, however, increase the proportions of ingested blastospores seen to be producing germ tubes and in the experiments described this proportion

correlated well with the calculated proportion of blastospores remaining viable inside PMNs. (These findings should, however, be considered in respect of the subjective nature of the visual counting method used). It appears, however, that germ tube production by ingested blastospores, at least in the very early stages of the interaction between C. albicans and PMNs has little significance in resistance to intracellular killing and that other defensive mechanisms may protect the fungus. If, however, ingested blastospores survive the initial contact with the PMN's hydrolytic and oxidative killing mechanisms it would seem that germ tube production may permit the fungus to grow out of the PMN and therefore escape intracellular killing.

The results for phagocytosis and killing of 6 h old blastospores suggest that no real correlation exists between the ability of C. albicans 73/055 blastospores to produce germ tubes in vitro and their resistance to intracellular killing. The proportion of ingested blastospores producing germ tubes after 2 h incubation was similar for stationary and logarithmic phase cells. These results agree with those of De Brabander et al. (1980) who also suggested that the ability to produce germ tubes in vitro did not correlate with the ability of ingested blastospores to produce germ tubes inside leukocytes.

Once produced inside a PMN, germ tubes will continue to elongate and may rupture the PMN membrane. Short, pre-formed germ tubes, 2.5 or 10 um long, can be completely ingested by human PMNs and may be killed by the phagocytic cells before they grow to a sufficient length to be able to puncture the PMN membrane. Germ tubes 15 µm long, although short enough to be totally phagocytosed, were killed significantly less well than blastospores. Although the biomass of fungus in these assays had not significantly changed by the time the germ tubes were mixed with PMNs it is possible that the increase in the physical size of the ingested germ tubes was sufficient to reduce the efficiency of the intracellular killing mechanisms.

The importance of the continuation of development of hyphae in the resistance to phagocytosis and killing by human PMNs is emphasized by the results obtained with ketoconazole-treated germ tubes and hyphae. Ketoconazole treatment had little effect on the phagocytosis and killing of germ tubes by human PMNs. Control and ketoconazole-treated germ tubes differed little in morphology or growth rate. Ketoconazole-treated hyphae were, however, shorter than control hyphae and also differed morphologically and in their rate of growth and elongation (Chapter 2). These observations may account for the significantly increased values for phagocytosis and killing observed when ketoconazole-treated hyphae were tested.

As reported in earlier studies, results presented here suggest that human PMNs differ in their phagocytic and intracellular killing abilities from mouse peritoneal macrophages (Table 1:4 & Chapter 1). Mouse peritoneal macrophages were less efficient in relation to the phagocytosis and killing of all three morphological forms of C. albicans tested. All three forms were, however, phagocytosed and killed to a similar extent. It is possible that the differences observed in the results for the two types of phagocytic cells may reflect differences in a number of factors, e.g. species from which the leukocytes were obtained, as well as the inherent phagocytic and killing abilities of PMNs and macrophages. In addition the low levels of phagocytosis seen could be due to the fact that phagocytosis and killing assays were performed with human rather than mouse serum which was not readily available in any quantity. It is possible that the composition of human serum may differ from that of mouse serum and this difference resulted in lower percentage phagocytosis figures. (see below). The low killing figures of all three morphological forms of C. albicans by mouse peritoneal macrophages do, however, suggest that these phagocytic cells may lack the ability to effectively kill C. albicans.

. . . . . .

Variation in the composition of serum in relation to its content of individual serum components was also shown to affect the efficiency of

phagocytosis and killing by human PMNs. The relative importance of individual components, however, differed between morphological forms. Phagocytosis of blastospores was reduced when foetal calf serum, containing no antibody, was used in the assay system but was not affected when complement-depleted serum was used. Although the extent of phagocytosis was reduced in the absence of serum, some blastospores were still phagocytosed contrasting with the observations of other workers e.g. Bridges et al. (1980), who reported that blastospores were not phagocytosed at all in the absence of serum. The efficiency of killing of blastospores was reduced when antibody or complement-depleted serum was used, but not, paradoxically, in the absence of both of these components. Phagocytosis of germ tubes was reduced in complement-depleted serum but killing was unaffected suggesting that for germ tubes complement was important only in relation to phagocytosis of the fungus. Serum antibody did not appear to be important in the phagocytosis or killing of germ tubes. Complement was the most important serum component in relation to phagocytosis of hyphae although both complement and serum antibody were involved in the intracellular killing of this form.

As shown for blastospores phagocytosis of germ tubes and hyphae still occurred in the absence of serum but was significantly less than observed in the presence of control serum.

The failure of antisera raised against C. albicans to increase the extent of phagocytosis and killing of 73/055 blastospores could suggest that very low levels of non-specific antibody or serum components other than immunoglobulins are sufficient for the optimal opsonization of C. albicans blastospores for phagocytosis. As serum complement appears to be the important component in relation to phagocytosis of C. albicans germ tubes and hyphae the lack of effect of anti-C. albicans antisera on the phagocytosis of these morphological forms is perhaps not surprising. It should be noted that the reproducibility of results of assays performed with rabbit serum was poorer than was seen when human serum was used in similar assays and that the results recorded with rabbit serum were generally lower for both

phagocytosis and killing. It is again possible that variation in the serum composition between different species could account for some of these observations.

The results of these assays suggest that although C. albicans blastospores, germ tubes and hyphae are phagocytosed by human PMNs to a similar degree, blastospores differ from the filamentous forms in their resistance to intracellular killing. These morphological forms have also been shown in the present study to differ in their chemotactic properties for human PMNs. When tested at cell concentrations similar to those used for the phagocytosis and killing assays, no chemotactic responses were observed. At higher cell concentrations (800 ng ATP ml^{-1}) germ tubes were found to be significantly less chemotactic than blastospores and 6 h old hyphae. Culture filtrates of blastospores or hyphae grown for 6 h in EMEM were equally chemotactic although less so than the fungal cells suggesting that some chemotactic soluble cell product was being released during the growth of both blastospores and hyphae. The failure of 2 h old blastospore or germ tube culture filtrates to attract PMNs could indicate that after only 2 h growth the concentration of the chemotactic product is insufficient to elicit a chemotactic response or possibly that this product is only released from C. albicans cells of a minimum age. Cutler (1977) reported that culture filtrates of C. albicans blastospores were chemotactic for guinea pig neutrophils only after 12 h incubation of the fungus in the growth medium.

The results presented here do, however, differ from those presented by Denning & Davies (1973), who reported that C. albicans blastospores were more chemotactic for human PMNs than hyphae. The hyphae used in the present study were only 6 h old compared to 3 day old hyphae used by Denning & Davies (1973) and presumably the hyphae used in this and the present study differed in metabolic terms and possibly in their rate of production of chemotactic factors. In addition the variation in incubation times and growth media used could have affected the cell wall composition and possible chemotactic

properties of the blastospores and hyphae. Although the blastospores and hyphae used in the present study in chemotaxis experiments were also grown in two different growth media for different periods of time, analysis of the polysaccharide composition of the blastospores and hyphae used in these assays (Chapter 3) showed that both morphologies had a similar polysaccharide composition.

CHAPTER 5

Concluding discussion

Although dimorphism in C. albicans has been extensively studied in vitro many aspects of this phenomenon are still poorly understood. The mechanisms by which environmental influences on morphology are mediated within the fungal cell and the relationship of C. albicans morphology to virulence have received much attention. Investigation of environmental aspects of dimorphism have tended to concentrate on the initial events related to dimorphism, blastospore budding and germ tube synthesis and little study has been made of the effects of environmental factors on the subsequent development of C. albicans hyphae in relation to blastospore budding and hyphal branches. Correlation between cell wall composition and morphology of C. albicans has previously been established but the possibility that cell wall composition of the fungus could also change with the age of blastospores and hyphae has not been investigated directly despite indirect evidence suggesting that this was the case. Results of experiments designed to test the relative virulence of C. albicans blastospores and hyphae in vitro and in vivo have given contradictory results. Although interactions of C. albicans blastospores and filamentous forms with phagocytic cells, one of the major host defence mechanisms against infection, has previously been investigated in vitro, previous studies have been hampered by a lack of adequate methods for the standardization of fungal biomass and for the objective assessment of phagocytosis and killing.

The principal objectives of the present study were, therefore, to investigate the effects of environmental conditions on the morphology and content of the major cell wall polysaccharides of C. albicans in relation to the initial and subsequent development of the fungus and to investigate the interaction of different morphological forms of C. albicans with phagocytic cells.

The results of the study have re-emphasized the complex nature of the interaction of C. albicans and environmental factors involved in the control of dimorphism. No single environmental factors could be shown to be responsible for the effects seen. Variations in morphology correlated well with changes in cell polysaccharide content and these results were in general agreement with those of earlier studies (Chattaway et al. 1968). Chitin had previously been implicated as an important cell wall component involved in dimorphic shifts and the results of the present study support this view. Chitin content was found to be significantly higher in germ tubes and hyphae than blastospores at all phases of the growth cycle, an observation that had not previously been reported. The increase in chitin content of germ tubes and hyphae correlated well with an increase in the activity of chitin synthase in these morphological forms. The results of the present study also support the findings of earlier workers (Chattaway, Shenolikar & Barlow, 1974), which suggested indirectly that the cell wall composition of C. albicans blastospores and hyphae changed with the age of the fungus and also suggest that medium composition may affect cell polysaccharide composition of C. albicans.

The mechanisms by which the effects of environmental factors on C. albicans morphology are mediated, are, for the most part, unknown. Since the shape of the fungus is determined by the shape of the cell wall it has been suggested that changes in the morphology of C. albicans mey result from changes in the activities of enzymes involved in the production of substrate for cell wall synthesis and of cell wall polysaccharide synthases, in particular chitin synthase. The results of two earlier investigations have supported this hypothesis (Chattaway et al. 1973; Braun & Calderone, 1978). The observation that dimorphic changes in C. albicans do not require de novo synthesis of proteins (Brown & Chaffin, 1981) supports the view that changes in endogenous enzyme activity, rather than synthesis of new enzymes, may be important in the initiation and control of dimorphism. The activity of cell wall synthase enzymes, once germ tube production or budding is initiated,

could presumably be regulated by conventional mechanisms including concentration of enzyme activators and inhibitors, substrate concentration and end product repression. Environmental influences on the activity of these enzymes could be mediated through some common intermediary metabolite or co-factor. cAMP has been shown to mediate the effect of environmental factors on many different types of cells (Chattaway et al. 1981) and these workers have proposed a role for cyclic nucleotides in the control of dimorphism in C. albicans.

All these observations suggest that morphological development in C. albicans is predominantly under metabolic control. However, other evidence suggests that regulation of cell shape may be under genetic control.

The observation in the present and earlier studies e.g. Manning & Mitchell (1980) that different isolates of C. albicans differ in their morphological development under similar incubation conditions suggests that isolates may differ genetically. Evans, Odds & Holland (1975) and Mitchell & Soll (1979) have shown that once a C. albicans blastospore initiates germ tube or bud formation it is committed to that particular mode of development and becomes resistant to other environmental factors known to influence cell morphology until its cell 'cycle' is complete. The hypothesis that environmental factors acted as a 'trigger' to initiate a genetically pre-determined series of events was put forward.

Although cell wall synthesis may be controlled by metabolic or genetic regulation of the activity of the enzymes involved in cell wall synthesis once blastospore budding or germ tube production has been initiated, the mechanism by which development is initiated is unknown. Since stationary phase blastospores of C. albicans are capable of producing blastospore buds or germ tubes the mechanisms by which the two modes of development are initiated are likely to differ. This possibility is supported by the results of earlier studies that have shown that the timing of DNA synthesis differs during germ tube formation from that during blastospore bud formation (Wain

et al. 1976). DNA synthesis coincides with bud emergence but occurs after germ tube initiation. In the monomorphic fungus S. cerevisiae the cell cycle has been shown to consist of two separate phases. A 'division cycle' involved in DNA synthesis and a 'growth cycle' responsible for synthesis of other cell components required for cell growth e.g. cell wall components (Mitchison, 1971). If a similar system operated in C. albicans, the available data would suggest that blastospore budding involves closely synchronised 'cycles' of division and growth. Germ tube initiation would involve an effective uncoupling of cell division from cell growth as postulated by Nickerson (1948). Environmental factors could therefore be postulated to act on different control mechanisms within the C. albicans blastospore. Under environmental conditions favouring blastospore budding, control of development could be envisaged to follow a similar pattern to that seen for budding of S. cerevisiae and could be genetically controlled. Germ tube initiation may be more directly controlled by metabolic mediation of environmental factors although subsequent development of hyphae may involve genetic control.

The observation that the site on the parent blastospore surface where new cell wall material is incorporated is carefully controlled for blastospore budding, but apparently random for germ tube initiation (Mackenzie, 1964) also suggests differences in the control of these two processes. Blastospore buds are produced at specific sites on the blastospore surface, adjacent to bud scars, only one bud being produced at any one time. Germ tubes may be produced at any site on the blastospore surface and several germ tubes may be produced within a short time. Localised activation of chitin synthase at sites of highest chitin concentration i.e. bud scars and therefore possibly highest concentration of substrate for new chitin synthesis could explain the spatial distribution of buds on the parent cell surface. A random distribution of chitin, therefore variable amounts of possible substrate for new cell wall synthesis, throughout the blastospore lateral walls could possibly explain the random distribution of the sites

of germ tube initiation. The mechanism by which the shape of the new cell wall material incorporated into the cell wall is determined is also unknown. It has been suggested that the shape is governed by the concentration of the inhibitors of synthase enzymes at the growing apex (Farkas, 1979) or that cell shape is determined by the rate at which newly synthesized wall is rigidified (Odds, 1979). The latter hypothesis also suggests that new cell wall material may be incorporated as the cell ages and is supported by the experimental findings in the present study that suggest that the cell polysaccharide composition may change with the age of C. albicans cell.

Although bud and germ tube initiation may involve similar enzyme systems, differences in morphology may result from differences in the extent to which these enzymes are controlled under different environmental conditions. In stationary phase blastospores the activity of cell wall synthases is low, presumably as a result of high levels of synthase inhibitors. It may be postulated that under environmental conditions favouring blastospore budding the concentrations of synthase inhibitors remains relatively high and the activity of synthase enzymes relatively low. The level of inhibitor may be genetically controlled and metabolic mediation through concentrations of substrate may be involved in the regulation of this gene product. Under environmental conditions favouring germ tube initiation where DNA synthesis and possible genetic control of concentrations of enzyme inhibitor may not occur, enzyme activity could increase resulting in more rapid, possibly more random cell wall synthesis. A similar argument could be proposed for the role of enzyme activators in the control of dimorphism.

Once initiated blastospores will continue to bud until nutrients become limiting and cultures enter the stationary phase of growth. In S. cerevisiae blastospores reach a minimum 'critical cell mass volume' before new buds are produced (Mitchison, 1971). Cell volume increases with the age of blastospores and it is thought that initiation of new DNA synthesis occurs only when the internal cell concentration of a particular cell product, synthesized during cell growth, rises to a critical minimum level. Blastospore volume has also

been found to affect the rate at which buds are produced by C. albicans (Chaffin & Wheeler, 1981). Results of the present study suggest that development of hyphae may be dependent on the isolate of C. albicans under test and on the environmental conditions suggesting that development may be controlled by a combination of genetic and metabolic factors. DNA synthesis occurs in elongating hyphae (Soll, Stasi & Bedell, 1978) and the possibility that in older hyphae gene products may be formed which result in a reduction in cell wall synthesis leading to secondary blastospore production must be considered. That the site of production of secondary blastospores and hyphal branches is more carefully controlled in hyphae is suggested by the observation that these processes always occur at septal junctions of hyphae supporting the hypothesis that chitin may be important in determining the site at which new cell wall material is incorporated.

The observation that, in the present study, isolate 73/055 could be induced to produce either secondary blastospores or hyphal branches by changing the environmental conditions suggests that genetic control of secondary blastospore and hyphal branch formation may be influenced by metabolic processes. The finding that medium composition affected polysaccharide content of blastospores of isolate 73/055 and the qualitative appearance of hyphae support this view.

Further analysis of the mechanisms by which cell wall shape and composition and dimorphic shifts are controlled in C. albicans could be performed with a series of C. albicans mutants. The results of the studies of other workers have suggested that difficulties may be encountered in producing such mutants and these difficulties have been attributed to the observation that C. albicans is diploid rather than haploid (Olaiya & Sogin, 1979; Whelan, Partridge & Magee, 1980). Some success in producing C. albicans mutants has, however, been reported (Bish & Sarachek, 1967; Kwon-Chung & Hill, 1970; Macdonald & Odds, 1982) and morphological mutants could theoretically be isolated by similar methods.

Although chitin has been implicated in cell wall morphogenesis the results of the present study also suggest that the content of the other cell wall polysaccharides may be morphology related. Analysis of the cell wall composition of mutants by thin-layer-chromotography of hydrolysates of chemical extracts or isolated cell walls could indicate the relative importance of individual cell wall components in the initiation and continued development of C. albicans blastospores and hyphae. Variations in the morphological development of such mutants could also be correlated with differences in the activities of the synthase enzymes involved in cell wall synthesis from those of the parent isolate. The importance of individual components of the cell wall in controlling the cell shape could further be investigated by studying cell wall regeneration of spheroplasts produced from these mutants. Mutants lacking the ability to synthesize a particular cell wall component may differ in their ability to regenerate a complete cell wall and could therefore differ from the parent isolate in their response to environmental conditions and in their ability to produce blastospore buds or germ tubes. Study of the morphological development of mutants that differed from the parent isolate in the site on the blastospore surface at which new cell wall synthesis is initiated coupled with studies of the activities of cell wall synthase enzymes and cell wall composition could indicate if individual polysaccharides were important in the control of the initial choice of such sites.

In addition to the determination of the activity of cell wall synthase enzymes in C. albicans blastospores and hyphae of different ages, studies of the incorporation of the substrates of these enzymes into whole cells may also indicate changes in the patterns of synthesis as cells age. If substrates were labelled with different radioactive markers individual cell wall fractions could be obtained and the distribution of label in various fractions determined by scintillation counting of hydrolysed cell wall fractions separated by thin-layer chromatography. This procedure could also be used to study the effect of substrate concentration and the availability of substrate on the quantitative cell wall composition of blastospores and hyphae. These studies may also be furthered by the use of

inhibitors of cell wall synthase enzymes e.g. 2-deoxy-glucose which is known to inhibit glucan synthesis in S. cerevisiae (Biely, Kovarik & Bauer, 1973).

A series of C. albicans mutants that differed in their morphological development could also allow simple genetic studies of the processes involved in dimorphism. The relative importance of individual processes involved in the initiation and control of cell wall synthesis could be assessed by studying the morphological development of recombinants produced by the fusion of spheroplasts of different C. albicans mutants. The possibility of transfer of the ability to produce germ tubes from C. albicans to other Candida species would also be worth investigating by this method. Finally the molecular basis of dimorphism could be further investigated by recombinant DNA technology although a great deal of work would be involved. The similarity between the protein contents of C. albicans blastospores and germ tubes suggested by the results of other workers (e.g. Manning & Mitchell, 1980a) suggests that the mRNA content of both forms and the cDNA libraries synthesized from the mRNA would be very similar in blastospores and germ tubes and detection of gene products specific to either morphological form would therefore be very difficult.

The imidazole antifungal ketoconazole, was shown to inhibit hyphal branching in C. albicans at very low concentrations. This compound, however, had no inhibitory effect on germ tube production even at higher concentrations. Effects of ketoconazole on morphology were only apparent after an initial lag period of approximately two hours. The appearance of ketoconazole-treated blastospores under the phase contrast microscope indicated that the effects of ketoconazole were not restricted solely to hyphal forms of C. albicans.

The mode of action of ketoconazole on C. albicans has been extensively studied. Van den Bossche et al. (1978) attributed the activity of imidazole antifungals to interference with sterol biosynthesis in C. albicans and further investigations with ketoconazole have supported this view (Marriott, 1980; Van den Bossche et al. 1980). Inhibition of sterol synthesis could

affect the composition and permeability of the C. albicans plasmalemma resulting in an inhibition of growth. As the enzymes chitin synthase (Braun & Calderone, 1978) and mannan synthase (Marriott, 1977) have been shown to be sited on the plasmalemma changes in sterol composition could influence the activity of these enzymes and possibly cell wall synthesis. This possibility was supported by the observations of Pesti, Campbell & Peberdy, (1981) who reported that chitin synthase activity was higher in ergosterol deficient mutants of C. albicans than in the parent isolate, and of Chiew, Sullivan & Shepherd (1982) who found that ergosterol inhibited both germ tube production and chitin synthase activity in C. albicans.

Very recently, however, Uno, Shigematsu & Arai (1982) have suggested that the effect of ketoconazole on sterol synthesis may not be the primary site of action of this compound. These workers reported that low concentrations of ketoconazole inhibited both endogenous and exogenous respiration in C. albicans blastospores and further work (Shigematsu, Uno & Arai, 1982) indicated that ketoconazole was a non-specific inhibitor of NADH and succinate oxidases. These workers therefore suggested that inhibition of sterol synthesis was a secondary effect of a general inhibition of electron transport and cell respiration.

The lack of inhibition of germ tube production in ketoconazole treated blastospores agrees with these observations since Land et al. (1975a) have reported that during germ tube initiation respiration is inhibited in C. albicans. These results also correlate with the findings of the present study that ketoconazole was most effective when added to C. albicans cultures during the early stages of growth and could also explain the lag period between addition of ketoconazole and the observation of any effects on cell growth and morphology. The pleomorphic nature of ketoconazole-treated hyphae and the lack of hyphal branching could be attributed to interference with enzyme activities or their regulation, resulting in changes in the arrangement rather than the composition of the C. albicans cell wall. Finally an inhibition of cell respiration could explain the reduction in

growth rate observed in ketoconazole-treated hyphae and blastospores of C. albicans and the finding that the effects of ketoconazole were not restricted to the hyphal form of the fungus.

The results presented in this study, using carefully standardized fungal biomass and an objective radiolabel assay for the assessment of phagocytosis and intracellular killing suggest that filamentous forms of C. albicans, although phagocytosed to the same extent as blastospores, may in fact be killed more readily than the blastospore form. The significance of germ tube and hypha production in relation to resistance to phagocytosis and intracellular killing by human PMNs is therefore put in doubt. The lack of correlation between germ tube production inside PMNs and the initial resistance to intracellular killing suggests that factors other than germ tube production may be important in this respect.

Inducible proteinases have previously been demonstrated in C. albicans (Staib, 1965) and have been extensively characterized (Macdonald & Odds, 1980; Ruchel, 1981). Recently Macdonald & Odds (1982) found that blastospores of a proteinase-producing isolate of C. albicans and of a proteinase deficient mutant differed in their resistance to phagocytosis and intracellular killing by human PMNs. Blastospores of both isolates were phagocytosed and killed to a similar extent when both were grown in SAB broth. However, when grown on a proteinase-inducing medium blastospores of the proteinase producing parent isolate were phagocytosed and killed significantly less well than those of the proteinase-deficient isolate. The possibility that proteinase could be produced inside PMNs, resulting in a reduction in intracellular killing should also therefore be considered since isolate 73/055 also produces an inducible proteinase (F.C.Odds personal communication).

It is also possible that blastospores of C. albicans may stimulate a less vigorous response of the enzyme systems involved in the intracellular killing of ingested organisms than filamentous forms. Sasada & Johnston 1980 reported that C. parapsilosis stimulated a stronger metabolic burst in mouse macrophages than C. albicans and was therefore killed more

effectively. Similar variations in the intracellular responses towards different morphological forms of C. albicans could explain the results of the present study.

The possibility that resistance of individual blastospores to intracellular killing may vary within a population is supported by the observation of Richardson & Smith (1981). These workers reported heterogeneity in relation to resistance to intracellular killing of C. albicans blastospores by mouse PMNs. Progeny of surviving blastospores were significantly less well phagocytosed and killed than blastospores of the parent isolate. Heterogeneity within a population could be significant since blastospores that were able to escape intracellular killing would presumably be able to persist in the host tissues for longer periods of time. The possibility that germ tubes and hyphae produced by these blastospores could also differ from the parent isolate in their resistance to intracellular killing should also be considered.

Although the results of experiments in the present study suggest that germ tube and hypha production may not be significant in relation to the resistance to intracellular killing, production of filamentous forms may still be significant in other aspects of candidosis.

The increased surface area per cell unit presented by germ tubes and hyphae may be important in relation to the attachment of filamentous forms to membrane surfaces. In addition the ability of hyphae to form clumps and possibly to cause emboli in vivo may be significant. It is also possible that on membrane surfaces where C. albicans may to some extent be out of the reach of host defence mechanisms such as phagocytic cells, germ tube and hypha production may be more significant than in systemic infection where contact between the fungus and phagocytic cells may be more frequent. The observation in the present study, that germ tubes of isolate 73/055 were less chemotactic for human PMNs than blastospores and hyphae may also be relevant if such chemotactic responses occur in tissues in vivo.

Further study of the significance of dimorphism, and other virulence factors in relation to the pathogenesis of candidosis could again be facilitated by the use of C. albicans mutants. Comparison of parent and germ tube-negative mutants of C. albicans in phagocytosis and killing and attachment assays and in relation to virulence for laboratory animals when administered systemically and locally could provide useful information. Mutants unable to produce germ tubes and/or proteinase could similarly indicate the relative importance of these two virulence factors in the establishment and maintenance of C. albicans in host tissues.

Study of the mechanisms by which different morphological forms of C. albicans are killed by PMNs could be extended by investigation of the individual steps involved in these processes. Morphology related differences in the attachment of PMNs to C. albicans, the engulfment of the fungus and of the fusion of phagolysosomes with ingested fungal cells could explain the results of the present study. Comparison of the results of such studies with C. albicans with those of the other pathogenic Candida species may indicate differences related to their relative virulence.

It should also be remembered that PMNs represent only a single aspect of the host tissue response towards C. albicans and that the responses observed in vivo may be more complex than those tested in vitro. T-lymphocytes are known to produce a range of lymphokines when exposed to antigen and these compounds may be involved in controlling and eliminating pathogenic micro-organisms such as C. albicans. Differences in the response of T-lymphocytes to different morphological forms of C. albicans may, therefore, be important in the initial response to infection and in subsequent control. In addition, although macrophages were shown in the present and earlier studies to be less efficient than PMNs in relation to the phagocytosis and killing of C. albicans these phagocytic cells may also be important in the processing of antigens before exposure to lymphocytes and differences in the responses of macrophages to different morphological forms of C. albicans may be significant in this respect and should be investigated further.

Investigation of the possibility that individual blastospores within populations differed in their resistance to intracellular killing by classical cloning of individual blastospores for use in such assays would also be worthwhile. If differences were apparent between such clones it could be possible to produce new isolates of C. albicans by combining clones of different properties in varying proportions. Variation in the behaviour of different C. albicans isolates in relation to properties such as virulence could also be investigated in this way.

The phagocytosis and killing assays performed in the present study used C. albicans suspensions of one morphological form. In infected tissues in vivo a combination of morphological forms may be present. It is conceivable that the response of phagocytic cells towards a mixture of C. albicans of differing morphologies and ages may differ from that towards monomorphic cultures. Phagocytosis and killing assays performed with combinations of different forms of C. albicans of different ages may indicate if this could be the case.

Bibliography.

1. Arai, T., Mikami, Y. and Yokoyama, K. 1977. Phagocytosis of Candida albicans by rabbit alveolar macrophages. Sabouraudia. 15, 171-177.

2. Archer, D.B. 1977. Chitin biosynthesis in protoplasts and subcellular fractions of Aspergillus fumigatus. Biochem. J. 164, 653-658.

3. Arnold, W.N. 1973. Volume and enzyme content of the periplasmic space in yeast. Physiol. Chem. & Physics. 5, 117-123.

4. Audrey, C. 1877. Sur l'évolution du champignon du muguet. Rev. Med. 7, 586-595.

5. Bacon, J.S.D., Davidson, E.D., Jones, D. and Taylor, I.F. 1966. The location of chitin in the yeast cell wall. Biochem. J. 101, 36c.

6. Bacon, J.S.D., Farner, V.C., Jones, D. and Taylor, I.F. 1969. The glucan components of the cell wall of bakers yeast (Saccharomyces cerevisiae) considered in relation to its ultrastructure. Biochem. J. 114, 557-567.

7. Balint, S., Farkas, V. and Bauer, S. 1976. Biosynthesis of β-glucans catalysed by a particulate enzyme preparation from yeast. FEBS Lett. 64, 44-47.

8. Balish, E. and Philips, A.W. 1966. Growth, morphogenesis and virulence of Candida albicans after oral inoculation in the germ free and conventional chick. J. Bacteriol. 91, 1736-1743.

9. Ballman, G.E. and Chaffin, La Jean. W. 1979. Lipid synthesis during reinitiation of growth from stationary phase cultures of Candida albicans. Mycopathologia. 67,39-43.

10. Bell, W.M. and Chaffin, La Jean, W. 1981. Nutrient limited yeast growth in Candida albicans: effect on yeast-mycelial transition. Can. J. Microbiol. 26, 102-105.

11. Bennet, J.H. 1844. On the parasitic vegetable structures found growing in living animals. Trans. R. Soc. Edinburgh. 15, 277-294.

12. Beran, K. and Rehacek, J. 1969. Content and structure of chitin in cell walls of the yeast Saccharomyces cerevisiae. Antonie Van Leeuwenhoek. 35, Suppl. B3-B4.

13. Berg, F.T. 1846. Om Torsk hos Barn. Stockholm: L.J. Hjerta.

14. Biely, P. Kovarik, J. and Bauer, S. 1973. Lysis of Saccharomyces cerevisiae with 2-deoxy-2-fluoro-D-glucose, an inhibitor of the cell wall glucan synthesis. J. Bacteriol. 115, 1108-1120.

15. Biely, P., Kovarik, J. and Bauer, S. 1973a. Cell wall formation in yeast. An electron microscopic autoradiographic study. Arch. Mikrobiol. 94,365-367.

16. Bish, J.T. and Sarachek, A. 1067. Influences of temperature and adenine concentration upon the cultural instability of a red adenine auxotroph of C.albicans. Mycologia. 59, 671-688.

17. Bishop, C.T., Blank, F. and Gardner, P.E. 1960. The cell wall polysaccharides of Candida albicans; glucan,mannan and chitin. Can. J. Chem. 38, 869-881.

18. Borgers, M. and DeNollin, S. 1974. The preservation of subcellular organelles of Candida albicans with conventional fixatives. J. Cell. Biol. 62, 574-581.

19. Borgers, M. Van den Bossche, H. De Brabender, M. and Van Cutsem, J.M. 1979. Promotion of pseudomycelium formation of Candida albicans in culture: a morphological study of the effects of miconazole and ketoconazole. Postgrad. Med. J. 55, 687-691.

20. Braun, P.C. and Calderone, R.A. 1978. Chitin synthesis in Candida albicans: comparison of yeast and hyphal forms. J.Bacteriol. 135, 1472-1477.

21. Braun, P.C. and Calderone, R.A. 1979. Regulation and solubilisation of Candida albicans chitin synthetase. J. Bacteriol. 140, 666-670.

22. Bridges, C.G., Dasilva, G.L., Yamamura, M. and Valdimarsson, H. 1980. A radiometric assay for the combined measurement of phagocytosis and intracellular killing of Candida albicans. Clin. Exp. Immunol. 42, 226-233.

23. Brown, L.A. and Chaffin, La Jean, W. 1981. Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in Candida albicans. Can. J. Microbiol. 27, 580-585.

24. Budtz-Jorgensen, E. 1971. Proteolytic activity of Candida spp. as related to the pathogenèsis of denture stomatitis. Sabouraudia. 12, 266-271.

25. Budtz-Jorgensen, E., Stenderup, A. and Grabowski, M. 1975. An epidemiologic study of yeasts in elderly denture wearers. Comm. Dent. Oral. Epidemiol. 3, 115-119.

26. Cabib, E. 1975. Molecular aspects of yeast morphogenesis. Ann. Rev. Microbiol. 29, 191-214.

27. Cabib, E. and Farkas, V. 1971. The control of morphogenesis. An enzymatic mechanism for the initiation of septum formation in yeast. Proc. Nat. Acad. Sci. USA. 68, 2052-2056.

28. Cassone, A. 1973. Improved visualisation of wall ultrastructure in Saccharomyces cerevisiae. Experientia, 29, 1303-1305.

29. Cassone, A., Kerridge, D. and Gale, E.F. 1979. Ultrastructural changes in the cell wall of Candida albicans following cessation of growth and their possible relationship to the development of polyene resistance. J. Gen. Microbiol. 110, 339-349.

30. Cassone, A., Mattia, E. and Boldrini, L. 1978. Agglutination of blastospores of Candida albicans by Concanavalin A and its relationship with the distribution of mannan polymers and the ultrastructure of the cell wall. J. Gen. Microbiol. 105, 263-273.

31. Cassone, A., Simonetti, N. and Strippoli, V. 1973. Ultrastructural changes in the wall during germ tube formation from blastospores of Candida albicans. J. Gen. Microbiol. 77, 417-426.

32. Chaffin, La Jean, W. and Sogin, S.J. 1976. Germ tube formation from zonal rotor fractions of C.albicans. J. Bacteriol. 126, 771-776.

33. Chaffin, La Jean. W. and Wheeler, D.E. 1981. Morphological committment in Candida albicans. Can. J. Microbiol. 27,131-137.

34. Chattaway, F.W., Bishop, R., Holmes, M.R., Odds, F.C. and Barlow, A.J.E. 1973. Enzyme activities associated with carbohydrate synthesis and breakdown in the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 75, 97-109.

35. Chattaway, F.W., Holmes, M.R..and Barlow, A.J.E. 1968. Cell wall composition of the mycelial and blastospore forms of Candida albicans. J. Gen. Microbiol. 51, 367-376.

36. Chattaway, F.W., Odds, F.C. and Barlow, A.J.E. 1971. An examination of the production of hydrolytic enzymes and toxins by the pathogenic strains of Candida albicans. J. Gen. Microbiol. 67, 255-263.

37. Chattaway, F.W., O'Reilly, J., Barlow, A.J.C. and Aldersley, T. 1976. Induction of the mycelial form of Candida albicans by hydrolysates of peptides from seminal plasma. J. Gen. Microbiol. 96, 317-322.

38. Chattaway, F.W., Shenolikar, S. and Barlow, A.J.E. 1974. Release of acid phosphatase and polysaccharide and protein containing components from the surface of dimorphic forms of Candida albicans by treatment with dithiothreitol. J.Gen. Microbiol. 83, 423-436.

39. Chattaway, F.W., Shenolikar, S., O'Reilly, J. and Barlow, A.J.E. 1976a. Changes in the cell surface of the dimorphic forms of Candida albicans by treatment with hydrolytic enzymes. J.Gen Microbiol. 95, 335-347.

40. Chattaway, F.W., Wheeler, P.R. and O'Reilly, J. 1981. Involvement of adenosine 3':5' cyclic monophosphate in the germination of blastospores of Candida albicans. J. Gen. Microbiol. 123, 233-240.

41. Chiew, Y.Y., Shepherd, M.G. and Sullivan, P.A. 1980. Regulation of chitin synthesis during germ tube formation in Candida albicans. Arch. Microbiol. 125, 97.

42. Chiew, Y.Y., Sullivan, P.A. and Shepherd, M.G. 1980. Chitin synthesis during germ tube formation in Candida albicans. Proc. Univ. Otago. Med. School. 58, 31-32.

43. Chiew, Y.Y., Sullivan, P.A. and Shepherd, M.G. 1982. The effects of ergosterol and alchols on the germ tube formation and chitin synthase in Candida albicans. Can. J. Biochem. 60, 15-20.

44. Chilgren, R.A., Hong, R. and Quie, P.G. 1968. Human serum interactions with Candida albicans. J.Immunol. 101, 128-132.

45. Costa, A.L., Costa, G., Misefari, A. and Amato, A. 1968. Sull' attiva enzymatica di taluni miceti. Atta societa peloritana di scienze fisiche matematiche naturali. 14, 93-101.

46. Cutler, J.E. 1977. Chemotactic factor produced by Candida albicans. Infect. Immun. 18, 568-573.

47. Cutler, J.E., Friedman, L. and Milner, K.C. 1972. Biological and chemical characterization of toxic substances from Candida albicans. Infect. Immun. 6, 616-627.

48. Cutler, J.E. and Poor, A.H. 1981. Effect of mouse phagocytes on Candida albicans in in vivo chambers. Infect. Immun. 31, 1110-1116.

49. Dabrowa, N., Howard, D.H., Landau, J.W. and Shechter, Y. 1970. Synthesis of nucleic acids and proteins in the dimorphic forms of Candida albicans. Sabouraudia. 8, 163-169.

50. Davies, R.R. and Denning, T.J.V. 1972. Candida albicans and the fungicidal activity of the blood. Sabouraudia. 10, 301-312.

51. De Brabander, M., Aerts, F., Van Cutsem, J., Van den Bossche, H. and Borgers, M. 1980. The activity of ketoconazole in mixed cultures of leukocytes and C.albicans. Sabouraudia. 18, 197-210.

52. De Luca, M.A. 1978. Methods in Enzymology Vol LVII. Academic Press, New York.

53. De Nollin, S. and Borgers, M. 1974. The ultrastructural morphology of Candida albicans after in vitro treatment with miconazole. Sabouraudia. 12, 341-351.

54. Denning, T.J.V. and Davies, R.R. 1973. Candida albicans and the chemotaxis of polymorphonuclear neutrophils. Sabouraudia. 11, 210-221.

55. Diamond, R.D., Krzesicki, R. and Jao, W. 1978. Damage to pseudohyphal forms of Candida albicans by neutrophils in the abscence of serum in vitro. J. Clin. Invest. 61, 349-359.

56. Diamond, R.D., Oppenheim, F., Nakagawa, Y., Krzesicki, R. and Haudenschild, C.C. 1980. Properties of a product of C.albicans hyphae and pseudohyphae that inhibits contact between the fungi and human neutrophils in vitro. J. Immunol. 125, 2797-2804.

57. Domanski, R.E. and Miller, R.E. 1968. Use of a chitinase complex and --(1,3)-glucanase for sphaeroplast production from Candida albicans. J. Bacteriol. 96, 270-271.

58. Evans, E.G.V., Odds, F.C. and Holland, K.T. 1975. Resistance of the Candida albicans filamentous cycle to environmental change. Sabouraudia, 13, 231-238.

59. Evans, E.G.V., Odds, F.C., Richardson, M.D. and Holland, K.T. 1975. Optimum conditions for initiation of filamentation in Candida albicans. Can. J. Microbiol. 21,338-342.

60. Evans, E.G.V., Richardson, M.D., Odds, F.C. and Holland, K.T. 1973. Relevance of antigenicity of Candida albicans growth phases to diagnosis of systemic candidosis. Br. Med. J. 4, 86-87.

61. Evans, Z.A. 1981. Tissue responses to the blastospores and hyphae of Candida albicans in the mouse. J. Med. Microbiol. 14, 307-319.

62. Evron, R. 1980. In vitro phagocytosis of Candida albicans by peritoneal mouse macrophages. Infect. Immun. 28, 963-971.

63. Farkas, V. 1979. Biosynthesis of cell walls of fungi. Microbiol. Rev. 43, 117-144.

64. Farkas, V., Kovarik, J., Kosinova, A. and Bauer, S. 1974. Autoradiographic study of mannan incorporation into the growing cell walls of Saccharomyces cerevisiae. J. Bacteriol. 117, 265-269.

65. Ferrante, A. and Thong, Y.H. 1979. Requirement of heat labile opsonins for maximal phagocytosis of Candida albicans. Sabouraudia. 17, 293-297.

66. Fleet, G.H. and Manners, D.J. 1976. Isolation and composition of an alkali soluble glucan from the cell walls of Saccharomyces cerevisiae. J. Gen. Microbiol. 94, 180-192.

67. Gentles, J.C. and La Touche, C.J. 1969. Yeast as human and animal pathogens. In: A.H. Rose and J.S. Harrison (Eds.), The Yeasts.Vol 1. 108-182. London: Academic Press.

68. Hammond, S.M. and Kliger, B.N. 1974. Polyene resistance in Candida albicans: a proposed nutritional influence. Microbios. 10, 97-101.

69. Harrington, C.R. and Douglas, J.L. 1980. Inhibition and activation of mannan synthesis in Saccharomyces cerevisiae spheroplast lysates. J. Bacteriol. 142, 852-858.

70. Hasilak, A. 1974. Inactivation of chitin synthase in Saccharomyces cerevisiae Arch. Microbiol. 101, 295-301.

71. Hauser, F. and Rothman, S. 1950. Monilial granuloma: report of a case and review of the literature. Arch. Dermatol. Syphilogr. 61, 297-310.

72. Hazen, C.K. and Cutler, J.E. 1979. Autoregulation of germ tube formation by Candida albicans. Infect. Immun. 24, 661-666.

73. Henrici, A.T. 1940. Characteristics of fungous disease. J. Bacteriol. 39, 113-138.

74. Herbert, D., Phipps, P.J. and Strange, R.E. 1971. Chemical analysis of microbial cells. In: J.R. Norris and D.W. Ribbons. Methods in Microbiology. Vol. 5B. 209-344.

75. Hippocrates. Circa 460-377 BC. Epidemics, book 3. Translated by F. Adams, 1939. Baltimore: Williams and Wilkins.

76. Holder, I.A. and Nathan, P. 1973. Effect in mice of injection of viable Candida albicans and a cell free sonic extract, on circulatory platelets. Infect. Immunol. 7, 468-472.

77. Holt, P.J., Higgs, J.M., Munro, J and Valdimarsson, H. 1972. Chronic mucocutaneous candidiasis: a model for the investigation of cell mediated immunity. Br. J. Clin. Pract. 26, 331-336.

78. Horisberger, M. and Vonlanthen, M. 1977. Localization of mannan and chitin on thin sections of budding yeasts with gold markers. Arch. Microbiol. 115, 1-7.

79. Hurley, J.V. 1963. Incubation of serum with tissue extracts as a cause of chemotaxis of granulocytes. Nature. London. 198, 1212-1213.

80. Hurley, R. and Stanley, V.C. 1969. Cytopathic effects of pathogenic and non pathogenic species of Candida on cultured mouse epithelial cells: relation to the growth rate and morphology of the fungi. J. Med. Microbiol. 2, 63-74.

81. Isenberg, H.D., Allerhand, J., Berkman, J.I. and Goldberg, D. 1963. Immunological and toxic differences between mouse virulent and mouse avirulent Candida albicans. J. Bacteriol. 86,1010-1018.

82. Iwata, K. and Uchida, K. 1969. Canditoxin, a new toxic substance from a strain of Candida albicans. Antonie.Van Leeuwenhoek. 35, Suppl. E39-40.

83. Johnson, S.A.M., Guzmon, M.G., and Agurlera, C.T. 1954. Candida (Monilia) albicans. Effect of amino acids, glucose, pH, chlotetracycline (auteomycin), dibasic sodium and calcium phosphates and anaerobic and aerobic conditions on its growth. Arch. Dermatol. 70, 49-60.

84. Jones, J.H, and Russel, C. 1974. The history of chronic candidal infection of the rats tongue. J. Pathol. 113, 97-100.

85. Joshi, K.R., Gavin, J.B. and Wheeler, E.E. 1973. A scanning EM study of the morphogenesis of Candida albicans in vitro. Sabouraudia. 11,263-266.

86. Kagaya, K. and Fukazawa, Y. 1981. Murine defense mechanism against Candida albicans infection. II. Opsonisation, phagocytosis and intracellular killing of C.albicans. Microbiol. Immunol. 25, 807-818.

87. Kagaya, K., Shinoda, T. and Fukazawa, Y. 1981x. Murine defense mechanism against Candida albicans infection. I. Collaboration of cell-mediated and humoral immunities in protection against systemic C.albicans infection. Microbiol. Immunol. 25, 647-654.

88. Kerridge, D., Koh, T.Y., Marriott, M.S. and Gale, E.F. 1976. In:(Eds.) J.F. Peberdy, A.H. Rose, H.J. Rogers and E.C. Cocking. Microbial and plant protoplasts. 23-38. London: Academic Press.

89. Kessler, G. and Nickerson, W.J. 1959. Glucomannan - protein complexes from cell walls of yeasts. J. Biol. Chem. 234, 2281-2285.

90. Kidby, D.K. and Davies, R. 1970. Invertase and disulphide bridges in the yeast cell wall. J. Gen. Microbiol. 61, 327-333.

91. Kimura, L.H. and Pearsall, N.N. 1978. Adherence of Candida albicans to the human buccal epithelial cells. Infect. Immun. 21,64-68.

92. Kimura, L.H. and Pearsall, N.N. 1980. Relationship between germination of Candida albicans and increased adherence to human buccal epithelial cells. Infect. Immun. 21, 464-468.

93. King, R.D., Lee, J.C. and Morris, A.L. 1980. Adherence of C.albicans and other Candida species to mucosal epithelial cells. Infect. Immun. 27, 667-675.

94. Klebanoff, S.S. and Clark, R.A. 1978. The neutrophil: Function and clinical disorders. Elsevier/North Holland. Amsterdam.

95. Kosinova, A., Farkas, V., Machala, S. and Bauer, S. 1974. Site of mannan Synthesis in yeast. An autoradiographic study. Arch. Microbiol. 99, 255-263.

96. Kozinn, P.J. and Taschdjian, C.L. 1962. Enteric candidiasis. Diagnosis and clinical considerations. Pediatrics. 30, 71-85.

97. Kozinn, P.J. and Taschdjian, C.L. 1966. Candida albicans: saprophyte or pathogen? A diagnostic guideline. J. Am. Med. Assoc. 198, 170-172.

98. Kreger, D.R. and Kopecka, M. 1973. In: Yeast, mould and plant protoplasts (Eds.) J.R. Villaneura, J.R. Garcia-Acha, S. Gascon and F. Uruburu. 117-130. London: Academic Press.

99. Kurup, P.V. 1963. Histochemical demonstration of alkaline phosphatase in Candida. Curr. Sci. 32, 408-409.

100. Kwon-Chung, K.H. and Hill, W.B. 1978. Studies on the pink, adenine deficient strains of Candida albicans. I. Cultural and morphological characteristics. Sabouraudia. 8, 48-59.

101. Lampen, J.O. 1968. External enzymes of yeast: their nature and formation. Antonie. Van. Leewenhoek. 34, 1-18.

102. Land, G.A., Mcdonald, W.C., Stjernholm, R.L. and Friedman, L. 1975a. Factors affecting filamentation in Candida albicans: relationship of the uptake and distribution of proline to morphogenesis. Infect. Immun. 11, 1014-1023.

103. Land, G.A., Mcdonald, W.C., Stjernholm, R.L. and Friedman, L. 1975a. Factors affecting filamentation in Candida albicans: changes in respiratory activity of Candida albicans during filamentation. Infect. Immun. 12, 119-127.

104. Landau, J.W., Dabrowa, N. and Newcomer, V.D. 1965. The rapid formation in serum of filaments by Candida albicans. J. Invest. Dermatol. 44, 171-179.

105. Langenbeck, B. 1839. Auffingung von Pilzen aus der Schleimhaut der Speiserohre einer Typhus-Leiche. NeueNot. Geb. Natur-u-Heilk(Froriep). 12, 145-147.

106. Larriba, G., Morales, M. and Ruiz-Herrera, J. 1981. Biosynthesis of -- glucan microfibrils by cell-free extracts from Saccharomyces cerevisiae. J. Gen. Microbiol. 124, 375-383.

107. Lee, K.W. and Balish, E. 1981. Resistance of germ-free mice to systemic candidosis. J. Reticuloendothel. Soc. 29, 241-248.

108. Lee, K.L., Buckley, H.R. and Campbell, C.C. 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of Candida albicans. Sabouraudia. 13, 148-153.

109. Lee, K.L., Reca, M.E., Watson, R.R. and Campbell, C.C. 1975a. Identification of yeast phase of pathogenic fungi by the specificity of their aminopeptidases. Sabouraudia. 13, 132-141.

110. Lehrer, R.I. 1969. Antifungal effects of peroxidase systems. J. Bacteriol. 99, 361-365.

111. Lehrer, R.I. 1972. Functional aspects of a second mechanism of candidicidal activity by human neutrophils. J. Clin. Invest. 51, 2566-2572.

112. Lehrer, R.I. 1975. The fungicidal mechanisms of human monocytes. 1. Evidence for myeloperoxidase independant candidicidal mechanisms. J. Clin. Invest. 55, 338-346.

113. Lehrer, R.I. and Cline, M.J. 1969a. Interaction of Candida albicans with human leukocytes and serum. J. Bacteriol. 98, 996-1004.

114. Lehrer, R.I. and Cline, M.J. 1969b. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to Candida infection. J. Clin. Invest. 48, 1478-1488.

115. Lehrer, R.I., Ferrari, L.G., Patterson-Delafield, J. and Sorrell, T. 1980. Fungicidal activity of rabbit alveolar and peritoneal macrophages against Candida albicans. Infect. Immun. 28, 1001-1008.

116. Linnemans, W.A.M., Boer, P. and Elbers, P.F. 1977. Localization of acid phosphatase in Saccharomyces cerevisiae: a clue to cell wall formation. J. Bacteriol. 131, 638-644.

117. Lopez-Romero, E. and Ruiz-Herrera, J. 1977. Biosynthesis of β-glucans by cell free extracts from Saccharomyces cerevisiae. Biochem. Biophys. Acta. 500, 372-384.

118. Louria, D.B. and Brayton, R.G. 1964. Behaviour of Candida cells within leukocytes. Proc. Soc. Exp. Biol. Med. 115, 93-98.

119. Lowry, O.H., Rosenborough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein estimation with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

120. Macdonald, F. 1979. Inducible hydrolases in the pathogenesis and serodiagnosis of systemic candidosis. Ph.D. Thesis. University of Leicester.

121. Macdonald, F. and Odds, F.C. 1980. Inducible proteinase of Candida albicans in diagnostic serology and in the pathogenesis of systemic candidosis. J. Med. Microbiol. 13, 423-435.

122. Macdonald, F. and Odds, F.C. 1982. Virulence for mice of a proteinase-secreting strain of Candida albicans and a proteinase deficient mutant. J. Gen. Microbiol. In Press.

123. Mackenzie, D.W.R. 1964. Serum tube identification of Candida albicans. J. Clin. Pathol. 15, 563-565.

124. Mackenzie, D.W.R. 1964. Morphogenesis of Candida albicans in vivo. Sabouraudia. 3, 225-232.

125. Maisch, P.A. and Calderone, R.A. 1980. Adherence of Candida albicans to a fibrin-platelet matrix formed in vitro. Infect. Immun. 27, 650-656.

126. Maisch, P.A. and Calderone, R.A. 1981. Role of surface mannan in the adherence of Candida albicans to fibrin-platelet clots formed in vitro. Infect.Immun. 32, 92-97.

127. Maiti, P.K. 1980. Candidicidal activity of mouse macrophages in vitro. Infect. Immun. 27, 477-

128. Manning, M. and Mitchell, T.G. 1980..Strain variation and morphogenesis of yeast and mycelial phase Candida albicans in low-sulphate,,synthetic medium. J. Bacteriol. 142, 714-719.

129. Manning, M. and Mitchell, T.C. 1980x. Morphogenesis of Candida albicans and cytoplasmic proteins associated with differences in morphology, strain and temperature. J. Bacteriol. 144, 258-273.

130. Mardon, D.N., Balish, E. and Philips, A.W. 1969. Control of dimorphism in a biochemical variant of Candida albicans. J. Bacteriol. 100, 701-707.

131. Mardon, D.N., Gunn, J.L. and Robinette, E. 1975. Variation in the lethal response in mice to yeast like and pseudomycelial forms of Candida albicans. Can. J. Microbiol. 21, 1681-1687.

132. Marriott, M.S. 1975. Enzymatic activity of purified plasma membranes from the yeast and mycelial forms of Candida albicans. J.Gen. Microbiol. 89, 345-352

133. Marriott, M.S. 1975x. Isolation and chemical characterization of plasma membranes from the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 86, 115-132.

134. Marriott, M.S. 1977. Mannan protein location and biosynthesis in plasma membranes from the yeast form of Candida albicans. J. Gen. Microbiol. 103, 51-59.

135. Marriott, M.S. 1980. Inhibition of sterol biosynthesis in Candida albicans by imidazole containing- antifungals. J. Gen. Microbiol. 117, 253-255.

136. Martin, M.V. 1979. Germ tube formation by oral strains of Candida tropicalis. J. Med. Microbiol. 12, 187-193.

137. Mattia, E. and Cassone, A. 1979. Inducibility of germ tube formation in Candida albicans at different phases of yeast growth. J. Gen. Microbiol. 113, 439-442.

138. McCourtie, J. and Douglas, J.L. 1981. Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infect. Immun. 32, 1234-1241.

139. McMurrough, I. and Rose, A.H. 1967. Effect of growth rate and substrate limitation on the composition and structure of the cell wall of Saccharomyces cerevisiae. Biochem. J. 105, 189-203.

140. Mims, C.A. 1982. The pathogenesis of infectious disease. London: Academic Press.

141. Mitchell, L.H. and Soll, D.R. 1979. Committment to germ tube or bud formation during release from stationary phase in Candida albicans. Exp. Cell. Res. 120, 167-179.

142. Mitchison, J.M. 1971. The biology of the cell cycle. Cambridge University Press.

143. Montes, L.F. and Wilborn, W.H. 1970. Acid phosphatase activity during phases of growth in Candida albicans. Int. J. Dermatol. 9, 220-225.

144. Morrison, R.P. and Cutler, J.E. 1981. In vitro studies of the interaction of murine phagocytic cells with Candida albicans. J. Reticulo. Endothel. Soc. 29, 23-34.

145. Mundkur, B. 1960. Electron microscopical studies of frozen-dried yeast. Exp. Cell. Res. 20, 28-42.

146. Necas, O. 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35, 149-170.

147. Nickerson, W.J. 1948. Enzymatic control of cell division in micro-organisims. Nature. 162, 241-245.

148. Nickerson, W.J. 1949. Mechanism of cell division in pathogenic fungi. Am. J. Bot. 36, 812.

149. Nickerson, W.J. 1951. Physiological basis of morphogenesis in animal disease fungi. Trans. N.Y. Acad. Sci. 13, 140-145.

150. Nickerson, W.J. 1963. Symposium on biochemical basis of morphogenesis in fungi. Bacteriol. Rev. 27, 305-325.

151. Nickerson, W.J. and Falcone, G. Identification of the protein disulphide reductase as a cellular division enzyme in yeasts. Science. 124, 722-723.

152. Niimi, M., Niimi, K., Tokunaga, J. and Nakayama, H. 1980. Changes in the cyclic nucleotide levels and dimorphic transition in C.albicans. J. Bacteriol. 142, 1010-1014.

153. Northcote, D.H. and Horne, R.W. 1952. The chemical composition and structure of the yeast cell wall. Biochem. J. 51, 232-236.

154. Odds, F.C. 1979. Candida and candidosis. Leicester University Press.

155. Odds, F.C. 1980. Laboratory evaluation of antifungal agents: a comparative study of the imidazole derivatives of clinical importance. J. Ant. Chemo. 6, 749-761.

156. Odds, F.C. 1981. The pathogenesis of candidosis. Hospital Update. 7, 935-945.

157. Odds, F.C., Evans, E.G.V., and Holland, K.T. 1975. Detection of Candida precipitins. A comparison of double diffusion and counter immuno-electrophoresis. J. Immunol. Meth. 7, 211-218.

158. Odds, F.C. and Hierholzer, J.C. 1973. Purification and properties of a glycoprotein acid phosphatase from Candida albicans. J. Bacteriol. 114, 257-266.

159. Odds, F.C. and Trujillo-Gonzales, A. 1974. Acid phosphatase levels in the genus Candida and their application to the taxonomy and identification of pathogenic Candida species. Sabouraudia. 12, 287-294.

160. Olaiya, A.F. and Sogin, S.J. 1979. Ploidy determination of Candida albicans. J. Bacteriol. 140. 1043-1049.

161. Partridge, B.M., Athor, M.A. and Winner, H.I. 1971. Chick embryo inoculation as a pathogenicity test for Candida species. J. Clin. Pathol. 24, 645-648.

162. Pepys, S. 1665. The diaries of Samuel Pepys. (Eds.) R. Latham and W. Mathews. Bell: London.

163. Pesti, M., Peberdy, M.C.A. and Campbell, J.F. 1981. Alteration of ergosterol content and chitin synthase activity in Candida albicans. Curr. Microbiol. 5, 187-190.

164. Peterson, R.M. and Calderone, R.A. 1978. Inhibition of specific amino acid uptake in Candida albicans by lysosomal extracts from rabbit alveolar macrophages. Infect. Immun. 21, 506-513.

165. Phaff, H.J. 1971. Structure and biosynthesis of the yeast cell envelope. In: (Eds.) A.H. Rose. and J.S. Harrison. The Yeasts. 2, 135-210. London: Academic Press.

166. Pluss, J.M. and Kadas, L. 1954. A case of monilial granulomas associated with widespread moniliasis and epidermophytosis. J. Invest. Dermatol. 23, 317-328.

167. Poulain, D., Tronchin, J., Dubremetz, J.F. and Biguet, J. 1978. Ultrastructure of the cell wall of Candida albicans blastospores: study of the constitutive layers by the use of a cytochemical technique revealing polysaccharides. Ann. Microbiol. 129, 141-151.

168. Pugh, D. and Cawson, R.A. 1975. The cytochemical localization of phospholipase A and lysophospholipase in Candida albicans. Sabouraudia. 13, 110-115.

169. Ray, T.L. and Wuepper, K.D. 1976. Experimental cutaneous candidiasis in rodents. J. Invest. Dermatol. 66, 29-33.

170. Ray, T.L., Hanson, A., Ray, L.F. and Wuepper, K.D. 1979. Purification of a mannan from Candida albicans which activates serum complement. J. Invest. Dermatol. 73, 269-274.

171. Rebora, A., Marples, R.M. and Kligman, A.M. 1973. Experimental infection with Candida albicans. Arch. Dermatol. 108, 69-73.

172. Reiss, E., Stone, S.H. and Hasenclever, H.F. 1974. Serological and cellular immune activity of peptidoglucomannan fractions of Candida albicans cell walls. Infect. Immun. 9, 881-890.

173. Remold, H., Fasold, H. and Staib, F. 1968. Purification and characterization of a proteolytic enzyme from Candida albicans. Biochim. Biophys. Acta. 167, 399-406.

174. Richardson, M.D. and Smith, H. 1981. Resistance of virulent and attenuated strains of Candida albicans to intracellular killing by human and mouse phagocytes. J. Inf. Dis. 144, 557-564.

175. Richardson, M.D. and Smith, H. 1981b. Production of germ tubes by virulent and attenuated strains of Candida albicans. J. Inf. Dis. 144, 565-569.

176. Rippon, J.W. 1974. In: Medical Mycology. The pathogenic fungi and the pathogenic Actinomycetes. London: W.B. Saunders Co.

177. Rogers, K.B. 1966. Candida infections in paediatrics. In: (Eds.). H.I. Winner and R. Hurley. Symposium on Candida infections. 179-195. London: Livingstone.

178. Rogers, T. and Balish, E. 1976. Experimental Candida albicans infection in conventional mice and germ-free rats. Infect. Immun. 14, 33-38.

179. Roitt, R.M. 1974. Essential Immunology. Second edition. Blackwell Scientific Publications: Oxford.

180. Roux, G. and Linnosier, G. 1890. Recherches morphologiques sur le champignon du muguet. Arch. Med. Exp. Anat. Pathol. 2, 62-87.

181. Rüchel, R. 1981. Properties of a purified proteinase from the yeast Candida albicans. Biochim. Biophys. Acta. 659, 99-113.

182. Salvin, S.B. 1952. Endotoxin in pathogenic fungi. J. Immunol. 69, 89-99.

183. Sandin, R.L., Rogers, A.L., Patterson, R.J. and Benecke, E.S. 1982. Evidence for mannose-mediated adherance of Candida albicans to human buccal cells in vitro. Infect. Immun. 35, 79-85.

184. Sasada, M. and Johnston, R.B. 1980. Macrophage microbiocidal activity: correlation between phagocytosis-associated oxidative metabolism and the killing of Candida by macrophages. J. Exp. Med. 152, 85-98.

185. Scherwitz, C. and Martin, R. 1979. The phagocytosis of Candida albicans blastospores and germ tubes by polymorphonuclear leukocytes. Dermatologica. 159, 12-23.

186. Scherwitz, C., Martin, R. and Ueberberg, H. 1978. Ultrastructural investigations of the formation of Candida albicans germ tubes and septa. Sabouraudia. 16, 115-124.

187. Schwartz, D.S. and Larsh, H.W. 1980. An effective medium for the selective growth of yeast or mycelial forms of Candida albicans: Biochemical aspects of the two forms. Mycopathologia. 70, 67-75.

188. Shepherd, M.G. and Sullivan, P.A. 1976. The production and growth characteristics of yeast and mycelial forms of Candida albicans in continuous culture. J. Gen. Microbiol. 93, 361-370.

189. Shepherd, M.G., Ghazali, H.M. and Sullivan, P.A. 1980. N-acetyl-D-glucosamine kinase and germ tube formation in Candida albicans. Exp. Mycol. 4, 147-159.

190. Shepherd, M.G., Chiew, Y.Y., Ram, S.P. and Sullivan, P.A. 1980x. Germ tube induction in Candida albicans. Can. J. Microbiol. 26, 21-26.

191. Shannon, J.L. and Rothman, A.H. 1971. Transverse septum formation in budding cells of the yeast-like fungus Candida albicans. J. Bacteriol. 106, 1026 -1028.

192. Shigematsu, M.L., Uno, J. and Arai, T. 1982. Effect of ketoconazole on isolated mitochondria from Candida albicans. Antimicrob. Agents. Chemother. 21, 919-924.

193. Sierra, J.M., Sentandreu, R. and Villaneuva, J.R. 1973. Regulation of wall synthesis during Saccharomyces cerevisiae cell cycle. FEBS. Lett. 34, 285-290.

194. Sietsma, J.H. and Wessels, J.G.H. 1981. Solubility of (1-3) -β-D (1-6) glucan in fungal walls: importance of presumed links between glucan and chitin. J. Gen. Microbiol. 125, 209-212.

195. Sikl, D., Masler, L. and Bauer, S. 1964. Mannan from the extracellular surface of Candida albicans Berkhout. Experientia, 20, 456.

196. Simonetti, N. and Strippoli, V. 1973. Pathogenicity of the Y as compared with the M form in experimentally induced Candida albicans infections. Mycopathol. Mycol. Appl. 51, 19-28.

197. Simonetti, N., Strippoli, V. and Cassone, A. 1974. Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in Candida albicans. Nature. 250, 344 -346.

198. Singh, B. and Datta, A. 1979. Regulation of N-acetyl-D-glucosamine uptake in yeast. Biochim. Biophys. Acta. 557, 248-258.

199. Skinner, C.E. 1947. The yeast-like fungi Candida and Brettanomyces. Bacteriol. Rev. 11, 227-274.

200. Sohnle, P.J., Frank, M.M. and Kirkpatrick, C.H. 1976. Deposition of complement components in the cutaneous lesions of chronic mucocutaneous candidiasis. Clin. Immunol. Immunopathol. 5, 340-350.

201. Soll, D.R., Stasi, M. and Bedell, G. 1978. The regulation of nuclear migration and division during pseudo-mycelium outgrowth in the dimorphic yeast Candida albicans. Exp. Cell. Res. 116, 207-215.

202. Spiro, A.G. 1966. Analysis of sugars found in glycoproteins. In Methods in Enzymology.(Eds). E.F. Neufield. and V. Gunsburg. 8, 3-26.

203. Staib, F. 1965. Serum-proteins as nitrogen source for yeast-like fungi. Sabouraudia. 4, 187-193.

204. Staib, F. 1969. Proteolysis and pathogenicity of Candida albicans strains. Mycopathol. Mycol. Appl. 37, 345-348.

205. Stanley, V.C. and Hurley, R. 1967. Growth of Candida species in cultures of mouse epithelial cells. J. Pathol. Bacteriol. 94, 301-315.

206. Stanley, V.C. and Hurley, R. 1969. The growth of Candida species in cultures of mouse peritoneal macrophages. J. Pathol. 97, 357-366.

207. Strippoli, V. and Simonetti, N. 1971. Separation and characterization of morphological elements of Candida albicans by urovison density gradients. Mycopathol. Mycol. Appl. 45, 77-84.

208. Sundaram, S., Sullivan, P.A. and Shepherd, M.G. 1981. Changes in lipid composition during starvation and germ tube formation in Candida albicans. Exp. Mycol. 5, 140-147.

209. Syverson, R.E., Buckley, H.R. and Campbell, C.C. 1975. Cytoplasmic antigens unique to the mycelial or yeast phase of Candida albicans. Infect. Immun. 12, 1184-1188.

210. Taschdjian, C.L. and Kozinn, P.J. 1957. Laboratory and clinical studies on candidiasis in the newborn infant. J. Pediatr. 50, 426-433.

211. Taschdjian, C.L., Burchall, J.J. and Kozinn, P.J. 1960. Rapid identification of Candida albicans by filamentation on serum and serum substitutes. Am. J. Dis. Child. 99, 212-215.

212. Taschdjian, C.L., Seelig, M.S. and Kozinn, P.J. 1973. Serological diagnosis of candidiasis. Crit. Rev. Clin. Lab. Sci. 4, 19-59.

213. Tkacz, J.S. and Lampen, J.O. 1972. Wall replication in Saccharomyces species : use of fluorescein-conjugated concanavalin A to reveal the site of mannan insertion. J. Gen. Microbiol. 72, 243-247.

214. Trevelyan, W.E. and Harrison, J.S. 1956. Studies on yeast metabolism. Yeast carbohydrate fractions. Separation from nucleic acid, analysis and behavior during anaerobic fermentation. Biochem. J. 63, 23-33.

215. Trnovec, T., Gajdosik, A., Bezek, S., Sikl, D., Koprda, V., Zemanek, M. and Faberova, V. 1977. The effect of fractions isolated from Candida albicans on phagocytic activity of the reticuloendothelial system in mice. J. Reticuloendothel. Soc. 22, 111-120.

216. Tronchin, G., Poulain, D., Herbert, J. and Biguet, J. 1981. Localization of chitin in the cell wall of Candida albicans by means of wheat germ agglutinin. Fluorescence and ultrastructural studies. Eur. J. Cell. Biol. 26, 121-128.

217. Uno, J., Shigematsu, M.L. and Arai, T. 1982. Primary site of action of ketoconazole on Candida albicans. Antimicrob. Agents. Chemother. 21, 912-918.

218. Van den Bossche, H. 1974. Biochemical effects of miconazole on fungi 1. Effects on the uptake and/or utilization of purines, pyrimidines, nucleosides, amino acids and glucose by Candida albicans. Biochem. Pharmacol. 23, 887-899.

219. Van den Bossche, H., Willemsens, G. and Van Cutsem, J.M. 1975. The action of miconazole on the growth of Candida albicans. Sabouraudia, 13, 63-73.

220. Van den Bossche, H., Willemsens, G., Cools, W., Lauwers, W.F.J. and La Jeune, L. 1978. Biochemical effects of miconazole on fungi 2. Inhibition of ergosterol biosynthesis in Candida albicans. Chem. Biol. Inter. 21, 59-78.

221. Van den Bossche, H., Willemsens, G., Cools, W., Cornellisen, F., Lauwers, W.F. and Van Cutsem, J.M. 1980. In vitro and in vivo effects of the antimycotic drug ketoconazole on sterol synthesis. Antimicrob. Agents. Chemother. 17, 922-928.

222. Venezia, R.A. and Lachapelle, R.C. 1973. The use of ferritin conjugated antibodies in the study of cell wall components of Candida albicans. Can. J. Microbiol. 19, 1445-1448.

223. Wain, W.H., Price, M.F. and Cawson, R.A. 1975. A re-evaluation of the effect of cysteine on Candida albicans. Sabouraudia. 13, 74-82.

224. Wain, W.H., Price, M.F., Brayton, A.R. and Cawson, R.A. 1976. Macromolecular syntheses during the cell cycles of yeast and hyphal phases of Candida albicans. J. Gen. Microbiol. 97, 211-217.

225. Wain, W.H. and Polak, A.M. 1979. The effect of flucytosine on the germination of Candida albicans. Postgrad. Med. J. 55, 671-673.

226. Walters, M.N.I. and Papadimitriou, J.M. 1978. Phagocytosis: A review. Crit. Rev. Toxicol. 5, 377-421.

227. Warwood, N.M. and Blazenc, D.J. 1977. Comparison of cream of rice agar and horse serum for differentiating germ tubes of Candida albicans from filaments of Candida tropicalis. J. Clin. Microbiol. 5, 501-502.

228. Whelan, W.L., Partridge, R.M. and Magee, P.T. 1980. Heterozygosity and segregation in Candida albicans. Molec. Gen. Genet. 180, 107-113.

229. Whittle, C.H. and Gresham, G.A. 1960. Candida in vitro and in vivo. Mycopathol. Mycol. Appl. 12, 207-215.

230. Wieckowski, E. 1968. Determination of chitin on the basis of glucosamine content. Chem. Anal. (Warsaw). 13, 1311.

231. Winner, H.I. and Hurley, R. 1964. Candida albicans. London: Churchill.

232. Wolter, J.R. 1962. Endogenous fungal endophthalmitis caused by Candida albicans. Arch. Ophthalmol. 68, 337-340.

233. Yamaguchi, H. 1974. Dimorphism in Candida albicans. 1. Morphology dependant changes in cellular content of macromolecules and respiratory activity. J. Gen. Appl. Microbiol. 20, 87-99.

234. Yamaguchi, H., Kanda, Y. and Osumi, M. 1974. Dimorphism in Candida albicans. 2. Comparison of fine structure of yeast-like and filamentous phase growth. J. Gen. Appl. Microbiol. 20, 101-110.

235. Yamamura, M. and Valdimarsson, H. 1977. Participation of C_3 in intracellular killing of Candida albicans. Scand. J. Immunol. 6, 591-594.

236. Yamamura, M., Boler, J. and Valdimarsson, H. 1977. Phagocytosis measured as inhibition of uridine uptake by Candida albicans. J. Immunol. Meth. 14, 19-24.

237. Yu, R.J., Bishop, C.T., Cooper, F.P., Hasenclever, H.F. and Blank, F. 1967 Structural study of mannans from Candida albicans.(Serotypes A and B), C.parapsilosis, C.stellatoidea and C.tropicalis. Can. J. Chem. 45, 2205-2211.

Addendum.

238. Ginsburg, I., Sela, M.N., Morag, A., Ravid, Z., Duchan, Z., Ferne, M., Rabinowitz-Bergner, S., Thomas, P.P., Davies, P., Niccols, J., Humes, J. and Bonney, R. 1981. Role of leukocyte factors and cationic polyelectrolytes in phagocytosis of Group A Streptococci and Candida albicans by neutrophils, macrophages, fibroblasts and epithelial cells. Modulation by anionic polyelectrolytes in relation to pathogenesis of chronic inflammation. Inflammation. 5, 289-312.

239. Gontijo, P.P. and Wayne, L.G. 1978. Phagocytic and antimicrobial activities of rabbit peritoneal and alveolar macrophages activated with Mycobacterium bovis BCG on Candida albicans. Rev. Lat. Amer. Microbiol. 20, 41-44.

240. Leigh, P.C.J., Van den Barselaar, M.T. and Van Furth, R. 1977. Kinetics of phagocytosis and intracellular killing of Candida albicans by human granulocytes and monocytes. Infect. Immun. 17, 313-318.

241. Maity, P.K., Kumar, R. and Mohapatra, L.N. 1981. Phagocytosis and intracellular growth of Candida albicans in mouse peritoneal macrophages. Ind. J. Med. Res. 73, 319-324.