INDUCIBLE HYDROLASES IN THE PATHOGENESIS AND SERODIAGNOSIS OF SYSTEMIC CANDIDOSIS

by

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STATEMENT

This thesis submitted for the degree of Doctor of Philosophy, is based on original work carried out by the author in the Department of Microbiology at the University of Leicester in the period October, 1976 and September, 1979. None of the work has been submitted for another degree in this or any other university.

Signed: F Macdonald

Date: 21st September, 1979

TO MY PARENTS

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SUMMARY

The aim of this study was to search for inducible hydrolytic enzymes of *Candida albicans*, to investigate any link between such enzymes and the pathogenesis of candidosis - a potentially fatal disease with a poor prognosis - and to test these enzymes as infection-specific antigens in the serological diagnosis of deep-seated infection.

No inducible hyaluronidase or phospholipases could be detected under the conditions employed.

An acid proteinase, found in the culture filtrates of *C.albicans* grown at 26°C in a medium based on glucose and bovine serum albumin, was purified by a one-step chromatographic method. The enzyme was judged to be pure by serological and biochemical tests. It was shown to be a glycoprotein, containing 1.5% mannan, and to belong to the carboxyl proteinases.

Proteinase was detected in greatest amounts in the most virulent of the pathogenic members of the genus *Candida*. It was secreted *in vivo* and was detected by indirect fluorescent antibody techniques around *C.albicans* blastospores in kidney microabscesses of experimentally infected mice.

Purified proteinase appeared to be a very specific antigen when tested against sera from experimentally infected rabbits, since no anti-proteinase precipitins were detected in the sera of those rabbits in which tissue invasion was prevented by treatment with ketoconazole. Precipitin titres to purified proteinase exceeded 1:4 in 77% of sera from 13 patients with proven systemic *Candida* infection, in 23% of sera from 22 patients positive for *Candida* anti-cytoplasm antibodies but without corroborative diagnostic evidence of systemic candidosis, and in none of 28 sera from patients without either candidosis or anti-cytoplasm antibodies.

Although the results did not confirm unequivocally the role of proteinase as a factor in the virulence of *C.albicans*, they gave considerable support to this idea. Purified proteinase was not a qualitatively specific antigen for the serological diagnosis of candidosis, but it was shown to give quantitatively superior results to those obtained with traditional cytoplasmic extracts of the fungus.

CHAPTER 1

Introduction

In recent years there has been an increase in the number of cases of systemic candidosis - a potentially fatal disease caused by *Candida albicans* and six other pathogenic members of the genus *Candida*. These yeasts are frequently found as commensal organisms of the gut, mucous membranes and probably other sites of the body. The diagnostic significance of *Candida* in culture in cases of suspected candidosis, may therefore be open to doubt and since many of the symptoms of the disease are similar to those of systemic bacterial infection, a reliable specific diagnostic test is needed before therapy with antibiotics toxic to yeasts can be instituted (Odds, 1979).

The detection of antibodies to *Candida* in the serum might be expected to give correborative evidence of systemic infection. Many problems still exist, however, with the serological diagnosis of deep-seated candidosis particularly the lack of standardization of the antigens (Faux *et al.*, 1975) and test methods used (Merz *et al.*, 1977) and the frequently reported occurrence of both false positive and false negative antibody reactions to *Candida* antigens (Odds, 1979, Taschdjian, 1973).

A more reliable test is therefore required, which will give positive results only in cases of deep-seated infections. This must in turn depend on the use of antigens specific to the process of *Candida* infection. Such 'infection-specific' antigens could be used directly for detection of antibody produced as a result of tissue invasion by the fungus or indirectly for detection of circulating *Candida* antigens.

A better understanding of the mechanisms involved in the pathogenicity of *Candida* such as factors leading to tissue invasion may suggest suitable antigens specific for systemic candidosis.

This study began therefore as an investigation of the pathogenicity of *C.albicans* and the use of macromolecules involved in this process as sero-diagnostic antigens.

1.1 History

Some of the diseases caused by Candida species have long been recognized. Hippocrates in his 'Epidemics' describes two cases - one of erysipelas and one of enteric fever in which the patients had white patches (aphthae) and ulcers in the mouth. In his diaries of 1665, Samuel Pepys describes a man as having 'a fever, a thrush and a hick-up' suggesting that the disease was at least as well known in the 17th century as it is today.

The causitive organism of the disease however was not discovered until the 19th century. Langenbeck (1839) is usually credited with the discovery of the fungus, which he isolated from the mouth of a patient suffering from typhoid, although he suggested that the organism was the cause of the typhoid rather than the oral lesions of thrush. Bennett (1844) published diagramatic evidence of a yeast-like fungus - described by him as a cryptogamic plant - in the sputum and lungs of a patient with pneumothorax but again he did not relate the organism to thrush. Two years later, Berg (1846) related Candida directly to thrush in his monograph 'Thrush in Children'. Thereafter Candida and many forms of candidosis were reported in the medical literature.

However, although the disease was well described, the taxonomic status of the organism was the subject of much confusion. Over 100 synonyms for C.albicans are given in Lodder's 'The Yeasts' (1970) since it was named Oidium albicans by Robin in 1853. It was not until 1923 that Berkhout suggested the generic name Candida to include asporogenous yeasts with the ability to produce pseudohyphae. C.albicans was finally accepted as the nomen conservandum for the principal thrush fungus by the 8th Botanical Congress in 1954.

1.2 Factors predisposing to candidosis

Candida spp are frequently isolated from the human body. The yeasts are commensals of the gut and mucous membranes though rarely causing disease in healthy individuals (Odds, 1979).

The average frequency of isolation of C.albicans from normal individuals is 10% from the mouth, 18% from the faeces and 6% from the vagina (Odds, 1979).

However, the occurence rate is considerably higher in much of the literature since many surveys have been carried out on hospital patients who, one must suppose are, to some extent, predisposed by their illness to infection by opportunist pathogens. The average frequency of recovery of C.albicans from these patients is 36% from the mouth, 24% from the faeces and 18% from the vagina,which are notably higher than the mean carriage rates for normal subjects.

Isolation frequencies in different reports cannot, however, be easily compared as it has been noted, for example, that the oral yeast population fluctuates during the day (Wilkinson, 1968) and also that the frequency of isolation varies with the sampling method (Johnston and Bodey, 1970).

Although Candida spp exist as commensals, the balance between saprophytism and parasitism is a delicate one,which can sometimes be altered in favour of the fungus by quite small decreases in host resistance to infection. This connection between prior debilitation and candidosis was recognized in the last century by Bennett in 1844, by Trousseau in 1869 and by Parrott in 1877, all of whom stated that thrush is always the consequence of a predisposed state. In 1962, Wilson suggested that "C.albicans is a better clinician and can discover abnormalities in persons much earlier than we can with our chemical tests". Systemic forms of candidosis are rarely, if ever, found in patients who are not suffering from at least one factor recognized as predisposing to candidosis. The main predisposing factors are listed in Table 1.

Some of these factors are simply slight deviations from the physiological 'normal' state such as infancy, pregnancy or old age. Others are infectious, hormonal or other idiopathic states such as endocrine disorders - these being associated in particular with cases of chronic mucocutaneous candidosis (Holt, 1972, Valdimarsson et al., 1973).

It can be seen from the table that many of the factors known to predispose to candidosis are procedures of modern medicine which explains, in part, the increase in cases of systemic candidosis in the last few decades (Hart et al.,

Table 1 Factors predisposing to infection by Candida albicans (and other pathogenic species of Candida).

HORMONAL DISTURBANCES AND OTHER IDIOPATHIC STATES	INFECTIOUS DISEASE
Diabetes	Tuberculosis
Hypoparathyroidism	Chronic bronchitis
Hypoadrenocorticism	Influenza
Carcinoma	Typhoid and other enteric infections
Leukemia	Bacterial endocarditis
Pernicious anaemia	SURGERY
Aplastic anaemia	Open heart operations
Agranulocytosis	Bowel resections
Bronchiectasis	Colostomy
Malformation of the urinary tract	Tooth extractions
Ulceration of the digestive tract	Eye operations (corneal grafts)
Debility	Ear operations (skin grafts)
Malabsorption	ACCIDENTAL INTRODUCTION OF CANDIDA BY INTRAVENOUS INJECTIONS OR INDWELLING URINARY CATHETERS
Malnutrition	Blood transfusions
Moribund state	Glucose saline drips and other supportive fluids
PRE-EMINENTLY RECEPTIVE STATES	Drugs, especially in addiction
Pregnancy	ACCIDENTAL TRAUMA
Infancy and old age	Eye injury
Carbohydrate-rich diet	Burns
Maceration of skin	
Skin surface contact with carbohydrates	
DRUG THERAPY	
Antibiotics	
Corticosteroids	
Contraceptive drugs	

From Gentles and La Touche, 1969.

1969). Antibiotics - in particular the broad spectrum antibiotics such as tetracycline - are frequently implicated as predisposing factors to candidosis (Seelig, 1966: Winner and Hurley, 1964). These drugs remove the normal bacterial flora from the gastrointestinal tract and mucous membranes, allowing yeast overgrowth. Antibiotics have also been thought to stimulate growth of Candida, suppress phagocytosis and decrease the natural host immunity to infection. Experimental data on these aspects are however contradictory and most reports on antibiotic therapy cannot be clearly assessed since patients are already predisposed by illness or injury before therapy is started.

Perhaps the most common cause of systemic candidosis at the present time is the introduction into patients of intravenous catheters, which can remain in place for many days or weeks frequently becoming contaminated (Schönbeck, 1972: Stone et al., 1974). Patients undergoing parenteral hyperalimentation are often found to have Candida septicaemia (Freeman, 1972: Stone et al., 1974) - the solutions used being perfect growth media for Candida (Failla et al., 1975: Gelbart et al., 1973). Candida endocarditis was frequently noted in 'main-lining' drug addicts (Harris et al., 1972) who used contaminated needles. However, today, this disease is most often seen in patients after open-heart surgery mainly because of the antibiotic and immunosuppressive therapy involved, indwelling intravenous catheters and occasionally, infected homografts or sutures (Seelig et al., 1973: Seelig et al., 1974).

Although Table 1 lists malignant diseases and trauma, including burns as factors predisposing to candidosis, the techniques involved in the management of these cases - antibiotic and immunosuppressive therapy and intravenous catheterization (Law et al., 1972) - are also predisposing factors and it may be these that are responsible for the occurence of candidosis rather than the primary disease or injury.

1.3 Clinical Features of Candidosis

The clinical picture of the disease in patients who are predisposed by the above factors varies considerably from mild superficial infections to

potentially fatal systemic disease.

Superficial Candidosis

Different forms of superficial candidosis can be divided into two main categories - cutaneous infections, involving the skin, nails and nail folds, and mucocutaneous infections, in which the mucous membranes are principally affected. In both forms of the disease the infection is restricted to the upper layers of the skin, hyphae never penetrating deeper than the stratum corneum (Montes & Wilson, 1968).

Cutaneous candidosis is characterized by pruritic erythematous lesions, often with fissuring, and by hyperkeratotic changes in the nails when they become infected (Forman 1966: Gentles & La Touche, 1969). This form of the disease usually results from local occlusion or maceration of the tissues.

Erythematous lesions are also seen in mucocutaneous candidosis along with white curd-like deposits and frequently - in the case of vaginitis - a thick white discharge (Morton & Rashid,1977: Winner & Hurley, 1964). Oral candidosis is often noted as 'thrush' in infants who are infected by the mother at birth (Kozinn & Taschdjian, 1962) or as denture stomatitis and angular chelitis among adults (Budtz-Jörgensen & Bertram,1970). Candida vaginitis is probably the most commonly seen Candida infection in modern medicine, largely as a result of many of predisposing factors listed in Table 1, in particular, pregnancy, oral contraceptives, antibiotics and diabetes (Morton & Rashid 1977). Superficial infections are readily diagnosed and - with the exception of Candida vaginitis where recurrent infections are common - respond rapidly to local therapy.

Chronic mucocutaneous candidosis (CMC) is a rare form of superficial infection affecting both cutaneous and mucocutaneous areas of the body. As with other superficial forms of candidosis, the infection in cases of CMC is restricted to the epidermis. Clinically, most of the features are similar to those of cutaneous and mucocutaneous candidosis although in one form of the disease, known as Candida granuloma, hyperkeratotic lesions develop on the face and scalp (Imperato et al., 1968). However, unlike other superficial Candida infections, CMC is resistant to topical therapy - and

usually responds only to intravenously administered antifungal drugs. The infection usually develops early in childhood and, as mentioned above, is associated with endocrine disorders, genetically based abnormalities in cellular immune mechanisms and with other abnormal cellular immune responses (Kirkpatrick et al., 1971: Valdimarsson et al., 1973).

Attempts have been made to classify the various forms of CMC - notably by Wells (1973) and by Valdimarsson et al., (1973) - based on various criteria. The clinico-genetic classification of Wells is the one most commonly used in the literature. The classification of Valdimarsson et al., (1973) based on the underlying defects in cellular immunity - mainly the presence or absence of delayed hypersensitivity and production of macrophage inhibition factor - draws attention to the four main patterns of detectable immune abnormalities of the disease.

1.4 Systemic Candidosis

Systemic candidosis is the least commonly seen form of candidosis. Unlike superficial infections, it does not present with any characteristic signs and, as a consequence, the disease is still diagnosed most frequently post-mortem.

Virtually all forms of systemic candidosis arise due to haematogenous spread of the yeasts and several means of entry into the blood stream have been suggested (Seelig et al., 1973). A proportion of cases arise due to introduction of Candida via intra venous catheters where there is a continual seeding of the yeasts into the blood (Brennan et al., 1972). Surgical and burn wounds were considered as the main foci responsible for systemic infections by Law et al., (1972) but no fungal invasion from infected granulating wounds could be detected by Stone et al., (1974) indicating that this method of entry into the blood is unlikely.

The most common portal of entry into the bloodstream is probably by migration or absorption of Candida across the intestinal mucosa. In a study by Krause et al., (1968), ingestion by a healthy volunteer of 10^{12} C.albicans cells led to positive blood cultures three and six hours later. These results were confirmed by the animal experiments of Stone et al., (1974). It would therefore appear that any marked increase in the intestinal population of

Candida e.g. during antimicrobial therapy, leads to migration of the yeast through the intestinal wall and into the blood, resulting in a transient candidaemia. If this process continues in patients with depressed antimicrobial defences Candida septicaemia and disseminated candidosis are likely to result.

Candidosis of the urinary tract and Candida endocarditis are probably the two forms of primary systemic candidosis on which most detailed individual case reports have appeared. The kidney has long been recognized as the most frequently infected organ in experimental Candida septicaemia and endocarditis has received much more attention in recent years since the increase in the numbers of cases due to open-heart surgery. Infections of all other organs of the body as well as bones and joints, the central nervous system and the eye have been described (Odds, 1979). Although the disease may remain localized as a primary infection in any one of these organs, Candida may spread, via the bloodstream, resulting in disseminated candidosis. In clinical reports of disseminated candidosis, the prediliction of Candida for the kidney can be seen although lesions have been frequently noted in the intestine, lungs and brain.

Diagnosis in all forms of systemic candidosis usually proves difficult. Yet accurate diagnosis is essential since the few antifungal agents available for systemic candidosis are likely to cause severe side effects to the patient. Symptoms of the disease are generally similar to those of bacterial infections. Presence of yeasts and pseudohyphal forms of Candida in sections of biopsy material is significant and definitive diagnostic evidence but biopsies are rarely performed as a first resort especially in patients with septicaemia.

Direct isolation of Candida is not always significant because of the saprophytic nature of the organism. This is the case for sputum, which can be contaminated by yeasts from the oral cavity, for faeces and for the vagina. Isolation of the yeasts from normally sterile sites is of much more value since obviously Candida does not occur as a commensal in internal organs. Stone et al., (1974) have suggested that the presence of yeasts in the urine is a good indicator of disseminated candidosis.

When Candida is isolated in blood culture, it is still sometimes regarded as a contaminant, despite the fact that it is not considered part of the normal skin flora (Odds, 1979). Conversely, attempts to recover Candida from the bloodstream frequently fail (Seelig et al., 1973: Stone et al., 1974). Experiments by Stone et al., (1974) explained the failure to isolate Candida from venous blood as being due to a filtering effect by macrophages of many organs of the body, in particular the liver and kidney, and suggested that arterial blood cultures might be a better indicator of infection. However, withdrawal of the blood from an artery is technically much harder to perform than from a vein so that the technique may not find favour with medical staff. No reports on its value have as yet appeared.

Successful isolation of yeasts from the blood is also hampered by the mode in which Candida grows in vivo. Instead of forming a homogenous suspension of cells as happens with bacteria, Candida grows as a tangled mass of mycelium and blastospores, which cannot be readily picked up in a syringe.

Ellis and Spivack (1967) have reported cases of candidaemia, due to transient contamination of the bloodstream, which do not require antifungal therapy. However, they suggested that blood cultures be repeated since repeated isolations of Candida are indicative of true cases of disseminated candidosis.

Since it appears that the microbiological tests either fail or give equivocal results, serological tests may be expected to give additional supportive evidence of systemic infection. However, there are several problems associated with serological diagnosis and these will be discussed in a later section.

1.5 Pathogenesis of Candida infections

a) Experimental Candidosis

The pathogenicity of *Candida* species has been studied mainly in animals or tissue culture. Many of the symptoms induced in experimentally inoculated animals are similar to those in humans.

In the mouse, an intravenous injection of 10^6 *Candida* cells generally results in 100% mortality with lesions in the kidney, heart, brain and spleen. Smaller doses result in chronic systemic infection and in some cases no effects are noted (Adriano & Schwarz, 1955).

Inoculation via other routes e.g. intra peritoneally leads to similar pathological changes although a much larger dose is required.

The rabbit is more susceptible to infection than the mouse, with smaller doses/kg leading to similar lesions. Again the kidney is the major organ affected (Evans & Winner, 1954).

The guinea pig was used as an experimental model for candidosis when studying the effect of corticosteroids on susceptibility to infection as it was suggested that it was most closely related to the human situation particularly with regard to steroid therapy (Hurley & Fauci, 1975).

Partridge *et al.*, have suggested the use of the chick chorioallantoic membrane (CAM) for pathogenicity tests with *Candida* since it is more sensitive and easier to inoculate than animals (Partridge *et al.*, 1971). Various aspects of pathogenicity have been studied using mouse renal epithelial cells in tissue culture (Stanley & Hurley, 1967).

These two techniques, among others, have been used to compare the differential virulence of the seven pathogenic species of *Candida* that fulfil Koch's postulates, with the same results. *C.albicans* is the most pathogenic, followed in order by *C.tropicalis*, *C.stellatoidea*, *C.parapsilosis*, *C.pseudotropicalis*, *C.krusei* and *C.guilliermondii*.

b) Determinants of Pathogenicity

Pathogenic micro-organisms may possess a variety of properties

by means of which they may disable host defences and/or cause damage to host tissues. Resistance mechanisms to phagocytosis, intracellular killing and to humoral and cellular immune responses have been defined for the most important bacterial pathogens, and several bacteria produce toxins or hydrolytic enzymes capable of causing direct damage to host tissues. The virulence factors of C.albicans and other pathogenic yeasts are less well established, although some have been described (Mims, 1977).

Filamentation

C.albicans has long been recognized as able to produce mycelium as well as blastospores. Berg (1846) described yeast-like organisms in blood culture as well as mycelium, although at that time no one appreciated that the fungus was dimorphic and that both phases belonged to the same organisms. This was proven beyond doubt several years later (Audrey, 1887).

The mechanism responsible for mycelial-yeast conversion has still not been fully determined. For nearly every report suggesting one factor favouring filamentation there is a conflicting report suggesting that the same factor suppresses filamentation (Odds, 1979). The one exception to this,is that many authors have reported on the effect of single amino acids or a combination of several which stimulate filamentation, but even here there is no agreement as to which amino acids are responsible (Odds, 1979).

C.albicans is the only pathogenic Candida species capable of true mycelium production - the others produce pseudomycelium. This property has been suggested to explain the greater virulence of C.albicans over the other species since mycelium production has often been considered to be a major factor in the pathogenicity of Candida.

The ability to produce mycelium must give an organism a hightened ability to invade tissue. It has been noted in plant pathogens that the mechanical force of mycelium alone is responsible for the penetration of gold film (Miyoshi, 1895). Mycelium production also allows C.albicans to escape phagocytosis by growing out from the phagocyte (Louria & Brayton, 1964).

Although numerous reports have appeared directly relating mycelium production with pathogenicity, blastospores are nearly always noted in infected tissues as well as filaments (Winner & Hurley, 1964). Some groups of workers have stated that the mycelial form is more virulent, others have shown that it is the yeast form which has the greater pathogenic potential. Several people have been unable to find any difference in virulence between the two forms. It is difficult however to compare directly the virulence of the two forms since it is hard to tell how much mycelium is equivalent to a given quantity of blastospores. The two forms must also behave differently in the body - the yeast form is likely to be spread much more readily throughout the body than the mycelium which might in turn be able to penetrate cells more readily.

When Hurley and Stanley (1969) compared different pathogenic species of Candida on cultured mouse renal epithelial cells, they were able to correlate the ability to produce mycelia with the pathological effects of the fungi. However, they did suggest that the yeast phase initiates infection, accounting for the observation of pure blastospores in some lesions and that mycelium production and tissue damage arise later.

Similar results were obtained by Taschdjian and Kozinn (1957) who found only blastospores in smears from babies' mouths until clinical lesions of thrush appeared, when filaments were noted.

None of the experimental observations on the virulence of different forms of C.albicans are clear cut - neither form can be considered exclusively invasive. However, the ability to produce filaments must be considered as a gratuitous factor in the organism's pathogenicity (Odds, 1979). The greater ease with which yeast-like cells can spread through the body and the observation that these forms are noted early on in lesions leads to the idea that this form may be responsible for initiation of infection. It should be noted that the opportunist yeast-pathogen, C.glabrata (formerly known as Torulopsis glabrata) is incapable of producing even pseudomycelium.

Toxin Production

Endotoxins are known to be determinants of virulence in bacteria (Mims, 1977)

Certain pathological features of experimental candidosis led Henrici in 1940 and Salvin in 1952 to suggest the presence of an endotoxin in C.albicans. Cutler et al., (1972) carefully tested different fractions of C.albicans for toxic properties and discovered that whole cells and cell walls were pyrogenic for rabbits and lethal for actinomycin D-treated mice. Further reports substantiated the toxic properties of C.albicans glycoprotein (Iwata, 1976). However, the potency of this toxin is very much lower than that of any bacterial toxin and it is very hard to relate the large amounts necessary for a reaction in experimental animals to the disease in humans. There is also evidence of a pyrogenic effect in rabbits with the cell walls of the non-pathogen Saccharomyces cerevisiae (Cutler et al., 1972) which suggests that the pyrogenic effect of C.albicans cell walls may not be an essential component in the virulence of the latter. It is probable however that the cell wall glycoprotein is responsible in part for the fever noted in cases of systemic candidosis.

Iwata (1976) isolated from C.albicans a very unusual toxin, known as canditoxin, which is made up of four subunits - two with carboxypeptidase activity, one with phosphomonoesterase activity and a fourth unidentified unit. This toxin has only been isolated from one strain of C.albicans. Chattaway et al., (1971) were unable to identify any toxin-like substance in four different strains of C.albicans. Such a strain-specific toxin as canditoxin is therefore unlikely to play any common part in the pathogenesis of candidosis overall.

Hydrolytic enzymes

Hydrolytic enzymes have been implicated in the pathogenicity of some bacteria especially those known as exotoxins, secreted by some gram positive bacteria. Similar enzymes have been described in Candida, notably acid phosphatase, proteinase and phospholipase, although their role in the pathogenicity has not yet been proven and only the proteinase is secreted by the fungus.

Chattaway et al., (1971) demonstrated the presence in C.albicans of a

constitutive alkaline phosphatase which had been previously shown histochemically by Kurup (1963).

The presence of acid phosphatases have been described by several workers. Montes and Wilborn (1970) detected acid phosphatase activity distributed in small intracellular granules located in the cytoplasm of Candida cells. Chattaway et al., (1971) showed the presence of two separate acid phosphatases with pH optima at 3.6 and 5.6 and suggested that the former was located close to the cell surface. The phosphatase appeared at the cell surface in growth media devoid of phosphate and could be released by treatment of cells with dithiothreitol (Chattaway et al., 1974). These results contradict the histochemical studies of Montes and Wilborn (1970). It seems likely that the latter workers were looking at a constitutive enzyme located in the cytoplasm - perhaps the phosphatase with pH optimum of 5.6 - and had overlooked the inducible enzyme located at the cell surface. Odds and Hierholzer (1973) purified the pH 3.6 enzyme and found that it was a glycoprotein with a pH optimum of 4.5 in its purified form. Qualitatively this enzyme showed many similar properties to the acid phosphatase found in S.cerevisiae (Boer and Steyn-Parvé, 1966) for example in the glycoprotein nature of the enzyme and in the similar broad range of substrate specificity. Cell-surface phosphatases have also been demonstrated in Candida species other than C.albicans (Odds and Trujillo Gonzales, 1974: Sanger et al., 1975). The existence of similar enzymes in less pathogenic and non-pathogenic organisms suggest it is unlikely to be a major factor in the pathogenicity of C.albicans.

In 1968, Costa et al., reported phospholipase A and C activity in a pathogenic strain of C.albicans grown in a medium containing serum and erythrocytes. Pugh and Cawson (1975) were able to detect phospholipase A and lysophospholipase cytochemically at the site of bud formation in young cultures and at the periphery of the cell in older cultures where it is secreted into the medium, but some cells showed no activity. These workers could not detect phospholipase C. However, since their cultures were grown in Sabouraud's medium and those of Costa et al., were grown in a medium containing phospholipids in the form of

erythrocytes it is likely that phospholipase C may be an inducible enzyme in C. albicans. Phospholipase A and lysophospholipase were also examined biochemically by Price and Cawson (1977) in C.albicans grown in Whickerham's medium, using an assay method with $[^3H]$ - labelled lecithin as substrate. By this method, enzyme activity was detected only in the cytoplasm of cells although Price (1977) had previously claimed to show extracellular phospholipase activity with C.albicans grown on solid media containing egg yolk. The enzyme activity was apparent as zones of opacity around colonies. It may be objected, however, that this crude method would also similarly detect proteinase activity, which is known to be extracellular in C.albicans. The data cannot therefore be taken as unequivocal proof of extracellular phospholipase production by C.albicans and this would explain the inability of Price and Cawson (1977) to detect phospholipase extracellularly by formal biochemical assay. Pugh and Cawson (1975) showed some extracellular phospholipase activity cytochemically but this may have been due to release of enzyme from cells that were disrupted by the fixation process or by the presence of lysolecithin substrate which is known to attack cell membranes and damage cells (Pugh and Cawson, 1975). Cawson's group has repeatedly suggested that phospholipase may play a part in the invasion process of the fungus as well as being involved in phospholipid turnover in the fungal membrane. But the intracellular nature of the enzyme and its presence, as with acid phosphatase, in S.cerevisiae (Kokke, 1966) make it less likely to be responsible for pathogenicity than would be an inducible, extracellular phospholipase, if such an enzyme could be proved to be formed by C.albicans.

Two proteinases have been detected in C.albicans. The first is a constitutive enzyme with a pH optimum of 6.6 (Chattaway et al., 1971) and the second is inducible and extracellular with a pH optimum of 3.2 (Remold et al., 1968).

Staib (1965) first showed that C.albicans was able to grow at pH 4-5 on a solid medium containing serum albumin as the sole nitrogen source and he was able to detect proteolytic activity around the colonies. Buffering of

the medium reduced the proteolytic effect (Staib, 1966). Not every strain of C.albicans is capable of proteolysis (Chattaway et al., 1971: Staib, 1969) but Staib et al., (1972) found that after 10 days only 3% of strains tested showed no qualitative proteolytic activity although the capacity to precipitate and proteolyze protein varied from strain to strain. Only those strains capable of proteolysis caused pathological and histopathological changes in mice (Staib, 1969: Staib et al., 1972). Maximum proteolytic activity was found in the yeast phase of C.albicans (Staib et al., 1972) which is consistent with the results of Hurley and Stanley (1969) who suggested that the yeast phase initiated infection. Montes and Wilborn (1968) also suggested that destruction of tissue, which they observed by electron microscopy might be due to proteolysis. Budtz-Jörgensen's study (1971) supports the assumption that the virulence of yeasts is related in part to proteolytic activity. Over 82% of strains of C.albicans isolated from patients with denture stomatitis showed proteolytic activity, although the extent of enzyme activity in vitro was not related to the severity of infection.

Proteinase appears to be the enzyme most closely related so far to the pathogenicity of C.albicans, although no attempts appear to have been made to demonstrate the significance of the enzyme directly, for example by demonstration that it is produced in vivo in lesions that contain C.albicans.

Host Defences to Infection

Before any microorganism can cause disease it must overcome the host's defence mechanisms which include mechanical barriers, non-specific and specific immunity and antimicrobial substances in the serum and other body fluids. In hosts predisposed to Candida infection one or more of these factors are either missing or defective.

The first barrier to systemic infection is the mechanical barrier of the epithelia of the intestines, lungs, skin etc. The several means by which these barriers may be breached were discussed in section 1.4. It is clear that in many instances Candida may gain fairly easy access to the bloodstream.

If this does happen in a normal individual, yeasts are rapidly destroyed by phagocytes of the reticulo-endothelial system which is the major component of mammalian antimicrobial defences. The efficiency of this system was shown experimentally by Jeunet et al., (1970) who demonstrated the clearance of more than 70% of injected C.albicans cells from the bloodstream of rats within 5 minutes in particular by lung phagocytes. Any defects in this system, due to a congenital or acquired condition, will markedly reduce this 'mopping up' effect of phagocytes thus pre-disposing them to infection.

Stone et al., (1974) were able to show a reduction in the phagocytic ability of hepatic Kupffer cells by treatment of animals with gentamycin, steroids or bacterial exotoxin. It would therefore appear that an increase in the numbers of yeasts caused by antibacterial therapy or depression of the reticulo-endothelial system could lead to less efficient clearance of Candida from the bloodstream and hence result in candidaemia.

Once yeasts are engulfed by phagocytes they are normally killed by the peroxide/myeloperoxidase system. Again any defect in this function may lead to Candida infection (Lehrer & Cline, 1969). Of all the pathogenic Candida spp C.albicans has a heightened ability to resist phagocytosis due to its ability to produce mycelia and grow out from polymorphs (Louria & Brayton, 1964).

Specific acquired immunity probably plays a major part in host defences to invasion by Candida. Agglutinating antibodies in particular have been noted in normal subjects in up to 96% of persons studied although the average is about 45% (Odds 1979). Up to 94% of normal subjects show a delayed hypersensitivity reaction in skin tests to Candida antigens, a value so high that it is believed that this test can be used to assess the general capacity of a person to show delayed hypersensitivity (Shannon et al., 1966).

Serum inhibitors to Candida other than antibodies have also been described. A substance in serum that specifically inhibited C.albicans and C.stellatoidea was detected after incubating yeasts in serum for 24h and determining the numbers of surviving yeasts by a pour plate enumeration technique (Louria & Brayton, 1964). This inhibitory factor was absent in children with CMC and

associated endocrine disorders (Louria et al., 1967). Four years after the original report it was suggested that the decrease in the numbers of yeasts was due, not to a serum inhibitor, but to the phenomenum of clumping caused by yeasts and mycelial phase organisms in serum, the specificity of the effect can thus be easily explained (Chilgren et al., 1968). IgG antibodies directed against C.albicans are known to prevent the clumping effect of serum which would explain the 'lack of inhibitor' in patients with CMC or systemic candidosis (Oblack et al., 1976).

Further reports on serum inhibitors of Candida, that use the same counting technique as Louria & Brayton, or others affected similarly by clumping must therefore be regarded as false. No effect of serum on Candida has been noted when growth was measured by determination of cellular DNA or RNA or when clumps of mycelium were disposed by homogenization (Odds, 1979).

A further inhibitory effect of serum on C.albicans - the reversal of serum fungistasis by the addition of iron - has been described (Caroline et al., 1964). Although several other workers have noted similar stimulatory effects of iron, it is in direct contradiction to the fact that anaemia is regarded as a pre-disposing factor for candidosis. Iron therapy has also resulted in an improvement in 80% of patients with CMC and associated iron deficiency (Higgs & Wells, 1972). Since these reports are contradictory, this second inhibitory effect of serum cannot be clearly assessed and it must therefore be concluded that at the present time, specific and non-specific immune mechanisms, associated with phagocytosis, are the most important factors in host defence against candidosis.

1.6 Serological Diagnosis of Systemic Candidosis

Systemic candidosis is often difficult to diagnose on clinical and mycological grounds alone, so serological tests are frequently employed to give confirmatory evidence of infection. Opinions vary greatly as to the value of

serological tests. In 1977, Winner stated 'It is not difficult in most cases to diagnose Candida infections by traditional methods. My personal view (on the significance of antibodies) is that their significance in diagnosis is much exaggerated' - a strong view, not generally held by other workers. Stone et al., (1974) stated more cautiously several years previously that 'serological testing as a means for establishing the diagnosis of an invasive yeast infection has not been reliable'. A review of the problems of serological diagnosis of candidosis by Taschdjian et al., (1973) concluded, however, that good serological tests for deep Candida infection are urgently needed and appear to be promising but further progress is dependent on development of suitable standardized antigens and standardized test methods. The value of a sensitive and specific sero-diagnostic test using standardized antigens was also stressed by Axelsen (1976). He considered that a commonly available antigen standard was necessary because of the antigenic complexity of C.albicans.

a) Antigenic Structure of C.albicans and other species

Some of the first work on the antigenic structure of C.albicans was carried out by Tsuchiya et al., (1961) who found seven antigenic determinants on the surface of the yeast-phase cells by agglutination-adsorption experiments. All Candida spp. possessed at least one common antigen. This number of determinants was later increased to ten. (Murray and Buckley, 1966).

The same technique was used to demonstrate the existence in C.albicans of two serotypes, A and B. Serotype A was shown to possess at least one more antigen than B (Hasenclever and Mitchell, 1961).

Cell wall components are less important than extracts containing water soluble cytoplasmic antigens of C.albicans in tests for the serological diagnosis of systemic infection. Biguet et al., (1961) showed 10 and later 15 (Biguet et al. 1965) to 17 fractions (Tran Van Ky et al., 1969) in such extracts by classical immunoelectrophoresis. 14 enzymes were identified in the antigen extracts - one of which, malic dehydrogenase, was a component common to all seven species of Candida studied (Tran Van Ky et al., 1969).

This same group also studied the antigens excreted into the culture medium, most of which appeared to be glycoprotein in nature. Five of the excreted antigens were identical with components of the cytoplasmic extracts and two were found only in the culture filtrate (Tran Van Ky et al., 1963). It is to be suspected that these polysaccharides in the culture filtrate come from the cell wall since many reactions of identity have been noted between cell wall polysaccharides and those of culture filtrates (Chew and Theus, 1967) or cytoplasmic extracts (Taschdjian et al., 1967). Improved immunoelectrophoretic methods eventually led Axelsen (1973) to detect 78 water soluble antigens in the cytoplasmic extracts of C.albicans, by the sensitive method of crossed immunoelectrophoresis.

Cross reactions occur between C.albicans and other Candida species, other yeasts and a few bacteria. Biguet et al., (1962) demonstrated that C.albicans shared 80%, 47%, 7% and 13% of antigens respectively with C.stellatoidea C.tropicalis, C.pseudotropicalis and C.krusei, an observation confirmed by Axelsen (1973). C.glabrata is known to cross react with C.albicans (Kemp and Solotorovsky, 1964) but not with C.tropicalis (Lehner, 1966). Mycobacterium tuberculosis (Maekawa & O'Hara, 1966) and a few bacterial species (Odds, 1979). also cross-react with Candida albicans
The possibility of cross reaction with Mycobacterium is of particular importance when attempts are made to raise antisera to C.albicans with the use of Freund's complete adjuvant.

b) Serological Tests for Candidosis in practice

A serological test for the diagnosis of systemic candidosis should ideally be positive only in true cases of infection and should be able to distinguish between superficial and deep-seated infection. Most of the tests used in serodiagnosis fail, either because they give positive results in the absence of infection or because they are unable to distinguish superficial from systemic forms of candidosis. This is important because people with systemic candidosis often have one or more clear superficial Candida lesions so that serological tests must relate specifically to systemic forms of infection. If the test is equivocal, one can never say for certain whether the antibodies detected

relate to the superficial infection or to systemic infection.

Skin testing and the complement fixation reaction have proved to be the least specific of tests that have been used as methods for serological diagnosis. The majority of the population show delayed cutaneous hypersensitivity reactions to Candida antigens (Shannon et al., 1966) and although the complement fixation test was used to detect serum antibodies to Candida in the early half of the century it proved to be unreliable for clinical diagnostic use and was not sufficiently specific to be able to distinguish the two forms of candidosis (Taschdjian et al., 1973).

The indirect fluorescent antibody (IFA) reaction has been used to detect antibodies to Candida and although the specificity of this test is superior to those described above, it is still unable to distinguish superficial forms of candidosis from systemic forms.

Lehner (1966) showed that IFA titres greater than 1:16 were common in clinical cases of infection but many of his patients had superficial, not systemic candidosis. Titres up to 1:8 were also noted in sera from control subjects without infection. IFA titres of 1:160 or higher are rarely encountered in normal individuals but they do occur in patients with CMC (Taschdjian et al., 1973).

Although Lehner et al., (1972) were unable to distinguish between systemic candidosis and CMC by the IFA test, they did suggest that rising IFA titres in successive serum samples were more significant than single tests for precipitating or agglutinating antibodies. This suggestion was based on the discovery of precipitins and a rise in agglutinating titre but an absence of a corresponding rise in IFA titre in patients after cardiac surgery who had no signs of endocarditis. However, Lehner et al., did not exclude the possibility of systemic candidosis in sites other than the heart. Recently Warnock and Hilton (1976) were unable to distinguish serologically between women with vaginal candidosis and uninfected women by the IFA test.

In general the IFA test can be said to be of little value in the serological diagnosis of systemic candidosis since it is unable to distinguish between different forms of the disease and because of the presence of titres

as high as 1:64 in the absence of infection.

The problems associated with the whole-cell agglutination test for anti-Candida antibodies are similar to those of the above mentioned tests. In 1945, Fuentes and Guarton reported that the test usually indicated clinically important cases of candidosis, and found that only 0.2% of subjects tested had agglutinins to Candida (noted by Winner, 1955). This report is contrary to all others subsequently documented. Anderson (1968) found anti-C.albicans agglutinins in low titre in 47% of normal subjects, with an increase in titre associated with old age. This high prevalence of agglutinins was related to the commensal carriage of the yeast. The prevalence of agglutinins is normally higher in hospitalized patients without candidosis than normal subjects (Odds, 1979). Winner (1955) found agglutinins in 32% of hospital patients without candidosis. Filice et al., (1977) found more false positive reactions with the agglutination test in a nine month period than true positive reactions. Harding et al., (1976) also believed that the test was a misleading indicator of infection although they suggested that a titre of less than 1:40 ruled out infection - perhaps a rather sweeping statement in the light of the number of false negative reactions that occur in patients with terminal anergy.

Priesler et al., (1969) believed that a rise in agglutinating antibody titre was a good indicator of systemic infection since commensal Candida organisms were unlikely to cause a rise in antibody titre. However, the rise in titre they found was seldom more than twofold and rarely reached 1:160, which is the value suggested by Seeliger (1962) and accepted by Taschdjian et al., (1973) as diagnostically significant since titres lower than this are common in superficial infections and in non-infected persons. Cross agglutination reactions between Candida and bacterial and other fungal pathogens are also possible and may cause an apparent rise in titre in the absence of Candida infection. Holder et al., (1977) have particularly emphasized that negative titres are found in some patients with microbiologically proven candidosis. The prevalence of such false negative tests has been found to be as high as 20 to 25% (Taschdjian et al., 1973). Such considerations tend to preclude the use

of the agglutinin test alone in the serological diagnosis of deep-seated candidosis.

A development of the whole-cell agglutination test is the latex agglutination test (Stickle et al., 1972) in which soluble cytoplasmic Candida extracts are coated on latex particles and used as antigens. This test shows an improvement in diagnostic specificity over whole-cell agglutination and it was able to detect 95% of proven cases of systemic candidosis. However, false positive cross reactions were noted in sera from cases of cryptococcosis, tuberculosis and in one proven case of torulopsaemia. The sera of 5 out of the 9 cases of superficially infected patients were also positive by this test which suggests that this test, like the others described above does not have sufficiently impressive specificity for candidosis to justify its routine diagnostic use.

The precipitin reaction as a test for Candida antibodies appears, at present, to represent a considerable improvement on the above tests. Several Candida antigens have been used in this test including cell wall mannans, cytoplasmic extracts prepared by mechanical disruption of cells or occasionally culture filtrates. Several varients of the immunoprecipitation phenomenon have been used in tests - Preer tube precipitation (Chew and Theus, 1967), immunodiffusion (Stallybrass, 1964: Faux et al., 1975: Odds et al., 1975) or various forms of immunoelectrophoresis (Remington et al., 1972: Dee and Rytel, 1975: Odds et al., 1975).

Elinov and Zaikina (1959) were the first to examine the precipitin reaction, using as an antigen, a ground up suspension of Candida cells, mainly polysaccharide in nature, and their results led them to conclude that 'the precipitin reaction may be used in the diagnosis of systemic candidosis'. However, their rationale for the diagnosis of deep-seated infection depended on isolation of C.albicans from mucous membranes, which is by no means a reliable method for the diagnosis of systemic candidosis. Since their 'case sera' were obtained from patients whose diagnosis of systemic candidosis was based on this rationale, their

conclusion cannot be depended on. The results did show that the sera from patients diagnosed as cases of superficial candidosis were negative in the test.

Akiba et al., (1961) used a phenol-extracted antigen and demonstrated precipitins in 92% of sera from cases of systemic candidosis. They believed the test to be reliable in the diagnosis of systemic infection but their finding of positive reactions in 26% of the sera from superficially infected patients makes this conclusion less convincing.

Further work with the polysaccharide antigen also indicated a high proportion of precipitins in sera from cases of superficial candidosis. Chew and Theus (1967) demonstrated precipitins to cell wall mannam in 48% of unconcentrated serum samples from healthy adults and in 69% of sera from patients with mucocutaneous candidosis when tests were performed using the sensitive Preer immunodiffusion tubes: but they found a much lower number of sera were precipitin positive by Ouchterlony plate diffusion. The higher sensitivity of the Preer tube method explains why earlier workers using the Ouchterlony technique were unable to detect precipitins to mannan in many normal or superficially infected subjects. The results of Chew & Theus also demonstrate convincingly that polysaccharide antigens from the C.albicans cell wall are unsuitable for use in the serological diagnosis of systemic candidosis. The high proportion of normal subjects with precipitins to these antigens can be explained by the high prevalence of C.albicans as gut commensals which is likely to lead to development of humoral immunity to the fungi after years of exposure.

Stallybrass (1964) was the first to study precipitins to cytoplasmic Candida extracts. He used a double diffusion technique, after Rimbaud (1960) had shown that only intravenous injection of C.albicans cells resulted in serum precipitins to cytoplasmic extract. It was believed that C.albicans was only broken down after ingestion by R.E. and tissue macrophages whereas in superficial infections Candida cells remained intact. Therefore precipitins to cytoplasmic extract would only be produced in cases of systemic infection.

Stallybrass (1964) demonstrated precipitins to cytoplasmic extract only in the sera of patients with systemic candidosis and not in sera of healthy persons or patients with superficial candidosis, however extensive. He believed, however, that the precipitins were formed against polysaccharide rather than protein components of the antigen mixture and that this was the basis of the specificity (Stallybrass, 1965). In the light of more recent studies this view must be contradicted (Taschdjian et al., 1973).

Taschdjian and colleagues have used a cytoplasmic extract of Candida cells - called by them, the S antigen - in many studies on the value of precipitins in diagnosis of systemic candidosis. This antigen was similar in composition to that of Stallybrass (Taschdjian et al., 1973: Taschdjian et al., 1964a: Taschdjian et al., 1964b). These authors agreed with Stallybrass on the diagnostic specificity of the precipitin test using the S antigen : they found precipitins in 89% of sera from cases of systemic candidosis and only 8% in sera from patients in whom Candida was not involved (Taschdjian et al., 1967: Taschdjian et al., 1972). However, in contrast to the observations of Stallybrass, it was found that two precipitin bands were found in sera from infected cases - the outer diffuse band representing a reaction with polysaccharide. These authors believed that the specificity of the reaction lay in the second precipitin line, which was probably produced by a reaction with a protein component of S antigen (Taschdjian et al., 1967). Cross antibody reactions against the S antigen were noted only for patients infected with species of the genus Candida and in cases of torulopsosis (Taschdjian et al., 1967). This finding agrees with that of other workers (Stickle et al., 1972). Although precipitins to both the S antigen and Aspergillus antigens were found in the serum from one case of systemic mycosis, Aspergillus infection of the heart valve as well as the presence of Candida in the spleen and pancreas was noted at autopsy (Taschdjian et al., 1969). A commercial version of the S antigen from Hollister-Stier Laboratories has given similar results to those above. (Taschdjian et al.,

1969: Gaines and Remington, 1973).

The value of rising precipitin titres in serial specimens of serum has been noted to be of particular value in the diagnosis of deep-seated candidosis.

Although the precipitin reaction, using Candida cytoplasmic antigen, has generally been found to be the most specific test for the purposes of serological diagnosis, false positive and false negative reactions do occur.

A high proportion of the population has been shown to have precipitins to C.albicans cell wall mannan. (Chew and Theus, 1967). As this polysaccharide is readily soluble, it can easily contaminate the cytoplasmic and culture filtrate antigens (Chew and Theus, 1967: Hellwege et al., 1972) and thus give rise to false positive antibody reactions. It is probably this feature that has led to some authors questioning the diagnostic reliability of the precipitin test.

Venezia and Robertson (1974) prepared an antigen, by sonication of protoplasts of Candida, which they believed to be free of mannan. This antigen reacted in a similar manner with all sera tested to the sonicated antigen prepared by a similar method similar to that of Taschdjian et al., (1973), so they therefore concluded that 'the possibility of false positive reactions due to mannans in the antigen preparation seems unlikely'. However, the S antigen of Taschdjian et al., (1973) has clearly been shown to give a line of identity with cell wall mannan so the preparation of Venezia and Robertson cannot absolutely be assumed to be mannan free. Subsequent experiments by Syverson and Buckley (1977) using extracts of spheroplasts prepared in a similar manner showed the presence of two concanavalin A precipitable antigens, confirming the presence of polysaccharide in such preparations.

Several groups have reported the detection of precipitins to Candida in sera from superficially infected patients (Chew and Theus, 1967: Pepys et al., 1968: Stanley et al., 1972) but Taschdjian et al., (1973) have shown statistically that most of these false positive reactions are due to polysaccharide.

Positive reactions have been noted frequently in patients with no signs of candidosis who have undergone open-heart surgery. Murray et al., (1969) found high agglutinin and precipitin titres in such patients; they believed them to be due to undetected Candida infection. Similar results were noted by Parsons and Nassau (1974) who suggested that precipitins arose because of an increase in the yeast population of the alimentary tract. Evans (1975) confirmed that there is an increase in the number of yeasts recovered from the mouth and faeces post-operatively, and the data of Stone et al., (1974) suggested that an increase in the prevalence of serum precipitins to Candida might result from persorption of yeasts across the intestinal mucosa, causing a transient candidaemia. Evans and Forster (1976) found a similar relationship between the occurrence of commensal yeasts and the appearance of Candida antibodies post-operatively. The prophylactic use of antifungal antibiotics reduced this post-operative response.

The frequent occurence of false positive reactions is one of the main problems associated with the serological diagnosis of candidosis. The other problem - false negative reactions - has already been mentioned above. It is generally believed that the false negative results arise either because the patient has had insufficient time to maunt a detectable antibody response, or because the patient has lowered immune responses because of severe illness such as leukaemia or immunosuppressive therapy (Taschdjian et al., 1967). Preisler et al., (1969) could detect Candida precipitins in only 17% of patients with acute leukaemia, and on the basis of these findings it was suggested that steadily rising agglutinin titres in serial specimens of serum were a better indicator of infection than positive tests. However, the sera used in this study had been stored for some time and it has been noted that prolonged storage of sera may result in 75% to 100% loss of precipitating antibodies (Taschdjian et al., 1972). This is not the case with agglutinating antibodies and may explain the results leading to the authors' preferance for the agglutinin test. False negative antibody responses in cases of candidosis have been noted by other authors but at a lower frequency than was implicated by Priesler. (Gaines and

Remington, 1973: Rosner et al., 1971).

Double diffusion tests are often used to detect Candida precipitins, but they take several days to complete. Remington et al., (1972) suggested counterimmunoelectrophoresis (CIE) as an alternative test method, as results were available within several hours. They found that the results were reproducible and that all sera from patients with systemic infection were positive whereas control sera were negative. The main problem encountered by these workers was that the optimum range for antigen concentration was very narrow.

Dee and Rytel (1975) recommended CIE for detection of antibodies since this method permitted easy determination of precipitation titre and could thus be used quantitatively to distinguish significant candidosis from Candida colonization or transient candidaemia although false positive reactions still occured. Odds et al., (1975) found that CIE was more sensitive as well as more rapid than double diffusion and recommended its use to detect precipitins to C.albicans cytoplasmic antigen.

False negative antibody reactions are still noted with CIE, despite its increased sensitivity over double diffusion. Marier and Andriole (1978a) demonstrated that discontinuous CIE is both quicker and more sensitive than the traditional method and it may permit diagnosis of candidosis in some patients who are negative by other tests. As with previous workers (Dee and Rytel, 1975: Preisler et al., 1969: Rosner et al., 1971) Marier and Andriole (1978b) stressed the value of serial determinations of antibody titre. They showed that patients with candidosis had a rise in titre during the first two weeks of infection but they were still unable to distinguish systemic from local forms of candidosis. Some patients with candidosis remained antibody negative even by discontinuous CIE and it appears likely that the detection of circulating Candida antigens may be a more valuable diagnostic test in antibody-negative cases (Warren et al., 1977) than antibody tests of ever increasing sensitivity.

Danish workers have recommended the use of two dimensional crossed immunoelectrophoresis with intermediate gel as a sensitive and quantitative

method for the determination of Candida precipitins.

In this technique, individual antibody-antigen reactions are resolved as peaks, whose area is proportional to the relative concentrations of antibody and antigen. Axelsen (1976) has suggested that the number and size of these peaks - the precipitin score - varies with different forms of the candidal disease. Such as score may therefore be diagnostically significant for systemic candidosis. The two dimensional immunoelectrophoretic technique also allows the detection of free antigen in the serum in the same operation as antibody detection by inclusion of human serum in the intermediate gel and rabbit antiserum to Candida in the referance gel (Svendsen & Axelsen, 1972). Any free antigen in the human serum migrates into the rabbit antiserum resulting in a horizontal precipitation line when it reacts with its corresponding antibody (Axelsen & Kirkpatrick, 1973: Axelsen, Kirkpatrick & Buckley, 1974). Although this technique is very sensitive it takes longer to perform than CIE and it is also technically more demanding so that it still remains mainly a research tool.

Radioimmunoassay (RIA) is known to be under investigation as a sensitive technique for use in Candida serology although few formal reports on its use have yet appeared in the literature. In 1978, Weiner and Coates-Stephens reported the detection of mannan antigenaemia by RIA. However, circulating mannan was detected in the serum of only 15 out of 29 rabbits. In the few patients tested, mannan could be detected in 2 patients with systemic candidosis but not in superficially infected patients, in normals or in patients with other fungal infections. These results appear promising but obviously need to be carried out on a much larger sample of patients. A further report on the use of RIA has appeared recently (Huang et al., 1979) but again the authors carried out their experiments on the sera of infected rabbits so that the value of this test still cannot be clearly assessed for use in the diagnosis of systemic candidosis in humans. The main disadvantage with RIA is that it requires the use of a single, purified antigenic species for optimal results.

Enzyme linked immunoassay (ELISA) is an alternative to RIA which is just

as sensitive but cheaper and easier to use (Lancet, Editorial, 1976). The method, which is originally developed by Engvall and Perlmann (1971) can be applied to detection of antigen and antibody and is equally reproducible whether it is used in tests with single antigens or complex antigenic mixtures. Hommel et al., (1976) suggested the use of ELISA as a rapid and sensitive serodiagnostic test for the diagnosis of both candidosis and aspergillosis. Warren et al., (1977) found that the technique, as well as being efficient and simple, could be used to detect circulating Candida antigen earlier in the course of systemic infection than could CIE. They were also unable to demonstrate cross reactions with a number of bacterial organisms and toxins or with A.fumigatus. No further reports of this technique have yet appeared, although it is known to be under investigation.

Many authors have discussed the lack of standardization in the serological tests for diagnosis of candidosis and the problems this causes in assessing the diagnostic significance of precipitins to Candida (Axelsen, 1976: Marier and Andriole, 1978: Odds, 1979: Taschdjian et al., 1977).

Faux et al., (1975) compared four different extracts of C.albicans - all varying in their antigenicity - and four different double diffusion test protocols. They found that the number of positive precipitin reactions varied directly. A cooperative study between six laboratories examined three different serological tests using identical sets of coded sera, and again found substantially different number of false positive and false negative sera from test to test (Merz et al., 1977).

The results of these two studies clearly show that optimal conditions for performance of tests and introduction of standardized reagents both need to be established before serological tests for candidosis can improve their credibility. At the present time, it is clearly difficult to compare the significance of precipitins in different groups of patients when each worker uses a different method to obtain his or her results.

Several attempts have been made to select a diagnostically specific

antigen for use in serological tests for candidosis or to improve on the currently used cytoplasmic antigen by removal of non-specific components.

Axelsen's two dimensional crossed immunoelectrophoresis studies resulted in the demonstration of '11 deep candida infection specific' precipitins although no single precipitin was common to all cases of systemic infection (Axelsen, 1976).

Two groups of workers have attempted to find antigens specific to C.albicans hyphae as the filamentous form of C.albicans is more often associated with infection than colonization (Evans et al., 1973: Syverson et al., 1975: see discussion in section 1.5). Although antigenic components unique to the mycelial form have been demonstrated by both these groups of workers there have as yet appeared no clinical evaluations of their use in routine tests. Such antigens are, however, unlikely to be able to distinguish superficial from systemic infections, as for example, Budtz-Jörgensen (1971)has shown a high correlation between hyphae in oral smears with lesions of denture stomatitis.

Longbottom et al., (1976) and Syverson and Buckley (1977) have independently tried to increase the diagnostic specificity of Candida cytoplasmic extracts by removal of cell wall glycoproteins with concanavalin A. Four antigens were precipitated from soluble extracts of yeast and mycelial phases of C.albicans by concanavalin A. Two of these antigens were also present in extracts prepared from spheroplasts in a manner similar to that of Venezia and Robertson (1974). These components may have been cytoplasmic glycoproteins or easily solubilized cell wall material (Syverson and Buckley, 1977). Any cell wall material in cytoplasmic extracts appears to reduce the diagnostic specificity of the antigen. Tests with human sera have now shown that concanavalin A-treated cytoplasmic antigens reduced the number of false positive precipitin reactions and increase the predictive value of the precipitin test for systemic candidosis. 4.9% of patients with other fungal infections still reacted with the concanavalin A treated antigen; this must presumably reflect cross reactions with some cytoplasmic antigen other than a glycoprotein. 2% of patients in whom infection was absent still gave positive reactions against the

new antigen.

All the antigens described up to this point have been prepared from fungi grown in mycological broths. Such media are, at best, only poor analogues of the environment in which C.albicans multiplies when it invades tissues. It is therefore possible that hitherto undiscovered, inducible antigens might be found if the fungus were grown in media that contained tissue substances as sole sources of carbon and nitrogen.

In theory, it should be possible to grow C.albicans in tissues in vitro, but in practice such cultures are antigenically too complex to make isolation of single antigens a reasonable proposition. Instead it should be possible to grow Candida in a synthetic medium containing single macromolecular components of body tissue as carbon sources. Such a medium would allow easy identification and isolation of any hydrolytic enzymes produced.

Staib has acknowledged this point recently and his group has carried out preliminary tests in which culture filtrates of C.albicans grown with serum albumin as sole a nitrogen source have been used as antigens for the detection of precipitins (Staib et al., 1977a: Staib et al., 1977b). These culture filtrate antigens contain exocellular C.albicans proteinase enzyme but since no attempt was made by Staib et al., to purify this enzyme, it is not possible to determine whether antibody reactions to the filtrate were directed specifically at the proteinase or at other antigenic components.

The results obtained by Staib et al., with the crude proteinase culture filtrates do not so far demonstrate any great increase in diagnostic specificity of these preparations over cytoplasmic extracts. However, the principle that purified, inducible, exocellular enzymes of C.albicans, such as the proteinase, may be valuable as antigens specific for the detection of antibodies in systemic candidosis, remains untested. The experimental work described in this thesis was therefore designed to investigate the use of purified, inducible antigens of C.albicans as serodiagnostic reagents. The investigation was planned to proceed in four stages:

1) determination of the range of macromolecular tissue substrates that C.albicans could utilize as growth sources.

2) assay for appropriate enzymes induced in C.albicans by macromolecular growth sources

3) purification and characterization of any enzymes discovered

4) assessment of the purified enzymes as antigens for serological diagnosis of candidosis

The details of the investigation are described in the following chapters.

Chapter 2.

GROWTH OF C.ALBICANS WITH TISSUE MACROMOLECULES AS SUBSTRATES

2.1 Introduction

The examination of inducible enzymes, produced by C.albicans, must begin with the determination of the ability of the yeast to grow on a number of tissue macromolecules as either carbon and/or nitrogen sources.

2.2 Materials and Methods

Strains: C.albicans ATCC 28366, originally isolated from the mouth of a hospital patient was the strain used principally throughout the study. In some experiments other isolates of C.albicans and other species of Candida were used. These were:-

		Site of Isolation
C.albicans	FCO	mouth
	G.L.	"
	B.J.	"
	D.K.	"
	F.M.	"
	R.C.	"
	73/075	rectal swab
	/025	vaginal swab
	/054	mouth
	/004	vaginal swab
	/042	mouth
	/032	mouth
	/031	mouth
	/030	mouth
	/038	mouth
	/039	mouth
	P.L.	mouth
	S.B.	mouth
C.stellatoidea	73/014	-
C.tropicalis	73/006	-
C.parapsilosis	73/096	vagina
C.pseudotropicalis	73/096	rectum
C.krusei	73/102	mouth
C.guilliermondii	73/044	vagina
C.glabrata	73/069	vagina
Rhodotorula rubra	74/043	
C.intermedia	73/033	rectum
C.norvegensis	73/070	mouth

The fungi were maintained on slopes of Sabouraud Dextrose Agar (SDA - Difco) and all inocula were prepared from yeasts grown for 18-24h at 37^{o}C on this medium.

A yeast suspension for this study was prepared as follows. Cells were

washed from slopes of SDA with phosphate buffered saline (PBS), centrifuged at 750 x g for 15 min and resuspended in sterile distilled water. The yeast cell density was estimated by haemocytometer counts and C.albicans was routinely added to the growth medium to give an initial yeast concentration of 10^6/ml.

Medium A synthetic growth medium was used for all experiments into the ability of C.albicans to use a variety of enzyme substrates as carbon and nitrogen sources (Table 2).

Table 2. Synthetic growth medium

Carbon source	5g/l
Nitrogen source	0.01M (NH_4SO_4)/5g/l others
Na_2 H PO_4	0.0005M
Mg SO_4 $7H_2O$	0.001M
NaCl	0.001M
$CaCl_2$	0.001M
K I	0.001M
biotin	20µg/l
nicotinic acid	400µg/l
pyridoxal hydrochloride	400µg/l
riboflavin	200µg/l
thiamine hydrochloride	400µg/l

Media were buffered with 0.01M citrate in the pH range 3-7 and with 0.01M tris in the pH range 7-10. These substances were not utilized by C.albicans at pH 5.6 (citrate) and 7.5 (tris).

The medium was sterilized by membrane filtration of a 10-fold concentrate, which was diluted with sterile distilled water for use.

For all growth experiments 21 flasks containing 11 cultures were incubated at 37°C with gyrotary shaking at 160rpm.

Determination of Growth

Growth of C.albicans was measured by ATP photometry. A sample of culture or a standard solution of ATP (0.5ml) was added to 5ml of 0.02M tris HCl buffer. pH 7.8 that had been pre-incubated in a boiling water broth. The mixtures were boiled for 4min then cooled rapidly in ice-water. A Lab-Line Instruments Inc bioluminescence photometer was used for estimation of ATP. Fire-fly lantern

extract (Sigma), reconstituted in water, was used as a source of luciferase enzyme: 0.1ml quantities of sample and luciferase solution were mixed and the resulting luminescence was integrated for 60s after a 15s delay. All readings were corrected for endogenous luminescence by subtraction of the reading for a control containing tris buffer and luciferase alone.

Detection of Enzyme activities

A preliminary investigation of the enzymes present in C.albicans ATCC 28366 or secreted into the medium was made by inoculation of the fungus into APIZYM strips (API system, S.A., Montalieu Vercieu, France) or onto test agars routinely used in bacteriology. The API system was a semi-quantitative method designed to detect rapidly the activities of nineteen enzymes. The strip was composed of twenty cupules - one of which was a control, 19 of which contained enzyme substrates. It was prepared by inoculation of each cupule with two drops of the specimen - either a suspension of C.albicans blastospores or a 4-fold concentrated culture filtrate - and incubated in a humid plastic tray for 4h at $37^{o}C$.

The strip was developed and recorded according to the manufacturer's instructions. The substrates and enzymes assayed for, are shown in Table 3.

The agars used to detect enzyme activity were serum protein agar (Staib, 1965) lecithin agar as described by Chrisope et al., (1976) modified by the omission of glucose, tributyrin agar (Difco) and DNase test agar (Difco).

Other enzymes were assayed as follows: hyaluronidase by the method of both Di Ferrante (1956) and Linker (1966 and 1974); phospholipase by the method of Price and Cawson (1978) modified by use of phosphatidyl [N-methyl-^{14}C] choline (Radiochemical Centre, Amersham) as substrate instead of phosphatidyl [N-methyl-^{3}H] choline; proteinase by the method of Remold et al., (1968) modified by the determination of the amount of proteolysis by measuring the absorbance of the filtrate at 280nm against a citrate buffer blank. Samples for enzyme assay were either culture filtrates or aqueous suspensions of washed C.albicans cells. Culture filtrates were concentrated before assay by ultrafiltration with Diaflo

Table 3. Enzyme substrates included in APIZYM strips

NO	ENZYME ASSAYED FOR	SUBSTRATE	pH
1	Control		
2	Phosphatase, alkaline	2-naphtyl phosphate	8,5
3	Esterase (C 4)	2-naphtyl butyrate	6,5
4	Esterase Lipase (C 8)	2-naphtyl caprylate	7,5
5	Lipase (C 14)	2-naphtyl myristate	"
6	Leucine arylamidase	L-leucyl-2-naphtylamide	"
7	Valine arylamidase	L-valyl-2-naphtylamide	"
8	Cystine arylamidase	L-cystyl-2-naphtylamide	"
9	Trypsin	N-benzoyl-DL-arginine-2-naphtylamide	8,5
10	Chymotrypsin	N-glutaryl-phenyl-alanine-2-naphtylamide	7,5
11	Phosphatase, acid	2-naphtyl phosphate	5,4
12	Phosphoamidase	Naphtol-AS-BI-phospho-diamide	"
13	α galactosidase	6-Br-2-naphtyl-α-D-galactopyranoside	"
14	β galactosidase	2-naphtyl-β D-galacto-pyranoside	"
15	β glucuronidase	Naphtol-AS-BI-β D-glucu-ronate	"
16	α glucosidase	2-naphtyl-α D-glucopyra-noside	"
17	β glucosidase	6-Br-2-naphtyl-βD-gluco-pyranoside	"
18	N-acetyl-β glucosaminidase	1-naphtyl-N-acetyl-β D-gluco-saminide	"
19	α mannosidase	6-Br-2-naphtyl-αD-manno-pyranoside	"
20	α fucosidase	2-naphtyl-αL-fuco-pyranoside	"

DM5 or PM10 membranes (Amicon Corp).

2.3 Results

The APIZYM strips indicated that alkaline phosphatase, C4 esterase, C8 esterase/lipase, leucine, valine and cystine aminopeptidase, acid phosphatase, phosphoamidase and glucosaminidase were all present in C.albicans after 24h and 48h of growth in Sabouraud's broth (Fig. 1). No enzymes were detected in unconcentrated or concentrated filtrates

Phospholipase activity was detected in C.albicans ATCC 28366 on lecithin agar after 10 days growth but only 25% of other strains showed any activity (Table 4).

After 48h, 82% of C.albicans strains tested, including ATCC 28366, showed detectable proteinase activity and by 5 days ATCC 28366 and the 3 other strains tested, had caused clearing of tributyrin agar, indicative of lipase and/or proteinase activity (Table 4).

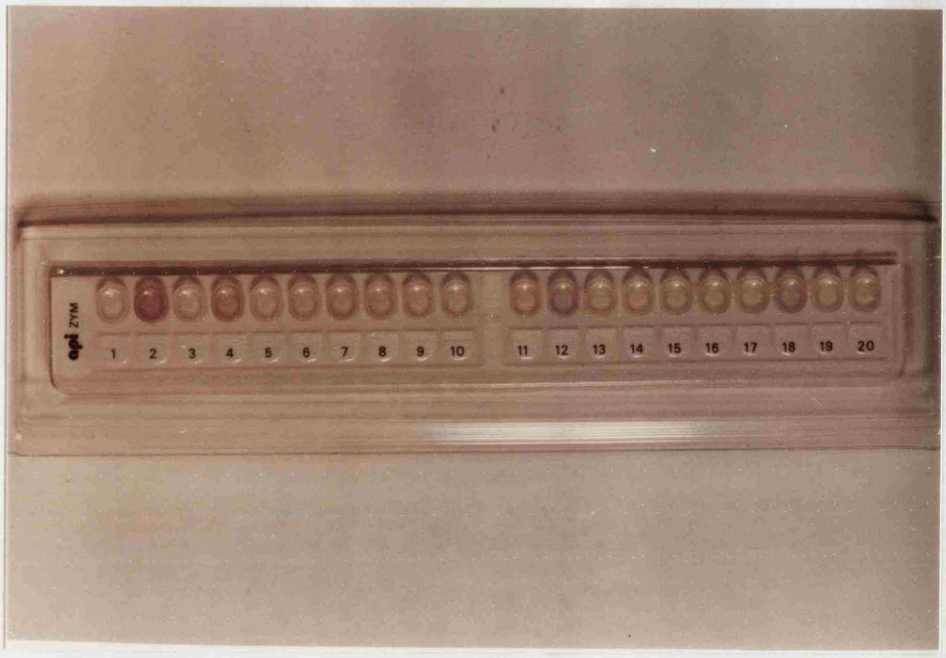
Preliminary tests showed that ATP photometer readings varied linearly with ATP concentration from 1µg/ml to 1ng/ml and with number of viable yeast cells in C.albicans suspension (Table 5). Because of the greater sensitivity of the ATP method over direct counts and OD550 readings, this method was used for all subsequent growth experiments.

Growth of C.albicans in media with macromolecular carbon and/or nitrogen sources

The ability of C.albicans to utilize various macromolecules as carbon and/or nitrogen sources was determined from ATP photometer readings made after 24h and 48h incubation.

A high yield of viable C.albicans was obtained in control cultures with glucose as the carbon source at all pH values tested except 9.5 (Table 6). Some growth was observed at pH 3.5 in the absence of nitrogen, probably due to carry-over of nutrients from the inoculating slope or a trace of nitrogenous compounds in the medium.

Fig. 1 Enzyme profile of C.albicans blastospores grown on SDA



The APIZYM strip was inoculated with a suspension of C.albicans blastospores, incubated for 4h at 37°C then developed as described in materials and methods. Weak enzyme activity was detected in cupules 2, 3, 4, 6, 7, 8, 11, 12 and 18.

Table 4. Test Agars for detection of Enzymes

Agar	Enzyme	Result
Serum protein	Proteinase	+
Tributyrin	Proteinase or Lipase	+
Lecithin	Phospholipase	+
DNase	DNase	-

Table 5. Relationship of ATP photometer reading to Viable Count and to ATP concentration.

ATP conc		ATP reading
10^{-6}	1µg/ml	81667
10^{-7}	100ng/ml	8380
10^{-8}	10ng/ml	622
10^{-9}	1ng/ml	69
10^{-10}	100pg/ml	54
10^{-11}	10pg/ml	36

No. of blastospores	OD550	ATP reading
Too high to count ($>10^6$)	1.34	146445
7×10^5	0.76	76284
4×10^5	0.48	38509
2×10^5	0.38	26645
1.8×10^5	0.30	17790
1×10^5	0.24	13178

ATP standards of varying concentrations were assayed by means of the ATP spectrophotometer. Serial dilutions of suspensions of C.albicans blastospores were counted with a haemocytometer, the OD550 was determined and each dilution was also assayed with the ATP spectrophotometer as described in materials and methods.

The first enzyme substrate examined as a possible carbon and nitrogen source was bovine serum albumin (BSA, fraction V, Sigma). The highest cell yield was obtained after 24h with $(NH_4)_2\ SO_4$ as nitrogen source at pH 3.5 (Table 7). In the absence of a low molecular weight nitrogen source, there was a lower yield of C.albicans at pH 3.5 and 5.5. At 48h there was evidence of cell death in cultures containing BSA and $(NH_4)_2\ SO_4$.

A little growth of C.albicans was apparent with chondroitin sulphate (Grade III, Sigma) as carbon source and $(NH_4)_2\ SO_4$ as nitrogen source at pH 3.5 and 5.5. but not at higher pH values (Table 8). In the absence of $(NH_4)_2\ SO_4$, there was some growth at pH 3.5 but again the yield was low.

Table 6. Relative biomass of C.albicans on synthetic medium with glucose as single Carbon source.

Nitrogen source	pH	ATP Spectrophotometer reading at 0h	24h	48h
NH_4^+	3.5	647	6575	638
	5.5	740	7657	2526
	7.5	1404	4915	1103
	9.5	684	187	321
None	3.5	236	1356	1523
	5.5	260	714	1706
	7.5	193	557	631
	9.5	292	801	804

Samples were removed from cultures at 0, 24 and 48h for ATP spectrophotometry.

Table 7. Relative biomass of C.albicans on synthetic medium with BSA as single Carbon source.

Nitrogen source	pH	ATP Spectrophotometer reading at 0h	24h	48h
NH_4	3.5	677	7425	2720
	5.5	798	3325	1716
	7.5	676	320	407
	9.5	1267	269	0
None	3.5	193	2467	3668
	5.5	126	516	1329
	7.5	-	-	-
	9.5	199	240	334

Table 8. Relative biomass of C.albicans on synthetic medium with chondroitin sulphate as single carbon source.

Nitrogen source	pH	ATP Spectrophotometer reading at 0h	24h	48h
NH_4	3.5	771	1012	911
	5.5	676	1112	513
	7.5	519	456	71
	9.5	556	393	60
None	3.5	332	1245	1375
	5.5	113	368	334
	7.5	217	360	216
	9.5	235	279	193

Table 9. Relative biomass of C.albicans on synthetic medium with erythrocyte ghosts as single carbon source

Nitrogen source	pH	oh	ATP Spectrophotometer reading at 24h	48h
NH_4	3.5	624	1864	361
	5.5	884	1568	851
	7.5	788	215	167
	9.5	759	222	213
None	3.5	240	1175	1810
	5.5	187	461	550
	7.5	227	127	284
	9.5	124	153	399

A suspension of erythrocyte ghosts prepared from human blood was repeatedly centrifuged at 750 x g for 30 min and washed with distilled water. The pellet was resuspended to the original physiological concentration with water then added to the medium at a concentration of 10% (v/v).

Similar results were obtained when C.albicans was grown with erythrocyte ghosts as carbon and/or nitrogen sources (Table 9). Growth in the presence of ammonium was apparent only at pH 3.5 and 5.5, and when the erythrocyte membranes were added as sole carbon and nitrogen sources an increase in yield was recorded at pH 3.5 only.

These preliminary tests were extended by measurement of growth rates and cell yields obtained in different media. pH 3.5 was selected as optimal for growth of C.albicans in cultures based on BSA, chondroitin sulphate and erythrocyte ghosts and pH 5.5 for cultures containing glucose and $(NH_4)_2SO_4$. Samples were removed from cultures at frequent intervals for ATP spectrophotometry and the data were plotted as growth curves for C.albicans. Growth of C.albicans at pH 5.5 and 37°C with glucose as carbon source was rapid with a maximum growth rate of $0.74h^{-1}$ (Fig. 2).

The most rapid growth for cultures based on BSA occurred in the presence of both BSA and ammonium with a maximum growth rate of $0.8h^{-1}$, but death also occurred rapidly after about 15h, perhaps because some autoinhibitory substance was produced by the fungus in the presence of ammonium ions. When BSA was the sole carbon and nitrogen source the growth rate was slower ($0.15h^{-1}$) and the yield less than in the presence of NH_4^+ (Fig. 3). Addition of glucose as a carbon source led to an increase in yield (Fig. 3) close to that of control, glucose based cultures (Fig. 2) and also to an increase in growth rate ($0.27h^{-1}$) over cultures containing BSA as sole carbon and nitrogen source, and no decrease in viability was noted for cells in the stationary phase of such cultures.

When chondroitin sulphate was used for a carbon source in the presence of ammonium,a high yield of C.albicans and a rapid growth rate ($0.8h^{-1}$) was obtained (Fig. 4). There was a lag phase of approximately 9h before growth was detected, which suggested the possible involvement of an inducible enzyme that had to be produced to degrade chondroitin sulphate before growth could start. When chondroitin sulphate was used as a carbon and nitrogen source or as a nitrogen source with glucose as carbon source,there was little detectable increase in cell yield (Fig. 4).

Fig. 2

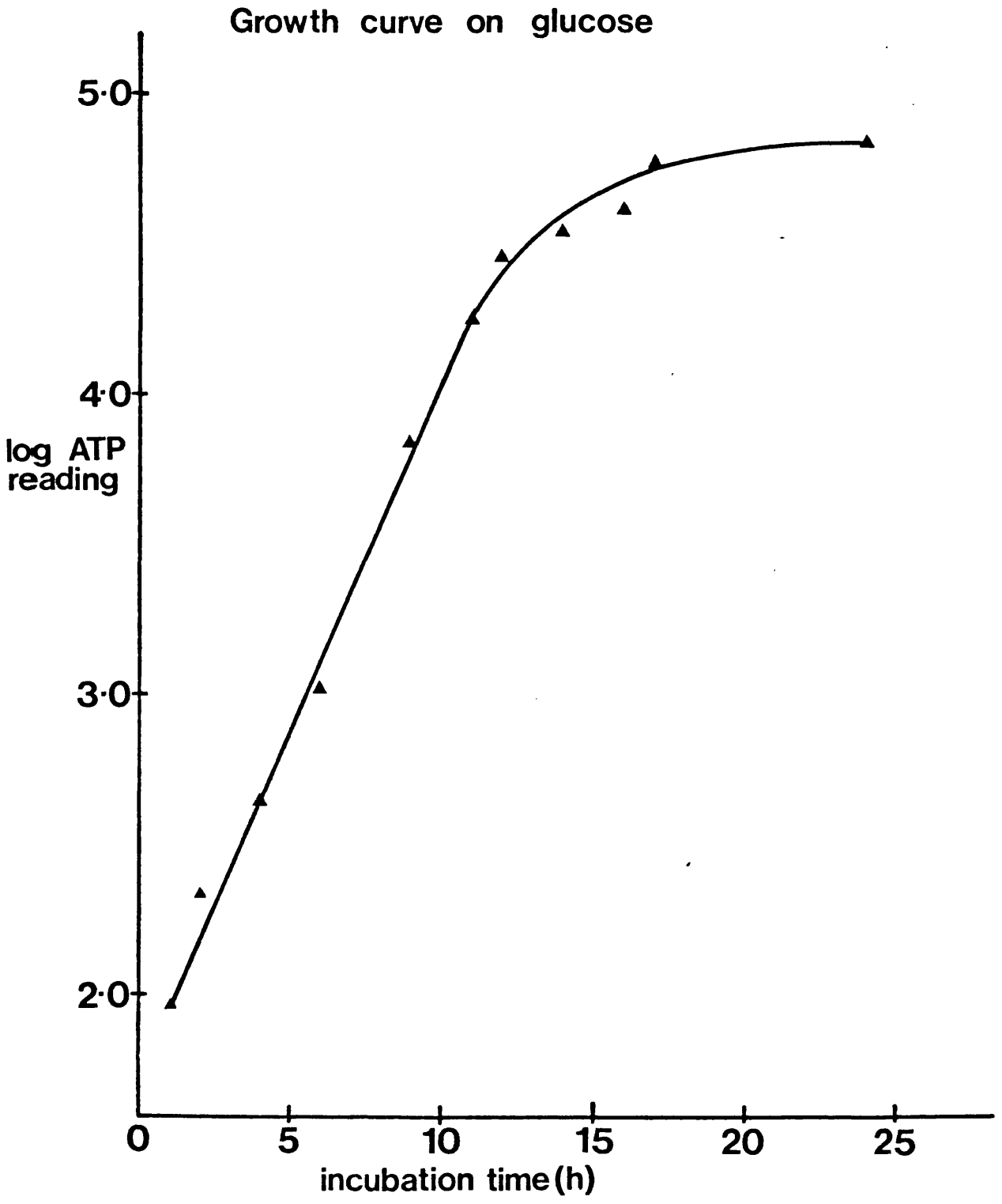


Fig. 2, 3, 4, 5 are each typical representatives of 4 separate experiments

Fig. 3

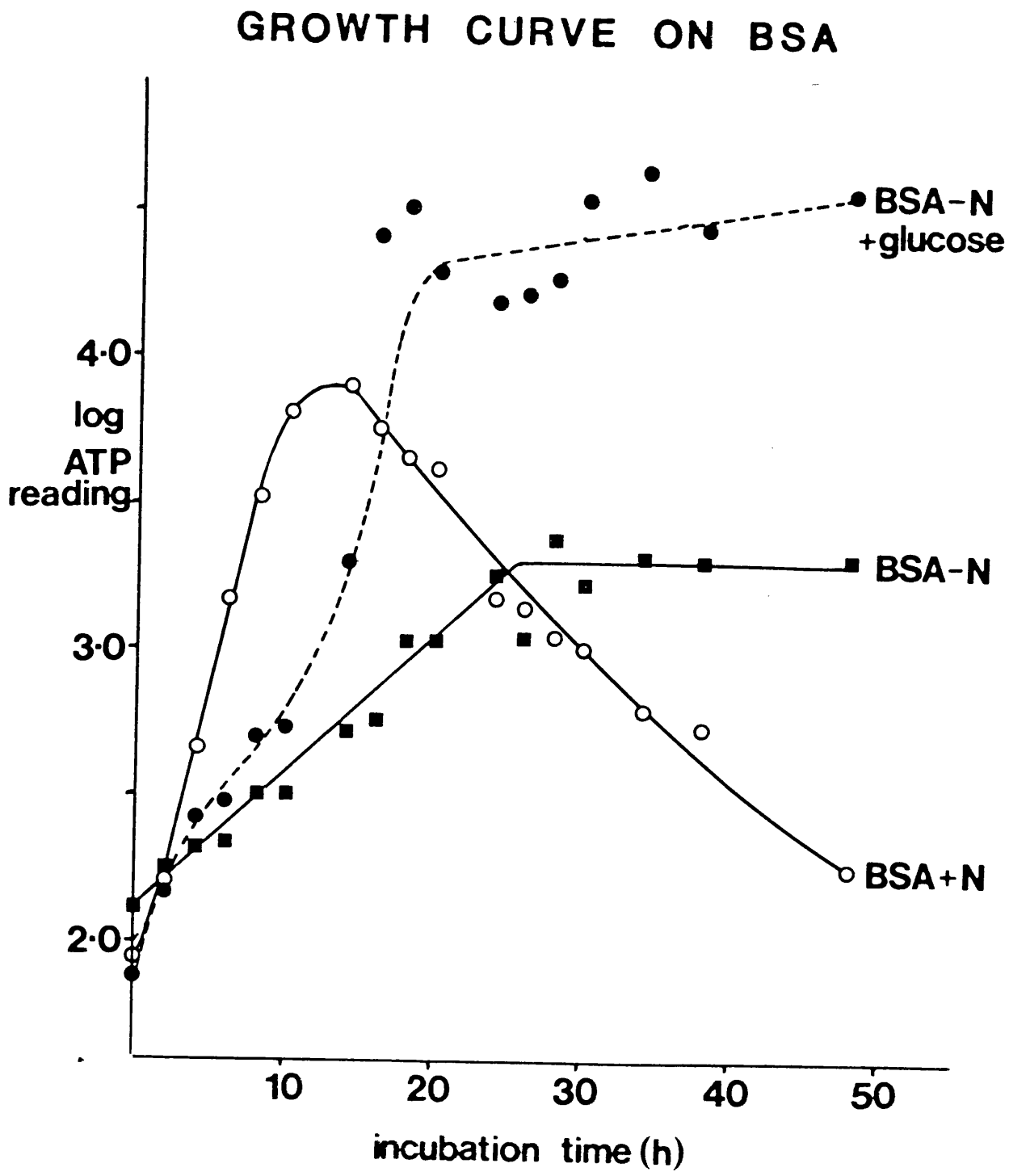
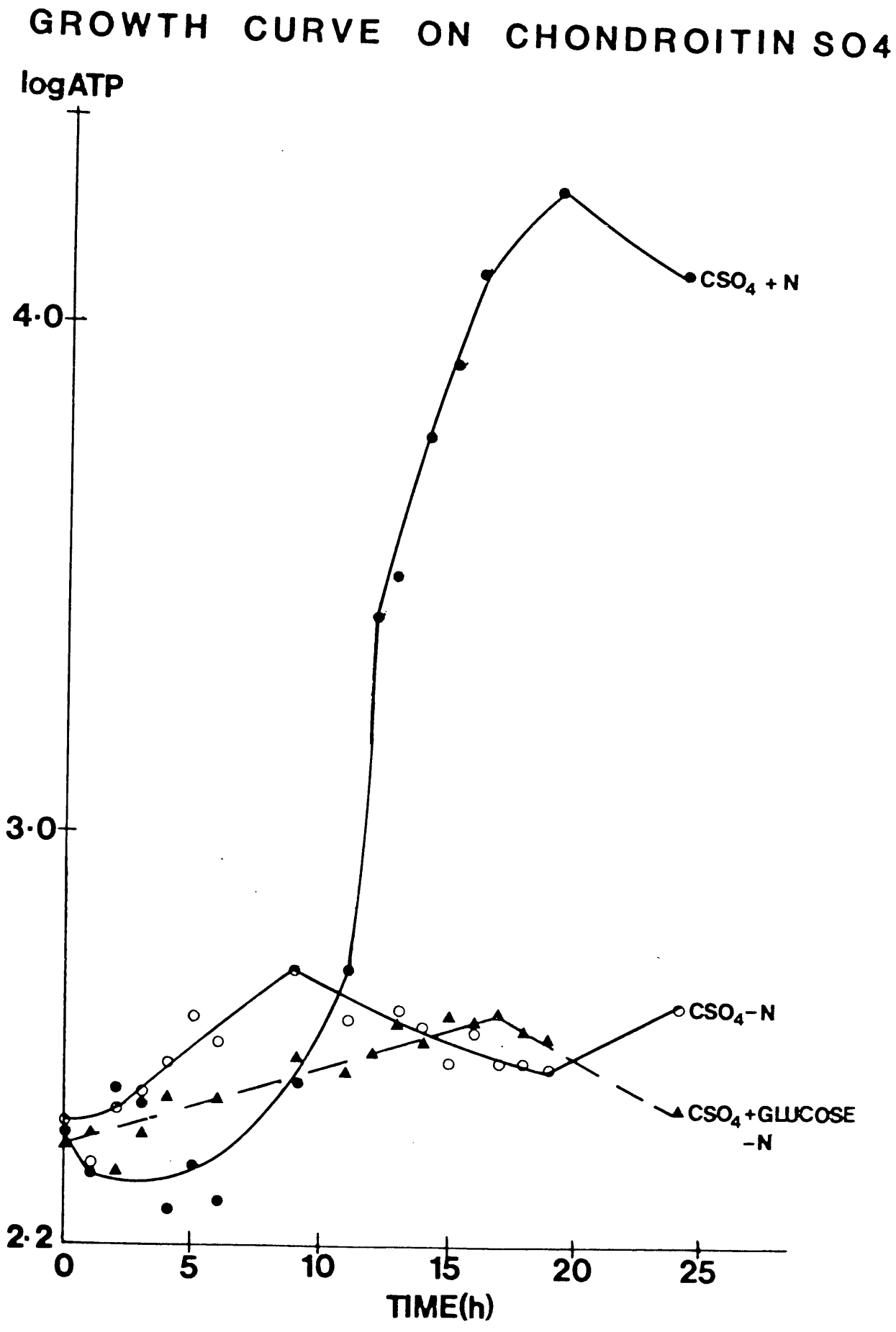

GROWTH CURVE ON BSA
BSA-N
+glucose
BSA-N
BSA+N
4·0
log
ATP
reading
3·0
2·0
10
20
30
40
50
incubation time (h)

Fig. 4.


GROWTH CURVE ON CHONDROITIN SO4
logATP
4·0
3·0
2·2
0
5
10
15
20
25
TIME(h)
CSO4 + N
CSO4 - N
CSO4 + GLUCOSE
-N

Growth curves for C.albicans with erythrocyte ghosts as carbon sources showed that a high yield - almost as high as with BSA - and a high growth rate ($0.5h^{-1}$) were obtained by 24h with ammonium sulphate as a nitrogen source (Fig. 5). However, numbers of viable cells declined rapidly after 48h. There was a detectable lag phase of about 15h before growth started. There was very little growth when erythrocyte ghosts were the sole source of carbon and nitrogen (Fig. 5).

Since Staib (1966) had previously reported that buffering of the growth medium resulted in decrease in proteolytic enzyme activity produced by C.albicans, growth curve experiments were repeated with the same carbon sources in unbuffered synthetic media. Although similar yields were obtained in media based on glucose and BSA, this time there was no detectable growth in chondroitin sulphate - or erythrocyte ghost-based media. The ability of C.albicans to utilize citrate buffer as carbon source was therefore re-examined and it was found that similar yields of yeast were obtained at pH 3.5 with citrate buffer or with chondroitin sulphate or erythrocyte ghosts in citrate-buffered media (Tables 8, 9 & 10). The same pattern of growth at pH 3.5 and 5.5 - an increase in biomass at 24h followed by a fall in biomass at 48h - was evident for all cultures that contained citrate and $(NH_4)_2SO_4$, irrespective of the presence of chondroitin sulphate or erythrocyte ghosts.

Hydrolytic enzyme production by C.albicans in synthetic medium with macromolecular carbon and/or nitrogen sources

Hyaluronidase was not detected in blastospores obtained at 24h, 48h, 72h or 120h from cultures that contained chondroitin sulphate nor was any activity present in culture filtrates of the same age. Since assays for hyaluronidase may sometimes give equivocal results (F.C. Odds, personal communication) the assays were conducted by three methods with negative results in all cases.

Fig. 5

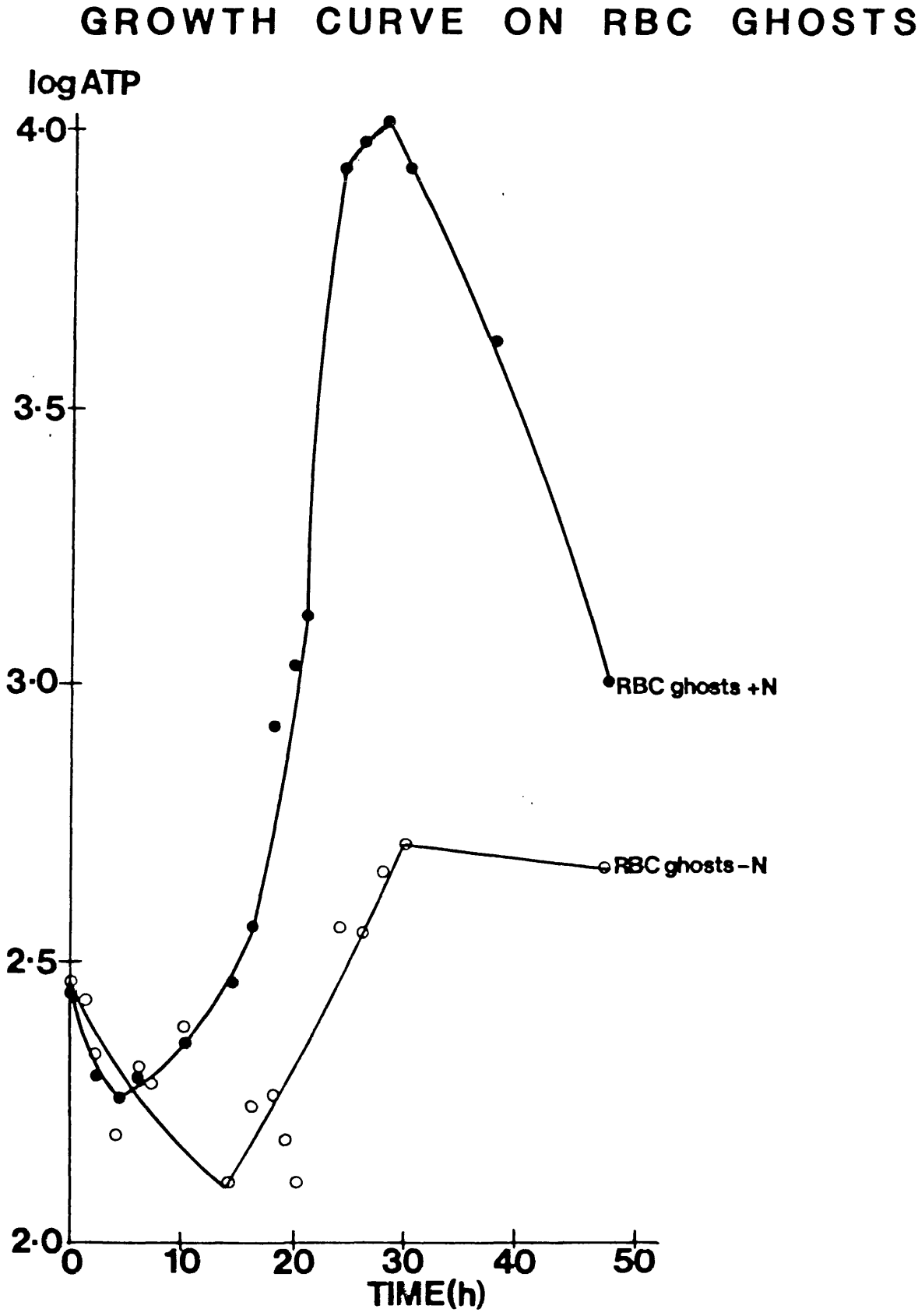

GROWTH CURVE ON RBC GHOSTS
log ATP
4·0
3·5
3·0
2·5
2·0
0
10
20
30
40
50
TIME(h)
RBC ghosts +N
RBC ghosts -N

Table 10. Relative biomass of C.albicans on synthetic medium with citrate buffer as sole carbon source

Nitrogen source	pH	oh	ATP Spectrophotometer reading at 24h	48h
NH_4^+	3.5	771	3201	758
	5.5	735	2377	341
	7.5	682	225	165
	9.5	425	93	280

No phospholipase activity was detected in either blastospores or culture filtrates from 24, 48, 72 or 120h cultures based on erythrocyte ghosts. Addition of calcium ions - known to stimulate phospholipase activity - and sonication of the lecithin substrate for periods up to 30 min, which makes it more susceptible to enzyme attack (Price and Cawson, 1977) did not result in the detection of phospholipase activity. Attempts were made to assay phospholipases in C.albicans grown in YNB according to the procedures described by Price and Cawson (1977) but no enzyme activity was detected despite 4 repetitions of the experiments. As apparent phospholipase activity had been detected around colonies of C.albicans grown on lecithin agar, the phospholipase activity of blastospores grown in a liquid version of this medium was examined but, again, no enzyme activity could be detected.

The absence of detectable hyaluronidase or phospholipase activity confirmed the inability of C.albicans to grow on either of the two appropriate enzyme substrates. Further examination of these two enzymes was therefore abandoned.

Proteinase activity was detected in greatest amounts (OD_{280} of 0.43/ml) in 4 fold concentrated culture filtrates after 5 days growth in cultures based on glucose and BSA.

2.4 Discussion

Although several hydrolytic enzymes were detected by means of APIZYM strips, these were not studied further in the present investigation since there was insufficient time. Several of the enzymes - acid phosphatase, alkaline phosphatase and leucine aminopeptidase have already been noted in the literature (Odds & Hierholzer, 1973; Chattaway et al., 1971; Kim et al., 1962) but esterases have received little attention so far and it may be of interest to study these further in the context of C.albicans pathogenicity.

The role of hyaluronidases in the pathogenicity of some bacteria has been described previously (Mims, 1977). In Staphylococci, where the enzyme has been most fully studied, the hyaluronidase does not possess toxic properties itself but appears to act as a 'spreading factor', breaking up

the connective tissue matrix and so permitting easier invasion by bacteria or viruses (Topley & Wilson, 1975). However, no inducible hyaluronidase was detected in C.albicans under the conditions employed in the present study; these findings confirm and amplify those of Chattaway et al., (1971) who could find no constitutive hyaluronidase in their investigation of C.albicans grown in a mycological broth.

A similarly negative result was obtained for phospholipases in both this study and that of Chattaway et al., (1971). However, constitutive phospholipase A was reported in C.albicans by Price and Cawson (1978). Attempts to reproduce the findings of Price and Cawson have proved unsuccessful, but irreproducibility in investigations with C.albicans is not an unknown phenomenon (Odds et al., 1978). It may be that phospholipase is a strain-specific enzyme, in which case its role in the pathogenicity of the organism is difficult to assess. Although Price (1977) suggested that phospholipases were secreted into the medium - based on an experiment which demonstrated the clearance of egg yolk agar around colonies of C.albicans - it is probable that extracellular proteinase was the cause of the reaction in this case, since proteolytic enzyme are known to cause clearance of egg yolk agar and because later experiments by Price and Cawson (1977) were unable to demonstrate extracellular phospholipase activity.

Proteinase was the only definite inducible extracellular enzyme detected in the present study. Its role in the pathogenicity of C.albicans was therefore studied further.

Chapter 3.

PURIFICATION AND CHARACTERIZATION OF PROTEINASE

3.1 Introduction

All the antigenic extracts previously described for the serological diagnosis of candidosis have been complex mixtures of compounds. The antigenic complexity of Candida necessarily leads to a wide ranging antibody response so that antigen-antibody reactions specific for deep-seated are likely to be obscured by the numbers of less-specific reactions. Therefore before any inducible hydrolytic enzyme is tested as an antigen, it should ideally be purified and used as a homogenous antigenic component in the serological tests rather than one of several components of the culture filtrate.

For this reason the next logical step in the investigation of the antigenic properties of C.albicans proteinase is its purification. Remold et al., (1968) have described a purification method for C.albicans proteinase but this contains several steps, takes several days to perform, leading to a loss of enzymatic activity, and it is not suitable for the preparation of large batches of enzyme. If proteinase is to be used routinely in laboratories, it would be an advantage to have a rapid purification method which could be used in large scale production of the enzyme.

Although growth of C.albicans in synthetic medium with glucose as carbon source and BSA as nitrogen source resulted in a high, stable yield of viable cells with detectable proteinase activity in the culture filtrate, it was suggested by Staib (1965) that buffering of the medium reduced the amount of enzyme produced by the fungus. An unbuffered medium was therefore tested to determine which medium resulted in the highest yield of proteinase.

3.2 Materials and Methods

Medium The medium eventually used for large scale proteinase purification was essentially that of Remold et al., (1968) modified by the use of a vitamin

solution of the same composition as in the synthetic medium instead of the 'Protovita' commercial preparation.

Proteinase purification: Proteinase was purified from the culture filtrate after 7 days growth of C.albicans at 26°C when the enzyme activity was at a maximum and the pH had fallen to 2.9. At this stage C.albicans cells were removed from cultures by centrifugation (5000 x g, 30 min). The culture filtrate was passed through 0.8 µm membrane filters to ensure removal of any remaining yeast cells. The pH of the filtrate was then adjusted to 6.5 with 3M NaOH.

Dry DEAE - Sephadex A-25-120 (Pharmacia) was added to the filtrate to a concentration of 20g/l. The mixture was shaken and left overnight at 4°C to ensure adsorption of all the enzyme and to allow the Sephadex to swell completely. The DEAE - Sephadex was removed by filtration on a Buchner funnel and transferred to a glass column to give a final bed size of 30 x 5.5 cm. No detectable proteina activity remained in the supernatant fluid.

Proteins were eluted from the column with batches of sodium citrate buffer, pH 6.5, of increasing ionic strength. 50ml fractions were collected at a flow rate of 25ml/min. The absorbance of each fraction at 280nm was determined against a citrate buffer blank and fractions containing UV absorbing material were assayed for proteinase activity by the method described in Chapter 2. The enzyme-rich fractions were pooled and concentrated 7-12 fold by ultrafiltration with DM5 Diaflo membranes (Amicon Corp.).

Brij 35 solution (100g/l) was added to the enzyme solution at a concentration of 2% (v/v) to stabilize the enzyme, which was then sterilized by membrane filtration and stored in plastic bottles in 5ml lots at 4°C.

Protein Measurement

Protein concentrations below 1mg/ml were determined by the method of Lowry et al., (1951). Spectrophotometric readings were made at 500nm and converted to µg protein/ml with a BSA standard curve. Protein concentrations above 1mg/ml were determined by the Biuret method (Gornall et al., 1949).

Carbohydrate Analysis

Samples were hydrolysed for 3h with 5N HCl at 100°C then dried *in vacuo* over phosphorus pentoxide and sodium hydroxide. Sugars in the hydrolysed sample were identified by 2 dimensional thin layer chromatography (TLC: Hotta & Kurokawa, 1968). Total hexose concentration was determined spectrophotometrically with anthrone reagent (Spiro, 1966): glucose and mannose were used as hexose standards.

Cytoplasmic Antigen Preparation

Cytoplasmic antigen was prepared from *C.albicans* ATCC 28366 which was grown for 48h at 37°C in Sabouraud's broth. Cells were harvested (MSE Coolspin: 8 min, 5000 x g) and washed twice with PBS then disrupted on a Dynomill to 95% breakage. The suspension was centrifuged at 20,000 x g for 1h then passed through 0.8 µm Millipore filter. The filtrate was then centrifuged at 30,000 x g for 1h then concentrated 4 fold by ultrafiltration with PM 10 membranes (Amicon Corp.). The concentrate was ultracentrifuged at 250,000 x g for 6h and the supernatant dialysed against PBS with PM 10 membranes. The gelatinous precipitate was redissolved in 100ml of PBS.

Polyacrylamide Gel Electrophoresis

Samples of purified proteinase (PP) were examined for purity by polyacrylamide gel electrophoresis (PAGE: Davies, 1964). Samples containing protein were prepared in sucrose (400g/l) with bromophenol blue as a marker and applied to 10% gels in tubes. Gels were electrophoresed at a constant current of 5mA per gel for 3h. They were stained overnight for protein with Page blue 83 (BDH: 2.5g/l) in a solution of trichloroacetic acid (50g/l in methanol/water 1:1) and destained with the dye-free solvent.

Samples of purified proteinase and *C.albicans* cytoplasmic antigen were compared by horizontal slab gel electrophoresis in a 10% gel. These gels were run at a constant current of 40mA for 5h then stained and destained as above.

Enzyme Inhibition

The effect of a variety of inhibitors on proteinase activity was investigated. The inhibitors were added to 0.5ml of enzyme solution from tenfold concentrated stock solutions to give the following final concentrations, dithiothreitol plus

EDTA (each 2mM), pepstatin (10 µg/ml: Sigma), phenanthroline (1mM), phenylmethyl sulphonyl fluoride (1mM), diazoacetylnorleucine methyl ester plus Cu^{2+} (each 1mM) and trypsin inhibitor (100 µg/ml, from soyabean: Sigma). The mixtures were pre-incubated for 30 min at 37°C, then activity was assayed as described in Chapter 2.

Serological tests

Conventional immunoelectrophoresis and crossed immunoelectrophoresis by the microplate method were carried out by methods previously described (Grabar and Williams, 1953: Weeke, 1973). Tandem crossed immunoelectrophoresis of proteinase and cytoplasmic antigens was performed as described by Krøll (1973). Antisera raised against the two antigens were compared by crossed immunoelectrophoresis with intermediate gel (Axelsen, 1973b).

Antisera

Antiserum to proteinase was raised in New Zealand White rabbits by a schedule that involved cutaneous and intravenous inoculations. For subcutaneous injection, equal volumes of PP solution (23mg protein/ml) and Freund's incomplete adjuvant were emulsified by ultrasonication. 0.5ml lots were administered twice weekly for 3 weeks. For intravenous inoculations, equal volumes of proteinase and a latex particle suspension (diluted 1:20 : Difco) were mixed and 0.5ml was injected weekly for 3 weeks. The animals were bled from the marginal ear vein 7 days after the last inoculation. The resulting serum was stored at -20°C before use.

Antiserum to CE antigen was raised by a schedule of subcutaneous inoculation only. Equal volumes of CE (20mg protein/ml) and Freund's incomplete adjuvant were emulsified by ultrasonication. 0.5ml lots of the emulsion were then given subcutaneously to rabbits twice weekly for 5 injections. Animals were bled 7 days after the final inoculation.

Precipitation of Proteinase with Anti-PP

Different volumes of proteinase antigen (23mg protein/ml) in the range 0-25µl were added to 250µl of anti-PP antiserum. Total volumes were kept constant at 500ul by addition of PBS. The tubes containing the antibody/antigen mixture were vortex mixed and incubated at 37°C for 1h then at 4°C for

48h.

The extent of precipitation was determined by measurement of the absorbance of the mixtures at 550nm against a PBS blank. The precipitate was removed by centrifugation and resuspended in 0.5ml of PBS - the proteinase activity in both the supernatant and precipitate were assayed as described in Chapter 2.

3.3. Results

Purification of proteinase

Maximum proteinase activity was detected after 168h at 26°C in the unbuffered medium at which time the highest viable cell yield was also achieved (Fig. 6). Although the growth curve at 37°C was similar to that at 26°C, enzyme activity was less at this temperature (Fig. 7).

C. albicans blastospores were also assayed for proteinase activity. After 5 days growth an assay of 0.07/ml was found compared with 0.16/ml for the culture filtrate (unconcentrated).

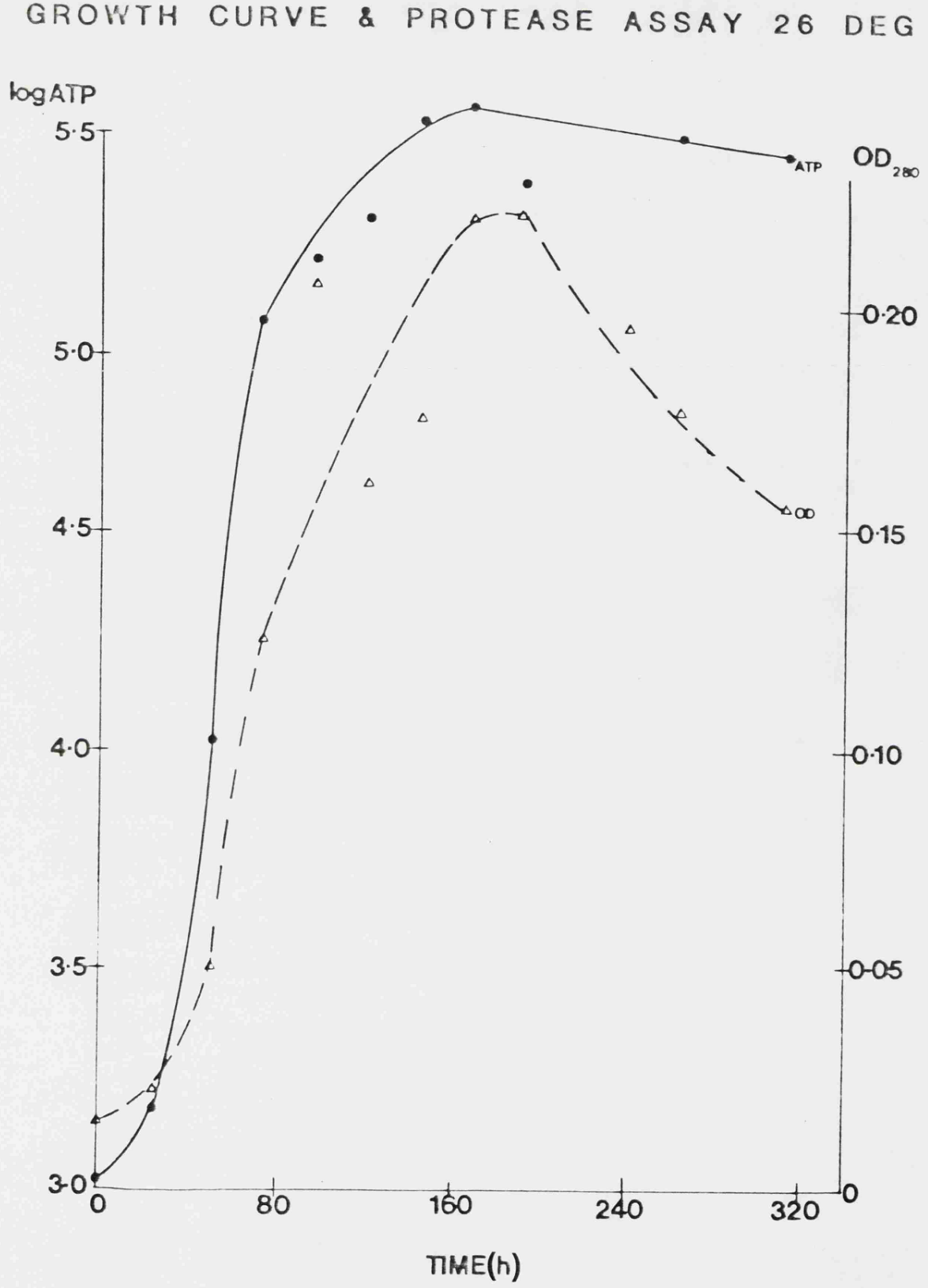
Proteinase was therefore purified from the filtrates of cultures grown at 26°C by a one-step column chromatographic procedure that involved elution with sodium citrate buffer (Fig. 8). The first large peak to be eluted with 0.03M buffer was presumed to contain principally albumin; proteinase was eluted as a single peak with 0.05M buffer. Fractions 57-64, containing enzyme, were pooled and concentrated. The details of purification are given in Table 11. The final yield of 4.7% was typical for 5 separate purification experiments.

Purity of enzyme

The final preparation was judged to be pure by several criteria. Only one band - diffuse in nature - was detected by PAGE; its Rf value was 0.49 (Fig. 9). A single precipitin arc was detected in immunoelectrophoresis tests of the enzyme (Fig. 10) and in two dimensional crossed immunoelectrophoresis only one rocket was seen (Fig. 11).

Although double diffusion experiments with undiluted PP and anti-PP suggested the existence of two precipitin arcs (Fig. 12), this was found to be an artefact due to a high concentration of reagents in the wells which gave rise to a split precipitin line. When both antibody and antigen were diluted, the lines were

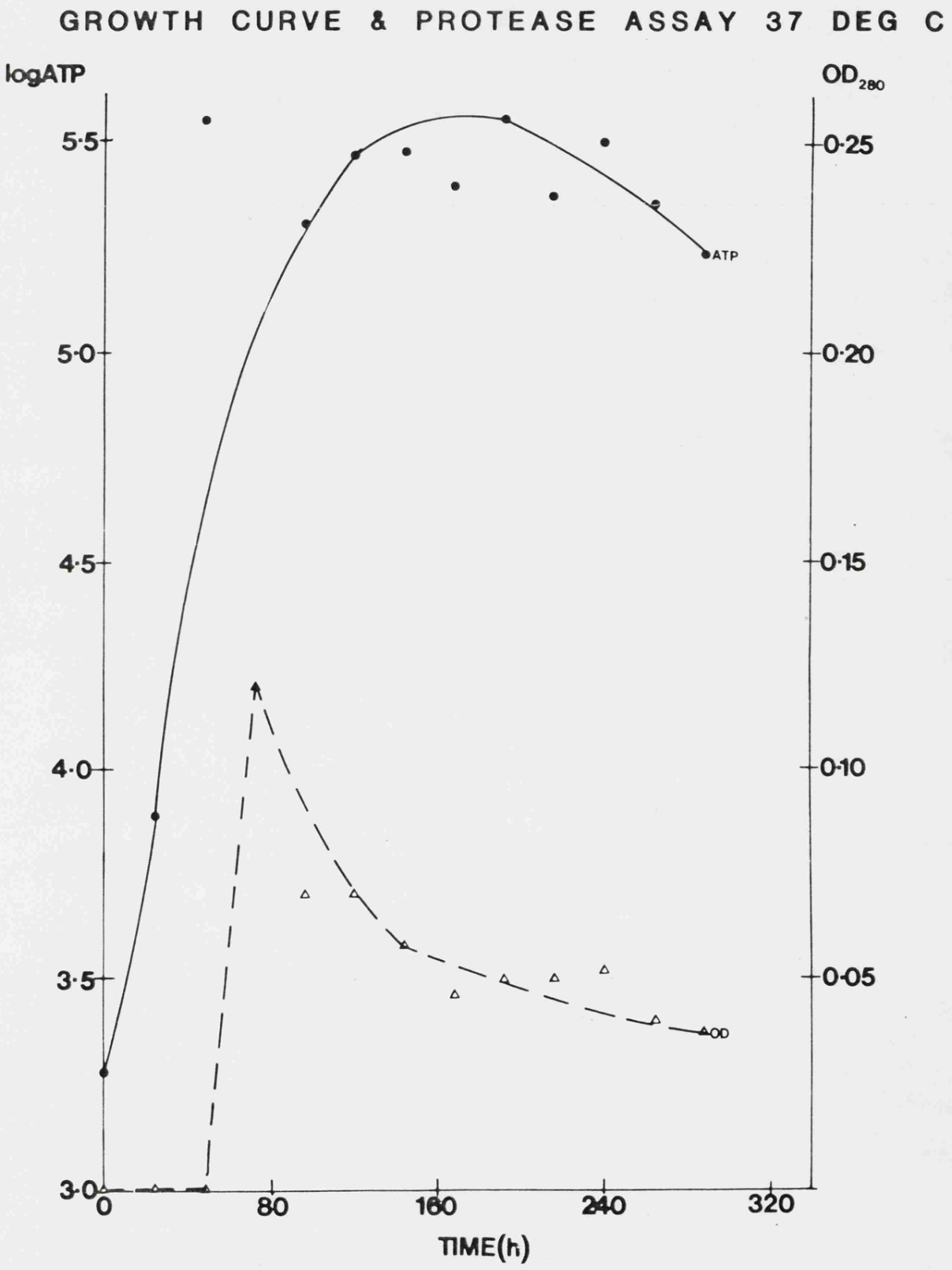
Fig. 6



Growth of C.albicans at 26°C in the medium of Remold et al., (1968). Samples were taken at 24h intervals for growth determination by ATP photometry and assayed for proteinase activity as described in Chapter 2. The graph shown is a typical representative of 6 separate experiments.

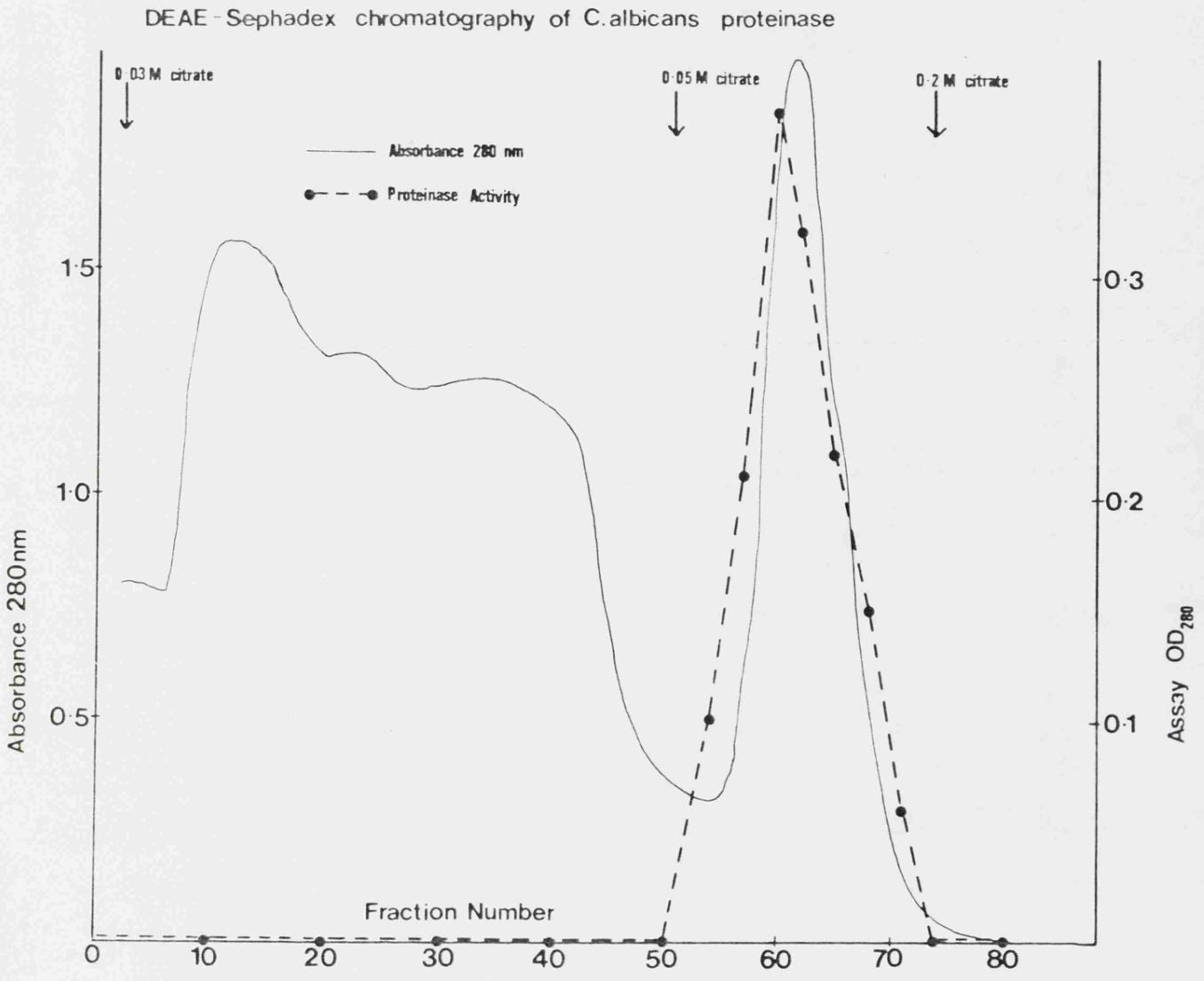
(Proteinase units = OD_{280}/30min/0.5ml)

Fig. 7



Growth of C.albicans at 37°C in the medium of Remold et al., (1968). Samples were taken at 24h intervals for growth determination by ATP photometry and assayed for proteinase activity as described in Chapter 2. The graph shown is a typical representative of 3 separate experiments.

Fig. 8



DEAE-Sephadex chromatography of C.albicans proteinase. Proteins were eluted from the column, 30x5.5 cm, with sodium citrate buffers, pH6.5, of increasing ionic strength. Fractions (50ml) were collected at a flow rate of 25ml/min. UV-absorbing fractions were assayed for proteinase activity. The purification procedure was repeated 5 times, with essentially similar results each time; proteinase was eluted as a single peak in 0.05M buffer.

Table 11. Purification of C.albicans proteinase

Sample	Volume (ml)	Protein Conc.[a] (mg/ml)	10^6xSpecific Activity[b]	Total Activity[c]	% Recovery
Unconcentrated medium	9000	1.6	3.3	47.5	-
Pooled DEAE fractions	400	2	6.3	5.1	10.6
Concentrated DEAE fractions	20	23	4.8	2.2	4.7

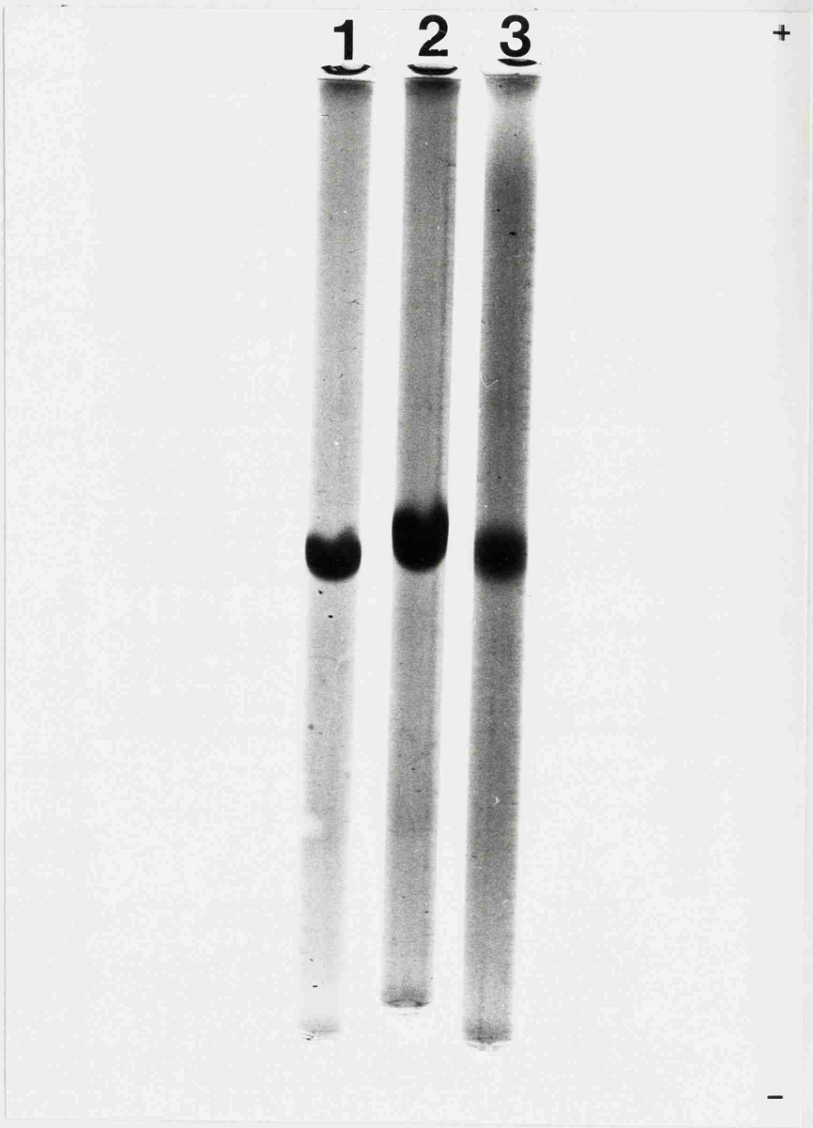
a) Protein concentration was determined by the method of Lowry et al., (1951). Spectrophotometric readings were made at 500nm and converted to µg Protein/ml with a BSA standard curve.

b) OD_{280}/min/µg protein.

c) Specific activity X Volume X protein concentration

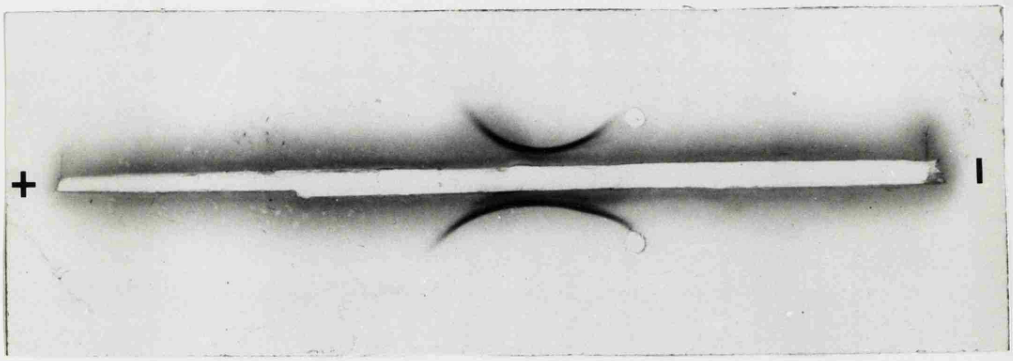
In early experiments 40 fold increases in specific activity were obtained when proteinase was purified according to Remold et al. Only 2 fold increases in specific activity were found when the enzyme was purified by the above method. Since subsequent tests for the purity of the enzyme confirmed its homogeneity, it is difficult to account for these differences in specific activity. It is possible that the enzyme purified by the 'DEAE - only' method was contaminated with low molecular weight material that gave a positive reaction in the Lowry test and hence a lower specific activity. Such material would have been removed in the first 2 steps of the Remold method, i.e. the concentration on larger pore ultrafiltration membranes and purification on Sephadex-G75. Evidence for the presence of low molecular weight, Lowry positive material comes from subsequent experiments carried out by myself that strongly Lowry-positive, proteinase-negative material is eluted after enzyme rich fractions on Sephadex G-25.

Fig. 9 Polyacrylamide gel electrophoresis of C.albicans proteinase.



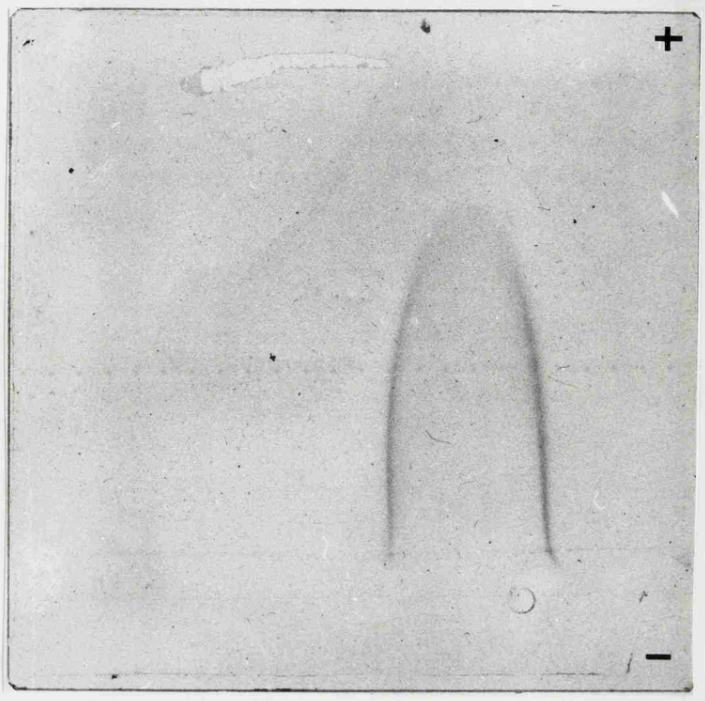
Samples of purified enzyme were examined for purity by PAGE. The sample applied to the gel in tube 3 contained 0.46mg protein/ml; the samples applied to gels 1 & 2 contained 1.15 mg protein/ml and came from 2 different batches of enzyme. Gels were electrophoresed at 5mA per tube for 3h. They were stained with Page blue 83, as described in materials and methods. Only a single, diffuse protein band was seen in any of the gels.

Fig. 10 Immunoelectrophoresis of proteinase.



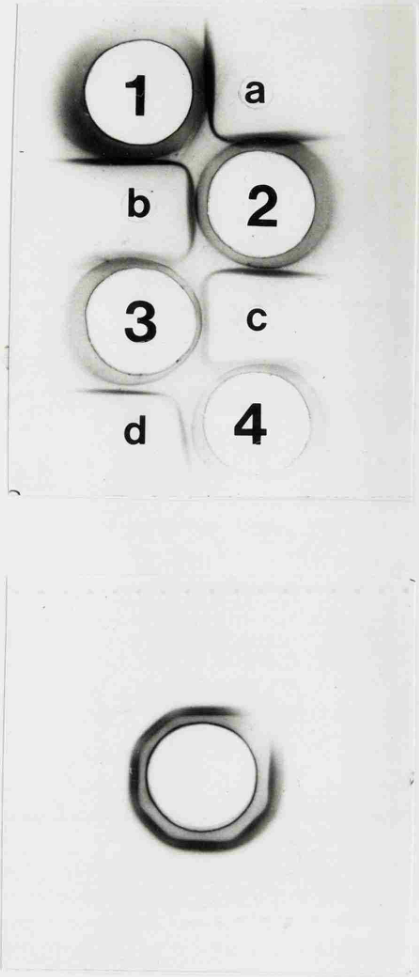
Immunoelectrophoresis was carried out as previously described (Grabar & Williams, 1953), except that the PP samples were subjected to electrophoresis for 60min instead of 90min. Proteinase from two separate purifications was added to the two wells. Anti-PP was added to the centre trough and the plate was kept in a humid box at room temperature for 48h. Plates were then dried and stained with Coomassie brilliant blue R. Only single precipitin arcs were observed in these tests.

Fig. 11 2D crossed immunoelectrophoresis of proteinase and anti-proteinase.



Crossed immunoelectrophoresis was carried out by the micromethod of Weeke, (1973). 2 µl of PP(23mg protein/ml) were electrophoresed in the first dimension for 60min at 10v/cm. Electrophoresis in the 2nd dimension was carried out into 3.3ml agarose (Sigma, Medium EEO) containing 75 µl of anti-PP, for 30h at 3v/cm. Plates were dried and stained with Coomassie brilliant blue R. Only a single precipitin 'rocket' was detected in several repetitions of the test, even when the relative proportions of antigen and antiserum were altered from those described.

Fig. 12



Double diffusion was performed as previously described (Odds et al., 1975). 12mm wells contained anti-PP, 4mm wells contained PP. Plates were stained with Coomassie brilliant blue R after 3 days at room temperature in a humid box. In the upper slide wells 1-4 contained doubling dilutions of antibody, wells a-d contained doubling dilutions of PP. The central well in the lower slide was filled with undiluted anti-PP and the peripheral wells contained different batches of PP (23mg protein/ml): the peripheral well in the top right hand corner of the bottom slide was filled with PBS as a negative control. It will be noted that apparently double precipitin lines seen in the bottom slide were found in the top slide only when undiluted antigen and antiserum were allowed to react: this observation suggests that the double lines resulted from splitting of a single precipitin band as a result of excessively high concentrations of reagents.

resolved as a single precipitin arc (Fig. 12). Ouchterlony and Nilsson (1978) had previously described this phenomenon of splitting of lines as a result of a high concentration of reactants in a zone with precipitation resulting in two areas where concentrations of antibody and antigen are optimal. The lower photograph in Fig. 12 shows a complete fusion of precipitin arcs. As 5 separate batches of PP were used as antigens, this observation indicates that each reagent was identical.

When different volumes of proteinase were mixed with antiserum raised against proteinase, a precipitate was formed (Fig. 13) and was at a maximum when 20µl of antigen was added to 250µl of antiserum. There was little detectable enzyme activity in either the precipitate or the supernatant. Control experiments made with normal serum showed this to be due to the presence of Candida proteinase inhibition in serum. It is not possible from these experiments to confirm whether the antibodies in the immune serum were capable of inhibiting or precipitating the enzyme.

Glycoprotein nature of purified proteinase

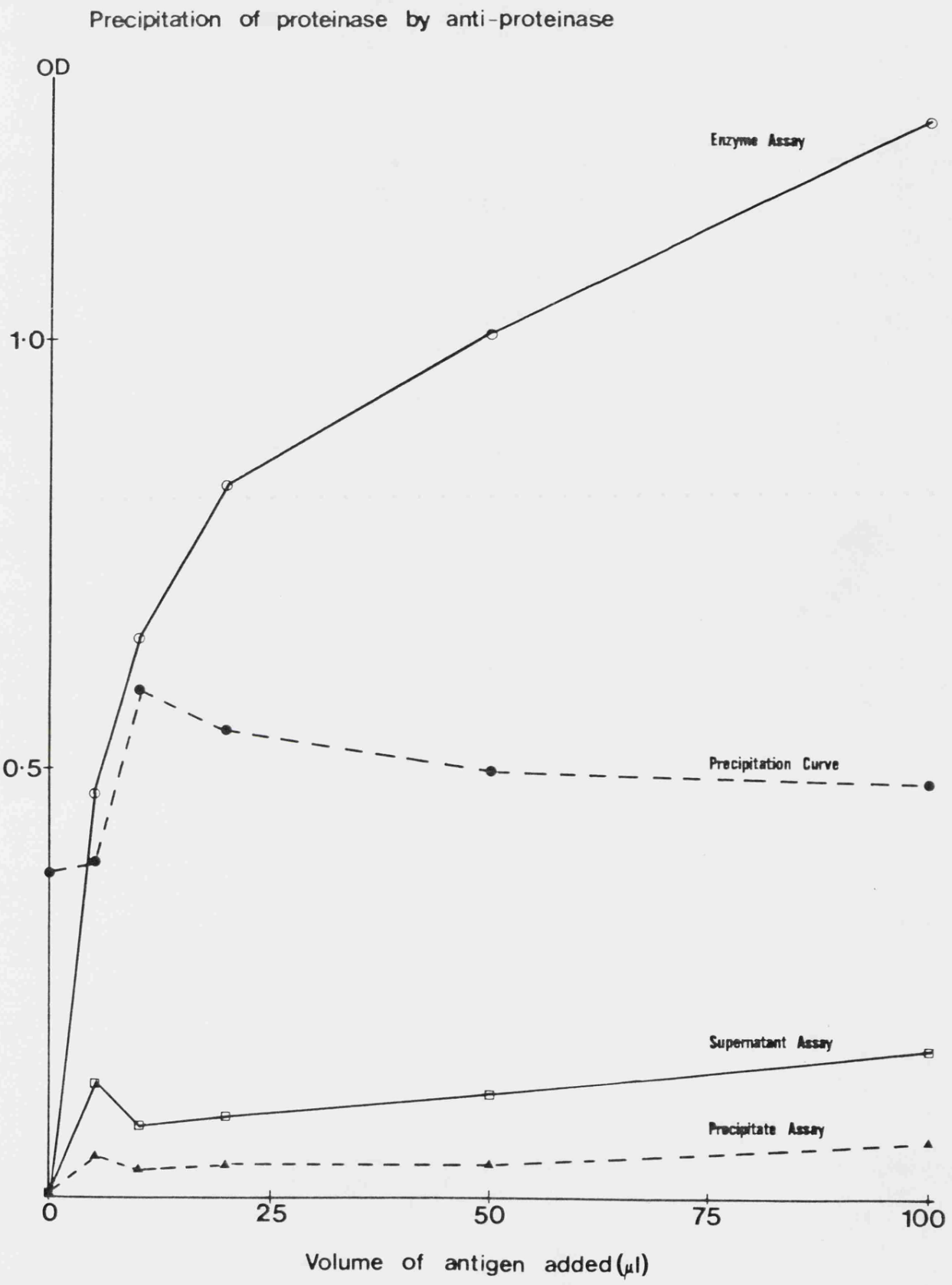
The diffuse nature of the bands in PAGE and of precipitin arcs suggested that C.albicans proteinase might be a glycoprotein rather than an exclusively protein molecule. A glycoprotein acid phosphatase from C.albicans has been previously described (Odds & Hierholzer, 1973). Quantitative tests with anthrone reagent confirmed the presence of hexose in the purified enzyme, and two-dimensional thin layer chromatography of hydrolysed proteinase identified the sugar as mannose. (Rf values were 0.312 in the 1st dimension and 0.136 in the second as compared with 0.32 and 0.131 respectively for a standard sample of mannose). Total hexose concentration of proteinase, as determined by the anthrone reaction, was 348µg of mannose/ml which means a protein : mannan ratio of 1:0.015.

Classification of purified proteinase

Proteinases can be classified into one of 4 types - carboxyl, metallo, thiol or serine on the basis of the reaction of their catalytic groups with a variety of inhibitors. Pepstatin was the only inhibitor to have any effect on

No significant rise in proteinase activity in the supernatant was noted as increasing amounts of enzyme were added, after the point of maximum precipitation. This observation suggests that there may have been Candida proteinase inhibitors in the serum. Subsequent experiments in which proteinase activity was measured in the presence of normal rabbit serum confirmed this result.

Fig. 13



Different volumes of proteinase were added to 250µl of anti-PP. The amount of precipitate formed was measured in terms of turbidity by determination of OD 550nm, and the proteinase activity of precipitates and supernatants were assayed and expressed as ODunits/min. o-o control curve showing enzyme activity in the absence of anti-PP. Proteinase activity in filtrates (□-□) and in the precipitate (▲-▲) was markedly reduced after immunoprecipitation of the enzyme with anti-PP.

see opposite

C.albicans proteinase, indicating that the enzyme belonged to the carboxyl proteinases (Fig. 14).

Unique nature of proteinase

Since up to 78 components have been detected in Candida cytoplasmic antigen extracts (Axelsen, 1976), it is possible that one of these may be the proteinase of C.albicans that has been purified from culture filtrates in the present study. To determine whether or not the proteinase was a unique antigen it was compared with the cytoplasmic antigen by PAGE and in serological tests.

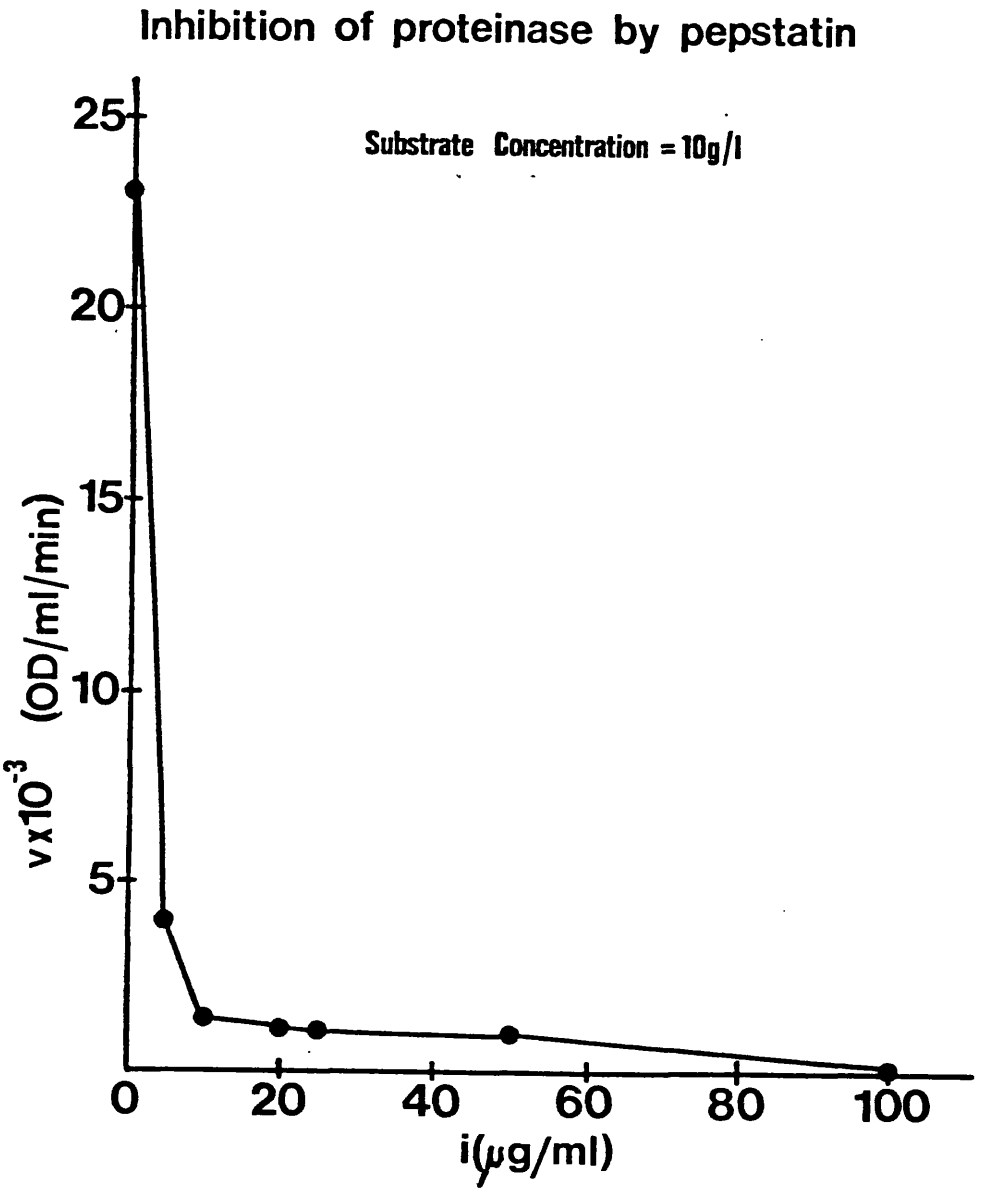
In PAGE, one band of the 16 detected in the cytoplasmic antigen had the same Rf value (0.47) as the band given by purified proteinase (Fig. 15). Subsequent evidence showed that it was unlikely that the coincident band in the CE was biochemically or serologically similar to the PP. When the cytoplasmic antigen was assayed for proteinase at pH3.2, no activity could be detected: in comparison of the two antigens by counter immunoelectrophoresis (CIE), PP was found to react only with anti-PP and CE reacted only with anti-CE: moreoever, in tandem crossed immunoelectrophoresis experiments PP and CE gave rise to no double precipitin peaks (Fig. 16). When the two antisera directed against each of the two antigens were compared by crossed immunoelectrophoresis with intermediate gel, no cross reactions could be detected between PP and anti-CE or between CE and anti-PP (Fig. 17). It was therefore concluded that PP was not a component of the CE preparation.

3.4 Discussion

A method for the purification of C.albicans proteinase that involves only one chromatographic step has been described and evaluated. The method is both technically easier to perform and more rapid than that of Remold et al., (1968). Although only up to 15l of culture filtrate were used for the preparation of the enzyme in the present study, larger volumes could be handled quite easily if yields of the enzyme sufficient for routine test purposes were required.

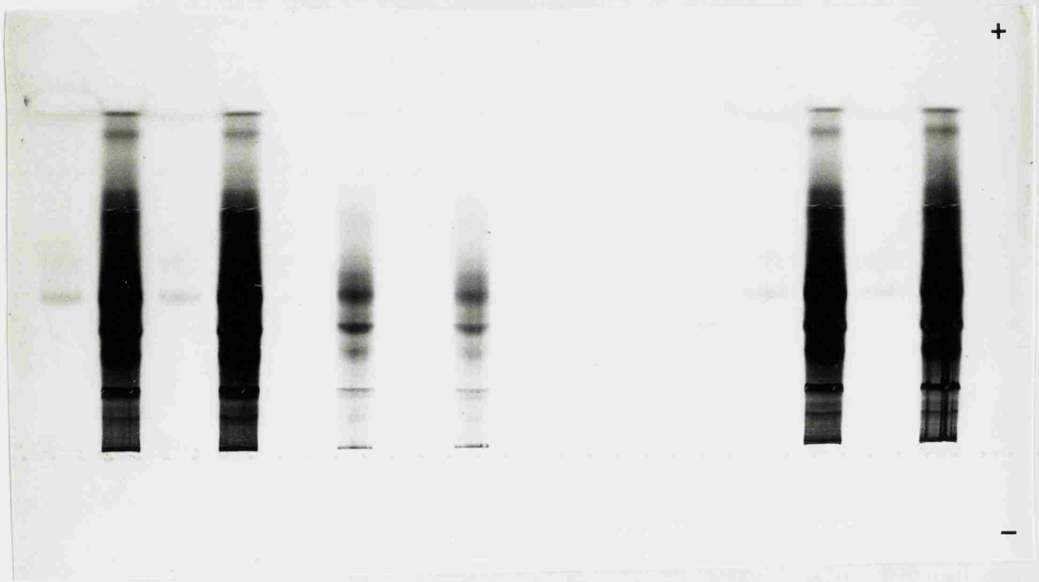
The purified proteinase was shown to be homogenous by polyacrylamide gel electrophoresis, in serological tests, by immunoelectrophoresis and two dimensional crossed immunoelectrophoresis. It is presumed that proteins

Fig. 14



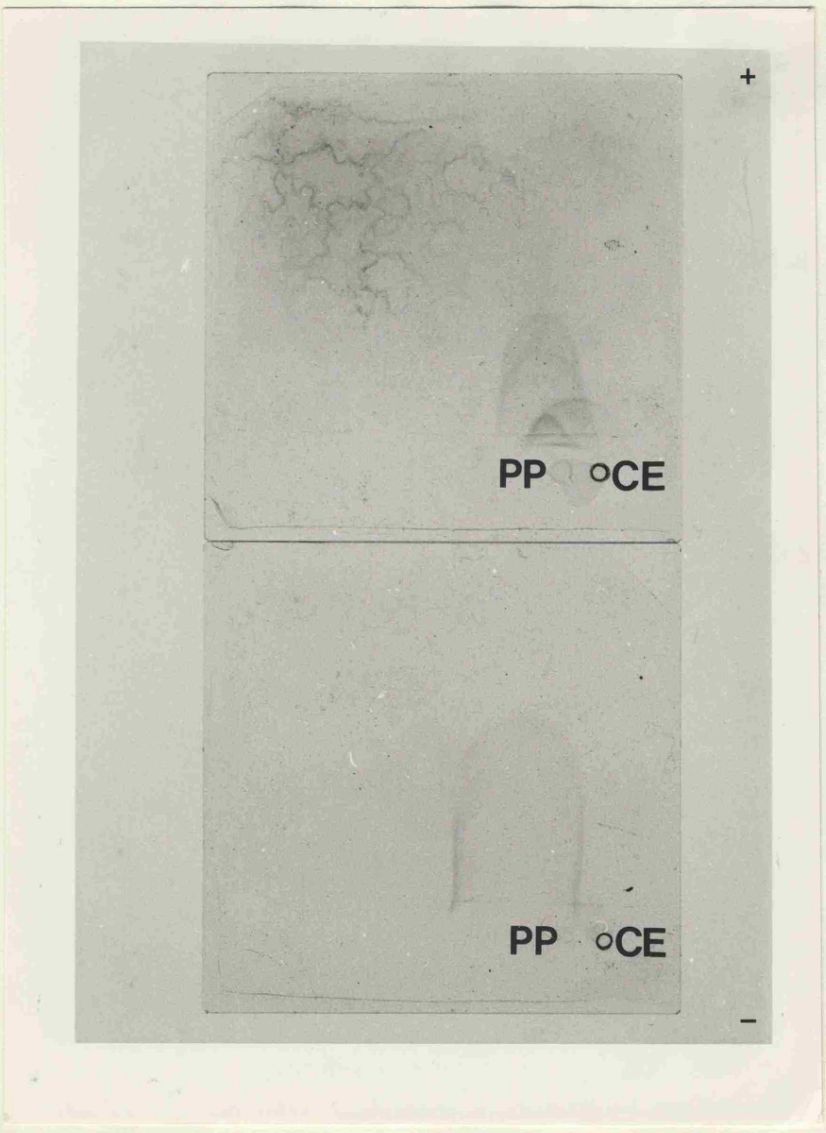
The effect of increasing concentrations of pepstatin on the activity of C.albicans proteinase was examined. Enzyme and inhibitor were pre-incubated at 37°C for 30min then the proteinase was assayed as previously described.

Fig. 15 Polyacrylamide gel electrophoresis of C.albicans proteinase and cytoplasmic extract.



Cytoplasmic antigen and proteinase were compared on horizontal slab polyacrylamide gels. CE and PP were placed in alternate wells. From the left of the figure, the first 4 wells contained undiluted antigens (23mg protein/ml); wells 5-8 contained 10-fold dilutions, and wells 9-12 contained 100-fold dilutions of CE and PP. The final 4 wells are duplicates of the first 4. Gels were run at 40mA for 5h then stained with Page blue 83 as described in materials and methods. 16 protein bands could be seen after electrophoresis of CE antigen, one of which appeared to migrate with the same Rf as the purified proteinase.

Fig. 16 Tandem crossed immunoelectrophoresis of proteinase and cytoplasmic antigen.



Antigens: 2µl of undiluted cytoplasmic antigen and proteinase were placed in adjacent wells.

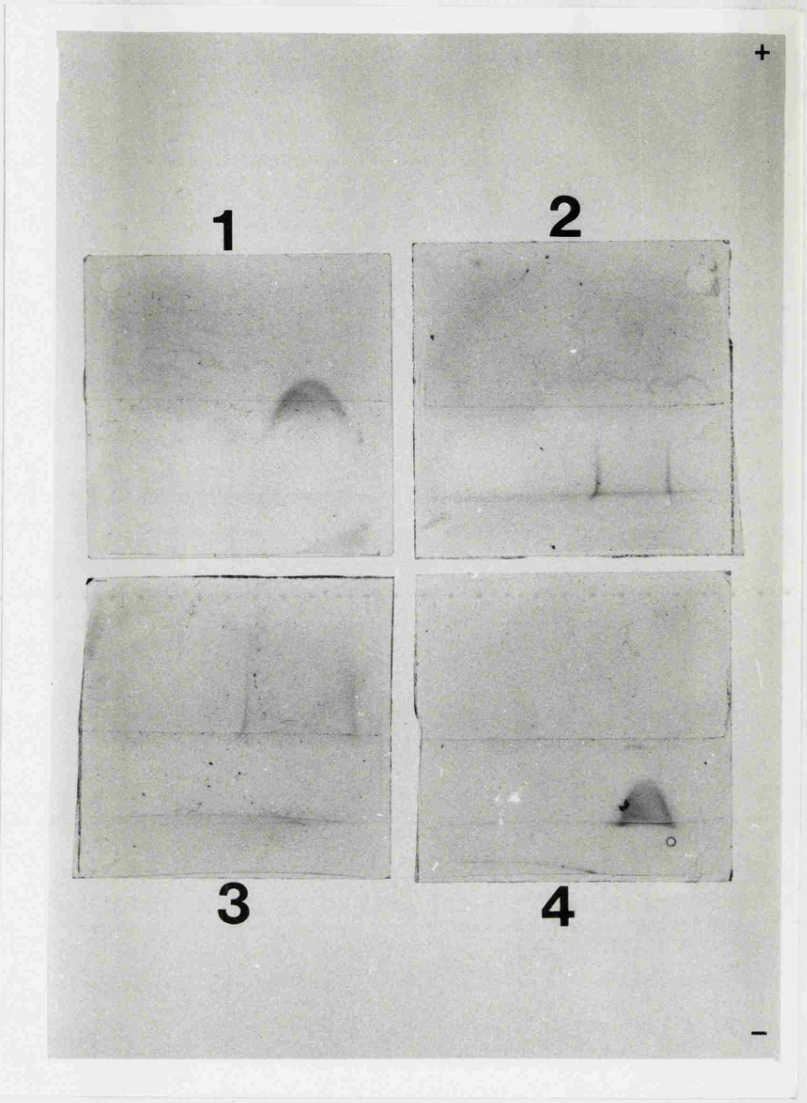
1st dimension: The run was done at 10v/cm for 60min.

Antibodies: In the upper slide, 3.3 ml of agarose contained 250µl of anti-CE. In the lower slide, 3.3 ml of agarose contained 75µl of anti-PP.

2nd dimension: The run was done for 30h at 3v/cm.

Slides were washed overnight with 0.4% Sodium borate/0.4% NaCl solution, then dried and stained with Coomassie brilliant blue R. Comparison of the 2 slides shows that a) the position of the PP precipitin peak did not coincide with any of the 5 peaks seen in the CE-anti-CE system, and b) there were no apparent double peaks; indicative of reactions of identity between PP and CE, in either test.

Fig. 17 Crossed immunoelectrophoresis with intermediate gel.



Antigens: Wells on slides 1 and 4 contained 2µl of cytoplasmic antigen (23mg protein/ml).

Wells on slides 2 and 3 contained 2µl of proteinase (23mg protein/ml).

1st dimensions: run was done at 10v/cm for 60min.

Antibodies: intermediate gel-slides 1 and 2 contained 50µl of anti-PP.

slides 3 and 4 contained 70µl of anti-CE.

reference gel-slides 1 and 2 contained 100µl of anti-CE.

slides 3 and 4 contained 50µl of anti-PP.

2nd dimension: run was done at 3v/cm for 60min.

Slides were washed overnight with 0.4% borate/0.4% NaCl solution then dried and stained with Coomassie brilliant blue R.

originally present in the culture filtrate were either unbound to DEAE-Sephadex or had been eluted with 0.03M citrate.

The purified proteinase was found to be a mannoprotein; this finding makes an interesting comparison with the results of Odds and Hierholzer (1973) who purified a glycoprotein acid phosphatase from C.albicans and with the recent results of Notario (1979) who purified a glucanase from C.albicans which was also found to be a glycoprotein. However, the ratio of mannose to protein in the phosphatase was shown to be 7:1 and serological data suggested that the mannan protion of the glycoprotein phosphatase was responsible for the antigenicity of this molecule. Since the purified proteinase in the present study contained only 1.5% mannose, it is likely that the polysaccharide plays little part in the molecule's antigenicity in comparison with the much larger protein moiety. The fact that no antigenic identity could be demonstrated between PP and CE suggests that the diffusion and electrophoretic mobilities of the PP are independent of its mannan component, since CE is known to contain mannan (see Chapter 1). The extent to which the mannan portion of the PP may interfere in tests with patients sera can only be assessed in further serological tests to be described in later chapters.

Proteinase was shown to be a carboxyl proteinase on the basis of its inhibition of pepstatin. Remold et al., (1968) had previously shown that the enzyme had a pH optimum of 3.2, and it is known that pH optima for carboxyl peptidases are usually in the range 2-5 (Barrett, 1977) which further confirms the identity of the C.albicans enzyme with proteinases of the carboxyl type. Diazoacetyl norleucine methyl ester plus Cu^{2+} has been described as an inhibitor of carboxyl proteinases(Barrett, 1977) but it had no effect on C.albicans proteinase. A likely explanation for the absence of inhibitory effect is that reaction of the inhibitor with the enzyme was slow. This has previously been described for some other carboxyl proteinases (Barrett, 1977).

Antiserum raised against proteinase was able to precipitate the enzyme but re is, as yet, no evidence that it will neutralize the activity Some authors have previously described enzyme activities in precipitin bands obtained by reaction of C.albicans antigen mixtures with homologous antisera (Tran Van Ky et al., 1969).

It may not therefore be assumed that antibodies to C.albicans enzymes inevitably neutralize the enzymes. Unfortunately, proteinase activity was unamenable to direct staining assays in agarose gels so that no assays could be conducted this way in the present study.

Although one protein component of the cytoplasmic antigen detected by PAGE migrated with the same Rf value as the proteinase, no proteinase activity was detected in the cytoplasmic extract and there were no serological cross-reactions between PP and anti-CE and vice versa. The proteinase is therefore a novel antigenic component which is not present in the cytoplasmic extracts currently employed in serological tests for the diagnosis of candidosis, and its evalulation as a diagnostic antigen is therefore warranted. Although it is possible that a basal level of proteinase is present in C.albicans in the absence of BSA as inducer, the enzyme was not present in the cytoplasmic extract in sufficient quantity to be detected by the very sensitive tandem and crossed immuno-electrophoresis with intermediate gels and it is therefore unlikely to be involved in any of the precipitin reactions that may be noted in less sensitive CIE tests when these are used to detect anti-C.albicans antibodies in human sera.

Chapter 4

PROTEINASE AS A FACTOR IN THE VIRULENCE OF C.ALBICANS

4.1 Introduction

If it could be proven that C.albicans proteinase was truly involved in the pathogenesis of candidosis, the case for its development as a diagnostic antigen, specific to tissue invasion by the fungus, would be greatly enhanced. So far, evidence for a pathogenic role of proteinase in published accounts is only circumstantial. Staib et al., showed that only 3 out of 100 strains of C.albicans were not able to proteolyze after 10 days observation (1972). Actively proteolysing strains injected into mice caused granulomatous lesions of the kidney and heart as well as peritonitis and septicemia. However, similar granulomatous lesions are seen when strains are grown in glucose/peptone media (Adriano and Schwarz, 1955) and it is not known whether these strains may have been capable of proteolysis once inside animals. In 1968, Remold et al., demonstrated that only those strains capable of proteolysis were lethal to mice. Budtz-Jörgensen (1971) reported that proteolysis was most frequently seen in the more pathogenic Candida species (C.albicans and C.tropicalis) than in less pathogenic species but he could not relate the severity of the inflammatory lesions of denture stomatitis to the proteolytic activity of yeasts recovered from the lesions.

Further proof of the involvement of proteinase in the pathogenicity of candidosis may come from the demonstration of extracellular enzyme production in lesions caused by Candida in vivo. A decrease in the extent to which proteinase is produced corresponding to the decrease in the virulence of the seven pathogenic species would also help to confirm the role of proteinase. Although Budtz-Jörgensen (1972) went some way to showing this, he only included 4 of the pathogenic species in his study as well as C.glabrata and two non-pathogenic species.

4.2 Materials and Methods

Mice were injected intravenously with 10^6 C.albicans, a dose likely to be fatal within 1 week (Adriano and Schwarz, 1955) and sacrificed after 1, 3 and 5 days. The kidneys were removed, embedded in Tissue Tek embedding fluid (Raymond Lamb) and frozen in liquid nitrogen. Tissue sections, 6um thick, were cut on a cryostat, fixed in a stream of hot air and stained for proteinase by an indirect immunofluorescent method. Three drops of a 1:10 dilution of anti-PP antiserum, prepared as described in Chapter 3, were added to each section on a slide and the slides were incubated at room temperature in a humid box for 30 min. The slides were washed for 30min with PBS then stained for 30min with a 1:80 dilution of swine fluorescein-conjugated anti-rabbit immunoglobulins (Dako). After a final washing with PBS as before, the slides were mounted in glycerol and examined for fluorescence with a Leitz U/V microscope.

Control slides were incubated with negative serum instead of anti-PP antiserum.

Photographs were taken with an Ilford HP5 film (ASA 400) and developed with Microphen (Ilford) to give the film a final rating of 1600 ASA.

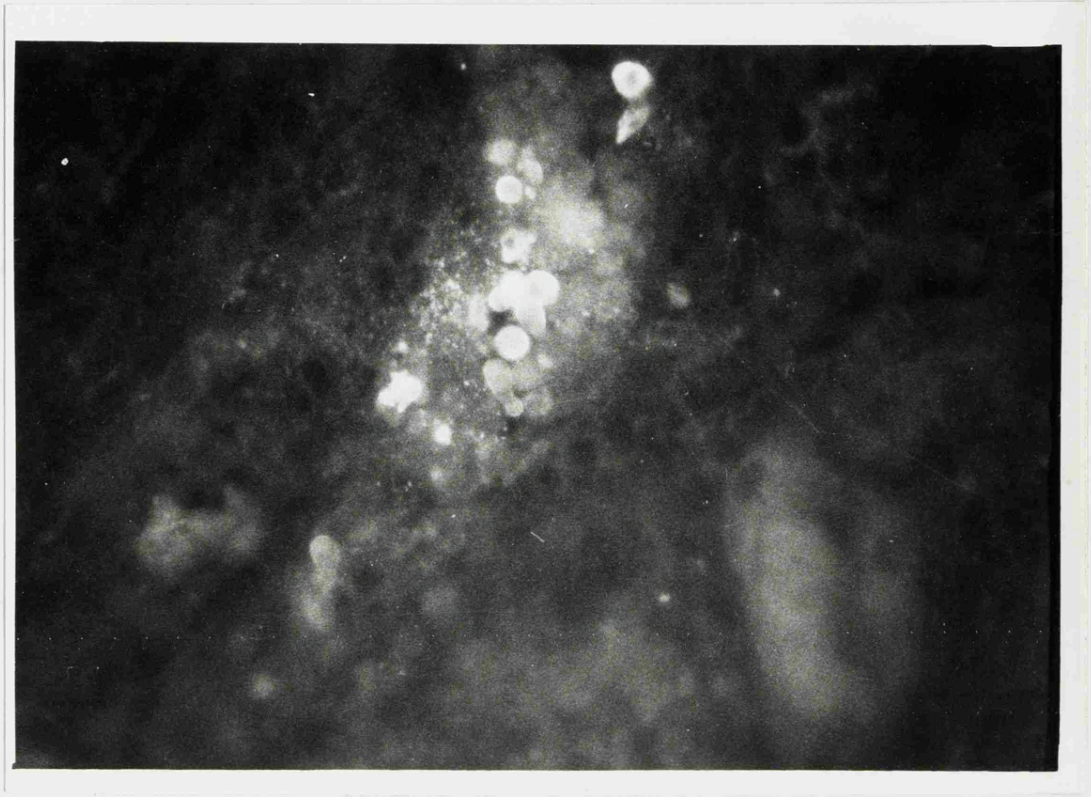
4.3 Results

C.albicans blastospores in the kidneys of mice sacrificed 3 days after inoculation fluoresced moderately brightly in the indirect fluorescence test for proteinase (Fig. 18). There was a halo of weaker fluorescence around the blastospores seen against the dark background of the rest of the kidney. No lesions were seen in mice sacrificed 1 day after inoculation but those sacrificed after 5 days had lesions similar to those described above. Only negligible fluorescence was seen in kidneys of control, uninoculated mice that were sacrificed at the same time as the experimental mice.

Blastospores grown in the medium of Remold et al., (1968) also fluoresced when stained for proteinase by the indirect fluorescent technique.

The most virulent of the pathogenic Candida species were shown, by measurement of absorbance at 650nm, to grow best on the medium which induces

Fig. 18 Immunofluorescent staining of Candida lesions in the mouse kidney.



Microabscesses caused by intravenous inoculation of C.albicans were stained for proteinase by an indirect fluorescent technique as described in materials and methods. C.albicans blastospores in the kidney 3 days after inoculation can be seen to fluoresce and there is a zone of weaker fluorescence around the fungal cells. Similar results were noted in 5 other lesions in different areas of the kidney. x 625.

proteinase (Table 12) especially C.albicans and C.stellatoidea. These two species were the only ones to show any measurable proteinase activity in unconcentrated culture filtrates (Table 12).

The seven pathogenic species were also inoculated onto serum protein agar. Zones of proteolysis were seen by 72h around colonies of C.albicans, C.stellatoidea and C.tropicalis and by 96h around C.parapsilosis. After 10 days colonies of all other species including C.glabrata were surrounded by zones of proteolytic activity.

4.4 Discussion

Indirect fluorescent staining has demonstrated that proteinase is probably secreted by C.albicans in lesions in vivo. Blastospores were also shown to fluoresce in vivo and in vitro. Since proteinase activity could be demonstrated in blastospores - although to a lesser degree than is found in culture filtrates (see Chapter 3) - this result is not surprising. The enzyme detected in blastospores in the assay and demonstrated by fluorescence is presumably at or near the surface of cells and is likely to be in the process of being secreted by them.

The most virulent species of Candida grew best on glucose/BSA medium and these species produced detectable proteinase activity. The same species were the first to show proteolytic activity on serum protein agar. This experiment was only a preliminary investigation into the proteolytic activity of Candida species other than C.albicans. More detailed experiments comparing the time of maximum production of proteinase in all the species and a comparison of the properties of the enzymes themselves should provide an interesting area of future experimentation.

The results described in this chapter - demonstration of proteinase production in vivo and a greater production of the enzyme in the more virulent species - provide a strong prima facie case that proteinase really may be involved in the pathogenicity of C.albicans. Admittedly the data presented above can only be considered as preliminary and the results need to be verified with a more extensive examination of tissues from experimentally infected animals and of

Table 12 Proteinase Activity of Pathogenic Candida species

Candida spp	OD_{650}	Proteinase Units
C.albicans	2.65	.36
C.tropicalis	0.51	.02
C.stellatoidea	2.50	.26
C.parapsilosis	0.57	.01
C.pseudotropicalis	0.78	-
C.kusei	0.32	-
C.guilliermondii	0.42	-
C.glabrata	0.24	-
C.intermedia	0.14	-
Rhodotorula	0.20	-

The medium of Remold et al., (1968) described in Chapter 3 was inoculated with different Candida species. After 5 days growth at 26°C the absorbance at 650nm was measured and the proteinase activity of culture filtrates was determined.

Proteinase unit = OD280/30min/0.5ml

material obtained from human cases of candidosis. Nevertheless, these encouraging findings in this chapter represent the first attempt to demonstrate an extracellular virulence factor of C.albicans directly in vivo.

It may be worthy of note that the two rabbits that were injected with proteinase to raise antiserum (Chapter 3) died of peritonitis 12 weeks after they were first inoculated. Peritonitis was also the main symptom noted by Staib et al., (1972) when actively proteolysing cultures of C.albicans were injected into mice. Further experiments still have to be done to determine if the proteinase was the cause of peritonitis or whether the onset of peritonitis was coincidental. Post mortem investigation of the two rabbits revealed no unequivocal cause of their illness.

Chapter 5

PROTEINASE AS AN ANTIGEN IN THE SEROLOGICAL DIAGNOSIS OF SYSTEMIC CANDIDOSIS

5.1 Introduction

The cytoplasmic extracts of C.albicans currently used as antigens in serological tests for candidosis frequently give rise to false positive reactions and are relatively non-specific, thereby resulting in positive reactions in superficially, as well as systemically infected patients (Odds, 1979). Several attempts have been made to find 'infection-specific' antigens, including a preliminary examination of C.albicans proteinase as a serodiagnostic reagent (Staib et al., 1977a, 1977b). However, Staib et al., have tested only culture filtrates, from C.albicans grown in glucose/BSA medium, as antigens. Their results thus far appeared to be promising but are still far from conclusive. The crude culture filtrate antigen presumably contains several antigenic components including, for example, the soluble mannan from the cell wall and the 3 enzymes detected in glucose/BSA and Sabouraud's broth filtrates (see Chapter 2), so that the precipitin reactions detected by Staib et al., could be directed against any one or several of these constitutive components.

A rapid purification method has been developed in this study (Chapter 3) so the purified proteinase enzyme can be tested as an antigen. The results described in Chapter 4 suggest that proteinase is secreted in vivo in experimentally infected animals. If a similar situation occurs in patients with systemic candidosis, the antigenic properties of proteinase may be very specific for deep-seated infections.

5.2 Materials and Methods

Serological tests

Precipitating antibodies to CE and PP antigens were detected by counter immunoelectrophoresis (CIE: Odds et al., 1975). Sera were serially diluted and the highest dilution that gave a line of precipitation to the test antigen was recorded as the precipitin titre of the serum.

Antisera

Antisera from rabbits experimentally infected with C.albicans and from hospital patients were tested retrospectively for anti-CE and anti-PP precipitins.

Three rabbits had been infected intravenously with C.albicans at $4x10^5$ yeasts/kg. This dose led to a chronic systemic Candida infection: the animals were bled 10, 14 and 42 days after infection. These rabbits are referred to as group A. Six further rabbits had been infected intravenously with $5x10^6$ C.albicans/kg. The effect of this dose was to produce a rapidly fatal, acute disseminated candidosis. Three of these infected rabbits were treated orally with the water-soluble antifungal ketoconazole from 1 to 7 days after infection: these animals survived the C.albicans infection for at least 30 days (Odds et al., 1979): blood was obtained from them 6, 10, 14, 22 and 30 days after infection and this group of rabbits is referred to as group B. The remaining 3 animals were untreated and died 6 days after infection: no sera were available from these untreated rabbits.

Human sera came from 3 groups of patients and were obtained through the co-operation of the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine; the Mycology Unit, Leeds General Infirmary and the Public Health Laboratory Service, Leicester. All sera had been stored at -20^oC or -70^oC for periods of up to 5 years before the present study. The 28 patients in group 1 had no microbiological, histological or serological evidence of deep-seated Candida involvement. Group 2 contained 17 who had undergone cardiac surgery 1-3 weeks before serum was obtained from them and 5 patients whose case history was uncertain due to lack of available relevant information. Sera of all 22 patients contained precipitating antibodies to C.albicans CE antigen but no additional information or evidence of deep-seated candidosis was available. Sera from patients in this group were termed 'false-positive' although it is recognized that some of the patients in this group may have had systemic Candida infection. Group 3 contained 13 patients all diagnosed as cases of systemic candidosis on microbiological and/or pathological grounds. They included 6 cases of disseminated candidosis, 3 cases of endocarditis, one of which was diagnosed as C.glabrata endocarditis, one case each of renal candidosis, oesophageal

candidosis, chronic mucocutaneous candidosis and secondary pulmonary candidosis. Serial serum samples were available from two of the cases of disseminated candidosis, one of the cases of endocarditis, and from the case of secondary pulmonary candidosis. For the purposes of statistical comparison of single precipitin titres to CE and PP antigens, only the second specimen in each series was considered. In addition to the above, an additional 10 sera from cases of systemic candidosis were available for study. These were all obtained in America between 1965-72 and had been frozen and thawed and lyophilized many times since then. However, the precipitin titre to CE antigen with these sera had altered considerably from the original value in some cases - prolonged storage is known to reduce the level of precipitating antibodies (Taschdjian et al., 1972). These sera were not considered in the statistical comparison.

All data were analysed statistically by the Mann-Whitney U test.

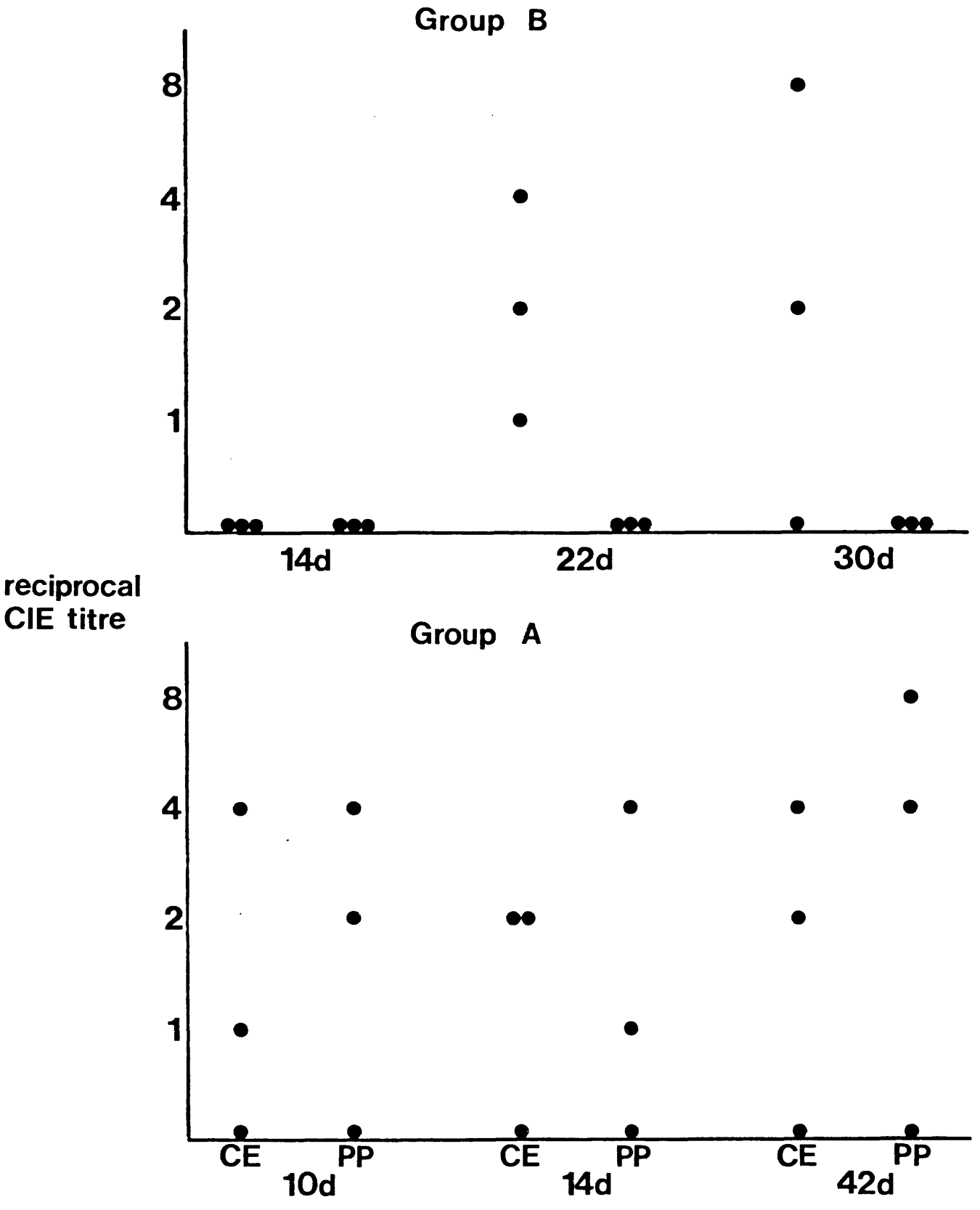
5.3 Results

Precipitins to CE and PP were detected in the sera from experimentally infected rabbits in Group A (Fig. 19). In the acutely infected, treated group of rabbits (Group B), the sera obtained on day 14 did not react with either antigen but by days 22 and 30 titres as high as 1:8 against CE antigen were found in the serum. No titres against PP antigen were detected in any serum sample from rabbits in Group B.

Of the 28 sera from patients without candidosis (Group 1) who were precipitin negative to CE antigen, 3 undiluted sera showed a faint reaction to PP (Table 13). Of the 22 'false-positive' sera (Group 2), all of which were precipitin positive to CE, 17 showed reactions against PP (Table 14). Titres to PP were higher than those to CE in 50% of the sera and were 1:8 or greater in 5 sera (Table 16). The geometric mean titre to both antigens was 1:4 in the 'false-positive' group of sera (Table 13).

In the 13 sera in Group 3, which came from patients diagnosed as having

Fig. 19



Precipitin titres to CE and PP antigens in the sera of two groups of experimentally infected rabbits. Rabbits in Group A received 4×10^5 C.albicans/kg; those in Group B received 5×10^6 yeasts/kg and were treated with ketoconazole (see materials and methods for details).
Titres to CE and PP were determined by CIE. Titres to CE antigen were detected in the sera of both groups of rabbits. No titres to PP were noted in the sera of animals treated with ketoconazole.

Table 13. Precipitin titres against CE and PP in patients with and without candidosis.

Precipitin Titre	Number of patients with titres in Group 1 CE	Group 1 PP	Group 2 CE	Group 2 PP	Group 3 CE	Group 3 PP
negative	28	25	0	5	1	0
1:1		3	13	6	1	0
1:2			1	2	4	1
1:4			4	4	3	2
1:8			2	3	1	4
1:16			2	1	0	2
1:32				1	1	2
1:64					2	2
Mean Titre	0	0	1:4	1:4	1:15	1:20

Table 14. 'False-Positive' Sera

Serum No.	Diagnosis	Precipitin titre to CE.	PP.
SH0907	Cardiac Surgery patient	1:1	1:2
SH2004	"	1:8	1:16
SH2704	"	1:1	1:4
SH2902	"	1:4	1:1
SH3803	"	1:4	0
SH3902	"	1:4	1:8
SH4704	"	1:4	0
78/097	"	1:1	0
78/004	"	1:1	1:1
77/028	"	1:1	0
76/111	"	1:1	1:1
76/015	"	1:1	0
78/096	"	1:1	1:4
78/078	"	1:1	1:1
77/023	"	1:1	1:1
76/084	"	1:8	1:1
7448	"	1:2	1:4
78/048	? SBE	1:1	1:8
78/127	Chronic Lung Fibrosis	1:16	1:8
77/054	Mitral Valve replacement	1:16	1:32
7319	Asthma	1:1	1:4
276	Prostatitis	1:1	1:2

Table 15. Case Sera

Serum No.	Diagnosis	Precipitin titre to CE.	PP.
77/101	CMC	1:4	1:16
76/078	Endocarditis	1:4	1:8
76/048	Renal candidosis	1:1	1:8
79/009	Oesophageal candidosis	1:2	1:8
78/020	Pulmonary candidosis	1:32	1:64
387	Disseminated candidosis	1:2	1:32
851	Endocarditis	1:4	1:16
6372	Endocarditis (C.glabrata)	1:8	1:32
6214	Disseminated candidosis	1:64	1:64
4261	Disseminated candidosis	1:64	1:4
6059	Disseminated candidosis	1:2	1:4
6213	Disseminated candidosis	1:2	1:8
4791	Disseminated candidosis	0	1:2
	American Sera		
s15x	Disseminated candidosis	1:16	1:2 (1:32)
S144	Renal candidosis	0	0
S32	Renal candidosis	1:8	1:1
S198	Endocarditis	1:4	1:8
S20	Disseminated candidosis	1:16	1:16 (1:16)
S18	Endocarditis	1:32	1:16 (1:32)
S14	Disseminated candidosis	1:16	1:2
S37a	Endocarditis	1:4	0
S17	Renal candidosis	1:4	0 (1:2)
S19	Disseminated candidosis	0	1:1 (1:8)
S15	Disseminated candidosis	0	0 (1:32)
S21	Disseminated candidosis	0	1:1 (1:4)

Previous Precipitin titre to cytoplasmic antigen is given in brackets where known

Table 16. Summary of precipitin reactions to CE and PP in patients with and without candidosis.

	Group 1		Group 2		Group 3	
	No.	%	No.	%	No.	%
PP titre>CE titre	3	11	10	45	11	85
PP titre≥1:8	0	0	5	23	10	77
PP titre≥1:8 and>CE titre	0	0	4	18	9	75

Group 1 consisted of patients with no microbiological, histological or serological evidence of deep-seated Candida involvement.
Group 2 consisted of patients with anti-CE titres but no additional evidence of systemic candidosis.
Group 3 consisted of patients all diagnosed as cases of systemic candidosis.
Precipitin titres were determined by CIE.

systemic candidosis, all but one showed titres to CE and all 13 had titres to PP of 1:2 or greater (Table 15). Anti-PP titres were higher than anti-CE in 11 of the sera and were 1:8 or greater in 10 (Table 16). The geometric mean titre to CE was 1:5 and was 1:20 to PP. Statistical comparison of Groups 2 and 3 showed that there was no significant difference between the groups with respect to CE but that anti-PP titres were significantly higher in the sera from patients with systemic candidosis than in the 'false-positive' group ($p < 0.01$).

Serial serum samples were available from 4 patients. In figure 20, patients 1 and 2 had disseminated candidosis, patient 3 had Candida endocarditis. In all three patients the anti-PP titre was equal to or higher than anti-CE with one exception. This was serum no.3 from patient 2, which had no titre against PP. This sample had been sent to the laboratory for the determination of its amphotericin B concentration.

The fourth patient from whom serial serum samples had been obtained was diagnosed as having pulmonary aspergillosis. C.guilliermondii had also been isolated from a chest drain. These sera were tested retrospectively for precipitins to CE and PP (Fig. 21). Anti-PP titres were, as in the three previous cases, higher than anti-CE by at least one serum dilution. After April, the pattern of titres to both the C.albicans antigens and to A.fumigatus antigen was similar. No cross reaction between proteinase and A.fumigatus cytoplasmic antigen could be detected by CIE or by double diffusion. Four other patients with A.fumigatus precipitins did not react to PP in serological tests.

All sera from both human cases and from the rabbits were examined by CIE for circulating proteinase but none was detected.

Proteinase and CE were compared by CIE against several patients' sera. No cross reactions were noted in any case (Fig. 22).

5.4 Discussion

No anti-PP antibodies were detected in the serum of experimentally infected rabbits treated with ketoconazole although such antibodies were present in untreated animals that had been infected with a lower dose of C.albicans.

Fig. 20

Precipitin titres to CE and PP antigens in the sera of three patients with candidosis

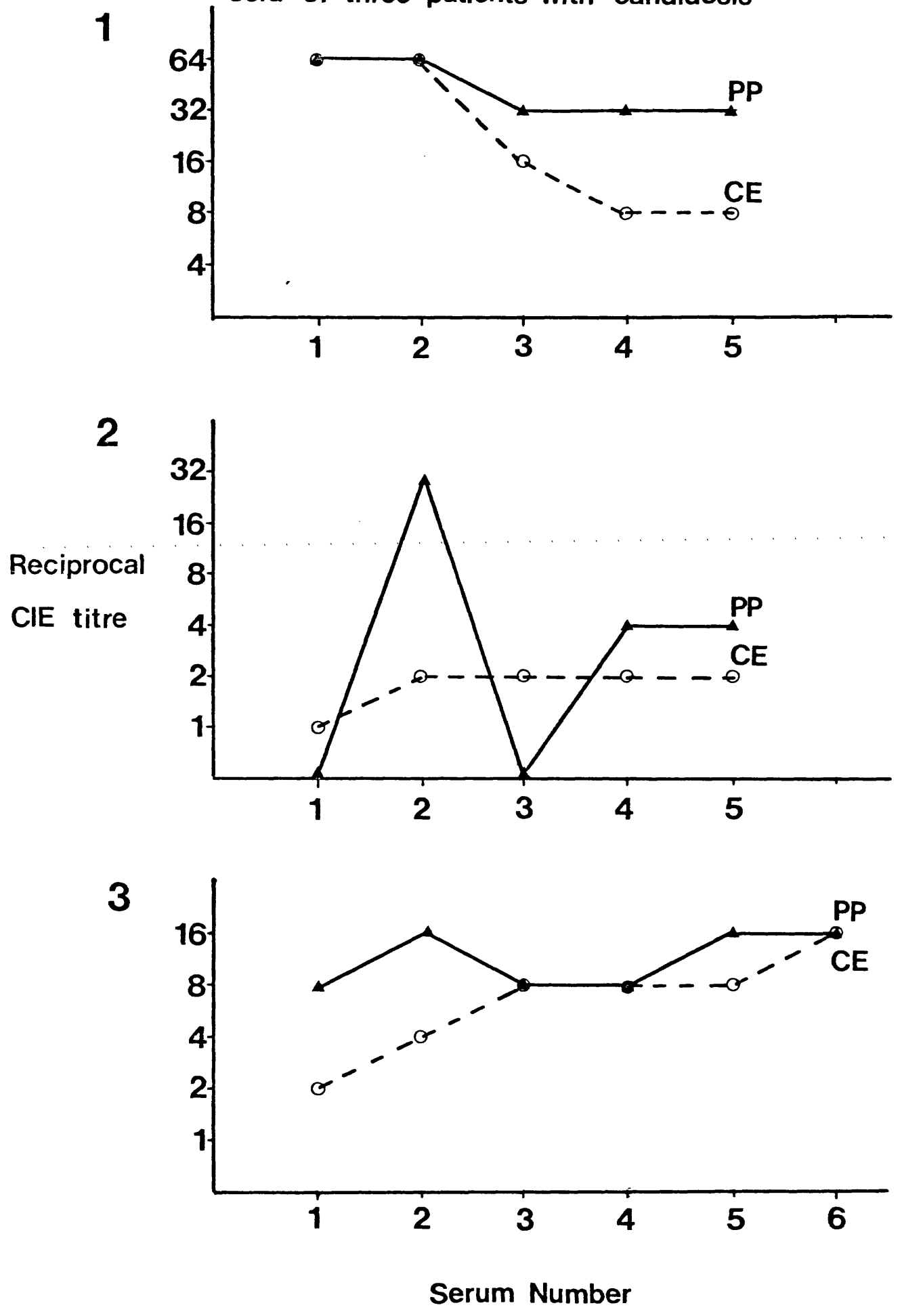
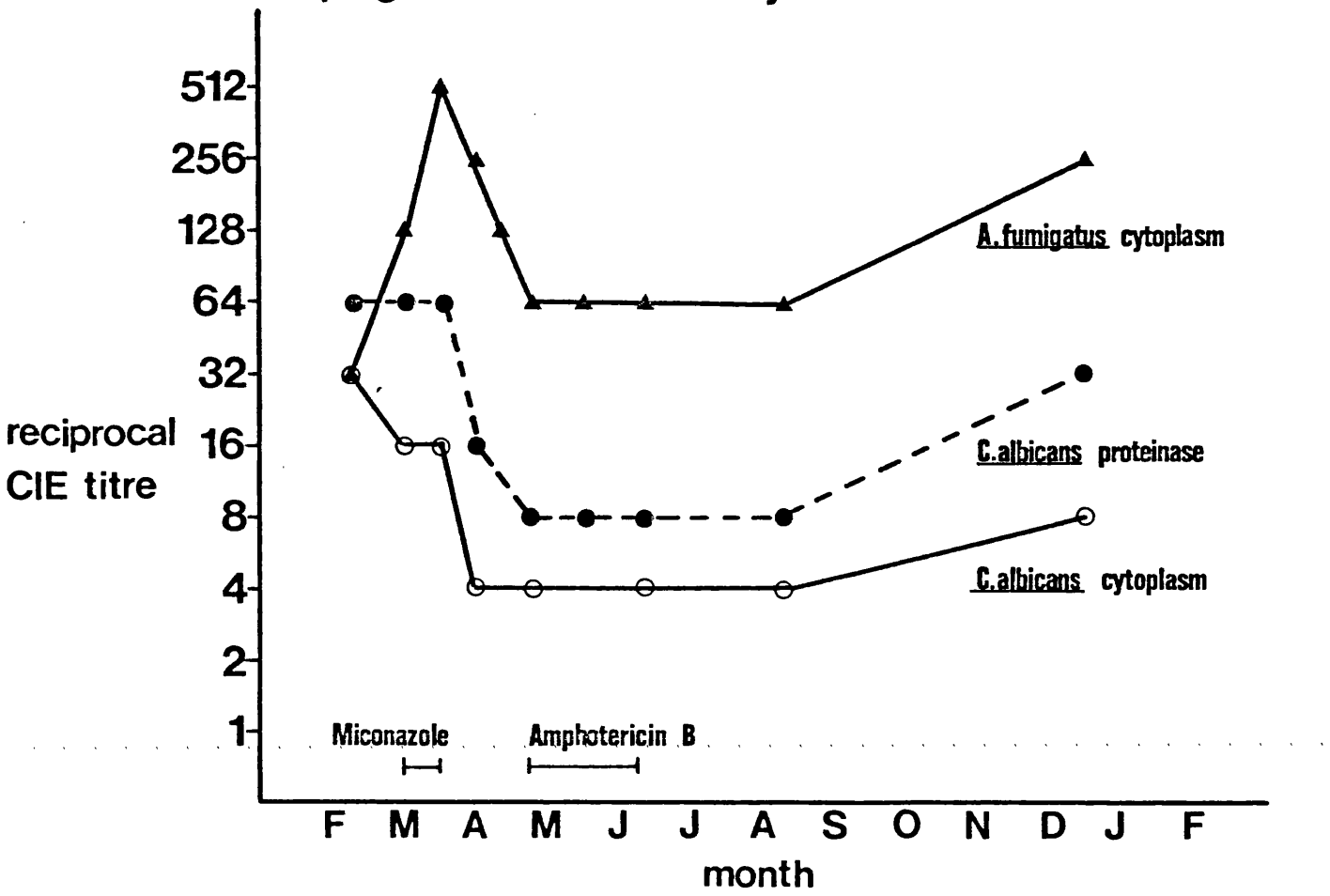


Fig. 21

Precipitin titres to 3 antigens in a case of pulmonary aspergillosis with secondary candidosis.



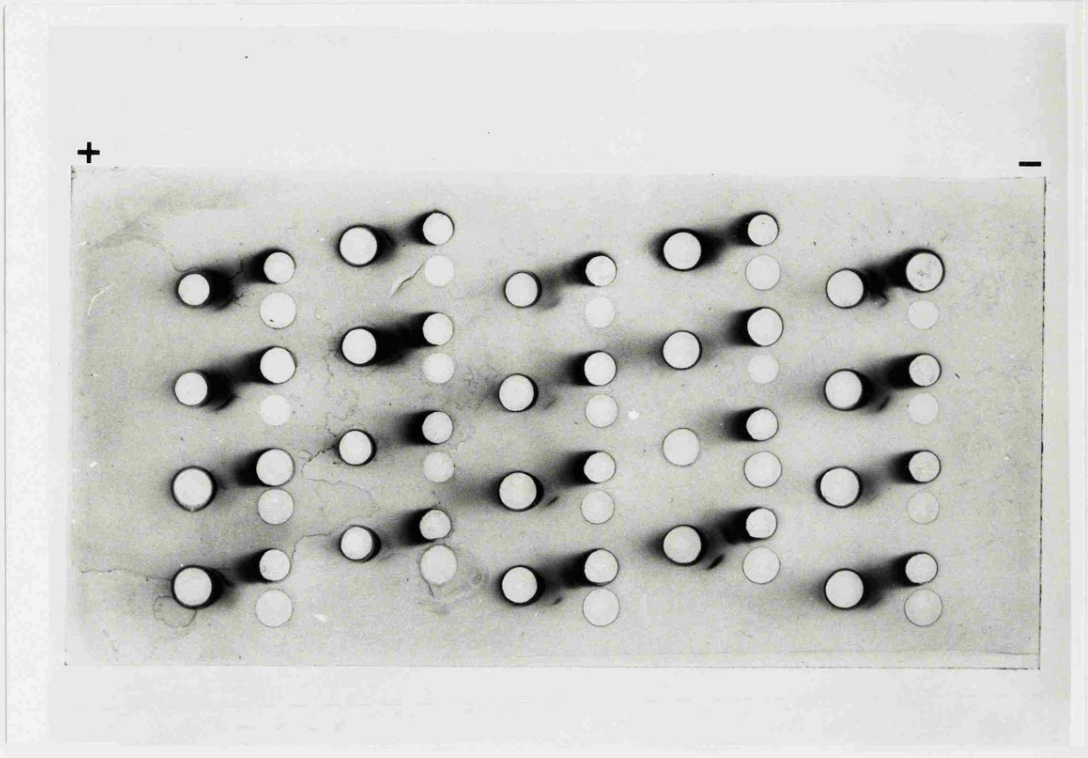
Titres to A.fumigatus and C.albicans cytoplasmic antigens and to C.albicans proteinase antigen in serial serum samples of one patient were determined by CIE. The patient was a 27-year-old man admitted to hospital with a diagnosis of pyopneumothorax. He was treated with a wide range of antibiotics including anti-tuberculous chemotherapy for the first 5 days. Attempts to re-expand the lung failed and a formal decortication was carried out 20 days after admission.

3 days later A.fumigatus was isolated from the chest drain and continued to be isolated for 29 days. 12 and 20 days after the decortication C.guilliermondii was isolated from the chest drain.

14 days after A.fumigatus was first isolated intravenous miconazole therapy was started and continued for 10 days. On the 13th day the patient suffered an anaphylactic reaction to the drug. Intravenous amphotericin B therapy was started 44 days later and continued for 43 days - a total dose of 990mg in 6 weeks

Serological investigations were started after the first isolation of A.fumigatus - titres to this antigen reached 1:512 in April and fell after anti-fungal therapy was started. The serum samples were tested retrospectively for antibodies to C.albicans cytoplasmic and proteinase antigens. After the first month of the patient's illness, the pattern of titres to all 3 antigens followed each other closely; the titre to C.albicans proteinase antigen was always higher than the titre to cytoplasmic antigen.

Fig. 22 Counterimmunoelectrophoresis of patients sera against CE and PP



Undiluted sera were placed in the wells in alternate rows starting at the anodal side of each plate. CE was placed in the upper well and PP in the lower well of each pair of antigen wells. Electrophoresis was carried out as described in materials and methods. No cross-reactions were noted between any pair of antigen wells, further confirming the unique properties of proteinase as an antigen.

If the antifungal prevented a systemic infection from occuring - as appears to be the case,since none of the rabbits showed any symptoms of candidosis, whereas an untreated group injected with the same dose of C.albicans at the same time died after 6 days - this absence of detectable anti-PP antibodies suggests that no proteinase was produced by the infecting fungus. These observations suggest that antibodies to purified proteinase are detectable only when clinical infection with C.albicans is established. In the experimental infections described the PP therefore appears to have behaved as a specific antigen for cases of systemic infection although so far only a relatively few animals have been tested.

The results for human cases of candidosis were nowhere near so clear cut. In the sera from the group of patients without candidosis and negative for precipitins to CE antigen, three showed a faint reaction to PP antigen. Anti-PP precipitins were also detected in 70% of 'false-positive' sera that were all anti-CE positive. The mean titres and the statistical results do show however a tendancy to higher precipitin titres to PP in proven cases of candidosis and on this basis the PP would appear to be a superior antigen in comparison with CE. 75% of these sera had titres that were both greater than 1:4 and higher than the titre to CE. The four patients from whom serial serum samples were available all showed parallel changes in titres to the two antigens and also confirmed the tendancy for the anti-PP titre to be higher than the anti-CE titre in instances of mycologically proven Candida involvement.

Five of the patients in the 'false-positive' group showed titres greater than 1:4 against PP. Although the positive reactions to Candida antigens in this group of patients are thought to be due to a transient candidaemia of little clinical importance, it is nevertheless possible that the 5 patients giving high titres to PP may have had a systemic Candida infection which was either undetected or of which we were unaware,due to a lack of relevant clinical information. In these cases, tissue invasion involving proteinase may have occurred, resulting in positive PP titres.

PP was also shown to react with the serum of a patient with C.glabrata endocarditis. Cross reactions between C.albicans and C.glabrata have been noted

previously (Hasenclever and Mitchell, 1960: Stickle et al., 1972). Although the strain of C.glabrata examined in the present study did not appear to secrete proteinase (see Chapter 4) it is possible that a fresh clinical isolate may do so. This is a possibility for further investigation.

Serum sample no.3 from patient 2 (Fig. 20) which was sent to the laboratory for amphotericin B estimation showed no detectable anti-PP activity. This negative result was confirmed in a repeat of the CIE experiment. There is no obvious reason why amphotericin B should suppress proteinase activity immediately the drug is given. Although antifungal therapy certainly prevented any detectable anti-PP precipitins in experimental animal infection, the drug was given right at the start of the infection, thus preventing infection from developing to give clinically apparent tissue invasion. This was not the case for patient 2, since anti-PP titres returned to 1:4 in the next serum sample tested. This single negative serum from a patient otherwise consistently positive for PP antibodies cannot be explained. The effect of amphotericin B and other antifungals on anti-PP precipitins must be examined carefully in the future.

The results of case no.4 (Fig. 21), the patient with a principal diagnosis of pulmonary aspergillosis, are unusual. As no cross reactions were found between proteinase and A.fumigatus cytoplasm, it would appear that precipitins to C.albicans antigens were genuine. No other patients with aspergillosis had anti-PP precipitins. Simultaneous Aspergillus and Candida infections are very rare (Winner and Hurley, 1964). It is unlikely that the patient suffered a transient candidaemia since the high anti-PP titres - 1:8 or greater - would suggest a more severe infection. C.guilliermondii was isolated from pleural fluid on two occasions, 8 days apart, which does not suggest a transient infection. The results seem to indicate therefore that the patient had both fungal infections. This patient, who remains unwell some 20 months after onset of his illness, is still under review.

The unique antigenic properties of proteinase, demonstrated in Chapter 3, were further confirmed by the absence of cross-reactions between CE and PP tested against 20 sera from patients.

Proteinase is not a qualitatively specific antigen for the diagnosis of systemic candidosis, as demonstrated by the positive reactions in anti-CE negative sera and in 'false-positive' sera. However, the tendency towards higher anti-PP titres in confirmed cases of candidosis does suggest that it could be valuable for the diagnosis of systemic candidosis. However, the tests in the present study involved sera that had been deep-frozen for up to 5 years, and cannot therefore be expected to give such accurate results as fresh sera. The decrease in anti-CE titre in some of the American case sera demonstrated the effect of repeated freezing and thawing of sera. It must also be assumed that the anti-PP titre in these sera had also decreased and, for this reason, the results were not considered in the statistical comparisons of the two antigens. The value of proteinase as a diagnostic antigen can be studied better with fresh prospectively obtained serum samples, and it is hoped that more sera can be obtained in the future to allow further evaluation of the specificity of this antigen.

Chapter 6. DISCUSSION

The aim of this study was to evaluate the involvement of inducible hydrolytic enzymes in the pathogenesis of candidosis and their use as specific serodiagnostic antigens in the diagnosis of deep-seated infection. The enzymes concentrated on were those which might conceivably be induced in the fungus *in vivo* and which might help the fungus to invade tissues of the body. The substrates were therefore macromolecular components of mammalian tissue surfaces, such as phospholipids or proteins, or components of connective tissue, such as chondroitin sulphate.

In early experiments, *C.albicans* appeared to be able to utilise either erythrocyte ghosts or chondroitin sulphate as carbon sources. However, it was later shown that growth in the presence of these substrates was due to the ability of *C.albicans* to use citrate buffer rapidly and fully at pH 3.5 and partially at pH 5.5. Since preliminary experiments had indicated that *C.albicans* ATCC 28366 could not utilise citrate at pH 5.5 and since it became able to do so later, it is presumed that the ability of this strain to assimilate citrate changed with time. The ability of different strains of *C.albicans* to assimilate citrate is described by Van Uden and Buckley (1970) as 'variable': the findings in this study suggested that this variability may be seen from time to time for a single strain of *C.albicans* as well as from strain to strain.

The inability of *C.albicans* to use erythrocyte ghosts and chondroitin sulphate as nutrient sources was confirmed by the lack of detectable phospholipase and hyaluronidase in either blastospores or mycelial forms.

Hyaluronidase could not be detected by any of three different assay methods, confirming and extending the results of earlier workers (Chattaway *et al.*, 1971). Hyaluronidase was not considered further as an enzyme of interest in this investigation. However, investigators inability to detect it, thus far, does not mean that hyaluronidase might not be induced under slightly different conditions of growth.

A negative observation naturally never excludes the possibility that different experimental conditions might give rise to a positive result:

however, in the case of C.albicans and hyaluronidase, the absence of detectable enzyme in vitro also reflects the fact that the fungus in vivo does not show great pathogenicity for connective tissue. In mucocutaneous infections the fungus rarely appears to penetrate beneath the outermost layers of epithelial tissue. This clinical observation itself suggests that C.albicans is unlikely to possess or secrete hyaluronidase in pathologically significant quantities.

Price and Cawson (1977) described intracellular phospholipase A and lysophospholipase in C.albicans. Costa et al., (1968) were able to detect phospholipase A and C around colonies of C.albicans grown on Sabouraud agar containing serum and sheep erythrocytes. However, none of these enzymes were detected in the present study in cells or culture filtrates of C.albicans grown with erythrocyte ghosts as sole carbon source, in Sabouraud's broth, in Wickerhams medium or in a medium containing lecithin as a carbon source. Addition of Ca^{++}, which is known to be necessary in some cases for phospholipase activity, had no effect here. The results of Price and Cawson (1977) could not be repeated under the same conditions with the strain of C.albicans used throughout the present investigation, although the assay could be shown to work with purified phospholipases obtained commercially. Chattaway et al., (1971) were similarly unable to detect any phospholipase activity in their strains of C.albicans. These contradictory results are difficult to interpret unless some of the observations are erroneous. It is possible that the enzymes may be strain specific, in which case they must be considered unlikely to play a major role in the pathogenicity of Candida, since the strains used both in this study and in that of Chattaway et al., (1971), were known to be pathogenic to animals. Phospholipase A has been detected in S.cerevisiae (Kokke, 1966), a yeast generally regarded as a non-pathogen despite reports of several rare cases of infection caused by it (Rippon, 1974). The existence of phospholipase in a non-pathogen lends further weight to the notion that it is not involved, to any great extent, in the pathogenesis of candidosis.

Price (1977) described extracellular phospholipase around colonies of C.albicans grown on a solid medium containing egg-yolk. The opacity noted around colonies on this medium could be due to a proteolytic enzyme, since egg-yolk agar

will detect both phospholipase and proteinase activity. This would explain why Price was unable to detect phospholipase activity in culture filtrates by the assay method described by Price and Cawson (1977). As Price was able to detect enzyme activity on egg-yolk agar only for C.albicans, C.stellatoidea and C.tropicalis, her results concerning extracellular phospholipase in fact agree with those of the present study; it seems likely that she was detecting proteinase instead of phospholipase.

Although no phospholipase activity was found in any liquid media examined, opacity around C.albicans colonies was detected on solid media with pure lecithin as sole carbon source. Whether this was a reflection of true phospholipase activity or the result of another enzyme,or some other effect,is not known at the present. Further experiments on phospholipases in Candida still have to be carried out since, as was the case with hyaluronidase, inability to detect the enzyme this far does not necessarily mean that it is never there. The results of the experiments described above do suggest however that phospholipase is unlikely to be involved in the virulence of Candida.

The acid proteinase of C.albicans originally described by Staib et al., (1965) and purified by Remold et al., (1968), became the major object of examination in this study. Several reports in the literature have suggested that the enzyme might be involved in the pathogenicity of C.albicans and Staib's group has proposed it might be also a specific antigen for the serological diagnosis of candidosis. However, none of the data in the literature has positively confirmed these ideas, and it must be said that the experiments carried out in this study, although lending much further weight to the argument of proteinase in pathogenesis, still do not absolutely substantiate the role of the enzyme as a virulence factor of C.albicans.

Remold et al., (1968) described a method to purify C.albicans proteinase, but the method was not suitable for large scale productions of the enzyme, such as would be required if it were to be used as a serodiagnostic antigen in routine tests for candidosis. A rapid one-step method for purification of the proteinase

was therefore developed, which yielded an enzyme that was judged to be pure by both biochemical and serological tests. Purification was made easier by the absence of large numbers of different proteins in the culture filtrate of C.albicans grown in a glucose/BSA medium. Any other proteins that bound to DEAE-Sephadex at pH 6.5 all appeared to be eluted with 0.03M citrate buffer along with albumin, since only one peak was eluted with higher concentrations of buffer, and this was shown to be pure proteinase.

The final yield of proteinase by this method of purification was only 4%, compared with 8% when the enzyme was purified as described by Remold et al., (1968). Activity was lost both at the first step - the elution of proteinase from DEAE-Sephadex - and at the concentration stage, either because of non-specific binding of proteinase to the walls of the ultrafiltration cell or because of loss of the enzyme through the ultrafiltration membrane. Hopefully this loss of proteinase can be reduced substantially by modification of purificat conditions in the future. Although the final yield is smaller with the one step purification method, much larger volumes of culture filtrate can be used for the purification of the enzyme and the method is therefore more suitable for its preparation as an antigen.

Alteration of the composition of the medium used for purification of proteinase, i.e. the proportions and concentrations of glucose and BSA, might lead to higher yields of enzyme, as may alteration of the strain of C.albicans used. Only one strain of C.albicans was examined in detail as regards proteinase activity; however, since it is known that a few strains of the fungus do not produce enzyme at all, it is also likely that some strains will produce more enzy than others. If differences in the amount of enzyme between producer strains prove to be negligible, treatment of the yeast with a chemical or physical mutagen may yield a proteinase over-producer strain, which would substantially reduce the need for large-scale cultivation of the fungus to produce enzyme.

Inhibition of the enzyme by pepstatin suggested that C.albicans proteinase belonged to the carboxyl class of endopeptidases. Pepstatin, unlike some other inhibitors, has little or no effect on other classes of proteinases, so the class of the enzyme in C.albicans can be assigned with reasonable certainty.

This class of endopeptidases, which includes pepsin and also the proteinases from several other fungi, is characterized by dependence of the enzymes on acidic environments, so the pH optimum of 3.2 for the C.albicans enzyme agrees with this classification.

Purified proteinase was found to be a glycoprotein containing 1.5% mannan. The acid phosphatase (Odds and Hierholzer, 1973) and the glucanase (Notario, 1979) of C.albicans are also both glycoproteins as is the acid phosphatase of S.cerevisia (Boer and Steyn-Parvé, 1966) and a lipase from Rhizopus arrhizus (Sémériva et al., 1969) so the notion of glycoprotein enzymes in fungi is not unusual. In all cases the sugar was shown to be mannose and with the exception of the C.albicans proteinase, the ratio of mannose to protein in the enzyme was at least 1:1.

The significance and function of the carbohydrate portion of the molecules in vivo is not known although it does not appear to play any role in the catalytic function, at least not in the lipase of Rh.arrhizus. It may possibly play some part in the protection of the protein portion from digestion by other enzymes, or it may be present as an inevitable result of the enzymes' location at, or secretion through, the cell wall.

Several reports in the literature have suggested that lytic enzymes might be factors in the virulence of C.albicans and the results obtained in this study have further supported this idea. Stanley and Hurley (1967) suggested that the differential virulence of Candida species might result from differences in lytic enzymatic activity. In their ultrastructural studies, Montes and Wilborn (1968) commented on the fact that large vacuoles were seen in C.albicans invading epithelial cells, and suggested that these might contain enzymes responsible for tissue degradation. Remold et al., (1968) demonstrated that only those strains of Candida capable of proteolysis were virulent to mice. Staib et al., (1972) found that almost all strains of C.albicans isolated from the human body were capable of proteolysis and that actively proteolysing strains injected into mice caused septicaemia and peritonitis. Budtz-Jörgensen (1972) showed that proteolytic activity was most frequently demonstrated in the more virulent Candida species.

These latter results were confirmed and extended in this study by the demonstration that the most pathogenic species of Candida were best able to utilize BSA as a nitrogen source and that only C.albicans, C.tropicalis and C.stellatoidea produced any detectable proteolytic activity in unconcentrated BSA medium culture filtrates. These results would appear to suggest that the enzyme is related to the pathogenicity of the fungus. However, the culture filtrates were assayed only after 5 days and it may be that maximum activity is not reached,in all species,at this time. Further experiments must, obviously, be carried out to compare growth patterns of different species in BSA media, but there was no time to do this in the present investigation. If the proteinases produced by all species are shown to be antigenically similar molecules and the enzyme is used in serodiagnostic tests as an antigen, it would be, in theory, the only antigen necessary to establish a diagnosis ofcandidosis, instead of a range of ill-defined antigens in cytoplasmic extracts from all species. Since the treatment of candidosis is the same regardless of the infecting species, establishment of the identity of the invading species is not a clinical necessity. If there are antigenically distinct proteinases in different species, the notion of a simple, one-antigen test for candidosis would be invalid, but the enzymes might be a useful tool for epidemiological studies of the disease.

The role of proteinase as a factor in the pathogenicity of C.albicans was further supported by the observation that the enzyme is secreted into tissues around C.albicans in kidney microabscesses from experimentally infected mice. This implies that the enzyme is at least available to break down tissues, though demonstration that the enzyme does attack tissues will require further experimentation.

The facts that larger amounts of enzyme are produced at 26^{o}C than at 37^{o}C and that the enzyme has a pH optimum of 3.2 do not negate its postulated role in the virulence of C.albicans. It is likely that the enzyme is destroyed more rapidly at 37^{o}C than at 26^{o}C since proteinases are self-degrading and the rate of production of the proteinase at 37^{o}C was not studied over short periods of time. Patterns of growth of the fungus at the two temperatures are similar, which may

indicate that equal amounts of enzyme are being produced. Although proteinase has a very low pH optimum, many of the sites commonly affected by Candida e.g. the mucous membranes, have pH values about 5 or below (Staib, 1965).

The localization of proteinase synthesis in C.albicans is not yet known. Presumably the enzyme is made inside the cell and is secreted. It is possible that the enzyme may be located in the vacuoles described by Montes and Wilborn (1968). In S.cerevisiae, similar vacuoles have been shown to contain hydrolytic enzymes corresponding to lysosomes (Matile and Wiemken, 1967). The acidic nature of proteinase also suggests that the enzyme might be located in a lysosome or a similar type of structure. If the enzyme is in vacuoles, the vacuoles may fuse with the plasmalemma to secrete the proteinase. Cytochemical demonstrations may provide evidence for the intracellular location and route of secretion of the enzyme in the future.

Neither morphological form of C.albicans can be said to be exclusively invasive or non-invasive (see Chapter 1.5). However, Hurley and Stanley (1969) suggested that the yeast form initiated infection and that mycelium production occurred later. Budtz-Jörgensen et al., (1975) and Taschdjian and Kozinn (1957) also noted primarily blastospores in C.albicans - colonized individuals until clinical lesions were apparent when mycelia were also detected. In a study by Staib et al., (1972), yeast forms of C.albicans were noted in granulomata, whereas in larger necrotic areas, the fungus was also found in the mycelial form. Similar results were noted in the immunofluorescence experiments in this study. Primary lesions contained mainly blastospores whereas both forms were seen in larger lesions. Amino acids have been suggested as one of the several environmental factors that stimulate mycelium production (Odds, 1979). It is possible that the yeast form initiates infection secreting proteinase - this form is the one that produces maximal amounts of enzyme (Staib et al., 1972) - which in turn breaks down proteins in the tissue to their constitutive amino acids. These then stimulate conversion of the fungus to the mycelial phase leading to large necrotic lesions containing both phases of growth. This hypothetical argument fits the available evidence from in vitro and in vivo experimentation but requires verification by direct experimentation.

The antigenic specificity of proteinase for systemic candidosis appeared to be high when the enzyme was tested against the sera from two different groups of experimentally infected rabbits, since anti-proteinase precipitins were found only in those animals with a chronic Candida infection. In those rabbits that were treated with ketoconazole after infection to prevent establishment of tissue invasion, i.e. a situation similar to a transient candidaemia in patients, no anti-PP precipitins were found. It must be stressed that these sera were examined retrospectively and that the two groups of rabbits were given different doses of C.albicans to begin with for purposes other than this investigation. The results nevertheless appeared to be promising.

When proteinase was tested against the sera from human cases, the results were not so clear cut, since some sera from patients without candidosis and in those who had anti-CE antibodies but no other corroborative evidence of infection, were anti-PP positive. There was however a tendency to anti-PP titres greater than 1:4 and greater than anti-CE titres, in the sera from patients with proven systemic candidosis. On this quantitative basis, proteinase appears to be a superior antigen to a cytoplasmic extract of Candida for serological diagnosis, and these results were supported statistically. However, it is clear that proteinase is still not a qualitatively specific antigen for the diagnosis of candidosis. The results of tests carried out, this far, suggest that titres to proteinase greater than 1:4 are likely to be indicative of Candida infection but this can only be substantiated by tests on much larger numbers of sera from patients of many types with and without candidosis. Ideally, fresh sera should be used, rather than sera that have been stored over a long period of time. The drop in titre with some of the American sera confirms the observations of Taschdjian et al., (1972), that prolonged storage of sera results in a fall in titres of precipitating antibodies. Some of the sera showed no variation in titre from the original value, and it has been suggested (D.W. Mackenzie, personal communication) that this might have been due to the fact that some sera were treated either with merthiolate or sodium azide as preservatives. It was not known which sera were so treated, so the suggestion cannot be confirmed.

It is possible that those patients in the 'false-positive' group with high

anti-PP titres may have had a Candida infection which was either undetected or of which we were unaware because of lack of available clinical information. In other cases, the positive reactions may have been due to the presence of mannan in the proteinase molecule since this is known to be a major cause of 'false-positive' reactions (Chew and Theus, 1967). The mannan portion of C.albicans acid phosphatase was shown to be the major antigenic determinant of that particular inducible enzyme (Odds and Hierholzer, 1973). The ratio of mannan to protein in the phosphatase was 7:1, so it is not surprising that the sugar played an important part in the antigenicity of the enzyme. However, the much smaller proportion of mannan in proteinase - 1.5% - is unlikely to play such a large part in its antigenic properties. Nevertheless, its removal or the neutralization of its antigenic properties may reduce the numbers of anti-PP positive sera in groups 1 and 2.

Cross reactions with C.albicans proteinase were noted in the sera of two patients with candidosis caused by species other than C.albicans. One patient had Candida endocarditis caused by C.glabrata and patient No.4 had a secondary Candida infection caused, presumably, by C.guilliermondii since this was the only yeast isolated. Although proteinase activity was not detected in either of the strains of C.glabrata or C.guilliermondii tested, it is possible that freshly isolated strains may be proteolytic, as it is known that the properties of strains change as they are stored in the laboratory and that even species considered as non-pathogens become pathogenic after they are repeatedly inoculated into animals and recovered (R. Hurley, personal communication).

The patient with secondary candidosis is a very unusual case as he also has pulmonary aspergillosis, and these two infections are rarely seen together (Winner and Hurley, 1964). Although titres to A.fumigatus cytoplasmic extract have recently fallen to 1:64 and those to proteinase to 1:4, the patient is still under review.

More sensitive tests, such as the enzyme-linked immunosorbent assay (ELISA) may also be useful in the detection of anti-PP antibodies particularly early in the course of infection.

'False-negative' serological reactions are known to occur in systemic

candidosis (Taschdjian et al., 1973), either because the patients have had no time to mount a detectable antibody response or because they are immunosuppressed. In these circumstances, it may be possible to detect circulating Candida antigens instead of antibodies. It is unknown whether concentrations of circulating proteinase antigen in such cases are likely to be high or low; the more sensitive ELISA test, rather than a precipitin test, would be the method of choice to detect the antigen, in case it does not appear in the bloodstream in concentrations likely to give visible precipitin reactions. Using this technique, Warren et al., (1977) were able to detect circulating C.albicans-related antigen several days earlier than they were able to do by means of CIE, although the significance of these findings is now in doubt (R.E. Warren, personal communication). No circulating antigen was detected in any of the human or animal sera tested by CIE in the present study. However, this was due, more probably, to the fact that proteinase in the serum would be destroyed by repeated freezing and thawing of the sera, than the lack of sensitivity of the CIE technique. Further studies should ideally be carried out on fresh serum samples.

It is possible that other enzymes of C.albicans are of value in the serological diagnosis of candidosis. A brief examination of both blastospores and culture filtrates by APIZYM strips revealed the presence of several other enzymes which have not yet been fully studied in the literature, and which were not examined in detail in the present investigation due to lack of time. The most notable of these, from the point of view of virulence factors, was the esterase. Lipolytic activities have been reported in some species of Candida (Kalle, 1972; Pospisil and Kabatova, 1976) although there are few reports of lipases or esterases in the pathogenic yeasts. Rudek (1978) has reported esterase activity in all the pathogenic species of Candida. Further examination of the properties of this enzyme may provide evidence for another antigen which, like the proteinase appears to have a role in the pathogenesis of candidosis, and potential diagnostic importance in serological tests for candidosis.

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INDUCIBLE HYDROLASES IN THE PATHOGENESIS AND SERODIAGNOSIS OF SYSTEMIC CANDIDOSIS

BY Fiona Macdonald

The aim of this study was to search for inducible hydrolytic enzymes of Candida albicans, to investigate any link between such enzymes and the pathogenesis of candidosis and to test these enzymes as infection-specific antigens in the serological diagnosis of deep-seated infection.

No inducible hyaluronidase or phospholipases could be detected under the conditions employed.

An acid proteinase, found in the culture filtrates of C.albicans grown at 26°C in a medium based on glucose and bovine serum albumin, was purified by a one-step chromatographic method. The enzyme was judged to be pure by serological and biochemical tests. It was shown to be a glycoprotein, containing 1.5% mannan, and to belong to the carboxyl proteinase.

Proteinase was detected in greatest amounts in the most virulent of the pathogenic members of the genus Candida. It was secreted in vivo and was detected by indirect fluorescent antibody techniques around C.albicans blastospores in kidney microabscesses of experimentally infected mice.

Purified proteinase appeared to be a very specific antigen when tested against sera from experimentally infected rabbits. Precipitin titres to purified proteinase exceeded 1:4 in 77% of sera from 13 patients with proven systemic Candida infection, in 23% of sera from 22 patients positive for Candida anti-cytoplasm antibodies but without corroborative diagnostic evidence of systemic candidosis, and in none of 28 sera from patients without either candidosis or anti-cytoplasm antibodies.

Although the results did not confirm unequivocally the role of proteinase as a factor in the virulence of C.albicans, they gave considerable support to this idea. Purified proteinase was not a qualitatively specific antigen for the serological diagnosis of candidosis, but it was shown to give quantitatively superior results to those obtained with traditional cytoplasmic extracts of the fungus.