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Studies On The Morphology And Growth Patterns Of Candida Albicans

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STUDIES ON THE
MORPHOLOGY AND GROWTH PATTERNS
OF CANDIDA ALBICANS

by

Anne Teresa Hendry

Department of Bacteriology and Immunology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada

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ABSTRACT

A more reproducible method than previously available was developed to verify the phenomenon that the growth of *Candida albicans* and *Cryptococcus neoformans* was inhibited by pooled human serum and that this inhibition was reversed by iron. Neopeptone-glucose (N-G) broth, employed as the basic medium, was found to be superior to other media, since Millipore filtration reduced its iron content. In comparison to dry weight, the measurement of growth by the optical density (O.D.) technique, was not only accurate but it also permitted a greater number of tests to be performed.

Cultural and environmental conditions such as pH, glucose concentration and the addition of cations were investigated for their influence on the growth-inhibiting effect of serum. The binding or dissociation of transferrin to iron is pH-dependent. This serum protein was found to be responsible for an increase in the inhibition of the growth of *C. albicans* in N-G broth containing serum when the pH was raised above 7.0. In contrast, growth was not significantly affected by pH values ranging from 5.0 to 9.0 in N-G broth alone or in broth containing serum plus iron. Growth of

C. neoformans was affected by serum in a similar manner, but this organism was also inhibited by an alkaline pH. These results showed that a pH of 7.0 was the proper value for use in all other experiments with both organisms.

Glucose was required by both organisms as a source of carbon. Nevertheless, it played no significant role in decreasing growth-inhibition by serum.

The cations, Zn^{2+} , Mo^{6+} , Mn^{2+} , Cu^{2+} , Mg^{2+} and Ca^{2+} had no significant effect on the growth of either organism in media containing serum.

Human albumin, γ -S-globulin or iron-free transferrin in N-G broth did not inhibit the growth of *C. albicans*. It was demonstrated that other effects of serum in addition to transferrin, acted to produce iron-reversible growth-inhibition. These effects caused changes in the morphology of the organism. Thus, the yeast-like growth was transformed to a mycelial form which required iron for growth. In N-G broth alone a large amount of growth occurred which increased very little with additions of iron. However, a marked response to iron additions was noted even when low concentrations of serum were present. The quantity of iron required to permit growth was greater for higher concentrations of serum.

The iron-reversible growth-inhibiting effect of the powerful iron-chelator desferrioxamine (DFOM) was much more evident in aerated cultures. This confirmed the fact that an oxidative metabolism (associated with the mycelial form) was more dependent on iron than was a fermentative one. The iron-dependent growth of *C. albicans* in media containing serum was also demonstrated by a negligible catalase activity of the sparse growth of cells harvested from such a medium. In contrast, cells harvested from broth, or broth containing serum and iron, had a considerable catalase activity.

Normal phagocytes possess an H_2O_2 -dependent system for killing ingested cells of *C. albicans*. Catalase activity of *C. albicans* cells may be indirectly involved in this system. In the blood of normal individuals cells of *C. albicans* first change to a mycelial form and then they may be ingested. Such cells would be killed since they could not synthesize catalase.

In contrast to numerous reports it was demonstrated that a high concentration of glucose, or other nutrients, did not inhibit chlamyospore formation by *C. albicans*. Furthermore, solid media were not required for their production. Numerous chlamyospores could be obtained at 25°C in liquid media containing serum and glucose. Partially purified preparations of chlamyospores could be produced consistently using a differential

settling method by which all blastospores were removed leaving masses of chlamyospores still attached to pseudomycelium. These chlamyospores retained their characteristic appearance for several months. Mature chlamyospores were not observed to bud or germinate *in vitro*, nor after they were injected into mice.

Cell walls of chlamyospores were observed to split in a characteristic way under slight pressure or even when left in media for varying periods. Globules of lipoidal material were emitted from split cells.

Addition of 0.3% phenethyl alcohol (PEA) to serum suppressed germ-tube formation by blastospores of *C. albicans*. Instead, other structures resembling buds were formed. This showed that the usual response of *C. albicans* to serum was a genuine morphological change.

INTRODUCTION

The susceptibility of certain individuals to candidiasis, in contrast to the apparent immunity of the majority of individuals who are carriers of *Candida albicans*, has not been fully explained. However, circumstantial evidence and clinical observations have indicated that individuals who have certain debilitating diseases such as acute leukemia or diabetes mellitus, or who may have defects in cellular immunological mechanisms, are more likely to acquire candidiasis. Indirect evidence indicates a possible role for cellular immune mechanisms in protection against candidiasis. In contrast, humoral antibodies do not serve in a protective capacity and are found in high titre as a result of established infection.

The literature on *C. albicans*-host relationships has steadily emphasized candidiasis in susceptible patients. An investigation of candidiasis on these terms requires an understanding of a number of serious, pathological disorders of the human body as well as an understanding of the organism itself. These difficulties can be lessened by studying the relationship of *C. albicans* to the healthy human body which so often harbors it. This, in fact, constitutes the normal *C. albicans*-host relationship. The simplest "model system" which can be devised for such a study could be based on the use of human serum as a medium for growth. A description of

any other suitable system has not appeared in literature to-date.

A well-known change that occurs in *C. albicans* cells after a short period of incubation in serum is the formation of germ-tubes which may develop into septate mycelia. The significance of this alteration in morphology has not yet been determined. In addition, there has been some controversy regarding germ-tube formation as to whether or not it constitutes the invasive form of the organism.

Virulent organisms play a dynamic role during their invasion of a host, whether or not this invasion results in disease. Smith (1968) pointed out that, in most cases, pathogenicity cannot be related to a single microbial product. Furthermore, one cannot properly study virulence *per se* unless the proper cultural conditions are determined for demonstrating a virulence factor. Avirulence may be due to the absence of only one of the factors which determine virulence (Smith, 1968). In candidiasis, however, such factors may be entirely of host origin, the active disease resulting from the response of the organism to these factors.

C. albicans is a member of the normal flora of skin, mucous membranes and intestines in mammals. Normally, a state of resistance exists, but the loss or absence of one or more defence factors results in infection with this organism. For example, loss of bacterial flora on mucous membranes or in the intestines may result in superficial

candidiasis. This occurs because cells of *C. albicans* multiply into a large 'inoculum size' which, together with the accumulation of irritating substances, cause the apparent infection.

The undoubted ability of *C. albicans* to reach the blood stream, or closed cavities of the body by penetrative growth or by persorption from the intestines, indicates that the normal body may have humoral and cellular mechanisms for the prevention of infection. The barriers that must be penetrated probably ensure that only a small 'inoculum size' reaches the blood stream at any one time. However, 'inoculum size' would be of no importance if the organism multiplied freely in the blood stream or other body fluids. Under these conditions the classical, immune defenses of the body would be overwhelmed permitting the rapid accumulation of living and dead cells as well as toxic products. Obviously, this situation does not occur in normal individuals.

The literature cited in this thesis indicated a role for the iron-unsaturated transferrin in controlling the growth of microorganisms reaching the blood stream and other body fluids. Iron is known to be essential for the most efficient metabolic functioning of aerobic microorganisms. Therefore, chelation of iron by transferrin, making the iron unavailable, results in growth inhibition. Part of the work included in this thesis attempted to verify this and to determine some of the factors influencing this growth inhibition for *C. albicans*.

A morphological transformation of *C. albicans* occurs in blood-serum or related substances. Present work attempted to determine to what extent this change may be analogous to the transformation in *Mucor rouxii* in which the mycelial phase tends to be oxidative and the yeast phase fermentative.

In addition to growth the metabolic functions of an organism may also be important in infection. Polymorphonuclear leukocytes (PMN) in blood engulf and kill invading microorganisms by a biochemical mechanism in which H_2O_2 is the toxic agent. Aerobic organisms produce catalase and therefore could render this H_2O_2 relatively innocuous to themselves or, at least, delay their death. Catalase is a heme protein and therefore a deficiency of iron would inhibit catalase production and other enzymes containing iron in their structures. In *C. albicans* the deficiency of iron for catalase production would enable the PMN to dispose of them quickly. This indicates that the pre-incubation of *C. albicans* cells in serum before phagocytosis may be of maximum benefit to the host. In this connection it should be noted that several reports in the literature have described the avirulence of filamentous strains of *C. albicans*. It is also of interest that the level of iron-saturation in leukemic patients is often high and these patients are unusually susceptible to candidiasis. The nature of certain constituents of the cytoplasm and their biochemical activity in cells of *C. albicans*, grown in media containing serum, has not yet

been investigated. Present investigations included studies of the catalase activity of the cells of *C. albicans*.

Finally, germ-tube formation by *C. albicans* in serum may prevent the number of infective units from increasing effectively even though nuclear division and increase of cytoplasmic volume may continue to occur.

In addition, present studies also sought an explanation for chlamydospore production in *C. albicans*. It is the writer's firm belief that when the significance of chlamydospore production is known *C. albicans* will be less of a mystery than it is at present.

HISTORICAL REVIEW

The asporogenous genera *Candida* and *Cryptococcus* are classed with the *Fungi Imperfecti*. Lodder and Kreger-Van Rij (1952) placed all such yeasts in the order *Cryptococcales*, containing one family, the *Cryptococcaceae*. *Candida* and *Cryptococcus* are two of the seven genera in the subfamily *Cryptococcoideae*, in which arthrospores are absent. Although *Candida albicans* (Robin) Berkhout 1923 and *Cryptococcus neoformans* (Sanfelice) Vuillemin 1901 are morphologically and physiologically different, they are two important fungal pathogens.

In general, *C. albicans* is composed of round or oval cells called blastospores. It also produces true mycelium, or more often, pseudomycelium with clusters of blastospores attached to it. *C. albicans* also produces, under certain conditions, characteristic cells called chlamydospores. These cells are large, round, thick-walled and are usually produced terminally on pseudomycelia. Metabolically, *C. albicans* can readily ferment glucose as well as several other sugars. Recently, Winner and Hurley (1964, 1966) have published two detailed reviews on *C. albicans* and candidiasis.

In contrast, *C. neoformans* is composed of round or oval cells, and rarely produces any filamentous structures. It is not a fermentative organism, although it can assimilate glucose and some other sugars. Littman and Zimmerman (1956) and Kao and Schwartz (1957) have described in detail the morphology and physiology of *C. neoformans* and the mycotic infections it causes in man and animals.

I. OPPORTUNISTIC INFECTIONS

C. albicans is the fungus most frequently isolated from a variety of mycoses in humans and animals. In addition, *C. albicans* is isolated as a saprophyte from skin, mucous membranes and alimentary tract of normal persons with no evidence of candidiasis (Marples & Di Menna, 1952; Winner & Hurley, 1964). *C. albicans*, *C. neoformans*, as well as other fungi cause what has been termed 'opportunistic infections'. This term at present is not favored by all medical mycologists because of its anthropomorphic inferences. A detailed discussion of opportunistic fungus infections to which a number of individual authors contributed has been published (Baker & Chick, 1962). Host and organism-mediated factors are relevant in any discussion of candidiasis. With few exceptions, these factors are little understood, least of all the organism-mediated factors.

A. Virulence of *Candida albicans*

Winsten & Murray (1956) reported that growth of *C. albicans* in a medium with cysteine suppressed filamentation and enhanced its virulence. Mourad & Friedman (1961) showed that strains of *C. albicans* differed in their virulence for mice. Soluble substances obtained from *C. albicans* cells, as well as from a nonpathogenic yeast, were also lethal for mice. Mankowski (1962) demonstrated that culture filtrates of *C. albicans* had a growth-depressing effect on newborn mice. Iwata & Uchida (1969) isolated an intracellular protein toxin from *C. albicans*. It was toxic for mice and also enhanced candidal infection in mice. Haley (1965) indicated that it was difficult to determine the existence of a *C. albicans* infection in humans. Although *C. albicans* cells in large numbers could be present in urine, this was not necessarily indicative of bladder infection. In addition, filaments, which are usually found in association with pathological lesions, could be present if the urine was acid.

B. Host Treatment

1. Hormone and Immunosuppressive Treatment. There is general agreement that steroids administered systemically promote the dissemination of deep fungal infections in laboratory animals. In humans the effect of steroids is difficult

to assess because patients receiving this treatment may already lack resistance to fungal infections and may be receiving antibiotics and cytotoxic agents as well (Burns, 1958). However, he reported that case histories of patients with mycotic infections do indicate a role for steroids in promoting systemic fungal infections. Rifkind, Marchioro, Schneck & Hill (1967) reported that immunosuppressive therapy played a role in fungus infections in renal transplant cases. They found systemic fungal infections in almost half of such patients. Most of these had bacterial and/or viral infections as well. *Candida* spp. caused the majority of the mycoses.

2. Antibiotic Treatment. Rabbits, whose bronchi were infused with *C. albicans* and tetracycline antibiotics, developed severe bronchopulmonary granuloma-like lesions, with hemorrhagic alveolitis, which did not occur under other experimental conditions (Felisati, Bastianini & De Mitri, 1959). Kashkin, Krassilnikov & Nekachalov (1961) stated that *Candida* infections may be the result of prolonged antibiotic therapy which causes shifts in microbial associations on mucous membranes. In addition, Seelig (1968) suggested that antibacterial antibiotic therapy may contribute to fungal diseases in otherwise normal or not seriously ill persons. Particularly notable was her emphasis on the importance of the intestine. It is in the intestine that an increased growth of *C. albicans* occurs under heavy antibiotic therapy.

In her view *C. albicans* penetrates the intestinal wall as mycelium and thus could be disseminated from this locus. Krause, Matheis & Wulf (1969) administered *per os* a large quantity of viable *C. albicans* cells to a human volunteer. The number of cells administered was equal to the number available in the intestine after antibiotic therapy and fungal overgrowth. Specimens of blood taken at 3 and 6 hours, and of urine at 2 3/4 and 3 1/4 hours were positive for *C. albicans*. In this case the rapid response indicated that transfer of the cells to the blood stream occurred by 'per-sorption' and not by penetrative growth.

C. Predisposing Host Factors.

Pre-existing host factors are of paramount importance for the establishment of some systemic fungal infections. Cancer hospital records of diagnosed mycoses showed an increasing incidence in succeeding years (Hutter & Collins, 1962). Of the mycoses, 56% were candidiasis, which usually occurred as a secondary infection. Half of these cases were diagnosed at autopsy. Bodey (1966) studied 454 acute leukemia patients by *post-mortem* examination and found that fungal infections had become a major complication. Most of the fungal infections were caused by *Candida* spp. He had determined that fungal infections had caused the death of 61% of the 161 patients with mycoses. He also noted that therapy and pre-existing

bacterial infections had played significant roles in the establishment of fungal infection. Salter & Zinneman (1967) reviewed the records of 185 patients with bacteremia or fungemia. These had been diagnosed entirely from positive blood cultures. The infection in nine of the patients was caused by *Candida* spp., and death in six was believed due to candidiasis.

A summary of the primary or iatrogenic conditions leading to the establishment of systemic candidiasis was recently published by Kozinn, Taschdjian, Seelig, Caroline & Teitler (1969). They pointed out that this infection is commonly diagnosed at *post-mortem*. They proposed a composite picture of the potential candidiasis patient which included the existence of a primary debilitating disease such as malignancy, diabetes, rheumatic heart disease or bacterial infection. The patient would have had prolonged therapy including immunosuppressive drugs, steroids, antibiotics, or other drugs by indwelling catheter. They cautioned that *C. albicans* should not be considered a saprophyte when isolated from blood, closed cavities or internal organs.

D. Environmental Factors

Scherr (1953) found that mice inoculated with *C. albicans* and kept either at a low temperature (5-12 C) or a high temperature (35-37 C) showed more severe dissemination

of infection and a higher death rate than mice kept at intermediate temperatures. Germ-free chicks inoculated with *C. albicans per os* subsequently displayed numerous hyphae in the alimentary tract, unlike conventional chicks which showed no evidence of infection (Balish & Phillips, 1966a). Conventional chicks suffered an invasion of the blood stream and kidneys by the yeast form of *C. albicans* when fed an x-ray treated diet that had been stored for six months at 25 C (Balish & Phillips, 1966a). Balish and Phillips (1966b) also found that severe crop infections with *C. albicans* occurred in germ-free chicks inoculated with *Streptococcus faecalis*, but only small lesions with few hyphae resulted if the chicks were pre-inoculated with *Escherichia coli*.

E. Phagocytosis

Phagocytosis may be intrinsically involved in the initiation of infection, as well as in aborting imminent infection. The experiment of Krause *et al* (1969) indicated that *C. albicans* cells rapidly reach the blood stream from the intestine. Introduction of *C. albicans* into the blood stream apparently does not result in systemic candidiasis being established. This tentative conclusion may be reached by noting that *C. albicans* is part of the normal body flora, but systemic candidiasis is secondary to other disease states (Kozinn *et al*, 1969). The normal host-*C. albicans* relationship is one in which no disease occurs. Published reports on the

behavior of *C. albicans* cells introduced into the peritoneum were conducted mainly with animals, inoculated with relatively large numbers of cells. After inoculation, *C. albicans* cells were seen to develop in a characteristic way and mobile body cells also acted in a characteristic fashion (Hill & Gebhardt, 1956; Young, 1958).

1. General Observations. Reynolds & Braude (1956) observed that *C. albicans* yeast cells grew into true mycelium upon incubation in human blood. This change occurred both in extracellular yeasts and in those within neutrophils. The filaments grew rapidly and punctured the walls of the leukocytes. Hill *et al* (1956) studied mycelium formation from *C. albicans* cells injected intraperitoneally and subcutaneously into the tissues at the back of the neck of mice. Phagocytosis was observed at two hours, and usually in the presence of germ-tubes, which were thought to hinder phagocytosis. Young (1958) also studied phagocytosis in mice and transformation of *C. albicans* into the filamentous form, when the yeasts were injected intraperitoneally. The cells showed mycelium formation in one hour. Monocytes were phagocytizing the cells at this time, especially those with filaments. Young (1958) describes the phagocytizing cells as seeming to be invaded by the filaments. By three hours the number of yeasts was much reduced, and rarely present at five hours. Polymorphonuclear leukocytes were present but

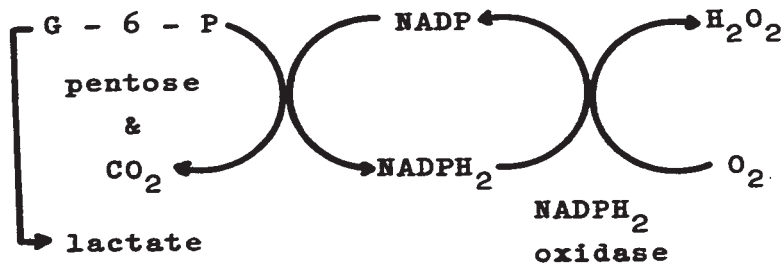
not seen phagocytizing. At two hours the pancreas was infected with many yeast cells with filaments, and by 24 hours invasion of this organ and of the kidneys was extensive. Mackenzie (1964) reported that phagocytosis had occurred, apparently by monocytes, within one hour after subcutaneous inoculation of mice with *C. albicans* cells.

Louria, Brayton & Finkel (1963) and Louria & Brayton (1964b) found that complete phagocytosis occurred in 30 minutes when any of six strains of *C. albicans* cells were incubated either in human blood or in washed human leukocytes in mouse plasma. Filaments of the cells of the least virulent strain were seen growing through the walls of the leukocytes much less frequently than those of the most virulent strain. The leukocytes may not have been perforated, for they were still functionally viable although reduced in phagocytic capacity. Four hours after phagocytosis occurred, the *C. albicans* cells remained viable. Stanley & Hurley (1969) found that mouse macrophages phagocytized all cells of *Candida* spp. in one hour in tissue culture fluid. Each macrophage contained one to five cells, and remained viable. Within two hours, almost all the intracellular yeast cells exhibited germ-tube formation. The filaments stretched the macrophage cell wall and occasionally were seen to break through it. However, even the remaining, viable macrophages which attempted to engulf the freed elements of *Candida*, died within 24 hours.

Rosner, Valmont, Kozinn & Caroline (1968) reported that neutrophils from leukemic patients phagocytized live *C. albicans* cells with a normal phagocytic index and percent phagocytosis. However, degranulation of the leukocytes was increased. The number and size of the germ-tubes formed by *C. albicans* was decreased. Leukemic patients' leukocytes lacked normal killing ability, while neutrophils from patients with chronic myeloblastic leukemia also had poor ingesting ability.

2. Molecular Basis of Phagocytosis. Polymorphonuclear leukocytes (PMN) from patients with chronic granulomatous disease fail to degranulate and release bactericidal and digestive substances into the phagocytic vacuole. Holmes, Page & Good (1967) found that such PMN cells did not show a normal increase in respiration during phagocytosis, although glucose consumption and lactate production were normal. The main differences between the patients' cells and normal cells was the lower resting level of $^{14}\text{CO}_2$ production from glucose $1\text{-}^{14}\text{C}$ in the first, without the stimulated production during phagocytosis that the latter showed. Normal cells oxidized several times the quantity of formate (an index of H_2O_2 production) during phagocytosis than the resting cells, but the patients' cells usually showed no such increase. McRipley & Sbarra (1967a) showed that H_2O_2 production is one reason for the increased O_2 consumption and increased metabolic activity by PMN during phagocytic activity. Killing of engulfed

bacterial cells was reduced under an N_2 atmosphere. Dialyzed PMN were reduced in H_2O_2 content and also showed reduced bacterial killing ability. The H_2O_2 -generating system was shown as:



McRipley & Sbarra (1967b) postulated that myeloperoxidase with H_2O_2 may constitute the killing system in PMN, because of the speed and nonspecificity of killing. The killing rate of bacteria was very high from pH 4 to 6. In the phagocytizing cell, the cytoplasmic granules (lysosomes), known to possess hydrolytic and proteolytic enzymes with acid pH optima, and antibacterial factors, decrease in number (= degranulation) due to their rupture into vacuoles containing the ingested material. The lysosomes also contain large amounts of myeloperoxidase. The authors postulated that a reduced pH would activate the release of NADPH₂ oxidase and myeloperoxidase. The H_2O_2 produced, plus myeloperoxidase killed bacteria synergistically. Inhibitors of myeloperoxidase also abolished the killing activity. Mandell & Hook (1969) found that when the killing activity of PMN for certain bacteria is low, as in chronic granulomatous disease, other bacteria were nevertheless killed. The difference was in these bacteria themselves, which produced adequate H_2O_2 to enable the otherwise H_2O_2 -deficient killing system to function.

Leukocytes from patients with chronic granulomatous disease acquired an improved killing activity of bacteria on ingesting latex particles to which glucose oxidase was bound (Johnston & Baehner, 1970). This oxidase could replace DPNH oxidase in generating H_2O_2 .

Lehrer (1969) found that the combination of myeloperoxidase, H_2O_2 and KI (or other halide salts) was rapidly lethal to *Candida* spp. and to other yeasts and fungi tested. The H_2O_2 could be replaced by an H_2O_2 -generating system of D-alanine, D-amino acid oxidase and FAD. Lehrer & Cline (1969) found that heat-labile serum constituents such as complement, promoted rapid phagocytosis, but different constituents promoted the subsequent rapid death of ingested *C. albicans* cells. A half-hour of pre-incubation of *C. albicans* cells in native or inactivated serum resulted in five to nine times the killing rate of subsequently phagocytized cells than pre-incubation in Hank's solution allowed. The authors observed that the poor killing ability of PMN of one patient was due to a deficiency of myeloperoxidase, and in three other cases the poor killing ability was due to other unknown factors.

II. IMMUNOLOGY

Adaptive immune responses to *C. albicans* antigens constitute a significant area of research on this organism and the disease it causes. Such research has been developed

in several ways, and includes studies on immunization methods, serological diagnostic procedures, the relationship of humoral and cellular defense mechanisms to resistance and studies leading to the establishment of an antigenic classification scheme.

Many reports published on immunization of animals against candidiasis indicate the difficulties encountered so far during these trials. Dobias (1964) summarized much of the literature on immunization of animals against *C. albicans* infection. Some protection was achieved by injecting mice with *C. albicans* or *Coccidioides immitis* spherule fragments (Hasenclever & Mitchell, 1963). Soles, Lim & Louria (1967) found that a partial, active homologous and heterologous immunity could be elicited from mice injected with *C. albicans* cells or other *Candida* spp. Passive immunization of mice against *C. albicans* demonstrated considerable protection, which included survival of one-third of them (Mourad & Friedman, 1968). Iwata *et al* (1969) purified a toxin from *C. albicans* that was an intracellular protein. The toxin was antigenic and produced a high titre of specific antitoxin in rabbits. The IgM fraction from the immune rabbit serum prevented experimental infection of mice by *C. albicans*. Al-Doory (1970) obtained complete protection of the highly susceptible baboon against any evidence of candidiasis by passive immunization. A lapse of time between serum administration and challenge was essential. However, he failed to find

a significant agglutinin or precipitin titre in the immune serum. He thought that either the protective factor was antibody, undetected by the usual procedures, or else was a substance producing a cell-mediating immunity in the recipient animal.

Patients with systemic candidiasis develop antibodies against *C. albicans*, and therefore a diagnostic test could be developed. The success of this test would depend on the use of suitable antigens. Comaish, Gibson & Green (1963) reported that skin tests for candidiasis did not reliably indicate the presence of this infection, but agglutinin titres of patients' sera did do so in most cases. Negrone (1968) found that complement-fixation and immunodiffusion titres were high in the sera of patients with systemic candidiasis, but negative for those with only superficial candidiasis. In contrast, skin tests were negative in candida septicemia and endocarditis, and positive in superficial and bronchopulmonary candidiasis. Preisler, Hasenclever, Levitan & Henderson (1969) reported that an agglutination test could assist in the diagnosis of systemic candidiasis patients. There is a great need for such diagnostic methods, for 23 of 33 acute leukemia patients were found on *post-mortem* to have visceral candidiasis, and only four had been correctly diagnosed. Fourteen of these patients showed a significant rise in agglutinin titre while alive. Taschdjian, Dobkin, Caroline & Kozinn (1964); Taschdjian, Kozinn & Caroline (1964) and Taschdjian, Kozinn, Fink, Cuesta, Caroline & Kantrowitz (1969) found

precipitating antibodies to cytoplasmic antigens of *C. albicans* in the serum of immunized rabbits and in systemic candidiasis patients. In some cases an apparent anergy was present.

Good, Peterson, Perey, Finstad & Cooper (1968, in Fujiwara, Landau & Newcomer, 1970) reported that patients with agammaglobulinemia are not unusually susceptible to fungal infections. This indicates that humoral defense mechanisms are less important in resistance to candidiasis than other defense mechanisms. Landau (1968) found that in some cases of recalcitrant cutaneous and systemic candidiasis, there were abnormalities of the thymus. This indicates that the existence of cellular immunological defects may predispose to candidiasis. In addition, Buckley, Lucas, Hattler, Zmijewski & Amos (1968) described the case of a patient with chronic mucocutaneous candidiasis, in which no agglutinins were present and no delayed hypersensitivity reactions could be shown. Serum immunoglobulins were normal. After being transfused with white blood cells, the immunological cellular defect was corrected, so that a delayed hypersensitivity to candida antigens developed, and the patient recovered from the infection. Fujiwara, Landau, & Newcomer (1970) reported that chicks whose bursae of Fabricius had been removed after hatching did not produce candidal agglutinins or precipitins upon challenge with nonviable cells, whereas thymectomized chicks failed to show a delayed hypersensitivity response.

The relative success of passive immunization in achieving protection against candidiasis may indicate that

antibodies directed against the antigens of a 'tissue phase' of *C. albicans* were present in the serum. However, Al-Doory proffered two explanations not based on this premise.

Reynolds *et al* (1956) observed that *C. albicans* cells rapidly undergo a morphological transformation in blood or plasma.

Antigens to detect antibodies against this altered form of *C. albicans* have not as yet been tested. Such studies could

assist in determining whether the yeast or mycelial form of *C. albicans* is the invasive and/or virulent form. A possible experimental model was suggested by Goos & Summers (1964), who showed that fluorescein-labelled antisera were bound to the cell walls of *C. albicans* cells. When these cells were incubated in unlabelled serum, germ-tubes developed whose cell walls also carried some of the labelled antibody.

Isenberg, Allerhand, Berkman & Goldberg (1963) showed serological differences in surface antigens of mouse virulent and avirulent strains of *C. albicans*. Tsuchiya, Fukazawa & Kawakita (1959) prepared specific antibodies against the antigens of *Candida*, to use for the rapid identification of the species. Fukazawa, Elinov, Shinoda & Tsuchiya (1968) obtained soluble specific polysaccharides from *C. albicans* and *C. tropicalis*. Fukazawa, Shinoda & Tsuchiya (1968) reported that the IgG fraction of rabbit immune serum was specific enough for use in agglutination and fluorescent work with *C. albicans*. The IgM fraction also had antibodies but they lacked specificity. Saltarelli (1968) found differences

between two strains and two mutants of *C. albicans* by electrophoresis and immunoelectrophoresis. Therefore, there is some evidence that a reliable, if complex, antigenic classification scheme could be developed for *C. albicans* and other *Candida* spp.

The development of high titres of humoral antibodies against *C. albicans* occurs as a result of infection. Active immunization is not yet feasible in normal individuals to achieve a high level of humoral antibodies as a protection against infection. However, in protection against some bacterial infections, nonspecific factors may play a role. Florman (1965) accidentally found that a staphylococcal extract gave protection to mice challenged within hours, too soon for antibody formation to have occurred. However, when the experiment was repeated, allowing a sufficient period of time to elapse before challenge, the extract failed to protect the mice. However, he did not suggest an explanation for the phenomenon. Kochan, Golden & Bukovic (1969) showed a relationship between serum hypoferremia and resistance to tuberculosis. Many other published reports also indicate the importance of the degree of iron-unsaturation of serum transferrin in resistance to infection by other microorganisms.

III. SERUM BACTERIOSTASIS AND FUNGISTASIS

A. Antibacterial Effect of Serum

In 1946 Schade & Caroline first investigated an iron-reversible bacteriostatic property of a serum fraction

using *Shigella dysenteriae*. This fraction was found to be rich in iron-binding protein. A possible role for this protein, called siderophilin or transferrin, in protection against infection, was not investigated for another decade. Jackson & Burrows (1956) reported that injection of large amounts of iron into mice with a non-virulent variant of *Pasteurella pestis* caused this bacterium to become lethal.

The bacteriostatic property of serum related to its content of iron-unsaturated transferrin was subsequently investigated by a number of workers who used the nonpathogenic *Bacillus subtilis*, as well as pathogenic bacteria. Martin (1962) noted that addition of iron to, or a lowering of the pH of serum resulted in a reversal of bacteriostasis for *B. subtilis*. He also found that the sera of four patients with agammaglobulinemia had a higher unbound iron-binding capacity of transferrin than normal, as well as a greater bacteriostatic activity. Bornside, Merritt & Weil (1964) found whole rabbit serum to inhibit the respiration of *B. subtilis*, and to be bactericidal. Iron in excess reversed the antirespiratory activity. Bornside & Getz (1967) found that a B_{2A} globulin was the most important antirespiratory serum protein for *B. subtilis*. Iron reversed the activity of this protein but not that of other serum proteins. Jackson & Morris (1961) found iron compounds to enable the avirulent strain of *P. pestis*, as well as *P. pseudotuberculosis* and *Salmonella typhimurium* to grow well in serum.

Schade (1963) reported that *Staphylococcus albus* strains failed to grow in serum unless iron was added to saturate the unbound iron-binding capacity. *S. aureus* grew in serum and was stimulated by the addition of iron. Moreover, the degree of saturation of siderophilin (transferrin) determined the length of the log phase and rate of growth. Growth rate at 90% saturation was four times that at 50% saturation, thus indicating that a high degree of saturation in a patient's serum may signify unusual susceptibility to this organism. Iron uptake by *S. aureus* at 95% saturation was four times that at 51%. The availability of iron to the cells was not merely proportional to the iron-saturation of the transferrin. Schade (1963) also found that the color of the cells and catalase activity indicated their iron content. Catalase activity, uptake of iron and growth of *S. aureus* was related to the degree of iron saturation of conalbumin, which has an iron-chelating activity related to that of transferrin. Bullen & Rogers (1969) found that reduction of oxygen tension shortened the response time in serum to iron addition for *P. septica*. Serum bacteriostasis evident at pH 7.5 was lost at pH 6.8. They reported that bacterial iron metabolism was important for *E. coli* and *P. septica* infections, in which serum transferrin, as well as complement and specific antibody may be involved.

A detailed investigation relating serum transferrin to a bacteriostatic effect *in vitro*, as well as to resistance

to infection has been reported, in which *Mycobacterium tuberculosis* was the test organism. Kochan & Raffel (1960) demonstrated an inhibition of the growth of *M. tuberculosis* in culture by sera from infected and BCG-vaccinated guinea pigs, but not by sera from normal guinea pigs. This property was not related to antibodies or complement. Kochan (1969) reported that transferrin in serum, through its unsaturated iron-binding capacity, was responsible for the tuberculostatic effect of serum, to the exclusion of any other factors. Iron sufficient to saturate transferrin completely neutralized the antimycobacterial activity of serum. Youdim (1969) also reported that iron-chelation by transferrin in human serum was responsible for tuberculostasis in culture. Normal guinea pig serum has a high level of iron-saturation of its transferrin, and it was not found to be tuberculostatic (Kochan *et al* 1969). This may explain the high susceptibility of guinea pigs to tuberculosis. Treatment of guinea pigs with lipopolysaccharide or tuberculous cell wall preparation caused their sera to become hypoferremic and also tuberculostatic. The tuberculostasis could be neutralized with iron. These authors suggested that the iron-transferrin system constitutes a humoral factor in resistance to tuberculosis.

A number of publications have reported on the virulence-enhancing effect of iron treatment of animals injected with bacteria. Martin, Jandl, & Finland (1963) found that iron administered to rats and mice enhanced the virulence of

Klebsiella pneumoniae. Some protection was offered by injections of iron-free transferrin. Iron compounds enhanced the virulence of *Escherichia coli* in guinea pigs (Bullen, Leigh & Rogers, 1968) and of *Listeria monocytogenes* in mice (Sword, 1966). In animals treated with iron compounds, the bacteria multiplied rapidly, resulting in serious disease and death of the animals. The strain of *E. coli* apparently possessed all the factors necessary for rapid growth *in vivo* except the ability to overcome a defense mechanism associated with iron. This mechanism was thought by Bullen *et al* (1968) to include both beta-2 and gamma globulins. The severe growth-limiting effects of the lack of iron was shown *in vitro* by Ratledge and Winder (1964) for *E. coli*. The growth rate was proportional to iron added in a medium free of trace metals. They suggested that in an iron-poor medium, a greater dependence on anaerobic fermentation resulted, and the growth rate was fixed by the amount of intracellular iron available.

Experiments which indicate indirectly that the transferrin factor may function together with antibodies have been reported. Bullen, Wilson, Cushnie & Rogers (1968) found that injection of iron compounds with *P. septica* into mice abolished the protective action of passive immunization. The bacteriostatic effect of horse anti-*Clostridium welchii* serum and normal horse serum was abolished by iron ions (Rogers, 1967). An immediate bacteriostatic effect not

reversible by iron was given by a mixture of B₂ and gamma globulins, but a delayed effect, reversible by iron, was shown by a mixture of the two proteins with transferrin. Bullen, Cushnie & Rogers (1967) showed that intravenously administered iron in *Clostridium welchii*-infected guinea pigs abolished the protective effect of antiserum, allowing the organisms to multiply in spite of excess anti-toxin being present in the plasma and tissues.

Resistance of mammals to bacterial pathogens may depend on the functioning of one or more factors. Unsaturated transferrin has been shown to act as a defense mechanism (Kochan *et al*, 1969). The failure of transferrin as a protective mechanism weakens the protection given by other mechanisms (Bullen *et al*, 1967, 1968). Transferrin may perform its protective function by inhibiting the growth of an invading organism during the time that other mechanisms involved in the killing and removal of the bacteria were functioning (Bullen *et al*, 1968). In addition, strains of some bacteria may be avirulent to test animals because the metabolism of the organism is unable to obtain iron *in vivo* (Jackson *et al*, 1956; Bullen *et al*, 1968).

The role of metallic ions as virulence factors for bacteria and the activity of these factors in the defense of the host against bacterial and fungal diseases has been reviewed by Weinberg (1966). He stated that, "Iron is the

metallic ion that presently appears to be most critical in determining whether an infectious agent is to be permitted to multiply in mammalian host tissues."

B. Serum Fungistasis

1. Serum Fungistasis Against Dermatophytes. Ayers & Anderson (1934) reported that 8% serum taken from patients with dermatophytid reactions and added to Sabouraud's medium, prevented the growth of dermatophytes. Serum from normal persons did not inhibit the growth of these fungi. Peck, Rosenfield & Glick (1940) found that sera in Sabouraud's broth possessed varying degrees of fungistatic activity toward *Trichophyton gypseum* (= *T. mentagrophytes*), and that sera from patients with dermatophytid reactions did not differ from others. Usually fungistasis was evident only at 30% or more of serum concentration. Lewis & Hopper (1948, in Lorincz, Priestley & Jacobs, 1958) also found that some sera were fungistatic regardless of the presence or absence of dermatophytid reactions of the patients. Lorincz, Priestly & Jacobs (1958) implanted Millipore chambers and dialysis bags containing *T. mentagrophytes* inoculum intraperitoneally into mice, and found that no growth of the fungus occurred although the inoculum remained viable. This indicated that a humoral and dializable factor was operating. This factor was unstable, because inoculum in dialysis bags placed in serum grew unless the serum was renewed daily.

Roth, Boyd, Sagami & Blank (1959) tested 156 sera and found most of them to be fungistatic to *T. mentagrophytes* and *T. rubrum*, with a mean inhibitory titre of 44.7%. Inactivation of serum did not alter the fungistatic capacity, but dialysis against water lowered it. Cord sera were less inhibitory for *T. mentagrophytes* than maternal sera. The serum of one patient with subcutaneous abscesses and granuloma due to *T. rubrum* was not inhibitory at 80%. Blank, Sagami, Boyd & Roth (1959) reported that *T. mentagrophytes* and *T. rubrum* will not penetrate the living dermis of skin in tissue culture if high concentrations of serum are used in the nutrient medium. Inhibition of the fungi was lowered if the serum was dialyzed against Hank's solution, or was inactivated. A number of proteins and fractions from serum singly or in combination did not show inhibition of penetration of the dermatophytes.

2. Anti-Cryptococcal Activity of Serum and Related Substances. Allen & Evans (1955) noted that the growth of *C. neoformans* was inhibited when mixed with animal sera. Dilution of human serum with neopeptone broth caused a gradual loss of its activity. Chicken serum was found to be fungicidal and fungistatic toward this yeast, but both activities were lost when 1% hog gastric mucin was added to the serum. Gadebusch (1961) reported that the anticryptococcal activity of mouse or guinea pig serum was not related to the pro-perdin system. It was Pillemer, Blum, Lepow, Ross, Todd & Wardlaw (1954) who first described this system as an apparently antimicrobial protein. Baum & Artis (1961a,b) reported

that sera from seven patients and three normal persons inhibited the growth of *C. neoformans* to different extents. Baum & Artis (1963) also found that the sera from three patients with lymphocytic leukemia, multiple myeloma and Hodgkin's disease, respectively, lacked an inhibitory effect against *C. neoformans*.

Howard (1961) found that although *C. neoformans* could be inhibited by a slightly alkaline pH, this factor was not the only reason for the inhibitory activity of 40% serum. Inactivation of serum, or absorption with live cells of *C. neoformans* did not alter the anticryptococcal activity of serum. A partially purified globulin extracted from the serum had no activity. Gadebusch & Gikas (1963) found that thiamine deficient mice were more susceptible to cryptococcosis, although *C. neoformans* has a requirement for this vitamin. The growth of the organism was equally inhibited by sera from normal and thiamine-deficient mice. Summers & Hasenclever (1964) reported that 10% mouse ascites fluid was inhibitory to the growth of *C. neoformans*, but that some reversal occurred when the concentration of nutrients was increased.

Igel & Bolande (1966) found no relation between the properdin system, complement or antibodies and the anticryptococcal effect of serum. Although he did not identify the inhibitor of growth in serum, it was protein, non-dialyzable and associated with a B-globulin fraction. A

number of cationic factors tested were not implicated, although phosphate decreased the anticryptococcal activity.

Szilagyi, Reiss & Smith (1966) found that addition of iron to serum may reduce its anticryptococcal effect, but they suggested that other unspecified factors besides the iron saturation of transferrin were acting. Of several serum fractions, the alpha-2-globulin and gamma-2-globulin were inhibitory. Reiss & Szilagyi (1967) found horse, sheep and other mammalian sera to have about the same anticryptococcal activity as human serum. Poultry sera showed only a short period of activity, while pigeon serum showed an enhancement of growth of *C. neoformans*.

3. Anti-Candidal Activity of Serum and Related Substances. Before the significance of the iron-unsaturated transferrin became known for fungi, some workers investigated the growth-depressing effects of serum in media for *C. albicans* and other yeasts. Roth *et al* (1959) used a turbidometric method to show that growth of *C. albicans* in the presence of cord sera was much less inhibited than in the presence of maternal sera. Under the conditions of these experiments, cord sera inhibited growth at 80%, maternal sera at 30% or less. Sera from infants became increasingly inhibitory up to the age of two. Absorption of sera with live or dead cells, or inactivation did not alter its activity. Roth & Goldstein (1961), in testing 250 normal adult sera, found that 5 or 10% was inhibitory to *C. albicans*. The growth of

C. albicans was less inhibited than that of other *Candida* spp. or *Saccharomyces cerevisiae*. The fungistatic effect of serum from acute leukemia patients was much less than normal. This was also true of Hodgkin's disease and multiple myeloma and in one patient with erythemic myelosis. Sera from a number of animals were also inhibitory. Different test media or media constituents failed to alter the titre pattern substantially. Inactivation or dialysis of serum also failed to have an effect. Roth (1961) reported that sera from patients with cutaneous candidiasis, diabetes, systemic mycoses, and metastatic carcinomas had a normal fungistatic activity against *C. albicans*. The fungistatic factor in serum was not thought to be related to classical antibody. It was destroyed by heating serum at 60 C for one to two hours.

Summers *et al* (1964) found that inhibition of growth of *C. albicans* in mouse ascites fluid was maximal at 10%. The serum from these mice, which had been treated with Freund's adjuvant to obtain the ascites fluid, was also fungistatic, but that from normal mice was not. Increased concentrations of the medium used, or increased concentrations of glucose reduced the fungistasis of ascites fluid. Addition of iron also reversed the fungistasis. Sera from mice treated with *Salmonella enteritidis* endotoxin, sublethal doses of *C. albicans* or dead *Coccidioides immitis* spherule fragments also inhibited *C. albicans in vitro*. Caroline, Taschdjian,

Kozinn and Schade (1964) reported that iron sufficient to saturate the unbound transferrin in human serum reversed the fungistatic action of the serum for *C. albicans*. In a low-iron medium made with egg white, iron additions had no effect until the unbound iron-binding capacity of the conalbumin was saturated. In serum, uptake of labelled iron by *C. albicans* cells was negligible at 50% saturation of the transferrin, but was high when excess iron was added.

Hasenclever *et al* (1963) showed that serum from mice immunized with *C. immitis* had more fungistatic effect on the growth of *C. albicans* than normal mouse serum. Louria *et al* (1964a) studied a factor in undiluted serum which was lethal for *C. albicans* and *C. stellatoidea* but not for other *Candida* spp., *C. neoformans* or *Saccharomyces cerevisiae*. The activity of the lethal factor was observed as decreased cell count determined by plating after an incubation period. The authors estimated a molecular weight of 10-20,000 for the substance, which was also susceptible to trypsin. It was thought to be present in the alpha and beta-globulin fractions after starch column electrophoresis.

Esterly, Brammer & Crouse (1967a) studied the anticandidal effect of a large number of sera. Cord sera, but not maternal sera, permitted growth to occur. Sera from young infants, from hemaglobinopathies, sera from patients with acute leukemia, hypoplastic anemia, acidosis, and

hypoparathyroidin spherocytosis were not fungistatic, although sera from patients with candidiasis alone were. Esterly, Brammer & Crouse (1967b) found that even high concentrations of transferrin alone, used in place of serum, failed to inhibit the growth of *C. albicans* in broth. A number of sera which were not inhibitory became so after transferrin was added. However, this did not occur for cord sera. Addition of excess iron to sera allowed growth to occur. Louria, Shannon, Johnson, Caroline, Okas & Taschdjian (1967) studied the sera of patients with endocrine hypofunctions and mucocutaneous candidiasis. Compared to the sera of normal persons, the sera of these patients were usually lacking in anti-candidal activity. Some of the sera also had substances interfering with the candidacidal activity of normal sera. Caroline, Rosner & Kozinn (1969) found that sera from many acute leukemic patients allowed a much greater growth of *C. albicans* than did normal sera. This was related to the iron saturation of the transferrin, because addition of transferrin to highly iron-saturated sera caused them to act as did normal sera.

In contrast to many other reports, Baum *et al* (1961) found no fungistatic effect of 50% serum on *C. albicans*, when tested by means of a serial inoculum dilution method.

The indirect effect of unsaturated transferrin has been shown to inhibit the growth of *C. albicans*. Experiments

to show the relationship of unsaturated transferrin to protection against infection *in vivo* are still lacking. However, this may be due to infection with *C. albicans* resulting in the persistent presence of the cells in the animal body. It is difficult to establish an experimental model under these conditions.

4. Serum Fungistasis Against Other Fungi. Baum *et al* (1961) found *Sporotrichum schenckii* and *Histoplasma capsulatum* to be inhibited by 50% serum. Roth *et al* (1961) reported that the growth of *Saccharomyces cerevisiae* was inhibited by human serum. Summers *et al* (1964) noted that mouse ascites fluid was as inhibitory to *Torulopsis glabrata* as it was to *C. albicans*.

Gale & Welch (1961) investigated the serum fungistatic activity against *Rhizopus oryzae*, for which growth was measured by dry weight. Five to 10% of pooled human serum stimulated the growth, but 20-30% inhibited it. Classical antibodies, complement, lysozyme or properdin were apparently not involved. Owens, Shacklette & Baker (1965) found that normal human serum inhibited *Rhizopus rhizopodiformis* at levels above 20%. Pooled sera from diabetes mellitus patients with ketoacidosis failed to have inhibitory activity, as did sera from acute leukemia patients.

English & Stanley (1966) reported that the growth of *Aspergillus niger* was more inhibited by serum than was

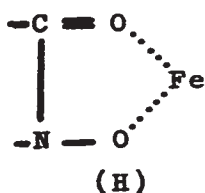
that of *A. fumigatus*, *terreus* and *flavus*. Weary (1968) showed that *Pityrosprum ovale* was partially inhibited by low concentrations of serum. Human gamma-globulin was also inhibitory, but not albumin.

C. Properties of Serum Transferrin.

Laboratory methods for the measurement of serum iron and serum iron-binding capacity have been described by Giovanniello & Peters (1963), Williams & Conrad (1966) and Giovanniello, DiBenedetto, Palmer & Peters (1968).

Holmberg & Laurell (1945) found that serum iron was nondialyzable at blood pH, and was bound to a protein. Acidified serum released its iron. Schade *et al* (1946) investigated Cohn fractions of serum for their ability to inhibit the growth of *Shigella dysenteriae*, and found that fractions II and III, and IV-3,4 were inhibitory, while V was slightly so. Only fraction IV-3,4 showed reversibility of inhibition upon addition of iron. This fraction also showed a salmon-pink color formation upon addition of ferrous iron. Schade, Reinhart & Levy (1949) found that maximum color formation occurred when one molecule of siderophilin (transferrin), the iron-binding protein of serum, reacted with 2 Fe^{2+} and two molecules of bicarbonate under aerobic conditions. Some titratable H^+ was freed for each iron molecule bound. Anaerobically, the color formed was yellow. Because of similarities of absorption maxima of conalbumin, siderophilin, aspergillitic acid and hydroxamine with iron,

a hydroxamic group was postulated by Fiala & Burk (1949) to be responsible for the iron-binding properties of these compounds.



Rath & Finch (1949) found serum iron-binding capacity to average 300 gammas in normal persons, with 200 gammas of unsaturated capacity per 100 ml serum. Considerable differences in serum iron and total capacity from the average value were found in the serum of patients with a variety of clinical conditions. More recently, Sinniah & Neill (1968) found the mean serum iron of normal persons to be 138.3 ± 4.3 ug/100 ml, and the mean total iron-binding capacity to be 470.1 ± 6.8 ug/100 ml. The mean saturation was 30.0%. There was a normal distribution and no sex differences.

Surgenor, Koechlin & Strong (1949) concentrated and crystallized the iron-binding protein of serum from fraction IV-7. The protein was a B₁-globulin. Both iron and copper were bound to this protein, and zinc was also thought to combine at neutral pH. Iron could displace copper from the complex. Above pH 6.5 the protein-iron complex developed its maximum color. The copper-protein complex had a green color, with maximum binding near pH 8.5. Koechlin (1952) reported physical and chemical properties of transferrin. Each molecule

combined with two molecules of ferric iron, but oxygen was required for ferrous iron to be bound. Transferrin was found to be very soluble in water. Iron, bound to the protein, could not be displaced by any other cation at any pH alkaline to 6.5. Rubin, Houlihan & Princiotto (1960) found that certain synthetic chelators were unable to remove iron from siderophilin in rabbit serum, but siderophilin was also unable to remove iron from the more tightly bound chelates. EDTA and HOEDTA at iron-binding ratios of 20-25/1 and 3-5/1 respectively, to that of siderophilin, competed on an equal basis for added iron.

There are several detailed reviews available concerning the chemistry and the physiological significance of transferrin. Among these are the reports by Giblett (1962) and by Bothwell & Finch (1962). Some of the properties of transferrin can be summarized as follows: transferrin binds two molecules of ferric iron in an ionic, not covalent bond. This binding requires one bicarbonate ion per ferric ion. The complex has a stability coefficient of about 10^{30} , and is most stable at alkaline pH. Transferrin is a glycoprotein with a carbohydrate content of about 5% (hexose, hexosamine and sialic acid) and has a molecular weight of 90,000. The isoelectric point is 5.45 when saturated with iron, and 5.80 when iron-free. Normal human subjects have a transferrin content of about 200-300 mg per 100 ml of plasma. The total iron-binding capacity ranges from 250-400 ug per 100 ml, but the normal concentration of iron is about

80 to 150 ug, leaving a capacity to bind 170-250 ug iron per 100 ml. Transferrin is not a single molecular entity, because on starch block electrophoresis, several distinct bands can be distinguished. Most individuals have the single transferrin C, the rest of the known types are uncommon. The level of transferrin and its degree of saturation can differ greatly from average values in various clinical conditions, and these can also vary in the same individual.

Peterson, Wyckoff & Sober (1961) found after 50% $(\text{NH}_4)_2\text{SO}_4$ precipitation, that several of the proteins of serum, including transferrin, were found in the supernatant fluid and in the precipitated portion. Bezkorovainy (1966) reported that the molecules of iron-saturated transferrin are more elongate and have a lower V_e than the iron-free transferrin.

In addition to iron and copper, transferrin also binds chromium, manganese and cobalt specifically (Aisen, Aasa and Redfield, 1969). Each metal is bound in the trivalent state, with bicarbonate. In addition, the binding with chromium indicated that the two binding sites of transferrin are different. However, Mann, Fish, Cox & Tanford (1970) found the two binding sites of transferrin to be identical. The molecule has two identical carbohydrate groups. They observed a molecular weight of 77,000, and all data indicated the existence of only a single polypeptide chain.

The unsaturated iron-binding capacity of blood serum can be altered *in vivo*. Endotoxin, or heat-killed

cells of gram negative bacteria injected into mice caused a hypoferremia which was specific, reproducible and dose-dependent (Baker, & Wilson, 1965).

Martin & Jandl (1960) reported an antiviral effect of transferrin in tissue culture. This effect was expressed as an inhibition of viral synthesis.

Two proteins similar to transferrin have been reported. These are milk transferrin (Baker, Jordan, Tuffery & Morgan, 1969) and gastroferrin (Rudzki & Deller, 1969). There are many other known iron-binding compounds of biological derivation. Some of these have been described by Weinberg (1957). In this review he reported that metallic ions may suppress or enhance the antimicrobial activity of many antimicrobial substances. Bickel, Gäumann, Keller-Schierlein, Prelog, Vischer, Wettstein & Zähler (1960) isolated a group of iron-containing, growth-stimulating compounds from streptomycetes. They proposed that these ferrioxamines be grouped with ferrichrome, coprogen and terregens factor in a new class of growth factors to be called the sideramines. Moeschlin & Schnider (1963) developed the potent drug desferrioxamine (DFOM) useful for the treatment of iron poisoning and hemochromatosis, by using an iron-containing compound from the group of sideramines. DFOM has a stability constant with iron slightly higher than that of transferrin-iron.

D. Conalbumin.

Schade & Caroline (1944) found that the growth of *Shigella dysenteriae*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* was inhibited in the presence of egg white. Iron alone of 31 elements tested had the ability to reverse the inhibition of growth in the presence of egg white. Alderton, Ward & Fevold (1946) partially purified an iron-binding substance, called conalbumin, from egg white. Bain & Deutsch (1948) purified conalbumin and determined its molecular weight to be 87,000. Fraenkel-Conrat & Feeney (1950) and Feeney & Nagy (1952) found that a number of bacteria were inhibited in growth by conalbumin. The inhibition was shown by an increased lag phase, and a slower rate of growth thereafter. Silva & Buckley (1962) investigated the antifungal activity of egg white in Sabouraud's agar and found that of 52 species of pathogens tested, only two *Rhizopus* spp. were not inhibited. Iron neutralized the antifungal activity for some fungi, and biotin did so in some cases.

IV. MORPHOLOGY AND PHYSIOLOGY OF CANDIDA ALBICANS

A. Metabolism of *Candida albicans*

Johnson (1954) found that no change occurred in the pH when *C. albicans* was grown in serum to which glucose was added. This was attributed by him to the buffering action of the serum. Staib (1964, 1965) found that some

strains of *C. albicans* proteolyzed albumin in serum agar at an acid pH and that this could be used for strain differentiation. Some strains utilized albumin as the sole source of nitrogen. He observed that since skin and mucous membranes may have a low pH, proteolysis may be a factor in mucocutaneous candidiasis. Staib (1969) also reported that only those strains of *C. albicans* showing proteolysis on serum protein agar caused extensive peritonitis and visceral infection in mice. No keratinolytic activity was found in *C. albicans* by Weary & Canby (1966). However, they used alkaline conditions in their experiments.

Rao, Ramakrishnan & Sirsi (1960) concluded that *C. albicans* had enzymes of both glycolysis (Embden-Meyerhof pathway) and the hexose monophosphate shunt. Kim, Adachi & Chow (1962) and Montes & Constantine (1968) demonstrated considerable leucine aminopeptidase activity in *C. albicans*. Cytochemical studies of *C. albicans* have demonstrated the presence of succinic dehydrogenase, NADH₂-tetrazolium reductase and NADPH₂-tetrazolium reductase activities (Mizuno & Montes, 1966).

Dastidar, Purandare & Desai (1967) found that *Candida* spp. grew in the mycelial form when growth conditions were poor. Best growth occurred when the pH was 5.0 - 6.0, and in media with inorganic ammonium compounds. Montrocher (1968) found that *C. albicans* required only biotin, and was

independent of requirements for several other factors. A thorough review of the nutritional requirements of *C. albicans* and other fungi was published by Gilardi (1965).

B. Chlamydospore Formation.

An early reference to chlamydospore formation is by Shrewsbury (1934). He distinguished between the plant *Monilia* spp. and the monilias of medical importance, now known as *Candida* spp. He also suggested the use of the term 'gemma' instead of chlamydospore for the terminal, thick-walled cells formed by a budding process. In addition, he maintained that the term 'chlamydospore' should be used for intercalary cells formed from existing cells. Linossier & Roux (1889, as reported by Shrewsbury, 1934) found that old gemmae ruptured through a V-shaped split, and they thought that the large globule therein was an endospore with the power to germinate. Shrewsbury (1934) disagreed with this observation. He noted that fat globules were often confused with endospores by early workers with fungi. He also observed V-shaped splits in gemmae of the medical monilias and reported that they produced new vegetation by budding daughter cells.

The formation of chlamydospores by *C. albicans* on a variety of media is, to the present, the most valid criterion for the identification of this species. Gordon, Bradley & Grant (1952) determined that agar made with yellow corn meal was superior for this purpose to that made with other corn

meals. Taschdjian (1953) indicated that an agar made with rice was easy to prepare and was comparable to corn meal agar. Nickerson & Mankowski (1953) devised a medium containing trypan blue and polysaccharide free of reducing sugars, with ammonium sulfate as the nitrogen source. Liu & Newton (1955) devised an alkaline agar medium containing no source of carbon and nitrogen. Pavlatou & Marselou (1957) found that substances such as bile, organic acid salts, albumin or tween '80' increased the production of chlamydospores of *C. albicans*. Walker & Huppert (1960) found that corn meal agar with tween '80' was a superior medium for chlamydospore production and for rapid identification of other *Candida* spp. Bakerspigel (1962) reported that a medium containing sodium taurocholate was excellent for the production of chlamydospores. Wolin, Bevis & Laurora (1962) found that media with a pH above 6.0 were very poor, and that a temperature of 26 C for incubation was better than 30 or 37 C. They found that combination of L-histidine, $(\text{NH}_4)_2\text{SO}_4$ and a small amount of glucose which was found stimulatory to the production of chlamydospores, could be used with tween '80' to make a medium better than corn meal.

This sampling of the literature covering several decades illustrates that little definitive work was reported on the nature and significance of the chlamydospore, *per se*.

C. Morphology and Cytology of *Candida albicans*

It is only during the last decade that several detailed studies have been published concerning the morphology of the blastospores and chlamyospores of *C. albicans*. For example, during the study of a large number of *C. albicans* strains, Brown-Thomsen (1968) found 15 forms, most of which could be differentiated by their macromorphology. Strains resembling *C. tropicalis* appeared as variants of *C. albicans*. Three types of pseudomycelium formation, and considerable differences in the reduction of triphenyltetrazolium chloride were also observed.

Van der Walt (1967) reported the isolation of sexually active strains of *C. albicans*, which he described as being homothallic. These homothallic strains apparently conjugated with their buds, and the result of this conjugation was the formation of monokaryotic zygotes. Zygotes could convert directly to chlamyospores or presumably form a diploid generation. A haplophase could then arise from small conidia. Examination of the photomicrograph showing van der Walt's 'chlamyospore' (Fig. 8, p. 249), reveals that it is not similar morphologically to chlamyospores produced by *C. albicans* on a variety of media. On the basis of his observations, van der Walt (1969) suggested that *C. albicans* should be placed in the genus *Syringospora* Quinquad emend. According to van der Walt, this yeast-like fungus now belongs with the *Hemibasidiomycetes*, which

is a group of smut fungi.

Bakerspigel (1963) isolated several unusual strains of *C. albicans* which produced numerous chlamydospores on Sabouraud's dextrose agar and also on other media containing up to 50% dextrose. Electron micrograph studies were facilitated since these strains produced numerous chlamydospores after a short period of time (Bakerspigel, 1964). Similar studies were made of blastospores by Bakerspigel (1964) and by Montes, Patrick, Martin & Smith (1965). Furthermore, in 1966, Bakerspigel also described the behavior of the blastospore nucleus during its division.

D. Dimorphism

1. Germ-tube Formation. Reynolds *et al* (1956) observed that *C. albicans* cells formed germ-tubes in plasma or serum to a dilution of 1/64. This was not due to agglutinins, and inactivation or dialysis did not alter this property of serum. Other workers also applied this phenomenon for identification of *C. albicans*. Taschdjian, Burchall, Kozinn (1960) found that germ-tubes could be produced in 90 min to 4 hr, at temperatures between 37 and 42 C. Mackenzie (1962) confirmed this finding, and noted other mammalian sera to be useful for this purpose. He also reported that too large an inoculum inhibited germ-tube formation, but age was not important. He determined the effective temperature to be between 31 and 41 C.

Landau, Dabrowa, Newcomer & Rowe (1964) stated that germ-tubes appeared to represent true mycelia rather than pseudomycelia since they elongate by apical growth and develop septa. They found that more cells produced germ-tubes in serum with the transferrin saturated with iron than in normal serum. The unsaturated transferrin formed an inhibitory factor against germ-tube formation. Landau, Dabrowa & Newcomer (1965) found that amphotericin B, Nystatin, cysteine, or Na_3EDTA reduced the percent germ-tube formation, whereas other antibiotics, antineoplastic agents, certain chemicals or dextrose had no effect. Absorption of serum with *C. krusei* or *C. albicans* cells greatly reduced the germ-tube formation. These authors were unable to determine the exact property of serum responsible for germ-tube formation, beyond noting that it was heat-stable, independent of complement and properdin, non-dializable, unrelated to agglutinins or precipitins, and that it was present in all sera tested. Grigoriu & Grigoriu (1969) found that complex media of plant or animal origin such as serum or Sabouraud's medium, allowed rapid germ-tube formation. A solution of plasma proteins failed to permit germ-tube formation. Dabrowa, Landau & Newcomer (1965) found that a significantly lower percentage of germ-tubes from *C. albicans* resulted in serum at various dilutions when the diluent was saline rather than distilled water.

Hill *et al* (1956) observed that in 60 min, cells

of *C. albicans* injected into mice formed what they termed pseudomycelia, although their photographs indicate that these were similar to germ-tubes. In four hours septa were present, and later, also lateral blastospores. Young (1958) studied *C. albicans* from peritoneal washings of mice injected with the cells. In one hour 60% of the cells showed mycelial formation, which, from his photographs, were germ-tubes. In two hours, 80% of the cells showed filaments. Louria & Brayton (1964b) showed that in blood, essentially all the *C. albicans* cells of several strains could be accounted for within viable polymorphonuclear leukocytes in 30 min. At four hours, the more virulent strains tended to longer filaments, either penetrating or distorting the phagocytes. Gresham & Whittle (1961) stated that the invasive form of *C. albicans* is mycelium and not pseudomycelium. The difference between these two forms was stated by him to mean metabolic continuity in the former and a string of functionally disconnected units in the latter. The mycelium of *C. albicans* taken from lesions showed RNA accumulations at the hyphal tip and at the septa, similar to that seen in other fungi.

The relationship of the tissue phase of *C. albicans* to the germ-tube formation observed in serum, *in vitro*, was also reported by Mackenzie (1964). He described the filaments formed in mice in one hour as being cylindrical outgrowths 0.6-1.2 μ in width, and up to 10 μ in length. Unlike buds, they lacked a constriction at the point of origin.

Mackenzie (1964) called the structures pseudogerm-tubes, to distinguish them from germ-tubes produced by authentic fungal spores. Furthermore he noted that the alteration in morphology was abrupt, with no transitional period. Mackenzie (1965) also studied the formation of germ-tubes by *C. albicans* cells on blood-free organ extract of rabbits. He found the lengths, and the numbers formed to vary in different organs.

2. Dimorphism of *C. albicans*. Germ-tube formation in this organism is now being used as a criterion for identification. However, it may have a significance related to the pathogenicity of *C. albicans*. Any definition of dimorphism, as it might apply to *C. albicans*, is inadequate at present because researchers differ about what constitutes the alternate morphological form. A number of workers, for example, Mardon, Balish & Phillips (1969), whose research was confined to cultural work with this organism, refer only to blastospore-pseudomycelium dimorphism. Scherr & Weaver (1953) state that pseudomycelium in *C. albicans* is an intermediate form between yeasts and hyphae.

A mutant of *C. albicans*, termed "filamentous", was studied by Nickerson (1954, 1963), Nickerson & Chung (1954), and Ward & Nickerson (1958). However, in a drawing published by Nickerson *et al* (1954), it is shown that the mutant formed pseudomycelium and not true mycelium such as is formed when cells of *C. albicans* are incubated in serum (Mackenzie, 1964). Nickerson (1963) found that protein disulfide reductase (for splitting S-S links in the glycoprotein network of the wall) was lower in concentration in the mutant than in normal

strains. Nickerson *et al* (1954) reported that growth of the mutant strain of *C. albicans* had become uncoupled from cellular division. The divisionless mutant was made more yeast-like by the addition of cysteine to the medium or by being streaked on agar next to a normal strain. However, Lingappa, Prasad, Lingappa, Hunt & Beimann (1969) reported that *C. albicans* produced autoantibiotics (phenyl alcohol and tryptophol) which caused its growth to become yeast-like. It is not known if the divisionless strain of Nickerson *et al* (1954) was deficient in these autoantibiotics. Ward *et al* (1958) observed that the filamentous mutant of *C. albicans* reduced oxygen more rapidly than the normal strain did. Bernander & Edebo (1969), in studying filamentation of *C. albicans* in Dubos' medium, suggested that the albumin in the medium may bind substances promoting yeast-cell formation.

Nickerson, Taber & Falcone (1956) reported that a filamentous mutant of *C. albicans* became virulent on regaining its yeast-like morphology. Gresham *et al* (1961) found that iso-nicotinic acid hydrazide (INAH) reduced the severity of candidiasis in rabbits. *In vitro* this substance prevented the mycelial development of the organism. Taschdjian & Kozinn (1961) reported that invasion by *C. albicans* is associated with inhibition of cell division, resulting in mycelium. In addition, they showed that a yeast-like tissue phase may be different from that seen in culture. They noted that *C. albicans* grown in serum did not evolve CO₂, although it rapidly

consumed glucose. They observed that triphenyltetrazolium chloride was not reduced by actively growing cells or mycelium of *C. albicans*. Phillips & Balish (1966) and Balish & Phillips (1966a) found that *C. albicans* became mycelial in the gut of germ-free mice and chicks, but remained in the yeast form in conventional mice and chicks. Taschdjian *et al* (1969) reported that *C. albicans* in the tissues and organs of candidiasis patients was in the yeast form. They concluded that the yeast form was the carrier of pathogenicity and was the invasive form, while mycelia were formed secondarily. Only yeast cells were seen in the macrophages. However, these workers studied terminal cases of candidiasis, which would indicate that their observations refer only to exceptionally susceptible patients, and to lesions in an advanced state of development. Romano (1966) reviewed the dimorphism of *C. albicans* in relation to its pathogenicity.

A fungus in which dimorphism occurs and which has been the subject of much study is *Mucour rouxii*. The information gained from these studies may be applied in the future to an understanding of other organisms like *C. albicans*, in which a less well-defined dimorphism exists. Haidle & Storck (1966) found that yeast-like cells of *M. rouxii* were converted to filaments upon exposure to air, whereupon synthesis of cytochrome oxidase was induced. Substances inhibiting the action or synthesis of this oxidase also inhibited the morphological change. Bartnicki-Garcia (1968) found that

M. rouxii converted to the filamentous form under a CO₂ atmosphere in low concentrations of glucose, but in high concentrations of glucose, a yeast-like morphology developed. Terenzi & Storck (1969) reported that the germination of spores of *M. rouxii* into filaments was inhibited by phenethyl alcohol (PEA). PEA did not inhibit synthesis of cytochrome oxidase or O₂ uptake, although a fermentative activity was stimulated. The activity of PEA was suggested to be due to uncoupling of oxidative phosphorylation. Elmer & Nickerson (1970) reported that a large inoculum of spores of *M. rouxii* under N₂ resulted in the formation of some yeast-like cells, whereas only filaments developed from a small inoculum.

EXPERIMENTAL

Introduction

The historical review presented here reveals the complexity of *Candida albicans* as a living organism as well as the complexity of its relationship to man and animals. *C. albicans* is among the easiest of microorganisms to culture on common laboratory media but its 'normal' habitat is the mucous membranes and intestines of animals, particularly man. However, at times this relationship with man changes so that it does harm to its host--it causes an infection. Completely satisfactory explanations for these changes have not as yet been given.

The literature on *C. albicans* may be divided into characteristic areas. One area is confined to *in vitro* studies. This includes studies of chlamydospore formation on a variety of media and some studies of its nutritional requirements. Another area encompasses numerical determinations of the carrier rate with regard to man. Still another area is concerned with the variety of infections caused by *C. albicans*. In general, these areas have not been studied simultaneously by individual workers, resulting in a segmentation of knowledge regarding *C. albicans*.

Some studies have been made on germ-tube formation by *C. albicans* in serum, (see Historical Review, P. 46-49), and on filament formation by this organism in culture, in serum and in living tissue (P. 49-51). Furthermore, several recent studies have now demonstrated that the growth of *C. albicans* in the presence of serum may be influenced by the unbound iron-binding capacity of the serum (P. 32-34). The results obtained from these studies have afforded an opportunity to understand better the role played by *C. albicans* and to connect the various areas of study.

Present studies were undertaken to understand the effect of the unbound iron-binding capacity of serum on *C. albicans* as well as the significance of germ-tube formation by this organism in serum. The possibility that these two aspects were linked was also investigated. A further link was sought in the area of chlamydospore formation.

Initially, it was essential to establish a working laboratory model and for this purpose, the growth of *C. albicans* in media containing serum was selected. It was also necessary to investigate the possible effect of pH; glucose concentration; aeration; cations and other variables on this model.

Serum fungistasis had been reported in a number of organisms in several previous publications (see Historical Review, P. 28-36). However, explanations for this phenomenon were lacking. Only one report showed by direct experiments that iron-binding by serum transferrin may

be responsible for serum fungistasis for *C. albicans*. (Caroline *et al*, 1964). Other reports suggested that this might occur. Therefore, it was necessary to determine whether or not serum fungistasis could indeed be demonstrated consistently. Thus, it was necessary to devise an experimental technique which could be employed in the analysis of serum fungistasis.

I. INTRODUCTORY EXPERIMENTATION

Materials and Methods

The organisms used in these experiments were a strain of *C. neoformans*, an oxidative yeast isolated by Bakerspigel, Campsall & Hession (1958); *C. albicans*, a fermentative yeast of variable morphological form; and *Histoplasma capsulatum*, a dimorphic fungus which is mycelial at room temperature and whose tissue phase is yeast-like. Stock cultures were maintained on neopeptone-glucose (N-G) agar containing 0.5% Difco neopeptone, 1% glucose and 1% agar. The yeast phase of *H. capsulatum* was maintained on blood agar slants at 37 C. For the first series of tests N-G agar slants were prepared which contained filtered, pooled, human serum from 1 to 6% and from 5 to 25% (Tables 1-5). The filtered serum, sterile distilled water and double-strength melted agar medium were mixed aseptically at 55 C to give single-strength medium. Slants were subsequently inoculated with yeast cells suspended in water and one drop was allowed to flood the surface of each slant which was then incubated at 25 C.

Other tests were performed in flat-bottomed petri dishes in which drops of iron salts were added to the surface of the agar after it had been inoculated by flooding and then dried. In these tests 20% human serum was used. In addition, 10, 25 and 50% serum without N-G were used for *H. capsulatum*.

Testing of Liquid Media

In order to measure or read growth by optical density methods it was necessary to devise liquid media to act as diluents for the serum. Littman's (1958) liquid medium was modified by being prepared double strength and by omitting the iron salt. Glucose was substituted for the main carbon sources. This liquid medium was then adjusted to pH 7.0 and sterilized by filtration. Another liquid medium used was casamino acids-glucose broth. It was prepared double strength and contained 1% casamino acids and 2% glucose. Another medium consisted of double strength broth containing 1% Difco neopeptone and 2% glucose (N-G broth). The growth of *C. albicans* and *C. neoformans* were tested in 25 and 10% serum respectively with each of these three media as diluents to make single-strength media. The serum was adjusted to pH 7.0 and sterilized by Millipore filtration. These tests were done in 13 x 100 mm tubes containing two ml of medium and additions of serum and water up to 4 ml. The media were also prepared with additions of FeCl_3 to make the final concentrations $1\mu\text{M}$,

10 μ M and 100 μ M. The inoculum consisted of 0.05 ml of suspensions containing 1.3×10^5 cells/ml and the tubes were incubated at 37 C. At 10, 15 and 21 hour intervals the tubes of *C. albicans* were shaken up on the vortex mixer and read at 500 mu on the Bausch and Lomb Spectronic 20 spectrophotometer. For *C. neoformans* readings were done daily for 7 days. Tests were also run in the same way with serum, diluted only with water, and containing 0.5% glucose.

Effect of pH

Growth of *C. albicans* and *C. neoformans* was determined in N-G broth adjusted to pH 6.0, 6.5, 7.0, 7.5, and 8.0 before inoculation. After a long period of growth (30.5 hrs.) the final pH was measured. In addition, growth in this medium was also determined when 25% human serum adjusted to the corresponding pH values was added. This test was performed in the same way as described for the testing of liquid media except for the longer incubation time.

Results

Human serum in an agar medium had a distinct fungistatic effect against *C. albicans*, *C. neoformans* and *H. capsulatum* (Tables 1-5). Tables 1 and 4 show that *C. neoformans* was inhibited by 2% serum and could, on prolonged incubation, overcome the fungistasis of 5 but not

TABLE 1

Growth of *C. neoformans* on N-G Agar
Containing 0-6% Serum

Serum %	3 days	5 days	10 days
0	+	+	+
1	+	+	+
2	<u>+</u>	+	+
3	-	+/-*	+
4	-	+/-*	+
5	-	-	+
6	-	-	+/-*

* Growth visible at top of slant.

TABLE 2

Growth of *H. capsulatum* on N-G Agar
Containing 0-6% Serum

Serum %	3 days	6 days	10 days	26 days
0	-	+	+	+
1	-	?	+	+
2	-	?	+	+
3	-	-	+	+
4	-	-	+	+
5	-	-	<u>+</u>	+
6	-	-	-	+

TABLE 3
Growth of *C. albicans* on N-G Agar
Containing 0-25% Serum

Serum %	4 days	5 days	11 days
0	+	+	+
5	+/-*	±	+
10	-	±	+
15	-	±	+
20	-	±	+
25	-	±	+

* Growth visible at top of slant.

TABLE 4
Growth of *C. neoformans* on N-G Agar
Containing 0-25% Serum

Serum %	4 days	11 days	13 days	18 days
0	+	+	+	+
5	-	+/-*	+/-*	+
10	-	+/-*	+/-*	+/-*
15	-	-	-	-
20	-	-	-	-
25	-	-	-	-

* Growth visible at top of slant.

TABLE 5
Growth of *H. capsulatum* on N-G Agar
Containing 0-25% Serum

Serum %	5 days	11 days	13 days	18 days	22 days
0	+	+	+	+	+
5	-	+	+	+	+
10	-	-	-	+	+
15	-	-	-	±	+
20	-	-	-	±	+
25	-	-	-	±	+

TABLE 6

pH Changes in X-G Broth During Growth of *C. albicans* for 30.5 hr.

Initial pH	<u>25% Serum</u>		<u>No Serum</u>	
	O.D.	pH, 30.5 hr	Initial pH	O.D. pH, 30.5 hr
6.0	1.2	4.65	6.0	1.1 4.3
6.5	1.0	5.05	6.5	1.1 4.2
7.0	.74	5.60	7.0	1.1 4.45
7.5	.61	5.90	7.5	1.1 4.6
8.0	.59	6.00	8.0	1.1 4.75

TABLE 7

pH Changes in N-G Broth During Growth of *C. neoformans* for 6 days

<u>25% Serum</u>			<u>No Serum</u>			O ₂
Initial pH	O.D.	pH, 6 days	Initial pH	O.D.	pH, 6 days	
6.0	.138	6.25	6.0	.485	4.45	2
6.5	.132	6.75	6.5	.360	4.75	
7.0	.082	7.30	7.0	.240	5.45	
7.5	.050	7.60	7.5	.250	5.80	
8.0	.050	7.90	8.0	.222	6.15	

of 6% serum. Table 3 shows that *C. albicans* was inhibited by 5% serum but could overcome the fungistatic effect of 25% serum on prolonged incubation under the conditions of the test. Tables 2 and 5 indicate that serum had a fungistatic activity against *H. capsulatum* at 3% although the organism progressively overcame this activity up to 25% upon continued incubation.

All three organisms grew well on N-G agar containing serum in the area where 0.05% ferric ammonium sulfate or 0.01% FeCl_3 were placed. *C. albicans*, however, overgrew the entire agar surface in a relatively short time obliterating the areas showing reversal by iron. *H. capsulatum* also grew well on agar containing serum without neopeptone or glucose in the area where 0.1% FeCl_3 was placed.

N-G broth permitted excellent growth of *C. albicans*. With the addition of serum, a considerable inhibition of growth was demonstrated. In addition, 100 μM FeCl_3 greatly stimulated the growth of this organism in serum-containing N-G broth. Casamino acids medium also supported the growth of *C. albicans* and allowed both a demonstration of serum fungistasis and its reversal with FeCl_3 . However, this medium was less satisfactory for growth than was N-G broth. The modified Littman's liquid medium was neither satisfactory for growth of *C. albicans* nor for demonstrating serum fungistasis. *C. neoformans* grew more slowly in these media but all proved satisfactory for growth and for demonstrating both serum fungistasis and its

reversal with iron for this organism. With the addition of thiamin and biotin to N-G broth this medium was found to be more satisfactory for *C. neoformans* than the others. Both organisms showed a considerable ability to grow in diluted serum containing glucose when iron was added.

A number of other experiments were also conducted with *C. albicans* and *C. neoformans* in which it was determined that various inorganic iron salts such as $\text{Fe}(\text{NO}_3)_3$, FeCl_3 , ferrous sulfate, ferric ammonium sulfate and ferric ammonium citrate were interchangeable. The final choice was FeCl_3 because it did not contain nitrogen in any form and because it could be obtained commercially in a pure form. Furthermore, it was soluble under conditions where other salts were not. Hemin was also tested as an iron source but reliable results could not be obtained because of poor solubility and the resulting color which interfered with optical density readings. However, use of .0008% hemin showed some reversibility of serum fungistasis for *C. neoformans*.

Tables 6 and 7 show the effect of initial pH of the medium on the final pH achieved after a long period of growth. *C. albicans* could lower a high pH value of N-G broth considerably as well as achieve considerable growth (Table 6). In the presence of serum the final pH achieved was not as low as it was without serum and, in addition, was correlated with much poorer growth above pH 7.0. In this experiment, *C. neoformans* did not

achieve considerable growth although it was much better at low pH values than at values above 7.0 (Table 7). In the medium without serum, *C. neoformans* also lowered the pH considerably after a period of growth.

Discussion and Conclusions

The growth of *C. albicans*, *C. neoformans* and *H. capsulatum* was inhibited by human serum incorporated into an agar medium. *C. albicans* and *H. capsulatum* showed a tendency to reverse the inhibition of the serum on prolonged incubation. A number of experiments revealed that iron could readily reverse the inhibition of serum. (P. 63,64).

N-G medium as a diluent for serum was found to be most satisfactory and was, therefore, chosen for further tests.

Although *H. capsulatum* showed an inhibition of growth on serum-containing agar media and a reversal with iron no further tests were done with this organism. No liquid medium completely suitable for measuring the fungistatic activity of serum could be devised for this organism. Furthermore, *H. capsulatum* could not be made to grow consistently in one phase in the presence of serum and the presence of masses of mycelium made optical density readings impractical. However, even the results obtained from the preliminary tests indicate that the

effect of serum with regard to iron-chelation could be a profitable area for future study on this fungi.

The results obtained in these preliminary studies paved the way for more detailed experimentation on *C. neoformans* and particularly on *C. albicans* (see next section). The results obtained on solid media should not be confused with those in liquid media because of obvious differences in micro-environment where the cells were grown. On solid media growth occurs as a surface layer, while in liquid media it occurs throughout the medium.

II. INVESTIGATIONS OF SERUM FUNGISTASIS

Materials and Methods

Inoculum

The organisms used in these studies were a strain of *C. neoformans* and *C. albicans* (7396). The strain of *C. albicans* was different from the one used in the introductory studies although it showed considerable chlamydo-spore production on appropriate media. The determination of inoculum size used in the following tests is recorded in Appendix I. Inoculum was grown in N-G broth, except in the case of tests with media reduced in iron where growth from N-G broth was suspended in sterile distilled water. Cells for inoculation were grown in half strength N-G broth at 37 C to obtain 1.3×10^5 cells/ml.

Medium

The medium most commonly used was double-strength N-G broth, containing 1% neopeptone (Difco); 2% glucose; 2 ppm thiamin and 0.02 ppm biotin. All of the neopeptone used in the medium was obtained from the same container. The iron content of the neopeptone was determined using standard methods (see Appendix 4). The medium was first sterilized by filtering through large-pored Millipore membranes and then through 0.45 or 0.22 μ - filters. In order to determine

possible loss of any iron from this medium during its preparation, the membranes were saved until 6000 ml had been filtered. They were then analysed for the presence of iron using the method described in Appendix 4. As a result all of the neopeptone media used were known to contain only 0.074 ug of iron in 5 ml of the single-strength medium used per tube in the tests. Although the concentration was extremely small, experimental results with serum and with iron additions (described later) showed that: 1) this concentration was adequate for optimum or nearly optimum growth of *C. albicans* and *C. neoformans* and 2) this amount was apparently made unavailable by as little as 2% pooled human serum.

Thiamin was added to the medium because *C. neoformans* requires it for growth (Littman, 1958). Where necessary, the medium was adjusted to pH 6.0, 7.0, or 8.0 or other values, using 1N NaOH or HCl.

Additional, special treatments of the N-G broth or other media, are noted in the experiments in which they were used.

Glass-distilled water was used in all of the experiments.

Human Serum Fractions

Pooled serum, in at least 150 ml lots, was obtained from a blood bank. The serum was first filtered through coarse Millipore filters, the pH adjusted to a required

value, then filtered through 0.2 μ Millipore filters. In some experiments, saline or water dilutions of the serum were prepared so that 1 ml additions to the test-tube, for the final tests, resulted in 1,2,3,4,5,10,15,20 and 25% solutions in single-strength N-G broth. In most experiments however, the serum was not diluted beforehand but the volumes in the test-tubes were adjusted by additions of water. The following were also employed in various tests; iron-free human transferrin (Calbiochem, Calif.); crystallized human albumin and γ -S globulin (Mann Research Lab., N.Y.); 5% Difco bovine albumin solution; Difco ascites fluid; and Difco horse serum. Serum dialyzed against water and against Tyrode solution were also used. Unsterilized preparations were sterilized by filtration.

General Procedure

Disposable 13 x 100 mm test tubes were used in most cases. When examined electrophotometrically (filled with cobalt chloride solution) they showed less variation in their optical qualities than did standard cuvettes. This was due to their very thin walls and the method of manufacture. It was essential to use these tubes because others were ruined by iron if autoclaved under the conditions used in some tests. Thus, difficulties encountered in obtaining very large numbers of matching test-tubes per test; scratched tubes or tubes spoiled by foreign substances were completely eliminated.

Tubes were soaked in 1N HCl and then thoroughly rinsed with glass-distilled water. The materials were pipetted aseptically into the sterile, foam-plugged tubes to a volume of 4 ml. This volume included 2 ml of double-strength broth with additions of various quantities of serum and water to make 4 ml. Some series of tubes contained 1 ml of 100 μ M FeCl₃ solution per tube. Other test materials were substituted for water as required. Thus, in some tests, the final, 4 ml of single-strength broth was also a 25 μ M solution of FeCl₃. This general procedure will hereafter be described as "standard". In some tests smaller amounts of more concentrated materials were added so that the final volume was 4 ml, if the number of materials to be added required it. Each tube was then inoculated with 0.05 ml of inoculum. This will henceforth be referred to as "standard" inoculum size. Test tubes were incubated at 37 C. In some experiments inoculated test tubes were also incubated at 25 C.

Optical density (O.D.) values of the tubes shaken in a vortex mixer were read on a Bausch & Lomb Spectronic 20 spectrophotometer at 600 mu. This wavelength was chosen after 'spectra' of each organism and of the broth were examined over a large number of wavelengths on this instrument. Separate blank tubes were prepared for each level of serum used.

The methods described here were selected following numerous tests to determine the best inoculum size; incubation

periods; relative rates of growth and their inter-relationships. The most useful readings were those which could be obtained over the most accurate range of the optical density scale on the spectrophotometer. These were taken at 9, 15, and 18 hours for *C. albicans* and at 2, 4, and 6 days for *C. neoformans*. Each experiment included a large number of tubes containing different concentrations of serum under at least two different conditions. Furthermore, they were read at three or more time intervals.

Initial experiments clearly demonstrated that there were no demonstrable effects resulting from the order in which ingredients were added to the tubes, or from the time that elapsed between additions and before inoculation. A convenient order was the addition of the broth and any water, followed by serum and then iron. Inoculation was made several hours after the last addition.

It should also be emphasized that the exact time of reading was irrelevant in these tests. Only the overall patterns of growth and the relation of the points to each other at any particular time interval were considered to be significant.

Specific Tests

1. Temperature

The effect of incubation temperature (37 C and 25 C) was determined for both *C. albicans* and *C. neoformans* when grown in N-G broth containing various concen-

trations of serum at pH 7.0. Readings were taken at appropriate intervals.

2. Inoculum Size

The effect of varying inoculum size by 9 and 3 times the "standard" was determined for both organisms grown in N-G broth containing various concentrations of serum at pH 7.0. Readings taken after 10, 14 and 18 hrs of incubation for *C. albicans*, and 2, 4 and 6 days for *C. neoformans*.

3. FeCl₃ Concentration

The effect of three widely differing concentrations of FeCl₃ (25μM, 10μM, and 2.5μM) was tested on the growth of both organisms in N-G broth containing various concentrations of serum at pH 7.0 by standard procedure. Readings were taken after 10, 14 and 17.5 hrs for *C. albicans* 4, 6 and 8 days for *C. neoformans*.

4. Dry Weight

A comparison of results was made after 16 hours of incubation when growth of *C. albicans* in N-G broth containing various serum concentrations was measured by both optical density and dry weight of the cells. The standard procedure was used except that four replications were pooled after incubation. Formalin was then added to make a final concentration of 2%. After 24 hours, the O.D. values were determined. The cells were then deposited on tared, dried Millipore membranes

by suction and washed. The membranes were dried in vacuo over P_2O_5 to a constant weight. The net weight of the cells at each serum concentration was calculated as a percentage of the weight of the cells in the control tube. For both dry weight and O.D., the control tube was the one without added serum or iron and was given the value of 100%. All other tubes were compared to this tube.

5. Cations

The effect of cations on *C. albicans* and *C. neoformans* in N-G broth containing various concentrations of serum was determined by standard procedure with and without $25\mu M$ $FeCl_3$. The effect of the following cations at pH 7.0 was tested: $25\mu M$ solutions of Zn^{2+} (as $ZnSO_4 \cdot 7H_2O$), Mo^{6+} (as $NaMoO_4 \cdot 2H_2O$), Mn^{2+} (as $MnSO_4 \cdot H_2O$); $2.5\mu M$ solution of Cu^{2+} (as $CuSO_4 \cdot 5H_2O$); $16\mu M$ solutions of Ca^{2+} (as $CaCl_2$), and Mg^{2+} (as $MgCl_2 \cdot 6H_2O$). Readings were taken after 9.5, 12.5 and 15.5 hours of incubation for *C. albicans* and after 2.5, 4.5 and 6.5 days for *C. neoformans*.

6. pH

The effect of the initial pH of the medium on the growth of *C. albicans* and *C. neoformans* in various concentrations of serum was studied by: 1) standard procedure at pH 6.0, 7.0, and 8.0; 2) use of 25% serum in broth for *C. albicans* and 10% serum in broth for

C. neoformans over several pH's. In two tests these were pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5; and pH 5.0, 6.0, 7.0, 8.0, and 9.0. The growth of the two organisms was also examined at these pH's in N-G broth alone and in broth, serum and 100 μ M FeCl₃. Further variations of similar tests included the use of phosphate buffers and tris buffers. Readings of all these tests were taken at three appropriate intervals.

7. Glucose

The effect of different concentrations of glucose on the growth of the two organisms in various concentrations of serum was determined by standard procedure where 0.1, 0.25, 0.5, 1.0, and 2.0% glucose was used. Further tests, in which a larger number of concentrations of glucose were used, were carried out where 25% serum in broth was used for *C. albicans* and 10% serum in broth for *C. neoformans*. The effect of glucose concentrations was also tested in N-G broth alone and in broth, serum and 100 μ M FeCl₃. Similar tests of the effect of glucose concentrations were conducted where N-G broth was omitted.

All these tests were conducted at pH 7.0 and readings were taken at three appropriate time intervals.

8. Growth in Serum (without broth)

The effect on the growth of both organisms of various

concentrations of serum at pH 7.0 in 1% glucose was determined by a modification of the standard procedure in which the neopeptone was omitted. The effect of $25\mu\text{M FeCl}_3$ was also determined in this system. The extent of growth was determined at numerous intervals and total incubation time was prolonged over the usual procedure. *C. albicans* was measured at $9\frac{1}{2}$, $12\frac{1}{2}$, $15\frac{1}{2}$, and $33\frac{1}{2}$ hours. *C. neoformans* was measured at $2\frac{1}{2}$, $4\frac{1}{2}$ and $6\frac{1}{2}$ days.

9. Effect of Aeration

The effect of aeration (by shaken culture) was compared to the effect of still culture on the growth of either organism at pH 7.0. These experiments were performed in foam-plugged, 18 x 150 mm test tubes, using 6 ml volumes. The test otherwise was performed according to standard procedure with similar proportions of constituents. Readings were taken at numerous intervals, i.e., for *C. albicans* (shaken & still) at 10, 11.5, 13, and 14.5 hrs; for *C. neoformans* (still) at 23.5, 29.5, 37.5, 43.5, 52.5 and 77 hrs and shaken at 19.5, 22.5, 25.5 and 28.5 hrs. All of these experiments were done at various concentrations of serum with and without the addition of $25\mu\text{M FeCl}_3$.

10. Effect of Pre-exposure of *C. albicans* to Serum

In this experiment, inoculum was exposed to $\frac{1}{2}$ ml of human serum at 37 C and 25 C for $\frac{1}{2}$, 1, 2, 3, 4, 5, 6

and 7 hours. This was achieved by inoculating the serum at intervals during the day and then centrifuging the tubes, removing the supernatant, and washing the cells once with N-G broth. The standard inoculum was taken from a culture of *C. albicans* in N-G broth. This culture was kept at the required concentration of cells by appropriate dilution at intervals. Four ml of N-G broth, with and without 25 μ M FeCl₃, was then added to the series of tubes at each pre-exposure temperature at pH 7.0. Incubation was at 37 C for 17½ hours.

11. Effect of Dialysis of Serum

The effect of human serum dialyzed against distilled water at 4 C (4 changes) and serum dialyzed similarly against tyrode solution lacking glucose was determined by the standard procedure for *C. albicans*. The pH of the sera and N-G broth was 7.0. Readings were taken at 23 hours.

12. The Effect of Ascitic Fluid, Horse Serum and Albumin

A number of substances were substituted for similar amounts of human serum in the standard procedure. Their effect, with and without the presence of 25 μ M FeCl₃, was determined on *C. albicans* and *C. neoformans*. These substances were Difco horse serum; Difco ascitic fluid, and Difco 5% beef albumin. Readings were taken at 9, 13 and 15 hours.

13. The Effect of Desferrioxamine (DFOM--CIBA)

This strong iron-chelator of microbial origin is described in Appendix 2. The effect of increments of this chelator was determined for both organisms, in shaken and still culture, with and without the presence of $25\mu\text{M}$ FeCl_3 in N-G broth at pH 7.0. In still culture, readings of the growth of *C. neoformans* were taken at 3, 5 and 7 days and of *C. albicans* at 10, 13 and 16 hrs. In shaken culture, readings of *C. neoformans* were taken at 41 and 54 hrs and of *C. albicans* at 11 and 13 hrs.

14. The Effect of Serum Proteins

The effect of increments of iron-free human transferrin at 11 concentrations from 0 to 2 mg (in 6 ml total volumes) was determined for *C. albicans* with and without $25\mu\text{M}$ FeCl_3 . The method used was similar to that already described as standard procedure. Readings were taken at 10.5, 13.5 and 16.5 hrs. Human γS -globulin increments from 0 to 6 mg and human albumin increments from 0 to 30 mg were also tested for their effect on the growth of *C. albicans* with and without $25\mu\text{M}$ FeCl_3 . These were read at the same times as the tests with transferrin.

15. The Effect of Iron Increments on Growth in Serum

Increments of FeCl_3 were added to N-G broth and to N-G broth containing 4 and 10% human serum, to determine the effect of these increments on the growth of

C. neoformans. The pH was 7.0 and 4 ml volumes were used. Growth was measured after 2.5, 4.5, and 6.5 days. Growth response of *C. albicans* to increments of FeCl_3 up to 100 μM concentration in the final volume was determined in N-G broth at pH 7.0 and in N-G broth plus 2, 5, 10 and 25% serum. In these tests, the N-G broth had been 'reduced' in iron by either autoclaving it with alumina (Ratledge & Winder, 1964) or by extraction with 8-hydroxyquinoline (Nicholas, 1957). In one set of tests, in which the inoculum concentration had been reduced by $\frac{1}{2}$, readings were taken at 16, 20.5 and 24 hours. In a second series of tests readings were taken at 17 and 21 hours. In these two series the N-G broth had been reduced in iron by 8-hydroxyquinoline extraction. In tests using ordinary N-G broth, or alumina-treated broth, readings were taken at 17, 19.5, and 24.5 hrs.

16. Relative Catalase Activity of *C. albicans* Yeast Cells

No references could be found describing studies of catalase or catalase activity of *C. albicans*. The investigation reported here was undertaken with the assumption that catalase, a heme-protein, would not be synthesized in cells grown in media deficient in iron. Growth in media containing serum was apparently subjected to a deficiency of iron.

A technique was developed which could be applied to the study of catalase activity in *C. albicans*. Cells of

C. albicans were harvested after 40 hr of incubation at 37 C from 200 ml of media of the following compositions: N-G broth; N-G broth containing 25% serum; N-G broth containing 25% serum and 20 μ M FeCl₃. These media were placed in 500 ml flasks. Growth in the medium containing serum was poor, therefore, additional flasks of this medium were required to supply a sufficient number of cells. Each flask was inoculated with 2.5 ml of inoculum, and incubated with shaking for some experiments and without shaking for others. After batch centrifugation the cells were washed 2X with ice-cold distilled water. To a homogenous slurry of cells in water, freezer-chilled acetone (-20 C) was added, then mixed well and centrifuged. The use of chilled acetone in enzyme studies of microorganisms has been described by Gunsalus (1955). The resulting 'powder' was washed 2X in acetone and the cells were finally placed under vacuum to remove acetone fumes. The volume of each slurry was determined by the volume of the cells and it was noted that the acetone required was about 10X the volume of the slurry. This 'acetone-drying' treatment had the effect of disrupting the integrity of the cell membrane, thereby allowing ready access of the enzymes to H₂O₂ in the tests. Chilling prevented denaturation of the enzyme. The assays had to be conducted within 3 to 5 hours after preparation of the cells because the activity of the

enzyme was gradually lost even though the powder was stored in a desiccator. The reaction mixture for the assay consisted of 20 ml of 0.00353M H_2O_2 solution; 4 ml 0.076M phosphate buffer at pH 7.0 and 6 ml of a suspension containing either 45 mg or 15 mg of acetone-dried cells per ml of buffer. The reagents and the flask containing the reaction mixture were kept in ice-water (0 C). Five ml aliquots of the reaction mixture were added to 5 ml amounts of 20% v/v H_2SO_4 at a 0 min and at 5 min intervals. The aliquots in H_2SO_4 were titrated against standardized 0.004M $KMnO_4$. A value was obtained at 25 min by correcting for a 4 ml aliquot. Additional information on the catalase assay can be found in Appendix 5.

Results

Figure 1 shows growth of *C. albicans* at 25°C in N-G broth with and without human serum, and with and without 25µM FeCl₃. The growth rate was very small in 20% serum unless FeCl₃ was also added. The growth rate in broth alone was not affected by the addition of FeCl₃. Almost similar results are shown in figure 2 where temperature of incubation was 37°C. Rate of growth of *C. albicans* was not substantially different in 10 to 25% serum at 37°C.

C. neoformans was affected similarly by 2 to 10% serum, either at 25°C or 37°C (Fig. 3, 4). At either temperature a greatly increased rate of growth resulted when 25µM FeCl₃ was also present.

Figure 5 shows the effect of three different concentrations of inoculum of *C. albicans* at 14 and 18 hrs over a number of different serum concentrations. A third set of measurements at 10 hrs was not recorded on the graph to avoid confusing the lines. However, it is apparent that the rate of growth of the cells in the higher concentrations of inoculum was greater than that in the standard inoculum.

Figures 6-8 show that the serum inhibition pattern

for *C. neoformans* was not obliterated regardless of the concentration of inoculum. These three figures show the growth obtained by this organism after 2, 4 and 6 days of incubation.

Figures 9-11 show that the serum inhibition pattern of *C. albicans* in N-G broth containing various concentrations of serum was altered by different degrees by the addition of three concentrations of FeCl_3 . These figures record the results at 10, 14 and 17.5 hrs of incubation respectively and, together, can be used to determine relative growth rates of the organism under the different conditions tested.

Similarly figures 12-14 show the effect of three different concentrations of FeCl_3 on the serum inhibition pattern of *C. neoformans* at 4, 6 and 8 days.

Although measurements of growth by dry weight and O.D. do not result in identical curves at all serum concentrations, a distinct pattern was given for *C. albicans* for serum inhibition and its reversal by FeCl_3 regardless of the means used to measure it (Fig. 15). The results obtained in this experiment verify those previously reported by Hendry *et al* (1969) for a similar experiment. The O.D. method of measuring amount of growth is probably less accurate than dry weight determinations. Nevertheless, under the conditions of the experiment, it is much more accurate than counts of colony-forming units

or direct counts in a hemocytometer. In contrast to dry weight determination the O.D. method is much preferred when handling a pathogenic yeast or when it is necessary to monitor growth over a period of time. It is also preferred when it is necessary to measure a large number of tubes rapidly.

Figures 16-21 show that there was no significant effect on the growth of *C. albicans* following the addition of any cation (other than Fe^{3+}) to N-G broth with or without various concentrations of serum and with or without $25\mu\text{M FeCl}_3$. The combined use of Fe^{3+} with other cations had not been previously reported in the literature. The results noted in the first four figures were obtained during the course of one experiment. The last two figures were obtained during another experiment. To avoid repetition, only the results obtained after 12.5 hrs of incubation are reported here.

Similarly, figures 22-27 show that cationic factors other than Fe^{3+} had no significant effect on the growth of *C. neoformans* in serum. Again, only the results obtained after 6.5 days have been recorded.

There is no information available relating the growth of *C. albicans* or *C. neoformans* in serum to pH. Therefore, a series of experiments were performed to clarify these relationships. Figure 28 shows the effect of pH 6.0, 7.0 or 8.0, on the growth of *C. albicans* in N-G broth alone, or containing various concentrations of

serum. It is apparent that pH was much more important in determining growth in the broth containing serum than in the broth itself. Figure 29 shows that pH was also very important in determining growth when $25\mu\text{M FeCl}_3$ was present. Figures 30-32 show some of the same information described in figure 28 and 29 except that measurements for 2 other time-intervals are included and some of the information for various concentrations of serum are deleted. Increase in pH from 6.0 to 8.0 reduced the rate of growth of *C. albicans* in 2 and 5% serum but rate of growth in 10% or more of serum was poor at any pH. Addition of $25\mu\text{M FeCl}_3$ permitted an optimum rate of growth in 2 and 5% serum at pH 8.0. From these experiments it can be concluded that the growth of *C. albicans* is independent of pH in N-G broth and is greatly influenced by pH if serum present. Figures 33 and 34 verify these effects of pH on the growth of *C. albicans*. In these two experiments 25% serum was used with or without $100\mu\text{M FeCl}_3$ in N-G broth and a larger number of pH values were employed. These figures clearly indicate that:

- 1) *C. albicans* grown in N-G broth is largely independent of pH over values from 5.0 to 9.0.
- 2) *C. albicans* in N-G broth containing 25% serum and $100\mu\text{M FeCl}_3$ also is largely independent of pH values from 5.0 to 9.0.
- 3) However, the growth of *C. albicans* in N-G broth containing 25% serum is markedly and increasingly depressed as pH values are increased to 7.0 from 5.0. Thus,

growth of *C. albicans* in broth containing 25% serum is as poor at pH 7.0 as it is at pH 8.0.

These results are significant with regard to those known properties of serum transferrin in which dissociation of the transferrin-iron complex decreases to almost a minimum value as pH is raised to 7.0 (see Historical Review).

Figures 35-37 include some information similar to that recorded in Fig. 33 as well as values obtained at two other time intervals. It can be seen that rate of growth of *C. albicans* at pH 5.5 is much greater than at pH 7.0 in broth containing 25% serum (Fig. 35). Figure 36 shows that rate of growth of *C. albicans* is similar at all tested pH values in broth alone. Fig. 37 shows that the organism grows in a similar manner at all tested pH values in broth containing 25% serum and 100 μ M FeCl₃.

Figures 38-42 show the results of experiments for *C. neoformans* which were done in a manner similar to those for *C. albicans* (Fig. 28-32). *C. neoformans* is definitely more inhibited by a high pH value than is *C. albicans*. Addition of FeCl₃ produced a less marked reversal of serum inhibition at pH 8.0. Figures 43 and 44 show that growth of *C. neoformans* was greatly inhibited at pH 8.5, or over, under any conditions. They do show, however, that iron had some effect in reversing this inhibition. Figures 45-47 record some of the same results

as shown in figures 43 and 44 and also include measurement at four other time-intervals. Figure 45 shows that *C. neoformans* grew much better at pH 5.5 in 10% serum than at any other pH value. Figure 46 reveals that addition of 100 μ M FeCl₃ to 10% serum in broth permitted optimum rates of growth to occur at acid pH. Smaller growth rates occurred when the pH increased from 7.0 to 8.5. Furthermore, this figure shows the considerable nutrient value of serum for *C. neoformans* which N-G broth alone does not have (Fig. 47). Figure 47 shows the greater rates of growth with increasing pH in N-G broth.

Phosphate buffers used in a number of experiments, similar to those described above, generally revealed similar results. This was also true of 'tris' buffers. However, use of 'tris' buffers was limited to pH values over 7.0. Phosphate buffers are not recommended for use in conjunction with serum since, under these conditions, precipitates occur.

Experiments revealed that glucose alone did not have a significant effect in promoting the growth of *C. albicans* and *C. neoformans* in a medium containing serum. Figures 48-52 show the effect of 0.1, 0.25, 0.5, 1.0 and 2.0% glucose respectively, on *C. albicans* in N-G broth containing 0 to 25% serum. In addition, these figures also show the amount of growth achieved after 10, 14 and 17.5 hrs of incubation. It is apparent that the rate of

growth of *C. albicans* at 1 or 2% glucose was much more rapid in the broth alone or in only 1 or 2% serum, than it was in 10 to 25% serum (Fig. 51 and 52). However, 0.1% glucose was insufficient to permit any considerable rate of growth in broth with or without serum (Fig. 48).

The effects of glucose on the growth of *C. albicans* was further demonstrated in figures 53-55 which show results taken at 11, 17 and 23 hrs, respectively. The response of *C. albicans* to a number of different glucose concentrations, in N-G broth alone, was similar to the response to the glucose in broth containing 25% serum and 100 μ M FeCl₃. The rate of growth was optimal at about 1.0% glucose concentration. In contrast, this organism responded to glucose in broth containing 25% serum and no iron with only a very slow rate of growth (Fig. 53-55).

In a similar experiment the response of *C. albicans* to glucose increments was tested just in 25% serum with or without 100 μ M FeCl₃ (Fig. 56). Measurements were made at 11, 17 & 23 hrs. Serum was shown to have considerable nutrient value for *C. albicans*. Under these conditions the rate of growth was optimal at 1.0% glucose, provided that FeCl₃ was also present. This result indicated that, although glucose was required as a carbon source for *C. albicans* growing in serum, little growth occurred unless iron was made available.

In experiments paralleling those with *C. albicans* (Fig. 48-52), *C. neoformans* was shown to respond to glucose concentrations as low as 0.1% in N-G broth alone showing a greater rate of growth here than in broth containing 2 to 15% serum (Fig. 51-61). Measurements were taken at 4, 6 and 8 days in these experiments.

Figures 62 and 63 show that *C. neoformans* required glucose as a carbon source and would respond to glucose additions in N-G broth alone or containing 10% serum plus 50 μ M FeCl₃. There was no measurable growth of this organism between 5.5 and 7.5 days in broth containing 10% serum if iron was not made available. Additional experiments (not recorded here) also revealed that *C. neoformans* can utilize serum as a source of certain nutrients requiring, however, the addition of glucose and FeCl₃.

Figure 64 shows that aeration of *C. albicans* during growth in N-G broth has an enhancing effect on inhibition of growth even by 1% serum and on reversal of growth if FeCl₃ is present. This figure shows the measurement at 13 hrs. However, the iron was probably not so readily available to the organism under aerated conditions and this may explain the sharp drop in growth at 4% serum. Measurement of growth of *C. albicans* by O.D. after aeration is not as reliable since cells have a greater tendency to clump. However, a study of the effect of aeration on *C. neoformans* was not troubled

by clumping and revealed that the growth of this organism is much more rapid under aeration. The response of *C. neoformans* to serum and iron under aeration was similar to that of *C. albicans*.

Pre-exposure of *C. albicans* cells to serum for varying periods of time revealed no apparent differences in the subsequent rate of growth in N-G broth (Fig. 65).

Experiments in which fluids other than pooled human serum were tested for their effect on the growth of *C. albicans* and *C. neoformans* revealed that horse serum and human serum were equivalent in their effects. Ascitic fluid up to 25% did not significantly alter growth rate of *C. albicans* (Fig. 66) but it did have an inhibitory effect on *C. neoformans* (Fig. 67). This effect was not iron-reversible. Difco albumin solution had no significant effect on the growth of either organism.

The effect of serum on growth of *C. albicans* and *C. neoformans* was apparently due to a deficiency of iron made acute at neutral or alkaline pH by the highly active iron-chelating protein, transferrin (see Historical Review). Experiments in which the potent iron chelator, desferrioxamine (DFOM), was added to N-G broth revealed no effect on the growth of *C. neoformans* in quantities up to 10ug in 4 mls (Fig. 68). Figure 69 shows that the same quantities of DFOM had a partial effect in decreasing the rate of growth of *C. albicans* in still culture. However,

there appeared to be no significant decrease in growth rate between 10 and 16 hrs, the decrease having occurred previous to 10 hr. In contrast, figure 70 shows that aeration resulted in a considerable decrease in growth rate of *C. albicans* by 7.5 ug or more of DFOM in 6 ml volumes. At 11.25 ug of DFOM virtually no growth occurred between 11 and 13 hours. Figures 69 and 70 show that any inhibition of growth by DFOM was reversed by 25 μ M FeCl₃.

Figure 71 shows that iron-free transferrin did not decrease the growth rate of *C. albicans* and there was no iron-reversible inhibition. Human γ S-globulin to 6 mg and human albumin to 30 mg also had no effect on the growth rate of *C. albicans* and showed no iron-reversible inhibition.

After it was found that the inhibition of growth of *C. albicans* and *C. neoformans*, in a medium containing serum, was reversed by addition of iron it became necessary to determine the quantity of iron required to accomplish this reversal. Figures 72 and 73 show the effect of a range of iron concentrations on the rate of growth of *C. neoformans* in N-G broth alone or containing 4 and 10% serum. Measurements for 2.5 days were omitted from figure 72 to avoid a confusion of lines. It is apparent that certain minimum concentrations of iron are sufficient to allow growth in a medium containing serum.

Quantities of iron required to negate the inhibitory effect of serum on the growth of *C. albicans* were determined under more precise conditions. Figures 74-78 show the marked inhibition of 2, 5, 10 and 25% serum in N-G broth on the rate of growth of *C. albicans* and the marked reversal of this inhibition when certain minimum quantities of FeCl_3 were added. The N-G broth was extracted with 8-hydroxyquinoline in an effort to reduce its iron content but, it is believed that this treatment could not further reduce the low iron concentration that was present. The accuracy of this experiment was increased by reducing the inoculum size to one-half the standard value. The rate of growth of this organism was negligible in the medium containing 2, 5, 10 or 25% serum where insufficient iron had been added (Figs. 75-78).

Figure 74 shows that *C. albicans* grew in the N-G broth alone at approximately the same rate regardless of iron addition revealing that the broth had sufficient iron for optimum growth of this organism. The results shown in figures 79-83 parallel those in figures 75-79 but reveal a less sharp response to iron addition because of the larger (standard) inoculum size. Results obtained using the regular N-G broth, or N-G broth autoclaved with alumina to reduce iron, were similar to those noted above.

The significance of the response of *C. albicans*

to iron for growth in media containing serum appeared to depend partly on the tendency of *C. albicans* to grow in a mycelial form in media containing serum (see following section describing morphological studies). Previous investigations did not describe or suggest an association between the morphological alteration of *C. albicans* in serum to changes in its iron metabolism. An index of iron metabolism in *C. albicans*, or in any organism which can undergo an oxidative (aerobic) metabolism, is the enzyme catalase. Catalase is a heme-protein and, therefore, requires iron for its synthesis. A deficiency of iron may be traced through a deficiency of catalase activity as well as through any other iron-containing substance in the cells. Typical results of numerous trials are shown in figures 84-86 where catalase activity of *C. albicans* cells, grown under different conditions, was measured. Figure 84 shows catalase activity of the cells harvested from N-G broth after incubation under aeration or as a still culture. Fig. 85 shows the lack of catalase activity of cells harvested from N-G broth containing 25% serum also after incubation with aeration or as a still culture. Fig. 86 shows that addition of iron apparently enables *C. albicans* to synthesize catalase even though it was grown in broth containing 25% serum.

Discussion

Hendry *et al* (1969) reported that concentrations of pooled human serum as low as 1 or 2% in N-G broth had

an inhibitory effect on the growth of *C. albicans* and *C. neoformans*. This is consistent with the finding that the iron content of filtered N-G broth was very low (Appendix 4) and with the knowledge that 2% pooled human serum in the broth was sufficient to bind this iron (Appendix 2). However, present results show that the N-G broth itself had almost optimum iron content for the growth of *C. albicans* and *C. neoformans*. In many experiments, the organisms did not increase in growth with additions of FeCl_3 to the broth.

The demonstration of an anticryptococcal and anticandidal effect of serum was therefore best achieved by studying the growth of the organisms in filtered N-G broth. The test method developed was more detailed and reproducible than any method described previously. The use of optical density methods was justified when the results obtained by both O.D. measurements and dry weight were compared.

Published reports on anticandidal activity of serum record several methods for testing the growth inhibition of *C. albicans* by serum although none were adaptable for use in present studies. Optical density readings were used by Roth *et al* (1959, 1961) to determine the inhibitory activity of different concentrations of individual sera. Caroline *et al* (1964) and Esterly *et al* (1967a, b) also determined the inhibitory activity of serum, diluted in liquid media, by optical density measurements. Hasenclever *et al* (1963) and Summers *et al* (1964)

incubated *C. albicans* in media containing serum and subsequently examined the growth by plating. Louria *et al* (1964, 1967) and Caroline *et al* (1969) added cells of *C. albicans* to undiluted serum and determined the candidacidal activity of the serum by plate counts immediately after and at several later intervals. Caroline *et al* (1964) determined the growth of *C. albicans* in serum by optical density values as well as by direct cell counts. The possibility that clumping of *C. albicans* in serum was responsible for a reduced number of colonies was discounted by Louria *et al* (1967). In addition, they indicated that undiluted serum often contained a factor which interfered with the candidacidal activity serum.

Various methods for testing the anticryptococcal activity of serum have also been reported. Allen *et al* (1955) plated out serum seeded with *C. neoformans*. Baum *et al* (1961, 1963) studied the anticryptococcal activity of serum by using an inoculum dilution method with subsequent plating. Igel *et al* (1966) studied the inhibition of *C. neoformans* in serial dilutions of serum in Sabouraud's broth. The cells were then counted in a hemocytometer. Szilagyi *et al* (1966) studied the growth of *C. neoformans* in Littman's capsule broth with optical density methods.

The importance of a standard pH in studying serum fungistatic activity against *C. albicans* and *C. neoformans* was previously discussed by Hendry *et al* (1969). Present results indicate that a medium with a pH as low as 6.0

could be acceptable for *C. neoformans* because it is inhibited by an alkaline pH (Howard, 1961). A pH of 7.0 is the lowest pH suitable for the testing of the growth of *C. albicans* in media containing serum because of its ability to lower the pH of a medium. These pH values are consistent with the known properties of serum transferrin and its iron-binding activity since chelation becomes greatest at pH 7.0 or over. The use of standardized pH for studies of the anticandidal activity of serum has been rarely reported in literature. Although Esterly *et al* (1967b) used a medium with a pH of 6.6, the actual pH of her test in which 20% serum was added, was not standardized. Caroline *et al* (1969) used a pH of 7.3 for their inhibition tests but these were performed in undiluted serum. Thus, to date, there has been no determination of the effect of pH combined with the effect of serum on the growth of either organism.

Schade *et al* (1944) reported that the antibacterial activity of egg-white was reversed by yeast extract due to its content of iron. Present results (not recorded here) also showed that 1% yeast extract was effective in reversing the anticandidal and anticryptococcal activity of serum. Therefore, the use of yeast extract in media for studying the antifungal activity of serum is not recommended. Nevertheless, yeast extract has been used in media for this purpose (Roth *et al*, 1959, 1961; and Esterly *et al*, 1967a, b).

Present experiments (Fig. 71), performed with a different experimental approach, confirmed some of the results recorded by Esterly *et al* (1967b) who showed that the growth of *C. albicans* was not inhibited by high concentrations of transferrin alone when added to media. Esterly *et al* (1967b) also stated that another factor, in addition to transferrin, is active in serum and that it may be partially responsible for the growth-inhibiting property of serum. However, present results indicate that one such "other factor" may be related to the altered morphology and metabolism of *C. albicans* when grown in the presence of serum. The evidence for this will be discussed more fully under 'Morphological Studies of *C. albicans*'. However indirect evidence for the importance of transferrin has been given, by Esterly *et al* (1967b) and Caroline *et al* (1969), who found that the addition of transferrin to sera, low in iron-binding capacity, restored the anticandidal activity of such sera, with the exception of cord sera. Present studies (P. 189) have shown that transferrin alone did not induce germ-tube formation by blastospores of *C. albicans*, nor did transferrin added to N-G broth cause growth inhibition of this organism (Fig. 71). This is presented as indirect evidence that a tendency towards morphological change to the mycelial form is required in order to demonstrate inhibition of growth by serum. *C. albicans* possesses the enzymes of both the hexose-monophosphate shunt and the Embden-Meyerhoff pathway (Rao *et al*, 1960). The high iron requirement for growth in the presence of serum might be due to unknown mechanisms

inhibiting the fermentative pathway, stimulating oxidative metabolism, and altering the morphology to the mycelial form. The enhancing effect of aeration (in media containing DFOM) supports this hypothesis (Fig. 69,70). However, DFOM does not substitute for serum. Aeration of media containing DFOM may cause *C. albicans* to use oxygen as its terminal electron acceptor.

The growth response of *C. albicans* to additions of glucose in serum when iron was present (and not in the absence of iron) (Fig. 53-56) shows that glucose is required as a carbon source, but that its presence does not result in significant growth in the presence of serum. The response of *C. neoformans* to glucose increments in media containing serum (Fig. 63) is probably less complex because this organism does not possess any strong fermentative abilities.

Availability of oxygen is an important requirement limiting the growth of *C. albicans* in media containing serum. Indeed, Mardon, Balish & Philips (1969) found that a deficiency of O₂ reduced the growth of filamentous forms of *C. albicans*. Although these authors apparently referred only to pseudomycelial growth this may indicate the relatively greater dependence of any filamentous form of this organism on an oxidative metabolism. It also suggests that the ability to "ferment" may be decreased when *C. albicans* is inoculated into serum. Present results (P. 189) showed that no filament formation occurred in inorganic media. N-G broth appears to complement the filament-inducing properties of serum (P. 189).

Iron is essential for the synthesis of heme-proteins or other iron-containing compounds, by organisms with an oxidative metabolism. An important example of a heme-protein is the enzyme catalase. Activity of this enzyme was negligible in the sparse growth of *C. albicans* harvested from N-G broth containing serum. In contrast, addition of iron to the medium containing iron not only resulted in considerably more growth of *C. albicans* was also in a high catalase activity.

A number of other *Candida* species tested (results not recorded here) also showed an iron-reversible growth inhibition in the presence of serum. The apparent universality of serum fungistasis indicates that, if there were any specific immunological factors responsible for growth inhibition of such widespread pathogens as *C. albicans* or *C. neoformans* by serum, these would likely be of less importance *in vitro* than the transferrin-mediated mechanism. The reason for this conclusion is that a high titer of specific antibodies for nonpathogenic yeasts is not likely to be present, nevertheless the growth of nonpathogenic yeasts was also inhibited by serum. Wood, Burgess & Morginson (1961), Martin (1962) and Bornside *et al* (1964, 1967) used the nonpathogenic bacterium *B. subtilis* in studies of the bacteriostatic and antirespiratory activity of serum. It is also highly unlikely that specific antibodies against this nonpathogenic bacterium may be found in human serum.

Good *et al* (1968) Landau (1968) and Fujiwara

et al (1970) stated that the cellular immune defences of an individual may be important in his resistance to candidiasis. However, Louria *et al* (1967) found that children with recalcitrant mucocutaneous candidiasis, who also suffered from endocrine hypofunctions, had normal cellular and humoral immune functions. On immunoelectrophoresis their serum proteins appeared normal. Nevertheless, their sera in most cases had a reduced anticandidal activity. This again underscores the need to search beyond those well-known cellular and humoral defenses of the acquired type in order to explain some types of resistance to infection.

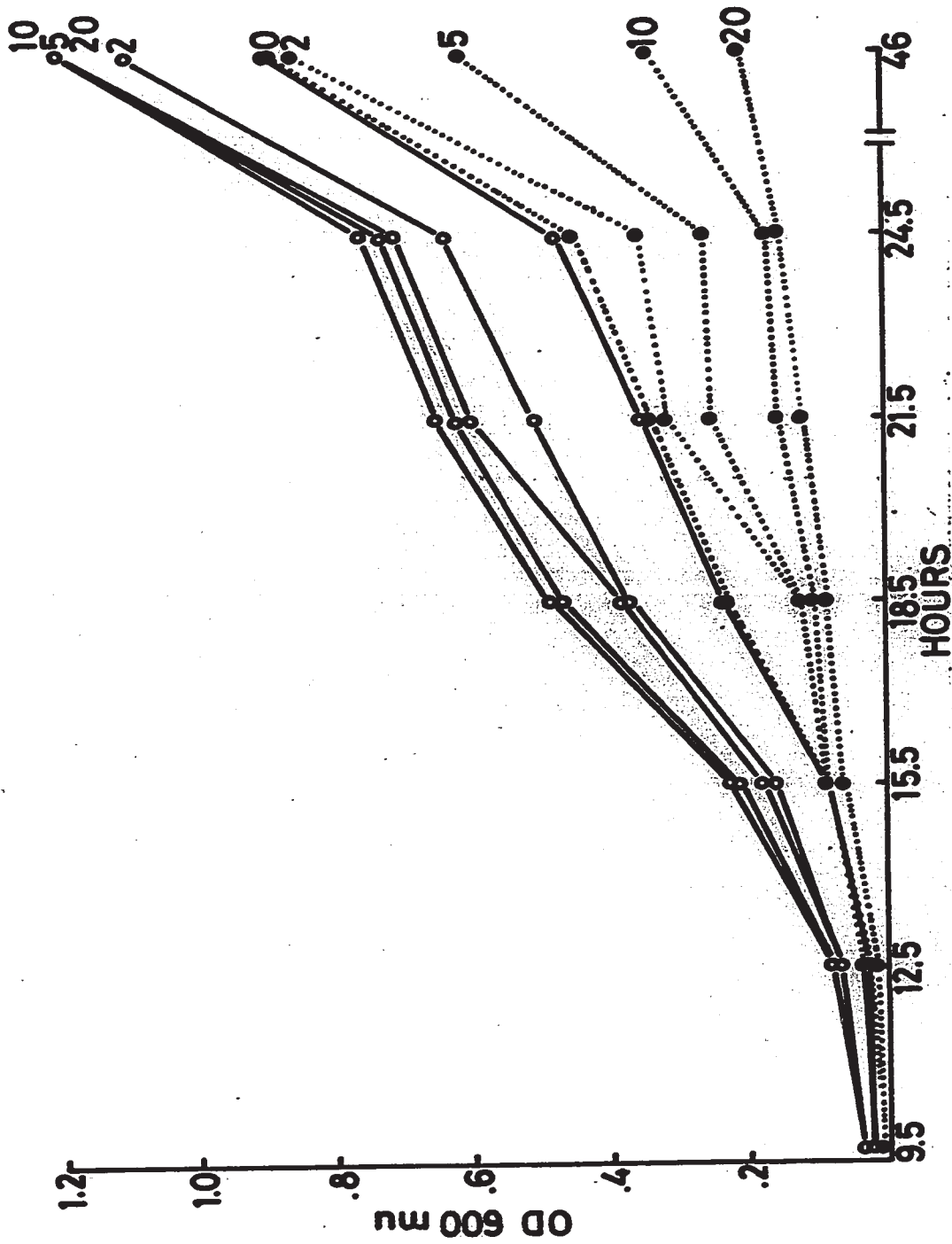


FIGURE 1. Rate of growth of *C. albicans* at 25°C in N-G broth. Numbers to the right of the lines indicate % serum. 25 μM FeCl₃ (○—○); no FeCl₃ added (●.....●).

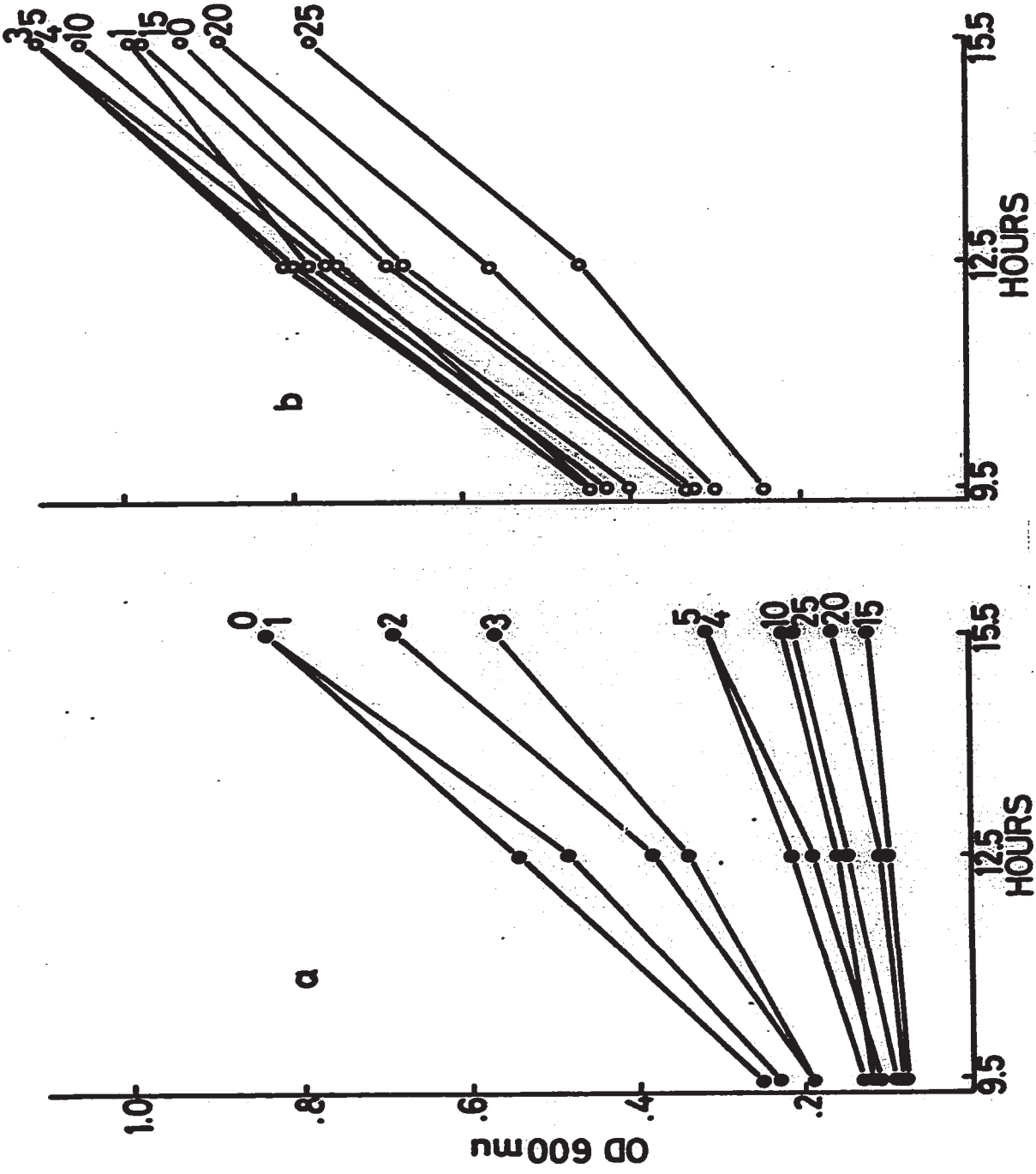


FIGURE 2. Rate of growth of *C. albicans* at 37°C in N-G broth. Numbers to the right of the lines indicate % serum. a, no added FeCl₃; b, 25µM FeCl₃.

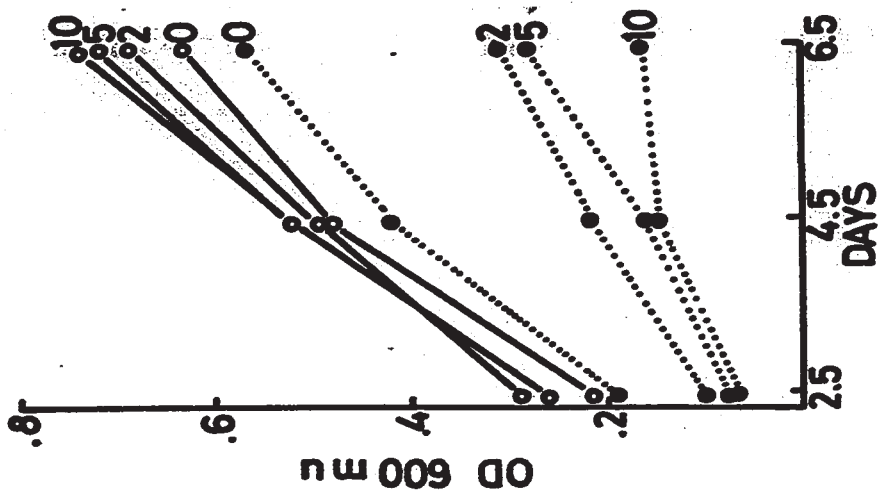


FIGURE 3. Rate of growth of *C. neoformans* at 25°C in N-G broth. Numbers to the right of the lines indicate % serum. 25μM FeCl₃ (○—○); no added FeCl₃ (●.....●).

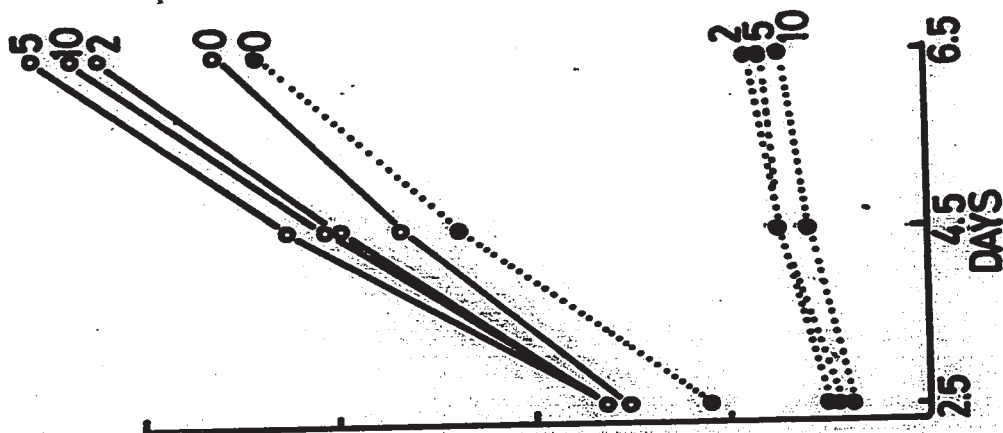


FIGURE 4. Rate of growth of *C. neoformans* at 37°C in N-G broth. Numbers to the right of the lines indicate % serum. 25μM FeCl₃ (○—○); no added FeCl₃ (●.....●).

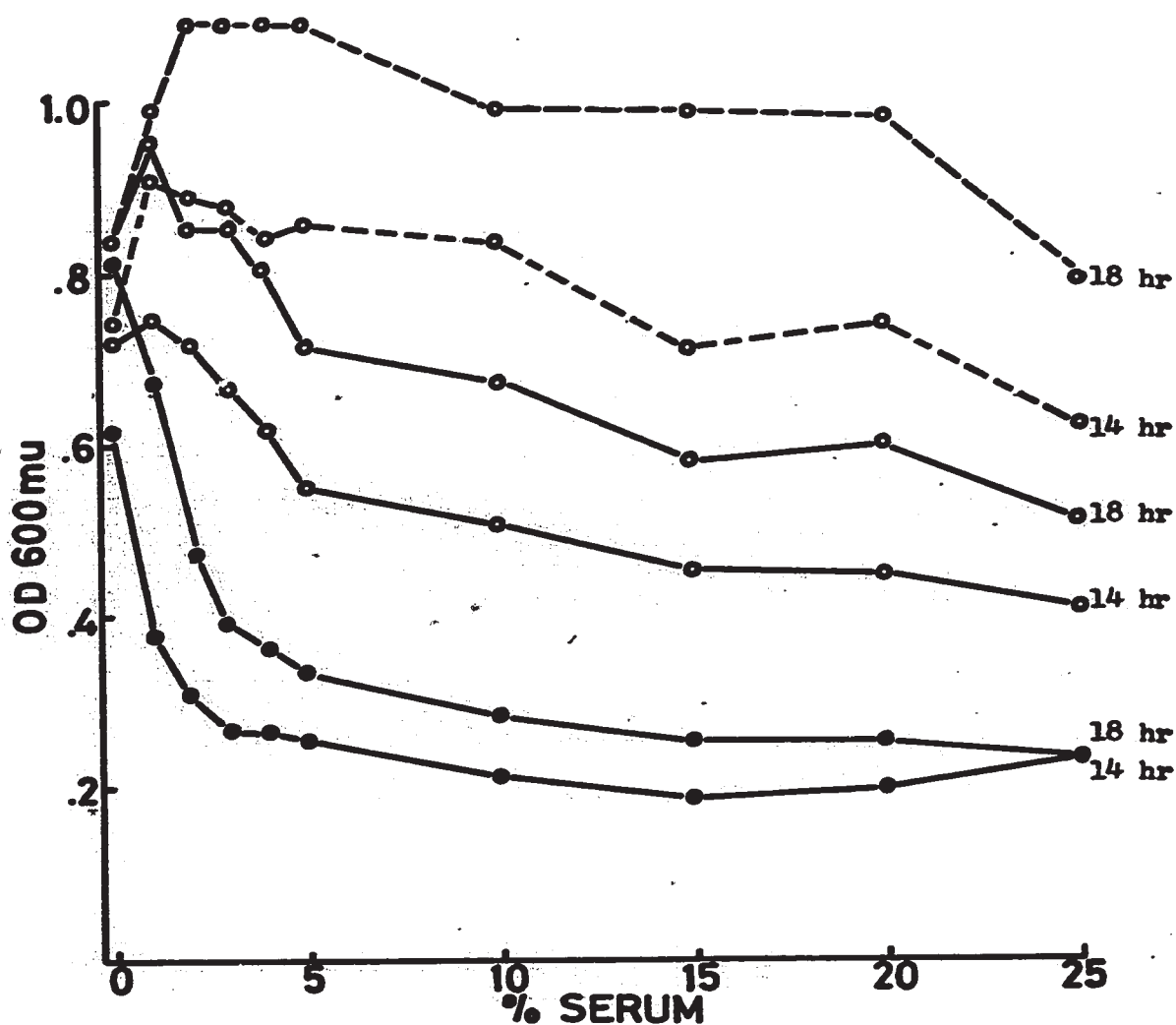


FIGURE 5. Effect of 9X (○---○) and 3X (●—●) the standard inoculum size (●—●) on the growth of *C. albicans* in H-G broth containing serum. 14 and 18 hr. Standard inoculum size = 6×10^3 cells in 0.05 ml of inoculum added per tube.

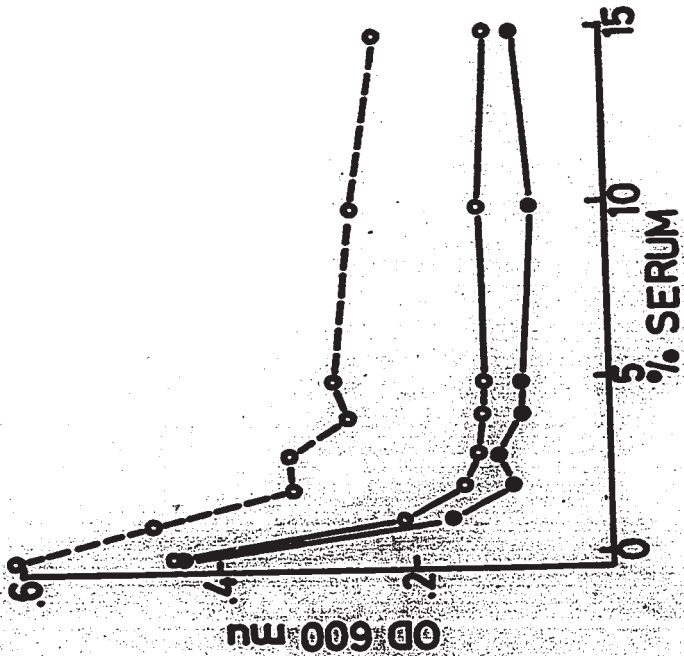


FIGURE 7. Effect of 9X (o---o) and 3X (o---o) the standard inoculum size (o---o) on the growth of *C. neoformans* in N-G broth containing serum; 4 days. Standard inoculum size = 6×10^3 cells in 0.05 ml of inoculum added per tube.

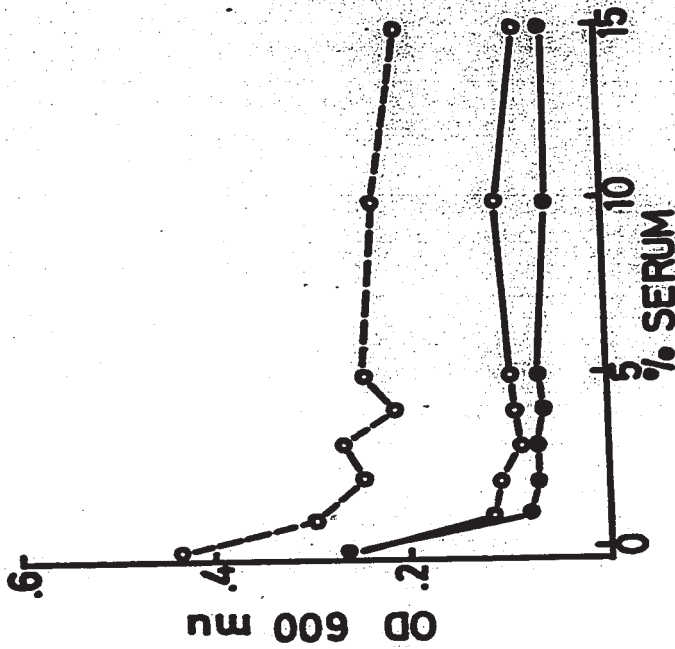


FIGURE 6. Effect of 9X (o---o) and 3X (o---o) the standard inoculum size (o---o) on the growth of *C. neoformans* in N-G broth containing serum; 2 days. Standard inoculum size = 6×10^3 cells in 0.05 ml of inoculum added per tube.

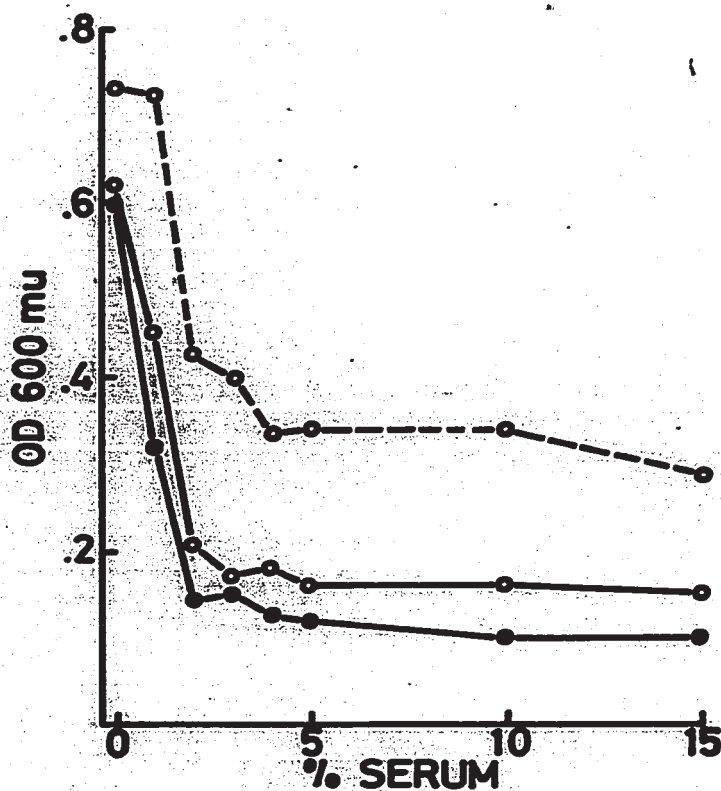


FIGURE 8. Effect of 9X (○---○) and 3X (○—○) the standard inoculum size (●—●) on the growth of *C. neoformans* in N-G broth containing serum; 6 days. Standard inoculum size = 6×10^3 cells in 0.05 ml of inoculum added per tube.

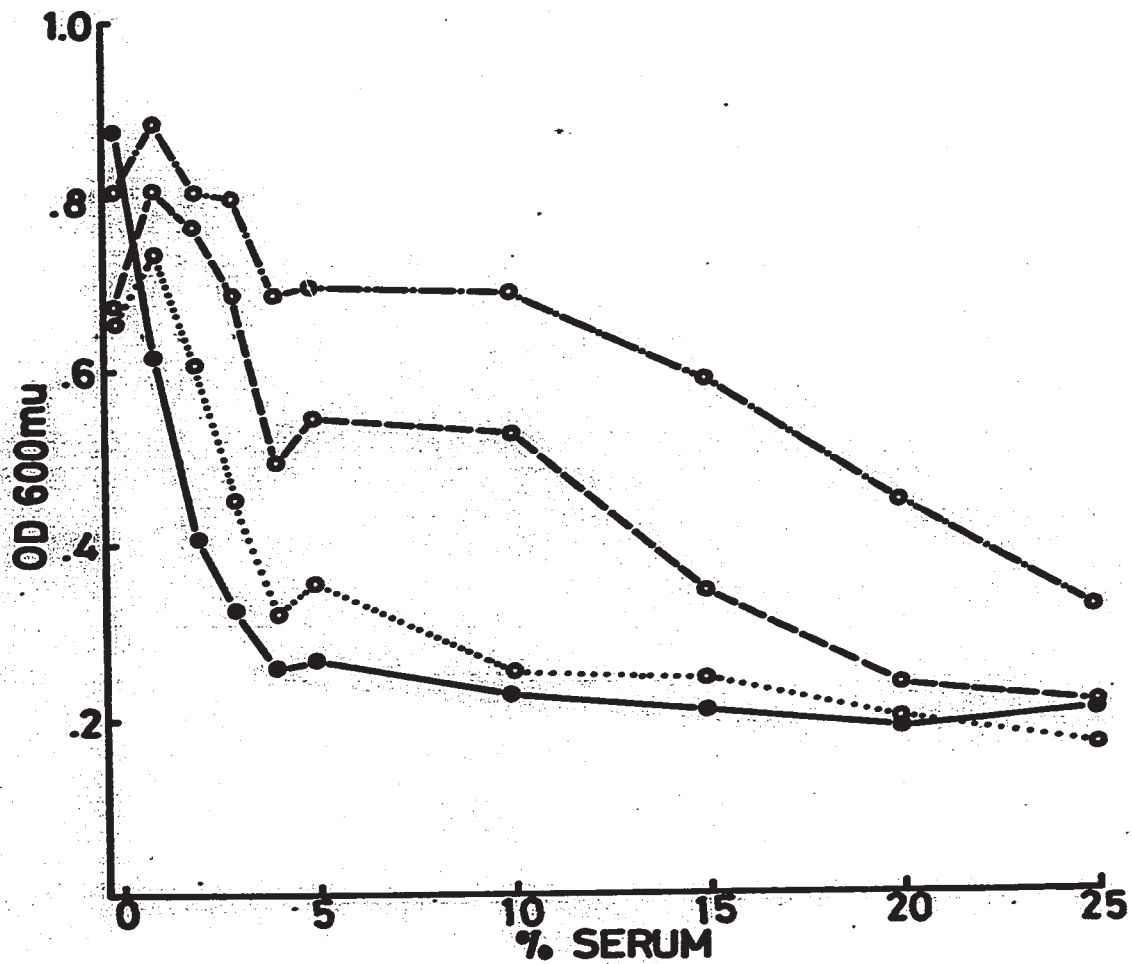


FIGURE 10. Effect of iron on the growth of *C. albicans* in N-G broth containing serum; 25µM FeCl₃ (—○—○—), 10µM FeCl₃ (—○—○—), 2.5µM FeCl₃ (·····○·····), no added iron (—○—○—); 14 hr.

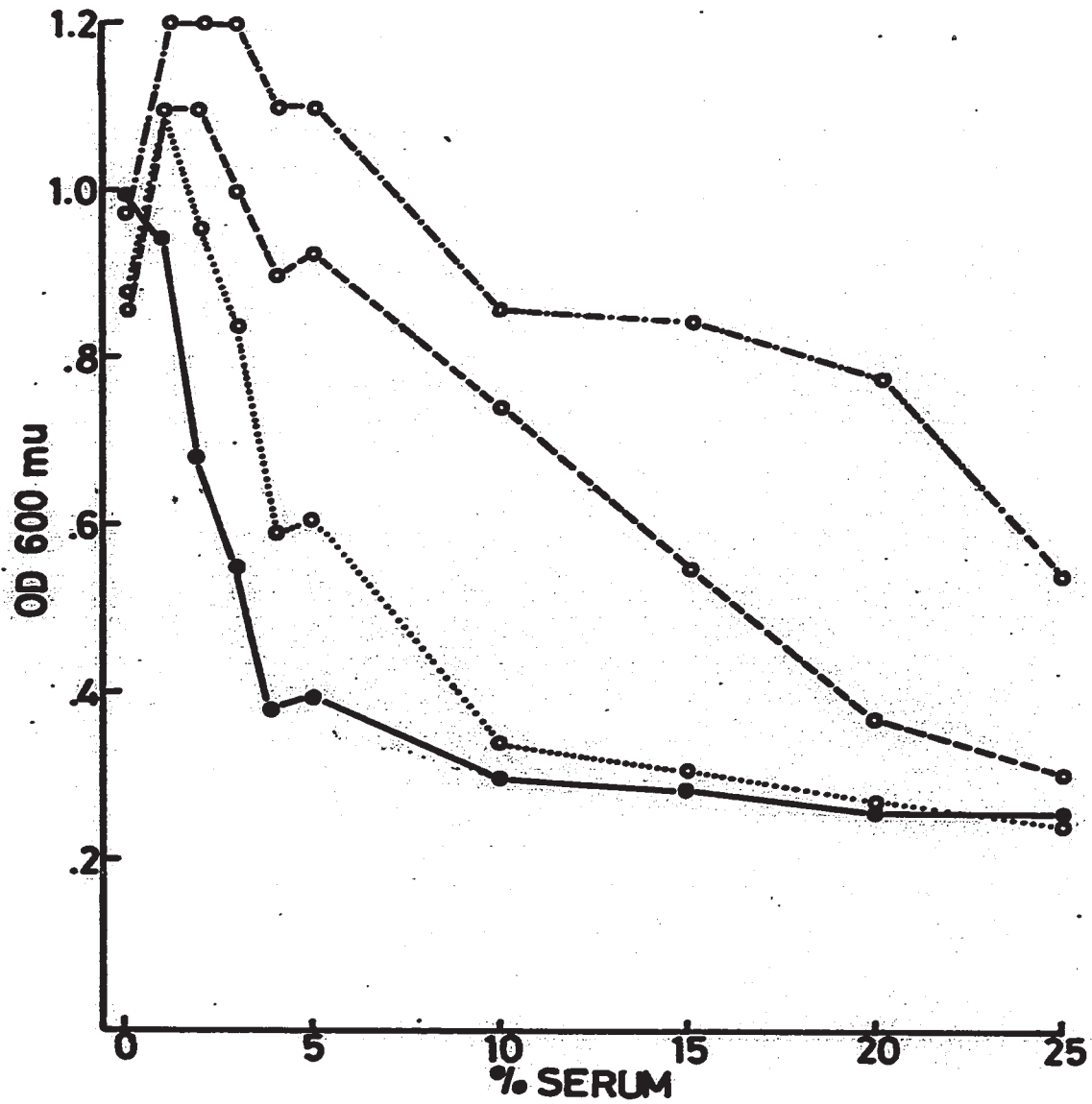


FIGURE 11. Effect of iron on the growth of *C. albicans* in N-G broth containing serum; 25 μ M FeCl₃ (○- - -○), 10 μ M FeCl₃ (○- · - ·○), 2.5 μ M FeCl₃ (○ · · · · ·○), no added iron (●- - -●); 17.5 hr.

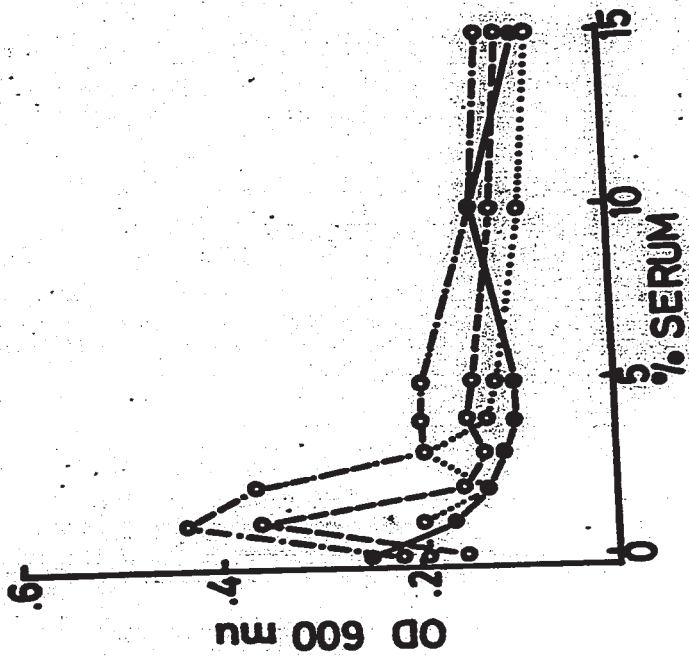


FIGURE 12. Effect of iron on the growth of *C. neoformans* in N-G broth containing serum; 25µM FeCl₃ (●—●), 10µM FeCl₃ (○- -○), 2.5µM FeCl₃ (○.....○), no added iron (●- - -●); 4 days.

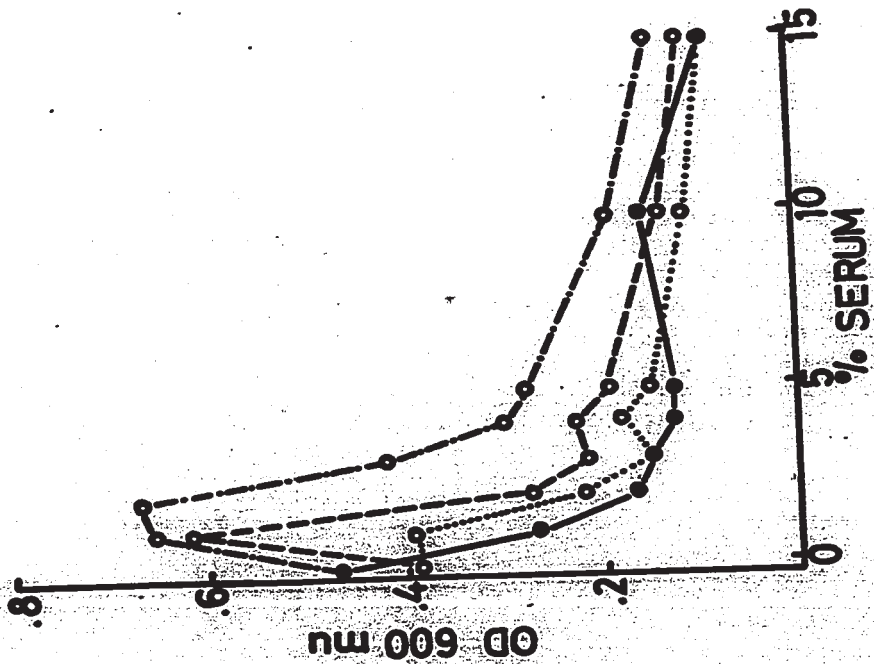


FIGURE 13. Effect of iron on the growth of *C. neoformans* in N-G broth containing serum; 25µM FeCl₃ (●—●), 10µM FeCl₃ (○- -○), 2.5µM FeCl₃ (○.....○), no added iron (●- - -●); 6 days.

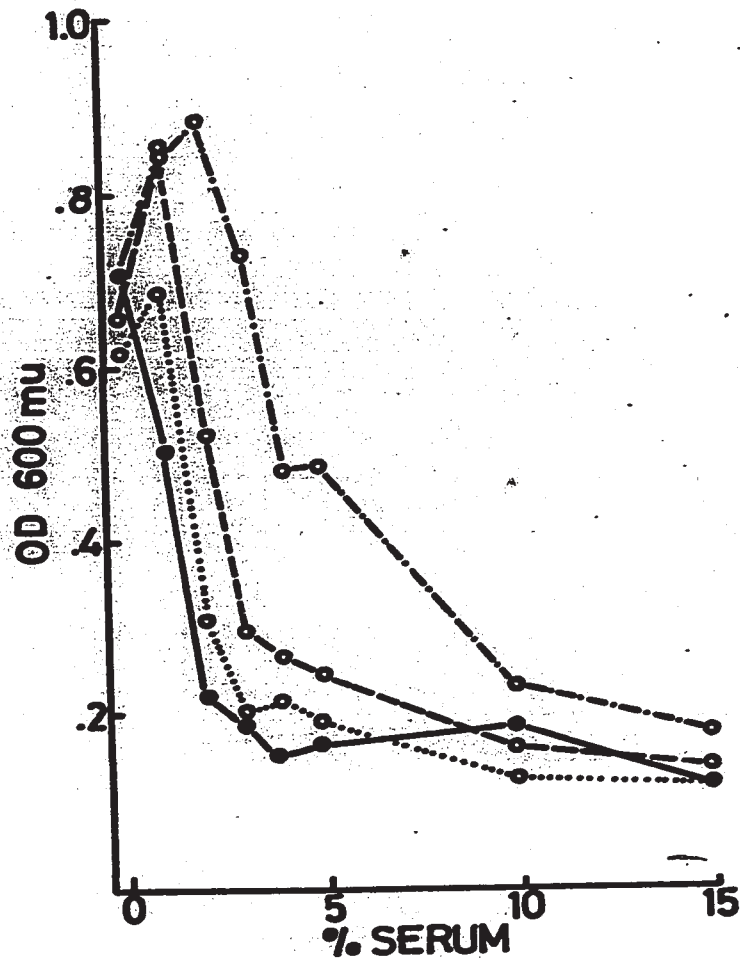


FIGURE 14. Effect of iron on the growth of *C. neoformans* in N-G broth containing serum; 25 μM FeCl₃ (○—○), 10 μM FeCl₃ (○---○), 2.5 μM FeCl₃ (○.....○), no added iron (—·—·—); 8 days.

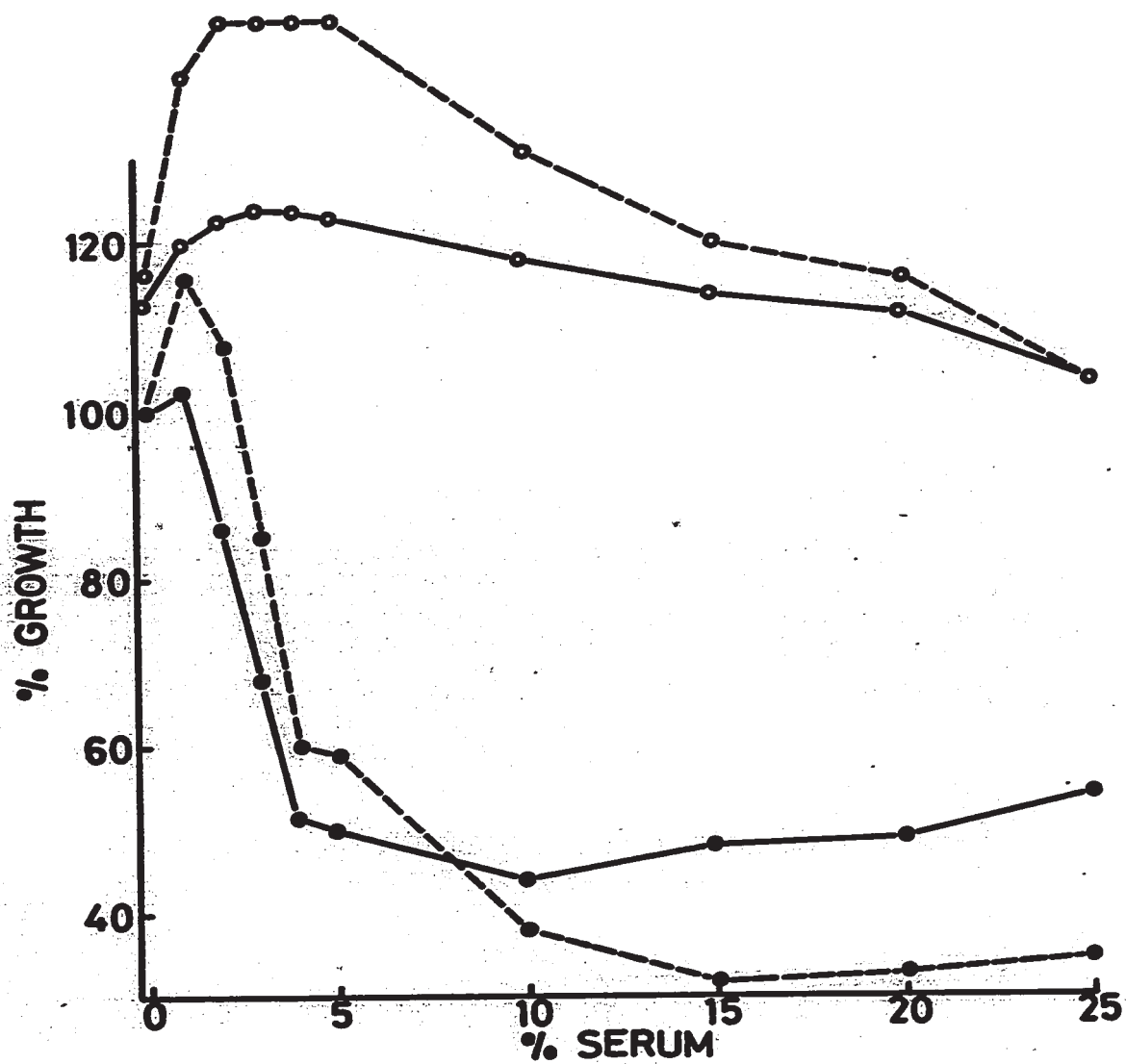


FIGURE 15. Inhibition of the growth of *C. albicans* in N-G broth containing serum (●); reversal by 25 μ M FeCl₃ (○); dry weight determinations (—), 0.D. (---); 16 hr.

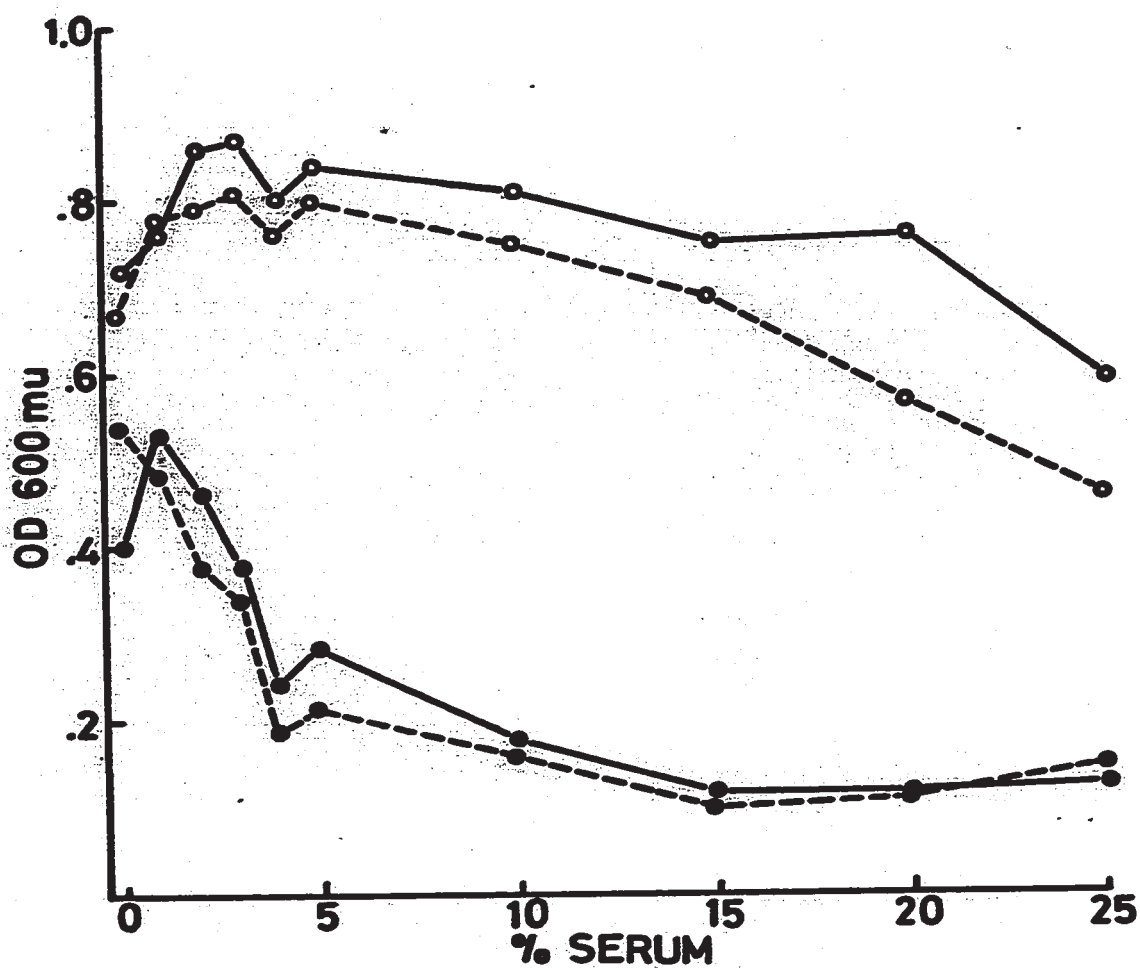


FIGURE 16. Effect of $25\mu\text{M Zn}^{2+}$ (—) on growth of *C. albicans* in N-G broth containing serum (•—•), or serum plus $25\mu\text{M FeCl}_3$ (○—○); 12.5 hr.

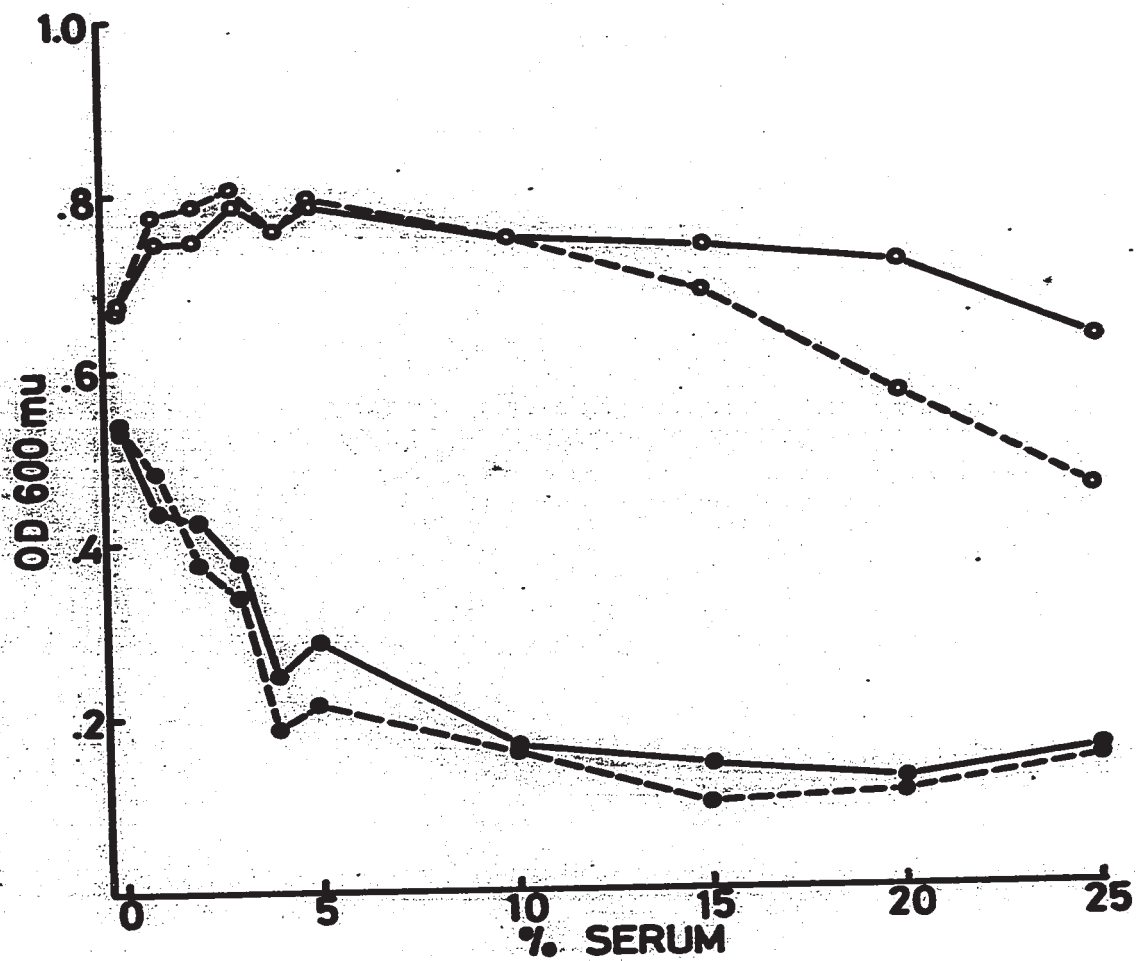


FIGURE 17. Effect of $25\mu\text{M Mo}^{6+}$ (—) on growth of *C. albicans* in N-G broth containing serum (●---●), or serum plus $25\mu\text{M FeCl}_3$ (○---○); 12.5 hr.

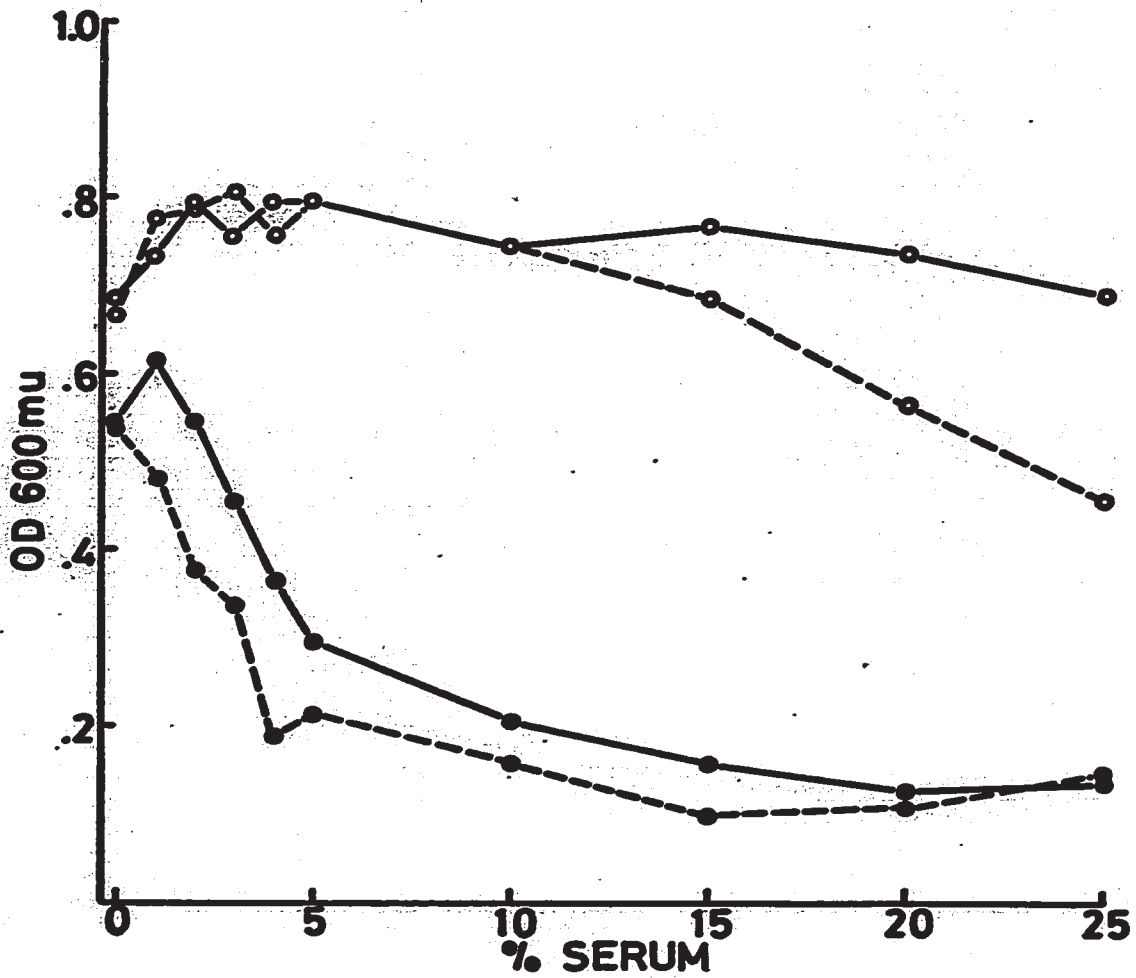


FIGURE 18. Effect of $25\mu\text{M Mn}^{2+}$ (—) on the growth of *C. albicans* in N-G broth containing serum (○—○), or serum plus $25\mu\text{M FeCl}_3$ (○---○); 12.5 hr.

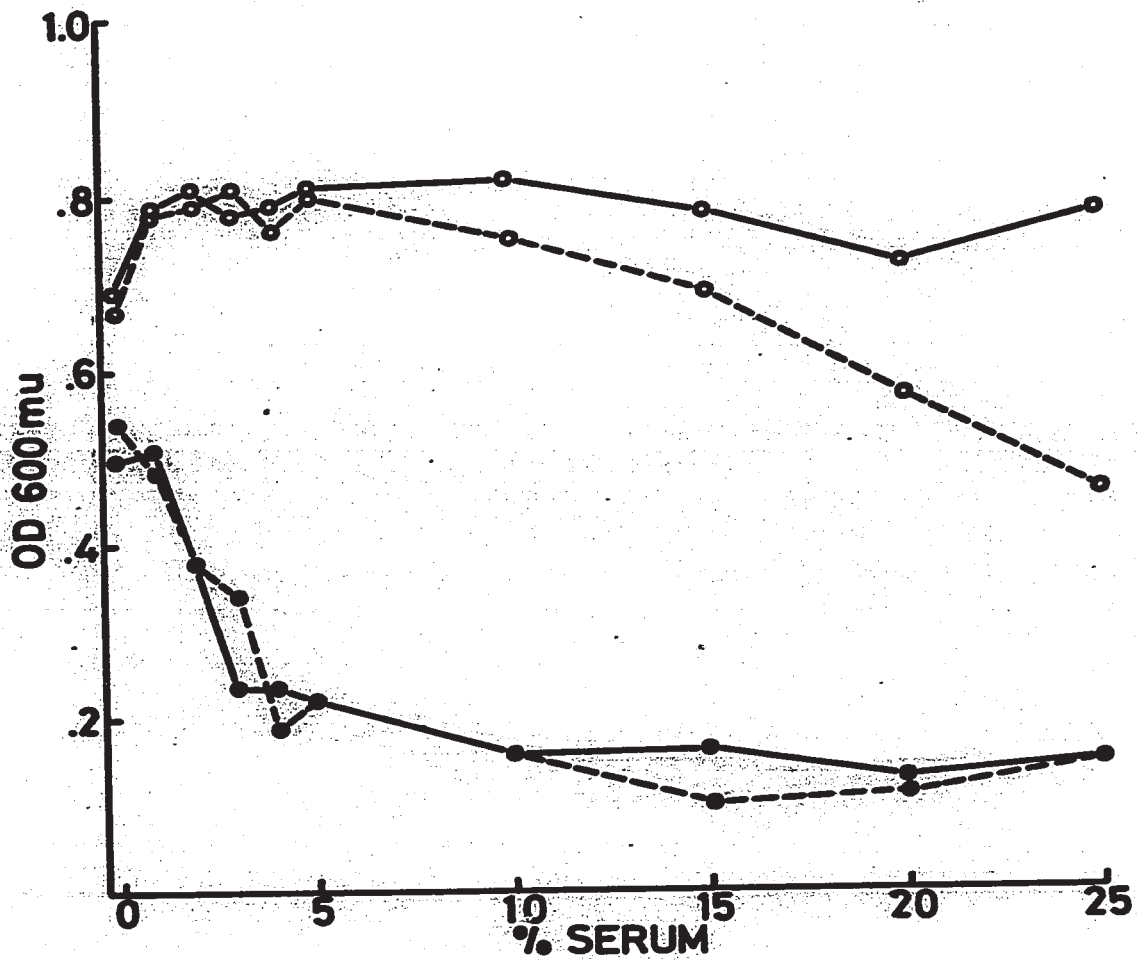


FIGURE 19. Effect of $2.5\mu\text{M Cu}^{2+}$ (—) on growth of *C. albicans* in N-G broth containing serum (○---○), or serum plus $25\mu\text{M FeCl}_3$ (●---●); 12.5 hr.

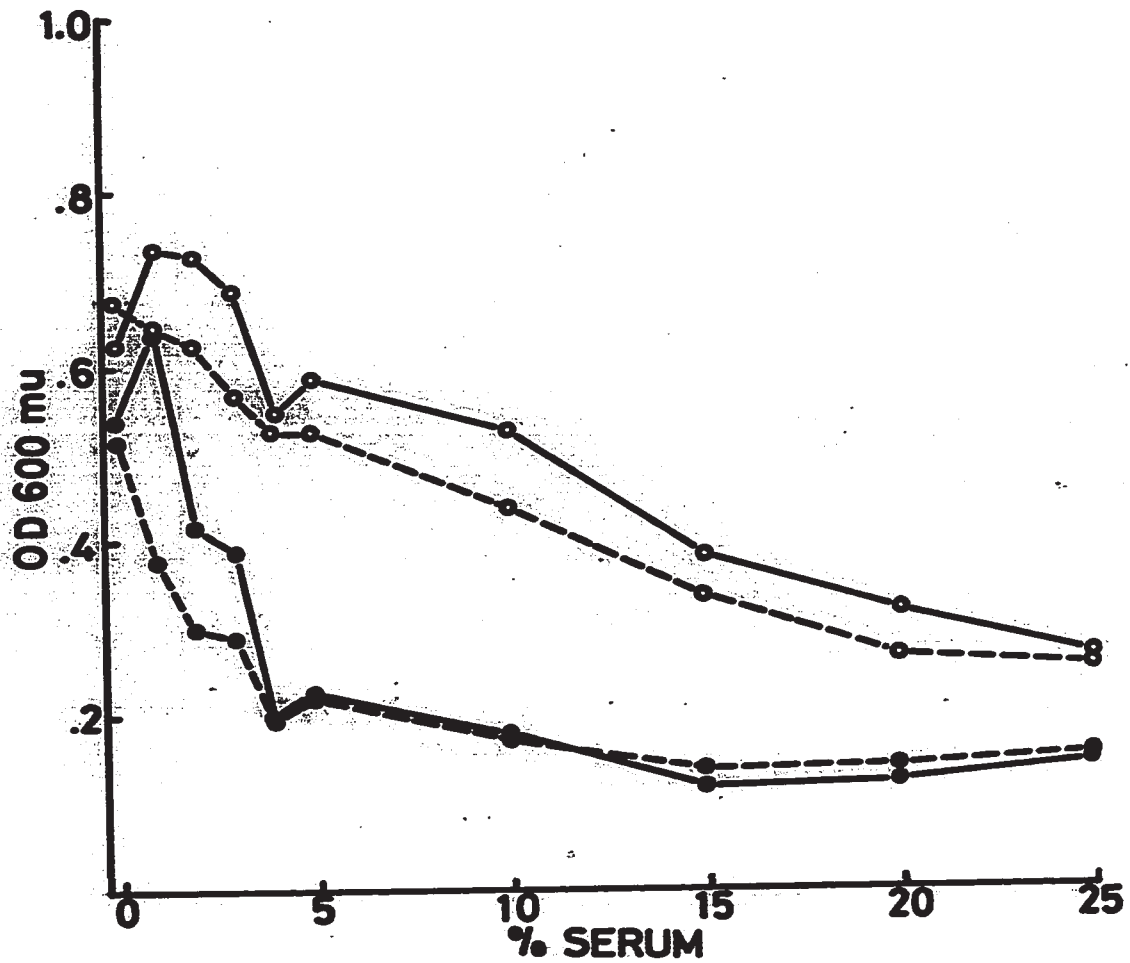


FIGURE 20. Effect of $25\mu\text{M Ca}^{2+}$ (—) on growth of *C. albicans* in N-G broth containing serum (●---●), or serum plus $25\mu\text{M FeCl}_3$ (○---○); 12.5 hr.

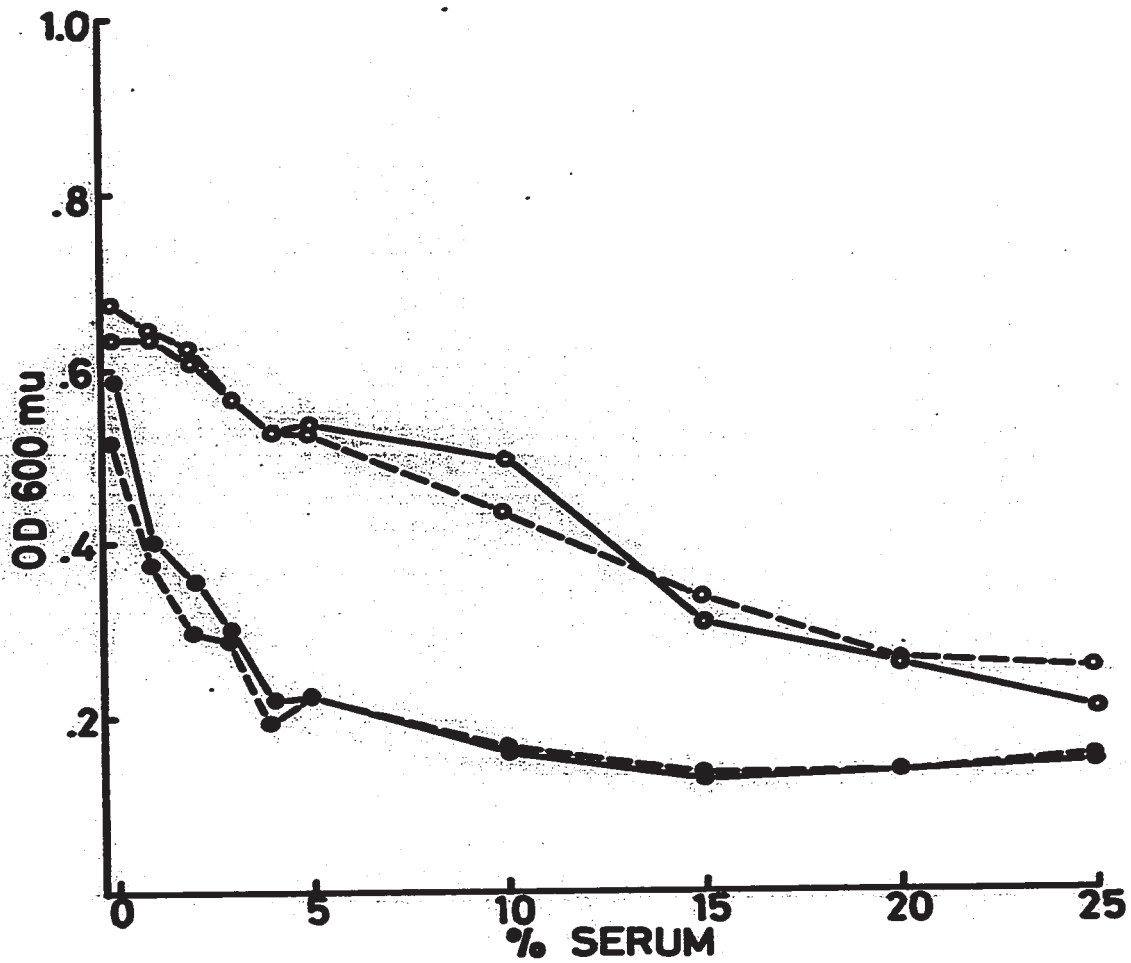


FIGURE 21. Effect of $25\mu\text{M Mg}^{2+}$ (—) on growth of *C. albicans* in N-G broth containing serum (○---○), or serum plus $25\mu\text{M FeCl}_3$ (○---○); 12.5 hr.

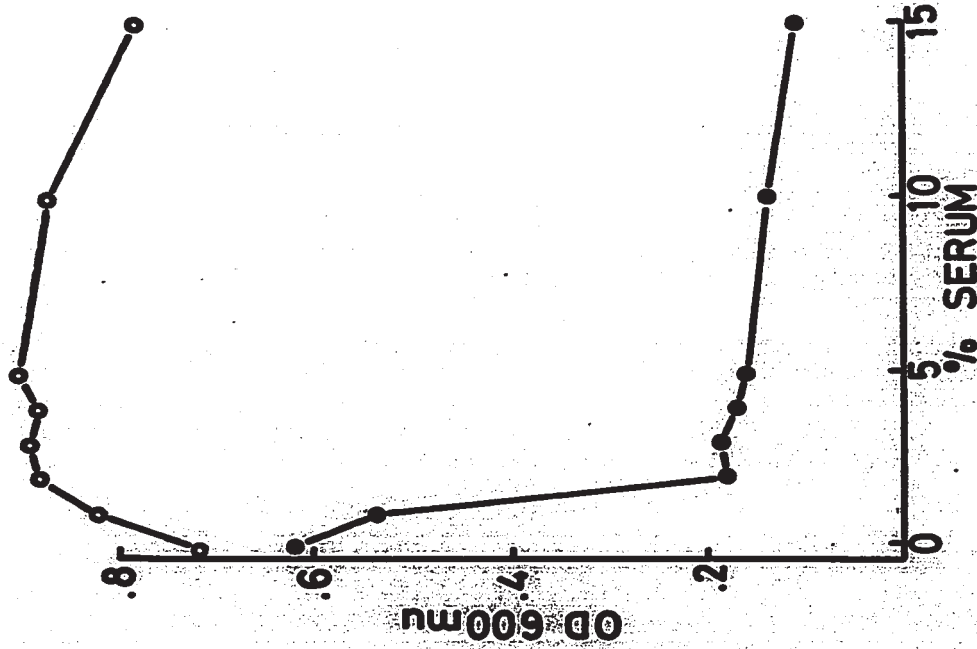


FIGURE 22. Effect of $25\mu\text{M Zn}^{2+}$ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus $25\mu\text{M FeCl}_3$ (○); 6.5 days. See Fig. 22a.

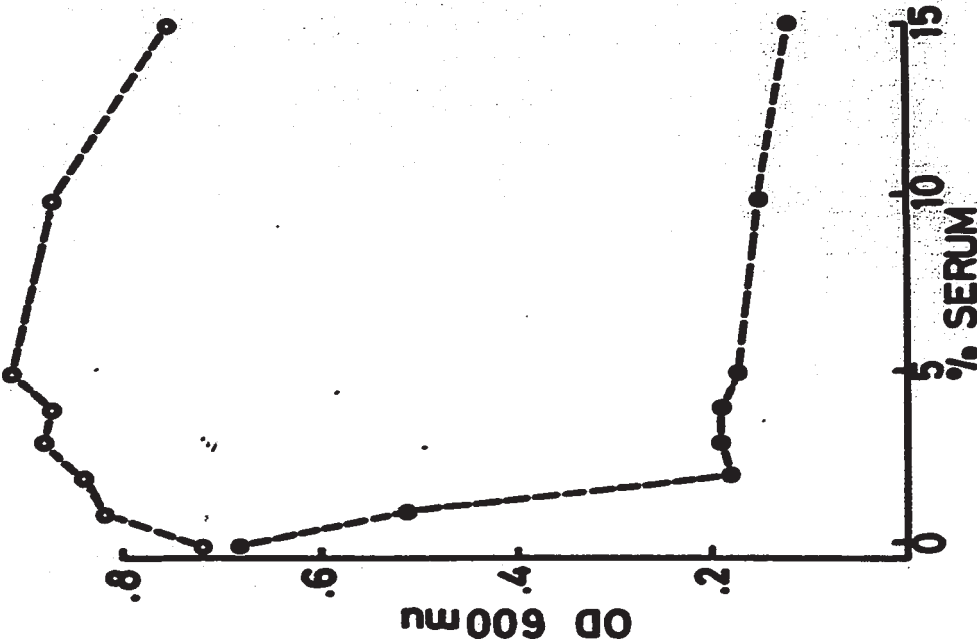


FIGURE 22a. Effect of $25\mu\text{M FeCl}_3$ (○) on the growth of *C. neoformans* in N-G broth containing serum (●); 6.5 days. These results constitute the control for Fig. 22-27.

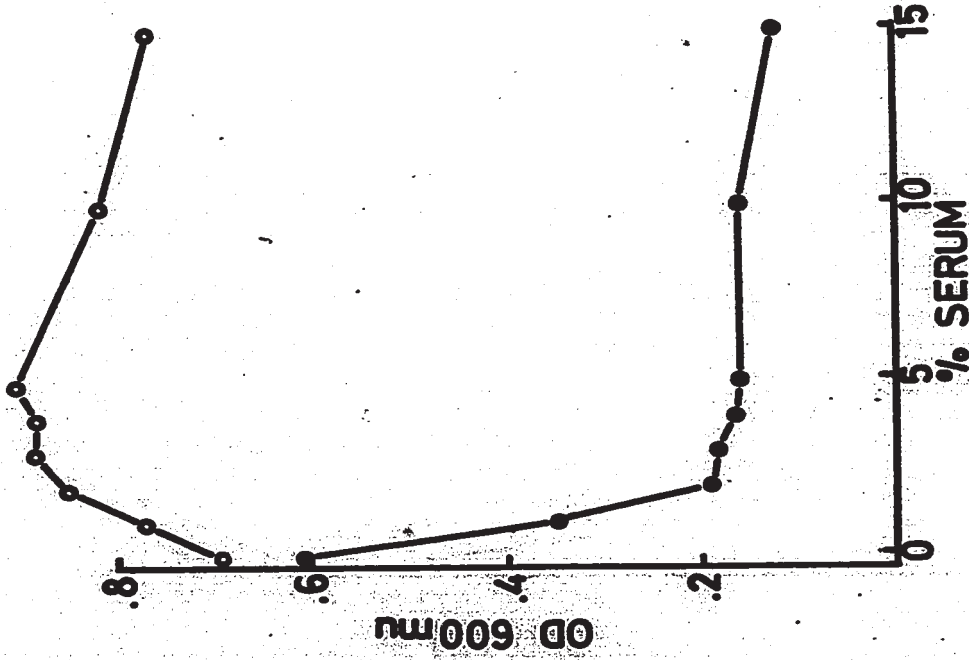


FIGURE 24. Effect of 25µM Mn²⁺ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus 25µM FeCl₃ (○); 6.5 days. See FIG. 22a.

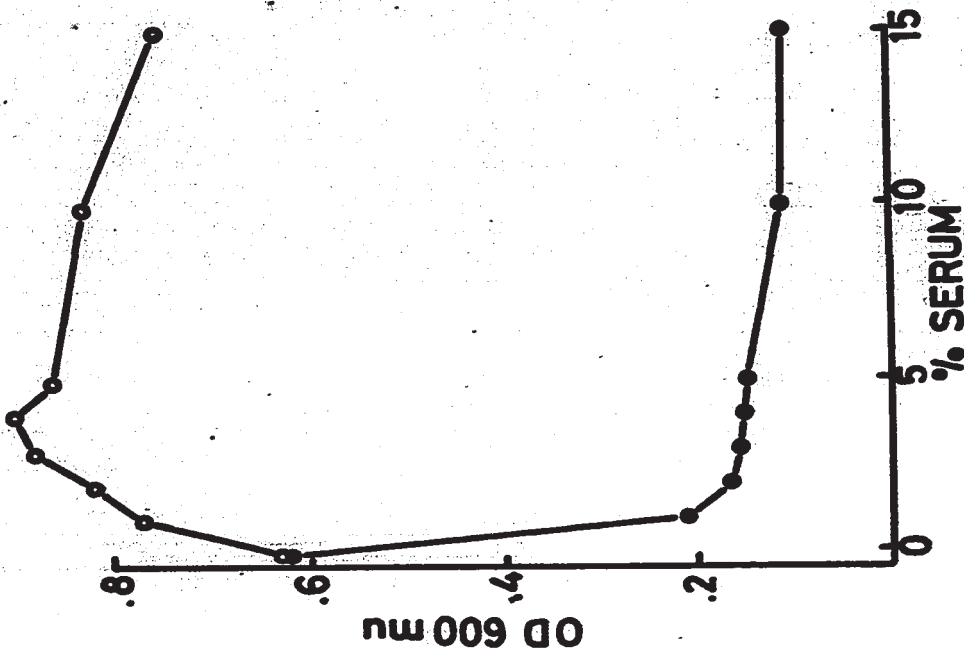


FIGURE 23. Effect of 25µM Mo⁶⁺ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus 25µM FeCl₃ (○); 6.5 days. See FIG. 22a.

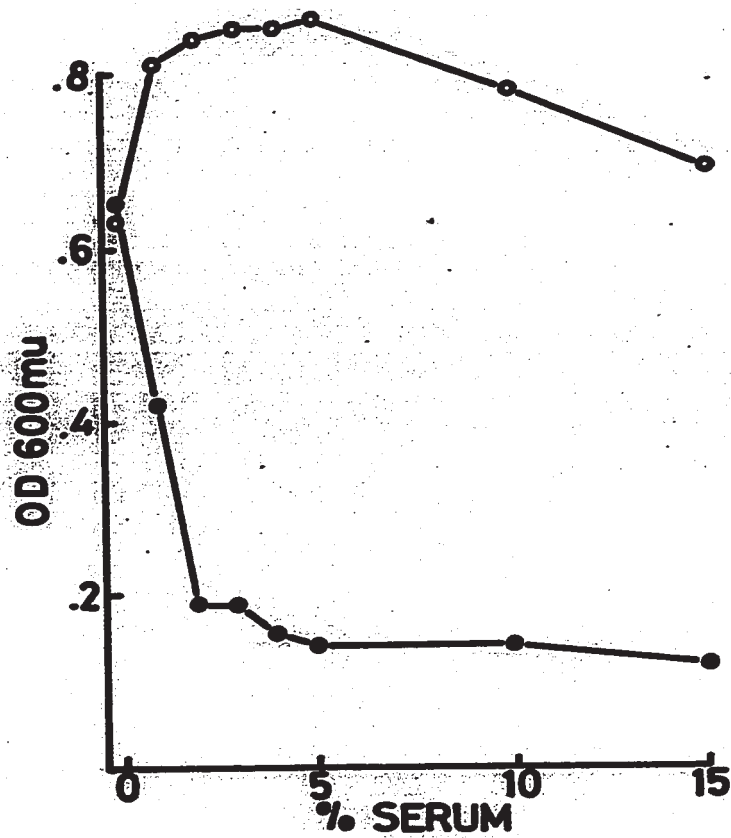


FIGURE 25. Effect of $2.5\mu\text{M Cu}^{2+}$ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus $25\mu\text{M FeCl}_3$ (○); 6.5 days. See Fig. 22a.

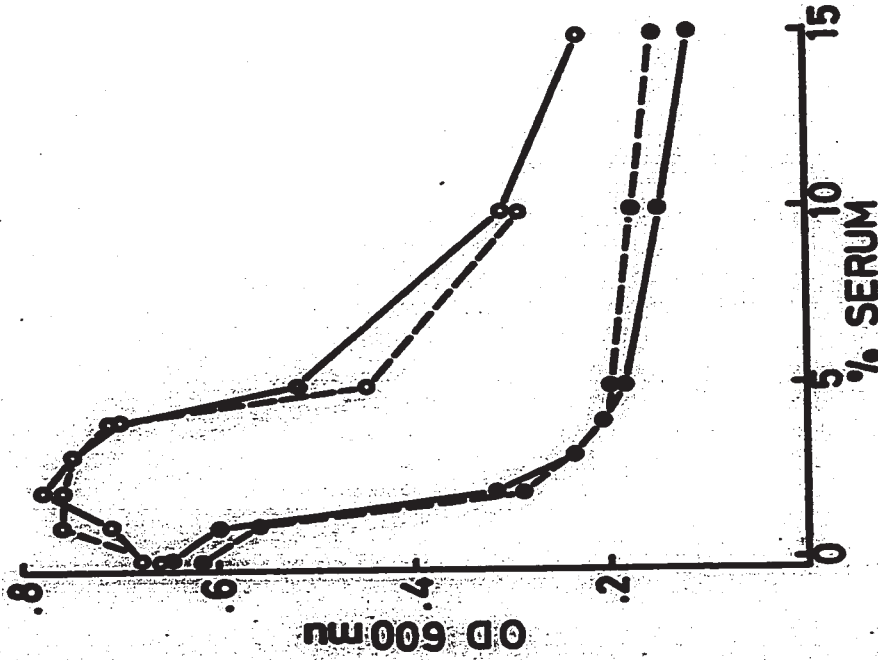


FIGURE 27. Effect of 25µM Mg²⁺ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus 25µM FeCl₃ (○); 6.5 days. See Fig. 22a.

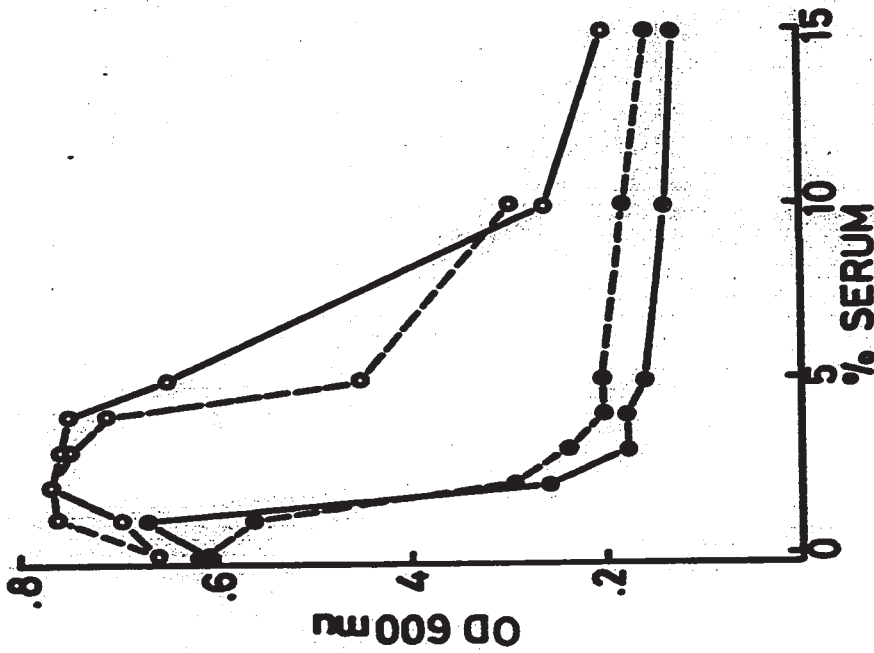


FIGURE 26. Effect of 25µM Ca²⁺ on growth of *C. neoformans* in N-G broth containing serum (●), or serum plus 25µM FeCl₃ (○); 6.5 days. See Fig. 22a.

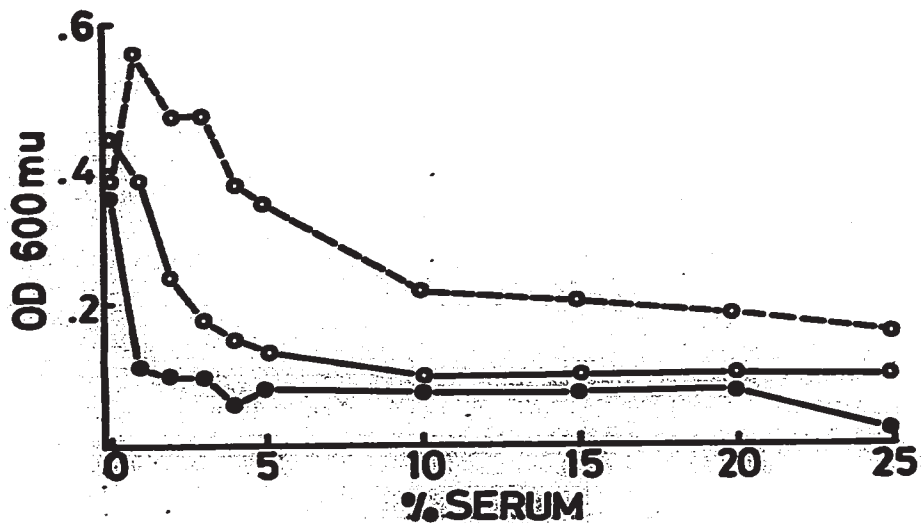


FIGURE 28. Inhibition of growth of *C. albicans* in increasing amounts of serum in N-G broth at pH 6.0 (●—●), pH 7.0 (○—○), pH 8.0 (—○—); 12.5 hr.

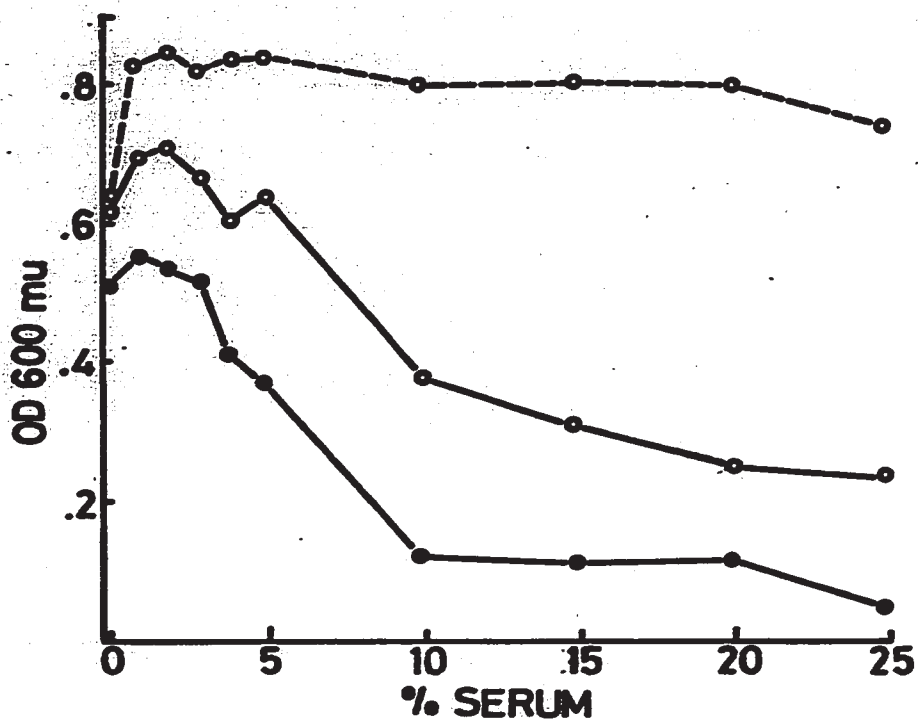


FIGURE 29. Reversal of inhibition of growth of *C. albicans* in increasing amounts of serum in N-G broth containing $25\mu\text{M FeCl}_3$, at pH 6.0 (●—●), pH 7.0 (○—○), pH 8.0 (—○—); 12.5 hr.

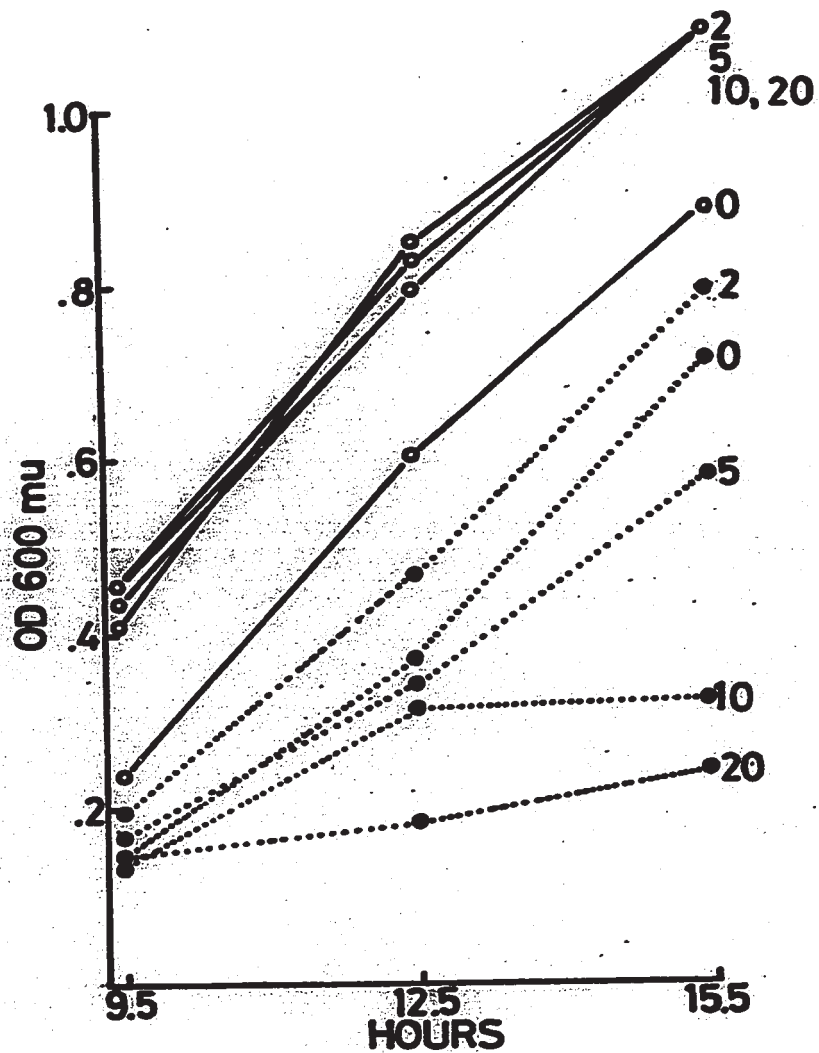


FIGURE 30. Rate of growth of *C. albicans* in N-G broth containing various concentrations of serum; pH 6.0; no added FeCl₃ (●.....●), 25 μM FeCl₃ (○—○). Numbers to the right of the lines indicate % serum.

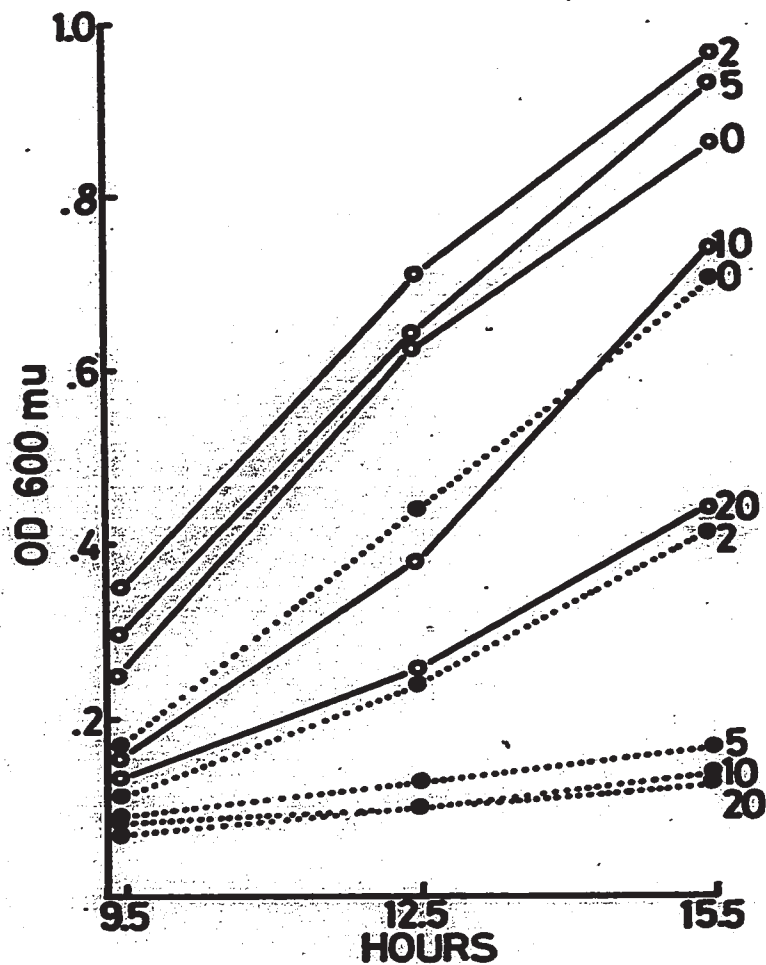


FIGURE 31. Rate of growth of *C. albicans* in N-G broth containing various concentrations of serum; pH 7.0; no added FeCl₃ (●.....●), 25μM FeCl₃ (○—○). Numbers to the right of the lines indicate % serum.

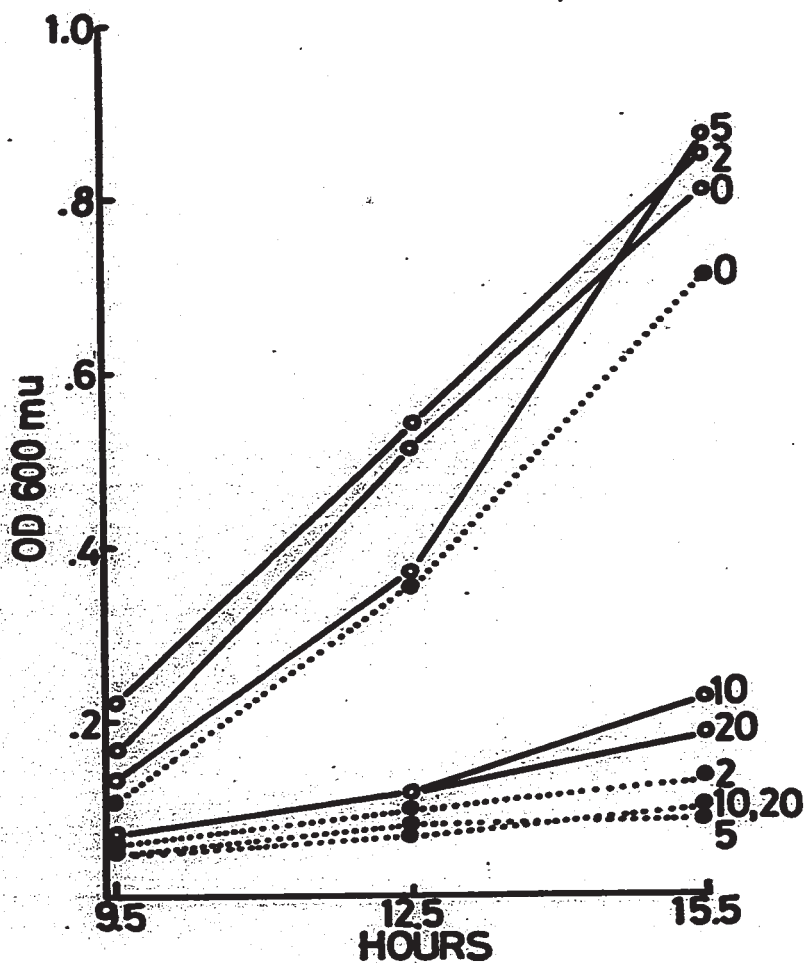


FIGURE 32. Rate of growth of *C. albicans* in N-G broth containing various concentrations of serum; pH 8.0; no added FeCl₃ (●.....●), 25 μM FeCl₃ (○—○). Numbers to the right of the lines indicate % serum.

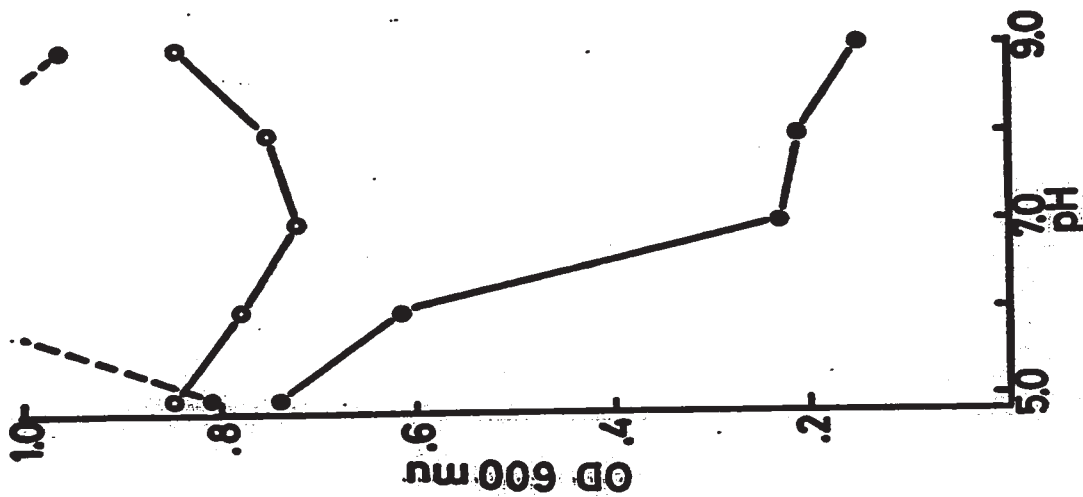


FIGURE 34. Effect of pH on the growth of *C. albicans* in N-G broth (○), with 25% serum (●—●), with 25% serum and 100µM FeCl₃ (○---○); 16 hr.

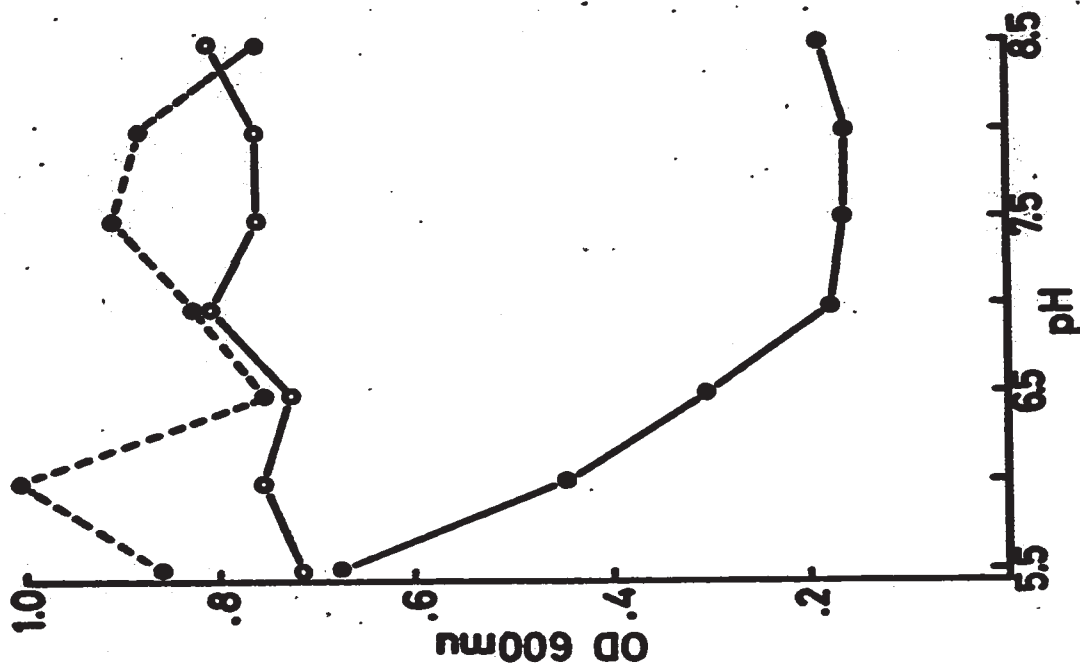


FIGURE 33. Effect of pH on the growth of *C. albicans* in N-G broth (○), with 25% serum (●—●), with 25% serum and 100µM FeCl₃ (○---○); 14 hr.

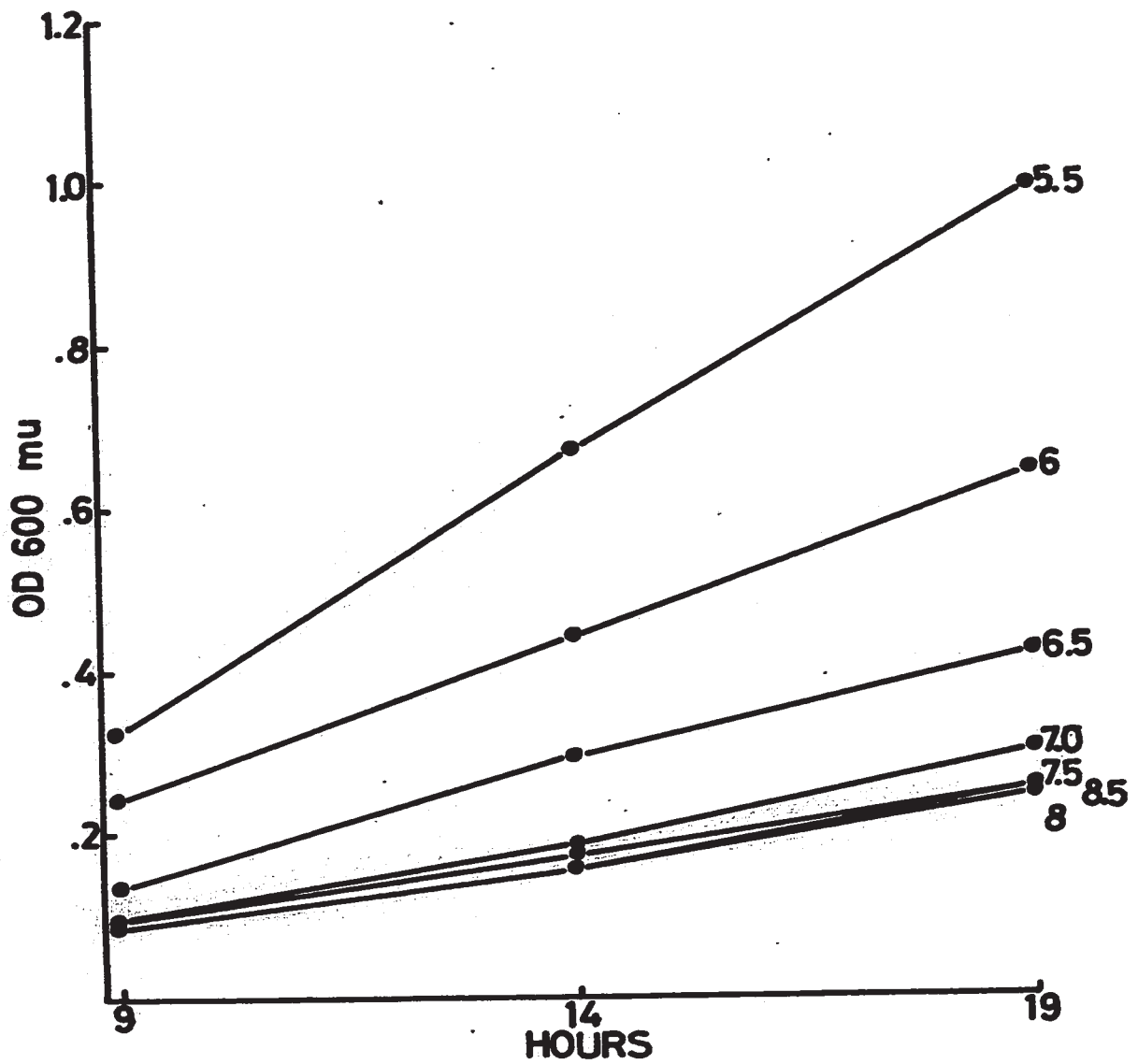


FIGURE 35. Effect of pH on rate of growth of *C. albicans* in N-G broth containing 25% serum. Numbers to the right of the lines indicate pH.

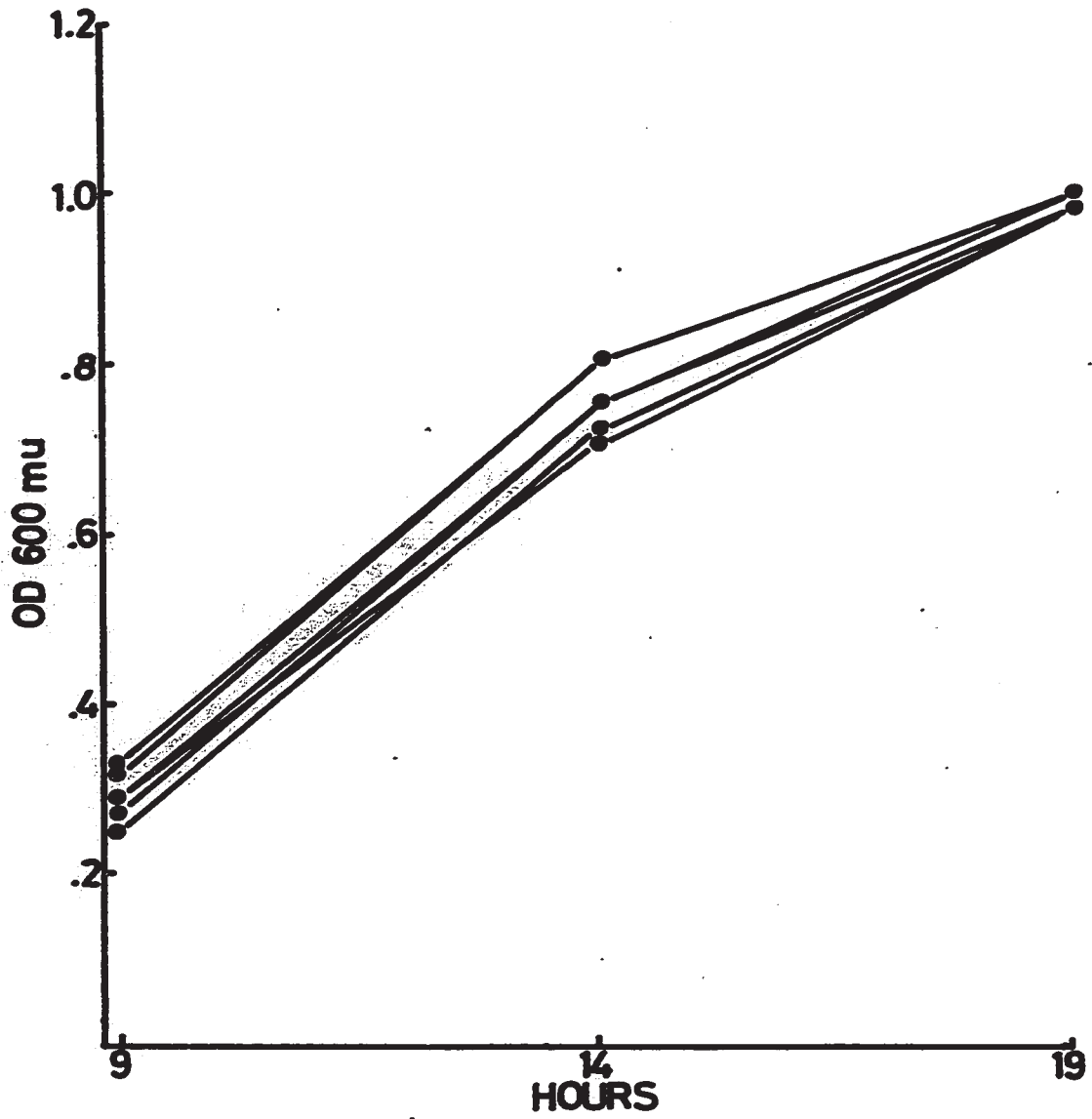


FIGURE 36. Effect of pH on rate of growth of *C. albicans* in N-G broth, at pH values from 5.5 to 8.5.

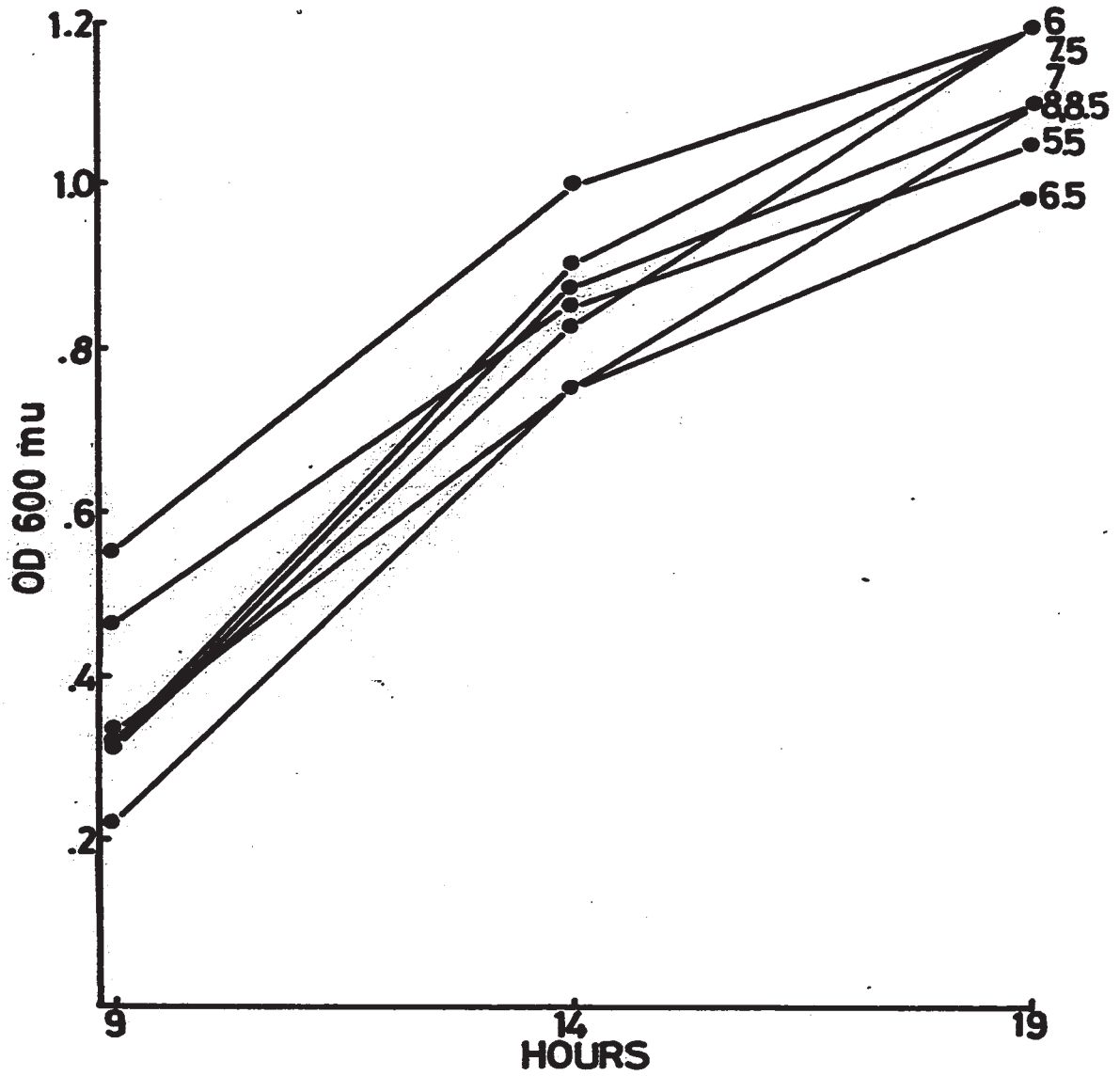


FIGURE 37. Effect of pH on rate of growth of *C. albicans* in N-G broth containing 25% serum plus 100 μ M FeCl₃. Numbers to the right of the lines indicate pH.

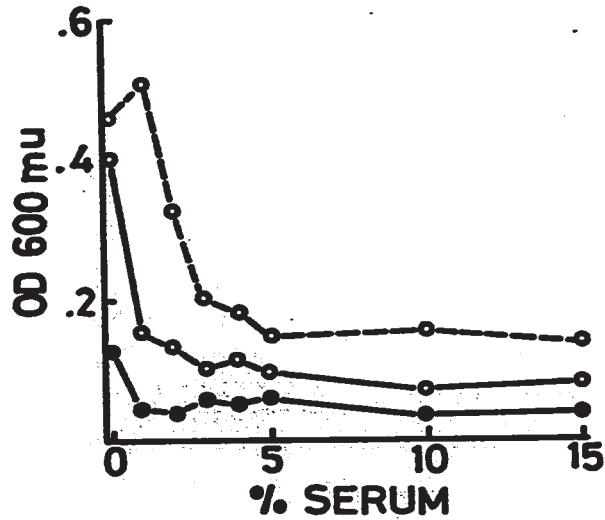


FIGURE 38. Inhibition of growth of *C. neoformans* in increasing concentrations of serum in N-G broth at pH 6.0 (○---○), pH 7.0 (○—○), pH 8.0 (●—●); 4.5 days.

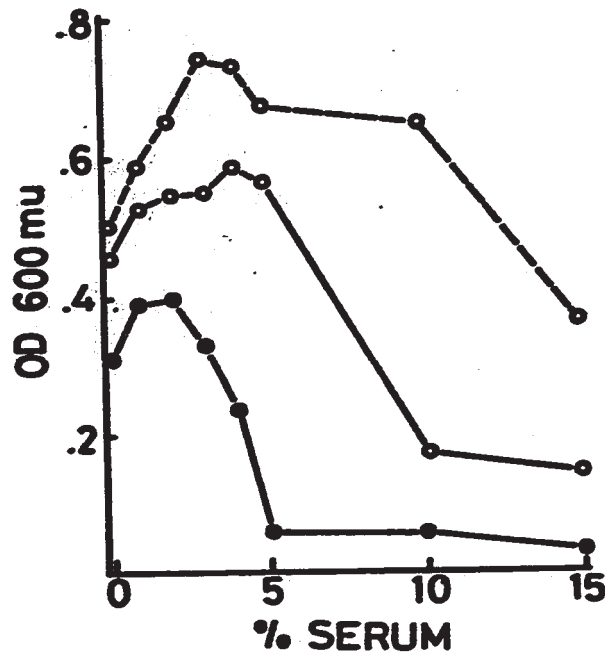


FIGURE 39. Growth of *C. neoformans* in increasing concentrations of serum in N-G broth plus 25 μ M FeCl₃ at pH 6.0 (○---○), pH 7.0 (○—○), pH 8.0 (●—●); 4.5 days.

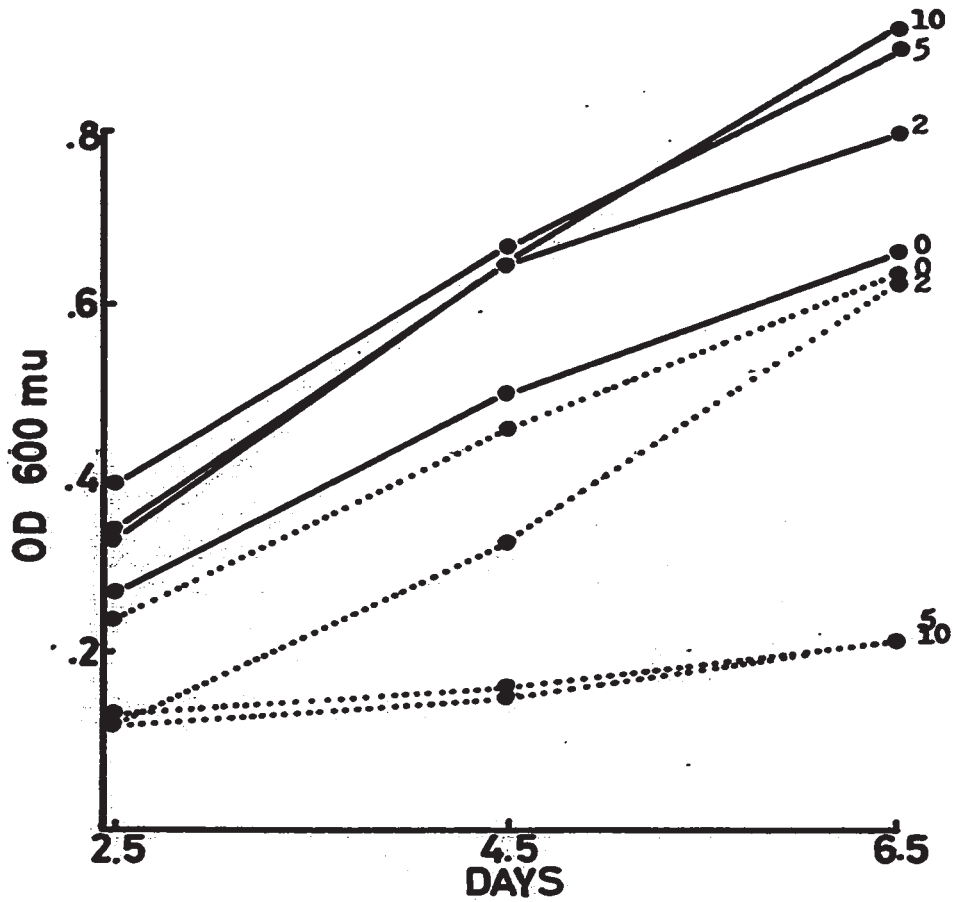


FIGURE 40. Effect of pH on the rate of growth of *C. neoformans* in N-G broth containing various concentrations of serum, pH 6.0; no added FeCl₃ (●.....●), 25µM FeCl₃ (●—●). Numbers to the right of the lines indicate % serum.

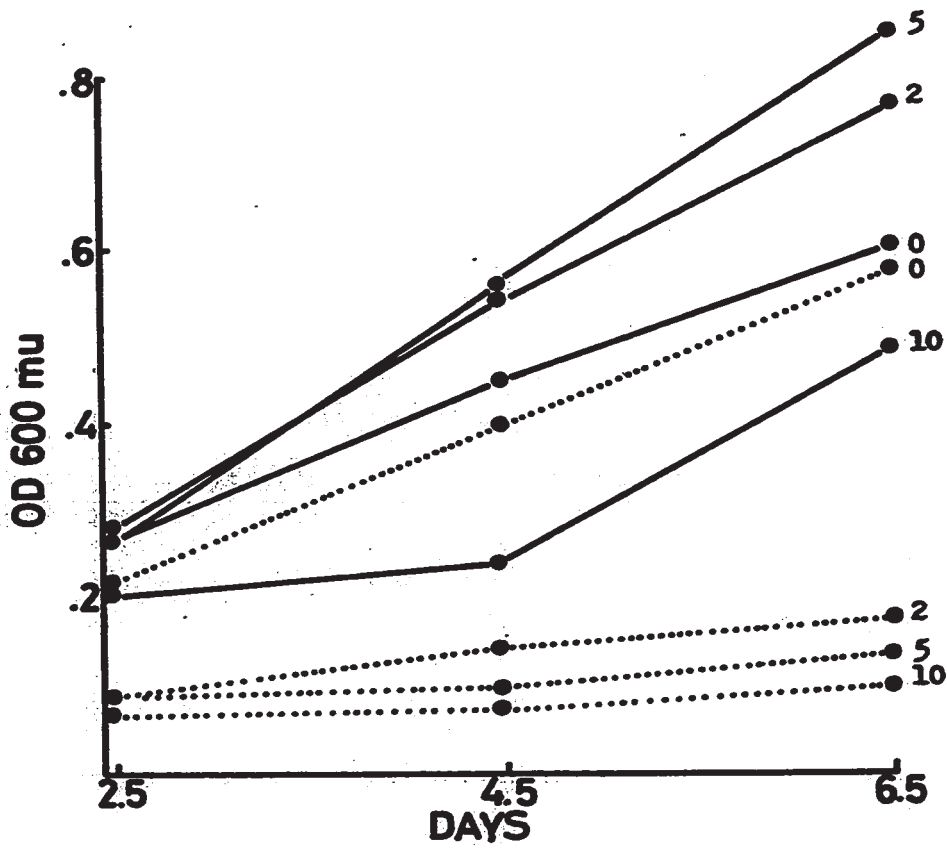


FIGURE 41. Effect of pH on the rate of growth of *C. neoformans* in N-G broth containing various concentrations of serum, pH 7.0; no added FeCl₃ (●.....●), 25µM FeCl₃ (●—●). Numbers to the right of the lines indicate % serum.

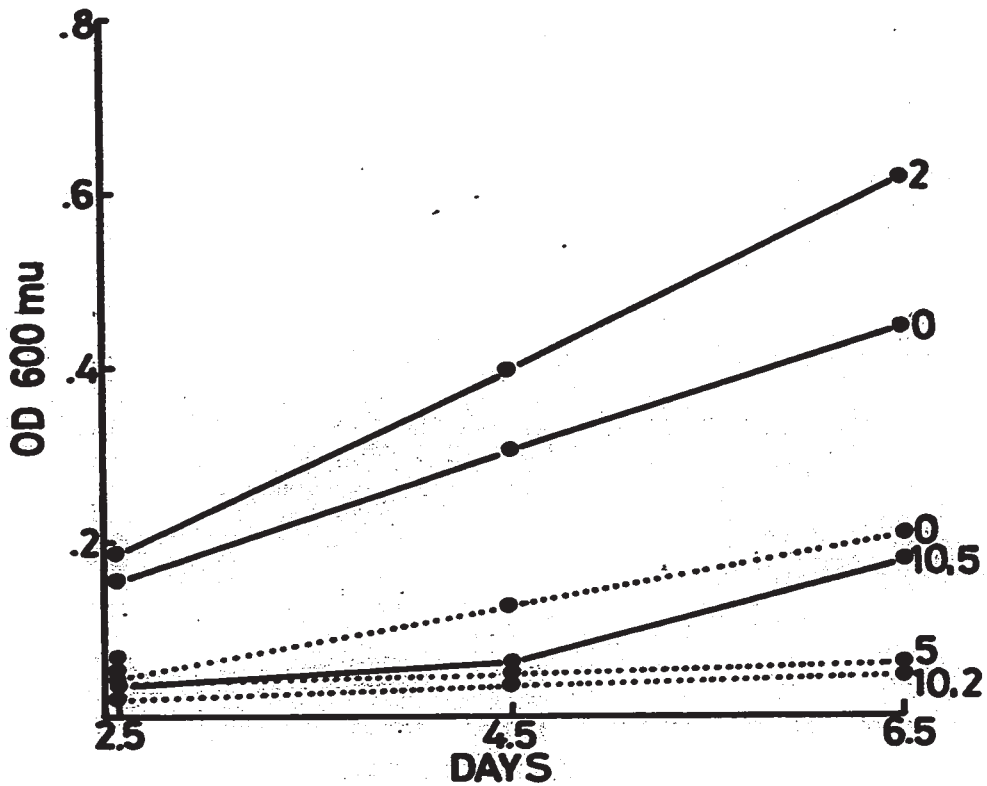


FIGURE 42. Effect of pH on the rate of growth of *C. neoformans* in N-G broth containing various concentrations of serum, pH 8.0; no added FeCl₃ (●.....●), 25µM FeCl₃ (●—●). Numbers to the right of the lines indicate % serum.

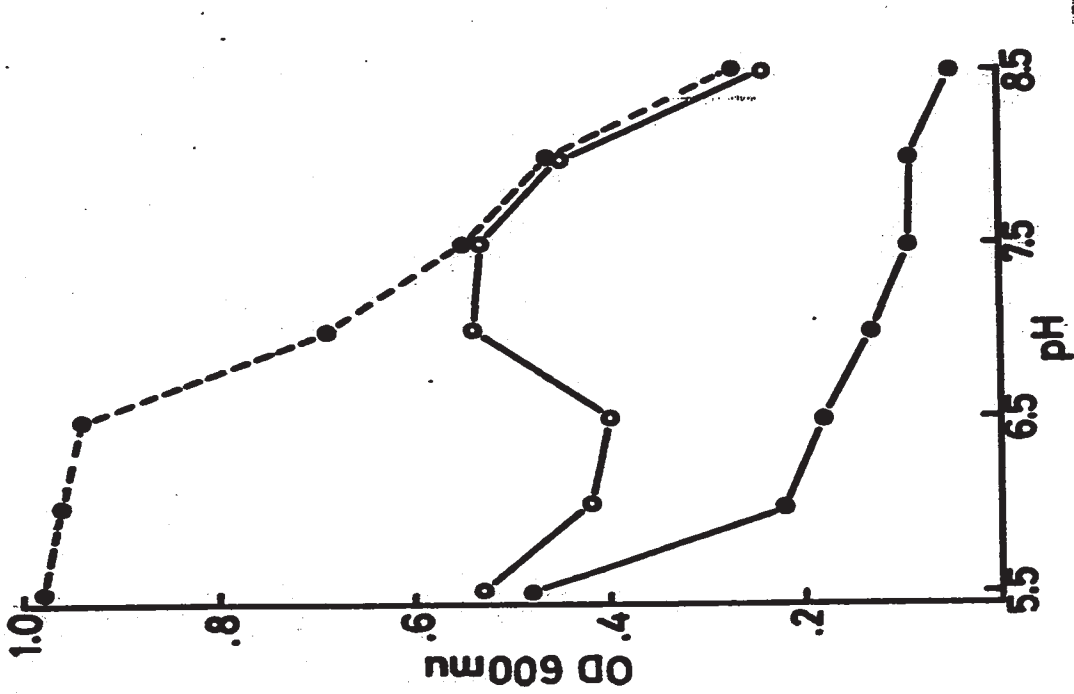


FIGURE 43. Effect of pH on the growth of *C. neoformans* in N-G broth (○), with 10% serum (○- - -○), with 10% serum and 100µM FeCl₃ (●- - -●); 7.5 days.

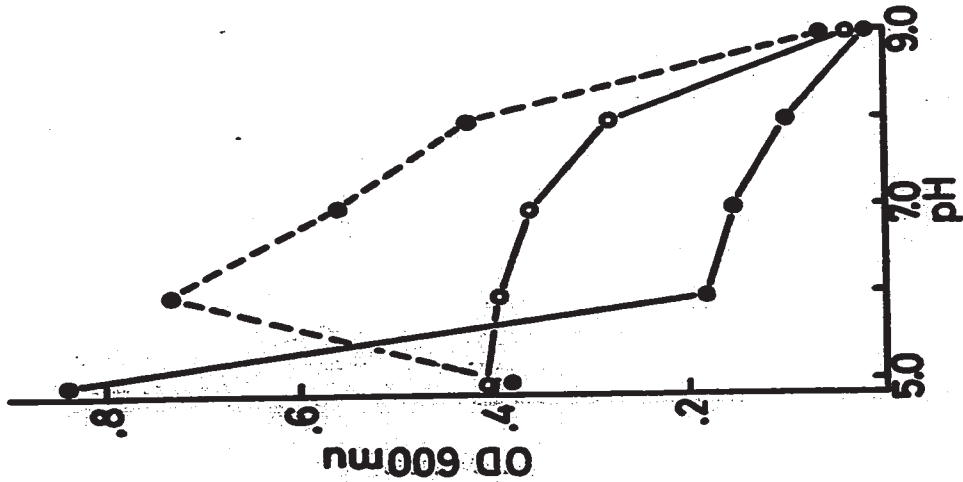


FIGURE 44. Effect of pH on the growth of *C. neoformans* in N-G broth (○), with 10% serum (○- - -○), with 10% serum and 100µM FeCl₃ (●- - -●); 6 days.

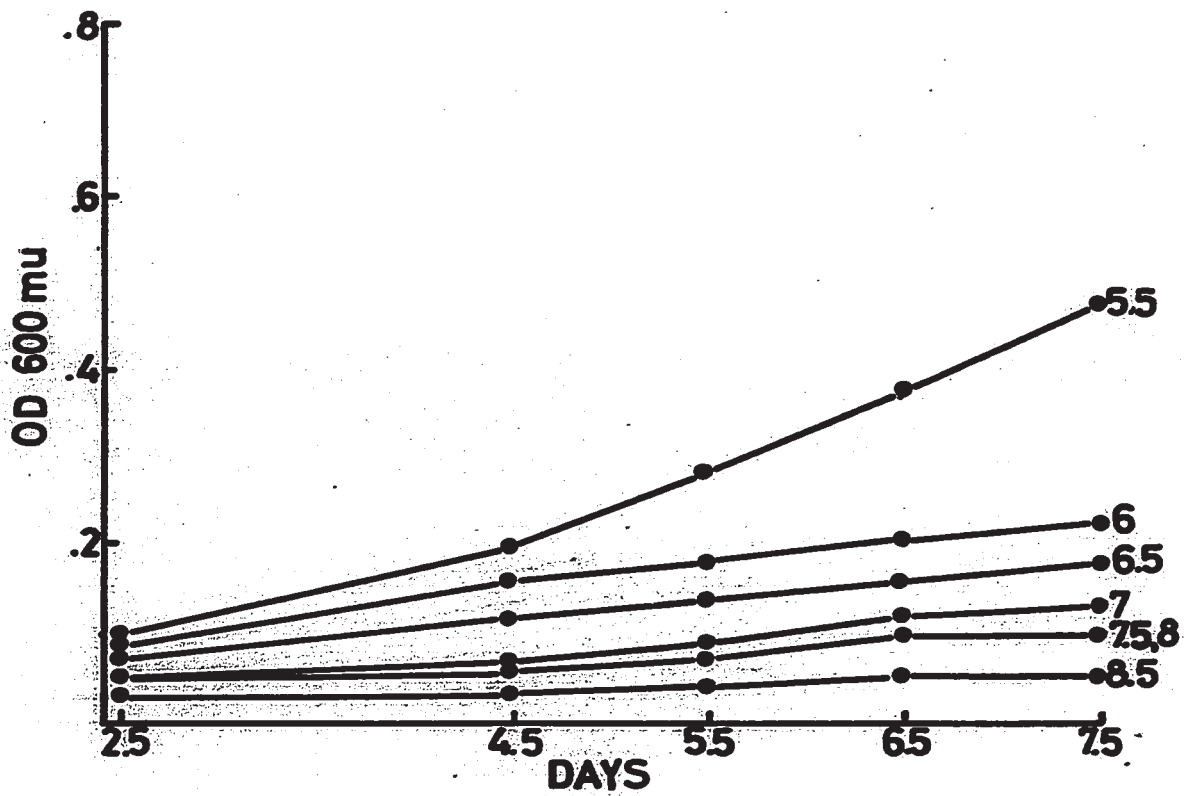


FIGURE 45. Effect of pH on the rate of growth of *C. neoformans* in N-G broth containing 10% serum. Numbers to the right of the lines indicate pH.

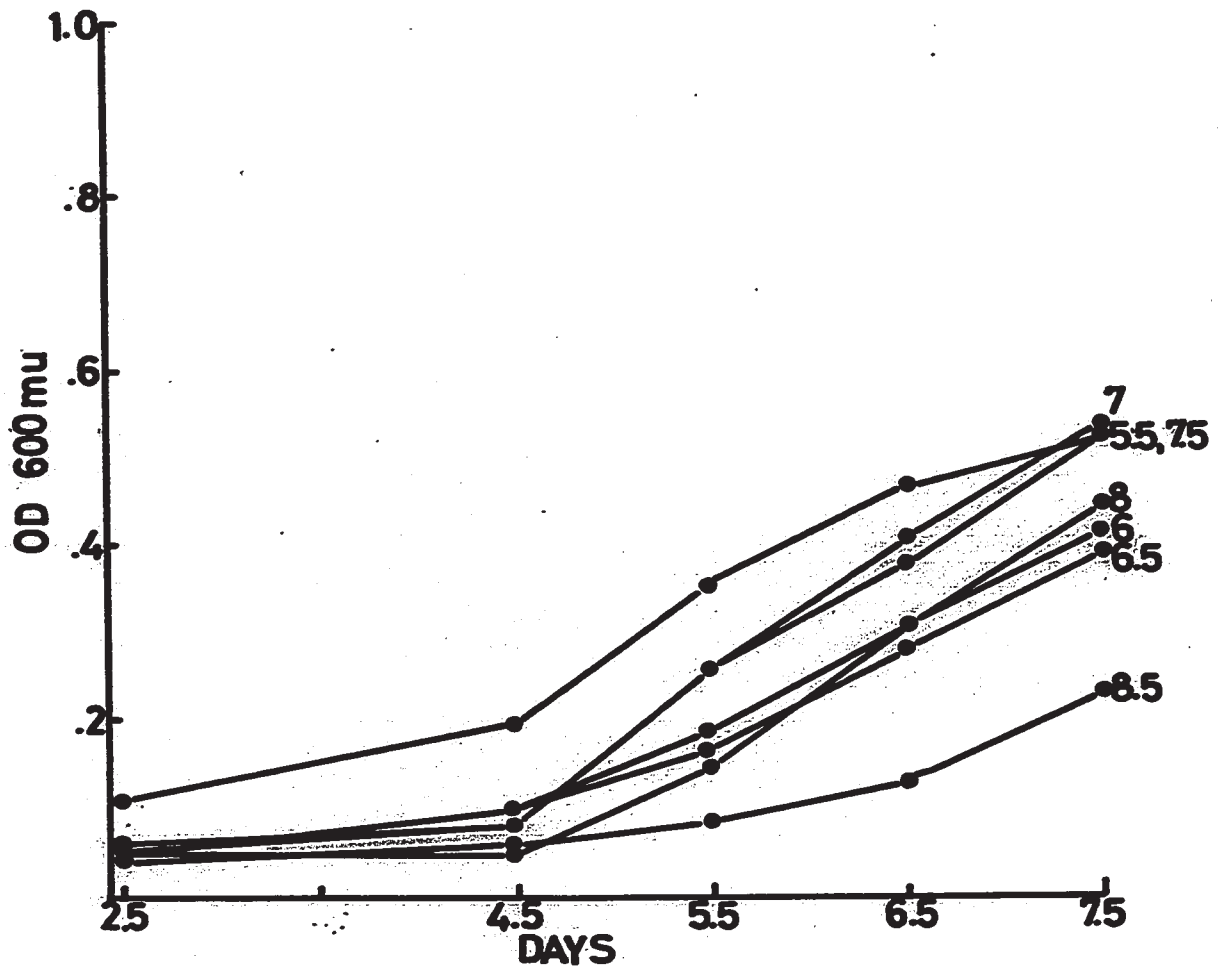


FIGURE 46. Effect of pH on the rate of growth of *C. neoformans* in N-G broth. Numbers to the right of the lines indicate pH.

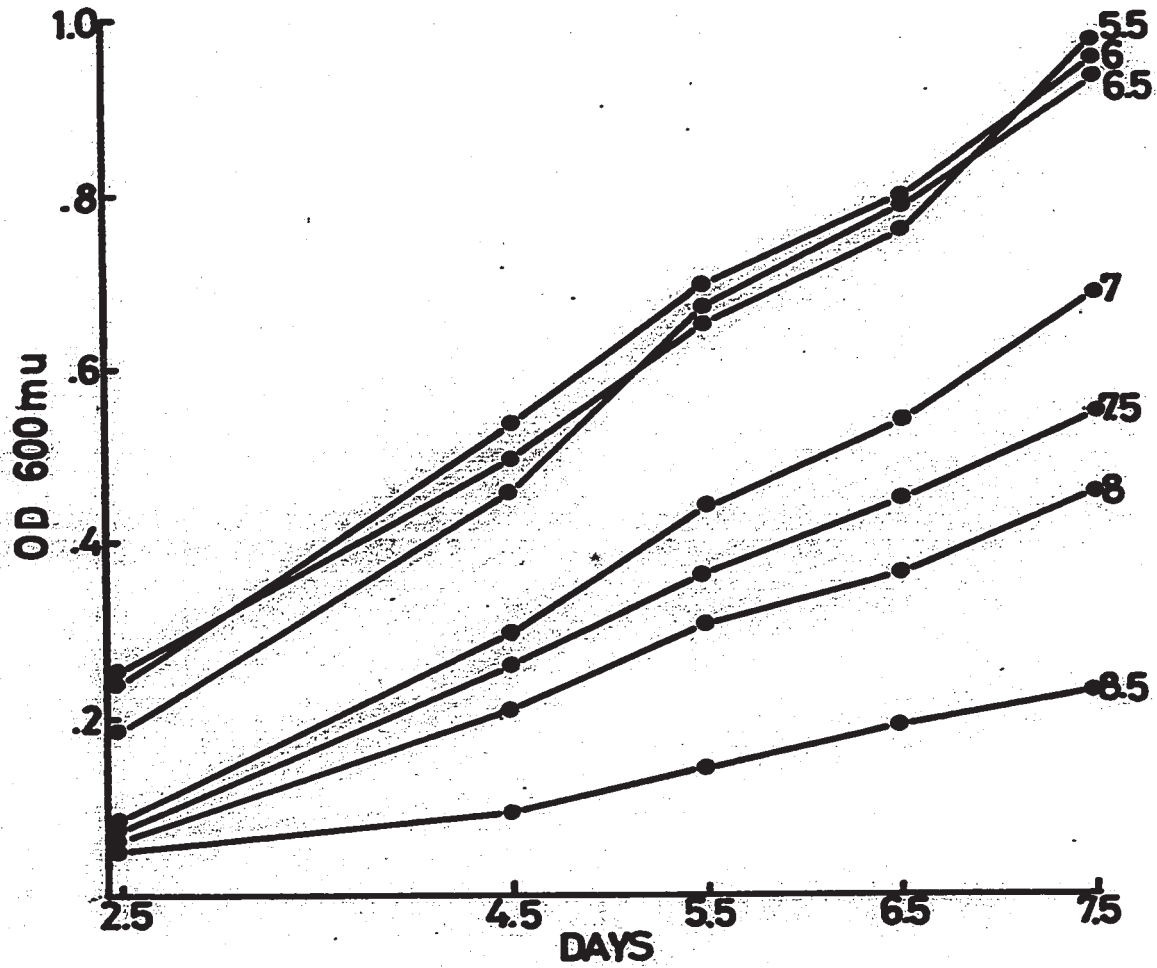


FIGURE 47. Effect of pH on the rate of growth of *C. neoformans* in N-G broth containing 10% serum plus 100 μ M FeCl₃. Numbers to the right of the lines indicate pH.

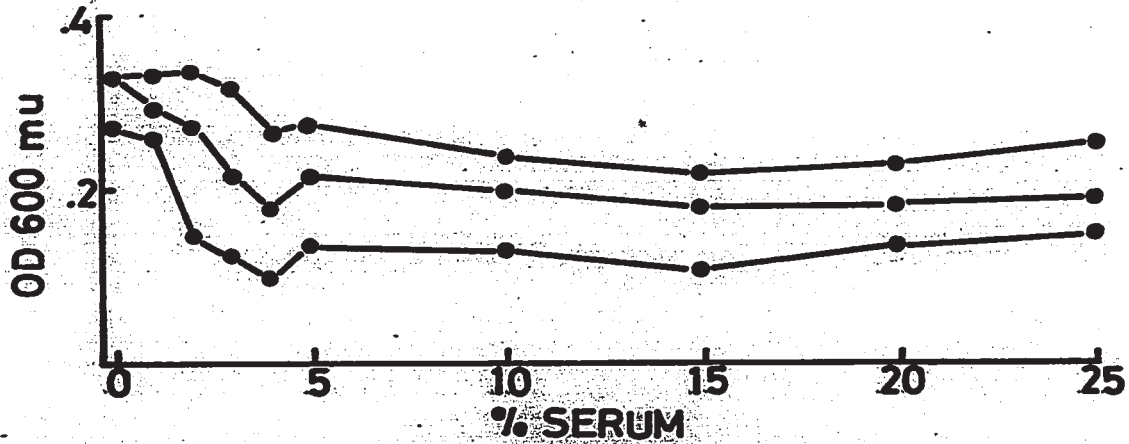


FIGURE 48. Growth of *C. albicans* in neopeptone solution containing 0.1% glucose and various concentrations of serum; Lines from top to bottom, 17.5, 14, 10 hr., resp.

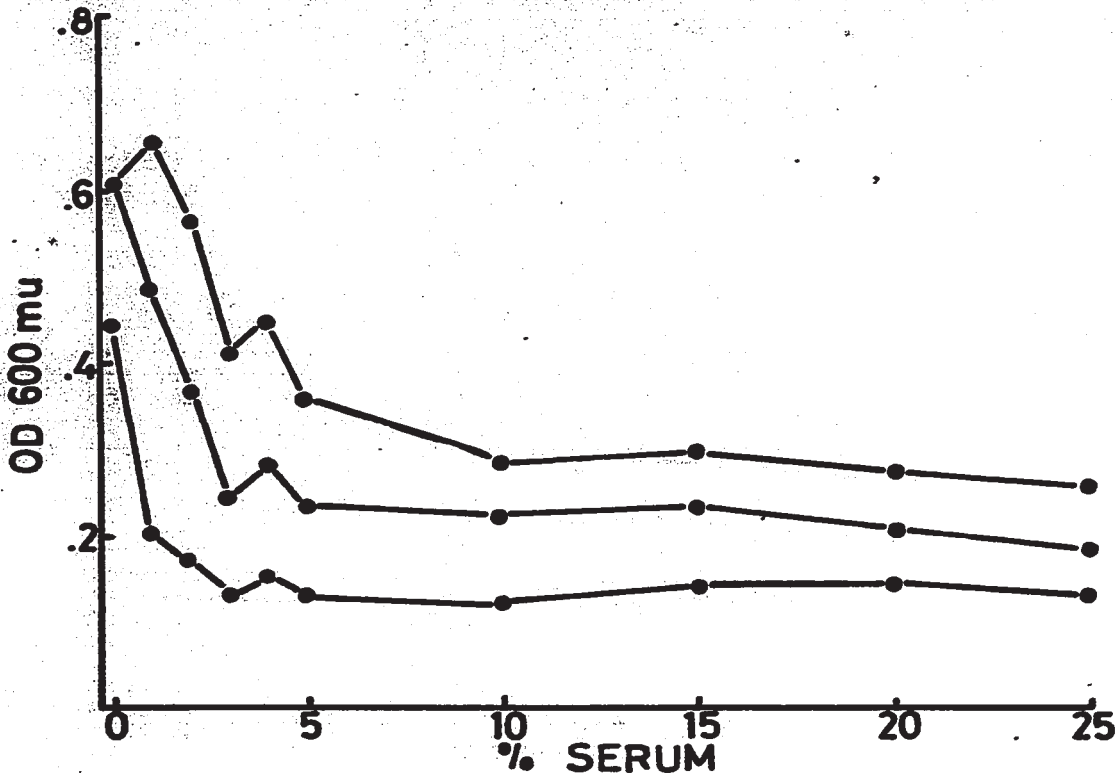


FIGURE 49. Growth of *C. albicans* in neopeptone solution containing 0.25% glucose and various concentrations of serum; Lines from top to bottom, 17.5, 14, 10 hr., resp.

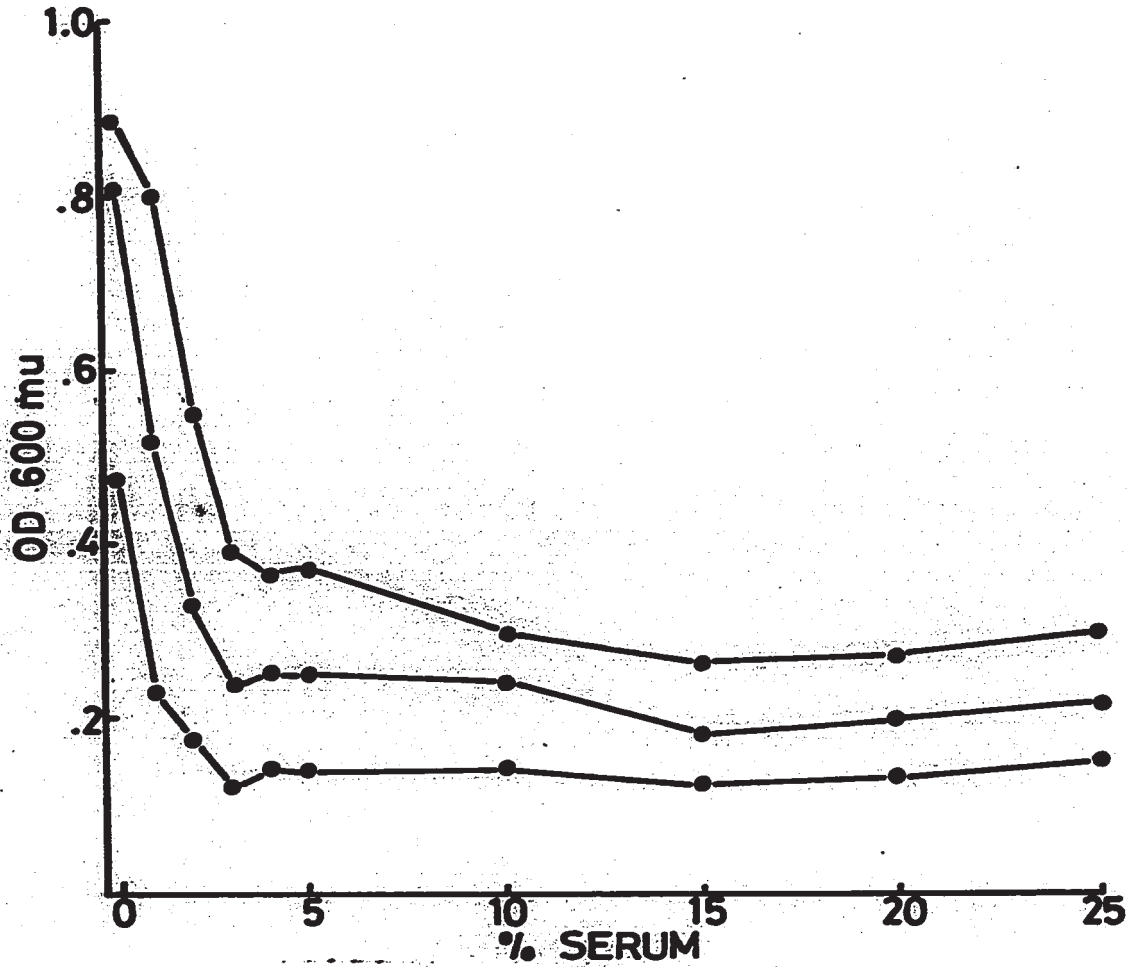


FIGURE 50. Growth of *C. albicans* in neopeptone solution containing 0.5% glucose and various concentrations of serum; Lines from top to bottom, 17.5, 14, 10 hr., resp.

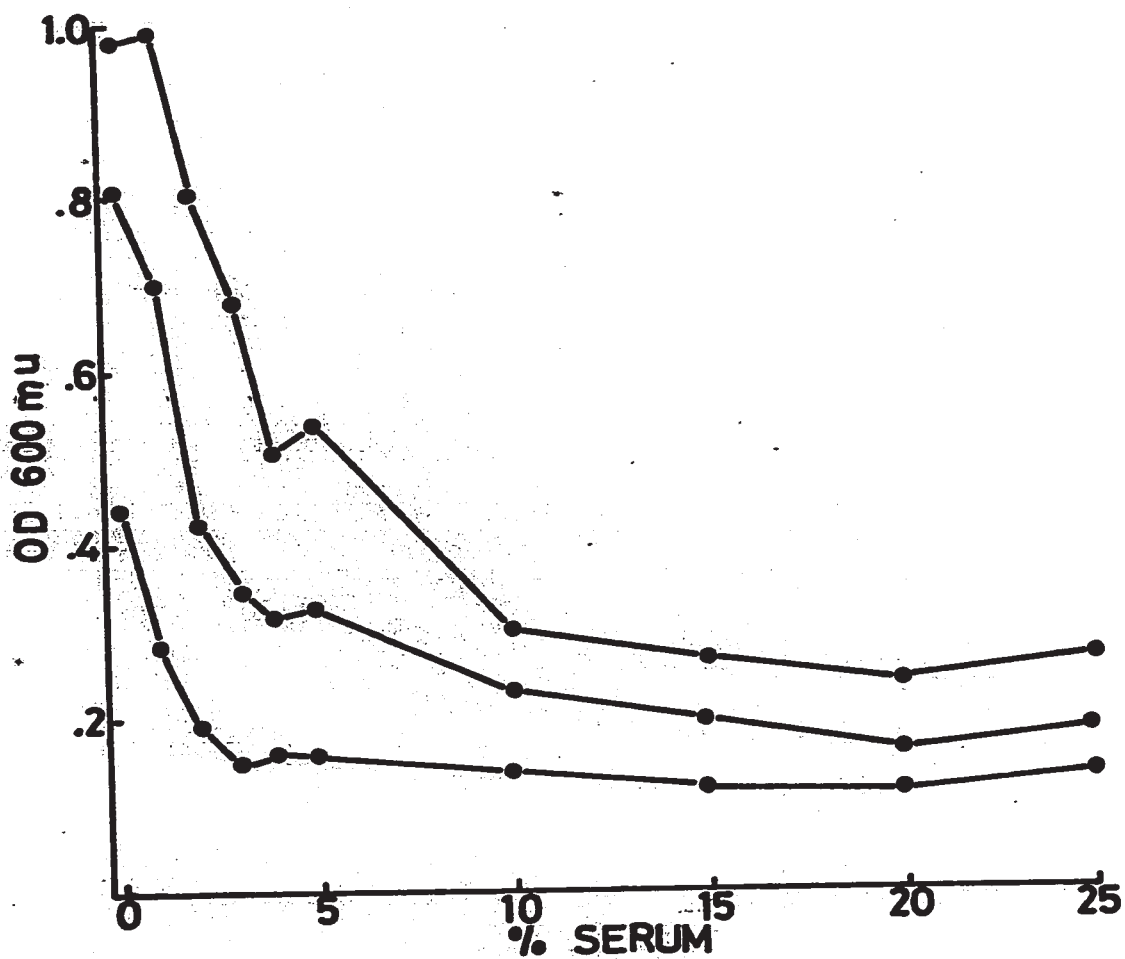


FIGURE 51. Growth of *C. albicans* in neopeptone solution containing 1.0% glucose and various concentrations of serum. Lines from top to bottom, 17.5, 14, 10 hr., resp.

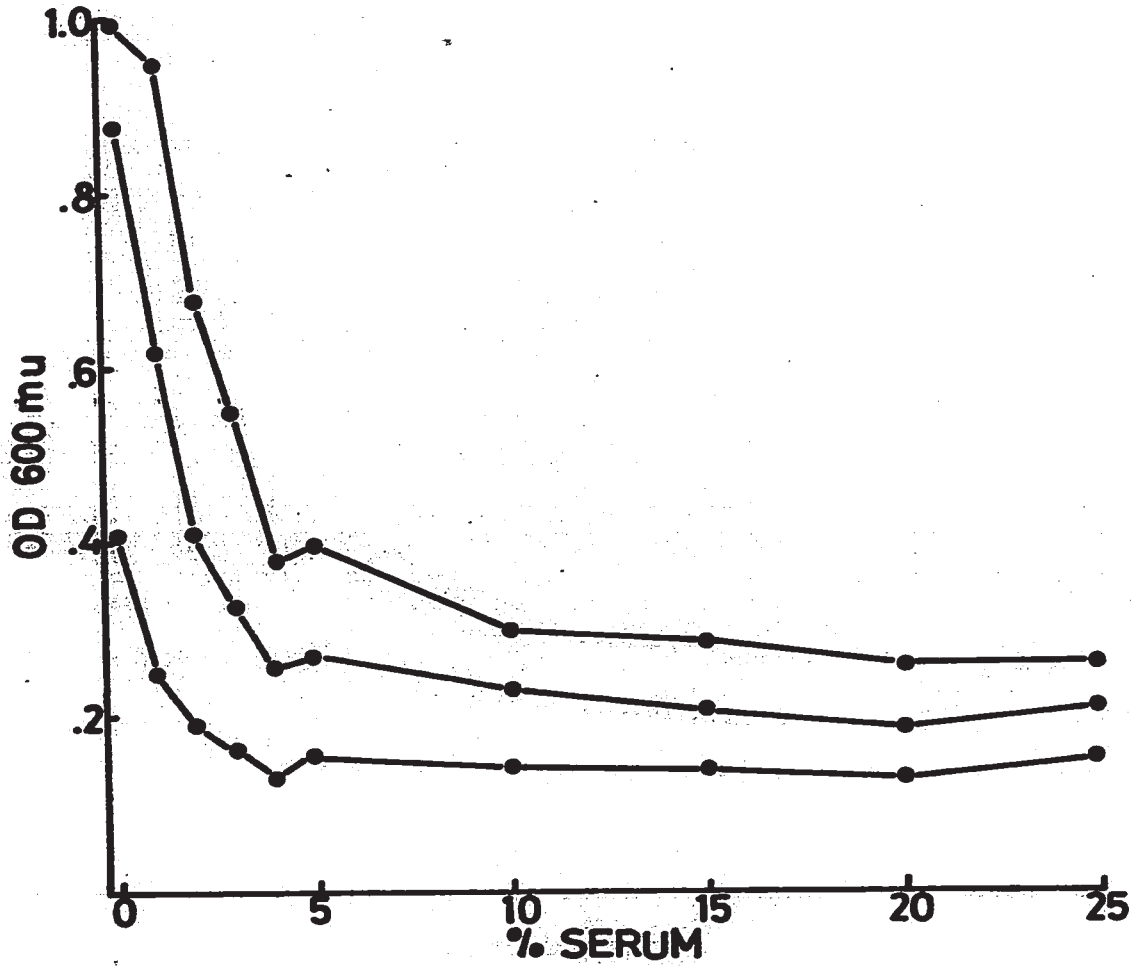


FIGURE 52. Growth of *C. albicans* in neopeptone solution containing 2.0% glucose and various concentrations of serum. Lines from top to bottom, 17.5 hr., 14 hr., 10 hr., resp.

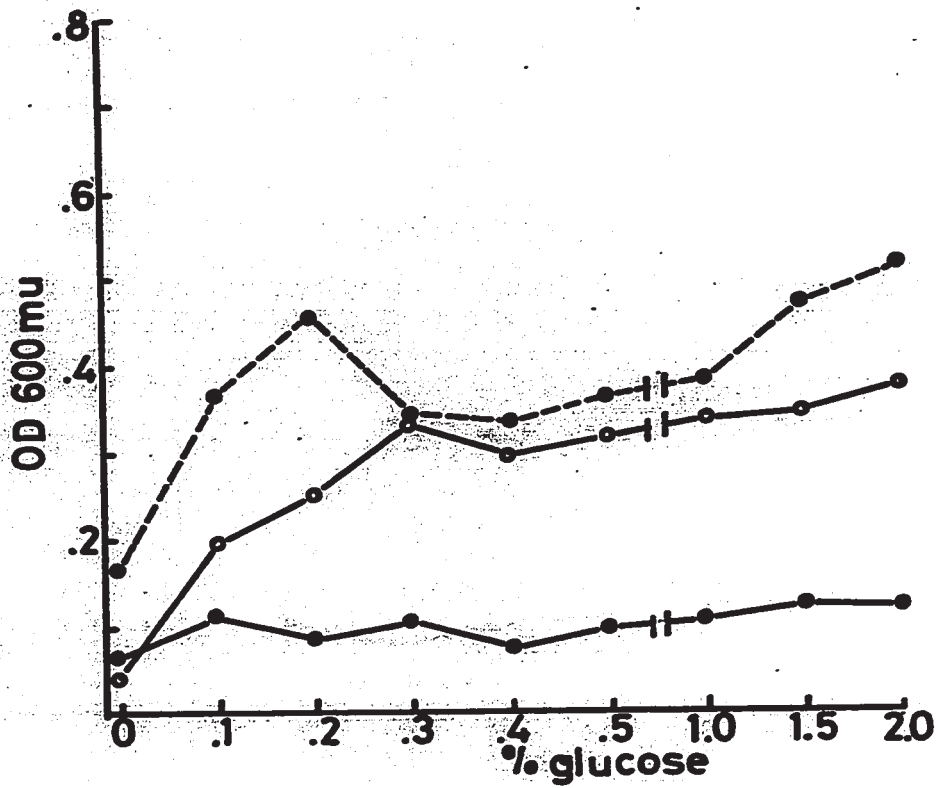


FIGURE 53. Effect of increasing amounts of glucose on the growth of *C. albicans* in neopeptone solution (○—○), in neopeptone solution plus 25% serum (●—●), in neopeptone solution plus 25% serum and 100µM FeCl₃ (●—●); pH 7.0; 11 hr.

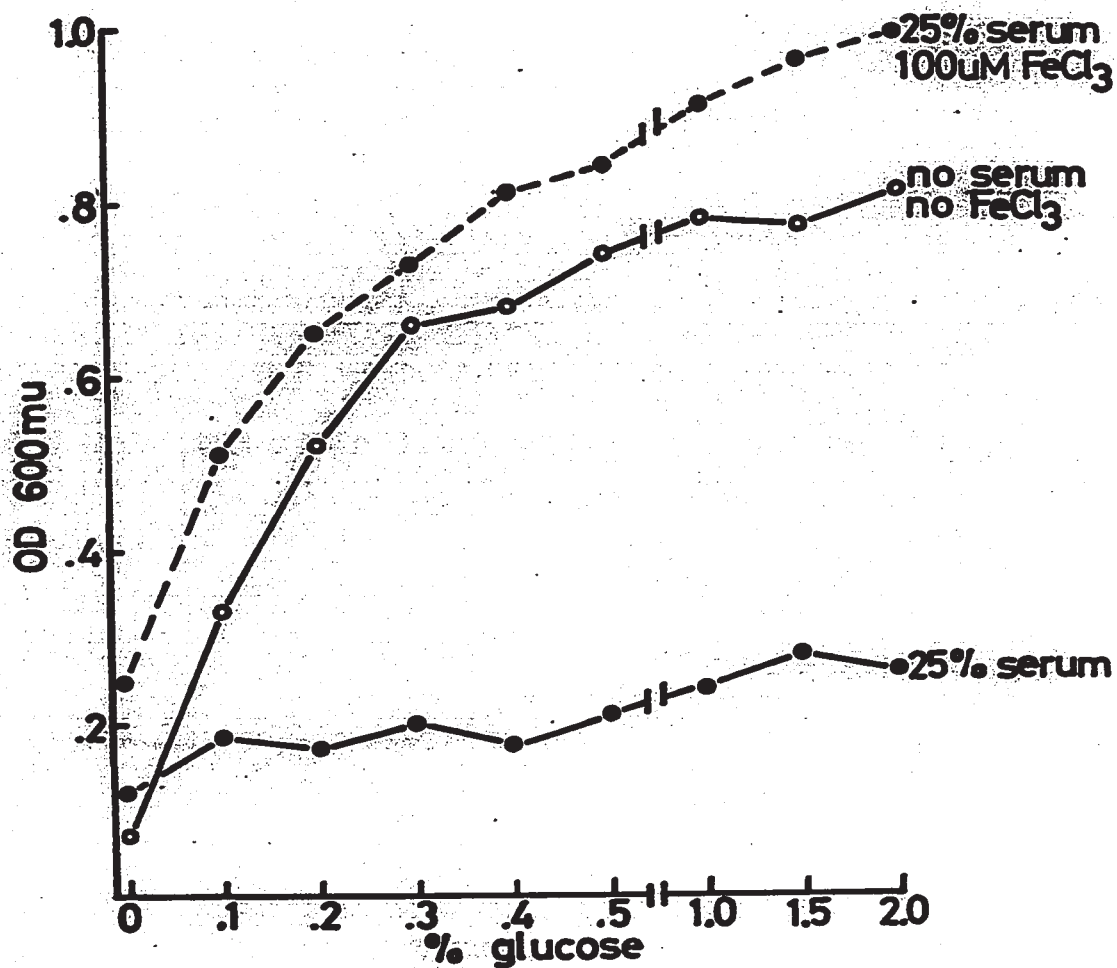


FIGURE 54. Effect of increasing amounts of glucose on the growth of *C. albicans* in neopeptone solution (○—○), in neopeptone solution plus 25% serum (●—●), in neopeptone solution plus 25% serum and 100μM FeCl₃ (●---●); pH 7.0; 17 hr.

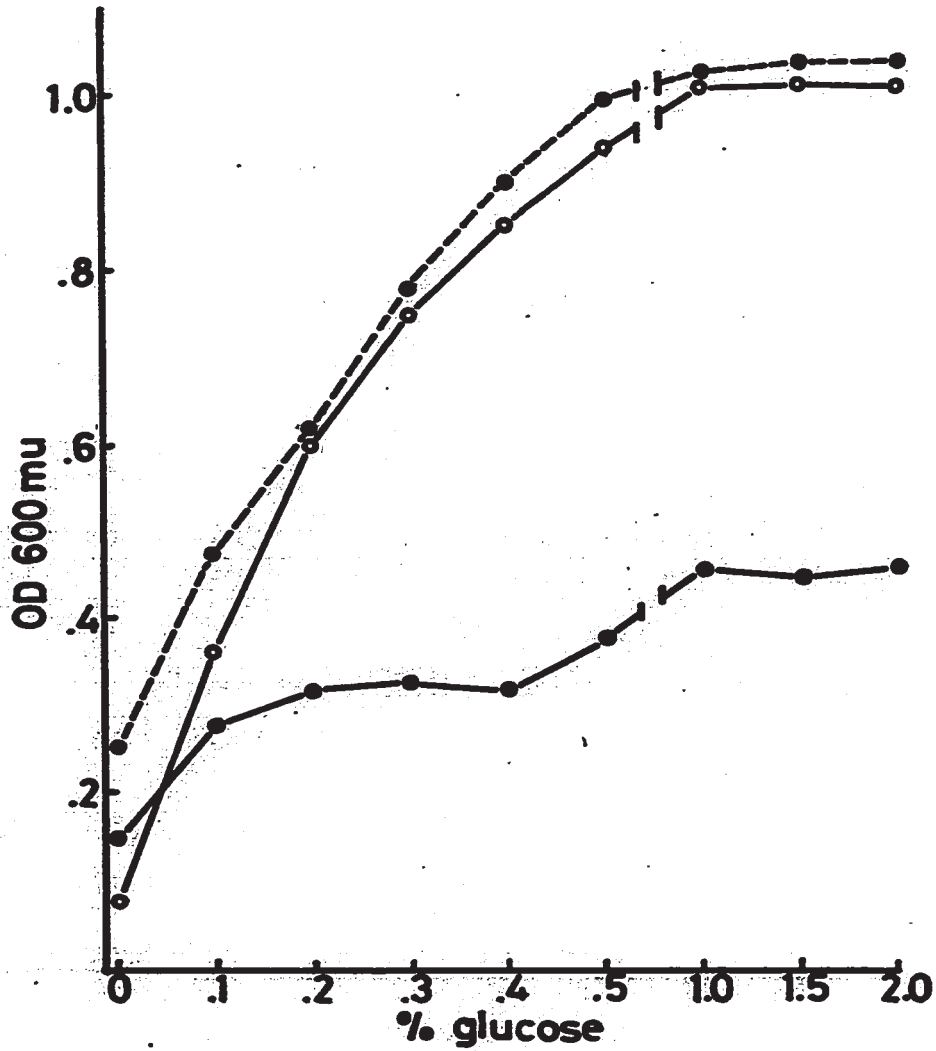


FIGURE 55. Effect of increasing amounts of glucose on the growth of *C. albicans* in neopeptone solution (○—○), in neopeptone solution plus 25% serum (●—●), in neopeptone solution plus 25% serum and 100µM FeCl₃ (●—●); pH 7.0; 23 hr.

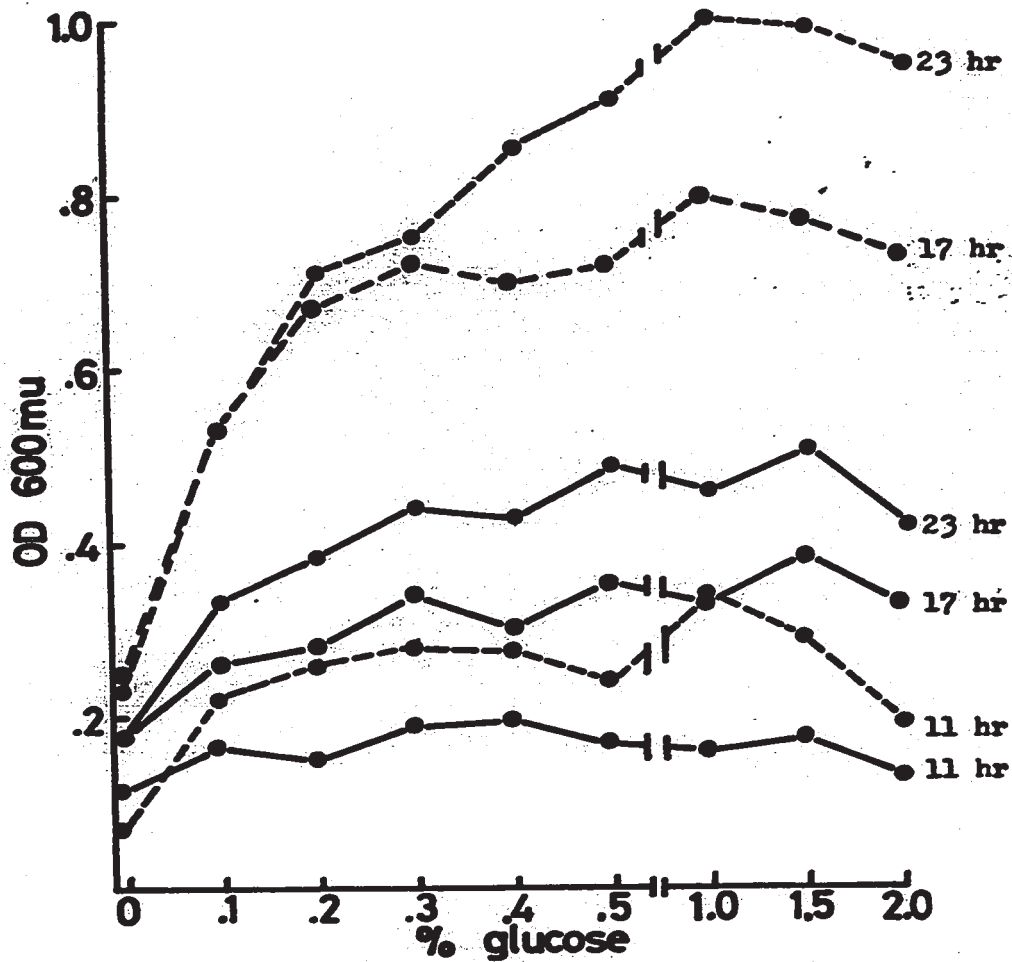


FIGURE 56. Effect of increasing amounts of glucose on the growth of *C. albicans* in water containing 25% serum (●—●), in water containing 25% serum and 100 μM FeCl₃ (●---●); pH 7.0; 11, 17, 23 hr.

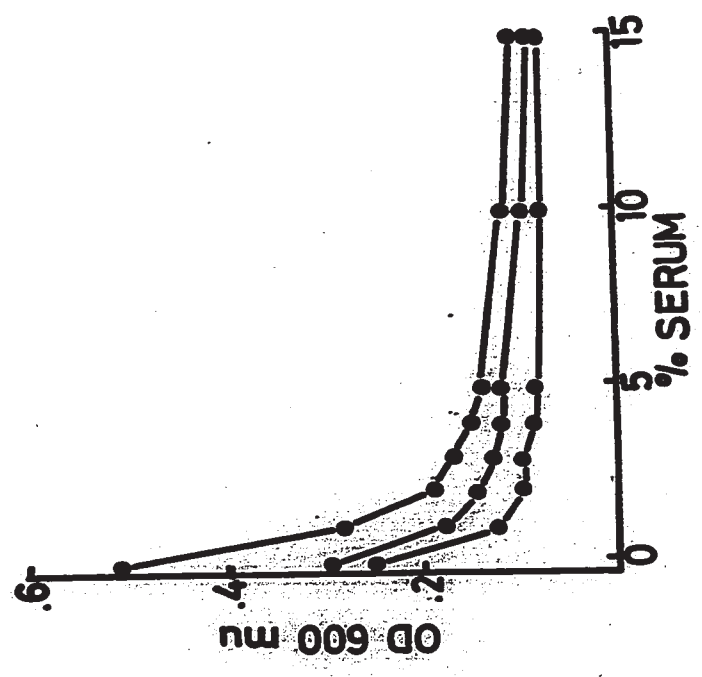


FIGURE 58. Growth of *C. neoformans* in neopeptone solution containing 0.25% glucose and various concentrations of serum. Lines from top to bottom, 8, 6, 4 days, resp.

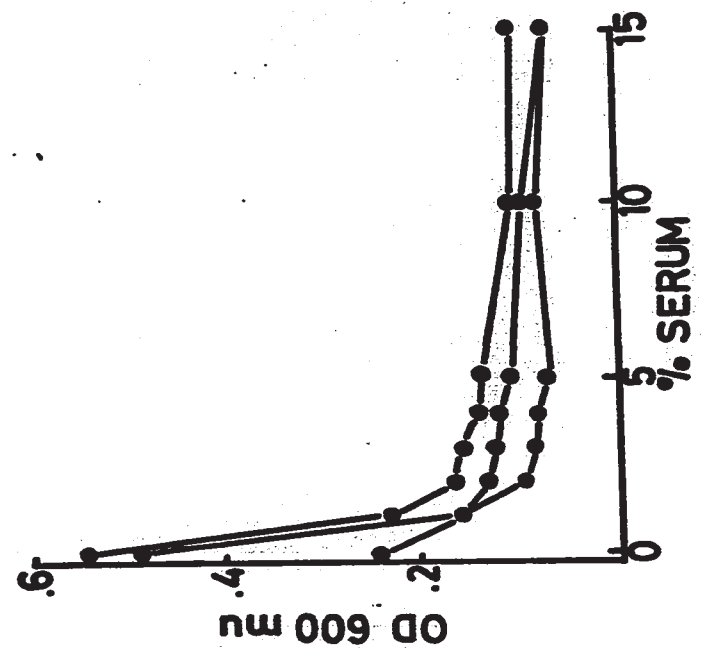


FIGURE 57. Growth of *C. neoformans* in neopeptone solution containing 0.1% glucose and various concentrations of serum. Lines from top to bottom, 8, 6, 4 days, resp.

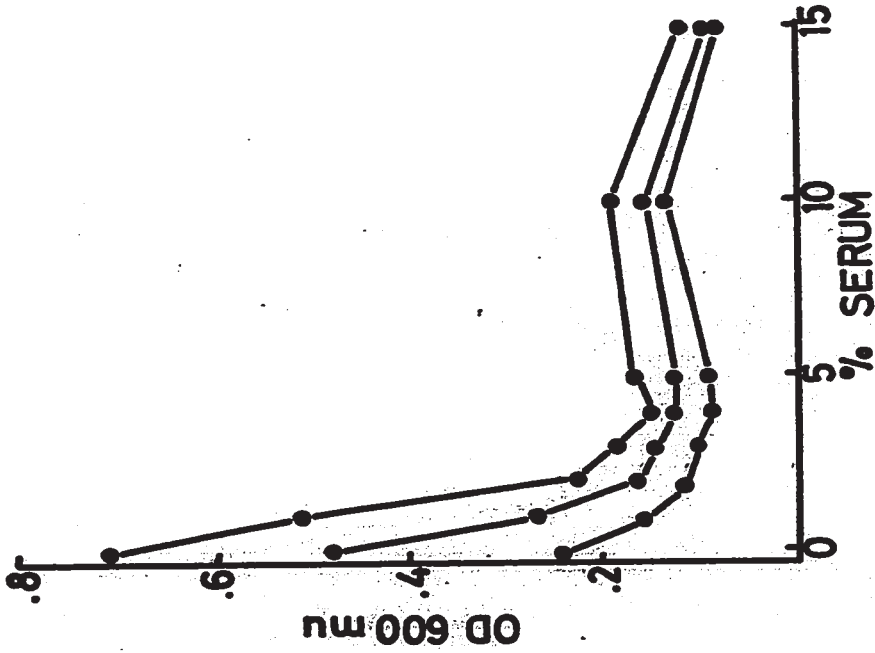


FIGURE 60. Growth of *C. neoformans* in neopeptone solution containing 1.0% glucose and various concentrations of serum; lines from top to bottom, 8, 6, 4 days, resp.

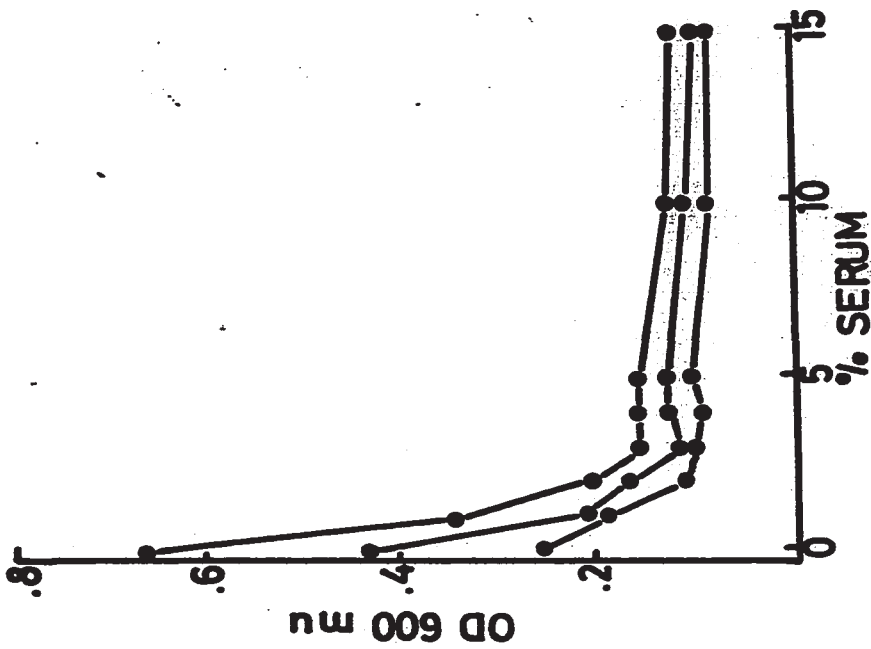


FIGURE 59. Growth of *C. neoformans* in neopeptone solution containing 0.5% glucose and various concentrations of serum; lines from top to bottom, 8, 6, 4 days, resp.

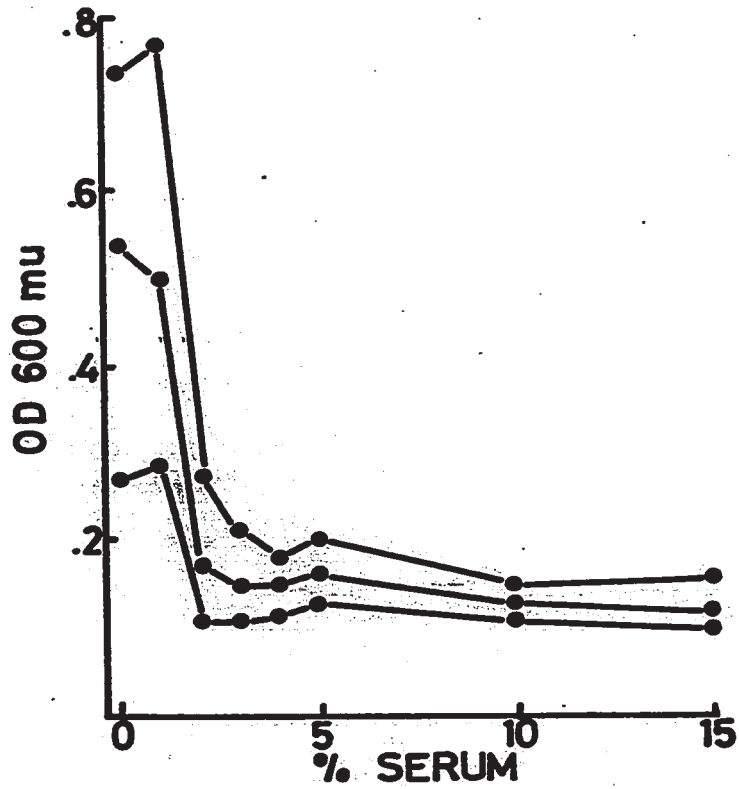


FIGURE 61. Growth of *C. neoformans* in neopeptone solution containing 2.0% glucose and various concentrations of serum; lines from top to bottom, 8, 6, 4 days, resp.

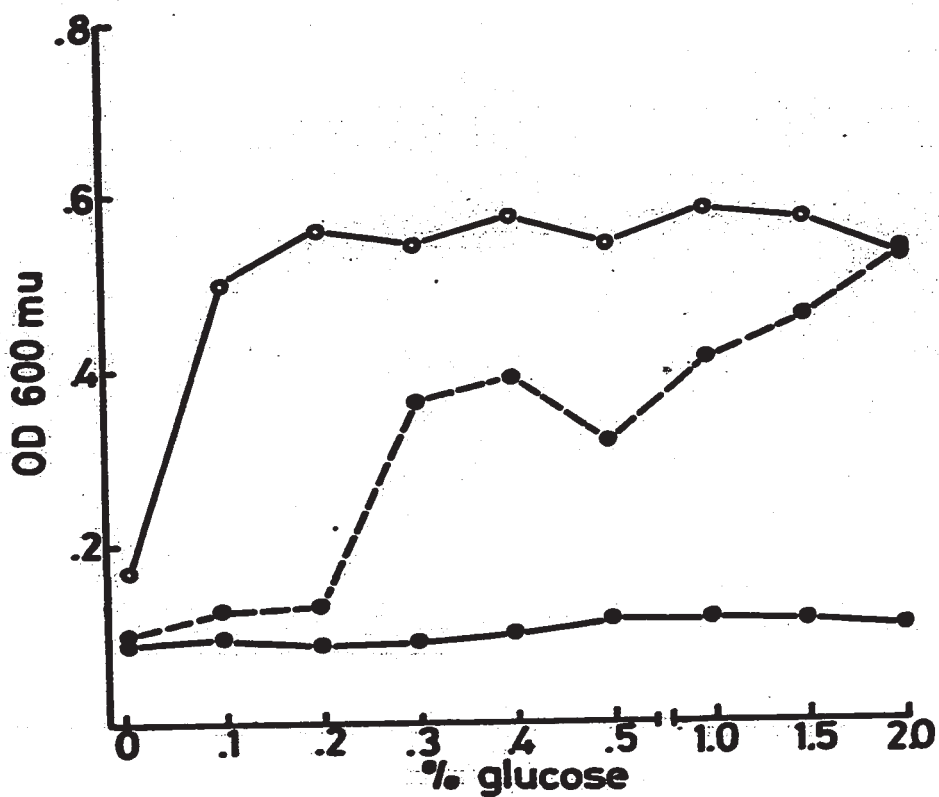


FIGURE 62. Effect of increasing amounts of glucose on the growth of *C. neoformans* in neopeptone solution (○—○), in neopeptone solution plus 10% serum (●—●), in neopeptone solution plus 10% serum and 100 μM FeCl₃ (●---●); pH 7.0; 5.5 days.

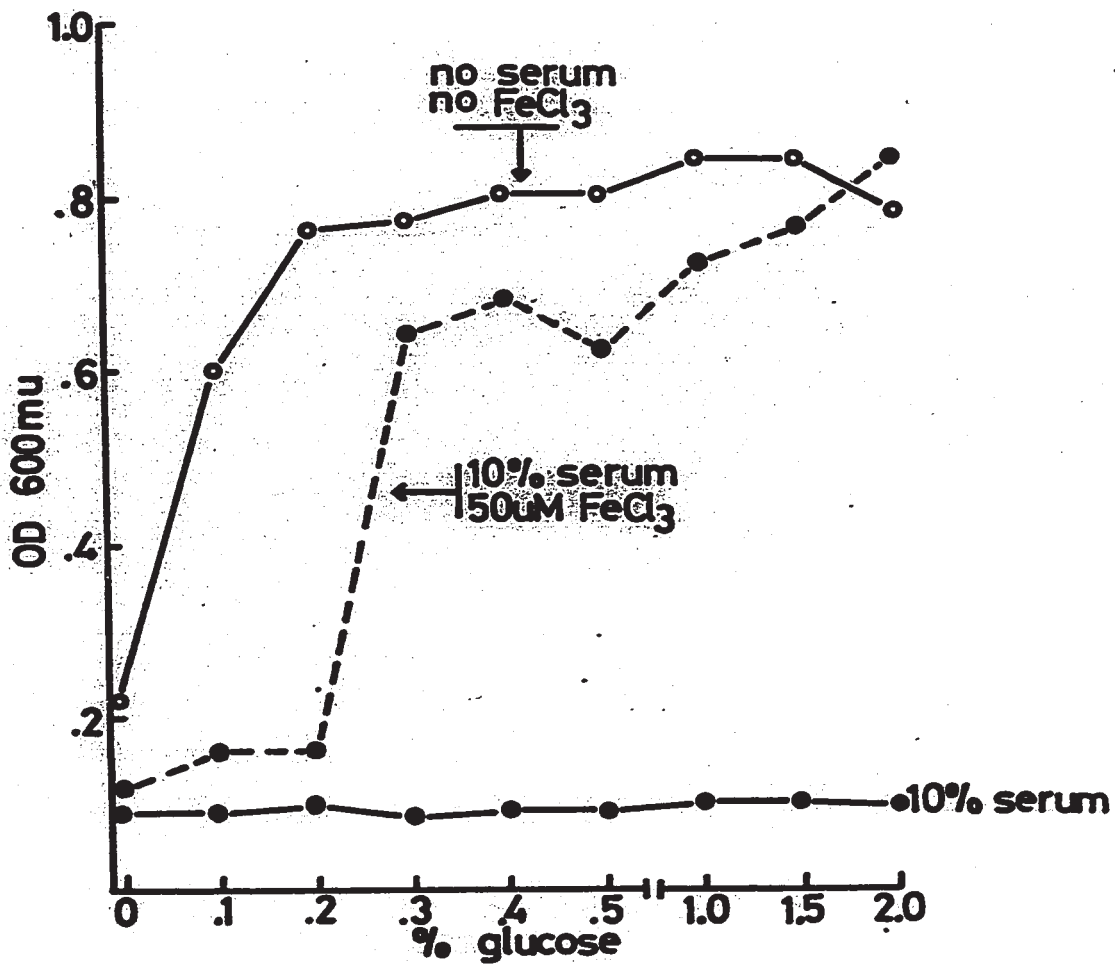


FIGURE 63. Effect of increasing amounts of glucose on the growth of *C. neoformans* in neopeptone solution (○—○), in neopeptone solution plus 10% serum (●—●), in neopeptone solution plus 10% serum and 100µM FeCl₃ (●—●); pH 7.0; 7.5 days.

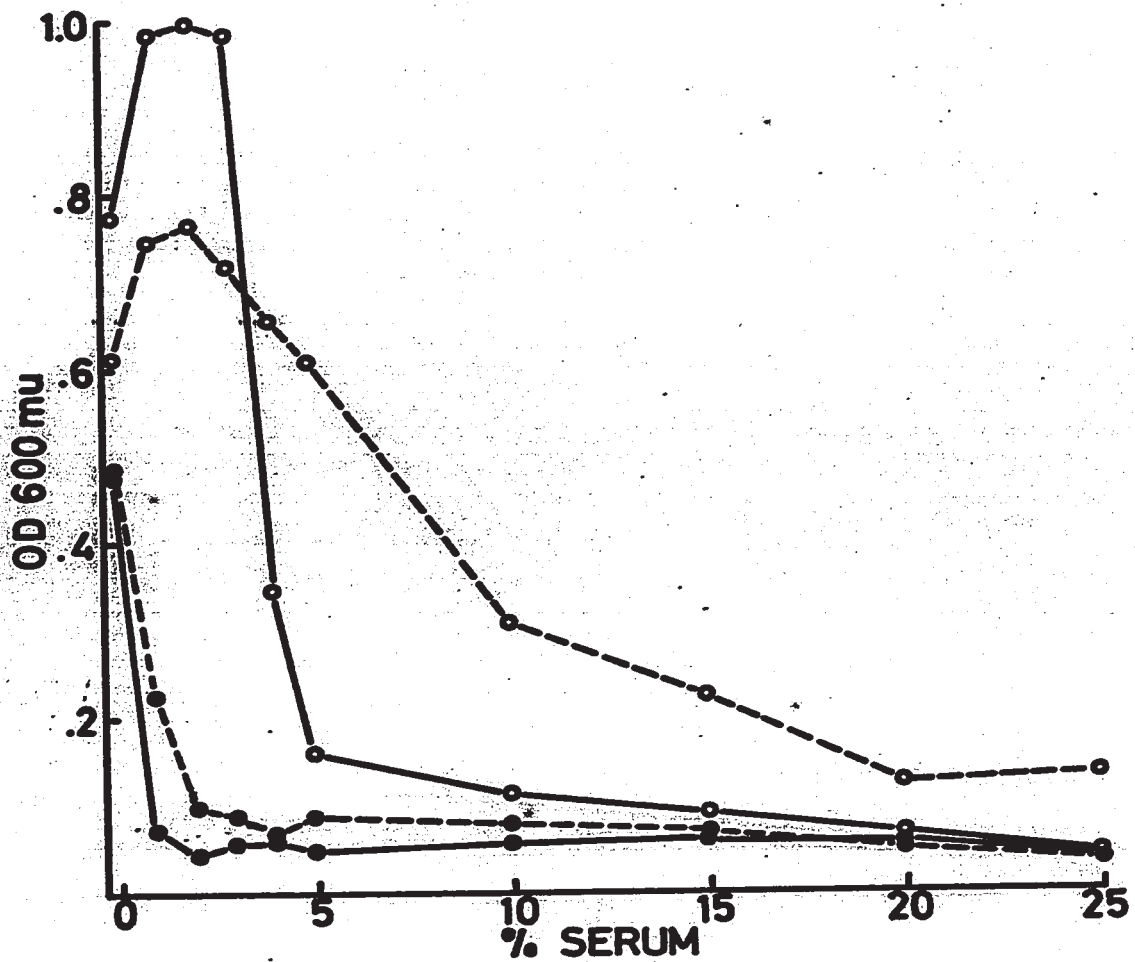


FIGURE 64. Effect of aeration on the growth of *C. albicans* in N-G broth containing various concentrations of serum, no FeCl₃ (●—●), with 25 μM FeCl₃ (○—○). Un-aerated controls (—) with (○) and without (●) FeCl₃. 13 hr, in 6 ml volumes.

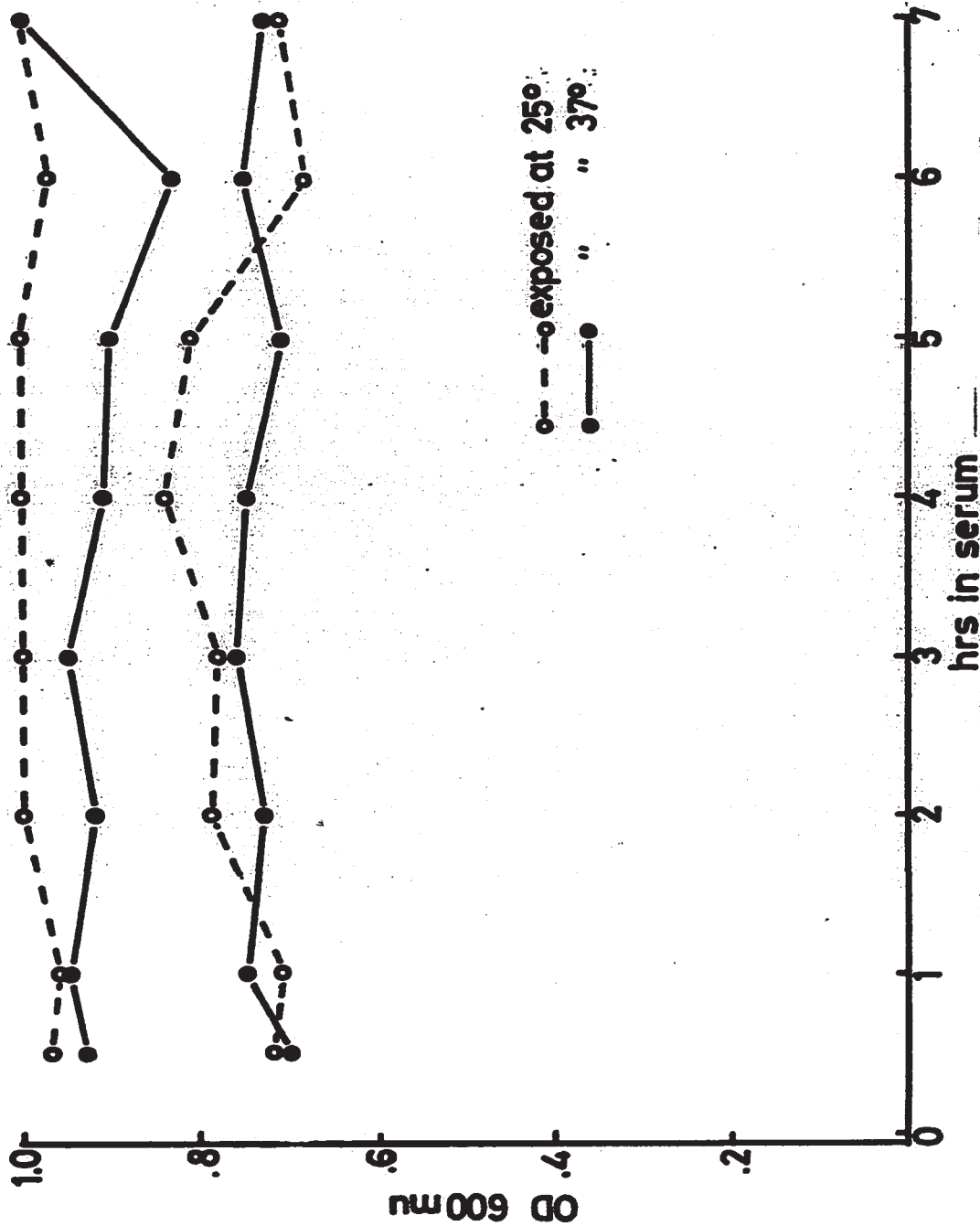


FIGURE 65. Growth of *C. albicans* in N-G broth after exposure of the cells to serum for various periods of time, at 25°C or 37°C. For the top two lines, the broth contained 25µM FeCl₃; 17.5 hr.

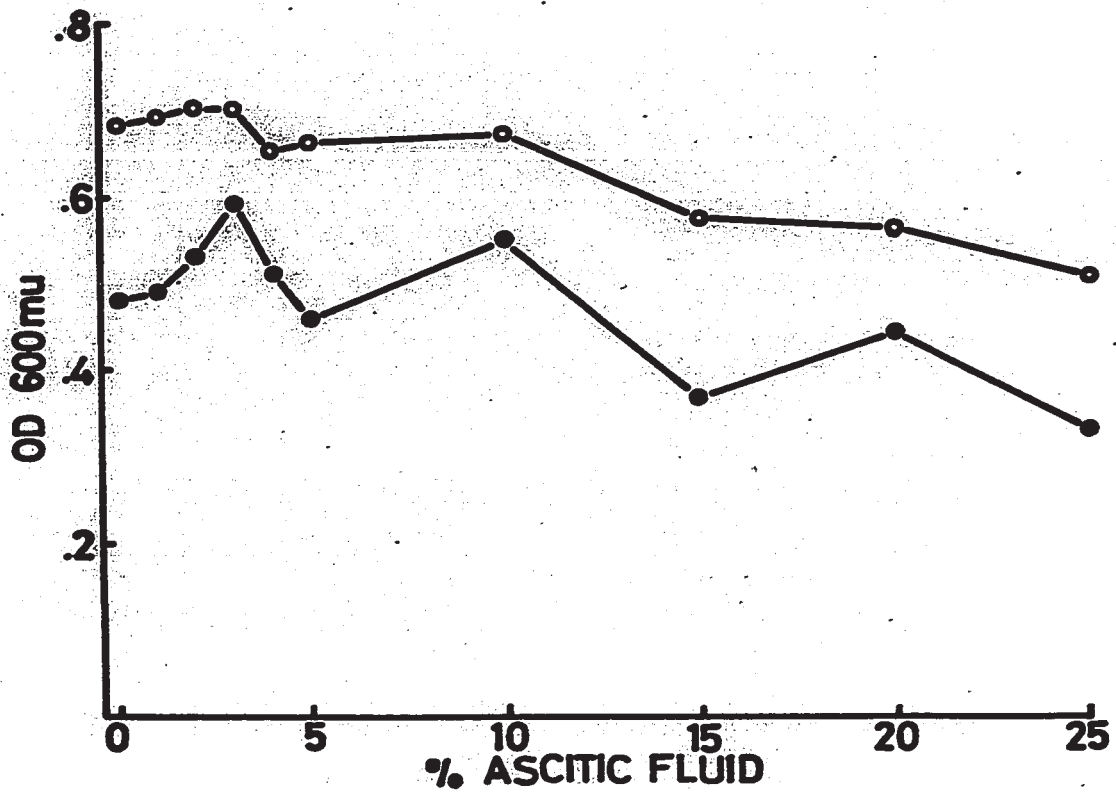


FIGURE 66. Growth of *C. albicans* in N-G broth containing various concentrations of ascitic fluid (●); with 25 μM FeCl₃ (◐); 13 hr.

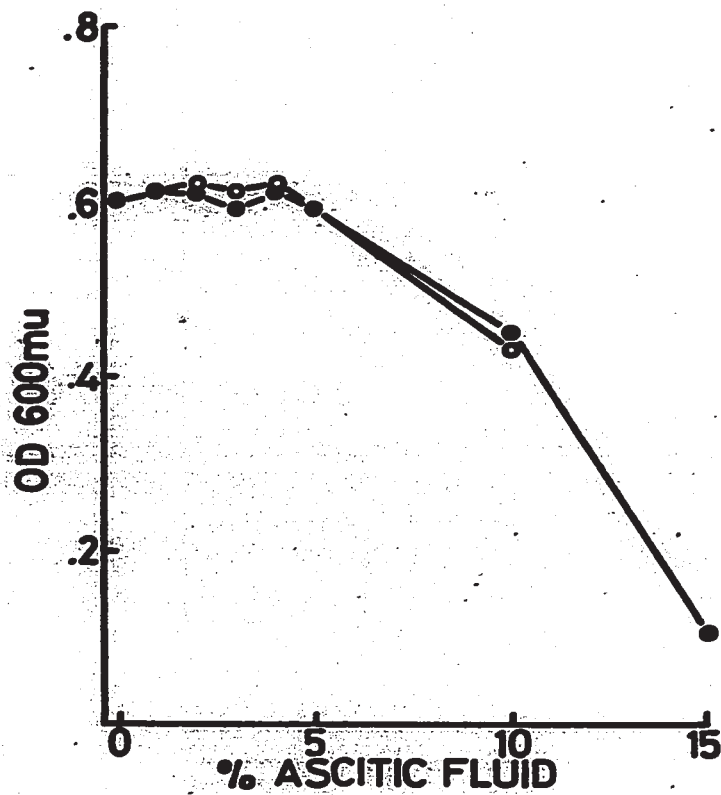


FIGURE 67. Growth of *C. neoformans* in N-G broth containing various concentrations of ascitic fluid (●); with 25 μ M FeCl₃ (○); 4 days.

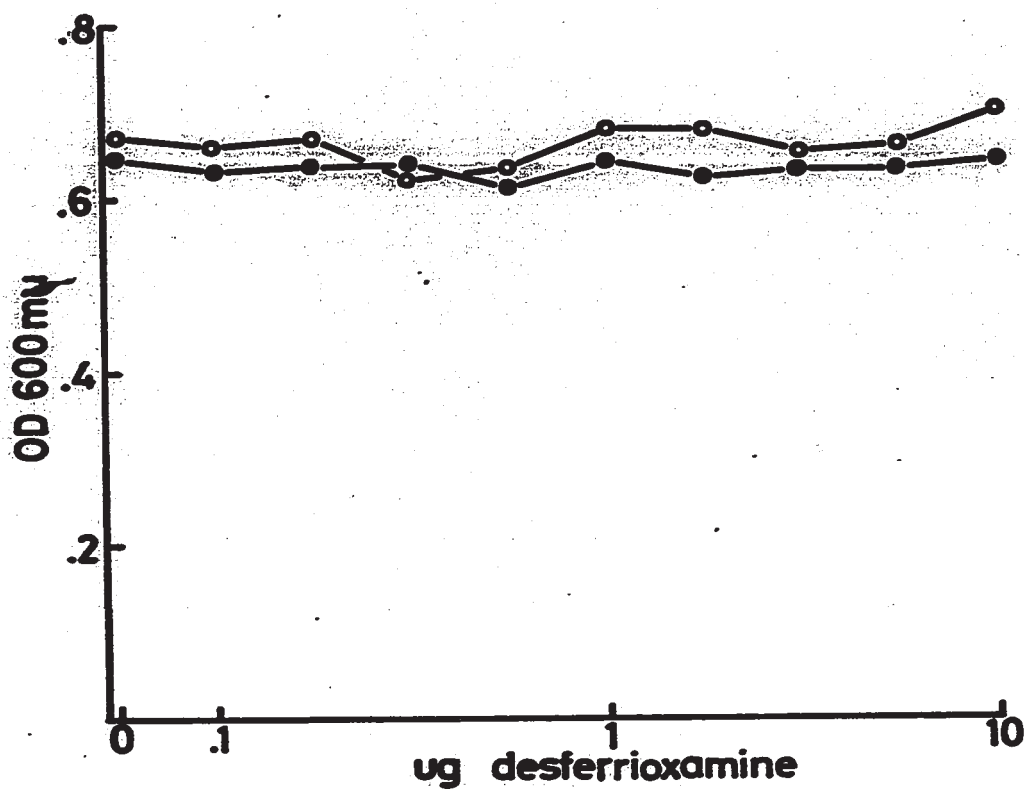


FIGURE 68. Growth of *C. neoformans* in N-G broth containing various concentrations of desferrioxamine (○); with 25µM FeCl₃ (●).

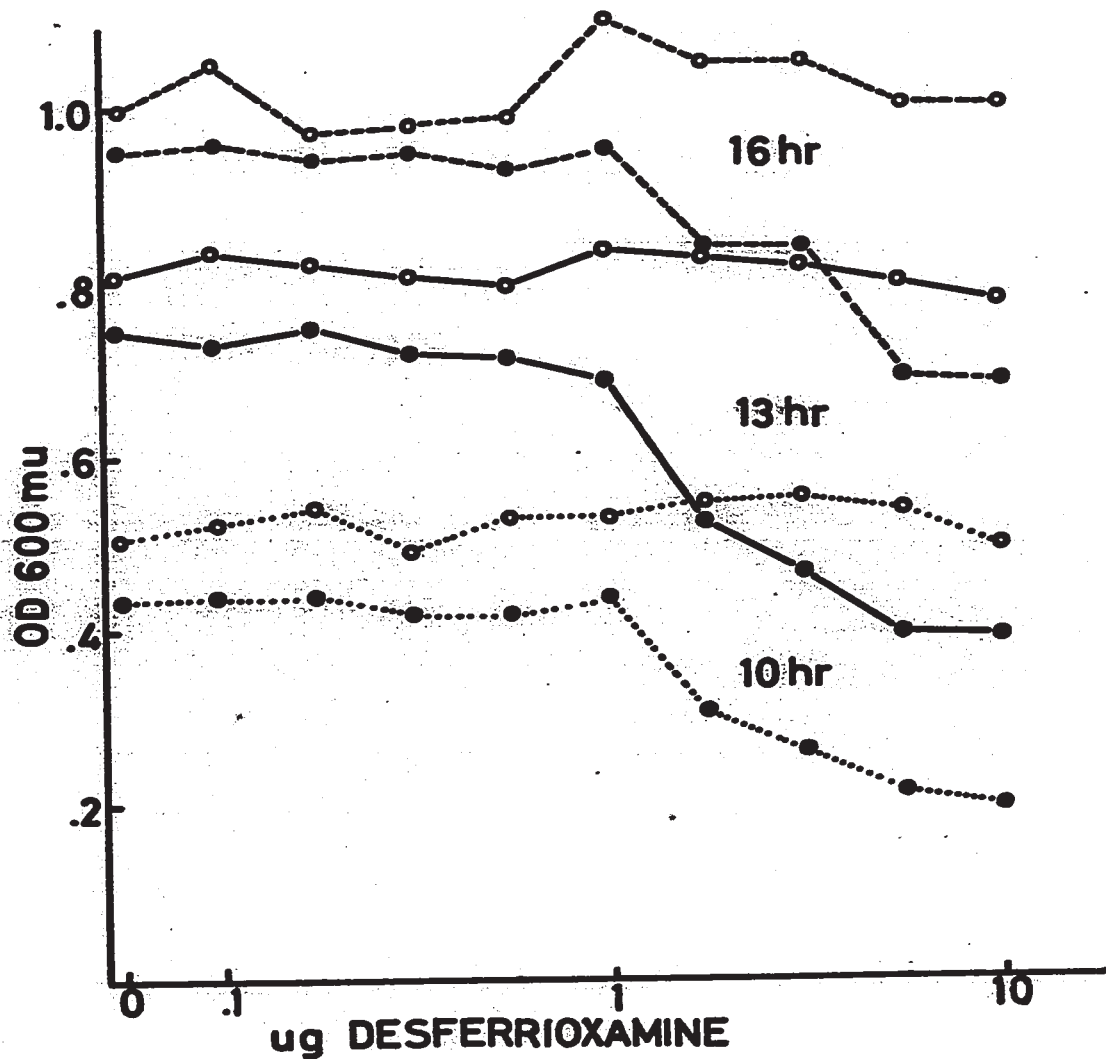


FIGURE 69. Growth of *C. albicans* in N-G broth containing various concentrations of desferrioxamine (●); with 25 μM FeCl₃ (○); 10, 13, 16 hr; still culture.

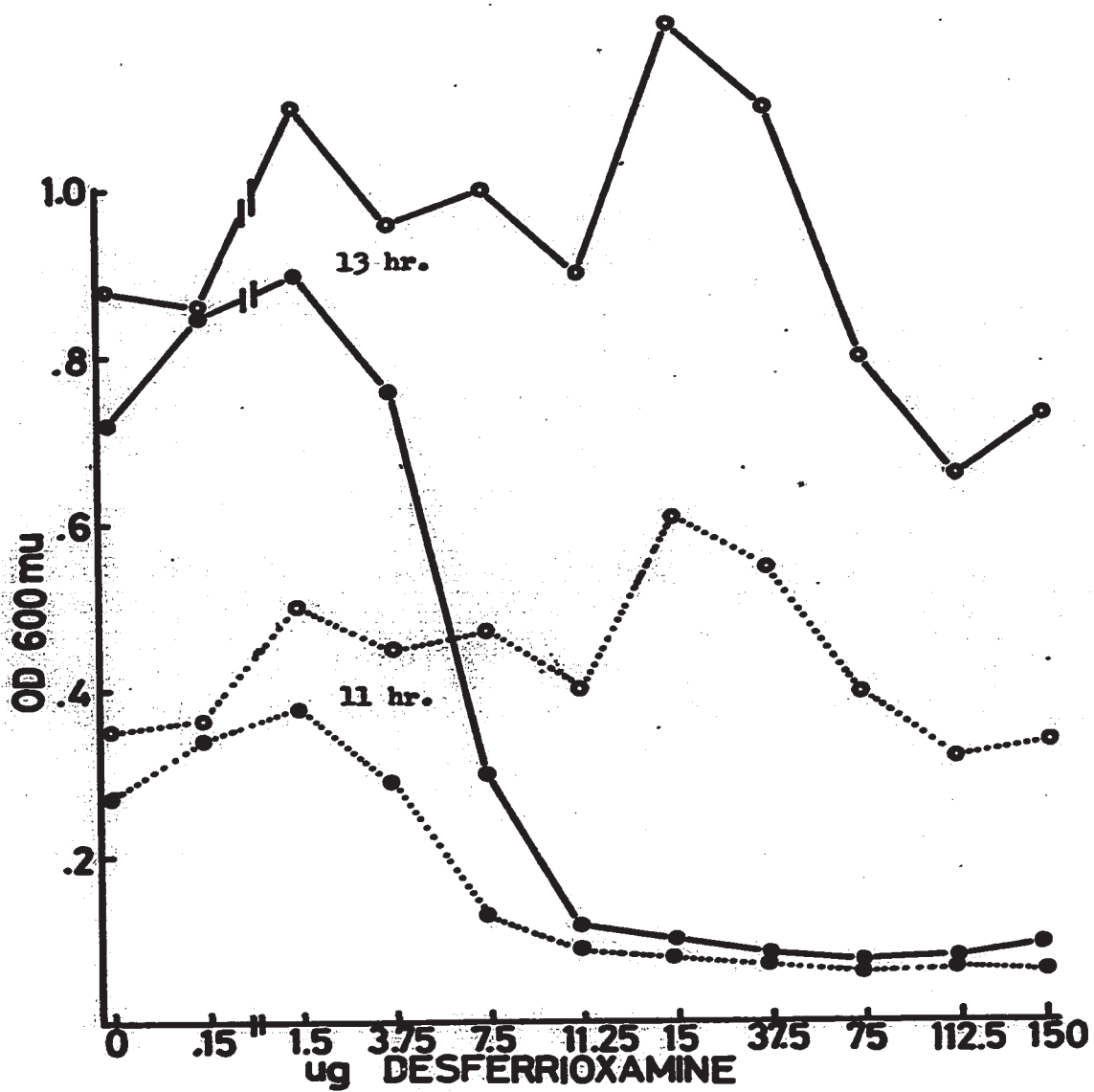


FIGURE 70. Growth of *C. albicans* in N-G broth containing various concentrations of desferrioxamine (●); with 25 μ M FeCl₃ (○); 11, 13 hr.; shaken culture.

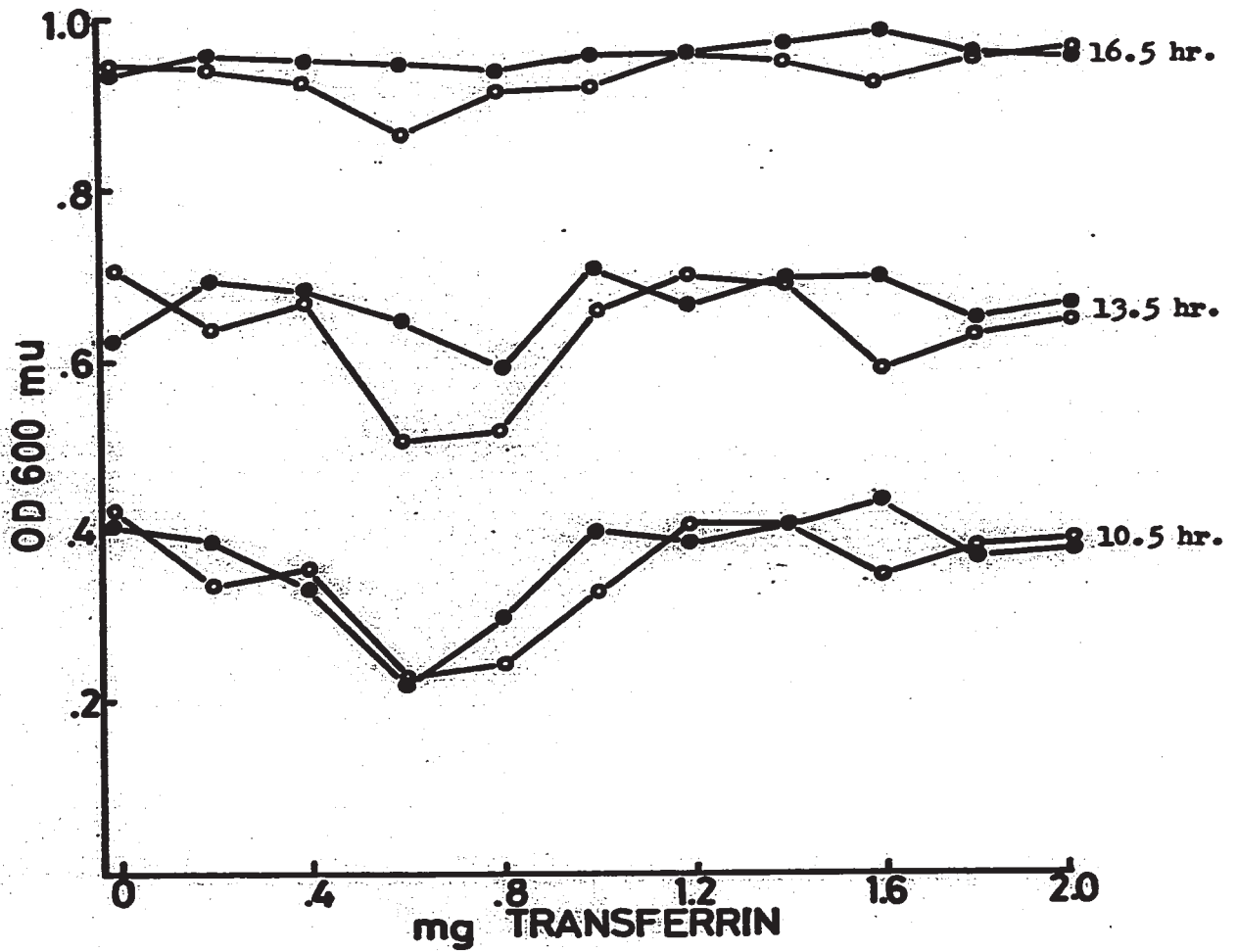


FIGURE 71. Growth of *C. albicans* in N-G broth containing various concentrations of iron-free transferrin (●); with 25 μM FeCl₃ (○); 10.5, 13.5, 16.5 hr.

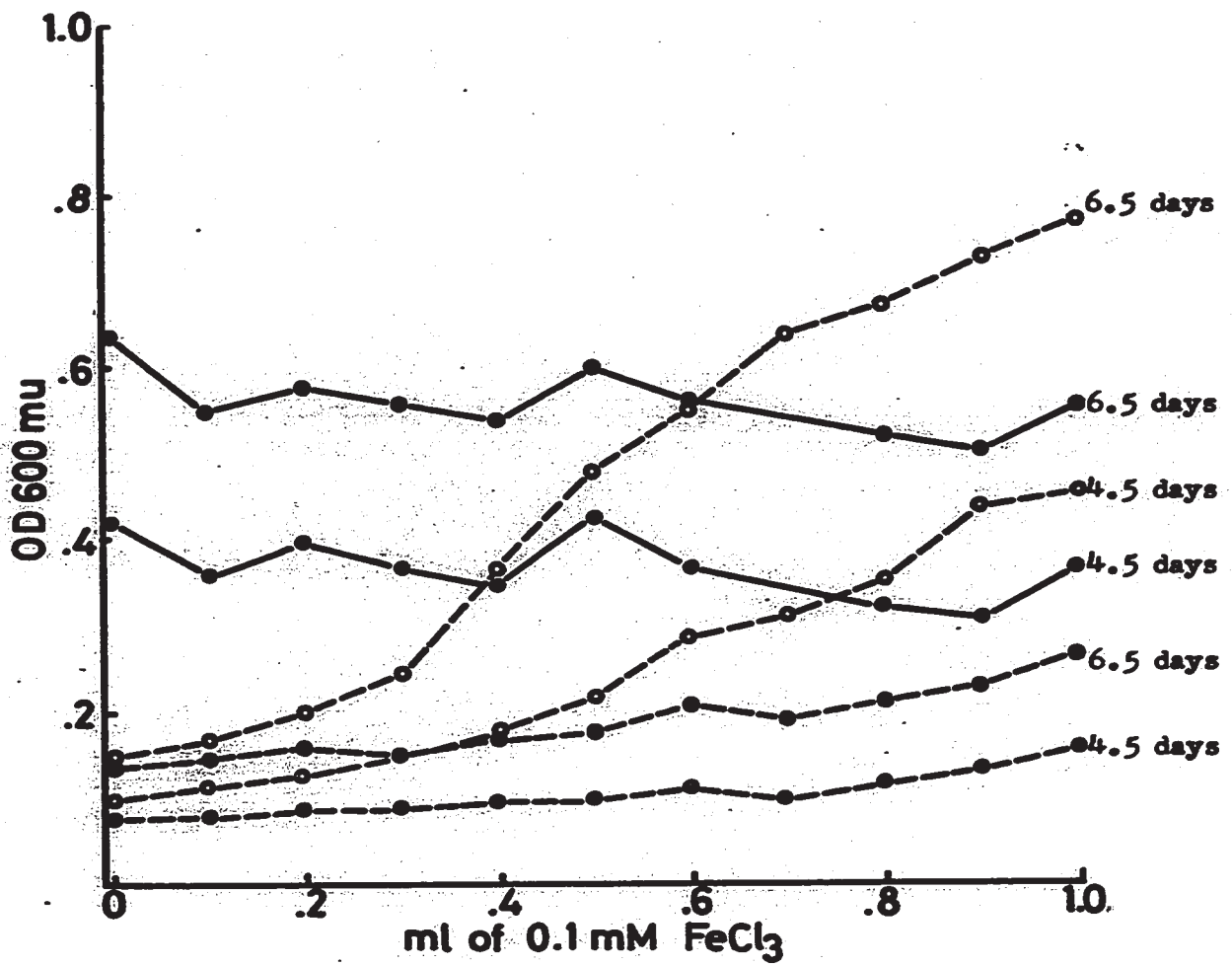


FIGURE 72. Growth response of *C. neoformans* to FeCl_3 in N-G broth (●—●), with 4% serum (○—○), with 10% serum (●—●); 4.5, 6.5 days.

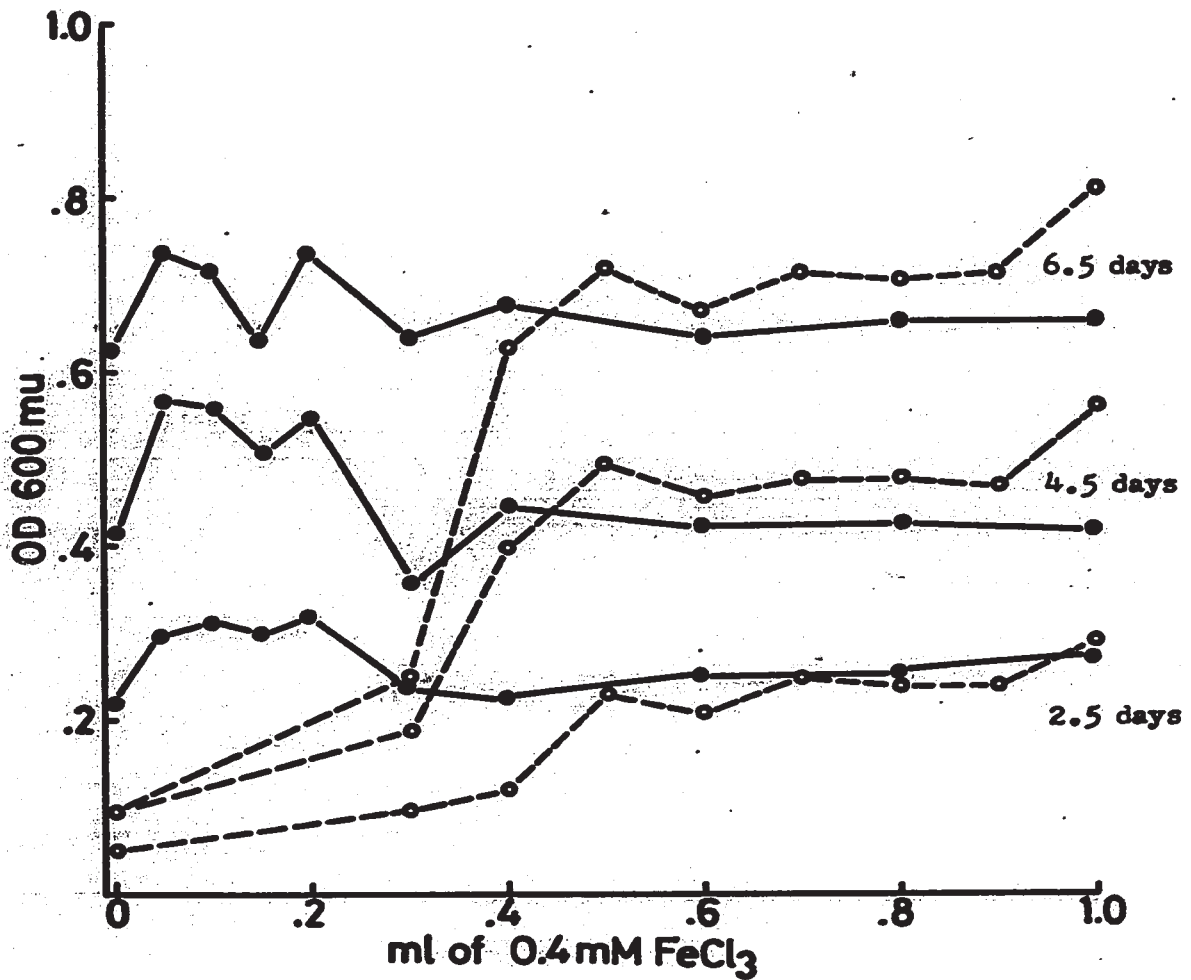


FIGURE 73. Growth response of *C. neoformans* to FeCl₃ in N-G broth (●—●), with 10% serum (○—○); 2.5, 4.5, 6.5 days.

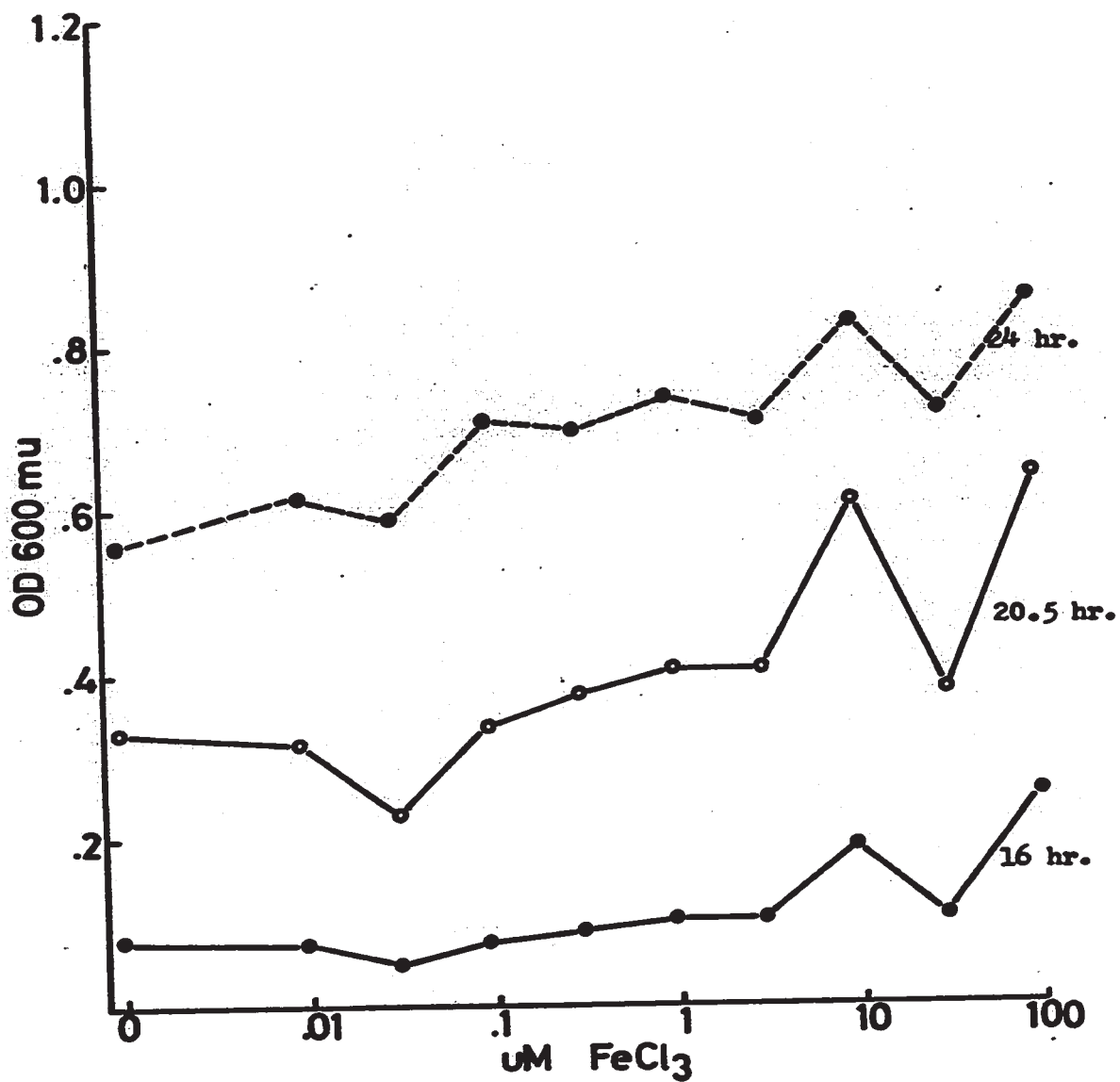


FIGURE 74. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline. One-half standard inoculum.

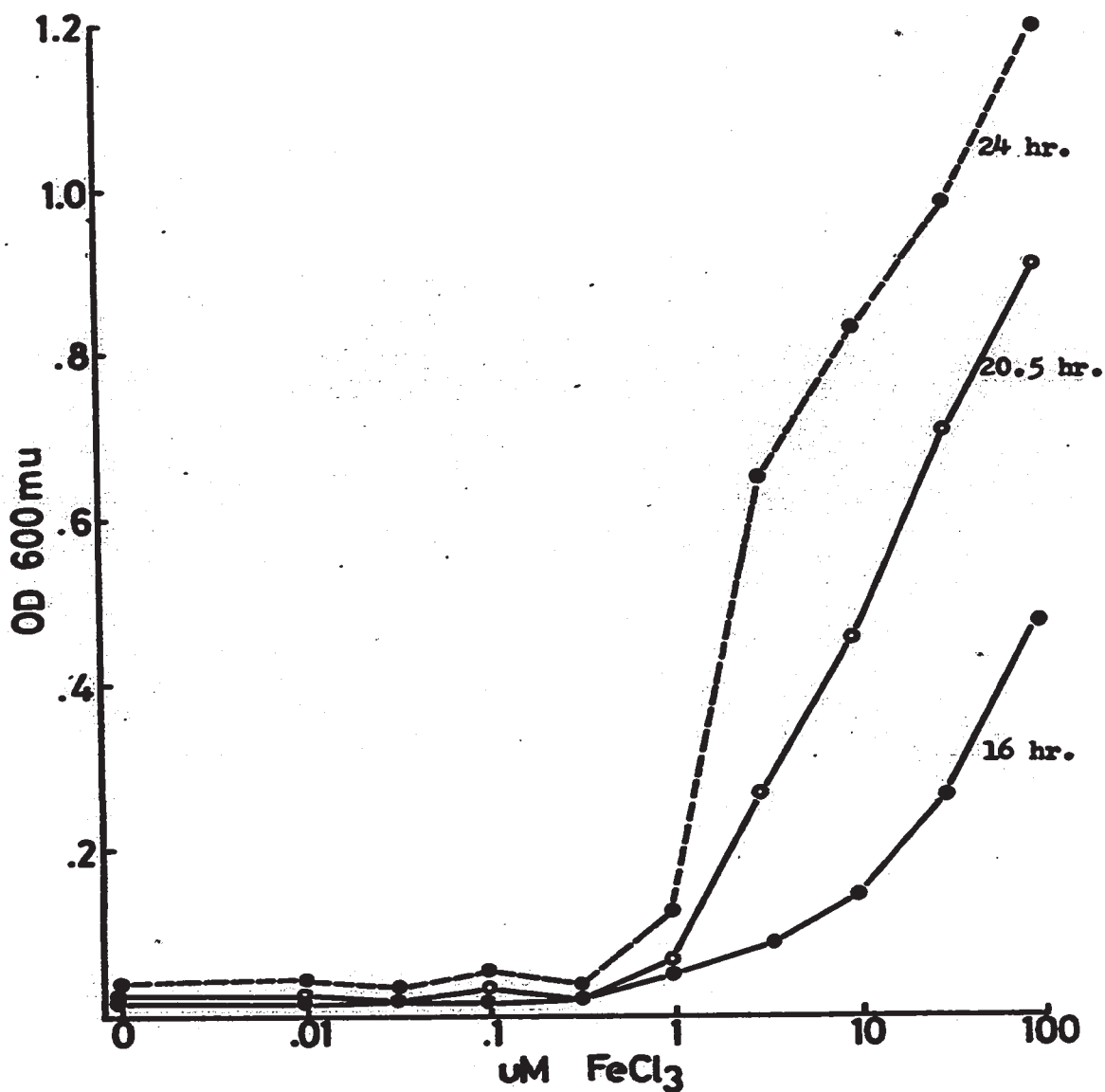


FIGURE 75. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in H-G broth extracted with 8-hydroxyquinoline, and containing 2% serum. One-half standard inoculum.

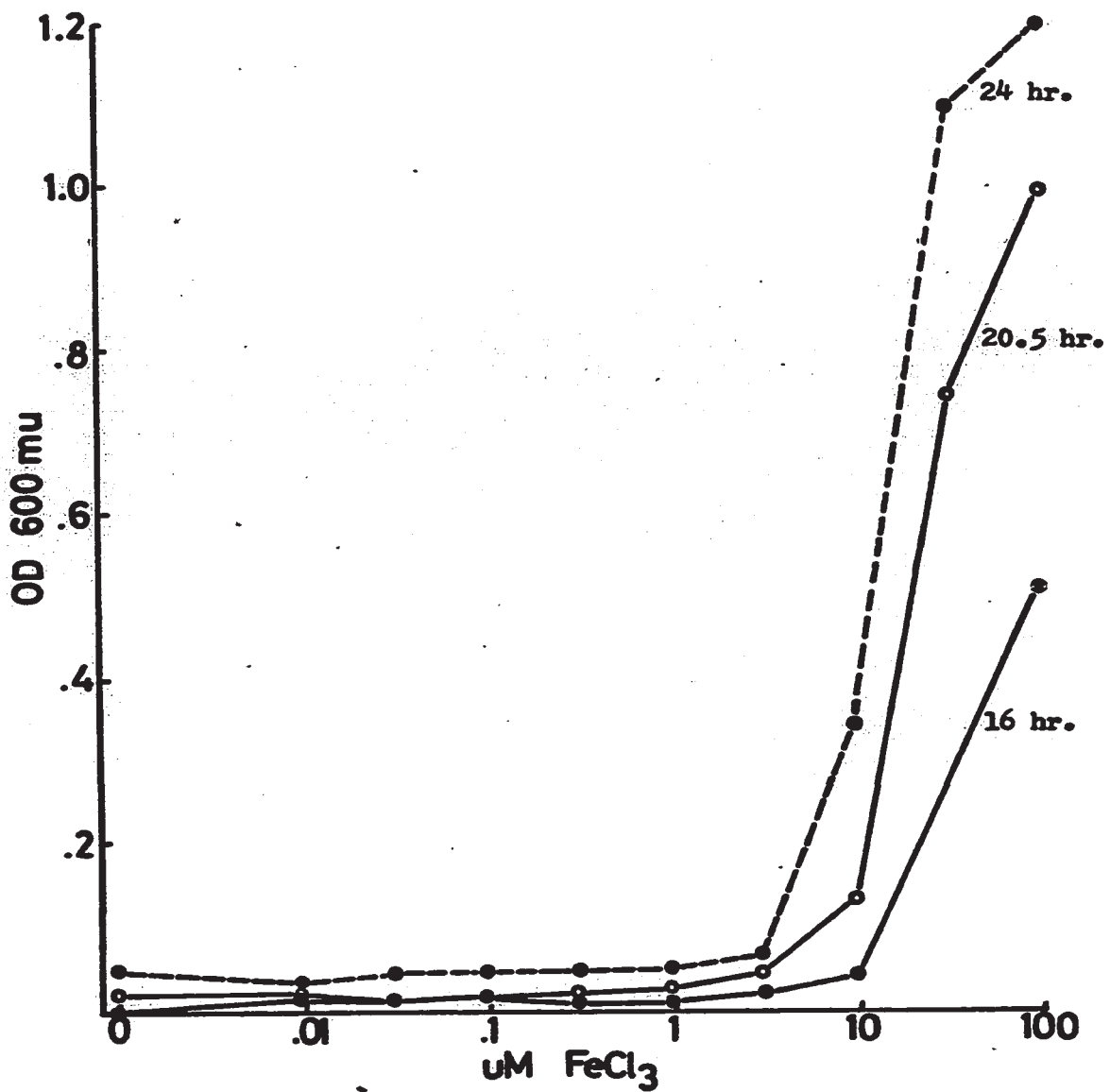


FIGURE 76. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline, and containing 5% serum. One-half standard inoculum.

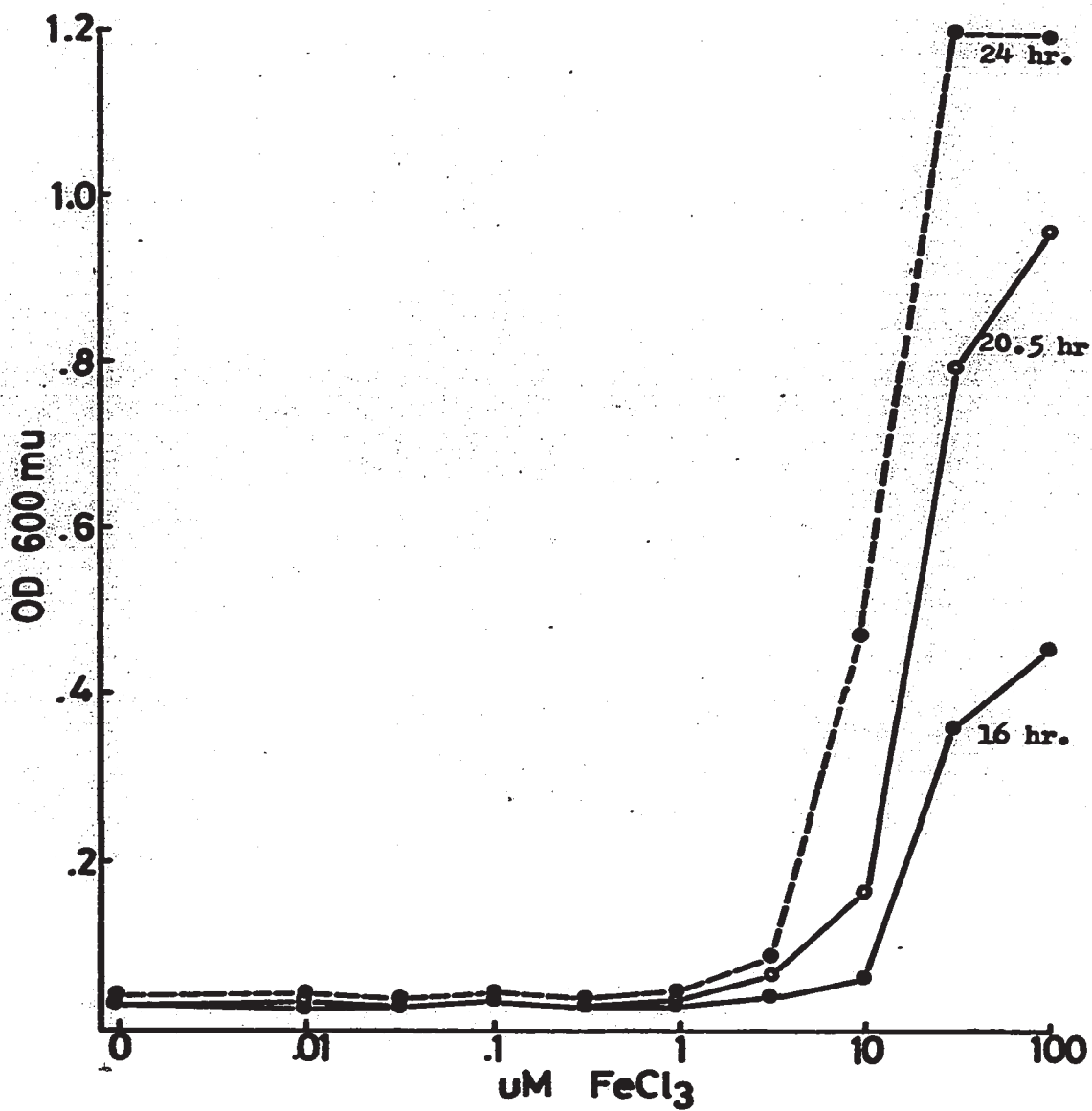


FIGURE 77. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in H-G broth extracted with 8-hydroxyquinoline, and containing 10% serum. One-half standard inoculum.

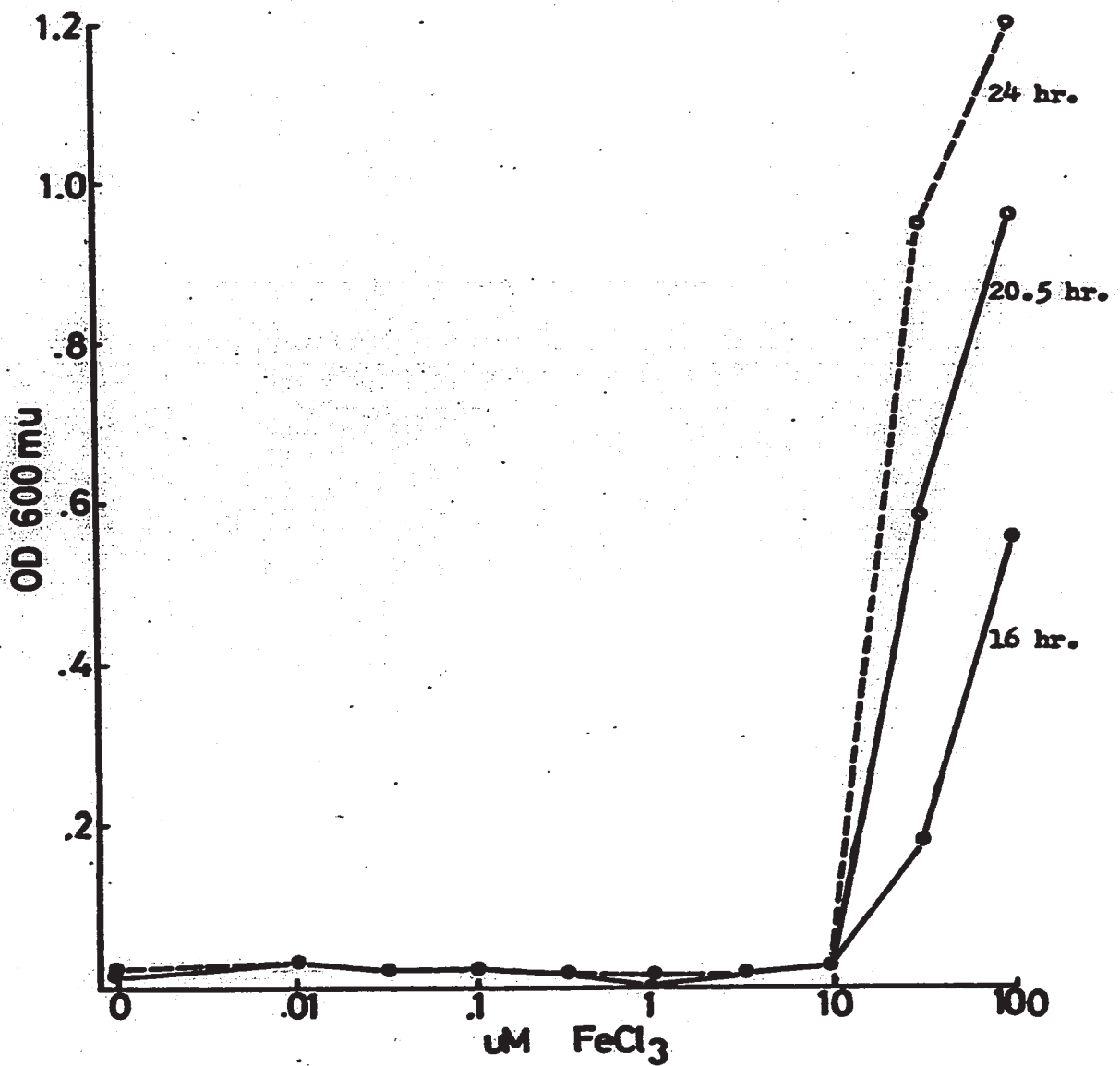


FIGURE 78. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline, and containing 25% serum. One-half standard inoculum.

Candida albicans, NO SERUM

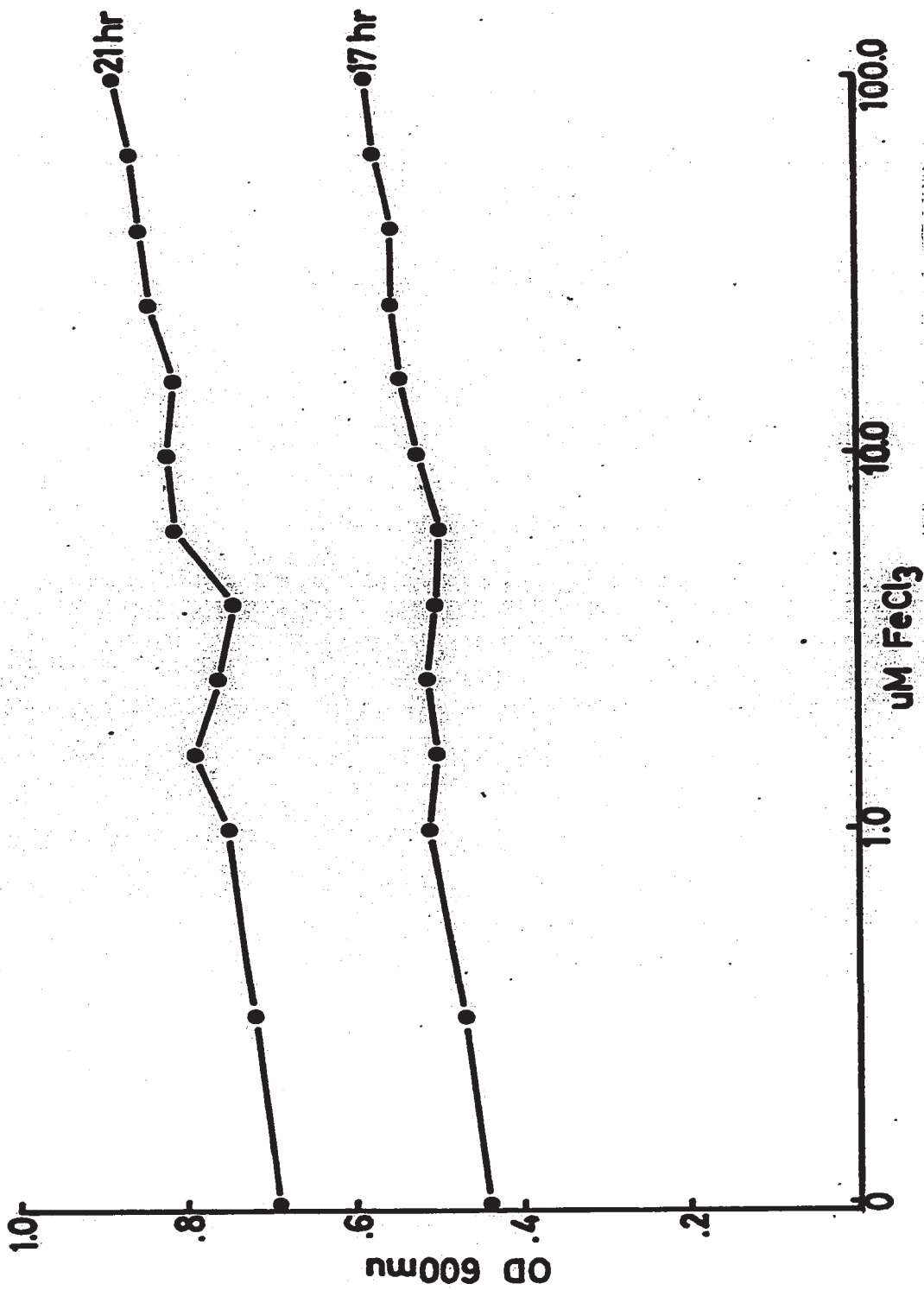


FIGURE 79. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline.

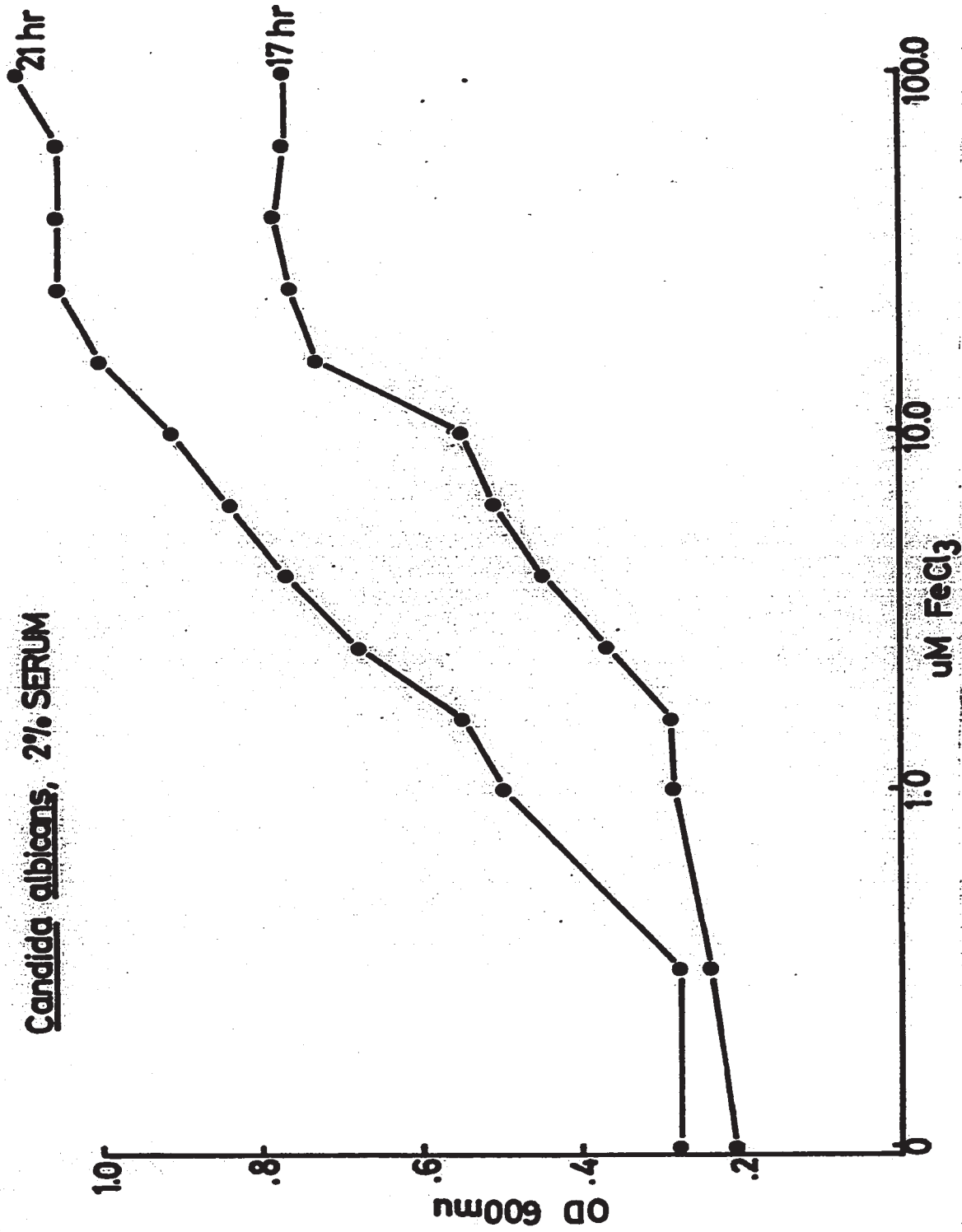


FIGURE 80. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline, and containing 2% serum.

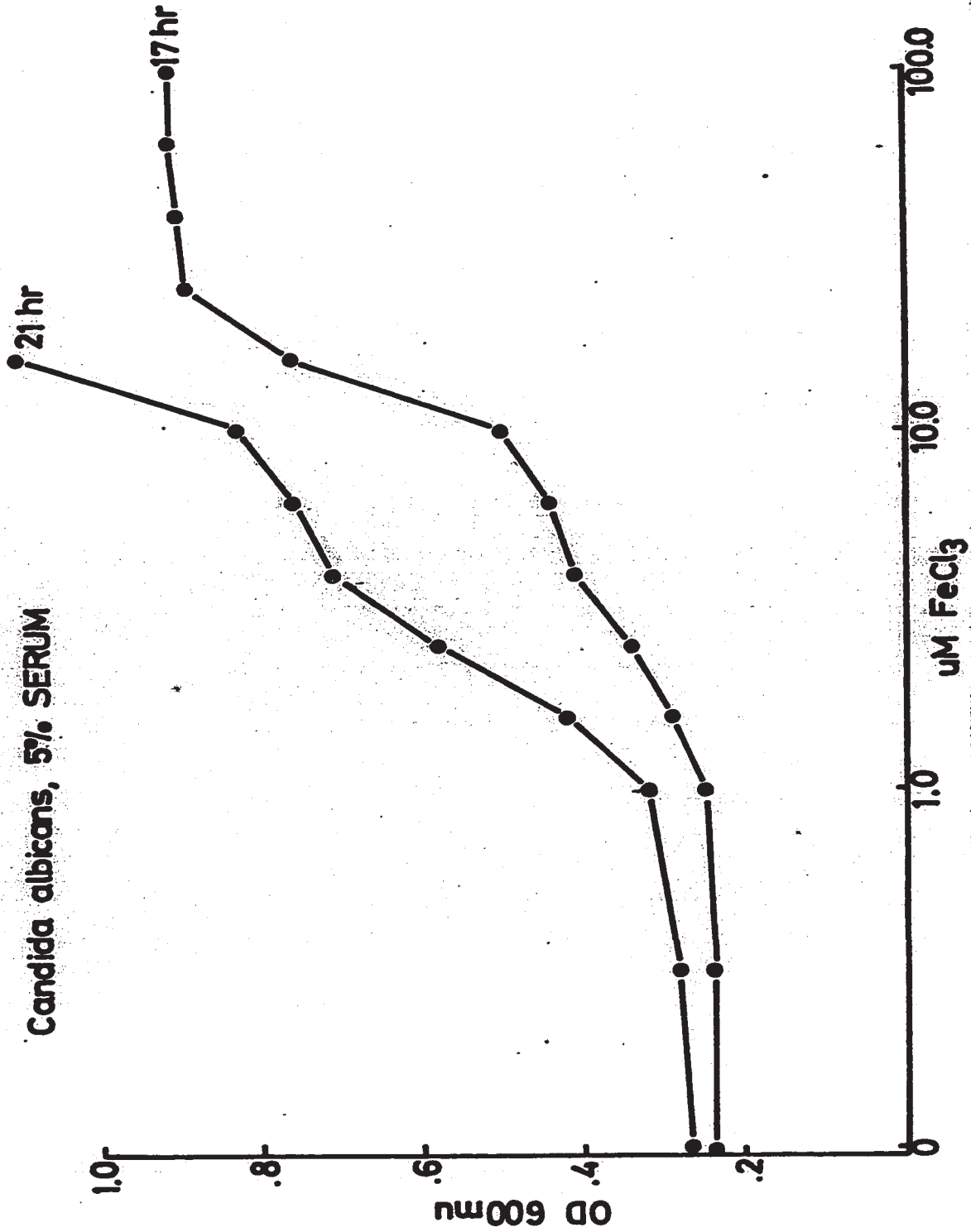


FIGURE 81. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline, and containing 5% serum.

Candida albicans, 10% SERUM

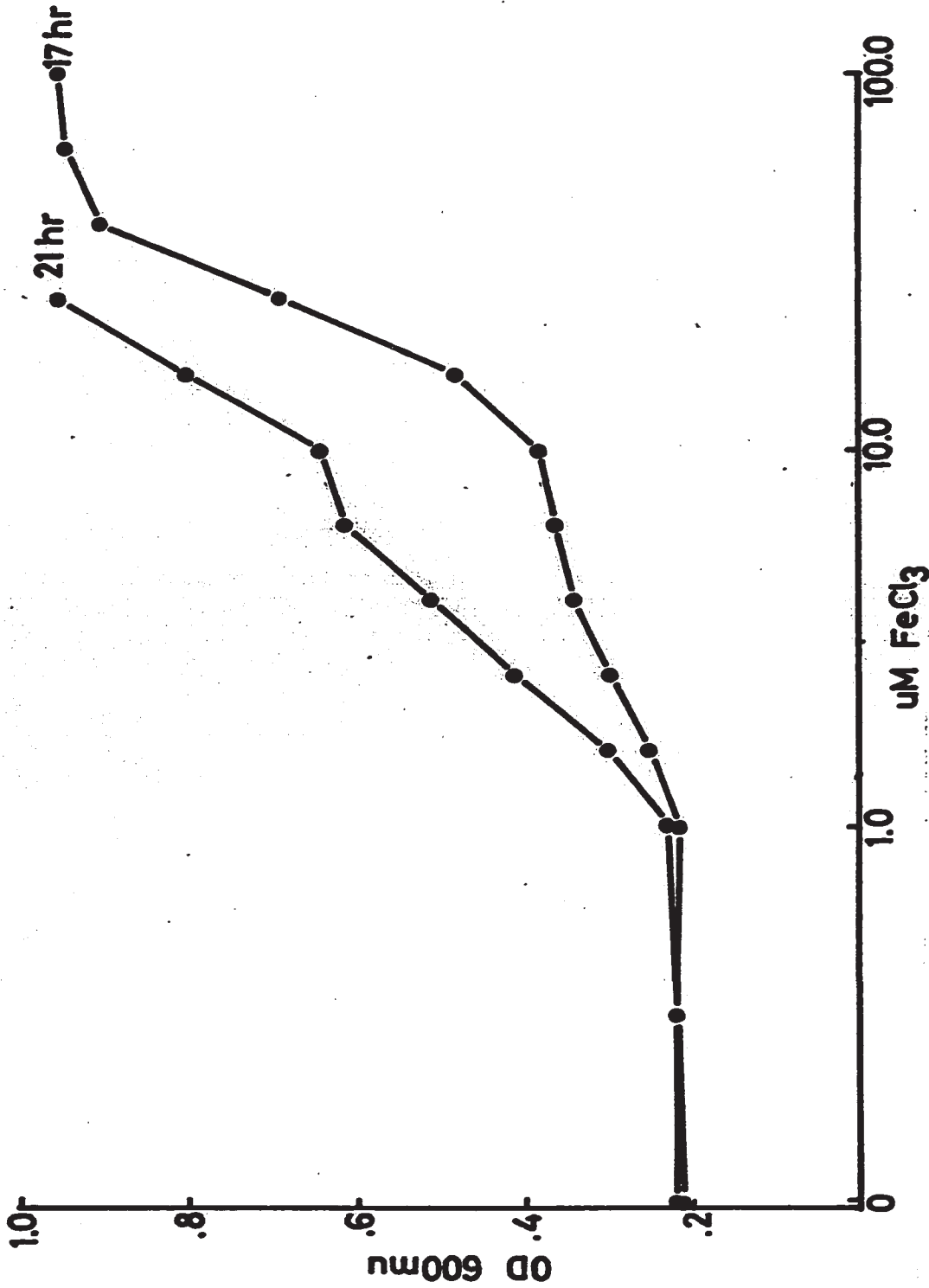


FIGURE 82. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-G broth extracted with 8-hydroxyquinoline, and containing 10% serum.

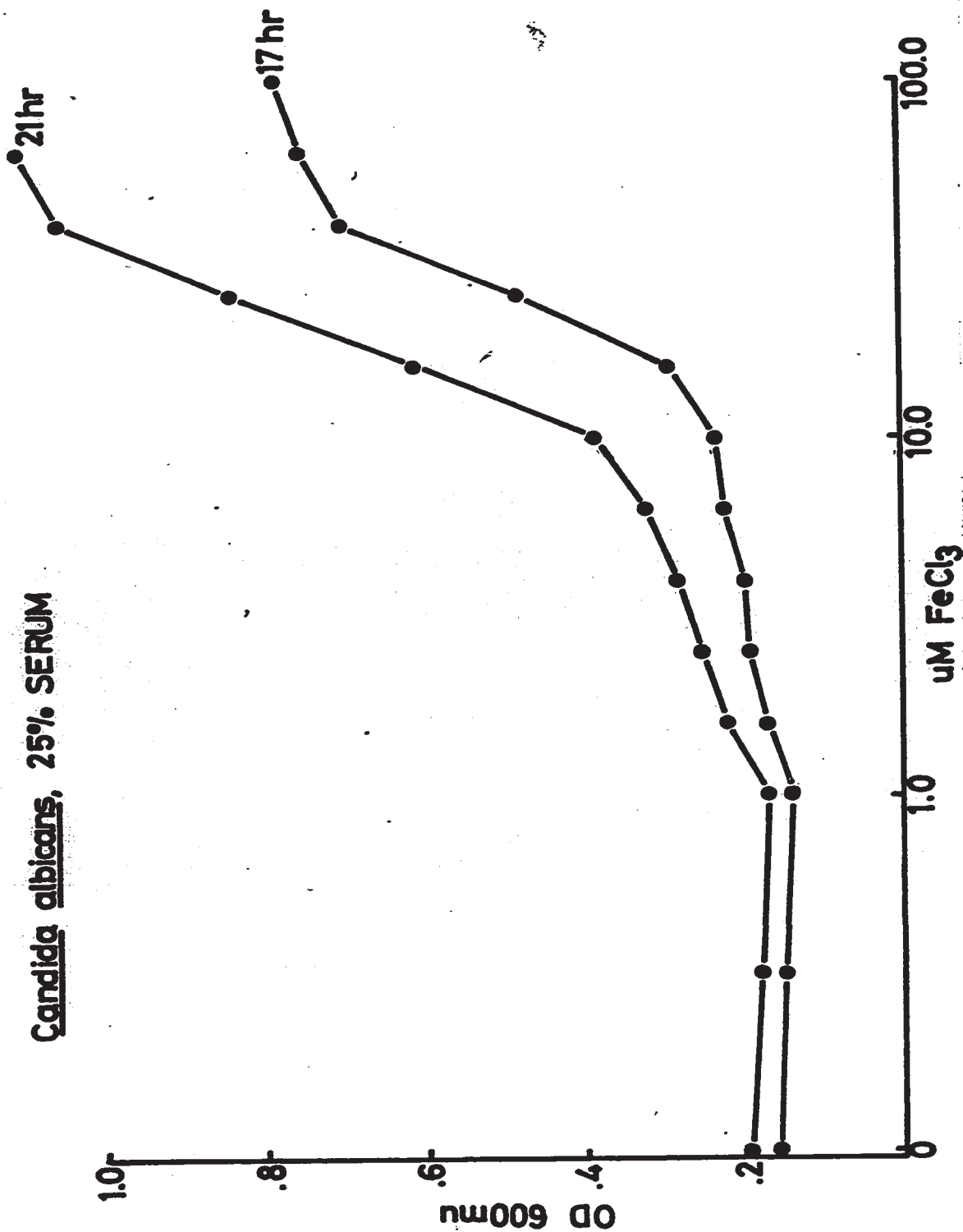
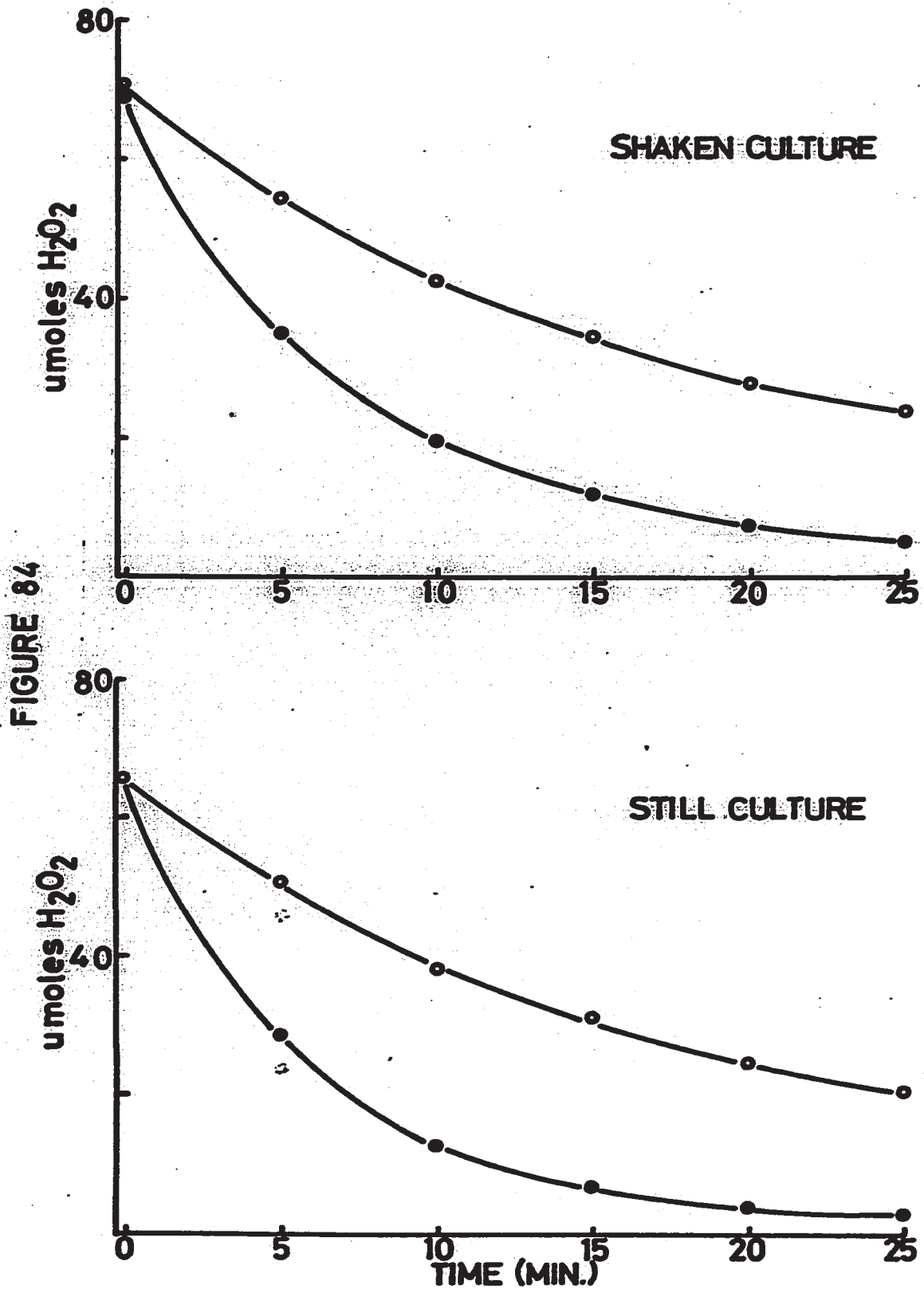


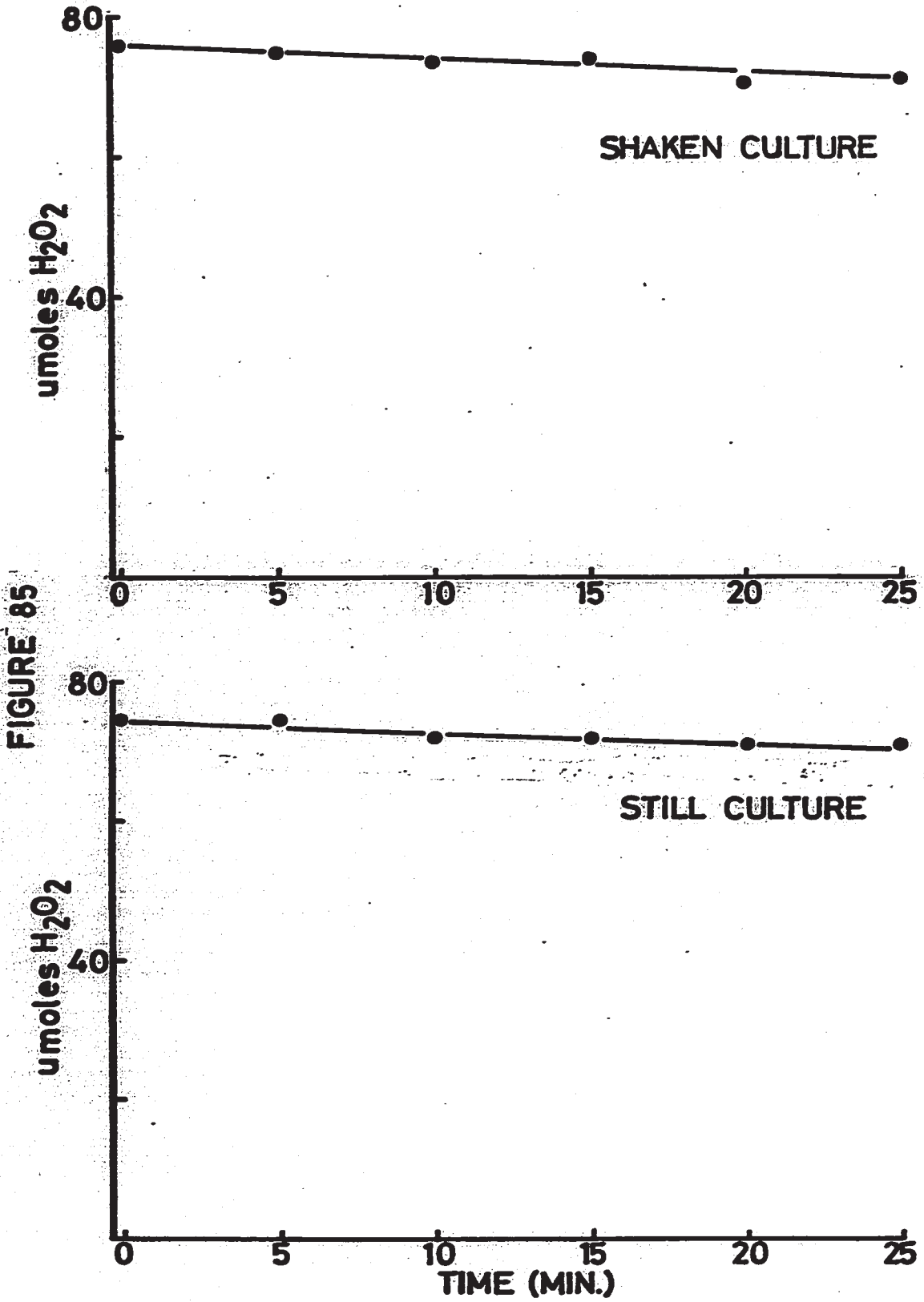
FIGURE 83. Effect of increasing concentrations of FeCl₃ on the growth of *C. albicans* in N-O broth extracted with 8-hydroxyquinoline, and containing 25% serum.

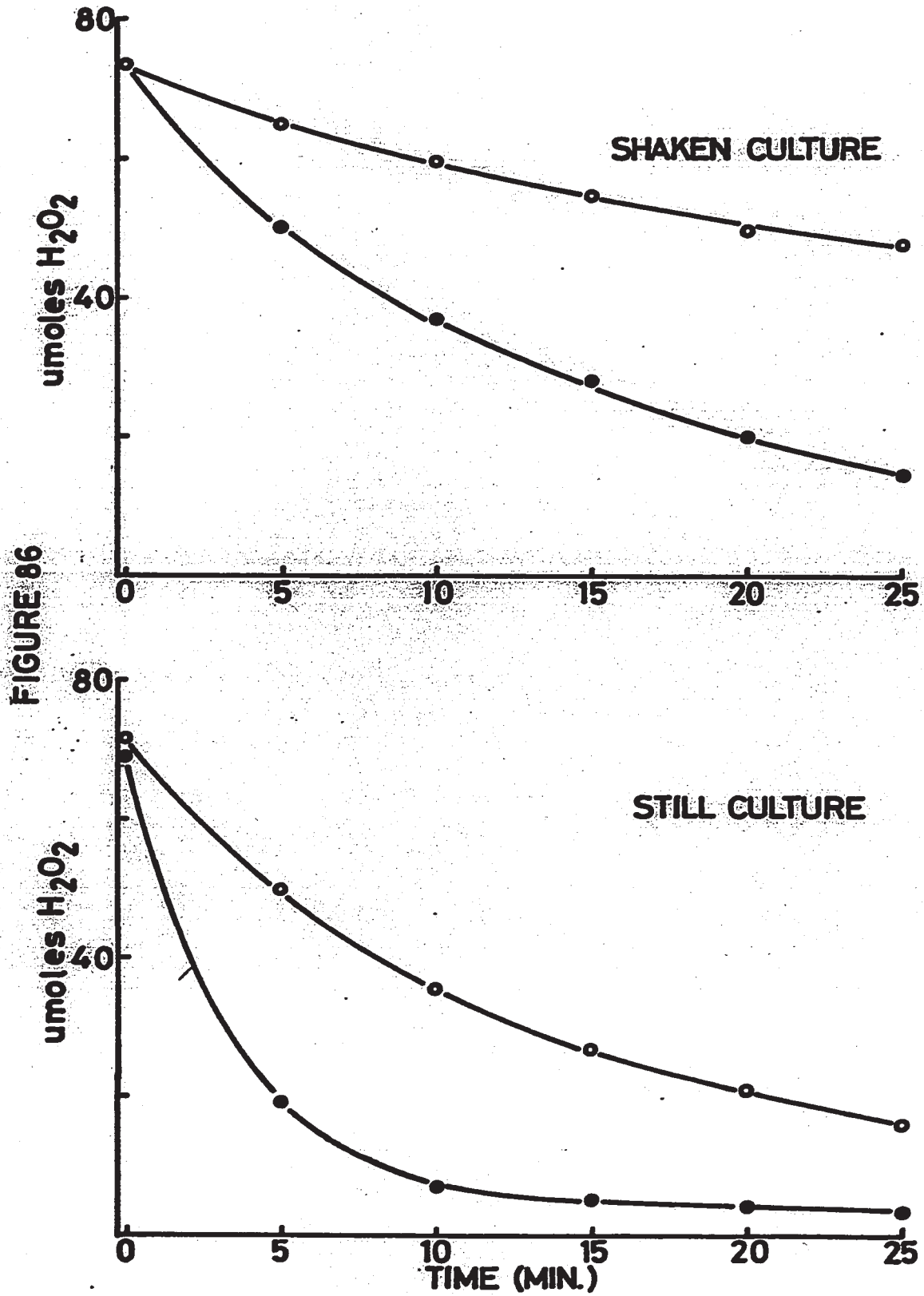
FIGURE 84. Catalase activity of *C. albicans* grown in N-G broth; 1.5 and 4.5 mg acetone-dried cells/sample, upper and lower curves, respectively.

FIGURE 85. Catalase activity of *C. albicans* grown in N-G broth containing 25% serum; 4.5 mg acetone-dried cells/sample.

FIGURE 86. Catalase activity of *C. albicans* grown in N-G broth containing 25% serum and 20 μ M FeCl₃; 1.5 and 4.5 mg acetone-dried cells/sample, upper and lower curves, respectively.







MORPHOLOGICAL STUDIES OF *C. ALBICANS*Introduction

The production and morphology of chlamyospores and germ-tubes were studied in order to understand better the process of infection by *C. albicans*. Chlamyospore formation is a phenomenon occurring best at 24-26 C (Bakerspigel, 1962), while germ-tube formation is more commonly studied at 37 C. Therefore, chlamyospore formation is not likely to function in infection. However, chlamyospores may keep the organism viable in unfavorable environments, and may act as infectious units, although they are not presently known to do so.

Chlamyospores were tested to determine whether they function as propagating and infectious units. For this purpose, purified preparations of chlamyospores were required, and attempts were made to obtain preparations free of blastospores and mycelium. Such preparations could then be used for metabolic, genetic, chemical, serological and structural studies, as well as for electron microscopy. Obviously, a genuine understanding of *C. albicans* cannot be achieved until the true nature of the chlamyospore itself is revealed.

C. albicans readily forms germ-tubes at 37 C in serum and in many other substances, but the exact reason for this response is unknown at present. Germ-tube formation apparently constitutes the initiation of mycelium (Gresham *et al.*, 1961; Mackenzie, 1964).

Materials and Methods

C. albicans (7396) produced abundant chlamydo-spores on sodium taurocholate agar medium, as well as in a liquid medium consisting of Yeast Nitrogen base with 20% human serum and 1% glucose. This medium was buffered at pH 6.0 with 0.05M MES buffer (Calbiochem, Calif.). In this medium, the ability of the organism to grow well was directly related to its ability to produce numerous chlamydospores. A pH value above 7.0, or the absence of glucose, resulted in poor growth.

A partially purified preparation of chlamydospores was obtained in the liquid medium. Optimum cultural conditions were obtained in 10 ml volumes of medium in 125 ml flasks at 25 C. After a week of growth, the cultures were transferred to large tubes, diluted with saline or water, shaken on a vortex mixer and allowed to settle for 25 min. The supernatant which was removed and which contained mostly blastospores, was discarded. Masses of chlamydospores were associated with pseudomycelium which settled to the bottom of the tubes before the blastospores. The procedure of dilution with water or saline and the settling was repeated

several times, until the supernatant became relatively free of blastospores. No method was found which would free the chlamydospores from the mycelium without damaging them. Apparently they were more firmly fixed to the mycelium than were the blastospores. However, the mycelium as a contaminant, was relatively unimportant. On standing, mycelium became 'empty' within two weeks, whereas chlamydospores retained their characteristic appearance for periods up to six months. These chlamydospores were relatively large, spherical, thickwalled, refractile, and filled with cytoplasm and globules of lipoid material.

The possibility that chlamydospores could germinate was also tested at temperatures between 37 and 43 C in N-G broth, in 1% yeast extract, in Yeast Nitrogen base, and in these media with and without 1% glucose. The possibility that chlamydospores could function as infectious units was tested by injecting them into 'granuloma pouches' on the backs of the necks of white mice (Mackenzie, 1964). These mice were injected with 0.2 ml of a heavy suspension of chlamydospores alone; with 0.2 ml of 1% cysteine (to establish reduced conditions); 1.0 ml of 0.004M FeCl₃; 0.1 ml croton oil (a chemical irritant causing inflammation), or with 0.2 ml of 5% hog gastric mucin (to enhance infection). The mice injected with chlamydospores alone were sacrificed at 2 and 5 hr and at 1, 2, 3, 4 and 8 days. The other mice were sacrificed at 3 days. To examine the tissues for the presence of chlamydospores, pieces of connective tissue were lifted and laid onto glass slides. Slides were also prepared by swabbing or scraping the tissues under the

skin. The slides were then stained with Giemsa.

Germ-tube formation by blastospores of *C. albicans* was examined in human serum at pH 7.0; in the dialyzed supernatants and precipitates of $(\text{NH}_4)_2\text{SO}_4$ -fractionated serum; in Difco bovine albumin; in pleural and abdominal fluids; in dialyzed serum; in serum boiled for $\frac{1}{2}$ hr; in 20% gelatin and in neopeptone solution. The effect of increasing the concentration of phenethyl alcohol (PEA) was tested on germ-tube formation in 50% serum at pH 7.0. These tests were examined at intervals between 2 and 4 hours, and after 20 hr incubation.

Results and Discussion

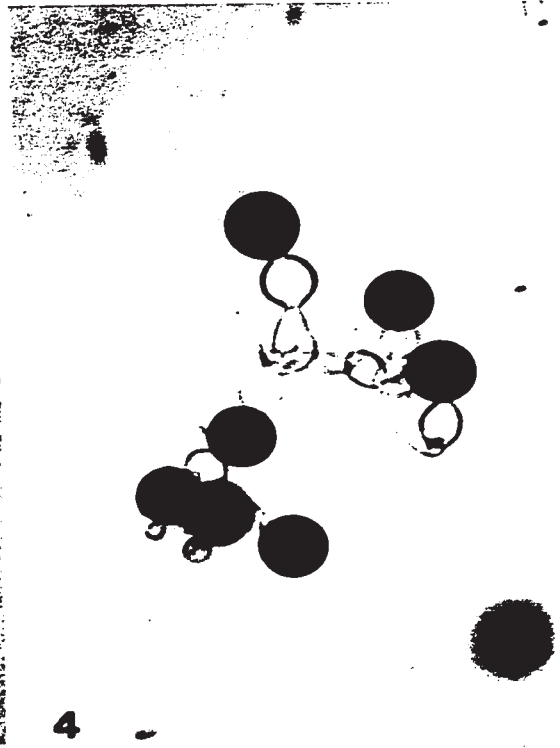
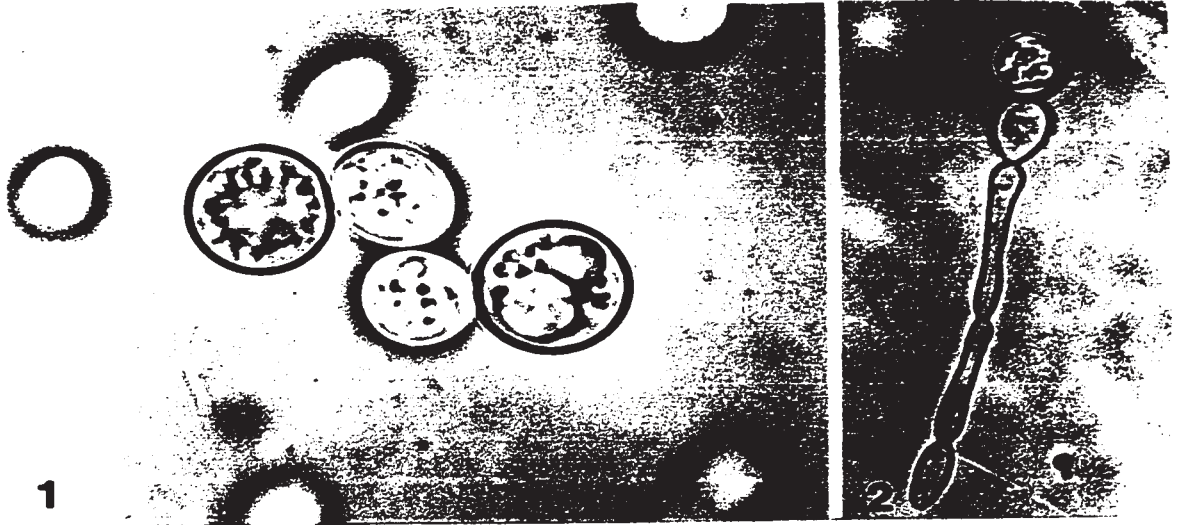
Chlamyospores

Observations revealed that chlamyospores were usually associated with pseudomycelial formation, and could arise in several different ways (Plate I, Fig. 1-3). One way (Fig. 1) that chlamyospores may arise is by budding directly from a single blastospore. The two chlamyospores shown in the figure were observed by Dr. Bakerspigel during their formation. Fig. 2 shows the characteristic appearance of a chlamyospore which budded from a 'round cell', which had itself arisen as a bud from elongated cells ('pseudohyphae'). Numerous observations have shown that usually the original blastospores budded two or three times in sequence before chlamyospores were formed. This chain of cells

PLATE I

- Fig. 1 Two mature chlamydospores of *C. albicans* (58/60), each having arisen from a single blastospore. Unstained, X3024.
- Fig. 2 A single, mature chlamydospore of *C. albicans* (58/60) on a four-celled 'chlamydophore'. Unstained, X1344.
- Fig. 3 A group of four chlamydospores, produced by *C. albicans* 7396, two of which are borne terminally on short, elongated cells ('pseudohyphae'). Unstained, X1344.
- Fig. 4 A group of 7 chlamydospores, produced by *C. albicans* 7396, aged 4 months, stained selectively with thionin. Note the empty cells attached to these chlamydospores. X1344.

PLATE I



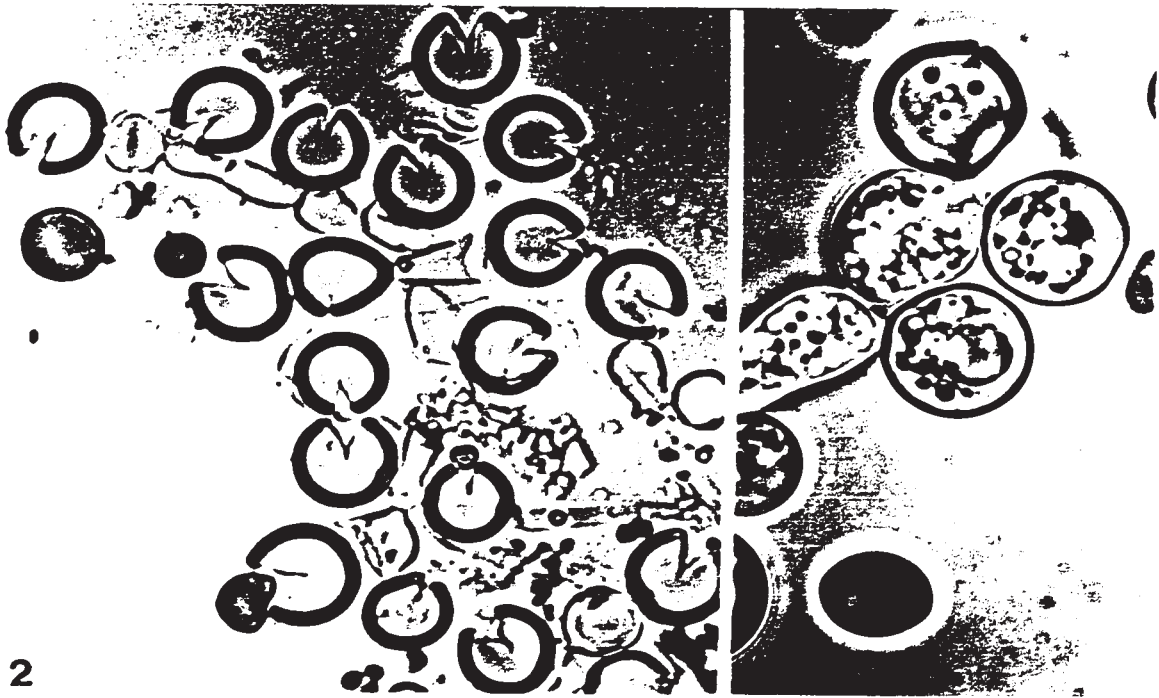
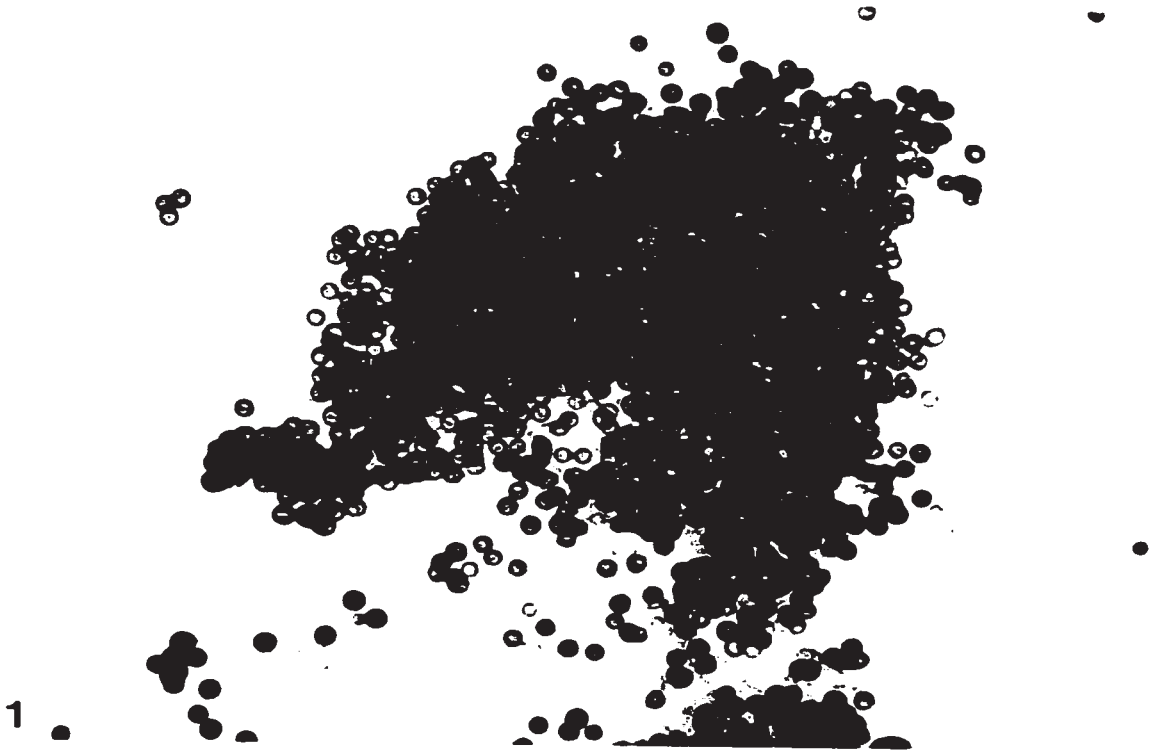
(Fig. 2) was termed a 'chlamydo~~phore~~' by Bakerspigel (1954). Since the chlamydo-spores are the final cells produced, and since they have not been observed to bud, they were designated as being 'terminal'. Although a 'rounded cell' was usually formed before the chlamydo-spore, this sequence did not occur at all times. Fig. 3 shows two chlamydo-spores borne in a manner described here as 'sessile'. The single rounded cells subtending the chlamydo-spores shown in Fig. 2 and 4 are similar to the cells which Lodder *et al* (1952) called 'pro-tochlamydo-spores'. However, it was observed in these studies that 4 or more of these cells in a chain formation could subtend a chlamydo-spore. Chlamydo-spores stained with thionin, reveal that they are clearly defined and do not appear to share any protoplasmic continuity with the subtending cells (Fig. 4). This is also clearly shown in that chlamydo-spores at any time may be completely separated from such cells in a wet mount on a glass slide by a gentle rubbing motion of the cover slip. Furthermore, it should be noted that the subtending cells become empty (Fig. 4).

Numerous chlamydo-spores can be readily produced in serum-containing media (Plate II, Fig. 1). Preparations of chlamydo-spores can be freed of blasto-spores by the differential-settling method described above. Within two weeks, the mycelium also becomes empty and collapses in many cases. Because the chlamydo-spores apparently do not share any cytoplasmic continuity with subtending cells, they may be

PLATE II

- Fig. 1 Numerous chlamydospores produced by *C. albicans* 7396 in serum-containing medium, 4 months. Thionin-stained, X336.
- Fig. 2 A group of slightly crushed chlamydospores of *C. albicans* 7396, showing V-shaped openings distal to the point of attachment. The spherical and bean-shaped globules, which were extruded from the chlamydospores, are thought to be lipoid in character. Unstained, X1344.
- Fig. 3 Several chlamydospores of *C. albicans* (58/60). Note the small opening in the highly refractile cell wall in the chlamydospore distal to the point of attachment. Unstained, X3024.

PLATE II



removed from them in large numbers, if a suitable procedure for this step could be found. This would obviate the need to age them in order to render the mycelium unimportant. The type of chlamyospore preparations that can now be made would not only permit electron microscope examinations, but also would facilitate many other procedures.

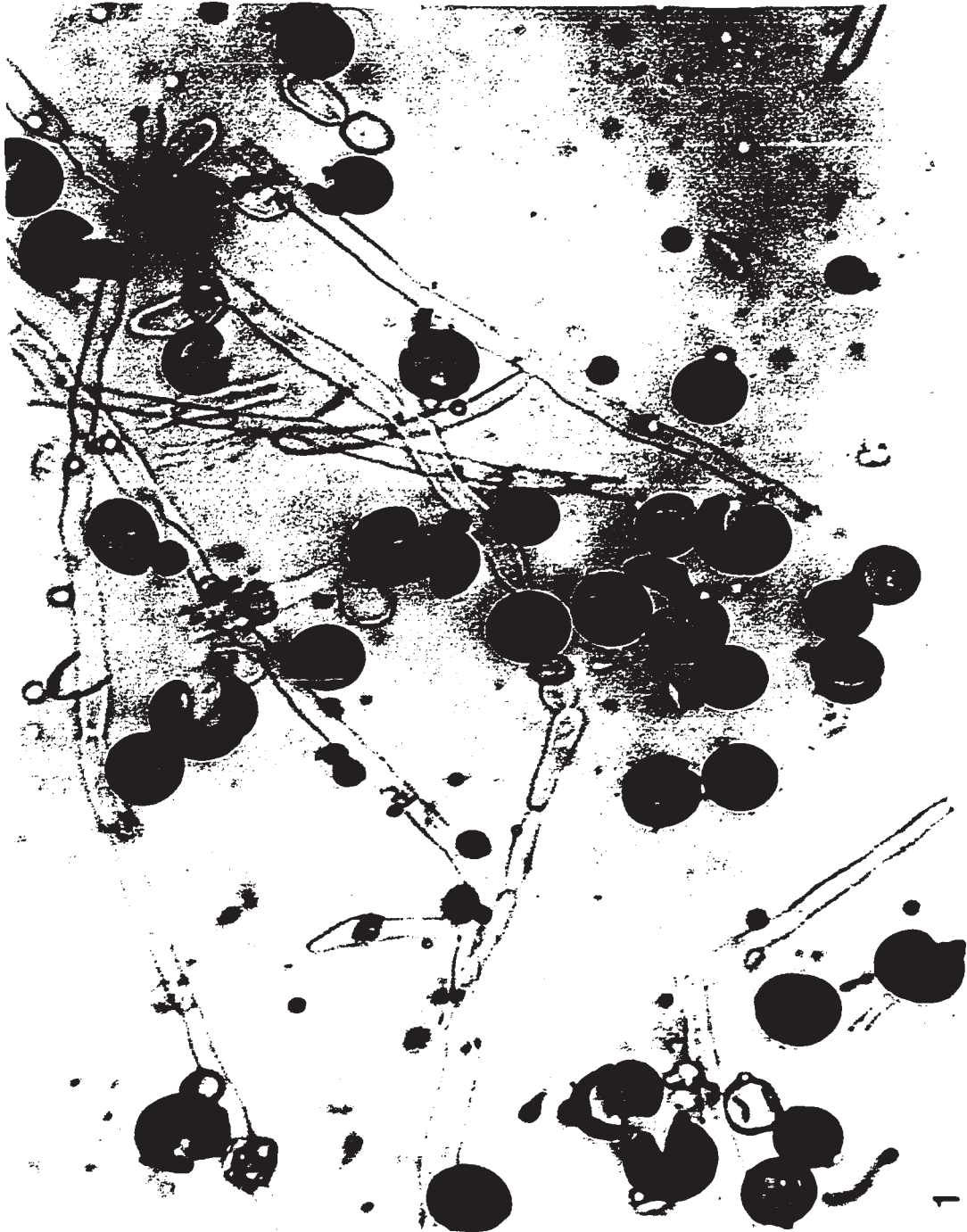
Fig. 2 (Plate II) and Fig. 1 (Plate III) show that slight crushing of chlamyospores causes a V-shaped rupture to occur at a weak area. The ruptured cells emit globules of a hydrophobic material, whose exact nature has not yet been determined. Depending on the force used to crush the cells, the globules may be disseminated, or they may coalesce to form the larger globules seen in Fig. 2 (Plate II). Crushed cells clearly reveal the thick cell wall, which was described in more detail by Bakerspigel (1964). Fig. 3 also shows a chlamyospore with an opening in its cell wall. Fig. 2 and 3 (Plate II) show that weak areas or openings do not occur in the region where the chlamyospores are attached to subtending cells. It is suggested that these areas may permit the exchange of materials from within the cell to the environment and *vice versa*. Furthermore, other types of cells may arise from chlamyospores at these areas.

Although free and unattached chlamyospores were observed for as long as 30 days on sealed glass slides, none were noted to bud or germinate. Observations made in

PLATE III

Fig. 1 A group of chlamydozoetes produced by *C. albicans* 7396, showing V-shaped splitting and lunar-shaped fragments of cell walls which appear to be regular in shape. Thionin-stained, X1344.

PLATE III



a number of liquid media did not reveal chlamydospores which had germinated. Increases in the number of blastospores or mycelial elements were ascribed to budding and to the germination of other viable elements. However, the possibility that chlamydospores could function as propagating units in some other ways cannot be dismissed. For example, chlamydospores might gradually revert to thinwalled cells before they germinate. It was observed that during the formation of chlamydospores, cell walls gradually thickened over a period of 24 hr or more. This process was probably assisted by the action of one or more enzymes. The reverse process may require the same or similar enzymes. Suspensions of chlamydospores kept for six months were observed to contain an increasing number of thinwalled cells. However, more precise explanations will depend upon further studies of mature chlamydospores.

Attempts to initiate germination of chlamydospores by injecting them into mice have thus far been unsuccessful. Tissue taken from the mice revealed abundant chlamydospores to be present at various times, although the numbers appeared to diminish after three days. No germinating chlamydospores were seen, although some showed V-shaped splits. In general, chlamydospores were engulfed by macrophage-like cells and giant cells. Inoculation of the chlamydospores into a mouse with croton oil resulted in the production of purulent lesions in which many hyphal elements were present though not associated with chlamydospores. The mouse receiving cysteine

developed numerous hemorrhages in the nape tissues, but again no chlamydospores were observed. These results are similar to those obtained in rabbits by Bakerspigel (personal communication) in a series of experiments using the oral route of infection.

It is difficult to accept the possibility that the chlamydospore is a cell which does not fulfil a specific function. The presence of the thick wall suggests a role in survival under unfavorable conditions. In addition, the optimum temperature for their formation is 25 C, indicating that an environment outside the warm-blooded body favors their production. It is suggested that they are dormant cells whose latency may be broken by some mechanism which is unknown at present. Their high content of lipid material also suggests that they could have an extended survival period, if the cells also had a low endogenous metabolism.

The results obtained from these studies of chlamydospores have refuted some previously held opinions on the environment and the medium required for their production. For example, any claim that 37 C was suitable for the production of chlamydospores (Liu *et al*, 1955) cannot be taken seriously, because certain other cells may be mistaken for chlamydospores. Another source of such an error could be due to previously formed chlamydospores being carried over onto a new medium. Liu *et al* (1955) also stated that solid media which are poor in nutritional value are necessary for chlamydospore formation. This statement is unfounded,

since the serum-containing liquid medium definitely contains many nutrients, including 1% glucose. Nickerson *et al* (1953) reported that reducing sugar inhibited chlamyospore production. Wolin *et al* (1962) found that a small amount of reducing sugar stimulated the production of chlamyospores. In the serum-containing medium, chlamyospore production was related to pseudomycelium production, which was readily formed in it. Furthermore, Liu *et al* (1955) reported that a low pH favored pseudomycelium production and inhibited chlamyospore production. Present studies revealed that a low pH favored the growth of this organism, and resulted in the abundant production of chlamyospores.

Germ-Tubes

Germ-tubes were readily formed by blastospores at 37 C in serum or its fractions; in gelatin; in abdominal and pleural fluids, and in Difco bovine albumin solution. In contrast, synthetic media such as YNB or purified serum proteins such as transferrin, γ -s-globulin or crystalline albumin did not induce germ-tube formation. Fig. 1 and 2 (Plate IV) show germ-tubes which were formed in serum at pH 7.0 after 3 1/2 hr. Upon further incubation, germ-tubes continued to elongate and become septate mycelium on which blastospores were produced (Fig. 1, Plate V). Germ-tubes were also produced in neopeptone solution, with or without 1% glucose. The appearance of germ-tubes was determined

PLATE IV

- Fig. 1-4 Cells and mycelium produced by *C. albicans* 7396 in serum, 37 C. Unstained, X1344.
- Fig. 1,2 Elongated germ-tubes grown for 3 1/2 hr at pH 7.0. Note septum in germ-tubes shown in Fig. 2.
- Fig. 3 A distorted germ-tube produced after 6 hr in serum containing 0.2% PEA.
- Fig. 4 Absence of characteristic germ-tube formation after 6 hr in serum containing 0.3% PEA.

PLATE IV



PLATE V

Fig. 1 Mycelium obtained within 20 hr at pH 7.0 by *C. albicans* 7396 in serum at 37 C. Note also septa, blastospores and the original cells. Unstained, X1344.

PLATE IV

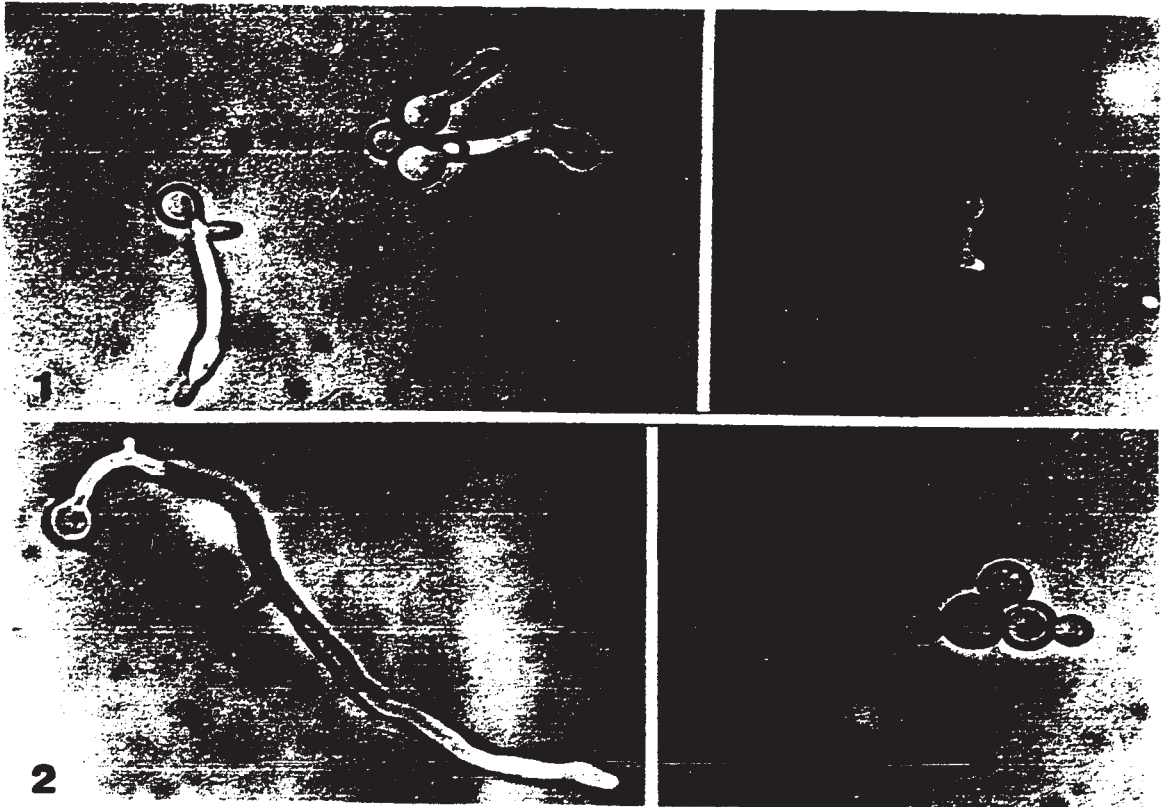


PLATE V

Fig. 1 Mycelium obtained within 20 hr at pH 7.0 by *C. albicans* 7396 in serum at 37 C. Note also septa, blastospores and the original cells. Unstained, X1344.

PLATE V



by a number of factors such as concentration of inoculum, and the particular fraction of serum used. In preparations containing high proportions of albumin and in gelatin, the germ-tubes were narrow and delicate in appearance, with no tendency to swell at the tip. It is suggested that such substances might be used for producing germ-tubes to study cytological changes occurring in the cells. The germ-tubes could be produced under conditions which are apparently free of exogenous nutrients. A relatively concentrated inoculum in serum resulted in germ-tubes that swelled at the tips. Fig. 3 (Plate IV) shows a distorted germ-tube produced in the presence of 0.2% PEA, in 50% serum at pH 7.0. With 0.3% PEA, typical germ-tubes were not formed within the usual period. However, in approximately 6 hr, rounded structures were formed, resembling buds, as shown in Fig. 4 (Plate 4). On further incubation, these continued to develop and bud again, so that after 20 hr, each original cell had two or more buds. Concentrations of PEA higher than 0.3% also inhibited budding to a considerable extent.

In agreement with Gresham *et al* (1961) and Mackenzie *et al* (1964) it is concluded that germ-tube formation represents the initiation of mycelial growth. In N-G broth, the formation of germ-tubes is transient, the growth being modified to a yeast-like form by autoantibiotics (Lingappa *et al*, 1969). In serum the tendency to mycelial formation is prolonged (Fig. 1, Plate 5). Thus the use of N-G broth together with serum permits a mycelial

form of growth. Under optimum growth conditions, such as a pH of 7.0, and adequate iron availability, the growth appears to become more yeast-like. However, it is likely that many of the yeasts formed are blastospores such as those shown on the mycelium in Fig. 1 (Plate V).

C. albicans did not produce germ-tubes in synthetic media such as YNB. In studies of chlamydospore production, such synthetic media were found to be ideal when used with serum for abundant pseudomycelial formation. Thus it appears that the mycelium-promoting activity of serum is modified when the diluent is a synthetic medium rather than an organic one.

GENERAL DISCUSSION

Mycotic infections among cancer patients occur frequently, but only a specific search for these infections reveals this incidence. Thus, in a series of *post-mortem* examinations of 454 leukemic patients, Bodey (1966) found 161 to have mycotic infections. The majority of these patients died from these infections rather than from leukemia. Cancer may also predispose to an increased incidence of *C. albicans* in the body. Vanbreuseghem, Coremans-Pelseneer, Swinne-Desgain & Janssens-van Dyk (1969) made a detailed study of 100 randomly selected cancerous cadavers and found 90 of them to be harboring fungi. Nearly 80% of the samples were positive for *C. albicans* on culture, and these samples came from 71 cadavers. *C. albicans* was most frequently found in the alimentary tract, particularly in the stomach. This was thought to be the main reservoir of *C. albicans* in the body. Marples *et al* (1952) found *C. albicans* in the mouths of one half of normal adults and of one-third of the children examined by them. *C. albicans* was present in almost one-third of their fecal specimens. Therefore, *C. albicans* is also present in normal individuals who do not present any symptoms of candidiasis.

The study of patients with candidiasis is more

often the subject of published reports (e.g., Taschdjian *et al*, 1969) than are studies of resistance to infection by *C. albicans* in normal individuals (Mackenzie, 1964). A distinction must be made between candidiasis as a clinical entity and resistance to candidiasis. The sequence of events which follows the introduction of *C. albicans* into the blood stream of healthy animals, as described by Mackenzie (1964), may not parallel the sequences that lead to the establishment of infection in susceptible individuals. Thus, those patients who already have candidiasis presumably lack one or more defense mechanisms. Since these mechanisms cannot be studied directly in such patients, it should be evident that the response of normal individuals should be studied instead. This rationale has not been reported in literature to date.

In addition, the changes which occur in *C. albicans* itself and in the body as well may be difficult to study during the progress of the infection. Smith (1968) stated that virulence of an organism is determined by differences which may be fully expressed only during growth *in vivo*. In the present studies, it was the nature of *C. albicans* that was of primary interest and, for this purpose, laboratory-controlled experiments were designed. Nevertheless, present results indicate two ways in which the normal human body may resist systemic infection with this organism.

Martin (1962) found that a high, unbound iron-binding capacity in the serum of patients with agamma-

globulinemia may help to compensate for a lack of protection against infection through the deficiency of humoral antibodies. Serum from such patients showed a greater than normal bacteriostatic effect against *B. subtilis*. Wood *et al* (1961) reported that some patients with low gamma globulin levels had serum antibacterial values exceeding normal values and, that the serum of normal individuals and of patients with hypogammablobulinemia who had acute (but not chronic) infections had elevated antibacterial activity against *B. subtilis*.

The growth of *C. albicans* is inhibited in the presence of human serum and this inhibition appears to be a normal defense mechanism of the body which prevents inoculum that has reached the blood stream from multiplying. Indirect evidence that serum transferrin, which is a powerful iron chelator at physiological pH, is responsible for this growth inhibition or at least for part of it has been given by Hendry *et al* (1967b) and by Caroline *et al* (1964). Present studies (Fig. 71), and those of Esterly *et al* (1967b), have demonstrated that transferrin by itself does not have a growth-depressing effect on *C. albicans* in culture. Reasons for this lie in the failure of transferrin alone to cause a morphological change in the growth. A change of this sort is due to other serum factors, unknown at present. However, experiments with DFOM have shown that iron chelation alone does affect the growth of *C. albicans*, particularly under aerobic conditions. Landau *et al* (1964) indicated that serum with

its transferrin saturated, induced a greater percentage of *C. albicans* cells to form germ-tubes. However, results obtained from present studies indicate that the transferrin-mediated growth-inhibition of *C. albicans* operates only in relation to the altered nature of the growth in the presence of serum.

In a medium containing serum growth and relative catalase activity of *C. albicans* cells were both greatly decreased. The significance of catalase activity is two-fold. Catalase is an enzyme essential for aerobic or oxidative metabolism. A deficiency of iron would thus inhibit the synthesis of this enzyme as well as that of other heme-proteins or iron-containing enzymes. The deficiency of iron would, therefore, inhibit the growth of any organism which cannot for any reason reduce its dependence on an oxidative pathway in its metabolism. The deficiency of catalase activity in the cells, and the coincidentally reduced over-all growth in a medium containing serum, indicates that the metabolism of *C. albicans* had indeed become more dependent on its iron-containing enzymes.

Catalase deficiency of *C. albicans* cells grown in a medium containing serum has another significance in relation to its role as a potential pathogen. This significance is concerned with phagocytosis. Young (1958) and Louria *et al* (1963, 1964) have shown that germ-tubes formation and phagocytosis readily and rapidly occur when *C. albicans* cells are injected into human blood. Lehrer

(1969) and Lehrer *et al* (1969) reported that polymorphonuclear leukocytes kill ingested *C. albicans* cells by a combination of myeloperoxidase, H_2O_2 , and a halide. *C. albicans* germ-tube formation which occurs in blood (Reynolds *et al*, 1956) may thus be a prerequisite for the most efficient killing of ingested cells. Present results show that cells which have produced germ-tubes are deficient in catalase activity due to the lack of iron in plasma and, thus, would be less able to detoxify the H_2O_2 . Any residual iron in the cells in the inoculum would be overtaxed to produce enzymes needed for the maintenance of oxidative metabolic functions.

Caroline *et al* (1969) observed that leukemic patients have a highly iron-saturated transferrin in their serum as well as a greater susceptibility to candidiasis. Individuals whose PMN leukocytes are deficient in killing ability (Lehrer *et al*, 1969) may also be more susceptible to candidiasis. However, cellular immune defects may also contribute to the establishment of candidiasis (Fujiwara *et al*, 1970, and Landau, 1968).

Other factors which may be important in candidiasis include the virulence of the strain of *C. albicans* (Winston *et al*, 1956), the presence or absence of microflora in the host (Balish *et al*, 1966a, b) and the size of the inoculum. Numerous cells reaching the blood stream at any one time may contribute large amounts of candidotoxin. This toxin, which was isolated by Iwata

et al in 1969 from blastospores of *C. albicans*, enhanced the infection in mice. It was noted by Mackenzie (1962) that the proportion of cells forming germ-tubes in serum was decreased with increasing concentration of cells. This observation may be analogous to that of Taschdjian *et al* (1969) who reported that yeast cells were present in the organs of patients who died of candidiasis. In addition, *C. albicans* may alter its mycelial growth to the yeast-like form by producing autoantibiotics, phenethyl alcohol and tryptophol, for this purpose (Lingappa *et al*, 1969). A large inoculum may thus suppress germ-tube formation by introducing such autoantibiotics in high concentrations. Under such circumstances autoantibiotics might be called 'virulence factors'. Indeed, Taschdjian *et al* (1969) stated that the yeast phase of *C. albicans* was the carrier of pathogenicity. It appears likely that factors promoting the yeast-phase in *C. albicans* will enhance infection with this organism and that mycelial formation is associated with either an unsuccessful infection or the availability of iron *in vivo* in abnormal individuals.

Although the cause of germ-tube formation by *C. albicans* in serum is unknown at present, Bernander & Edebo (1969), in studying mycelial formation of this organism in Dubos medium, suggested that serum albumin may bind yeast cell-promoting substances in the inoculum. In this connection it may be that the autoantibiotics phenethyl

alcohol and tryptophol (Lingappa *et al*, 1969) are the substances which are bound. However, Bernander *et al* (1969) also suggested that impurities present in the albumin solution may be required by the blastospores for conversion to mycelium.

Ramirez & Ransom (1964) reported that the exhaustion of carbohydrates and nitrogenous compounds from the medium was responsible for the inhibition of pseudomycelial formation at the edges of parallel colonies of *C. tropicalis*. However, the findings of Lingappa *et al* (1969) make this opinion untenable.

Scherr *et al* (1953) stated that pseudomycelium is a growth form intermediate between yeasts and mycelium. However, there is some indirect evidence available to indicate that the problem is much more complicated. For example, Mardon, Balish & Phillips (1969) studied yeast-pseudomycelium dimorphism in *C. albicans* with a culture that did not form germ-tubes in serum. On the other hand, present results indicate that PEA inhibits germ-tube formation by *C. albicans* in serum.

An additional problem related to dimorphism in *C. albicans* concerns the use of certain substrates for its growth. Thus, Mardon *et al* (1969) found that a $\text{CO}_2:\text{O}_2$ ratio of 2:1 favored a higher proportion of pseudomycelium to yeast cell formation. They also found that any one of several amino acids when used as a sole N-source favored

pseudomycelial formation and the best ones were methionine, phenylalanine and tyrosine. However, Johnson (1954) noted that no single amino acid used as a source of nitrogen permitted *C. albicans* to grow as well as did a mixture of amino acids. Since poor growth generally favored mycelial formation in this organism (Dastidar *et al*, 1967) it is, therefore, not relevant to state that any one amino acid will favor pseudomycelial formation. In addition, Dastidar *et al* (1967) found that phenylamine in Czapek Dox broth (containing NaNO_3 as a source of nitrogen) supported excellent growth and favored the yeast phase. However, they also found methionine and tyrosine permitted only a relatively poor growth and mycelial formation. Thus, there is no proper evidence showing that the reasons for poor growth and/or mycelial formation are simply related to the presence or absence of nutritional substances.

Chattaway, Ellis & Barlow (1963) found that 5-20% human serum inhibited proteolytic activity of enzymes extracted from dermatophytes. This inhibition occurred only above pH 6.0. Staib (1964, 1965) reported that some strains of *C. albicans* exhibited a proteolytic activity against serum albumin not demonstrable above pH 5.0. However, Staib did not determine if this was an inhibition of the proteolytic activity above this pH in the presence of serum or if it involved pH-sensitive enzymes. If the real situation in this respect for *C. albicans* is analogous to that described by Chattaway

et al (1963) then it might mean that an antienzymatic activity of serum may also be involved in susceptibility or resistance to candidiasis. This activity could presumably operate at physiological pH but allow growth of *C. albicans* at any pH below normal.

Present experiments on the iron-reversible anticandidal activity of serum should not necessarily be interpreted to mean that introduction of excess iron, *in vivo*, will promote candidiasis. These experiments were designed primarily to gain more information about *C. albicans per se*.

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ADDENDUM

Additional References

In addition to other tests performed on the sera of patients, Barlow (1967) attempted to determine the extent of serum fungistasis on *C. albicans* using Kjeldahl nitrogen determinations. Turbidity methods for measuring growth were ruled out since mycelial pellets were formed in broth while cultures were being aerated during incubation. Plate counts were considered to be inaccurate, and too little serum could be obtained per patient to permit dry weight determinations. However, total nitrogen determinations also failed to yield any usable data because of interference from serum nitrogen.

Similar problems were encountered during present studies in attempting to determine the best methods to use in measuring fungistasis. Several methods were not employed because of anticipated difficulties. Use of pooled serum solved the problem concerning the requirement for sufficient quantities of serum. Growth in liquid media did not form pellets if the tubes were not shaken during incubation. In spectrophotometric measurements relatively little error was introduced in relating mass to the degree of decrease in light transmittance by mycelial strands. This claim cannot be made

for plate counts of colony-forming units or for direct microscopic counts of cells and filaments produced by this organism.

Bienias & Szymczykiewicz (1967) attempted to measure relative fungistatic activity of individual serum samples by a method whereby blastospores of *C. albicans* were inoculated into the undiluted serum. After 24 hrs a measured quantity of this serum was inoculated into broth and, following a further period of incubation, a direct count of cells was made. However, Bienias *et al* (1967) were apparently unaware that they were probably measuring two, quite distinct, effects of serum. Thus, incubation in undiluted serum may have had a lethal effect on the cells in the inoculum. Incubation in the broth, which would now contain almost 5% serum carried over with the inoculum, may have had a growth inhibiting effect. In view of the data presented in this thesis 5% serum could be a critical amount depending on the type of broth and the qualities of the serum. In addition, it is very likely that filament formation would interfere with any reliable measurement of growth based on microscopic counts. Nevertheless, none of these problems were discussed by Bienias *et al* (1967).

The reports by Barlow *et al* (1967) and Bienias *et al* (1967) were apparently published before those which first attributed the fungistatic activity of serum to its content of iron-unsaturated transferrin. Nevertheless,

they illustrate the need to establish reliable testing methods for determining the extent of fungistasis in patients' sera as well as the difficulties encountered in the performance of this task.

The recent reports of Jansons & Nickerson (1970a, b) are evidence of the continuing interest in, and the significance attached to, the chlamydospores produced by *C. albicans*. Some of the results in these reports are similar to observations in this thesis and, in some instances, confirm them. Jansons *et al* (1970a) reported the preparation of chlamydospores free of blastospores although still attached to pseudomycelium. This result had also been achieved in the present work (see Morphological Studies of *C. albicans*). However, Jansons *et al* (1970a) failed to produce chlamydospores in a liquid medium and, therefore, their purification method was more complex than the one employed in the present work. Jansons *et al* (1970a) observed germination of chlamydospores, provided these were younger than 24-40 hrs. In the present studies chlamydospores could not be induced to germinate at any time. However, spherical cells which might have become chlamydospores were often observed to bud and these buds subsequently formed other spherical cells. Differences in interpretation may also be due to the use of agar on slides by Jansons *et al* (1970a) whereas, in the present studies, thin mounts in liquid media were used. Furthermore, it should be emphasized that spherical cells were not termed

"chlamydospores" until they achieved a characteristic appearance with maximum refractility of their cell walls.

Jansons *et al* (1970b) observed that granules found outside of chlamydospores, when split by slight pressure, were deeply stained by lipid dyes. Several cytochemical procedures were also performed by Jansons *et al* (1970b) on chlamydospores still attached to pseudomycelium. In present studies it was observed that chlamydospores could be detached from pseudomycelium without breaking them although a method was not found to accomplish this on a 'test-tube' scale. Jansons *et al* (1970b) were unable to localize a nucleus in the chlamydospore. Bakerspigel (1964) encountered a somewhat similar difficulty when he attempted to demonstrate nuclei in fixed chlamydospores. However, chlamydospore nuclei, stained by the HCl-Giemsa technique, have been observed which reveal among other things that the chlamydospores are uninucleate (Bakerspigel, Personal Communication).

Van der Walt (1970) described a method for obtaining diplophase and haplophase cells of *C. albicans*. The life cycle of *C. albicans*, as proposed by Van der Walt, has been discussed under "Morphological Studies of *C. albicans*". Van der Walt (1970) also described a method for inducing germination of chlamydospores on a non-nutritive agar containing salts. According to him

chlamydospores germinated by forming haploid buds, which gave rise to the sexually active haplophase.

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APPENDICES

1. Determination of Inoculum Size

A suspension of cells of *C. neoformans* was used which showed an O.D. reading of 0.89 on the Bausch and Lomb Spectronic 20 spectrophotometer at 600 m μ , in 16 X 150 mm tubes. In a hemocytometer, this gave a count of 1.2×10^5 cells per ml. Therefore, the inoculum size was 6×10^3 cells in the 0.05 ml used per tube of medium in present experiments.

Similarly, a suspension of cells of *C. albicans* was used which showed an O.D. value of 0.350, with a count of 1.3×10^5 cells per ml. The inoculum was 6.5×10^3 cells in 0.05 ml.

The quantity of cells in the inoculum was nearly the same for both organisms. The difference in the optical density readings was probably due to the capsular material accumulated by *C. neoformans*.

The spectrophotometer gave a linear response to arithmetic increases in cell concentration over the O.D. values from 0.1 to 0.9.

2. Desferrioxamine (DFOM)

The molecular weight of DFOM is 597 (Moeschlin *et al*, 1963) and each molecule binds 1 molecule of Fe^{3+} which has a molecular weight of 55.85. Therefore, there is approximately a 10:1 relationship by weight. It was calculated that about 0.75 ug of DFOM would be required to bind the iron in 4 ml of single strength N-G broth (Appendix 4). The binding coefficient of DFOM with iron is known to be even greater than that for transferrin with iron at physiological pH.

3. Serum Transferrin

The mean total iron-binding capacity in serum was found by Sinniah & Neill (1968) to be 470 ug per 100 ml, of which 30% was saturated. This left 329 ug of unbound capacity per 100 ml, or 3.29 ug per ml. That is, 1 ml samples of serum from most individuals examined bound quantities of iron not greatly above or below 3.29 ug. The unbound capacity of a pool from a large number of individuals can be expected to approximate this value.

Table 9 shows that the unbound iron-binding capacity of 0.04 ml pooled human serum (using the mean value) would be sufficient to bind the iron present in 4 ml single-strength N-G broth (Appendix 4). In the majority of present experiments, 5.6 ug Fe ($25\mu\text{M FeCl}_3$ in the final volume) were added to 4 ml of broth, or

broth containing serum. This is sufficient to bind the iron requirement of much more than 25% serum concentration, the maximum used in many of the present experiments. It is beyond any reasonable margin of error assumed by arbitrarily using the best published mean value for unbound serum iron-binding capacity.

4. Iron content of FeCl_3 Solution and N-G Broth

In determining the iron content of FeCl_3 solutions, the O-phenanthroline-hydroxylamine method (Horowitz, 1960) was used. The Bausch and Lomb spectronic 20 spectrophotometer was used to measure density of color. The standard curve was prepared from Fisher Scientific purified iron powder. A 0.004M FeCl_3 solution was diluted 10 times and half was filtered through a 0.22u Millipore membrane. The unfiltered solution was found to contain 96.21% of the expected iron concentration, in comparison to the standard solution, and the filtered solution 95.32%. Thus, in diluting 0.004M solutions of FeCl_3 for addition to media, a value of 95% was used as a correction factor.

The iron content of neopeptone was determined by the method of Ballentyne & Burford (1957). This method was also spectrophotometric. The Difco value for iron in neopeptone is 0.0041 g per 100 g (about 40 ug per g). However, on analysis, a 2 g sample of one batch of neopeptone was found to contain 11.1 ug per g, or

0.222 ug of iron for the 0.02 g neopeptone contained in 4 ml medium. This same batch was used throughout all the experiments. Therefore the iron content of N-G broth was known in all present experiments.

In addition, Millipore filtration of 6000 ml N-G broth (containing 60 g neopeptone) showed that 7.4 ug of iron for every g of neopeptone was retained on the filters. No iron was found in the same type of filters used to obtain blank values. This loss represents 66.67% of the iron concentration of this batch of neopeptone. Therefore, by subtraction, 0.074 ug of iron remained in each 4 ml of filtered N-G broth (IX strength). This concentration is in fact, a very low value which made Millipore filtered N-G broth an exceptionally good medium to use in determining the effect of serum on organisms.

5. Catalase Determinations

See also Lück (1956) and Chance & Maehly (1955). A 0.004M KMnO_4 solution, standardized with sodium oxalate, was used. Oxidation of H_2O_2 is shown by the formula: $2\text{KMnO}_4 + 4\text{H}_2\text{SO}_4 + 5\text{H}_2\text{O}_2 = 2\text{KHSO}_4 + 8\text{H}_2\text{O} + 5\text{O}_2 + 2\text{MnSO}_4$. In the catalase tests, the average of all blank values in the experiments reported (31 determinations) showed that 7.35 ml of 0.004M KMnO_4 oxidized 3.33 ml of H_2O_2 solution. This value gives

TABLE 8

Iron-Binding Capacity of Serum
(mean value of Sinniah *et al*, 1968)

Serum %	ml in 4 ml Medium	Iron-binding capacity
1	0.04	0.1316 ug
2	0.08	0.2632
3	0.12	0.3948
4	0.16	0.5264
5	0.2	0.658
10	0.4	1.316
15	0.6	1.974
20	0.8	2.632
25	1.0	3.29

the molarity of the H_2O_2 solution used as 0.00353M.
There is a relationship of 1 mole KMnO_4 per 2.5 moles
of H_2O_2 (from the equation). Thus, 7.35 ml of 0.004M
 KMnO_4 have 29.4 umoles of KMnO_4 , equivalent to $2.5 \times$
 $29.4 = 73.5$ moles of H_2O_2 . Therefore, 1 ml of KMnO_4
solution breaks down 10 umoles H_2O_2 .